A PATHOBIONT OF THE MAMMALIAN MICROBIOTA BALANCES INTESTINAL INFLAMMATION AND COLONIZATION

Thesis by

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ABSTRACT

Humans and mammals are colonized by a multitude of microbial organisms that have co-evolved with their hosts for millions of years. The majority of these microbes reside in the gastrointestinal (GI) tract as a complex and dynamic consortium. Though most associations with the host are symbiotic or commensal, some resident bacteria have the potential to cause disease under certain conditions. We refer to these bacteria as 'pathobionts.' Pathobionts are distinct from opportunistic pathogens, which are often acquired from the environment and cause acute infections. Bacterial type VI secretion systems (T6SSs) are one mechanism for mediating close host-microbial interactions. Herein we report that the T6SS of *H. hepaticus*, a pathobiont of the murine intestinal microbiota, mediates critical protective functions during association with its mammalian host. In cell cultures, infection of intestinal epithelial cells (IECs) with *H. hepaticus* T6SS mutants results in increased bacterial association compared to wild-type bacteria. In animals, T6SS mutants colonize the lower GI tract to a higher degree. Most importantly, *H. hepaticus* defective in type VI secretion is unable to restrain potent innate and adaptive immune responses in an animal model of experimental colitis. In addition, the *H. hepaticus* T6SS directs an anti-inflammatory gene expression profile in IECs, and CD4+ T cells from mice colonized with T6SS mutants produce increased proinflammatory interleukin-17 cytokine in response to IECs presenting *H. hepaticus* antigens. Thus, our findings reveal that *H. hepaticus* has evolved a T6SS as a mechanism to actively maintain a non-pathogenic, symbiotic relationship in the GI tract by regulating bacterial colonization and host inflammation. Disturbances in the

dynamic interaction between gut bacteria and the intestinal immune system may lead to exacerbated host inflammation. As intestinal bacteria profoundly influence host biology, our findings support an emerging hypothesis that alterations in the composition of the microbiota, known as dysbiosis, is a critical factor in various human disorders such as inflammatory bowel disease and colon cancer.

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INTRODUCTION

Overview of the intestinal microbiota

The human body is colonized by microbial organisms on virtually all environmentally exposed surfaces. The vast majority of these microbes are harbored in the gastrointestinal (GI) tract where over 1,000 microbial species thrive and commensal bacteria can outnumber host cells 100 to one. Many important nutritional, metabolic, and immunologic functions are provided by the gut microbiota including the synthesis of vitamins, digestion of complex sugars, maintenance of the intestinal epithelial barrier, and resistance to pathogen colonization (Backhed et al., 2005). Millions of years of co-evolution have interdependently linked the health of mammals to their microbiota, as commensal bacteria have been shown to play an important role in coordinating the immune system (Lee and Mazmanian, 2010).

Even though 55 divisions of Bacteria exist, the selective pressures of the human gut appear to be quite rigorous as the microbiota is dominated by members of only two divisions, Bacteroidetes and Firmicutes (Ley et al., 2006). Within these two phyla, there is considerable variation in the microbiota between individuals due to numerous variables including hygiene, diet, and host genetics. To successfully colonize the GI tract long-term, members of the microbiota must be able to survive host immune system responses, competition from other bacteria, peristalsis of the gut, and the availability of a limited variety of nutrients.

Type VI secretion systems

Microbial secretion systems are universal mechanisms dedicated to molecular interactions between bacterial and host cells, and can range from simple transporters to large, membrane-spanning structures. Type III secretion systems (T3SSs), T4SSs and T6SSs function as multi-subunit complexes that translocate effector substrates across the double membrane of Gram-negative bacteria, and act as a 'needle and syringe' to inject molecules directly into eukaryotic cells (Raskin et al., 2006). The temporal and spatial profile of secreted molecules can have profound effects on host biology. Therefore, these secretion systems represent well-evolved microbial strategies capable of mediating molecular interactions between the microbiota and mammals.

T6SS genes are present in over 25% of all sequenced bacterial genomes, and have been largely associated with bacterial pathogenesis. T6SSs are almost exclusively found in α-, β-, and γ-proteobacteria (Bingle et al., 2008). *Helicobacter hepaticus* is unique in that it appears to contain the only bacterial genome in the epsilon (ε) subgroup of proteobacteria which encodes for a T6SS. In recent seminal studies, Mekalanos and colleagues reported that *Vibrio cholerae* requires T6SS genes to cause cytotoxicity in macrophages (Pukatzki et al., 2006). A set of molecules were identified as the first T6SS substrates: Hcp (hemolysin co-regulated protein) and 3 VgrG (valine-glycine-repeat) proteins. Further evidence supporting a role for T6SSs in bacterial virulence has been demonstrated in other Gram-negative pathogens including *Pseudomonas aeruginosa* (Mougous et al., 2006), enteroaggregative *E. coli* (Dudley et al., 2006), and *Aeromonas hydrophila* (Suarez et al., 2008). However, the

function of bacterial T6SSs is not limited to eliciting disease. A growing body of research has shown that T6SSs may modulate virulence (Bladergroen et al., 2003; Das et al., 2002; Roest et al., 1997). Therefore in some contexts, T6SSs may modulate bacterial virulence in order to 'balance' the relationship between microbe and host.

Helicobacter hepaticus

Helicobacter hepaticus, a spiral microaerophilic bacterium, is a commensal bacterium of the mammalian microbiota that is capable of promoting inflammation in specific animal models of colon cancer and experimental colitis (inflammation of the colon). Interestingly, *H. hepaticus* only causes disease in immunocompromised animals that lack immune regulation and mount inflammatory responses toward intestinal bacteria (Erdman et al., 2009; Kullberg et al., 2001; Kullberg et al., 1998). Pathologies elicited by *H. hepaticus* remarkably resemble that of human disease based on molecular, cellular, and histological parameters. Furthermore, colonization with *H. hepaticus* causes a chronic inflammatory response similar to human inflammatory bowel disease (IBD), in contrast to infections with *Citrobacter rodentium* or *Salmonella typhimurium* which induce acute and self-limiting infections. Therefore, the *H. hepaticus*-*Mus musculus* relationship represents a unique and invaluable tool in the study of intestinal pathologies highly similar to human disorders such as IBD and colon cancer.

Interestingly, multiple studies have shown that *H. hepaticus* sustains long-term colonization of the lower GI tract of wild-type mice without causing intestinal disease (reviewed in Solnick and Schauer, 2001). In fact, *H. hepaticus* colonization is highly endemic in most laboratory animal facilities but is largely unnoticed due to

the absence of clinical disease. We have previously proposed that *H. hepaticus* acts as an intestinal pathobiont— i.e., a symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host (Mazmanian et al., 2008; Round and Mazmanian, 2009). Pathobionts are distinct from opportunistic pathogens which are often acquired from the environment and lead to acute infections. The concept of pathobionts is supported by clinical data which reveal that in IBD patients with underlying genetic mutations, inflammation is targeted to specific members of the microbiota and not to infectious pathogens (Packey and Sartor, 2009). T cell responses and antibody reactivity during IBD target certain subsets of symbiotic microbes such as *Escherichia*, *Clostridium* and *Enterococcus* species that are found in all people. Since *H. hepaticus* displays a potentially pathogenic association with its murine host similar to specific microbes found in humans, and the T6SS appears to mediate both symbiotic and pathogenic outcomes, we investigated a role for the *H. hepaticus* T6SS during intestinal inflammation.

CHAPTER 1: *H. hepaticus* modulates colonization of intestinal epithelial cells

Introduction

The genome of *Helicobacter hepaticus* contains a 71-kilobase genomic island, termed HHGI1 for *H*elicobacter *h*epaticus *g*enomic *i*sland 1. HHGI1 has been characterized as a pathogenicity island based on the presence of genes homologous to virulence factors (Suerbaum et al., 2003). Isolates missing the pathogenicity island are highly attenuated for inducing disease and deletion of a 19 gene segment of HHGI1 reduces virulence (Ge et al., 2008). However, the functions of the gene products encoded within HHGI1 remain unclear. In recent years, studies have identified a protein secretion system referred to as a type VI secretion system (T6SS) (Raskin et al., 2006). We report herein that HHGI1 encodes for a canonical and functional T6SS.

It is well established that many enteric pathogens such as *Listeria monocytogenes* and *Shigella flexneri* enter intestinal epithelial cells (IECs) during infection. In several cases, these bacteria possess T3SSs which either facilitate entry or are required for internalization (Galan and Wolf-Watz, 2006). Because T6SSs have been shown to mediate close host-microbial interactions, and because *H. hepaticus* colonizes the mucosal surface overlaying IECs in the gut, we investigated whether *H. hepaticus* enters IECs. Using an intestinal epithelial cell line, MODE-K, we colonized host cells with wild-type or T6SS mutant *H. hepaticus* bacteria and used confocal microscopy to identify internalization.

Results

1.1 *H. hepaticus* encodes for a canonical type VI secretion system

H. hepaticus encodes for a set of T6SS genes that are homologous to those found in *V. cholerae*, *P. aeruginosa*, and other Gram-negative microorganisms (Table 1). *H. hepaticus* contains 12 homologous T6SS genes that are clustered and arranged in a genomic organization similar to other T6SSs (Figure 1A). A homolog of the *icmF* gene, thought to encode for a structural transmembrane protein of virtually all identified T6SSs, lies within this genomic locus (*H. hepaticus* gene HH0252). Furthermore, HHGI1 encodes for homologs of Hcp (HH0243) and 3 VgrG proteins (HH0237, HH0242, HH0291), highly conserved translocated substrates that play key roles in T6SSs.

We constructed insertional mutants in the *icmF* or *hcp* genes, as previous studies in several pathogens have shown that deletion of these genes leads to T6SS defects. An antibiotic selection marker (erythromycin acetyl transferase, *eryR*) (Mehta et al., 2007) was inserted within each open reading frame by homologous recombination. PCR amplification verified integration of the *eryR* gene to create mutants ΔIcmF and ΔHcp (Figure 1B). To validate a functional defect in the T6SS, *in vitro* cultured bacteria were assayed by immunoblot for Hcp. In wild-type bacteria, Hcp was detected in the culture supernatant and cell pellet fraction as expected (Figure 1C). However, in the ΔIcmF mutant, Hcp was absent from supernatant fractions and accumulated to a higher degree in the cell pellet, indicating Hcp is still produced but not secreted in the absence of a functional T6SS. Therefore, deletion of

the *icmF* homolog results in a secretion defect for Hcp, demonstrating functional inactivation of the T6SS.

Bacteria possessing T6SSs bind host cells into which substrates can be injected. Since *H. hepaticus* colonizes the murine gut and is in close association with intestinal crypts, we investigated the association of *H. hepaticus* with murine IECs. *H. hepaticus* was co-cultured with the mouse intestinal epithelial cell line MODE-K (Vidal et al., 1993), and examined for translocalization of VgrG by confocal immunofluorescence microscopy. Labeling of MODE-K cells with antisera specific for the T6SS substrate VgrG showed diffuse staining upon incubation with wild-type bacteria (Figures 1D and 1E). Conversely, VgrG labeling of MODE-K cells incubated with ΔIcmF mutant showed punctate staining patterns suggesting VgrG produced by T6SS mutants is bacterially-associated. As whole bacterial antisera raised against *H. hepaticus* reveals a punctate staining pattern as well (Figure 1D), the diffuse staining of VgrG in wild-type bacteria suggests substrate translocation in the presence of MODE-K cells (Figure 1E).

1.2 *H. hepaticus* is internalized into host intestinal epithelial cells

Infection of an intestinal epithelial cell line, MODE-K cells, revealed that both wild-type and T6SS mutants were capable of entering host cells (Figure 2A). Confocal microscopy demonstrated that all strains were found associated with the cell surface as well. Z-stacks reconstructed from confocal images further revealed significant numbers of intact bacteria within the intracellular compartment of IECs. To determine if uptake was an active process, we incubated wild-type and T6SS

mutants with MODE-K cells for 30 min in the presence or absence of cytochalasin D, an inhibitor of actin polymerization. Cells were subsequently treated with gentamicin to kill extracellular bacteria and plated for colony forming units (CFUs). Figure 2B reveals that while all strains could be recovered from the intracellular compartment of MODE-K cells, cytochalasin D inhibited bacterial uptake. Therefore, *H. hepaticus* appears to be actively internalized into cultured IECs through a requirement for actin rearrangement.

1.3 T6SS mutants display increased cell association within intestinal epithelial cells

We sought to determine if the T6SS affects bacterial internalization. MODE-K cells were incubated with *H. hepaticus*, treated with (or without) gentamicin, and plated for bacterial enumeration. At early time points (30 min), no differences were observed in the proportions of intracellular and cell-associated bacteria between wild-type and T6SS mutants (Figures 2C and 2D). Remarkably, by 3 hr and 6 hr, ΔIcmF and ΔHcp had significantly higher levels of intracellular bacteria (Figure 2C). In addition, T6SS mutants displayed greater numbers of MODE-K-associated bacteria after 6 hr (Figures 2D). Total numbers of bacteria recovered from co-cultures (which includes all non-cell associated bacteria) as well as cultures grown in the same media without MODE-K cells were comparable (Figure S1A), demonstrating that the mutations did not affect bacterial growth. These results reveal that the T6SS of *H. hepaticus* limits intracellular bacterial numbers within IECs.

To investigate if the increased cell association by T6SS mutants was due to increased adherence, we blocked internalization of *H. hepaticus* into MODE-K cells. Cytochalasin D was added for the duration of the co-culture (6 hrs). In the absence of bacterial internalization, T6SS mutants still exhibited an increase in adherence to the extracellular surface of MODE-K cells (Figure 2E). An increase in cell adherence in the absence of internalization was also observed for T6SS mutants when MODE-K cells were fixed with 4% paraformaldehyde prior to co-incubation. Despite the increase in MODE-K adherence, the T6SS mutants did not affect the viability of MODE-K cells compared to wild-type bacteria. Staining for MODE-K cell apoptosis with Annexin V and propidium iodide showed that neither wild-type nor T6SS mutants had a significant effect on IEC viability (Figure S1B).

1.4 T6SS mutants display increased colonization of the murine intestine

Germ-free animals (devoid of all microorganisms from birth) were employed to measure *in vivo* intestinal colonization. Mice were mono-colonized with wild-type, ΔIcmF, or ΔHcp *H. hepaticus* (ranging from 2-13 weeks of colonization), at which time RNA was isolated from colonic tissue. Colonization levels were determined by quantitative RT-PCR (qRT-PCR) for *H. hepaticus*-specific 16S ribosomal RNA, as previously described (Nanda Kumar et al., 2008). Interestingly, RNA extracted from the ceca and colons of mono-colonized animals revealed that *H. hepaticus* colonization by ΔIcmF and ΔHcp mutants was increased compared to wild-type (Figure 3A). *In vitro* analysis of RNA (and DNA) levels demonstrated that mutating the T6SS does not affect 16S rRNA expression (Figure S2A). Accordingly, plating for

viable bacterial CFUs from homogenized colonic tissue confirmed significantly elevated levels of colonization for T6SS mutants (Figure 3B). We observed a similar increase for T6SS mutants following colonization for up to three months in two strains of germ-free mice, outbred Swiss Webster and inbred C57Bl/6 mice. Images of colon sections from mono-colonized animals showed that both wild-type and ΔIcmF were found intimately associated with host cells (Figure 3C). Consistent with previous reports for wild-type *H. hepaticus* (Kullberg et al., 2002), we did not observe any cases of intestinal disease in colonized animals. Examination of colonic tissues (e.g., epithelial hyperplasia, infiltration of leukocytes, crypt abscesses) revealed no signs of histopathology (Figure S2B and data not shown). qRT-PCR analysis of colonic tissues for pro-inflammatory cytokines showed comparable levels of tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) between germ-free and mono-colonized animals (Figure S2C). In addition, the percentage of $CD4^{+}TCR\beta^{+}$ cells in the mesenteric lymph nodes (MLNs) did not differ between groups (Figure S2D).

To investigate the intracellular association of *H. hepaticus* within IECs *in vivo*, we colonized SPF (specific pathogen free) C57Bl/6 mice with wild-type, ΔIcmF or no *H. hepaticus*. Colonic IECs were purified and treated with gentamicin either prior to or following IEC lysis. Internalized bacteria were enumerated as the gentamicin resistant population. Interestingly, ΔIcmF showed increased intracellular numbers in IECs compared to wild-type bacteria (Figure 3D). This increase in bacterial internalization by ΔIcmF was also observed in IECs recovered from the colons and ceca of C57Bl/6 *Rag1*$^{-/-}$ and *IL-10*$^{-/-}$ mice (Figure S2E). IECs lysed with saponin prior

to incubation with gentamicin showed no viable *H. hepaticus* bacteria, demonstrating the complete killing of bacteria (Figure 3D).

T6SS homologues in other bacterial species

H. hepaticus	*V. cholerae*	*P. aeruginosa*
HH0243 Hcp	VCA0017 (62/77)[a] Hcp	Hcp (58/69)
	VC1415 (62/77) Hcp	
HH0244	VCA0111 (29/43)	PA0089 (25/41)
HH0245	VCA0110 (30/50)	PA0088 (24/41)
HH0246	—	—
HH0247	VCA0108 (60/78)	PA0084 (33/56)
HH0248	VCA0107 (52/68)	PA0083 (30/50)
HH0249	VCA0113 (24/41)	PA0080 (26/46)
HH0250	VCA0114 (22/46)	PA0079 (23/44)
HH0251	VCA0115 (39/63)	PA0078 (29/49)
HH0252 IcmF	VCA0120 (22/41)	PA0077 (19/38) IcmF1
HH0237 VgrG	VC1416 (25/46) VgrG-1	PA0095 (21/48)
	VCA0018 (21/47) VgrG-2	
	VCA0123 (25/45) VgrG-3	
HH0242 VgrG	VC1416 (22/36) VgrG-1	PA0095 (21/39)
	VCA0018 (21/36) VgrG-2	
	VCA0123 (23/37) VgrG-3	
HH0291 VgrG	VC1416 (27/43) VgrG-1	PA0095 (25/48)
	VCA0018 (24/40) VgrG-2	
	VCA0123 (24/38) VgrG-3	

Table 1. Comparison of T6SS genes from *H. hepaticus* and Enteric Pathogens.

[a] (% identity / % similarity)

Proteins were analyzed using BLAST.

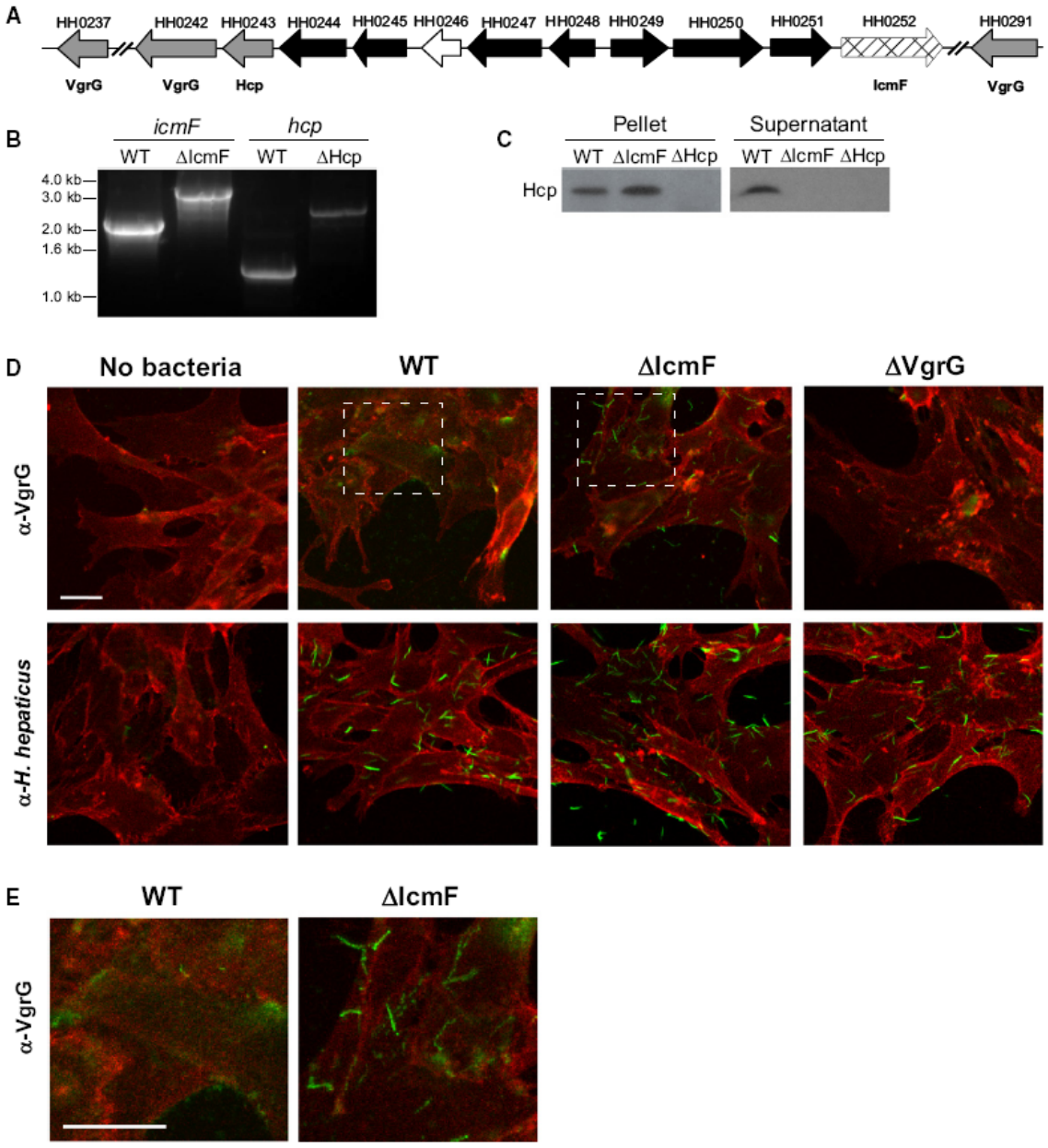


Figure 1. *H. hepaticus* Encodes for a Functional T6SS.

(A) Schematic diagram of the genetic organization of *H. hepaticus* T6SS genes. Gray arrows represent *Hcp* and *VgrG* genes, a cross-hatched arrow indicates *IcmF* homologue, black arrows represent T6SS homologues of unknown function, and a white arrow indicates a gene non-homologous to other T6SS genes.

(B) Genomic DNA collected from mid-log cultures of WT, ΔIcmF, or ΔHcp *H. hepaticus* was amplified by PCR using *icmF* or *hcp* specific primers. Insertion of the *eryR* gene was detected by a 1.1kb-increase in the resulting PCR band.

(C) Hcp is undetected in supernatants from ΔIcmF and ΔHcp T6SS mutants. Equal amounts of mid-log bacterial cultures of WT, ΔIcmF, or ΔHcp were centrifuged to separate bacterial pellets and supernatant. Supernatants were subsequently filtered to ensure removal of all bacteria. Bacterial pellets and supernatants were analyzed by Western blot. Membranes were blotted with anti-Hcp antibody.

(D, E) Confocal images of bacteria incubated with MODE-K cells. WT, ΔIcmF, or ΔVgrG (ΔHH0242) *H. hepaticus* were incubated with MODE-K cells for 5hr. MODE-K cells were subsequently rinsed with PBS, fixed in 4% PFA, and stained for the eukaryotic cell membrane marker wheat germ agglutinin (red) and either *H. hepaticus* or VgrG (green). Outlined regions for WT and ΔIcmF in (D) are shown at higher magnification (E). Scale bar represents 20μm.

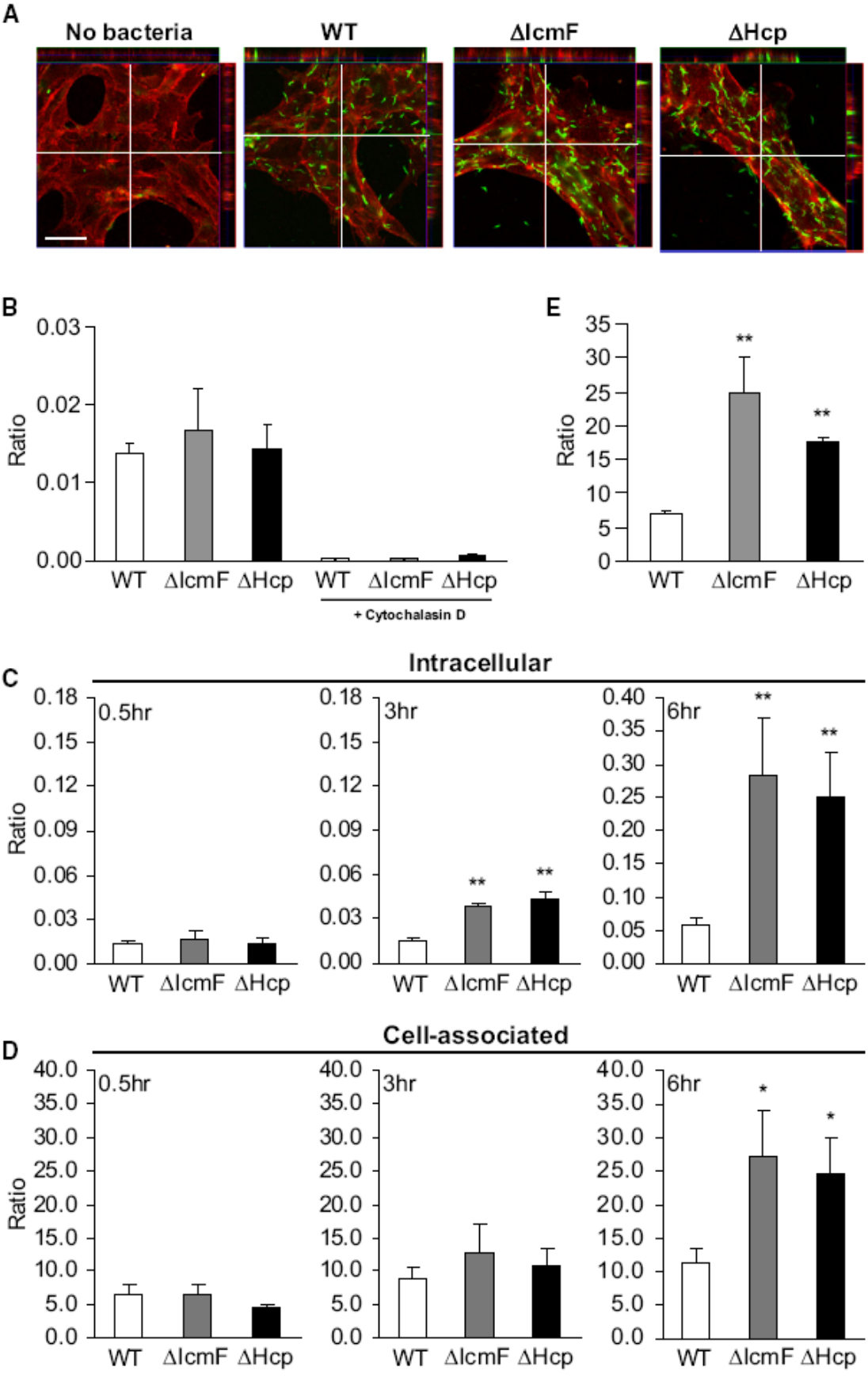

A
No bacteria
WT
ΔIcmF
ΔHcp
B
Ratio
0.03
0.02
0.01
0.00
WT
ΔIcmF
ΔHcp
WT
ΔIcmF
ΔHcp
+ Cytochalasin D
E
Ratio
35
30
25
20
15
10
5
0
**
**
WT
ΔIcmF
ΔHcp
C
Intracellular
Ratio
0.5hr
3hr
6hr
0.18
0.15
0.12
0.09
0.06
0.03
0.00
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
**
**
**
WT
ΔIcmF
ΔHcp
D
Cell-associated
Ratio
0.5hr
3hr
6hr
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
*
WT
ΔIcmF
ΔHcp

Figure 2. T6SS Mutants Display Higher Intracellular and Cell-associated Accumulation in MODE-K cells.

(A) Confocal image of bacteria inside MODE-K cells. WT, ΔIcmF, or ΔHcp was incubated with MODE-K for 6hr. MODE-K cells were rinsed with PBS, fixed in 4% PFA, and stained for *H. hepaticus* (green) and the eukaryotic cell membrane marker wheat germ agglutinin (red). Scale bar represents 30μm.

(B) Cytochalasin D inhibits uptake of *H. hepaticus*. Prior to incubation with bacteria, MODE-K cells were treated with 10μM cytochalasin D for 1hr. Bacteria were added at an MOI of 100. After 0.5hr incubation at 37°C under microaerophilic conditions, cells were treated with 100μg/ml gentamicin, and intracellular bacteria plated for enumeration. Results are expressed as colony-forming units (CFUs) of intracellular bacteria divided by number of MODE-K cells. Error bars indicate SEM from 3 experiments.

(C, D) Gentamicin protection assay in which MODE-K cells were incubated with bacteria at a multiplicity of infection (MOI) of 100. After 0.5hr, 3hrs, or 6hrs incubation, media was replaced with 100μg/ml gentamicin for enumeration of intracellular bacteria (C) or without gentamicin for cell-associated bacteria (D). Cells were washed and bacteria plated for quantification. Ratios are expressed as CFUs of bacteria divided by number of MODE-K cells. Error bars indicate SEM from 3-5 experiments. *$p<0.05$, **$p<0.01$ vs WT.

(E) Increased adherence of T6SS mutants is not dependent on bacterial internalization. Prior to co-culture, MODE-K cells were treated with 10μM cytochalasin D. Bacteria were added at an MOI of 100 for 6hrs at 37°C under

microaerophilic conditions. Bacteria were plated for enumeration. Results are expressed as CFUs of bacteria divided by number of MODE-K cells. Error bars indicate SD from 2 experiments. **p<0.01 vs WT.

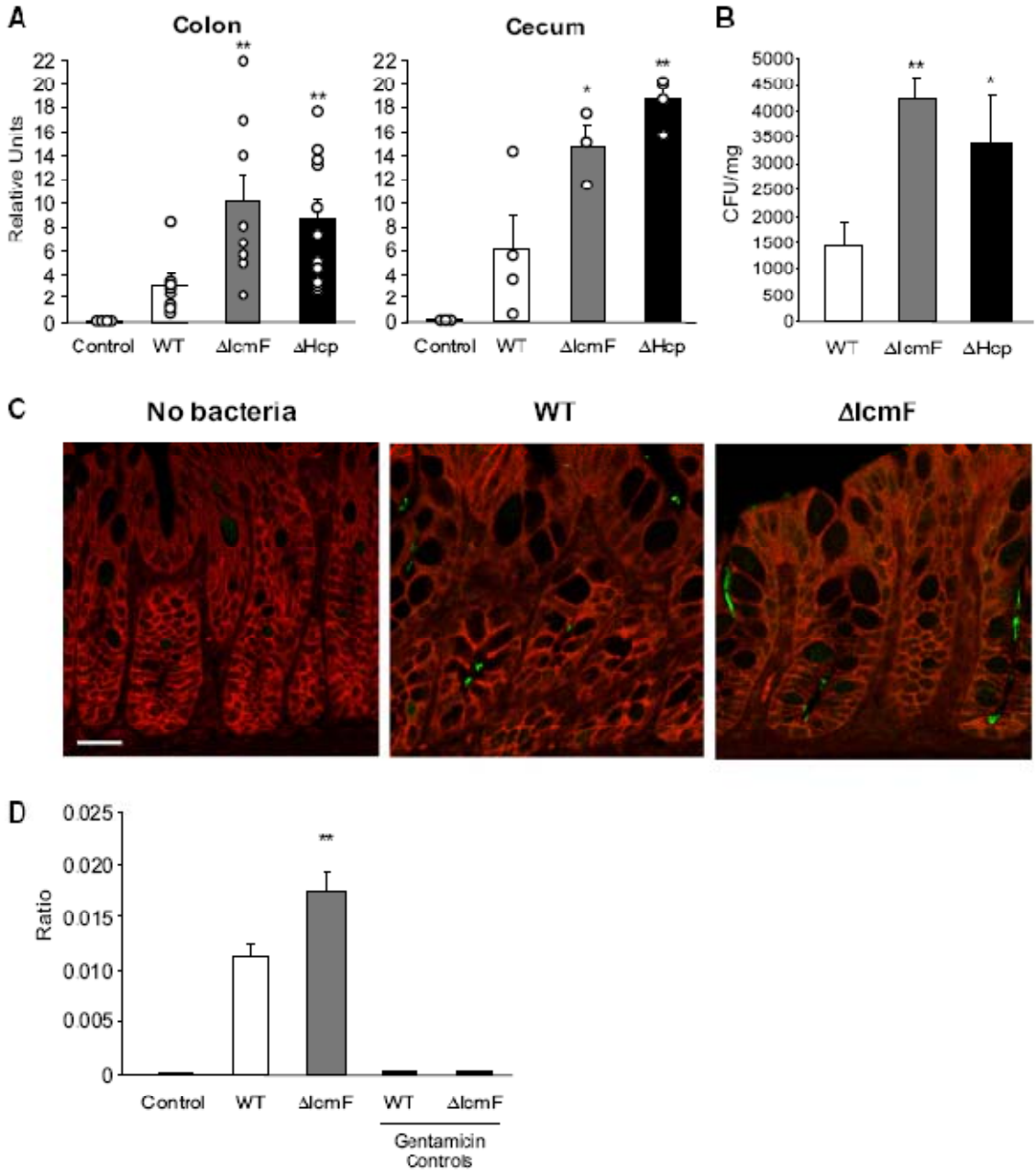


Figure 3. T6SS Mutants Have Increased Colonization Levels within the Colon.

(A) ΔIcmF- and ΔHcp-mono-colonized animals have greater amounts of *H. hepaticus* 16S rRNA in the colon and cecum compared to WT-mono-colonized animals. RNA was collected from colon and cecum. Levels of 16S were quantified by qRT-PCR

using *H. hepaticus*-specific 16S primers. Error bars show SEM, n=4-11 animals per group. *p<0.05, **p<0.01 vs WT.

(B) ΔIcmF- and ΔHcp-mono-colonized animals have higher levels of viable *H. hepaticus* in the colon compared to WT-mono-colonized animals. Colon tissues were homogenized in BHI and plated for quantification. Bacterial numbers were normalized to colon tissue weights. Units are expressed as CFUs per mg of tissue. Error bars show SEM from n=4 animals per group. *p<0.05, **p<0.01 vs WT.

(C) *H. hepaticus* is found in colonic intestinal crypts. Colons from WT- and ΔIcmF-mono-colonized mice were paraffin-embedded and sectioned. Colon sections were stained for *H. hepaticus* (green) and E-cadherin (red). Animals were colonized for 8 weeks. Scale bar represents 20μm.

(D) ΔIcmF-colonized animals have increased levels of intracellular *H. hepaticus* compared to WT-colonized animals. Purified IECs were treated with gentamicin either prior to or following lysis with saponin (which selectively permeablizes eukaryotic membranes). Bacteria were enumerated by plating on TVP (trimethoprim, vancomycin, polymyxin B) plates which are known to select for *H. hepaticus*, as confirmed by control mice which received no *H. hepaticus*. In 'Gentamicin Controls,' IECs were lysed and then treated with gentamicin. Ratio is expressed as CFUs of bacteria divided by number of IECs. Error bars indicate SEM from n=12 animals per group. **p<0.01 vs WT.

Discussion

Taken together, our results demonstrate that *H. hepaticus* contains a functional T6SS. Deletion of critical T6SS genes results in mutants unable to secrete effector substrates during bacterial growth. Furthermore, in co-cultures with IECs, T6SS mutants are unable to secrete VgrG (HH0242), a highly conserved T6SS protein that has been shown to play both structural and effector roles in other bacteria. In *V. cholerae*, the C-terminal of VgrG1 contains an actin cross-linking domain that is capable of cross-linking actin in eukaryotic cells (Pukatzki et al., 2007). Tropomyosin-like, pertactin-like or YadA-like activities have been identified in other VgrG C-terminal domains (Cascales, 2008). Future investigations will be needed to characterize what functional domain(s) HH0242 may possess, in addition to identifying other effectors. Outside of the family of VgrG proteins, only a few T6SS substrates have been identified and these still remain to be characterized in depth for their roles in mediating host interactions. As these bacterial molecules are thought to modulate host signaling pathways and other cellular mechanisms, future studies may provide insight on novel biological processes.

Our studies are the first to highlight the ability of *H. hepaticus* to enter host cells. Disruption of communication via the T6SS may lead to critical disturbances in host-microbial interactions, such as increased colonization observed in our results. Our findings are consistent with previous reports (for other bacteria) suggesting that T6SSs limit entry of additional microorganisms once bacteria have already gained access to the host cytoplasm (Ma et al., 2009). Contrary to most secretion systems of enteric bacteria which promote infections, the *H. hepaticus* T6SS limits

bacterial numbers during colonization of the mouse intestine. It has been proposed that intestinal commensal bacteria may limit potentially negative host immune responses by colonizing the lumen of the gut (Hooper, 2009). As intestinal epithelial cells are covered by a thick layer of mucus and other glycoproteins, creating such a spatial barrier may prevent inflammatory responses from being elicited by bacterial antigens. Typically only pathogens are believed to infect the mucus layer. However our data suggests that certain commensals (or pathobionts) of the gut may successfully colonize intimately with host tissues by actively limiting bacterial numbers.

Supplementary Figures

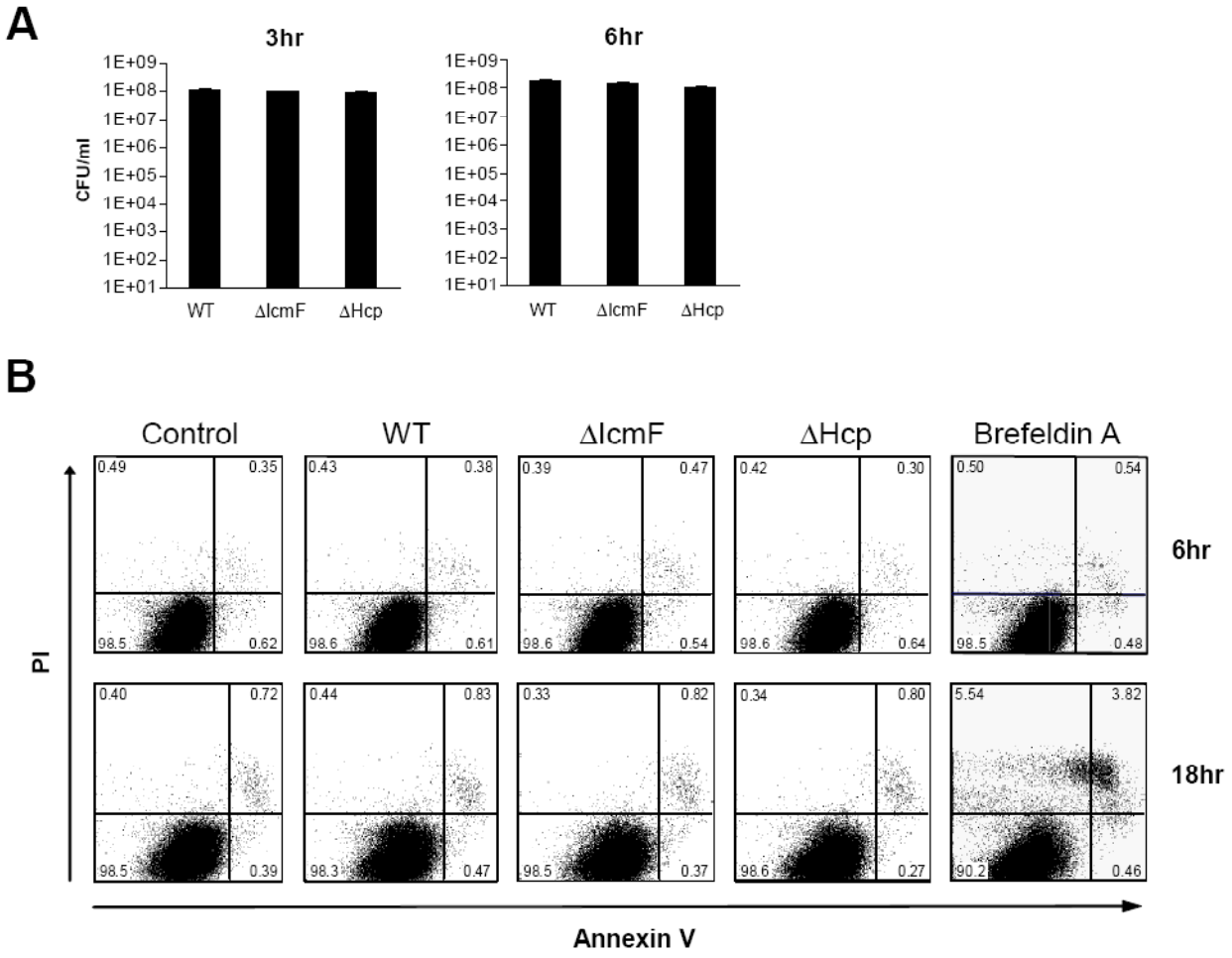


Figure S1. T6SS mutants do not affect the viability of MODE-K cells.

(A) Total numbers of *H. hepaticus* during co-culture with MODE-K cells are comparable between WT and T6SS mutants. MODE-K cells were incubated with wild-type, ΔIcmF, or ΔHcp *H. hepaticus* at a MOI of 100 at 37°C under microaerophilic conditions. After 3hrs or 6hrs incubation, total bacteria were plated on Brucella blood agar plates for bacterial quantification. Error bars represent SEM from 3 independent experiments.

(B) T6SS mutants do not affect the viability of MODE-K cells. MODE-K cells were incubated with wild-type, ΔIcmF, ΔHcp, or no *H. hepaticus* at a MOI of 100 at 37°C

under microaerophilic conditions for either 6hr or 18hr. MODE-K cells were stained with antibodies to annexin V and propidium iodide (PI). Cells were analyzed by flow cytometry. Early apoptotic cells are marked as annexin V^+ PI^- whereas late apoptotic and necrotic stages are marked by annexin V^+ PI^+ staining. Cells were also treated with 10ug/ml Brefeldin A as a positive control for apoptosis.

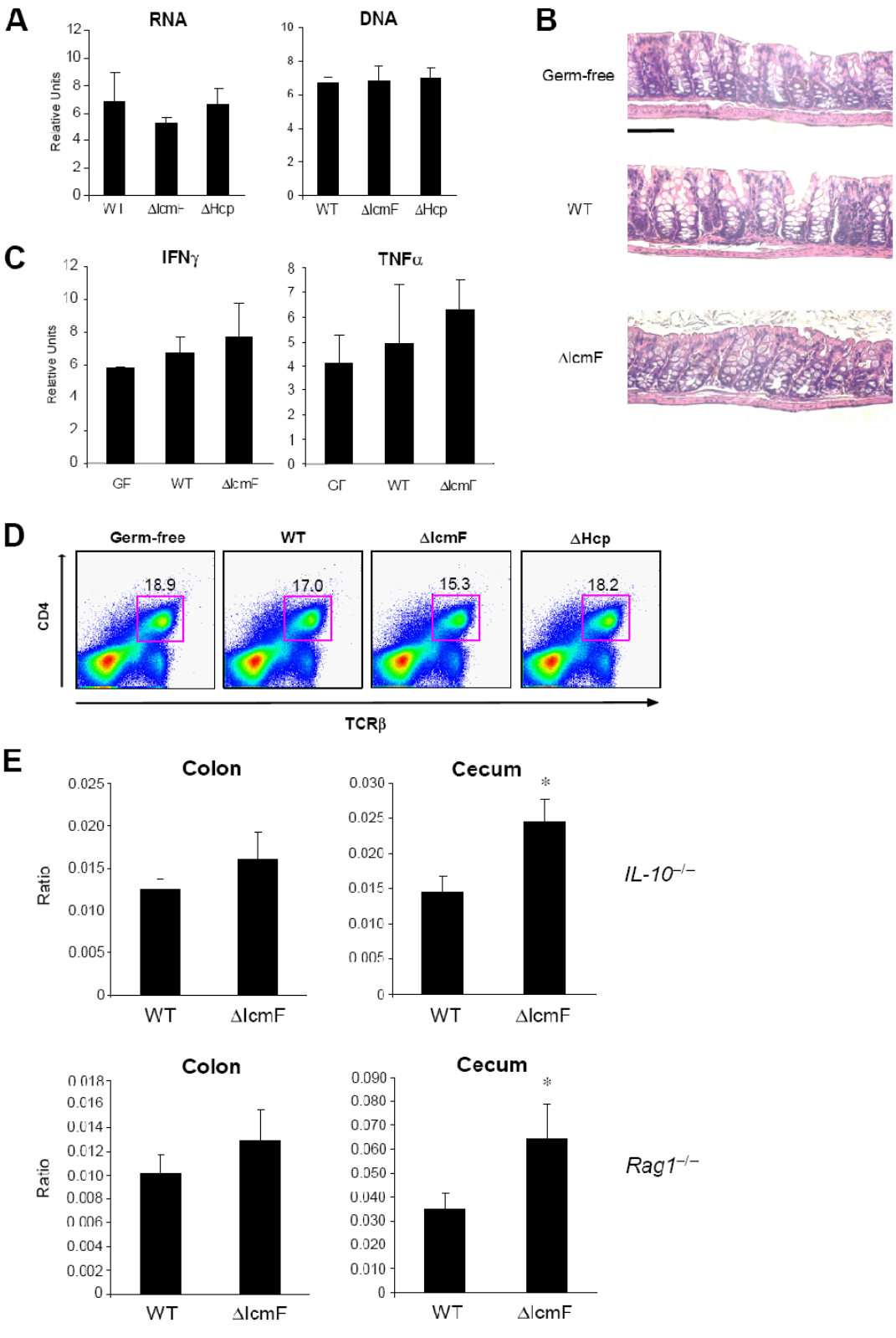

A
RNA
DNA
Relative Units
WT
ΔIcmF
ΔHcp
B
Germ-free
WT
ΔIcmF
C
IFNγ
TNFα
Relative Units
GF
WT
ΔIcmF
D
Germ-free
WT
ΔIcmF
ΔHcp
18.9
17.0
15.3
18.2
CD4
TCRβ
E
Colon
Cecum
Ratio
WT
ΔIcmF
IL-10−/−
Rag1−/−

Figure S2. T6SS Mutants Have Increased Colonization Levels within the Colon.

(A) WT, ΔIcmF, and ΔHcp bacteria produce comparable amounts of 16S ribosomal RNA and DNA. Bacterial RNA and genomic DNA were collected from mid-log wild-type, ΔIcmF, or ΔHcp bacterial cultures using TRIzol or Promega Wizard Genomic DNA Purification kit, respectively. The amount of 16S ribosomal RNA or DNA was quantified by qRT-PCR using *H. hepaticus*-specific 16S primers. Error bars represent SEM from 3 independent samples.

(B) WT- and ΔIcmF-mono-colonized animals show no obvious signs of intestinal pathology. Colons from germ-free and WT- and ΔIcmF-mono-colonized Swiss Webster mice were fixed in Bouin's fixative, paraffin-embedded, and sectioned. Colon sections were stained by H&E. Animals were colonized for approximately 8 weeks. Scale bar represents 100μm.

(C) Germ-free and WT- and ΔIcmF-mono-colonized animals show comparable levels of inflammatory cytokine transcripts. RNA from the colons of germ-free and WT- and ΔIcmF-mono-colonized Swiss Webster mice was analyzed by qRT-PCR for inflammatory cytokines. Experimental values were normalized against L32. Results are representative of 4 experiments, with n=4 per group. Error bars show SEM.

(D) Germ-free and WT- and ΔIcmF-mono-colonized animals have similar amounts of $CD4^+$ T cells in the MLNs. Total MLN cells from germ-free and WT- and ΔIcmF-mono-colonized Swiss Webster mice were stimulated for 4 hr with PMA and ionomycin. Cells were subsequently stained for CD4 and TCRβ. For each group, cells from n=4 mice were pooled together. Results are representative of 2 experiments.

(E) ΔIcmF-colonized SPF animals have increased levels of intracellular *H. hepaticus* compared to WT-colonized SPF animals. SPF *IL-10*$^{-/-}$ (n=4 per group) and *Rag1*$^{-/-}$ (n=9 per group) animals were colonized with either wild-type or ΔIcmF *H. hepaticus* for 2-4 weeks. IECs were isolated and treated with gentamicin and then lysed and plated for bacterial enumeration. Ratios are expressed as CFUs of bacteria divided by the number of IECs. Error bars indicate SEM. *p<0.05 vs WT.

CHAPTER 2: *H. hepaticus* limits intestinal inflammation

Introduction

During homeostasis, a defined balance exists among commensal, symbiotic, and pathobiotic bacteria in the intestinal microbiota. We and others have hypothesized that disturbances in this equilibrium may lead to intestinal disorders in humans (Packey and Sartor, 2009; Round and Mazmanian, 2009). Dysbiosis can result from an overgrowth of pathobionts or a loss of beneficial commensal bacteria, leading to elevated host inflammation toward the microbiota. We therefore tested this hypothesis through association of animals with T6SS mutants to induce dysbiosis by experimentally increasing colonization of a defined pathobiont.

Furthermore, in the GI tract, IECs form a monolayer barrier that separates luminal contents from underlying host cells. Though IECs contribute to innate immunity by sensing through TLRs and secreting antimicrobial peptides, IECs are generally not believed to mediate adaptive immunity, unlike professional antigen-presenting cells (APCs) such as dendritic cells and macrophages. Interestingly, studies have shown that IECs are capable of expressing major histocompatibility complex (MHC) class II proteins (Bland and Warren, 1986; Mayer et al., 1991), and IEC presentation of antigens to $CD4^+$ T cells via MHC class II molecules can result in lymphocyte proliferation (Westendorf et al., 2009). Thus, we investigated whether IECs could present *H. hepaticus* antigen to T cells to elicit an adaptive immune response.

Results

2.1 The T6SS directs an anti-inflammatory gene expression profile in intestinal epithelial cells

Based on the evidence from these studies of T6SS mutants, it appears that *H. hepaticus* intimately interacts with IECs. To appreciate the magnitude of this interaction, we examined the gene expression profile of MODE-K cells incubated with either wild-type or ΔIcmF bacteria. GeneChip analysis showed clear down-regulation of numerous transcripts in MODE-K cells co-cultured with wild-type bacteria possessing a functional T6SS (Figures 4A and 4B). Examination of various gene families exhibited an unmistakable pattern of transcriptional repression by wild-type *H. hepaticus*, including many immune-related genes (Figure 4C). Previous studies have shown *H. hepaticus* reduces activation of innate immune molecules such as Toll-like receptor 4 (TLR4) in IECs (Sterzenbach et al., 2007). We reveal by both microarray and qRT-PCR analysis of MODE-K RNA that suppression of TLR4 expression by *H. hepaticus* is T6SS-mediated (Figures 4D and 4E). Moreover, wild-type bacteria reduced the transcript levels for several additional mediators of innate (NF-κB, TLR3) and adaptive immunity (interleukin-17 receptor; IL-17RA). *H. hepaticus* promotes colon cancer in animal models; the molecular mechanisms underlying this outcome are unknown. Intriguingly, our data show that T6SS may serve a protective activity in the development of colorectal cancer, as a critical oncogene in hereditary colon cancers (Adenomatosis polyposis coli; APC) and a marker for cell proliferation during epithelial hyperplasia (Ki-67) are significantly reduced by wild-type bacteria (Figures 4D and 4E). The extent of the contribution

by T6SS to *H. hepaticus*-mediated cancer remains to be determined. Collectively, these studies strongly support a model whereby the T6SS of *H. hepaticus* functions to reduce immune responses elicited by IECs during bacterial colonization.

2.2 *H. hepaticus* T6SS mutant elicits elevated intestinal inflammatory responses

The T cell transfer model of experimental colitis highlights the intricate interaction between mice and *H. hepaticus*. In this model, T and B cell deficient *Rag1*$^{-/-}$ mice are reconstituted with naïve, pathogenic CD4^{+}CD45Rb^{high} T cells to provide immune cells that can react to the microbiota. In the absence of *H. hepaticus*, SPF T cell recipient animals remain healthy. However, in the presence of *H. hepaticus*, mice develop a profound inflammatory response in the colon (Mazmanian et al., 2008). To test the effects of T6SS, we colonized T cell reconstituted *Rag1*$^{-/-}$ mice with wild-type or ΔIcmF strains of *H. hepaticus*. Initially, we examined colonization levels and again observed a significant increase by the T6SS mutant (Figure 5A). Most importantly, colonization with ΔIcmF led to higher levels of intestinal inflammation compared to wild-type (Figure 5B). qRT-PCR of RNA from colons revealed that animals colonized with the T6SS mutant displayed considerably increased cytokine transcript levels of numerous innate and adaptive immune responses. Many cytokines associated with the inflammatory T-helper 17 (T_H17) arm of immunity were increased in response to ΔIcmF—IL-17, IL-21, IL-23, and IL-23R—suggesting the T6SS may help in controlling exaggerated T_H17 responses toward bacteria. The pro-inflammatory cytokine TNFα and inducible

nitric oxide synthase (iNOS) were also significantly increased in response to the T6SS mutant. TNFα and iNOS are highly expressed during *H. hepaticus*-induced colon inflammation and carcinogenesis, and are believed to be strong contributors to disease (Erdman et al., 2009).

Interestingly, there was no marked difference in intestinal pathology by measures of cell proliferation, cellular infiltrates, and abscess formation between wild-type and ΔIcmF mutant-colonized animals (Figure S3A). Histopathology analysis by a blinded pathologist verified similar colitis scores between wild-type and mutant-colonized animals (Figure S3B). Although more subtle phenotypes cannot be excluded, the lack of increased disease by T6SS is not surprising given the fact that wild-type bacteria elicit very pronounced disease in the T cell transfer model. To measure inflammatory protein levels, organ cultures in which un-stimulated colon sections are cultured *ex vivo*, and supernatants assayed by ELISA showed an increase in the inflammatory molecules TNFα, IL-1β and IL-17 in tissues harvested from ΔIcmF mutant-colonized animals compared to wild-type (Figure 5C)**.** Furthermore, MLN cells from ΔIcmF-colonized animals that were re-stimulated *in vitro* with PMA/ionomycin or T cell stimuli (α-CD3/α-CD28) released increased IL-17 during *in vitro* cultures (Figure 5D). Analysis of the ceca also showed similar patterns of increased T6SS mutant colonization and elevated pro-inflammatory cytokine responses (Figures S3C-S3E).

2.3 Intestinal epithelial cells are capable of stimulating CD4+ T cell responses with *H. hepaticus* antigen

MODE-K cells increase MHC class II expression in response to IFNγ treatment, when stained with antibodies directed against the I-A^k haplotype (Figure 6A) or to a non-polymorphic region of the I-A molecule (Figure S4). The T cell activating co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) were also increased. To determine if IECs could present *H. hepaticus* antigens to T cells, MODE-K cells treated for 7 days with IFNγ were pulsed with *s*oluble *Hel*icobacter *a*ntigens (SHelAg). After 24hrs, MODE-K cells were washed and co-cultured with $CD4^+$ T cells from MLNs of SPF *Helicobacter*-free C3H/HeJ animals. Total RNA was analyzed by qRT-PCR for cytokine levels. Co-cultures that had been treated with SHelAg showed an increase in numerous pro-inflammatory cytokine transcripts, such as IL-17, IFNγ, and TNFα (Figure 6B). These responses are T cell-specific, as no cytokine production was observed in the absence of T cells.

2.4 Antigen-specific CD4+ T cell responses are increased in animals colonized with *H. hepaticus* T6SS mutants

We investigated the hypothesis that IEC presentation of *H. hepaticus* antigens elicits increased pro-inflammatory responses from $CD4^+$ T cells from animals colonized with T6SS mutants. MODE-K cells were pulsed with SHelAg or left untreated, washed, and incubated with purified $CD4^+$ T cells from mice colonized with either wild-type, ΔIcmF, ΔHcp or no *H. hepaticus*. In the presence of SHelAg, T cells from uncolonized and wild-type *H. hepaticus*-colonized animals produced comparable

levels of IL-17 (Figure 6C). This illustrates that previous exposure to *H. hepaticus* antigens does not augment immune responses relative to naïve animals, which is consistent with our findings that wild-type bacteria do not induce inflammation. In contrast, $CD4^+$ T cells harvested from both T6SS mutant-colonized animals elicited significantly increased levels of IL-17 compared to cells from wild-type-colonized animals. This response was antigen-specific, as no cytokine was produced in the absence of SHelAg. Therefore, colonization of animals with T6SS mutants leads to the generation of an increased Th17 cell response in the gut, revealing that *H. hepaticus* evolved this molecular mechanism to restrain unwanted intestinal inflammation.

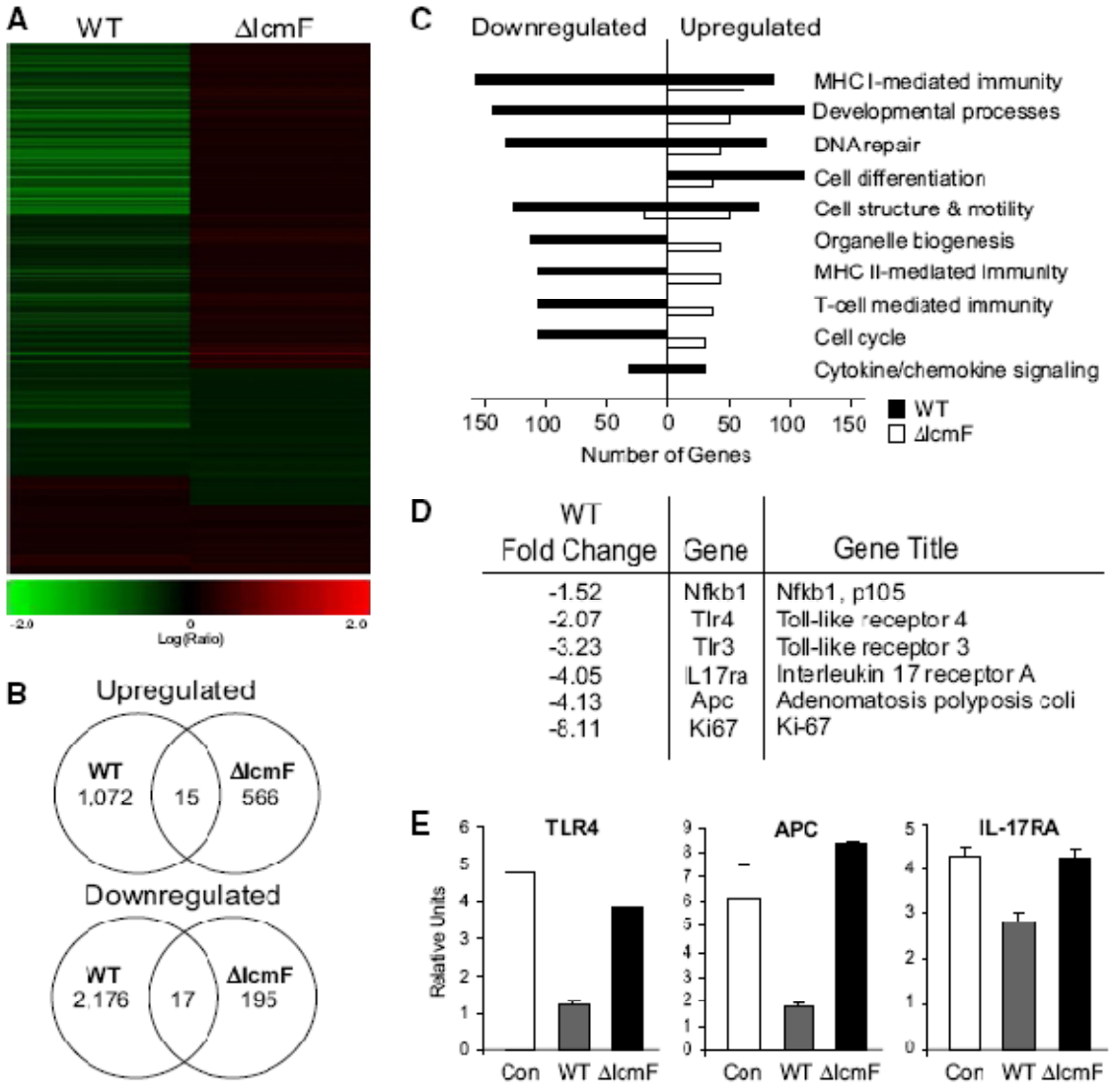


Figure 4. Wild-type *H. hepaticus* Induces a Wide Repertoire of Responses in MODE-K cells.

RNA was harvested from MODE-K cells incubated for 6hr with either wild-type *H. hepaticus*, ΔIcmF mutant, or no bacteria and analyzed by an Agilent Whole Mouse Genome Microarray. Gene expression of MODE-K cells incubated with bacteria was compared to transcript levels from RNA of untreated MODE-K cells. Only genes with a p-value <0.5 and fold change >1.5 were used for subsequent analysis.

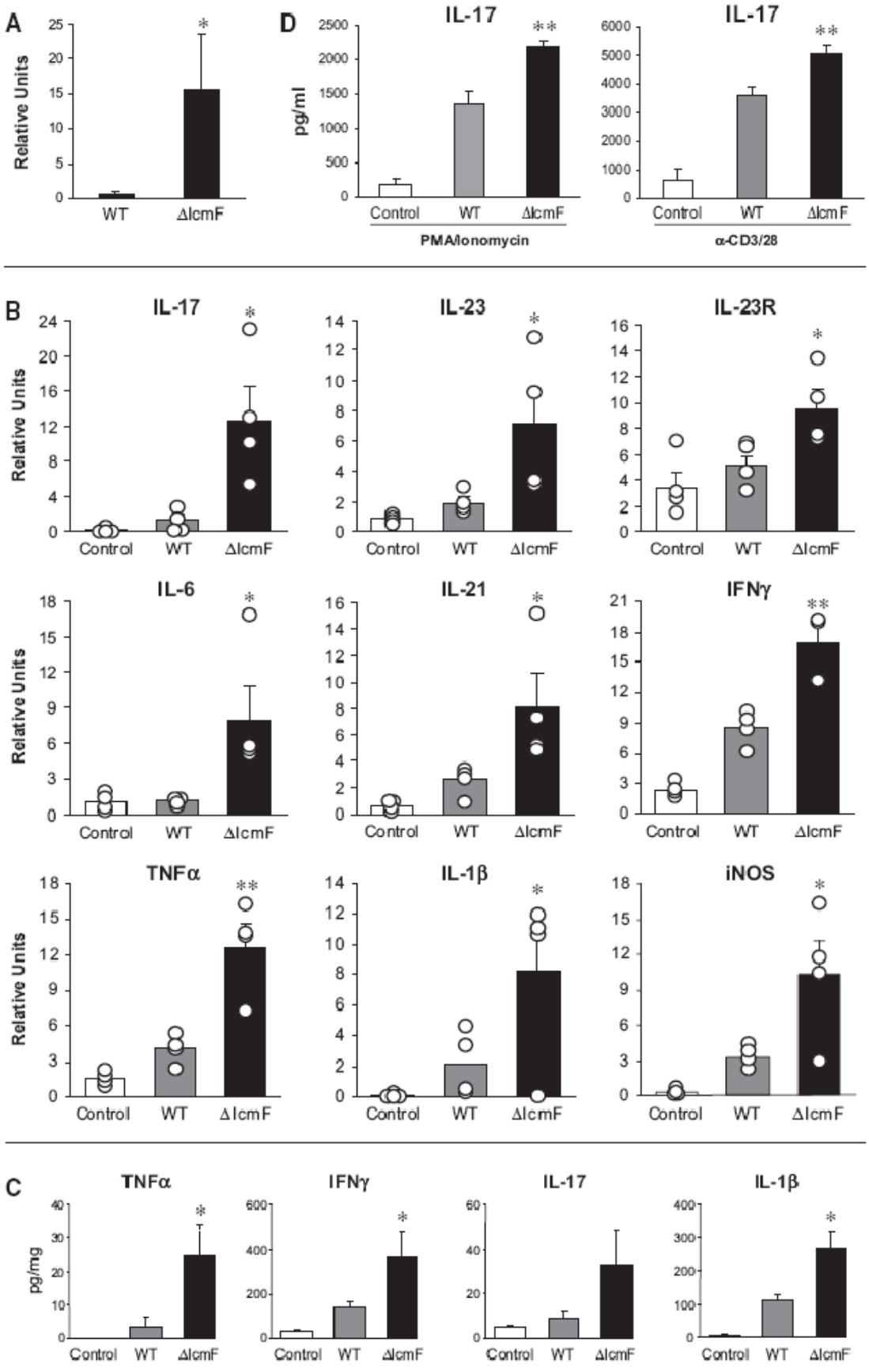
(A) Heat-map analysis of MODE-K gene expression in the presence of WT or ΔIcmF shows massive down-regulation by wild-type *H. hepaticus*.

(B) Venn diagram showing up- and down- regulation of gene expression in the presence of WT or ΔIcmF.

(C) Gene ontogeny analysis of changes in MODE-K transcript levels for various functional groups. Wild-type bacteria down-regulate numerous cellular pathways.

(D) Fold change of select innate and adaptive immune genes in the presence of WT *H. hepaticus*.

(E) qRT-PCR analysis of RNA from MODE-K cells for genes associated with inflammation and colon cancer. Error bars show SEM.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Relative Units
WT
ΔlcmF
D
IL-17
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Control
WT
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PMA/Ionomycin
IL-17
α-CD3/28
B
IL-17
IL-23
IL-23R
IL-6
IL-21
IFNγ
TNFα
IL-1β
iNOS
C
TNFα
IFNγ
IL-17
IL-1β
pg/mg

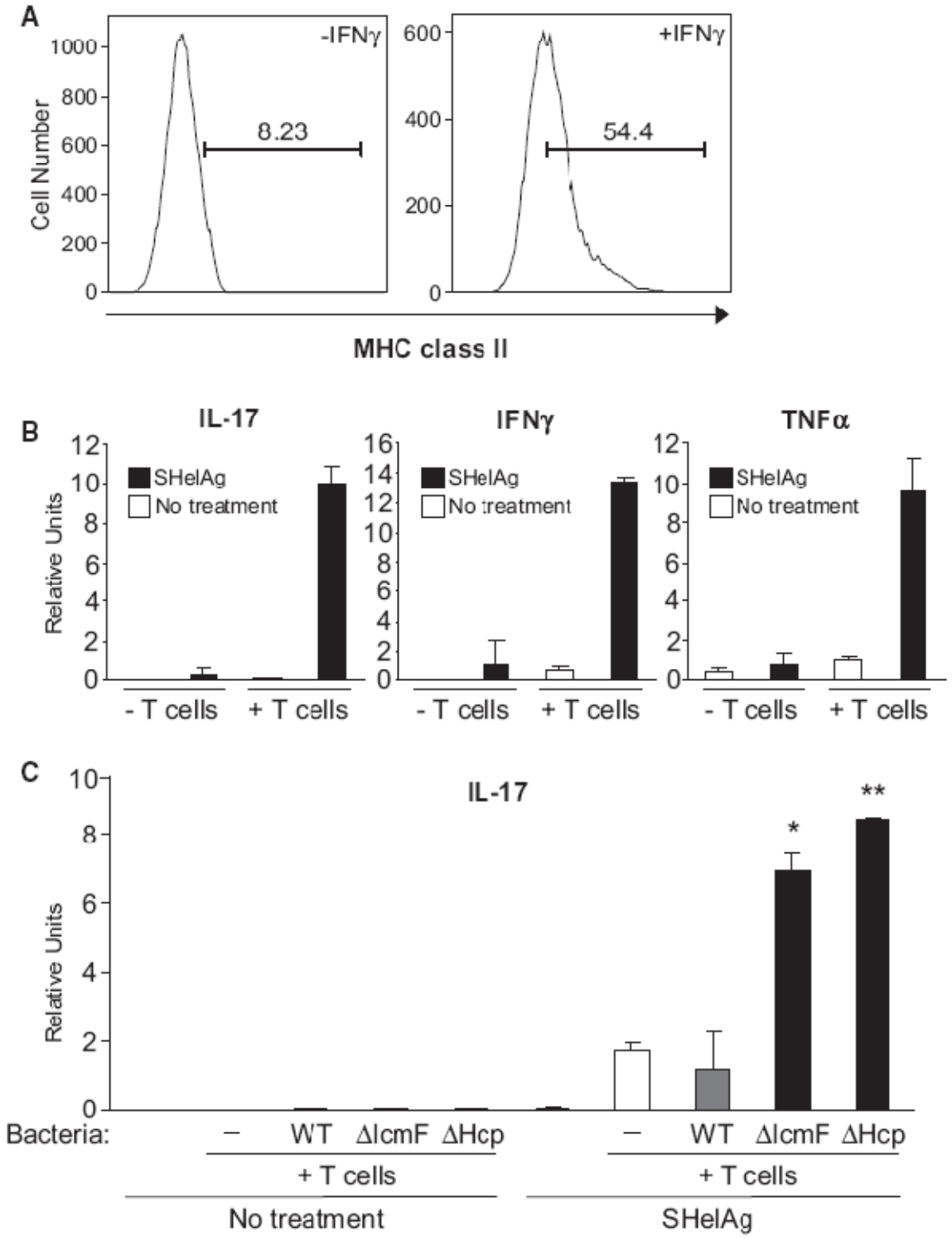
Figure 5. T6SS Mutant Leads to Greater Inflammation in the Colon in the Rag Adoptive Transfer Model of Colitis.

(A) ΔIcmF-colonized animals have greater amounts of *H. hepaticus* 16S rRNA in the colon compared to WT-colonized animals. RNA was collected from colons. Levels of 16S were quantified by qRT-PCR. Data are representative of 3 experiments, with n=4 per group. Error bars show SEM. *p<0.05 vs WT.

(B) RNA from the colon was analyzed by qRT-PCR for inflammatory cytokines. Experimental values were normalized against L32. Results are representative of 5 experiments, with n=4 per group. Each circle represents an individual animal. Error bars show SEM. *p<0.05, **p<0.01 vs WT.

(C) Colons were cultured *ex vivo* for 24hrs. Supernatants were assayed for cytokine by ELISA. Samples were normalized to total protein. Data are representative of 2 experiments, with n=4 mice per group. Each circle represents an individual animal. Error bars show SEM. *p<0.05 vs WT.

(D) Mesenteric lymph nodes pooled from each experimental group were restimulated with either PMA and ionomycin, or α-CD3 and α-CD28 for 24hrs. Supernatants were assayed by ELISA. Results are from 2 experiments, with n=4 mice per group. Error bars show SEM. **p<0.01 vs WT.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-IFNγ
+IFNγ
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54.4
MHC class II
B
IL-17
IFNγ
TNFα
SHelAg
No treatment
Relative Units
- T cells
+ T cells
C
IL-17
Relative Units
Bacteria:
–
WT
ΔIcmF
ΔHcp
+ T cells
No treatment
SHelAg
*
**

Figure 6. MODE-K Cells are Capable of Stimulating CD4+ T cell Responses with *H. hepaticus* Antigens.

(A) MODE-K cells were treated with 100U/ml of IFNγ for 7 days or left untreated. Cells were removed from the plate with trypsin, stained for MHC class II antigens, and analyzed by flow cytometry.

(B) MODE-K cells pre-treated with 100U/ml of IFNγ for 7 days were pulsed with either 20 μg/ml of soluble *H. hepaticus* antigen (SHelAg) from wild-type bacteria or no SHelAg for 24hrs. Media was changed prior to addition of CD4+ T cells harvested from syngeneic C3H/HeJ *Helicobacter*-free animals or no T cells. The ratio of MODE-K:T cells was 1:10. Cells were co-cultured for 3d. RNA was collected from total cells and analyzed by qRT-PCR. Error bars indicate SD from 2 independent experiments.

(C) MODE-K cells pre-treated with IFNγ were pulsed with either 20 μg/ml of SHelAg from wild-type bacteria or no SHelAg for 24hrs. Media was changed prior to addition of CD4+ T cells collected from animals colonized with wild-type, ΔIcmF, ΔHcp, or no *H. hepaticus*.

The ratio of MODE-K:CD4+ T cells was 1:10. Cells were co-cultured for 3d. RNA was collected from total cells and analyzed by qRT-PCR. Error bars indicate SD from 2 independent experiments. *$p<0.05$, **$p<0.01$ vs WT treated with SHelAg.

Discussion

Collectively, these results reveal that experimentally induced dysbiosis results in increased inflammatory responses from both the innate and adaptive immune system, and the T6SS of *H. hepaticus* functions to reduce intestinal inflammation during colonization. As Th17 cell responses are important mediators of IBD and colon cancer in experimental animals (Hue et al., 2006; Kullberg et al., 2006), our data suggest that increased gut inflammation in reaction to dysbiosis of the microbiota may be crucial in the onset and/or progression of human intestinal diseases**.** Previous studies have shown that *H. hepaticus* can reduce activation of TLR4 and TLR5 (Sterzenbach et al., 2007). Intriguingly, Kullberg et al. demonstrated that anti-inflammatory regulatory T cells (Tregs) from *H. hepaticus*-infected animals are able to prevent intestinal inflammation when transferred to naïve mice (Kullberg et al., 2002). As Treg cells are known to suppress Th1 and Th17 cell responses, this data correlates well with our findings.

However, the molecular mechanism(s) underlying these observations have remained elusive. A large deletion of the HHGI1 has been reported to reduce inflammation caused by *H. hepaticus* resulting in the absence of typhocolitis in *IL-10*$^{-/-}$ mice (Ge et al., 2008). Though differences arising from animal models cannot be excluded, we speculate that additional virulence factors (not T6SS components) within HHGI1 may elicit pro-inflammatory responses. In support of this notion, several HHGI1 genes bear homology to known toxins. Prospective studies will determine the precise function of gene products within HHGI1, and their effects on the induction of pathology in animal hosts. Collectively, however, we reveal herein

that type VI secretion functions to attenuate both innate and adaptive immunity to *H. hepaticus*.

Supplementary Figures

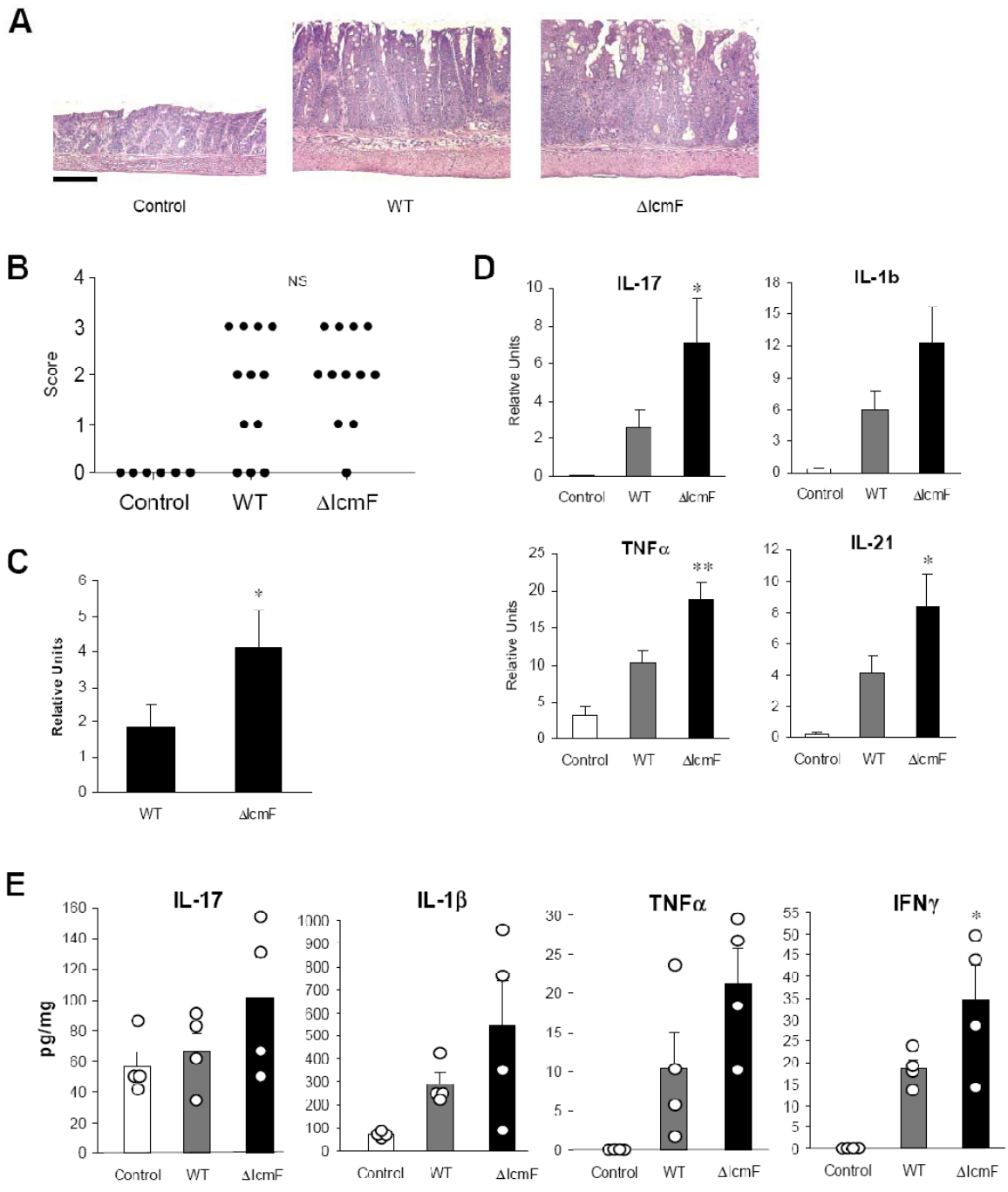

Figure S3. T6SS Mutant Leads to Greater Inflammation in the Colon and Cecum in the Rag Adoptive Transfer Model of Colitis.

(A) T cell reconstituted-*Rag1*$^{-/-}$ animals show no significant differences in intestinal pathology when colonized with WT or ΔIcmF *H. hepaticus*. Colons from WT- and ΔIcmF-colonized *Rag1*$^{-/-}$ mice were fixed in Bouin's fixative, paraffin-embedded, and sectioned. Colon sections were stained by H&E. Scale bar represents 100μm.

(B) T cell reconstituted-*Rag1*$^{-/-}$ animals show no significant differences in intestinal pathology when colonized with WT or ΔIcmF *H. hepaticus.* Colons from WT- and ΔIcmF-colonized *Rag1*$^{-/-}$ mice were fixed in Bouin's fixative, paraffin-embedded, and sectioned. Colon sections were stained by H&E. Sections were evaluated in a blinded fashion by the same pathologist, and scored accordingly: 0 = normal; 1 = mild epithelial hyperplasia; 2 = pronounced hyperplasia and significant inflammatory infiltrates; 3 = severe hyperplasia and infiltration with significant decrease in goblet cells; 4 = severe hyperplasia, severe transmural inflammation, ulceration, crypt abscesses, and substantial depletion of goblet cells. Each circle represents an individual animal. Statistics were calculated using a two-tailed Mann-Whitney *U* test. NS, not significant.

(C) ΔIcmF-colonized animals have greater amounts of *H. hepaticus* 16S rRNA in the cecum compared to WT-colonized animals. RNA was collected from cecum tissue of WT- or ΔIcmF-colonized animals, and levels of 16S were quantified by qRT-PCR. Data shows combined results of 2 experiments, with n=8 mice per group. Error bars show SEM. *$p<0.05$ vs WT.

(D) RNA from the cecum of WT- or ΔIcmF-colonized animals was analyzed by qRT-PCR for various inflammatory cytokines. Experimental values were normalized against L32. Data shows combined results of 2 experiments, with n=8 mice per group. Error bars show SEM. *p<0.05, **p<0.01 vs WT.

(E) Cecum tissues from WT- or ΔIcmF-colonized animals were homogenized in RIPA lysis buffer. Supernatants were assayed for cytokine by ELISA. Graphs are representative of 2 experiments, with n=4 mice per group. Each circle represents an individual animal. Error bars show SEM. *p<0.05 vs WT.

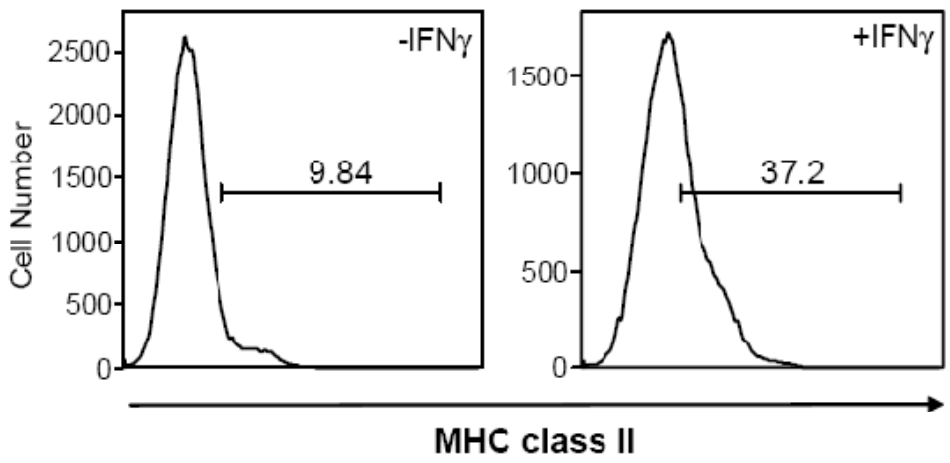

Figure S4. MODE-K cells are capable of expressing MHC class II antigens.

MODE-K cells were treated with 100U/ml of IFNγ for 7 days or left untreated. Cells were removed from the plate with trypsin, stained for MHC class II antigens, and analyzed by flow cytometry.

CHAPTER 3: The role of *H. hepaticus* in colorectal cancer

Introduction

It is well established that chronic inflammatory conditions can contribute to the development of some cancers by promoting cell proliferation, cell survival, and/or angiogenesis (Mantovani et al., 2008). Individuals with IBD (in particular ulcerative colitis) have an increased risk of developing colorectal cancer (McConnell and Yang, 2009). Colorectal cancer is the second most common cause of malignant tumors in the United States (Wilmink, 1997), and often has life-threatening consequences. Moreover, epidemiologic and clinical data show that the incidence of colon cancer is dramatically increasing in Western countries. A genetic basis for cancer is well established; however, it is clear that non-genetic (environmental) components are also crucial to the disease process.

Multiple studies have demonstrated that the presence of intestinal bacteria may be required for carcinogenesis. In an experimental animal model of colitis-associated cancer, *IL-10-/-* mice treated with the chemical carcinogen azoxymethane were devoid of tumors when raised under germ-free conditions (Uronis et al., 2009). Similar results were found in other animal models of spontaneous colon cancer. Germ-free rederivation of *TCRβ-/-p53-/-* mice and *TGFβ1-/-* mice eliminated the formation of intestinal tumors (Engle et al., 2002; Kado et al., 2001). In addition, clinical studies have identified a higher incidence of adherent and intracellular *E. coli* in biopsies from carcinoma patients compared to controls (Martin et al., 2004; Swidsinski et al., 1998).

Several studies have implicated *H. hepaticus* in the induction of colorectal cancer in rodent disease models. Colonization of 129/SvEv *Rag2-/-* mice with *H. hepaticus* initiates development of hyperplasia and dysplasia in the large intestine (Erdman et al., 2003). In this model, increased levels of TNFα and nitric oxide appear to be critical for the progression of inflammation to cancer. Since our previous work indicated that the T6SS of *H. hepaticus* is capable of reducing inflammatory responses in the colon, we investigated the role of the *H. hepaticus* T6SS in the induction and progression of colorectal cancer in two distinct rodent models.

Results

3.1 The role of *H. hepaticus* in a colitis-associated model of colon carcinogenesis

Strong evidence suggests that chronic inflammation of the colon can lead to disease progression into colorectal cancer (CRC). As *H. hepaticus* has been associated with the induction of chronic inflammation, we explored the functional impact of *H. hepaticus* and its T6SS on disease initiation and progression in an inflammation-driven model of CRC. Specifically, animals were treated with a colonotropic carcinogen, azoxymethane (AOM), followed by repeated cycles of an inflammatory agent dextran sodium sulfate (DSS) added to the drinking water (Neufert et al., 2007).

Intraperitoneal administration of AOM initiates cancer by alkylating DNA, thereby facilitating base mispairings. AOM itself is not the final carcinogenic metabolite, but rather requires further stepwise activation that is dependent on the

microbiota (Fiala, 1977; Reddy et al., 1974). Furthermore, the detergent DSS is toxic to the epithelial lining of the colon, resulting in severe colitis when administered by itself. Thus, the AOM/DSS model of CRC leads to an inflammation-driven tumor progression that causes rapid growth of multiple colon tumors within 10 weeks.

Pathology in the AOM/DSS model of CRC highly resembles that found in spontaneous CRC in humans. In both cases, tumors predominantly localize to the distal colon and exhibit polypoid growth. However one striking difference is that tumors in the AOM/DSS model often lack mucosal invasiveness and rarely metastasize. AOM/DSS-induced tumors resemble tumors found in human CRC at the molecular level as well. Common features included: aberrant protein expression for adenomatosis polyposis coli (APC); mutations and altered cellular localization of β-catenin; dysregulation in *cellular myelocytomatosis oncogene* (*c-Myc*), *cyclin D1*, and *cyclin-dependent kinase 4*; and elevated levels of enzymes involved in prostaglandin and nitric oxide synthesis.

In our studies, adult mice were colonized with either wild-type, T6SS mutant ΔIcmF, or no *H. hepaticus* for one week prior to injection with AOM and administration of DSS. Approximately three months after disease induction, tumors in the colon were quantified and measured for size. Surprisingly, there was no significant difference in the size or total number of tumors among all groups of mice, suggesting that colonization with *H. hepaticus* had little impact on the progression of disease (Figure 7). As tumor analysis was conducted at the end of the experiment, any existing differences in early stages of tumor progression may have gone undetected. In addition, DSS alone is often used as an inflammatory agent to induce

colitis. Thus it is possible that the strong inflammation induced by multiple cycles of DSS treatment may have 'overwhelmed' the immune system and masked any inflammatory (or anti-inflammatory) effects mediated by *H. hepaticus* and its T6SS.

3.2 The impact of *H. hepaticus* on cancer progression in *Apc*$^{Min/+}$ mice

The *Apc*$^{Min/+}$ mouse is a well-established animal model of human familial adenomatous polyposis (FAP). Individuals with FAP often have mutations in the tumor suppressor *APC* gene and develop tumors in the colon as early as their teenage years. In *Apc*$^{Min/+}$ mice, a dominant mutation in *APC* predisposes animals to multiple intestinal neoplasia by three months of age (Moser et al., 1990). Similar to tumors in FAP, tumors in *Apc*$^{Min/+}$ mice often are benign in early stages but can eventually become locally invasive in the colon with time. On the contrary, in *Apc*$^{Min/+}$ mice, most tumors are found in the distal small intestine, whereas in patients with FAP, tumors are predominantly in the colon.

APC functions as a tumor suppressor through its effects on multiple cell processes including cell migration, differentiation, and apoptosis (Goss and Groden, 2000). The role of APC in the Wnt pathway (which has been shown to have a key role in the induction of many gastrointestinal cancers) is to regulate cytoplasmic levels of β-catenin by controlling β-catenin degradation in the proteasome. Excessive β-catenin accumulation in the cytosol can lead to subsequent translocation to the nucleus where it may activate Tcf/Lef family members to initiate downstream gene transcription (Clements et al., 2003). Tumor growth appears to be dependent on the continued activation of oncogenes, such as *c-Myc*, as

genetic deletion of *c-Myc* in the IECs of *Apc*$^{Min/+}$ mice abolishes tumor growth (Sansom et al., 2007).

The role of the microbiota in mediating disease in *Apc*$^{Min/+}$ mice is currently unclear. It has been reported that under germ-free conditions, *Apc*$^{Min/+}$ mice develop two-fold fewer adenomas than conventional controls in the medial small intestine, but show no significant difference in the rest of the intestinal tract, suggesting microbial status does not strongly alter tumor burden in this mouse model (Dove et al., 1997). On the other hand, the TLR signaling adaptor protein MyD88 was found to be important for the progression of colon carcinogenesis in *Apc*$^{Min/+}$ mice (Rakoff-Nahoum and Medzhitov, 2007). Furthermore, previous studies have shown that colonization with *H. hepaticus* can increase the number of intestinal tumors by two-fold (Rao et al., 2006).

Thus, we sought to uncover the role of the *H. hepaticus* T6SS in tumor progression in *Apc*$^{Min/+}$ mice. Adult mice were colonized for eight weeks with either wild-type, T6SS mutant ΔIcmF, or no *H. hepaticus*. Consistent with previous reports, in the small intestine a nearly 2-fold increase in tumors was observed in the presence of wild-type *H. hepaticus* (Figure 8). Interestingly, however, this increase in tumor number was not found in mice colonized with T6SS mutant bacteria. Furthermore, in the colon, tumor burden was similar in mice colonized with or without wild-type *H. hepaticus*, whereas again in mice colonized with the T6SS mutant, a significant reduction in the number of tumors was observed (although it should be noted that overall tumor numbers were much lower in the colon than in the small intestine).

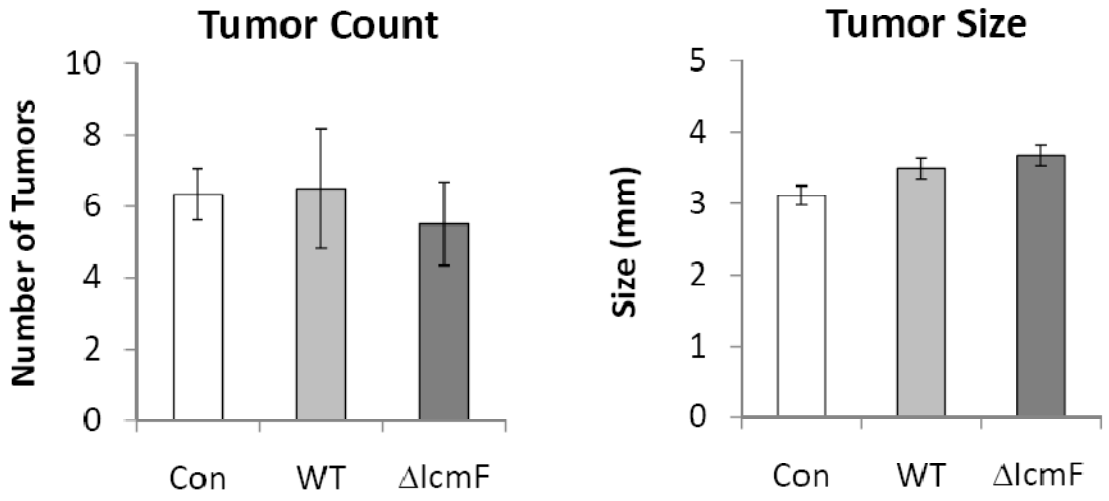

Figure 7. Colonic tumor burden in mice treated with AOM/DSS.

Mice were colonized with either wild-type, T6SS mutant ΔIcmF, or no *H. hepaticus* for one week prior to injection with AOM and 3 cycles of DSS treatment. Tumors in the colon were quantified and measured for size. Data shows combined results of 2 experiments, with n=8 mice per group. Error bars show SEM.

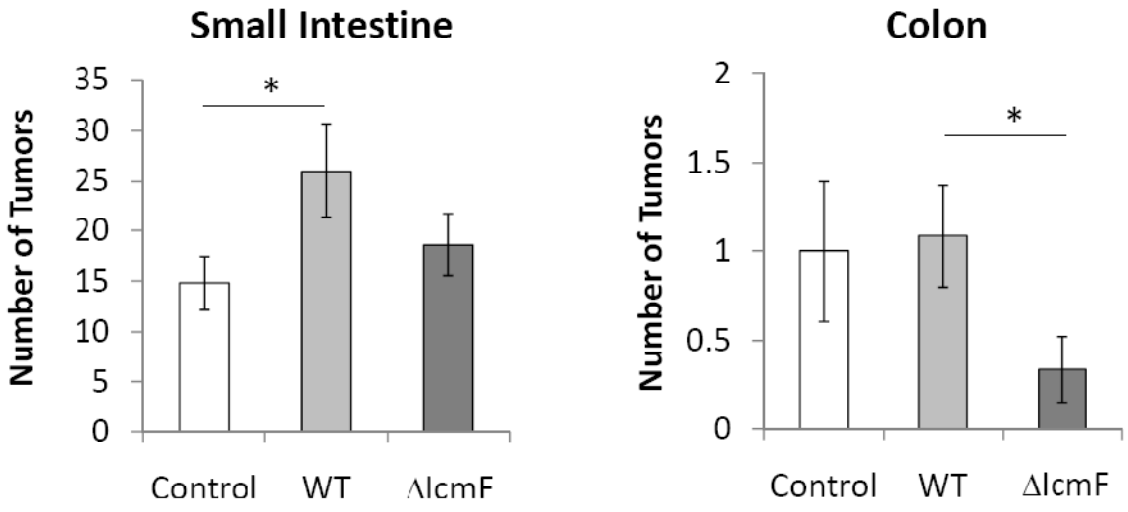

Figure 8. Intestinal tumor burden in *Apc$^{Min/+}$* mice.

Apc$^{Min/+}$ mice were colonized with either wild-type, T6SS mutant ΔIcmF, or no *H. hepaticus* for 8 weeks. Total tumor numbers in the colon and small intestine were quantified. Data shows combined results of 2 experiments, with n=10-12 mice per group. Error bars show SEM. *p<0.05

Discussion

Chronic inflammation is believed to be capable of promoting tumorigenesis by leading to microenvironments that foster genetic mutations and cell survival (Rakoff-Nahoum, 2006). Consistent with this idea, clinical data has demonstrated that anti-inflammatory medication can lead to regression of large bowel adenomas (Baron and Sandler, 2000). Based on our previous data showing that *H. hepaticus* T6SS mutants elicit increased inflammation in the colon, we predicted that colonization of mice with *H. hepaticus* T6SS mutant bacteria would increase tumor progression compared to colonization with wild-type strains. However, our results indicate that in $Apc^{Min/+}$ mice, colonization with T6SS mutant bacteria decreases tumor burden compared to wild-type *H. hepaticus*.

The paradoxical dual-function of the immune system in regards to cancer development may explain these findings. It is believed the immune system has the ability to eliminate tumor formation in addition to promoting and even selecting for tumor growth (Schreiber et al., 2011). Specifically, the idea of 'cancer immunosurveillance' has been put forth to describe the ability of the immune system to identify transformed cells and target them for destruction (Smyth et al., 2006). Tumor antigen-specific cytotoxic T-lymphocytes (CTL) appear to be the major effectors in the immune response against tumor cells. Interestingly, the same components of the immune system that promote oncogenic activity can also help induce tumor immunity depending on when they are recruited during the cancer development process. MyD88 has been shown to be required for tumorigenesis in mouse models (Rakoff-Nahoum and Medzhitov, 2007); however, MyD88 appears to

be important for antitumor activity as well by facilitating antigen presentation from dying tumor cells (Apetoh et al., 2007). In addition, in *Drosophila melanogaster* TNFα has been shown to have both tumor-promoting and tumor-suppressing activities (Cordero et al., 2010).

During cancer development, an overly 'tolerogenic' environment may prevent effective killing of cancerous cells. In support of this hypothesis, Foxp3+ regulatory T cells were found to be important for driving tumorigenesis in a mouse model of lung cancer (Granville et al., 2009). Furthermore, preliminary studies from our laboratory have suggested that in a T cell transfer model of colitis, CD4+ T cells from mice colonized with wild-type *H. hepaticus* may be more immunosuppressive compared to CD4+ T cells from mice colonized with T6SS mutant bacteria. Thus, if T cells from animals colonized with T6SS mutant bacteria are inherently less suppressive, this may help in combating diseases such as colon cancer where Tregs can possibly prevent the immune system from recognizing and destroying transformed cells and tissues.

Understanding the role of intestinal pathobionts during the induction and progression of cancer will be critical as mounting evidence suggests that commensal organisms of the gut may play a causative role in disease. Classified as a class I carcinogen, *H. pylori* has been shown to lead to gastric adenocarcinoma in 1% of infected individuals. While 50% of the human population is thought to be colonized with *H. pylori*, only a small percentage actually develop gastric disorders (Dorer et al., 2009). Furthermore, *T-bet-/-Rag2-/-* (TRUC) mice spontaneously develop dysbiosis and colitis, which can eventually progress into colorectal cancer (Garrett

et al., 2009); remarkably, microbiota transfer from these donors into wild-type mice can confer disease (Garrett et al., 2007). Subsequent studies have identified two commensal proteobacteria over-represented in TRUC mice, *Proteus mirabilis* and *Klebsiella pneumonia*, as the colitogenic microbes (Garrett et al., 2010). Whether there is a causal relationship between the microbiota, intestinal inflammation, and colon carcinogenesis will require further investigation.

CONCLUSION

Host-bacterial interactions (whether beneficial or harmful) are defined by a dynamic exchange of molecules that mediate various biological outcomes. Although T6SSs have been largely studied in the context of bacterial virulence, growing evidence supports the notion that T6SSs may have also evolved for non-pathogenic purposes in symbiotic bacteria. We characterize herein that the T6SS of *H. hepaticus* limits colonization of animals and actively suppresses innate and adaptive immune responses. A summary of these findings is depicted in Figure 9, illustrating that the T6SS of *H. hepaticus* may shape an immunologically tolerant host immune system (i.e., reducing TLR, Th17 responses and promoting Tregs) through its interaction with IECs. Though numerous aspects of this host-bacterial interface remain to be determined, the T6SS appears to promote a symbiotic relationship between *H. hepaticus* and mammals.

Understanding how *H. hepaticus* is able to maintain a long-term, non-pathogenic symbiosis with its murine host will be critical for insight into the biology of human intestinal *Helicobacters*. A handful of studies have implicated at least two species with tropism for humans, *Helicobacter cinaedi* and *Helicobacter fennelliae*, as being associated with enterocolitis, diarrhea, and bacteremia in a portion of infected patients (Flores et al., 1990; Fox et al., 2000; Totten et al., 1985). Moreover, studies have shown a higher association of *Helicobacter* species in the intestinal tract of Crohn's disease and IBD patients compared to healthy patients, suggesting a potential role in pathogenicity (Bohr et al., 2004; Laharie et al., 2009; Man et al., 2008; Zhang et al., 2006). An important observation from these studies is that

intestinal *Helicobacter* species are also detected in healthy patients. Similar to *Helicobacter pylori* whereby only 1% of colonized people develop gastric ulcers or gastric cancer, intestinal human *Helicobacters* do not appear to be pathogens as the majority of colonized people are asymptomatic. Unlike opportunistic pathogens that may not permanently colonize a host, understanding the dynamic molecular relationship between *Helicobacters* and mammals may provide paradigms for studies into human diseases caused by dysbiosis of pathobionts.

The complex consortium of microbes within our GI tract actively shapes mammalian immune responses (Mazmanian et al., 2005). The microbiota has been implicated in numerous human disorders such as IBD, colon cancer, allergies, asthma, and type 1 diabetes (Kinross et al., 2008; Mazmanian et al., 2008; Penders et al., 2007; Wen et al., 2008), highlighting the importance of understanding the individual species that make up a 'healthy' microbiota. Numerous investigations have shown a significant alteration in the microbiota of patients with IBD (Frank et al., 2007; Lepage et al., 2005; Scanlan et al., 2006). A recent metagenomic (culture-independent analysis) case-control study comparing the microbiota of patients with IBD to that of non-IBD controls, revealed a statistically significant difference between the microbial compositions (Frank et al., 2007)**.** Our understanding of how dysbiosis affects IBD is still preliminary. Further, genetic factors play an important role in the pathogenesis of IBD. Polymorphisms in bacterial sensing (*NOD2*/*Card15*) (Hampe et al., 2001), autophagy (*ATG16L1*) (Hampe et al., 2007) and T cell immunity (*IL-23R*) (Duerr et al., 2006) genes have highlighted the connection between microbes and inflammation in IBD (Figure 9). However, genetic variations

appear to predispose, but not predict, disease development as concordance rates between monozygotic twins are only 30% for IBD, and many people with polymorphisms for IBD-related genes are healthy. Thus, environmental factors play a significant role in disease.

Mounting evidence predicts that IBD, at least in part, results from dysbiosis of the normal microbiota (O'Hara and Shanahan, 2006). The convergent contributions of the host genetic landscape and epigenetic variables (i.e., the microbiota) should therefore be considered in evaluating the cause of complex immunologic diseases in humans. Our findings predict that discrete and identifiable bacterial species of the microbiota can drive intestinal inflammation if their 'balance' with the host is altered. From this perspective, therapeutics which selectively target pathobionts may prove invaluable as a treatment for intestinal diseases such as IBD and colon cancer.

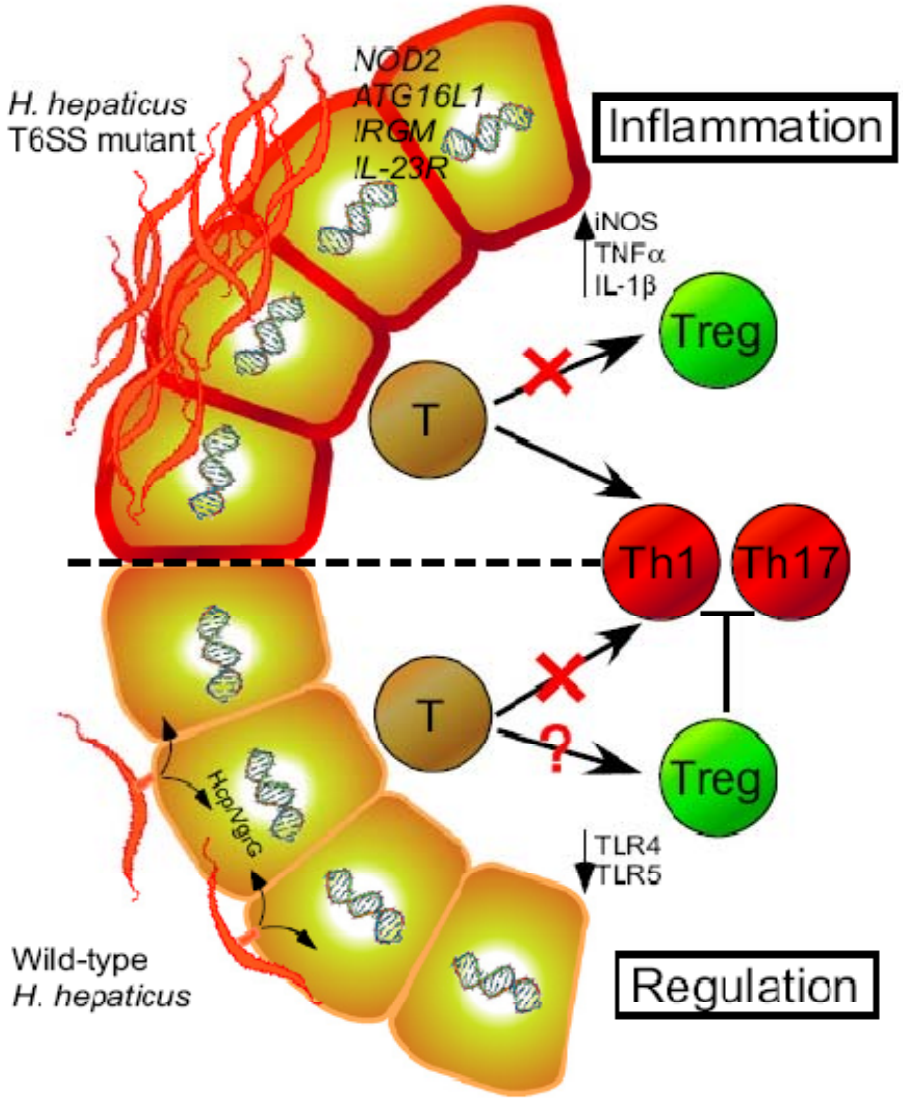

Figure 9. Proposed Interactions between *H. hepaticus* T6SS and the Intestinal Immune Response during Colonization.

During prolonged intestinal colonization of animals, *H. hepaticus* intimately contacts the epithelium and uses its T6SS to create a tolerogenic immune environment (possibly through down-regulating TLR expression and/or promoting Treg development). Crosstalk between host and bacteria maintains a balanced symbiotic interaction. This balance can be disturbed by genetic mutations associated with IBD

(*NOD2*, *ATG16L1*, *IRGM*, *IL-23R*) and/or dysbiosis caused by external disturbances (e.g., antibiotics, enteric infections, diet, etc.), which may result in elevated immune responses (increased Th17) in genetically susceptible hosts. Based on our and previous studies, it appears that the combination of host genotype and microbial status contributes to intestinal disease.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Helicobacter hepaticus ATCC51449 (ATCC) was cultured on Brucella agar plates with 5% sheep's blood (Teknova) or in BHI with 10% FBS. Cultures were grown at 37°C in 1% O_2, 10% CO_2, and 10% H_2. For construction of mutants, ~2kb fragments of *HH0252* (*IcmF*), *HH0243* (*Hcp*), and *HH0242* (*VgrG*) were PCR amplified and ligated into pGEMT (Promega). An erythromycin resistance gene digested from pSLB167 (Mehta et al., 2007) was inserted within the ORFs. Plasmid construction was carried out using *E. coli* JM109 with erythromycin and ampicillin used at 150μg/ml and 100μg/ml, respectively. Plasmid was introduced into *H. hepaticus* by electroporation. Mutants were selected on plates with 5μg/ml erythromycin.

MODE-K Cell Culture

MODE-K cells were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 50U/ml penicillin, 50μg/ml streptomycin, and 10mM hepes at 37°C in 5% CO_2 incubator. Cells were passaged using trypsin-EDTA. For bacterial incubations, media were changed to UltraDOMA-PF media (Lonza) supplemented with 10% FBS, 2mM L-glutamine, 10mM hepes, non-essential amino acids, 1mM sodium pyruvate, and 0.5mM β-mercaptoethanol. Cytochalasin D (Sigma) was used at 10μM and added 1hr prior. For gentamicin protection assays, bacteria were added at an MOI of 100. Incubations were carried at 37°C in 1% O_2. MODE-K cells were washed in PBS, and media added with or without 100μg/ml gentamicin for

2hrs at 37°C. MODE-K cells were rinsed with PBS, lysed with 0.1% saponin for 15min at 25°C, and plated for bacterial quantification.

Generation of *H. hepaticus* Antibodies

~900bp fragments *HH0243* (*Hcp*) and *HH0242* (*VgrG*) were cloned into pQE30 6xHis-tagged expression vector (Qiagen) and transformed into *E. coli* JM109. *E. coli* were grown at 25°C with 0.5mM IPTG for 5hrs. Peptides were purified using Ni-NTA columns (Qiagen) and injected into chickens for antibody production (QED Bioscience). Antibodies were collected from eggs using the EGGstract IgY kit (Promega).

Immunohistochemistry

MODE-K cells were fixed in 4% PFA. Paraffin-embedded tissues were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval in 10mM sodium citrate, pH 6.0 was carried out for 20min at 95°C. PBS with 5% FBS was used to block and dilute antibodies. Wheat germ agglutinin conjugated to tetramethylrhodamine was used at 1:1000 for 1hr at 4°C (Invitrogen). Anti-*H. hepaticus* and anti-VgrG were incubated at 20μg/ml overnight at 4°C. Rabbit anti-mouse E-cadherin antibody was diluted at 1:250 (Santa Cruz Biotech). Samples were imaged using a Zeiss LSM 510 Upright confocal microscope.

Microarray Hybridization and Data Analysis

RNA was prepared using TRIzol. RNA was labeled and hybridized to Agilent microarrays (Whole Mouse Genome Microarray) following manufacturer's instructions. Microarrays were scanned using an Agilent DNA Microarray Scanner G2565CA, and data were acquired using Agilent's Feature Extraction Software version 10.1.1.1. Significant genes were selected based on $p<0.05$ and fold change >1.5. For enrichment analysis of biological process ontology, probe lists were analyzed in DAVID and selected based on $p< 0.01$.

Animal Housing

7- to 10-week-old animals were used for all experiments. SPF C57Bl/6 and C3H/HeJ wild-type mice were purchased from Taconic Farms and Jackson Laboratories, respectively. SPF C57Bl/6 *Rag1*$^{-/-}$ and C57Bl/6 *IL-10*$^{-/-}$ mice were bred and maintained in our facilities. Germ-free Swiss Webster and C57Bl/6 mice were kept in sterile isolators. Germ-free animals were screened weekly for bacterial, viral, and fungal contamination. *Apc*$^{Min/+}$ mice on a C57Bl/6 background were either purchased from Jackson Laboratories or received as a gift by Sergio Lira. *Apc*$^{Min/+}$ mice were colonized with *H. hepaticus* for 8 weeks and analyzed at the end of the colonization period. Animals were cared for under established protocols and IACUC guidelines of California Institute of Technology.

Induction of AOM/DSS-mediated colorectal cancer

C57Bl/6 mice were colonized with *H. hepaticus* for 1 week prior to intraperitoneal injection with 10mg of azoxymethane (AOM) per kg of body weight on day 0, followed by 3 periods of 7-day-long oral administration of 2.5% dextran sodium sulfate (DSS) in drinking water. DSS cycles began on Day 0, 21 and 54. Animals were sacrificed on Day 81.

Intestinal Epithelial Cell Isolation

Colons were cut longitudinally and 1cm fragments were incubated twice in HBSS (no calcium or magnesium) with 5mM EDTA and 10mM Hepes for 20min at 37°C with gentle agitation. Cells were then treated with 5% FBS, 3U/ml Dispase, and 100ug/ml DNAse for 30min at 37°C. IECs were subsequently treated with 200ug/ml gentamicin for 2hrs at 37°C, lysed with 0.5% saponin, and plated on TVP plates for selection of *H. hepaticus*.

Adoptive Cell Transfer

Single-cell suspensions of spleens from sex-matched mice were treated with red blood cell lysing buffer (Sigma). $CD4^+$ T cells were isolated using a negative selection $CD4^+$ isolation kit (Miltenyi Biotec). Cells were stained with 5μg/ml anti-CD4-FITC and 2μg/ml anti-CD45Rb-PE (eBioscience). $CD4^+CD45Rb^{hi}$ cells were isolated by fluorescence activated cell sorting (FACS). $2x10^5$ cells were injected intraperitoneally into *Rag1*$^{-/-}$ animals. Two weeks later mice were orally gavaged with $1x10^8$ wild-type, ΔIcmF, or ΔHcp *H. hepaticus*. Animals were sacrificed 2-4

weeks after. Colon tissues were fixed in Bouin's fixative and sent out for paraffin-embedded sectioning and H&E staining (Pacific Pathology, San Diego).

Quantitative real-time PCR

RNA was extracted using TRIzol. RNA was treated with DNase (Sigma) prior to cDNA conversion using iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Reactions were carried out on a Bio-Rad iCycler IQ5. For cytokine analysis, samples were normalized to the housekeeping gene L32.

Colon Organ Culture and MLN Re-stimulation

Colon tissues were washed in PBS and cultured in 48-well plates in serum-free complete RPMI for 24hrs. Supernatants were collected and normalized to total protein concentration using Bradford reagent. Samples were analyzed by ELISA (eBioscience). For re-stimulation assays, MLNs were disrupted into single-cell suspensions and cultured in 48-well plates at $1x10^6$ cells/ml in complete RPMI. Cell stimulants were added: PMA at 50ng/ml, ionomycin at 500ng/ml, anti-CD3 at 2μg/ml, and anti-CD28 at 2μg/ml. Supernatants were collected after 1d and analyzed by ELISA.

MODE-K Antigen Presentation

MODE-K cells were treated with 100U/ml of IFNγ for 7 days prior to experiments. MODE-K cells were pulsed with 20μg/ml of SHelAg for 24hrs. Cells were rinsed

prior to the addition of $CD4^+$ T cells collected from the MLNs of SPF C3H/HeJ mice colonized with wild-type, ΔIcmF, ΔHcp, or no *H. hepaticus* for 2 weeks. The ratio of MODE-K to T cells was 1:10. After 72hrs, RNA was collected, and cytokine levels assayed by qRT-PCR. Preparation of SHelAg consisted of sonicating wild-type bacteria and centrifuging lysate to remove insoluble material. MODE-K cells were stained with anti-MHC class II antibodies obtained from eBioscience or ATCC (10.2.16) and analyzed by flow cytometry.

Primers for generation of *H. hepaticus* mutants

Primers used are as follows from 5' to 3': IcmF Fwd - GAC TTG TTA GAG GGT ATG CG; IcmF Rev - GGC AGG ATT TGG CAC ATA GG; Hcp Fwd - GAA TCA ACA GAA TAG TTT AGG AT; Hcp Rev - GAT AGT TTG TGT TGC CAT AGG; VgrG Fwd - ATG AGT GCT TTT CTT TCT TTG G; VgrG Rev - CTC TGT TTG ATG ATA CTT GCA T.

Apoptosis Labeling

MODE-K cells were briefly rinsed once with PBS and removed from the plate using trypsin. Cells were stained for annexin V and propidium iodide using the Annexin V Apoptosis Detection kit (eBioscience) following manufacturer's instructions.

Primers for qRT-PCR

Mouse primer sequences were used from previous studies (Mazmanian et al., 2008). Primer sequences for the *H. hepaticus* 16S gene are as follows from 5' to 3': F-GAC ATA GGC TGA TCC TTT AG; R-TAG GTT ATG TGC CCT TTA GT.

Cecum homogenate

Cecum tissue fragments were washed in PBS and homogenized in RIPA lysis buffer (Pierce) supplemented with protease inhibitors (Roche). Supernatants were normalized to total protein concentration and analyzed for cytokines by ELISA.

Statistical Analysis

Student's *t* test was used for evaluating statistical significance. $p<0.05$ was considered significant.

Accession Numbers

Microarray data have been deposited in the GEO database with the accession number GSE20434.

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