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Breaking Down the Barrier: Novel Insights Into the Role of Intestinal Microvilli,
M cells, and Exploiting “Innate” B Cells as Therapeutic Targets

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Kaila Marie Bennett

December 2014

Dissertation Committee

Dr. David D. Lo, Chairperson

Dr. Sharon L. Walker

Dr. Dimitrios Morikis

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The Dissertation by Kaila Marie Bennett is approved:

Committee Chairperson

University of California, Riverside

Acknowledgments

There are so many people that have helped me both professionally and personally, without whom this dissertation would not have been possible. First, I would like to thank my advisor Dr. David Lo for his unending support during my PhD and taking a chance on a bioengineer who didn't even know what the mucosal immune system was when I first joined the lab. Thank you for always giving me the freedom to explore the scientific questions that I found interesting, which has allowed me to become an independent thinking scientist. I truly believe that this will aid me immensely in my future career endeavors and for that I will be eternally grateful. I would next like to thank both members of my thesis committee, Dr. Sharon Walker and Dr. Dimitrios Morikis for their guidance, starting when I was a mere undergrad, providing me with great scientific role models, and continuing throughout my PhD. Also, thank you both for being great collaborators, providing new innovative ideas to take my research to the next level. I would especially like to thank Dr. Meera Nair, who, although I didn't meet till later in my PhD, both challenged and supported me, and is someone who inspires me and I aspire to be like, scientifically.

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The text of this dissertation, in part, is a reprint of the material as it appears in:

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The co-author David Lo directed and supervised the research, which forms the basis for this dissertation. Other co-authors listed provided methodological and technical expertise.

Dedication

I dedicate this dissertation to
my future husband Ronald Gorham,
and my parents,
for making me believe that the impossible is possible,
and for pushing to me to reach for my dreams

“Somewhere, something incredible is waiting to be known.”

~Carl Sagan

ABSTRACT OF THE DISSERTATION

Breaking Down the Barrier: Novel Insights Into the Role of Intestinal Microvilli, M cells,
and Exploiting “Innate” B Cells as Therapeutic Targets

by

Kaila Marie Bennett

Doctor of Philosophy, Graduate Program in Bioengineering
University of California, Riverside, December 2014
Dr. David Lo, Chairperson

The mucosal barrier is a well-armed opponent against the microbial world that works to limit entry of pathogenic bacteria, while maintaining a level of tolerance towards the resident microbiota. This paradoxical function is carried out by three distinct barriers i.e. physical, innate, and adaptive. Microvilli, a component of the physical barrier, while noted for their capacity to take up nutrients, have been overlooked as playing a role in the prevention of bacterial entry. We report that microvilli generate an electrostatic barrier that directs pathogen binding based on their zeta potential (proxy of surface charge). By knocking out microvilli in an intestinal cell line we were able to probe how bacterial adherence/uptake was affected by the absence of microvilli. This electrostatic barrier may have important functional consequences at the surface of the follicle-associate epithelium (e.g. Peyer’s patch), where interdispersed between adjacent enterocytes reside M cells, which lack microvilli. M cells are a feature of the adaptive barrier, and are known for their ability to transcytose luminal antigens in the small intestine. However, their role in the large intestine has been neglected. We describe the

induction of M cells in the colonic epithelium during intestinal inflammation, which seems to be reliant on the pro-inflammatory cytokine TNF α , as treatment with anti-TNF α largely blocked M cell induction. This was confirmed using a novel reporter mouse that was engineered to express DsRed in both neutrophils and M cells. Thus, M cells may play a greater role during inflammation. Furthermore, M cells also mediate adaptive immune response through associated B lymphocytes. The follicle of the Peyer's patch is heavily populated with B lymphocytes that help to generate secretory IgA. Large portions of these B cells are of the B1 subset, known for their capacity to stimulate T cell independent antibody response in the presences of polymeric antigens. We describe the design of a T cell independent antigen using polymeric flagellin as a vaccine backbone to stimulate the T cell independent B cells. We incorporated the envelope protein from dengue virus 2 into the D3 domain of flagellin and these hybrid filaments were able to produce a humoral response in both T cell dependent and independent models.

Table of contents

Chapter 1: General Introduction: The mucosal barrier and how homeostasis is maintained	1
1.1 List of abbreviations	2
1.2 Overview of the mucosal barrier	3
1.3 Physical barriers: mucus layer and the intestinal epithelium	4
1.4 Microvilli development and its function at the barrier	8
1.5 Pathogenic attachment and effacement mechanism	10
1.6 Innate barrier: homeostasis towards the resident microbiota	12
1.7 Adaptive barrier: the follicle-associated epithelium	13
1.8 M cells and how they can be exploited	16
1.9 GALT immune cells and their role in mucosal immune responses	19
1.10 Inflammatory bowel disease and murine models	22
1.11 Thesis aims	26
1.12 References	27
Chapter 2: Epithelial microvilli establish an electrostatic barrier to microbial adhesion	41
2.1 Introduction	42
2.2 Results	44
2.3 Discussion	55
2.4 Acknowledgments	56
2.5 Materials and methods	57
2.6 References	67
Chapter 3: Induction of colonic M cells during intestinal inflammation	71
3.1 Introduction	72
3.2 Results	75
3.3 Discussion	90
3.4 Acknowledgments	92
3.5 Materials and methods	93
3.6 References	100
Chapter 4: General introduction: T cell independent antibody response and antigens	106
4.1 List of abbreviations	107
4.2 T cell dependent vs T cell independent antibody response	107
4.3 T cell independent antigens with emphasis on flagellin	112
4.4 Flavivirus with emphasis on Dengue virus	114
4.5 Thesis aim	117
4.6 References	118

Table of contents (continued)

Chapter 5: Hybrid flagellin as a T cell independent vaccine scaffold	126
5.1 Introduction	127
5.2 Results	129
5.3 Discussion	138
5.4 Acknowledgments	139
5.5 Materials and methods	140
5.6 References	148

Chapter 6: Perspectives and future directions	151
6.1 Perspectives and future directions for chapter 2	152
6.2 Perspectives and future directions for chapter 3	154
6.3 Perspectives and future directions for chapter 5	157
6.4 References	160

Appendix	162
Appendix A: Supplemental material for chapter 2	163
Appendix B: Supplemental material for chapter 3	168
Appendix C: Supplemental material for chapter 5	172
Appendix D: Curriculum Vitae	178

List of Figures

Chapter 1: General Introduction: The mucosal barrier and how homeostasis is maintained

1.1 The mucosal barrier of the small intestine	4
1.2 Protein network of microvilli formation	9
1.3 Attaching and effacing mechanism of enteropathogenic <i>E. coli</i>	11
1.4 Organization of SI PP follicle	14

Chapter 2: Epithelial microvilli establish an electrostatic barrier to microbial Adhesion

2.1 Development of microvilli minus (MVM) cell lines using Caco2BBe	44
2.2 Quantification of cell glycoprotein content in cell with or without microvilli	46
2.3 Epithelial cell uptake of live bacteria	47
2.4 Adherence of fixed bacteria to epithelial cell monolayers	48
2.5 Epithelial cell adherence of synthetic nanoparticles	50
2.6 Bacterial binding to epithelium under flow conditions	51
2.7 Loss of microvilli in cells expressing Tir	52
2.8 Bacterial adherence to TirBBe cells	53
2.9 Schematic of the electrostatic barrier generated by apical microvilli	55

Chapter 3: Induction of colonic M cells during intestinal inflammation

3.1 PGRP-S ⁺ mice are susceptible to DSS chemically-induced inflammation	74
3.2 DSS inflammation induces M cells in colonic epithelium	76
3.3 Colonic M cells are not quiescent	78
3.4 Colonic M cells were associated with poorly organized follicles	80
3.5 Anti-TNF α ameliorates M cell induction during DSS treatment	81
3.6 Anti-TNF α causes more severe pathology than DSS alone	83
3.7 Intestinal inflammation caused by <i>C. rodentium</i> induces colonic M cells	85
3.8 Colonic M cell genes are regulated during <i>C. rodentium</i> infection and M cells are associated with poorly organized follicles	87
3.9 Induced M cells are capable of transcytosis	88

List of Figures (continued)

Chapter 4: General introduction: T cell independent antibody response and antigens	
4.1 Mechanism of TD and TI antibody production	109
4.2 Schematic of FliC filament	113
Chapter 5: Hybrid flagellin as a T cell independent vaccine scaffold	
5.1 Design of hybrid flagellin protein	128
5.2 Assessment of secondary structure of hFliC protein	130
5.3 Analysis of hFliC filament	131
5.4 Humoral immune response in non-TI restricted mouse models	132
5.5 Ig isotype produced in WT mice	134
5.6 hFliC filaments are capable of crosslinking	135
5.7 Humoral immune response in TI mouse model	136
Chapter 6: Perspectives and future directions	
6.1 Genes level modulation of microvilli	153
6.2 Modulation of microvilli during intestinal inflammation	155
6.3 Schematic of the Ebola virus	157
Appendix A: Supplemental material for Chapter 2	
A.1 Characterization of MVM cell model	163
A.2 Bacterial MATH analysis	164
A.3 Epithelial cell uptake of mutant <i>S. Typhimurium</i> strains	165
A.4 Quantification of bacterial zeta potential	166
A.5 Schematic of flow chamber for dynamic adherence assay	167
Appendix B: Supplemental material for Chapter 3	
B.1 Further characterization of the colonic M cells and follicle	168
B.2 Analysis of cytokine expression	169
B.3 Validation of <i>C. rodentium</i> infection	170
B.4 Analysis of follicles in <i>C. rodentium</i> infection	171

List of Figures (continued)

Appendix C: Supplemental material for Chapter 5

C.1 Purification of hybrid flagellin	172
C.2 Further analysis of hFliC filaments	173
C.3 Production of purification of recombinant FliC and DENV2	174
C.4 Recombinantly produced DENV2 specific monoclonal IgA	175
C.5 ELISA curves with full dilution series	176
C.6 B10.D2 mice immunized with hFliC administered via IP injections	177

Chapter 1

General Introduction

The mucosal barrier and how homeostasis is maintained

1.1 List of abbreviations

GIT	Gastro-intestinal tract
PP	Peyer's Patch
GALT	Gut associated lymphoid tissue
NALT	Nasal associated lymphoid tissue
MALT	Mucosa-associated lymphoid tissue
SI	Small intestine
FAE	Follicle-associated epithelium
CMIS	Common mucosal immune system
LP	Lamina propria
M cell	Microfold cell
BB	Brush border
IEC	Intestinal epithelial cell
Th	T helper cell
DC	Dendritic cell
MΦ	Macrophage
fDC	Follicular dendritic cells
rFB	Reticular fibroblast
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn's disease
DSS	Dextran sodium sulfate
<i>C. rodentium</i>	<i>Citrobacter rodentium</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. Typhimurium</i>	<i>Salmonella</i> serovar enterica <i>Typhimurium</i>
<i>Y. enterocolitica</i>	<i>Yersinia enterocolitica</i>
EHEC	Enterohemorrhagic <i>E.coli</i>
EPEC	Enteropathogenic <i>E.coli</i>
TJ	Tight junctions
AJ	Adherens junctions
A/E	Attaching and effacing
LEE	Locus of enterocyte effacement
T3SS	Type three-secretion system
Tir	Translocated intimin receptor
EPI64	EBP50 and PDZK interactor of 64 kDa
ERM	Ezrin/Radixin/Moesin family of proteins
AMP	Anti-microbial peptide
PGRP-S	Peptidoglycan recognition protein-S
TNFα	Tumor necrosis factor α
NF-κB	Nuclear factor κB
CXCL	C-X-C motif ligand
CCL	CC chemokine ligand
GP2	Glycoprotein-2
SIgA	Secretory IgA

1.2 Overview of the mucosal barrier

Lining regions of the gastro-intestinal (GI), respiratory, and urogenital tracts the mucosal barrier acts as the body's first line of defense against invading pathogens. Moreover, during development the GI and respiratory tracts become colonized with symbiotic microorganisms collectively referred to as "microbiota", which in the context of the GI, aid the host in the digestion and absorption of nutrient. In fact, as you travel down the GI-tract from the small intestine (SI) to large intestine (colon) the concentration of microbiota grows denser, reaching to a density of 10^{12} bacteria per gram of luminal content, outweighing eukaryotic cells 10:1 (1). Thus, the barrier is charged with the momentous task of tolerating commensal or good bacteria and producing appropriate immune responses against pathogenic bacteria. This task is largely accomplished by three distinct barrier functions, which work together synergistically to maintain a state of homeostasis and thus all are to some extent influenced by inflammation (Figure 1.1). Firstly, a physical barrier is established by polarized enterocytes (also referred to as intestinal epithelial cells (IEC)) and mucus producing goblet cells that limit paracellular transport and direct bacterial contact to the epithelium respectively. Secondly, the innate immune barrier comprised of paneth cells that secrete a variety of anti-microbial peptides to help maintain the appropriate bacterial density. Finally, the adaptive barrier established by the follicle-associated epithelium (FAE), M (microfold) cells, and underlying lymphocytes, help to generate immune response against invading pathogens. Loss of barrier function results in intestinal dysbiosis and inflammation and inappropriate regulation of any barrier component results in the onset and progression of chronic inflammatory disease such as inflammatory bowel disease (IBD). Thus, appropriate barrier function is crucial for maintenance of homeostasis.

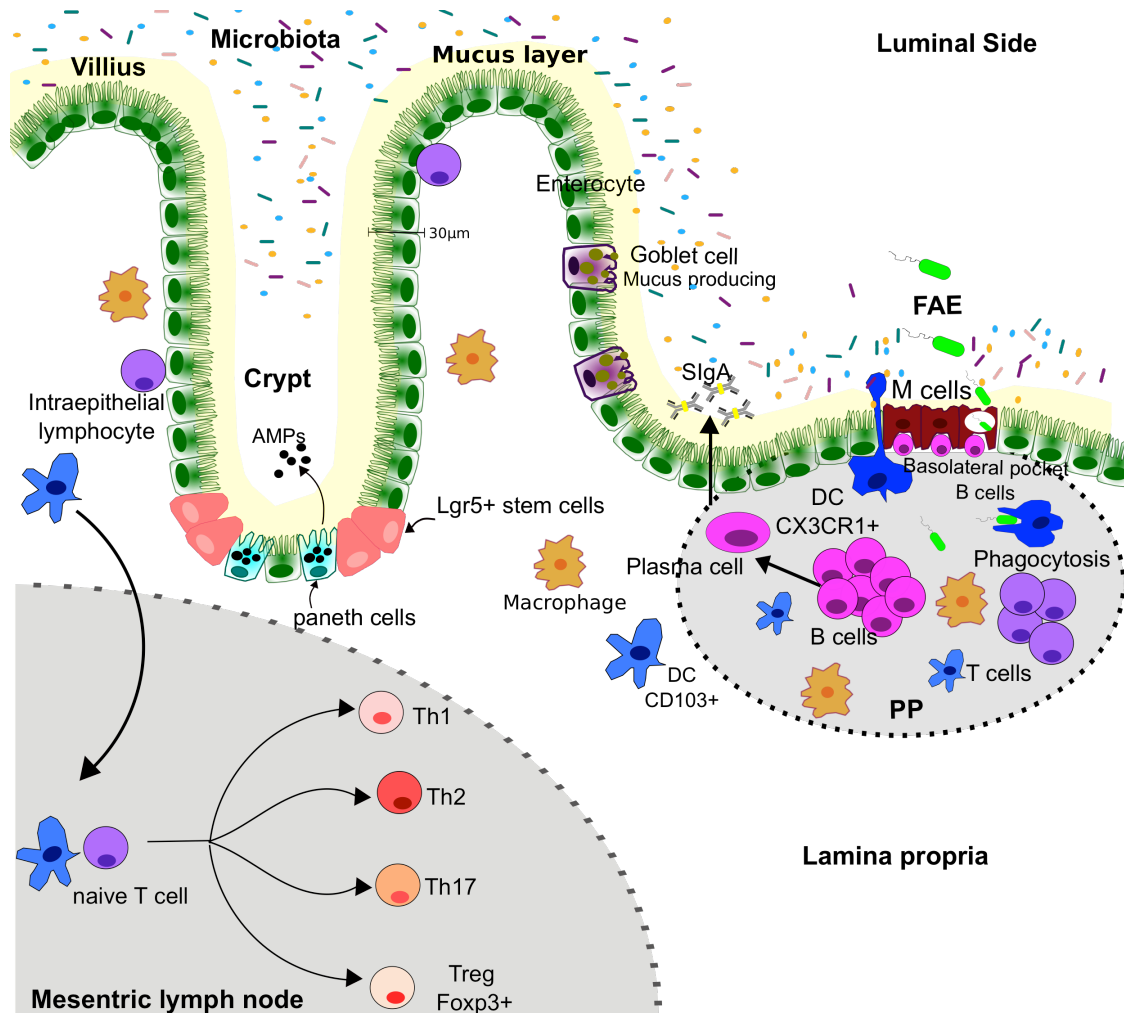


Figure 1.1 The mucosal barrier of the small intestine. The physical barrier provided by both the IECs, which form the epithelial junctional complex and apical microvilli, and goblet cells producing associated mucus layer. The innate barrier provided by paneth cells at the bottom of the crypt, producing AMPs. The adaptive barrier provided by the FAE, where M cells, DCs, MΦ, B cells and T cells in the lamina propria help to generate mucosal associated immune response.

1.3 Physical barriers: the mucus layer and the intestinal epithelium

The mucus layer present throughout the GI, is the first barrier in which luminal microbe's encounter and is established by specialized secretory cell subset of the epithelium known as goblet cells. This mucus is comprised primarily of secreted Mucin-2, a large glycosylated protein (2.5 MDa), stored within goblet cell granules (2). While

this mucus layer is present throughout the stomach and the intestines, it is thickest in the colon. In fact it has been estimated to be 200 μm in the colons of humans (50 μm in mice), and is divided into two structurally distinct layers, an outer mucus layer containing most of the luminal bacteria and a firmly attached inner layer that is largely devoid of bacteria (3; 4). Unlike the colon, the SI mucus layer is discontinuous with some surfaces, such as the tips of the villi and the FAE lack mucus altogether, which may suggest why secretory IgA (SIgA) and antimicrobial peptides (AMPS) play a more prominent role in the SI compared to the colon (5; 6).

Since goblet cells and the associated mucus layer are present throughout the GI, they are crucial for maintenance of intestinal homeostasis. In line with this notion, one study found that in mice, the absence of Mucin-2 (Mucin-2 $-/-$), resulted in both a disordered mucus layer, and spontaneous development of IBD (5; 7). This was largely attributed to increased bacterial contact with the colonic epithelium and subsequent bacterial infection. Moreover, *in vitro* treatment of goblet cell lines (LS180) with pro-inflammatory cytokines like $\text{TNF}\alpha$ and IL-6 increased the expression of various mucin proteins (8; 9), suggesting that perhaps increased mucus production is required to regain intestinal homeostasis, and that defects in this process further drive inflammation. Recently, it was found that mice deficient in the inflammasome related protein NLRP6 (Nod-like receptor family pyrin domain containing 6) were unable to clear *Citrobacter rodentium* (*C. rodentium*) infection (10), and this was not attributed to defects in the host immune response but rather the inability of the goblet cells to secrete Mucin-2. Taken together these studies indicate that both the goblet cell and its secreted mucus help to limit bacterial epithelial contact, and its absence makes the host more susceptible to bacterial infection, which in turn drives intestinal inflammation.

Just below the mucus layer lies the intestinal epithelium, covering a surface area of 300m², which is crucial for separating the luminal microenvironment from the underlying lamina propria (LP) and the inside of the host (11). This barrier is established by a single monolayer of polarized intestinal epithelial cells (IEC also referred to as enterocytes) that is approximately 30 µm thick. These enterocytes, making up ~90% of the epithelium, are polarized in the sense that their basolateral and apical faces exhibit a unique phenotype from one another, due to the expression a unique sets proteins, and receptors. The apical side of mature IECs contains finger-like F-actin protrusions referred to as microvilli that work to increase the surface area of the cell; a feature that was once solely linked nutrient absorption (brush border epithelium: 12). Meanwhile, the basolateral pole is attached to connective tissue in the LP, known as the basolamina. Remarkably, the intestinal epithelium is the most rapidly proliferating tissue in the body, where in humans it is renewed every 5 days and in mice every 3 days from the Lgr5⁺ multipotent stem cell progenitors located near the base of the crypts of Lieberkühn (13; 14). All cells migrating from the crypt will differentiate into one of four main cell lineages; enterocytes, goblet cells, paneth cells or enteroendocrine cells. Terminally differentiated IECs at the tip of the villi in the SI and the cuffs of the colon undergo apoptosis and slough off, and will be replenished by new cells migrating up the crypt villus axis (15). It is now believed that this dynamic self-renewal mechanism plays a protective role during bacterial infection and inflammation, in which damaged or infected cells will be removed and subsequently replaced.

Polarized enterocytes form strong cell-to-cell contacts with adjacent cells to limit paracellular transport of luminal content, and are divided into three distinct complexes that together make up the epithelial junctional complex; the most apical tight junctions

(TJs), the adherens junctions (AJs) and the most basolateral desmosomes (Figure 1.3: (16)). Remarkably, during homeostasis this process of epithelial replacement occurs without jeopardizing the epithelial junctional complex and subsequent intestinal permeability. The TJs are comprised of a heterogeneous complex of transmembrane proteins, including occludins, claudins (24 members identified), and junctional adhesion molecules (16-18). Intracellular scaffolding proteins including zonula-occludens members link these molecules to the actin cytoskeleton, which provide structural integrity. TJs are largely influenced by both dietary factors and luminal microbiota. In fact various commensal bacteria species, have been found to enhance barrier function, this has been observed for Lactobacilli and members of the firmicutes phyla (19; 20). Moreover, a breach in TJ integrity may precede the development of colitis, which is associated with elevated luminal levels of pro-inflammatory cytokines (21-23). This has been corroborated by *in vitro* treatment of intestinal epithelial cells with IFN- γ , which resulted in a marked decrease in epithelial resistance (24). Moreover, in contrast to commensal bacteria, many enteric pathogens have evolved mechanism to perturb the TJs to increase their uptake across the epithelium. One of the most well studied examples of this is in the case of the enteropathogenic *E. coli* (EPEC) infection, which causes the dephosphorylation of occludins, and subsequently leads to a decreased TJ integrity (25; 26). Meanwhile certain strains of Salmonella have shown to increase the expression of claudin-2 (leaky claudin), which again disrupts TJ integrity (27).

Below the TJs lie the AJs, which are largely comprised of the transmembrane protein E-cadherin that link to the actin cytoskeletal network (28). Thus AJs have largely been studied in the context of their ability to help maintain cell polarity during tissue morphogenesis serving as foci for actin polymerization (16; 28; 29). Finally, the last

component of the epithelial junctional complex is the desmosomes, which are structurally similar to AJs and are comprised of cadherin family members desomglein and desomcolin (16; 30). However, unlike both TJs and AJs, desmosomes are not spatially restricted and can be found anywhere in the lower half the lateral membrane, and instead of aiding in structural integrity, they are crucial for cell-to-cell communication (30). Thus, the physical barrier provided by the intestinal epithelium and mucus layer are crucial for maintenance of intestinal homeostasis by limiting bacterial contact and uptake across the epithelium. Any breach of either barrier results in intestinal dysbiosis and inflammation.

1.4 Microvilli development and its function at the barrier

One defining feature of terminally differentiated IECs is the presence of a densely packed array of microvilli (~1,000 microvilli per cell) on its luminal surface, known as the brush border (BB: (31)). These microvilli are thought to increase the surface area of the enterocyte to increase nutrient adsorption and water transport (12; 31). Excitingly, it is now becoming increasingly apparent that the BB plays an active role in defending host tissues against bacterial binding. In the context of the nasal passages, the BB epithelium is replaced by the ciliated epithelium, which turns over at a slower rate compared to the intestine (32). Interestingly, the differentiation program of the ciliated epithelium relies largely on the forkhead transcription factor FoxJ1, however no such master regulator has been identified driving the BB differentiation program (33; 34). While many efforts have focused on the development of mouse models lacking BB in order to gain insight into the *in vivo* functional consequence of a lack of BB, still little is known about the molecular mechanism controlling microvillar length and packing on the apical surface (35; 36).

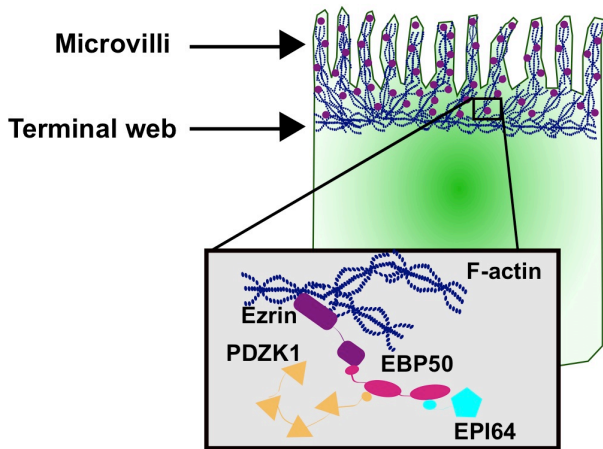


Figure 1.2 Protein network of microvilli formation. Apical microvilli of polarized enterocytes are largely generated and stabilized by Ezrin that bind F-actin along the length of the microvilli. Ezrin upon activation via phosphorylation will interact with EBP50, PDZK1 and EPI64. This protein complex stabilizes the interaction between ezrin and F-actin

The protein network governing microvilli formation and stabilization has largely been characterized, however new information is still emerging to further our understanding of BB development (37). First the ERM (Ezrin/Radixin/Meosin) membrane cytoskeletal linker family member Ezrin is crucial for tethering the central F-actin bundle of the microvilli to the apical plasma membrane via its C-ERMAD domain (C-terminal ERM-association domain Figure 1.2: (38)). Remarkably, while Ezrin associates with F-actin along the length of the microvilli, the loss of Ezrin *in vivo* does not completely abrogate BB formation, suggesting other factors may compensate for the loss of Ezrin in BB development (39). Moreover, Ezrin is conformationally regulated, and upon phosphorylation will not only interact with F-actin but also to the scaffolding protein EBP50 (ERM-binding phosphoprotein of 50 kDa: Figure 1.2) via its PDZ (postsynaptic density 95/discs large/zona occludens-1) domain (39). Finally EBP50 interacts with both PDZK1 and EPI64 (EBP50-PDZ interactor of 64 kD), first identified from extracts of placental microvilli (Figure 1.2: (40)). Interestingly, Hanono *et al* identified *in vitro*, using a dominant negative form of EPI64, that EPI64 is required for the stabilization of the protein network mentioned above, and in its absence, BB development is abolished (41).

Other factors influencing microvilli stabilization and length have recently been identified. First, an elegant study by Crawley and colleagues determined that protocadherin-24 is critical for the assembly and organization of the BB (42). The protein Cordon bleu, first identified to be present at the apical surface of IECs via a proteomic analysis from the lab of Matthew Tyska, has now been suggested to govern microvilli length (43). Disorders (i.e. microvillous-inclusion disease) and bacterial infection (enteric pathogens) in which, enterocyte microvilli formation is abrogated, results in intestinal dysbiosis and in the most severe cases can lead to death, suggesting microvilli are a crucial component of the physical barrier. Thus, microvilli associated proteins are important for maintenance of intestinal homeostasis, and are probably implicated/modulated during intestinal inflammation, such as in IBD.

1.5 Pathogenic attachment and effacement mechanisms

Certain enteric pathogens, including EPEC, EHEC, murine homolog *C. rodentium* and *Salmonella* serovar enterica *Typhimurium* (*S. Typhimurium*), have developed mechanisms to specifically target microvilli to increase their uptake across the epithelium (Figure 1.3). These pathogens will use their type 3 secretion system (T3SS), and through a process called attachment and effacement (A/E), will inject effector proteins that hijack host proteins to knockout microvilli formation. In *E.coli* strains these A/E effectors are collectively located on the pathogenicity island, known as the locus of effacement effectors (LEE). One important effector protein that initiates the infection and A/E process of enteric *E. coli* is translocated intimin receptor (Tir), which inserts itself into host cell membranes, where it binds to the *E. coli* associated protein intimin, allowing the bacterium to bind to the epithelium (26). However, how this bacterium makes it through initially to the mucus layer to bind to the epithelium still remains poorly

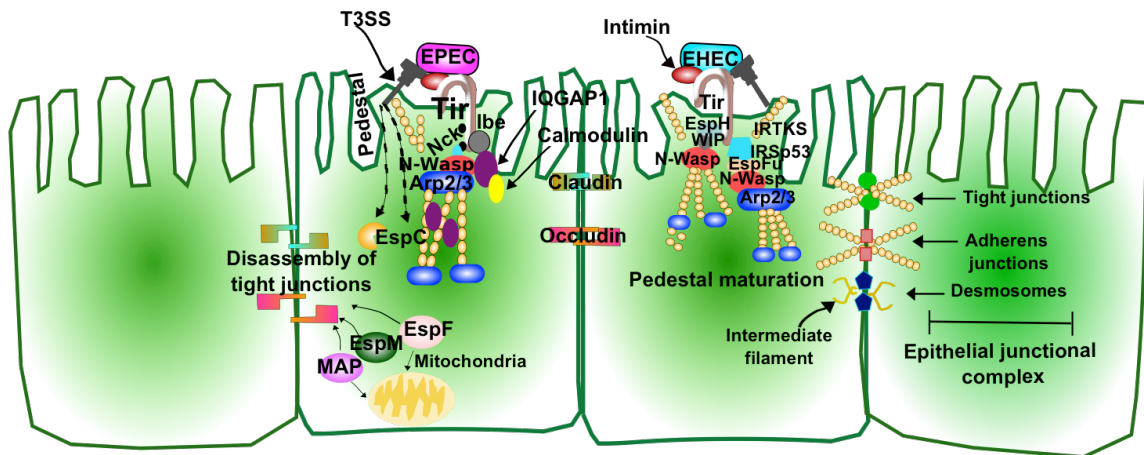


Figure 1.3 Attaching and effacing mechanism of enteropathogenic *E. coli*. This A/E mechanism abolishes microvilli formation and results in pedestal formation by hijacking the actin machinery. EHEC and EPEC also hijack other host protein to break the epithelial junction complex.

understood. Unlike *E. coli* pathogenesis, our understanding of how *S. Typhimurium* manipulates the host cell cytoskeleton is still rapidly evolving. SopB from *S. Typhimurium* was recently found to be capable of transforming a lineage committed IEC to an M cell (44). Unfortunately, it was not discussed whether these cells were genetically manipulated into a M cell phenotype by *S. Typhimurium* or whether these cells only phenotypically look like M cells due to the lack of microvilli. Nonetheless, SopB seems to be indispensable for Salmonella to gain entry into the host. A study from Keestra and colleagues found that endosomal receptor Nod1 (Nucleotide-binding oligomerization domain-1) senses the cytosolic delivery of *S. Typhimurium* effector proteins and in turn activates small Rho GTPases (Rac1 and CDC42: (45)), which have been implicated in maintenance of the host cytoskeleton and microvilli. Taken together, these studies suggest microvilli may themselves offer a barrier to limit bacterial binding and subsequent uptake. However, it is unclear whether microvilli are purely a steric hindrance to prevent entry, or if a more complex physicochemical mechanisms work in concert to limit pathogen adherence and subsequent entry.

1.6 Innate barrier: homeostasis towards the resident microbiota

During birth, we become colonized with a large microbial community, and it is well understood the route of delivery impacts the initial colonization of microbiota. Vaginal delivery results in the vertical transfer of the mother's microbiota to the infant, an effect not seen in babies delivered via Cesarean section (46; 47). Thus, the resident commensal microbiota have an immense influence on the development of the intestinal architecture (48). In fact, it is been documented that mice born under germ-free conditions compared to conventionally reared mice, have both a thinner mucus layer and slower rate of intestinal turnover (49; 50). It is no wonder shifts in the microbial ecology within the GI tract have been linked to the onset and progression of IBD. Maintenance of both the appropriate bacterial density and ratios of bacterial species is paramount preserving intestinal homeostasis.

The large intestine, as mentioned earlier, is protected from changes in the microbial community due to the presence of a thick mucus layer. However, in the SI the mucus layer is both thinner, and more discontinuous. Thus, the SI needs additional mechanisms to maintain the appropriate microbial architecture. In the SI high concentrations of AMPs act as both a chemical and innate line of defense against pathogenic and commensal bacteria that have penetrated the physical barrier provided by the mucus layer. AMPs are predominately secreted by paneth cells that lie in small clusters at the base of the crypts of Lieberkühn throughout the SI (Figure 1.1: (51; 52)). Paneth cells are distinguished from other associated epithelial cells due to their extensive endoplasmic reticulum and Golgi network, which direct anti-microbial rich secretory granules to the apical surface where they are released into the crypt lumen (53). These AMPs then can diffuse away from the crypt and disseminate into the mucus

layer. Interestingly, paneth cells have recently been found to secrete ligands that act as trophic factors (wnt and notch) that help to maintain the $Lgr5^+$ crypt stem cell niche (53). In fact, direct interactions between stem cell and paneth cell populations are crucial for proper intestinal differentiation (54).

Although paneth cells are usually restricted to the SI, they have been identified in the colonic epithelium during intestinal inflammation. Moreover, in the presence of inflammatory stimuli, such as $TNF\alpha$ and $IFN\gamma$, paneth cells increase their rate of AMP secretion. Interestingly, in ileal Crohn's disease (CD) a major category of inflammatory bowel disease is associated with increased bacterial association to the epithelium and abnormal composition of colonizing microbiota. Not surprising, dysfunction in paneth cell biology has been linked pathogenesis of ileal Crohn's disease (CD) and in fact many paneth cell specific genes have been identified as susceptibility genes for CD. A study from Wehkamp and colleagues demonstrated that paneth cell α -defensin, an important AMP, had significantly reduced expression levels in CD patients compared to healthy individuals (55). However this decrease was not linked to intestinal inflammation but rather an early event in disease pathogenesis. Moreover, individuals carrying single point source mutations in *Nod2*, an intercellular pattern recognition receptor, and *Tcf4*, a transcription factor pivotal for paneth cell differentiation, have lower α -defensin levels (55-57). Taken together, paneth cells and their associated products are pivotal for the maintenance of small intestinal homeostasis, and dysfunction can lead to intestinal dysbiosis.

1.7 Adaptive barrier: the follicle-associated epithelium

The mucosal immune system is charged not only with the momentous task of maintaining a symbiotic relationship with the resident microbiota, but also with initiating

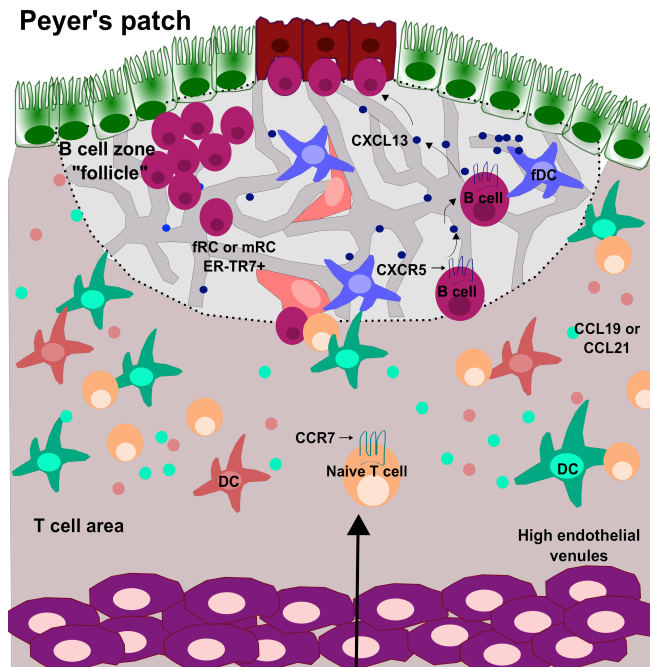


Figure 1.4 Organization of SI PP follicle. SI PP during organogenesis develops a highly organized stromal cell network that aids in guiding in lymphocytes to form the follicle. B cells are driven into the B cell (CXCR5⁺) zone by CXCL13 gradient meanwhile T cells (CCR7) stay in the T cell zone via the expression of CCL19 and CCL20. During inflammation this stromal cell network further aids lymphocyte infiltration. Other stromal cells that makeup the follicle include fRC, mRC and other fibroblasts.

an appropriate response against non-self antigens. The contributions of the physical and innate barriers for maintaining intestinal homeostasis have been previously discussed. This section will now concentrate on the contributions provided by the adaptive arm of the mucosal system for perpetuation of homeostasis. The adaptive barrier is established by organized mucosa-associated lymphoid follicles (MALT), in which overlying microfold (M) cells within the FAE help to generate antigen-specific immune responses to non-self antigens (Figure 1.1), and play an important role in the generation of SIgA. Remarkably, the two main components of MALT, Peyer's patches (PP) in the gut and nasal associated lymphoid tissue (NALT), contain all the immunocompetent lymphocytes and antigen presenting cells (APCs) required to drive a mucosal immune response (58). Thus, MALT tissues act as major inductive sites for mucosal mediated immune response to ingested or inhaled antigens in the intestine and respiratory tracts. These inductive sites (i.e. the lamina propria of the intestinal and respiratory tract) are linked to effector sites via the common mucosal immune system (CMIS: (58; 59)). The CMIS terminates in

the generation of antigen specific T helper 1 (Th1), Th2, Th17, cytotoxic T lymphocytes responses, and IgA-producing plasma cells (58). While NALT and PP have similar functions, in the context of mucosal mediated immune response, their developmental programs are quite distinct from one another (58).

In mice, PPs are observed as dome shaped lymphoid follicles in developing embryos at pre-natal day 15 (60; 61). Meanwhile, nasal associated lymphocytes are not seen until post-natal day 7 and are not fully developed into the bell-shaped NALT until 5-8 weeks after birth (62). Not only are the gestational periods in which PP and NALT develop differ, an effect that could potentially be attributed to their anatomical location in the body, the factors driving their development are distinct as well. In PP, lymphotoxin (LT) and TNF α and their cognate receptors (LT- β R and TNFRp55/TNFRp75) generate a cytokine rich environment termed “programmed inflammation”, that drive organogenesis of the PP as well as other secondary lymphoid organs (63; 64). Mechanistically, this cytokine rich environment induces NF- κ B stromal cell expression of a series of chemokines, including CCL19, CCL21, and CXCL13, which facilitate the accumulation and maintenance of the lymphoid follicle (Figure 1.4: (65-67)). LTs have proven to be crucial in secondary lymphoid follicle generation, and deletion of any one of its family members results in the complete loss of PP development (64). Moreover, the cytokine interleukin (IL)-7 has also been linked to the genesis of the PP, as well as providing additional growth signals to the nearby intraepithelial $\gamma\delta$ T lymphocytes (68; 69). One study indicated that PP development was dependent on the IL-7R⁺CD3⁻CD4⁺CD45⁺ cell subset, and were deemed the PP inducers (70). These cells were also found to express high levels of chemokine receptor CXCR5, the receptor for CXCL13, an important chemokine component of the PP stromal network, as well as LT- β (70; 71). Interestingly,

in mice lacking transcriptional regulators ID2 (inhibitor of DNA binding-2) and ROR γ (retinoic-acid-receptor-related orphan receptor γ) both PP and lymph nodes were absent (72; 73).

NALT, although still reliant on CD3⁺CD4⁺CD45⁺ inducer cell subset for its development, in the absence of ROR γ , genesis of NALT still occurs (74). This suggests that although NALT and PP development seems dependent on the same inducer cell phenotype; in PPs these cells are under the regulation of both ID2 and ROR γ , while in NALT these cells are solely regulated by ID2. Moreover, unlike PP genesis, NALT formation seems to occur independently of IL-7R signaling pathway and LT- β R cascade (74). Regardless of the mechanism of development, PP and NALT play a pivotal role in maintaining intestinal homeostasis by being the sole components of the mucosal barrier able to generate an adaptive immune response. It is becoming increasingly apparent that SI PP may act as the major inductive site of ileal Crohn's Disease (75; 76), while deficiencies in NALT function has been implicated in such autoimmune diseases like asthma (77). Thus, defects in PP and NALT genesis may lead to intestinal inflammation and dysbiosis.

1.8 M cells and how they can be exploited

M cells, are a specialized subset of epithelial cell that play a central role in mucosal immune surveillance, by actively capturing and transporting luminal microbial particulates (i.e. bacteria, viruses etc.) to underlying immune cells, where mucosal mediated immunity is generated (Figure 1.1: (78; 79)). Thus, M cells are an essential component of MALT for the generation of antigen-specific immune response. The prevailing capture-receptor hypothesis is thought to make M cells efficient at pathogen capture. One noteworthy study was the identification of GP2 (Glycoprotein-2) found to

specifically bind fimbriae on various commensal and enteric bacteria (80). Interestingly, GP2 was found to be expressed exclusively by M cells and is now used as a functional marker of M cells. Another study suggested that *Yersinia enterocolitica* (*Y. enterocolitica*) enters the host by binding β 1-intergrin on the apical surface of M cells (81). Due to their ability to efficiently capture and transcytose bacteria, it is no wonder that many studies have exploited M cells for mucosal-targeted drug therapies. Recently, our lab made use of the highly specific targeting ligand clostridium perfringens enterotoxin, a ligand for claudin-4 expressed on the apical surface of M cells, to increase the uptake of vaccine microparticles (82; 83). However, studies of M cell biology have been hindered due to lack of unique surface markers. Recently, our lab identified that PGRP-S (Peptidoglycan recognition protein-S), a protein typically associated with the tertiary granules of neutrophils, was also expressed by murine M cells (84-86). Making use of this, we developed a transgenic mouse in which the PGRP-S promoter was used to drive DsRed expression in both M cells and neutrophils (86). This reporter mouse should increase our understanding of M cell regulation during homeostasis and inflammation.

M cells are phenotypically distinct from the surrounding enterocytes in that they lack organized apical microvilli, a feature that may be linked to their ability efficiently capture antigens and will be the subject of chapter 3 of this thesis. M cells are scattered among the enterocytes in the FAE. This distribution is highly regulated with a characteristic checker board pattern, which has recently been found to be under the influence of Notch signaling (87), where conditional deletion of Notch resulted in both increased M cell density and clustering. M cells are largely restricted to the FAE. The cytokine rich environment provided by the follicle drives development and maturation of

M cells (58). In line with this notion, a study from our lab indicated that treatment of an intestinal cell line (Caco2BBE) with high concentrations of pro-inflammatory cytokines, resulted in the increase of M cell specific genes (88). These results suggested that pro-inflammatory cytokines could shift the lineage commitment of an IEC to that of M cell, indicating that M cells may be regulated during IBD, where high levels of pro-inflammatory are present.

The basolateral side of M cells is also distinct from the surrounding enterocytes. In fact, the basolateral surface of an M cell contains a large invagination termed the basolateral pocket, large enough to contain a mature B lymphocyte. Interestingly, this basolateral pocket is intimately tied to M cell function. In B cell deficient mice, it has been reported that the basolateral pocket is misshaped, and as a result the M cell cannot mature and develop transcytosis function. Moreover, in these animals the PP follicle was much smaller compared to WT animals (89; 90). Meanwhile, in T cell deficient animals, PP follicle size was similar to WT animals, suggesting that B cells play a more prevalent role in follicle and subsequent M cell formation.

Not all M cells are restricted to the FAE. The recently identified villous M cells, as the name suggests, develop along the SI villi in the absence of a follicle (91). Although these villous M cells have been shown to potentially transcytose luminal antigens like other FAE restricted M cells, it is unclear whether they develop the required basolateral pocket for transcytosis function. Moreover, villous M cell formation seems to be a product of various inflammatory stressors. A study from our lab reported that treatment of PGRP-S reporter mouse with cholera toxin resulted in a marked increase of villous M cells (86). Interestingly, these villous M cells did not express DsRed and were only identified using the marker UEA-1 (Ulex europaeus agglutinin stains both M cells and

goblet cells). These results suggest that FAE associated M cells and villous M cells may have unique differentiation programs from one another. However, regardless of the differentiation program, M cells play an important role in regulating the mucosal immune response, and may play a greater role the pathogenesis of inflammatory conditions.

1.9 GALT immune cells and their role in mucosal immune responses

Luminal antigens are constantly sampled by both M cells in the FAE and professional APCs (DCs and macrophages (MΦ)) of the underlying follicle and LP (Figure 1.1). Interestingly, both DCs and MΦ can encounter antigens either through direct luminal sampling or within the lymphoid follicle where antigens have been transcytosed by M cells. Once activated LP DCs can migrate to the mesenteric lymph nodes (MLN) and help to initiate a T cell response (Figure 1.1: (92)). Moreover, DCs in the follicle prime naïve T cells and drive their differentiation and clonal expansion. This section will focus on the various DC and MΦ phenotypes present in the GALT and their contributions to the mucosal immune response.

DCs have been implicated in the maintenance of tolerance toward the resident microbiota, and generation of protective immune responses against variety of pathogens, and in the context of the intestines, reside in the LP, PP, and draining lymph nodes (92). DCs typically migrate from peripheral tissues to lymphoid tissues where they can present antigen to T cells (92). Typically, most tissue resident DCs are immature and are poorly immunogenic due to their low expression of Major histocompatibility (MHC) molecules. DCs mature due to some immunological stressor, and these mature DCs can then interact naïve CD4⁺ T cells. Depending on the DC phenotype and cytokine environment, these naïve T cells will differentiate into the various effector and regulatory T cell subsets (93). LP CD11c⁺ DCs are divided into subsets depending on their

expression of CD103 receptor (a receptor for E-cadherin), and are derived from two different blood cell precursors (93-95). CD103⁻ DCs, arrive as precursors from the blood (L-selectin expression), and are distinguished from the CD103⁺ DCs by their expression of the fractalkine receptor CX3CR1. This feature allows them to stay in close contact with the epithelium, as fractalkine is expressed on the basal membrane of the epithelium (96; 97). Moreover, CD103⁻CX3CR1⁺ DCs project dendrites through the IEC into the intestinal lumen forming a tight junction like structure with IECs (96). Interestingly, this complex not only allows the DCs to sample antigens, but is also a potential mechanism by which apoptotic IECs are engulfed and transported to the MLNs. CX3CR1⁺ DCs have also been found to constitutively express CD70, and once activated by commensal-derived ATP, results in the local differentiation of Th17 cells (98). Defects in CX3CR1 DCs function has been implicated in increased susceptibility to bacterial infection of enteric pathogens including *S. Typhimurium* and *E. coli* (97).

The other subset of DCs in LP is the CD103⁺CX3CR1⁻ DCs, and are important for converting naïve CD4 T cells to Foxp3⁺ regulatory T cells (Tregs), which play crucial for the production of the anti-inflammatory cytokine IL-10 (92). Interestingly, in mice, silencing of IL-10 resulted in colitis like disease, suggesting a protective role Tregs and IL-10 during inflammation (99). Recently, it was found that SI goblet cells were able to preferentially deliver low molecular weight antigens to the underlying CD103⁺ DCs, a mechanism that has now been linked to maintenance of intestinal homeostasis (100). Although these DCs cells are null for CX3CR1, they remain in the LP due to the fact they are negative for various migratory and homing receptors (CCR7 and CCR9: (92)). During intestinal homeostasis this DC subset has been implicated in the maintenance of a tolerogenic state toward the resident microbiota (101). However, the tolerogenic

property of this DC subset is dependent on their local tissue environment, in particular, factors derived from the IECs (TGF β and retinoic acid), which are induced in the IECs after apical association of an inflammatory stimuli (102; 103).

Finally, several DC subsets have been identified within the follicle of the SI PP, which perform various functions (92). First, in the dome region of the PP, CX3CR1⁺ DCs stay in close contact with the FAE, which like the LP CD103⁻CX3CR1⁺ DCs, can transcytose luminal antigens. Unlike LP DCs, PP CX3CR1⁺ DCs were suggested not to play a role in inducing T cell response. Interestingly, a newly identified DC subset in the PP, CD103⁻ DCs, have been found to prime IFN γ and IL-17 T cells with gut homing capabilities (92; 104). Also residing largely in the T cell region of the PP follicle, are DC subsets that express the migratory receptors CCR6 and CCR7, which aid in their migrations into the follicle (97). Once in the follicle these DC subsets help in CD4⁺ T cell activation and defense against various intestinal pathogens. CCR6⁺ DC populations are unique to PP, even though IECs express CCL20, the ligand for CCR6, and in fact during infection these DCs migrate toward the FAE to engulf transcytosed pathogens (97; 105). Moreover, as mentioned earlier, the PP is important for the production of SIgA, which is in part mediated through the CD11b⁺ DC subset. Interestingly, this same DC population is involved in IgA class switching in a T cell dependent manner (106-108). However, it has been suggested that 70% of SIgA at the mucosal barrier is produced in a T cell independent manner (the focus of Introductory Chapter 4: (109)).

The mucosal barrier is the largest reservoir of tissue resident macrophages, and like the LP DCs derive from blood monocytes via endogenous chemottractants in the non-inflamed intestine (110). During homeostasis these monocytes are exposed to stromal factors (TGF β) that cause differentiation of M Φ into a non-inflammatory M Φ

phenotype, characterized by downregulation of various innate response receptors, including IgA, IgG, complement receptor (CR) 3 and CR4 (110; 111). Interestingly, these MΦ maintain strong phagocytosis and bactericidal function. In fact, studies have indicated that these MΦ are strongly bactericidal against gram-negative bacterium, such as *S. Typhimurium* and *E. coli* (110). During inflammation, blood monocytes migrate into the intestine and differentiate into MΦ that retain their pro-inflammatory potential. In line with this notion, MΦ from patients with IBD, express high levels of CD14, a co-receptor for lipopolysaccharide, a potential explanation for increased levels of pro-inflammatory cytokines in patients with IBD (112; 113). Together both intestinal DCs and MΦ play a crucial role in maintaining intestinal inflammation, and if they are dysregulated inflammation ensues.

1.10 Inflammatory bowel disease and murine models

Inflammatory bowel disease is an umbrella term for ulcerative colitis (UC) and Crohn's disease, which are characterized as chronic inflammation and epithelial damage along the intestinal tract. However, CD and UC have differing clinical and pathological features (114; 115). Pathologically, CD causes patchy inflammation termed skip lesions along the length of the colon into the terminal ileum of the SI; meanwhile, in UC, inflammation is solely restricted to the colon. Although the exact mechanism has yet to be elucidated, it is thought that intestinal inflammation is initiated and propagated due to loss of immune tolerance toward the resident microbiota. Remarkably, the onset and propagation of IBD is multifactorial in nature, and is dependent on various genetic and environmental factors, such as diet. In fact a genome-wide-meta-analysis (GWAS) revealed several susceptibility genes for both UC and CD; 47 were identified for UC, 71 for CD, and 28 were shared between both diseases (116-118). Many of the genes

identified are implicated in barrier function, microbial defense and autophagy. Moreover, it is now well understood that western diet (high fat) can shift the microbial ecology of the intestine, reducing the number butyrate-producing bacteria, which can promote IBD by retarding barrier formation and increasing paracellular transport (29). Many studies have confirmed that *Faecalibacterium prausnitzii*, the major butyrate-producing bacteria in the intestine known for its anti-inflammatory properties, is severely decreased in obese population as well as in patients with IBD (20).

The immune response of IBD is characterized by an inappropriate mucosal immune response driven by chronic T cell activation and production of pro-inflammatory cytokines (119). However, both the T cell response and subsequent cytokine response differ between CD and UC. CD is characterized by a Th1 cytokine profile, while UC has a more Th2 phenotype (Figure 1.1: (119-121)). This was demonstrated by isolating LP CD4⁺ T cells from both CD and UC patients. When stimulated *in vitro* CD4⁺ T cells taken from CD patients produced high amounts of IFN γ ; in contrast this same stimulation of T cells isolated from UC resulted in a significant increase in IL-5 (120). The pro-inflammatory cytokine IL-12 has also been implicated specifically in the pathogenesis of CD, where both DC and M Φ have shown altered IL-12 (p35 and p40) production (122; 123). Another study found that IL-12 production by LP M Φ was significantly higher in CD patients compared to UC patients and this effect was abrogated by treatment with anti-IL-12 (124). In contrast to CD, it is becoming increasingly apparent that UC is associated with altered IL-13 response, resulting in loss of TJ integrity and IECs apoptosis, driving mucosal ulceration (125). Moreover, this atypical IL-13 in UC patients has been linked to increased presence of CD1d-restricted non-classical natural killer cells, which in turn drives epithelial damage (126).

Th17 are now being recognized as an important cell subset in the pathogenesis of the IBD (127). They are most abundant in the terminal ileum of the SI and are activated by various normal microbiota species, such as segmented filamentous bacteria (SFB; (128; 129)). GWAS studies identified polymorphism in the Th17 signaling pathway (IL-23R and others) in IBD patients (130; 131). Studies have revealed that LP Th17 cells from both UC and CD patients have increased production IL-17f, and IL-17a, and was further corroborated with the *C.rodentium* colitis mouse model (130; 132; 133). It has been suggested that in IBD the Th17 cytokine production is chronically stimulated, by such cytokine as IL-23, as well as other TNF family members (131). Moreover, Th17 cytokines, IL-17 upregulates other pro-inflammatory cytokines like TNF α , IL-6 and IL-1 β (134-136). Paradoxically, Th17 cells have also been found to upregulate the anti-inflammatory cytokines IL-22, which control epithelial cell proliferation and production of AMPs like Regenerating-islet derived family of proteins (137). Thus, taken together pro-inflammatory and anti-inflammatory cytokines by Th cells shape the pathogenesis of IBD. It is no wonder that current gold standard treatments for IBD therapies targeting cytokine, the most well studied being anti-TNF therapies (138). Currently on market there are several anti-TNF drugs, including infliximab (remicade), adalimumab, certolizumab, and several others are in the pipeline (114; 115; 139-143). Other therapies being explored for the treatment of IBD include ustekinumab, an agonist of IL-12 and IL-23, as well as tocilizumab, a soluble IL-6 agonist (143-145).

Various mouse models have been established to better our understanding of the factors that drive IBD, although no model can specifically recapitulate disease associated with UC or CD. The most well studied model is the chemically induced dextran sodium sulfate (DSS) model, first established in 1985 (146). Although the exact

mechanism has yet to be determined, the prevailing hypothesis is that DSS causes inflammation by binding medium chain fatty acids, and enter the IEC to drive toxicity (146; 147). Other proposed mechanisms include, DSS being taken up by intestinal macrophages, which results in the inflammation (148). Another popular chemically induced model of inflammation is the TNBS (2,4,6-trinitrobenzene sulfonic acid), which is a haptenated agent that causes a disease similar to CD, by producing such cytokines IFN- γ and IL-12 (149). Disease is hallmarked by neutrophil infiltration, loss of goblet cells, edema, and is most efficient at generating disease when administered rectally and mixed with ethanol. Pathogen-induced inflammation models are increasing in prevalence, as they more reliably imitate human disease as compared to chemical induced inflammation. The most widely used is the *C. rodentium* infection model, which is a rodent homolog of EHEC and EPEC, and induces both a robust Th1 and Th17 response (150-152). Other models of bacterial induced inflammation include *S. Typhimurium*, *Helicobacter supp*, *Brachyspira hyodysenteriae* infections (45; 153-156). IL-10 knockout mice, which is model of engineered intestinal inflammation has greatly furthered our understanding of the important role that Tregs play in dampening inflammation (157-159). Finally, as T cells play a crucial role in shaping the inflammatory response of IBD, a T cell transfer model, termed CD45RB^{hi} model is commonly employed to study intestinal inflammation, where CD45RB^{hi} T cells are adoptively transferred into *scid* mice. However, although the shortcoming of this model is that mice develop colitis like disease after three to five weeks, these mice only develop inflammation in colon, which may reliably model UC disease (160-162). Although, in recent years we have furthered our understanding of the inflammatory process of IBD,

there is still a lot to learn about intestinal inflammation and the pathogenesis of IBD and other intestinal disorders.

1.11 Thesis aims

The work described in this thesis aims to describe a new barrier function of the mucosal system in the intestine that has previously been overlooked. Moreover, this thesis aims to further understand the pathogenesis of IBD, and how a previous undefined alteration in the barrier during inflammation may help to reestablish homeostasis. In **chapter 2** we describe the generation of a negative electrostatic barrier provided by apical microvilli present on the polarized enterocyte. By engineering an intestinal epithelial cell line to lack microvilli, we identify that this charge barrier dictates the pathogen ability to bind to the intestinal epithelium. Moreover, in this study we provide new insight on why M cells are efficient at capturing pathogens, which we believe is a result of absences of microvilli and the associated charge barrier. Meanwhile in **chapter 3** we examine the induction of M cells in the colonic epithelium during intestinal inflammation. Using a unique reporter mouse in which M cells stably express DsRed, we find a significant induction of colonic M cells in two mouse models of intestinal inflammation. This effect was largely abrogated by anti-TNF α , however these mice became sicker, suggesting a protective role of M cells during inflammation.

1.12 References

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Chapter 2

Epithelial microvilli establish an electrostatic barrier to
microbial adhesion

2.1 Introduction

Polarized epithelial cells such as intestinal and kidney tubule epithelium are exposed to a wide range of aqueous environments, with rapidly changing ionic and osmotic conditions (1-4). Despite these dynamic conditions, these epithelia must retain their barrier function and provide resistance to microbial adhesion and invasion. In the mucosal and airway epithelium, a layer of mucus also augments the barrier, though the mucus is not an intrinsic component of epithelium and is not present at all epithelial surfaces. A common feature of the polarized epithelial cells is the development of apical brush border microvilli, which display various transporter proteins and ion channels to facilitate small molecule and ion movement across the cell membrane (5; 6). While the apical brush border increases the surface area for these functions, it may also risk the increased availability of attachment sites for microbial invasion. An apparent paradoxical situation is presented by intestinal epithelium M cells, which are commonly found overlying mucosal organized lymphoid tissues (7; 8); these cells are specialized for capture and uptake of luminal microbes, yet they are morphologically distinctive by their absence of apical microvilli.

We recently observed that M cell uptake of nanoparticles in aqueous suspensions in mouse nasal passages *in vivo* could be enhanced by the use of small targeting ligands, but the influence of ionic conditions could be far more potent (9). Interestingly, in lower ionic strength buffers M cell particle uptake was enhanced, yet under these conditions the negative charge of both the particles and mucosal epithelium should have resulted in substantial electrostatic repulsion occurring between them. Historically, using DLVO (Derjaguin and Landau, Verwey and Overbeek) based approaches it has been well documented that most if not all bacterial species are

negatively charged, and yet they are still capable of making their way across the mucosal barrier (10-12). Since brush border microvilli present a variety of charged molecular species, including polar carbohydrates and charged amino acid side chains, it was possible that the negative charge of the microvilli generates a high density electrostatic charge at the enterocytes' apical surface, driving particles toward the more neutral M cell surface. In line with this notion, a study from Mutsaers *et al* demonstrated that the proportion of microvilli on healing mesothelium alters the level of glycocalyx and relative membrane surface charge, and while intestinal epithelial cells have a glycocalyx layer that protrudes 400-500 nm past the tip of its microvilli, M cells lack this layer (13-15). Yet it has not been determined whether changes in global electrostatic charge due to apical cell surface morphology plays a role in dictating bacterial binding. Thus, we sought to test the hypothesis that brush border microvilli contribute to the mucosal epithelium barrier using electrostatic repulsion, and conversely, the ability of M cells to capture microbial particles is in part related to a lower electrostatic repulsion.

To develop an *in vitro* test for the potential contribution of surface charge as an effective electrostatic barrier, we compared a cell model of brush border epithelium with modified cell lines designed to specifically impair microvillus formation. Using a series of assays for the adhesion or uptake of microbes or synthetic particles by these epithelial cells, including both static and laminar flow conditions, we found that bacterial adhesion to epithelium was markedly affected by the presence of microvilli. Quantification of the effect showed an apparent relationship to the surface charge of the microbial particles. Our results support the notion that microvilli provide a barrier to microbial invasion through electrostatic repulsion, and in turn, microbial virulence can in some cases be related to the ability to manipulate this barrier effect.

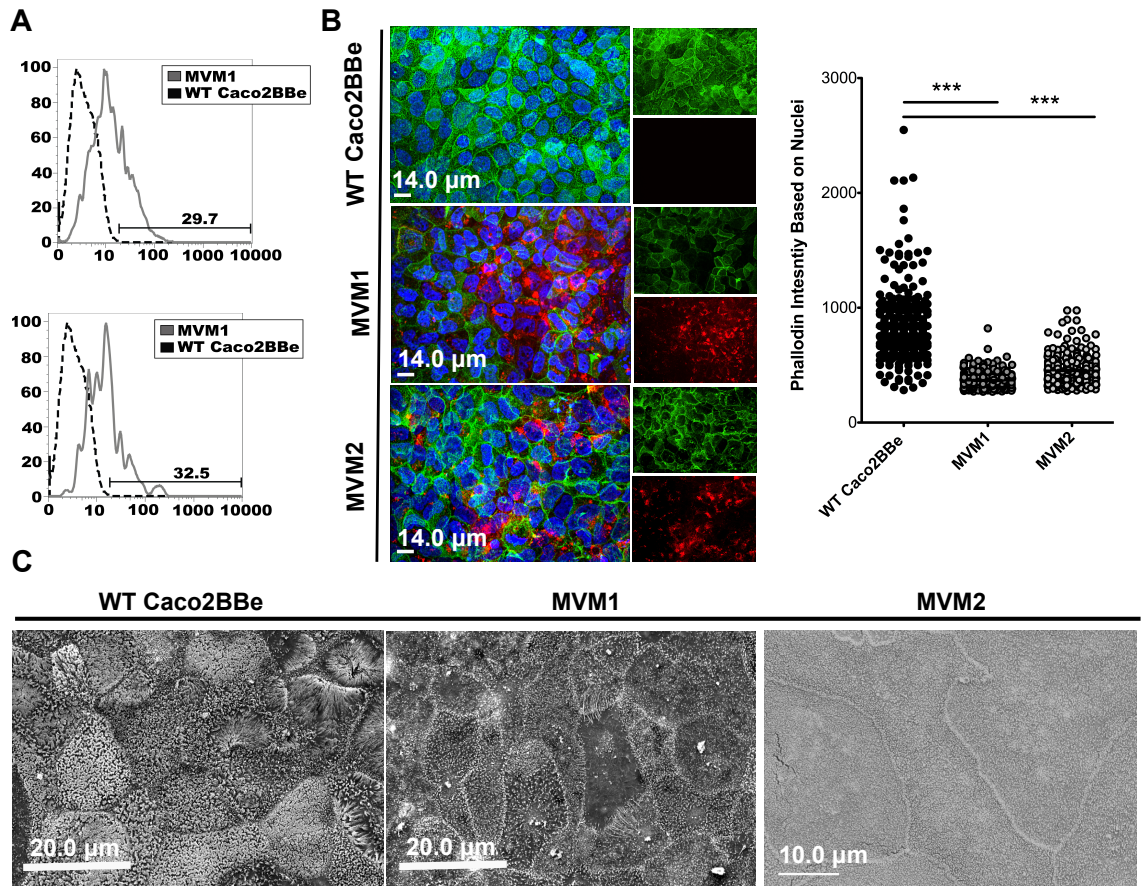


Figure 2.1 Development of microvilli minus (MVM) cell lines using Caco2BBE. (A) Flow cytometry histograms show that WT Caco2BBE transfected with EPI64LADsRed has stable expression of DsRed protein (solid line) as compared to WT unmanipulated control (dotted line). (B) Confocal micrographs showing DsRed (red) expression and decreased phalloidin/F-actin staining (green) compared to WT cells. Phalloidin intensity was quantified and significance was determined by unpaired Student's t-test. (C) Scanning electron micrographs (SEM) confirm that MVM cells have few apical microvilli compared to WT.

2.2 Results

2.2.1 Establishment of microvilli-minus cells

Our working hypothesis is that the increased apical membranes associated with microvilli provide increased charged molecular species at the apical surface, resulting in increased local electrostatic charge. Therefore, to directly alter the apical surface of

polarized epithelial cells, we generated “Microvilli-minus” (MVM) cells on the principle that reducing the apical microvilli without altering other features of the epithelium (e.g., tight junctions) would mainly affect the apical electrostatic charge. Although intestinal epithelium is also covered by mucus, this *in vitro* model does not incorporate mucus-producing cells; however, since many wetted epithelia (e.g., duct and tubular epithelium) do not produce mucus, our system tests only the effect of apical microvilli on particle adhesion. In addition, all of our studies were performed using epithelial monolayers on solid surfaces such as plastic dishes or glass; while growth of epithelial cells might be different from transwell filters where nutrients can reach the basolateral side of cells, these culture effects should affect all of the cell lines equally.

To modify the polarized epithelial cells by targeting only the microvilli, MVM cells were generated by stably transfecting Caco2BBe cells (16) with a mutant form of EPI64 (17-20) fused to DsRed (EPI64LADsRed), providing a dominant negative disruptor of microvilli actin filament formation (Figure 2.1A). Resulting MVM cells exhibited lower apical F-actin levels than WT Caco2BBe cells (Figure 2.1B) and reduced levels of apical microvilli using high-resolution scanning electron microscopy (SEM) (Figure 2.1C). Despite the loss of microvilli, the apical dimensions were similar to WT Caco2BBe cells and tight junction formation was not impaired as assessed by TJ protein localization and transepithelial electrical resistance (TEER) (Appendix Figure A.1A). Certain genes (CD137, GP2, β 1 integrin) have been identified as important for M cells in the binding and capture of pathogenic microbes (“capture receptor hypothesis”) (21-24); however, MVM cells showed no induction of these genes compared to a cytokine treated cell model (known to induce these genes Appendix Figure A.1B).

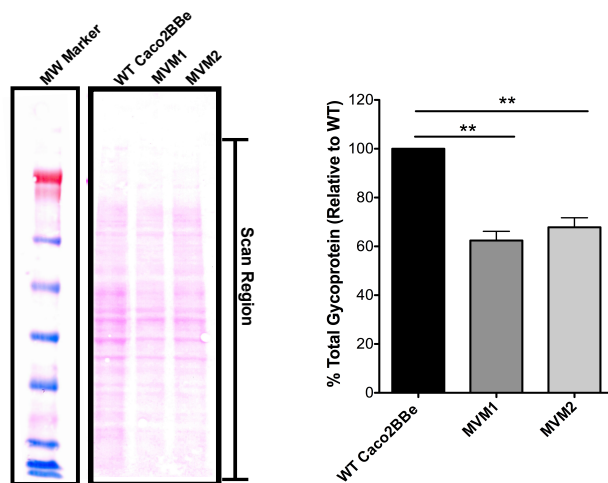


Figure 2.2 Quantification of cell glycoprotein content in cell with or without microvilli. Cell lysate total glycoprotein levels were quantified by integrating the full scanned density of each lane as indicated on the figure. MVM cells showed approximately 40% lower glycoprotein levels compared to WT cells. Error bars represent SEM (n=4); significance was determined using paired Student's t-test (relative values were taken).

Would manipulation of apical microvilli be sufficient to alter apical surface charge? Direct measurements of apical electrostatic charge are unfortunately particularly challenging for live cells. Surface charge can be measured for particles or cells in suspension (see below) but this cannot indicate local electrostatic charge on the apical surface of polarized epithelium. An indirect indication of the contribution of microvilli to the cell's apical surface charge might be derived by measuring the cell's glycoprotein content; this is based on the assumption that glycoproteins are mainly associated with the plasma membrane, and they most directly contribute to the cell surface charge. Accordingly, a stain for glycoprotein from cell extracts was used to measure glycoprotein content, normalized for total protein. The method is based on the Periodic acid –Schiff method (PAS), which detects proteins bearing sialic acid or other oxidizable carbohydrate groups. Integrating the stain signal along the whole gel lane of each cell type showed approximately 40% reduction of glycoprotein in MVM cells compared to WT Caco2BBE cells (Figure 2.2), consistent with the notion that the membrane extensions associated with microvilli are a major contributor to apical cell surface charge.

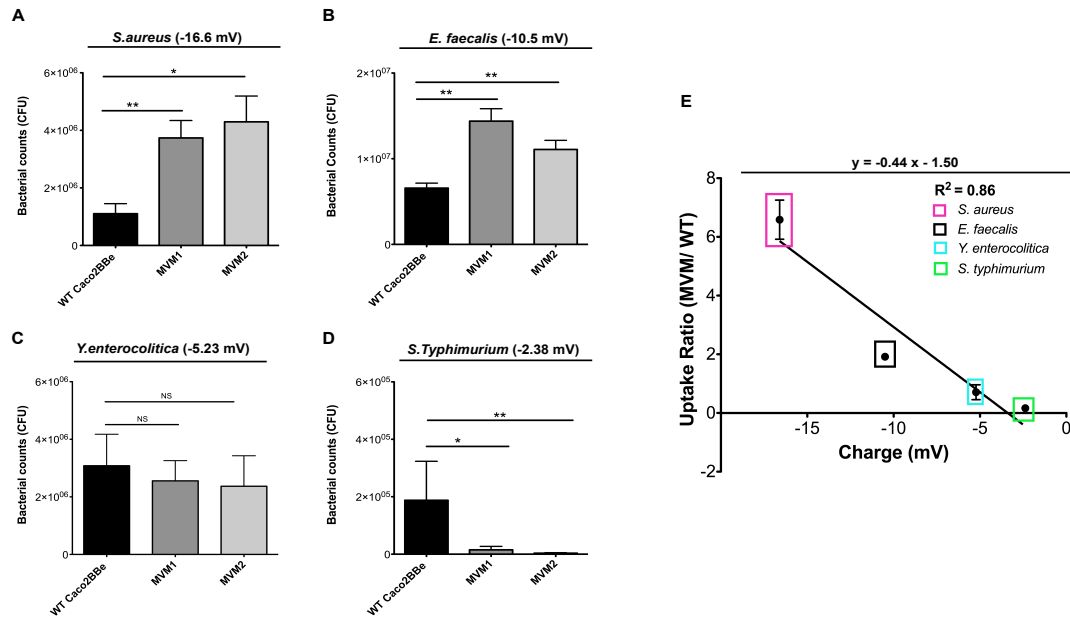


Figure 2.3 Epithelial cell uptake of live bacteria. Two-week monolayers of WT or MVM cells were assayed for endocytosis of bacteria (with measured zeta potential as indicated for each microbe). Uptake of *S. aureus* (A), *E. faecalis* (B), *Y. enterocolitica* (C), and *S. Typhimurium* (D). Error bars represent two individual experiments run in triplicate, significance was determined using unpaired Student's *t*-test. (E), Relationship between bacterial zeta potential and uptake. Ratios between the number of bacteria bound to MVM and WT cells were calculated and plotted against the bacterial zeta potential (values used were the mean of three individual experiments run in triplicate). Bacterial uptake exhibits a linear relationship with bacterial ZP ($R^2 = 0.86$). Error bars represent the SEM of duplicate experiments with two biological replicates ($n=4$).

To assess the effects of impaired microvilli formation on bacterial adhesion and uptake, we used a series of different assays intended to assess the ability of suspended particles to adhere to the surface of the epithelial cell monolayer. First, we used an indirect measure of adhesion relying on consequent endocytosis of live bacteria by the epithelial cells. Second, we performed a direct assay of adhesion of fluorescent particles using confocal microscopy image analysis. Finally, we used a flow chamber to test the adhesion of particles under dynamic flow conditions that resemble the physiological conditions of epithelial cells in mucosal tissues as well as in ducts and tubules.

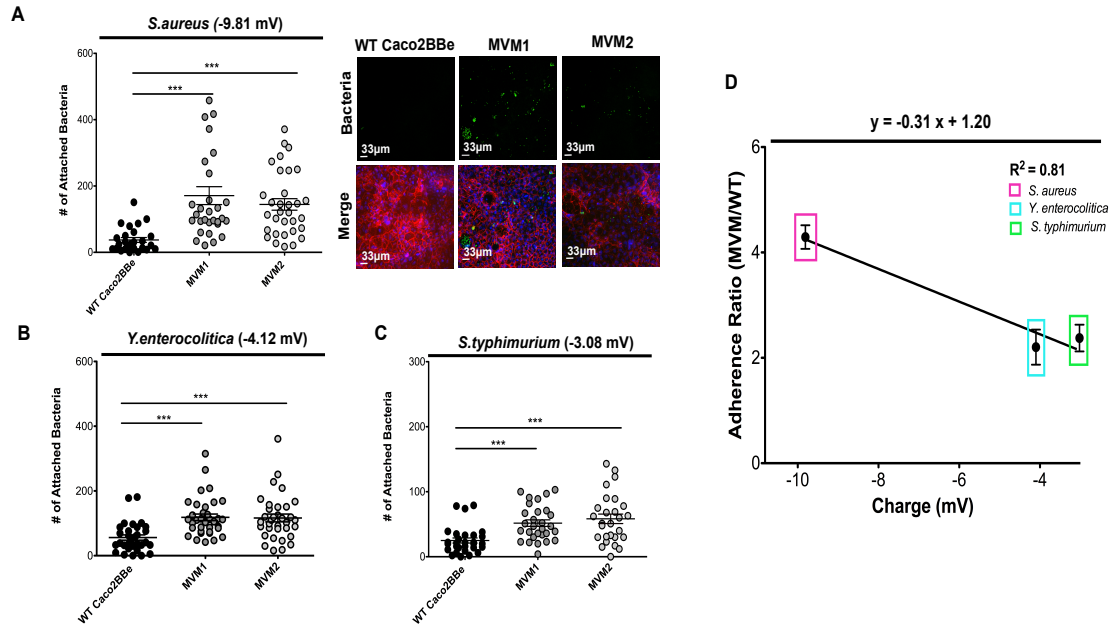


Figure 2.4 Adherence of fixed bacteria to epithelial cell monolayers. (A) *S. aureus* showed a preference to MVM cell compared to WT; confocal images to the right show bacteria (green) on monolayers, also stained for F-actin (phalloidin, red). Similar analysis for *Y. enterocolitica* (B), and *S. Typhimurium* (C). Error bars represent the SEM of three independent experiments with at least 10 images taken for each condition, significance was determined using unpaired Student's t-test. (D) Adherence ratios between the number of bacteria bound to MVM versus WT cells were plotted against bacterial zeta potential (values used were the mean of three individual measurements). Bacterial adherence exhibits a linear relationship with bacterial ZP ($R^2 = 0.81$). Error bars represent the sum of three individual experiments with two biological replicates each (n = 6)

2.2.2 Bacterial adhesion and uptake by MVM cells

Since bacterial adhesion to epithelial cells can be a preliminary step in colonization or invasion, we tested a variety of known mucosal bacterial inhabitant species (*S. Typhimurium*, *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* (*E. faecalis*), *Y. enterocolitica*) for their binding and endocytosis by established epithelial monolayers. In this assay, direct adhesion to the apical surface of the epithelial cells leads to endocytosis and resistance to a subsequent washing step with an antibiotic solution. Suspensions of bacteria were incubated with established epithelial monolayers, allowing time for bacteria to adhere and undergo endocytosis by epithelial cells. After

washing the epithelium with an antibiotic media to eliminate free bacteria, cells were homogenized and plated to count colonies formed from bacteria that were endocytosed by the epithelial cells (Figure 2.3A-D). The bacteria each showed consistent preferences for binding and uptake by MVM versus WT Caco2BBe cells, but to differing degrees depending on the bacterial species.

The characteristics of the bacterial particles affecting their adhesion and uptake by epithelium could involve several factors, including surface charge, hydrophobicity, bacterial or cell adhesion molecules, or other as yet unidentified factors. The charge of the bacteria can be assessed by measuring electrophoretic mobility (from which zeta potential (ZP) is calculated: (25)), interestingly, the degree of bacterial preference for MVM was proportional to their measured ZP, and could be expressed as a linear function of particle ZP (Figure 2.3E). To assess potential effects of hydrophobic interactions between the bacterial and the epithelium, we also compared hydrophobicity of the particles but found that most microbes were hydrophilic with the exception of *S. aureus* (Appendix Figure A.2), so no correlation between hydrophobicity and bacterial uptake was evident.

2.2.3 Particle adhesion by MVM cells

As noted above, bacterial adhesion to epithelium may be influenced by bacterial or epithelial adhesion protein interactions and other effectors, such as Type III Secretion Systems (Appendix Figure A.3: (26-28)). To minimize these effects, we used a second assay in which bacteria expressing fluorescent proteins were chemically fixed to disrupt the function of any adhesion proteins or other virulence factors, and the surface charge of these inert particles was again measured using electrophoretic mobility. It

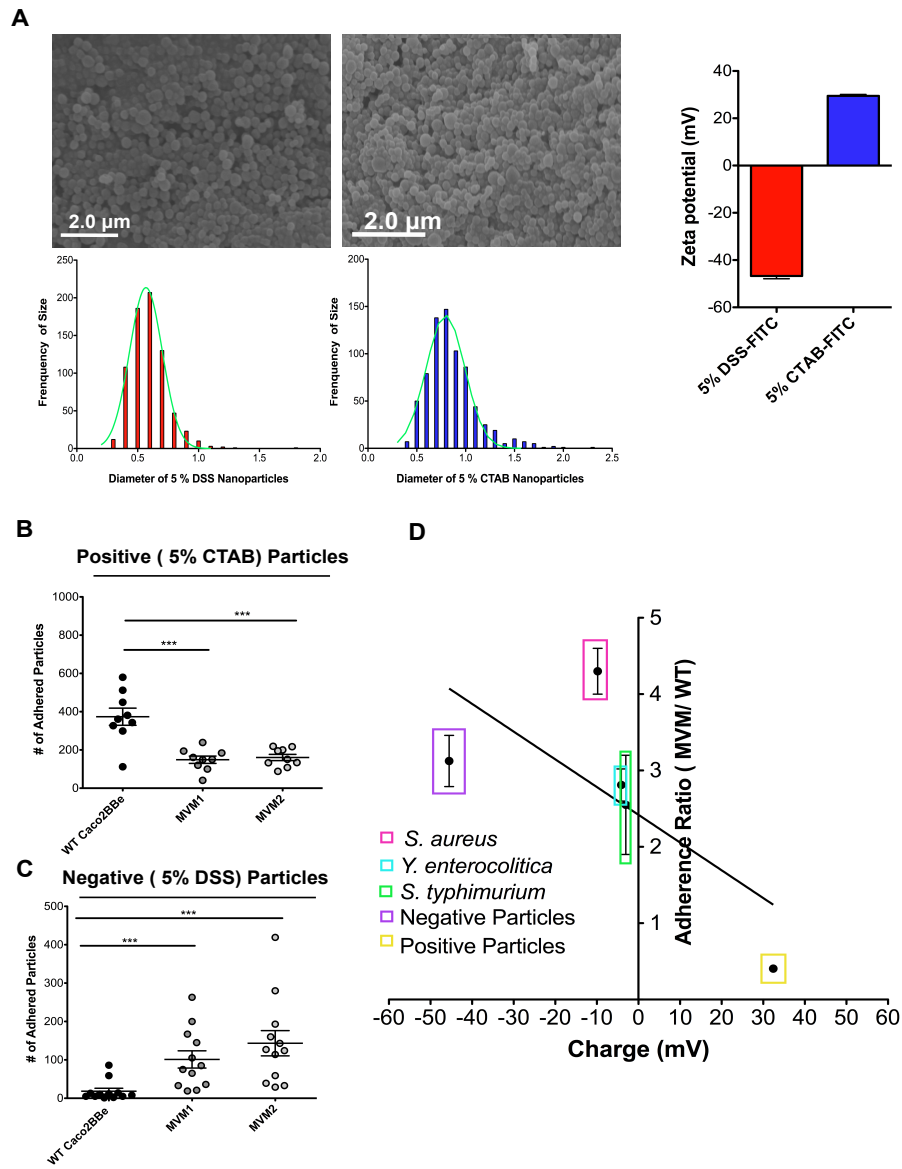


Figure 2.5 Epithelial cell adhesion of synthetic nanoparticles. (A) SEM images of synthetic nanoparticles (left, negatively charged PLGA particles loaded with 5% DSS; right, particles loaded with 5% CTAB), showing uniformity of particle shape and size. Histograms show the size distribution; with median calculated to be 0.74 μm for negatively charged particles, and 0.81 μm for positively charged particles. Zeta potentials of synthetic nanoparticles (far right) were measured in triplicate. (B),(C) Nanoparticle adhesion was assayed as performed with fixed bacteria; positively charged nanoparticles showed a higher preference to WT cells, while negatively charged nanoparticles showed preference for MVM cells. (D) Adhesion of fixed bacteria (from Fig. 6) and synthetic microparticles shown on same plot, showing similar relationship between adhesion and charge. Error bars represent the SEM of three individual experiments with two biological replicates ($n=6$); significance was determined using unpaired Student's t -tests.

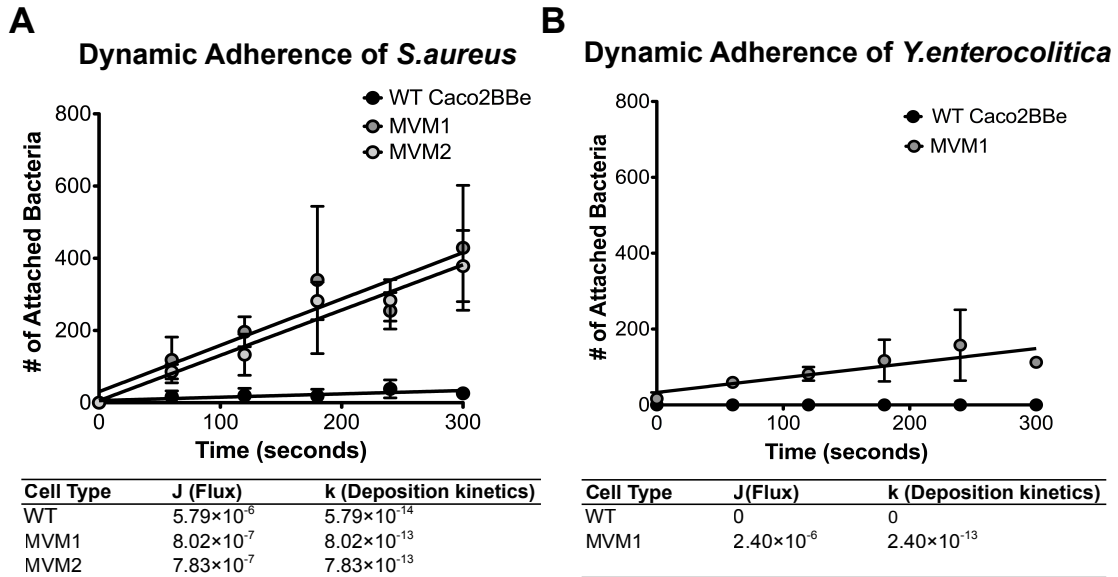


Figure 2.6 Bacterial binding to epithelium under flow conditions. (A) Deposition kinetics for *S. aureus*. Each MVM clone had two replicates each (n=2) compared to WT cells (n=3). (B) Kinetics for *Y. enterocolitica*, two replicates for MVM cells (n=2), two replicates for WT (n=2).

should be noted that *E. faecalis* was excluded from this assay because its calculated zeta potential was similar to that of *S. aureus* when chemically fixed (Appendix Figure A.4). These fixed particles were then added to cells, followed by microscopy image analysis of bound or endocytosed bacterial particles (Figure 2.4A-C). Here too, a clear linear relationship between cell adhesion preference and surface charge was noted (Figure 2.4D). This relationship between charge and extent of particle-epithelium binding was further corroborated using synthetic poly lactic-co-glycolic acid (PLGA) nanoparticles designed to display calibrated surface charges: positively and negatively charged particles were made using surfactants Cetyl trimethylammonium bromide (CTAB) and Dioctyl sulfosuccinate sodium (DSS), respectively (Figure 2.5A). The binding preferences of negatively charged particles resembled the behavior of bacteria; moreover, positively charged particles showed the reverse preference for the WT displaying different degrees of apical microvilli appear to be a direct linear function of the Caco2BBE cells (Figure 2.5B). Thus, the binding preferences of epithelial cells e charge

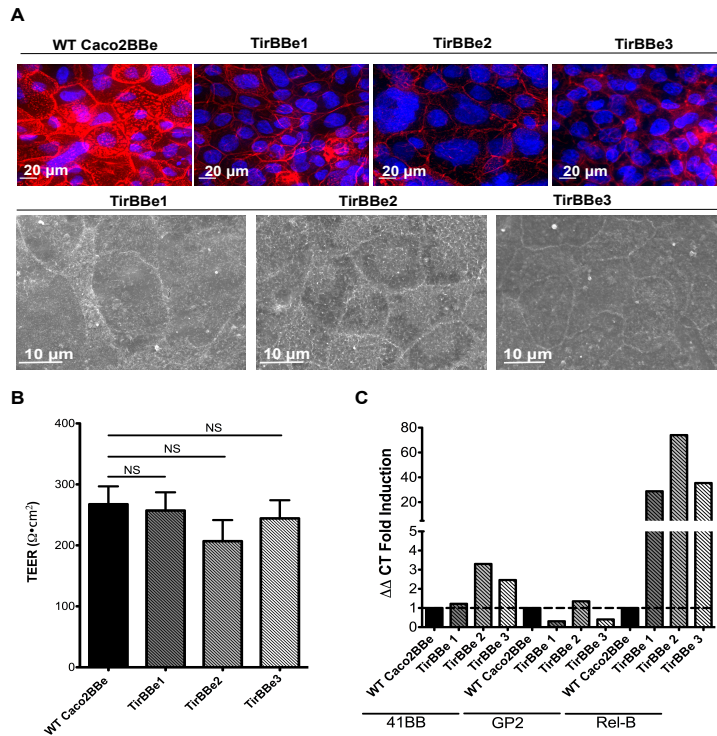


Figure 2.7 Loss of microvilli in cells expressing Tir. (A) Caco2BBE cells were transfected with *E.coli* O157:H7 effector Tir (TirBBE). F-actin (phalloidin, red) staining (top row) suggests loss of microvilli; SEM images (bottom row) confirm the absence of microvilli. (B) TirBBE cells showed similar electrical resistance (TEER) to WT cells, indicating tight junction formation is retained. Error bars represents the SEM of three individual experiments n=3. (C) qPCR shows that TirBBE cells had no induction of M cell related genes 41BB and gp2, but did show induction of Rel-B.

exhibited by both fixed bacterial particles and synthetic nanoparticles. In effect, the strength of the electrostatic barrier effect can be quantified, dependent on the presence of microvilli and particle surface charge.

2.2.4 Bacterial adhesion under laminar flow conditions

In the intestinal lumen, as well as in ducts and tubules, suspended particles move dynamically across the epithelial apical surface under laminar flow conditions. Therefore, to model the *in vivo* passage of luminal contents in the intestine, we adapted a flow chamber system (29) in which a suspension of fluorescent bacteria moves across an epithelial monolayer under low shear stress (1.5 mL/min corresponding to an average linear velocity 11.25 m/h). Accumulation of adhering particles was documented by video, and deposition kinetics was calculated (Appendix Figure A.5). Bacteria with high surface

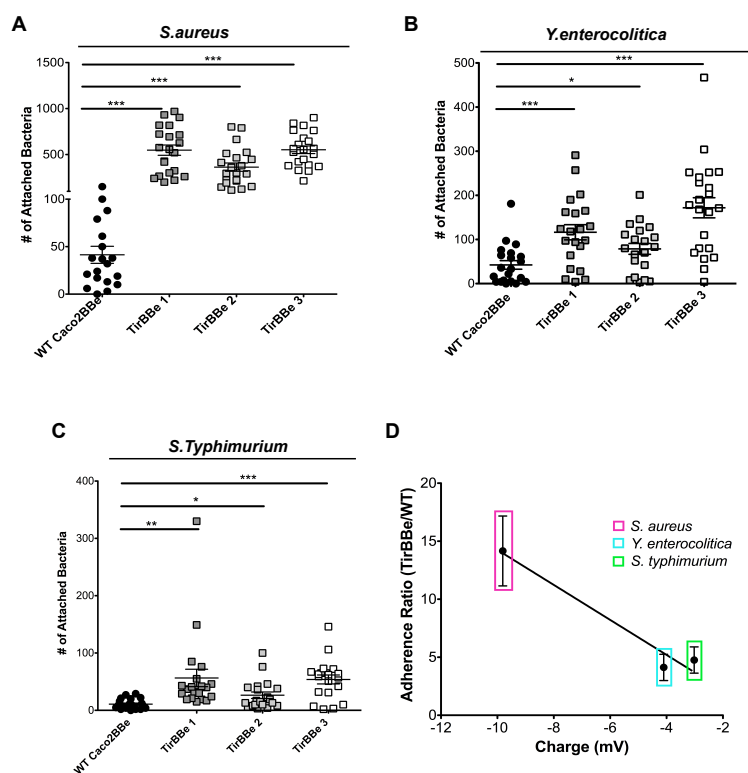


Figure 2.8 Bacterial adherence to TirBBe cells. Adhesion of fixed bacteria *S. aureus* (A), *Y. enterocolitica* (B), and *S. typhimurium* (C). All assays were run in duplicate (two individual experiments). D, Adherence ratios were plotted against bacterial surface charge, showing similar linear relationship seen with MVM cells. Error bars represents the SEM of two individual experiments with three biological replicates n=6.

charge (i.e., *S. aureus*) showed substantial deposition on MVM cells with almost no detectable accumulation on WT Caco2BBe cells (Figure 2.6A). Bacteria with lower charge (*Y. enterocolitica*) also showed preferential adhesion for MVM cells (Figure 2.6B), albeit at a lesser rate, consistent with the differences noted in the static binding assay. It is notable that under these dynamic conditions, deposition of any of the negatively charged particles onto WT Caco2BBe cells was essentially undetectable.

2.2.5 Bacterial effacement effector reduces microvilli and enhances microbial adhesion

Our results from three different assays of particle adhesion to epithelial cells suggest that epithelial resistance to microbial adhesion and invasion may rely in part on the presence of the dense layer of apical brush border microvilli. To counter epithelial barrier mechanisms, some invasive bacteria have developed virulence mechanisms

such as the Type Three Secretion System (TTSS) that actively inject effector proteins into epithelial cells causing effacement, or loss of microvilli (30). Adhesion of the bacteria to the cell plasma membrane is also associated with actin pedestal formation (31; 32), but it is possible that electrostatic effects also play a major role in this mechanism of bacterial virulence as well.

One known bacterial effacement effector protein is the Tir protein used by both EHEC and EPEC (33; 34), so we transfected Caco2BBe cells with Tir ("TirBBe" cells) to test its effect on electrostatic mechanisms. Although studies suggested that the effacement function of Tir is dependent on interactions with additional bacterial effectors (33), we found that stable transfection of Tir alone was sufficient to induce a striking loss of apical microvilli while retaining tight junction integrity, similar to the morphology seen with the MVM cells (Figure 2.7A,B). Here, Tir is expected to induce loss of microvilli by a mechanism distinct from the MVM model. TirBBe cells showed no induction of M cell genes, again paralleling the MVM cell model; however, TirBBe cells showed induction of RelB, suggesting non-canonical NF- κ B activation (Figure 2.7C).

Importantly, bacteria showed a preferential binding to TirBBe cells, again showing a linear relationship with surface charge similar to that seen with MVM cells (Figure 2.8A-D). These results support the notion that bacteria actively reengineer the epithelium apical architecture to manipulate the surface charge of the epithelial cells, ultimately enabling increased binding and colonization of the host. In this context, electrostatic interactions and bacterial charge can be viewed as a virulence factor.

2.3 Discussion

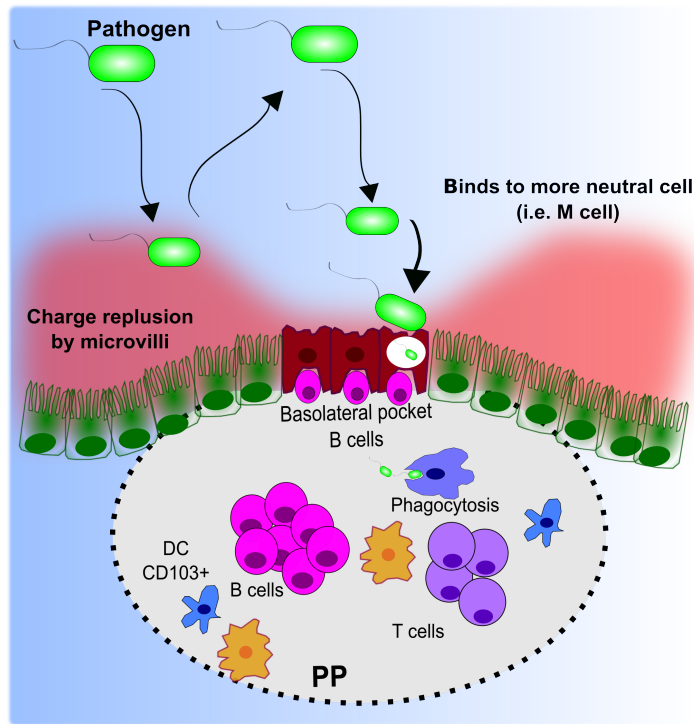


Figure 2.9 Model of the electrostatic barrier generated by apical microvilli on enterocytes. The apical microvilli not only work to increase the surface area for nutrient absorption by the enterocyte but based on the study presented herein the apical microvilli generate a net negative charge. Luminal pathogens encounter this barrier provided by the microvilli and are repulsed away from the enterocytes surface and are instead driven to the more neutral M cell lacking microvilli.

Microvilli are found in a variety of wet epithelia with diverse functions, such as mucosal barrier function and nutrient uptake, transport of material from secretory glands, transport of fluids in various stages of processing in the kidney, and so on. Our results suggest that despite their diverse locations and functions, microvilli in these tissues may share a broader function in establishing a barrier – via electrostatic forces – to adhesion and invasion by microbes. Conversely, M cells – lacking microvilli – can rely on their lower electrostatic charge to make it easier to capture luminal microbial particles. Pathological conditions may disrupt this protective mechanism and permit microbial invasion, as described above for effacement effector proteins. Inflammatory processes may also disrupt microvillus formation and maintenance of the electrostatic barrier. For example, inflammatory cytokines such as lymphotoxin and TNF α were found to reduce microvillus formation in Caco2BBE cells *in vitro* (24); since these cytokines are known

effectors in inflammatory bowel disease (35; 36), a similar effect on microvilli *in vivo* may be relevant to mucosal barrier function.

We note that the natural experiment – M cells in mucosal tissues – also provides the *in vivo* example of epithelial microvilli-minus cells that inspired this study. Since the generation of this electrostatic effect appears to be a function of the increased area provided by microvilli membrane extensions, it is also possible that other mucosal surfaces, such as ciliated airway epithelium, may also benefit from the membrane extensions such as cilia to generate a similar electrostatic force barrier. Thus, here too, loss of ciliated epithelium may be predicted to increase the susceptibility to invasiveness by luminal microbes. Finally, this view of the mucosal barrier does not ignore the additional contributions of the mucus layer at mucosal surfaces and accompanying intestinal motility or airway ciliary clearance. Indeed, our demonstration of resistance to adhesion under flow conditions suggests that the electrostatic barrier may actively complement the role of mechanical luminal clearance (Figure 2.9).

2.4 Acknowledgments

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Microscopy and Microanalysis for assistance with Scanning Electron Microscopy and Bahman Anvari for the use of the Malvern Zetasizer.

2.5 Materials and methods

2.5.1 Bacterial strains

Cloning of genes was performed with *Escherichia coli* strain DH5 α . Strains of *E.coli* were grown in Luria-Bertani broth (LB), and growth medium was supplemented with antibiotics, based on plasmid resistance. For functional assays, the following bacterial strains were used; *S. aureus* strain RN4220 transformed with a green fluorescent protein (GFP) reporter plasmid (kindly provided by A.R. Horswill), *Y. enterocolitica* strain (kindly provided by J. Meccas), clinical isolate of *E. faecalis* (kindly provided by N. Schiller), *E.coli* O157:H7, *S.typhimurium*, IR715 WT Strain, $\Delta invA$ IR715 strain and $\Delta sipA$, $\Delta sopE2$, $\Delta sopB$, $\Delta sopA$, $\Delta sopD$ ZA21 strain (kindly provided by M. Raffatellu). All *Salmonella* strains were transformed with a vector containing EGFP (kindly provided by C.R. Nagler) using AMAXA nucleofector system. For all assays performed in this study, bacteria were grown in normal LB broth at 37°C and agitated at 300 RPM overnight. In the case of adherence assays, bacteria cultures were pelleted at 2000g for 20 m, resuspended in 150 mM PBS and fixed with 1% paraformaldehyde/PBS solution for 10 minutes at room temperature. For uptake assays, overnight cultures were diluted 1:10, then grown to mid-log phase ($OD_{600} = 0.6$).

2.5.2 Cloning of genes for transfection

Cloning of the EPI64LA gene was accomplished using PCR amplification from cDNA isolated from Caco2BBE cell line, using a reverse primer encoding an alanine residue downstream of the C-terminal leucine residue and upstream of *KPN1* restriction

site, and a forward primer containing a *HINDIII* site upstream of the transcriptional start site of the EPI64 gene (accession code NCBI GenBank: AF331038.1). An overlap PCR was performed to fuse EPI64LA to DsRed, and was cloned from PCMV-DsRed-Express2 vector (Clontech). Finally the EPI64LA-DsRed was ligated into pcDNA vector (pcDNA-EPI64LA-DsRed). Tir was cloned from genomic DNA from *E.coli* O157:H7 using a forward primer containing upstream KPN1 site and a reverse primer containing a downstream Xho1 site (accession code NCBI Reference sequence: NZ_AERR01000033.1, GI: 485657470). Tir was then cloned into pcDNA plasmid (pcDNA-Tir). All genes were sequenced to verify correct DNA sequence.

2.5.3 Development of stably transfected lines

Caco2BBe (ATCC CRL-2102) was obtained from the American Type Culture Collection. Caco2BBe cells were cultured in media comprised of Advanced Dulbecco's Modified Eagle Medium (ADMEM, Gibco) plus 10% FBS (Biowest), 1% Penicillin-Streptomycin-Glutamine (Cellgro), 1% HEPES buffer (Cellgro) and grown at 37°C in 5% CO₂. An Amaxa Nucleofection kit T (Lonza) was used to transfect Caco2BBe cells with either pcDNA-EPI64LA-DsRed or pcDNA-Tir. One day after transfection, transfected cells were placed under selection; 1500 ng/μl of geneticin (Corning) for cells transfected with pcDNA-EPI64LA-DsRed, and 50 ng/μl of hygromycin (Life technologies) for cells transfected with pcDNA-Tir. Limit dilution was used to generate clonal cell populations, which were subjected to multiple rounds of screening to identify cells with expected phenotype.

2.5.4 Immunohistochemistry and confocal microscopy

In order to visualize cells using confocal microscopy, cell cultures were fixed with 1% paraformaldehyde/PBS and then permeabilized first with cytoskeleton buffer, followed by 0.5% Tween-20/PBS. Cultures were then blocked in 0.1% Tween-20 in casein solution (Thermo Scientific). Monolayers were stained with Phalloidin (647nm) (Life technologies) to visualize apical microvilli, fixed with 4% paraformaldehyde/PBS and mounted with Prolong Gold antifade reagent (Life technologies). DAPI was used as a nuclear counterstain. Images were obtained using a spinning disk BD CARVII Confocal Imager (BD Biosystems) attached to a Zeiss Axio Observer inverted microscope. Hardware, including microscope, confocal and digital camera (Diagnostic Instrument Xplorer – XS and Qimaging Rolera EMC²) was controlled using both BD IPLabs and Metamorph imaging software. Images were further optimized using Volocity deconvolution software. Phalloidin quantification was carried out using Volocity software, where nuclei were first identified and a diameter was set to 10 µm from each nuclei. The intensity of phalloidin over the nuclei and 10 µm away from the nuclei was determined.

2.5.5 Flow cytometry

Cells were grown in 96 well cluster plates in selective media. The day of the experiment, cells were detached from plates using a trypsin/EDTA solution (Gibco) and moved into 2ml round bottom tubes. Cells were washed three times in PBS and data was collected using a FACSCalibur (Becton Dickinson) equipped with CellQuest Pro software (BD Biosciences). Data analysis was performed using FlowJo Software (Tree star).

2.5.6 Quantitative and semi-quantitative PCR

Cell monolayers were cultured in 6 well cluster plates and RNA was extracted using RNAeasy Plus Mini kit (Qiagen). Using the Superscript III first-strand synthesis system (Life technologies), 1 or 3 μg of total RNA from each sample were used to generate cDNA. The SYBR Green assay (Bio-Rad) and the Biorad CFX96 detection system (Bio-Rad) were used to detect real-time PCR products from 10 μl of the reverse-transcribed RNA (1:10 dilution) samples (from a 20- μl total volume). Human GAPDH was used as a reference gene for all qPCRs. Primers used in qPCR assays are described in (27). The comparative $\Delta\Delta\text{CT}$ method was used to determine the relative amount of gene expression. In semi-quantitative PCRs, a reaction volume of 20 μl was used. In the PCR reaction a total 4 μl of template cDNA was added and 2x Taq master mix (NEB) was used to carry out the PCR reaction. Human GAPDH was used as the reference gene. For *Tir*, a cycle number of 35 was used and for GAPDH cycle number 30 was used.

2.5.7 Zeta potential measurements

The zeta potential of all bacteria were measured using laser Doppler electrophoresis (Zetasizer Nano ZS90, Malvern Instruments), based on the method described by *Rajapaska et al.* (9). Briefly, bacteria were diluted to a concentration of 8×10^7 bacteria per 1.5 ml. Diluted samples were moved to folded capillary cells (Malvern) and zeta potentials were quantified based on Smoluchowski-Helmholtz relationship equation, where U_E is the electrophoretic mobility, ϵ is the dielectric constant, $f(Ka)$ is the Henry function, set to 1.5 (Smoluchowski approximation), η is the viscosity of the buffer, and ζ is the zeta potential. For measurements of synthetic particles, 1 mg of lyophilized

particle pellet was suspended into 150 mM PBS. All measurements were run in triplicate in each individual experiment; experiments were repeated 3 times.

$$(1) \quad U_E = \frac{2\varepsilon_0 f(Ka)}{3\eta}$$

2.5.8 Microbial Adhesion to Hydrocarbons (MATH)

Based on a method previously describe by Rosenberg *et al* (37; 38), to determine bacterial particle hydrophobicity, bacterial cultures ODs were normalized to 1.0 at a wavelength of 400 nm. 4 ml of culture was added to various volumes of n-dodecane (Sigma-Aldrich). The tubes were vigorously vortexed for 2 min and left to stand for 15 min at room temperature to allow the organic and aqueous phase to separate. The aqueous phase was carefully removed and OD measured at 400 nm using SoftMaxPro SpectraMax M2 spectrometer. The percentage of bound bacteria to hydrocarbon was determined by subtracting the absorbance reading from 1. Hydrophobicity plots represent three individual experiments.

2.5.9 Scanning electron microscopy

Cell monolayers were grown on glass-bottom petri dishes. Cell monolayers were fixed by first adding 2.5% glutaraldehyde (Ted Pella) solution for 30 minutes. Samples were then washed in DDH₂O and 4% osmium tetroxide (Ted Pella) was added for 30 minutes. Samples were then dehydrated using 25%, 50%, 75%, 90%, and 100% ethanol for 5, 5, 5, 5 and 10 minutes, respectively. Dehydrated samples remained immersed in 100% ethanol until critical point drying was performed. Critical point drying was performed using Balzar's Critical Point dryer (CPD). Samples immersed in 100% ethanol (transferring liquid) were quickly moved to transfer baskets and the chamber of the CPD

was filled with enough ethanol to cover the basket; the transferring liquid was slowly swapped with CO₂ (drying gas). Once all traces of transferring liquid were removed from the chamber, the CO₂ within the chamber was slowly heated to 42⁰C and pressure was brought to 1230 PSI (31⁰C and 1072 PSI). Pressure was slowly released within the chamber, and samples were then mounted onto SEM pin stub mounts (Ted Pella) with carbon coated conductive tape, and finally sputter-coated with platinum/palladium for 60 seconds (Sputter coater Cressington 108 auto). The apical surface of cells was then viewed using XL-30 FEG Scanning electron microscope at 10 kV.

2.5.10 Glycoprotein staining and quantification.

Cell monolayers were grown in 6 well cluster plats, and cells were lysed with 1% Triton X- 100 solution. Cell lysate was shredded and agitated for 30 min at 4⁰C. Protein concentration of cell lysate was determined using a Bradford assay. All protein concentrations were normalized based on the lowest protein concentration. Samples were run on 4-12% Bis-tris gels (Life technologies), and transferred to nitrocellulose. The nitrocellulose membrane was then stained for glycoprotein content using a Pierce glycoprotein staining kit (Thermo Scientific). Briefly, membrane was first washed with 3% acetic acid for 10 min, transferred to an oxidizing solution for 15 min, and again washed in acetic acid. Membrane was then placed into the glycoprotein staining solution for 15 min, and then placed in the reducing solution. The membrane was washed with 3% acetic acid and DDH₂O, then scanned along the length of each sample lane (rather than any individual band) and the signal integrated for each lane was quantified using the ImageJ software (NIH). Staining was performed on four individual cell monolayers.

2.5.11 TEER measurements

Transepithelial electrical resistance (TEER) was measured using EVOMX Epithelial Volttohmmeter (World Precision Instruments). Monolayers were cultured on 5.0 μm pore polycarbonate membrane transwells, 1.12 cm^2 area (Costar); selective media was added to both the top and bottom of the transwell insert. Before TEER measurements the microelectrodes of the EVOX Volttohmmeter were washed in 70% ethanol for 15 min, and equilibrated in culture media. Microelectrodes placed straddling the transwell insert and resistance was determined for blank transwells (R_B), and transwells containing cell monolayer (R_{total}) and electrical resistance of the cell monolayer alone was determined by $R_c = R_{\text{total}} - R_B$. All measurements were run in triplicate in each individual experiment; experiments were repeated 3 times.

2.5.12 Synthetic microparticle formulations

PLGA particles were prepared using solvent evaporation/double emulsion (water-in oil-in water) based on a described in Rajapaska *et al* (9). Nanoparticles were then produced with calibrated surface charge, confirmed by Zetasizer: for preparation of cationic particles (positively charged), 2% CTAB (cetyl trimethylammonium bromide) was added to PLGA solution, and for anionic particle (negatively charged) preparation, 2% DSS (Dioctyl sodium sulfosuccinate) was added to the PLGA solution. Particle morphology was determined using SEM, and particle diameter was measured using ImageJ.

2.5.13 Epithelial cell uptake assays

For bacterial uptake assays (modified from (39)), cells were grown in 12 well cluster plates in selective media. New media lacking antibiotics was added on the day

that assays were performed. Bacteria were grown in antibiotic selective Luria-Bertani (LB) overnight. Overnight cultures were diluted (1:10) and grown to an OD₆₀₀ of 0.6. Bacteria were counted and added to monolayers at 50:1 infection ratio. Plates were centrifuged for 5 min at 500g and then incubated for 2 hours. Cell cultures were then washed 3 times with PBS and media containing 100 µg/ml gentamicin was added. Cells were again incubated at 37°C for 2 hours to kill off any extracellular bacteria. Cell monolayers were washed, and trypsin and 0.02 % triton-X 100 were added to detach and lyse cells. Cells were then plated onto LB agar plates and incubated at 37°C, and counted after 18 hours. Assays were duplicated with two biological replicates. Finally, ratios between MVM cells and WT cells were determined and plotted against the bacterial charge.

2.5.14 Epithelial cell adherence assay.

Assays were performed on monolayers were grown on collagen coated chamber slides in selective media (BD Biosciences). Bacteria were added at 200:1 infection ratio and were allowed to attach for 1 hour at room temperature (to limit effects of endocytosis). Slides were then imaged with spinning disk confocal microscope (BD CarvII system). Bacteria were counted using Volocity software, where intensity and size parameters were set to help identify bacteria from background. For particle adherence, the same procedure was followed, but 1 mg lyophilized particles was diluted in 2 ml of PBS. Ratios were determined and plotted against fixed bacteria zeta potential. Experiments were repeated 3 times.

2.5.15 Dynamic adherence assay using parallel flow chamber

Bacterial deposition under flow conditions were performed using a parallel-plate flow chamber comprised of a Plexiglass block, a flexible silicon elastomer gasket, and a microscope slide. Monolayers of cells were grown directly on microscope slides (SuperFrost (Fischer Scientific)). On the day of the experiment, cells were stained with Hoechst dye (Life technologies), for easy viewing of the cells. Chambers were sealed with a thin film of vacuum grease on the gasket. The parallel flow chamber was installed onto BD CARVII Confocal Imager (BD Biosystems). Fluorescent bacteria were diluted in 150 mM PBS to an approximate concentration of 10^7 bacteria/ml, and were added to a 30 ml syringe and attached to syringe pump (kd Scientific). Bacteria were injected at a constant flow rate (q) of 1.5 ml/min (or mean velocity of 11.25 m/h), estimated to be the physiological flow rate of the small intestine in fed state. Bacterial accumulation was documented for 10 min, with pictures taken every 60 seconds. Flow in the chamber was kept in the laminar regime (based on the Reynolds number) to reduce the effects of shear forces. Deposition rate (k) of bacteria was determined by first linear regressing on the raw bacterial counts over time to obtain the slope J (deposition flux) and then dividing the slope by the initial concentration of bacteria.

$$(2) \quad Re = \frac{\rho v L}{\mu} = \frac{v L}{\nu}$$

$$(3) \quad J = \frac{\text{Slope of curve}}{\text{Viewing area}}$$

$$(4) \quad k = \frac{J}{C_0}$$

2.5.16 Statistical test and analysis

All data was expressed by the mean $\pm$ standard error of the means (SEM). Normality was checked and either the unpaired Student's *t* test was used to make comparisons between two groups or the one-way analysis of variance (ANOVA) was used to make comparisons between three or more groups with one variable in common, as indicated in figure legends. All statistical calculations were performed using GraphPad Prism version 5.0. Graphs were made by using GraphPad Prism version 5.04. $p < 0.05$ was considered statistically significant, where * represents $p < 0.05$, ** represents $p < 0.005$, and *** represents $p < 0.0005$

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Chapter 3

Induction of colonic M cells during intestinal inflammation

3.1 Introduction

M cells are a specialized subset of epithelial cell that play a central role in mucosal immune surveillance, by actively capturing and transporting luminal microbial particulates (i.e. bacteria, viruses etc.) to underlying immune cells, where mucosal mediated immunity is generated (1; 2). M cells are distinct from surrounding enterocytes, in that they lack apical microvilli (F-actin protrusions), a feature that has been inversely correlated with their efficiency in luminal microparticle binding (3). Most M cells (Peyer's patch-type M cells) are found in the FAE overlying organized lymphoid follicles, such as SI PP and NALT; a distinct M cell phenotype is the inducible villous M cell (4-6). In contrast to villous M cells, the Peyer's patch-type M cells are associated with organized lymphoid follicles with an established stromal cell network comprised of fDCs, reticular fibroblasts, and marginal reticular cells (7; 8). During organogenesis, pro-inflammatory cytokines, such as TNF α and LT- β , induce NF-kB stromal cell expression of a series of chemokines, including CCL19, CCL21, and CXCL13, which facilitate the accumulation and maintenance of the lymphoid follicle (7; 9; 10). Moreover, during intestinal dysbiosis and inflammation this stromal cell network further provides scaffolding for chemokines to help guide the infiltration of appropriate subsets of mononuclear cells (7; 8).

The M cell differentiation program is thought to be driven by the cytokine rich microenvironment provided by the underlying immune lymphoid cells within the follicle. In line with this notion, previous studies found that treatment of the intestinal epithelial cell line Caco2BBE with TNF α and LT- β R agonist resulted in the induction of M cell associated gene expression (11). Since these cytokines are also found in inflammatory settings, inflammatory signals may be sufficient to induce M cell development. Interestingly, SI villous M cells, although not associated with FAE, are also thought to be

induced in presence of inflammatory stressors like cholera toxin (5; 11; 12). Thus, M cell development and function – regardless of phenotypic subset – seems to be intimately tied to local production of inflammatory cytokines.

Intestinal inflammation, as with IBD, which includes CD and UC, is predictably associated with production of inflammatory cytokines. UC and CD appear to have differing etiologies; UC is characterized by colonic inflammation driven in part by Th2 cytokines, while CD inflammation extends into the SI and is driven by Th1 cytokines (13-16). Both conditions are characterized by elevated TNF α levels (17-20); accordingly, the current standard clinical treatment for both UC and CD is anti-TNF α blockade (21-23). Although anti-TNF α therapies have shown some success, it remains unclear how efficacious they are for prolonged periods (i.e. chronic inflammation) and whether they are more beneficial for the treatment of Crohn's versus UC (24; 25).

Since similar cytokines may drive both inflammation and M cell development, we tested for the possibility that M cells would be induced in colonic epithelium during inflammation and whether this is related to increased TNF α production. To test this we made use of a reporter mouse in which the promoter for PGRP-S drives expression of DsRed in both neutrophils and M cells (26-28). In two models of colonic inflammation, we found significant induction of Peyer's patch-type M cells in the large intestine, though associated with poorly organized mononuclear infiltrates. Moreover, in the DSS colitis model this M cell induction was largely abrogated by the administration of anti-TNF α blockade. These results suggest that cytokine-induced development of colonic M cells may related to the pathogenesis of inflammatory bowel related diseases.

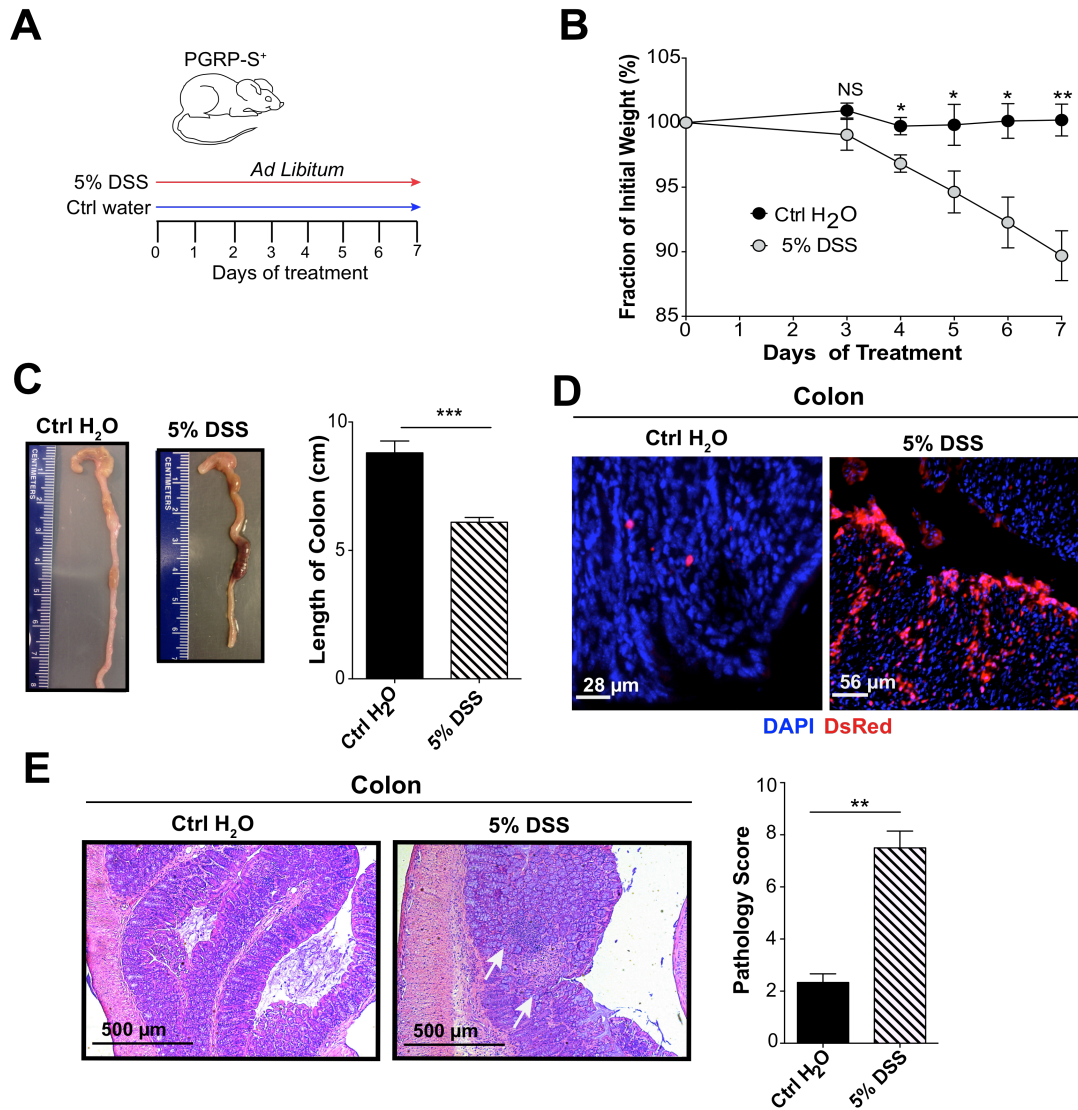


Figure 3.1 PGRP-S⁺ mice are susceptible to DSS chemically-induced inflammation. (A) The PGRP-S⁺ transgene and DSS treatment protocol. The PGRP-S promoter fused to DsRed, driving expression in both M cells and neutrophils. 5% DSS w/v was given to mice ad libitum for seven days. (B) PGRP-S⁺ treated with DSS showed a significant weight loss compared to control animals. Data represents the mean $\pm$ SEM with three mice per group, assessed by unpaired Student's t test. (C) DSS treated mice showed significant reduction in colon length, data represents the mean $\pm$ SEM with five mice per group, and assessed by unpaired Student's t test. (D) Confocal micrographs confirmed the presence of infiltrating neutrophils into the colonic lamina propria in DSS treated mice. (E). H and E staining indicating inflammation, ulceration, and loss of crypt architecture (indicated by arrows) in DSS treated mice. Blinded pathology scoring indicated increased pathology in DSS treated animals; data represents the mean $\pm$ SEM with 3-4 mice per group and assessed by unpaired Student's t test.

3.2 Results

3.2.1 Colonic inflammation induced by DSS is associated with M cells induction

The M cell differentiation program in the small intestine is dependent on the production of various inflammatory cytokines, yet while M cells are known to play an important role in mucosal immunity, studies have not yet assessed M cell induction during colonic inflammation. Using our reporter mouse in which the PGRP-S promoter drives expression of DsRed in both neutrophil and M cell populations, we sought to identify whether M cells become induced during chronic inflammation (Figure 3.1A), where inflammatory cytokines such as TNF α may be produced. We initially used DSS to chemically induce colonic inflammation (29; 30); this model is known to mimic the clinical and histological features of human IBD and produce a robust Th1 response. We first confirmed that our PGRP-S⁺ mice were susceptible to DSS colitis, by assessing the clinical signs and overall gross pathology as indicators of intestinal inflammation. One clinical measure of colonic distress is weight loss due to dehydration and diarrhea. During the course of treatment, mice given DSS exhibited a significant reduction from their starting weight by D4 and this continued until mice were sacrificed at D7 (Figure 3.1B). At postmortem, mice showed a significant shortening of the colon (Figure 3.1C). Making use of our reporter transgenic mouse, confocal microscopy confirmed the presence of infiltrating neutrophils into the colonic lamina propria (Figure 3.1D) as compared to control treated animals. Moreover, histological staining and scoring indicated increased pathology due to loss of crypt architecture and inflammation in DSS treated mice (Figure 3.1E). Taken together these data confirm that DSS reliably causes intestinal inflammation in PGRP-S⁺ mice.

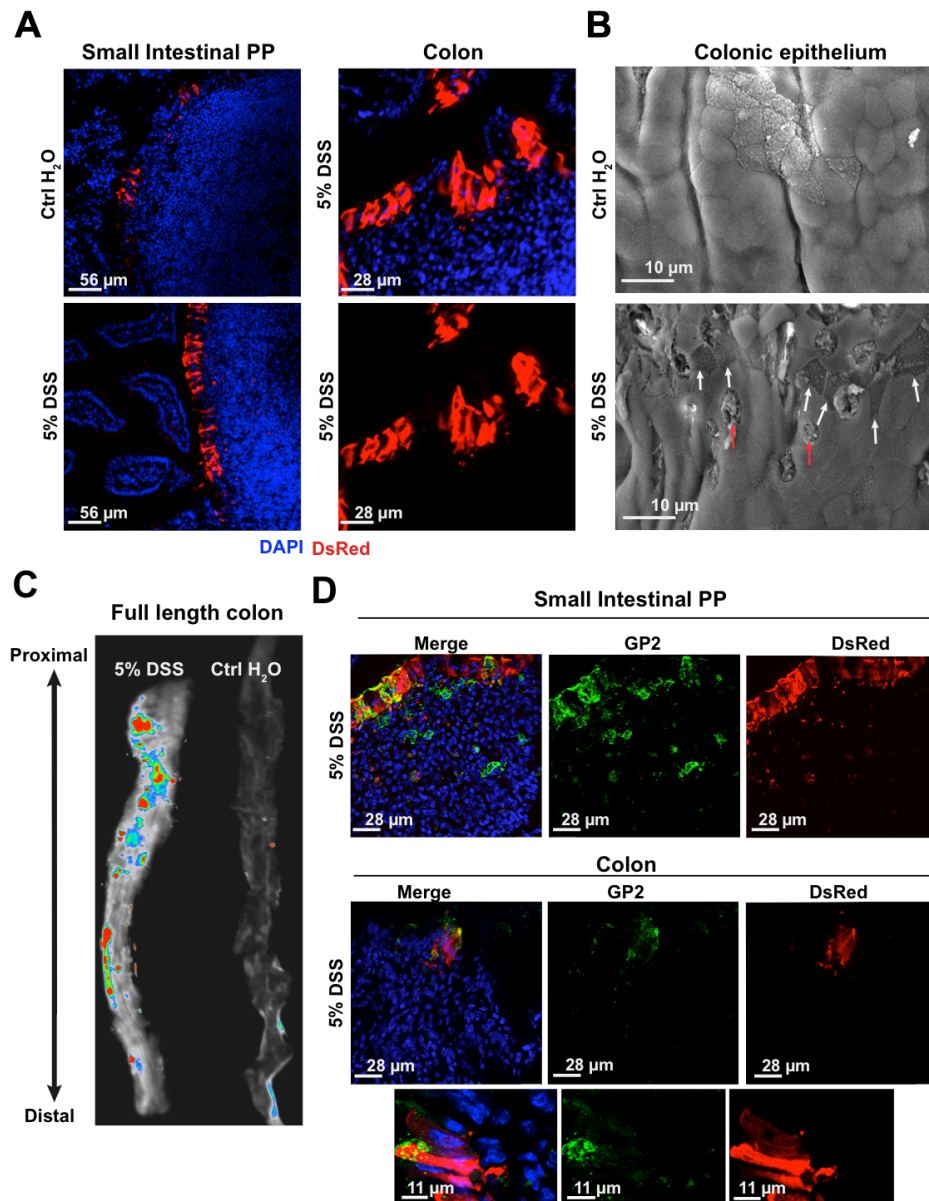


Figure 3.2 DSS inflammation induces M cells in colonic epithelium. (A) Confocal micrographs of small intestine PP with DsRed⁺ M cells from DSS (lower left) and control (upper left) treated animals. The right panels indicate the presence of DsRed⁺ colonic M cells, localized in epithelium and identified by morphology. (B) Scanning electron micrographs confirming the presence of colonic M cells (white arrows) in DSS treated mice, and increased goblet cells (red arrows). (C) Whole tissue imaging of the full-length colons taken from DSS and control treated animals. DSS treated colon had stronger DsRed fluorescence as compared to controls, due to appearance of DsRed⁺ neutrophils and M cells. (D) GP2 staining was used to more quantitatively identify colonic M cells. Top panel represents SI PP stained with GP2 as positive control. Bottom panel represents colonic M cells stained with GP2 showing similar staining patterns as compared to PP.

To determine whether M cells were induced during colonic inflammation, we employed a variety of microscopy techniques. We found that DSS treated animals contained a significant number of DsRed⁺ M cells throughout the colonic epithelium, while control treated animals essentially had no M cells (Figure 3.2A,B). Interestingly, DSS treatment also increased the numbers of PP M cells in the small intestine, which suggests that DSS effects are not solely restricted to the colon (Figure 3.2A). The presence of M cells within the colonic epithelium was further confirmed using scanning electron microscopy (SEM), where M cells were identified as the invaginated polygonal shaped cells (Figure 3.2B). Using whole tissue imaging, we observed that colons from DSS treated animals had increased DsRed fluorescence throughout their length as compared to control animals (Figure 3.2C).

Our initial identification of colonic M cells were based on qualitative parameters of DsRed expression as well as cell morphology, but the expression of the transgene DsRed is specific to Peyer's patch M cells, while induced villous M cells do not express the transgene DsRed (28). Therefore, to confirm the Peyer's patch-type M cell identity of the colonic M cells we also stained for GP2, which has also been shown to be a specific Peyer's patch-type M cell specific gene (31). Colonic M cells showed GP2 staining and when compared to SI PP M cells, these cells exhibited similar pattern of staining (Figure 3.2D). These data suggest that intestinal inflammation induces Peyer's patch-type M cells in the colonic epithelium.

3.2.2 Induction of colonic M cell gene expression and development among dividing cells

We found histological evidence of the Peyer's patch M cell gene GP2 in the induced M cells, but to assess the breadth of expression of inducing cytokines as well as M cell associated genes during the course of colonic inflammation, we also looked at

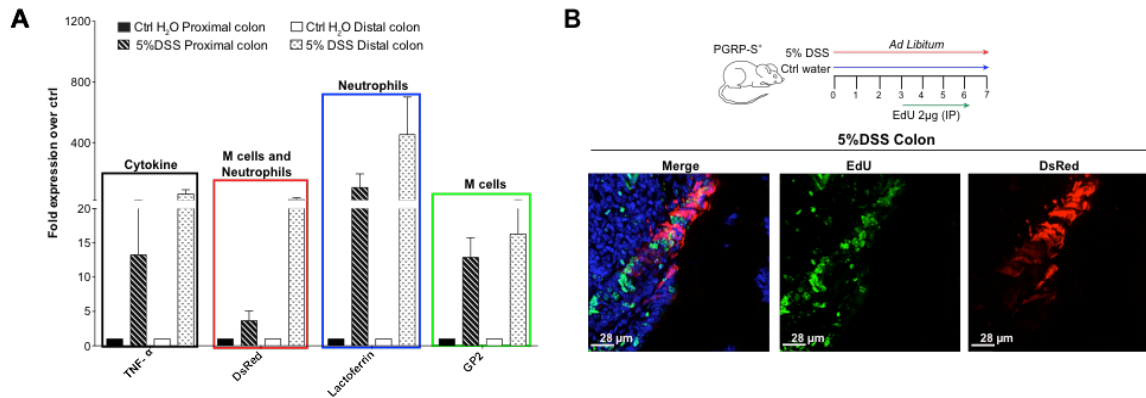


Figure 3.3 Colonic M cells are not quiescent. (A) qPCR was used to identify whether M cell associated genes were expressed during colonic inflammation. DSS treated animals showed upregulation of TNF α , DsRed, Lactoferrin, and GP2 compared to control. Data represents the mean $\pm$ SEM with 3 mice per group. (B) EdU labeling was used to identify if M cell population were quiescent. Representative confocal micrographs confirmed a large percentage of colonic M cells were double for DsRed and EdU.

transcriptional regulation of genes in the whole colon. First, TNF α mRNA was upregulated in both the proximal and distal colon of DSS treated mice, which suggests the inflammatory cytokine signals are present to drive M cell induction (Figure 3.3A). This was associated with increased transcriptional levels of Lactoferrin (Lacto), which is found in the second granule of neutrophils (32), DsRed, CCL20, and most specifically GP2 in the distal and proximal colons (Figure 3.3A and Appendix Figure B.1A). These data suggest that along with inflammatory cytokines, M cell associated genes are broadly induced during DSS colonic inflammation.

Associated epithelial cells, which include enterocytes, Paneth cells, goblet cells, and enteroendocrine cells, are under continuous self-renewal, deriving from the common Lgr5⁺ stem cells located near the base of the crypts in SI and colon (33; 34). In mice it is thought that the intestinal epithelium turns over every three to five days (33; 34). Moreover, to determine if the colonic M cells were from a proliferative population of cells during the course of colonic inflammation, we treated mice with EdU (5' ethynyl-2'-

deoxyuridine), a nucleoside analog of thymidine, during the late stage of DSS treatment (D3 to D6), to label dividing cells. Using confocal microscopy we found that a large portion of the identified colonic M cells were double positive for DsRed and EdU (Figure 3.3B). Taken together these data suggest that the M cell population induced by DSS treatment were generated by cells that divided during treatment and not quiescent. These results are consistent with M cell differentiation from dividing progenitor cells rather than from transdifferentiation of extant quiescent enterocytes.

3.2.3 Induced colonic M cells in the absence of organized follicles

Small intestinal PP's have a highly organized stromal cell network that helps guide mononuclear cells into the follicle via the expression of various chemokines (i.e. CXCL13, CCL19, CCL21: (9; 10)). By contrast, DSS-induced colonic M cells seemed to be associated with loose collections of infiltrating mononuclear cells. Since Peyer's patch-type M cells are more commonly found associated with organized follicles (in contrast with villous M cells), we wanted to determine whether these poorly organized infiltrates contained a stromal cell network. Since PP follicles are known to have higher percentage of B cells compared to other mononuclear cell subsets, we first stained for CXCL13, which is a ligand for CXCR5 on B cells. As expected, SI PP's from control treated animals exhibited strong CXCL13 staining, and PP from DSS treated mice showed an even stronger CXCL13 staining (Figure 3.4A). In contrast, in DSS treated mice the colonic infiltrates exhibited essentially no CXCL13 staining, correlating with a relatively low percentage of infiltrating B220⁺ B cells (Figure 3.4A and Appendix Figure B.1B).

We also stained for ER-TR7, a marker for reticular fibroblasts, and found SI PP from both control and DSS treated mice exhibited strong ER-TR7 staining (Figure 3.4B).

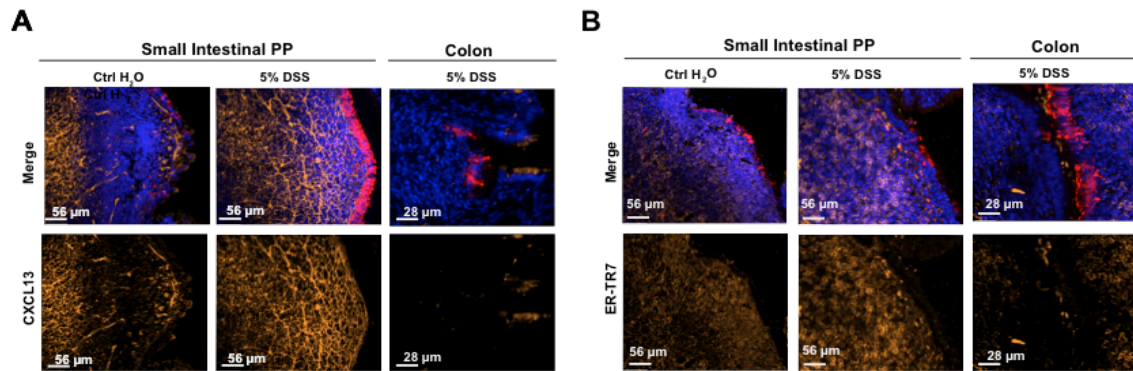


Figure 3.4. Colonic M cells were associated with poorly organized follicles. (A) CXCL13 expression in SI PP and colon infiltrates. Images show CXCL13 staining of SI PP in water and DSS treated animals respectively. CXCL13 staining is strong in SI PP of control and DSS treated animals. Right panels show that colonic infiltrates are essentially devoid of CXCL13. (B) ER-TR7 is a marker of reticular fibroblasts associated with the SI PP stromal network. Left and middle panels show ER-TR7 staining of SI PP in water and DSS treated animals respectively. Right panels show that ER-TR7 identifies a few cells among colonic infiltrates of DSS-treated mice.

However, the infiltrates from DSS treated animals included far fewer cells staining positive for ER-TR7 (Figure 3.4B). These data suggest that during colonic inflammation the induced M cell population is associated with a loose mononuclear infiltrate and a poorly organized stromal cell network compared to that of SI PP follicle. However, this less organized stromal cell network still may help guide mononuclear infiltrates.

3.2.4 Anti-TNF α abrogates M cell induction in DSS colitis model

Based on our *in vitro* studies (11) showing that Caco2BBE cells treated with inflammatory cytokines increased M cell-associated gene transcripts, we wanted to determine whether M cell induction during colonic inflammation was dependent on the presence of TNF α . We treated PGRP⁺CX3CR1^{Het} mice with mouse specific anti-TNF α on D0, D3, and D5 of DSS treatment (Figure 3.5A). DSS treated mice showed increased production of TNF α compared to control animals, with comparable levels to WT B6 mice (Figure 3.5B and Appendix Figure B.2A). In mice given both DSS and anti-TNF α ,

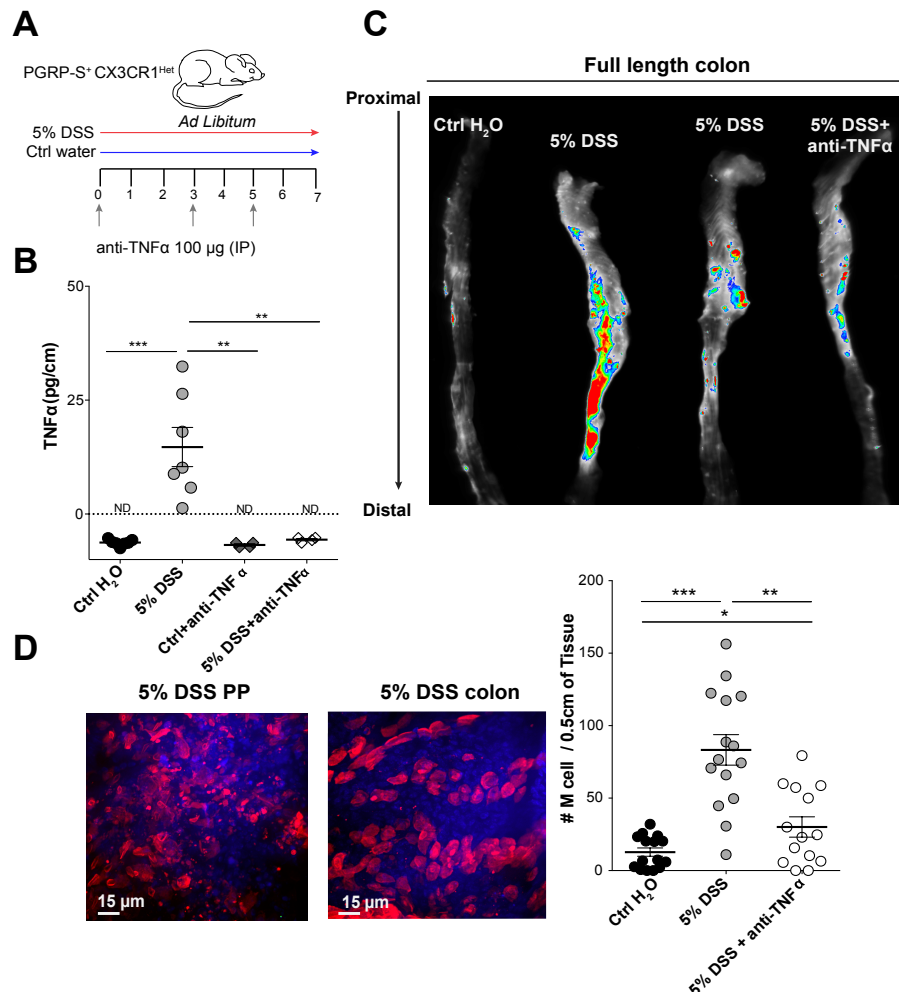


Figure 3.5 Anti-TNFα ameliorates M cell induction during DSS treatment. (A) Schematic representation the dual treatment of DSS and anti-TNFα in this study. (B) Reduction of TNFα in anti-TNFα treated animals was confirmed using ELISA. DSS treated animals showed increased TNFα levels in the colon as compared to controls; mice given anti-TNFα showed undetectable levels of TNFα. Data represents the mean ± SEM with 4-6 mice per group, assessed by unpaired Student's t test. (C) M cell numbers were quantified using whole mount tissue imaging. Top panel shows representative whole mount tissue micrographs from colonic tissue and SI PP. Bottom panels represents the quantification, DSS showed increased numbers compared to control and anti-TNFα treated animals show a reduction in M cell numbers. Counts were from individual confocal micrographs individual n = 13-15 images per mouse with 3 mice per group. Data is plotted as the mean ± SEM, and statistics was determined by unpaired Student's t test. (D) Whole tissue imaging indicates the DSS treated animals have increased DsRed fluorescence compared to control treated animal, and anti-TNFα treated animal exhibited reduced fluorescence compared to DSS animals. Left panel shows confocal images with SI PP M cells and induced colonic M cells, both expressing DsRed. Bottom right panel shows quantification of M cells; DSS showed increased numbers compared to control and anti-TNFα treated animals showed a reduction in M cell numbers.

TNF α levels were undetectable (Figure 3.5B). This correlated with an apparent reduction in infiltrating PGRP-S⁺ neutrophils (not shown). Additionally, colons of DSS treated mice had increased IL-6 and IL-1 β , which were again undetectable in anti-TNF α treated animals (Appendix Figure B.2B-D).

Whole tissue imaging again confirmed that colons from DSS treated animals had increased DsRed fluorescence compared to colons from control animals. By contrast, colons from mice treated with DSS along with anti-TNF α had reduced DsRed fluorescence (Figure 3.5C). We then quantified the number of M cells induced during inflammation using whole mount tissue imaging, similar to the method used to quantify the number of M cells in SI PP (Figure 3.5C,D). We found that DSS treated animals had significantly increased numbers of colonic M cells as compared to control animals (Figure 3.5C). In line with our hypothesis, treatment with anti-TNF α resulted in a marked decrease in M cell numbers compared to DSS alone (Figure 3.5C). Together these data suggest the M cell induction in the colon is largely dependent on the presence of TNF α .

3.2.5 Anti-TNF α failed to improve pathology in the DSS model

Anti-TNF α therapeutics are currently being used in clinical settings for the treatment of both CD and UC. Indeed, there are three FDA approved TNF α blockers, and at least seven in the pipeline for the treatment of CD and UC. Clinical studies suggest that anti-TNF α treatment is protective during intestinal inflammation, and the apparent reduction in neutrophil infiltration is consistent with results in the clinical setting. To determine whether anti-TNF α treated animals had better disease prognosis than

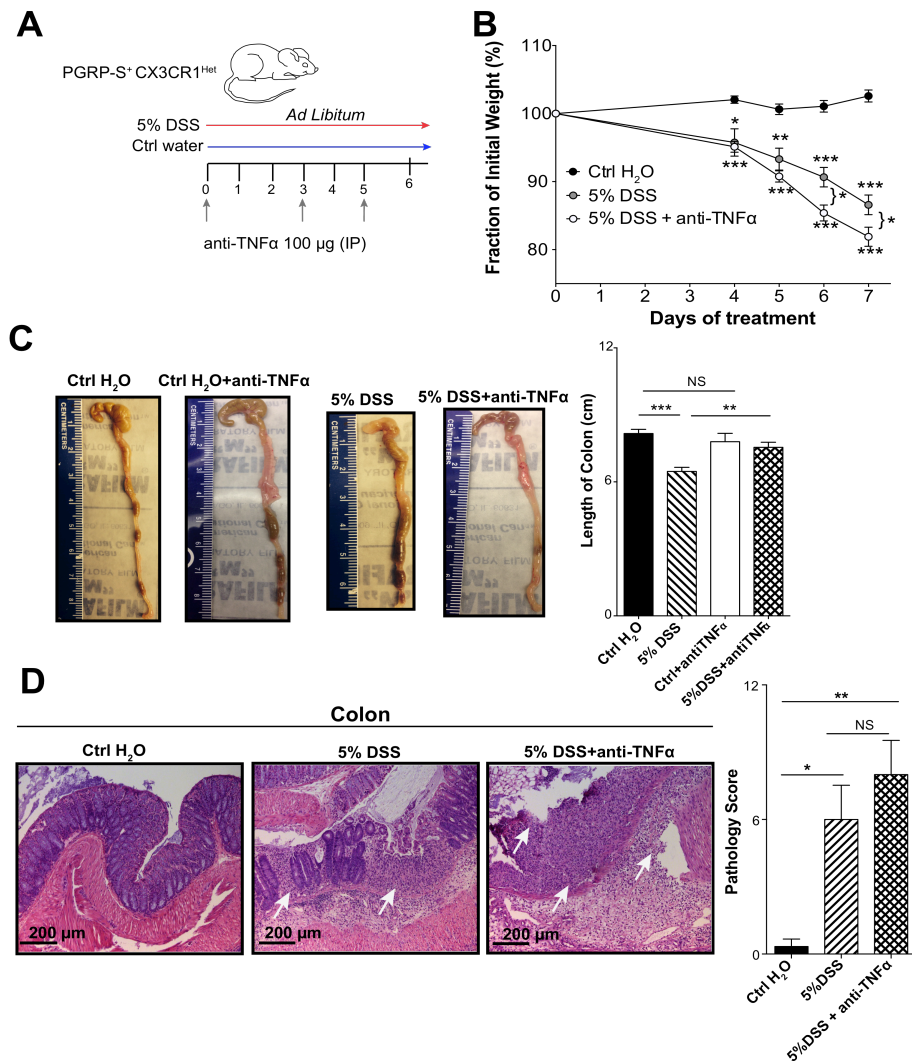


Figure 3.6 Anti-TNFα causes more severe pathology than DSS alone. (A) Schematic representation of the dual treatment of DSS and anti-TNFα in this study. (B) DSS treated mice showed significant weight loss as compared to control animals, and anti-TNFα showed greater weight loss compared to DSS treated mice. Data represents the mean ± SEM with 6-8 mice per group, and assessed by unpaired Student's t test. (C) DSS treated mice exhibited reduction in colon length as compared to control, while anti-TNFα treated mice showed similar colon length compared to control. Data represents the mean ± SEM and assessed by unpaired Student's t test. (D) H and E staining indicating inflammation, ulceration, and loss of crypt architecture (indicated by white arrows) in DSS treated mice. Meanwhile anti-TNFα treated animals exhibited regions with complete loss of epithelial architecture. Blinded pathology scoring indicated increased pathology in anti-TNFα treated animals compared to DSS and control; data represents the mean ± SEM with 3 mice per group assessed by unpaired Student's t test.

mice given DSS alone, we assessed the level of colonic inflammation using weight loss, colon shortening, and histopathology. DSS treated mice again showed significant weight loss compared to control treated animals (Figure 3.6B). Unexpectedly, mice given TNF α blockade during DSS treatment had weight loss as severe (and perhaps slightly worse) compared to mice given DSS alone (Figure 3.6A,B). Anti-TNF α had some effect on colon shortening; while DSS treated animals showed reduced colon length compared to control, anti-TNF α treated mice showed less shortening than the DSS group (Figure 3.6C). However anti-TNF α treatment was still associated with severe histopathology: while DSS treated mice exhibited inflammation, loss crypt architecture and ulceration, some mice given anti-TNF α showed a complete loss of colonic architecture (Figure 3.6D). These results may suggest two possibilities; TNF α may be needed to drive (1) immune response or (2) M cells, since associated with mucosal mediated immune responses may play a protective role in colonic inflammation. Thus, lack of colonic M cells due to TNF α blockade could be related to worsened disease.

*3.2.6 Intestinal inflammation caused by *C. rodentium* induces colonic M cells and parallels DSS injury-induced colonic inflammation*

Although the exact mechanism of IBD pathology remains elusive, evidence suggests that intestinal inflammation is initiated by dysregulated immune responses directed against the microbiota (35-37) and while DSS can mimic many histopathological features of human IBD, the mechanism triggering inflammation may be different. Thus, we employed *C. rodentium*, an infection-induced model of colonic inflammation, which may be a more relevant model of human disease. *C. rodentium* belongs to the group of attaching and effacing (A/E) bacteria and is a murine homolog of EHEC and EPEC,

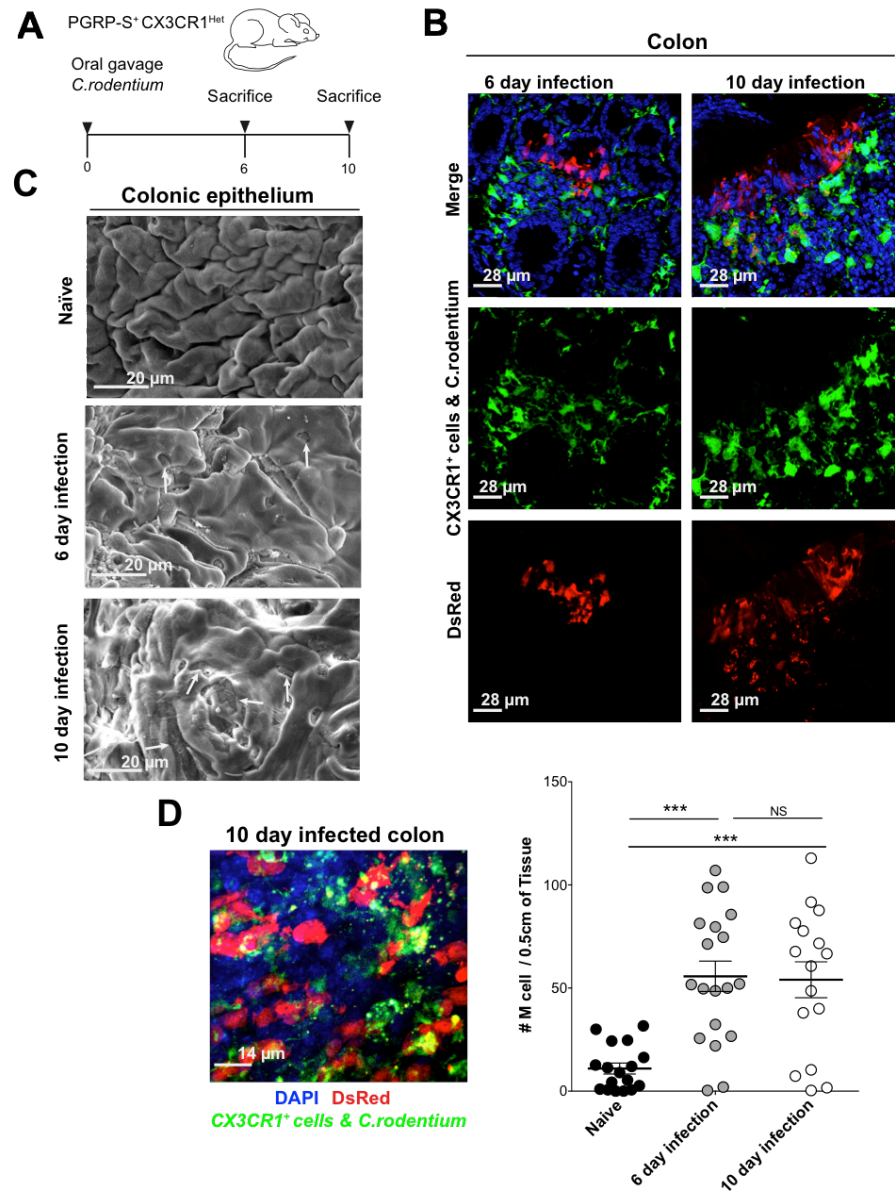


Figure 3.7. Intestinal inflammation caused by *C. rodentium* induces colonic M cells. (A) Schematic depiction of *C. rodentium* infection used in this study. (B) Representative confocal micrographs indicate the presence of DsRed⁺ M cells in the colonic epithelium of *C. rodentium* infected mice at D6 and D10 post infection. (C) Representative scanning electron micrograph further confirming the presences of colonic M cells in *C. rodentium* infected mice at D6 and D10 post infection, arrows indicating M cells. (D) M cell numbers quantified again using whole mount tissue imaging. Left image shows confocal image of DsRed⁺ M cells, and GFP-expressing bacteria (small green dots) as well as EGFP⁺ CX3CR1⁺ myeloid cells. Right graph shows M cell numbers increased at D6 and D10 post infection as compared to naïve mice. Data represents the mean ± SEM with 3 mice per group, assessed by unpaired Student's t test.

which are major causative agents of diarrheal diseases (38; 39). Moreover, *C. rodentium* inflammation is thought to be driven by both robust Th1 and Th17 responses (38-42). We wanted to determine whether M cells were also induced in the colon at the peak of *C. rodentium* infection (D6 and D10). Mice infected with GFP expressing *C. rodentium* (Figure 3.7A) exhibited high bacterial burdens at D6 and D10 post infection, including a large number of bacteria associating with the colonic epithelium (Appendix Figure B.3A and data not shown). At D6 and D10 post infection mice had severe crypt hyperplasia, moderate reduction in colon length, and a significant number of infiltrating DsRed⁺ neutrophils present in the colonic lamina propria compared to the naïve animals (Appendix Figure B.3B,C and data not shown).

Infected mice, at both D6 and D10 post infection, showed the presence of DsRed⁺ M cells within the colonic epithelium, evidenced by DsRed expression in qPCR (Figure 3.7A) and confocal microscopy (Figure 3.7B), further confirmed by SEM (Figure 3.7C). We then quantified the level of M cell induction at D6 and D10 post infection using whole mount tissue imaging. At both D6 and D10, mice displayed increased M cells numbers as compared to their naïve controls (Figure 3.7D).

To further study the parallels between DSS and *C. rodentium* models of colonic inflammation in the context of M cell induction, we studied the transcriptional regulation of M cell and neutrophil related genes. Similar to the DSS model, we saw upregulation of TNFa, DsRed and Lacto, at both D6 and D10 in both the proximal and distal colon (Figure 3.8A). Consistent with M cell induction, we again saw upregulation of the M cell specific functional gene GP2 at both D6 and D10 (Figure 3.8A). As with the DSS model, the M cells were associated with poorly organized mononuclear infiltrates, with a less robust stromal cell network as compared to SI PP (Figure 3.8B). At D6 and D10 these

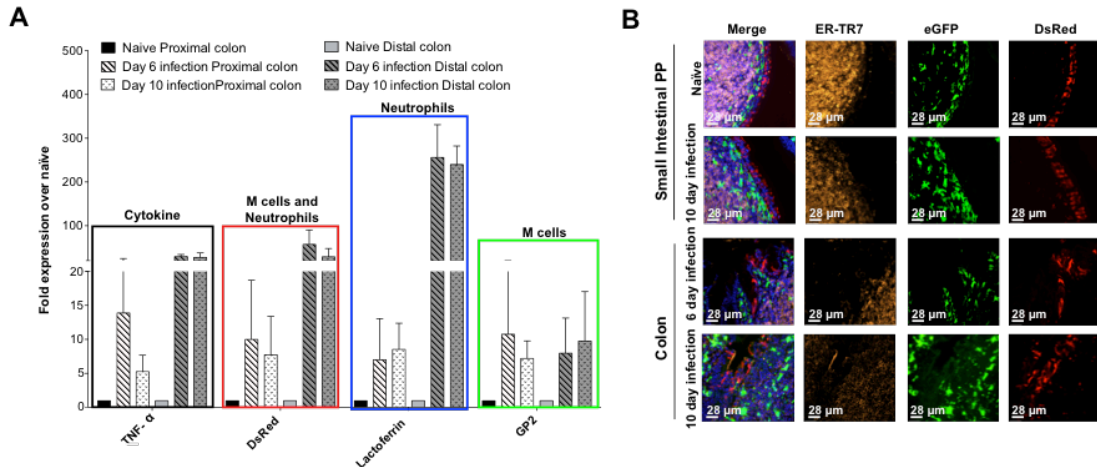


Figure 3.8 Colonic M cell genes are regulated during *C. rodentium* infection and M cells are associated with poorly organized follicles (A) qPCR was used to identify whether M cell functional genes were regulated during intestinal inflammation. D6 and D10 infected animals showed upregulation of TNF α , DsRed, Lactoferrin, and GP2 compared to naïve animals. These numbers were comparable to those seen in DSS treated animals. Data represents the mean $\pm$ SEM with 4 mice per group. (B) Induced M cells in *C. rodentium* infected were associated with poorly organized follicles, in D6 and D10 infected mice colonic follicles stained positive for ER-TR7 paralleling DSS induced colonic inflammation.

aggregates had essentially no CXCL13 staining, but stained positive for ER-TR7 (Appendix Figure B.4 and Figure 3.8B). Taken together, both the DSS and *C. rodentium* data suggest a role for M cells during colonic inflammation and their presence may be an important correlate for human disease.

3.2.7 De novo Induced M cells and transcytosis

The singular characteristic feature of M cells is their ability to transcytose luminal antigens, yet in the absence of basolateral pocket B-lymphocytes, M cells cannot develop this critical transcytosis function (43; 44). To determine whether it was important that induced colonic M cells were able to transcytose luminal microparticles, we treated PGRP-S⁺ and PGRP-S⁺IgH6^{KO} (B cell KO) with DSS (control treated mice were not used

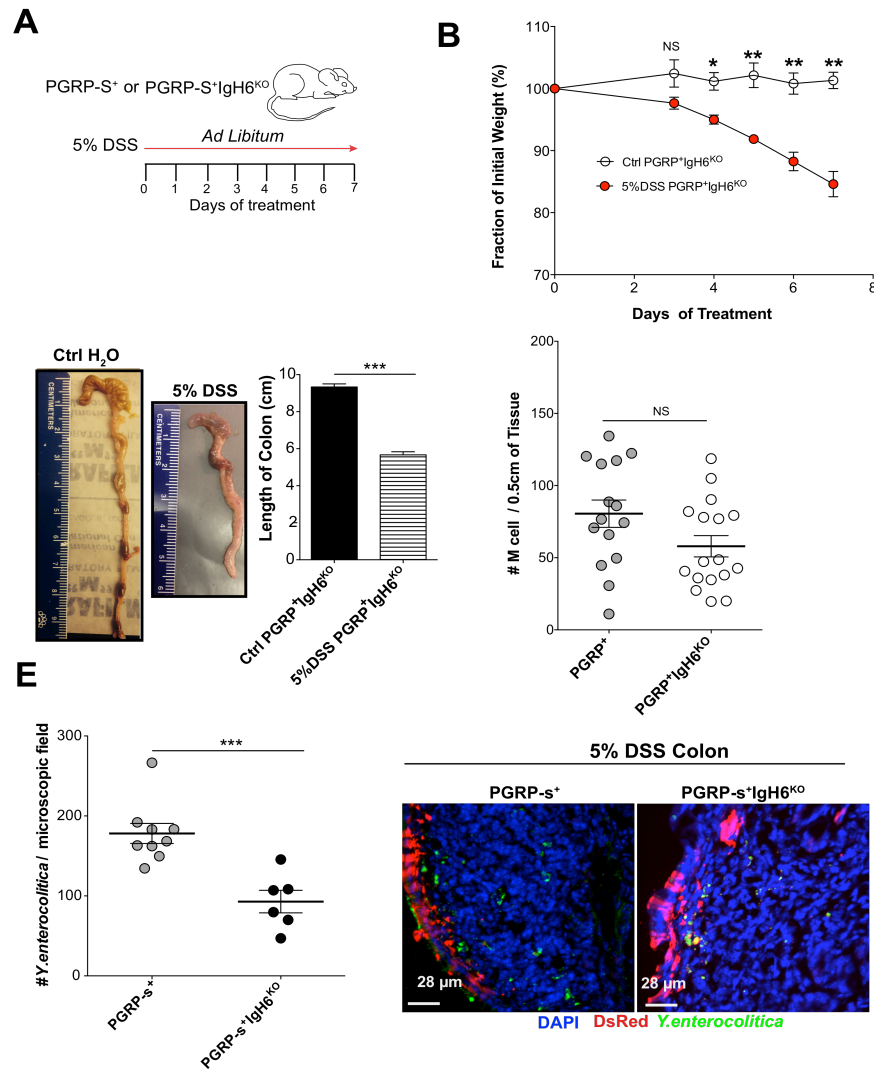


Figure 3.9 Induced M cells are capable of transcytosis. (A) Schematic representation of live uptake assays performed. (B) DSS treated PGRP-S⁺IgH6^{KO} mice exhibited increased weight loss as compared to DSS PGRP-S⁺ mice (data taken from Figure 1). Data represents the mean $\pm$ SEM, with 3 mice per group. (C) DSS PGRP-S⁺IgH6^{KO} treated mice exhibited reduction in colon length as compared to ctrl PGRP-S⁺IgH6^{KO}; data represents the mean $\pm$ SEM with 3 mice per group assessed by unpaired Student's t test. (D) DSS PGRP-S⁺IgH6^{KO} treated mice showed induction of M cells (data taken from Figure 1); data represents the mean $\pm$ SEM with 3 mice. (E) Left panel shows the quantification of *Y. enterocolitica* uptake; DSS PGRP-S⁺ treated animals showed increased *Y. enterocolitica* uptake compared to PGRP-S⁺IgH6^{KO}; data represents the mean $\pm$ SEM with 3 mice per group assessed by unpaired Student's t test. Right side images are representative confocal micrographs of DSS PGRP-S⁺ and PGRP-S⁺IgH6^{KO} showing uptake of *Y. enterocolitica*.

because they are essentially devoid of colonic M cells, Figure 3.9A). In these mice, the absence of mature B cells results in defective M cell transcytosis despite the ability to produce committed M cell precursors (28).

Interestingly, when assessed for clinical signs of colonic distress, DSS treated PGRP-S⁺IgH6^{KO} had more severe disease, similar to what was seen in anti-TNF α treated animals. DSS treated PGRP-S⁺IgH6^{KO} animals showed more severe weight loss compared to PGRP-S⁺ (Figure 3.9A,B). These mice also had more significant reduction in colon length (Figure 3.9C). These results are consistent with a recent study indicating that a MyD88 specific B cell KO mice had increased susceptibility to DSS induced colitis (45). Importantly, the B cell KO mice showed induction of colonic M cells similar to the numbers seen with control PGRP-S⁺ DSS treated mice (Figure 3.9D), though they would lack the transcytosis function of controls.

To confirm the defect in M cell-dependent transcytosis, *Y. enterocolitica* was used in uptake studies; it binds to β_1 integrin on the apical surface of M cells to enable endocytosis (46). Suspensions of *Y. enterocolitica* (GFP expressing) were injected right below the cecum of anesthetized DSS treated animals and after 30 mins mice were sacrificed and colons were excised. Regions of DsRed⁺ M cells were identified and *Y. enterocolitica* transcytosis was quantified. Areas with PGRP⁺ DsRed⁺ M cells showed increased numbers of bacteria in the lamina propria as compared to PGRP-S⁺IgH6^{KO} (Figure 3.9E). These data indicate the induced colonic M cell are not simply a phenotypic marker of inflammation and are likely to play an active role in mucosal mediated immune responses in the colon. Secondly, B cell deficient mice known to lack functioning M cells, have increased susceptibility to DSS induced inflammation consistent with the notion that functional M cells may be protective during inflammation.

3.3 Discussion

M cells in the small intestine play an important role in the transcytosis of luminal antigens, leading to the production of sIgA and other mucosal immune responses (1; 47). However, their role in the large intestine has largely been overlooked. This study presented here suggests a new role for M cells in view of their de novo induction during colonic inflammation in two different models of colitis. M cells, like other epithelial associated cell subsets i.e. goblet cell, Paneth cells, enteroendocrine cells, seem to be intimately linked to intestinal dysbiosis/inflammation, and their increased presence may help initiate or drive processes restoring tissue homeostasis. Two lines of evidence support this potential protective role of M cells. First, while anti-TNF α treatment proved to be effective at blocking the induction of colonic M cells during DSS treatment, it was unexpectedly associated with exacerbated disease, including increased weight loss, and increased pathology.

The effect of anti-TNF α treatment on disease severity was largely unexpected given that TNF α blockade therapeutics currently on market are being used for the treatment of IBD with some success, and other TNF α therapeutics are currently being explored for other autoimmune disorders such as rheumatoid arthritis (RA), multiple sclerosis (MS) and psoriasis (48). Interestingly, several TNF α blockade therapeutics have entered clinical trials for the treatment of MS and have been either halted or failed due to exacerbated disease progression (48-51). Although worsened disease due anti-TNF α treatment during DSS induced inflammation has also been reported by others (52-55), we now report a potential link between the inhibition M cell induction and exacerbated disease. This may mean that by blocking M cell induction, the system is

less able to sense luminal microbes, and consequently mucosal mediated immune responses (or immunoregulatory mechanisms) are delayed.

Consistent with this notion, B cell deficient mice also showed worsened disease with DSS treatment. In the absence of B cells, developing M cells fail to associate with basolateral pocket B cells, and are then unable to develop mature transcytosis ability (43; 44). While DSS treatment increased M cell precursor numbers in the B cell deficient mice, these M cells did not acquire transcytosis capability and also had disease levels comparable to mice treated with anti-TNF α . Thus, in the absence of functional M cells, inflammation worsens, suggesting these induced colonic M cells may be required to help drive immunoprotective and/or immunoregulatory responses and may play a restorative role in IBD.

In our studies on M cell phenotypes and lineage, we found that intestinal M cells that are induced acutely by inflammatory stimuli such as cholera toxin are not conventional phenotypic Peyer's patch-type M cells as found overlying organized lymphoid tissues (28). These cells instead fall into the category of "villous" M cells (5) with a distinct gene expression pattern, such as the absence of GP2 expression. Interestingly, the M cells identified here in the DSS and *C. rodentium* model showed the conventional Peyer's patch-type M cell phenotype (e.g., expression of PGRP-S/DsRed and GP2) despite the absence of fully organized lymphoid tissue. Thus, the chronic nature of DSS and *C. rodentium* colitis may be optimal for recruitment of Lymphoid Tissue Inducer cells (a.k.a., Innate Lymphoid T cells – 3; (56-58)) or the kinetics of local cytokine (including TNF α) production promotes a specific M cell phenotype.

Finally, IBD is difficult to clinically identify and diagnose. This is largely due to the fact that disease activity measurements are only indirect measurements and it still

remains unclear whether or not it is more reliable to assess inflammation systemically or from the bowel itself (59). Since we saw induction of colonic M cells in two disparate models of intestinal inflammation i.e. DSS and *C. rodentium*, with two distinct immune responses, this perhaps indicates that M cells could be used clinical biomarker of IBD. In fact one recently published study investigating the transcriptome of ileal Crohn's patients suggested that the terminal ileum, where 50% SI PP's are located, could be the major inductive site of CD, noting upregulation of IFN- γ /Stat-1 dependent Th1 signatures (60; 61). Thus, M cell presence in the colonic epithelium in clinical biopsies could be used to identify active intestinal inflammation. Moreover, studies from our lab have found that therapeutics can be directly targeted to the M cell surface making use of highly specific ligands (62; 63). This indicates that the induced colonic M cells could be exploited by directly targeting anti-inflammatory therapeutics to their surface, which could have a two-fold effect by (1) limiting deleterious off-target effects as has been seen with anti-TNF α drugs, or (2) increasing uptake of the anti-inflammatory therapeutics.

In conclusion, we have found that M cells are induced in the colonic epithelium during active inflammation in two separate murine models, using a unique reporter mouse. These M cells were associated poorly organized mononuclear infiltrates. Thus, this study provides novel insight into the potential protective role M cells play during intestinal inflammation and dysbiosis, offering a possible new biomarker for the diagnosis of IBD as well as a new target for anti-inflammatory therapeutics.

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Graduate Research Fellowship Program and Dissertation Year Fellowship at UCR. The authors thank Kathy Vu, Joseph Pham, Juliane Lieu, and Olivia Sakhon for technical assistance, Joan Meccas for the *Y. enterocolitica* isolates, Bruce Vallance for the *C. rodentium* isolates, and Mathias Rommelfanger, for assistance with the SEM and CPD. KMB, DDL, and MGN designed experiments. KMB and DDL wrote manuscript. MGN provided input on the manuscript. KMB performed experiments and analyzed data for DSS experiments. GC and MGN performed *C. rodentium* experiments. GC analyzed the *C. rodentium* data. MGN performed blind histopathology scoring. The authors claim no competing interest financial or otherwise. All authors have approved the final version of the manuscript.

3.5 Materials and methods

3.5.1 Mice

WT C57BL/6, CX3CR1-EGFP Knock-in (CX3CR1^{tm1Litt/J}), B-cell-deficient IgH-6 (IgH6^{tm1cgn} or Ighm^{tm1Cgn}) mice were purchased from The Jackson Laboratory. PGRP-S-DsRed (referred to as PGRP-S⁺) mice were generated as previously described in (Wang et al., 2011). PGRP-S-DsRed were crossed with CX3CR1-EGFP and only PGRP-S-DsRed⁺ × CX3CR1-EGFP^{Het} (referred to as PGRP-S⁺CX3CR1^{Het}) were used. PGRP-S mice were backcrossed for 12 generations with IgH-6 BALB/c mice (referred to as PGRP-S⁺IgH6^{KO}). All mice used were younger than 4 months and were bred in the University of California, Riverside vivarium under specific pathogen-free conditions and were handled in accordance with Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

3.5.2 Dextran sodium sulfate

DSS (M.W. 36,000-50,000 MP Biomedicals, Solon, OH) was added to the drinking water at 5% (w/v) during the course of the experiment. The mice were monitored daily for signs daily for morbidity (piloerection, lethargy), weight loss, and rectal bleeding based on a method described (42). Aged matched mice put on normal drinking water were used as controls.

3.5.3 *C. rodentium* infection model

Mice were infected by oral gavage with a 0.2ml overnight culture containing $\sim 2.6 \times 10^8$ GFP reporter *C. rodentium* (containing a chloramphenicol resistance gene) in Luria broth (LB) (64). To enumerate the bacteria, fecal pellets were weighed and homogenized in PBS; serial dilutions were plated onto MacConkey agar plates (Sigma-Aldrich), and incubated overnight at 37°C. Bacterial colonies were counted the next day.

3.5.4 Anti-TNF α blockade

Mice were either given DSS in their drinking water as described earlier or given control water. Mice then were given three intraperitoneal (IP) injections containing 100 μ g of a functional grade anti-mouse TNF α (eBioscience) at D0, D3, and D5 of DSS treatment. These mice were also assessed daily for signs daily for morbidity (piloerection, lethargy), weight loss, and rectal bleeding.

3.5.5 Tissue pathology and histological analysis

Mice were sacrificed and colons were excised, and split via a longitudinal incision. Colons were rinsed with PBS, and rolled distal to proximal and transected with a 27-gauge needle (65). The roll was then placed into a specimen cup containing Bouin's fixative (Sigma-Aldrich) and allowed to fix for 3 hours. The samples were then

washed several times using 70% ethanol to remove excess fixative. These samples were then processed for H/E staining. Finally, blinded clinical scoring was performed according to the following criteria: crypt hyperplasia (1–5), inflammation (1–3) and ulceration (1-3).

3.5.6 Immunohistochemistry

Colons were excised and rolled in the same manner as described in previous sections. Tissue was placed in 4% PFA and 30% sucrose-PBS solution for 2 hours on ice protected from light. Cryostat sections were treated with 0.5% Tween 20–PBS (Fisher Scientific), washed 3X in 0.1% Tween/PBS and then blocked using 0.1% Tween 20 in casein solution (Thermo Scientific). Primary antibodies, goat anti- mouse CXCL13 (R and D Systems: AF470), rat anti-mouse ER-TR7 (AbD Serotec: MCA2402), and rat anti-mouse GP2 (MBL: D278-3) were then added diluted in blocking solution. Samples were wash with 3X 0.1% Tween-20-PBS. Secondary antibodies were added Alexa Fluor @ 488 (donkey anti-rat: A21208), Alexa Fluor @ 647 (donkey anti-goat: A21447 and chicken anti-rat: A21472). Tissue was again washed, post-fixed with 4%PFA in PBS and mounted with Prolong Gold antifade reagent (Life Technologies) containing DAPI(4',6-diamidino-2-phenylindole), which was used as a nuclear counterstain. Images were obtained by using a BD CarvII spinning-disk confocal imager (BD Biosystems) attached to a Zeiss Axio Observer inverted microscope. Hardware, including the confocal microscope and digital camera (Qimaging Rolera EMC²), was controlled by Metamorph imaging software. Images were further optimized by using Volocity deconvolution software (PerkinElmer).

3.5.7 Whole mount tissue preparation

Colons were excised from sacrificed mice, and opened longitudinally, laid out flat and placed into a petri dish containing 4% PFA and 30% sucrose-PBS solution for two hours on ice protected from light. The samples were washed with 3 times in PBS and then placed into a solution containing 150 mM Tris-HCl @ pH 8 (Fisher Scientific), 2mM DTT (Life technologies), and 20% Ethanol for 45 mins. Samples were again washed and cut into ½ cm pieces, and placed onto a glass slide, where a gasket was created using two binder reinforcements placed on top of one another. The samples were mounted with Prolong Gold antifade reagent (Life Technologies) and coverslip was held in place by putty. Tissues were allowed to cure overnight and imaged; five randomly chosen frames were taken per ½ cm of tissue. The number of M cells was quantified by setting limits on both size and intensity using Volocity software. Each group contained 3 mice.

3.5.8 Nucleoside analog (Edu) labeling and detection

Mice were given DSS in their drinking water as described earlier or given control water. At D3, D4, D5, D6 of DSS treatment mice were given a 2 µg IP injection of EdU from Click-it Plus EdU Alexa fluoro @ 488 imaging kit (Life technologies). At necropsy colonic tissue was excised, fixed in 4% PFA (Electron Microscopy Sciences) and 30% sucrose (Fisher Scientific) solution, placed into O.C.T (Tissue-Tek), frozen and cryosectioned into 10µM sections. EdU incorporation was detected based on manufacturer protocol.

3.5.9 SEM fixation and viewing

Mice were sacrificed, and colonic tissue was excised and first fixed with 2.5% glutaraldehyde solution (Ted Pella) for 2 hrs. Samples were then washed in double-

distilled water (ddH₂O), and 4% osmium tetroxide (Ted Pella) was added for 30 min. Samples were then gradually dehydrated in 25, 50, 75, 90, and 100% ethanol for 5-min intervals. Dehydrated samples remained immersed in 100% ethanol until critical-point drying was performed. Critical-point drying was performed by using a Balzar critical-point dryer (CPD). Samples were then mounted onto scanning electron microscopy pin stub mounts (Ted Pella) with carbon-coated conductive tape and finally sputter coated with platinum/palladium for 60 s (Cressington 108 Auto sputter coater). The samples were viewed by XL-30 FEG scanning electron microscope at 10 kV.

3.5.10 Whole tissue imaging

Mice were euthanized and colonic tissue was excised and opened longitudinally. The tissues from treated and untreated mice were then placed onto parafilm and imaged on the Texas red channel using the iBox Explorer imaging microscope (UVP), and images were then pseudo colored to show DsRed fluorescence signal. Tissues from treated and control animals were imaged side by side and thus treated the same.

3.5.11 Real-time RT PCR

A 1cm piece taken from both the distal and proximal colon was flash frozen in liquid nitrogen and stored at -20°C until used. Colonic tissue RNA was isolated by Trizol (Invitrogen) in accordance with the manufacturer's instructions. RNA from DSS treated animals were further processed after initial Trizol precipitation based on a method described in (66). Briefly, the precipitated RNA was suspended in RNAase free H₂O and then incubated with 0.1 volume of 8M Lithium Chloride (LiCl: Sigma Aldrich) for 2 hrs on ice, and then centrifuged for 30min 14,000 g, and then suspended in RNAase free H₂O, and this entire step was repeated once more. The RNA was precipitated at -20°C for 30

min, in 0.1 volume of 3 M sodium acetate @ pH 5.2 and 2 volumes of 100% absolute ethanol. RNA was again centrifuged and washed once with 70% ethanol and finally dissolved in 50 µL of RNase free H₂O. RNA was reversed transcribed into cDNA using Super Script III First Strand synthesis kit (Invitrogen) in accordance with the manufacturer's protocol. cDNA was analyzed by real- time PCR using SYBR Green technology (Applied Biosystems). Primers used:

TNF α -F: ACGTCGTAGCAAACCAACCAA ,TNF α -R: ATAGCAAATCGGCTGACGGT

DsRed-F:AACGGCCACGAGTTCGAGAT, DsRed-R: CACTTGAAGCCCTCGGGGAA

Lacto-F:TCCGCCAGTCACAGGAGAAGT, Lacto-R: CACCATGTGACCCGGGCCTT

GP2-F:CACTGGGCAGGGAGGAAGGAT, GP2-R: TTCCTGGGCCTCCCATAACCTTG

The HPRT primer used as the housekeeping gene as reported by (Wang et al., 2009).

$\Delta\Delta$ CT method was used to determine the fold induction for each gene.

3.5.12 Quantitative cytokine ELISAs

At necropsy 1cm distal colonic tissue was excised and flash frozen in liquid nitrogen and stored at -20°C until used. Tissue was mechanically homogenized in a solution containing 0.05% sodium azide, 0.5% Triton-X 100 (Sigma), 1X protease inhibitor cocktail and 1mM PMSF. TNF α , IL-1 β , IL-6 and IL-10 ready-set-go ELISA kits (eBioscience) were used to quantify the level of each cytokine in accordance with the manufacture's instructions.

3.5.13 Semi-quantitative bacterial uptake assay

Mice were anesthetized by IP injection of avertin. Once the mouse was unconscious, a small 1cm incision just right of the xiphoid process was made, using dull forceps, the Cecum (beginning of large intestine) was exposed. 5×10^8 bacteria injected

just below the cecum and allowed to incubate for 30 mins, and the bacteria used were *Y. enterocolitica*, expressing GFP (kindly provided by Joan Mecsas). At the end of this 30 min period mice were humanely sacrificed and colons were excised and rolled, using the same procedure as in previous sections. The tissue was fixed 4%PFA and 30% Sucrose in PBS solution for 2 hrs and frozen in OCT. Sample were cryosectioned and imaged using confocal microscope as mentioned previously. An image was taken every time red M cells were seen, maintaining spatial locations, so as to minimize double counting. Bacteria were enumerated for every red M cell region using Volocity software, and size and intensity limits were set.

3.5.14 Statistical analysis

Results represent the mean $\pm$ SEM of individual animals or replicate wells. Normality of the data values was checked, and the unpaired Student t test was used to make comparisons between two groups. All graphs and statistical calculations were performed by using GraphPad Prism version 5.04. A P value of <0.05 was considered statistically significant. $p \leq 0.05$ (*), $p \leq 0.005$ (**), $p \leq 0.0005$ (***)

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Chapter 4

General Introduction

T cell independent antibody response and antigens

4.1 List of abbreviations

TD	T cell dependent
TI	T cell independent
TI-1	T cell independent antigen type 1
TI-2	T cell independent antigen type 2
MHC	Major histocompatibility
MZ	Marginal zone
Btk	Bruton's tyrosine kinase
LPS	Lipopolysaccharide
PS	Polysaccharide
TLR	Toll-like receptor
CR	Complement receptor
FliC	Flagellin
BCR	B cell receptor
TCR	T cell receptor
HIV	Human immunodeficiency virus
FV	Flavivirus
DENV	Dengue virus
DENV2	Dengue virus envelope 2
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
ADE	Antibody dependent enhancement

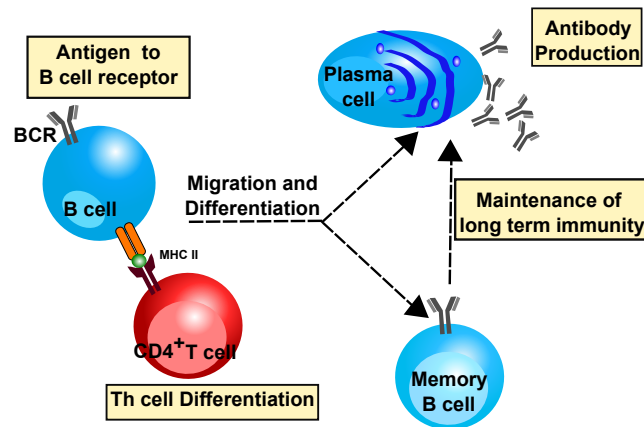
4.2 T cell dependent vs T cell independent antibody response

It is well understood that we are born both sterile and largely without the ability to generate a humoral antibody responses due to lack of an adaptive or “acquired” immunity (1). Antibody responses are reliant on canonical T and B lymphocytes (B2 B cells). *Ex utero*, naïve T and B lymphocytes develop from common lymphoid progenitor cells in the bone marrow. B2 B cells stay in the bone marrow to mature; meanwhile T cells leave the bone marrow and mature within the thymus (central lymphoid organ; (2)). Once mature, B2 B and T cells can circulate between the blood and peripheral lymphoid tissues where they will encounter antigens. T cells, as mentioned previously, after encountering antigens via DCs, can differentiate into various effector T cell subsets based on their cytokine niche (3). However unlike T cells, B cells, after antigen

stimulation, will exclusively differentiate into plasma cells, to generate antibodies against the encountered antigen. Mechanistically, plasma cell differentiation first begins by antigen binding to the B cell cognate receptor (BCR), which results in an antigen-BCR complex that becomes internalized, and is broken down into smaller fragments (4). One of the antigen-BCR fragments is presented via Major histocompatibility (MHCII) to the CD4⁺ Th cells. The Th cell then can provide the necessary signals to trigger both the differentiation of the B cell to a plasma cell, as well as drive its own clonal expansion (2). Moreover, during this B cell differentiation, the membrane bound BCRs undergo alternative splicing to become secreted antibodies. This process has been termed T cell dependent mode of antibody production (TD: Figure 4.1(4)).

Although the TD pathway is efficient at generating highly specific antibodies, in many disease states this pathway becomes ineffectual at conferring protection to the host. One of the most notable examples is in the case of human immunodeficiency virus (HIV)-AIDS pandemic. HIV is a retrovirus, and is able to specifically target host Th cells through binding of its major capsular envelope protein glycoprotein 120 to host CD4 molecules (mainly on Th cells) (5-7). Through additional binding of its co-receptors CCR5 and CXCR4, the virus is able to hijack the cell's transcription machinery, to continue infecting other Th cells. This process results in the inability of the Th cell to provide help to B cells (8-12). In addition to HIV, this TD mode of antibody production is also sluggish/ineffectual in neonates, the elderly, chemotherapy patients, as well as many more disease states. Luckily, these deficiencies in the TD pathway can be partially compensated by alternative modes of antibody production. Certain subsets of B cells collectively termed "innate-like" B cells are capable of producing antibodies in the absence of T cell help, and the T cell independent (TI) pathway (Figure 4.1: (13; 14)).

T cell dependent pathway



T cell independent pathway

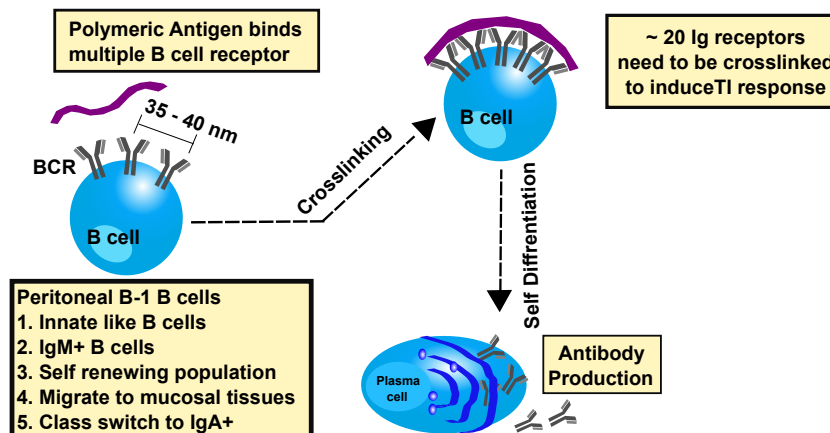


Figure 4.1 Mechanism of TD and TI antibody production. In TD, B and T cells interact with one another after the B cell presents antigen to T cells via MHCII molecules. This interaction causes the B cell to differentiate into plasma (antibody producing) cell. TI responses are driven by the presence of polymeric antigens which crosslink the BCR. This crosslinking event causes the B cell to undergo self-differentiation to plasma cells.

Three distinct populations of TI B cells have been identified in mice. Marginal zone (MZ) B cells, B1b B cells, and B1a B cells together only make up ~ 15% of the total B cell population (15). However distinguishing these B cell populations from the canonical

B2 B cell subset is difficult due to lack of unique surface markers (15). Thus, studies of these cells have largely been centered on identifying properties that distinguish them from the greater B2 B cell subset, rather than their actual functional role *in vivo*. MZ B cells, due to their expression of various integrins and lysophospholipid receptors, reside in the splenic marginal sinus (peripheral lymphoid tissue) where they can respond quickly to T1 type-2 antigens (discussion of next section: (16)). Studies have identified that the MZ compartment contains memory B cell phenotypes that are a result of TD stimulation, which suggest that these MZ B cells are not solely restricted to generating T1 response, but can also participate in TD responses ((17)). MZ B cells have been identified in humans and are referred to as IgM memory B cells. It is not surprising that these cells have been linked to hyper-IgM syndrome, a genetic deficiency that results in mutations in either CD40 or CD40L, such that the cognate T and B cell interaction cannot take place (18). Interestingly, other studies have linked these cells to the induction CD1d restricted NK cells, an innate cell analogous to cytotoxic T cells present in the adaptive immune system (17; 19). Together these studies suggest that MZ B cells have ties to both the innate and adaptive arms of the immune system. In humans, MZ B cells are large (abundant cytoplasm) CD27⁺ (TNF receptor) cells, which are further divided based on their IgD and IgM expression (20; 21); interestingly, unpublished observations by Gusti and Lo suggest that B1 B cells in mice are larger in size compared to the canonical B2 B cells, a characteristic that may help to distinguish them from B2 cells.

The second ‘innate-like’ B cell population is the B1 B cells, which are the main producer of natural antibodies (IgM and IgA) and develop early in embryogenesis in the fetal liver (22-24). Interestingly, it is still unknown whether these cells differentiate from a

disparate precursor cell compared to other lymphocytes (lineage hypothesis) or from the common lymphoid precursor (induced differentiation hypothesis: (25-27)). B1 B cells are further subdivided into B1a B and B1b B cells, where both subsets are noted for their self-renewing capabilities (28; 29). While B1 cells are found primarily in the pleural and peritoneal cavities, once activated they can migrate to secondary lymphoid tissues, including, mucosal sites, and the omentum (27; 30). However, to date it remains unclear whether a B1 phenotype exists in humans. One study by Griffin and colleagues suggested the identification of human B1 B cells in human umbilical cord, with a novel cell phenotype (CD20⁺CD27⁺CD43⁺CD70⁻: (31)). This human B1 phenotype has since been refuted by Descatoire *et al*, suggesting this cell phenotype is more similar to MZ B cells (32). Unlike MZ B cells activated in TI manner, B1 cells confer long lasting immunity in TI manner (33; 34). B1 B cells under steady state conditions are IgM⁺ cells, and in fact studies have found that 80% of serum IgM is derived from B1 cells (35). Interestingly, B1 cells *in vivo* undergo class switch recombination specifically from IgM⁺ cells to IgA⁺, however, where this occurs is unknown (36). In line with this notion, a seminal study from Kroese *et al* estimated that up to 50% IgA⁺ B cell in the intestinal lamina propria are of the B1 cell lineage (37). Moreover, as SIgA plays a crucial role in the immune exclusion process at the intestinal barrier, this study links B1 cells to regulation of intestinal homeostasis (37; 38). This has since been further corroborated by Macpherson and colleagues, who found that large proportion IgA produced by B1 B cells in the intestines has specificity to various species in normal microbiota (39). Taken together, these data suggest that “innate-like” B cells are important mediators of humoral immune response as well helping to maintain homeostasis in both the absence and presence of T cells.

4.3 T cell independent antigens with emphasis on flagellin

While all antigens can engage the BCR to induce various activation events, TI antigens are typically multivalent (polymeric) in nature (Figure 4.1). This repetitive nature of the TI antigen allows it to bind multiple BCRs, which in turns crosslinks the BCR and drives differentiation of the B cell to a plasma cell (40-43). However, TI antigens have not been classified as one distinct antigen class, but rather as two, TI antigen 1 (TI-1, and TI antigen 2 (TI-2). This classification was first made based on the antigen's ability to generate an antibody response in neonates, as well as hosts with congenital or induced mutations in Burton's tyrosine kinases (Btk: (13; 44; 45)). The bacterial antigen lipopolysaccharide (LPS) was able to induce a non-antigen specific B cell response in neonates and individuals carrying mutations in Btk, and was categorized as TI-1 antigen (46-48). Meanwhile polysaccharide (PS), another bacterial component, classified as TI-2 antigen, only had a limited ability to confer protection in TI manner in these individuals (13; 44). Interestingly, this study suggests that although TI-2 antigens are not MHCII restricted, a secondary signal may be required to induce antibody secretion in B cells activated by BCR crosslinking in the absence of T cells (49; 50). This secondary signal is thought to be reliant on various innate immune receptors, which include pattern recognition receptors (PRR) like, Toll-like receptors (TLR) and Complement and its associated complement receptors (CR). In fact it has been identified that certain CR enhances the TI-2 response by facilitating BCR antigen binding and subsequent crosslinking (45; 51-56). In line with this, CR2 null mice exhibited ~50% reduction in B1a B cells, suggesting CR2 is needed to maintain the B1 cell repertoire (57). Moreover C3d coated antigens have been found to enhance B1 cell signaling and BCR crosslinking

(58). However, the original classification criteria for TI-1 and TI-2 has since become less stringent, with less of a clear distinction between TI-1 and TI-2 antigens.

Studies have identified that TI-2 responses can produce neutralizing antibodies that have specificity toward viral glycoproteins, which are expressed on viral capsids in a very ordered fashion (59-61), further implicating TI-2 responses during pathogenic

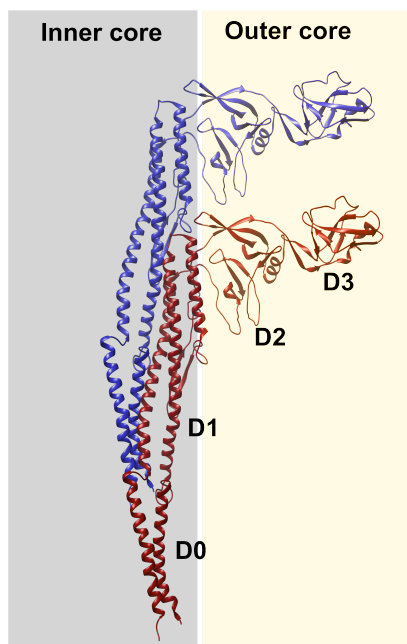


Figure 4.2 Schematic of FliC filament. Depicts FliC dimer in which the D0 and D1 are buried within the filament, while D2 and D3 are solvent exposed.

microorganism and viral infections. This has sparked interest in developing neonatal vaccines in which PS is conjugated to T cell dependent antigens (62-64). While TI-1 antigens seem to produce low avidity antibody response, TI-2 antigens when linked to TD antigens seem capable of producing highly specific antibodies. It is now well understood that TI antigens produce a unique antibody repertoire as compared to TD antigens. TI-2 antigens are not only able to strong IgM response, but seem able to influence the IgG subclass profile. A study by Mongini et al found that immunization of Scid mice with a classical

TI-2 antigen TNP (2,4,6-Trinitrophenyl)-Ficoll and found these mice could produce both high IgM and IgG3, and to lesser extent IgG1, IgG2a, IgG2B (65). Negligible levels of serum IgM and IgG3 corroborated this in Btk null mice; meanwhile other IgG isotype levels remained normal (46). Together these data suggest the TI antigens can be

engineered to generate antibodies with antigen specificity that are capable of neutralization.

Another bacterial component that is multivalent in nature and may be capable of producing a TI response is the bacterial protein flagellin (FliC). FliC is the major component of Flagellum (11 fold symmetry) used by various bacterial species for locomotion, which can grow up to 15 μm in length. FliC is a four-domain protein made up of two conserved domains and two variable domains in the shape of a greek letter Γ , and has been incorporated into vaccine formulations due to its adjuvant activity (Figure 4.2: (66)). The two conserved regions (D0 and D1) are helical in nature and are pivotal for stabilization of the filament. Interestingly, as a monomer, the D1 domain of FliC is fully exposed, and likewise its TLR5 and NLRC4 recognition sites located in D1 are also exposed, owing to its adjuvant activity (67-72), while only the variable (D2) and hypervariable domains (D3) are largely solvent exposed in filament formation (Figure 1.2: (66)). *In vitro* studies revealed that FliC is able to form filaments hundreds of nanometers in length (73). This is a critical feature for the success of TI antigen, as it has been identified that a TI antigen needs to be at least 500 nm in length to produce a potent TI response (74; 75). Moreover, FliC polymerization *in vitro* seems to be highly reliant on the ionic environment, and it has been identified that high molar salt conditions are more efficient in promoting FliC polymerization (73). Thus, FliC holds the potential for being a potent TI antigen as it has many of the features required to stimulate a TI response.

4.4 Flavivirus with emphasis on Dengue virus

As mentioned in the previous section, TI responses can produce neutralizing antibodies against various viral glycoproteins due to their repetitive nature. The genus

flavivirus (FV) is a large family of viruses in which their capsid proteins are highly organized and repetitive in nature and to date there are 70 identified members (76-78). All FV genomes are positive-strand RNA encoding three structural proteins (capsid, precursor of membrane, envelope (E)) and seven non-structural proteins (78). The E protein is the major protein of the capsid and is crucial for binding and fusion with host cells. It is not surprising most vaccine strategies for FV have attempted to target the E protein. Moreover, this class of viruses is largely hematophagous arthropod-borne viruses; for examples yellow fever virus, West Nile virus, Japanese encephalitis virus, and Dengue virus (DENV) are all transmitted via mosquitos, while other family members are transmitted to the host by ticks (77). As a result of both climate change and increased travel, these viruses have invaded new locations, posing a huge threat to human health (79). In fact the WHO estimates that 2.5 billion people (over 40% of the world population) are at risk for contracting Dengue.

While vaccines have been licensed for several members of FV, to date no vaccine has been approved for the treatment of DENV infection. DENV is unique among the FV as four major serotypes have been identified (DENV1 to DENV4), and recently a potentially new serotype was identified (80; 81). In its most mild form, DENV infection results in a flu like symptoms, however more severe infections lead to DENV hemorrhagic fever (DHF) or DENV shock syndrome (DSS: (80; 81)). DHF and DSS are not usually due to the primary infection but rather a result of a second infection by another DENV serotype. This process is termed antibody dependent enhancement (ADE: (82)). Efforts have been focused on identifying the mechanism that underlies the sudden onset of increased vascular permeability and hemorrhaging resulting in DHF/DSS. A seminal study by Kurane *et al* found that sera from patients with DHF have

increased levels of soluble CD4, CD8, and IL-2 (83), and based on this study it is believed that T cells play a defining role in promoting DHF/DSS. DHF/DSS patients also exhibit increased CCL2 (also known as monocyte chemotactic protein-1), which has been linked to reduction of TJs of the vascular endothelium (84). Paradoxically, a study from Yauch and colleagues found that DENV specific CD8⁺ T cells are protective in a mouse model of DENV ADE (85). Nonetheless, all these point to a role for T cells in DENV infection, however whether or not they play a protective or pathogenic role still remains unclear.

Mechanistically, ADE is a result of non-neutralizing antibodies remaining in the host after the primary DENV infection (82). This process of ADE has largely hindered vaccine development for DENV infections, as a vaccine is required to be equally effective against all four serotypes of DENV. In fact most DENV vaccines have failed in clinical trials due to lack of broad efficacy against the various serotypes. The lead vaccine strategy has been a mixture/formulation of four live virus strains representing the four DENV serotypes that were attenuated by serial passage in primary dog kidney cells. This live-attenuated tetravalent dengue virus vaccine (TDEN) was developed by the Walter Reed Army Institute (WAIR) and various formulations have entered clinical trials (86-89). Recently, two formulations of this TDEN entered phase II clinical trials where healthy adults were used to identify their efficacy and it was concluded that it was only modestly immunogenic for the four DENV serotypes (90). Other vaccine strategies have been based on chimeric viral vaccines design, in which the genome from live attenuated yellow fever virus was engineered to express the E protein of the four DENV serotypes (91-95). Recently, a tetravalent chimeric vaccine, the Sanofi's dengue vaccine, completed phase III clinical trials, but yielded disappointing results against DENV2

infection (96). Thus, a universal DENV vaccine still remains elusive and is still an ongoing area of research.

4.5 Thesis aim

Chapter 5 puts forth the design of a new TI antigen using bacterial FliC as vaccine scaffold. Native FliC from *S.Typhimurium* was reengineered such that the D3 domain was replaced with full functional viral antigens, specifically the E protein from DENV2. This new hybrid FliC was able to form polymers with immunogenic activity greater than its monomeric counterpart in both TD and TI restricted mouse models. Together these data suggest hybrid FliC can be used as TI antigen as well as having potential for use as DENV vaccine.

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Chapter 5

Hybrid flagellin as a T cell independent vaccine scaffold

5.1 Introduction

Adaptive immune antibody responses rely primarily on the interaction between Th cell populations promoting B cell activation, isotype switching, and development of plasma cells producing immunoglobulin, with subsequent pathogen opsonization/viral neutralization (1-3). However, in T cell-deficiency settings such as AIDS, this TD mode of antibody production is ineffective at conferring protection to the host. However, “innate-like” B cells, which include splenic marginal zone B cells (15% of total B cells) and peritoneal B1 B cells (B1a and B1b, 3% of total of B cells), can become activated after recognition of repetitive or polymeric antigenic epitopes causing B cell receptor (BCR) clustering, independently of Th subset (TI: (4; 5)). Efficient BCR clustering is crucial for the generation of a TI antibody response and in fact it has been determined that 10-20 BCRs must be crosslinked in order to mount a TI response (6; 7). On resting B cells, BCRs are spaced approximately 35nm apart, requiring TI antigens to be at least 500nm in length (7). Once a TI B cell has become activated, its antibody response is largely characterized by the production of IgM and certain IgG isotypes (IgG3 and possibly IgG1: (9)). B1 B cells have also been found to undergo class switch recombination from IgM to IgA. Although the anatomic site of this isotype switch remains elusive, this suggests that a significant proportion of IgA may be generated in a TI manner (10).

While many bacterial capsular polymeric components have been studied for their ability to stimulate a TI antibody response, it remained to be tested whether bacterial flagellin, a major antigen present on many commensal and pathogenic microbes, can be exploited as a scaffold for TI vaccines (11; 12). Indeed, one study suggested that polymeric flagellin, like its monomeric form, relies on T cell help to stimulate a humoral

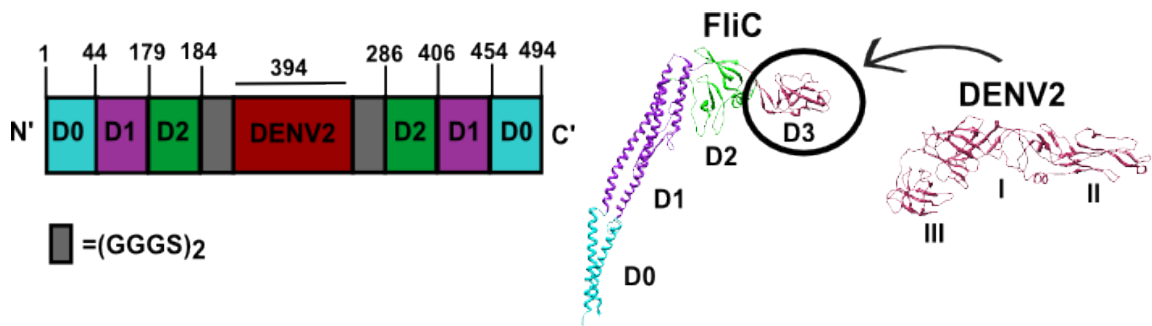


Figure 5.1. Design of hybrid flagellin protein. Schematic of the construction and design of hybrid flagellin (hFliC). The D3 domain from the parent FliC protein was deleted (residues 185 to 285). DENV2 plus GS linkers flanking the termini were inserted to replace the D3 domain. The final gene product was cloned into the pENTR plasmid for baculovirus expression of the hybrid protein

immune response. However, this study did not include IgM and IgG3 (two major antibody isotypes indicative of TI response) in their analysis (13). FliC from *S. Typhimurium* is the major protein making up the Flagellum and the flagellum can grow to 15 μm in length (8). Monomeric FliC is a four-domain (D0, D1, D2, D3) protein that structurally resembles the Greek letter Γ (Figure 1), and has been well characterized for its adjuvant activity due to its TLR5 and NLRC4 binding sites located in its D1 domain, a domain also responsible for polymerization (8; 14-16). In polymerized FliC filaments, the D3 domain of FliC is completely solvent exposed, while other domains remain mostly buried within the inner core. A study from Muskotal *et al* found that as a monomer the D3 domain is the most stable portion of FliC, where D0 and D1 completely lose their α -helical secondary structure; corroborating previous structural data which due to lack of secondary structure could not resolve the D0 domain. Moreover, by eliminating D3, this study found that D3 was dispensable for the overall stability of the flagellum filament (17), marking D3 as a possible insertion site for novel antigenic determinants. Our new approach was to test this site for insertion of a fully independent conformational domain as an antigenic vaccine target.

5.2 Results

5.2.1. Design and development of hFliC

Based on the study by Muskotal et al we sought to replace the D3 domain of FliC with a vaccine antigen to increase its exposure for TI mediated antibody response. Thus, to assess the ability of the FliC D3 loop to support an independent domain, we reengineered FliC by replacing D3 (from residue 185 to 285) with our vaccine antigen DENV2 (Dengue 2 envelope protein: Figure 5.1). DENV2 is one of three structural proteins produced by the Dengue 2 virus (there are now five identified Dengue serotypes, sharing 70-80% homology) and is the major protein component of the viral capsid, so it has been extensively studied for its antigenicity as a DENV2 vaccine (18). We added Gly-Ser flexible linkers to join FliC to the N- and C-terminus of DENV2 in order to provide the conformational freedom needed for both proteins to fold into their appropriate and respective three-dimensional structures. In addition, a C-terminal His₆-tag was added for purification purposes. The final hybrid FliC protein (hFliC) was produced using a baculovirus expression system, purified (Appendix Figure C.1A), and subsequently assayed for appropriate structure and function.

5.2.2. Characterization of hFliC monomers and polymers

First, circular dichroism (CD) was used to assess if the purified hFliC protein had the anticipated secondary structure (Figure 5.2) compared to free FliC and DENV2. Quantification of percent secondary structure from the CD spectra revealed that FliC (protein size and purity confirmed: Appendix Figure C.3A) was largely α -helical. Meanwhile, DENV2 (protein size and purity confirmed: Appendix Figure C.3B) had a larger β -sheet region, consistent with previous published spectra, and was in good

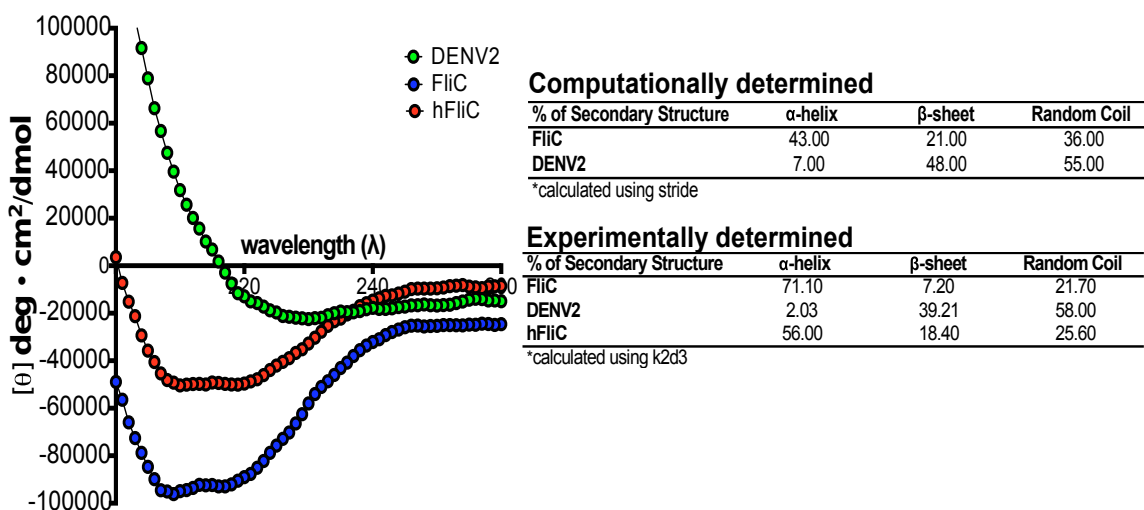


Figure 5.2. Assessment of secondary structure of hFliC Protein. Circular dichroism (CD) was used to assess secondary structural feature of the hFliC protein. CD spectra of FliC (blue curve) exhibit a characteristic α -helical region, while DENV2 spectra (green curve) exhibit a large β -sheet and coil regions. Free FliC and DENV2 percentage of secondary structure was quantified from the CD spectra, and was in good agreement with the predicated degree of secondary structure quantified from their respective crystallographic structure. hFliC CD spectra (red curve) was essentially a hybrid of the two proteins, and this was further confirmed by quantification of its percent of secondary structure.

agreement with computationally determined secondary structure percentages based on crystallographic structures (Figure 5.2: (19; 20)). Remarkably, the hybrid hFliC exhibited a spectrum of mixed α -helical and β -sheet character (Figure 5.2). Since hFliC monomers exhibited proper folding, we next wanted to determine if hFliC was able to form filaments. Using negative stain transmission electron microscopy (TEM), we determined that the hFliC protein was able to form filaments in high molar ammonium sulfate or sodium

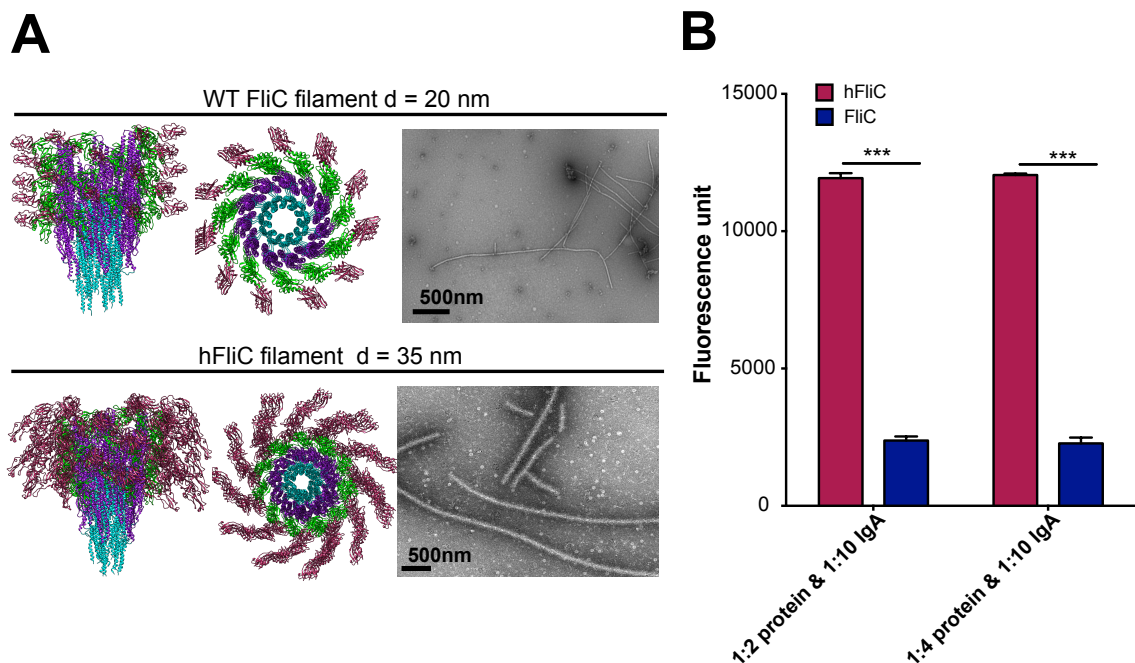


Figure 5.3. Analysis of hFliC Filament. (A) The top panel represents molecular structure of the parent FliC filament (8) (left panel: XY view and middle panel: Z view). Negative stain TE micrograph of WT FliC filament generated by recombinantly produced FliC (right panel); FliC filament diameter was calculated to be 20nm. The bottom panel represents a hypothetical molecular model of hFliC, where DENV2+GS linkers were modeled into native FliC filament to replace D3 domain. The diameter of hFliC was computationally determined to be 35nm. Negative stain TE micrographs confirmed resulting hFliC filaments had increased diameter compared to FliC filament. (B) Binding ELISA was performed using DENV2 specific monoclonal IgA antibody. hFliC showed binding to monoclonal IgA, compared to FliC alone, which showed no binding. Experiments were performed in triplicate (n = 3), and error bars represent mean +/- SEM, Student's t-test was used to determine statistics, and a p < 0.05 were considered statistically significant.

citrate (Figure 5.3A, and Appendix Figure C.1B), the purported molar conditions for *in vitro* WT FliC polymerization (21). The resulting filaments had an increased diameter of 35 nm compared to FliC filaments (diameter of ~20 nm (8)), which was consistent with the hypothetical molecular model of the hFliC filament (Figure 5.3A), it however remains unclear whether the hFliC forms prototypical 11-protofilament structure (suggested in Figure 5.3A), or rather a more fibril-like structure.

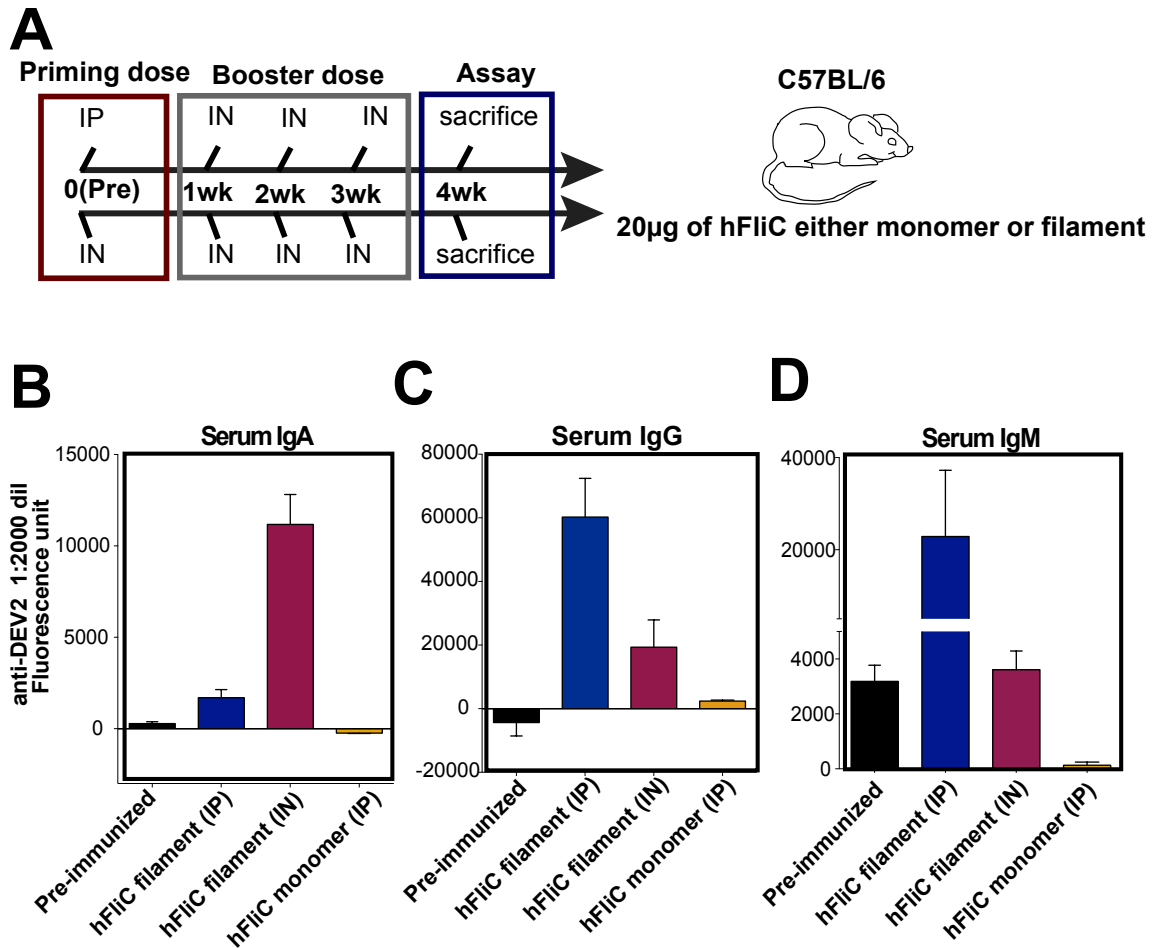


Figure 5.4. Humoral immune response in non-TI restricted mouse models. (A) Schematic diagram of *in vivo* immunization protocol used. The immunization period was five weeks, with four injections spaced a week apart. Priming injection at week zero was either administered IP or IN, and the three subsequent booster doses were all administered IN. (B) ELISA scans show serum IgA response in WT C57BL/6 mice, assayed after the five-week immunization period. C57BL/6 mice given polymeric hFluC IN throughout the entire immunization exhibited a strong serum IgA response, while all other mice showed no IgA titer. (C) ELISA scans for serum IgG response in WT C57BL/6, assayed after the five-week immunization period. C57BL/6 mice given polymeric hFluC IP showed a stronger IgG response compared to mice given monomeric hFluC IP. (D) ELISA scans for serum IgM response in C57BL/6 mice assayed after the five-week immunization period. Again, all groups of mice showed and again mice receiving polymeric hFluC IP showed strongest IgM response.

The inner core of the hFliC filaments exhibited a high degree of contrast, as compared to Tobacco Mosaic Virus (TMV) filaments (known to have high contrast) (Appendix Figure C.2A and Appendix Figure C.2B). However, the outer regions of the filaments displayed a low degree of contrast relative to the rest of the filament suggesting that the DENV2 antigen domain was flexible (further confirmed via cryoelectron microscopy, data not shown). This flexibility may aid in the avidity of the DENV2 antigenic domain of the polymeric hFliC vaccine. Finally, to test if the DENV2 portion was functional, we used a DENV2 specific recombinant IgA monoclonal antibody, (Appendix Figure C.4A and Appendix Figure C.4B) to test the binding ability of the DENV2. The DENV2 portion of the hFliC was able to bind the 4G2 IgA (Figure 5.3B, negative control FliC alone). We also observed that DENV2 portion of hFliC was able to bind DENV2 specific IgG, which was confirmed by immunogold labeling of the hFliC filament using a DENV2 specific IgG isolated from 4G2 B cell hybridoma (Appendix Figure C.2C and Appendix Figure C.2D).

5.2.3. In vivo activity hFliC in TD restricted model

Since hFliC exhibited proper structure and function, we next wanted to study its ability to produce a humoral immune response. Using an immunization protocol similar to that used by Lo *et al.* (22) but in this case without the addition of any additional adjuvant (such as cholera toxin), we tested the activity of both monomeric and polymeric hFliC in non-TI restricted mouse models (WT C57BL/6; Figure 5.4A). We first administered a priming dose (either intraperitoneal (IP) or intranasal (IN)), followed by three IN booster doses at one-week intervals. First, we found that hFliC filaments resulted in a stronger antibody response than their monomeric counterparts in C57BL/6

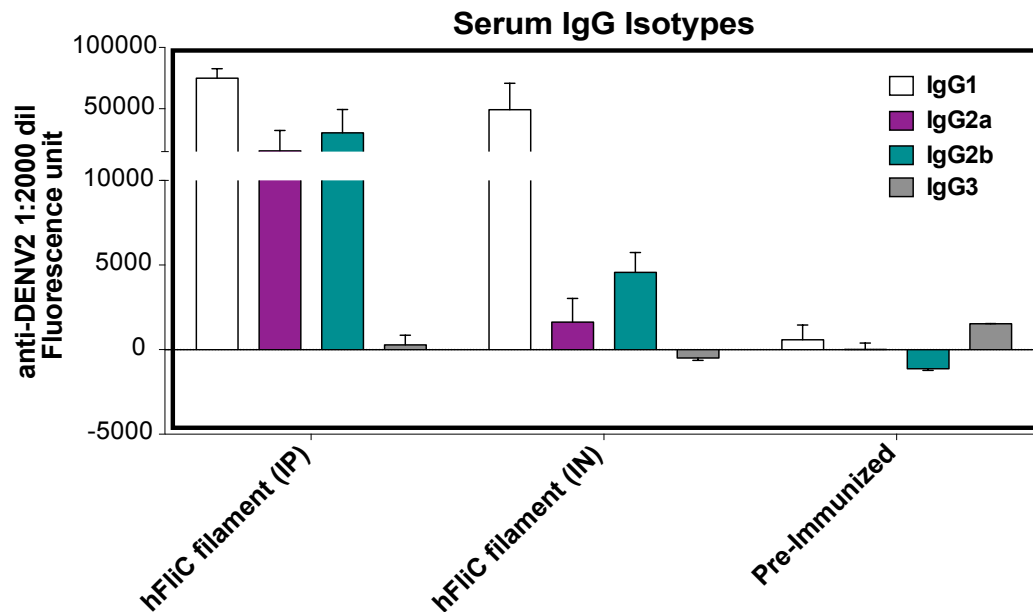


Figure 5.5. Ig isotype produced in WT mice. Serum IgG isotypes were assayed in C57BL/6 mice, mice given polymeric hFliC IP showed a robust IgG1 response. All groups in immunization protocols used an n = 3-5 mice per group, and error bars represent the mean $\pm$ SEM. Values were reported as the fluorescence measured in 1:2000 serum dilutions, with background fluorescence values subtracted.

WT mice (Figure 5.4B-D and Appendix Figure C.5). IP administration of polymeric hFliC during the priming injection resulted in robust serum IgG (total) and IgM responses (Figure 5. 4C, and Appendix Figure C.5A and Appendix C.5C). Interestingly, mice given IN priming and booster injections of polymeric hFliC throughout the immunization protocol showed a strong serum IgA response (Figure 5.2B and Appendix Figure C.5B). This might be helpful in the development of Dengue vaccines, as IgA could compete with IgG and reduce the potential for antibody dependent enhancement (ADE), a phenomenon that exacerbates the pathogenesis of the Dengue virus resulting in Dengue Hemorrhagic Fever (DHF: (23; 24)). We next wanted to test if polymeric administration of hFliC via different routes of delivery resulted in the production of different IgG

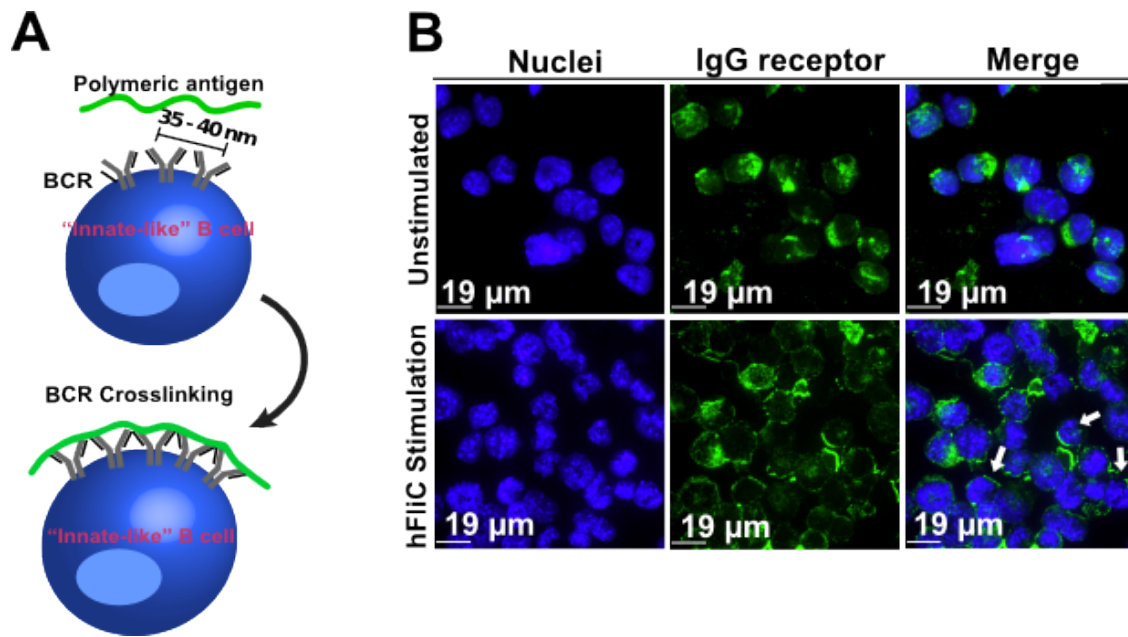


Figure 5.6. hFliC filaments are capable of crosslinking. (A) Schematic of how TI immune response is generated. Polymeric antigens bind to B cell receptors and crosslink them. Once this crosslinking event occurs, the B cell will self-differentiate into plasma cells to produce antibodies. (B) 4G2 B cell hybridoma (DENV2-specific IgG producing hybridoma) stimulated with polymeric hFliC. Stimulated cells showed more organized expression of Ig receptors compared to unstimulated cells, indicating cells were crosslinked.

subclasses. WT C57BL/6 mice given polymeric hFliC via IP priming injection produced a strong Th2-associated serum IgG1 response, with a lower serum IgG2b response (Th1-associated); meanwhile, IN administration resulted only in serum IgG1 (Figure 5.5).

5.2.4. *In vivo* activity hFliC polymers in TI restricted model

TI responses rely on the ability of antigens to crosslink a sufficient number of BCRs (Figure 5.6A). Thus, we first wanted to determine if hFliC filaments were capable of BCR crosslinking. We incubated 4G2 B cell hybridoma cells directly with hFliC filaments, and examined their effect on surface BCRs. Confocal micrographs of unstimulated cells exhibited a randomized distribution of Ig receptors, meanwhile cells

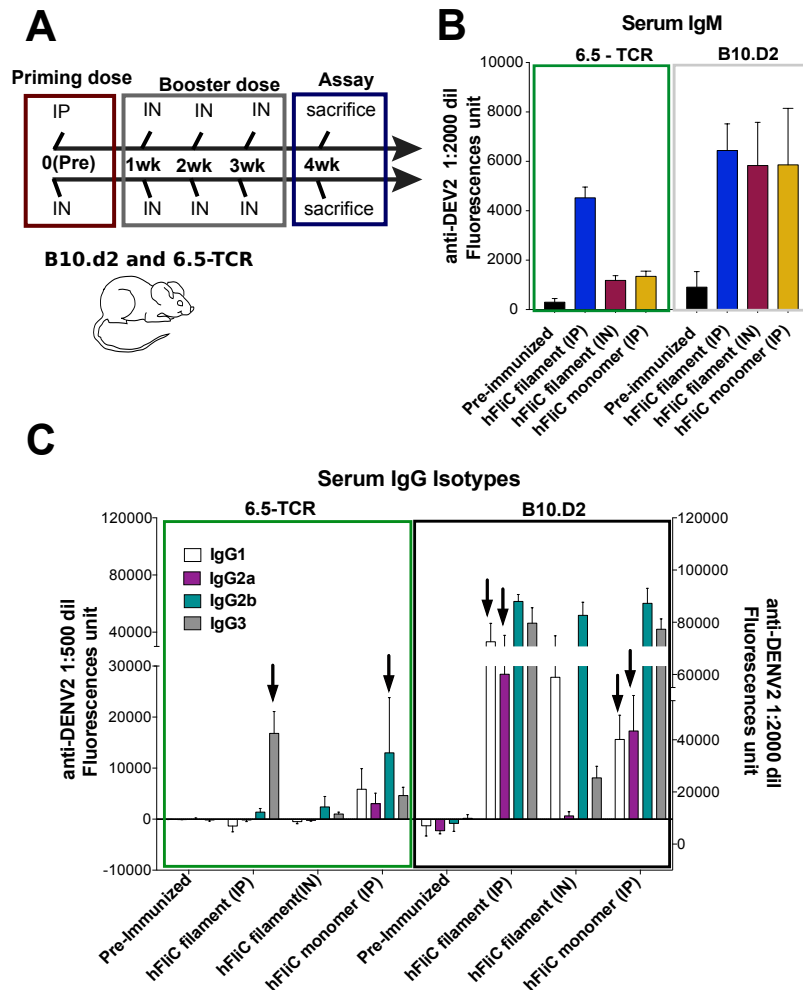


Figure 5.7. Humoral immune response in TI mouse model. (A) Schematic of immunization protocol. (B) ELISA scans of serum IgM in 6.5-TCR and B10.D2 over five- week immunization period. Only mice given polymeric hFlIC IP in the priming dose showed a serum IgM response. All groups B10.D2 mice immunized showed strong IgM response. Statistics were determined by unpaired Student's t-test, $p < 0.05$ were considered statistically significant, and error bars represent the mean $\pm$ SEM. (C) ELISA scans of serum IgG isotypes in 6.5-TCR, and B10.D2 mice. 6.5-TCR mice given polymeric hFlIC IP in the priming dose exhibited a specific IgG3 response, meanwhile B10.D2 mice showed broader subclass distribution (more balanced between Th1 and Th2). All groups in immunization protocols used an $n = 3-5$ mice per group, and error bars represent the mean $\pm$ SEM. Values were reported as the fluorescence measured in 1:2000 serum dilution in the case of IgM and IgG isotypes for B10.D2 immunized mice, and values were reported as the fluorescence measured in 1:500 serum dilution for IgG isotype for 6.5-TCR immunized mice, with background fluorescence values subtracted

stimulated with hFliC filaments exhibited a more organized distribution of the BCRs around the circumference of the cell (Figure 5.6B, showing staining of surface Ig, further confirmed by his-tag staining), suggesting that the receptors had in fact been clustered by the hFliC filament.

To test the potential for the polymeric vaccine to induce TI responses, we performed *in vivo* immunization, but this time in a T cell restricted model (Figure 5.7A). Here we used a T cell receptor transgenic mouse model (6.5-TCR, recognizing an influenza hemagglutinin peptide presented on MHCII I-Ed: (25; 26)), in which the mono-specific hemagglutinin-specific T cells would be unable to provide help to FliC- or DENV-specific B cells and the transgene also excludes development of other T cells, while all other components of the immune system would develop normally. In addition, since the 6.5-TCR mice were on a B10.D2 background, B10.D2 mice were as WT control. With an immunization protocol using hFliC filaments administered IP (priming dose), 6.5-TCR mice were still capable of producing a strong serum IgM response within the range of B10.D2 controls (within a two-fold dilution), though no detectable levels of IgA were observed in any condition (Figure 5.7B). Interestingly, B10.D2 mice treated with polymeric hFliC in three IP doses exhibited the most robust IgM and IgG of any group of mice used in this study (Appendix Figure C.6), raising the possibility that this protocol was specifically targeting peritoneal B1 populations. Finally, we wanted to determine if differences existed in the IgG isotypes produced in non-TI (B10.D2) or TI (6.5-TCR) restricted models, as certain IgG subclasses have been identified as more prominent in TI antibody responses. B10.D2 mice showed a robust Th1 serum IgG2b response, with a lower serum IgG3 response (Figure 5.6C). Interestingly, although IgG titers were much lower in the 6.5-TCR mice as compared to B10.D2 mice due to the absence of cognate.

T cell help, 6.5-TCR mice given hFliC filament IP still showed a detectable IgG3 response (Figure 5.6C).

5.3 Discussion

Taken together, these data suggest that hFliC filaments are indeed capable of producing a TI antibody response in a T cell restricted model. Development of therapeutics with dual function or with multivalent binding (clustering) has largely been accomplished by the linkage of two proteins in a linear fashion; however, this method has had limited success in certain diseases (27-29). Thus, the reengineering of FliC by replacing its solvent exposed D3 domain with antigen determinants, may hold promise for an entirely new vaccine framework, based on the design of vaccines with true dual functionality. This is especially important for development of vaccines capable of producing protective immune responses, especially in the early perinatal period, in people suffering from various T cell immunodeficiency disorders (HIV-AIDS, DiGeorge syndrome), patients undergoing chemotherapy, as well as elderly patients with sluggish adaptive immunity.

In the context of Dengue viral infection, to date no licensed vaccine exists for DENV infection. This is complicated by the concern that sub-neutralizing levels of anti-DENV antibodies might result in more severe disease (i.e., Dengue Hemorrhagic Fever). Thus, it is crucial to develop a vaccine framework that is equally effective against all five DENV serotypes. Live attenuated tetravalent viruses have shown only limited success in both non-human primates and humans, but it has been difficult to establish a proper balance between immunogenicity and adverse reactions with these formulations (30; 31). In addition, recombinantly produced DENV subunit vaccines have been found to induce neutralizing titers in mouse models when co-administered with adjuvant (32), and

although safer than live attenuated DENV vaccines, were found to be less efficacious. Our approach of reengineering FliC to replace its solvent exposed D3 domain with antigenic determinates may be able to combine the benefits of the immunological adjuvant effects of flagellin with the added immunogenicity of polymeric vaccine antigens. Moreover, independent replacement of D3 with envelope proteins from all five DENV serotypes, and then producing mixed filaments with stoichiometric amounts of each hFliC protein could lead to the development of a universal DENV vaccine with balanced broadly neutralizing capabilities (i.e. against all serotypes). This strategy may overcome the problem noted in a recent trial with a tetravalent recombinant vaccine, in which the response to one serotype was much lower than the response to the other serotypes(33).

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analyzed data as well as wrote the manuscript. DDL, helped design the experiment and wrote the manuscript. RDG and DM performed all computational analysis. VG and LT cloned, produced and analyzed DENV2 specific monoclonal IgA. The authors claim no competing financial interests.

5.5 Methods and Materials

5.5.1. Design of hFliC

Flagellin was PCR amplified from an earlier vaccine design, where the DENV2 portion was linked to FliC at the N' terminal domain through a GS linker. This FliC was first PCR amplified from genomic DNA isolated from a culture of *S. Typhimurium*. Flagellin was PCR amplified in two fragments. The first piece was amplified using a forward primer with flanking 5' BamH1 site and reverse primer with 3' flanking Kpn1, EcoR1 site, that had amplified only the first 184 residues. The second piece was amplified using a forward primer with 3' flanking Kpn1, EcoRI site and reverse primer with 5' Xho1 site, which amplified residues 285-494 plus a 6x-His tag. These two pieces were then PCR overlapped. DENV2 was PCR amplified from the initial vaccine construct, and flanking N' and C' terminal GGGSGGGS linkers were then ligated into FliC lacking D3 via the Kpn1 and Ecor1 sites. Gene constructs were checked for appropriate sequence throughout the cloning process. This final construct was then ligated into the pENTR 3C (life technologies) expression vector for recombination and subsequent expression.

5.5.2. Production and purification of recombinant proteins (hFliC, FliC and DENV2)

Recombinant protein was produced using Baculovirus expression system. First, cloned cDNA construct was transfected into SF21 insect cells (Life Technologies) in

Grace's supplemented or unsupplemented media (Thermo Scientific) based on manufacturer's protocol. Resulting protein viral stocks were then transduced into SF9 insect cells (Life Technologies), cultured in HyClone SFX-Insect media (Thermo Scientific), for larger scale production. The insect cells secreting the protein were cultured at 27°C, and the supernatant was harvested after an optimized incubation period. The insect culture media was filtered before purification. Western blot (Life Technologies) was used to verify protein expression and purity before precipitating protein from the media with 80% saturated ammonium sulfate (Fisher). After a 2 hr precipitation at 4°C, protein was centrifuged at 12,000 rpm for 30 mins to pellet the protein. The protein pellet was resuspended with 1XPBS + 0.05% Tween-20 and dialyzed over night in a porous membrane with a molecular weight cut off of 12-14,000 (SpectrumLabs). Protein was then bound to HisPur cobalt resin (Thermo Scientific) overnight. Bound protein was eluted from HisPur resin using increased concentrations of imidazole. Eluted protein was subjected to a final dialysis to rid the protein sample of any traces of Tween-20 and imidazole. The final protein was concentrated using polyethylene oxide (PEO: Sigma Aldrich). Concentrated protein was checked for purity using both Coomassie (Thermo Scientific) and Western blot (1° antibody rabbit- α -Ht (Santa Cruz Scientific) and 2° Donkey α -rabbit @680nm (Odyssey)), and concentration was quantified either using BCA or Bradford.

5.5.3. Molecular modeling of FliC and hFliC filaments

The structure of FliC (PDB code 3A5X) and DENV2 (PDB code 1OAN) were obtained from the Protein Data Bank(8; 18). The hFliC filament was built using the molecular structures of the FliC filament and the DENV2 protein. The D3 domain of a FliC monomer was deleted (residues 185-285), and the coordinates of DENV2 were

manually translated and rotated until the N- and C-termini of DENV2 were in close proximity to the C- and N-termini of the end of the D2 domain of FliC. The linkers between FliC and DENV2 (sequence GGGSGGGS) were added using Modeller (34).

5.5.4. Polymerization of hFliC or FliC

Based on previous studies, purified FliC or hFliC proteins samples were diluted in either 3.0M ammonium sulfate or sodium citrate(21; 35). A final buffer concentration of 1.5M was obtained for each buffer+protein sample. This molar condition has been suggested to be ideal for FliC polymerization. Each buffer+protein sample was allowed to polymerize overnight at room temperature. Pelleted with high-speed centrifugation, and washed in with 150mM PBS. These samples were then used for either TEM or vaccination.

5.5.5. Circular Dichroism

CD was used to determine secondary structure of the hFliC protein. CD spectra were collected for each protein (FliC, DENV2, hFliC) using Jasco J-815 CD Spectrophotometer. Measurements were performed at 25°C in a 1 cm path length cuvette (Varian), over a wavelength range of 190-260nm. Protein for CD measurements was diluted in water to eliminate the effects of NaCl absorption, which absorbs strongly at wavelengths below 190 nm.

5.5.6. Negative stain transmission electron microscopy

For transmission electron microscopy studies, formvar carbon coated grids (Ted Pella) were glow discharged for at least 20 seconds, 3µl of polymeric sample was then loaded onto formvar carbon coated grid, and excess liquid was removed. Grids were then washed 3 times in PBS. For viewing in TEM, grids were negatively stained with

either 4% uranyl acetate or 2% PTA (phosphotungsten acid) and placed into desiccator until imaged. Micrographs were obtained using JEM-1011 transmission electron microscope or PHILIPS TECNAI 12 transmission electron microscope.

5.5.7. Binding ELISA of hybrid flagellin

Binding ELISAs were performed to see if the hFliC was capable of binding DENV2 specific antibodies. Briefly, ELISA plates were coated with either purified hFliC or purified FliC (negative control) at two different dilutions (1:2 and 1:4) and incubated overnight at 4°C. Plates were then washed, blocked and DENV2 specific monoclonal IgA antibody (at two dilutions) was added and again incubated overnight. Detection was performed by adding anti-IgA antibody (Southern Biotech) conjugated to Alkaline Phosphatase, and 4-MUP (Life Technologies) was added for detection by fluorescence.

5.5.8. Design of DENV2 recombinant IgA

Mass spectrometry N-terminal protein sequencing results of purified D1-4G2-4-15 hybridoma (4G2; ATCC# HB112) antibody was used to determine the leader peptide nucleotide sequence of the variable regions. 4G2 Dengue-specific kappa light and IgG2a heavy chain sequence was cloned and deposited in Genbank; NCBI accession code for kappa light chain is KJ438784 and IgG2a heavy chain is KJ438785. The full-length kappa sequence was amplified from 4G2 cDNA and cloned into pCDNA3.1(+) (Life Technologies) using NheI and EcoRI sites. The heavy chain variable region amplified from 4G2 hybridoma cDNA was overlapped with the IgA heavy constant region amplified from BALB/c spleen cell cDNA(36). GS linker and 6XHis-tag PCR fragment was added with the using another round of overlap PCR. The complete Dengue specific IgA heavy chain was cloned into pCAG-eGFP-puro by replacing the eGFP with AgeI and NotI

before ligation and transformation(37). The Dengue specific IgA heavy chain clone was confirmed by sequencing.

5.5.9. Production and purification of DENV2 recombinant IgA

Dengue specific kappa light and IgA heavy chain DNA constructs was stably transfected into the Chinese hamster ovary cell line (CHO; CHO-K1; ATCC# CCL-61) and clonally selected via limit dilution under selection media (F-12K media (Corning) supplemented with 10% fetal bovine serum (Biowest), 1000µg/ml G418 (Corning), and 10 µg/ml puromycin (Sigma)). Final highest IgA-producing clone was adapted to serum free media (Corning) and used for production. The final clone was expanded in 40-T150-flasks and overgrown for one week prior to supernatant collection in serum free media. One liter of the supernatant media total protein was used to purify the rIgA (purification step as mentioned above). The production yield and purity was determined by Western blot, Coomassie, DENV2 protein binding ELISA, and total IgA ELISA quantification.

5.5.10. Immunogold labeling of hybrid flagellin filaments

Immunogold labeling was performed to further confirm that the DENV2 subunit was presented on the surface of the filaments. Here, filaments were loaded onto carbon coated formvar grids. The grids were then incubated in 1XTBST (tris-buffered saline with Tween) with 0.05% BSA (Bovine serum albumin) for 30 minutes. Grids were washed and incubated with 4G2 (Dengue specific IgG hybridoma) for two hours; again the grids were washed and incubated with Goat anti-mouse IgG conjugated gold particles (10nm: Ted Pella). Finally, the grids were stained with 2% uranyl acetate and viewed.

5.5.11. *B cell crosslinking*

Crosslinking studies were performed using a 4G2 B cell hybridoma (ATCC# HB112). 4G2 cell line was cultured/maintained, based on ATCC recommendation, using Dulbecco's modified Eagle's medium (4 mM L-glutamine, 4.5g/L of glucose, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum: Cellgro). Cells were maintained at a concentration 1×10^6 cells/ ml, and were passed every three days. To assess if hFliC filaments were able to crosslink the BCRs of the 4G2 cell line, cells were first stimulated with a 20 μ g dose of hFliC filaments (prepared as mentioned above) for 30 mins under ambient conditions. After the 30 mins incubation, cells were washed 3X in 1XPBS. Cells stimulated with hFliC filament antigen as unstimulated control were then spun onto glass slides (Superfrost plus, Fisher Scientific) using a Cytospin (Shandon). Cells were then fixed with 1% Paraformaldehyde/PBS and then permeabilized first with cytoskeleton buffer, followed by 0.5% Tween-20/PBS and washed with 3X 0.1% Tween-20/PBS. Cells were then blocked with 0.1% Tween-20 Casein solution (Thermo Scientific) for at least 30 mins. Cells were then stained with polyclonal IgG antibody (@488nm) for at least 1hr, and again were washed. Finally, cells were post fixed with 4% PFA/PBS solutions and mounted with Prolong Gold antifade reagent (Life Technologies), which contained DAPI to counterstain the nuclei. Images were obtained using a spinning disk BD CARVII Confocal Imager (BD Biosystems) attached to a Zeiss Axio Observer inverted microscope. Hardware, including microscope, confocal and digital camera (Qimaging Rolera EMC²) was controlled using Metamorph imaging software. Images were further optimized using Volocity deconvolution software.

5.5.12. Immunization protocol

C57BL/6J, B10.D2, 6.5-TCR (Tg(Tcra/Tcrb)1Vbo) mice were maintained under specific pathogen free colony conditions at the UC Riverside vivarium. All procedures were performed in accordance with institutional and NIH guidelines. Immunizations were performed over a five-week period, where the first dose (priming dose) was administered either through intranasal (IN) or intraperitoneal (IP) injections. For intranasal injections, a volume of 20µl containing 20µg protein was administered (10µl containing 10µg of protein into each nostril). For intraperitoneal injections, a volume of 200µl, again with a total protein concentration of 20µg, was injected into the peritoneum of mice using a 25 gauge needle. It should be noted all protein samples were diluted in 1XPBS to its final protein concentration. All mice were first anesthetized prior to injection. Three subsequent booster doses were given to all groups of mice via IN injections. Serum titers were assayed from peripheral blood collected by at the time points indicated. For fecal antibody analysis, dry fecal pellets were weighed, and extracted in a proportionate amount of extraction buffer (1 ml PBS with 0.1 mg/ml trypsin inhibitor (Sigma) per 100 mg fecal pellet). After incubation and centrifugation, 400 microliters of supernatant was mixed with 100 microliters glycerol/1 mM PMSF (Thermo Scientific) for storage. In all experimental groups, four mice were used per group. Mice were humanely sacrificed under anesthesia and cervical dislocation at the conclusion of the experiment.

5.5.13. ELISA for quantification of antibody titers

Black flat-bottom plates (Costar) were coated with 10µg/ml recombinant DENV2 (prepared as described earlier), in coating buffer (25 mM Na₂CO₃, 75 mM NaHCO₃, pH 9.5). Plates were then washed 3X using 1X TBST buffer (50 mM Tris pH 7.5, 0.28 M NaCl, 6 mM KCl, 0.1% Tween 20), using a Biotek ELx405 Automated Plate Washer.

Plates were blocked for 2 hrs at room temperature in 3% normal goat serum (Vector Labs). Samples were first diluted in blocking solution; serum was diluted 1:2000 and feces were diluted 1:10. Diluted samples were then two-fold serially diluted, and added to coated/blocked plates in triplicate. After washing, detection was performed with either Rat anti-mouse IgA-AP (Southern Biotech, diluted 1:1000), Goat anti-mouse IgM-AP (Southern Biotech, diluted 1:1000), Goat anti-mouse IgG-AP (Southern Biotech, diluted 1:2000), Goat anti-mouse IgG1-AP (Southern Biotech, diluted 1:1000), Goat anti-mouse IgG2A-AP (Southern Biotech, diluted 1:1000), Goat anti-mouse IgG2B-AP (Southern Biotech, diluted 1:1000), Goat anti-mouse IgG3-AP (Southern Biotech, diluted 1:1000), in 1X TBST. For final development, 10 mM 4-MUP (Molecular Probes) in DMSO diluted 1:25 in substrate buffer (50 mM K_2CO_3 2 mM $MgCl_2$, pH 9.8) was added. Fluorescence was detected 60 minutes later 360nm excitation and 460nm emission wavelengths on Molecular Devices SpectraMax M2e plate reader. Raw fluorescence values from the dilution series were then subtracted from background fluorescence values.

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Chapter 6

Perspectives and future directions

6.1 Perspective and future directions for Chapter 2

The intestinal epithelium has long been viewed as passive barrier functioning to limit pathogens from invading the host. It is now believed that this barrier plays a much more active role in directing pathogen binding meanwhile maintaining a mutualistic relationship with the resident microbiota (1). Microvilli, finger like structures present on the apical surface of polarized IECs, once solely linked to nutrient uptake, have now been implicated in pathogen invasion. In fact, microvilli seem to be the main target of various pathogens to gain entry into the host, one strong example of this is in the case of the attachment and effacement process used by various gram negative bacterium, in which microvilli are knocked out by bacterial effector proteins hijack host cytoskeletal machinery (see introductory chapter 1: (2)). This poses the question why does the pathogen expend all the energy to downregulate microvilli? And does this suggest that microvilli may have other functions not related to nutrient absorption, perhaps playing an active role in pathogen infection?

The field of colloidal chemistry has largely classified bacteria as charged particles, studying how changes in their surface charge might augment their ability to bind to various surfaces (3; 4). Remarkably, the GI tract is subject to local changes ionic strength, a property known to influence surface charge of bacteria and perhaps biological surfaces, like the epithelium. Thus, the intestine can be viewed as continuous colloidal system in which bacteria of various surface charges are suspended in dielectric medium, which might lead one to speculate that perhaps electrostatic forces, such as charge, may govern bacterial binding to the epithelium. In line with this, previous studies from our lab indicated that uptake of synthetic nanoparticles could be modulated in the nasal passages by simply altering the ionic environment (5). To further upon this notion

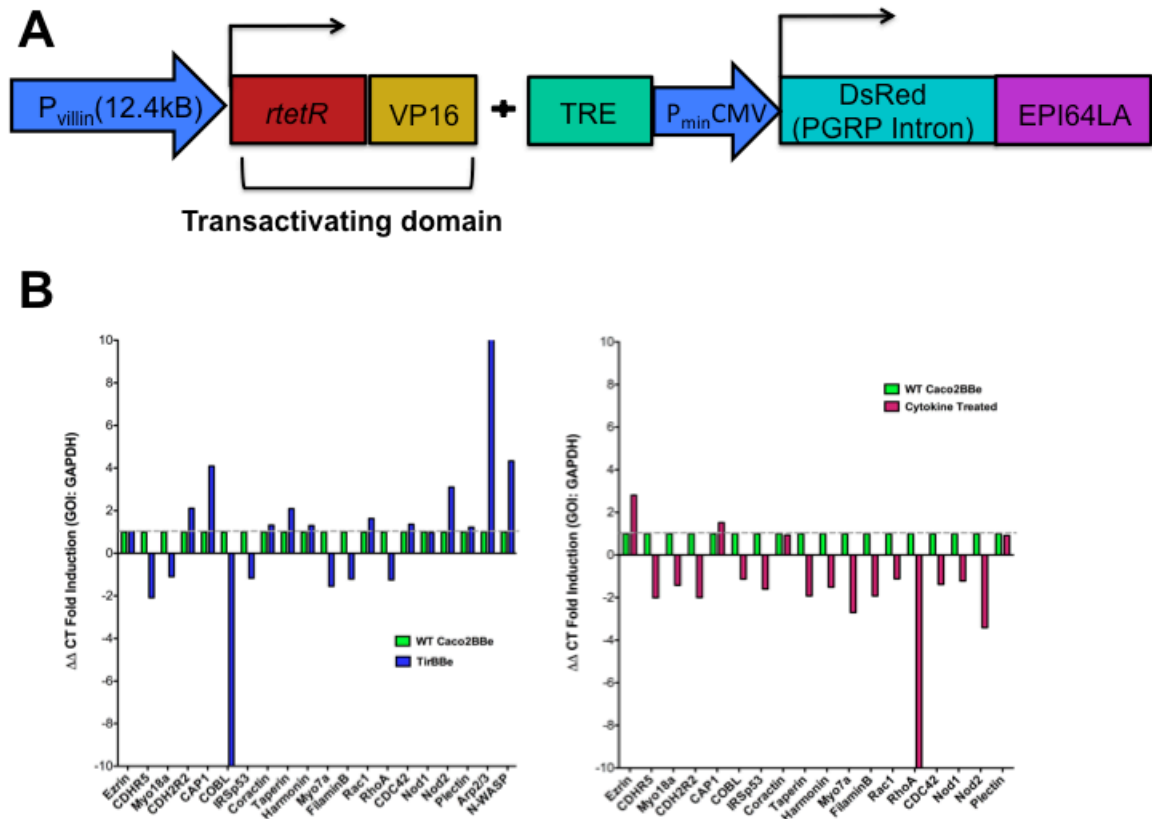


Figure 6.1 Genes level modulation of microvilli. (A) Schematic of inducible transgene to knockout microvilli formation *in vivo*. The IEC specific villin promoter drives expression of the transactivating domain (rtTA) once the mice are treated with tomaxifen the rtTA complex will bind to TRE and drive expression of the DsRed-EPI64LA transgene. (B) Transcriptional regulation apical associated proteins identified in proteomic study, in the Caco2BBE, TirBBE, and cytokine treated cell models.

we sought to identify whether polarized enterocytes due to the presence of microvilli, not only increase the surface area of the cell but also change the electrostatic potential of the cell. We found that in fact the presence of microvilli decreased bacterial binding in an *in vitro* setting, and this seemed to be linearly dependent on the bacterial surface charge. However, whether this effect is retained *in vivo* still remains to be determined. To test the *in vivo* relevance of this electrostatic barrier, a mouse model in which enterocytes lack the ability of generating microvilli must be established. Many attempts

to make such a mouse has only had limited success. Based on our *in vitro* studies, potentially either EPI64LA or Tir can be used to abrogate *in vivo* formation of microvilli (Figure 6.1A) and test whether this linear relationship is maintained.

To date, no single protein or transcription factor has been identified as the master regulator of microvilli formation. A beautiful proteomic study by McConnell and colleagues revealed several proteins that are specifically located at the apical surface of IECs (6). Interestingly, analyses of the promoter regions (i.e. 10 kb upstream of the transcriptional start) of these genes implicated in microvilli formation (i.e. Cordon Bleu, Protocadherin-24, etc: (7; 8)) seem to all contain putative Nkx2.5 and FoxJ2 binding sites (unpublished observation by Bennett and Lo). Perhaps these transcription factors can be studied for their role in microvilli formation. Moreover, the various cell lines reported in chapter 2 of this thesis, as well as other cell lines, in which microvilli formation has been abrogated in potentially disparate mechanism, exhibit transcriptional regulation of these apical associated proteins (Figure 6.1B: unpublished). Thus, transcriptional analysis of these cell lines could lead to the identification of the master regulator of microvilli, if one in fact exists. Additionally, in two mouse models of colonic inflammation we found a decrease in microvilli length (Figure 6.2), which may suggest that the proteins governing microvilli formation may potentially be susceptibility genes for IBD, and should be the focus of future studies.

6.2 Perspective and future directions for Chapter 3

M cells play a crucial role in mediating mucosal immune responses due to their ability to transcytose luminal antigens, where DCs and other APCs will then encounter these antigens and result in the priming of effector T cell populations (9). M cell

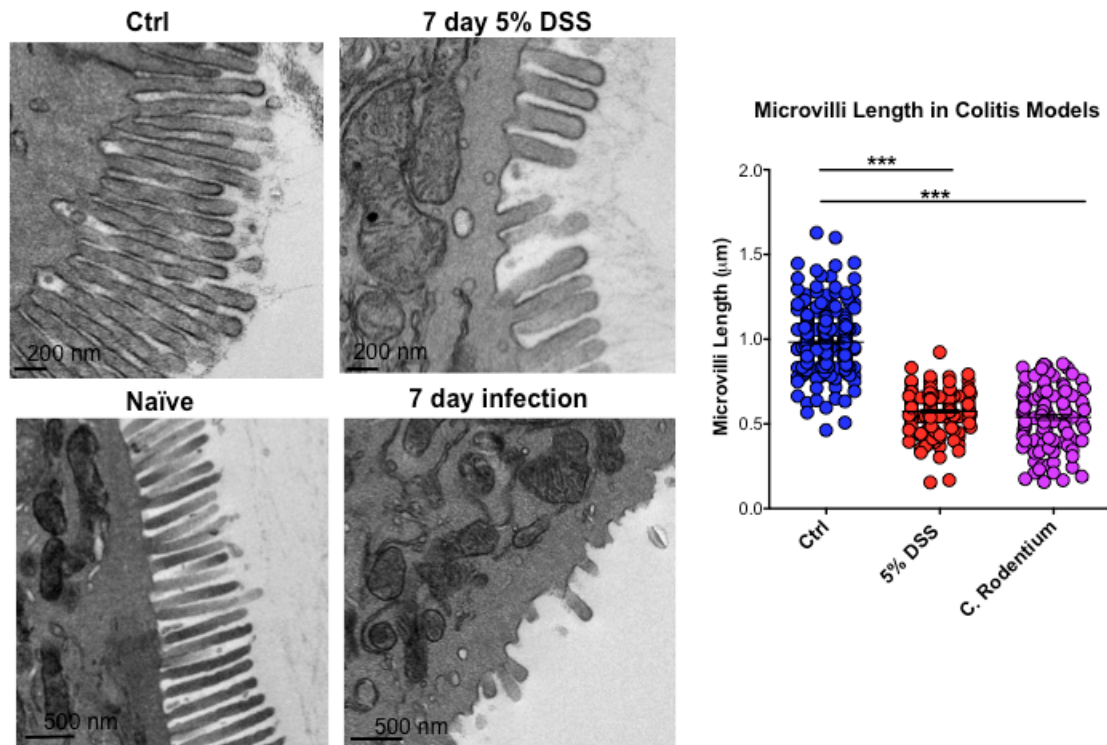


Figure 6.2 Modulation of microvilli during intestinal inflammation. Upper panel, transmission electron micrographs of colonic epithelium taken from water treated and DSS treated mice, mice treated with DSS exhibited significant reduction in microvilli length. Lower panel, transmission electron micrographs of colonic epithelium taken from naïve and 7 day infected *C.rodentium* infected animals. Mice infected with *C.rodentium* also exhibited a significant reduction in microvilli length. Right graph represents the quantification of microvilli length, linearly approximated using ImageJ

maturation and differentiation in the context of the SI FAE seem to be tied to local levels of pro-inflammatory cytokines (programmed inflammation). In chapter 3 of this thesis we identified that M cells become induced in the colonic epithelium during acute intestinal inflammation in two colitis models (8; 10). These M cells were associated with poorly organized follicles, in contrast to SI PP, which have a highly organized stromal cell network. One interesting question that arises is what happens to these disorganized colonic follicles during both chronic inflammation and recovery. Do they eventually generate a organized stromal cell network similar to SI PP that provide the necessary

signals to maintain the follicle and generate new M cells or do they recede once inflammatory signals are no longer present? Perhaps the induced M cell populations derive from the Lrg5⁺ crypt stem cells in the presence of inflammatory stimuli, and need that same level of inflammatory signals to persist. Alternatively, during active inflammation mononuclear cells including lymphoid tissue inducer (LTI) cell populations infiltrate into the colonic lamina propria and may persist providing trophic factors to maintain the follicle and generate new M cells. Thus, future studies should focus on M cell presence during epithelial recovery and perhaps chronic inflammation, which may help to further characterize M cell role in IBD.

Based on the study presented in chapter 3, colonic M cells appear to play a protective role during intestinal inflammation. This was largely substantiated by the fact that in the absence of functioning M cells inflammation and subsequent disease seemed to worsen (B cell deficient mice and mice treated with DSS and anti-TNF α). In addition, our data suggest that TNF α plays a crucial role in driving the induction of M cells in colonic epithelium. However, the exact mechanism that governs the M cell induction remains to be determined. Future studies should work to dissect the exact mechanism that drives the M cell differentiation and maturation in the colon. This process could perhaps also rely on other TNF family members as well as the TNF super family of receptors. Although M cells appear to play a protective role during intestinal dysbiosis, it remains unclear whether or not the presence of colonic M cells has any functional consequence on disease state. Thus, future studies should also try to fully elucidate whether M cells play a protective or pathogenic role during intestinal inflammation.

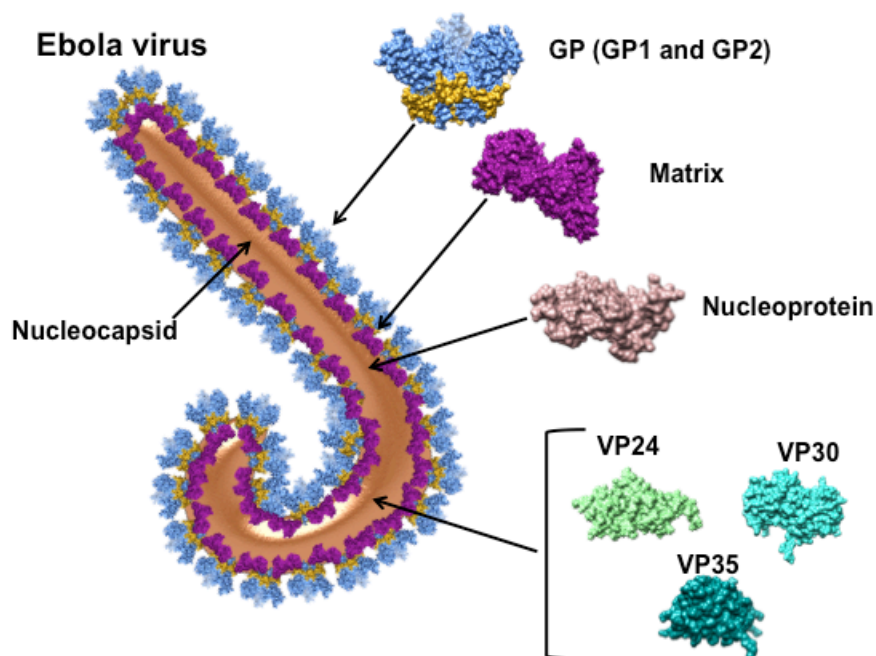


Figure 6.3 Schematic of the Ebola virus. The Ebola virus contains seven structural proteins, the GP complex (GP1,2), the matrix protein, the nucleoprotein, and the nucleocapsid proteins VP24, VP30, VP35. The nucleoprotein resides inside the nucleocapsid. The GP complex is responsible for binding to host cells.

6.3 Perspective and future directions for Chapter 5

TI responses are an important component of the humoral antibody response and can partially compensate for the TD antibody response when it is inhibited in various disease states (11; 12). In chapter 5 of this thesis, we identified that flagellin from *S.Typhimurium* could be reengineered to house whole vaccine antigens resulting in increased antigen exposure in filament formation for better stimulation of a TI mediated antibody response. This was confirmed by replacing the D3 domain of flagellin with E protein from Dengue 2 virus and studying its *in vivo* activity in TI restricted mouse model. The hybrid flagellin was able to produce a strong serum IgM response as well as IgG3 (TI IgG subclass), suggesting this hybrid flagellin could produce a TI restricted antibody

response. However it remains unclear if this design could be used as a universal dengue vaccine against all dengue serotypes, as sub-neutralizing antibody response could lead to exacerbated disease (13). Future studies should identify if the E proteins from the other three DENV serotypes could be inserted in a similar manner to the E protein from DENV2 and in effect produce a mixed filament vaccine. Furthermore, it should be determined if the mixed filament vaccine is in fact capable of producing neutralizing antibodies against all four DENV serotypes.

Ebola virus is a filamentous virus of the *Filoviridae* genus that like DENV infection results in hemorrhagic fever (14). Currently, there are no licensed vaccines for the treatment of Ebola virus, which has a 50% mortality rate and in light of the recent Ebola outbreak, has restored interest in trying to develop new vaccines strategies to combat Ebola infection. Although, we are still learning new details into the molecular pathogenesis of Ebola virus infection, it seems clear that T-lymphocytes may be important in controlling Ebola infection, even though Ebola virus does not directly infect them. In fact, analysis of serum taken from patients with lethal Ebola infection exhibited a significant reduction in the number of T-lymphocytes and natural killer (NK) cells (14-16). This might suggest that during Ebola infection that host is devoid of T cell help and a TI vaccine may be beneficial. The current vaccine strategies being explored for the treatment of Ebola infection rely on the use of subunit vaccines that incorporate one or more of the virus-encoded structural proteins to induce protective immunity to viral challenge (17; 18). The Ebola viral proteins, seven in total, have been tested for their ability to produce a protective effect (Figure 6.3:(19-24). Like HIV and the influenza virus, Ebola virus capsid is coated with GP (glycoprotein) complex (GP1 and GP2: Figure 6.3), which is responsible for binding to host cells and due it being fully exposed, the GP

complex should act as the obvious choice for a vaccine target (19). Potentially in the future, GP components of Ebola can be inserted into the flagellin backbone, and this may allow for a vaccine that is able to produce neutralizing levels of antibodies against Ebola.

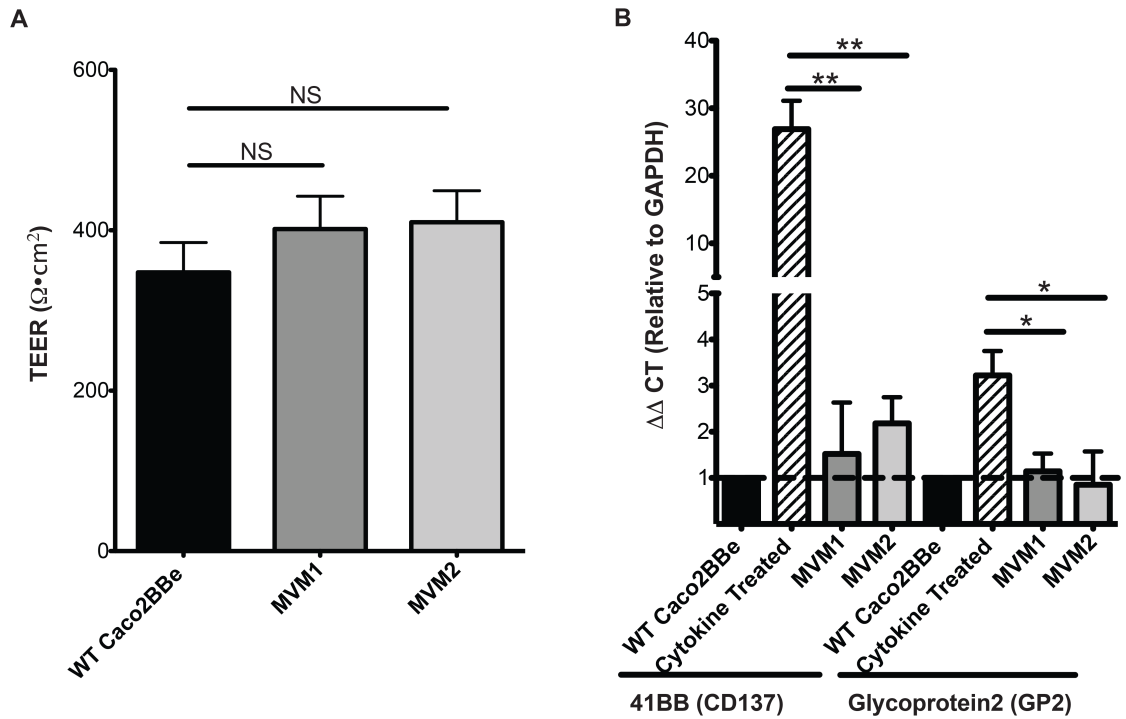
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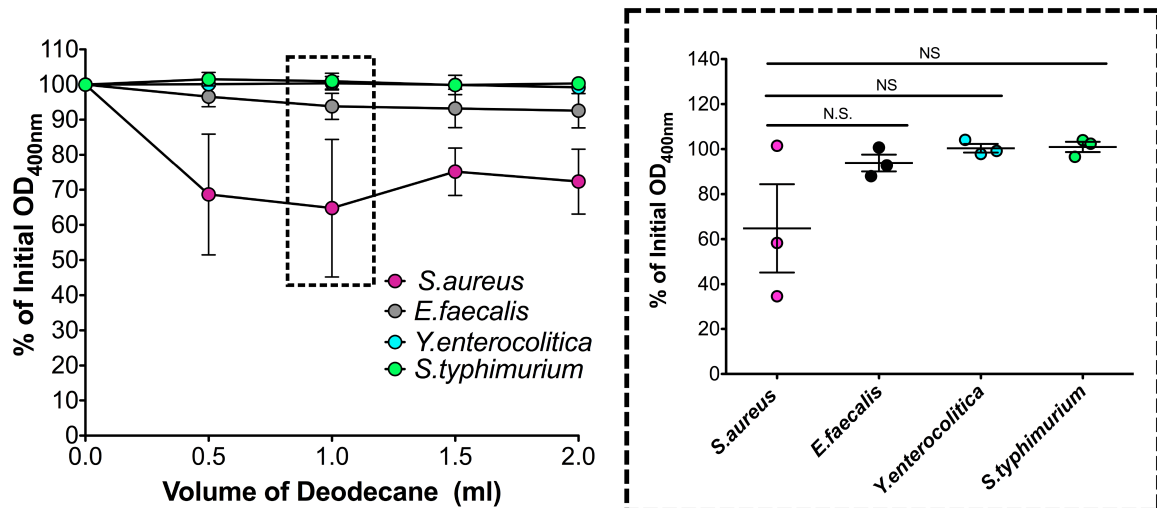
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Appendix

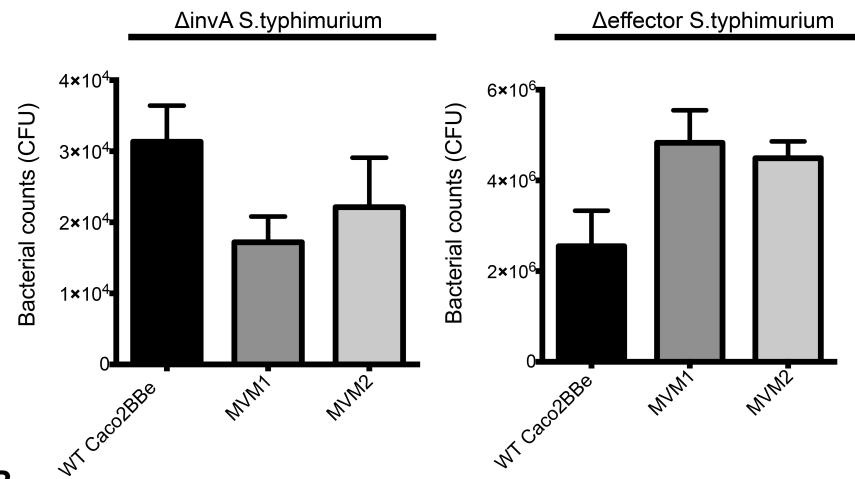
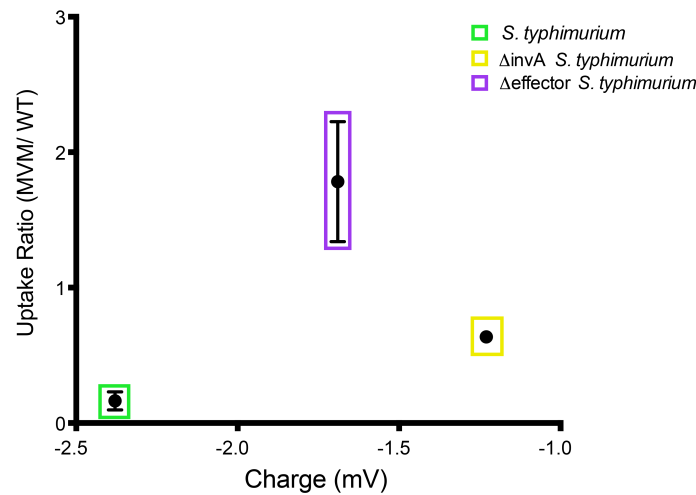
Appendix A: Supplemental material for Chapter 2



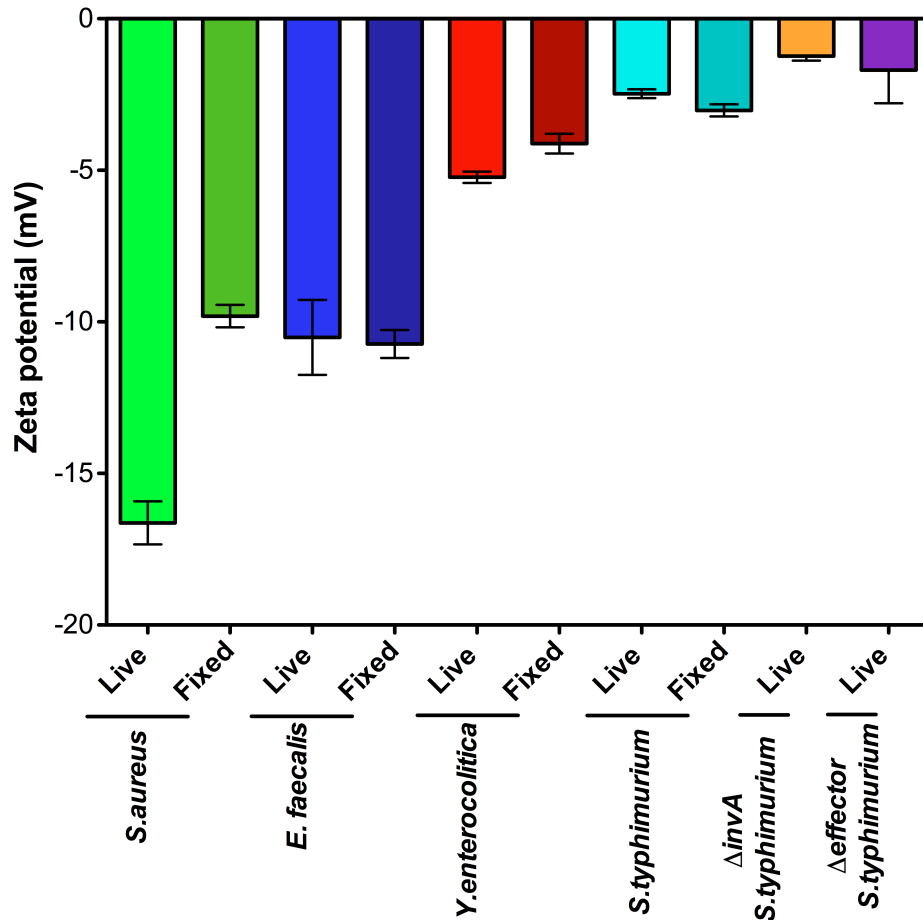
Appendix Figure A.1 Characterization of MVM cell model. (A) MVM cells showed similar transepithelial electrical resistance (TEER) compared to WT. Error bars represent SEM (n=3); significance was determined using paired Student's t-test. (B) MVM cells were assayed for induction of M cell related genes using qPCR. MVM cells showed no induction M cell related genes as compared to cytokine treated positive control. Error bars represent SEM (n=3); significance was determined using paired Student's t-test.



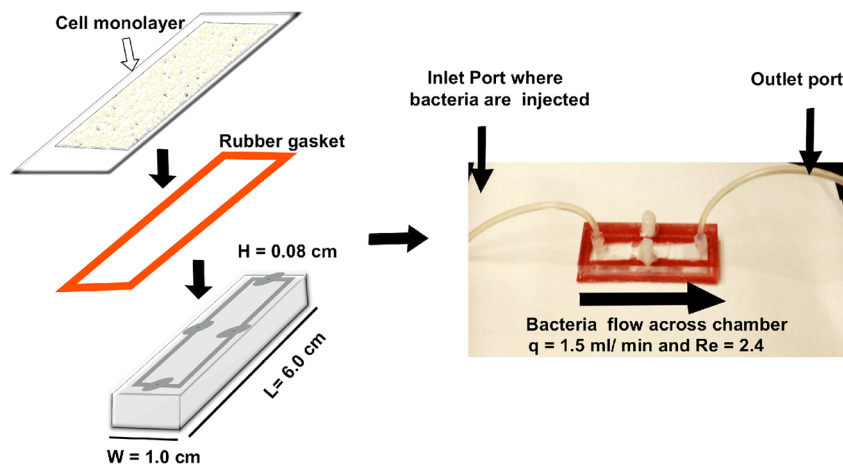
Appendix Figure A.2 Bacterial MATH analysis. Microbial Adhesion to Hydrocarbon (MATH) was used to determine the hydrophobicity of the bacteria used in the uptake/adhesion assay. All bacteria showed low hydrophobicity, with no significant differences among them. Error bars represent three individual experiments ran in triplicate and differences within the dataset were found to be non-significant using a one-way ANOVA analysis.

A**B**

Appendix Figure A.3 Epithelial cell uptake of mutant *S. Typhimurium* strains. (A) Uptake of mutant strains *S. Typhimurium* was performed to show that WT *S. Typhimurium* had preference toward WT cell due to effacement mechanism and not charge. *Salmonella* strain where effacement mechanism was knocked out shows a restored preference for MVM cells. (B) Ratios of uptake between MVM and Caco2BBE cells were quantified, *Δeffector S. Typhimurium* showed a higher preference toward MVM cells compared to WT and *ΔinvA S. Typhimurium*. Error bars indicate the sum of two individual experiments with two biological replicates.

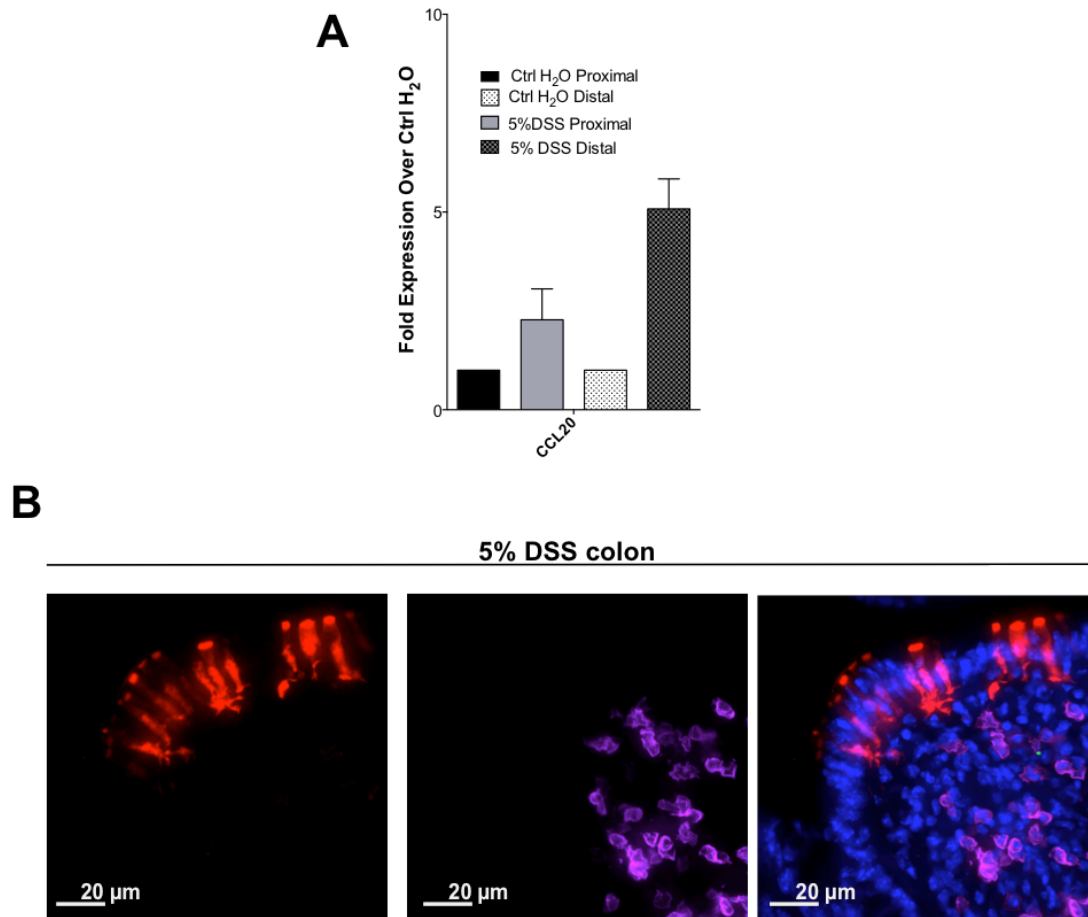


Appendix Figure A.4. Quantification of bacterial zeta potential. The magnitude of surface charge was approximated for each bacteria used in this study by measuring its zeta potentials using laser Doppler electrophoresis. Most Bacteria had similar zeta potentials regardless of being fixed with 1% PFA/PBS solution or a live, with the exception of *S. aureus*. Error bars represent (SEM) triplicate measurement of three individual bacterial cultures (n=9).

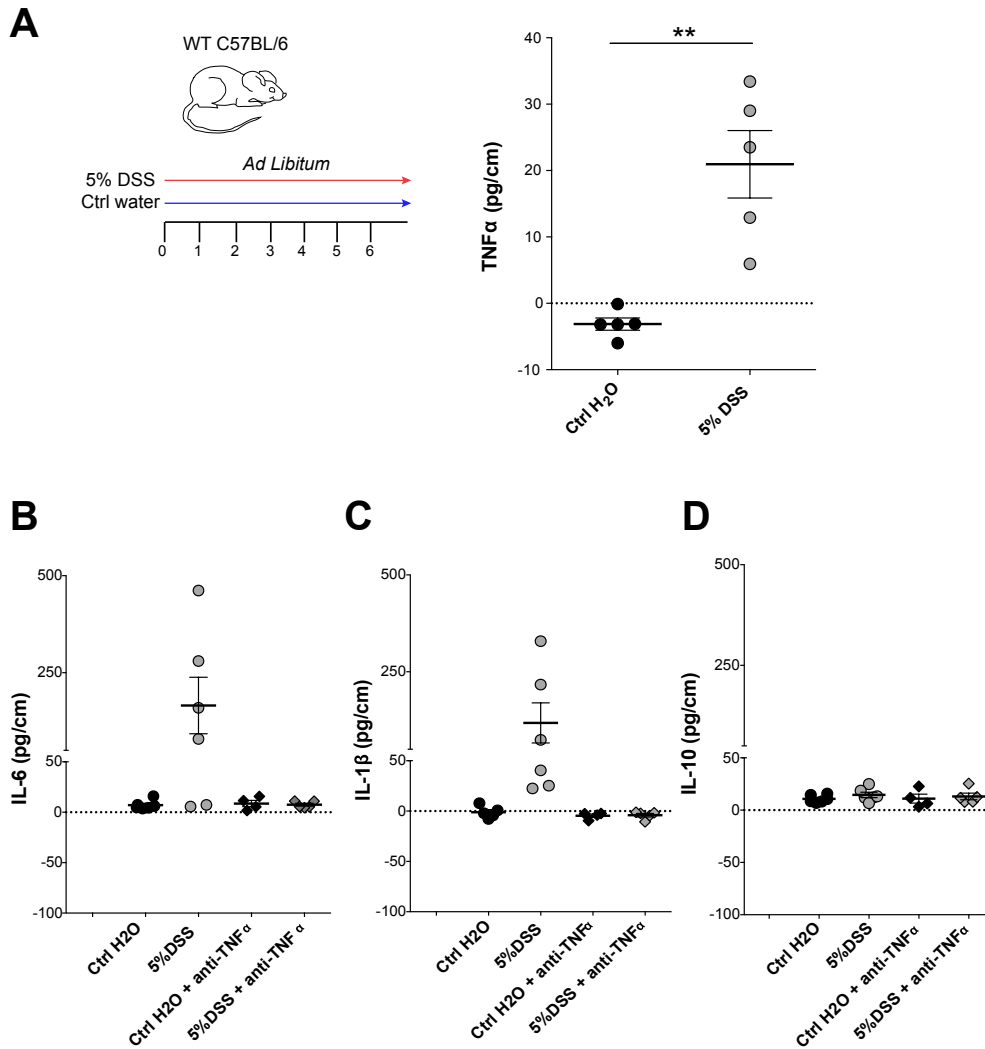


Appendix Figure A.5 Schematic of flow chamber for dynamic adherence assay. A flow chamber was clamped on to a glass slide on which a cell monolayer had been grown. The device was mounted on a confocal microscope for video documentation, and then a suspension of bacteria was injected into the chamber at a constant flow rate.

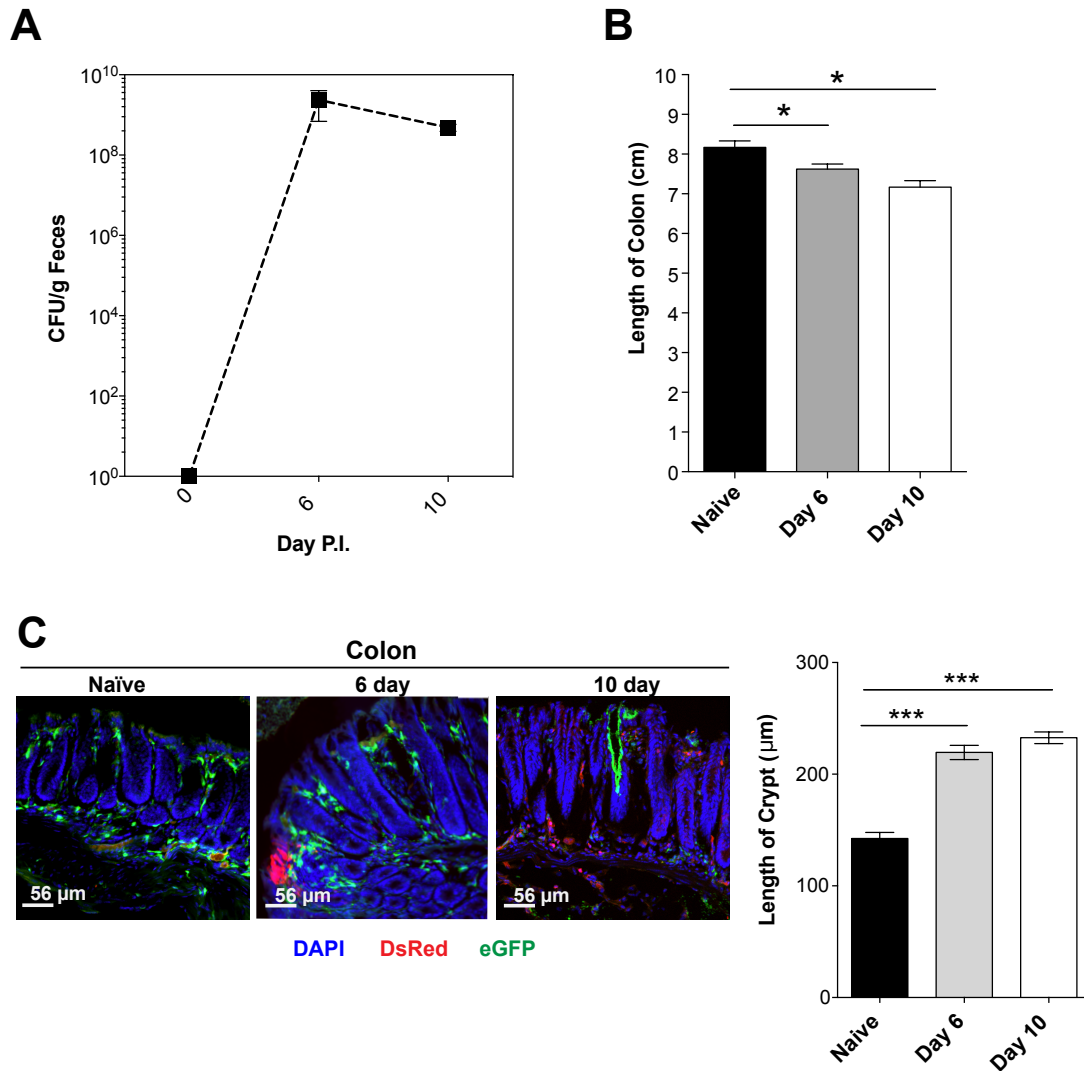
Appendix B: Supplemental material for Chapter 3



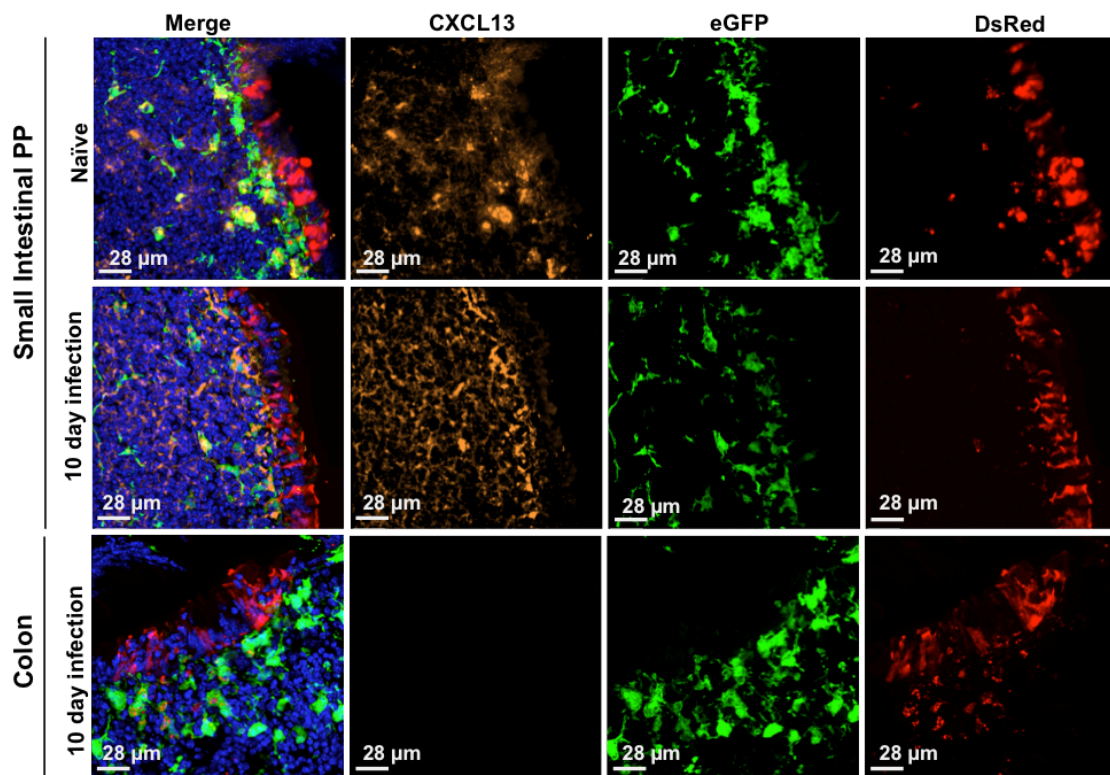
Appendix Figure B.1 Further characterization of the colonic M cells and follicle. (A) Mice treated with DSS exhibited increased CCL20 and gene that has been associated with the FAE. Data represents the mean $\pm$ SEM with 3 mice per group. (B) Low CXCL13 staining in the colonic follicle was associated with low B220 staining.



Appendix Figure B.2 Analysis of cytokine expression. (A) C57BL/6 mice treated with DSS exhibited similar TNF α levels compared to our reporter mice. Data represents the mean $\pm$ SEM with 5 mice per group. (B) Reporter mice treated with DSS exhibited increase IL-6 meanwhile mice treated with both DSS and anti-TNF α had non-detectable levels. (C) Reporter mice treated with DSS exhibited increase IL-1 β meanwhile mice treated with both DSS and anti-TNF α had non-detectable levels. (D) No mice exhibited detectable levels of IL-10 Data represents the mean $\pm$ SEM with 4-6 mice per group.

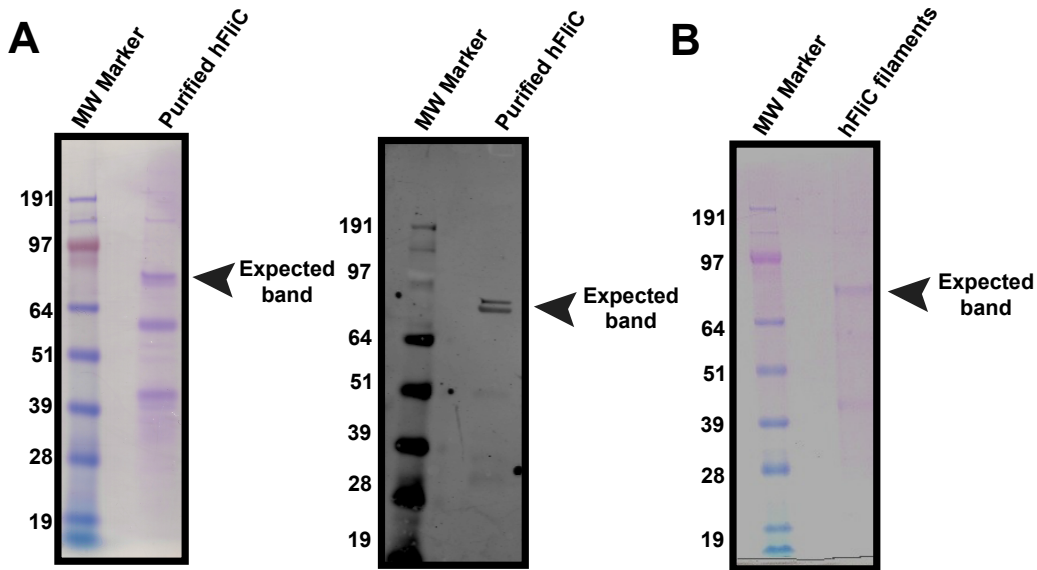


Appendix Figure B.3 Validation of *C.rodentium* infection. (A) Mice infected with *C.rodentium* exhibited high fecal bacterial burden at D6 and D10 post infection. Data represents the mean $\pm$ SEM with 3-4 mice per group. (B) Infected mice exhibited a modest reduction in colon length compared to Naïve mice. Data represents the mean $\pm$ SEM with 3-4 mice per group and statistics was determined by unpaired Student's t-test. (C) Infected at D6 and D10 post infection mice exhibited severe crypt hyperplasia as compared to Naïve mice. Data represents the mean $\pm$ SEM of 5 randomly chosen frames and statistics was determined by unpaired Student's t-test

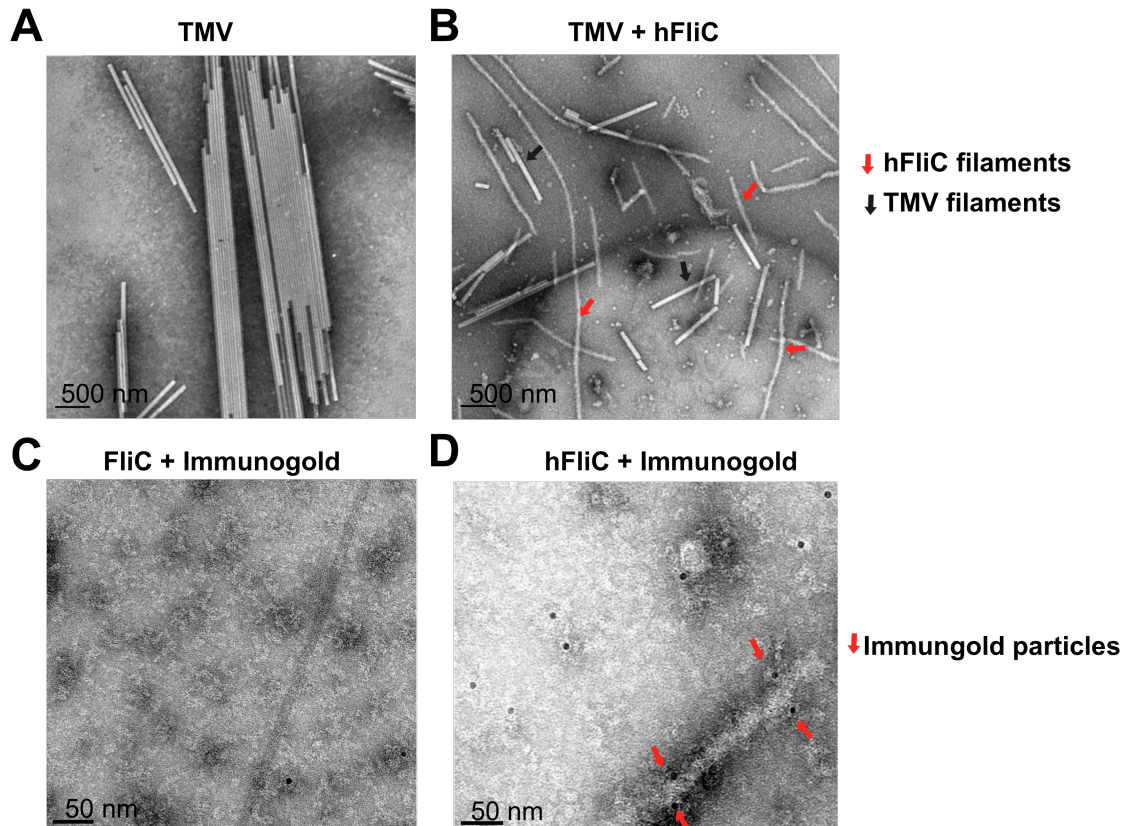


Appendix Figure B.4 Analysis of follicles in *C.rodentium* infection. *C.rodentium* induced colonic follicles showed no CXCL13 staining. Representative confocal micrograph confirmed that colonic follicles exhibit no CXCL13 as compared to the SI PP

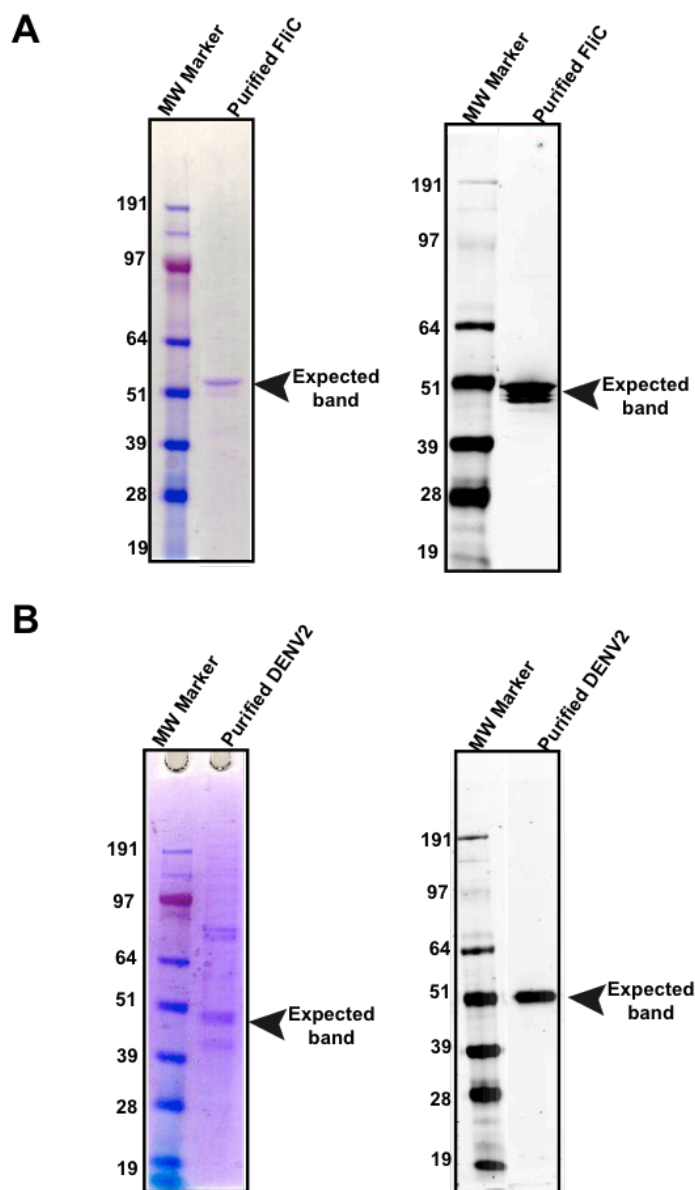
Appendix C: Supplemental material for Chapter 5



Appendix Figure C.1 Purification of hybrid flagellin. (A) hFliC protein produced in baculovirus expression system was checked for purity and anticipated molecular weight (~85 kDa) using both Coomassie stain (left panel, which labels all proteins) and Western blot (right panel). In the Western blot, protein was probed against the his-tag under denaturing conditions. Major bands from both blots indicate appropriate protein size, however some degradation products were present in Coomassie gel. (B) Purified hFliC protein was polymerized in high molar ammonium sulfate and resulting filaments were pelleted with high-speed centrifugation and resuspended in 150mM PBS. Under denaturing conditions the major band was only that of full-length hFliC.

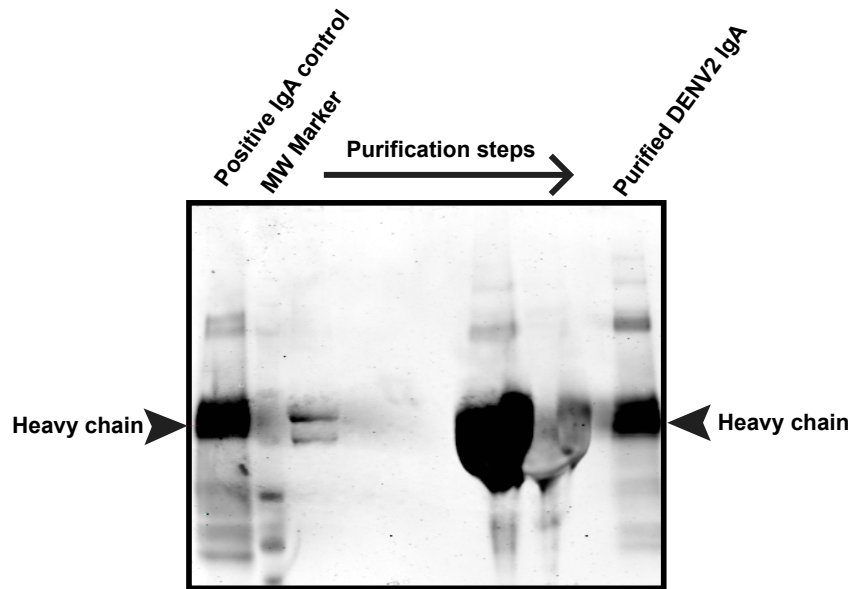


Appendix Figure C.2 Further analysis of hFliC filaments. (A) Negative stain transmission electron micrograph of TMV (tobacco mosaic virus), which was used as a positive control for contrast (high degree of contrast). (B) Negative stain transmission electron micrograph of hFliC filaments compared directly to TMV filaments. The micrograph indicates the inner core the hFliC filaments have a high degree of contrast, while the outer portion (i.e. DENV2) lacks contrast. Immunogold particle labeling against DENV2 specific IgG (primary antibody, isolated from 4G2 culture supernatant), was used to determine the placement of the DENV2 portion relative to the rest of the filament. (C) Negative stain transmission electron micrograph of immunogold labeling of WT FliC filaments, confirming that no immunogold particles bound to WT filament (lack DENV2). (D) Negative stain transmission electron micrograph of immunogold labeling of hFliC filaments, which indicates that the immunogold particles primarily associate with the outside of the hFliC filament.

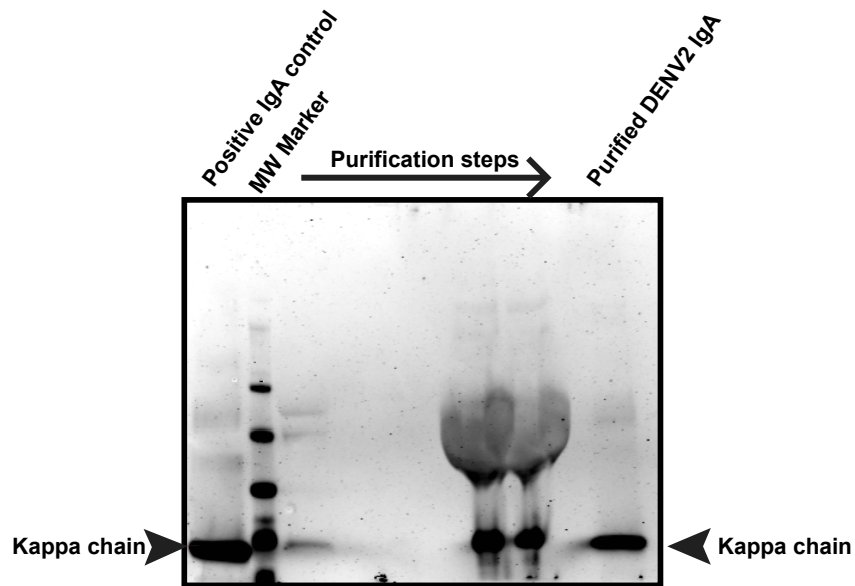


Appendix Figure C.3 Production of purification of recombinant FliC and DENV2. (A) Coomassie (left panel) and Western blot were used to assess purity and size (56 kDa) of recombinantly produced FliC protein. Both Coomassie and Western blot confirmed FliC size as well as its purity. (B) Same analysis was used to confirm DENV2 purity and size (50 kDa) and revealed recombinantly produced DENV2 had appropriate size and was pure.

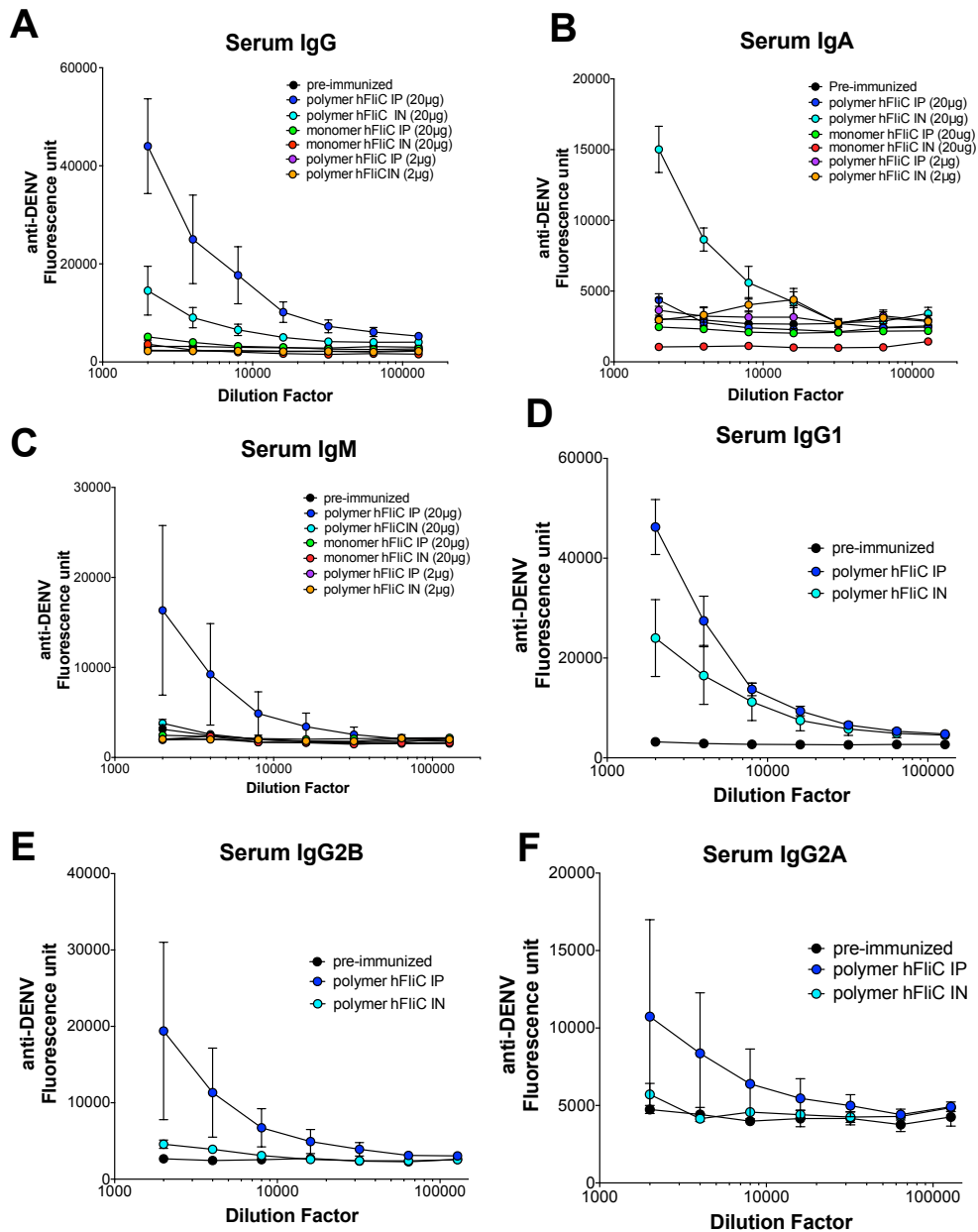
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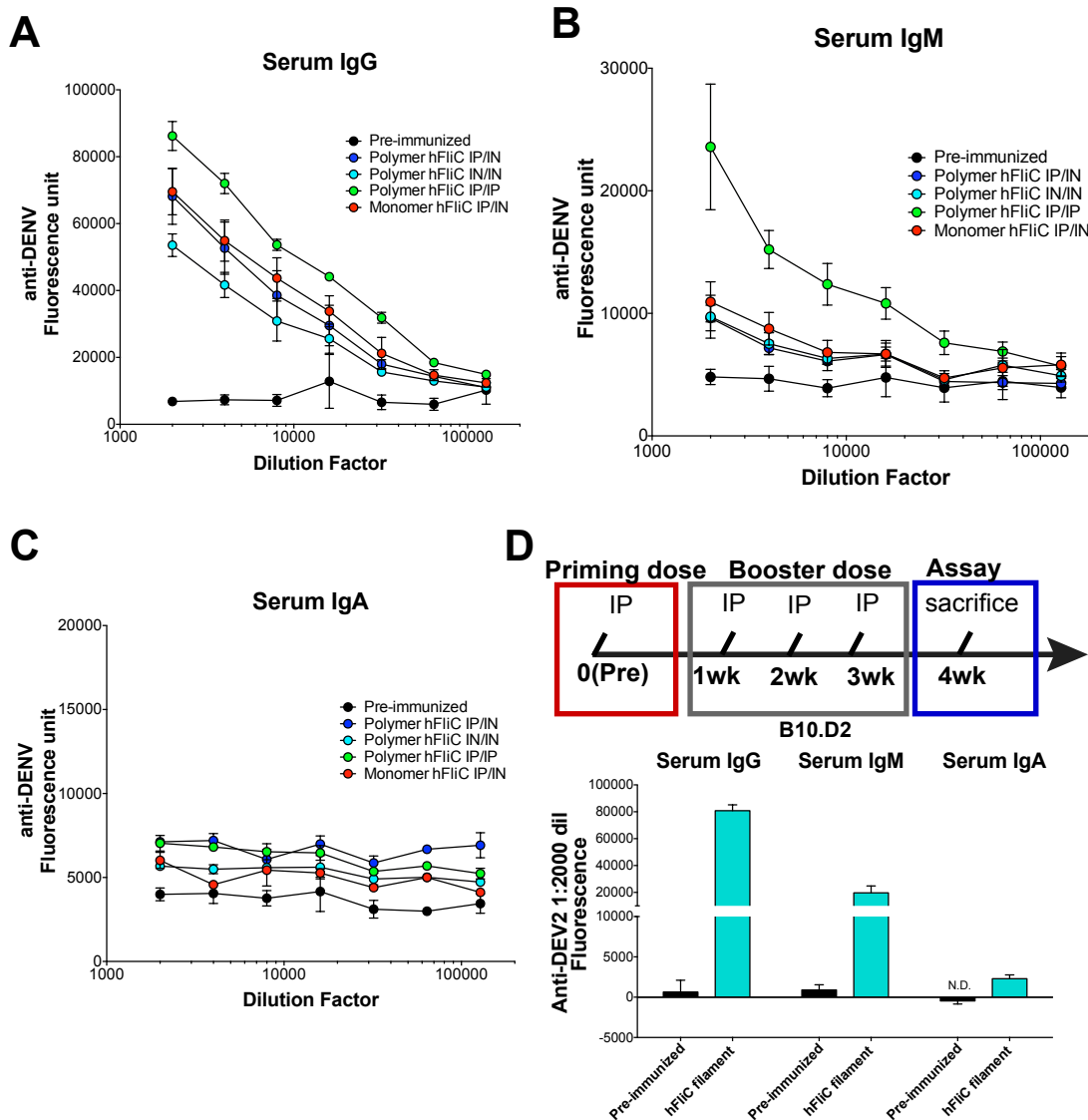
B



Appendix figure C.4 Recombinantly produced DENV2 specific monoclonal IgA. Positive IgA control used was purified mouse IgA, Kappa isotype control. (A) Western blot probing for IgA heavy chain blot indicates that DENV2 monoclonal IgA has anticipated size as compared to positive control. (B) Western blot probing for IgA kappa (light) chain, again blot indicates DENV2 monoclonal IgA has appropriate size as compared to positive control.



Appendix Figure C.5 ELISA curves with full dilution series. ELISA curves from immunized C57BL/6 mice, with either 20 or 2µg dose (which showed no response). All curves show the dilution series that was used. These values were subtracted from background fluorescence's and in most instances the 1:2000 dilution were used to make comparisons between groups. Same analysis was used for B10.D2 and 6.5-TCR immunized mice (but not shown).



Appendix Figure C.6 B10.D2 mice immunized with hFlIC administered via IP injections. (A) Schematic of the immunization protocol that was used where IP injection was not only used in priming injection but also in all subsequent injections. B10.D2 mice given on hFlIC filaments IP showed strongest IgG and IgM compared to all mice used in this study (B) Represents the raw dilution series, which indicates that these mice had the strongest IgM and IgG response. (C) Represents the response after background had been subtracted. (D) Mice exhibited a broad subclass IgG isotype response.

Appendix D: Curriculum Vitae

Kaila Marie Bennett

Address: Webber Hall 2101, University of California, Riverside, CA 92521

Email: kbenn001@ucr.edu or kaila.bennett18@gmail.com

Phone number: (951) 236-9801

I. Education

Degree: B.S. Bioengineering

Institution: University of California, Riverside

Degree: Ph.D. Bioengineering

Institution: University of California, Riverside

II. Research experience

Academic title: Ph.D. Candidate and NSF Fellow

Affiliation: School of Medicine and Division of Biomedical Sciences, UC Riverside

Advisor: Dr. David D. Lo, Distinguished Professor of Biomedical Sciences and Senior Associate Dean of Research

Doctoral Thesis title: Breaking down the barrier: novel insights into the role of intestinal microvilli, M cells, and exploiting “innate” B cells as therapeutic targets

Academic title: Visiting Whitaker Graduate Student Fellow

Affiliation: Graduate School of Frontier Biosciences, Osaka University, Suita Campus

Advisor: Dr. Keiichi Namba, Professor and Dean of the Graduate School of Frontier Biosciences

Project title: Structural Studies of Reengineered Flagellin, a Possible T cell Independent-Antigen

Academic title: Undergraduate Researcher and MYBEST Fellow

Affiliation: Department of Bioengineering, UC Riverside

Advisor: Dr. Victor G.J. Rodgers, Professor of Bioengineering

Project title: Measurement of Zeta Potential of Microparticles for Mucosal Vaccines

Academic title: Undergraduate Researcher

Affiliation: Department of Bioengineering, UC Riverside

Advisor: Dr. Dimitrios Morikis, Professor of Bioengineering

Project title: Development of Software Package for Determining Protein Titration Properties

III. Publications

4. Gusti V., Bennett K.M., Lo D.D. CD137 signaling enhances tight junction resistance in intestinal epithelial cells. *Physiological reports*. **2**:e12090, 2014
3. Bennett, K.M., Walker, S.L., Lo, D.D. Epithelial microvilli establish an electrostatic barrier to microbial adhesion. *Infection and Immunity*. **82**:2860-2871, 2014
(Highlighted in "IAI Spotlight" and image selected for cover)
2. Woodrow, K., Bennett, K.M., Lo, D.D. Mucosal vaccine design and delivery. *Annual Review Biomedical Engineering*. **14**:17-46, 2012
1. Rajapaska, T.E., Bennett, K.M., Stover-Hamer, M., Lytle, C., Rodgers, V.G.J. and Lo, D.D. Intranasal M cell uptake of nanoparticles is independently influenced by targeting ligand and buffer ionic strength. *Journal of Biological Chemistry*. **285**:23739-23746, 2010

IV. Publications in review

1. Bennett, K.M., Gorham, R.D., Morikis, D., Lo D.D. Bacterial flagellin for use as a T cell independent vaccine scaffold. Under review to *Biomaterials*
2. Bennett, K.M., Chen, G., Nair, M.G., Lo, D.D. Induction of colonic M cells during intestinal inflammation. Under review at *Mucosal Immunology*

V. Patents

THE USE OF HYBRID FLAGELLIN AS A NOVEL T CELL INDEPENDENT VACCINE
United States 1279-594P-140527 (Provisional Patent)

VI. Invited conference presentations

Conference: La Jolla Immunology Conference, La Jolla, California, 2014
Presentation: Podium talk

Conference: FASEB SRC Gastrointestinal Tract XV: Epithelia, Microbes, Inflammation and Cancer, Steamboat Springs, Colorado, 2013
Presentation: Podium talk

Conference: ACS Colloid & Surface Science Symposium, UC Riverside, 2013
Presentation: Podium talk

Conference: Southern California Eukaryotic Pathogen Conference, UC Riverside, 2012
Presentation: Poster

Conference: La Jolla Immunology Conference, La Jolla, 2012
Presentation type: Poster

Conference: AAPS NBC Conference, San Francisco, California, 2011
Presentation type: Podium talk (sponsored by Genetech) and Poster

Conference: UC Systemwide Bioengineering Symposium, UC Davis, 2010
Presentation type: Podium talk

VII. Awards

Award: UCR Dissertation Year Program Fellowship
Monetary value: \$30,000
Time funded: One quarter

Award: Whitaker International Summer Grant
Monetary value: \$12,000
Time funded: One summer

Award: NSF Graduate Research Fellowship Program
Monetary value: \$120,000
Time funded: 3 years

Award: AAPS Innovations in Biotechnology by Genentech
Monetary value: \$2,000

Award: Dean's Distinguished Fellowship
Monetary value: \$60,000
Time funded: 2 years

Award: Mentoring Year-Round in Biological Engineering, Science, and Technology (MYBEST) funded through NSF
Monetary Value: \$14,000
Time funded: 1 year

VIII. Mentorship

Undergraduate student: Juan Nevarez, a part of the Medical Scholars Program
Project: Zeta Potential of Commensal Bacteria
Time frame: 2 years

Undergraduate student: Lien Trinh, a part of the Medical Scholars Program
Project: Generation of Dengue 2 Specific Monoclonal IgA
Time frame: 3 years

Undergraduate student: Kathy Vu
Project: Generation of E2 Crimson, DsRed Expressing Caco2BBe Cells
Time frame: 2 years

Undergraduate student: Caleb Mohrhauser
Project: Production of hFliC
Time frame: 1 year

Undergraduate student: Ishan Naik
Project: Production of hFliC
Time frame: 1 year

IX. Service and outreach

- Orange County Science and Engineering Fair, Judge, March 2013
- Inland Empire Regional Science Olympiad Competition, Volunteer, February 2013
- Matthew Gage Middle School Science Olympiad Team, Coach, 2011-2012