

# Systems Analysis and Characterization of Mucosal Immunity

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## Abstract

During acute and chronic infectious diseases hosts develop complex immune responses to cope with bacterial persistence. Depending on a variety of host and microbe factors, outcomes range from peaceful co-existence to detrimental disease. Mechanisms underlying immunity to bacterial stimuli span several spatiotemporal magnitudes and the summation of these hierarchical interactions plays a decisive role in pathogenic versus tolerogenic fate for the host. This dissertation integrates diverse data from immunoinformatics analyses, experimental validation and mathematical modeling to investigate a series of hypotheses driven by computational modeling to study mucosal immunity. Two contrasting microbes, enteroaggregative *Escherichia coli* and *Helicobacter pylori*, are used to perturb gut immunity in order to discover host-centric targets for modulating the host immune system. These findings have the potential to be broadly applicable to other infectious and immune-mediated diseases and could assist in the development of antibiotic-free and host-targeted treatments that modulate tolerance to prevent disease.

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## Attributions

The work presented in this dissertation has been made possible through collaborative team science. Colleagues that contributed significantly to experimental design and execution, analysis, and written discussions deserve respective credit. Descriptions of these contributions are as follows:

### Chapter 1

Josep Bassaganya-Riera (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) and Raquel Hontecillas (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) contributed to the design, revision and final version of the presented text.

### Chapter 2

Josep Bassaganya-Riera and Raquel Hontecillas added ideas and helped with editing and writing sections of the final version of the manuscript

### Chapter 3

Josep Bassaganya-Riera and Raquel Hontecillas contributed to conceiving experimental designs and writing sections of the manuscript. Monica Viladomiu (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) and Mireia Pedragosa (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) were heavily involved in conducting *in vivo* animal experiments for data generation. Richard Gurrant (University of Virginia, Center for Global Health) and James Roche (University of Virginia, Center for Global Health) provided expertise in animal models of EAEC infection.

### Chapter 4

Josep Bassaganya-Riera and Raquel Hontecillas were involved in the design of computational and experimental projects in addition to writing sections of the final manuscript. Monica Viladomiu & Barbara Kronsteiner (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) assisted in conducting *in vitro* and *in vivo* validation experiments. Vida Abedi (Virginia Tech, Nutritional Immunology and Molecular Medicine Laboratory) contributed to clustering techniques for RNAseq analysis. Stefan Hoops (Virginia Tech, Modeling Immunity to Enteric Pathogens) aided in computational modeling efforts. Pawel Michalak (Virginia Tech, Modeling Immunity to Enteric Pathogens) performed the sequencing alignment and statistical analyses. Stephen Girardin (University of

Toronto, Laboratory of Medicine and Pathobiology) generously provided knockout mice and input for experimental designs.

## **Chapter 5**

Josep Bassaganya-Riera and Raquel Hontecillas aided in editing and writing portions of the text.

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# Chapter 1

## Introduction to immunoinformatics and systems immunology

Casandra W Philipson, Raquel Hontecillas, and Josep Bassaganya-Riera

### 1.1 Introduction

During acute and chronic infectious diseases hosts develop complex immune responses to cope with bacterial persistence that result in a variety of outcomes ranging from peaceful co-existence to detrimental disease. Innate immune cells, such as mononuclear phagocytes and epithelia, play decisive roles in host fate by controlling bacterial loads and modulating the immunological impact of this burden at the gut mucosa. Likewise, the adaptive immune system shapes the pathological impact of such perturbations by governing a delicate balance between effector and regulatory signaling mechanisms. This dissertation investigates a series of hypotheses driven by computational and mathematical modeling to study mucosal immunity. These findings have the potential to be broadly applicable to other infectious and immune-mediated diseases and could assist in the development of antibiotic-free and host-targeted treatments that modulate tolerance to prevent disease.

Complex mechanisms underlying immunity to bacterial stimuli span several spatiotemporal magnitudes and are not completely understood. Tissue-level pathologies

represent the summation of hierarchical interactions between the microbe, epithelial cells as well as infiltrating and resident acute (neutrophilic and mononuclear phagocytes) and chronic (lymphocytic) immune cells. These interactions involve changes that can persist for days (*e.g.* Enteroaggregative *E. coli*) to decades (*e.g.* *H. pylori*). Additionally, determining disease prognosis is patient-specific, time sensitive and often elusive yet crucial for deciding effective treatment and disease control. Current diagnostic techniques, ranging from serology panels to endoscopy, analyze byproducts of pathologies and often neglect to consider relationships occurring between biological scales (temporal and spatial) that enable sustained disease. Using an immunoinformatics platform represents a step towards integrating models educated by broad-based and time-sensitive patient-specific calibration datasets aimed to develop more efficacious and safer personalized medicine interventions. Computational and mathematical modeling provides an avenue for integrating and standardizing diverse data and knowledge to comprehensively understand unforeseen mechanisms.

## **1.2 General Approach for Constructing Computational Models**

Building network models: Models can be designed to encompass intracellular, cellular and tissue-level scales. Complex biological relationships among species of distinct scales defined in these models are represented as interaction networks. All models presented in this dissertation have been engineered and annotated using CellDesigner, a systems-biology markup language software. The network architectures have been developed based on experimental data, comprehensive literature searches and data mining of public repositories *e.g.* Gene expression omnibus. Recent tools have emerged that permit network inference based on patterns in data of extensively curated databases. In this work, we link network inference methods from Ingenuity Pathway Analyzer (IPA) with literature searches in order to: 1) quickly identify high priority molecules and canonical signaling cascades based on time and treatment of samples; 2) ensure accuracy of predicted reactions from IPA's highly curated knowledge base; and 3) allow easy customization of molecular networks based on hypotheses and Project 1 calibration datasets.

Representing immunological processes as reactions: Our present models contain a broad array of biological processes including ligand-receptor binding, protein-protein

interactions, transport between compartments, transcription, translation, and degradation. We have simulated these processes using deterministic models engineered with ordinary differential equations (ODEs). Three kinetic equations are commissioned for computational modeling of such biological processes, namely: 1) mass action, 2) Michaelis-Menten, and 3) Hill equation kinetics [78]. Additionally, dynamic behavior of biological networks often contains reoccurring wiring patterns known as “network motifs” that must be taken into consideration mathematically. One of the most integral network motifs is the feed-forward loop (FFL) that occurs during molecular cross-talk. FFLs are undoubtedly a common theme in our models and thus mathematics underlying signaling networks adopt a combination of equations and parameters as justified herein.

The Hill equation is a sigmoidal function that easily represents switch mechanisms, such as transcription factor binding. The Hill model provides an advantage for estimating in a precise fashion to what extent positive or negative cooperativity between molecules exists. Extensive studies have also demonstrated the benefits of the Hill equation for studying combinatorial regulation, especially those observed in FFLs, and therefore Hill equations will be used for characterizing cooperative roles among intracellular molecules. Additionally, molecules that are regulated by several inputs (i.e. one gene jointly regulated by two transcription factors) may be calibrated given the assignment of a Hill equation.

Mass action equations provide some advantages over Hill equations primarily related to their inherent ability to decipher mechanisms underlying molecular cooperativity rather than just positive or negative regulation (Hill model). Degradation rates and molecule transport are accurately represented by mass action equations. Mass action kinetics are also suitable for modeling multisite protein phosphorylation and are implemented in our studies due to their reliability in deterministic and stochastic simulations.

Parameter estimation has been performed on unknown constant values using the Genetic Algorithm and Particle Swarm algorithms in COPASI. Numerical boundaries are applied such that reaction rates are estimated in a biologically relevant range (e.g., transcription factor mRNA decay rates could be given a parameter search space of 1-3 hours). Fitting results have been thoroughly analyzed using COPASI’s objective functions and cross-referenced to experimental data for calculating standard error.

Models deemed calibrated by these standards are then used for *in silico* experimentation.

Verifying and Validating of computational models: The predictive power of modeling predictions lies in their ability to be experimentally validated. To test and confirm computational models developed within this text, we employ *in vitro* and *in vivo* methods using murine models of infection and cell culture techniques.

### **1.3 Summary**

This dissertation combines hypothetical and theoretical computational modeling to explore host immunity during acute and chronic infectious diseases. Chapter 2 presents a comprehensive overview of experimental and *in silico* models used to study host-microbe interactions at the epithelial barrier using Enteroaggregative *Escherichia coli* (EAEC) as an agent for mucosal perturbation. Chapter 3 further characterizes mucosal immunity during EAEC infection using a novel murine model of protein-energy malnutrition. Immunosuppression on adaptive immune cells and the capacity for the host to develop antibacterial responses is explored. Chapter 4 characterizes the role of macrophages in innate immunity toward *H. pylori* infection with respect to pathogenic burden and cytokine signaling. Lastly, Chapter 5 provides discussion-based text.

# Chapter 2

## Animal models of Enteroaggregative *Escherichia coli* infection

Casandra W Philipson, Josep Bassaganya-Riera, and Raquel Hontecillas

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### 2.1 Summary

Enteroaggregative *Escherichia coli* (EAEC) has been acknowledged as an emerging cause of gastroenteritis worldwide for over two decades. Epidemiologists are revealing the role of EAEC in diarrheal outbreaks as a more common occurrence than ever suggested before. EAEC induced diarrhea is most commonly associated with travelers, children and immunocompromised individuals however its afflictions are not limited to any particular demographic. Many attributes have been discovered and characterized surrounding the capability of EAEC to provoke a potent pro-inflammatory immune response, however cellular and molecular mechanisms underlying initiation, progression and outcomes are largely unknown. This limited understanding can be attributed to heterogeneity in strains and the lack of adequate animal models. This review aims to summarize current knowledge about EAEC etiology, pathogenesis and clinical manifestation. Additionally, current animal models and their limitations will be discussed

along with the value of applying systems-wide approaches such as computational modeling to study host-EAEC interactions.

## 2.2 Introduction

*Escherichia coli* are classified as motile, rod-shaped, non-spore forming, Gram-negative *Enterobacteriaceae*. The majority of *E. coli* strains co-exist in the gastrointestinal tract as harmless commensal symbionts that colonize the gastrointestinal tract within hours of life and remain the most predominant facultative anaerobe within the colonic microflora of humans [1]. However, disease-causing pathogenic *E. coli* strains have the ability to induce life-threatening illnesses that often require hospitalization and can result in death [2]. Pathotypes known to induce enteric disease have been categorized into six groups: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) [3].

Enterotoxigenic *Escherichia coli* (EAEC) was first identified in the late 1980s as an enteric pathogen that causes diarrhea [4]. Since its discovery, scientists have been studying host response to EAEC with aims to identify pathognomonic factors implicated in lesion formation in the gut and enteric disease. Increasing attention to EAEC has given rise to improved diagnosis of infection prompting more comprehensive epidemiological studies. For instance, a broad-based meta-analysis was conducted using all published literature about EAEC infections from 1987 through 2006 and revealed EAEC as a causative agent of diarrheal illnesses among many different subpopulations in both developing and industrialized regions worldwide[5]. Etiological efforts have uncovered striking numbers of infectious cases identifying EAEC as the causative agent of diarrhea in travelers, children (especially malnourished populations), and immunocompromised individuals (specifically HIV-infected patients) [6-8]. Also, EAEC has been identified as a common cause of acute diarrheal illness in children and adults in inpatient and emergency units throughout the United States [9]. The alarming rise in attention to EAEC led to its inclusion on the National Institutes of Health category B list of infectious organisms of potential importance as a bioterrorism weapon in 2002[10]. In May 2011 an outbreak of *E. coli* O104:H4 occurred in Germany where more than 4,000 people became victims to infection and 54 of these cases resulted in death;

the highest frequency of deaths ever recorded for an *E. coli* outbreak [11]. Nucleotide analysis of the genome sequence classified *E. coli* O104:H4 within the EAEC pathotype though it was Shiga-toxin (Stx2) producing. This hybrid strain acquired the phage-borne gene encoding Stx2, a characteristic associated with EHEC strains, likely through lateral gene transfer providing clear evidence for enhanced virulence and detrimental effects caused by emerging heterogeneity among strains[12].

Transmission of EAEC is most commonly associated with contaminated food and water. In Mexico, EAEC is the most common bacterial pathogen isolated from food [13]. Poor sanitation and crowded living conditions increase the propensity for EAEC to spread[14]. Recent research has identified food handlers, especially those working in tourist hotels, as primary carriers of EAEC. Over 65% of the isolates from these individuals are multidrug resistant thus posing a significant public health threat [15]. Furthermore, the prevalence of EAEC induced travelers' diarrhea throughout winter and summer seasons remains constant unlike other diarrheagenic *E. coli* strains such as ETEC, EPEC, and EIEC whose rate of infection significantly decreases in lower temperature [16]. Genetic predisposition has also been alluded to in EAEC susceptibility. Single-nucleotide polymorphisms (SNPs) in the IL-8 gene promoter have proven to be associated with increased incidences of EAEC-associated diarrhea while individuals with lactoferrin SNPs correlate with higher susceptibility to traveler's diarrhea [17, 18].

According to the CDC's 2011 estimates, diarrheal episodes and enteric infections caused by foodborne illness affect an estimated 47.8 million people annually in the United States alone, from which approximately 130,000 people seek hospitalization and 3,000 cases result in death ([www.cdc.gov](http://www.cdc.gov)). EAEC is one of the primary, if not most common, bacterial instigator of diarrheal illness in people from industrialized and developing countries around the globe including the United States, especially children [19-24]. Yet despite EAEC outbreaks and many years of high-level research, the disease pathogenesis remains widely unknown. This review will highlight known pathogenicity factors, describe host responses to disease, discuss current animal models, and emphasize the necessity for an integrated immunoinformatics approach that combines computational immunology and animal experimentation in order to advance towards safe and effective preventative and therapeutic treatments towards EAEC.

### **2.3 Host-EAEC interactions at the intestinal epithelium**

Understanding the complex interactions between host and bacterium is crucial for revealing disease pathogenesis of infectious diseases. The intestinal epithelium is constantly exposed to trillions of microorganisms and faces the challenge to peacefully coexist with harmless commensal bacteria while swiftly responding to pathogens [25]. The ability for a host to resist bacterial colonization or clear infection is determined by carefully arranged cellular and molecular interactions between the host and pathogen at the mucosal interface. A single layer of epithelial cells, the epithelial barrier, provides the first line of defense against pathogenic microorganisms. The epithelial barrier integrity is formed by “tight-junctions” between cells and the protective mucus-gel that coats the cells [26]. If an enteric pathogen passes through the mucus layers, evolutionarily conserved pathogen-associated molecular patterns (PAMPs) expressed on the microbial surfaces are recognized by pattern recognition receptors (PRRs) expressed on epithelial cell surfaces such as toll-like receptors (TLRs). TLRs activate potent innate responses by triggering signaling pathways that regulate gene transcription, such as NF- $\kappa$ B and MAPK, and activate the production of a large repertoire of pro-inflammatory mediators to orchestrate the influx of leukocytes [27]. More specifically, secretion of IL-8 and CXCL1 by enterocytes generates a chemotactic gradient promoting the recruitment of neutrophils to facilitate clearance of bacteria through phagocytosis [28]. Epithelial cells also secrete CCL20 in response to enteric pathogen to enhance infiltration of cells expressing CCR6. Dendritic cells expressing CCR6 are brought to the underlying lamina propria to hasten antigen presentation and activation of the adaptive immune system [29]. Th17 cells are CCR6+ and implicated as primary contributors to defense against extracellular bacterial infections. In addition to the secretion of cytokines to mediate cellular trafficking, epithelial cells produce potent antimicrobial proteins such as  $\beta$ -defensins, cathelicidins, and calprotectin in response to stimulation from enteric pathogens or proinflammatory cytokines for further defense against infection [28]. Importantly, a great amount of attention has recently shifted away from the host response and towards understanding the protective barricade created by commensal microbiota during infection [30]. The combined efforts of innate and adaptive immune responses with the beneficial influence of the gastrointestinal microbiome generally contribute to successful eradication of disease in healthy individuals.

Pathogenic bacteria such as EAEC have developed strategic mechanisms to conceal recognition and/or enhance survivability during interaction with host tissues and immune response, tactics predominantly driven by genetically encoded virulence factors. EAEC strains harbor a 60- to 65-MDa virulence plasmid (pAA) that encodes many of the known virulence factors including the aggregative adherence fimbriae (AAF), Pet toxin, the transcriptional regulator AggR, and the secretory protein dispersin[31]. The detection of pAA by probe, also known as the CVD432 probe, was initially trusted as a common broad-spectrum analysis used to identify the prevalence of EAEC isolates, however studies using this methodology have since exposed a large variation in accurate sensitivity towards EAEC ranging between 15 and 90% in separate cases [32, 33]. The golden standard for EAEC identification remains the highly specialized HEp-2 cell-adherence culture but, due to the assay's extensive requirements, the more common alternative is multiplex PCR though no molecular biological assays have been described with 100% specificity. Multiplex PCR evaluation of EAEC detects *aggR*, *aap*, and *aatA*, three EAEC plasmid-borne genes, and proven a suitable diagnostic test [34]. A key virulence factor harbored by pAA is the transcriptional activator AggR which is considered the master regulator of virulence due to its capability to activate a large cluster of virulence genes in EAEC permitting adherence while also promoting the production of cytotoxins and enterotoxins [35]. In fact, combined DNA microarray and real-time quantitative RT-PCR data confirm that AggR activates the expression of at least 44 genes in the EAEC prototype strain 042 [36]. To mediate protein secretion, EAEC possess a type VI secretion system (T6SS) that is chromosomally encoded on the pathogenicity island *pheU* and transcriptionally regulated by AggR. *Sci-1* and *sci-2* are two gene clusters present on *pheU* responsible for encoding T6S machines [37]. Additionally, the ETT2 gene cluster has been identified in the EAEC O42 genome sequence providing evidence for T3SS mechanism prevalence as well [38]. These secretion systems may play a key role in EAEC virulence due to expulsion of toxic proteins and association with biofilm formation [39]; their roles in pathogenesis remain widely unknown. Heterogeneity amongst EAEC strains remains an overarching issue that complicates elucidating pathogenic mechanisms underlying infection. Many virulence factors are not consistently expressed throughout various EAEC strains and the clinical manifestation of disease ranges significantly in severity. Moreover,

successful immunoregulatory responses by the host that potentiate EAEC clearance are limited in the literature. Nevertheless, numerous studies suggest that infection can be summarized in three general stages: 1. adherence and colonization, 2. increased mucus production, and 3. toxin release and host response [40].

## 2.4 Stages of pathogenesis

During the first step of pathogenesis, EAEC abundantly adhere to the intestinal mucosa in a stacked brick pattern termed aggregative adherence (AA) (Figure 1). The AA phenotype was first described using a biological co-culture of EAEC with HEp-2 cells. Biopsies from pediatric intestinal mucosa cultured with EAEC strains 17-2 and 221 portrayed the ability for EAEC to adhere to jejunal, ileal, and colonic mucosa [41]. Another early study provided evidence that fimbriae mediate EAEC adherence to HEp-2 cells [42]. Four AA fimbriae (AAF) have since been described. Characteristics of AAF vary between EAEC strains both in morphology and genetic code however all mediate the essential role of bacterial attachment to epithelial cells. Prototype strains EAEC17-2, 042, 55989, and C1010-00 express AAF-I, II, III, and IV respectively and all four strains develop the observed AA phenotype [43, 44]. Evidence from an *in vivo* intestinal cell model of EAEC infection shows that disruption between intestinal epithelial cells induced by strains 042 and JM221 is due to an AAF-dependent delocalization of tight junction proteins, claudin-1 and occludin. AafA, the major pilin protein of AAF fimbria, is directly linked to diminished transepithelial resistance [45]. The expression of AAF-I, -III, or -IV is sufficient for the induction of polymorphonuclear cell transmigration *in vitro*. More pertinently, human fetal intestinal xenografts implanted into SCID mice and inoculated with EAEC 042 and mutants verify an AAF-dependent inflammatory response [46]. AAF are highly hydrophobic thus favoring agglutination in an aqueous environment. In order to promote the spreading of EAEC for efficient colonization EAEC secretes a low molecular weight protein known as dispersin (aap). Dispersin is a positively charged hydrophobic surface protein that maintains electrostatic interactions with the outer lipopolysaccharide layer of the bacteria preventing the positively charged AAF from clinging to bacterial membrane [47, 48]. AAF fimbriae actually collapse in the absence of dispersin and lack functionality critical for adherence [49].

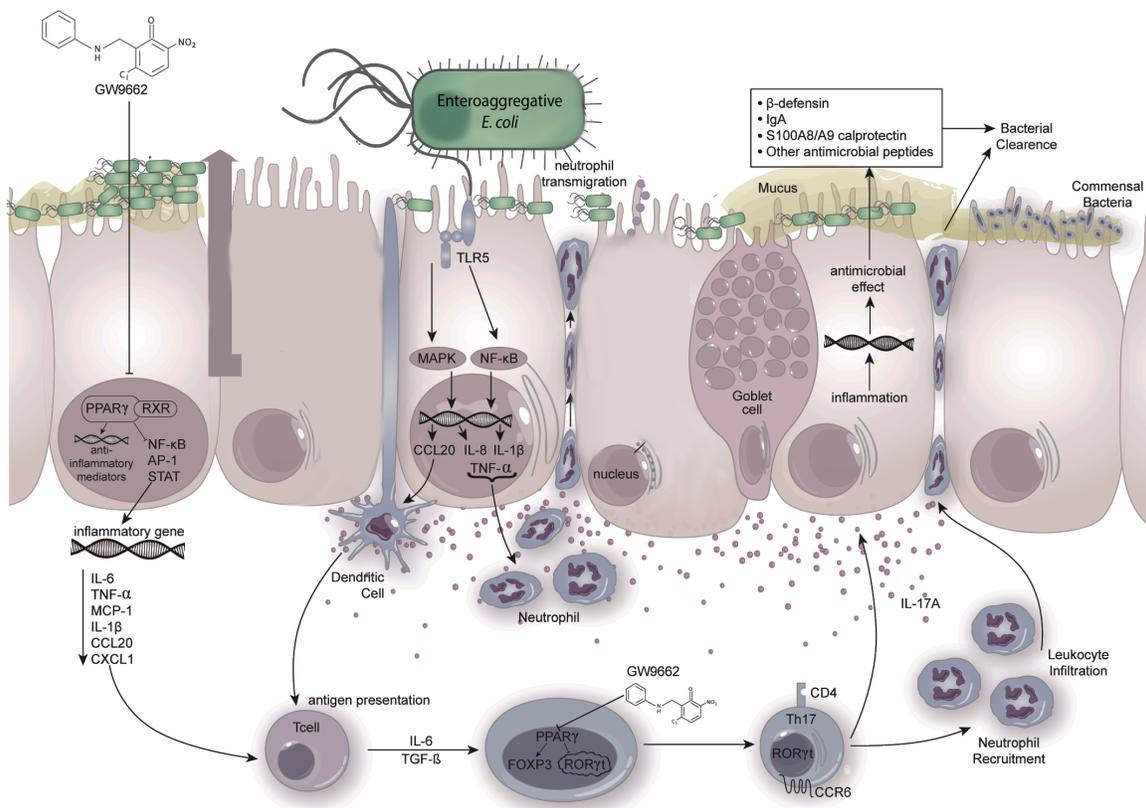
Other accessory molecules have been discovered and associated with EAEC colonization to include a serine protease autotransporter, Pic. Pic is encoded on the chromosomes of EAEC strain 042 and is suggested to mount a pivotal role in the colonization and growth of EAEC. Having hemagglutinin and mucinolytic activity, Pic is able to penetrate the intestinal mucus layer and possibly promote EAEC growth by enhancing the use of nutrients from mucin [49, 50]. Notably, Pic causes hypersecretion of intestinal mucus in EAEC infected rat ileal loops while also significantly increasing the number of mucus-containing goblet cells in intestinal villi [51]. Moreover, Pic efficiently cleaves extracellular glycoproteins on human leukocytes like CD43, a highly expressed surface marker found on almost all cells from a hematopoietic lineage. Interestingly, Pic protein is a key virulence factor in other enteropathogens including uropathogenic *E. coli* and *Shigella flexneri*, underscoring its importance in EAEC pathogenicity. Human neutrophils treated with purified Pic protein experience impaired chemotaxis and transmigration but increased activation of the neutrophil oxidative burst while activated T cells experience Pic-induced apoptosis [52].

Once EAEC successfully adhere, epithelial cells are stimulated to produce a thick mucus layer above the enterocytes forming a biofilm (Figure 2.1 first enterocyte). The presence of AAF is critical in biofilm formation though other unidentified factors including those regulated by *fis* and *AggR* gene expression are likely contributors as well[53]. The ability to form biofilm is closely associated with bacterial persistence and many chronic bacterial infections are now believed to be linked to biofilm production [54]. To enhance colonization, EAEC surround themselves with biofilm and recruit cells forming micro-colonies that are interspersed within fluid-filled channels. The biofilm then protects colonies by restricting antimicrobial penetration and protecting against attacks from the host's intestinal immune system prolonging infection [55].

During the third stage of pathogenesis EAEC secretes putative enterotoxins and cytotoxins that elicit a host inflammatory response. Mucosal toxicity can occur causing morphological changes in the architecture of the mucosa characterized by microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion [44]. Three primary enterotoxins have been discovered; namely EAEC heat-stable enterotoxin-1 (EAST1), Plasmid-encoded enterotoxin (Pet), and *Shigella* enterotoxin 1 (ShET1). EAST1 is a 4.1kDa toxin first detected in EAEC strain 17-2 that has now been associated with other diarrheagenic strains of *E. coli* providing evidence for its

relationship to enteropathogenic induced diarrhea [56]. The role of EAST1 in the molecular pathogenesis remains incompletely understood, however, scientists hypothesize that the toxin promotes the initial phase of watery diarrhea seen in many patients [57]. EAST1 binds to the extracellular domain of guanylate cyclase (GC) on the apical membrane of enterocytes (Figure 2.1 second enterocyte). EAST1 then induces high production levels of cGMP inside cells inhibiting the Na/Cl transport system. This significantly reduces the absorption of electrolytes and water from the intestine at the villus tips resulting in elevated secretion of water in crypt cells [54]. Pet is a serine protease autotransporter enterotoxin that generates a high toxicity in human epithelial cells resulting in structural damage to the cell. After internalization via receptor-mediated endocytosis, Pet is delivered to the cytoplasm by means of retrograde trafficking (Figure 2.1 fourth enterocyte). This is then accompanied by cleavage of spectrin, also known as the actin-binding protein fodrin, within microvilli cytoskeleton leading to cell elongation, rounding, and ultimately the release of cells from the substratum [58-60]. ShET1 appears to induce intestinal secretion via cAMP and cGMP however much of the biochemistry and mechanism of action surrounding this toxin remain elusive[61].

Most EAEC strains harbor genes encoding Class I and Class II serine protease autotransporter toxins (SPATEs). Class I SPATEs are cytotoxic to epithelial cells and include Pet, Sat, EspP, and SigA while the non-cytotoxic Class II category includes *pic* and *sepA*[62, 63]. Sat, originally discovered in uropathogenic and diffusely adhering *E. coli*, has been described as the most commonly detected SPATE among EAEC strains. Sat, like its homolog Pet, is believed to cleave the intracellular protein spectrin and cause cytoskeletal damage to tight junctions between intestinal epithelial cells [64]. Likewise, SigA, a SPATE largely associated with *Shigella flexneri* pathogenesis, is capable of inducing fodrin degradation causing catastrophic morphological changes in cells [65]. Interestingly, although only moderately prevalent in EAEC strains, SepA is the SPATE most strongly associated with severe diarrheal illness [63] though its role in EAEC pathogenesis remains largely uncharacterized.



**Figure 2.1. Enterotoxigenic *Escherichia coli* (EAEC) pathogenesis and host response at the colonic mucosa.** The clinical manifestation of EAEC infection is the outcome resulting from complex host-pathogen-microbiota interactions regulated at a molecular level. EAEC attach and aggregate on colonic epithelial cells in a stacked brick pattern by means of AAF fimbria and the secreted protein encoded by *aap* known as dispersin. EAEC form a thick biofilm enabling protection against host or interventional antimicrobial responses. FliC surface flagella are then recognized by TLR5 receptors expressed on the apical surface of enterocytes. Bacterial-epithelial cell contact triggers a cascade of events activating NF- $\kappa$ B and MAPK pathways that result in the upregulation of proinflammatory cytokines IL-8, TNF- $\alpha$ , and CCL20 responsible for recruiting dendritic cells and neutrophils to the site of infection. Small red spheres underneath the colonic epithelial layer portray the chemokine gradient indicative of inflammation. EAEC harbors the transcriptional regulator AggR responsible for the expression of virulence factors including Pic, Pet, EAST-1, *aap*, and ShET1 portrayed in the amplified image of the bacteria. EAST-1 toxin binds to extracellular guanylate cyclase (GC) on enterocytes and stimulates overproduction of intracellular cyclicGMP (cGMP) ultimately impairing Na/Cl transport. This causes water to be secreted from the enterocyte and contributes to the watery diarrhea seen in infected individuals. ShET1 is also proposed to affect intracellular cGMP levels however much of the biochemistry surrounding this enterotoxin remains unknown. Pet enters the cell via clathrin-mediated endocytosis and is translocated into the cytosol after being transferred from the Golgi complex to the endoplasmic reticulum through retrograde trafficking. In the cytosol, Pet cleaves the actin-binding protein  $\alpha$ -Fodrin inducing cytotoxic disruption of the cytoskeleton. Systemic administration of PPAR  $\gamma$  antagonist GW9662 to malnourished EAEC infected hosts enhances an upregulation of inflammatory gene expression and potentiates a beneficial early T helper 17 (Th17) response that successfully facilitates neutrophil recruitment and antimicrobial production that clears the

infection and ameliorates disease. A healthy enterocyte is depicted on the far right cohabitating peacefully with the beneficial microflora.

## 2.5 Shiga Toxin producing EAEC strains

*E. coli* O104:H4, reported as a causative agent of diarrhea since 2001 and the disease causing strain in the 2011 German outbreak, is an EAEC strain that has adopted the ability to produce Shiga-toxin (Stx2) [66], a chromosomally encoded cytotoxic verotoxin that targets globotriaosylceramide (Gb3) receptors located on host intestinal and kidney cells. Death from infection with Stx2-producing EAEC strains is strongly linked to the development of hemolytic uremic syndrome (HUS), a life-threatening disease induced by Stx2 shortly after the onset of diarrhea. Stx2 undergoes retrograde transport to induce endothelial cell apoptosis causing significant gastrointestinal damage. Additionally, Stx2 is able to enter systemic circulation and induce glomerular occlusion as blood is filtered through the capillary arrangement in the kidney. The resulting hemolytic anemia and acute renal failure are complications that most commonly affect children and contribute to the increasing mortality rates [67, 68]. The 2011 EAEC O104:H4 outbreak an unusually high proportion of adult patients (especially women) and significantly increased incidence of HUS (25% of patients) [66]. Interestingly, death occurred in patients who had not developed HUS; these cases most commonly occurred in elderly with female gender having a significant predisposition [69].

Whole genome-phylogenesis confirmed strain O104:H4 as an EAEC strain. Acquisition of a Stx2 bacteriophage is the leading factor for hypervirulence. This phenomenon could have occurred in mammalian intestines or an environment where both human and ruminant feces were present [70]. Alignment of an EAEC O104:H4 isolate TY2482 against the prototype EAEC strain 55989 chromosome ultimately revealed the presence of the large conjugative plasmid pAA which resembled the AAF gene-coding cluster from strain 55989. Interestingly, pAA TY2482 encoded for AAF/I rather than the more common AAF/III. The isolate lacked the locus of enterocyte effacement (LEE; responsible for bacterial adherence), intimin adherence factor, and a type III secretion system normally identified in enterohemorrhagic *E. coli* (EHEC) strains [66]. Since EAEC virulence factors are encoded on plasmids, bacteriophages, and genetic pathogenicity islands, the traits are easily transferred to new emerging strains [71].

Survivability and Shiga toxin production alone are not likely the sole causes of HUS in EAEC infected patients. EAEC O104:H4 adherence to the intestinal mucosa is mediated by AAF/I and potentially more aggressive than EHEC LEE mediated adherence. Additionally, EAEC infections induce proinflammatory responses and epithelial barrier disruption possibly enhancing systemic dissemination of shiga-toxin and HUS induction providing an explanation for the strain's hypervirulent activity. In addition to Stx2 gaining systemic accessibility, severe epithelial damage induced by the toxin could have allowed bacterial components to enter peripheral blood exaggerating inflammation systemically leading to death by sepsis in non-HUS patients. Most importantly, the genome sequence of TY2492 illuminates the ability for Shiga toxin-producing *E. coli* to produce various adhesion mechanisms portraying the ability for pathotypes to overlap and evolve into more virulent strains. Rapid responses in sequencing efforts during the EAEC O104:H4 outbreak suggests that genomic epidemiology will become a standard molecular strategy to elucidate infectious disease outbreaks [72].

## **2.6 Host response to colonization and virulence factors of EAEC**

Measured immune responses in infected subjects represent the result of a delicate balance between host–microbial interactions. Additionally, responses are specific and dependent on variables found among hosts and EAEC strains. For instance, genetic variability seen in both host and EAEC strains can significantly impact the susceptibility and outcome of EAEC infection. The capacity for specific EAEC strains to produce Stx2 and cause HUS-induced mortality demonstrates enhanced virulence. In other instances, host age dictates disease severity portrayed when children are more susceptible to persistent EAEC infection compared to healthy travelers. Regardless, studies have proven that bacterial-epithelial contact is a key determinant of host response to EAEC [73]. The EAEC bacterial surface protein flagellin (FliC) has been shown to mediate NF- $\kappa$ B and p38 MAP kinase activation in epithelial cells by cellular signaling through Toll-like receptor 5 resulting in IL-8 production (Figure 2.1 third enterocyte) [74, 75]. FliC is the major inducer of IL-8 release however other AggR regulated factors contribute and AAF adhesion is required for full IL-8 induction [76]. Interleukin (IL)-8 is a cytokine associated with infection with EAEC and other enteric pathogens. IL-8 production is involved in recruitment of neutrophils and the transmigration of these cells into the intestinal mucosa

disrupts epithelial tight junctions ultimately inducing colitis; a mechanism of action common among diarrhea-inducing pathogens. Some research suggests that the induction of IL-8 and subsequent disruption of the epithelial barrier is beneficial for EAEC pathogenicity enhancing toxic effects on the host though *in vivo* models are yet to validate this theory. Elevated levels of fecal IL-1 $\beta$ , another cytokine that can induce neutrophil migration [77], have also been reported in adults diagnosed with EAEC induced traveler's diarrhea [78]. Lactoferrin, an iron-binding antimicrobial glycoprotein[79], has been a target in other studies that demonstrate significantly increased levels of this protein alongside fecal leucocytes in EAEC infected patients[80]. Not surprisingly, CCL20, a dendritic cell recruiter, is also known to be upregulated after persistent EAEC stimulus [73].

Recently, our group published *in vivo* data reporting for the first time the importance of T helper (Th)17 cells in host responses to EAEC and EAEC clearance[81]. By pharmacologically and genetically disrupting the activity of the transcription factor peroxisome proliferator-activated receptor (PPAR)  $\gamma$ , we modulated mucosal inflammation that resulted in enhanced Th17 phenotypes and disease amelioration (Figure 2.1 first and fifth enterocytes, underlying mucosa). PPAR  $\gamma$  regulates anti-inflammatory responses through its ability to inhibit signaling pathways such as NF- $\kappa$ B, AP-1, and STAT in epithelial cells, macrophages, and T and B lymphocytes [82, 83]. Our studies suggest a more potent early inflammatory response may be required to ameliorate enteric disease during EAEC infection, especially in malnourished children. Mice infected with EAEC strain JM221 that were treated with a potent PPAR  $\gamma$  antagonist, GW9662, or those that lacked functional PPAR  $\gamma$  in T-cells (PPAR  $\gamma$  fl/fl CD4-cre+ mice) cleared EAEC significantly faster than untreated wild type mice. Colonic gene expression for inflammatory cytokines and chemokines, including TNF- $\alpha$ , IL-6, MCP-1, CCL20, CXCL1, and IL-1 $\beta$  was significantly upregulated early during infection in PPAR  $\gamma$  deficient mice when compared to wild-type counterparts. During the chronic phase of infection, PPAR  $\gamma$  deficiency significantly enhanced IL-6, TGF- $\beta$ , and IL-17A expression in the colon suggesting the presence and importance of CD4+Th17 cells during EAEC infection. Flow cytometry validated higher percentages of Th17 cells in colonic lamina propria of PPAR  $\gamma$  deficient mice. Histopathological analysis of colons also provided consistent evidence that PPAR  $\gamma$  blockade enhanced the inflammatory response without causing collateral tissue damage at the gut mucosa. Th17 responses

are known to enhance antimicrobial inflammatory responses by increasing the expression of antimicrobial peptides and effectively recruiting and activating neutrophils that contribute to destroying invading extracellular pathogens [84, 85]. Notably, EAEC clearance was directly correlated to the upregulation of colonic calprotectin, in GW9662 treated mice. The beneficial role of Th17 cells to enhance effector mucosal responses during EAEC infections is a pivotal finding and future studies should focus on how EAEC induces these responses.

EAEC appears to strategically orchestrate an inflammatory response in the host's intestinal mucosa regardless of the presence or absence of diarrhea [7, 86]. Infiltration of innate immune cells, disruption of the epithelial barrier, and increased mucus production best explain the most commonly reported symptoms including watery diarrhea, with or without blood and mucus, abdominal pain, nausea, and fever [10, 58]. However, this response inflammatory is sometimes not sufficient to facilitate pathogen clearance, thereby resulting in extended host tissue damage. New data suggests that EAEC may also induce a Th17 response and that enhancing this response in a malnourished host early during the infection process is beneficial to overcome disease. Of note, EAEC can persist subclinically facilitating a chronic inflammatory state that can impair nutrient absorption and developmental processes at the intestinal wall [87]. Over the past few decades, a few key animal models have been developed in order to help gain a better understanding of how EAEC modulates mucosal immune responses.

## **2.7 Current animal models used in examining EAEC infection**

The first reported animal model used to study EAEC infection was published in 1988 [88]. Specifically, a team of scientists commenced the preliminary infectious trials using ligated intestinal loops in NZB rabbits and Fischer 344 rats. EAEC infection resulted in intestinal lesions, limb paralysis, and even death in some animals. These studies provided ample evidence that EAEC exhibited sufficient distinct characteristics in comparison to other diarrheagenic *E. coli* (DEC) strains to become its own discrete category. These studies also proposed EAEC virulence was likely accompanied by toxins [88]. Currently there is a substantial deficiency in the development of reliable and reproducible animal models that effectively portray immunological responses toward EAEC at the gut mucosa. The developing animal models can be subdivided into two

general categories. The first group includes models that use young animals while the second uses adults. Known animal models used to date will be described in more detail below.

**Gnotobiotic piglets:** One approach to study the pathogenicity of EAEC in neonates uses 24-hour-old germ-free piglets. This model clearly demonstrates detrimental effects caused by EAEC at the colonic mucosa and most closely resembles pathology observed in humans. Infected animals suffer from severe diarrhea and sometimes mortality. Importantly, in this model EAEC adhere to the mucosal epithelium in the described “stacked-brick” pattern verified during *in vitro* studies and elicit edema and lesions in the intestinal lamina propria. The gnotobiotic piglet appears to be one of the best whole-animal models reported however confines to using the model are high [89]. Piglet models have low scalability and are extremely labor intensive in comparison to rodent models. Also, large animal biosafety level 2 (ABSL2) facilities are not as readily available as mouse ABSL2 facilities. Lastly there is a large deficiency in the production of swine carrying targeted gene mutations (conditional knockout animals) restricting studies to “wild type” animals. More than a decade has passed since advances in a swine model of EAEC infection have been reported. Despite boundaries, swine remain an ideal model for studying human diseases, especially those affecting the gastrointestinal tract. Pigs are monogastrics and leverage the gastrointestinal, nutritional, metabolic, and immunologic similarities of humans [90-92].

**Neonatal and weaned C57BL/6 mouse model with or without malnutrition:** Another neonatal model was developed more recently using 6-day-old C57BL/6 mice to study EAEC induced infantile diarrhea. Mice are challenged orally with a bacterial inoculum of prototype EAEC strains 042 or JM221 and remain with their dam for regulated time periods until weaning. Additionally, to comparatively analyze vulnerability to EAEC in young children, a weaned mouse model was established in parallel with an emphasis on the effects of malnutrition. In these studies, weaned C57BL/6 mice are fed either regular (20% protein) or malnourished (2% protein) diet throughout the duration of infection. Both neonatal and weaned mice experience significant developmental stunting due to infection and malnutrition intensifies this effect, especially in mice infected with strain JM221. Mild histopathological changes in the colonic epithelium including localized inflammation and goblet cell hyperplasia are noticed as early as four days post infection. Due to protein malnutrition, the infectious burden can become chronic and bacterial

shedding persists for over three weeks post infection. While these models have opened the door to potentially divulge novel characteristics of EAEC induced childhood infection, the model has limited translational value. Experimental limitations include the fact that symptoms are only mild in relation to reported human infections and mice do not develop overt diarrhea. Nonetheless, these experiments were successful in portraying heterogeneity between separate EAEC strains (042 and JM221) that remain novel and beneficial to the scientific community though research advancements using this model remain remarkably underreported [93].

Antibiotic treatment of adult mice in combination with infection: Adult mice over the age of 6-weeks are treated with 5g/liter streptomycin for up to 48 hours prior to infection and for the duration of the experiment. Some experimental designs also include treatment with sodium bicarbonate just prior to infection in order to neutralize gastric acid. This model has been successful at recapitulating *in vitro* studies demonstrating the ability for Pic, a serine protease autotransporter secreted by EAEC, to enhance EAEC growth in mouse colons by its utilization of nutrients from mucin [49]. Another study using this model provides insight on EAEC promoter induction *in vivo* through luminescence imaging [94]. Though advancements in understanding a key role of Pic in EAEC pathogenesis have resulted from using this approach, mice in this model do not develop clinical signs or histopathological abnormalities according to authors using this protocol therefore gaining no expansion in the ability to study mechanisms of inflammation at the gut mucosa.

Immunodeficient mice and human intestinal xenograph implantation: Most recently, the need for an effective *in vivo* model prompted a unique investigation using severe-combined immunodeficient (SCID) mice and xenographs from fetal intestinal tissue to generate a model with intact and functional human tissue during infection. Fetal tissue was implanted into the subscapular region of SCID mice and then infected by direct intraluminal inoculation. Findings using this approach demonstrated the ability for AAF to trigger polymorphonuclear cell (PMN) migration across mucosal surfaces of the intestinal epithelial barrier [46]. However, the availability of fetal tissue is rare and restricted, which will significantly constrict the broad acceptability and use of this model. Also, this approach does not allow scientists to address critical interaction between innate and adaptive immunity during EAEC infection leaving a significant portion of the pathogenesis story untold.

The development of animal models as a means of studying chronic and acute EAEC infections has provided insight into novel features related to EAEC pathogenesis, however all established animal models are underused and limited in their ability to fully characterize immunological responses to EAEC at the intestinal mucosa. This is likely due to many factors and scientists studying this particular infectious disease have proposed two probable causes. Firstly, disease severity is mild at most during *in vivo* trials likely resulting from dampened immune responses compared to human studies. Secondly, it is likely that EAEC pathogenesis has primarily adapted to human intestinal tissue enhancing variability in clinical manifestation that does not imitate natural disease [4, 46]. Collectively, there is still a desperate need for a reproducible and comprehensive animal model that results in significant disease activity, weight loss and intestinal pathology.

## **2.8 Computational modeling approaches to understand EAEC pathogenesis**

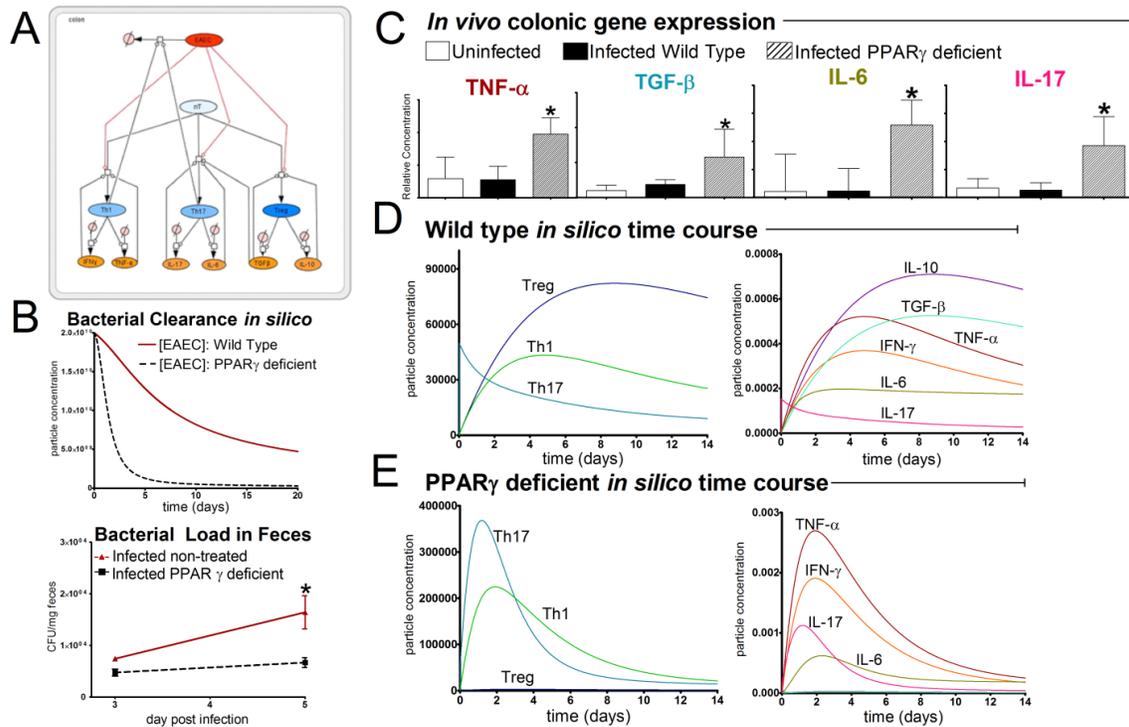
Systems biology is an emerging field that transcends biology, engineering and computer science, and aims to elucidate mechanistic functions in complex systems through the integration of computational work and biological research using mathematical models [95, 96]. These models range in size, purpose, and specificity to include infectious mechanisms at the cellular level, tissue level, and individual level, to population-scale disease spread [95, 97, 98]. The first step in creating a model consists of a comprehensive literature search and construction of a network portraying mechanisms of interest. A specific mathematical modeling approach is selected and applied to the network allowing calibration to begin. Model refinement continues in a cyclic process to incorporate experimental validation or add additional dynamics.

Two common mathematical approaches used to implicitly model the kinetics of biological processes include stochastic and deterministic modeling systems [99, 100]. Stochastic models hold an advantage in accounting for randomness in a system and they produce results based on probability mimicking individual variation more realistically. Agent based models (ABM) are an example of a powerful stochastic modeling technique for predicting and simulating biological events. In ABM, each entity, or agent, in the system assesses its status and makes a decision based on the current environment. This

constant sensing generates randomness in the data thus providing the most realistic approach for modeling systems that are nonlinear and discrete. However, stochastic models are extremely mathematically complex requiring extensive time for development, fitting, and calibration, requiring mathematical expertise. This is explained by the fact that, as the name implies, ABM simulate individual agents and thus simulating the behavior of large systems with many entities as one unit is extremely computationally intense and often require high-performance computing solutions [101]. In contrast, deterministic models require less data, are considered more user-friendly, and multiple software programs currently exist to assist the user in construction [102, 103]. Deterministic models can be built around the law of mass action, a fundamental law that governs rates of reactions in biochemistry. This is performed by assigning rates of creation and degradation for each species. Ordinary differential equations (ODEs) then combine the set of functions assigned to each species and, through numerical integration using given data, express the rate of change of each molecule; a species' concentration over time. Since deterministic models are equation-based systems, the evaluation and execution of a simulation will be consistent each time the task is performed unless the user manually changes the rates and parameters. This minimizes the complexity and time requirement for simulations when compared to the ABM systems. Deterministic models have been vastly useful for scientists inquiring mechanisms behind infection, especially using a series of ODEs [95, 96, 99].

The Modeling Immunity to Enteric Pathogens (MIEP) program at Virginia Tech has developed a complex network representing host interactions with EAEC at the colonic mucosa and CD4+ T cell differentiation using the graphical software package, CellDesigner. Interactive annotated EAEC and CD4+ T cell differentiation models developed by MIEP are deposited and archived on the team website, [www.modelingimmunity.org/models](http://www.modelingimmunity.org/models), available to download. The EAEC model is comprised of four compartments including the colonic lumen, epithelium, lamina propria, and mesenteric lymph node (MLN). EAEC enters the system at the lumen and immediately contacts epithelial cells causing a cascade of reactions triggering cytokine secretion, neutrophil activation, and macrophage differentiation in the lamina propria. Additionally, dendritic cells sample the EAEC present in the lumen and subsequently migrate to the MLN facilitating naïve T cell differentiation activating adaptive immunity. Phagocytic macrophages and neutrophils play primary roles in expediting EAEC death in

the model. Th1 and Th17 CD4+ cell populations are known to possess antimicrobial properties and provide defense against extracellular bacteria [97, 98]. Thus after T cell migration into the LP, Th1 and Th17 cells also assist in bacterial clearance. Initial steps in EAEC model calibration involved isolating parameters regulating T cell differentiation and bacterial clearance to reconstruct a smaller network. A calibration database was generated using in house gene expression, flow cytometry, and bacterial shedding data from malnourished mice infected with EAEC strain JM221; parameters were estimated in COPASI. The model successfully portrays chronic bacterial burden in malnourished EAEC infected mice and significantly reduced T cell populations, lack of effector responses, and low concentrations of proinflammatory cytokines signifying immunodeficiency. To assess the model's ability to predict immune responses to EAEC in PPAR $\gamma$  deficient mice, we reduced the ability for naïve T (nT) cells to differentiate into Treg and enhanced Th1 and Th17 differentiation. The model successfully predicts a dominant proinflammatory Th17 effector response correlated with successful bacterial clearance by day 5 post infection. The systems biology approach to predict the modulation of PPAR $\gamma$  on CD4+ T cell responses using this approach has been extensively developed and provided novel unintuitive characterizations of unforeseen mechanistic pathways [104]. We are confident that this new approach will deliver favorable predictive results with new revelations surrounding innate and adaptive host responses toward EAEC (Figure 2.2).



**Figure 2.2: Modeling immune responses to EAEC.** The EAEC T cell differentiation model network created in CellDesigner using systems biology markup language (A) was linked to COPASI software for the calibration. A calibrated wild type system was created using in house data from malnourished EAEC strain JM221 infected mice (C) (asterisks indicate statistical significance;  $p < 0.05$ ) [81]. PPAR $\gamma$  deficiency was simulated by modulating the parameters regulating T cell differentiation into separate phenotypes; Th1 and Th17 cell differentiation was enhanced equally while Treg differentiation was decreased in an equal magnitude. Bacterial clearance predicted *in silico* mimicked EAEC quantification in feces from infected mice (B). *In silico* simulations of a time course infection over 14 days were performed using COPASI. The wild type system (D) portrays immunodeficiency while the PPAR $\gamma$  deficient system (E) predicted enhanced effector responses.

## 2.9 Discussion and Conclusions

EAEC has been recognized as a causative agent of persistent diarrheal illness worldwide for over two decades. A better understanding of the cellular responses, particularly the adaptive immunity, involved in host response toward EAEC is critical for the development of treatments to ameliorate disease. The ability to validate that effector Th17 responses are induced by EAEC would have great value on targeting cellular responses and specific molecular mechanisms during therapeutic treatments in chronically ill or immunocompromised patients. To date, no such studies are presented and this is likely attributed to the lack of a reliable animal model. Limitations of animal models have hindered advancements in novel discoveries of pathophysiology and

preclinical testing for therapeutics. Thus there is still a desperate need for highly reproducible animal models that provide an outlet to examine cellular responses at the intestinal mucosa during EAEC infection. Lastly, transdisciplinary immunoinformatics approaches that combine omics data and computational modeling to compile complex and heterogeneous data regarding host responses to EAEC holds great potential in unveiling dynamic commonalities in mechanisms of infection that have otherwise been undetected.

# Chapter 3

## The Role of Peroxisome proliferator-activated receptor $\gamma$ in Immune Responses to Enteroaggregative *Escherichia coli* infection

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### 3.1 Summary

Enteroaggregative *Escherichia coli* (EAEC) is recognized as an emerging cause of persistent diarrhea and enteric disease worldwide. Mucosal immunity towards EAEC infections is incompletely understood; due in part to the lack of appropriate animal models. This study presents a new mouse model and investigates the role of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) in the modulation of host responses to EAEC in nourished and malnourished mice. Wild-type and T cell-specific PPAR $\gamma$  null C57BL/6 mice were fed protein-deficient diets at weaning and challenged

with  $5 \times 10^9$  cfu EAEC strain JM221 to measure colonic gene expression and immune responses to EAEC. Antigen-specific responses to *E. coli* antigens were measured in nourished and malnourished mice following infection and demonstrated the immunosuppressive effects of malnutrition at the cellular level. At the molecular level, both pharmacological blockade and deletion of PPAR $\gamma$  in T cells resulted in upregulation of TGF- $\beta$ , IL-6, IL-17 and anti-microbial peptides, enhanced Th17 responses, fewer colonic lesions, faster clearance of EAEC, and improved recovery. The beneficial effects of PPAR $\gamma$  blockade on weight loss and EAEC clearance were abrogated by neutralizing IL-17 *in vivo*. Our studies provide *in vivo* evidence supporting the beneficial role of mucosal innate and effector T cell responses on EAEC burden and suggest pharmacological blockade of PPAR $\gamma$  as a novel therapeutic intervention for EAEC infection.

### **3.2 Introduction**

Enteroaggregative *E. coli* (EAEC) is a Gram-negative, rod-shaped bacterial pathogen of the *Enterobacteriaceae* family recognized as an emerging causative agent of gastroenteritis and diarrhea in developing and industrialized countries worldwide [105, 106]. EAEC infections can cause diarrheagenic symptoms in immunocompromised adults, travelers, victims of food borne illness [107], and particularly severe cases in children with malnutrition [5, 35]. The relationship between malnutrition and diarrheagenic infection has been described as a vicious cyclic pattern hindering the host's ability to clear bacteria and ameliorate disease [108]. Malnutrition predisposes individuals to infection by impairing epithelial barrier integrity and suppressing immune responses [109]. Adverse effects to intestinal absorption are exacerbated during infection generating a catabolic state that depletes nutrients needed for tissue synthesis

and growth further increasing the likelihood of pathogens breaching the epithelial barrier [110]. Malnutrition impairs host responses thereby amplifying infection and pathology [111]. More importantly, EAEC infections hinder the functionality of the epithelial barrier disrupting nutrient absorption worsening malnutrition and potentiating growth retardation [112].

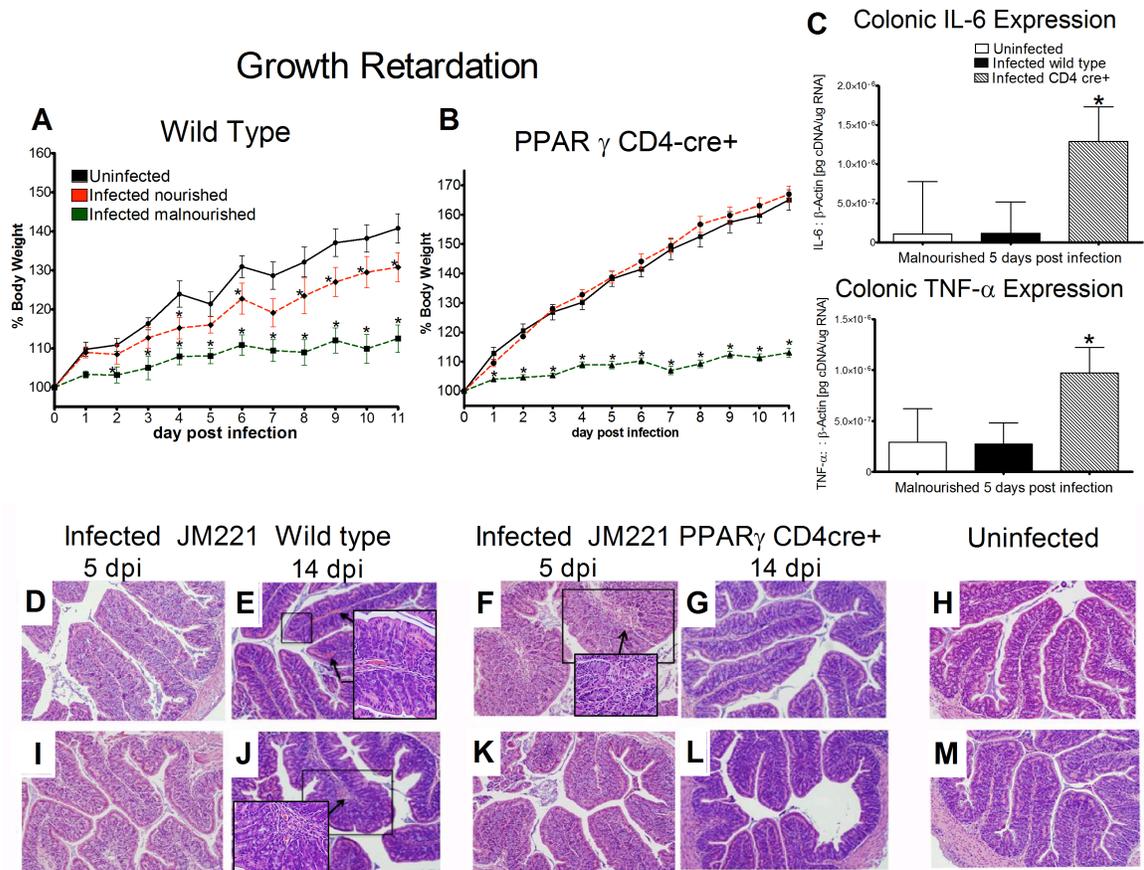
*E. coli* pathovars use a multi-step scheme for pathogenesis consisting of mucosal colonization, evasion of host defenses, replication, and host damage. Direct contact with the epithelium is a key determinant of the host's innate immune response to EAEC [73]. Specifically, AAF fimbriae are presumably the primary pathognomonic virulence factor contributing to the manifestation of EAEC infection. Aggregated adherence to enterocytes by means of the AAF fimbriae fosters an environment prone to increased colonization. Upon aggregating, EAEC has the capability to disrupt epithelial tight junctions, subsequently leading to penetration of bacterial toxins and induction of the host's mucosal immune response [45]. Interaction between EAEC flagellin and Toll-like receptor 5 on host epithelial cells elicits a proinflammatory response extensively characterized by secretion of IL-8 from epithelial cells [74, 75]. Proinflammatory responses induced by EAEC are thought to contribute to the pathogenesis of EAEC. IL-8, a principal chemoattractant for polymorphonuclear leukocytes and the migration of these cells into the intestinal mucosa, is a hallmark of inflammatory infectious diarrhea including EAEC-induced disease [113]. Recruitment and transmigration of neutrophils to the gut mucosa causes intestinal damage that may promote EAEC colonization [114]. The role of T cells, dendritic cells (DC) and macrophages in mucosal responses to EAEC remains incompletely understood.

The mucosal immune system in the intestine peacefully coexists with 100 trillion commensal bacteria while responding swiftly to pathogens such as EAEC. These

studies aimed to characterize the role of mucosal inflammatory and effector responses during acute EAEC infection and their relation to clinical recovery in a mouse model of malnutrition-induced immunosuppression. We targeted the transcription factor peroxisome proliferator activated receptor (PPAR)  $\gamma$  pharmacologically and genetically to modulate mucosal inflammation and immunity [115] during EAEC infection to evaluate initiation, progression and outcomes. Specifically, we used the compound 2-chloro-5-nitrobenzanilide (GW9662), a potent PPAR $\gamma$  antagonist [116], and conditional PPAR $\gamma$  knockout mice to delineate the impact of PPAR $\gamma$  during infection with EAEC in nourished and malnourished mice.

### **3.3 The loss of PPAR- $\gamma$ in T cells diminishes growth retardation during EAEC infection**

Detrimental growth shortfalls were observed in infected malnourished mice of all genotypes as early as day three post-infection (PI). Malnourished mice never gained more than 15% of their body weight due to severe protein deficiency. PPAR $\gamma$  null CD4cre<sup>+</sup> mice on a control diet grew at rates similar to uninfected mice while nourished infected wild type (WT) mice experienced significant retardation in growth up to 11 days after challenge (Figure 3.1).



**Figure 3.1. Early beneficial effects of PPAR $\gamma$  deficiency in T cells during enteroaggregative *Escherichia coli* (EAEC) challenge.** Growth retardation in wild type (A) and T cell specific PPAR $\gamma$  deficient mice (B) is expressed as percent growth from day 0 after challenge. Gene expression for IL-6 and TNF- $\alpha$  in colonic tissue of malnourished C57BL/6 and PPAR $\gamma$  CD4cre+ mice was analyzed using quantitative real-time RT-PCR on day 5 PI (C). Representative photomicrographs of colonic specimens of infected mice at 5 or 14 days PI in infected wild type mice (D,E,I,J), infected mice lacking PPAR $\gamma$  expression in T cells (F,G,K,L), and uninfected controls (H,M). The top panel corresponds to nourished mice whereas the bottom panel corresponds to malnourished mice. Original magnification 200 $\times$ . Boxes and arrows are areas where an amplified image (400 $\times$ ) is provided to emphasize examples of leukocyte infiltration, mucosal thickening, goblet cell hyperplasia, and vasodilation. Mice per group: n=8. Asterisks indicate values where differences are statistically significant ( $p < 0.05$ ).

### **3.4 Histological analysis demonstrates faster recovery in mice lacking PPAR $\gamma$ in T-cells whereas wild type mice experience prolonged inflammation**

On day 5 PI CD4cre<sup>+</sup> mice had significantly higher levels of mucosal thickening (illustrated by arrow Figure 3.1) and leukocyte infiltration (representative of boxed area Figure 3.1) while alterations in tissue architecture were negligible in colons of WT mice. Importantly, although significant colonocyte hyperplasia occurred in CD4cre<sup>+</sup> mice 5 days PI, the epithelial layer showed no signs of erosion or harmful cell death signifying epithelial barrier integrity was not negatively compromised due to inflammation. Conversely, at dpi 14 WT mice experienced increased levels of mucosal thickness (depicted by arrow Figure 3.1), vasodilation, goblet cell hyperplasia (boxed-in example Figure 3.1), and leukocyte infiltration while CD4cre<sup>+</sup> mice had no significant signs of inflammation or chronic burden of disease and resembled uninfected colons (Figure 3.1).

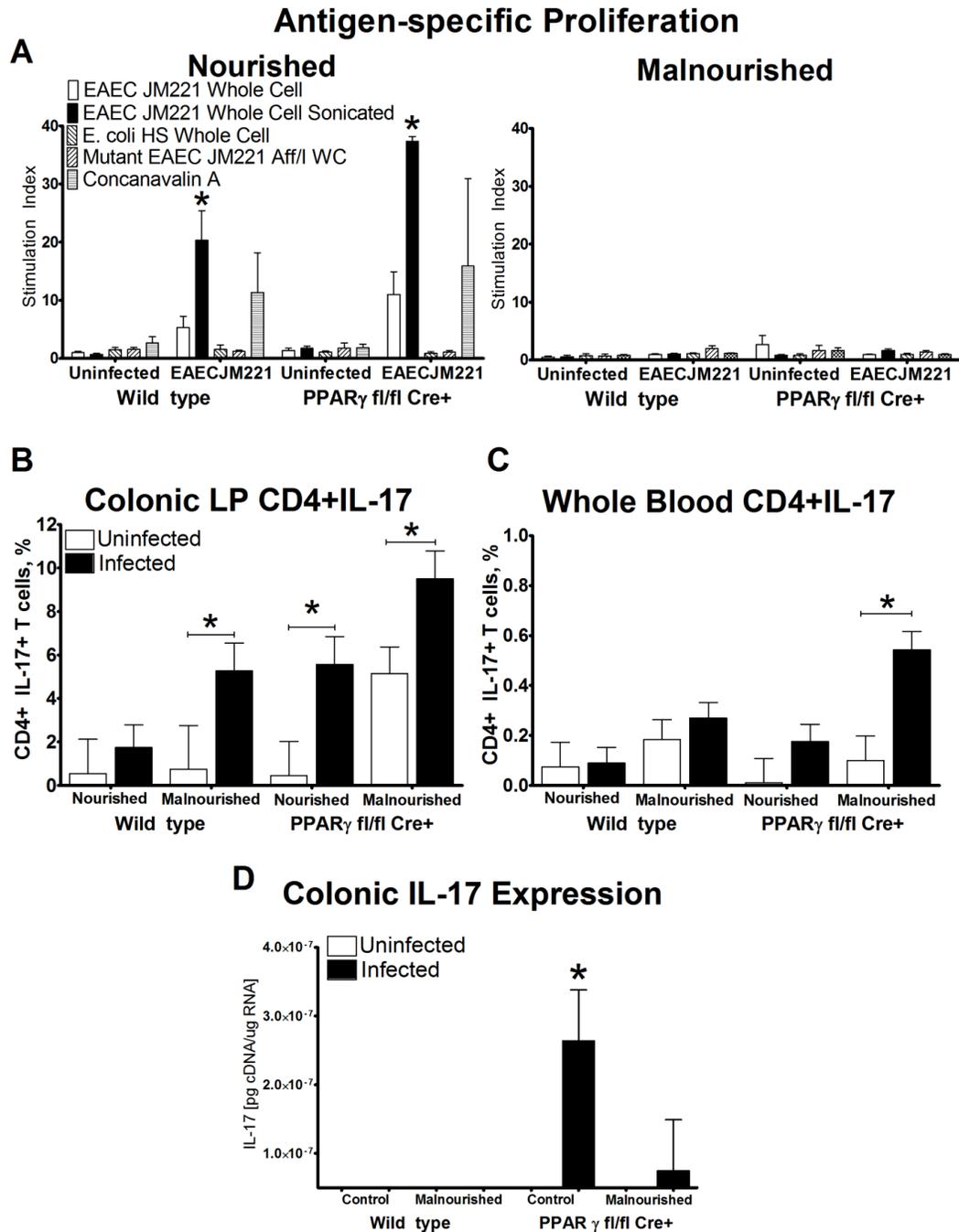
### **3.5 PPAR $\gamma$ deficiency in T cells enhanced mucosal effector response characterized by significant increases in proinflammatory gene expression early during infection**

At day 5 PI, malnourished CD4cre<sup>+</sup> mice expressed significantly elevated levels of IL-6, a pro-inflammatory cytokine responsible for neutrophil and monocyte recruitment early during acute infections [117]. TNF- $\alpha$ , another inflammatory cytokine and activator of neutrophils [118, 119], was also significantly upregulated in colonic tissue from CD4cre<sup>+</sup> mice early during infection (Figure 3.1).

### **3.6 Enhanced antigen-specific proliferative recall responses in mice lacking PPAR $\gamma$ in immune and epithelial cells accompanied by increased levels of IL-17 associated with dampened EAEC burden at 14 days PI**

Overall lymphocyte proliferation was assessed in splenocytes using *ex vivo* antigen stimulation and the incorporation of titrated thymidine in a lymphocyte blastogenesis test. The loss of PPAR $\gamma$  enhanced the magnitude of antigen-specific recall responses to EAEC in nourished mice, whereas malnutrition abrogated responsiveness to antigens or to the mitogen ConA regardless of genotype. In addition, PPAR $\gamma$  deficiency led to an

increase in colonic IL-17 expression and Th17 responses. IL-17 is one of the first cytokines released during innate responses and plays an essential role in mucosal defense against extracellular bacteria through neutrophil trafficking [120] which is critical for host defense against various pathogens [121]. Tissue from the whole colon was analyzed for IL-17 gene expression 14 days PI. Regardless of infection or diet several mice expressed low basal levels of IL-17 in the colon, however nourished MMTV-cre+ mice expressed significantly elevated levels of colonic IL-17 compared to all other groups. Malnourished MMTV-cre+ mice also had a higher tendency to express IL-17. Flow cytometric analysis provided evidence that percentages of local CD4+ T cells expressing IL-17 (i.e., Th17 cells) at the colonic mucosa were increased in nourished and malnourished MMTV-cre+ and malnourished WT mice. The systemic levels of Th17 cells were also significantly elevated in malnourished MMTV-cre+ mice. These combined results suggest that EAEC infection may induce Th17 responses and the loss of PPAR $\gamma$  enhances the magnitude of Th17 responses. All mice except for the wild-type malnourished completely cleared colonization by day 14 post infection providing evidence that the enhanced effector responses facilitated bacterial clearance (Figure 3.2).



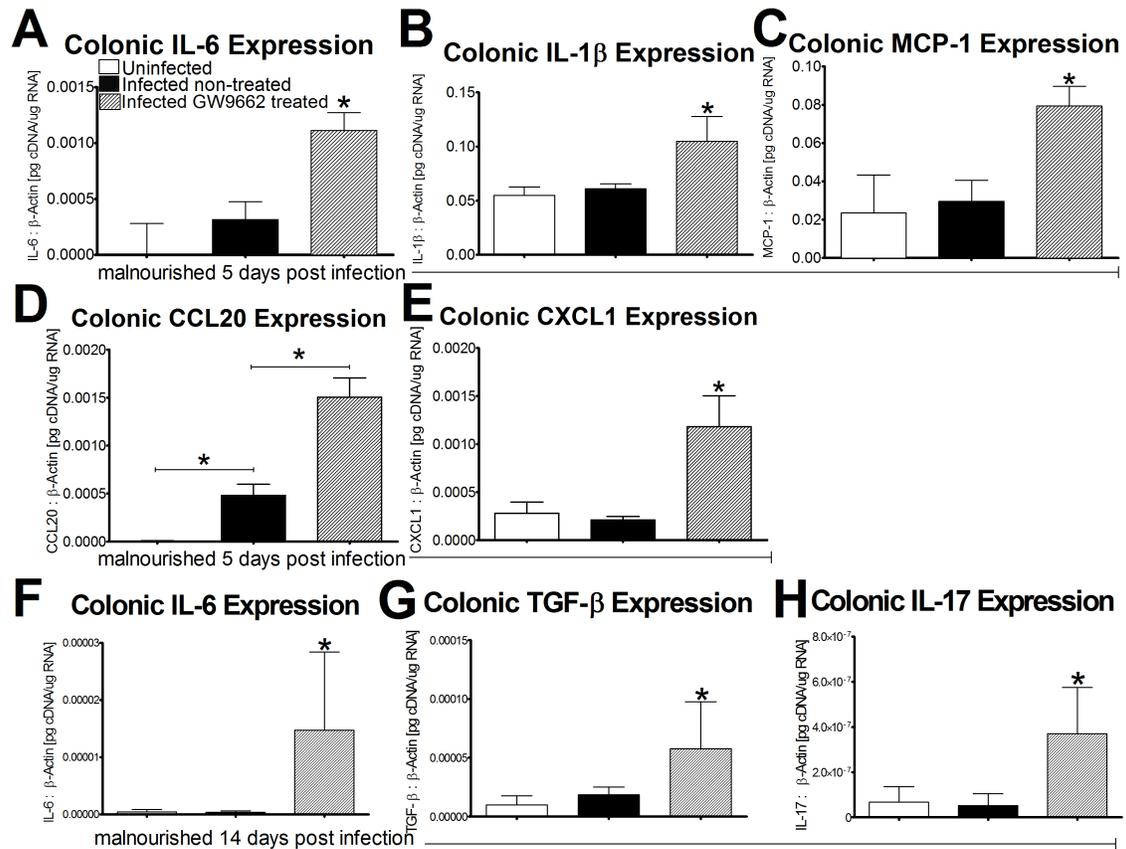
**Figure 3.2. Immune responses during enteroaggregative *Escherichia coli* (EAEC) infection in peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )-deficient mice associated with bacterial clearance.** Antigen specific recall responses of splenocytes from mice infected with EAEC were measured ex vivo using the lymphocyte blastogenesis test. EAEC JM221 whole cell and whole cell sonicate were used in parallel to two negative controls, *E. coli* HS and mutant EAEC Aff/I strains as well as one positive control, concanavalin A (ConA). Lymphocyte proliferation is expressed stimulation indexes which are calculated by dividing the counts per minute (CPM) of antigen-stimulated wells by the CPM of unstimulated wells (A). IL-17 expression was assessed in colonic lamina propria (B) and whole blood (C) CD4+ T cells by flow cytometry

and in the colon by quantitative real time RT-PCR (D) 14 days PI. Mice per group: n=10. Asterisks indicate values where differences are statistically significant ( $p<0.05$ ) while bars connect groups where comparisons are made.

### **3.7 Pharmacological blockade of PPAR $\gamma$ induces effector and antimicrobial mucosal responses and facilitates bacterial clearance early during infection**

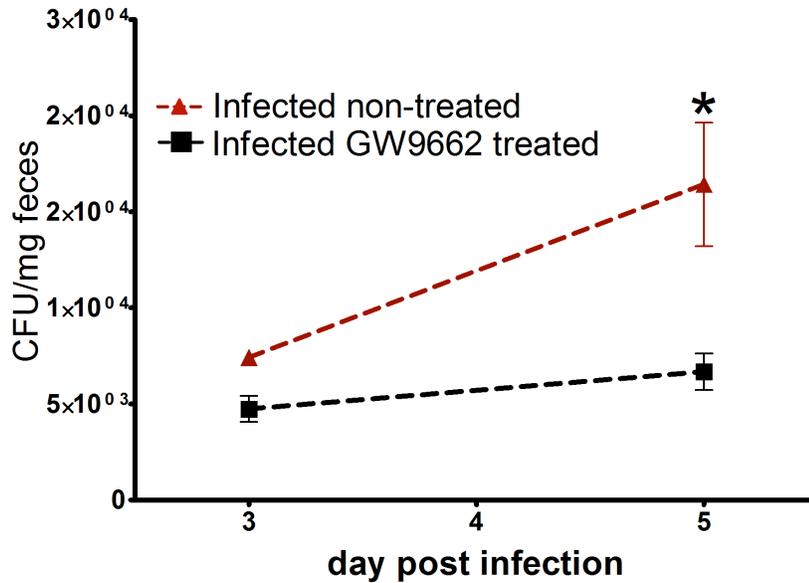
Mice that received GW9662 (1 $\mu$ M) treatment expressed significantly higher levels of proinflammatory cytokines in the colon, including IL-1 $\beta$ , IL-6, CXCL1, and MCP-1, when compared to the untreated group at day 5 PI. CCL20 was significantly upregulated in both treated and non-treated infected mice compared to uninfected controls. Additionally, GW9662 treated mice expressed significantly higher levels of colonic CCL20 when compared to the untreated infected mice (Figure 3.3). A significant decrease in IL-10 expression exists in both infected groups at day 5 PI however no significant differences were observed for expression of IL-12p35 and IL-4. Proinflammatory cytokine responses in GW9662 treated mice were associated with significantly larger percentages of infiltrating cells to the colonic lamina propria at 5 days post infection. Percentages of CD3+CD4+ T-cells and MHCII+CD11b-CD11c+ DC were significantly higher in GW9662 treated mice while untreated mice experienced higher levels of GR1high+CD11b+ neutrophils. Although no significance in MHCII+F4/80+CD11b+ macrophages was detected between groups, mice treated with GW9662 tended to have higher percentages of this cell phenotype. Fecal bacterial shedding results demonstrated a significant EAEC burden in infected untreated mice at the peak of infection, 5 days PI, while GW9662 treated mice experienced a mild level of EAEC shedding throughout the duration of infection (Figure 3.4). S100A8 and S100A9, proteins that form the antimicrobial peptide complex known as calprotectin, were also significantly upregulated in the colon of mice treated with GW9662 on day 5 PI displaying an enhanced antimicrobial response associated with bacterial clearance. By day 14, calprotectin levels were nearly undetectable in all mice when compared to expression values on day 5 portraying a reduction in antimicrobial responses after the peak of infection (data not shown). Remarkably, by day 14 PI GW9662 treated mice had sustained the stark increase in IL-6 while simultaneously expressing significantly elevated levels of colonic TGF- $\beta$  and IL-17 further suggesting a Th17 effector response late during infection. Colonic gene

expression for IL-10, IL-12p35, and IL-4 revealed no significant differences among groups suggesting that regulatory T cells (Treg), Th1, and Th2 phenotypes were unaffected during infection.

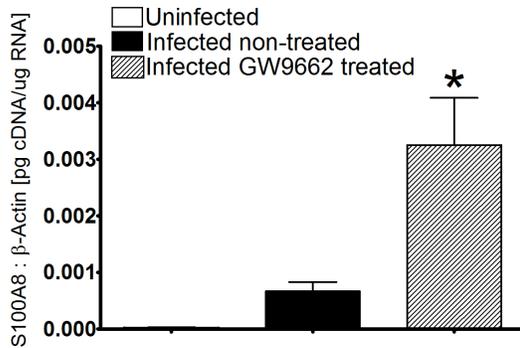


**Figure 3.3. Gene expression suggests a T helper 17 response in mice when peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is antagonized.** Gene expression data from colonic tissue of malnourished C57BL/6 mice was analyzed using quantitative real-time RT-PCR and reported as values normalized to  $\beta$ -actin. IL-6, IL-1 $\beta$ , MCP-1, CCL20, and CXCL1 were quantified at day 5PI (mice per group: n=10) (A–E) while IL-6, TGF- $\beta$ , and IL-17 were quantified 14 days PI (n=10) (F–H). Asterisks indicate values where differences are statistically significant ( $p < 0.05$ ) while bars connect groups where comparisons are made.

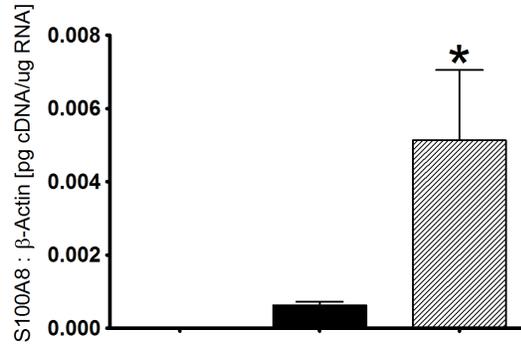
## A Bacterial Load in Feces



## B Colonic S100A8 Expression



## C Colonic S100A9 Expression

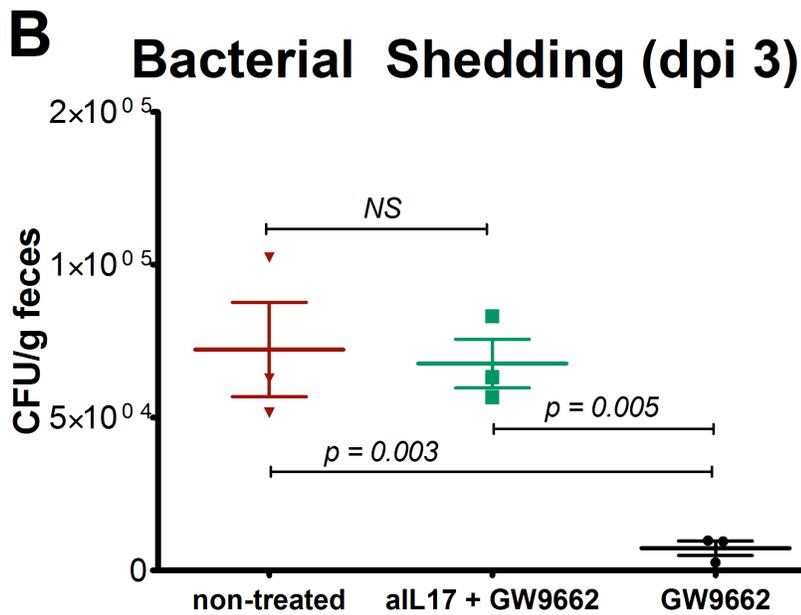
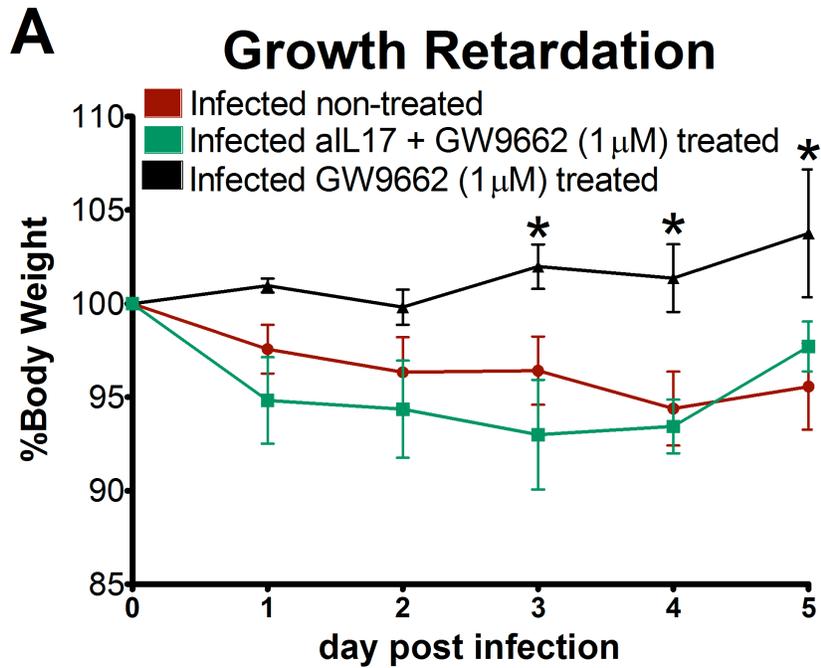


malnourished 5 days post infection

**Figure 3.4. Pharmacological blockade of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) associated with antimicrobial response and bacterial clearance.** Enteroaggregative *Escherichia coli* (EAEC) burden in colon was assessed by quantitative real time RT-PCR using bacterial DNA isolated from feces of infected mice treated with PPAR $\gamma$  antagonist GW9662 (n=9) or left untreated (n=9). Data is presented as CFU/mg of tissue. S100A8 and S100A9 gene expression was analyzed in colonic tissue from C57BL/6 malnourished mice at day 5 days PI (n=10) using quantitative real-time RT-PCR (B and C). S100 proteins are presented as values normalized to  $\beta$ -actin. Asterisks indicate values where differences are statistically significant ( $p < 0.05$ ).

### **3.8 Neutralizing anti-IL-17A antibody treatment abrogates the beneficial role of GW9662 in weight loss and EAEC burden**

Simultaneous treatment with anti-IL17A and GW9662 (1 $\mu$ M) resulted in significant differences in body weight beginning on day 3 post-infection (Figure 3.5). The pattern of weight loss in mice treated with both anti-IL17A and GW9662 resembled that of untreated mice. Mice solely receiving GW9662 grew at significantly faster rates than the other two groups beginning 3 days after infection. More importantly, significant weight loss coincided with increased bacterial burdens on day 3 PI in mice from non-treated and anti-IL17A+GW9662 groups when compared to GW9662 treatment alone. These data suggest that the beneficial effects on the host resulting from PPAR $\gamma$  blockade are largely mediated by IL-17A. An additional study performed to assess dose-response effects of GW9662 on IL-17 production revealed that increasing concentrations of GW9662 during infection significantly upregulated colonic IL-17A mRNA expression.



**Figure 3.5. Neutralization of IL-17 abrogates the beneficial effects of GW9662 on weight loss and bacterial burden.** Growth retardation in infected wild type mice is expressed as percent growth from day 0 after challenge (A). Enteroaggregative *Escherichia coli* (EAEC) burden in the colon was assessed by quantitative real time RT-PCR using bacterial DNA isolated from feces of infected mice treated with 1 μM PPARγ antagonist GW9662 (n=3), 50 μg anti-IL17 and 1 μM GW9662 combined (n=3) or untreated (n=3). Asterisks indicate values where differences are statistically significant ( $p < 0.05$ ), NS signifies no significant difference, and bars are present to indicate significance between groups.

### 3.9 Discussion and Conclusions

Malnutrition is the most common cause of immunodeficiency worldwide [122]. Protein energy malnutrition is associated with significant impairments in cell-mediated immunity, phagocytosis, cytokine production, and other necessary immune processes that contribute to host protection [123]. Malnourished children are exceptionally susceptible to pathogenic diarrhea that persists and enhances the burden of malnutrition [109, 124]. We provide evidence that malnourished wild type mice exhibited an impaired ability to induce proinflammatory cytokines during EAEC infection. Indeed, cytokine gene expression in the colon of infected, untreated, malnourished, wild type mice was no different than uninfected mice and the lymphoproliferative recall responses of splenocytes to EAEC were impaired. The immunodeficiency observed suggests that these malnourished mice are unable to mount protective innate or adaptive immune responses to EAEC in the gastrointestinal tract. Histopathological analysis indicated minimal inflammation in colons of untreated mice in response to EAEC challenge early during infection even though significantly higher percentages of neutrophils were detected in the colonic lamina propria at that time. By day 14 PI untreated mice began showing signs of chronic pathological burden in the colon noted by elevated bacterial loads, increased goblet cell hyperplasia, and leukocyte infiltration, though the response was still impaired.

PPAR $\gamma$  is a widely expressed transcription factor (i.e., expressed in epithelial cells, macrophages, T and B lymphocytes) and a potent immune modulator that suppresses effector and inflammatory responses [83]. The anti-inflammatory effect of PPAR $\gamma$  activation has been extensively studied and known to be mediated by the inhibition of signaling pathways such as NF- $\kappa$ B, AP-1 and STAT [125]. Agonists of PPAR $\gamma$  have shown therapeutic efficacy in mouse models of colitis [126, 127] and clinical

inflammatory bowel disease (IBD) [128]. In contrast to IBD, where suppression of inflammation is the desired outcome, our data suggests that a more potent inflammatory and effector response early following infection might be required to clear EAEC infection. We diminished the functionality of PPAR $\gamma$  using a targeted deletion in knockout mice and pharmacological blockade through administration of GW9662, a PPAR $\gamma$  antagonist. Our data indicates that EAEC-infected PPAR $\gamma$  deficient mice developed stronger inflammatory and effector responses towards EAEC early following the challenge ultimately leading to faster recovery from infection. Significant increases in IL-17 were observed locally and systemically while additional potent proinflammatory cytokines (e.g., IL-6, TNF- $\alpha$ , IL-1 $\beta$ ) were also significantly upregulated in mice that lacked PPAR $\gamma$ . Epithelial cells secrete a distinct array of proinflammatory mediators including IL-6 [129], TNF- $\alpha$ , and MCP-1 in response to bacterial invasion [130]. During the early stage of infection, PPAR $\gamma$  deficiency significantly upregulated IL-6 and TNF- $\alpha$  expression in malnourished mice. These two proinflammatory cytokines are involved in neutrophil and monocyte recruitment and activation. Likewise, IL-1 $\beta$ , a proinflammatory cytokine responsible for macrophage activation, and CXCL1, a neutrophil-recruiting chemokine, were enhanced significantly [131, 132]. Although IL-1 $\beta$  is translated as an inactive precursor that becomes cleaved by caspase-1 to form its secreted state and caspase-1 activation is dependent upon the formation of a multimolecular scaffold inflammasome, the relative induction of IL-1 $\beta$  mRNA is significant [133]. Additionally, pharmacological blockade of PPAR $\gamma$  increased the colonic expression of MCP-1 five days PI in malnourished mice. MCP-1 expression is induced by IL-1 $\beta$  and remains a primary chemoattractant for monocytes [131]. MCP-1 binds to CCR2 stimulating monocyte differentiation towards the classically activated proinflammatory M1 phenotype [134]. Intestinal epithelial cells also secrete CCL20 in response to enteropathogenic bacteria

[135]. EAEC-infected GW9662 treated mice significantly upregulated levels of CCL20 in the colon. CCL20 has potent chemotactic properties contributing to the recruitment of CCR6-expressing cells including dendritic cells, B-cells, and some T cell subsets in mucosal tissue [136, 137]. IL-1 $\beta$ , TNF- $\alpha$ , and IL-17A enhance the release of CCL20 [138]. The interrelationship between these cytokines, whose transcription is regulated by the NF- $\kappa$ B pathway in epithelial and immune cells [139], provides evidence for an orchestrated inflammatory response promoting leukocytic infiltration due to the lack of PPAR $\gamma$ .

This significant increase in pro-inflammatory and effector markers directly correlates to our histological analysis showing significant increases in leukocyte infiltration of T cells, dendritic cells, and macrophages 5 days PI in PPAR $\gamma$  deficient mice. Most significantly, when the bacterial load was quantified over the course of infection, malnourished wild type untreated mice experienced a significant EAEC burden on days 3 and 5 PI while mice administered GW9662 shed low levels of EAEC never amounting values comparable to the WT group. In comparison to the untreated infected counterparts whose innate response was almost undetectable, the significant upregulation of proinflammatory cytokines in malnourished mice lacking PPAR $\gamma$  functionality suggests a beneficial effect of antagonizing PPAR $\gamma$  early during infection to evoke a more potent acute inflammatory response to EAEC. Moreover, PPAR $\gamma$  antagonism was associated with significantly increased levels of calprotectin mRNA expression on day 5 PI. Calprotectin is a phagocyte-derived protein with antimicrobial properties abundantly present in neutrophils, monocytes, and macrophages whose expression is directly correlated with bacteriologically positive infectious diarrhea [140, 141]. Calprotectin is commonly used as a biological marker of neutrophilic intestinal inflammation and infiltrating tissue macrophages, thus the significant upregulation of this protein in

GW9662 treated mice provides evidence for an enhanced infiltration of phagocytic leukocytes with antimicrobial properties [142]. The upregulation of calprotectin directly correlates with low levels of EAEC shedding while untreated infected mice that were unable to produce significant amounts of calprotectin experienced enhanced pathological burden at day 5 PI.

In the later phase of infection, WT mice remained immunosuppressed lacking the ability to mount an effective immune response to bacterial invasion noted by the inability to produce proinflammatory genes such as IL-6 and IL-17 at 14 days PI. This too correlated with histopathological findings illustrating a subordinate inflammatory response when compared to the inflammation seen in knockout mice at day 5. Wild type mice continued to lack sufficient responses, however, PPAR $\gamma$  deficient mice transitioned into developing effector responses. By day 14 PI, the significant increase in IL-6 remained and was coupled with upregulation of TGF- $\beta$  in PPAR $\gamma$  deficient mice. Most importantly, IL-17 was also concurrently expressed in these mice at considerably higher concentrations both locally in the whole colon and colonic lamina propria, as well as systemically in blood by day 14 post-infection. Interestingly, increasing concentrations of GW9662 administered to mice provides evidence for a direct correlation between PPAR $\gamma$  blockade via dose concentration and IL-17A mRNA levels. The colonic gene expression of IL-10, IL-12p35, and IL-4 remained suppressed throughout the duration of infection alluding to the limited impact of colonic Treg, Th1, and Th2 cells in this process [143-145]. Recent data is exposing the crucial protective role of IL-17A in immunity to extracellular pathogens because of its abilities to enhance tight junction integrity, mobilize neutrophils, and increase cytokine production by epithelial cells [146]. The primary source of IL-17A varies depending on the immunoregulatory environment. Innate sources of IL-17 appear in a matter of hours after epithelial damage. In the gut, rapid IL-17 production is

attributed to  $\gamma\delta$  T cells, CD8+ T cells, NK cells, NKT cells, and paneth cells [147-149], whereas mast cells [150], alveolar macrophages [151] and neutrophils [152] might also produce IL-17 in certain conditions. If infection persists and the initiation of adaptive immunity occurs, the production of IL-17 is more commonly attributed TCR $\alpha\beta$  CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup> Th17 cells [153]. Since the recent discovery of Th17 effector responses, countless studies are concluding the importance of Th17 responses toward extracellular bacteria [154], but to date no studies have examined Th17 responses in EAEC infection. The Th17 population bridges innate and adaptive immunity to produce a robust antimicrobial inflammatory response essential for an effective mucosal and epithelial response to enteric pathogens [84]. Importantly, a Th17 response can effectively recruit and orchestrate neutrophil activation inducing the killing and clearance of extracellular invading pathogens [85]. Our data demonstrated antigen-specific lymphocyte proliferation of splenocytes from infected nourished knockout mice. These results suggest that the blockage of PPAR $\gamma$  was beneficial in generating an effective EAEC-specific effector response from lymphocytes. The data also implicates antigen presentation from innate immune cells was highly effective in mice lacking PPAR $\gamma$  in all immune and epithelial cells. To investigate the effects of enhanced mucosal effector responses on EAEC burden we quantitatively determined the bacterial burden once again at day 14 PI. Malnourished wild type mice endured persistent elevated levels of EAEC while all other mice had ameliorated disease.

A possible explanation for the upregulation of IL-17 in our studies is an early (4-7 day PI) innate IL-17 response initiated by EAEC followed by a robust Th17 response seen 10-14 days PI similar to previously characterized responses to other pathogenic bacteria [155]. Importantly, CCR6, whose ligand is CCL20, has been established as the homing receptor for Th17 cells and regulates the migration of Th17 cells in the intestine [156].

The significant increase in colonic CCL20 expression in mice treated with GW9662 during infection combined with flow data displaying significantly higher percentages of DC and T cells in the colonic lamina propria further supports an enhanced active recruitment of these cells early during infection. In response to bacterial infection, dendritic cells produce IL-6, TGF- $\beta$ , and IL-1 $\beta$  that drive IL-17 production from innate lymphocytes [157]. A limitation of our colonic IL-17 expression analyses is that we have not characterized the cell that produces this cytokine. However, our flow cytometry data indicate systemic and mucosal production of IL-17 by CD4<sup>+</sup> T cells, suggesting for the first time a role for Th17 responses in the clearance of EAEC infections. We observed significantly higher levels of CD4<sup>+</sup> T cells present in the colonic lamina propria of mice treated with GW9662 illustrating an early recruitment of T cells. Th17 differentiation is induced by a combination of TGF- $\beta$  and IL-6 or IL-21 along with the expression of the ROR $\gamma$ t transcription factor [158]. Colonic gene expression data from day 14 PI showed significant upregulation of IL-6 and TGF- $\beta$  providing further evidence that Th17 cells are present late during EAEC infection. Treatment of mice with GW9662 and anti-IL-17 abrogated the beneficial effects of GW9662 on weight loss and EAEC burden, suggesting that the blockade of PPAR  $\gamma$  ameliorates EAEC infection and disease through an IL-17-dependent mechanism. Future studies will characterize the origin of mucosal IL-17 to determine whether this is an EAEC-mediated Th17 response or an innate response as well as the potential role of the CCL20/CCR6 pathway in mediating infiltration of Th17 cells in the colonic LP.

Previous studies suggest potential harmful effects of inflammation during EAEC infection in healthy adult volunteers due to transmigration of neutrophils disrupting the epithelial barrier [17, 74]. In contrast, we show that malnourished mice were unable to generate effective innate or adaptive responses towards EAEC on their own which resulted in

higher bacterial burden throughout the course of infection. We speculate that mucosal effector responses in the malnourished mouse were impaired due to malnutrition and were therefore unable to control EAEC. By blocking PPAR $\gamma$ , we promoted proinflammatory cytokine production and leukocyte infiltration at day 5 PI. Pharmacological blockade of PPAR $\gamma$  through administration of increasing concentrations of GW9662 provides evidence for a direct correlation between dose concentration and IL-17A mRNA levels. More importantly, PPAR $\gamma$  deficient mice were able to generate a Th17 effector response by day 14 post-infection. These responses were likely involved in bacterial clearance.

In conclusion, we report for the first time the importance of Th17 responses in clearing EAEC infections and the beneficial role for PPAR $\gamma$  blockade and subsequent upregulation of colonic effector and inflammatory responses during EAEC infection. More specifically, PPAR $\gamma$  blockade significantly enhanced lymphoproliferative recall responses, upregulated expression of IL-17, anti-microbial peptides and inflammatory cytokines at the colonic mucosa, and decreased EAEC fecal shedding. Thus, PPAR $\gamma$  antagonism represents a novel host-targeted therapeutic approach for EAEC infections.

### **3.10 Materials and Methods**

*Animal Procedures:* Wild-type, PPAR $\gamma$  tissue-specific conditional knockout mice exhibiting Cre recombinase targeted to the CD4 promoter (PPAR $\gamma$  fl/fl, CD4-cre+) or hematopoietic and epithelial cells (PPAR $\gamma$  fl/fl MMTV-cre+) in a C57BL/6 background were weaned at 21 days of age and assigned to groups that were fed regular purified AIN-93G rodent diet (20% protein) or protein deficient diet (2% protein). Three days post weaning each mouse was challenged intragastrically by gavage with  $5 \times 10^9$  CFU EAEC strain JM221. In follow up studies C57BL/6 mice were administered GW9662 (0.5, 1, or

2  $\mu$ M dose; 13.8mg/kg, 27.6mg/kg, and 55.3mg/kg respectively); Cayman Chemical, Ann Arbor, MI) orogastrically beginning at the time of infection and continuing daily for up to seven days post infection. Anti-IL17A neutralizing antibody (50 $\mu$ g; R&D Systems, Minneapolis, MN) was administered intraperitoneally on days 0, 2, and 4. Body weights and disease activities were monitored daily. Fecal collection for bacterial shedding quantification was performed.

Ethics Statement: All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 10-087VBI) and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act. Animals were under strict monitoring throughout the duration of infection and all efforts were made to minimize suffering. Mice were euthanized by carbon dioxide narcosis followed by secondary cervical dislocation.

Histopathology: Colonic sections were fixed in 10% buffered neutral formalin, later embedded in paraffin, sectioned (6  $\mu$ m) and stained with H&E. Tissue slides were examined in an Olympus microscope (Olympus America Inc., Dulles, VA). Colons were scored for leukocyte infiltration, epithelial erosion, and mucosal thickness.

RNA isolation and real-time polymerase chain reaction of cytokines: Total RNA from colon was isolated using the Qiagen RNA isolation kit (Qiagen) according to manufacturer's instructions, and used to generate the cDNA template using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was performed as previously described [126].

DNA isolation and quantification of EAEC from feces: Fecal samples were weighed and DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions. Primer sequences were designed using the Beacon Designer (PREMIER Biosoft) and used to quantify the dispersin gene (*aap*), using real-

time RT-PCR. The protocol for standard cycling ran as follows: 1) 95°C, 5min 2) 95°C, 30sec 3) 61.5°C, 30sec 4) 72°C, 40 sec (repeat step 2 for 40 cycles) 5) Repeat steps 2-4 39 more times 6) 72°C, 10min 7) Melt curve 65-95°C increment 0.5°C for 5 seconds, plate read. The correlation between DNA quantification data and CFU values was developed as follows: DNA was isolated from eight EAEC JM221 cultures with concentrations of 10 CFU up to 10<sup>8</sup>, each increasing by a factor of 10. Real-time RT-PCR was ran to quantify *aap* in DNA from the known CFU cultures. An eight point standard curve was generated that converted DNA quantities to CFU values. CFU values were divided by the weight of the feces (in milligrams) to obtain a final value for CFU/mg feces.

Bacterial Growth: EAEC strain JM221 was streaked onto LB Agar (Fisher) and left to grow for 24 hours at 37°C in a static incubator. One colony was then picked from the plate and used to inoculate 5mL LB media (Fisher) with 0.5% dextrose to create a preinoculum which grew in a shaking incubator at 37°C for 12 hours. The preinoculum was used to inoculate a larger volume of LB media (1:1000 dilution). Optical density (OD) measurements were monitored over time at 600 nm. When bacteria reached optimal growth, media was centrifuged at 4000 rpm for 10 minutes and bacteria pellets were resuspended in LB media at a concentration of 2x10<sup>10</sup>cfu/mL. Mice received 0.1 ml of the inoculum.

Flow Cytometry: Colonic lamina propria mononuclear cells and whole blood were seeded onto 96-well plates and used for flow cytometry staining. Cells were incubated in the dark at 4°C for 20min with the following fluorochrome-conjugated primary antibodies: CD4-PECy7, CD3 PeCy5, IL-10 PE, IL-17A-PerCPCy5.5, IFN $\gamma$ -PE, MHC-II Biotin-Texas Red, CD11b AlexaFluora700, F4/80 PeCy5, Gr1 PeCy7, CD11c FITC. Flow results were

computed using a BD LSR II flow cytometer and data analysis was performed with FACS Diva software (BD).

Lymphocyte proliferation assay: Splenocytes were stimulated in 96-well round bottom plates with media alone (non-stimulated) or medium containing enteroaggregative *Escherichia coli* (EAEC) strain JM221 sonicated antigens, *Escherichia coli* strain HS (non-pathogenic) and mutant Enteroaggregative *Escherichia coli* strain JM221 *Aff1*<sup>-</sup> (lacking Aff1 fimbria) whole cell antigens (5 µg/mL). Concanavalin A (5 µg/mL) was used as a positive control for proliferation. Antigen-specific proliferation was measured on day 5 of culture. Cultures were pulsed for the last 20 h with 0.5 µCi of [<sup>3</sup>H]-Thymidine. Overall lymphocyte proliferation was presented as stimulation indices, which were calculated by dividing the counts per minute (cpm) of antigen-stimulated wells by the cpm of non-stimulated wells.

Inactivation of *E. coli* strains and antigen preparation: To obtain inactivated whole cell (WC) antigens from EAEC that was grown as described above, centrifuged and washed twice with 1xPBS. Formaldehyde was added to a concentration of 0.4% and the suspension was incubated at 37°C to inactivate bacteria. After 48 hours of formaldehyde incubation, the inactivated bacteria was centrifuged and washed three times with 1xPBS. To confirm bacterial inactivation, 100µL of the suspension was plated, incubated for 48 hours at 37°C and analyzed for bacterial growth; no growth was observed. To obtain whole cell sonicated (WCS) antigens, the inactivated bacteria were sonicated 5 times on ice for 20 seconds with 1 minute intervals prior. Protein quantification was performed using the Bradford assay (DC protein assay kit, Bio-Rad Laboratories).

Statistics: To determine statistical significance in the model, analysis of variance (ANOVA) was performed using the general linear model procedure of Statistical Analysis Software (SAS), and probability value (*P*)<0.05 was considered to be significant.

Experiments 1 and 2 were analyzed as (2 x 2 x 2) factorial arrangement within a completely randomized design. ANOVA was utilized to determine the main effects of the dietary treatment (nourished vs. malnourished), mouse genotype (wild-type vs. PPAR  $\gamma$  knockout), or the infection status (uninfected vs. infected) and the 2-way and 3-way interactions between dietary treatment, mouse genotype, and infection status. Experiment 3 was analyzed as a completely randomized design. When the model was significant, ANOVA was followed by Fisher's Protected Least Significant Difference multiple comparison method.

# Chapter 4

## **Modeling the Regulatory Mechanisms by which NLRX1 Modulates Innate Immune Responses to *Helicobacter pylori* infection**

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Philipson C.W., Bassaganya-Riera J., Viladomiu M., Kronsteiner B., Abedi V., et al. (2015) "Modeling the Regulatory Mechanisms by which NLRX1 Modulates Innate Immune Responses to *Helicobacter pylori* infection" *PLoS One* (Under Review)

### **4.1 Summary**

*Helicobacter pylori* colonizes half of the world's population as the dominant member of the gastric microbiota resulting in a lifelong chronic infection. Host responses toward the bacterium can result in asymptomatic, pathogenic or even favorable health outcomes, however, mechanisms underlying the dual role of *H. pylori* as a commensal versus pathogenic organism are not well characterized. Recent evidence suggests mononuclear phagocytes are largely involved in shaping dominant immunity during infection mediating the balance between host tolerance and succumbing to overt disease. We combined computational modeling, bioinformatics and experimental validation in order to investigate interactions between macrophages and intracellular *H. pylori*. Global transcriptomic analysis on bone marrow-derived macrophages (BMDM) in a gentamycin

protection assay at six time points unveiled the presence of three sequential host response waves: an early transient regulatory gene module followed by sustained and late effector responses. Kinetic behaviors of pattern recognition receptors (PRRs) are linked to differential expression of spatiotemporal response waves and function to induce effector immunity through extracellular and intracellular detection of *H. pylori*. We report that bacterial interaction with the host intracellular environment caused significant suppression of regulatory NLRC3 and NLRX1 in a pattern inverse to early regulatory responses. To further delineate complex immune responses and pathway crosstalk between effector and regulatory PRRs we built a computational model calibrated using time-series RNAseq data. Our validated computational hypotheses are that: 1) NLRX1 expression regulates bacterial burden in macrophages; and 2) early host response cytokines down-regulate NLRX1 expression through a negative feedback circuit. This paper applies modeling approaches to characterize the regulatory role of NLRX1 in mechanisms of host tolerance employed by macrophages to respond to and/or to co-exist with intracellular *H. pylori*.

## 4.2 Introduction

*Helicobacter pylori* is a microaerophilic Gram-negative, spiral-shaped bacterium that colonizes 50% of the world's population establishing a decades-long infection as the dominant member of the human gastric microbiota [159]. *H. pylori* colonization results in health outcomes ranging from protection against allergies, diabetes, asthma and chronic inflammatory autoimmune diseases to favoring peptic ulcers and cancer. A small proportion (10-15%) of *H. pylori*-infected individuals experience serious pathologies [160]. Therefore investigating the complex tolerance mechanisms that facilitate the co-existence between *H. pylori* and its human host may yield a deeper understanding of mechanisms of immunoregulation at mucosal sites. Furthermore, characterizing innate immune mechanisms that reduce the negative impact of *H. pylori* infection on host fitness without directly affecting microbial burden may result in host-based precision medicine strategies that do not rely on anti-microbial treatment.

Mucosal pattern-recognition receptors (PRRs) expressed by epithelial cells and mononuclear phagocytes (MNP; macrophages and dendritic cells) are essential for

detection of *H. pylori* and required for initiation of both effector and regulatory immune responses following infection [161]. The loss of a single PRR can cause dramatic changes in immunological parameters and disease outcomes. For example, loss of TLR2 signaling during *H. pylori* infection results in dysregulated host responses and severe immunopathology [162]. Conversely, NOD1-deficiency is associated with significantly reduced interferon production at the expense of higher *H. pylori* burden in the gastric mucosa [163]. Thus, characterizing the mechanisms underlying the modulation of PRR expression and signaling by *H. pylori* infection at the systems level may lend valuable new insights into the regulation of inflammatory and anti-inflammatory host responses, bacterial burden and tissue damage at the gut mucosa. Additionally, modulating host immunity through genetic manipulation of PRRs represents an effective means to better understand the mechanisms by which *H. pylori* endures as a commensal or pathogenic organism.

Although traditionally recognized mainly as an extracellular microbe, more recent evidence suggests that *H. pylori* can thrive as a facultative intracellular bacterium and replicate within gastric epithelial cells and mononuclear phagocytes (reviewed [164]). In our novel pig model of *H. pylori* infection we demonstrated presence of the bacterium inside myeloid rich lymphoid aggregates as well as the induction of cytotoxic CD8+ T cell responses by *H. pylori* infection [164, 165]. Additionally, we recently published a tissue-level computational and mathematical model that explores the role of macrophage phenotypes on disease pathogenesis [166-168]. In a follow up study, we established an *in vitro* co-culture system to study intracellular *H. pylori* and validated the importance of myeloid cells in maintaining bacterial loads by demonstrating reduced colonization following macrophage depletion *in vivo*. Although acknowledged, mechanisms underlying host responses to intracellular *H. pylori* are widely unresolved.

This study was designed to investigate immunomodulatory mechanisms of macrophages infected with *H. pylori*. Using global transcriptome sequencing we identify three temporal molecular modules (early, sustained and late) significantly altered in *H. pylori* infected bone marrow derived macrophages (BMDM) from mice. In addition, we characterize a cohort of genes significantly down-regulated during co-culture. Analysis of PRR expression in co-culture studies confirms that *H. pylori* infection suppresses regulatory nucleotide-binding domain and leucine-rich repeat-containing protein X1 (NLRX1). Many

canonical PRR and cytokine signaling pathways modulated by *H. pylori* converge at NLRX1. Limited mechanistic insight exists for cell-specific immunomodulatory roles of NLRX1 as well as host-mediated or pathogen-driven suppression of this regulatory NLR. We present a validated computational model for characterizing novel feedback loop tolerance mechanisms implicated in the regulation of microbial burden and innate immune responses in macrophages.

### **4.3 *H. pylori* induces distinct temporal waves in innate immunity in macrophages**

To study macrophage-specific responses to intracellular replication we co-cultured primary wild type (WT) bone marrow derived macrophages (BMDM) with *H. pylori* using a gentamycin protection assay and examined global gene expression at six discrete time points (0, 60, 120, 240, 360 and 720 minutes post-co-culture). Our analysis identified 1,077 differentially expressed genes due to infection in a time-dependent manner (FDR p-value < 0.05). Clustering was used to categorize significantly altered transcripts into 12 gene modules (Clusters 1-12). Within each cluster, genes have similar temporal magnitudes, synchronized expression patterns and common biological roles when analyzed by Gene Ontology (GO) enrichment [169, 170]. Chronologically, the profiles of the clusters broadly capture early transient (C4), sustained (C3, C9, C12), late (C6, C7, C10 C11) and suppressed (C8) patterns (Figure 4.1).

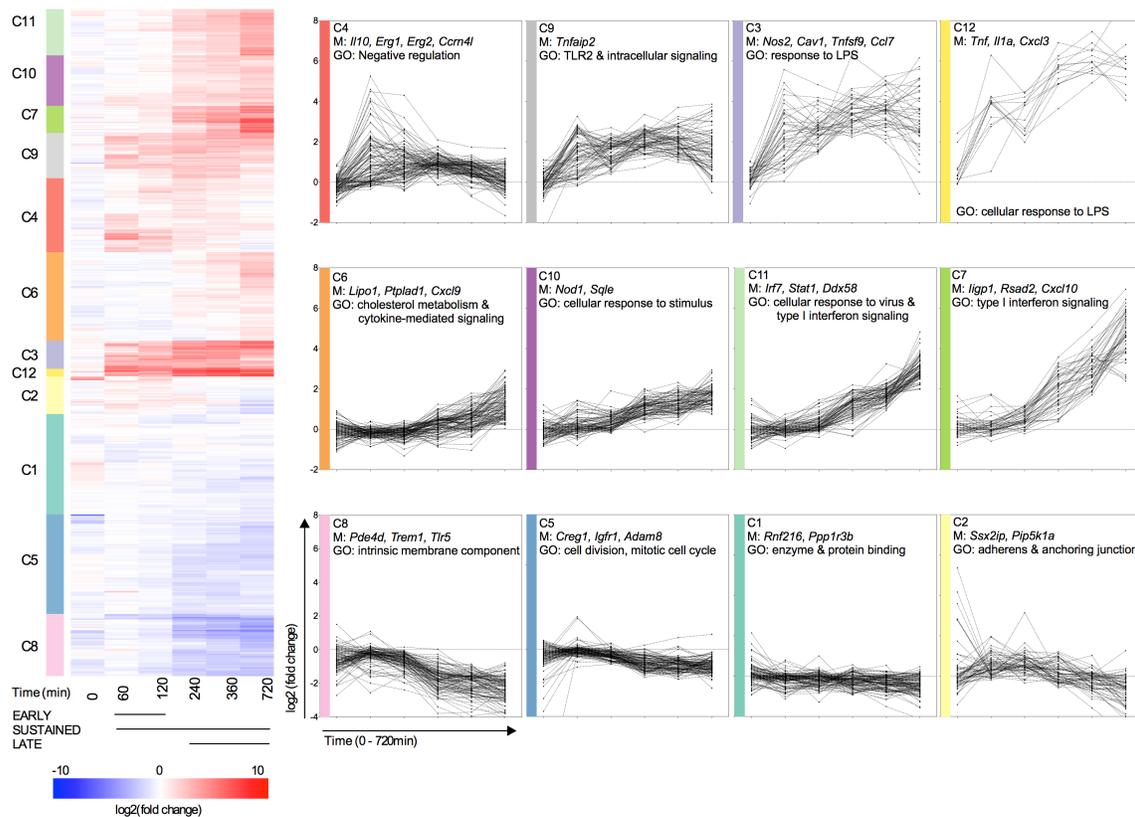
Genes with an early transient peak (C4) fall in a sub-cluster embodying regulatory molecules that negatively influence cellular stress and inflammation. *H. pylori* infection is known to induce a regulatory response in dendritic cells (DCs) characterized by enhanced production of IL-10. This dataset provides evidence that *H. pylori* infected macrophages up-regulate *Il10* mRNA through similar means early during infection (C4). We have further characterized this regulatory signature by identifying increased *E2f5*, *Erg1* and *Csrnp1*, molecules with anti-tumor properties, and *Prdm1*, whose gene product BLIMP-1 is a negative regulator of interferon (IFN) beta signaling. *Ddit4* (also referred to as *Redd1*), a glucose-regulated repressor of Akt/mTORC1 signaling and promoter of autophagy, was also among the rapidly induced transcripts [171-173]. Although dominated by immune suppressors, the early peak also contained pro-inflammatory mediators *Csf2* and *IL23a*.

The sustained innate response wave includes genes whose transcriptional profile is induced around 60 minutes and remains expressed throughout the duration of the experiment. Functionally, this phase corresponds to effector immunity elicited by recognition of extracellular bacteria and inflammatory cytokines. TLR2-dependent signaling is significantly enriched (C9) and likely governs the sustained response in a manner similarly described for *H. pylori* infected DCs [174]. Proinflammatory TLR2-mediated cytokines *Tnf*, *IL1a*, *Saa3* (C12) as well as *Il1b*, *Il6*, *Nos2*, *Ccl22* and *Ccl7* (C3) were all induced early and remained at high levels throughout the duration of the co-culture. The transcript for IL-12, an immunomodulatory cytokine involved in orchestrating T cell responses, clustered in C3. Antimicrobial peptides (AMP) are an important innate defense mechanism for epithelial cells to kill *H. pylori* and are generally enhanced with pro-inflammatory cytokines [175]. Interestingly we found that AMP were largely unchanged in our macrophage dataset; *Ltf* and *Lyz* cluster in C2 and were slightly down-regulated and neither  $\beta$ -defensins nor S100 genes were significantly altered. This suggests some degree of cell-specificity in response to intracellular versus extracellular *H. pylori* stimulation as well as differences in induction of host response between macrophages and epithelial cells.

The late wave contains genes with simultaneous onset of expression beginning at 240 minutes that continues to rise through the last collection. Late clusters are representative of host response to intracellular bacterial stimuli resulting in type I interferon signaling (C6-7, C10-11). *Nod1* (C10) and its responsive genes *Irf7* and *Stat1* (C11) were elevated late during infection as well as interferon-induced genes *Cxcl10*, *Ifit1-3*, *ligp1* and *Rsad2* (C7). Similar to sustained genes, the later transcriptional pattern is not unique to macrophages and is observed in *H. pylori* treated DCs [174].

Down-regulated transcripts also coherently group together (C8) and signify genes that may be modulated by *H. pylori* to thrive in the host environment. Most C8 transcripts are intrinsic membrane associated molecules involved in signal transduction. Clusters 1,2 and 5 included genes associated, enzyme and protein binding, cell adhesion, and cell division respectively but were relatively unaltered. Moreover, these clusters contain transcripts with the highest p-values indicating that these three clusters may not be significant in this study.

Together these data suggest that mechanisms of mononuclear phagocyte immunity to *H. pylori* occur sequentially and may depend on the location of the bacterium with respect to the host cell. Additionally, *H. pylori* induces a unique regulatory gene module with an early transient peak during infection.



**Figure 4.1. Intracellular *Helicobacter pylori* induces temporal waves in macrophage immunity.** Clustering analysis was performed on differentially expressed genes (FDR p-value < 0.05) obtained from RNAseq performed on macrophages co-cultured with *H. pylori*. Values are presented as the log<sub>2</sub> (ratio) of average infected (n=3) to average non-infected (n=3) for each time point (0, 60, 120, 240, 360 and 720 min post-co-culture). Transcripts were grouped into 12 gene-modules based on expression levels and temporal behavior. Gene expression profiles for each cluster are plotted with representative members (M) and similarities in function identified by Gene Ontology (GO).

#### 4.4 Time-sensitive innate immune responses to *H. pylori* are induced by intracellular and extracellular PRRs in macrophages

Innate PRR families facilitate the recognition of unique components from microbes, host response and/or the environment to subsequently induce classic molecular cascades

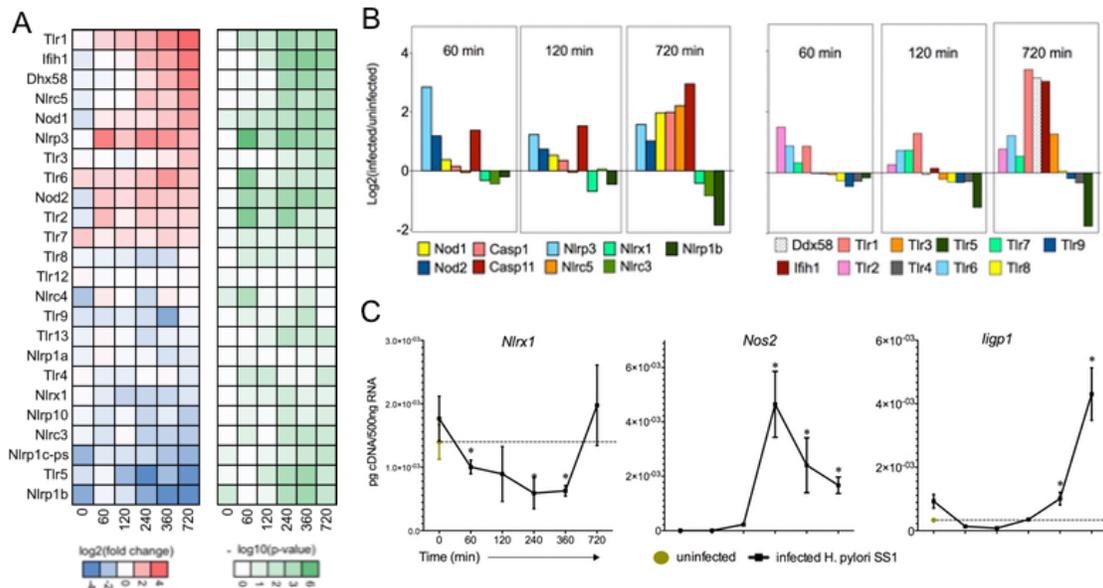
that efficiently modulate immunity. TLRs 2 and 9, RIG-I, NOD1, NOD2, NLRP3, NLRC4, NLRC5, NLRP9, NLRP12 and NLRX1 have all been reported in the context of *H. pylori* infection with roles ranging from detection of *H. pylori* to initiation of inflammation or regulatory responses at the gastrointestinal mucosa [174, 176]. We analyzed the three-phase response clusters for known PRRs in an effort to dissect complex induction of immunity and potential molecular crosstalk specific to macrophages co-cultured with *H. pylori*. Statistical comparison between uninfected and infected samples at every time point highlighted significant temporal behavior for each PRR (FDR p-value < 0.05). Every measured PRR transcript, except *Tlr12*, demonstrated differential expression at one or more time points. Up-regulated PRRs conformed to either sustained or late expression patterns; no transient peaks were observed (Figure 4.2).

Extracellular receptors *Tlr2* and *Tlr6* were significantly co-expressed beginning at 60 minutes and remained relatively stable over time. *Tlr1* was the only other up-regulated cell-surface receptor and was more strongly expressed later during infection. TLR2 is essential for recognizing *H. pylori* by dendritic cells and therefore it is not unexpected to find its transcript at significantly high levels during our co-culture. Moreover, TLR2 represents the only known mechanism whereby *H. pylori* induces a significant regulatory signature, which is in line with our clustering results above (Figure 1, C4) [177, 178]. Lipoproteins from *H. pylori* and other microbes can signal through either a TLR1/2 or TLR2/6 heterodimer however this has not been extensively investigated [179, 180]. Other extracellular PRR mRNAs were repressed (*Tlr4*, *Tlr5*), unchanged (*Tlr12*), or undetected (*Tlr10*, *Tlr11*) upon bacterial colonization.

With regards to intracellular PRRs, *Nlrp3* and *Nod2* both ranked as highly significant transcripts and were induced early but maintained relatively stable levels over time. *Nod1*, *Nlrc5*, *Ifih1* and *Dhx58*, were induced in a pattern akin to the late wave and begin immediately after the peak of intracellular *H. pylori* replication measured by bacterial re-isolation. NOD1 is recognized as a primary contributor of host response to *H. pylori* through the detection of intracellular peptidoglycan, a component of the bacterium's cell wall, and is likely a key detection method used by macrophages to identify infiltrated bacteria in our project [163]. The roles and influence of the other aforementioned intracellular receptors have been well documented in the context of *H. pylori* infection and recently reviewed [181]. We expanded our analysis to encompass inflammasome

components and found that indeed *Casp-1* and *Casp-11* were both significantly induced at late time points. Notably, Caspase-11 was recently identified as an innate receptor for intracellular LPS and although *H. pylori*-derived components do not directly induce Caspase-11 it has not been confirmed whether the live bacterium can be recognized through this novel mode of detection [182]. Additionally, caspase proteins require post-translational modifications to become active and their involvement in immune responses to *H. pylori* must be confirmed at the protein level.

The majority of PRRs participate in host immunity by inducing inflammatory responses in immune cells. Indeed the patterns of extracellular and intracellular receptors are in line with intracellular *H. pylori* replication and time-specific activation of sustained and late cytokine waves. However, one novel subgroup of NLRs, whose members include NLRC3, NLRP12 and NLRX1, functions to attenuate inflammation by regulating activation of NF- $\kappa$ B and MAVS signaling pathways [183]. Our transcriptomic profiling demonstrates that macrophages treated with *H. pylori* have significantly repressed *Nlrc3* and *Nlr1* transcripts, especially early post-infection; a finding further validated by qRT-PCR along with sustained and late-wave genes. These data are aligned with recent reports demonstrating that *H. pylori* infection suppresses *Nlr1* and *Nlrp12* in a human monocytic cell-line [176]. *Nlrp12* was detected at 360 minutes only in our dataset and it was slightly increased. Suppression or deficiency of regulatory NLRs has been linked to worsened disease due to exacerbated inflammation and cell death however mechanisms underlying the negative-feedback loops that govern this phenomenon are not well understood [184-186].



**Figure 4.2. Pattern recognition receptors are differentially regulated by *Helicobacter pylori*.** Genes involved in pathogen detection were extracted from the RNAseq dataset and analyzed at each time point for significance (A; left – expression, right – significance). Expression of effector PRRs conformed to sustained or late waves whereas regulatory molecules were suppressed (B). Dynamic expression of transcripts observed by RNAseq was validated by quantitative real time RT-PCR (C). Transcriptomic data (A,B) are presented as average  $\log_2(\text{ratio})$  whereas validation gene expression is presented as pg cDNA/500ug RNA. Asterisk indicates  $P$ -value  $< 0.05$  based on ANOVA.

#### 4.5 Modeling macrophage responses to intracellular replication of *H. pylori* reveals possible mechanisms of regulation of innate responses by NLRX1

Due to substantiated evidence that NLRX1 can be suppressed in an *H. pylori* dependent manner [176], we hypothesized that NLRX1 represents a central molecular checkpoint involved in the fate of bacterial burden and macrophage responses during infection. To investigate the roles of effector versus regulatory PRRs during *H. pylori* infection in macrophages we generated a calibrated ODE-based model representing the pathways implicated in NLRX1-mediated immunity.

Network Model: Despite the highly specific nature of PRRs, our bioinformatics analysis suggests high levels of redundancy, crosstalk and synergism among these sentinel inflammatory receptors; a common pattern that manifests in host response to bacteria

[174]. For instance, *H. pylori* detection through TLR2 and NOD1 can both result in ample NF- $\kappa$ B activation and inflammatory cytokine production. However, detection by PRRs is dependent upon bacterial location (e.g. TLR2 is extracellular whereas NOD1 is intracellular) resulting in pathway activation at different spatiotemporal scales. Cytokine signaling triggers positive feed-forward loops that further activate the same molecular profile (i.e. TRAF6 phosphorylation occurs due to detection of bacteria (e.g. TLRs) and cytokines (e.g. TNFR)). Additionally, NF- $\kappa$ B signaling is tightly controlled through numerous self-regulated negative feedback mechanisms, including PPAR $\gamma$  and NLRX1 [187]. To generate a comprehensive model structure we first performed network analysis on differentially expressed genes using the Ingenuity Pathway Analysis (IPA) tool [188]. Relationships in top altered canonical pathways were merged to encompass the three temporal gene-expression waves. We integrated IPA canonical pathways with feedback loops and generated a systems biology markup language (SBML)-compliant model that incorporates recognition of *H. pylori* through TLR2 (via adaptor molecule MyD88), NOD1 and RIG-I. Pathogen detection initiates second-tier inflammatory cascade branches that activate either NF- $\kappa$ B or IRF3/7 (IRF) and ultimately result in the production of early (Cyt<sub>o</sub>E), sustained (Cyt<sub>o</sub>S) and late (Cyt<sub>o</sub>L) cytokine responses. Negative feedback from NF- $\kappa$ B is represented cumulatively by inhibitory factor X. NLRX1 functions to regulate inflammation by inhibiting MAVS and the activation of NF- $\kappa$ B by TRAF6 (Figure 3.3).

Causative agents for suppression and resolution of NLRX1 are unknown. Therefore, we engineered our model in an effort to describe such behavior. Based on our analysis of expression patterns described in previous sections, *Nlr1* decreases when early transient responses are high and increases as sustained and late cytokines rise. Thus we added hypothetical negative and positive feedback loops from undefined transcription factors induced by early and sustained responses, respectively, to NLRX1. These feedback loops represent two key predictions of NLRX1 modulation embedded in our computational model. Out of the 120 early gene set, 17 have transcription factor or nucleic acid binding capabilities and represent potential candidates for negative feedback. In addition to genes extracted from the early cluster above, *Bcor*, *Irf4*, *Myc* and *Rel* fall within this category. Positive feedback could be linked to several genes in the sustained or late waves such as NF- $\kappa$ B molecules, *Stat5* (both in C9), *Stat1* or *Irf7* (C11).

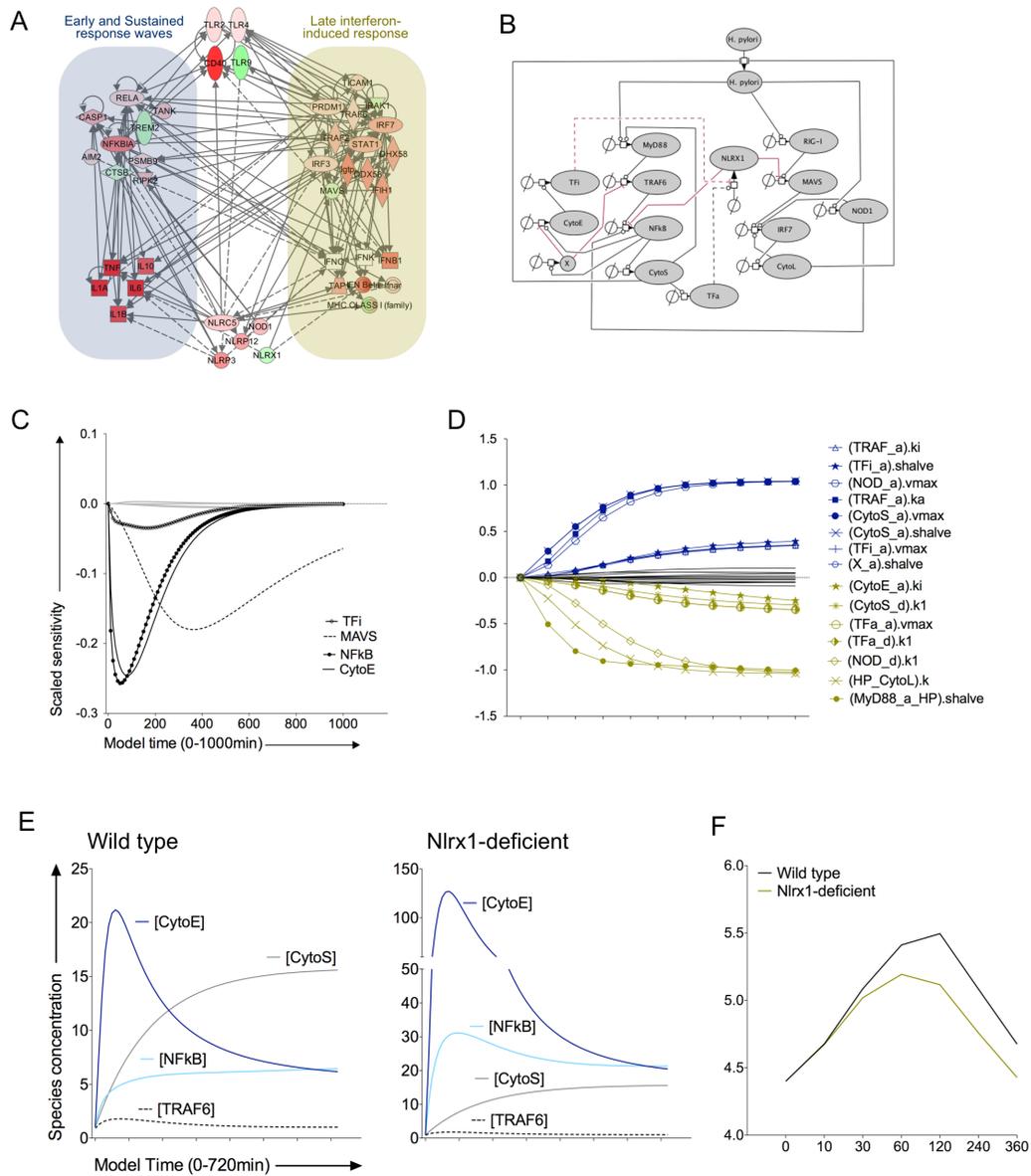
Training data and parameter fitting results: In order to study the kinetic behavior of our reaction network, we generated a deterministic mathematical model wherein each species was assigned an ODE. We combined in-house bacterial re-isolation with select data from our transcriptomic profiling results to calibrate the system of equations. Each model species was trained using data measured at six distinct time points for its respective gene transcript. Unknown rate values were estimated using COPASI [102, 189]. Specifically the model was optimized to fit experimental data by minimizing a weighted sum of squared residuals using a Genetic Algorithm. After training, the model accurately simulates the three distinct waves of macrophage immunity to *H. pylori*.

Sensitivity Analyses: After calibration we assessed the local sensitivities of NLRX1 on transient concentrations of other model species. Our results suggest that NLRX1 plays an inhibitory role in all three host-response waves. Initially NLRX1 will negatively regulate NF $\kappa$ B signaling and early response factors. However NLRX1 will serve as a repressor of MAVS signaling at the onset of late signaling. We calculated a second sensitivity analysis to determine the effects of local model parameters on the temporal behavior of NLRX1. Activation of MyD88 signaling by *H. pylori* had the largest negative impact on NLRX1 expression over time. Although high quantities of *H. pylori* are inhibitory in the beginning, accelerated bacterial clearance due to late cytokines causes the second largest negative impact on NLRX1. This suggests a complex pattern of interaction in that the presence of *H. pylori* may both promote and inhibit NLRX1 expression depending on spatiotemporal detection and prevailing immune responses. In contrast, model parameters required for sustained cytokine production were positively influential for NLRX1. The importance of NOD1 in positively regulating NLRX1 was an unexpected finding and further supports the existence of crosstalk between effector and regulatory NLRs. Interestingly, parameters controlling NF $\kappa$ B, RIG-I and MAVS kinetics had very little influence on NLRX1.

In silico NLRX1-deficient system: We next generated an *in silico* knockout system to predict the effects of NLRX1-deficiency on macrophage immunity to intracellular *H. pylori*. To create the knockout system, initial and transient concentrations of NLRX1 were fixed at a constant value of 0. The most dramatic differences in the NLRX1-null system are depicted by elevated NF- $\kappa$ B activity. Interestingly, increased NF- $\kappa$ B levels caused a strong response skewed toward up-regulated early cytokines while sustained cytokines

remained unchanged. Although the loss of NLRX1 did exaggerate MAVS concentrations, downstream IRF and late cytokines were unaffected. Lastly, increased NF- $\kappa$ B and early wave molecules in the NLRX1<sup>-/-</sup> model were directly associated with reduced *H. pylori* burden in our model. Specifically, bacterial replication peaks around 60 minutes in the NLRX1-deficient system compared to 120 minutes in the WT system with the most dramatic differences occurring 120 minutes onward.

To summarize, our modeling and simulation studies generated four computational hypotheses surrounding the immunomodulatory mechanisms of macrophage-NLRX1 during *H. pylori* infection. First, *H. pylori* rapidly induces an early wave response that is largely responsible for NLRX1 suppression in infected macrophages. Second, sustained inflammatory mediators are required for the induction of NLRX1 expression during infection. Third, the loss of NLRX1 results in a sharp increase of NF- $\kappa$ B and early cytokines but little changes are observed in sustained or late cytokines. Lastly, NLRX1-deficiency results in lower levels of intracellular *H. pylori* when compared to wild type simulations.



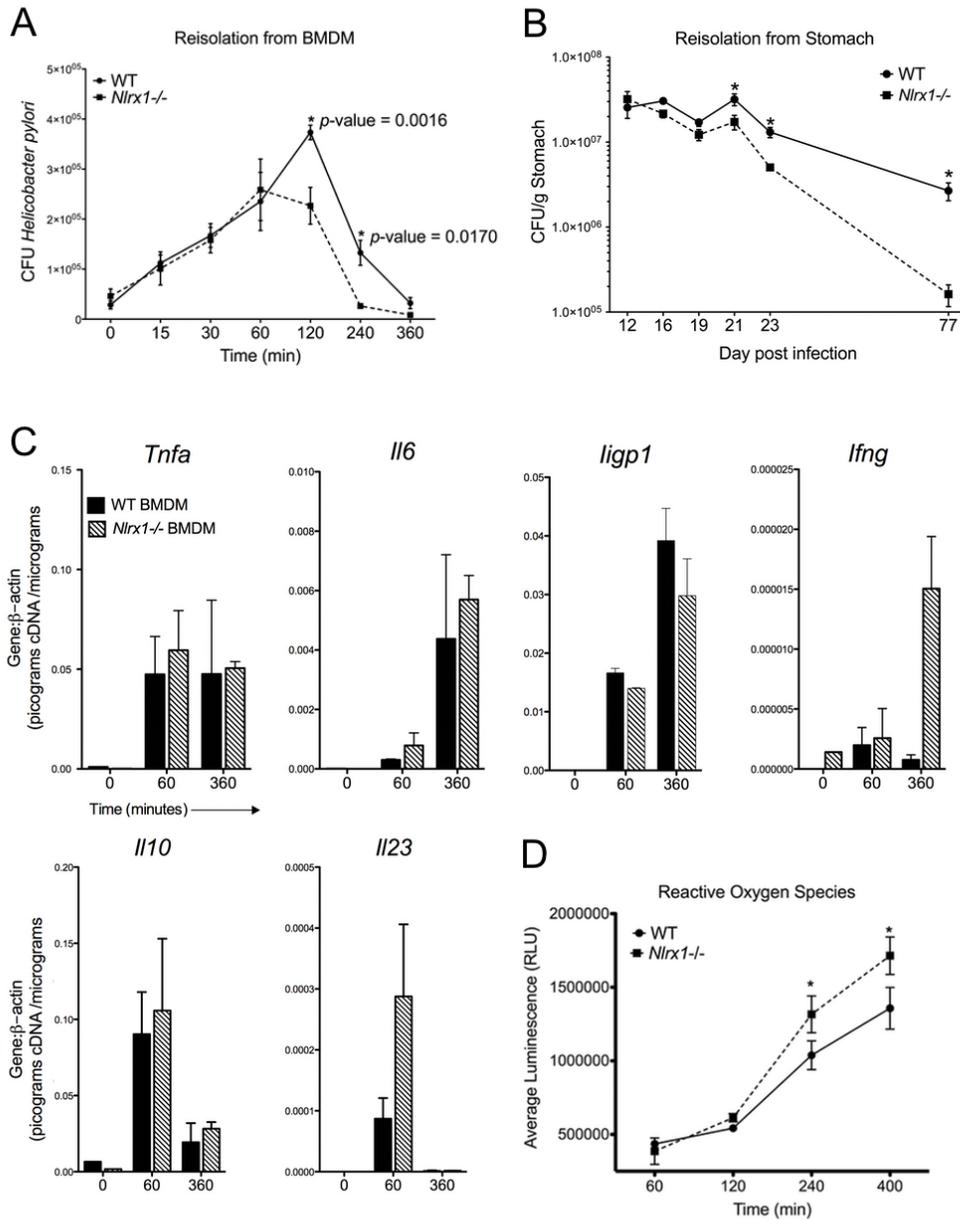
**Figure 4.3. Modeling macrophage immunity to *Helicobacter pylori* infection reveals an essential role for NLRX1 in favoring bacterial burden.** Differentially regulated genes from the RNAseq dataset were analyzed for molecular crosstalk using Ingenuity Pathway Analyzer and a preliminary interaction network was created. Red indicates increased expression whereas green indicates reduced expression at time 360 min. Molecules in white are not detected (A). Early, sustained and late wave responses were included. Feedback circuits were added to canonical pathways and a biological system was engineered in Systems Biology Mark-up Language (B). In B: red lines represent negative regulation, positive reactions are black, solid lines are experimentally validated and dashed lines are hypothetical feedback loops. Two sensitivity analyses were performed on the calibrated mathematical model: effects of model species on NLRX1 (C); and effect of NLRX1 on local parameters (D). Time course model simulations for a wild type compared to Nlr1-deficient system demonstrate differences in NF- $\kappa$ B signaling (E) and *H. pylori* burden *in silico* (F).

#### 4.6 Validation studies demonstrate that NLRX1-deficiency reduces intracellular *H. pylori* loads

Using computational modeling predictions as a governing framework, we performed a series of validation experiments to confirm the immunomodulatory role of NLRX1 in macrophage immunity to *H. pylori*. BMDM were generated from wild type and *Nlr1-/-* mice, subjected to the gentamycin protection *H. pylori* co-culture, and then assayed for bacterial re-isolation or quantitative gene expression. We validated the computational hypothesis that NLRX1 deficiency alone is sufficient for reducing bacterial burdens when compared to wild type cells. Moreover, bacterial levels indeed peak earlier, around 60 minutes, in *Nlr1-/-* versus wild type BMDM mirroring our *in silico* predictions. *Nlr1-/-* mice experienced significantly lower gastric bacterial loads when compared to their wild-type counterparts beginning 21 days post infection. Differences in *H. pylori* colonization between genotypes became more accentuated over time and diverged over ten-fold by 77 days post-infection. Due to the lack of conditional NLRX1 knockout animals, our murine studies do not confirm cell-specific roles of *H. pylori* infected macrophages *in vivo* however this presents an opportunity for future validation. Next, gene expression was assayed using real-time RT-PCR at 0, 60, and 360 minutes during co-culture to assess early (*Il10*, *Il23*), sustained (*Tnf*, *Il6*) and late (*lpg1*) response transcripts. NLRX1-deficient BMDM behaved similar to wild-type samples and up-regulated appropriate genes in a time-dependent manner. Almost no differences were observed in genes measured in sustained and late waves emulating observations presented by *in silico* experiments. Differences were observed in *Il23* at 60 minutes validating model predictions, however differential regulation of *Il10*, another early cytokine, was not observed. Since IL-10 and IL-23 are differentially regulated by bacterial stimulation, these data do not necessarily negate one another [190]. Since *H. pylori*-infected epithelial cells and monocytes often produce IFN- $\gamma$  as a means of antimicrobial host immunity we measured its transcript even though it is not included in our model [191]. Interestingly, *Ifng* mRNA levels increase dramatically at 360 minutes in macrophages lacking NLRX1 presenting another possible mechanism whereby NLRX1-deficient cells reduce pathogen loads. Finally, we measured reactive oxygen species (ROS) production due to its involvement in NLRX1-mediated immunity and *H. pylori* resistance to host mechanisms [191]. Similar to *Ifng*, BMDM lacking NLRX1 produced significantly higher

amounts of ROS when compared to wild type macrophages during infection with intracellular *H. pylori* (Figure 3.4).

These studies have provided novel mechanistic insights toward characterizing the cell-specific roles of NLRX1 in macrophages. Collectively our data validates the computational prediction that NLRX1-deficient macrophages infected with *H. pylori* exert dysregulated host responses resulting in a microenvironment that favors bacterial clearance. We also established that reduced *H. pylori* colonization due to the lack of NLRX1 is paralleled *in vivo*. Our findings support computational hypotheses that point away from classically associated NF- $\kappa$ B and type-I-interferon signaling and suggest a novel role for type-III-interferons (IFN- $\gamma$ ) and ROS production in NLRX1-mediated regulation of *H. pylori*. Moreover, they help form a picture for a role of NLRX1 in mediating host tolerance to *H. pylori*.



**Figure 4.4. Experimental validation studies confirm role for NLRX1 in *Helicobacter pylori* burden and host immunity.** Bone marrow derived macrophages were obtained from wild type and *Nlr1*<sup>-/-</sup> mice then challenged with *H. pylori* using the gentamycin protection assay. Cells were collected incrementally from 0 to 360 minutes post co-culture and assayed for intracellular *H. pylori* replication via re-isolation (n=3) (A), gene expression via quantitative real-time RT-PCR (C), or production of reactive oxygen species (n=3) (D). Stomachs from *H. pylori* infected mice were collected from 12 to 77 days post infection and bacterial burden was determined via re-isolation (n=7). Asterisk indicates *P*-value < 0.05 based on ANOVA.

## 4.7 Discussion and Conclusions

*H. pylori* has co-evolved with its human host for over 60,000 years [192, 193], diverged with ancient human migrations [194], and infects half of the world's population. Individual responses toward the bacterium vary tremendously with respect to strain, host, genetic, and socioeconomic factors resulting in differences ranging from asymptomatic superficial gastritis to invasive adenocarcinoma [160, 195]. Approximately 10-15% of infected individuals develop peptic ulcers, gastric carcinoma or mucosa-associated lymphoid tissue lymphoma as a result of improperly controlled gastritis at the stomach mucosa [196]. In contrast, a larger cohort of carriers remains asymptomatic or develops beneficial immunity due to *H. pylori* colonization. Specifically, emerging data reveal *H. pylori*-dependent protection against esophageal and cardiac pathologies [197-201], childhood asthma and allergies [202-205], inflammatory bowel disease [206] and obesity in developed countries [207, 208] demonstrating symbiotic properties of this bacterium. Cell populations and molecular mechanisms that mediate host tolerance by actively shaping immune responses to minimize detrimental effects of co-existence with high levels of *H. pylori* are likely critical for orchestrating pathogenic versus commensal roles of this bacterium [209].

This study aimed to broadly elucidate immunomodulatory properties of intracellular *H. pylori* on macrophages. Similarly we sought to determine prevailing immunity employed by macrophages to either favor or counterpoise intracellular invasion. To do so, we generated the first time-series global gene expression dataset for *H. pylori* infected macrophages and identified three novel response modules: early transient, sustained and late waves. Additionally, we describe how temporal behavior of differentially expressed transcripts is induced by redundant, synergistic and antagonistic detection mechanisms of extracellular and intracellular PRRs suggesting a level of spatiotemporal dependency. From these studies we confirm that macrophage-*H. pylori* co-culture suppresses NLRX1 and present novel evidence that NLRC3, a member in the same subset of regulatory NOD-like receptors, is also significantly repressed. By developing a calibrated computational model of host responses to *H. pylori* in macrophages we predict, then experimentally validate, that NLRX1 is a key determinant for maintaining elevated *H. pylori* loads at the cellular and organismal levels. Importantly, we propose a novel mechanism underlying *H. pylori*-induced reduction of NLRX1 governed by a

negative-feedback loop from the early-induced transient response element. Taken together, this work provides a novel and comprehensive framework for dissecting perturbations in innate immunity caused by *H. pylori* and could help reveal novel broad-based mechanisms implicated in the maintenance of host tolerance to a variety of organisms.

Considerable evidence has surfaced demonstrating that MNP populations are highly influential in governing prevailing mucosal immunity, pathogen loads and gastric pathology during *H. pylori* infection. Cells from a hematopoietic origin have been shown to provide protection against T-cell mediated gastritis in *H. pylori* infected mice [210]. Enrichment of certain phenotypes such as CD103+CD11b-IL10+ dendritic cells during infection bestows protective systemic immunity in *H. pylori*-colonized hosts. Our team recently identified a novel subset of CD11b+F4/80hiCD64+CX3CR1+ MNPs that expand upon infection, secrete high levels of IL-10 and are required for maintaining elevated levels of *H. pylori* in the stomach mucosa early during infection. Modulating macrophage phenotypes by genetic ablation of transcriptional machinery, such as hypoxia-inducible factors [211] or peroxisome proliferator-activated receptor gamma, results in loss of protection characterized by substantially lower *H. pylori* colonies at the expense of mucosal damage. Molecular mechanisms prompting myeloid cells to provide protective immunity and restrict inflammatory signals in the gastric mucosa are largely unexplored. In terms of overall cellular response, our data suggests that effector responses from BMDM co-cultured with *H. pylori* occur in patterns similar to dendritic cells [174], especially TLR2 induced genes and NOD1-mediated immunity. One principal advantage of our dataset is that we capture the entire early transient signaling wave and thus we describe the regulatory element of macrophage immunity in more detail than previous studies [174, 212]. Beyond IL-10 we have described five new regulatory pathways and validating their physiological roles in host tolerance will be valuable next steps for understanding potential mechanisms governing protective or deleterious effects of pathogens and members of the human microbiota.

Chronic persistence of *H. pylori* is generally attributed to the microbe's numerous immune-evasion tactics [194]. CagA+ *H. pylori* strains are rapidly ingested by macrophages but survive in the intracellular compartment due to their ability to resist phagocytic killing and form viable colonies in megasomes [213]. Additional evidence

demonstrates that *H. pylori* can induce autophagic vesicles that can serve as a replication reservoir for the bacterium [214-216]. More recently, emphasis has been on anti-inflammatory responses induced by *H. pylori* that promote tolerance and are incidentally related to the protective immunomodulatory properties of the bacterium [217]. In this study we took a host-centric view and investigated dynamic macrophage-dependent mechanisms involved in detecting and regulating intracellular survival of *H. pylori*. We analyzed the activity of PRRs and found that all measured PRR transcripts were significantly altered at one or more time points with the exception of *Tlr12*. Presently, NOD1 and TLR2 are the main receptors associated with detection of *H. pylori*, and indeed we observed upregulation of these molecules in our dataset. Importantly, we report for the first time that *H. pylori* stimulation of macrophages significantly enhances mRNA levels of *Caspase11*, a non-canonical caspase protein recognized as a receptor for cytosolic lipopolysaccharide (LPS) [182, 218]. *H. pylori* has low efficacy for many PRRs partially as a result of the molecular evolution of its surface molecules [161, 219]. *H. pylori* LPS has achieved structural mimicry akin to human blood antigens by resembling polysaccharides found in mammalian hosts and thus is nearly undetectable by the LPS-receptor TLR4 [220]. Hence, detection of *H. pylori* LPS through an alternative pathway would yield a significant contribution in understanding how the host can recognize intracellular *H. pylori* and should be validated at the protein level. Interestingly, many effector PRRs were repressed over time. Modulating expression and cellular distribution of PRRs following host-microbe interaction is a fundamental tactic used by immune cells to alter sensitivity to bacteria and reduce the inflammatory impact of stimulation. Classically this property is affiliated with the intestinal environment where adjusting expression and bio-geographical distribution of PRRs by epithelial cells and MNP is directly involved in the balance between homeostasis and development of autoimmune diseases such as inflammatory bowel disease [221]. Based on our findings it is tempting to speculate that macrophages modify PRR abundance during *H. pylori* infection as a mechanism to circumvent susceptibility to overt inflammatory disease.

One key finding of our study was reduced expression of regulatory NLRs, NLRC3 and NLRX1, following perturbation of innate immune responses by *H. pylori*. Suppression of regulatory NLRs is associated with worsened pathology during inflammatory diseases but mechanisms of negative regulation have not been described [184-186]. We and

others have demonstrated that NLRX1 can be suppressed in an *H. pylori* dependent manner [176], however limited mechanistic insight exists to explain this behavior or its contribution to immune responses and bacterial burden. To further investigate the roles of NLRX1 during *H. pylori* we engineered a calibrated ODE-based computational model that encompassed the three-wave innate immune response identified by our clustering analysis. Using *in silico* knockout simulations we predicted and validated the novel hypothesis that, despite being suppressed during peak microbial loads, NLRX1 is required for maintaining high levels of *H. pylori* in both macrophages and the murine stomach. Analytical results on our computational model propose a novel scenario for immunomodulation in which host responses rapidly induced following *H. pylori* colonization of macrophages regulate NLRX1 suppression; a prediction is being investigated intensively in ongoing validation studies.

Another key validation experiment confirmed model predictions that sustained (i.e. *Tnf* and *Il6*) and late (i.e. *ligp1*) effector molecules were largely unchanged in NLRX1-deficient BMDM compared to wild type during the gentamycin protection assay. Our studies do show significant differences in the induction of *Ifng* and ROS in NLRX1-null macrophages occurring through an undefined mechanism. Importantly, one model-driven hypothesis is that loss of NLRX1 would result in higher levels of early cytokines. We indeed observed higher levels of the cytokine transcript for *Il23* in *Nlrp1*<sup>-/-</sup> BMDM. *Il-23* has been studied with several infectious disease models and is largely involved in regulating robust innate immunity required for containing bacterial burden [222]. On the other hand, early *Il10* response measurements were very similar between genotypes. *IL-23* and *IL-10* signal through different mechanisms and are differentially regulated in mononuclear phagocytes infected with various pathogens, thus these pathways may be working independently [190] and future studies could investigate the contrast in signaling with respect to NLRX1. It remains unclear whether lower bacterial loads represent more efficient killing of *H. pylori* by macrophages, reduced replication rates due to altered intracellular signaling (i.e. autophagy), or both. NLRX1 is believed to antagonize NF- $\kappa$ B activity, modulate reactive oxygen species (ROS), promote autophagy and interrupt interferon signaling [223] via direct interaction with intermediate transduction proteins, TRAF6 and MAVS. These findings, however, remain controversial and vary among murine models of infectious and immune-mediated diseases. For example, NLRX1 has

been identified as a regulator of mitochondrial antiviral immunity through interference with RIG-I-MAVS and TRAF6-NF- $\kappa$ B signaling to attenuate inflammation during Sendai virus and influenza infections and LPS induced shock [224-227]. In contrast, *Nlrp1*<sup>-/-</sup> mice have reduced interferon responses during influenza independent of MAVS interactions [228, 229]. Similarly, the debate that NLRX1 potentiates ROS production [230, 231] or not [232] is ongoing. Since NLRX1 serves as a cytosolic receptor for host and microbe components, it is likely that NLRX1 acts in a pathogen-specific manner, potentially contributing to conflicting results. Our results support the notion that NLRX1 negatively influences ROS production. Additionally we present IFN- $\gamma$  as a unique identifier for altered immunity resulting from loss of NLRX1.

A potential limitation of our findings could result from the slight misfit of early cytokines in our model. Experimental data suggests that the early response wave reaches values closer to baseline and more rapidly than our model. One possible explanation to rationalize this result is the lack of an unforeseen feedback loop that negatively regulates early responses independent of NF- $\kappa$ B. Some early genes, such as *Ddit4*, are involved in autophagy and regulated by lipid and glucose metabolism suggesting roles for both classical NF- $\kappa$ B and alternative enzymatic signaling [233]. Future model extensions could incorporate metabolic and autophagy modules to enhance fitting of initial transient signaling components and provide clarity for induction of this novel regulatory element. Likewise, we did not predict the result of IFN- $\gamma$  since type III interferon signaling was not included in our present model; this branch of immunity could be added in future renditions. Lastly, it is possible that the inverse behavior observed between early response elements and NLRX1 is coincidental rather than causal. Given over 100 genes in the early response element, it is not feasible to experimentally test each molecule's ability to control transcriptional activity of NLRX1. Future model versions with factorial simulations or sparse sensitivity analyses designs will help narrow more likely candidates for validation.

In conclusion, we combined transcriptomic analyses, mathematical modeling and experimental validation of key computational predictions to characterize novel macrophage-dependent mechanisms involved in detection and response to intracellular *H. pylori*. The regulatory response element that we describe involves genes that have not yet been connected with disease outcomes during *H. pylori* infection thus presenting

an exciting new avenue for potential investigation. Through analytical studies of a calibrated mathematical model, we determine that the dynamic behavior of one molecule, NLRX1, is tightly regulated and essential for *H. pylori* colonization. Concisely, persistently high levels of microbial colonies can only occur when NLRX1 is involved in a feedback loop that suppresses sustained and late immune waves. Finally, our integrated computational and experimental validation studies also shed light on an undescribed role for IFN- $\gamma$  in NLRX1 immunity as well as a unique scenario for host-mediated suppression of NLRX1. Broadly, our model of host response to *H. pylori* infection represents an invaluable tool to study the mucosal immune system as an information processing system. Moreover, our findings present new data that could assist in the development of host tolerance-based precision medicine strategies that do not rely on anti-microbial treatment.

#### **4.8 Materials and methods**

*Ethics Statement:* All experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech and met or exceeded the requirements of the Public Health Service, National Institutes of Health and the Animal Welfare Act. Animals lived in a 12:12 light/dark cycle and were under strict monitoring throughout the duration of the project. All efforts were made to minimize unnecessary pain and distress. Animal experimentation was performed under IACUC protocol 12-174 VBI.

*Isolation and culture of bone marrow derived macrophages (BMDM):* Bone marrow was isolated from hind legs obtained from wild type and *Nlr1*<sup>-/-</sup> mice in sterile conditions as previously described [234]. Briefly, muscle was removed and bones were soaked in 70% ethanol for 1 minute. Bones (femur and tibia) were cut proximal to each joint and flushed with 10mL cold sterile complete macrophage medium (RPMI 1640, 10% FBS, 2.5% HEPES, 1% Sodium pyruvate, 1% Pen/Strep, 1% L-Glutamine) using a 25-G needle inserted into the bone marrow cavity. Cells were filtered through a 100  $\mu$ m strainer and centrifuged for 8min at 1,200 rpm at 4°C. Red blood cells were eliminated by osmotic lysis. Single cell suspensions of bone marrow progenitors were plated in untreated petri dishes with media supplemented with 25ng/mL murine macrophage colony stimulating factor (M-CSF, Peprotech). Cells were fed on day 3 with fresh media containing M-CSF.

On day 6 of culture, nonadherent cells were removed whereas adherent cells were collected by scraping.

Gentamicin protection assay: BMDM cell suspensions were resuspended in antibiotic-free cRPMI and seeded at a density of 250,000 in 12-well plates for bacterial reisolation or  $2 \times 10^6$  in 6-well plates for RNA isolation and left overnight to adhere at 37°C, 95% humidity and 5% CO<sub>2</sub>. The following day, cells were co-cultured with *H. pylori* strain SS1 at an MOI 10 and synchronized by quick spin to ensure immediate bacteria-cell contact. After a 15-minute incubation, extracellular bacteria was killed and removed by washing cells with PBS/5%FBS containing 100ng/ml Gentamicin. Complete macrophage media supplemented with 100ng/mL Gentamicin was added to cells and the co-culture proceeded for the indicated times. Time-point 0 represents the initial collection beginning after Gentamicin washes. At desired time points, cells were washed with 1xPBS and collected by scraping for downstream assays. For re-isolation of *H. pylori* from cells, BMDM were collected in 100 $\mu$ l of sterile Brucella broth and sonicated for 5 sec to release intracellular bacteria; serial dilutions were plated. *H. pylori* growth plates were prepared with Difco Columbia blood agar base (BD Biosciences) supplemented with 7% horse blood (Lampire) and *Helicobacter pylori* selective supplement (containing 10 mg/liter vancomycin, 5 mg/liter trimethoprim, 5 mg/liter amphotericin, and 5 mg/liter polymyxin from Oxoid) at 37°C under microaerophilic conditions as previously described.

Global transcriptomic gene expression analysis by RNAseq: Total RNA was isolated from WT BMDM collected at time-points 0, 30, 60, 120, 240, 360 and 720 minutes post-coculture with *H. pylori* strain SS1. Each time point contained triplicates for *H. pylori* treated and non-infected controls. RNA was submitted for whole transcriptome gene expression assessment using Illumina Hiseq (Virginia Bioinformatics Institute Core Lab Facilities). Fastq files containing 100bp-long pair-end reads were assessed and poor quality reads (>40% of bases with PHRED score <10; percentage of N greater than 5%; and polyA reads) were filtered out. Remaining high quality reads were mapped to RefSeq (mm10 from <http://genome.ucsc.edu/>) using Bowtie [235] (version: 1.0.0) with parameters set to '-l 25 -l 1 -X 1000 -a -m 200'. Gene expression levels were calculated using an expectation-maximum algorithm, RSEM [236]. FPKM [237] (fragments per kilobase per million sequenced reads) values were used as expression level

measurements. The present RNAseq dataset has been submitted to NCBI's GEO database (Accession Number GSE67270).

Clustering: Hierarchical based clustering was used to analyze the time series RNAseq data. Expression patterns of significant genes (FDR p-value < 0.05) were used to group genes into different clusters. The clustering was performed in R (version 3.1.3) using the *hclust* method with Ward's minimum variance method and Manhattan distance metric. The clustering and Gene Ontology analysis on the sub-clusters were performed iteratively to group genes in ideal sets; 12 sets were found to be ideal for a well separated functional group from this dataset. Clustering was considered an additional layer for data processing and filtering following statistical analysis.

Computational Modeling: Unknown parameter values were estimated using COPASI [102, 189]. Specifically the model was optimized to fit experimental data by minimizing a weighted sum of squared residuals using a Genetic Algorithm. The experimental training dataset used for model was calibrating was transcriptomic levels obtained by RNAseq at six distinct time points for wild type BMDM stimulated with *H. pylori* as outlined above. Two or three replicates were provided per transcript per time point for training. Due to high distribution in FPKM readouts, replicates are representative of the fold change of *H. pylori* infected cells normalized by time-matched untreated cells. Thus concentrations of model species over time is indicative of fold change (SS1 treated/untreated). Model species-transcript pairing was as follows: NLRX1-*Nlrp1*; NOD1-*Nod1*; RIGI-*Ddx58*; MAVS-*Mavs*; IRF7-*Irf7*; Cytol-*ligp1* and *-Ifi47*; MyD88-*Myd88*; TRAF6-*Traf6*; NFkB-*Nfkb*; CytE-*Il10* and *-Il23*; Cytol-*Il1b* and *-Il6*. Sensitivity analyses were also calculated in COPASI as previously described [238]. In this case, sensitivity analysis of NLRX1 on local parameters was performed with respect to reaction rates in the system whereas sensitivity analysis on transient concentrations of NLRX1 was performed with respect to kinetic fluxes of local model species.

Validation of gene expression by qRT-PCR: RNA was isolated from BMDM using the RNeasy Minikit following manufacturer's instructions (Qiagen). mRNA concentrations were quantified by optical density at 260 nm with a Nanodrop spectrophotometer (Invitrogen). One microgram of RNA per sample was used to synthesize cDNA using the iSCRIPT cDNA Synthesis kit (Bio-Rad). Quantitative reverse transcription (RT)-PCR was performed (CFX96; Bio-Rad) to assess absolute expression of genes. Standard curves

were created by diluting primer-specific PCR amplicons of known concentrations (5 to  $5 \times 10^{-6}$  pg per reaction volume).

Detection of secreted reactive oxygen species: ROS was measured by monitoring bioluminescent activity of hydrogen peroxide species in culture via the ROS-Glo H<sub>2</sub>O<sub>2</sub> Assay (Promega). Briefly, the enzymatic substrate provided by the kit was added to cells in culture during the gentamycin protection co-culture following manufacturer's instructions for direct cell-based detection. The assay was performed in a 96-well plate with a density of 10,000 cells in 100uL media. Cells were protected from light and incubated for up to 6 hours with luminescent ROS-Glo reagents.

Murine model of *H. pylori* infection: Age and sex matched 8-12 week-old wild type and NLRX1<sup>-/-</sup> mice from a C57B/6 background were challenged with *Helicobacter pylori* strain SS1 by orogastric gavage after a 6-hour fast. *H. pylori* inoculum was prepared fresh in sterile 1X PBS and was administered to mice on days 0 and 2 of the study (500  $\mu$ l,  $5 \times 10^7$  CFU/mouse). Between weeks 2 and 11 post-infection mice were euthanized and stomach was excised for bacterial re-isolation. Gastric tissue was thoroughly rinsed in sterile 1X PBS, weighted, then homogenized in Brucella broth using a BioVortexer and pestle (Thomas Scientific). Serial dilutions were plated onto Difco Columbia agar base plates as described above.

Statistics: For statistical analysis on our RNAseq dataset, all genes with a median expression level in all samples greater than 0 were included in a 2-way (treatment and time) ANOVA analysis. Normal quantile transformation (qqnorm from R [239]) was used to normalize the FPKM to fit the normality assumption of ANOVA (tested with Kolmogorov-Smirnov test). Two-way ANOVA analysis was performed in R [239]. FDR [240] and Bonferroni adjustments were used to identify differentially expressed genes.

# Chapter 5

## Concluding Remarks

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Complex mechanisms underlying immunity to bacterial stimuli span several spatiotemporal magnitudes resulting in a variety of outcomes ranging from asymptomatic commensalism to pathological disease. This vast difference is clearly exemplified when studying differential mucosal immunity initiated by EAEC and *H. pylori*. EAEC is representative of an acute infection causing overt disease predominantly in immunosuppressed individuals. In contrast, *H. pylori* colonizes nearly half of the world's population and establishes a decades-long infection without causing severe pathology. In this dissertation we have demonstrated that these microbes can be used to perturb mucosal immunity for discovering host-centric, rather than anti-bacterial, targets for therapeutic intervention. Moreover, we have combined computational and mathematical modeling to accelerate the development of valuable hypotheses leading to novel findings.

EAEC is a leading cause of diarrhea world-wide, especially in immunosuppressed individuals such as children. In Chapter 2 we present a comprehensive overview covering the most up to date *in vitro*, *in vivo* and *in silico* models used to study host-EAEC interactions. We developed a novel mathematical model representing effects of immunosuppression on adaptive immunity and predicted mechanisms to boost anti-bacterial Th17 responses in malnourished animals. Similarly, chapter 3 further details findings from our malnourished murine model of EAEC infection. As predicted by computational modeling, we provide evidence that skewing inflammatory signaling in the colon through pharmacological blockade of an anti-inflammatory molecule, PPAR-gamma, leads to elevated immune signaling and pathogen clearance. This was characterized by beneficial infiltration of immune cells and the production of antimicrobial peptides in the colon which occurred through an IL-17A-dependent mechanism.

In Chapter 4 we switch gears and investigate macrophage immunity and *H. pylori* infection. Dysregulated host response toward *H. pylori* can cause overzealous inflammation leading to the development of peptic ulcers and cancer in extreme cases. Interestingly however, only 15% of individuals develop severe *H. pylori*-dependent disease. Therefore we investigated potential mechanisms that balance host tolerance versus defense. We describe novel findings on the role of the regulatory molecule NLRX1 in shaping innate immune responses to intracellular *H. pylori*. Specifically, we validate model-driven predictions that NLRX1 is required for successful intracellular colonization.

Taken together this dissertation presents one full cycle in the systems immunology approach: from literature searches to model construction, then predictions to experimental validation yet leaving a new set of testable predictions for future development. We employ high-throughput techniques and data mining to obtain robust calibration datasets and engineer biological systems. Using mathematical modeling we generate hypotheses that are novel and experimentally testable. Finally, our model predictions are supported by a wide variety of experimental studies ranging from the cellular to tissue level scales. The knowledge and methods generated through the concerted integration of experimental and analytical approaches are broadly applicable for the discovery of more efficacious host targeted cures for infectious and immune-mediated diseases.

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