Vi suppresses early inflammatory responses from human intestinal epithelial cells
Introduction

The results described in the previous chapter suggested that Vi capsular polysaccharide of *S. typhi* can interact with host cells through a specific recognition complex containing prohibitin family of molecules as major components. Prohibitin is an anti-proliferative protein that has been shown to regulate mammalian cell cycle by repressing E2F family of transcription factors. These transcription factors are also repressed by another tumor suppressor molecule, the retinoblastoma (Rb) protein (Wang *et al.*, 1999a). However, these two molecules target different regions of E2F for repression and respond to different upstream signals. The repression of E2F by Rb is modulated by cyclin-dependent kinases, whereas release of prohibitin-mediated repression of E2F apparently involves mitogen-activated protein kinase (MAP-kinase) pathway (Wang *et al.*, 1999b). Since MAP-kinase pathway also plays an important role in inflammatory responses during infection with many pathogenic organisms including *Salmonella*, the question we asked in this study was if engagement of cells with Vi could modulate inflammatory responses from intestinal epithelial cells.

Intestinal epithelial cells respond to enteric pathogens by producing a number of proinflammatory cytokines and chemokines that participate in the recruitment of immune cells to the site of infection. Infection of epithelial monolayers with strains of invasive bacteria such as *Salmonella dublin, Shigella dysenteriae, Yersinia enterocolitica, Listeria monocytogenes* and enteroinvasive *E.coli* results in the secretion of cytokines such as interleukin-8 (IL-8), monocyte chemoattactant protein 1 (MCP-1), granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) (Jung *et al.*, 1995). IL-8, one of the best characterized chemokines, is involved in the recruitment of neutrophils to the site of infection (Fleckenstein and Kopecko, 2001). In *Salmonella* and many other pathogenic bacteria, the key inducer of IL-8 from intestinal epithelial cells is flagellin (Gewirtz *et al.*, 2001a). Flagellin, one of the pathogen associated molecular patterns (PAMP), is recognized by the Toll like receptor-5 (TLR-5) and evokes a variety of inflammatory and innate immune responses from various cell types including intestinal epithelial cells, dendritic cells and macrophages (Hayashi *et al.*, 2001, Gewritz *et al.*, 2001b). Stimulation of these cells with flagellin results in the activation of transcription factors such as NF-κB and AP-1.
The experiments described in this chapter analyze the effect of Vi on inflammatory responses from intestinal epithelial cells.

RESULTS

Engagement of Caco-2 cells with Vi suppresses early inflammatory responses
To study the role of Vi in the modulation of inflammatory responses, Caco-2 cells were incubated with Vi and then infected with capsule-negative *S.typhi*. As can be seen from Figure 16a, cells incubated with Vi secreted significantly reduced amounts of IL-8 upon infection with Vi-negative *S.typhi*; the inhibition was dose-dependent. The effect was not due to blockade of binding of Vi-negative *S.typhi* to Caco-2 cells by Vi, as comparable number of bacteria bound to Caco-2 cells in the presence or absence of Vi (Fig. 14). The reduction in IL-8 secretion in the presence of Vi was also seen in response to stimulation of Caco-2 cells with PMA (Figure 17a). The inhibition was specific to Vi as LPS derived from *S.typhi* did not bring about any significant decrease in IL-8 secretion. The specificity of this effect was also established by the ability of anti-Vi monoclonal antibodies to reverse Vi-mediated suppression of IL-8 secretion (Figure 17b).

The effect of Vi on IL-8 secretion was best seen under serum-free conditions. In fact, the degree of inhibition mediated by Vi was reduced upon increasing concentration of the serum in the stimulant i.e., PMA (Figure 18). Furthermore, modulation of IL-8 secretion by Vi in Caco-2 cells was dependent on the confluency of cells. The degree of inhibition decreased as the confluency of cells increased (Fig. 19a). This could be due to lesser accessibility of Vi-interacting molecules to Vi in highly confluent monolayers. A comparison of binding of Vi to Caco-2 cells differing in confluence states revealed that this in fact was the case. Vi showed significantly reduced binding to Caco-2 cells grown as a tight confluent monolayer (Figure 19b). Importantly, even anti-prohibitin antibodies bound poorly to Caco-2 cells which were grown to confluency. Consistent with this binding data, Vi-mediated inhibition of IL-8 secretion was more pronounced with freshly detached Caco-2 cells as compared to those cells that had been plated for 4h at 37°C (Figure 20).
Figure 16. Vi inhibits IL-8 secretion from Caco-2 cells. (a) Freshly detached Caco-2 cells were incubated with varying concentrations of Vi for 1h at 37°C followed by infection with Vi S.typhi (moi 10) for 1h. Thereafter, cells were washed to remove unbound bacteria and incubated with RPMI-1640 containing gentamycin (200μg/ml). Supernatants were collected after 5h for IL-8 analysis. (b) shows a representative standard curve obtained with known concentrations of IL-8.
Figure 17. Vi suppresses PMA-induced IL-8 secretion from Caco-2 cells. (a) Freshly-detached Caco-2 cells were incubated with Vi or LPS in serum-free RPMI-1640 and 1h later stimulated with PMA (25ng/ml) for 5h at 37°C. IL-8 was determined in the supernatants by ELISA. (b) Vi-induced IL-8 inhibition can be reversed by antibodies to Vi. Cells were treated with Vi in the presence of anti-Vi MoAb followed by stimulation with PMA (10ng/ml) for 5h. Dotted lines represent IL-8 levels from unstimulated cells.
Figure 18. Serum reverses Vi-induced inhibition of IL-8 secretion. Caco-2 cells were incubated with Vi for 1h in serum-free RPMI-1640 followed by stimulation with PMA diluted in serum-free RPMI-1640 (RPMI), in RPMI-containing 0.5% FCS (RPMI-0.5), 1% FCS (RPMI-1) or 5% FCS (RPMI-5). The culture supernatants obtained after 5h incubation at 37°C were analyzed for IL-8.
Figure 19a. Inhibition of IL-8 secretion by Vi is dependent on the confluency of Caco-2 cells. Caco-2 cells were plated overnight at different cell densities. After incubation with Vi (12.5 µg/ml) or PBS for 1h at 37°C, cells were stimulated with PMA (50 ng/ml). Supernatants were collected after 6h and analysed for IL-8 by ELISA.

Figure 19b. Vi binds poorly to highly confluent Caco-2 cells. Cells were plated overnight and incubated with Vi or PBS for 1h at 4°C. Cells were washed, incubated with biotinylated anti-Vi MoAb followed by FITC-anti-rabbit Ig antibody for 1h at 4°C. Subsequently, cells were permeabilised with chilled methanol containing 0.01% TritonX-100 for 5 minutes at 4°C, followed after washing with rabbit anti-prohibitin Ab and PE-labeled streptavidin. Thereafter, cells were washed, anti-fade reagent was added and cells were observed under a fluorescence microscope. Images were captured with a digital camera using ACT software and transferred to Adobe photoshop for printing.
Figure 20. Suppression of IL-8 by Vi is more potent in freshly-detached cells. Freshly detached Caco-2 cells or cells plated at 37°C for 4h, were incubated with Vi or LPS diluted in serum-free RPMI-1640 for 1h at 37°C, following which these were infected with Vi S.typhi at moi 10. After 1h, unbound bacteria were removed and cells were incubated with RPMI-1640 containing gentamycin (200μg/ml). The culture supernatants were collected after 5h and analyzed for IL-8 by ELISA. The dotted lines represent IL-8 levels from uninfected cells.
Vi⁺ *S. typhi* induces less IL-8 secretion from Caco-2 cells, as compared to Vi⁻ *S. typhi*.

The results described above suggested that incubation of Caco-2 cells with Vi reduced their ability to secrete IL-8 in response to infection with capsule-negative *S. typhi*. The significance of these findings was investigated further by comparing the abilities of Vi positive and Vi negative *S. typhi* to induce IL-8 secretion from Caco-2 cells. The results showed that infection of Caco-2 cells with Vi-positive *S. typhi* produced significantly lower levels of IL-8 as compared to infection with Vi-negative *S. typhi* (Figure 21). This was consistent with the ability of soluble Vi to downregulate IL-8 secretion from Caco-2 cells (Figure 16a). However, it was important to rule out that the reduced ability of Vi positive *S. typhi* to evoke inflammatory responses from Caco-2 cells was not because encapsulated bacteria were less invasive as compared to capsule-negative *S. typhi* (Zhao et al., 2001) or because Vi-positive *S. typhi* produced less flagellin during interaction with Caco-2 cells. The first possibility was analyzed by studying the effect of bacterial internalization on IL-8 secretion from Caco-2 cells. As can be seen from Figure 22, treatment of Caco-2 cells with cytochalasin D, a well known inhibitor of actin polymerization, inhibited internalization of *S. typhi* by more than 90% but did not significantly affect IL-8 secretion from these cells. The data suggested that internalization of bacteria may not play a major role in IL-8 secretion in response to infection with *S. typhi*. These results are consistent with many previous studies which have shown that adhesion rather than invasion might be the critical determinant for activation of transcription factors required for the secretion of proinflammatory cytokines by intestinal epithelial cells (Tang et al., 1994; Hobbie et al., 1997; Savkovic et al., 2001). Weinstein et al. (1998) showed that secretion of IL-6 from intestinal epithelial cell lines in response to infection with *S. typhi* was not dependent on internalization of bacteria by these cells. Similarly, Eaves-Pyles et al. (1999) have demonstrated that inhibition of invasion of *S. dublin* or *S. typhimurium* does not abolish degradation of IκB, activation of NF-κB or IL-8 secretion from intestinal epithelial cells.

To test the second possibility, an immunoprecipitation analysis was carried out to detect flagellin during infection of Caco-2 cells with Vi positive or Vi negative *S. typhi*. Even though, there were differences in the amounts of flagellin released by the two strains (Figure 23), those could not account for the differences seen in IL-8 secretion. In
Figure 21. Caco-2 cells infected with Vi+ *S. typhi* produce less IL-8 as compared to cells infected with Vi− *S. typhi*. Cells were infected with bacteria (10 and 100 moi) in the absence (A) or presence (B) of serum (1% FCS). After 1h, unbound bacteria were removed and cells were incubated with gentamycin-containing RPMI-1640 or RPMI-10. Supernatants were collected after 5h and analysed for IL-8 by ELISA. The dotted line represents IL-8 levels from uninfected cells.
Figure 22. Internalization of bacteria is not essential for inducing secretion of IL-8 from Caco-2 cells. Cells were plated overnight and thereafter incubated with varying concentrations of cytochalasin D for 30 minutes at 37°C. Subsequently, cells were infected with Vi S.typhi at moi 10. After 1h, unbound bacteria were removed and cells were incubated with RPMI-1640 containing gentamycin (200μg/ml). (a) Culture supernatants were collected after 5h for IL-8 analysis. Dotted line represents IL-8 levels from uninfected cells. (b) Simultaneously, cells were washed, lysed with lysis buffer and plated on SS agar plates. The number of colony forming units were determined after overnight incubation at 37°C.
Figure 23. Reduced IL-8 secretion with Vi⁺ S.typhi was not due to decreased production of flagellin. The culture supernatants collected from Caco-2 cells 1h after infection with Vi⁺ S.typhi or Vi⁻ S.typhi were incubated with protein G-Sepharose beads preloaded with anti-S.typhi flagellin MoAb. The beads were washed, boiled with Laemmli sample buffer (non-reducing) and run in a 12.5% SDS-PAG. Proteins were transferred to a NC membrane and blotted with anti-flagellin MoAb. A-flagellin from cells infected in the absence of serum, B-flagellin from cells infected in the presence of serum. Lanes: 1-Protein G-Sepharose + anti-flagellin MoAb, 2- as in lane 1+ culture supernatant (CS) from uninfected cells, 3 and 4- as in lane 1+ CS from cells infected with 10 and 100 moi Vi⁺ S.typhi, 5and 6- as in lane 1+ CS from cells infected with 10 and 100 moi Vi⁻S.typhi.
fact, Vi-positive *S.typhi* at 100 moi produced significantly more flagellin than Vi-negative *S.typhi* at 10 moi, yet the latter induced more IL-8 secretion from Caco-2 cells. These results suggest that Vi might inhibit IL-8 secretion by suppressing signaling through TLR-5, the receptor for flagellin. It should be mentioned here that flagellin is a crucial molecule for inducing inflammatory responses as deletion of this molecule from *S.typhimurium* has been shown to abrogate the ability of this pathogen to induce IL-8 secretion from intestinal epithelial cells (Zeng *et al.*, 2003). In the presence of serum, which triggered more flagellin release from bacteria, Vi positive *S.typhi* did induce significant amounts of IL-8 from Caco-2 cells but the levels were still lower than those induced by Vi negative *S.typhi*. This might be, as described earlier (Figure 18), due to antagonistic effect of serum on the ability of Vi to inhibit IL-8 secretion from Caco-2 cells. That might also explain why differences in IL-8 secretion in response to Vi positive and Vi negative *S.typhi* were more pronounced in the absence of serum.

**Vi targets MAP-kinase pathway**

The induction of inflammatory responses in intestinal epithelial cells upon infection with enteric pathogens including *Salmonella* involves activation of a number of intracellular signaling pathways including NF-κB and MAP-kinase pathway (Hobbie *et al.*, 1997). The ERK signaling module of the MAP kinase is a vital mediator of a number of cellular fates including growth, proliferation and survival (Johnson *et al.*, 2002). It has also become clear that several inflammatory processes involve ERK1/2 activation (Hommes *et al.*, 2003). To understand the mechanism by which Vi mediated inhibition of IL-8 secretion, effect of activation with the polysaccharide on phosphorylation of ERK was investigated. Cells were incubated with Vi and subsequently activated with PMA. As can be seen from Figure 24a, cells pre-stimulated with Vi showed slightly increased ERK phosphorylation in response to PMA at earlier time point but this was followed by significant reduction in ERK phosphorylation. Phosphorylation of ERK was also inhibited in cells which were incubated with Vi and subsequently infected with Vi-negative *S.typhi* (Figure 24b). However, the latter ERK phosphorylation was reduced at all time points indicating that there might be subtle differences in the way Vi affected signaling in Caco-2 cells in response to PMA and bacteria. Nevertheless, the data
Figure 24. Stimulation with Vi modulates MAP-kinase pathway in Caco-2 cells. Freshly detached cells (1x10^6) were incubated with Vi (12.5μg/ml) for 1h in serum-free RPMI-1640 and stimulated with PMA (100ng/ml) for different time periods (a), or infected with Vi S.typhi moi 50 (b). Cells were lysed with TKM buffer containing 1% Triton X-100, the lysates were run in a 12.5% SDS-PAG and transferred to a NC membrane. The NC sheet was blotted with anti-phosphoERK antibodies and developed using ECL. Subsequently, the NC sheet was incubated with a low pH solution (0.1M acetic acid containing 0.15M NaCl) to strip the bound antibodies and blotted with anti-ERK antibodies. (a) Lanes: 1-3, Caco-2 cells incubated with medium for 5’, 15’ and 60’; lanes 4-6, cells activated with PMA for 5’, 15’ and 60’; 7- cells incubated with Vi for 60’ and lanes 8-10, cells incubated with Vi for 60’ followed by activation with PMA for 5’, 15’ and 60’. (b) Lanes 1-3, Cells incubated with medium for 5’, 15’ and 60’; lanes 4-6, cells infected with Vi S.typhi for 5’, 15’ and 60’ and lanes 7-9, cells incubated with Vi for 1h followed by infection with Vi S.typhi for 5’, 15’ and 60’.
suggested that MAP-kinase pathway might be one of the targets modulated by Vi to dampen early inflammatory responses. To confirm that activation of ERK was indeed involved in the secretion of IL-8, Caco-2 cells were infected with *S.typhi* in the presence of a specific MEK inhibitor, PD98059. PD98059 inhibited IL-8 secretion in a dose-dependent manner (Figure 25).

**Effect of Vi on cytokine secretion from a human macrophage cell line**

The importance of macrophages and polymorphonuclear leukocytes in early responses to *Salmonella* is well documented (O'brien *et al*, 1979). Once across the intestinal epithelium, salmonellae encounter macrophages in the sub-mucosa. The bacteria survive and replicate inside these macrophages. These infected macrophages secrete a number of proinflammatory cytokines and chemokines (Rosenberger *et al*., 2000). Analysis of binding of Vi to a human monocytic cell line, U937 showed that Vi could bind to these cells in a dose-dependent fashion (Figure 4). More importantly, the interaction with these cells was mediated through a complex very similar to that obtained with Caco-2 cells, (Figure 7) which suggested that Vi might modulate inflammatory responses from mononuclear phagocytes as well. This was investigated by analyzing the effect of Vi on the secretion of IL-8 and TNFα in response to stimulation of U937 cells with PMA. The results showed that U937 produced significantly lower levels of IL-8 as well as TNF-α when preincubated with Vi (Figure 26a and b).

**Discussion**

Pathogenic microorganisms have evolved different strategies to modulate defense mechanisms in order to establish themselves within a susceptible host. These include interference with intracellular signal transduction pathways that are important in the regulation of expression of various cytokines and other immune mediators, downregulation of early innate immune responses etc (Hornef *et al*., 2002). These modulations are mediated through host-pathogen interactions involving pathogen-specific molecules and corresponding receptors on host cells (Guo, *et al*., 1997; Hayashi *et al*., 2001). Capsular polysaccharides expressed by many pathogenic bacteria have been known to play an important role in the pathogenesis of these microorganisms. These
Figure 25. Effect of MEK inhibitor, PD98059, on IL-8 secretion by Caco-2 cells in response to infection with *S.typhi*. Freshly detached Caco-2 cells were incubated with PD98059 for 1h before infecting with Vi-positive *S.typhi* (A) or Vi-negative *S.typhi* (B) at moi 10. After 1h, unbound bacteria were removed and cells were incubated for another 5h in RPMI-1640 containing gentamycin. IL-8 was determined in the culture supernatants by ELISA. The dotted line represents IL-8 levels from uninfected cells.
Figure 26. Vi inhibits PMA-induced secretion of IL-8 and TNF-α from U937 cells. Cells were incubated with Vi for 1h at 37°C followed by stimulation of with PMA. The culture supernatants were collected after 5h were analyzed for (a) IL-8 or (b) TNF-α by ELISA. (a) and (b) represent two different experiments. Dotted lines represent cytokine levels from unstimulated cells.
molecules can protect bacteria against complement mediated lysis, enhance virulence by rendering bacteria resistant to phagocytosis, promote bacterial colonization and persistence in mucosal surfaces, and can also induce inflammatory responses (Domenico et al., 1994; Hirose et al., 1997; Kozel et al., 1982; Marques et al., 1992; Pietrella et al., 2003; Powell et al., 1997). Vi polysaccharide that forms a capsule around *S. typhi* has also been suggested to contribute to virulence by inhibiting complement mediated killing and by rendering this pathogen resistant to intracellular killing mechanisms of macrophages (Looney and Steigbigel, 1986). The results described in this chapter show that in addition to these effects, Vi can also bring about a crucial modulation early on during interaction of *S. typhi* with the intestinal epithelium. The data demonstrate that engagement of a human intestinal epithelial cell line with Vi can significantly reduce the ability of theses cells to produce IL-8 upon infection with capsule negative *S. typhi*. Consistent with these results, it was also observed that capsule-positive *S. typhi* induced significantly less IL-8 secretion from intestinal epithelial cells as compared to capsule-negative *S. typhi*. This was not related to the reduced ability of Vi positive *S. typhi* to invade intestinal epithelial cells indicating that the production of inflammatory responses may not necessarily depend on bacterial invasion. Similar results have been reported with other bacterial pathogens including *Helicobacter pylori*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Enterohaemorrhagic E.coli* (Tang et al., 1994; Hobbie et al., 1997; Savkovic et al., 2001). The reduced inflammatory response observed with encapsulated *S. typhi* was seen even when this pathogen produced sufficient amounts of flagellin during interaction with intestinal epithelial cells, suggesting that incubation of cells with Vi might interfere with TLR-5 signaling. TLR-5 is the receptor for bacterial flagellin which is considered to be the major proinflammatory determinant of pathogenic *Salmonella* in the gut (Gewirtz et al., 2001b). Interestingly, the polysaccharide-mediated inhibition of IL-8 secretion was best seen with freshly detached Caco-2 cells or cells that had been plated at lower cell densities for shorter duration (not grown to confluency). This could be due to sequestration of Vi-interacting receptor complex in tight junctions in highly confluent cultures, as revealed by reduced binding of Vi to cells grown to confluency (Figure 19b). Furthermore, Vi-induced suppression of IL-8 secretion was more pronounced under serum-free conditions and could in fact be reversed by serum,
suggesting that the cascade activated by serum might antagonize the pathway triggered by Vi.

The results discussed above suggested that interaction of intestinal epithelial cells with Vi could dampen early inflammatory responses during infection with *S. typhi*. How did Vi bring about this inhibition? Given that MAP-kinase pathway plays an important role in inflammatory responses as well as in the regulation of prohibitin-mediated effects on cell cycle, experiments were carried out to investigate the effect of stimulation with Vi on ERK phosphorylation. There was a significant reduction in the induction/sustenance of ERK phosphorylation when Caco-2 cells were treated with Vi before being infected with Vi-negative *S. typhi* or stimulated with PMA, suggesting that MAP-kinase pathway might be a target for Vi-mediated suppression of inflammatory responses. The secretion of IL-8 upon infection with *S. typhi* was also reduced in the presence of MEK inhibitor PD98059 demonstrating that activation of ERK was indeed involved in the induction of this inflammatory chemokine by *S. typhi*. The identification of ERK as a target for Vi-mediated inhibition of inflammatory responses would also explain why serum could reverse these effects as serum is known to activate Raf-1/MEK/ERK cascade in cells (Dhillon *et al.*, 2002). Significantly, serum has also been shown to reverse prohibitin-mediated suppression of E2F transcriptional activity and this reversal is believed to be mediated through activation of MAP-kinase pathway (Wang *et al.*, 1999b).

The findings described in this study identify prohibitin and its closely related homologs as novel host targets that can be engaged by a pathogen-derived molecule to modulate cellular responses. The exact mechanism by which Vi, through interaction with cell surface associated prohibitins, reduced ERK phosphorylation and in turn downregulated inflammatory responses is not clear at the moment and should be the focus of future studies. Nevertheless, the results clearly demonstrate that Vi can suppress early inflammatory responses from intestinal epithelial cells, which might play a crucial role in the establishment of infection by *S. typhi*. In vivo, Vi-mediated suppression described here could be brought out by soluble Vi, which is released in abundance by *S. typhi* during *in vitro* growth and has also been reported in the serum and urine samples of typhoid patients (Rockhill *et al.*, 1980; Barret *et al.*, 1982), or by pathogen associated Vi. Vi released from *S. typhi* may also contribute to infection in a fashion similar to that
reported recently with filamentous hemagglutinin of *Bordetella pertussis*. The release of this adhesin by the pathogen facilitated mucosal colonization by the pathogen (Coutte *et al.*, 2003). Preliminary data reported in this thesis suggests that Vi-mediated inhibition of IL-8 secretion may not be restricted to intestinal epithelial cells as secretion of this chemokine following stimulation of a human monocytic cell line, U937 with PMA was also downregulated in the presence of Vi. It is therefore possible that Vi might also modulate various cellular responses after the pathogen has disseminated into blood and various lymphoid organs. Considering that macrophages play a central role in host defense against pathogenic *Salmonella* (and other pathogenic organisms), the role of Vi in the modulation of macrophage functions should be investigated in greater detail in future.