Deciphering Role of *Salmonella typhi* Virulence Polysaccharide in the Modulation of Immune Cell Functions

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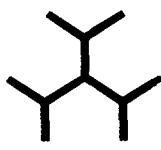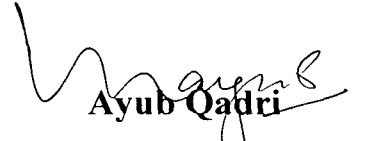for the degree of

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CERTIFICATE

This is to certify that thesis entitled "**Deciphering role of *Salmonella typhi* virulence polysaccharide in the modulation of immune cell functions**" embodies the work done by **Rohini Garg** in partial fulfillment of the Ph.D. degree of the **Jawaharlal Nehru University** under my guidance at the **National Institute of Immunology**. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

Ayub Qadri

(Supervisor)

dedicated to
...my mother

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translating my dreams into reality. I would like to give my special thanks to my brother Davesh for his best wishes and endless love. I bow before the Almighty for everything that has been, is and ever will be. Last but not the least; I would like to thank my husband, Mukesh, for always being with me in thick and thin moments of my life and pepping up my flagging spirits.

Rohini Garg

ABBREVIATIONS

AP	Alkaline Phosphatase
APC	Antigen presenting cells
BAP-37	B cell receptor-associated protein-37
BSA	Bovine serum albumin
CD	Cluster of differentiation
DC	Dendritic cells
°C	degree Celsius
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	relative centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte monocyte colony stimulating factor
h	hour/hours
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
I-κB	Inhibitor of κB
IL	Interleukin
IP	Immunoprecipitaion
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen-activated protein kinase
min	minutes
NC	Nitrocellulose
NF-κB	Nuclear factor- κB

PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PECs	Peritoneal exudate cells
PGUA	Polygalacturonic acid
Phb	Prohibitin
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene Fluoride
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
RU	Response unit
shRNA	short hairpin RNA
siRNA	small interfering RNA
SPR	surface plasmon resonance
TCR	T-cell receptor
TLRs	Toll-like receptors
Vi	Virulence polysaccharide derived from *S.typhi*
WB	Western blot

CONTENTS

Introduction

INTRODUCTION

Salmonella pathogenesis is a complex phenomenon involving a large number of host-pathogen interactions. The infection is initiated *via* invasion of intestinal epithelium which may be followed by systemic dissemination of the pathogen. Bacteria can be isolated from blood, spleen, liver, bone marrow and gall bladder during systemic infection. These bacteria reside mainly inside mononuclear phagocytes (Wick, 2004). The molecules that are required for invasion of intestinal epithelial cells, intracellular survival of the pathogen within macrophages, induction of cellular cytotoxicity and several other manifestations, are to a large extent conserved amongst different *Salmonella* species (Cotter and DiRita, 2000). However, in spite of this conservation, many *Salmonella* serovars exhibit a high degree of host specificity. *Salmonella enterica* serotype Typhi is a strictly human adapted - pathogen that causes systemic infection, typhoid, almost exclusively in humans. Typhoid is characterized by mononuclear cell infiltration and hypertrophy of the reticulo-endothelial system including Peyer's patches, mesenteric lymph nodes, liver, spleen, and bone marrow. In contrast, non-typhoidal *Salmonella* species such as *Salmonella typhimurium* or *Salmonella enteritidis* cause gastroenteritis that usually remains localized to the intestine and mesenteric lymph nodes. The differences in the host responses and disease manifestations of typhoid fever and gastroenteritis suggest that serotype Typhi and non-typhoidal *Salmonella* serotypes cause disease by different mechanisms. Unlike in humans, *S.typhimurium* produces a systemic infection in mice that is analogous to human typhoid. This mouse model of *S.typhimurium* infection has been used for many years to understand *S.typhi* pathogenesis (Jones and Falkow, 1996; Hensel *et al.*, 1995; Gulig and Curtiss, 1988; O'Brien, 1982). However, even though this model has considerably increased our understanding of how pathogenic *Salmonella* species invade intestinal epithelial cells or cause cellular cytotoxicity, it has not provided any clues about the reasons that might be responsible for the host specificity exhibited by *S.typhi*. Clearly, one or more molecules not conserved between *S.typhi* and *S.typhimurium* must be playing a vital role in producing different manifestations by these two closely related *Salmonella* serovars. These molecules may also determine the quality and magnitude of inflammatory and immune responses produced during infection with these two pathogens.

Following oral ingestion, *Salmonella* colonizes and invades intestinal mucosa. The invasion of enterocytes and M cells results in the secretion of a large number of chemoattractants which bring about recruitment of polymorphonuclear leukocytes (PMN) to the infected mucosa (Sansonetti, 2002; Eckmann *et al.*, 2001; Waylis and Galyov, 2000; Yang *et al.*, 1997; Jung *et al.*, 1995; McCormick *et al.*, 1993). *S.typhi* is believed to generate lesser inflammatory responses in the gut as compared to *S.typhimurium*, which may allow it to invade deeper tissues of the gut (Young *et al.*, 2002). Once past intestinal epithelium, *S.typhi* disseminates systemically into secondary lymphoid organs. Cells of the monocytic lineage cargo the pathogen from the gut to the spleen, liver and bone marrow (Wick, 2004). The ability of this bacterium to survive and replicate within mononuclear phagocytes constitutes a key pathogenic factor in the development of systemic infection.

Macrophages and dendritic cells have the ability to recognize pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) or Toll-like receptors (TLRs). TLR activation in phagocytes produces proinflammatory cytokines and chemokines that contribute directly to elimination of infectious agents and activation of adaptive immune responses (Palm and Medzhitov, 2009; Takeda ana Akira, 2005). Of various TLR ligands expressed by *Salmonella*, LPS and flagellin mount an efficient inflammatory reaction through their interaction with TLR4 and TLR5 respectively (Freudenberg *et al.*, 2001). These responses play a vital role in early innate immunity against *Salmonella*. However, like other pathogens, *Salmonella* has also evolved strategies to modulate these responses in order to establish infection (Rosenberger and Finlay, 2003). *S.typhimurium* expresses the avirulence (avr) gene that can suppress NF-kB - mediated immune responses thereby promoting establishment of infection (Collier-Hyams *et al.*, 2002). *S.typhi* does not contain the avr genes, however it possesses a region within *Salmonella* pathogenicity island 7 (SPI7), termed the *viaB* locus that is absent in serotype Typhimurium (Parkhill *et al.*, 2001). This locus contains genes required for the biosynthesis and the export of the Vi capsular polysaccharide. The latter is a linear polymer of 1,4 (2-deoxy)-2-*N*-acetylgalacturonic acid variably *O*-acetylated at the C3 position (Felix and Pitt, 1934a). The expression of Vi is associated with resistance of *S.typhi* to the action of anti-O antibody and complement-mediated killing (Looney and Steigbigel, 1986; Robbins and Robbins, 1984; Felix and Pitt, 1934b). Vi also enhances survival of *S.typhi* in cultured macrophages (Hirose *et al.*, 1997). Typhoid rates are significantly higher in volunteers infected with capsulated serotype Typhi strains than in those infected with

passaged derivatives lacking the Vi antigen. Although non-capsulated *S.typhi* can still cause typhoid fever, *in vivo* data suggest that the loss of the Vi antigen results in considerable attenuation (Hornick *et al.*, 1970a, 1970b). Recently, Vi has been shown to suppress inflammatory responses during infection of intestinal epithelial cells with *S.typhi* by targeting prohibitin family of molecules. Engagement of prohibitin complex with Vi resulted in reduced MAP-kinase activation from IEC infected with capsule negative *S.typhi* (Sharma and Qadri, 2004).

Prohibitin belongs to a family of highly conserved molecules that play a crucial role in various cellular functions. The mitochondrial and the nuclear prohibitin and its closely related homolog, the B cell receptor associated protein – 37 (BAP-37, also called prohibitin-2), are involved in the stabilization of mitochondrial respiratory chain complexes and regulation of cell cycle respectively, whereas membrane associated prohibitin has been shown to be a critical component of Ras-mediated activation of the Raf/MEK/ERK signaling pathway (Mishra *et al.*, 2005; Rajalingam and Rudel, 2005). Thus, targeting of membrane prohibitin by Vi may constitute an important strategy by *S.typhi* to subvert host defenses. Considering that prohibitin is expressed in all cell types and Vi has been reported in circulation during typhoid (Barret *et al.*, 1982; Rockhill *et al.*, 1980), this strategy may not be restricted to IEC but may also operate during infection of other cell types including macrophages and dendritic cells. These cell types constitute an important component of innate immunity against pathogenic *Salmonella*. The present study was therefore undertaken to carry out a detailed investigation on the interaction of Vi with mononuclear phagocytes and analyze consequences of this interaction on the induction of inflammatory and innate immune responses from these cells.

Antibodies against Vi render protection against typhoid fever, and Vi is currently one of the vaccines available for use in humans (Lin *et al.*, 2001; Klugman *et al.*, 1996; Plotkin *et al.*, 1995; Acharya *et al.*, 1987; Klugman *et al.*, 1987; Robbins and Robbins, 1984). Immunization with this polysaccharide produces both IgM and IgG antibodies, with the latter playing a crucial role in determining the vaccine efficacy. However, Vi being a T-cell – independent antigen, the mechanism by which IgG kind of antibodies are produced is not clear. In the second part of this study, the role of Vi-immune cell interaction in modulating anti-Vi antibody response was investigated.

Review of Literature

REVIEW OF LITERATURE

Salmonella

The genus *Salmonella* contains a group of closely related organisms that are pathogenic for humans and other vertebrates. Principal clinical syndromes caused by *Salmonella* serotypes are typhoid fever and gastroenteritis. *Salmonella enterica* serotype Typhi is an enteroinvasive gram-negative bacterium that causes typhoid fever in humans. It is a strictly human adapted pathogen responsible for some 200,000 deaths and 22 million cases of typhoid fever annually (World Health Organization, 2009; http://www.who.int/ vaccine_research/diseases/diarrhoeal/en/print.html). In contrast, *Salmonella enterica* serotypes Typhimurium and Enteritidis produce self-limiting gastroenteritis that usually remains localized to the gut. It has a short incubation period (12 to 72 h) which is followed by a succinct episode of disease (4 to 7 days). Typhoid fever on the other hand is characterized by a considerably prolonged incubation period (median of 5 to 9 days) and longer duration of symptoms (fever persists for approximately 3 weeks). Serotype Typhi and non-typhoidal *Salmonella* serotypes further differ in their abilities to survive and persist in human tissues suggesting that these two closely related pathogens may cause disease by different mechanisms. In mice, *S.typhimurium* infection results in a systemic disease with pathogenesis similar to typhoid fever in humans. Murine infection with *S.typhimurium* has therefore been used as a model to understand systemic infection with pathogenic *Salmonella*.

Typhoid fever: pathogenesis and disease

Typhoid fever is a systemic infection caused by intake of food or water contaminated with *Salmonella* serotypes Typhi or Paratyphi. After ingestion, bacteria invade gut epithelium *via* M cells followed by colonization of Peyer's patches and lamina propria of intestine. Bacteria multiply here and are released *via* lymph to the thoracic duct from where they enter blood circulation (transient primary bacteremia) and are disseminated to liver, spleen and bone marrow. Clinical onset of disease is marked by re-entry of bacteria in blood (secondary bacteremia). *S.typhi* thrives and multiplies in the bile and re-infects the intestinal mucosa and Peyer's patches (**Figure 1**). The onset of disease takes 5 to 20 days. The pathophysiological hallmark of typhoid fever is mononuclear cell infiltration and

hypertrophy of secondary lymphoid organs. The disease is characterized by sudden onset of fever, severe headache, nausea, anorexia, abdominal discomfort, constipation or in some cases diarrhea (Everest *et al.*, 2001; Jones and Falkow, 1996). The symptoms vary from one individual to another depending upon health of the individual and number of organisms ingested. Fever rises in step wise fashion and is sustained for upto two weeks. Progression of untreated typhoid fever occurs in four stages. First week is characterized by slowly rising temperature with relative bradycardia, malaise, headache and cough.
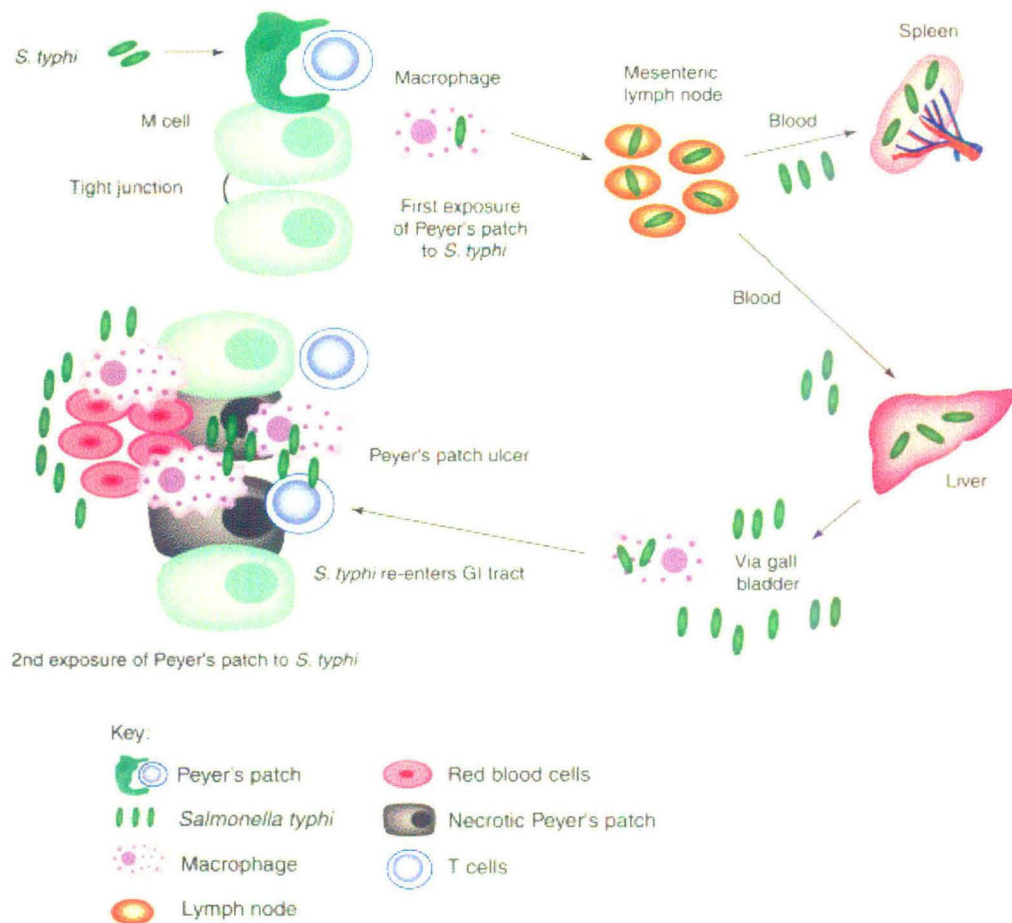
Figure. 1 *Salmonella typhi* infection. After oral ingestion, *Salmonella typhi* infects *via* the Peyer's patches (PP) of the small intestine. Bacteria migrate to mesenteric lymph nodes and arrive *via* the blood in the liver and spleen (and bone marrow, not shown). After multiplication in these sites a second bacteremic episode occurs and bacteria re-enter the intestinal tract *via* the gall bladder, exposing the PP to bacteria a second time. PP tissue damage occurs resulting in ulceration, bleeding, necrosis and, in extreme cases, full-thickness perforation.
Adapted from Everest *et al.*, 2001.

Blood cultures are positive for *S.typhi* in first week whereas classical Widal test is negative (Bhan *et al.*, 2005). Second week is characterized by high fever plateauing around 40°C, along with bradycardia, delirium, abdominal pain and appearance of rose spots in lower chest and abdomen in some patients. The spleen and liver are enlarged and there is elevation of transaminases. The Widal test is positive with high titres of anti-O (anti-LPS) and anti-H (anti-flagellar) antibodies. Intestinal perforations, septicemia and hemorrhage can occur during third week of infection along with high fever (Beutler *et al.*, 1991; Bitar and Tarpley, 1985; Butler *et al.*, 1985; Roy *et al.*, 1985). By fourth week of infection, the symptoms of untreated typhoid fever begin to resolve, although relapse can occur in 10% of patients (House *et al.*, 2001). In these patients, reinfection of the intestinal tract occurs *via* gall bladder. Case-fatality rates can be reduced from 10-20 % to 1% with appropriate antibiotic therapy.

Salmonella typhi

S.typhi is a gram negative, non-spore forming, motile and facultative anaerobic bacterium of about 2-4 μm by 0.5 μm in size. It is an aerobe and facultative anaerobe and grows well in ordinary culture media. MacConkey's agar and Deoxycholate citrate agar (DCA) are used as selective media for *Salmonella*. The important antigens of *S.typhi* which have been suggested to play a role in the pathogenesis or in the development of immune response are:

Lipopolysaccharide

LPS is a major component of the outer membrane of gram-negative bacteria, contributing greatly to the structural integrity of the outer membrane of bacteria. LPS is an endotoxin, and induces a strong response from normal animal immune systems. It comprises of three parts: lipid A, core oligosaccharide and O-antigen. Lipid A represents the conserved molecular pattern of LPS and is the main inducer of immunological responses to LPS. The presence of characterstic O antigens on the bacterial surface forms the basis of classification of *Salmonella* into various serotypes. According to this classification *S.typhi* possesses O9 and O12 antigenic determinants on its surface (van der Woude and Baumler, 2004). O side chains are easily recognized by the antibodies of the host (Qadri *et al.*, 1988).

Flagellar antigen

The flagellar antigen (flagellin) is a monomeric protein of 50-55kDa that multimerizes to form the flagellum. Bacterial flagellum is a highly complex structure that extends from the outer membrane and is important for bacterial motility. Flagellum also aids in bacterial attachment to host cells as well as in bacterial invasion of these cells (Bardy *et al.*, 2003; Josenhans and Suerbaum, 2002). Flagellin of H1-d type is encoded by *fliC* gene in *S.typhi* (Frankel *et al.*, 1989). Flagellin is also a potent proinflammatory factor.

Capsular antigen

The *S.typhi* chromosome contains a 134kb DNA region termed *Salmonella* pathogenicity island 7 (SPI7), that is absent in the serotype Typhimurium (Parkhill *et al.*, 2001). The *viaB* locus located on SPI7 contains genes involved in the regulation, the biosynthesis and the export of the Vi capsular antigen (Virlogeux *et al.*, 1995). Vi is a highly acidic polymer of O- and *N*-acetylated galactaminuronic acid units connected through α (1-4)-linkages (Felix and Pitt, 1934a). The molecular weight of Vi ranges from 5×10^6 to 20×10^6 Daltons. Vi has been shown to posseses two antigenic determinants, one constituted by O-acetyl moiety of galacturonic acid and other by N-acetyl and carboxyl groups together (Qadri *et al.*, 1990; Sweczyk and Taylor, 1980). The expression of Vi confers virulence to *S.typhi* by shielding the pathogen against the action of anti-O antibody and action of serum complement (Looney and Steigbigel, 1986; Robbins and Robbins, 1984; Felix and Pitt, 1934b).
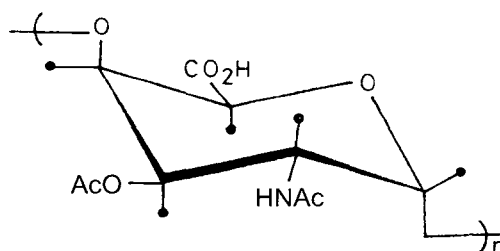
Figure 2. Monomeric unit of Vi polysaccharide is N-acetylgalacturonic acid linked through α 1,4 linkage and variably O-acetylated at C-3 position.
Adapted from Stone and Szu, 1988.

Vi has been extensively studied for its potential as a vaccine against infection with *S.typhi* as antibodies against this antigen are protective and currently it is one of the vaccines available for use in humans (Acharya *et al.*, 1987; Klugman *et al.*, 1987; Robbins and Robbins, 1984). It has been shown that Vi can interact with intestinal epithelial cells (IEC) through a specific cell-surface associated recognition complex containing tumor suppressor protein, prohibitin, and prohibitin-related molecule B cell receptor- associated

protein 37 (BAP-37), as major constituents (Sharma and Qadri, 2004). Engagement of IEC with Vi inhibited their ability to produce an inflammatory response upon infection with Vi⁻ *S.typhi*. Consistent with this effect, infection of Caco-2 cells with capsulated *S.typhi* produced less IL-8 compared with non-capsulated bacteria. It has also been shown that the presence of *viaB* locus is associated with reduced pathogen-induced cytokine secretion, neutrophil recruitment and fluid accumulation elicited by *Salmonella* either by modulating flagellin secretion or by preventing LPS recognition through TLR4 (Wilson *et al.*, 2008; Winter *et al.*, 2008; Raffatellu *et al.*, 2007).

Prohibitin

Prohibitin (Phb1; 30–32 KDa) and B-cell-receptor-associated protein 37 (BAP-37) or prohibitin 2 (Phb2) are ubiquitously expressed proteins highly conserved across species (Mishra *et al.*, 2006; Mishra *et al.*, 2005; Rajalingam and Rudel, 2005). Phb1 and Phb2 form a high molecular weight complex in the mitochondria and the plasma membrane (**Figure 3**) (Rajalingam *et al.*, 2005; Sharma and Qadri, 2004; Nijtmans *et al.*, 2000; Terashima *et al.*, 1994). Phb was originally thought to have a central role in the inhibition of cell-cycle progression by binding to E2F family of transcription factors (Wang *et al.*, 1999; McClung *et al.*, 1995). The best-characterized function of this complex is as a chaperone involved in the stabilization of mitochondrial inner membrane proteins (Nijtmans *et al.*, 2000; Coates *et al.*, 1997). Phb has also been identified as a component of the cell-surface-associated molecular complex in Caco-2 human intestinal epithelium, which binds to Vi and attenuates *S.typhi*-induced IL-8 secretion (Sharma and Qadri, 2004). The targeting of pro-apoptotic peptides to Phb in the adipose vasculature caused ablation of white fat suggesting that membrane-associated Phb might be a useful target for the delivery of therapeutic compounds to enhance fat resorption (Kolonin *et al.*, 2004). Phb also plays a role in the activation of c-Raf by Ras and in modulating epithelial cell adhesion and migration. Phb is important for the displacement of 14-3-3 from c-Raf that facilitates plasma membrane localization and phosphorylation of this kinase at serine 338. Phb-silenced epithelial cells (HeLa) failed to migrate in response to epidermal growth factor (EGF) on collagen suggesting that Phb might be obligatory for cell migration (Rajalingam *et al.*, 2005). Phb localization in the mitochondria, nucleus and plasma membrane, in addition to its potential to interact with tumor-suppressor molecules, transcription factors

and associated cofactors; suggest that it has important biological roles that have yet to be completely elucidated.
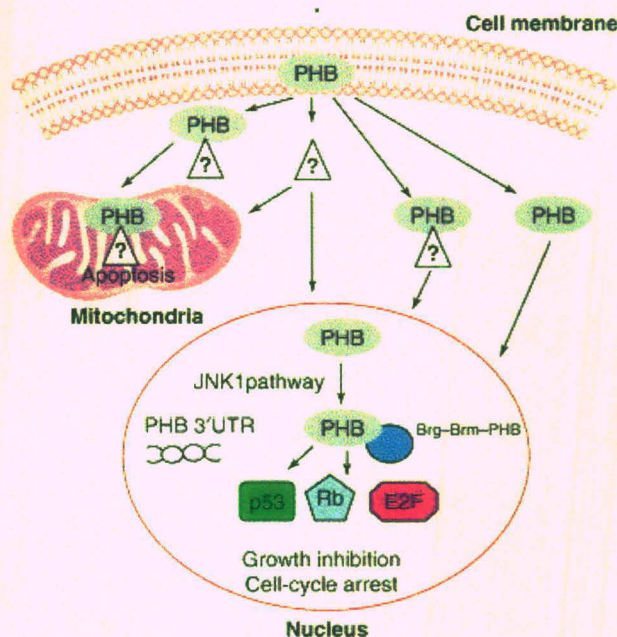
Figure 3. The subcellular localization of prohibitin (PHB) and potential mechanisms involved in PHB action. PHB is present in the cell membrane, mitochondria and nucleus. In the nuclear compartment, PHB might function as a modulator of transcription and cell cycle regulation. Mitochondrial prohibitin is required for maintenance of inner membrane structural integrity. Membrane-bound PHB might be involved in MAP kinase and other signaling pathways.
Adapted from Mishra *et al.*, 2005.

Prophylaxis

Effective control of typhoid fever can be achieved by improved hygiene, clean drinking water, adequate sanitation, and efficacious vaccines. Vaccines that have been developed against typhoid include inactivated whole cell vaccine, a subunit vaccine comprising of Vi and a live attenuated oral vaccine.

Inactivated whole cell vaccine

The heat-killed, phenol-preserved, injectable whole-cell *S.typhi* vaccine was utilized as far back as 1896 in England and Germany (Shandera *et al.*, 1985). This vaccine resulted in 73% efficacy over three years (Engels *et al.*, 1998). Though this vaccine was able to confer protection against *S.typhi*, it was considered unsuitable because of its tendency to cause fever, headache and severe local pain (Engels *et al.*, 1998; Ashrocft *et*

al., 1964). This vaccine also induced poor cell mediated immunity (Bhaskaran *et al.*, 1990; D'Amelio *et al.*, 1988) therefore it has been discontinued.

Vi polysaccharide vaccine

The subunit polysaccharide vaccine of *S.typhi* was developed in the 1980s in the laboratory of John Robbins at the NIH (Robbins and Robbins, 1984). The vaccine is based on Vi antigen that is purified from *S.typhi* by treatment with Cetavlon, so as to retain the immunogenicity of the polysaccharide (Wong *et al.*, 1974; Wong *et al.*, 1972). The vaccine (commercially available as Typhim™, Typbar™) is administered as a single dose of 25µg by the intramuscular or subcutaneous route, revaccination is recommended every 2 years. Its efficacy was demonstrated in multiple randomized trials and it ranged from 60-75% (Klugman *et al.*, 1996; Plotkin *et al.*, 1995; Acharya *et al.*, 1987; Klugman *et al.*, 1987). Four fold rise in anti-Vi titers two weeks post immunization is considered protective (Hessel *et al.*, 1999). Vi capsular polysaccharide is safe except for few side effects that include pain, redness, fever and induration at the site of injection.

A drawback of Vi vaccine is that it does not stimulate mucosal immunity and because of its T-independent nature does not generate immunological memory (Keitel *et al.*, 1994). As is the case with other polysaccharide (PS) vaccines, Vi is poorly immunogenic in infants and cannot be used to vaccinate children less than 2 years of age. This has prompted development of Vi – protein conjugate vaccines. Vi linked to recombinant *Pseudomonas aeruginosa* exotoxin A as the protein carrier (Vi-rEPA) has shown 91% efficacy over 27 months and 89% over 46 months of follow-up (Lin *et al.*, 2001; Kossaczka *et al.*, 1999). This vaccine also stimulated a booster response in 2-4 year old children. Another conjugate vaccine comprising of Vi - diphtheria toxoid (Vi-DT) has been developed at the International Vaccine Institute in Seoul and a third Vi conjugate vaccine with *S.typhi* – derived OmpC as the carrier is under development at the All India Institute of Medical Sciences, New Delhi (World Health Organization, 2009; http://www.who.int/vaccine_research/diseases/diarrhoeal/en/print.html).

Live attenuated Ty21a oral vaccine

The attenuated *S.typhi* strain Ty21a was generated by chemical mutagenesis of wild-type strain Ty2 and developed as the first live oral typhoid fever vaccine. This strain lacks a functional galactose-epimerase (*galE*) gene and the Vi antigen (Germanier and

Fuer, 1975). The *galE* phenotype contributes to strain attenuation *in vivo*. The vaccine (Vivotif ™) is currently manufactured by Crucell as enteric-coated capsules to be swallowed every other day for one week. Protection with this vaccine is mediated by mucosal and serum antibodies (IgA and IgG) as well as by cell mediated immune responses (Salerno-Goncalves *et al.*, 2002). The efficacy of Ty21a was 67-80% for upto 7 years as assessed in large scale clinical trials (Levine *et al.*, 1990; Levine *et al.*, 1987). An important feature of Ty21a is generation of herd immunity (Levine *et al.*, 1989). This vaccine has been shown to be extremely safe with remarkably few side effects. Reversion to virulence has not been observed *in vivo* or *in vitro*.

Other live attenuated *S.typhi* vaccines

Several live attenuated *S.typhi* strains are being developed to be used as oral vaccines. In preliminary clinical trials, these strains seem to be even more immunogenic than Ty21a (Garmony *et al.*, 2002). Perhaps the most advanced of these live attenuated candidate vaccines is Ty800, a phoP/phoQ deletion mutant of Ty2, which is developed by AVANT Immunotherapeutics (Hohmann *et al.*, 1996). The vaccine has been shown to stimulate vigorous IgA and serum O antibody responses in volunteers. Another attenuated strain is CVD 909, an aroC/aroD/htrA deletion mutant which was engineered to constitutively express the *S.typhi* Vi antigen. This strain, which induces anti-Vi antibodies in orally vaccinated subjects, is being developed as a live attenuated typhoid fever vaccine by Acambis and Crucell (Witherell, 2003). In addition, an attenuated *S.typhi* Ty2 strain with deletions in ssaV and aroC genes has been developed for oral vaccination against typhoid fever (Khan *et al.*, 2007).

Salmonella-host cell interactions

Salmonella typhi is a strictly human pathogen and fails to produce systemic infection in rodents; therefore most of our understanding of *Salmonella* pathogenesis is based on *S.typhimurium* infection in mice. In addition, *in vitro* infection studies with human and murine cells have also been helpful in understanding molecular interactions that take place during infection with pathogenic *Salmonella*.

Interaction of *Salmonella* with intestinal epithelial cells

The entry of bacteria into intestinal epithelial cells (IEC) is the first important step in the initiation of *Salmonella* infection (**Figure 4**). *Salmonella* adheres to and invades M cells of intestinal epithelium (Jones *et al.*, 1994). Bacterial fimbriae contribute to bacterial adherence to the apical epithelial surface (Baumler *et al.*, 1996). Shortly after adherence,
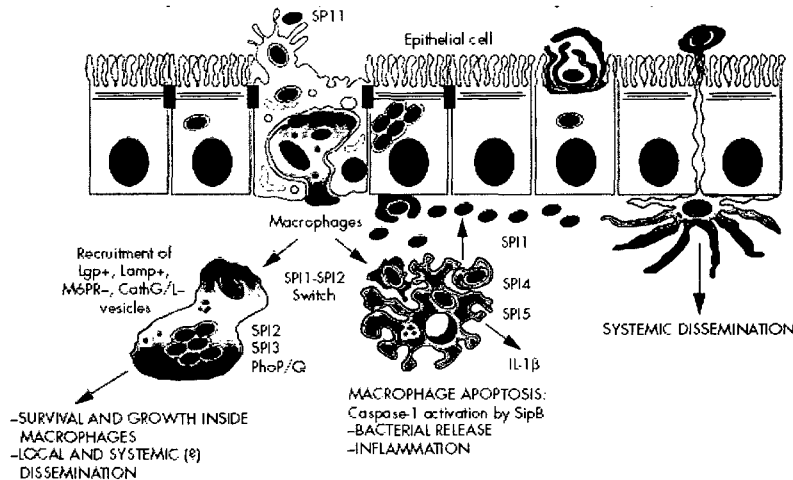
Figure 4. Physiopathological scheme of *Salmonella* infection Adapted from Sansonetti, 2002.

salmonellae invade epithelial cells by pathogen-induced endocytosis that brings about profound cytoskeleton rearrangements in host cells followed by disruption of normal epithelial brush border and induction of membrane ruffles. The latter enclose adherent bacteria in large vesicles resulting in macropinocytosis and triggering of a cascade of intracellular signaling events inside the epithelial cell (Galan *et al.*, 1996a, 1996b; Bliska *et al.*, 1993; Francis *et al.*, 1992). Following entry into M cells, bacteria are transcytosed to immune cells underlying the epithelium. The virulence factors that mediate host cell invasion and induce inflammatory cytokine secretion are encoded by *Salmonella* pathogenecity island 1 (SPI-1) (Galan, 2009). Two effector proteins translocated by SPI-1 type III secretion system (TTSS), SopE and SptP, target members of Rho family of G proteins that that are important for regulating cytoskeleton rearrangement. SopE activates cdc42 and Rac-1 *in vitro* by acting as GDP/GTP exchange factor (Hardt *et al.*, 1998). However SptP inactivates cdc42 and Rac-1 by acting as GTPase –activating protein (Fu and Galan, 1999) thereby bringing about recovery of epithelial brush border morphology shortly after invasion and thus ensuring residence and replication of *Salmonella* inside IEC. The other SPI-1 TTSS effectors SipC and SipA act in concert with SopE and SptP to initiate nucleation, bundling and stabilization of actin filaments at the site of initial membrane contact between the pathogen and the host cell (Hayward *et al.*, 1999; Zhou *et*

al., 1999). SopB, a phosphoinositide phosphatase, mediates bacterial internalization (Zhou *et al.*, 2001), stimulates production of nitric oxide (Drecktrah *et al.*, 2005), activates Akt (Steele-Mortimer *et al.*, 2000), and modulates vesicular trafficking of the bacteria-containing vacuole (Mallo *et al.*, 2008; Hernandez *et al.*, 2004). This protein is also involved in intracellular bacterial replication at the phagosomes (Patel *et al.*, 2009). Flagellin, the structural component of bacterial flagella is secreted by *Salmonella* following contact with epithelial cells (Subramanian and Qadri, 2006). It is an important mediator of intestinal inflammatory response (Gerwitz *et al.*, 2001b). In the absence of flagellin, *Salmonella* are unable to generate inflammatory response from IEC *in vitro* suggesting that this TLR ligand might be the major proinflammatory determinant of this pathogen in the gut (Zeng *et al.*, 2003).

IEC respond to enteric pathogens by producing inflammatory and antimicrobial responses. One important component of the antimicrobial response is secretion of cytokines and chemokines that are important for innate immunity against *Salmonella*. These include IL-8, GROα, GROβ (MPI-2α), GROγ, IP-10, MCP-1, MIP-1β, MIP-3α and RANTES (Eckmann *et al.*, 2001; Yang *et al.*, 1997; Jung *et al.*, 1995; McCormick *et al.*, 1993). *Salmonella* - induced IL-8 is secreted basolaterally by epithelial cells upon activation by bacterial flagellin. IL-8 along with pathogen elicited epithelial chemoattractant PEEC attracts neutrophils at the site of infection (Hobert *et al.*, 2002; Criss *et al.*, 2001; McCormick *et al.*, 1995; McCormick *et al.*, 1993). Flagellin also induces production of nitric oxide synthase (iNOS), nitric oxide, matrilysin (MMP-7), human β-defensin 2 and CXCL2 (MIP-2α) (Zeng *et al.*, 2003; Eaves-Pyles *et al.*, 2001; Sierro *et al.*, 2001). These factors participate in anti-microbial activity, recruitment of professional killer cells and antigen presenting phagocytes. The cytokine storm ensuing from the amplification of inflammatory responses results in the activation of antimicrobial responses like secretion of β-defensin 2 and lipocalin-2 in the intestinal mucosa (Godinez *et al.*, 2009; Raffatellu *et al.*, 2009). IP-10, Mig and I-TAC attract specialized subset of T-cells and MIP-3α attracts T-cells and immature dendritic cells to the site of infection (Dwinell *et al.*, 2001; Izadpanah *et al.*, 2001).

Salmonella - macrophage interactions

Subsequent to invasion of IEC, *Salmonella* traverses the epithelium to reach the lamina propria, where bacteria infect phagocytes (neutrophils and mononuclear cells) and

possesses an intracellular location within phagocytes (**Figure 4**). Macrophages form the preferred niche for survival of *Salmonella*. Once inside phagosomes *Salmonella* senses phagosomal environment by PhoP/PhoQ two-component regulatory system that allows it to resist or evade nutrient poor and microbicidal environment of the phagosome (Miller *et al.*, 1989). PhoP/PhoQ system regulates gene expression in response to environmental cues by sensing through PhoQ, and signal relay and transcription control by PhoP. PhoP-activated genes are required for survival within macrophages, whereas genes like SPI-1 TTSS that are not required during this phase are repressed (Pegues *et al.*, 1995). PhoP regulated lipid A modification promotes resistance to antimicrobial peptides (Guo *et al.*, 1998). In addition, *Salmonella* virulence factors that mediate survival within macrophages are encoded by *Salmonella* pathogencity island 2 (SPI-2) which is activated inside macrophages (Ochman *et al.*, 1996). The SPI-2 effector proteins interfere with maturation of phagosomes and prevent phago-lysosomal fusion hence preventing action of microbicidal effectors within macrophages (Mukherjee *et al.*, 2001; Uchiya *et al.*, 1999). Resistance of salmonellae to reactive oxygen species is mediated by superoxide dismutase and to reactive nitrogen species by homocysteine (Fang *et al.*, 1999; De Groote *et al.*, 1996). In addition, *Salmonella* adapts to nutrient-deprived conditions within phagosomes by inducing multiple biosynthetic genes necessary for *de novo* synthesis of essential metabolites (McFarland and Stocker, 1987; Hoiseth and Stocker, 1981).

The infection of macrophages with pathogenic *Salmonella* triggers a cascade of intracellular signaling events and expression of F4/80, Gr-1, MHC-II and co-stimulatory molecules on macrophage cell surface (Kirby *et al.*, 2002). It also leads to upregulation of expression of genes encoding pro-inflammatory mediators (e.g. iNOS, chemokines, IL-1β), cell surface receptors and adhesion molecules (e.g. TNF-α R, CD40 and ICAM-1) and anti-inflammatory mediators (e.g. TGF β1 and 2) (Rosenberger *et al.*, 2000). The induction of proinflammatory cytokines like TNF-α, IL-1, IL-6, IL-8 and IL-12 is brought about either by activation of Toll-like receptors or by TTSS-delivered specific bacterial effectors. *Salmonella* is known to be cytotoxic to macrophages (Richter-Dahlfors *et al.*, 1997) and upregulates genes involved in cell death or apoptosis including caspase-1 (Monack et al., 2001, 2000). The disease outcome following infection with *Salmonella* is linked to the ability of the pathogen to both persist within and elicit damage in host's macrophage cells. Macrophage cell death is mediated by SPI-1 effector protein SipB which activates caspase-1 (Hersh *et al.*, 1999). Caspase-1 activation results in secretion of pro-inflammatory IL-1β

and might be important for systemic spread of *Salmonella* (Rosenberger and Finlay, 2003; Boise and Collins, 2001; Brennan and Cookson, 2000; Watson *et al.*, 2000). Recently, flagellin sensing *via* Ipaf has been shown to induce caspase-1 - dependent cell death of infected macrophages and secretion of IL-1β (Franchi *et al.*, 2006; Miao *et al.*, 2006).

Salmonella - dendritic cell interactions

Dendritic cells (DC) are a key link between innate and adaptive immunity. Mucosal DCs play a critical role in regulating the complex interaction between gut microflora, pathogens and immune system leading to either tolerance or immunity. *Salmonella* has been shown to infect DCs both *in vivo* and *in vitro*, bringing about activation and secretion of cytokines from these cells (Svensson *et al.*, 2000; Marriot *et al.*, 1999; Hopkins and Kraehenbuhl, 1997). DCs can internalize and present *Salmonella* - derived antigens along with MHC-I and MHC-II to CD4+ or CD8+ T-cells respectively (Yrlid and Wick, 2002; Yrlid *et al.*, 2001; Svensson *et al.*, 2000, Svensson and Wick, 1999; Svensson *et al.*, 1997). In addition, these cells can also serve as bystander antigen presenting cells by engulfing apoptosed macrophages harboring *Salmonella* and presenting pathogen-derived antigens to T cells. This ability is unique to DCs and absent in macrophages (Yrlid and Wick, 2000). DCs can produce IL-12p40 and TNF-α upon *Salmonella* infection (Yrlid and Wick, 2002; Kirby *et al.*, 2001; Svensson *et al.*, 2000). The survival of *S.typhimurium* in DCs does not depend on virulence factors required for intracellular survival in macrophages (Niedergang *et al.*, 2000). However, *Salmonella* can induce apoptosis of dendritic cells by caspase-1 - dependent mechanism (van der Velden *et al.*, 2003).

Adaptive immune responses during infection with *Salmonella*

Although early innate mechanisms of the immune system are highly effective in restricting initial growth of *S.typhimurium* for several days, these mechanisms fail to eliminate bacteria from host tissues. The effective clearance of *Salmonella* from tissues is brought about by adaptive immune responses produced by T and B lymphocytes. These responses are highly specific and generate immunological memory which provides immunity against subsequent infection with *Salmonella* (Mastroeni and Menager, 2003). Studies with several immunodeficient mouse strains like nude mice, αβ T cell deficient mice or MHC-II deficient mice have demonstrated importance of T cells in clearance of *Salmonella* infection. These mice strains succumb to infection with attenuated *Salmonella*

strains that would otherwise be eradicated in wild type mice (Sinha *et al.*, 1997; Weintraub *et al.*, 1997; Hess *et al.*, 1996). Both CD4+ and CD8+ T cells have been suggested to be important for protection, however relative contribution of the two cell types is debatable. CD4+ T cells have been suggested to be more important in the development of protective immunity to *Salmonella* in adoptive transfer experiments (Pie *et al.*, 1997; Nauciel, 1990). Experiments with MHC class-I-deficient mice suggests that CD8+ T cells are important for protection as these mice can resolve initial infection with vaccine strains of *Salmonella* but, unlike wild-type mice, there is no protection against re-challenge with virulent *Salmonella* (Lo *et al.*, 1999). CD8+ T lymphocytes are fundamental in controlling *Salmonella* infection by promoting the lysis of infected macrophages and dendritic cells. Consequently, this event would then release bacteria from the replicative habitat, rendering them accessible to activated macrophages and to the humoral immune response. In animals and humans exposed to live *Salmonella*, cellular responses are of the Th1 type, as indicated by delayed-type hypersensitivity (DTH) and by the predominant production of IL-2 and IFN-γ upon *in vitro* restimulation of immune T cells (Harrison *et al.*, 1997; Sztein *et al.*, 1994). The importance of IFN-γ in control of *Salmonella* infection is evident by the fact that IFN-γ $^{-/-}$ mice are more susceptible to infection with attenuated *Salmonella* (Lalmanch and Lanteir, 1999). In addition, a recent study has shown that IFN- γ neutralization causes reactivation of persistent systemic *S.typhimurium* infection (Monack *et al.*, 2004). The concerted action of several cytokines including TNF-α, IFN-γ, IL-12, IL-15 and IL-18, is essential for the adaptive phase of the immune response (Vazquez-Torres *et al.*, 2001, Mastroeni et al., 1998). Given the importance of T cells for bacterial clearance, it seems likely that *Salmonella* have evolved mechanisms to inhibit cellular immunity. Indeed, numerous studies have demonstrated that *Salmonella* can suppress T cell activation to evade adaptive immunity (Bueno *et al.*, 2007). It has been shown that *Salmonella* has a direct, contact-dependent inhibitory effect on T cells (van der Velden *et al.*, 2005). Recently it is reported that there is progressive culling of *Salmonella* specific CD4+ T cells in *Salmonella*-infected mice (Srinivasan *et al.*, 2009). Thus, *Salmonella* is able to limit the development of cellular immunity by targeted inhibition of CD4+ T cells after clonal expansion has occurred.

In addition to T-cell mediated immunity, B-cells have been shown to be important in mediating immunity to *Salmonella*. B cell deficient mice fail to control growth of virulent salmonellae during secondary infections (Mastroeni *et al.*, 2000a; McSorley & Jenkins, 2000; Mittrucker *et al.*, 2000). Infection with *Salmonella* results in potent antibody

response against a variety of antigens like flagellin, porins, outer membrane proteins as well as LPS (Brown and Hormaeche, 1989). Protection by antibodies could be mediated either by inhibition of epithelial cell infection, by enhancing engulfment *via* Fc-receptor - mediated phagocytosis or by activating classical complement pathway (Michetti *et al.*, 1994; Brown *et al.*, 1991). The best example of antibody - mediated protection against *Salmonella* is Vi polysaccharide vaccine that provides immunity through antibodies (Acharya *et al.*, 1987).

   Salmonella is able to infect and survive within B lymphocytes and fibroblasts, and infected B lymphocytes are able to present *Salmonella* antigens by MHC-II molecules to CD4+ T cells (Yrlid *et al.*, 2001; Sztein *et al.*, 1995). BCR mediated internalization of *Salmonella* activates IgM secretion and efficient CD4+ T cell help, which boosts *Salmonella*-specific antibodies production. BCR-mediated internalization of *Salmonella* is superior over extracellular antigen extraction in inducing rapid and specific humoral immune responses (Souwer *et al.*, 2009).

Limitations of the mouse model

   The model of systemic infection with *S.typhimurium* in mice sometimes referred to as murine typhoid has given important insights into *Salmonella* pathogenesis in general. However, studies carried out in this model have not provided any explanation for the host specificity that is exhibited by *S.typhi*. Considering that *S.typhi* and *S.typhimurium* cause distinct clinical manifestations in humans, the conclusions drawn from the mouse model about *S.typhi* pathogenesis should be interpreted cautiously. These different clinical manifestations might be produced by one or more as yet unidentified pathogen - specific interactions. It has been suggested that two pathogens might elicit different host responses at the site of infection in intestinal mucosa. *S.typhi* uses cystic fibrosis transmembrane conductance regulator (CFTR) as the receptor for entry into intestinal epithelial cells whereas *S.typhimurium* does not (Pier *et al*, 1998). Further, *S.typhi* induces IL-6 secretion from human IEC whereas *S. typhimurium* does not generate this response from human and mouse IEC (Wienstein *et al.*, 1997). In mice, *S.typhimurium* entry through M cells is associated with extensive destruction of epithelium and bacteria gain access to subepithelial lymphoid tissues where they encounter macrophages, DCs, lymphocytes and neutrophils. In comparison, *S.typhi* enters murine M cells less efficiently, does not destroy

epithelial layer and is cleared from the Peyer's patches soon after M cell entry (Pascopella *et al.*, 1995).

S.typhi and *S.typhimurium* also differ in their ability to survive and persist within macrophages and cause cytotoxic damage. *S.typhi* is less cytotoxic to human and murine macrophages than *S.typhimurium*. The ability of *S.typhi* to survive in macrophages without causing significant death may account for its systemic dissemination during typhoid fever in humans (Schwan *et al.*, 2000). Infection of human colonic tissue explants with *S.typhimurium* results in IL-8 secretion whereas such a response is not observed with *S.typhi* (Raffatellu *et al.*, 2005). Comparative genome analysis of *S.typhi* and *S.typhimurium* revealed presence of multiple pseudogenes in *S.typhi* (McClelland *et al.*, 2001; Parkhill *et al.*, 2001) that include mutations in 7 of 12 bacterial attachment factors. It is believed that this loss of multiple determinants in *S.typhi* might be associated with preferential targeting of the pathogen to particular cell types such as DCs or CD18+ cells for systemic dissemination while avoiding non-specific targeting to epithelial cells which leads to local gut inflammation (Vazquez-Torres *et al.*, 1999). The inactivation of single genes, as well as the acquisition or loss of single genes or large islands of DNA, may have contributed to host adaptation and specificity of *S.typhi* (Young *et al.*, 2002). Furthermore, the presence of Vi capsular polysaccharide in *S.typhi* might also contribute to modulation of inflammatory responses at the level of gut (Raffatellu *et al.*, 2007; Raffatellu *et al.*, 2005; Sharma and Qadri, 2004).

Pattern Recognition Receptors

Salmonella resides and replicates within the cells of the monocytic lineage including macrophages, dendritic cells and neutrophils (Hueffer and Galan, 2004; Sandquist *et al.*, 2004; Wick, 2004). These cells are considered to be one of the first lines of host defense because of their microbicidal mechanisms. These cells sense conserved pathogen associated molecular patterns (PAMPs) through germline encoded pattern recognition receptors (PRRs) (Takeda and Akira, 2005; Janeway and Medzhitov, 2002; Medzhitov, 2001). The activation of these receptors results in the induction of inflammatory and innate immune responses from these cells (Takeda and Akira, 2005). The PRRs include the Toll-like receptors (TLRs) which are either expressed at the cell membrane or in the endosomes, the Nod-like receptors (NLRs) which are present in the

cytoplasm and circulating PRRs such as pentraxins which are secretory in nature (Akira, 2009).

Toll-like receptors

Toll-like receptors (TLR) are evolutionarily conserved from insects to mammals. Toll was identified in *Drosophila* as a molecule important for the development of embryonic dorso-ventral polarity, and was later shown to play a vital role in the antifungal response of flies (Lemaitre *et al.*, 1996). Subsequently homologues of TLRs have been identified in mammals and to date 13 members of the TLR family are known in mammals (Hornef and Bogdan, 2005; Muzio *et al.*, 2000). TLRs are type-I transmembrane proteins comprising of an extracellular domain of leucine rich repeats (LRR) and a cytoplasmic domain homologous to that of the interleukin-1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neil, 2000). TLR1, TLR2, TLR4, TLR5 and TLR6 are present on the plasma membrane, whereas TLR3, TLR7, TLR8 and TLR9 are found in intracellular compartments such as endosomes (**Figure 5**) (Nishiya *et al.*, 2005; Latz *et al.*, 2004; Nishiya and DeFranco, 2004; Matsumato *et al.*, 2003; Hemmi *et al.*, 2000).

TLRs sense either extracellular or membrane-encased foreign organisms. TLR1, TLR2 and TLR6 recognize lipopeptides and peptidoglycans (Lien *et al.*, 1999). TLR4 is an essential receptor for lipopolysachharide (LPS), whereas bacterial flagellin is the ligand for TLR5. TLR3 recognizes double-stranded RNA (dsRNA), whereas TLR9 is essential for recognition of CpG DNA (Takeda *et al.*, 2003). TLR11 present in mice recognizes profilin-like protein from protozoa (Yarovinsky *et al.*, 2005). Mouse TLR7 and human TLR8 recognize synthetic antiviral imidazoquinoline components (R848, Imiquimod etc) and some guanine nucleotide analogs (loxoribine etc) as well as uridine-rich or uridine/guanosine-rich ssRNA of both viral and host origin (Diebold *et al.*, 2004; Heil *et al.*, 2004; Hemmi *et al.*, 2002). The endosomal localization of TLRs is important for efficient recognition of microbial components as well as for prevention of self-nucleotide recognition.
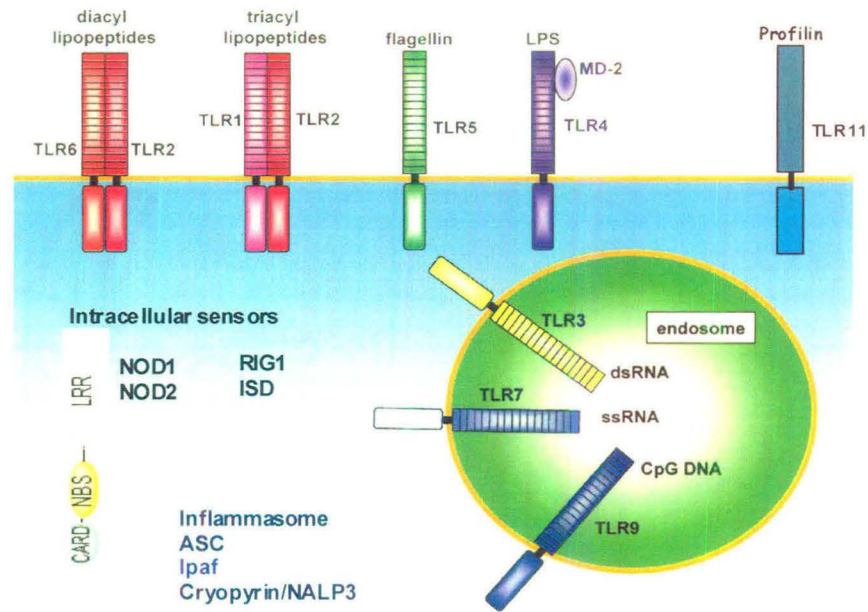
Figure 5. Toll-like receptors and cytosolic sensors. TLR2, in collaboration with TLR1 or TLR6, discriminates between the molecular structures of triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR5 and TLR11 recognize bacterial flagellin and profilin respectively. TLR3, TLR7, TLR8 and TLR9 reside in endosomal compartments and recognize nucleic acids; TLR3 recognizes viral dsRNA, whereas TLR7 and TLR8 recognize viral ssRNA. TLR9 recognizes bacterial and viral CpG DNA motifs.
Intracellular sensors Nod1, Nod2 recognizes γ–D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively. RIG1 and ISD are sensors for viral dsRNA, poly I:C and intracellular DNA. Ipaf, NALP3 and cryoprin sense flagellin, bacterial RNA, ATP and uric acid crystals.
Adapted from Takeda and Akira, 2005.

TLRs are expressed in various types of cells including dendritic cells, T cells, neutrophils, eosinophils, mast cells, mononuclear phagocytes and epithelial cells (Crellin *et al.*, 2005; Takeda and Akira, 2005; Iwaski and Medzhitov, 2004; Marshall *et al.*, 2003). Monocytes/macrophages express mRNA for most TLRs except TLR3 (Muzio *et al.*, 2000). TLR expression differs in different subsets of dendritic cells. Myleoid dendritic cells express TLR1, 2, 4, 5 and 8 whereas plasmacytoid DCs express TLR7 and 9 (Jarrosay *et al.*, 2001; Kadowaki *et al.*, 2001; Krug *et al.*, 2001). Expression of TLR1, 2, 4 and 5 decreases as DCs mature (Mellman and Steinman, 2001; Visintin *et al.*, 2001) whereas TLR3 is expressed in mature DCs (Muzio *et al.*, 2000). TLR5 is expressed on the basolateral side of intestinal epithelia (Gerwitz *et al.*, 2001a). TLR4 is expressed at relatively low levels in intestinal epithelial cells which explain unresponsiveness of IEC to LPS. The expression of TLRs is modulated in response to microbial pathogens and a variety of cytokines (Takeda *et al.*, 2003). The activation of TLRs by pathogen - derived

products causes induction of innate host defenses that include secretion of proinflammatory cytokines (like IL-8, TNF-α, IL-12p40, IFN-γ), recruitment of neutrophils, upregulation of costimulatory molecules, upregulation of phagocytosis and killing of microbes (Takeda and Akira, 2005). TLRs also control B-cell response to T-dependent and T-independent antigens (Pasare and Medzhitov, 2005).

TLR signaling

The engagement of TLRs by PAMPs brings about activation of signaling cascades leading to induction of genes involved in the activation of innate and adaptive immune responses. Following ligand recognition, TLRs dimerize and undergo a conformational change required for recruitment of downstream signaling molecules. TLR2 heterodimerizes with TLR1 or TLR6 but other TLRs are believed to homodimerize (Jin *et al.*, 2007; Akira and Takeda, 2004; Ozinsky *et al.*, 2000). Dimerization is followed by recruitment of adapter proteins to the cytoplasmic TIR domain of TLRs. These TIR domain containing adaptor proteins are myeloid differentiation primary response protein 88 (MyD88), TIR-associated protein (TIRAP)/Myd88-adaptor like molecule (MAL), TIR-domain-containing adaptor protein-inducing IFN- β (TRIF)/TIR-domain-containing molecule 1 (TICAM1) and TRIF-related adaptor molecule (TRAM) (Akira and Takeda, 2004). Activation of different TLRs produces different responses by associating with different sets of adaptor molecules. Based on association with MyD88, TLR signaling pathway is either MyD88-dependent or MyD88-independent (**Figure 6**).

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MyD88 - dependent signaling pathway

MyD88 - dependent signaling pathway is common to all TLRs except TLR3 (Takeda *et al.*, 2003). Following TLR stimulation, MyD88 associates with cytoplasmic TIR domain of TLR and recruits IRAK4 (IL-1R-associated kinase) to this complex *via* interaction of the death domain of both the molecules (Akira and Takeda, 2004; Dunne *et al.*, 2003). This association facilitates phosphorylation of IRAK1. Activated IRAK1 associates with TRAF6 and triggers two distinct signaling pathways. One pathway leads to activation of AP-1 transcription factor through activation of MAP kinase and the second pathway activates TAK1/TAB (TGF- β-activated kinase / TAK1-binding protein 1) complex, which then activates I-κB kinase (IKK) complex. Activation of IKK complex induces phosphorylation and subsequent degradation of I-κB followed by nuclear

translocation of NF-κB. NF-κB nuclear translocation leads to induction of inflammatory cytokines such as TNF-α, IL-8, IL-6 and IL-12p40. TIRAP is involved in MyD88-dependent signaling *via* TLR2 and TLR4 (Takeda and Akira, 2005).
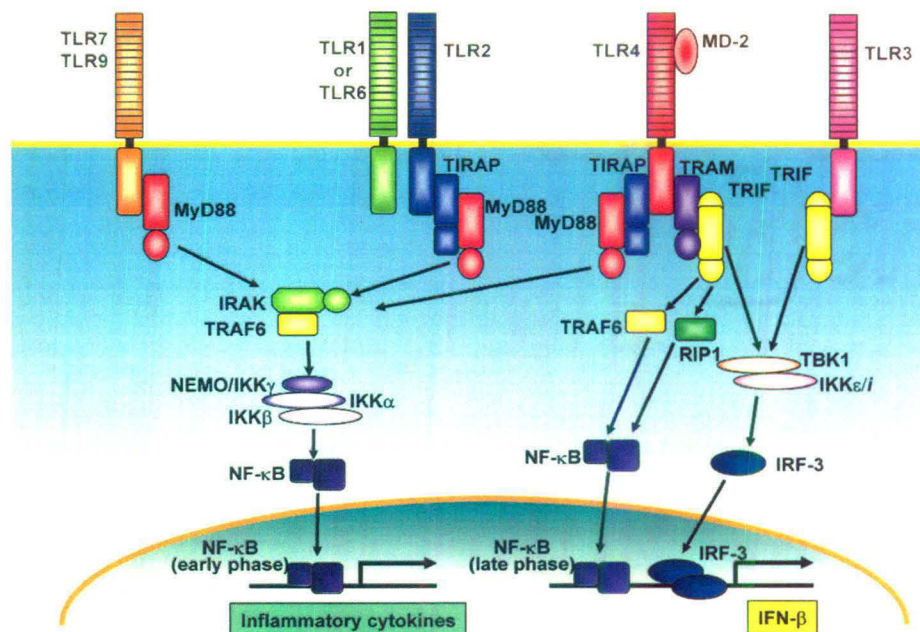
Figure 6. TLR signaling pathway. TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the I-κB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates I-κB, resulting in nuclear translocation of NF-κB which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway *via* TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKKi/IKKε and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.
Adapted from Takeda and Akira, 2005.

MyD88-independent signaling pathway

TLR3 and TLR4 utilize MyD88-independent signaling pathway to induce production of IFN-β (Doyle *et al.*, 2002; Yoneyama *et al.*, 1998). In TLR4 - mediated MyD88-independent pathway, TRAM associates with TLR4 and recruits TRIF to this complex. However, TRIF can directly bind to TLR3. The N-terminal portion of TRIF associates with IKKi/IKKε and TBK1, which mediate IRF-3 - dependent IFN- β induction (Sato *et al.*, 2003; Fitzgerald *et al.*, 2001). The C-terminal portion of TRIF associates with RIP1 and activates NF-κB in response to TLR3 ligand (Meylan *et al.*, 2004). The N-

terminal portion of TRIF can also associate with RIP1 in a TRAF6 - dependent manner and activate NF-κB in response to TLR4 ligand (Sato *et al.*, 2003).

TLR4-LPS interaction

TLR4, the first TLR identified in mammals, is an essential receptor for recognition of LPS. LPS is a complex glycolipid with the hydrophobic lipid domain (lipid A) harboring the biological activity of the molecule. TLR4 recognition of LPS is a multistep process that involves a series of interactions with several proteins which include plasma-derived LPS binding protein (LBP), CD14, MD-2 and TLR4 (**Figure 7**) (Gioannini and Weiss, 2007; Nagai *et al.*, 2002). LBP is soluble shuttle protein which binds to LPS and transfers it to CD14 (Wright *et al.*, 1989). CD14 is a glycophosphatidyl (GPI)-anchored protein, which facilitates transfer of LPS to the TLR4/MD-2 complex and modulates TLR recognition (Akashi *et al.*, 2000). MD-2 is a soluble protein that associates with TLR4 non-covalently but can directly form a complex with LPS in the absence of TLR4 (Miyake, 2007). LPS binding to MD-2 (bound to TLR4) induces a conformational change in MD-2 that in turn induces dimerization of TLR4 (Brodsky and Medzhitov, 2007; Kim *et al.*, 2007). Dimerization of TLR4 triggers recruitment of specific adaptor proteins to the intracellular domain of the receptor, thus initiating a signaling cascade.
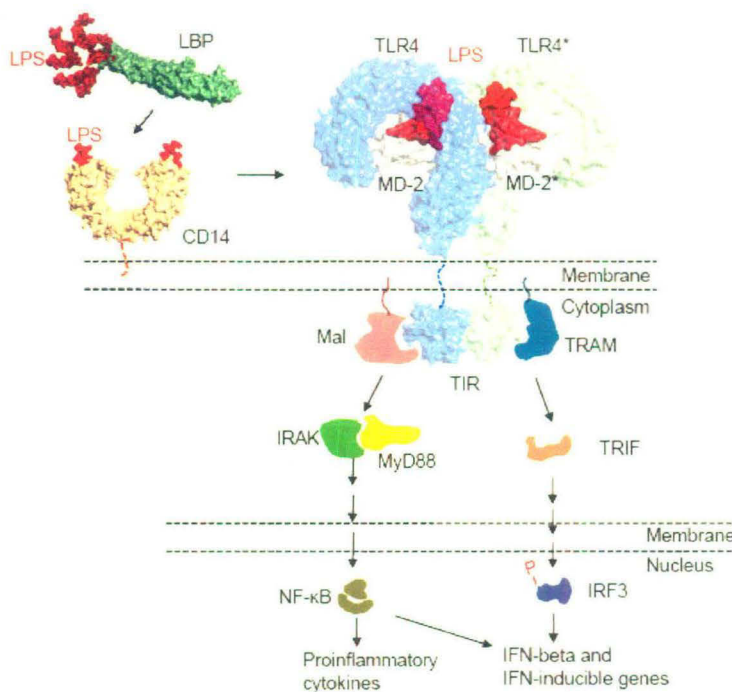
Figure 7. Ligand recognition by the TLR4. LPS-binding protein (LBP) binds plasma LPS and conveys it to the surface of cells in a bioactive form to CD14. CD14 has no intracellular signaling domain and delivers LPS to the TLR4-MD-2 complex. TLR4 and MD-2 form a heterodimer that recognizes a common 'pattern' in structurally diverse LPS molecules. The receptor multimer is composed of two copies of the TLR4–MD-2–LPS complex arranged in a symmetrical fashion. Binding of agonistic ligands causes dimerization of the extracellular domains and is believed to trigger the recruitment of specific adaptor proteins to the intracellular domains, thus initiating a signaling cascade. Adapted from Park *et al*; 2009.

TLR5-flagellin interaction

TLR5 recognizes flagellin from both gram positive and gram negative bacteria. It detects a specific conformation, which is exposed only in monomeric flagellin and not the filamentous form of flagellin, the main form that exists on the surface of bacteria. That is the reason why only monomeric flagellin can activate TLR5 (Smith *et al.*, 2003). Binding of flagellin to TLR5 initiates MyD88 - dependent intracellular signaling leading to activation of MAP kinases and I-κB degradation followed by activation of NF-κB (Matsumura *et al.*, 2004; Mizel and Snips, 2002). Flagellin stimulates epithelial cells to secrete chemokines like IL-8 and MIP-2α (Gerwitz *et al.*, 2001; Hybiske *et al.*, 2001) as well as upregulate expression of iNOS, matrizylin and human β-defensin 2 (Zeng *et al.*, 2003; Sierro *et al.*, 2001; Steiner *et al.*, 2000). Flagellin stimulates monocytes and macrophages to secrete TNF-α, IL-6 and nitric oxide (Moors *et al.*, 2001; Mcdermott *et al.*, 2000), and brings about maturation of dendritic cells (Means *et al.*, 2003). In addition, flagellin has been shown to synergize with TCR-dependent and independent stimuli to up-regulate secretion of IFN-γ, IL-8 and IL-10 by human CD4+ T cells (Caron *et al.*, 2005).

TLR2

TLR2 recognizes a variety of microbial components that include lipoproteins and glycolipids from various pathogens (Lien *et al.*, 1999; Takeda and Akira, 2005). In addition, TLR2 mediates cellular responses to yeast cell wall, lipoarabinomannan from mycobacteria, peptidoglycan, *Treponema* glycolipid, *Tryponosoma cruzi* GPI-anchor (Takeda *et al.*, 2003). The recognition of a wide variety of microbial components by TLR2 is attributed to its ability to form heterodimers with TLR1 and TLR6. In addition, TLR2 has been shown to functionally collaborate with distinct type of receptors such as dectin-1. TLR2 along with TLR6 recognizes peptidoglycan, zymosan and diacylated lipopeptides such as MALP-2; whereas TLR2 along with TLR1 recognizes tri-acylated lipopeptides from mycobacterium and *B. burgdoferi* (Brodsky and Medzhitov, 2007, Takeda *et al.*, 2003; Akira *et al.*, 2001). TLR2 has also been shown to recognize carbohydrate antigen PSA of *Bacteroides fragilis* (Wang *et al.*, 2006). Recently it has been shown that serum protein vitronectin specifically recognizes bacterial lipopeptides (BLP) and facilitated delivery of BLP to the vitronectin receptor, which is a part of TLR2 activation complex (Gerold *et al.*, 2008).

Cytoplasmic pattern recognition receptors

In addition to TLRs, immune cells are equipped with cytoplasmic sensory system that can distinguish between intracellular and extracellular infections. The cytoplasmic PRRs survey the cytoplasm for signs of intracellular invaders and are classified into two classes based on their mechanism of activation. The first class of receptors directly detects cytosolic PAMPs whereas the second class consists of members that are involved in formation of large multimeric complexes known as inflammasomes. Nucleotide-binding oligomerization domain (Nod) - like receptor (NLR) family members Nod1 and Nod2, retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and IFN stimulatory DNA (ISD) sensors are members of the first class, whereas NALP3 (Nacht domain-, LRR- and PYD- containing protein 3) and Ipaf (ICE protease activating factor) form the second class of intracellular PRRs (Palm and Medzhitov, 2009; Meylan *et al.*, 2006).

Nod1 and Nod2 detect γ–D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide respectively (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003). RIG-I and MDA5 recognizes cytoplasmic viral dsRNA (Yoneyama *et al.*, 2004). In addition, these viral sensors bind poly I:C. Similar to dsRNA, viral DNA is also recognized in the cytoplasm by a mechanism independent of TLRs (Ishii *et al.*, 2005). NALP3 or cryopyrin is involved in recognition of bacterial RNA, ATP and uric acid crystals (Kanneganti *et al.*, 2006; Mariathasan *et al.*, 2006; Martinon *et al.*, 2006). Activation of NALP3 inflammasome in macrophages requires two signals, one (e.g. LPS) primes for activation and induces pro-IL-1β production while the second signal (e.g. potassium efflux) activates the inflammasome resulting in caspase-1 activation and IL-1β processing and secretion. Ipaf senses cytosolic flagellin and triggers caspase-1 - dependent cell death of macrophages and secretion of IL-1β (Franchi *et al.*, 2006; Miao *et al.*, 2006).

Soluble pattern recognition receptors

In addition to cell surface associated TLRs and cytosolic PRRs, a number of PRRs are secreted in plasma as humoral proteins. These soluble PRRs are involved in detection of pathogens in body fluid and their opsonization for facilitating phagocytosis. Carbohydrate recognizing lectins or collectins (mannose-binding lectin, MBL; surfactant proteins A and D, SP-A, SP-D), ficolins and pentraxins are examples of soluble PRRs. Collectins can be divided into mannose/glucose-type or galactose-type based on relative

monosaccharide specificity. Ficolins bind to acetylated molecules, including *N*-acetylgalactosamine and *N*-acetylglucosamine (Sahly *et al.*, 2008). These lectins are involved in activation of lectin-dependent complement pathway (Zelensky and Gready, 2005; van der Watering *et al.*, 2004). In addition, binding of lectins to surface of microorganisms leads to massive aggregation and enhancement of phagocytosis of microorganisms by opsonization or *via* upregulation of the activity of the mannose receptor. Collectins enhance the oxidative burst in phagocytes and modulate the secretion of cytokines. MBL increases membrane permeability of microorganisms *via* activation of the lectin pathway of complement activation; however mechanism of increased membrane permeability by SP-A and SP-D is not known (van der Watering *et al.*, 2004).

Pentraxins are a superfamily of acute phase proteins like C-reactive protein (CRP), serum amyloid P-component (SAP) and PTX3 (Bottazi *et al.*, 2009). CRP and SAP constitute short pentraxins, while PTX3 is a long pentraxin. These receptors do not induce production of pro-inflammatory cytokines on their own but they form an important component of humoral arm of innate immunity. CRP is secreted by hepatocytes in response to inflammatory mediators (Pepys and Hirschfield, 2003). PTX3 is released by PMNs or produced by DCs and monocytes in response to microbial recognition by cellular PRRs (Garlanda *et al.*, 2005). PTX3 - binding has been observed with conidia of *Aspergillus fumigatus*, *Paracocciodes brasiliensis*, zymosan, *S.typhimurium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, OMP-A of *K.pneumoniae* (Jeanin *et al.*, 2005; Diniz *et al.*, 2004; Garlanda *et al.*, 2002). PTX3 enhances phagocytic ability of macrophages by acting as an opsonin and its binding to OMP-A amplifies pro-inflammatory responses (Jeannin *et al.*, 2005). PTX3 also provides resistance to human and murine cytomegalovirus (HCMV and MCMV); and influenza virus infections by reducing viral entry and infectivity (Reading *et al.*, 2008; Bozza *et al.*, 2006).

Role of PRRs in adaptive immunity

The innate and adaptive arms of the immune system use two fundamentally different strategies to recognize invading microbes. Innate immune system detects infection using a limited number of germ-line encoded receptors that recognize molecular structures unique to classes of infectious microbes. However, adaptive immune system uses randomly generated, clonally expressed highly specific receptors of varied specificities. Combination

of these two strategies makes the immune system highly efficient in targeting immune effectors against non-self. Cells of the innate immune system such as macrophages and DCs prominently express PRRs. The ability of innate immune cells to distinguish infectious self from non-infectious self and differentially sense various classes of pathogens (intracellular *vs* extracellular) through different sets of PRRs enables the innate immune system to regulate the magnitude as well as the quality of adaptive immune responses. For example, IFN-γ-producing Th1 cells are activated against intracellular bacteria, IL-4-secreting Th2 cells are generated against helminthes and Th17 responses are produced during infection with extracellular bacteria (Reiner, 2007). The activation of different kinds of T-cell responses depends on production of different sets of cytokines by antigen presenting cells following recognition of microbial components through various PRRs. IL-12 is made during bacterial infections; interferons are produced in response to viral infections and thymic stromal lymphopoietin (TSLP), IL-4 and IL-13 secreted during helminthic infection (Reiner, 2007). The extent and persistence of infection is controlled by the magnitude and duration of response through PRRs. In addition, signals transduced through PRRs also play a critical role in generating immunological memory and antibody switching in response to T-dependent and T-independent antigens (Palm and Medzhitov, 2009). Thus, recognition of microbial components by PRRs is a key element in innate immunity and also plays a vital role in instructing the adaptive immune response. Not all PRRs are equal in their ability to activate adaptive immune response, while some PRRs (e.g TLRs) are sufficient to induce both T- and B-cell responses, others such as mannose receptor and scavenger receptors are not competent to activate adaptive immunity by themselves (Iwaski and Medzhitov, 2004).

Materials and Methods

MATERIALS AND METHODS

MATERIALS

Cell lines

The human monocytic cell line, THP-1 and human promyleomonocytic cell line, U937 were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). These cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified CO_2 (5%) incubator. Human Embryonic Kidney epithelial cell line (HEK293-T) was provided by Dr. Vinay Kumar Nandicoori, National Institute of Immunology, New Delhi. Bone marrow derived LPS-hyporesponsive mouse macrophage cell line, GG2EE, was kindly made available by Dr. Steven B. Mizel, Wake Forest University, North Carolina.

Bacterial strains

S.typhimuriun SL1344 wild type strain was provided by Prof. Emmanuelle Charpentier, Department of Microbiology and Genetics, University of Vienna, Austria.

Antibodies

Anti-mouse Ig and anti-rabbit Ig antibodies labeled with horseradish peroxidase (HRP) or fluorescein isothiocyanate (FITC) were purchased from Jackson Laboratories (West Grove, Pennsylvania). Alkaline phosphatase (AP)–conjugated isotype-specific antibodies were procured from Southern Biotech (Birmingham, Albama). Rabbit anti-prohibitin antibodies were obtained from Neomarkers, USA. Antibodies specific to prohibitin and BAP-37 were prepared by immunizing rabbits with c-terminal peptides (conjugated to keyhole limpet hemocyanin [KLH]) which are different between prohibitin and BAP-37 (Coates *et al.*, 1997). Monoclonal anti-TLR2 antibody (T2.5) was obtained from eBiosciences (San Jose, California). Anti-hemoglobin antibodies, anti-human GAPDH antibody and anti-TLR2 blotting antibody were procured from Santa Cruz Biotechnology (Santa Cruz, California). Anti-phospho-p44/42 MAP-kinase (pERK) antibody, anti-p42/p44 MAP kinase (ERK) antibody, anti-phospho-p38 antibody and anti–I-kB antibodies were obtained from Cell Signaling Technology Inc. (Danvers,

Massachusetts). Mouse anti-actin antibody was obtained from Calbiochem (Darmstadt, Germany). Monoclonal antibodies to Vi were previously generated in our laboratory (Qadri *et al.*, 1990).

Chemicals and reagents

Vi capsular polysaccharide (Vi) derived from *S.typhi* was obtained from Bharat Biotech International Limited (Hyderabad, India). It was dialyzed against PBS before use in cellular studies. LPS isolated from *S.typhosa*, polygalacturonic acid (PGUA), 3,3',5,5'-Tetramethyl benzidine (TMB), phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), human hemoglobin (Hb) and Proteinase K were procured from Sigma Chemicals Co. (St. Louis, Missouri). O-acetyl derivative of polygalacturonic acid (PGUA) was prepared as described by Sweczyk and Taylor (1980). Protein-G-Sepharose, Ficoll-Paque Plus and Amersham Enhanced Chemiluminescence (ECL) reagents were obtained from GE Healthcare Bio-sciences (Piscataway, New Jersey). p38 inhibitor, SB203580 and JNK inhibitor were obtained from Calbiochem (Darmstadt, Germany). MEK inhibitor, PD98059, was purchased from Santa Cruz Biotechnology (Santa Cruz, California). RPMI-1640 and DMEM were obtained from GIBCO (Carlsbad, California). Fetal calf serum (FCS) was purchased from Biological Industries (Kibbutz, Israel). ELISA kits for detection of mouse IL-6, TNF-α, IL-12p40, IFN-γ and human IL-8, IL-6, TNF-α were all purchased from BD biosciences (San Jose, California). RT-PCR kits, Trizol reagent, and Platinum HiFidelity Taq DNA polymerase were purchased from Invitrogen (Carlsbad, California). Primers were synthesized at Sigma chemicals (Bangalore, India). The reagents used in electrophoresis and Western blotting were purchased from Sigma Chemical Co. (St. Louis, Missouri). Nitrocellulose membrane was obtained from Advanced Microdevices Pvt. Ltd. (Ambala, India). Chemicals used in the preparation of buffers and other solutions were of analytical grade, and unless otherwise stated, were obtained from E. Merck Ltd. (Mumbai, India). Vector (pSUPER.neo.gfp) and oligos for RNA interference experiments were purchased from Oligoengine (Seattle, Washington).

Animals

C57BL/6, BALB/c, C3H/HeJ, TCR β null and TCR δ null mice were obtained from Jackson laboratories, USA and maintained at the small animal facility of the National Institute of Immunology. TCR β null and TCR δ null mice were maintained in germ-free

conditions. Animal experiments were carried out according to the guidelines provided by the Institutional Animal Ethics Committee.

Preparation of buffers and other reagents

Cell culture medium

RPMI-1640 or DMEM, supplied as dry powder, was reconstituted according to the instructions provided by the suppliers. One liter RPMI-1640 medium was supplemented with 2 g $NaHCO_3$ and 2.38 g HEPES, and one liter DMEM medium was supplemented with 3.7 g $NaHCO_3$ and 2.38 g HEPES. Complete medium was prepared by adding FCS (10% final concentration) to RPMI-1640 or DMEM subsequently referred to as RPMI-10 or DMEM-10.

Phosphate-buffered Saline (50 mM phosphate, 150 mM NaCl, pH 7.4)

Na_2HPO_4	40.5 mM
NaH_2PO_4	9.49 mM
NaCl	150 mM

PBS-Tween: Tween 20 was added to PBS to a final concentration of 0.05%.

Acetic acid-NaCl solution, pH 3.0

Acetic Acid	0.1 M
NaCl	0.15 M

TKM buffer

Tris-HCl, pH 7.4	50 mM
KCl	25 mM
$MgCl_2$	5 mM
EDTA	1 mM
NaN_3	0.02%

Lysis buffer

TKM buffer was supplemented with Triton X-100 (1% final concentration), a cocktail of protease inhibitors (Roche Diagnostics, Germany) and sodium orthovanadate (100 µM) just before use.

Buffers for ELISA

Carbonate buffer, pH 9.5

Na_2CO_3	32 mM
$NaHCO_3$	74 mM

Sodium phosphate buffer, pH 6.5

Na_2HPO_4	66.3 mM
NaH_2PO_4	103.1 mM

Citrate phosphate Buffer, pH 5.6

Citric acid	22.1 mM
Na_2HPO_4	51.4 mM

Substrate for ELISA

TMB-TBABH solution

3, 3', 5, 5'-Tetramethyl benzidine (TMB)	41 mM
Tetrametylammonium borohydride (TBABH)	8.2 mM
N, N-Dimethylacetamide (DMA)	10 ml

The solution was stored at 4°C in dark in a glass container. 200 µl of TMB-TBABH solution and 3 µl of 30% H_2O_2 were added to 8 ml of citrate phosphate buffer immediately before use.

Alkaline phosphatase substrate

Tris-Cl (100 mM, pH 8.0)	5.0 ml
NaCl (100 mM)	5.0 ml
$MgCl_2$ (5 mM)	250 µl
p-nitophenyl phosphate (PnPP)	50 mg
H_2O	39.75 ml

The solution was prepared immediately before use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using Laemmli buffer system. The solutions were prepared according to the following composition:

Resolving Gel, 12.0% (10 ml)

Acylamide 30%, bis-acrylamide 0.8%	4.0 ml
Tris-HCl (1.5 M Trizma base), pH 8.9	2.5 ml
H_2O	3.35 ml
SDS (10%)	100 µl
APS (10%)	50 µl
TEMED	8 µl

Stacking Gel (8 ml)

Acylamide 30%, bis-acrylamide 0.8%	0.65 ml
Tris-HCl (1.0 M Trizma base), pH 6.8	0.65 ml
H_2O	3.65 ml
SDS (10%)	50 µl
APS (10%)	25 µl
TEMED	6 µl

SDS- Sodium dodecyl sulfate; APS- Ammonium persulfate; TEMED- N, N, N', N'-Tetramethylethylenediamine.

Laemmli sample buffer (non-reducing)

Tris-HCl buffer, pH 6.8	0.16 M
SDS	2.3%
Glycerol	10%
Bromophenol blue	0.1%

Electrode buffer

Glycine	192 mM

Trizma base	25 mM
SDS	3.5 mM

Coomassie staining solution

Coomassie brilliant blue	0.25%
Methanol	40%
Glacial acetic acid	10%

Destaining solution

Methanol	40%
Glacial acetic acid	10%

The resolving gel was polymerized in a Hoefer or BioRad Protean-3 mini gel apparatus for 30-45 min. The thickness of the gel was 1.5 mm. The stacking gel prepared afresh was layered on top of the resolving gel and allowed to polymerize for 15-20 min. Samples to be analyzed were mixed with Laemmli sample buffer and placed in a heating block at 100°C for 5 min before loading into wells. Electrophoresis was carried out at a constant current of 30 mA.

Western blotting
Transfer buffer

Glycine	192 mM
Trizma base	25 mM
Methanol	20%

Ponceau-S (1X)

Ponceau S	0.1% (w/v)
Acetic acid	5%

Western blotting was carried out by the method described by Towbin *et al.* (1979). The sample to be analyzed was separated in a 12% SDS-PAG and transferred to a nitrocellulose (NC) membrane at a constant current of 300 mA for 2 h using a Bio-Rad transfer apparatus (BioRad, USA). The transfer of proteins was ascertained by staining the NC membrane with Ponceau-S (1X). The membrane was blocked for 1 h at room

temperature with 1% non-fat milk protein prepared in PBS and subsequently probed with the appropriate primary antibody, followed by HRP-labeled secondary antibody and developed using Enhanced Chemiluminescence (ECL) reagent.

METHODS

Bacterial culture conditions

Bacteria were cultured in Luria-Bertani (LB) medium at 37°C in a shaker incubator at 220 rpm for 12-14 h. Bacteria were centrifuged at 5000×g for 20 min. The bacterial pellet was washed twice with PBS. The pellet was resuspended in PBS and processed as needed.

Maintenance of cell lines

Cells were maintained in RPMI-1640 supplemented with 10% heat inactivated FCS (RPMI-10) at 37°C in a humidified CO_2 (5%) incubator. Cells were centrifuged at 315×g for 5 min, washed twice with serum-free RPMI-1640, resuspended in RPMI-10 and grown in 75 cm^2 tissue culture flasks.

Isolation of flagellin from *Salmonella typhimurium*

Salmonella typhimurium (*S.typhimurium*) was grown overnight in LB medium and flagellin was isolated as described by Smith *et al.* (2003). Briefly, bacteria were pelleted by centrifugation at 8000×g for 10 min, washed twice with PBS, resuspended in PBS and sheared for 2 min at high speed in a waring blender. The sheared suspension was centrifuged at 8000×g for 10 min. The supernatant was collected and centrifuged at 100,000×g for 1 h to pellet flagellin filaments. The filaments were resuspended in PBS, left overnight at 4°C and centrifuged at 100,000×g for 1 h. This washing step was repeated twice. The resulting pellet of flagellin filaments was resuspended in PBS and heated at 70°C for 15 min. This treatment depolymerizes flagella to give flagellin monomers. The solution was centrifuged again at 100,000×g to pellet flagellar filaments which did not depolymerize. The supernatant was collected and used as the source of monomeric flagellin. Protein concentration was determined using the Pierce Micro BCA™

(bicinchoninic acid) protein assay kit (Thermo scientific; Rockford, Illinois) according to the manufacturer's instructions.

Purification of monoclonal antibodies

Monoclonal antibodies were purified from culture supernatants obtained from B-cell hybridomas by affinity chromatography using Protein G-Sepharose. Briefly, the culture supernatant was filtered through a 0.2 μm membrane and passed through a column containing Protein G-Sepharose beads. The column was washed with Tris-HCl buffer (pH 7.4) and the antibody bound to beads was eluted with a low pH solution (acetic acid-NaCl solution, pH 3.0). The eluate was neutralized immediately with Tris-HCl buffer (pH 8.9) and dialyzed against PBS. The purity of the antibody was checked by SDS-PAGE. The antibody was concentrated using Centricon concentrator (Millipore; Bedford, Massachusetts) and protein concentration was determined using the Micro BCA™ protein assay kit.

Analysis of Vi binding to cells

The binding of Vi to U937, THP-1 and human PBMCs was analyzed by flow cytometry. Briefly, cells were taken at a density of 10^5 cells/well in a round bottom 96-well plate and incubated for 1 h at 4°C with varying concentrations of Vi diluted in PBS. Cells were washed with PBS and incubated with anti-Vi monoclonal antibody ($P_2B_1G_2/A_9$, Qadri *et al.*, 1990) or an isotype matched control antibody for 1 h at 4°C. After washing, cells were incubated with FITC-conjugated anti-mouse Ig antibody (diluted in PBS-1% BSA) for 1 h at 4°C. Control cells were incubated with anti-Vi monoclonal antibody and FITC-conjugated anti-mouse Ig antibody. Cells were washed with PBS-BSA and resuspended in PBS. 10,000 cells were analyzed in flow cytometer (Becton Dickinson; San Jose, California) using Cell Quest software. The data was plotted using WinMDI software (Scripps Research Institute; La Jolla). Binding was also analyzed with Vi diluted in RPMI-1640, RPMI-1640 supplemented with different concentrations of serum or RPMI-1640 supplemented with Proteinase K- digested FCS. Proteinase K digestion of serum was carried out at 37°C for 1 h. The digested product was heated for 1 h at 90°C to inactivate Proteinase K.

Internalization of Vi

To study internalization of Vi, cells were incubated with Vi ($1\mu g/ml$) for 1 h at 4°C, washed extensively and transferred to 37°C for different time periods. Subsequently, cells were incubated with anti-Vi antibody and FITC - labeled anti-mouse Ig antibody followed by flow cytometric analysis.

Identification of Vi - binding molecules

The cell surface associated prohibitin family of molecules involved in binding to Vi were identified by immunoprecipitation (Sharma and Qadri, 2004). Briefly, cells (2×10^7) were incubated with Vi (1 $\mu g/10^6$ cells) for 1 h at 4°C. Cells were washed with PBS and lysed in TKM lysis buffer. The lysate was centrifuged at $15,000\times g$ for 20 min. The supernatant was passed through a 0.45 μm filter before loading on Protein-G-Sepharose beads preloaded with anti-Vi monoclonal antibody for 4 h at 4°C. After washing with TKM lysis buffer, beads were boiled with Laemmli sample buffer (non-reducing) and run in a 12% SDS-PAG. The immunoprecipitated proteins were transferred to a NC membrane. The NC membrane was blocked with 1% milk protein in PBS and incubated with rabbit anti-prohibitin antibodies diluted in 1% PBS-BSA for 1 h at room temperature. Subsequently, the NC membrane was treated with HRP-labeled anti-rabbit IgG antibodies. The blot was washed extensively with PBS-Tween and developed using ECL.

Analysis of chemokine/cytokine secretion from monocytes and macrophages

Cells were incubated with flagellin (isolated from *S.typhimurium*) in the presence or absence of Vi for 6 h at 37°C in a 96 well plate in triplicate. Stimulations were carried out in RPMI-1640 or RPMI-10. The supernatants were analyzed for IL-8 by commercially available ELISA kit (Opt EIA, BD Pharmingen). In some experiments, cells were treated with flagellin in RPMI for 1 h, and then incubated for another 5 h with Vi in RPMI followed by determination of IL-8 in the supernatants.

THP-1 cells were incubated with different concentrations of Vi in presence of serum. The supernatants were collected at 8 h (for IL-8) and at 24 h (for TNF-α). For IL-6 analysis, THP-1 cells were first activated with PMA (100 ng/ml) for 24 h and then incubated with Vi for another 24 h in presence of serum. Cytokines were analyzed by commercially available ELISA (Opt EIA, BD Pharmingen). In some experiments, cell

stimulation was carried out in presence of polymyxin B (10 µg/ml; throughout stimulation) or in presence of inhibitors of various signaling pathways (PD98059 for MEK, SB203580 for p38-MAPK and JNK II for JNK; for 30 min before incubating with Vi in RPMI-10 for another 5 h).

Human *ex-vivo* PBMCs were also stimulated with flagellin or Vi in the absence or presence of serum, and supernatants were assayed for IL-8 and TNF-α. Immature murine DCs (isolation described later) were also stimulated with flagellin along with Vi in the absence or presence of serum. Cells were seeded in a 24-well plate at 5×10^5 cells per well and incubated with flagellin and/or Vi in RPMI or RPMI-10 at 37°C in 5% CO_2 for 24 h. The supernatants were assayed for IL-6 and TNF-α. Peritoneal exudates cells obtained from C3H/HeJ mice were incubated with different concentrations of Vi in presence of serum. The culture supernatants were collected after 24 h for determination of IL-6 by ELISA.

Isolation of peripheral blood mononuclear cells (PBMC) from human blood

Blood was collected by veinpuncture from healthy human volunteers into heparin-coated vacutainers. PBMC were isolated by Ficoll-Paque density gradient centrifugation. Briefly, fresh heparinized blood was diluted with an equal volume of RPMI-1640 and slowly layered over Ficoll-Paque solution in 15 ml conical centrifuge tubes. 3 ml of Ficoll-paque was used per 10 ml of blood / RPMI mixture. The tubes were centrifuged at 2000×g for 30 min at 20°C. The upper layer containing plasma and most platelets was removed and the mononuclear cell layer at the plasma/Ficoll-Paque interface was collected in a separate tube. These cells were washed with RPMI-1640 and used for *in vitro* stimulation experiments.

Generation of dendritic cells (DC) from bone marrow derived cells

Cells were isolated from femur and tibia bones of C57/Bl6 mice. Bones were cut from both ends and bone marrow was flushed out with ice cold RPMI-1640. Cells were pelleted down at 400×g for 5 min and the cell pellet was incubated with 0.15 M NH_4Cl solution to lyse RBC. Cells were washed with RPMI, resuspended in RPMI-1640 containing 20% FCS along with 2% culture supernatant derived from GM-CSF – transfected P885L and transferred to 24-well plates (2.0×10^6 cells/well). To generate

immature DC, adherent monocytes were replenished with fresh medium containing GM-CSF every alternate day and cultured for 6-8 days at 37°C in 5% CO_2. On day 7, cells were washed thoroughly with RPMI-1640 and used for cell stimulation experiments.

Determination of cytokines in culture supernatants

The supernatants were assayed for IL-8, IL-6, IL-12p40, TNF-α and IFN-γ by commercially available ELISA kits (Opt EIA Set, BD Pharmingen) according to the manufacturer's instructions with slight modifications. Briefly, a 96-well microtitre plate (Maxisorp, Nunc) was coated overnight at 4°C with 50 μl capture antibody (diluted 1:250 in either 100 mM carbonate buffer, pH 9.5 or 200 mM sodium phosphate buffer, pH 6.5). The plate was washed twice with PBS-Tween (PBST) and blocked with PBS-BSA (PBS containing 1% BSA; 100 μl/well) for 1 h at 37°C. Culture supernatants to be assayed for a particular cytokine were added to each well (50 μl/well) alongside standards for that cytokine, and the plate was incubated for 1 h at 37°C. Subsequently, the plate was washed and incubated with detection reagent mix (detection antibody + avidin-HRP; diluted 1:250 in PBST-BSA-1%) for 1 h at 37°C. The plate was washed thoroughly with PBST and the reaction was developed by adding freshly prepared substrate solution containing TMB/TBABH/H_2O_2 (75 μl/well). The reaction was stopped with 125 μl of 2M H_2SO_4 and the absorbance was read at 450 nm. The concentration of cytokine in each sample was determined from a standard curve plotted for each cytokine.

Analysis of intracellular signaling

U937 cells were washed with RPMI-1640 and incubated with Vi (10 μg/ml/10^6 cells) under serum free conditions. After 1 h, cells were stimulated with flagellin (100 ng/ml) for different time periods. Stimulation was stopped by adding chilled PBS. Cells were centrifuged at 5000×g and the pellet was lysed in 50 μl lysis buffer. Cell lysates were boiled with non-reducing Laemmli sample buffer before loading in a 12% SDS-PAG. The proteins were transferred to a NC membrane and probed with phospho-specific antibodies. The blot was developed with ECL. For re-blotting, the NC membrane was stripped with a solution containing 0.1 M acetic acid and 0.15 M NaCl for 2 h followed by extensive washing with PBST and subsequent probing with another antibody.

THP-1 cells were incubated with Vi (10 µg/ml) in presence of serum at 37°C for different time periods. Cell lysates were run on SDS-PAG, transferred to NC membrane and analyzed for phospho-ERK, phospho-p38, ERK and I-kB by western blotting with respective antibodies.

Knock-down of prohibitin expression

To obtain stable repression of prohibitin, oligonucleotide sequences targeting coding regions of prohibitin were cloned into pSUPER.neo.gfp expression vector system (Oligoengine) (**Figure 1**). The pSuper.neo.gfp expression vector uses the polymerase-III H1-RNA gene promoter and produces small RNA transcripts without a poly (A) tail. The transcripts have transcriptional start site and a termination signal consisting of five thymidine residues. The recombinant vector can produce transcripts which fold back to form short hairpin loop like structures. The short hairpin loop precursor transcript is quickly cleaved to produce functional siRNA in cells to degrade target mRNA. The presence of neomycin resistance gene allows selection of stable lines.
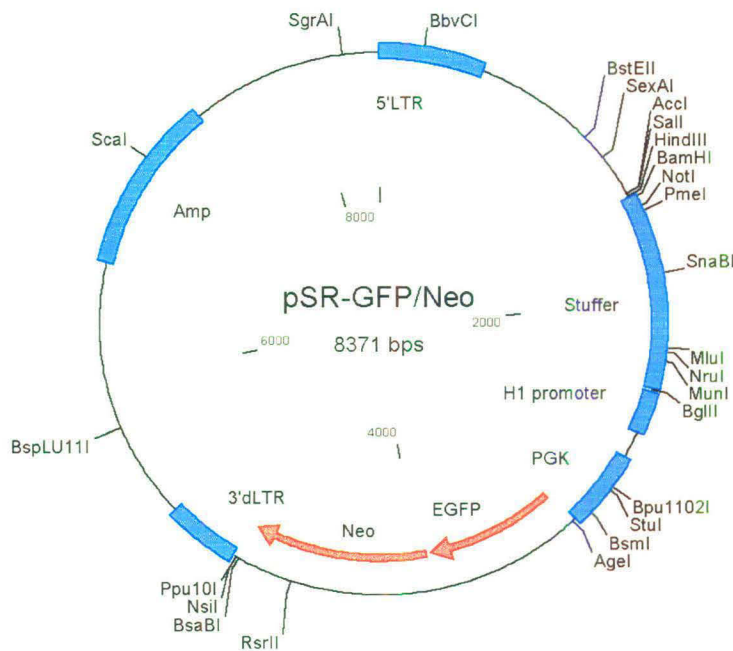

Figure 1. pSuper.gfp.neo construct obtained from Oligoengine.

pSuper.neo.gfp-Phb

The 60 base pair long forward and reverse oligonucleotides were hybridized to form double stranded DNA sequence and cloned into pSUPER.neo.gfp vector between *Bgl*II and

*Hin*dIII restriction sites. The target sequences used in this study Phb1: 5'-UGU CAA CAU CAC ACU GCG C-3', Phb2: 5' AAT GTG GAT GCT GGG CAC AGA- 3' were chosen from previous studies (Rajalingam *et al.*, 2005; Wang *et al.*, 2004). Oligos (0.05 μM) were dissolved in 50 μl sterile ddH$_2$O. pSUPER.neo.gfp vector was digested using *Bgl*II and *Hin*dIII enzymes to linearize vector. Phb1 and Phb2 oligonucleotides were then ligated with the linearized vector. Ligation reaction was performed overnight at 4-8°C as follows:

Restricted vector	1 μl (100 ng)
Restricted dsDNA	5 μl (1 μg)
10× buffer for T4 ligase	1 μl
T4 ligase	1 μl
ddH$_2$O	2 μl
Total	10 μl

Bacterial transformation

Heat shock transformation method was used to transform competent *E.coli* (DH5α) with the plasmid DNA or ligation mixture. Approximately 100 ng plasmid DNA or the whole ligation mixture was added to 100 μl freshly thawed competent cells and incubated for 20 min on ice. Bacteria were subjected to heat shock at 42°C for 90 s followed by incubation on ice for 2 min. One ml of LB medium was added to the mixture and incubated at 37°C with constant shaking at 250 rpm. After 1 h, 250 μl of the culture was spread on LB agar plates containing suitable antibiotic. The plates were incubated at 37°C overnight. The pSUPER.neo.gfp-Phb clones were digested with *Bgl*II, *Eco*RI and *Hin*dIII to establish presence of correct size inserts.

Transfection of cells

U937 cells were transfected using Amaxa Nucleofection Tranfection kit V (Lonza) according to the manufacturer's instructions. Briefly, 100 μl of nucleofector solution V was added to a pellet of 5×10^6 cells. This cell suspension was mixed with 5 μg DNA and the mixture was transferred to an amaxa certified cuvette. The cuvette was inserted into the nucleofector and transfection was carried out using W-001 program. 500 μl of pre-warmed culture medium were added to the cuvette, cells were transferred to a culture dish and incubated at 37°C. Two days post-transfection, cells were put on G418 (1 μg/ml) to select

stable transfectants. Unhealthy cells were removed by Ficoll-Paque density gradient centrifugation. Knock-down of prohibitin expression was confirmed by Western blotting.

RT-PCR analysis

Semiquantitative RT-PCR was performed to measure various transcripts. RNA was isolated from cells transfected with Phb1 and Phb2 or control vector using TRIzol® Reagent The cDNA was prepared by reverse transcription of 1 µg total RNA. The total RNA was mixed with oligo (dT) primer, incubated at 65°C for 5 min and immediately transferred to ice. The contents were mixed with RT master mix containing M-MLV reverse transcriptase, buffer, RNase inhibitor and dNTPs. The complete RT reaction mixture was incubated at 50°C for 1 h and the cDNA was denatured at 85°C for 5 min. cDNA was subsequently treated with RNaseH for 20 min at 37°C.

To measure transcripts of interest, semi-quantitative PCR was performed using gene-specific primers with the Veriti Gradient Thermocycler (Applied Biosystems). The PCR for the target and the house keeping genes was performed in parallel as follows.

1 cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 56°C for 30 s and 70°C for 1 min followed by final extension step for 10 min at 70°C. At the end of the PCR, each sample was subjected to agarose gel electrophoresis.

Primers

BAP-37   Forward 5'-ATGGCCCAGAACTTGAAGGACT-3'

             Reverse 5'-GCTTTCTTACCCTTGGATGAGGCTGTC-3'

GAPDH   Forward 5'- ACCACCATGGAGAAGGCTGG -3'

             Reverse 5'- CTCAGTGTAGCCCAGGATGC -3'

β-Actin   Forward 5'-TATGCCAACACAGTGCTGTCTGG-3'

             Reverse 5'-TACTCCTGCTTGCTGATCCACAT-3'

Transfection with TLR

HEK293-T cells were transfected with various TLR2 constructs (human TLR2, hTLR2/1, hTLR2/6 obtained from Invivogen; **Figure 2**) using Lipofectamine 2000 (Invitrogen) as transfection reagent. Stable cell lines were obtained after selection with blasticidine-S-hydrochloride (10 µg/ml; Sigma). For analysis of TLR2 expression, cells were incubated with anti-TLR2 antibody for 1 h followed by FITC-labeled anti-mouse Ig antibody and analyzed by flow cytometry.
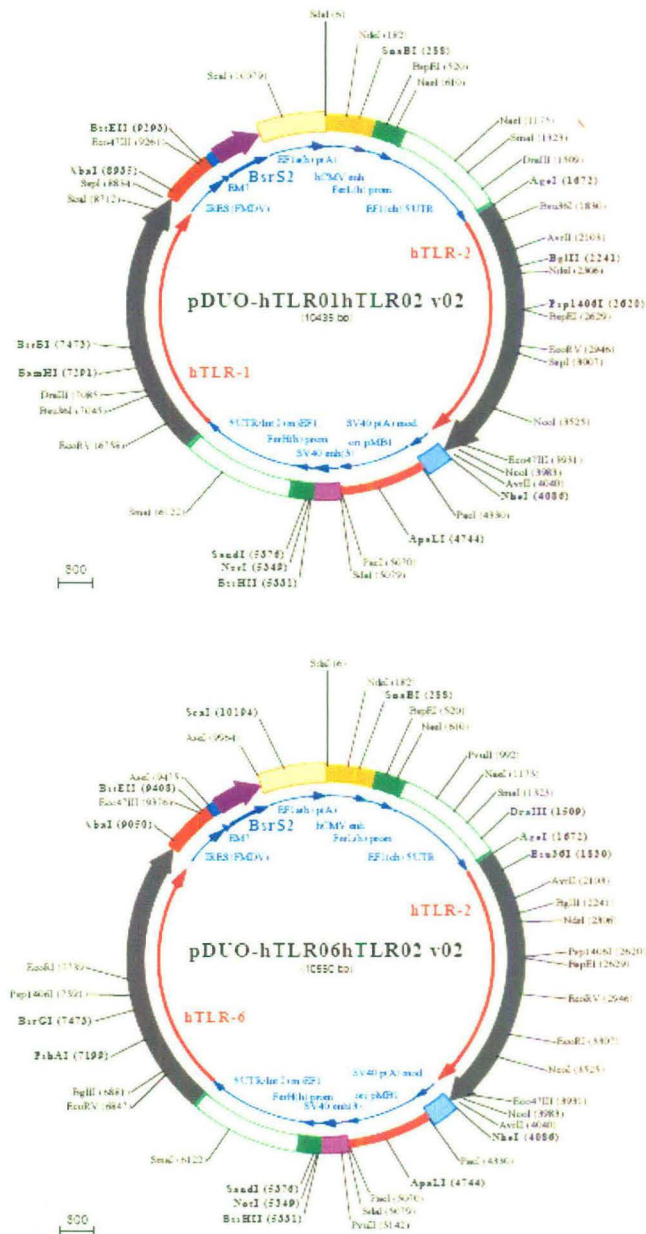
Figure 2. Human TLR2/TLR1 and TLR2/TLR6 constructs obtained from Invivogen.

Identification of serum proteins interacting with Vi

Vi was incubated for 48 h at 37°C with different concentrations of serum in presence of anti-Vi antibodies. 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 10% SDS-PAG. The gels were either stained with coomassie brilliant blue or silver stained as described by Morrissey (1981). Simultaneously, the proteins were transferred to a PVDF membrane and

molecules precipitated specifically with Vi were subjected to N-terminal protein sequencing.

Biotinylation of Vi

Vi was biotinylated as described by Ferry *et al.*, 2004. Briefly, Vi (1mg/ml) was dialyzed against water at 4°C overnight. Potassium periodate was added to it at a final concentration of 10 mM and the mixture was incubated on ice for 5 min in dark before dialyzing overnight in coupling buffer (0.1M sodium acetate buffer, pH 5.5). This dialyzed Vi-preparation was mixed with 50 mM biotin hydrazide solution along with 1 mM magnesium chloride and incubated in dark for 2 h at room temperature. Subsequently, the reaction was terminated by adding 1 mM sodium borohydride. This biotinylated preparation was again dialyzed against PBS overnight at 4°C and stored at -20°C.

Surface plasmon resonance (SPR)

The binding of Vi to hemoglobin was studied by SPR using BIA*core*2000 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 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 HBS 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 HBS buffer and analyzed at various concentrations at a flow rate of 30μl/min. An activated flow-cell without immobilized ligand was used to evaluate non-specific binding. Results were calculated using BIAevaluation version 4.1 software (BIA*core*).

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 (Torrance, California) connected to a Shimatzu HPLC system, with PBS as the mobile phase (flow rate, 0.25 ml/min). Alternatively, Vi-Hb mixture was subjected to Proteinase K digestion for 30 min at 37°C before subjecting to gel filtration chromatography. Fractions were checked for their pro-inflammatory activity on THP-1 cells. These fractions were also run in a 10% native PAG and transferred to NC membrane. The presence of Vi and Hb in different fractions was visualized by blotting the membrane with antibodies to Vi and Hb.

Deacetylation of Vi

Vi was incubated with different concentrations of NaOH for 30 min. The pH of the solution was neutralized with 0.1M HCl and it was dialyzed overnight against PBS at 4°C. 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 (Qadri *et al.*, 1990). Briefly, native and NaOH-treated derivatives of Vi, were coated in a 96-well microtitre plate overnight at 4°C (50 μl/well; 10 μg/ml diluted in 100 mM carbonate buffer, pH 9.5). The plate was washed two times with PBS-Tween (PBST) and blocked with PBS-BSA (PBS containing 1% BSA; 100 μl/well) for 1 h at 37°C. Anti –Vi antibodies (IgM OAc, $P_5A_2G_4/B_{11}$; IgM NAc, $P_5B_2D_8/A_9$) were added to the wells (50 μl/well) and the plate was incubated for 1 h at 37°C. Subsequently, the plate was washed and incubated with HRP-labeled anti-mouse Ig antibodies diluted in PBST-BSA-1% for 1 h. The plate was washed thoroughly with PBST and the reaction was developed by adding freshly prepared substrate solution containing TMB/TBABH/H_2O_2 (75 μl/well). The reaction was stopped with 125 μl of 2N H_2SO_4 and the absorbance was recorded at 450 nm.

Analysis of anti-Vi antibody responses in mice

Mice were immunized intra-peritoneally or intradermally with 10 μg of Vi, N-deacetylated Vi (NDeVi), or N- and O- deacetylated Vi (DeVi) dissolved in sterile PBS. Sera were collected from blood samples obtained before immunization (day 0) and day 14 after immunization. Five-eight mice were taken for each group. IgM and IgG anti-polysaccharide antibodies were determined by ELISA as described above. Briefly, Vi and its different derivatives (10 μg/ml) were coated in a 96-well microtitre plate overnight at 4°C (50 μl/well diluted in 100 mM carbonate buffer, pH 9.5). The plate was washed twice with PBST and blocked with 1% PBS-BSA for 1 h at 37°C. Sera samples diluted in 1% PBS-BSA were added to each well (50 μl/well) and the plate was incubated for 1 h at 37°C. Subsequently, the plate was washed and incubated with alkaline phosphatase (AP)-labeled anti-mouse Ig antibody diluted in PBST-BSA-1% for 1 h. The plate was washed thoroughly with PBST and the reaction was developed by adding freshly prepared substrate solution containing PnPP (75 μl/well). The plates were incubated at room temperature for 30 min - 4 h and read at 405 nm.

Results and Discussion

CHAPTER 1

Interaction of Salmonella typhi virulence polysaccharide, Vi, with monocytes: modulation of inflammatory responses

CHAPTER 1

Introduction

Microbial pathogens are sensed by the immune system through germline encoded pattern recognition receptors (PRRs) including Toll-like receptors (TLR), nucleotide binding and oligomerization domain (NOD) - like receptors (NLR) and lectins (Akira, 2009; Ishii *et al.*, 2008). These receptors recognize conserved pathogen associated molecular patterns (PAMPs) which include lipids, polysaccharides, proteins as well as nucleic acids (Akira, 2009). 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 (Bottazzi *et al.*, 2006; Hasty *et al.*, 2006; Gorczynski *et al.*, 2004). The responses produced through engagement of PRRs not only contribute to inflammation but also constitute an important component of host defense against a large number of pathogens (Albiger *et al.*, 2007; Gerold *et al.*, 2007).

Many pathogens have devised ways to counter host defense mechanisms 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 (O'Neill, 2008). Vaccinia virus-encoded protein A46R has been shown to interfere with TLR4-mediated activation of nuclear factor-kB (NF-kB) (Stack *et al.*, 2005; Bowie *et al.*, 2000). A46R contains TIR domain that interferes with TLR signaling by interacting with MyD88. Recently, the TIR-like protein A from *Salmonella enterica* serovar Enteritidis was shown to impair TLR- and MyD88-mediated activation of NF-kB leading to increased intracellular bacterial accumulation (Newman *et al.*, 2006). In another study by Cirl *et al.*, it was demonstrated that uropathogenic *Escherichia coli* and *Brucella melitensis* secrete TIR domain containing–proteins (Tcps) that are internalized into macrophages and impede TLR signaling by targeting MyD88 adaptor protein (Cirl *et al.*, 2008). Potent inhibitory effect of di-acylated lipoarabinomannan derived from *Mycobacterium bovis* BCG on LPS-induced macrophage activation has also been reported (Emilie *et al.*, 2007). These observations suggest that pathogens are capable of directly modifying the TLR-dependent host defenses.

Vi capsular polysaccharide is a major virulence factor of human typhoid-causing bacterium *Salmonella typhi*. It is a linear homopolymer of N-acetylated galacturonic acid

that is variably O-acetylated at the C3 position. The virulence of *S.typhi* correlates with the expression of Vi as typhoid rates are significantly higher in volunteers infected with Vi^+ *S.typhi* than in those infected with Vi^- *S.typhi* suggesting that the loss of Vi results in considerable attenuation (Hornick *et al.*, 1970a, 1970b). Vi encapsulated strains of *S.typhi* have been shown to survive better than Vi negative strains in cultured macrophages *in vitro* (Hirose *et al.*, 1997). Expression of Vi capsule in *S.typhi* prevents recognition by anti-O antibody thereby rendering the pathogen resistant to phagocytosis and complement-mediated killing (Hirose *et al.*, 1997; Looney and Steigbigel, 1986; Robbins and Robbins, 1984; Felix and Pitt, 1934b). Recently, this polysaccharide has been shown to interact with membrane associated prohibitin family of molecules in intestinal epithelial cells (IEC) and downregulate early chemokine secretion during infection of these cells with *S.typhi in vitro*. This downregulation was associated with reduced activation of extracellular regulated kinase (ERK) (Sharma and Qadri, 2004).

Vi is not only expressed on the surface of *S.typhi* but it is also released during *in vitro* growth of this pathogen. More importantly, it has been reported in the sera and urine samples of typhoid patients (Barret *et al.*, 1982; Rockhill *et al.*, 1980a, 1980b) which raised the possibility that this molecule could interact with cells of the immune system during systemic dissemination of *S.typhi* and bring about immune modulations. The present study reports interaction of Vi with mononuclear phagocytes and effects of this interaction on inflammatory responses from these cells.

Results

Vi inhibits inflammatory responses from human monocytes

Flagellin which is a major pro-inflammatory determinant of pathogenic *Salmonella* (Tsolis *et al.*, 2008; Winter *et al.*, 2008; Zeng *et al.*, 2003; Wyant *et al.*, 1999) and activates responses through TLR5 was used as a model stimulus to analyze possible modulatory effects of Vi on inflammatory responses produced by mononuclear phagocytes. The human monocytic cell lines, THP-1 and U937, were incubated with flagellin in the presence or absence of Vi and secretion of IL-8 was determined. The secretion of this chemokine from these cells in response to activation with flagellin was inhibited by Vi (**Figure 1A**). This inhibition was seen even when the polysaccharide was added to cells 1 h post stimulation with flagellin (**Figure 1B**), indicating that the inhibition was not due to blockade of binding
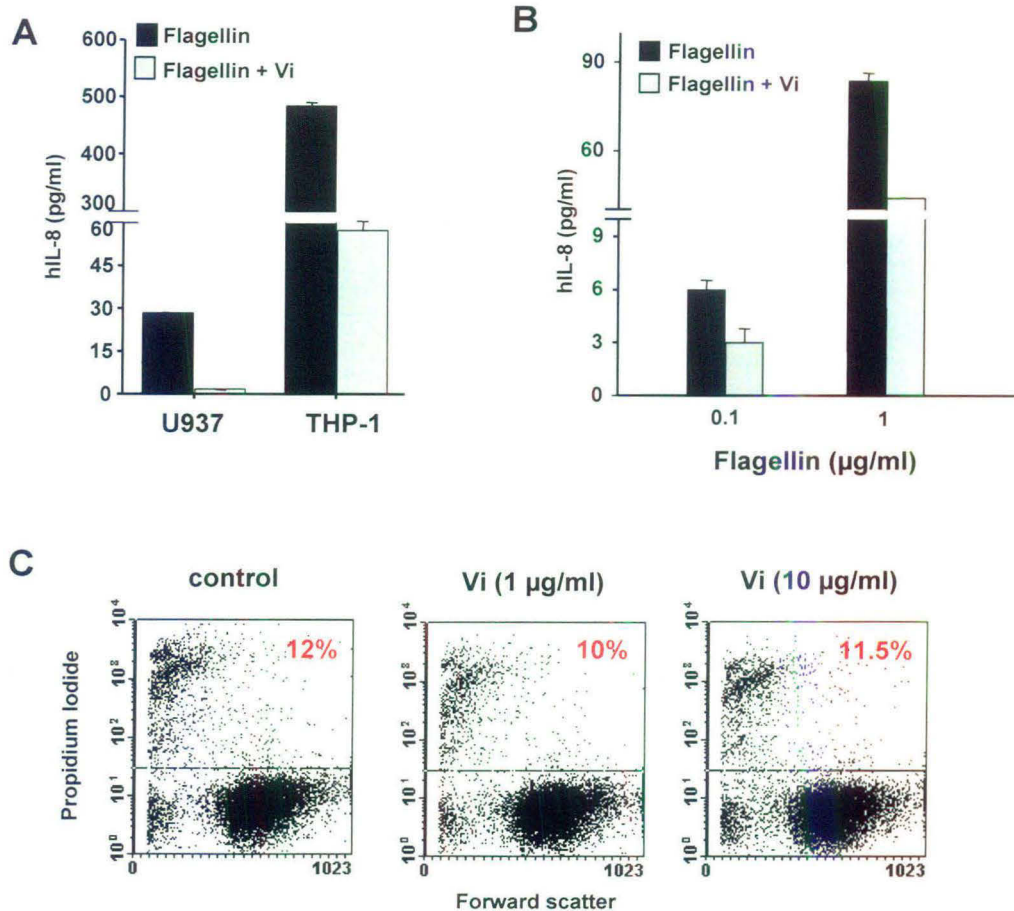
Figure 1. Vi inhibits flagellin-induced IL-8 secretion from human monocytes. (**A**) U937 and THP-1 human monocytic cell lines were incubated with or without Vi (10 μg/ml) in serum-free medium and 1 h later stimulated with *S. typhimurium* flagellin (0.1 μg/ml) in the absence of serum for 6 h. IL-8 was determined in the supernatants by ELISA. (**B**) 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. (**C**) U937 cells were incubated with Vi in absence of serum for 6 h and cell death was determined by propidium iodide uptake. Control represent cells incubated with PBS.

of flagellin to cells by Vi. Vi did not bring about cell death in U937 as determined by propidium iodide uptake (**Figure 1C**). Importantly, the suppression mediated by Vi was not restricted to monocytic cell lines as inhibition in flagellin - activated IL-8 was also observed with *ex-vivo* human peripheral blood mononuclear cells (**Figure 2A**) suggesting that inhibition of TLR5 responses with Vi would be relevant during *in vivo* infection with *S.typhi*. Moreover, the effect of Vi was not specific to human cells or IL-8 as bone marrow derived mouse dendritic cells also showed reduced IL-6 and IL-12p40 secretion when activated with flagellin in presence of Vi (**Figure 2B**).

Vi modulates flagellin - induced intracellular signaling events in monocytes

The results shown in **Figure 1** suggested that Vi might inhibit flagellin induced IL-8 secretion by modulating intracellular signaling events activated through TLR5. Treatment of cells with flagellin brings about activation of NF-kB and MAP-kinase pathways of intracellular signaling and inhibition in any one of these pathways leads to suppression of TLR5-induced inflammatory responses (Khan *et al.*, 2004; Tallant *et al.*, 2004). To investigate possible effects of Vi on the activation of these pathways, phosphorylation of MAP-kinases and degradation of I-kB were analyzed in U937 cells stimulated with flagellin in presence or absence of this polysaccharide. Cells activated with flagellin showed phosphorylation of ERK, p38 MAP-kinase and degradation of I-κB in a time-dependent fashion (**Figure 3**). However, cells stimulated with flagellin in presence of Vi displayed markedly reduced activation of p38 MAP-kinase and delayed degradation of I-κB (**Figure 3**) demonstrating modulation of key intracellular signaling pathways by this polysaccharide, thereby dampening flagellin activated inflammatory responses. Stimulation of cells with Vi in absence of serum did not activate any of these intracellular signaling pathways.

Prohibitin is required for Vi - mediated suppression of TLR5 - induced IL-8 secretion

Vi has been previously shown to interact with membrane associated prohibitin family of molecules in human IEC and suppress IL-8 secretion during infection of these cells with *S.typhi* (Sharma and Qadri, 2004). To analyze if Vi engaged similar molecules on macrophages, interaction of Vi with U937, THP-1 and PBMC was carried out. Vi
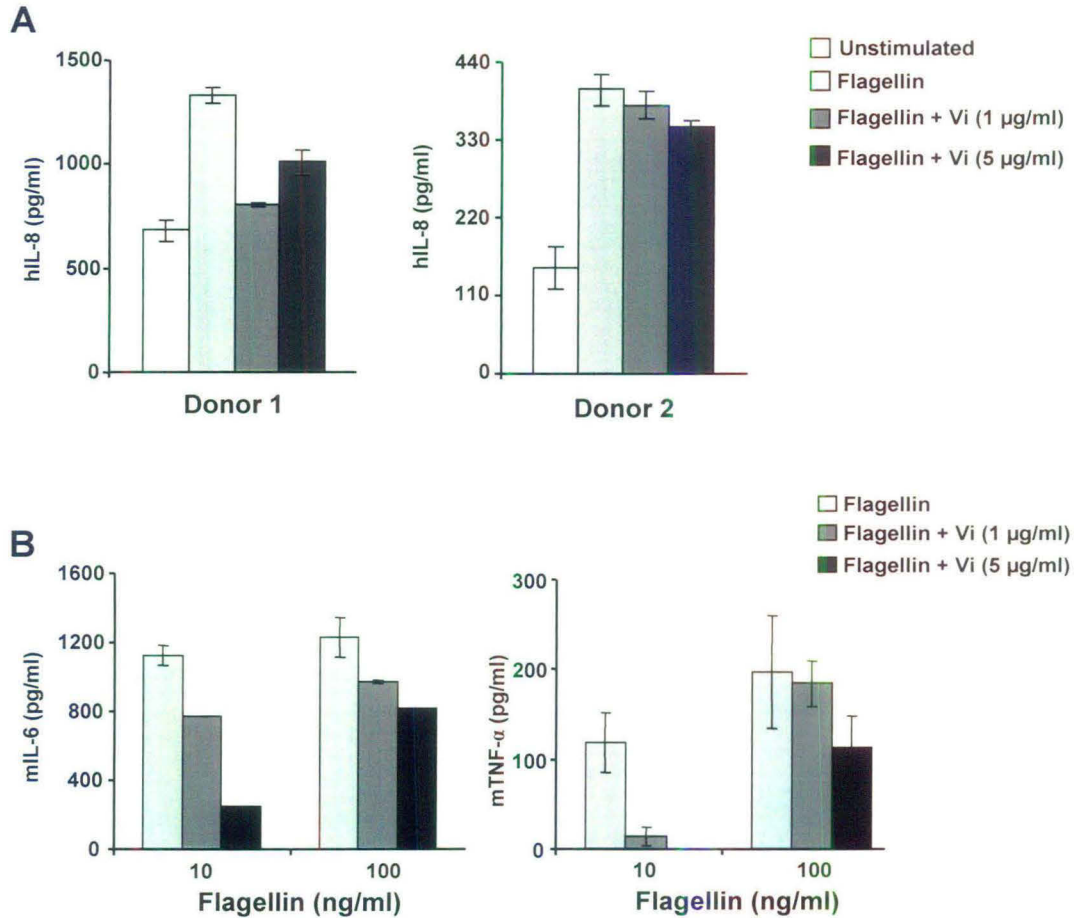
Figure 2. Vi inhibits flagellin-induced cytokine secretion from human peripheral blood mononuclear cells and mouse dendritic cells. (A) Human PBMC were incubated with or without Vi in serum-free medium and 1 h later stimulated with *S. typhimurium* flagellin (0.1 µg/ml) in the absence of serum for 24 h. IL-8 was determined in the supernatants by ELISA. (**B**) Mouse BMDCs were incubated with or without Vi in serum-free medium and 1 h later stimulated with *S. typhimurium* flagellin in the absence of serum for 24 h. IL-6 and TNF-α were determined in the supernatants by ELISA.
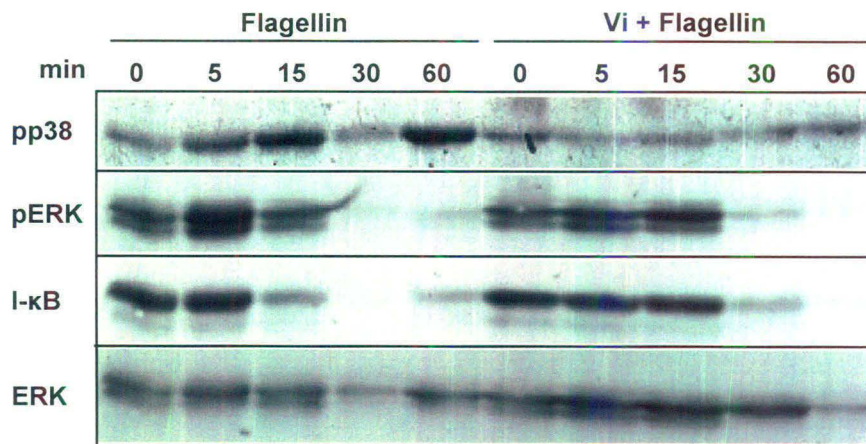
Figure 3. Vi reduced flagellin-induced activation of p38 MAP kinase and degradation of I-κB. U937 cells were stimulated with *S. typhimurium* flagellin (100 ng/ml) in the absence or presence of Vi (10 μg/ml) for various time points. The cell lysates were run in an SDS-PAG, transferred to a nitrocellulose membrane and probed with antibodies to phospho-p38, phospho-ERK, I-κB and ERK. The blot was developed using ECL reagent.

showed a dose – dependent binding to these cells (**Figure 4A**). Immunoprecipitation analysis with U937 revealed interaction of Vi with cell surface associated prohibitin and prohibitin related molecules (**Figure 4B**). Although BAP-37 was not readily detectable in the U937 cell lysate and Vi-immunoprecipitated complex when analyzed with commercially available anti-prohibitin antibody (**Figure 4B**), subsequent probing with prohibitin and BAP-37-specific antibodies generated against unique C-terminal peptide stretches showed comparable levels of prohibitin and BAP-37 in these cells and both could be immunoprecipitaed with Vi (see **Figure 6B,** data not shown). The possibility that Vi might be pulling down mitochondrial prohibitin was ruled out because Vi didn't get internalized in these cells (upto 24 h of incubation at 37°C) (**Figure 4C**) plus immunoprecipitations were always carried out with lysates prepared from intact viable cells incubated with Vi.

To establish the role of prohibitin in Vi - mediated suppression, the expression of prohibitin was knocked down in U937 cells by RNA interference using pSUPER.neo.gfp vector system that allows persistent suppression of gene expression. Oligonucleotides (Phb1 and Phb2) targeting the exons 1 and 3 of prohibitin were cloned into the pSUPER.neo.gfp vector system (**Figure 5A**). The presence of correct size inserts was established by restriction digestion (**Figure 5B**). Cells stably co-transfected with Phb1 and Phb2-containing pSUPER.neo.gfp vectors (siPhb1 and siPhb2) were monitored for gfp expression by flow cytometry (**Figure 6A**). Cells co-transfected with siPhb1 and siPhb2 showed reduced prohibitin protein levels as compared to vector-transfected cells (**Figure 6B**). Knock-down of prohibitin expression also led to reduction in the expression of BAP-37 protein without affecting its RNA levels (**Figure 6B and C**). This happens because prohibtin and BAP-37 are always present as heterodimer and BAP-37 monomers undergo degradation in absence of prohibitin (Ross *et al.*, 2008). Knockdown of prohibtin did not however change constitutive expression of actin and GAPDH showing that siRNA against prohibitin are targeting-specific (**Figure 6B and C**).

The reduction in the expression of prohibitin resulted in reduced binding of Vi to siPhb-transfected cells as compared to vector-transfected cells (**Figure 7A**). Consequently, the inhibition in flagellin-induced IL-8 secretion brought about by Vi was also reduced in prohibitin knock-down cells (**Figure 7B**). These results demonstrated that Vi can bring about inhibition of pro-inflammatory cytokine secretion elicited by TLR5-activation through its interaction with prohibitin.
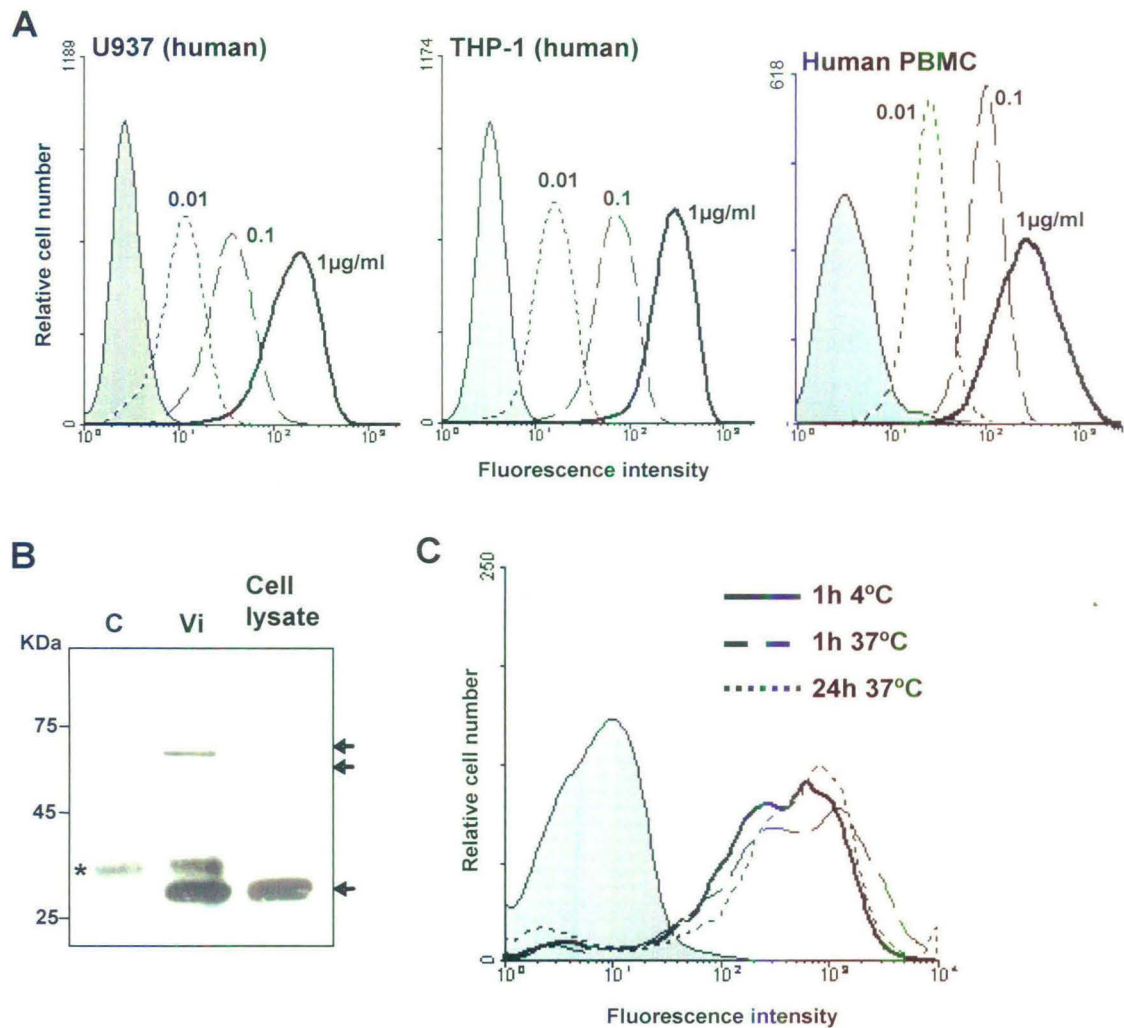
Figure 4. Vi interacts with membrane prohibitin in human monocytes. (**A**) Cells were incubated with different concentrations of Vi followed by anti-Vi monoclonal antibody (MoAb). Subsequently, cells were stained with PE-conjugated anti-mouse Ig antibody and analyzed by flow cytometry. (**B**) U937 cells were incubated with Vi (10 µg/ml) in PBS at 4°C for 1 h. Cell lysates were immunoprecipitated (IP) with anti-Vi MoAb preloaded on Protein-G-Sepharose beads and immunoblotted with anti-prohibitin antibodies. Control- IP from untreated cells, Vi-IP from Vi - treated cells. * indicates a non-specific band from Protein-G-Sepharose beads. (**C**) THP-1 cells were incubated with Vi (10 µg/ml) in RPMI at 4°C for 1 h. Subsequently, cells were washed and transferred to 37°C for 1 h or 24 h and incubated with anti-Vi MoAb followed by PE-conjugated anti-mouse Ig antibody and analyzed by flow cytometry. Shaded histogram represent control cells incubated only with anti-Vi MoAb and PE-conjugated anti-mouse Ig antibody.
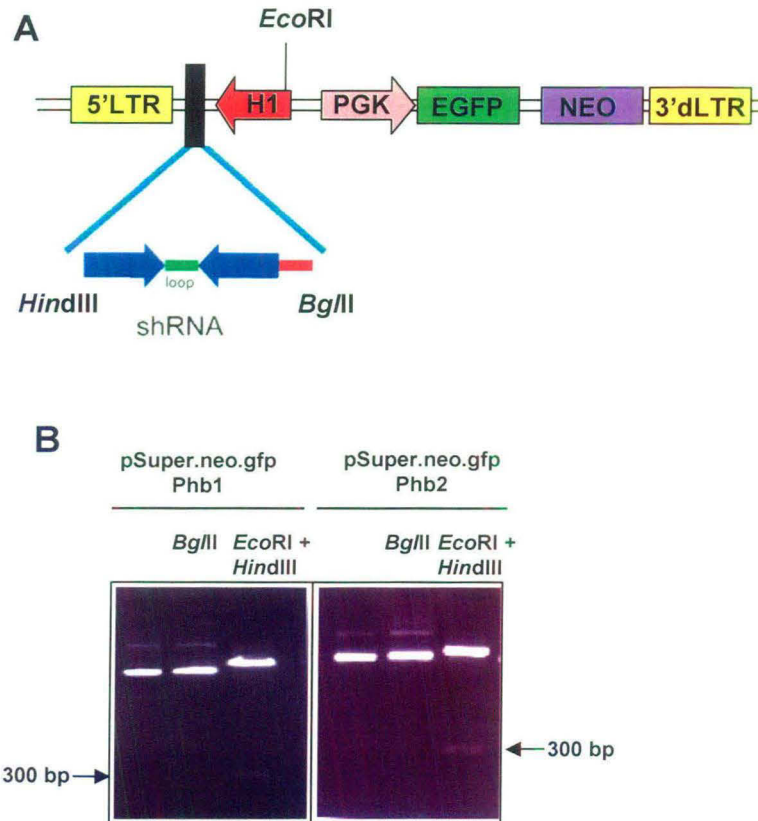
Figure 5. Cloning of oligonucleotides encoding prohibitin siRNA. (A) To generate a constitutively active knock-down of prohibitin, a set of different oligonucleotides encoding siRNAs targeting exons 1 and 3 (Phb1 and Phb2) were cloned into *Bgl*II and *Hind*III sites of the pSuper.neo.gfp vector under the control of the polymerase III promoter H1. **(B)** Positive clones (i.e., vector containing oligo insert) were screened by digesting with *Eco*RI and *Hind*III for release of ~300 base pair (bp) insert. Positive clones remain uncut with *Bgl*II. shRNA: short interfering hairpin RNA, siRNA: short interfering RNA.
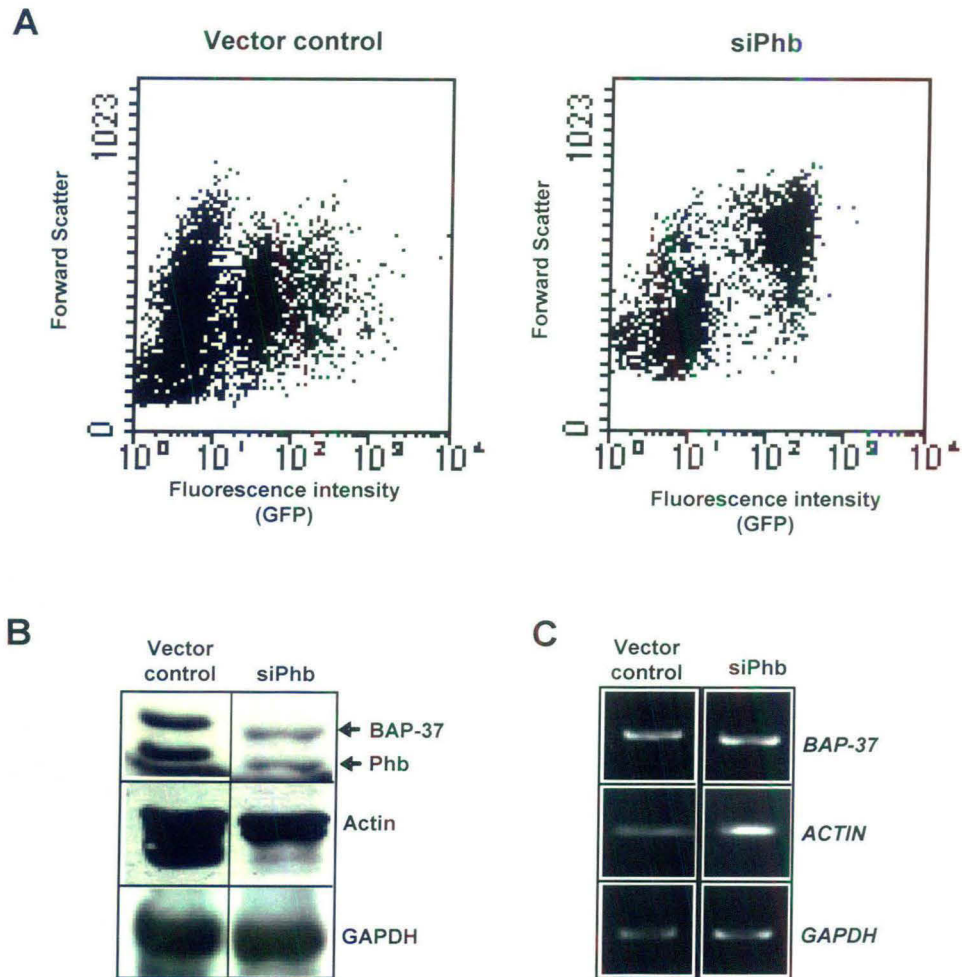
Figure 6. Knock-down of prohibitin expression. (A) U937 cells were transfected with empty vector or prohibitin siRNA expression vectors (siPhb1 and siPhb2). Cells were analyzed for gfp expression by flow cytometry in positive clones. **(B)** Cell lysates were prepared from U937 transfectants and run in a SDS-PAG, transferred to nitrocellulose membrane and blotted with antibodies to prohibitin (Phb), BAP-37, actin and GAPDH. **(C)** Total RNA was prepared from U937 transfectants and the relative amounts of endogenous mRNA for BAP-37, actin and GAPDH were analyzed by quantitative-reverse transcription–PCR (RT-PCR) using gene specific primers. siRNA: short interfering RNA; Vector control: cells transfected with empty vector; siPhb-cells transfected with prohibitin siRNA expression vectors.
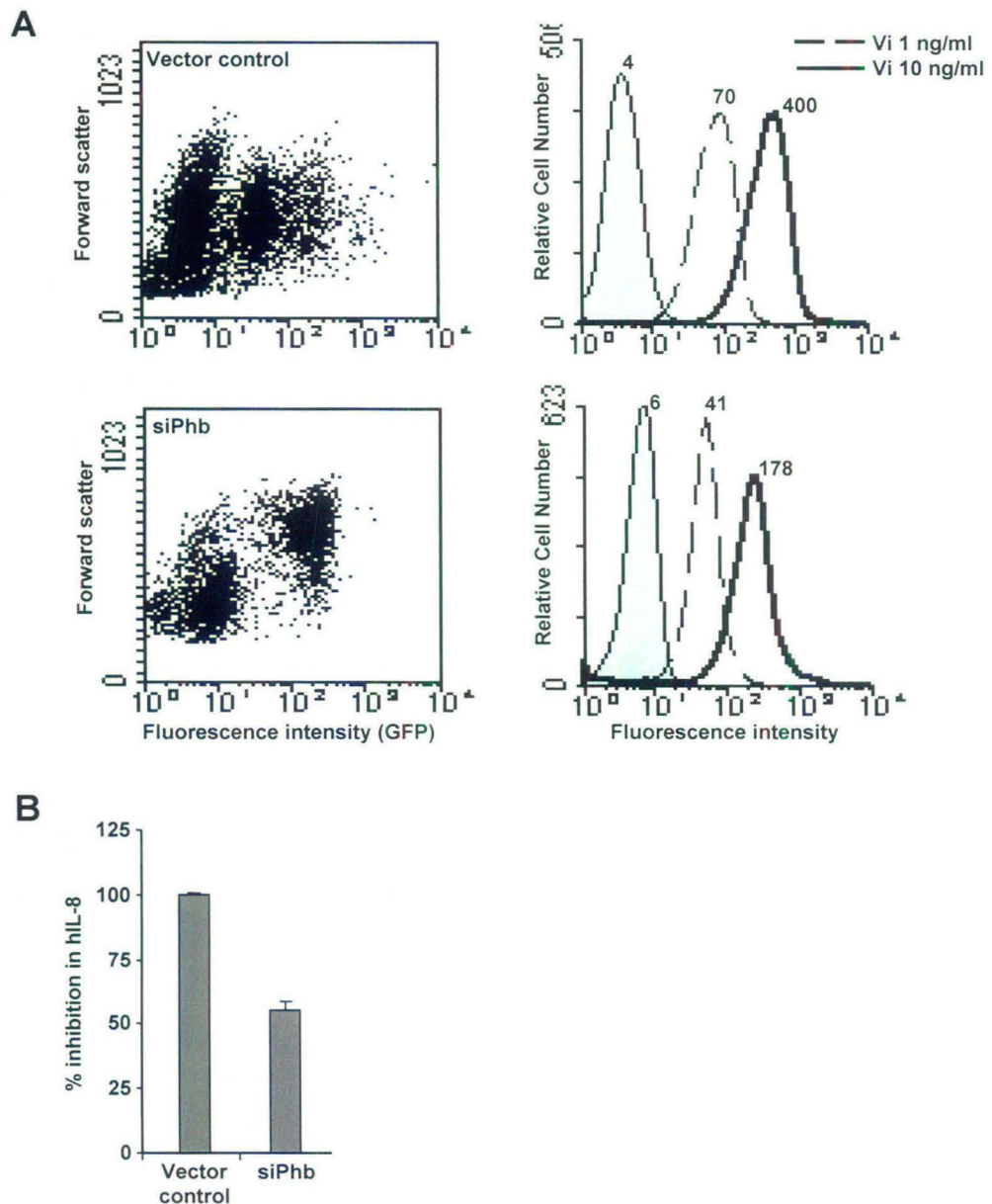
Figure 7. Prohibitin is required for inhibition of TLR5-induced IL-8 secretion by Vi.
(**A**) U937 cells transfected with an empty vector or prohibitin siRNA expression vectors (siPhb) were incubated with different concentrations of Vi followed by anti-Vi MoAb. Subsequently, cells were stained with PE-conjugated anti-mouse Ig antibody for 1 h and analyzed by flow cytometry. Numbers on the histogram represent mean fluorescence intensities. Shaded histogram show control cells incubated with anti-Vi MoAb and PE-labeled anti-mouse Ig antibody. (**B**) 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 secretion by Vi was calculated for each set. Vector control- cells transfected with empty vector.

Serum brings about switch of Vi from an anti- to a pro- inflammatory molecule

The inhibition produced by Vi in TLR5-triggered IL-8 secretion was best seen when stimulations were carried out under serum-free conditions. Serum abrogated anti-inflammatory effect of Vi in both U937 and THP-1 (**Figure 8A**). This phenomenon was previously observed with IEC as well (Sharma and Qadri, 2004). The loss of anti-inflammatory effect was in fact a result of Vi switching to a proinflammatory state, bringing about secretion of IL-8, TNF-α and IL-6 from THP-1 in a dose-dependent manner (**Figure 8B**). Such responses were not seen with IEC (Sharma and Qadri, 2004). The ability to promote IL-8 secretion with Vi was not restricted to FCS but was also observed with human and mouse serum (**Figure 8C**). Serum shows a concentration-dependent effect in enhancing Vi mediated cytokine secretion (**Figure 8D**). The induction of pro-inflammatory cytokines with Vi in presence of serum was not restricted to cell lines as stimulation of human PBMC with Vi under similar conditions also led to release of IL-8 and TNF-α (**Figure 9A**). Moreover, mouse bone marrow derived dendritic cells also showed secretion of pro-inflammatory cytokines when activated with Vi in presence of serum (**Figure 9B**).

Serum is known to upregulate LPS – induced inflammatory responses through TLR4. LPS interacts with serum-derived LPS-binding protein (LBP) which transfers LPS to the TLR4-CD14-MD2 complex and brings about a dramatic increase in LPS-induced cellular responses (Gioannini and Weiss, 2007; Wright *et al.*, 1989). To ascertain that IL-8 secretion produced by Vi was not due to any residual LPS in the vaccine preparation used in the present study, THP-1 stimulations were carried out in the presence of polymyxin B. Polymyxin B abolished LPS-induced responses but not Vi-induced responses (**Figure 10A**), demonstrating that the response with Vi was not due to the presence of small amounts of LPS, if any, in the Vi vaccine. However, depletion of Vi from the vaccine preparation using anti-Vi Ab-coated Protein-G-Sepharose beads, led to loss of cytokine stimulation from THP-1 (**Figure 10B**). Treatment with Lipase or Proteinase K did not abrogate cytokine secretion with Vi ruling out any contaminating protein or lipid in the vaccine preparation that might be responsible for inflammatory responses (**Figure 11A and B**).

The conversion of Vi from anti-inflammatory to proinflammatory state could be a result of reduced interaction of this polysaccharide with membrane prohibitin alongwith
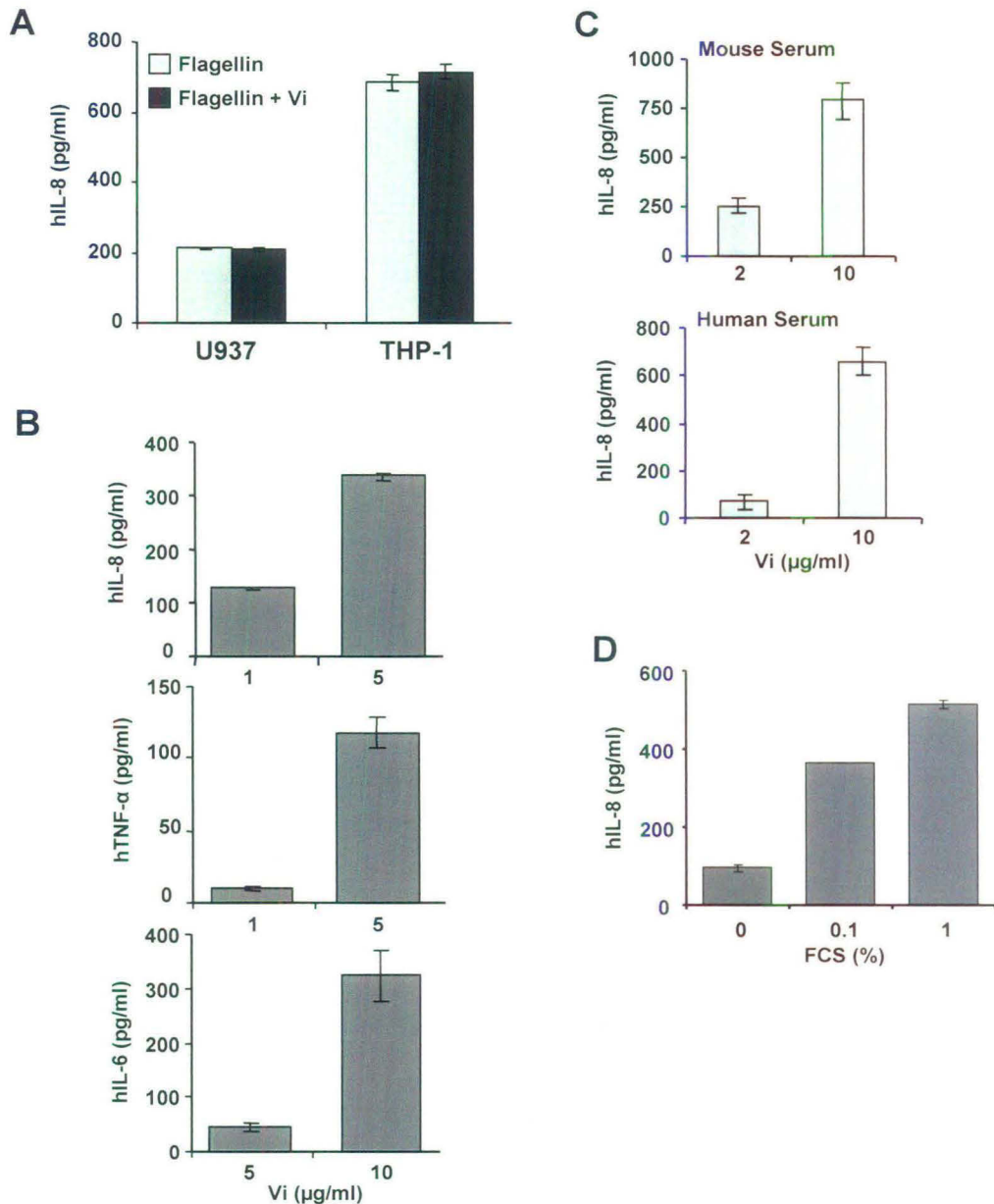
Figure 8. Serum converts Vi into a pro-inflammatory molecule. (**A**) Cells were incubated with Vi (10 µg/ml) in the absence of serum and 1 h later stimulated with flagellin (100 ng/ml) in presence of serum for 6 h. IL-8 was determined in the supernatants by ELISA. (**B**) THP-1 cells were incubated 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 - prestimulated THP-1 cells were incubated with different concentrations of Vi in the presence of serum for 24 h. (**C**) THP-1 cells were incubated with different concentrations of Vi in the presence of heat inactivated human or mouse serum. Supernatants were collected after 6 h for IL-8 analysis. (**D**) THP-1 cells were incubated with Vi (5 µg/ml) in the presence of different concentrations of serum (FCS) for 6 h. IL-8 was determined in the supernatants by ELISA.

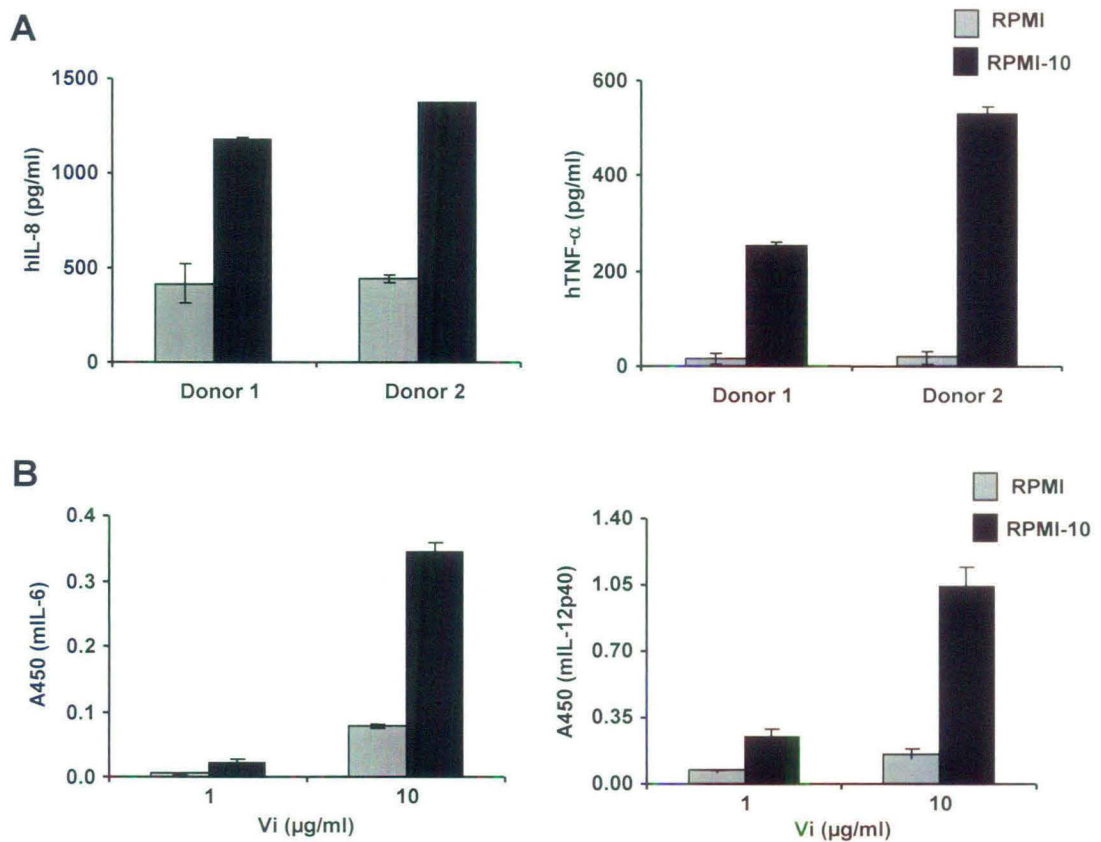**Figure 9. Vi induces pro-inflammatory cytokine secretion from human PBMC and mouse BMDC.** (**A**) Human PBMC were incubated with Vi (5 μg/ml) in the absence or presence of serum for 24 h. IL-8 and TNF-α were determined in the supernatants by ELISA. (**B**) Mouse BMDC were incubated with Vi (1 and 10 μg/ml) in the absence or presence of serum for 24 h. IL-6 and IL-12p40 was determined in the supernatants by ELISA.
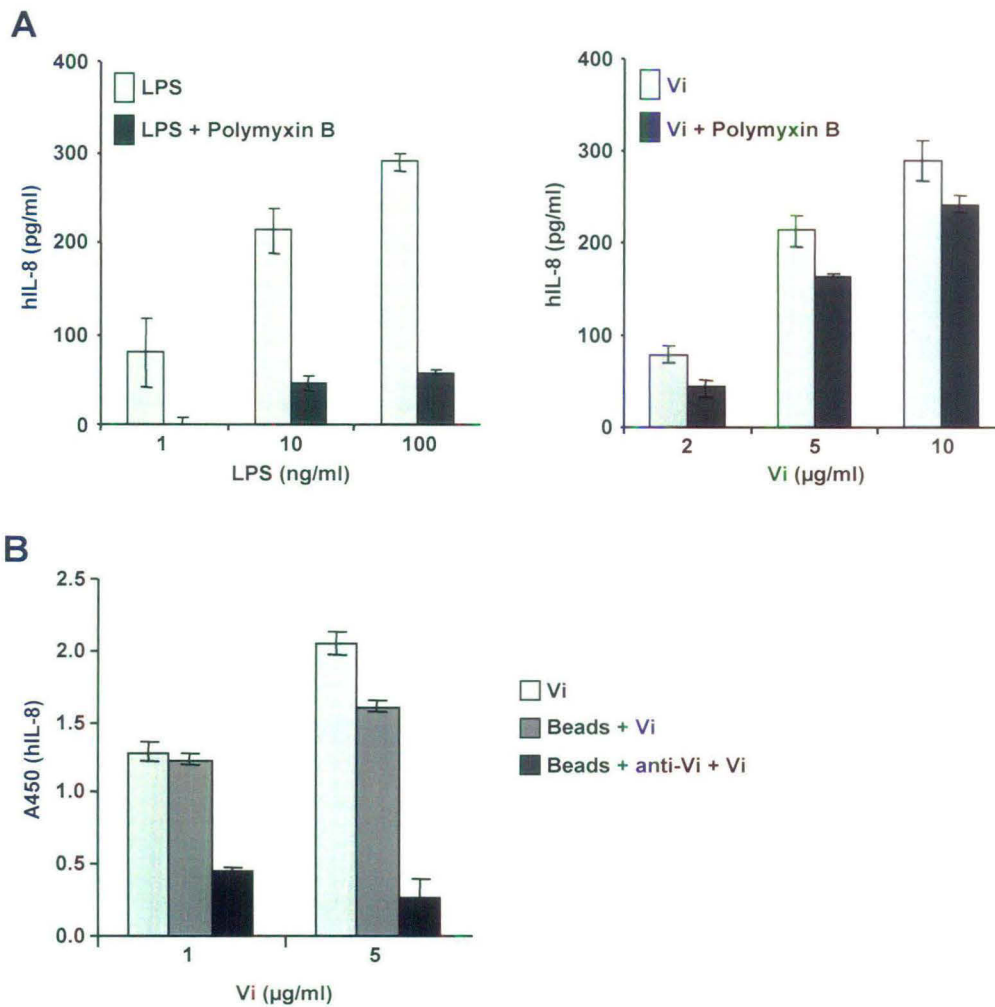
Figure 10. Polymyxin B does not abrogate induction of IL-8 secretion by Vi. (A) THP-1 cells were incubated with different concentrations of LPS or Vi in the presence or absence of polymyxin B (10 μg/ml) for 6 h. IL-8 was determined in the supernatants by ELISA. **(B)** Vi vaccine (50 μg/ml) was incubated 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.
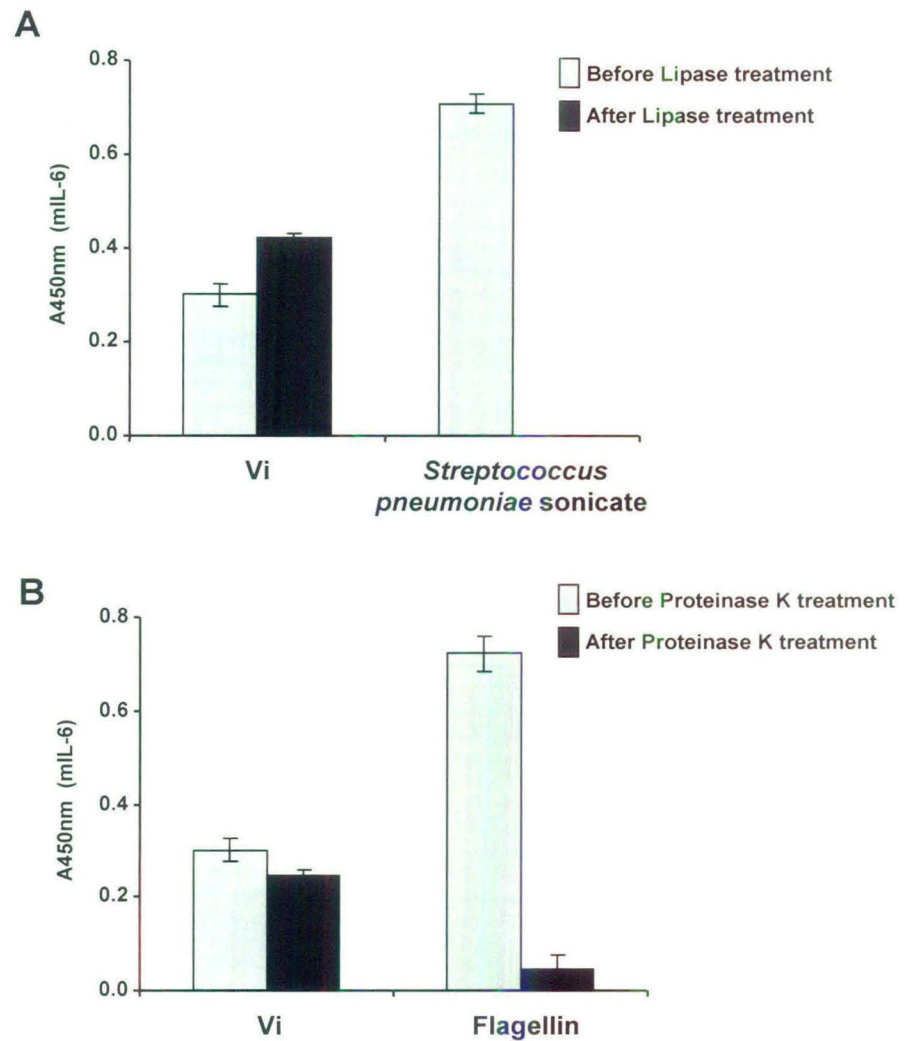
Figure 11. Treatment with Proteinase K or Lipase does not abrogate induction of IL-6 secretion by Vi. (**A**) Vi (5 µg/ml) or heat killed *Streptoccocus pneumoniae* sonicate (10 µg/ml) were incubated with Lipase for 1 h at 37°C followed by heat denaturation of the enzyme for 1 h at 80°C. Untreated and Lipase-treated preparations were incubated with mouse macrophage cell line GG2EE for 24 h at 37°C. IL-6 was determined in supernatants by ELISA. (**B**) GG2EE cells were incubated with untreated or Proteinase K-treated Vi (5 µg/ml) or *S.typhimurium* flagellin (50 ng/ml) for 24 h at 37°C. Supernatants were collected after 24 h for IL-6 analysis.

simultaneous engagement of another cellular receptor that would enable it to produce inflammatory responses. Vi showed reduced interaction with cells in presence of serum in a concentration-dependent manner (**Figure 12A**). This interaction with serum led to conversion of Vi into a form that did not bind prohibitin readily (**Figure 12B and C**). These results suggested that modification by serum might indeed facilitate interaction of Vi with an activating receptor while abrogating its binding to the inhibitory prohibitin complex.

Serum-derived hemoglobin converts Vi into a proinflammatory molecule

The molecule(s) in serum responsible for transforming Vi into a proinflammatory molecule was proteinaceous in nature as digestion with Proteinase K abrogated the ability of serum to promote inflammatory responses with Vi (**Figure 13A**). Vi bound to cells in presence of Proteinase K – digested serum as readily as in the absence of serum (**Figure 13B**) again suggesting that transformation of Vi into a proinflammatory state was dependent on its interaction with a proteinaceous molecule in serum. To identify the nature of this molecule, an immunoprecipitation was carried out by incubating Vi with different concentrations of FCS in the presence of anti-Vi antibodies. This resulted in the formation of precipitates which were not readily seen when Vi was incubated with anti-Vi antibodies in the absence of serum. This precipitate formation was specific to Vi as incubation of LPS with anti-LPS antibodies (same isotype as anti-Vi antibody) did not result in any visible precipitate. Silver staining of precipitated components showed that Vi could specifically interact with three serum proteins (**Figure 14**); the higher molecular weight major proteins and rest of the minor bands were derived from anti-Vi antibody. N-terminal amino acid sequence analysis identified these three proteins as hemoglobin, apolipoprotein A-I and fetuin (**Figure 14**). These data suggested that one or more of these proteins might be involved in generating inflammatory responses with Vi. This possibility was tested by carrying out stimulation of THP-1 with Vi in the presence of these proteins. The results showed that only purified adult hemoglobin could promote Vi-induced IL-8 secretion in a dose-dependent manner (**Figure 15A**). Of the two chains of hemoglobin, β chain was significantly more efficient than α chain at potentiating Vi-activated IL-8 secretion from THP-1 (**Figure 15B**). However, purified heme did not have any effect on Vi-mediated IL-8 secretion. The induction of inflammatory responses with Vi in presence of hemoglobin
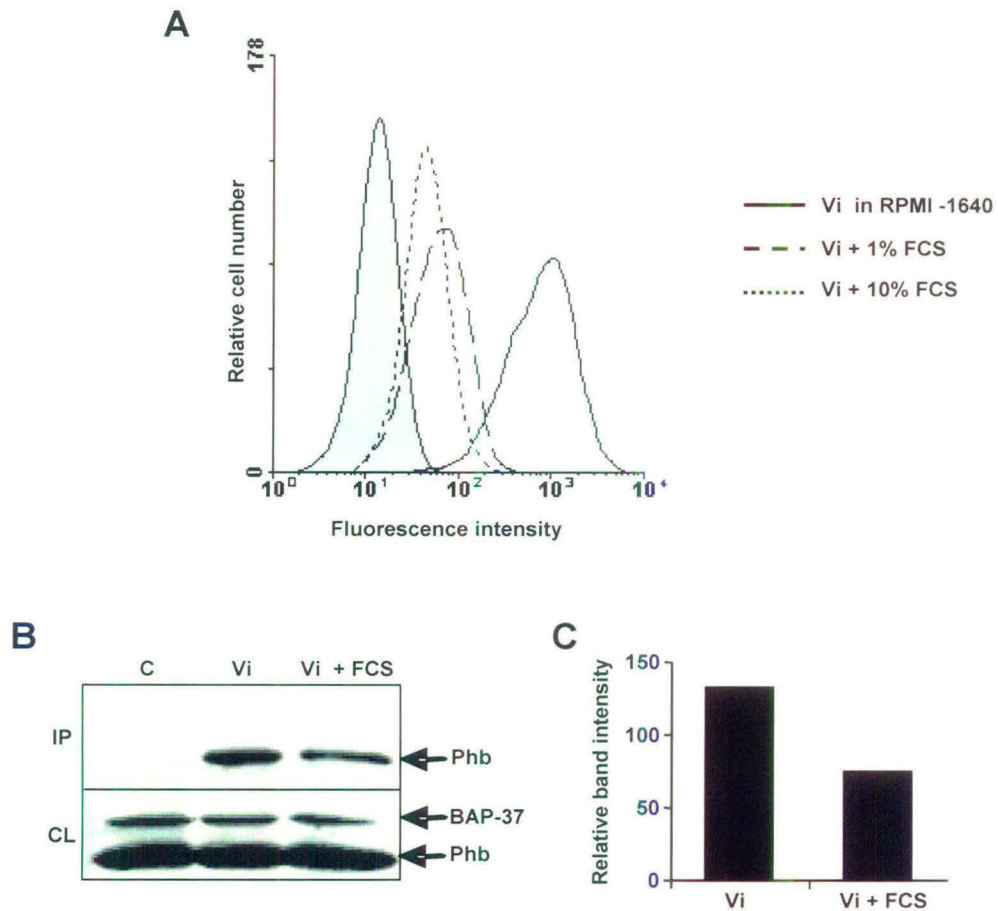
Figure 12. Serum-modified Vi shows reduced binding to prohibitin. (**A**) THP-1 cells were incubated with Vi (1 µg/ml) in the absence or presence of different concentrations of FCS followed by anti-Vi MoAb. Subsequently, cells were stained with FITC-conjugated anti-mouse Ig antibody for 1 h and analyzed by flow cytometry. Shaded histogram shows staining in cells incubated with anti-Vi MoAb and FITC-conjugated anti-mouse Ig antibody. (**B**) THP-1 cells were incubated with Vi (10 µg/ml) in RPMI or RPMI supplemented with 1% FCS at 4°C for 1 h. Cell lysates were immunoprecipitated with anti-Vi MoAb loaded on Protein-G-Sepharose beads and immunoblotted with anti-prohibitin antibodies. C – lysate from untreated cells, Vi – lysate from cells treated with Vi in RPMI, Vi + FCS – lysate from cells treated with Vi in RPMI-1% FCS, IP - immunoprecipitation, CL – cell lysates. (**C**) Relative band intensity of prohibitin immunoprecipitated with Vi and Vi + 1% FCS in (**B**) was determined by densitometry scanning using ImageJ software.

A

RPMI-1640
Vi in RPMI-1640
Vi + FCS
Vi + Proteinase K-digested FCS

B

Vi in RPMI-1640
Vi + FCS
Vi + Proteinase K-digested FCS

Fluorescence intensity
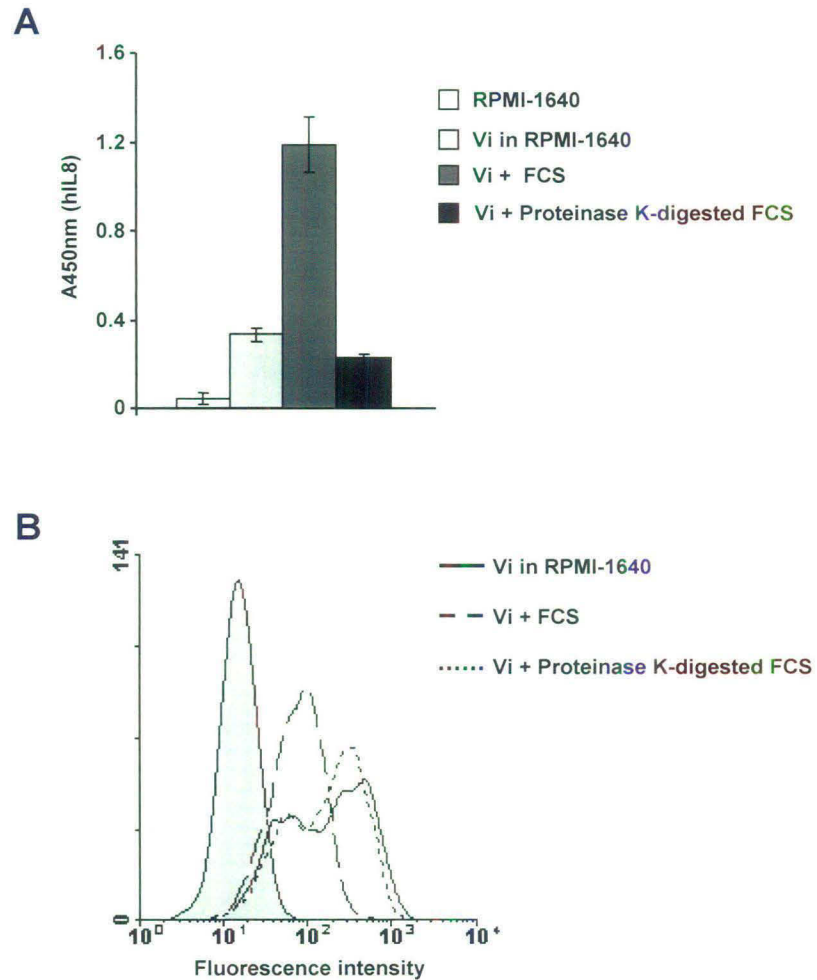
Figure 13. Serum factor interacting with Vi is proteinaceous in nature. (**A**) THP-1 cells were incubated with Vi (1 µg/ml) in the absence or presence of undigested or Proteinase K-digested FCS (0.1%) for 6 h. IL-8 was determined in the supernatants by ELISA. (**B**) THP-1 cells were incubated with Vi (100 ng/ml) in the absence or presence of undigested or Proteinase K-digested FCS (0.1%) followed by anti-Vi MoAb. Subsequently, cells were stained with FITC-conjugated anti-mouse Ig antibody for 1 h and analyzed by flow cytometry. Shaded histograms show staining in cells incubated with anti-Vi MoAb and FITC-conjugated anti-mouse Ig antibody.
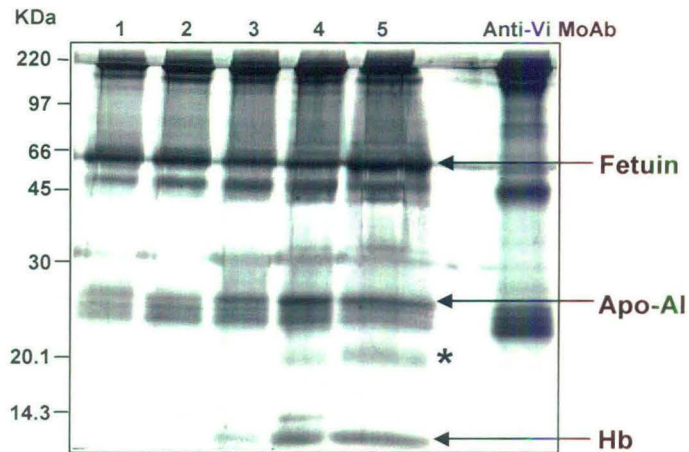
Figure 14. Vi interacts with serum-derived fetuin, Apo-AI and hemoglobin. Vi was incubated for 48 h with different concentrations of serum in presence of anti-Vi monoclonal antibody (MoAb). Precipitates were pelleted down at 12,000xg, electrophoresed in a 12.5% SDS-PAG and silver stained. Vi-interacting proteins were identified as hemoglobin (Hb), apolipoprotein A-I (Apo-AI) and fetuin by N-terminal sequencing. Lanes 1-5: 1%, 5%, 10%, 50%, and 80% serum respectively incubated with anti-Vi antibody and Vi. * identified as antibody light chain fragment.
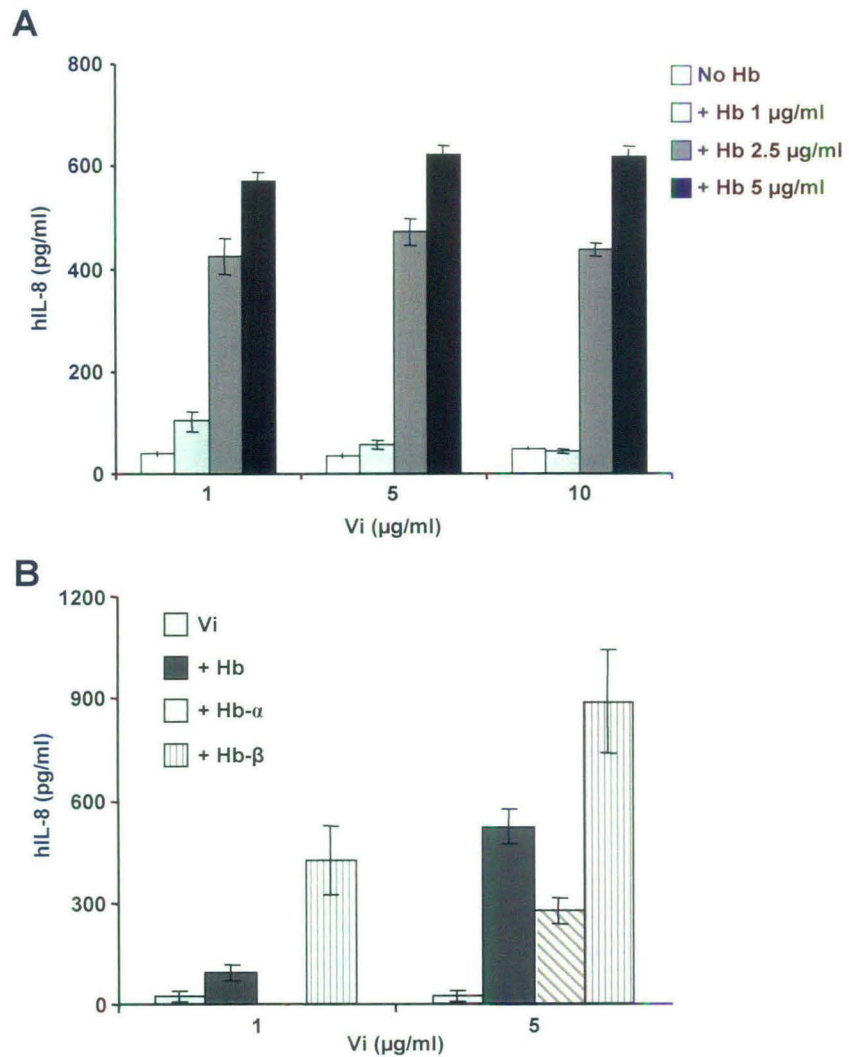
Figure 15. Serum-derived hemoglobin converts Vi into a proinflammatory molecule. THP-1 stimulations were carried out with Vi in the absence or presence of hemoglobin (Hb; **A**), hemoglobin α chain (Hb-α) or β chain (Hb-β; 5 µg/ml each; **B**) for 6 h. IL-8 was determined in the supernatants by ELISA.

suggested that these two might form a molecular complex. Hemoglobin bound to biotinylated Vi loaded on avidin beads (**Figure 16A**). The formation of a molecular complex between Vi and hemoglobin was revealed by a change in the mobility of Vi in a non-denaturing gel. Vi being a very high molecular weight polymer showed poor migration into gel. However, when it is incubated with hemoglobin or its β chain, it migrated readily into the gel as revealed by western blotting with anti-Vi monoclonal antibody (**Figure 16B**), indicating change in the physical state of Vi that resulted very likely from disaggregation (**Figure 16B**). This shift in migration of Vi was not readily observed with the α chain of hemoglobin or apolipoprotein A-I (**Figure 16B**). The binding of Vi to hemoglobin or its β chain was also associated with a mobility shift in hemoglobin molecules (**Figure 16B**). The interaction between Vi and hemoglobin was further established by surface plasmon resonance (SPR) in which Vi showed a dose-dependent binding to hemoglobin immobilized on a sensor chip (**Figure 16C**).

To understand whether the inflammatory response was produced by hemoglobin modified Vi or by Vi – hemoglobin complex, the two molecules were allowed to bind to each other and this mixture was subjected to gel filtration chromatography. Native gel analysis of fractions obtained on gel filtration of Vi-hemoglobin mixture demonstrated that interaction with hemoglobin led to generation of a novel Vi species that separated later than native Vi which came out in the void volume (**Figure 17A**). This species was not seen when Vi alone was subjected to gel filtration chromatography (**Figure 17A**).This faster migrating Vi, which retained reactivity with N- as well as O- acetyl recognizing anti-Vi monoclonal antibodies, was capable of activating IL-8 secretion from THP-1 (**Figure 17B**). However, if the mixture of Vi-hemoglobin was digested with Proteinase K before running in the gel filtration column, generation of this new species was completely abrogated (**Figure 17B**). These results suggested that dynamic interaction between Vi and hemoglobin was essential for maintaining the modified state of Vi and hence transformed its pro-inflammatory character. It is likely, that hemoglobin modifies Vi in a manner similar to how it modifies LPS (Howe *et al.*, 2007; Jurgens *et al.*, 2001). It has been suggested that interaction between hemoglobin and LPS leads to change of the three-dimensional arrangement of LPS aggregates from unilamellar into cubic LPS aggregates that resulted in increased biological activity of LPS (Howe *et al.*, 2008; Kaca *et al.*, 1994).
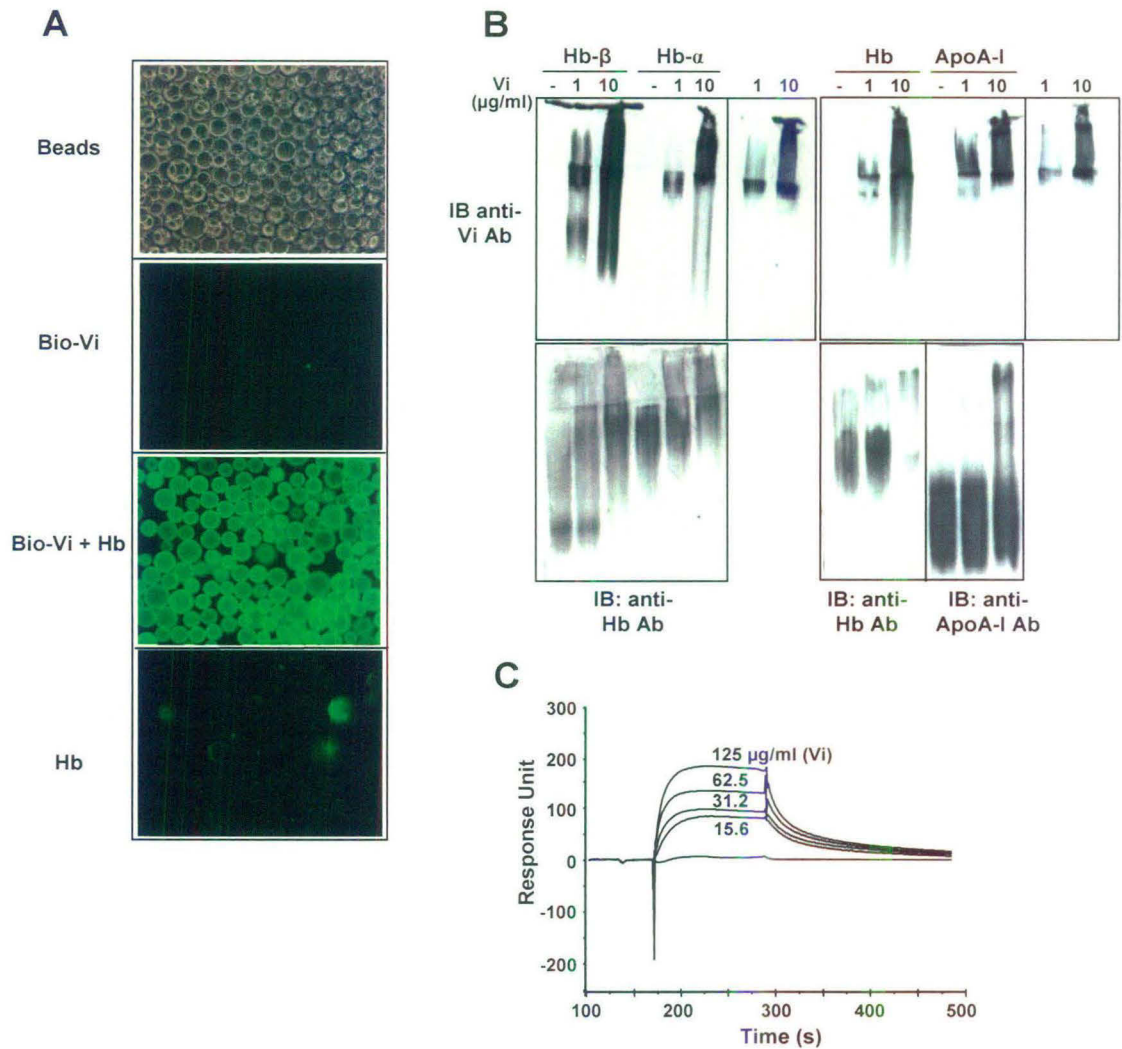
Figure 16. Interaction of Vi with hemoglobin. (**A**) Biotinylated Vi was incubated with Hb at room temperature. After 1 h, avidin beads were added to this mixture and allowed to incubate for another 1 h. As controls, beads were incubated with biotinylated Vi (bio-Vi) and Hb separately. After extensive washing, beads were probed with mouse anti-Hb antibodies and subsequently with FITC conjugated anti-mouse Ig antibody. Beads were visualized under a fluorescent microscope. (**B**) Vi was incubated with Hb-β, Hb-α, Hb or ApoA-I (1 µg each), run in a native polyacrylamide gel and transferred to nitrocellulose (NC) membrane. The NC membranes were probed with antibodies to Vi, Hb and ApoA-I. IB - immunoblot. (**C**) Hb was immobilized on a CM-5 sensor chip and incubated with different concentrations of Vi (µg/ml). The binding was continuously monitored in a surface plasmon resonance (SPR) biosensor.

A

Fractions 4 5 6 7 8 9 M

Vi

Vi + Hb

IB: anti-Vi Ab

B
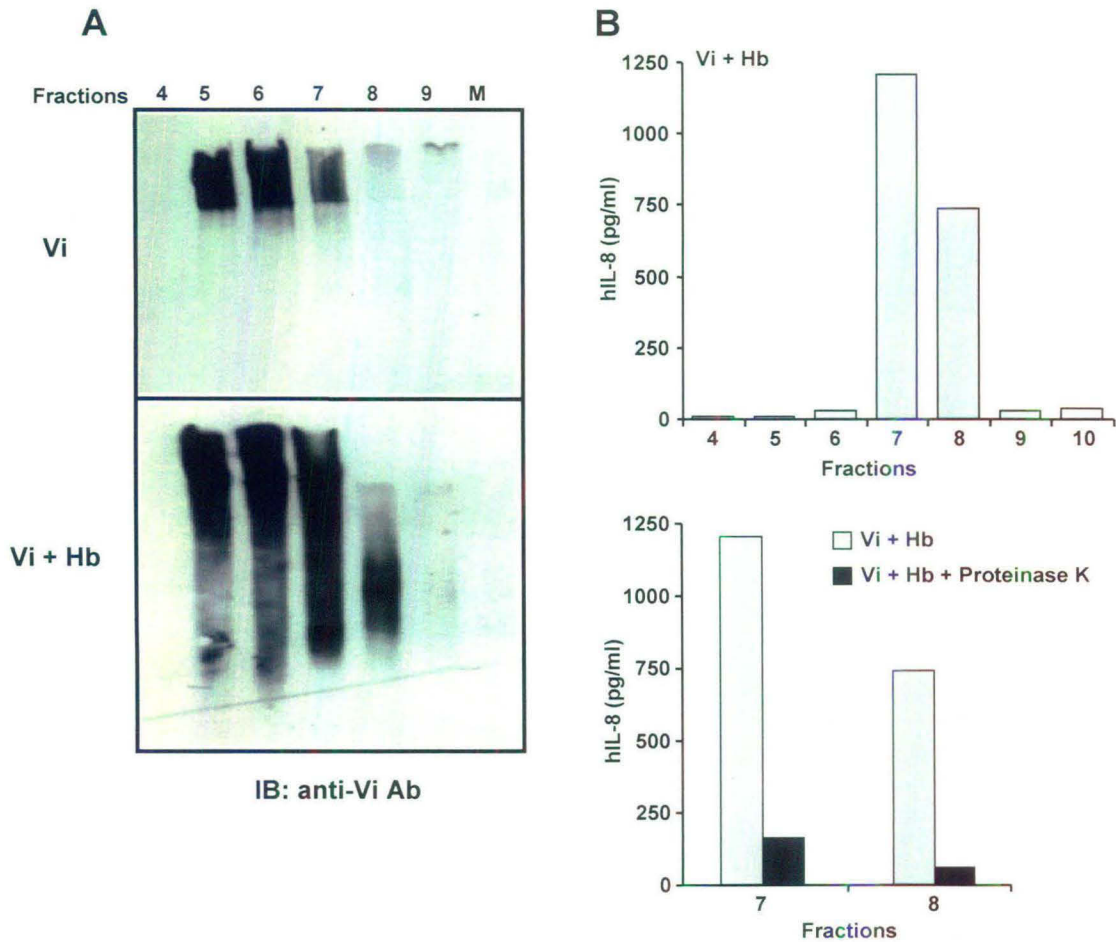
Figure 17. Conversion of Vi into a proinflammatory molecule requires dynamic interaction with hemoglobin. Vi was separated on a gel filtration BioSep-Sec-S2000 column. Alternatively, Vi was incubated with Hb for 1 h followed by gel filtration or Vi + Hb mixture was digested with Proteinase K-before before loading on GFC column. (A) Fractions of Vi alone or Vi + Hb were analyzed in a native gel, and probed with anti-Vi antibodies. IB – immunoblot. (B) THP-1 cells were stimulated for 6 h with various fractions obtained after passing untreated Vi + Hb mixture or Proteinase K-digested Vi + Hb mixture through GFC column. IL-8 was determined in the supernatants by ELISA.

Proinflammatory Vi engages TLR2 to activate cellular responses

The ability of Vi to induce inflammatory responses in presence of serum was also observed with 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 (**Figure 18A**). This data suggested that TLR4 may not be involved in the induction of cytokines by Vi. This observation coupled with previous studies implicating TLR2 in recognition of polysaccharides (Wang *et al.*, 2006; Gantner *et al.*, 2003), prompted us to analyze role of TLR2 in inflammatory responses produced by Vi. The secretion of IL-8 from THP-1 stimulated with Vi was specifically blocked by anti-TLR2 monoclonal antibody (**Figure 18B**); an isotype matched antibody did not inhibit responses with Vi. These results suggested that Vi might engage TLR2 on THP-1 cells to generate inflammatory responses. The serum / hemoglobin – promoted IL-8 secretion with Vi was however not seen with U937 even though it expressed TLR2 albeit at lower levels than THP-1 (**Figure 19A and B**). U937 was also unresponsive to known TLR2/TLR1 agonist Pam$_3$CSK$_4$ but responded well to *Streptococcus pneumoniae* sonicate (**Figure 19B**) suggesting that this cell line might be responsive to TLR2/TLR6 ligands but not to TLR2/TLR1 ligands. Together, these results suggested that Vi might stimulate IL-8 secretion through specific engagement of TLR2/TLR1. To establish role of this heterodimer in induction of inflammatory responses with Vi, HEK293-T cells, which are otherwise unresponsive to TLR ligands, were transfected with human TLR2/TLR1 or TLR2/TLR6 constructs (**Figure 20A**). Vi induced IL-8 secretion from HEK293-T cells transfected with TLR2/TLR1 but not from untransfected cells or cells transfected with TLR2/TLR6 (**Figure 20B**). Importantly, the ability of hemoglobin to mimic serum in promoting Vi-induced IL-8 secretion was also evident in TLR2/TLR1 transfected HEK293-T (**Figure 20B and C**). The induction of some amount of IL-8 from TLR2/TLR1 transfected HEK293-T (**Figure 20B**) and human PBMCs (**Figure 9A**) with Vi in absence of serum might be attributed to higher sensitivity of these cells as compared to THP-1 as these cells responded better to known TLR2 agonist Pam$_3$CSK$_4$. Serum-independent response also suggested that the vaccine preparation might have small amounts of pre-existing proinflammatory Vi present in it.
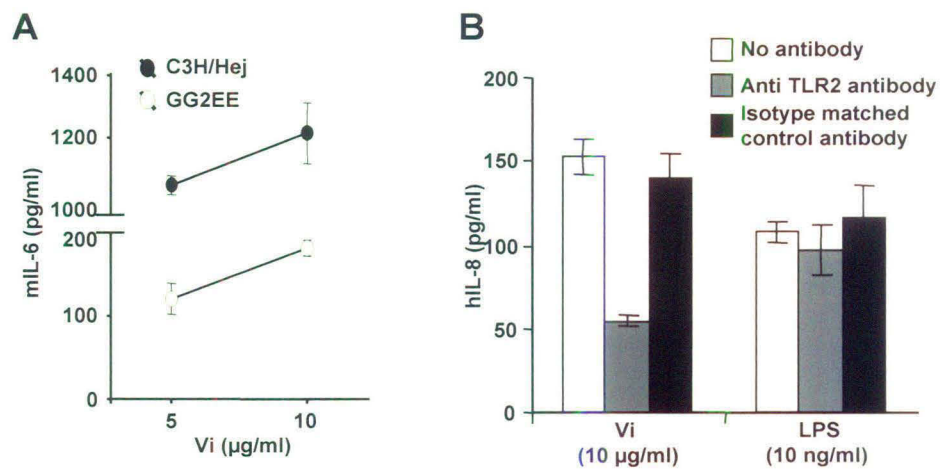
Figure 18. Vi produces inflammatory responses through interaction with TLR2. (A) LPS-hyporesponsive bone marrow-derived macrophage cell line GG2EE or peritoneal macrophages from LPS-hyporesponsive C3H/HeJ mice were incubated with different concentrations of Vi in the presence of serum for 6 h after which supernatants were analyzed for IL-6. **(B)** THP-1 cells were incubated with Vi or LPS in serum-supplemented medium in the presence or absence of neutralizing anti-TLR2 antibody or an isotype control antibody (30 µg/ml) for 6 h. IL-8 was determined in the supernatants by ELISA.
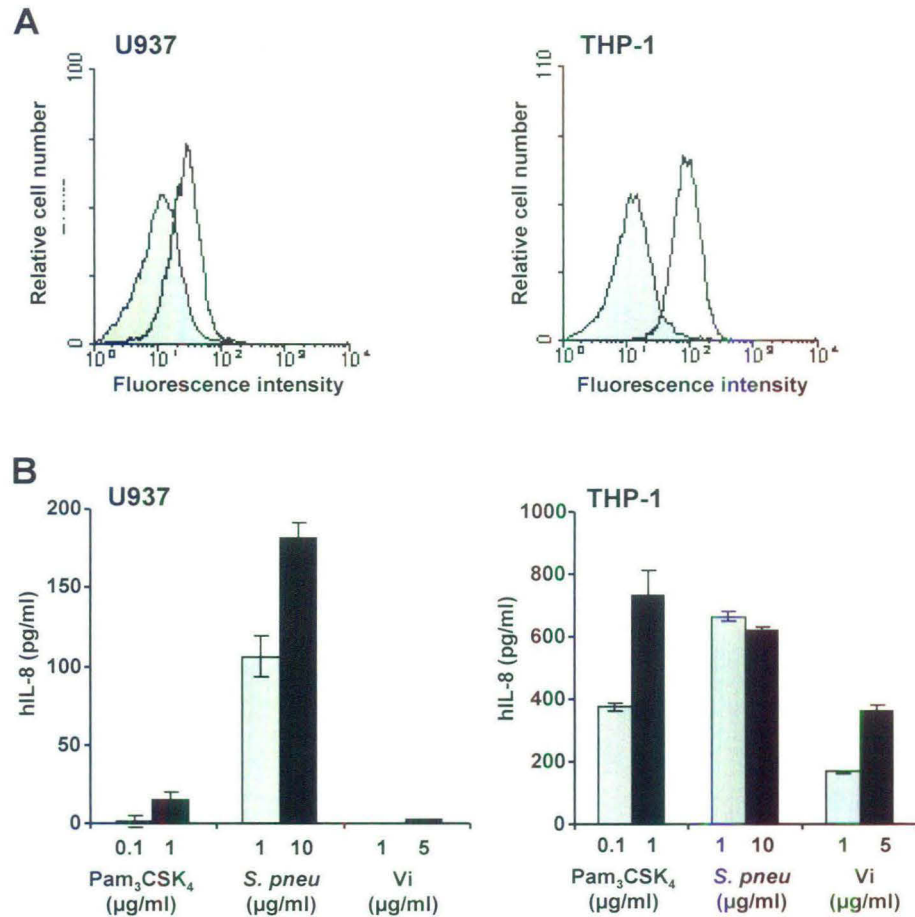
Figure 19. THP-1 but not U937 is responsive to TLR2/TLR1 agonist. (**A**) Cells were incubated with anti-TLR2 MoAb. Subsequently, cells were stained with FITC-conjugated anti-mouse Ig antibody and analyzed by flow cytometry. Shaded histograms show staining with isotype control antibody. (**B**) Cells were stimulated with Pam$_3$CSK$_4$, heat killed *Streptococcus pneumoniae* (*S. pneu*) sonicate or Vi in presence of serum for 6 h after which supernatants were collected for IL-8 analysis.
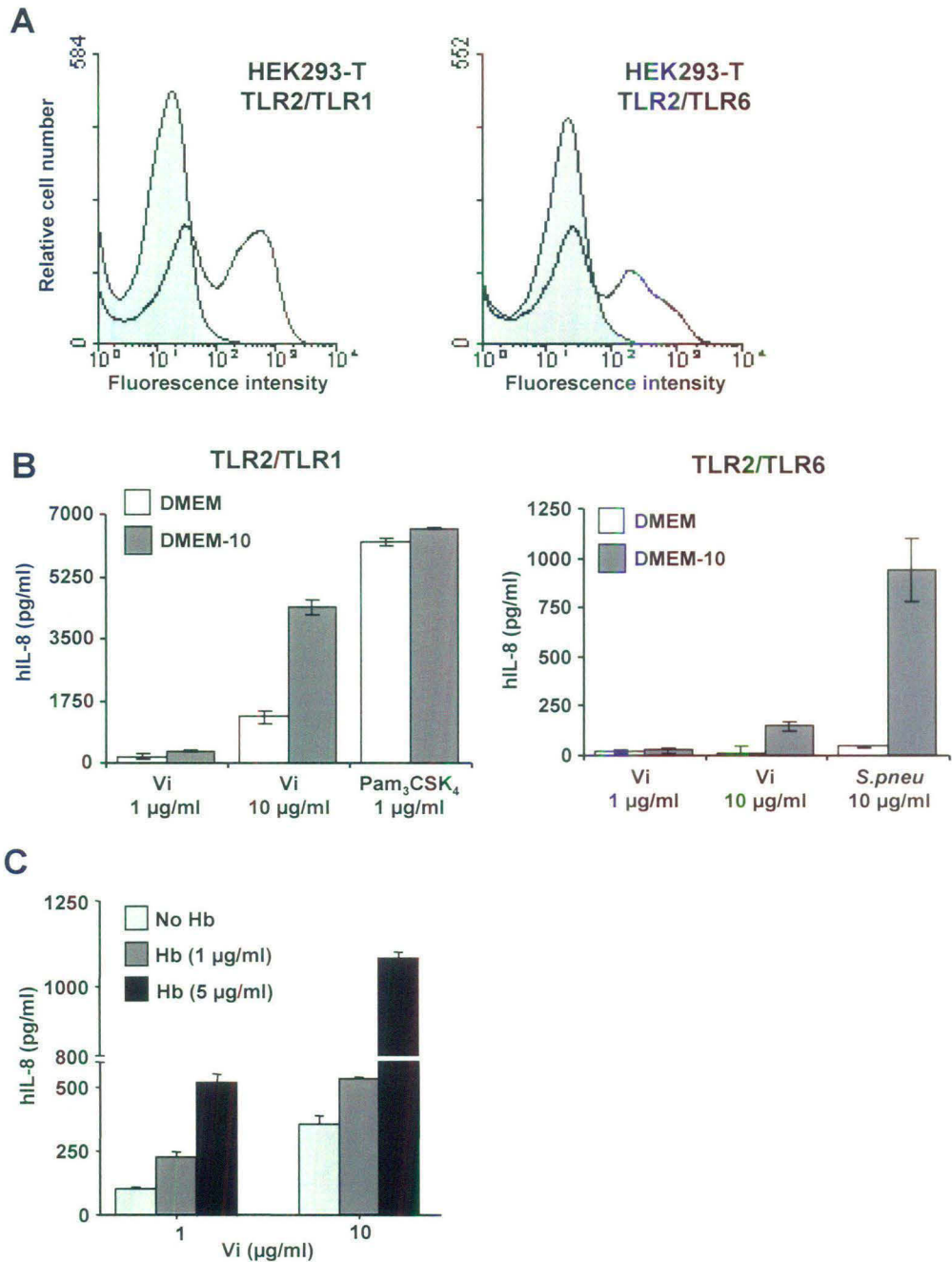
Figure 20. Vi interacts with TLR2/TLR1 heterodimer to produce inflammatory responses. (**A**) HEK293-T cells transfected with TLR2/TLR1 or TLR2/TLR6 constructs were incubated with anti-TLR2 MoAb followed by FITC-conjugated anti-mouse Ig antibody and analyzed by flow cytometry. Shaded histograms show staining with isotype control antibody. (**B**) HEK293-T cells transfected with TLR2/TLR1 or TLR2/TLR6 were incubated with Vi, Pam_3CSK_4, and heat killed *Streptococcus pneumoniae* (*S.pneu*) sonicate in presence or absence of serum for 6 h after which supernatants were collected for IL-8 analysis. (**C**) HEK293-T cells transfected with TLR2/TLR1 were incubated with various concentrations of Vi in presence or absence of hemoglobin. IL-8 was determined in culture supernatants by ELISA.

Vi brings about activation of MAP-kinase and NF-kB pathways of intracellular signaling in presence of serum

Engagement of TLR2 on monocytes is known to induce secretion of cytokines and chemokines through activation of MAP-kinase and NF-kB signaling pathways (Takeda and Akira, 2005). Analysis of these pathways in cells stimulated with Vi showed that this polysaccharide could trigger degradation of I-kB and phosphorylation of MAP-kinases, ERK and p38, when cell stimulations were carried out in presence of serum (**Figure 21A**). Consistent with these data, Vi - induced IL-8 secretion from THP-1 was suppressed in the presence of inhibitors PD98059 and SB203580 which target activation of MEK and p38 MAP-kinase respectively (**Figure 21B**). In addition JNK II inhibitor also downregulated Vi-mediated IL-8 secretion (**Figure 21B**) suggesting that JNK signaling might also be contributing to induction of cytokines during activation of TLR2 by Vi.

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

Acetyl groups have been previously shown to be important for generating antibody responses with Vi (Szu *et al.*, 1991). To study the role of these groups in the induction of inflammatory responses, Vi was either partially deacetylated to remove acetyls from N-acetyl groups (NDeVi) or fully deacetylated to remove acetyls from both N- as well as O-acetyl groups (DeVi). N- and O- deacetylation was monitored by reactivity with anti-Vi monoclonal antibodies recognizing different determinants on Vi (Qadri *et al.*, 1990; **Figure 22**). 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 (**Figure 23A**). However, partially deacetylated Vi (NDeVi) which retained most of the O-acetyl groups (**Figure 22**) did trigger IL-8 secretion from macrophages (**Figure 23A**) indicating that O-acetyls might be the critical functional determinants involved in the induction of chemokine secretion by Vi. Significantly, deacetylation of Vi was also associated with inability to interact with hemoglobin. DeVi did not bring about mobility shift in hemoglobin nor did it bind to hemoglobin bound to a chip (**Figure 23 B, C**). On the other hand, NDeVi showed a dose - dependent binding to hemoglobin (**Figure 23 B, C**). Consistent with these results commercially available polygalacturonic acid which is chemically similar to Vi except for the lack of acetyl groups also did not trigger any IL-8 secretion from THP-1 cells (**Figure 23A**). Interestingly, even O-acetylated
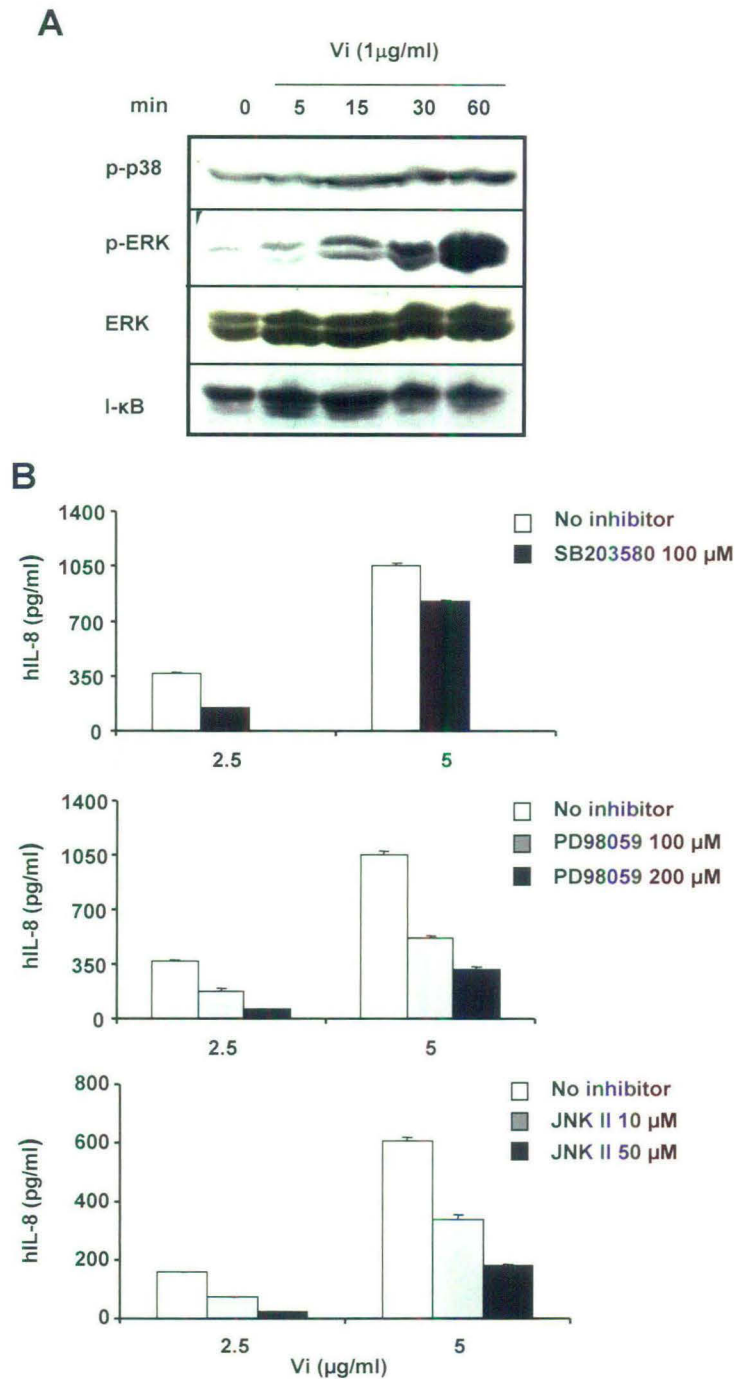
Figure 21. Vi induces cytokine secretion through activation of NF-κB and MAP Kinases. (A) THP-1 cells were incubated of Vi (1 μg/ml) in the presence of serum for various time points and cell lysates were probed with antibodies to phospho-p38, phospho-ERK, ERK and I-κB after running in SDS-PAG and transferring to nitocellulose membrane. **(B)** THP-1 cells were incubated with inhibitors to MEK (PD98059), p38-MAPK (SB203580) and JNK II for 30 min before stimulating with Vi for 6 h. Supernatants were analyzed for IL-8 by ELISA.
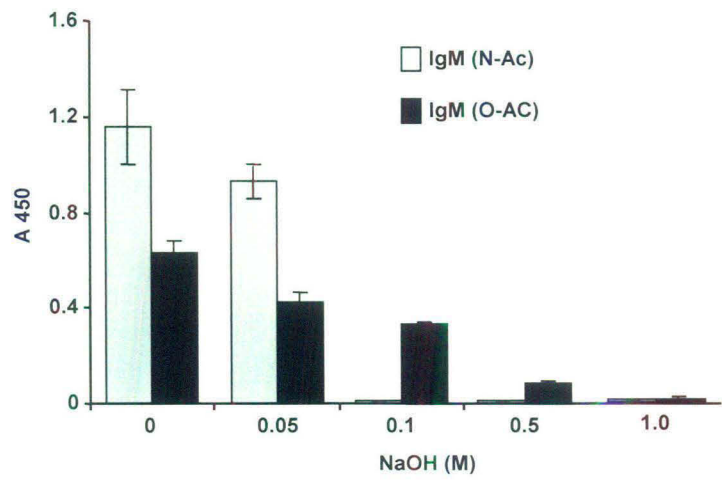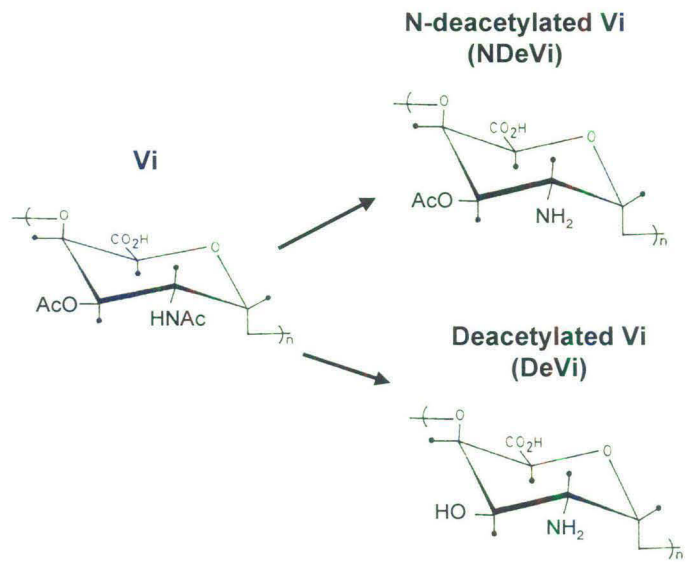
Figure 22. Deacetylation of Vi with NaOH. Vi was incubated with different concentrations of NaOH (M) for 30 min. Deacetylation of N- and O-acetyl groups was ascertained by reactivity in ELISA with anti-Vi monoclonal antibodies directed against O-acetyl (IgM-OAc) and N-acetyl (IgM-NAc) – dependent antigenic determinants in Vi.
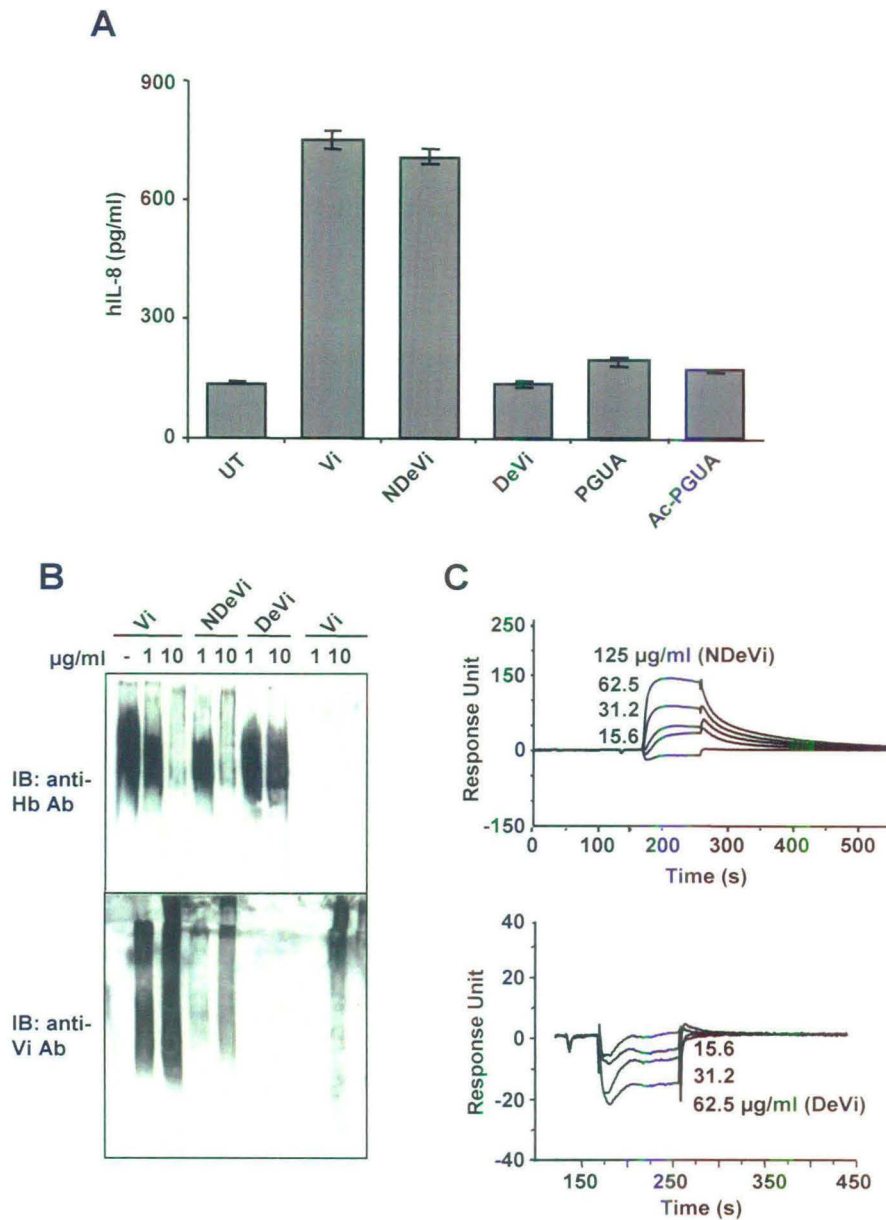
Figure 23. Acetyl groups are required for the induction of inflammatory responses with Vi. (**A**) THP-1 cells were incubated with Vi, N-deacetylated Vi (NDeVi), totally deacetylated Vi (DeVi), polygalacturonic acid (PGUA) or O-acetylated polygalacturonic acid (Ac-PGUA) (10 µg/ml each) in the presence of serum for 6 h. Supernatants were analyzed for IL-8 by ELISA. (**B**) Vi, NDeVi and DeVi (1 or 10 µg) were incubated with hemoglobin (1 µg) for 1 h at room temperature, run in a native polyacrylamide gel and transferred to a NC membrane. The NC membrane was immunoblotted (IB) with antibodies to Hb and Vi. (**C**) Hb was immobilized on a CM-5 sensor chip and incubated with different concentrations of NDeVi and DeVi (µg/ml). The binding was continuously monitored in an SPR biosensor.

polygalacturonic acid did not activate IL-8 secretion from THP-1 suggesting that O-acetylation at C-3 might not be sufficient to produce inflammatory responses with Vi (**Figure 23A**). Therefore, in addition to O-acetyl groups, a specific configuration of Vi that might be produced following its interaction with hemoglobin is likely required to impart proinflammatory character to this polysaccharide.

Vi also induced IL-6 and IL-12p40 from murine splenocytes *ex vivo* and similar to THP-1, proinflammatory cytokine secretion from mouse splenocytes was observed with NDeVi but not with DeVi (**Figure 24**). To establish pro-inflammatory character of Vi *in vivo*, C57Bl/6 mice were injected with Vi and IL-6 and IL-12p40 were analyzed. Both IL-6 and IL-12p40 were detected in the sera and peritoneal exudates of mice injected with Vi (**Figure 25**). These cytokines were also detected in mice immunized with NDeVi but not in DeVi-injected mice (**Figure 25**). These results were consistent with *in vitro* data which showed that DeVi incapable of interacting with hemoglobin and activating TLR2 was defective at inducing IL-8 secretion from human monocytes, and demonstrated that acetyl groups were also required for Vi-induced cytokine secretion *in vivo*.

Discussion

The inflammatory and innate immune responses produced during microbial infections play a vital role in determining the outcome of infection. A large number of these responses are generated *via* recognition of conserved PAMPs by germline encoded PRRs expressed on immune cells and many other cell types (Palm and Medzhitov, 2009). Many pathogens have devised ways to modulate these responses in a manner that would promote establishment of infection (O'Neil, 2008; Tsolis *et al.*, 2008). *S.typhi* produces systemic infection exclusively in humans. The infection is initiated in the gut following which bacteria are disseminated into secondary lymphoid organs. Due to its extreme host specificity there is no suitable animal model for this disease and therefore the host pathogen interactions during infection of humans with this bacterium remain poorly understood. It has been previously reported that the virulence polysaccharide, Vi, of *S.typhi* can inhibit inflammatory responses from intestinal epithelial cells during their infection with this pathogen *in vitro*. Vi interacted with membrane bound prohibitin on these cells and targeted MAP kinase pathway to suppress inflammatory responses (Sharma and Qadri, 2004).
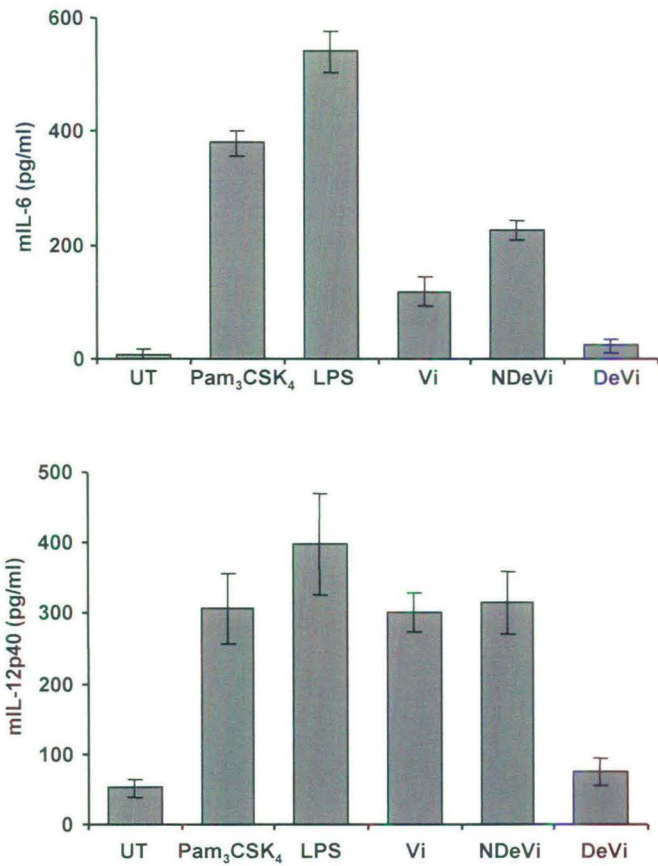
Figure 24. Vi and NDeVi induces proinflammatory cytokines from mouse splenocytes *ex vivo*. Splenocytes from C57Bl/6 mice were incubated at 37°C for 3 days with Pam_3CSK_4 (100 ng/ml), LPS (1 µg/ml), Vi (10 µg/ml), NDeVi (10 µg/ml) and Devi (10 µg/ml) in presence of serum. Culture supernatants were analyzed for IL-6 and IL-12p40 by ELISA.

A

B

C
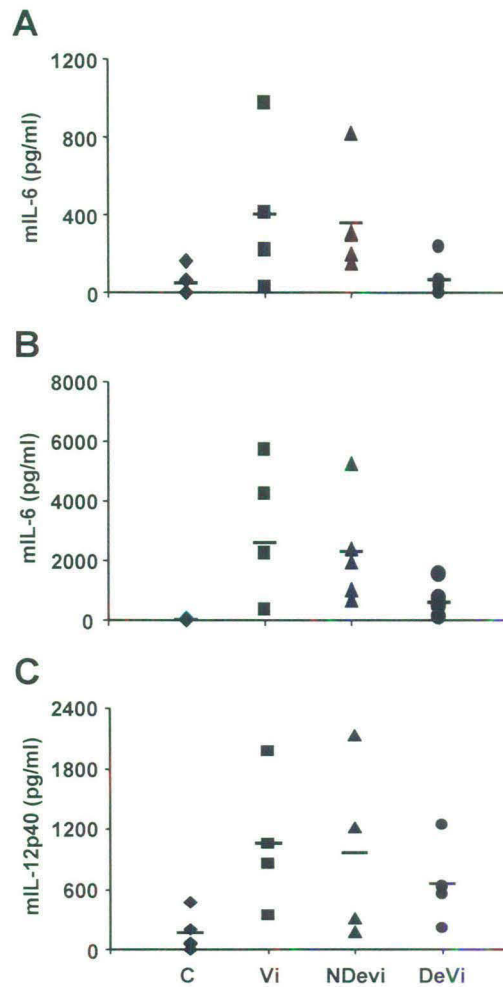
Figure 25. Vi induces inflammatory responses *in vivo*. C57Bl/6 mice were injected intraperitoneally with Vi, NDeVi or DeVi (10 µg/mouse). Sera and peritoneal exudates of control (C; PBS - injected) and Vi/ NDeVi/ DeVi - injected mice were collected at 2 h for cytokine analyses by ELISA. IL-6 was analyzed in sera (**A**) and peritoneal exudates (**B**); and IL-12p40 was analyzed in peritoneal exudates (**C**).

The data described in the present study show that this molecule can also inhibit inflammatory responses from human monocytes activated through TLR5 with flagellin, a dominant proinflammatory determinant of pathogenic *Salmonella*. This inhibition was dependent interaction of Vi with prohibitin. Vi mediated this suppression by inhibiting MAP kinase and NF-κB pathways of intracellular signaling that are activated in response to TLR activation. Recent studies by Sharma and Qadri (Ph.D. thesis "Molecular analysis of cellular responses induced by *Salmonella* flagellin"; 2008); and Srikanth and Qadri (Ph.D. thesis "A study on the interactions of *S.typhi* with T lymphocytes-comparison with *S.typhimurium*"; 2007) have shown that induction of inflammatory responses from human T cells with flagellin is dependent upon p56^{lck} and lck activation is modulated by Vi. Therefore, it is possible that at the level of membrane proximal signaling treatment with Vi modulates related src-kinase lyn activity, which has been previously reported to play a role in TLR4 and TLR2 -mediated responses (Medvedev *et al.*, 2007; Kannan *et al.*, 2006).

Remarkably, when Vi was presented to monocytes along with serum or serum-derived hemoglobin, it no longer inhibited inflammatory responses but instead it produced potent inflammatory responses on its own. These results suggest that a bacterial virulence factor can produce totally opposite responses depending upon how it is presented to the immune system. In the absence of circulating hemoglobin, Vi is a potent inhibitor of inflammatory responses but following its interaction with serum – derived hemoglobin this polysaccharide switches to a proinflammatory state (**Figure 26**). The inflammatory Vi produced cellular responses *via* specific engagement of TLR2/TLR1. This transformation involved binding of the polysaccharide to hemoglobin followed by its conversion into a proinflammatory molecule. The proinflammatory Vi retained both N- and O-acetyl groups, the latter being crucial for its interaction with hemoglobin and for the induction of inflammatory responses. However, the presence of these groups on the polygalacturonic acid backbone was not sufficient to impart proinflammatory character because O-acetylated polygalacturonic acid was non-inflammatory. Moreover, it was observed in the course of this study that N- and O-acetyl – containing low molecular weight polysaccharides present in small amounts in the vaccine preparation were non-stimulatory even in the presence of serum or hemoglobin. Therefore, a specific size or/and a specific configuration of Vi might be required for inducing TLR2-dependent inflammatory responses. The requirement for TLR2 in the induction of inflammatory responses with Vi also explains lack of IL-8 secretion in our previous study with human model IEC line, Caco-2, as the latter has been

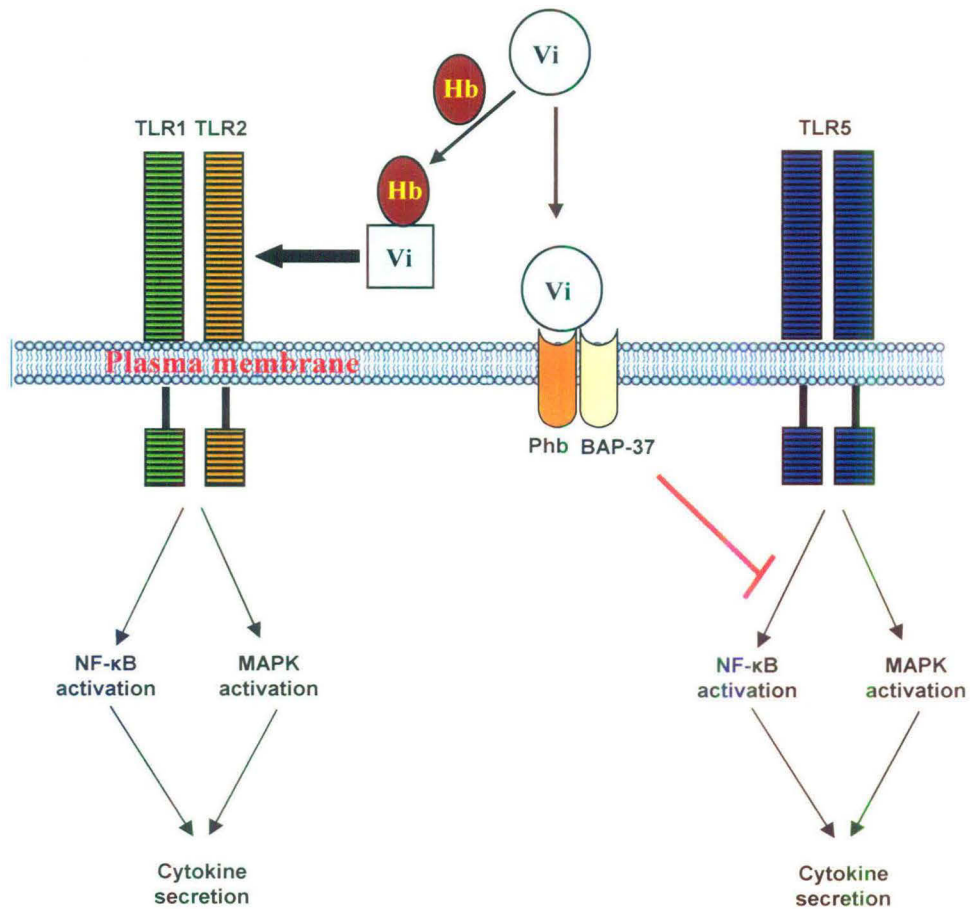**Figure 26. Schematic representation of a model showing anti- and pro-inflammatory activities of Vi.** Native Vi interacts with a complex of prohibitin and BAP-37 and brings about downregulation of TLR5-induced NF-κB and MAPK activation and subsequent inflammatory responses. However, in the presence of hemoglobin, Vi is modified in a manner that it can interact with TLR2/TLR1 complex and activate secretion of cytokines.

reported not to respond to TLR2 ligands (Melmad *et al.*, 2003). Even though Vi was capable of interacting with prohibitin in the presence of serum derived hemoglobin, interaction of anti-inflammatory Vi with prohibitin might not be sufficient to bring about inhibitory effects on TLR signaling activated by simultaneous engagement of TLR2 with proinflammatory Vi.

Hemoglobin, which is known to exist extracellularly at concentrations ranging from 115-155 µg/ml (Hasty *et al.*, 2006; Boyanton *et al.*, 2002), has been previously reported to potentiate cellular responses with LPS and LTA (Cox *et al.*, 2007; Hasty *et al.*, 2006; Gorczynski *et al.*, 2004; Kaca *et al.*, 1994). However, a striking difference between those studies and the present one is that in here hemoglobin not only unveiled proinflammatory capability of Vi but it also abrogated its anti-inflammatory capability. The exact mechanism by which hemoglobin promotes inflammatory responses with Vi is not clear at the moment. However, it looks likely that hemoglobin does it by transferring Vi to TLR2/TLR1 much the same way as LBP delivers LPS to TLR4 complex or serum vitronectin delivers BLP to TLR2 (Gerold *et al.*, 2008; Gioannini *et al.*, 2007). It is important to mention that both THP-1 and HEK293-T do not express any detectable levels of hemoglobin receptor CD163 (Babs *et al.*, 2009; Schaer *et al.*, 2006) therefore hemoglobin is not directly contributing to any intracellular signaling event that might synergize with Vi-induced intracellular signals to activate inflammatory responses. Thus, hemoglobin action is strictly restricted to its modification of the ligand. Irrespective of the exact mechanism by which hemoglobin modifies Vi, our study indicates that this soluble PRR might play a decisive role in the regulation of inflammatory and innate immune responses to this key virulence determinant of *S.typhi*. The anti-inflammatory character of native Vi and its conversion to an inflammatory molecule in the presence of hemoglobin might have important consequences for the induction of inflammatory and innate immune responses during infection with *S.typhi*. The absence of Vi in non-typhoidal *Salmonella* serovars such as *S.typhimurium* which causes different clinical manifestations in humans, assigns even more significance to Vi-macrophage interaction described in the present study. The anti-inflammatory modulation mediated by circulating Vi through interaction with membrane prohibitin would be in addition to the effects of bacterial surface-associated Vi on regulating accessibility and/or release of pro-inflammatory effectors from *Salmonella* reported recently (Wilson *et al.*, 2008; Winter *et al.*, 2008). It is possible that the anti-inflammatory effect of Vi prevails during early stages of infection with *S.typhi* in the gut

while the proinflammatory effects become apparent in the course of systemic dissemination of the pathogen during which circulating hemoglobin might be more readily available. Additionally, hemolytic activity mediated by SPI-1 TTSS and ClyA of *Salmonella* during the course of infection might lead to increased local concentrations of hemoglobin (Miki *et al.*, 2004; Oscarsson *et al.*, 2002). The proinflammatory face of Vi might also have implications for the induction of antibodies with this polysaccharide vaccine. These results yet again highlight multiple mechanisms by which inflammatory and innate immune responses might be regulated during infection with a microbial pathogen. In summary, the findings presented in this chapter reinforce a key role to hemoglobin as a soluble PRR in the regulation of innate immune responses to microbial stimuli and unveil a previously unrecognized face of Vi in host-pathogen interaction.

CHAPTER 2

Regulation of antibody response to Vi

CHAPTER 2

Introduction

Antibodies to polysaccharides are known to play a vital role in controlling systemic infection with encapsulated bacteria (Raff *et al.*, 1988; Robbins and Robbins, 1984; Brown *et al.*, 1983; Robbins, 1978; Felix and Pitt, 1934b). These antibodies are generated independent of T cell help that is normally provided to B cells during antibody response against protein antigens (Ueda *et al.*, 2007; Martin *et al.*, 2004; Lin, 2001; Koassacka *et al.*, 1999; Lane, 1996; Szu *et al.*, 1994, 1991; Acharya *et al.*, 1987; Klugman *et al.*, 1987). Therefore, these antigens are referred to as T-independent antigens. These are further classified into type I and type II. The former include molecules such as LPS and CpG that can bring about polyclonal proliferation of B cells, whereas the latter are polysaccharides that do not have this mitogenic property but can engage B-cell receptor and generate antigen-specific B cell responses (Vos *et al.*, 2000; Mond *et al.*, 1995). The antibody response to T-independent antigens is predominantly IgM; IgG antibodies are produced at low frequency (Snapper and Mond, 1996). T-independent B cell responses against these polysaccharide antigens confer long lasting humoral immunity in the absence of recall responses and unlike T-dependent protein antigens, these responses are not known to generate memory B cells (Garcia de Vinuesa *et al.*, 1999). Many of these polysaccharide antigens carry repetitive determinants which enables them to simultaneously engage multiple antigen receptors on B-cells. This engagement of multiple B-cell receptors provides first signal for B-cell proliferation (Szuler and Perelson, 1997; Snapper and Mond, 1996). However, there is a need for a second signal to B-cells for immunoglobulin secretion and class switching (Vos *et al.*, 2000). A number of studies have shown that this second signal can be provided by tumor necrosis factor family members such as B lymphocyte stimulator / B cell activating factor (Blys/BAFF) and a proliferation inducing ligand (APRIL) (Stein *et al.*, 2002; Scheimann *et al.*, 2001; Do *et al.*, 2000). These molecules bind the transmembrane activator calcium-modulator and cyclophilin ligand interactor (TACI) and also two other receptors, B cell activating factor receptor (BAFF-R) and B cell maturation antigen (BCMA) (Xu *et al.*, 2001; Miller *et al.*, 1992). TACI has been shown to promote efficient plasma cell differentiation in response to T-independent type II antigens (Mantchev *et al.*, 2007). In addition, other cytokines such as IL-5 and IFN-

γ have been shown to bring about immunoglobin secretion and isotype switching in response to type II polysaccharides (Snapper *et al.*, 1992).

TLRs play a key role in determining the quality of T cell response and in turn the quality of B cell response to protein antigens. Ligation of TLRs on antigen presenting cells such as DCs brings about upregulation of costimulatory molecules essential to T cell activation and induce secretion of different cytokines that can instruct differentiation of naive CD4+ T cells into T helper 1 (Th1) cells or T helper 2 (Th2) cells (Palm and Medzhitov, 2009; Pasare and Medzhitov, 2005). Th1 cells produce interferon-γ (IFN-γ) which is known to bring about switching of IgM to IgG2a from protein antigen-specific B cells while Th2 cells, which produce IL-4, promote switching to IgG1 (Snapper *et al.*, 1997). Recent studies suggest that TLR activation might also contribute to antibody responses against polysaccharide antigens (Sen *et al.*, 2005). Several reports have shown that TLRs might play a role in B cell responses against T-independent type II antigens either through cross talk with B-cell receptor or through activation of dendritic cells and macrophages. The latter provide soluble mediators that can promote B cell differentiation and immunoglobin secretion (Pasare and Medzhitov, 2005; Sen *et al.*, 2005; Bondada *et al.*, 2000).

Vi polysaccharide forms a capsule around *S.typhi*. It is a major virulence factor of this pathogen (Felix and Pitt, 1934b). Vi is currently in use as a vaccine against typhoid in humans. Antibodies to this polysaccharide have been shown to confer protection against infection with *S.typhi* for 3-4 years (Klugman *et al.*, 1996; Plotkin *et al.*, 1995; Acharya *et al.*, 1987; Klugman *et al.*, 1987). Vaccination with Vi elicits IgM as well as IgG kind of antibodies (Ferry *et al.*, 2004). However, like other type II T-independent (TI-2) antigens, the mechanism by which IgG type of antibodies are generated in the absence of "classical T cell help" is not understood. The results described in the previous chapter showed that Vi could produce inflammatory cytokines when presented to monocytes in the presence of circulating hemoglobin. The hemoglobin-modified Vi specifically engaged TLR2 to generate inflammatory cytokines including IL-6, TNF-α and IL-12p40. The ability of some of these cytokines and other TLR2 – induced signals to bring about isotype switching (Sen *et al.*, 2005) prompted us to investigate the role of inflammatory capability of Vi in B cell responses to this polysaccharide.

Results

Immunization with Vi generates IgM and IgG3 antibodies

To study antibody response to Vi, BALB/c and C57BL/6 mice were immunized with this polysaccharide intradermally through the foot pad. Sera collected before immunization and 14 days after immunization with the polysaccharide were used for determination of anti-Vi IgM and IgG antibodies. Both strains of mice produced IgM as well as IgG antibodies with IgM levels being much higher than IgG (**Figure 1**). However, Vi was more immunogenic in C57BL/6 than in BALB/c mice. Analysis of isotypes in sera obtained from C57BL/6 mice showed that IgG anti-Vi antibodies were mainly of IgG3 isotype (**Figure 2**).

Non-inflammatory Vi evokes poor IgG antibody response

The data presented in the previous chapter showed that Vi could bring about secretion of inflammatory cytokines *in vitro* and *in vivo* in the presence of serum. The induction of these responses required interaction of Vi with circulating hemoglobin and specific engagement of TLR2/TLR1 on monocytes. The interaction of Vi with hemoglobin as well as induction of cellular responses through TLR2 were dependent upon presence of O-acetyl groups in the polysaccharide; deacetylated Vi (DeVi) was non-inflammatory *in vitro* as well as *in vivo*. To investigate possible role of TLR2 – induced signals in the induction of B cell responses to Vi, antibody responses were studied in mice immunized with Vi and its deacetylated derivative (DeVi). As expected, Vi induced both IgM and IgG type of antibodies (**Figure 3A**). On the other hand, mice immunized with DeVi produced anti-Vi IgM antibodies but no IgG antibodies to Vi (**Figure 3A**). The possibility that IgG antibodies in sera from these mice were directed primarily against DeVi and hence unable to recognize native Vi was checked by determining their reactivity with DeVi. The data revealed that even with DeVi only IgM kind of antibodies could be detected (**Figure 3B**). These results suggested that non-inflammatory DeVi was inherently not capable of bringing about switching of antibodies to IgG isotype. Thus, TLR2-activating ability of Vi is required for isotype switching during antibody response against this polysaccharide.
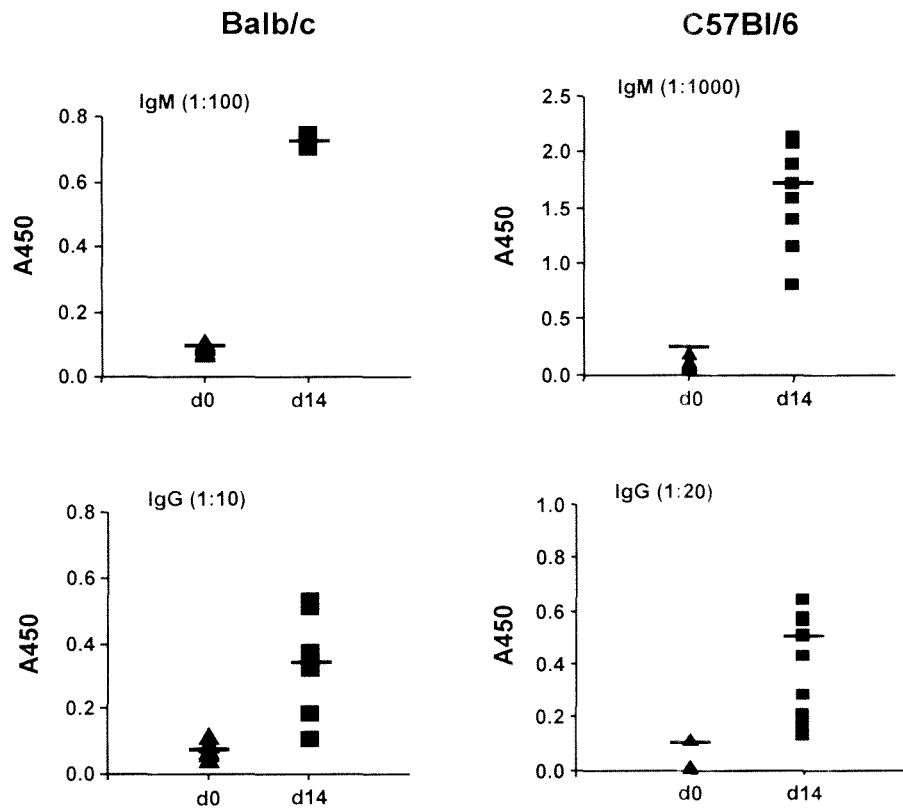
Figure 1. Vi induced IgM and IgG antibodies in Balb/c and C57Bl/6 mice. Mice were immunized with Vi (10 µg/mouse) intradermally and sera was analysed for Vi-specific IgM and IgG antibodies by ELISA. Values in brackets show sera dilutions used in ELISA. d0 - sera collected before immunization; d14 - sera collected 14 days after immunization with Vi.
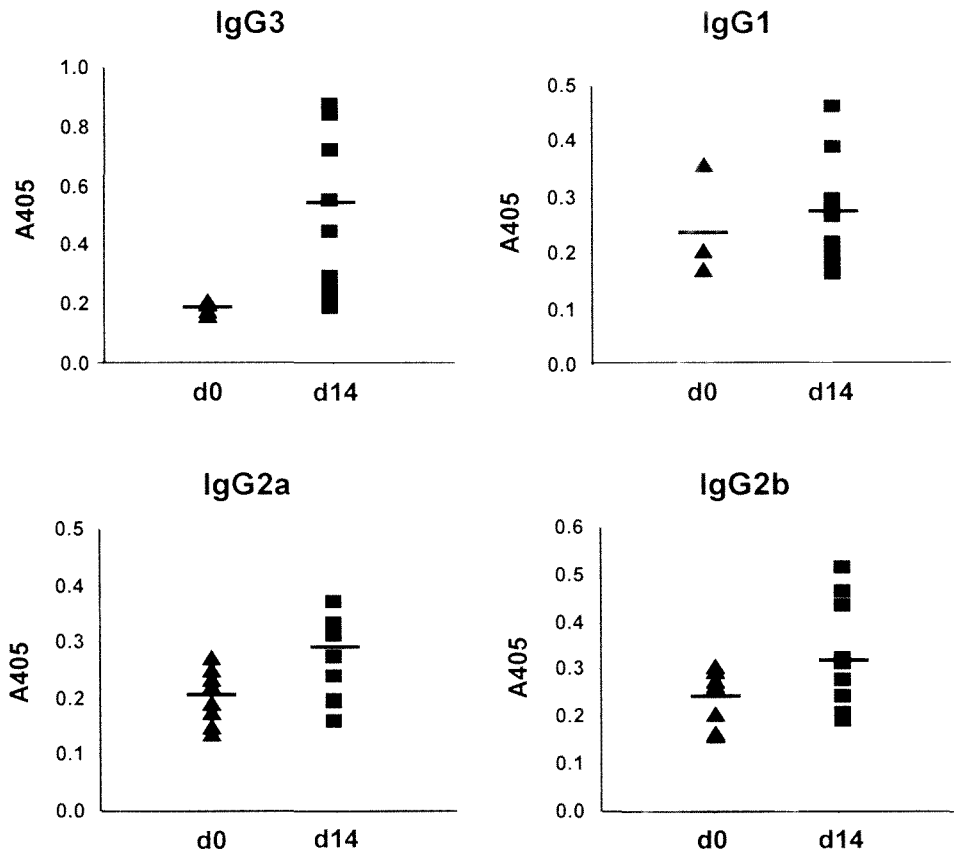
Figure 2. IgG anti-Vi antibodies in C57Bl/6 mice were mainly of IgG3 isotype. Mice were immunized intradermally with Vi and sera were analyzed for Vi-specific IgG isotypes by ELISA. d0 and d14 represent sera collected before immunization and 14 days after immunization with Vi. Sera were analyzed at 1:20 dilution.
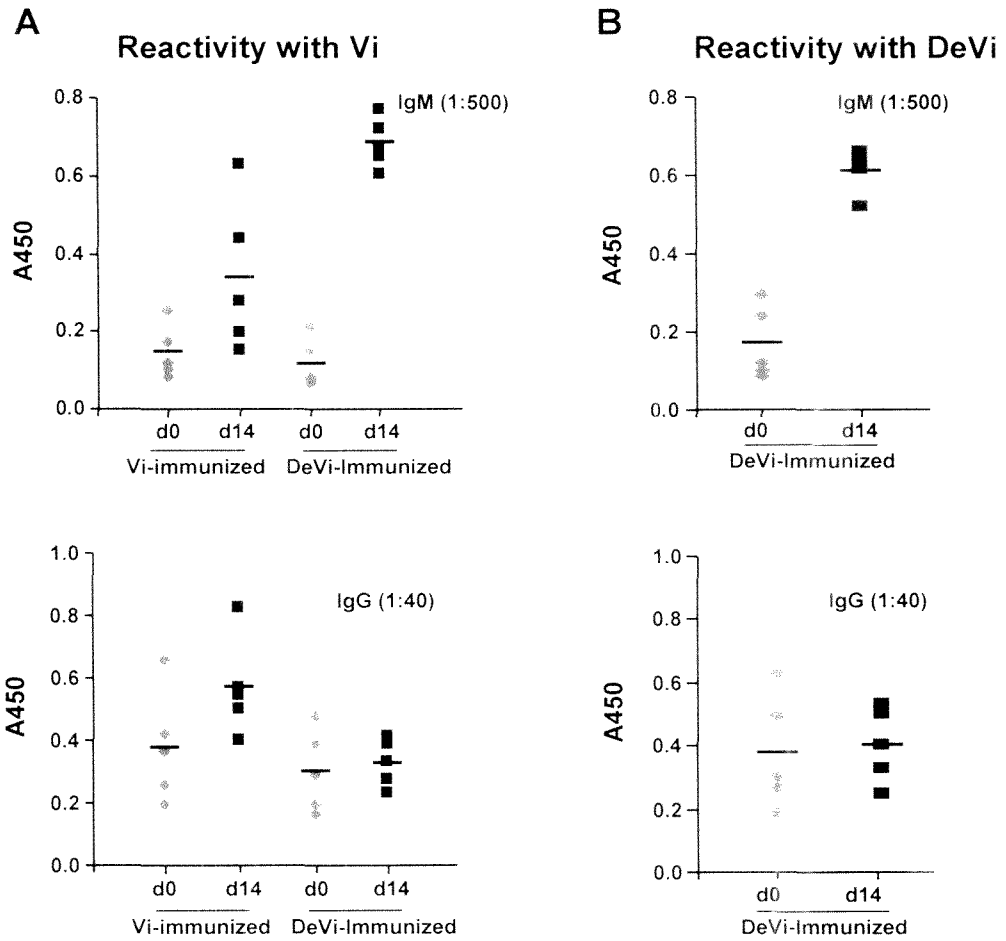
Figure 3. Deacetylated Vi (DeVi) did not elicit IgG kind of antibodies. C57Bl/6 mice were injected with Vi or DeVi (10 μg/mouse). IgM and IgG antibodies against Vi (**A**) and DeVi (**B**) were determined by ELISA. Values in brackets show sera dilutions used in ELISA.

Cytokines from macrophages contribute to switching of anti-Vi antibodies to IgG

Recent studies suggest that some bacterial polysaccharides with special chemical features might be able to bring about activation of T cells which secrete IFN-γ and IL-17 (Mazmanian and Kasper, 2006; Chung *et al.*, 2003; Tzianabos, 2000). Similarly, antibody responses to pneumococcal polysaccharide were shown to be compromised in MHC class I – deficient and CD8-deficient mice suggesting a role for T cells in these responses (Kobrynski *et al.*, 2005). Interestingly, zwitterionic form of Vi has been shown to produce inflammatory responses in animal models similar to those produced by other zwitterionic polysaccharides derived from *Bacteroides fragilis* and *Streptococcus pneumoniae*; these responses are dependent on activation of T cells by the polysaccharide (Tzianabos, 2000; 1993).

To investigate possible involvement of T cells in the activation of B cell responses against Vi, anti-Vi antibody responses were analyzed in wild type (WT), TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ mice. WT and TCR $\beta^{-/-}$ mice elicited comparable levels of IgM anti-Vi antibody responses, whereas TCR $\delta^{-/-}$ mice showed slightly reduced responses (**Figure 4A**). However, IgG anti-Vi antibodies were substantially higher in TCR $\beta^{-/-}$ as compared to wild-type and TCR $\delta^{-/-}$ mice (**Figure 4B**). These antibodies were mainly of IgG3 type although low levels of IgG1 antibodies could also be detected (**Figure 5**). IgG anti-Vi antibodies were comparable in WT and TCR $\delta^{-/-}$ which suggested that $\gamma\delta$ T cells might not be directly involved in bringing about switching of IgM anti-Vi antibodies to IgG. Examination of splenocyte populations in these mice showed 4 fold increase in the number of CD11b+ cells in TCR $\beta^{-/-}$ mice as compared to wild type and TCR $\delta^{-/-}$ mice (**Figure 6**). In addition, both TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ mice showed higher B220+ cells in comparison to wild type mice (**Figure 6**). TCR $\beta^{-/-}$ mice are known to show deregulated cellularity with time and have been shown to develop inflammatory bowel disease possibly due to lack of regulatory T cells (Mizoguchi, 2003; Podolsky, 1997; Mombaerts *et al.*, 1993). Analysis of cytokine responses from these splenocytes showed enhanced secretion of cytokines IL-6 and IFN-γ from TCR $\beta^{-/-}$ mice – derived spleen cells (**Figure 7**). Increase in number of B220+ cells in TCR$\delta^{-/-}$ mice does not impart them with increased antibody titers as well as cytokine production in response to Vi, as compared to wild type cells. Thus, enhanced anti-Vi IgG antibody secretion in TCR $\beta^{-/-}$ mice could be attributed to increased numbers of

A

IgM (1:1000)

B

IgG (1:20)
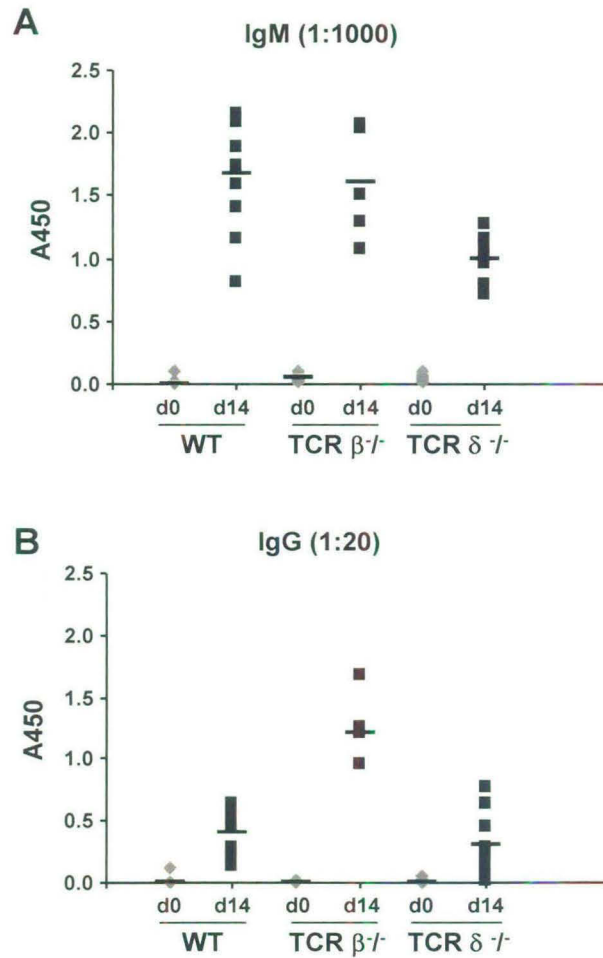
Figure 4. TCR β-/- mice generated higher IgG anti-Vi antibody response as compared to wild type and TCR δ-/- mice. Mice were immunized intradermally with Vi (10 µg/mouse) and anti-Vi IgM (**A**) and IgG (**B**) antibodies were determined in sera collected before immunization (d0) and day 14 after immunization (d14) by ELISA. Numbers in brackets show sera dilutions used in ELISA.
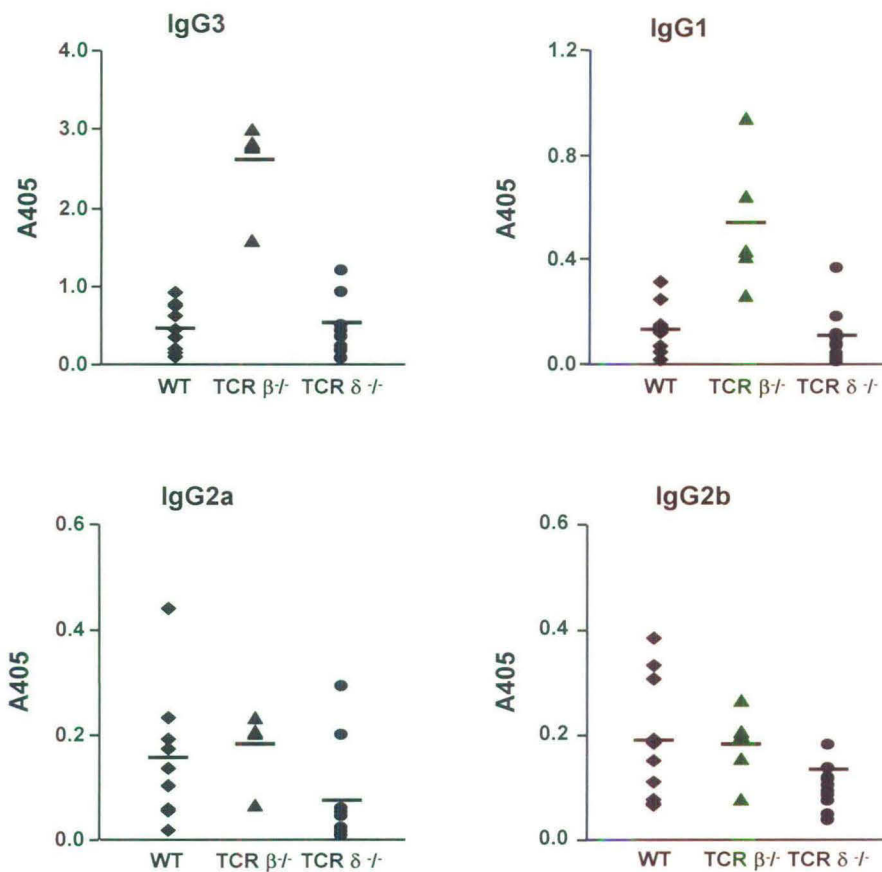
Figure 5. Vi elicited higher IgG3 and IgG1 antibodies in TCR β-/- mice as compared to wild-type and TCR δ-/- mice. Wild type (WT), TCR β-/- and TCR δ-/- mice were immunized with Vi (10 μg/mouse) and anti-Vi IgG isotypes were determined by ELISA in sera obtained on day 14 after immunization. Sera were analyzed at 1:20 dilution.
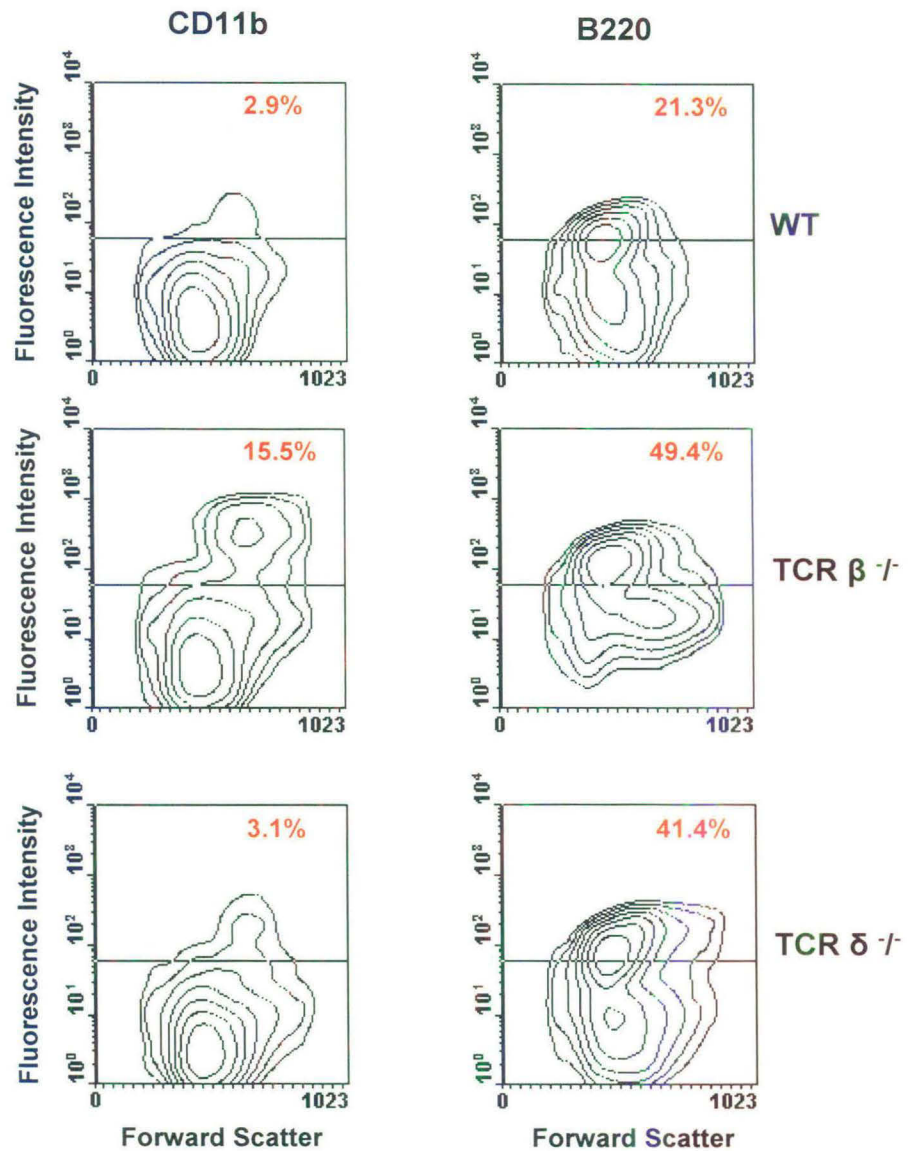
Figure 6. Spleens from TCR β-/- mice showed higher percentage of CD11b positive cells as compared to splenocytes from wild type and TCR δ-/- mice. Splenocytes from wild type, TCR β-/- and TCR δ-/- mice were stained with FITC-labeled anti-CD11b, FITC-conjugated anti-B220 antibody or isotype matched fluorescent antibodies. Cells were washed and analyzed by flow cytometry.
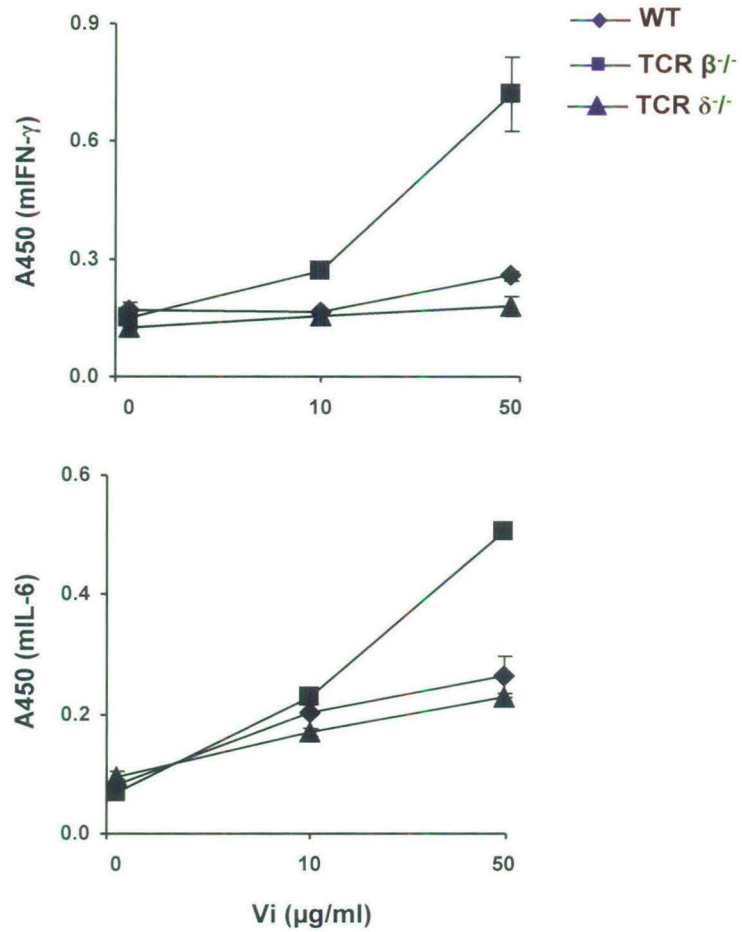
Figure 7. Vi induced higher amounts of IL-6 and IFN-γ in splenocytes from TCR β-/- mice. Splenocytes from wild type and TCR β-/- mice were incubated at 37°C for 48 h with the indicated concentrations of Vi, and supernatants were analyzed for IL-6 and IFN-γ by ELISA.

CD11b+ cells of monocytic lineage capable of producing cytokines upon stimulation with Vi and not because of increased numbers of B220+ cells.

Discussion

Capsular polysaccharides are key virulence determinants of many pathogenic bacteria and antibodies against these molecules provide protection against systemic infection with these pathogens (Raff *et al.*, 1988; Robbins and Robbins, 1984; Brown *et al.*, 1983; Robbins, 1978). Vi capsular polysaccharide of *S.typhi* contributes to virulence by inhibiting complement – mediated killing and by shielding this pathogen from the action of anti-O (LPS) antibodies (Hirose *et al.*, 1997; Looney and Steigbigel, 1986; Robbins and Robbins, 1984; Felix and Pitt 1934). More recently, this molecule has also been implicated in modulating inflammatory responses during infection of IEC with *S.typhi* (Sharma and Qadri, 2004). Vi is absent in non-typhoidal *Salmonella* serovars such as *S.typhimurium* (Parkhill *et al.*, 2001). Antibodies to Vi have been shown to protect humans against infection with *S.typhi* in two large scale clinical trials (Klugman *et al.*, 1996; Plotkin *et al.*, 1995; Acharya *et al.*, 1987; Klugman *et al.*, 1987) and currently, this polysaccharide is available as a vaccine against typhoid. Vaccination with Vi produces IgM as well IgG kind of anti-Vi antibodies and protection correlates with anti-Vi IgG antibody levels (Hessel *et al.*, 1999). However, the mechanism by which IgM anti-Vi antibodies switch to IgG isotype has not been elucidated. The results presented in this chapter suggest that generation of IgG antibodies against Vi in mice might be regulated by the ability of this molecule to produce inflammatory responses through activation of TLR2/TLR1. The induction of IgG antibodies was dependent upon the presence of O-acetyl groups at position C3 in Vi. These determinants are required for producing inflammatory cytokines with Vi. Vi lacking acetyl groups produced IgM antibodies alright but was defective at generating IgG kind of antibodies. These results are consistent with earlier studies reporting inability of acid-treated deacetylated preparation of Vi to protect volunteers challenged with *S.typhi* (Hornick and Woodward, 1966). However, Szu *et al.* (1991) showed that while partial deacetylation slightly increases immunogenicity of Vi; complete O- deacetylation eliminates immunogenicity of Vi which is in contrast to the results reported in the present study. The reason for this difference might be in the methodology used to prepare DeVi in the two studies. In our study, the deacetylated Vi was prepared by treating Vi with 1M

NaOH for 1 h. In the earlier study, prolonged treatment of Vi with NH$_4$OH used to prepare DeVi, might have led to loss of all the antigenic determinants resulting in abrogation of immunogenicity. That would be consistent with reports which suggest that O-acetyl is only a part of epitope that reacts with antibodies to Vi (Szewczyk and Taylor, 1980). The present study suggests a direct correlation between TLR2 agonistic activity of Vi that is dependent on O-acetyl groups at C-3 and production of IgG anti-Vi antibodies *in vivo*. While a direct role for TLR2 on B cells in antibody responses against Vi can't be ruled out, the data with TCR β $^{-/-}$ mice which showed higher IgG antibodies and increased secretion of IL-6 and IFN-γ following stimulation with Vi *in vitro* due to increased numbers of CD11b+ cells suggests that cells of monocytic lineage might be providing stimuli for switching of anti-Vi antibodies to IgG isotype. IFN-γ has been previously reported to promote isotype switching to IgG3 and IgG2a during immunization with type II- T-I antigen (Snapper *et al.*, 1992). Therefore, increased numbers of IFN-γ-producing CD11b+ cells in TCR β $^{-/-}$ mice might have contributed to isotype switching of anti-Vi antibodies to IgG kind. Interestingly, O-acetyl groups are known to be important in determining the immunologic properties of many bacterial polysaccharides including Vi, pneumococcal polysaccharide, menningococcal polysaccharide and many others (Szu *et al.*, 1991; Kabat *et al.*, 1988; Szu *et al.*, 1981). Our results with Vi suggest that these determinants might be directly or indirectly involved in activating innate immune responses and that is how they modulate specific B cell responses.

The role of TLRs in antibody responses against T-independent antigens has been suggested by several studies (Sen *et al.*, 2005; Vanlandschoot *et al.*, 2005; Latz *et al.*, 2004). Pasare and Medzhitov showed that optimal T-independent IgG3 response to flagellin, which is the ligand for TLR5, required TLR signaling through accessory cells and not B cells (Pasare and Medzhitov, 2005). Sen *et al.*, showed that humoral responses to isolated pneumococcal polysaccharides were dependent on the presence of associated TLR ligands (Sen *et al.*, 2005). Direct role of TLR agonists in promoting terminal plasma cell differentiation of B1 and marginal zone B cells which are specialized in T-independent responses, and enhanced B cell activation mediated by TLR and BCR cross-talk have also been demonstrated (Genestier *et al.*, 2007).

In summary, the results presented here suggest a mechanism by which Vi polysaccharide of *S.typhi* might be able to generate IgG kind of antibodies. Combined with

other studies, the present investigation calls into question the physiological relevance of classifying polysaccharide antigens as either T-I type 1 or T-I type II. The distinction between these two kinds of T-independent antigens may be only in the level of TLR activity. The in-built TLR agonistic activity in T-independent type II antigens enables them to activate the innate immune system while simultaneously recruiting antigen-specific B cells. By combining these two pathways, the T-I type-II response enables the host to rapidly produce antigen-specific antibodies and thereby prevent rapid multiplication of pathogenic microorganisms.

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Vi capsular polysaccharide is a major virulence factor of human typhoid-causing bacterium, *S.typhi*; it is absent in non-typhoidal *Salmonella* serovars such as *S.typhimurium*. Vi contributes to virulence by protecting *S.typhi* from complement-mediated killing and by inhibiting its phagocytosis. Recently, Vi was shown to interact with membrane associated prohibitin family of molecules in intestinal epithelial cells and inhibit inflammatory responses during infection of these cells with *S.typhi*. Given that prohibitin and its related homologs are highly conserved molecules which participate in crucial cellular functions, the present study investigated interaction of Vi with mononuclear phagocytes which host *S.typhi* during systemic infection and analyzed possible role of Vi-prohibitin interaction in the induction of inflammatory responses from these cells. The study also examined regulation of antibody response to Vi.

The results demonstrate that Vi is a potent inhibitor of innate immune activation of monocytes. Vi suppressed IL-8 secretion from human and mouse monocytes activated with flagellin which is a major proinflammatory determinant of pathogenic *Salmonella* and induces inflammatory and innate immune responses through TLR5. The polysaccharide produced this suppression by downregulating activation of NF-kB and MAP kinase pathways of intracellular signaling. This inhibition was dependent on the interaction of Vi with prohibitin as knock-down of prohibitin expression by RNA interference reduced the inhibitory effect with Vi. Remarkably, Vi not only lost its anti-inflammatory capability but it also turned into a proinflammatory molecule when monocyte stimulations were carried out in presence of serum. Immunoprecipitation of serum proteins with Vi followed by N-terminal sequencing revealed that Vi could interact with serum - derived hemoglobin, apolipoproteinA-I and fetuin. However, the molecule responsible for bringing about switch of Vi from an anti- to a pro- inflammatory state was serum-derived hemoglobin. Analysis by native gel electrophoresis showed that hemoglobin could bring about a mobility shift in Vi indicative of a change in the physical state of the polysaccharide. Hemoglobin-modified proinflammatory Vi produced cellular responses through specific activation of TLR2/TLR1 heterodimer. This was confirmed by carrying out blocking experiments with anti-TLR2 antibody and by investigating responses of Vi in HEK293-T cells transfected with various Toll-like receptors. Vi triggered secretion of IL-8 through TLR2/TLR1 but not through

TLR2/TLR6. Further analysis revealed that the O-acetyl groups present in the polysaccharide were critical for its interaction with hemoglobin and consequently for its ability to produce inflammatory responses. Derivatives of Vi which were unable to interact with hemoglobin were non-inflammatory *in vitro* as well as *in vivo*. Therefore, hemoglobin seems to play a critical role in converting Vi from an anti-inflammatory to a proinflammatory molecule. Together with studies reported with other bacterial virulence factors, these results also reiterate a key role for hemoglobin in the regulation of innate immune responses to microbial stimuli and suggest that it may be designated as a soluble pattern recognition receptor.

Analysis of antibody responses in mice immunized with Vi showed that TLR2 agonistic activity might also contribute to switching of anti-Vi antibodies to IgG isotype. Vi is a T-independent type II antigen and induces IgM as well as IgG antibodies. The IgG anti-Vi antibodies correlate with the efficacy of Vi vaccine in humans. Mice immunized with Vi produced both IgM and IgG antibodies, with IgG levels being much lower than IgM. Significantly, deacetylated Vi, which could not trigger inflammatory responses *in vitro* or *in vivo*, while elicited IgM antibodies similar to those produced by native Vi, did not generate any detectable level of IgG antibodies; indicating a direct relationship between inflammatory ability of Vi and generation of IgG anti-Vi antibodies. Further analysis suggesting that the signal for isotype switching of IgM to IgG might be provided by cytokines produced through activation of TLR2/TLR1 on monocytes by Vi.

The findings presented here suggest that Vi can be anti-inflammatory or proinflammatory depending upon how it is presented to the immune system. In the absence of hemoglobin the interaction of Vi with prohibitin brings about downregulation of TLR - induced inflammatory responses. However, in presence of hemoglobin, Vi is modified in a manner that it can now interact with TLR2/TLR1 complex and stimulate inflammatory responses. Hemoglobin very likely transfers Vi to TLR2/TLR1 complex the same way as LBP transfers LPS to TLR4-CD14-MD2 complex. The anti-inflammatory character of Vi might prevail during early stages of *S.typhi* infection in the gut where circulating hemoglobin levels would be low. The downregulation of inflammatory responses at this stage would promote establishment of infection. On the other hand, during systemic infection when circulating hemoglobin would be readily available, inflammatory responses with Vi might predominate. This kind of a regulation might have important consequences for the pathogenesis of *S.typhi* infection.

Taken together, the present study establishes a role for membrane prohibitin in the downregulation of innate immune responses from mononuclear phagocytes by *S.typhi* Vi polysaccharide and unveils a host factor-promoted proinflammatory character of this polysaccharide that can trigger inflammatory and innate immune responses through specific engagement of TLR2/TLR1 heterodimer. This study also assigns an important role to Vi-induced inflammatory responses in the induction of IgG antibodies against this polysaccharide.

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