Transdisciplinary Strategies for the Characterization of Mucosal Immune Responses

Monica Viladomiu Pujol

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Josep Bassaganya-Riera, Chair
Raquel Hontecillas-Magarzo, Co-chair
David R. Bevan
Pawel Michalak

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Abstract

The gastrointestinal mucosal immune system has the daunting task of maintaining immune homeostasis by eliminating potentially harmful microorganisms and limiting tissue injury while inducing tolerogenic responses to luminal antigens including innocuous food, commensal bacteria and self-antigens. This carefully orchestrated system depends on elaborate down-regulating mechanisms that mediate and maintain a state of tolerance under normal conditions. Changes in such delicate balance are linked to the development of gastrointestinal pathology as well as systemic disease states. Despite the rapid increase in our appreciation of the gastrointestinal immune system, there is still a major disconnect between the description of how mucosal immune responses are organized and controlled and an insufficient mechanistic understanding of how such responses shape and influence disease outcome and pathogenesis. By using model enteric microorganisms *Helicobacter pylori* and *Clostridium difficile*, this dissertation presents a systematic effort to generate novel mechanistic hypothesis based on computational predictions and experimentally elucidate the mechanisms of action underlying mucosal immune responses and pathology in the gut. In this thesis I present i) an overview on mucosal immunology and the need to develop novel therapeutics that limit the pathogenic effects of invading bacteria while maintaining their protective functions, ii) the role of miRNAs in the modulation of immune responses to enteric pathogens, iii) the mechanisms by which *Helicobacter pylori* is able to limit effector inflammatory responses required for bacterial clearance thus favoring tolerance over immunity, iv) intracellular mechanisms of immune evasion that contribute to bacterial persistence and chronic infection. The knowledge generated throughout this dissertation exemplifies how a combination of computational modeling, immunoinformatics and experimental immunology holds enormous potential for discovering unforeseen targets and developing novel vaccines and cures for infectious, allergic and immune-mediated diseases.

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Attributions

Among the chapters in this dissertation, several colleagues also contributed their own efforts for which credit is due. Descriptions of these contributions are as follows.

Chapter 1

Dr. Bassaganya-Riera and Dr. Hontecillas contributed to write the paper and helped designing the sections of the review.

Chapter 2

Dr. Bassaganya-Riera and Dr. Hontecillas contributed to write the paper, design the experiments, and helped designing the sections of the manuscript. Mireia Pedragosa was involved in the *in vivo* animal models. Adria Carbo and Stefan Hoops helped with the computational work. Pawel Michalak and Katarzyna Michalak performed the sequencing alignment and statistical analyses. Richard L. Guerrant, James K. Roche and Cirle A. Warren gave critical expertise in animal models of infection.

Chapter 3

Dr. Bassaganya-Riera and Dr. Hontecillas contributed to write the paper, design the experiments, and helped designing the sections of the manuscript. Casandra W. Philipson, Barbara Kronstreiner, Andrew Leber and Adria Carbo were involved in the *in vivo* animal models.

Chapter 4

Dr. Bassaganya-Riera and Dr. Hontecillas contributed to write the paper, design the experiments, and helped designing the sections of the manuscript. Barbara Kronstreiner and Casandra W. Philipson helped with the *in vitro* co-cultures. Pawel Michalak performed the sequencing alignment and statistical analyses.

Dr. Bassaganya-Riera is the director of the Nutritional Immunology and Molecular Medicine Laboratory (NIMML) and the Center for Modeling Immunity to Enteric Pathogens (MIEP) at the Virginia Bioinformatics Institute (VBI) at Virginia Tech.
Dr. Hontecillas is the NIMML co-director and MIEP Immunology Lead.
Dr. Hoops is a senior project associate at VBI and the MIEP Computational Lead.
Dr. Michalak is an associate professor at VBI and a MIEP member.
Katarzyna Michalak is a research assistant at VBI.
Dr. Carbo, Dr. Philipson and Mireia Pedragosa are former NIMML doctorate students and MIEP members.
Dr. Kronstreiner is a former NIMML post-doc and MIEP member.

Andrew Leber is currently a doctorate student at NIMML and a MIEP member.
Dr. Guerrant is a professor of medicine at University of Virginia (UVA) School of Medicine and a MIEP collaborator.
Dr. Roche was a retired associate professor at UVA School of Medicine.
Dr. Warren is an assistant professor in medicine at UVA School of Medicine.

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Chapter 1

Introduction: Understanding Mucosal Immune Responses to Enteric Pathogens

Monica Viladomiu, Raquel Hontecillas, and Josep Bassaganya-Riera.

1.1 Summary

Mucosal surfaces are consistently exposed to the external environment and are vulnerable to invasion by pathogenic micro-organisms. Such mucosal tissues are equipped with a specialized immune system – the mucosal immune system – that provides defense against invading pathogenic organisms by inducing a variety of inflammatory and anti-microbial responses. The immune system is particularly important in the gastrointestinal tract, where the divergent needs for efficient nutrient absorption, well-functioning host defense and tolerance towards commensal microbiota collide. Despite the rapid increase in our understanding and appreciation of the mucosal immune system, we are only beginning to decipher how the different molecules and cell populations shape innate and adaptive responses and influence disease pathogenesis.

Many pathogens have developed mechanisms to evade host immune responses and colonize the gut mucosa, leading to millions of cases of enteric infections a

year. *Helicobacter pylori* and *Clostridium difficile* are two examples of highly efficient enteric microorganisms that successfully induce inflammatory responses as a result of mucosal invasion. The studies discussed throughout this dissertation will use the model organisms *H. pylori* and *C. difficile* to study the mechanisms employed by the mammalian gastrointestinal immune system and how invading microorganisms respond, evade and subvert such mechanisms.

1.2 Introducing the mucosal immune system

The principal challenge of the gastrointestinal immune system is balancing the host response to pathogens while not responding to stimuli derived from commensal microbiota and food antigens [1]. Commensal bacteria residing in the intestinal lumen are thought to contribute to immune tolerance, although translocation of the same commensal microfloral antigens to the lamina propria results in effector and inflammatory responses. Also, mice are better protected from colitis in a germ-free environment [2], suggesting a role of the gut microbiota in the pathogenesis of inflammatory diseases such as Inflammatory Bowel Disease (IBD).

Mucosal immune homeostasis can be disrupted during gut inflammation due to alterations in: 1) barrier function of the epithelium; 2) the innate immune cells (e.g., macrophages and dendritic cells, DC) which provide the initial innate response to invading bacteria; and 3) lymphocyte function in both the lamina propria (LP) and the mesenteric lymph nodes (MLN), including the T cell population of the normal gut mucosa [3].

Absorptive and secretory cells, as well as different epithelial cell subsets such as microfold cells (M cells), goblet cells and Paneth cells, form a single cell layer that constitutes the epithelial barrier. Globet cells form a protective mucus layer, whereas Paneth cells secrete potent antimicrobial peptides known as defensins in the base of small intestinal crypts. M cells are found in the follicle-associated epithelium of the Peyer's patches sample intestinal mucosal contents and deliver them via transcytosis to DC and lymphocytes. The disruption of the epithelial barrier or the infiltration of pathogenic bacteria into the LP activate DC and macrophages, which process the antigen and present it on their surfaces through the MHC class II complex. These

antigen presenting cells (APC) are transported to the MLN, where they promote the differentiation of naïve T cells into effector and regulatory T cells. The cytokine environment secreted in part by the APC skews the differentiation of naïve CD4+ T cells into T helper (Th1, Th2, Th17, Th9, T follicular helper, Th22) or regulatory T (Treg) cell subsets. These now differentiated T cells migrate back to the LP, where they start the adaptive immune response towards the antigen. A predominance of dysregulated Th1 and Th17 responses in the colonic LP has been associated with *Clostridium difficile* infection [3]. **Figure 1.1** illustrates some key aspects of mucosal immunity relevant to gut inflammation in the context of nutritional protective mechanisms.

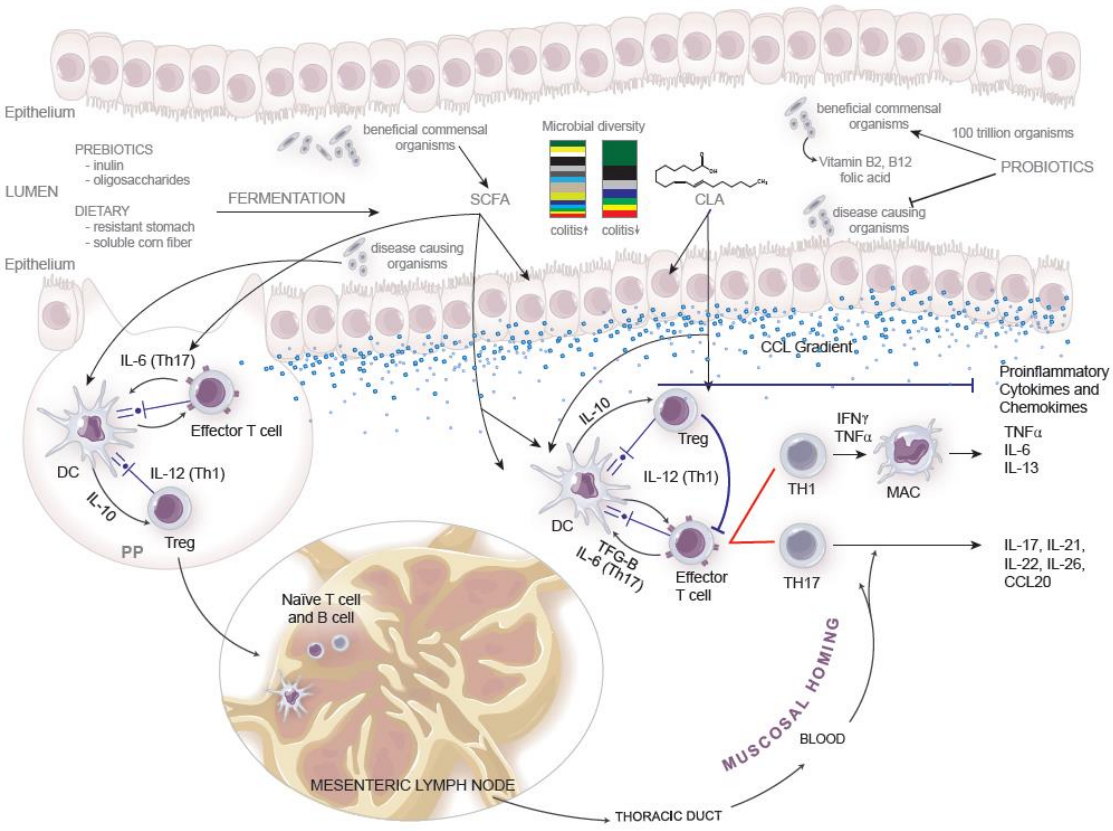

Figure 1.1. Mucosal immune mechanisms of nutritional protection against gut inflammation. The disruption of the epithelial barrier or the infiltration of pathogenic bacteria into the lamina propria (LP) or Peyer's patches (PP) activate dendritic cells (DC) and macrophages (MAC), which process the antigen and present it on their surfaces through the MHC class II complex. These antigen-presenting cells (APC) either stay in the PP or are transported to the mesenteric lymph nodes (MLN), where they promote the differentiation of naïve T cells into effector and regulatory T cells. The cytokine environment secreted in part by the APC skews the differentiation of naïve CD4+ T cells into T helper (Th1 and Th17) or regulatory T cell (Treg) subsets. Th1 cells differentiate in the presence of interleukin-12 (IL-12); IL-6 and transforming

growth factor (TGF) β induce a Th17 phenotype; whereas IL-10 induces a regulatory T (Treg) cell differentiation. These mature T cells migrate back to the lamina propria (LP), where they start the adaptive immune response towards the antigen, resulting in the production of effector and pro-inflammatory cytokines. The administration of prebiotics (inulin, oligofructose), dietary fibers (resistant starch, soluble corn fiber) and probiotics decrease effector responses and pro-inflammatory cytokine expression by the production of short chain fatty acids (SCFA), as well as conjugated linoleic acid (CLA), punicic acid (PUA), eleostearic acid (ESA) and abscisic acid (ABA), which activate peroxisome proliferator-activated receptor (PPAR) γ, a nuclear transcription factor that antagonizes pro-inflammatory pathways.

1.3 *Clostridium difficile* infection

Clostridium difficile is an environmental Gram-positive, anaerobic, spore-forming bacillus identified in 1935 in the stool of a healthy newborn [4]. In 1893, a severe form of *C. difficile*-associated disease characterized by inflamed colon and visible patches was first described. However, it was not until 1978 that *C. difficile* was identified as an opportunistic pathogen and causative agent of antibiotic-associated diarrhoea and pseudomembranous colitis (PMC) [5]. *Clostridium difficile* infection has rapidly increased since the 1990s with alarming rise since 2000, and it is currently the leading cause of diarrhea in the healthcare setting and becoming an increasingly common cause of diarrhea in industrialized countries [6]. Although it is not considered part of the human microbiome, *C. difficile* colonizes and can be isolated from about 1-5% of healthy adult population and 75% of infant mammals, with an increase to 39% in hospitalized patients [7]. However, only the toxin-producing strains are associated with the diarrheic disease *C. difficile* infection (CDI).

CDI is a nosocomial toxin-mediated intestinal illness whose clinical outcome ranges from asymptomatic colonization, mild to severe diarrhea, abdominal pain, fever and leukocytosis. Fulminant or more complicated CDI cases are associated with colitis with or without the presence of pseudomembranes, toxic megacolon or bowel perforation, sepsis, shock and even death [8]. Other rare manifestations associated with CDI are extra-intestinal infections [9], ileal infections [10], reactive arthritis [11], post-proctocolectomy enteritis [12] and bacteremia [13]. The severity of the symptoms mostly depends on the properties of the *C. difficile* strain, which is usually characterized by the genetic-dependent ability to produce toxins. Patients can acquire a non-toxigenic *C. difficile* strain [14], thus becoming asymptomatically colonized. On the other hand, individuals can develop *Clostridium difficile*-associated disease (CDAD) if they acquire a toxigenic *C. difficile* strain following antibiotic treatment and they fail to mount an

anamnestic serum immunoglobulin G (IgG) antibody response to toxins. However, if patients can mount an antibody response, they become asymptomatically colonized with *C. difficile*. Therefore, the outcome of CDI not only is defined by the pathogen itself, but also by the interaction of the pathogen with the host immune response.

C. difficile mostly grows in the intestine of individuals with altered commensal microflora [15, 16] due to antimicrobial therapy, immunosuppressants treatment, and cytostatic agents or proton pump inhibitors administration [17]. Ages above 65 years, co-morbidities, gastrointestinal disorders, or previous hospitalization also seem to predispose patients to CDI [18, 19]. In this regard, *C. difficile* is usually defined as the main cause of nosocomial infectious diarrhea developed in elderly patients with altered microflora, most usually after antibiotic treatment. However, the emergence of new hypervirulent strains has resulted in increased morbidity and mortality in younger populations with no previous contact with hospital environment or antibiotics [7, 20, 21].

Many countries have reported an increase in both incidence and severity of CDI over the last few years [22-24]. In 2005, a new *C. difficile* strain responsible for a large number of infections across North America was identified: BI/NAP1/027. Since its isolation, this strain has been documented in hospitals in 40 states in the United States, all provinces in Canada and most of the European countries [25-28]. However, rising rates of CDI are not limited to the spread of BI/NAP1/027, since other strains, including ribotypes 001, 027, 053 and 106, are also often associated with outbreaks and severe cases [28-30]. This increased virulence of *C. difficile* may be partly due to changes in the antibiotic susceptibility of emerging strains, since the emergence and spread of new *C. difficile* strains correlates with higher resistance to fluoroquinolone antibiotics such as gatifloxacin and moxifloxacin [31]. These hypervirulent strains are responsible for increased morbidity, mortality and healthcare costs in the US, Canada and Europe, primarily due to toxic megacolon, septic shock, intestinal perforation, and resistance to metronidazole [7, 20, 21, 25]. For instance, an outbreak in Quebec in 2002 lead to mortality of 17% and another in 2003 resulted in 13.4% mortality [32]. There have been several recurrent outbreaks especially in hospitals close to Montreal, with an estimate of 2,000 total deaths within the last decade. Moreover, an analysis of CDI-associated outcomes in nonsurgical patients in St. Louis in 2003 reported attributable mortality of 6% [33, 34]. According to the Agency for Healthcare Research and Quality Nationwide Inpatient Sample, there were approximately 139,000 patients discharged from acute care facilities who were diagnosed with *C. difficile* infection in 2000. This number

increased to 336,000 patients in 2008. In the United States alone, CDI was estimated to affect over 500,000 people and to cost the healthcare system over $3 billion in 2009 [8]. Thus, understanding the immunoregulatory mechanisms that allow *C. difficile* to be a leading cause of enteric disease is of enormous relevance for the improvement of current treatments and development of novel host-targeted therapeutics.

1.4 *Helicobacter pylori* infection

Helicobacter pylori is an ancient Gram negative, spiral-shaped bacterium that selectively establishes lifelong colonization of the gastric mucosa in more than 50% of the human population (Figure 1.2) [35, 36]. Prevalence can reach 90% in certain regions of Asia and Africa. However in developed countries of Europe, North America and in Australia, the prevalence has dropped dramatically to 15-20 % due to changes in life style including increased hygiene and the generalized use of antibiotics [37]. However, immigrants from South American countries are colonized at rates as high as 85% (e.g., Honduras).

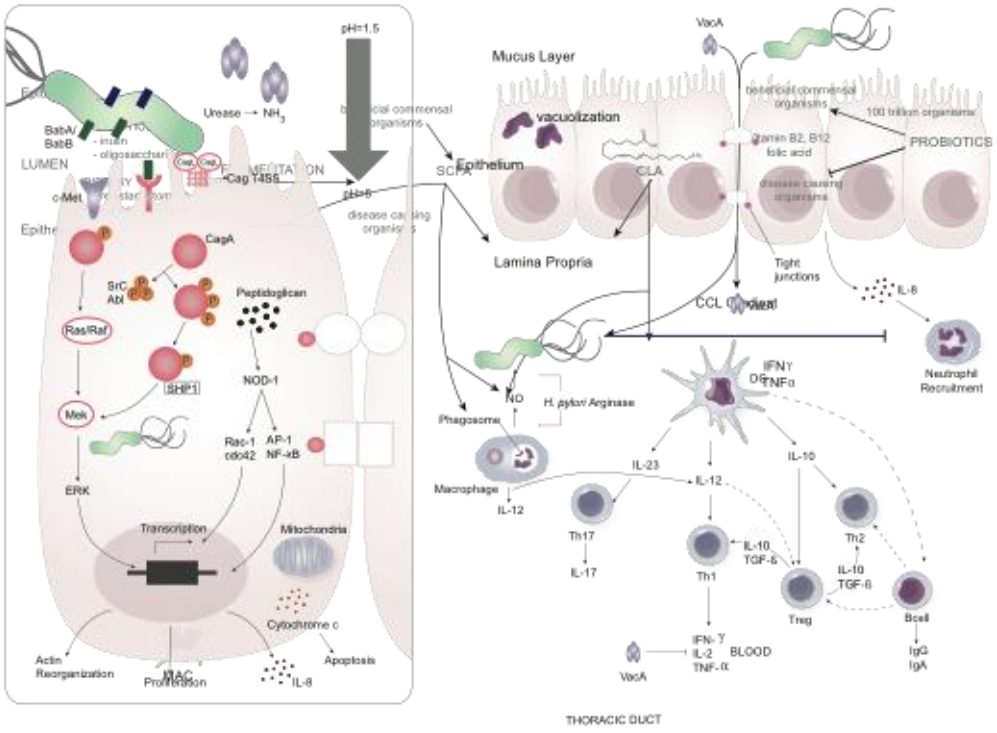

Figure 1.2. Schematic representation of *Helicobacter pylori*-host interactions.

H. pylori is highly specialized to naturally colonize the human stomach mucosa. It is an exceptionally successful microorganism and, if not treated, rapidly becomes dominant within the gastric niche [38]. Epidemiological data indicates that, in the vast majority of cases, *H. pylori* infection leads to asymptomatic chronic gastritis [39]. Specifically, it is estimated that about 85-90% of infected individuals do not present any adverse manifestations of disease throughout their life, while only 10-15% eventually develop gastric malignancies, including gastritis, peptic and duodenal ulcer, dysplasia, neoplasia, gastric-B-cell lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) and gastric adenocarcinoma (Figure 1.3) [39, 40]. Moreover, it has been estimated that *H. pylori* carriers have more than two-fold increased risk of developing gastric cancer, leading to about 11,000 deaths per year in the U.S. and 650,000 worldwide. In this regard, there have been targeted efforts to eradicate the bacteria via antibiotic treatment in people with a positive test [41], despite emerging evidence suggesting its beneficial effects in autoimmune, allergic diseases, and metabolic disorders [42-47].

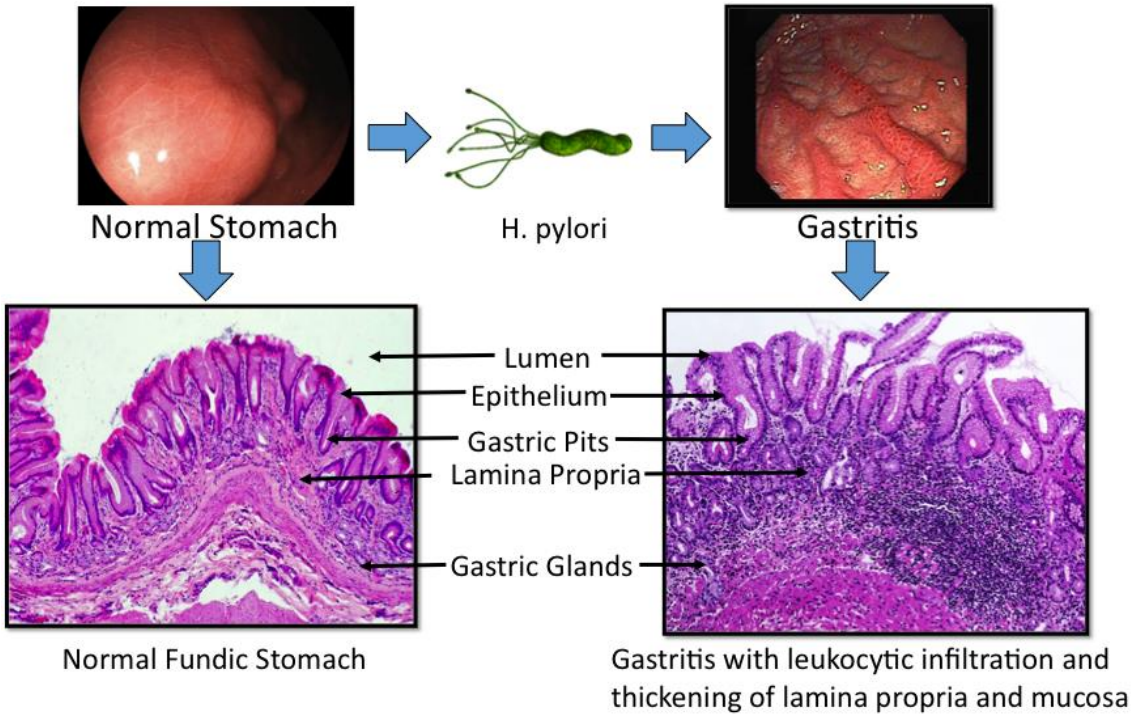

Figure 1.3. **Anatomy and histopathology of a healthy stomach (left) and an inflamed stomach following infection with *Helicobacter pylori* (right).**

The systematic elimination of *H. pylori* and its decreased presence as a predominant member of the human microbiome is controversial. There are still important efforts towards finding new therapies and vaccines against *H. pylori*. At the same time, it is believed that the 85% of carriers who will not develop gastric diseases have an advantage over non-carriers by the induction of regulatory immune responses, which protect from pathologies with an inflammatory component. For instance, mice infected with a strain of *H. pylori* lacking the *cag* (*cytotoxin-associated gene*) pathogenicity island (*cag* PAI) were protected from genetically and diet-induced type 2 diabetes when compared to non-infected mice [45]. Despite the above observations, the mechanisms by which *H. pylori* is able to evade the immune response and acquire protective versus pathogenic behaviors are incompletely understood. Hence, there is a need for further mechanistic studies to better characterize host-pathogen interactions in the context *H. pylori* infection.

1.5 Conclusions

Helicobacter pylori and *Clostridium difficile* are two examples of highly efficient enteric microorganisms that successfully induce inflammatory responses as a result of mucosal invasion. Antibiotic resistance along with limited treatment compliance has progressively decreased the efficacy of existing multidrug regimens used for eradicating enteric infections. Also, *C. difficile* and *H. pylori* vaccines are currently not available.

Investigating *C. difficile* and *H. pylori* pathogenesis will significantly contribute to our understanding of how pathogens are detected in the gastrointestinal tract and induce mucosal inflammation. A better characterization of the immunoregulatory mechanisms underlying immune responses to these model microorganisms has the potential to open a new host-targeted therapeutics-based era that limits the pathogenic effects of the invading bacteria and reduce disease incidence.

Chapter 2

Modeling the Role of Peroxisome Proliferator-Activated Receptor γ and MicroRNA-146 in Mucosal Immune Responses to *Clostridium difficile*

Monica Viladomiu, Raquel Hontecillas, Mireia Pedragosa, Adria Carbo, Stefan Hoops, Pawel Michalak, Katarzyna Michalak, Richard L. Guerrant, James K. Roche, Cirle A. Warren, and Josep Bassaganya-Riera.

Viladomiu M., Hontecillas R., Pedragosa M., Carbo A., Hoops S., et al. (2012) "Modeling de role of Peroxisome Proliferator-Activated Receptor γ and microRNA-146 in mucosal immune responses to *Clostridium difficile*". *PLoS One* doi:10.1371/journal.pone.0047525

2.1 Summary

Clostridium difficile is an anaerobic bacterium that has re-emerged as a facultative pathogen and can cause nosocomial diarrhea, colitis or even death. Peroxisome proliferator-activated receptor (PPAR) γ has been implicated in the prevention of inflammation in autoimmune and infectious diseases; however, its role in the immunoregulatory mechanisms modulating host responses to *C. difficile* and its toxins remains largely unknown. To characterize the role of PPARγ in *C. difficile*-associated disease (CDAD), immunity and gut pathology,

we used a mouse model of *C. difficile* infection in wild-type and T cell-specific PPARγ null mice. The loss of PPARγ in T cells increased disease activity and colonic inflammatory lesions following *C. difficile* infection. Colonic expression of IL-17 was upregulated and IL-10 downregulated in colons of T cell-specific PPARγ null mice. Also, both the loss of PPARγ in T cells and *C. difficile* infection favored Th17 responses in spleen and colonic lamina propria of mice with CDAD. MicroRNA (miRNA)-sequencing analysis and RT-PCR validation indicated that miR-146b was significantly overexpressed and nuclear receptor co-activator 4 (NCOA4) suppressed in colons of *C. difficile*-infected mice. We next developed a computational model that predicts the upregulation of miR-146b, downregulation of the PPARγ co-activator NCOA4, and PPARγ, leading to upregulation of IL-17. Oral treatment of *C. difficile*-infected mice with the PPARγ agonist pioglitazone ameliorated colitis and suppressed pro-inflammatory gene expression. In conclusion, our data indicates that miRNA-146b and PPARγ activation may be implicated in the regulation of Th17 responses and colitis in *C. difficile*-infected mice.

2.2 Introduction

Clostridium difficile typically is a harmless environmental sporulated gram-positive anaerobic bacterium [18, 48], but it has recently re-emerged as a significant enteric pathogen implicated in nosocomial diarrhea, colitis and even death, particularly after antibiotic treatment. *C. difficile* grows in the intestine of individuals with altered commensal microflora [15, 16] due to treatment with antimicrobials, immunosuppressants, cytostatic agents or proton pump inhibitors [17]. An increase in both incidence and severity of *C. difficile*-associated disease (CDAD) has been reported over the last years [22-24]. Previously, CDAD was a concern in older or severely ill patients, but the emergence of new hypervirulent strains such as NAP1/BI/027 has resulted in increased morbidity and mortality for other age groups in the United States, Canada and Europe [7, 20, 21]. The increased virulence of *C. difficile* is attributed to greater sporulation and production of binary toxins [49, 50] or to higher level of fluoroquinolone resistance [31]. Persistent or severe CDAD is currently being treated

with discontinuation of the antibiotic therapy that led to the disease, and vancomycin therapy [51]. Nevertheless, these therapeutic approaches do not restore the normal microflora and are not effective in clostridial clearance, but further prolong *C. difficile* shedding and destroy beneficial gut anaerobic bacteria [51, 52]. In contrast to targeting the bacterium and its toxins directly, the better understanding of the cellular and molecular basis underlying the host response will enable the rational development of host-targeted therapeutics for CDAD.

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor and ligand-activated transcription factor involved in glucose homeostasis and lipid metabolism. PPARγ antagonizes the activity of NF-κB, STAT and AP-1. Specifically, it suppresses NF-κB [53] by stabilizing the inhibitory κB (IκB)/NF-κB [54], thereby blocking pro-inflammatory gene transcription. More importantly, activation of PPARγ modulates mucosal immune responses and is involved in the prevention of inflammatory bowel disease (IBD) in mice [53, 55], pigs [56], and humans [57, 58]. Moreover, mice with a targeted deletion of PPARγ in epithelial cells, macrophages or T cells display increased pro-inflammatory gene expression and susceptibility to colitis [59-61]. PPARγ also suppresses Th1 responses [55] and blocks the differentiation of CD4+ T cells into a Th17 phenotype, thus potentiating a regulatory T (Treg) cell response [62]. However, no studies are available investigating the role of PPARγ in the pathogenesis and treatment of CDAD.

Another mechanism by which colonic gene expression can be tightly regulated is microRNA (miRNA)-driven RNA interference. MiRNAs are small (~22–24-nucleotide), non-coding, single-stranded RNA molecules that are processed from longer primary-miRNA transcripts. In the last decade, miRNAs have emerged as new potent genome regulators [63] and therapeutic targets [64]. MiRNAs are broadly found in plants, animals, viruses, and algae [65] and contribute to regulating gene expression. These molecules lead to translation inhibition of specific mRNAs depending on the type of base-pairing between the miRNA and its mRNA target [66]. In mammals, miRNA mostly affect the mRNA translation process, but mRNA target degradation also occurs. The role of miRNA has been explored in IBD and other immune-mediated diseases as a promising avenue for the discovery of novel mechanisms of pathogenesis, diagnostics, and therapeutics. Distinct miRNA expression profiles have been found in Crohn's disease and ulcerative colitis [67]. There is also mounting evidence that miRNAs contribute to orchestrate immune regulation and host responses to pathogen infections.

For example, miR-146a and miR-155 are involved in the regulation of T- and B-cell development [68], their differentiation and function [69]. Mice lacking miR-155 fail to control *Helicobacter pylori* infection as a result of impaired Th1 and Th17 responses [70]. Therefore, understanding the role of miRNAs in antibacterial immune and inflammatory responses holds promise of new molecular diagnostic markers as well as novel gene therapy strategies for treating hypervirulent bacterial infections and associated immunopathologies.

This study investigates the mechanisms underlying PPAR*γ* modulation of mucosal immune responses to *C. difficile*, including a possible relationship between nuclear receptors and miRNAs. Specifically, we applied mathematical and computational modeling approaches in combination with mouse challenge studies to study the mechanisms underlying the interactions between PPARγ activity and miRNA-146b to regulate colitis during *C. difficile* infection. Next, we investigated how either T cell-specific deletion or pharmacological activation of PPAR*γ* modulate colonic inflammatory cytokines and effector Th17 responses to *C. difficile* infection in mice. Our data indicate that T cell PPAR*γ* prevents colitis and down-modulates effector T cell responses in mice with CDAD and suggest a potential crosstalk between miRNAs and the PPAR γ pathway.

2.3 Increasing doses of *C. difficile* infection correlate with increasing CDAD and colonic inflammatory lesions

We first performed a dose-response study to identify the optimal infectious dose that results in greater inflammatory responses in wild type mice and found that clinical signs of disease appeared as early as 24 hours post-infection following challenge with *C. difficile* strain VPI10463. Increasing infectious doses of *C. difficile* from 10^5 to 10^7 cfu corresponded with greater disease severity and colonic inflammatory lesions. Colon, cecum, spleen and MLN were scored for inflammation-related gross pathology lesions during the necropsy. All *C. difficile*-infected mice presented gross pathological lesions in all the examined tissues when compared to uninfected mice (Figure 2.1). Colonic histopathological analyses showed increased epithelial erosion, leukocytic infiltration and mucosal thickness, with more severe inflammatory lesions corresponding to increasing

infectious doses of *C. difficile*. In addition, microscopic examination revealed extensive areas of necrosis of the mucosa and submucosal edema (Figure 2.2).

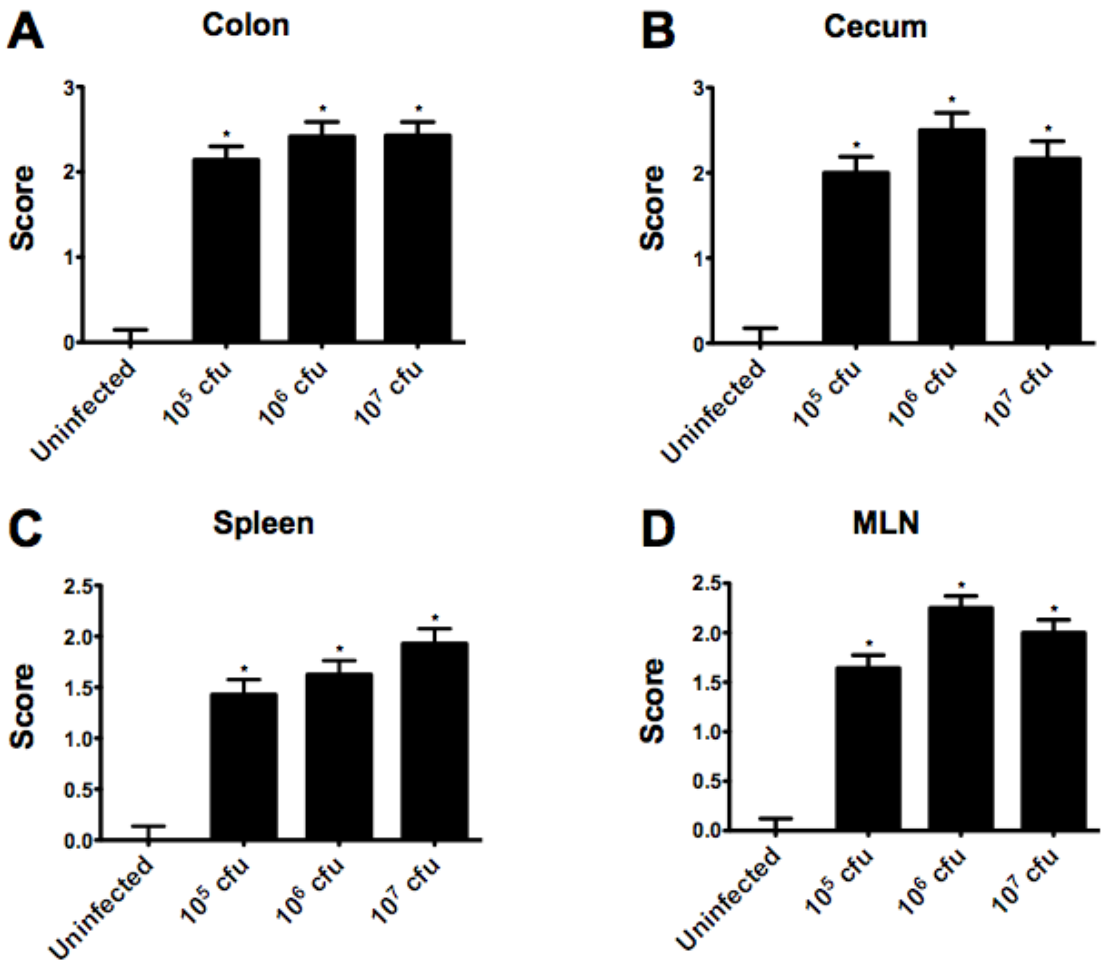

Figure 2.1. Effect of infection with *Clostridium difficile* strain VPI 10463 on macroscopic inflammation-related lesions in C57BL/6J wild-type mice. Colon (A), cecum (B), spleen (C) and mesenteric lymph nodes (MLN) (D) were macroscopically scored for inflammation during the necropsy (n=10). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (P<0.05).

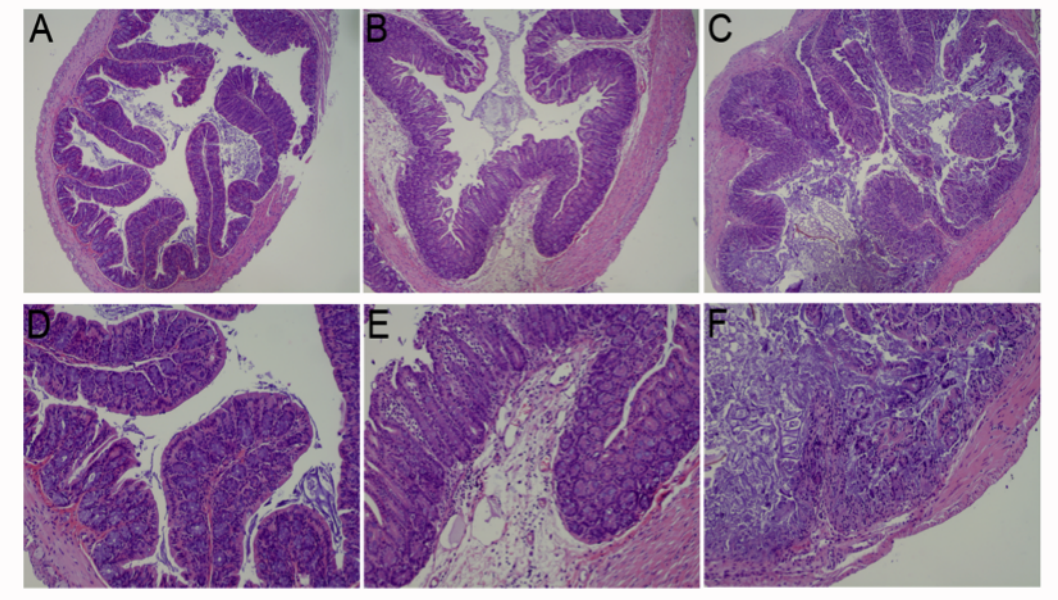

Figure 2.2. **Effect of infection with *Clostridium difficile* strain VPI 10463 on microscopic lesions observed following a 4-day challenge.** Representative photomicrographs of colons of uninfected (A and D), infected with 10^6 colony-forming units (cfu) of *C. difficile* (B and E) and infected with 10^7 cfu of *C. difficile* (C and F) (n=10). Original magnification at 40× (top panel) and 100× (bottom panel).

2.4 *C. difficile* upregulates colonic pro-inflammatory cytokine expression

RNA was extracted from colon and real time RT-PCR was performed to examine the effect of *C. difficile* infection on colonic gene expression. Mice challenged with 10^7 cfu of *C. difficile* had a significant increase in monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-17 and IL-1β when compared to all the other groups, indicating that the bacterial challenge induced a strong pro-inflammatory response in the gut (Figure 2.3). Although mice infected with 10^5 and 10^6 cfu had a higher disease activity score than the control mice, no increase in the colonic expression of such cytokines was seen in these groups, possibly due to a counterbalance mediated by a parallel regulatory response at lower doses of infection.

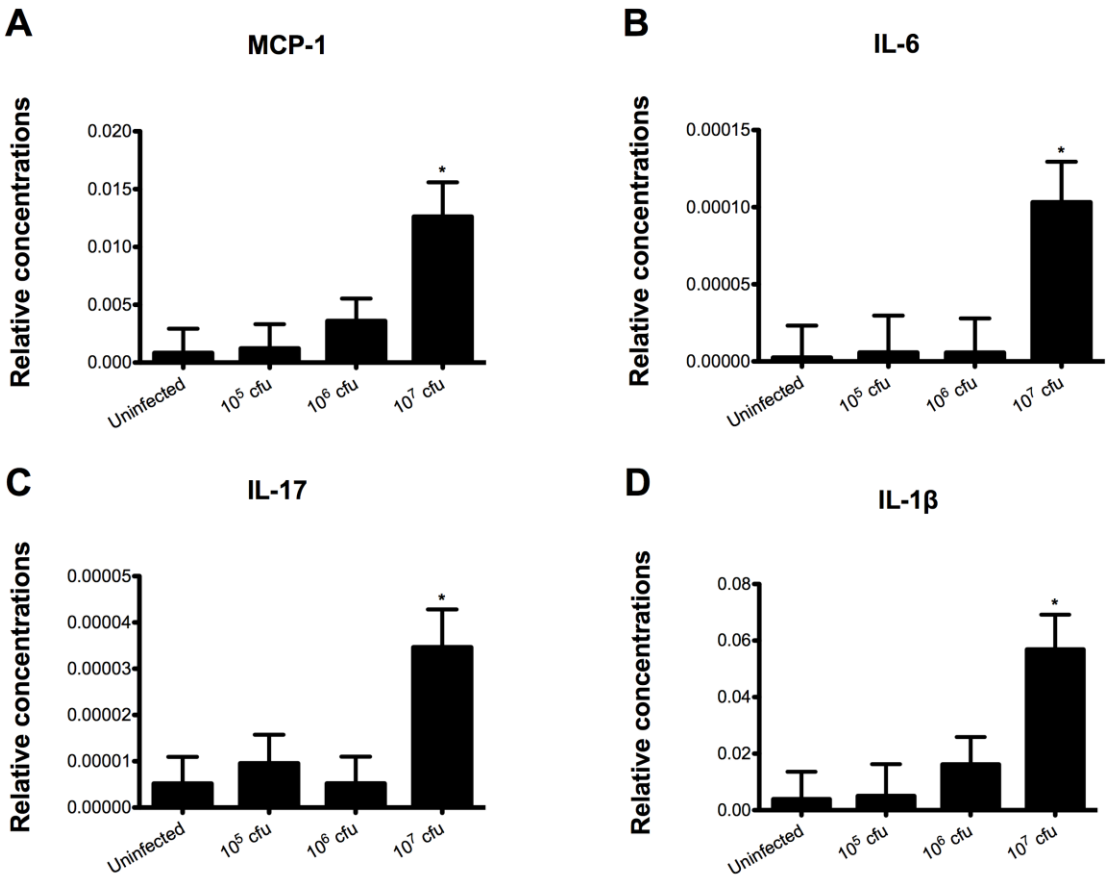

Figure 2.3. *Clostridium difficile* infection modulates colonic gene expression in mice. Colonic expression of monocyte chemotactic Protein 1 (MCP-1) (A), interleukin 6 (IL-6) (B), interleukin 17 (IL-17) (C) and interleukin 1β (IL-1β) (D were assessed by real-time quantitative RT-PCR in mice infected with *C. difficile* (n=10). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (P<0.05).

2.5 Infected mice overexpress miR-146b

MiRNA profiles of *C. difficile*-infected and uninfected mice were determined by Illumina sequencing. Specifically, a total of 454 miRNA types were detected among 8,902,783 Illumina reads from the six samples (3 non-infected and 3 infected with 10^7 cfu) multiplexed within two lanes. Three miRNAs were significantly overexpressed within infection: miR-146b, miR-1940, and miR-1298 (FDR P < 0.05) (Figure 2.4). The sequencing results were validated by real-time RT-PCR. Our data showed an upregulation of miR-146b correlating with the dose of *C. difficile* infection. NCOA4 was

computationally predicted as a target of miRNA-146b based on thermodynamics. In order to begin to validate such prediction, co-expression of miRNA-146b and NCOA4 within the colon and differential expression of NCOA4 with increasing doses of infection was assessed by RT-PCR. Interestingly, a significant decrease in the expression of colonic NCOA4 was found with increasing *C. difficile* infectious doses. NCOA4 is a coactivator molecule that interacts with the PPAR γ complex and facilitates its activation. Therefore, the decrease in NCOA4 expression results in a reduction of PPAR γ activation. In line with the suppression of NCOA4 expression we found that PPAR γ target genes CD36 and GLUT4 were significantly downregulated in colons of *C. difficile*-infected mice (Figure 2.5), indicating that colonic PPARγ activity is suppressed in mice infected with *C. difficile*.

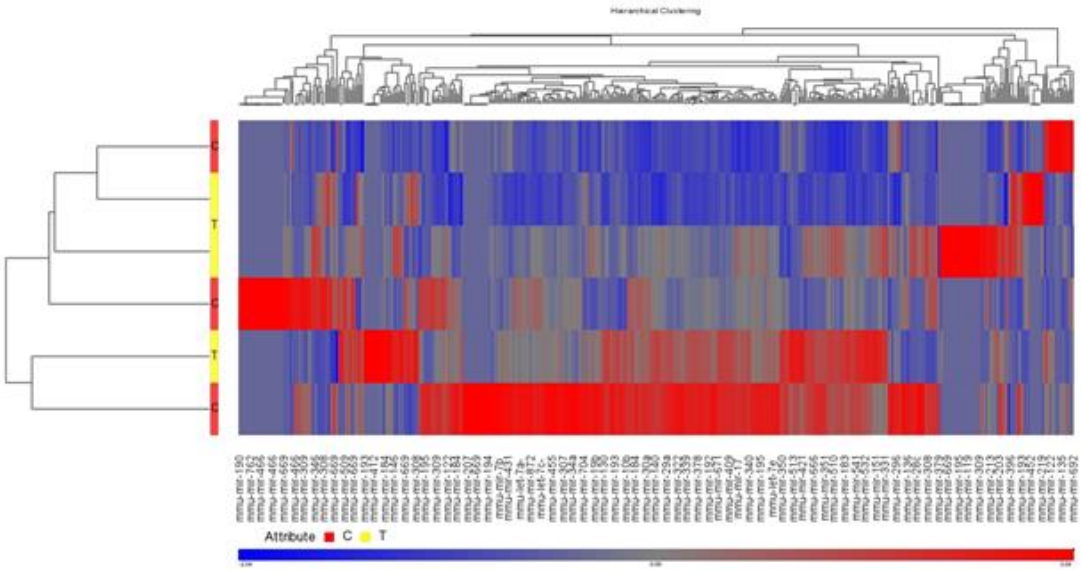

Figure 2.4. Effect of infection with *Clostridium difficile* strain VPI 10463 on miRNA differential expression in C57BL/6J wild-type mice. miRNA-seq heatmap of Clostridium difficile-infected (T) and uninfected control (C) mice (n=3).

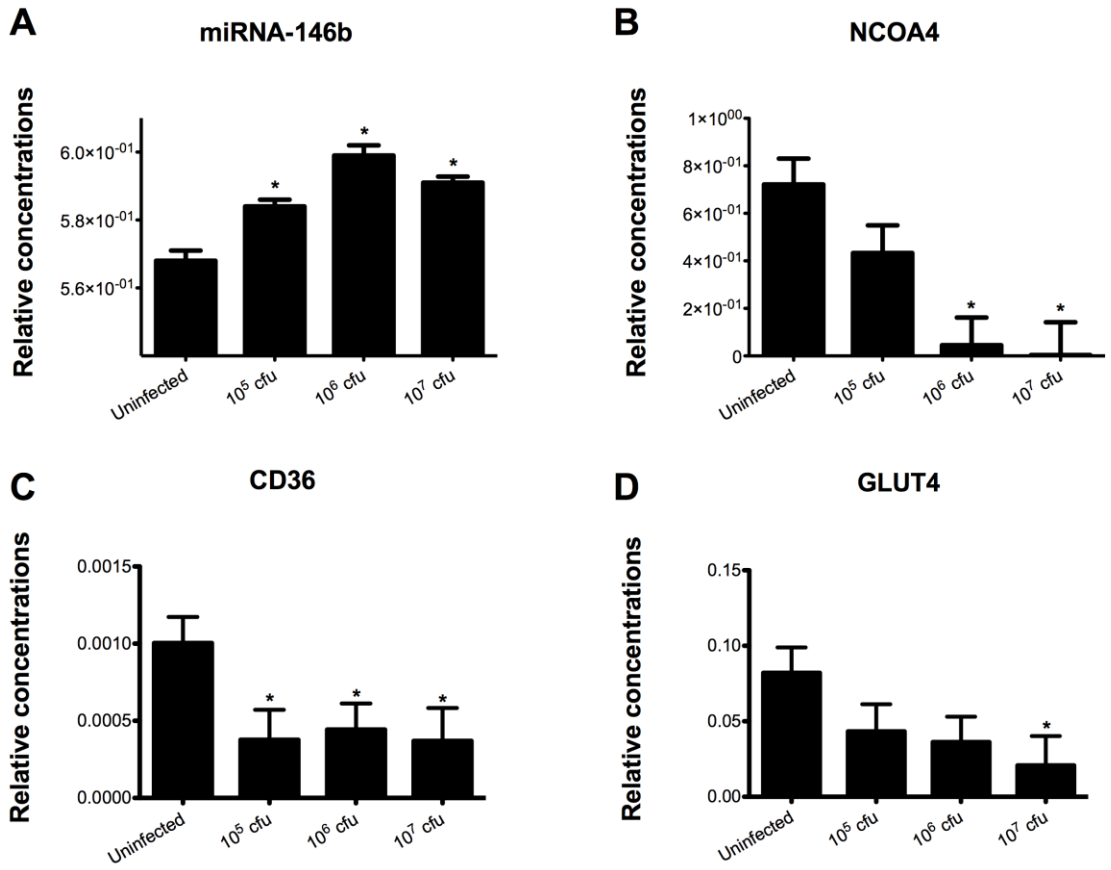

Figure 2.5. Effect of *Clostridium difficile* infection on the colonic expression of miR-146b and target genes NCOA4, CD36 and GLUT4 mRNA in mice. Colonic expression of miRNA-146b (A) as well as NCOA4 (B), CD36 (C) and GLUT4 (D) were assessed by real-time quantitative RT-PCR in mice infected with *C. difficile* (n=10). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (P<0.05).

2.6 Computational modeling of host responses to *C. difficile* infection

To further integrate and characterize the potential interactions occurring between *C. difficile*, miR-146b and PPARγ, we developed a computational and mathematical model of the colonic gene expression changes occurring in the colon following *C difficile* infection. This network was constructed based on our experimental findings and literature information (Figure 2.6A). By using this model, we explored *in silico* the mechanisms by which *C. difficile* modulates the expression of effector and inflammatory cytokines. Our computational simulation predicts an upregulation of miR-146b, and IL-17

and a down-regulation of NCOA4 and PPARγ in colons of mice after infection with *C. difficile* (Figure 2.6B).

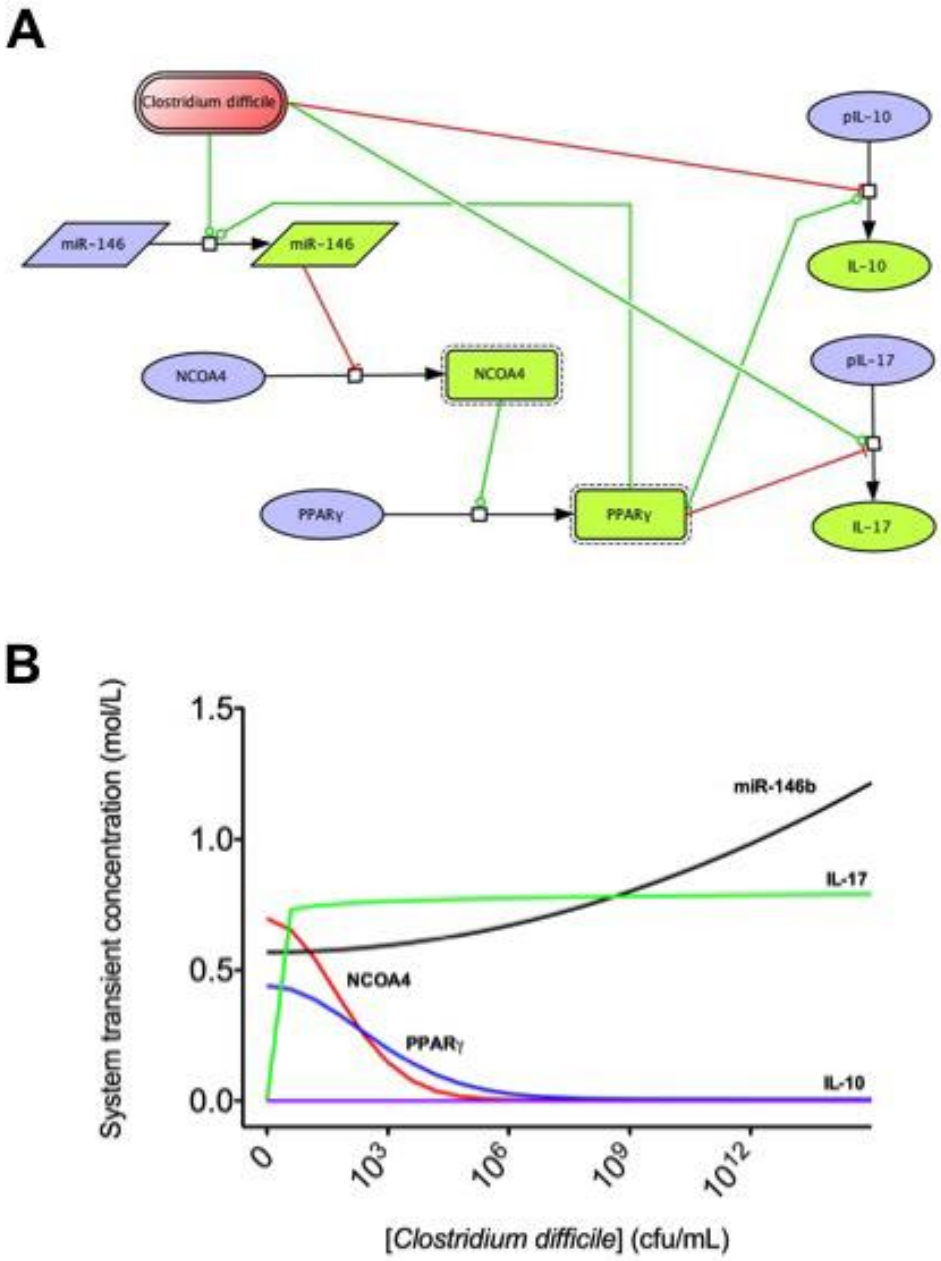

Figure 2.6. Computational modeling of mucosal immune responses to *Clostridium difficile* infection. CellDesigner-based illustration of the Complex Pathway SImulator model of the model for *Clostridium difficile* immune response (A). The model represents the interaction between *C. difficile*, miRNA-146, nuclear receptor coactivator 4 *(*NCOA4), peroxisome proliferator-activated receptor γ (PPAR γ*)*, interleukin 10 (IL-10) and interleukin 17 (IL-17) in Systems Biology Markup Language format. Inhibition is represented in red and activation in green. COPASI steady state scan showing the variation on the species concentrations with increasing computational concentration of C. difficile (B). *In silico* simulations show how increasing concentrations of *C.*

difficile increase miRNA-146b levels, thus decreasing NCOA4 and PPAR *γ.* In line with the experimental data, IL-17 expression also increases with the infection.

2.7 The loss of PPARγ in T cells significantly increased CDAD and colitis following *C. difficile* infection

Since our data indicates that the PPARγ pathway is downregulated at the colonic mucosa during *C. difficile* infection, we assessed the impact of the loss of PPARγ in epithelial and hematopoietic cells on *C. difficile* infection-associated weight loss. Our results show a more dramatic weight loss and more accentuated inflammatory lesions in tissue-specific PPARγ null mice following infection with *C. difficile* (data not shown). In a follow-up study we determined the impact of T cell-specific PPARγ deletion in the inflammatory response and disease caused by this facultative anaerobic bacterium. T cell-specific PPARγ null (i.e., CD4cre+) mice had higher disease activity scores than wild type mice did. They also showed a 20% body weight loss by day 4 post-infection which resulted in 30 % of mortality (Figure 2.7). Moreover, infected CD4cre+ mice had more severe colonic inflammatory lesions (Figure 2.8), suggesting that PPARγ expression in T cells plays and important role in ameliorating CDAD in mice. Uninfected CD4cre+ mice did not show any difference when compared to the uninfected WT group (data not shown). On the other hand, mice treated with the PPARγ agonist pioglitazone (70 mg/kg) had reduced inflammatory lesions, colonic histopathology (Figure 2.8), and had lower levels of colonic inflammatory mediators when compared to untreated mice infected with *C. difficile* (Figure 2.9). Also, uninfected WT mice treated with pioglitazone did not differ from uninfected and untreated group.

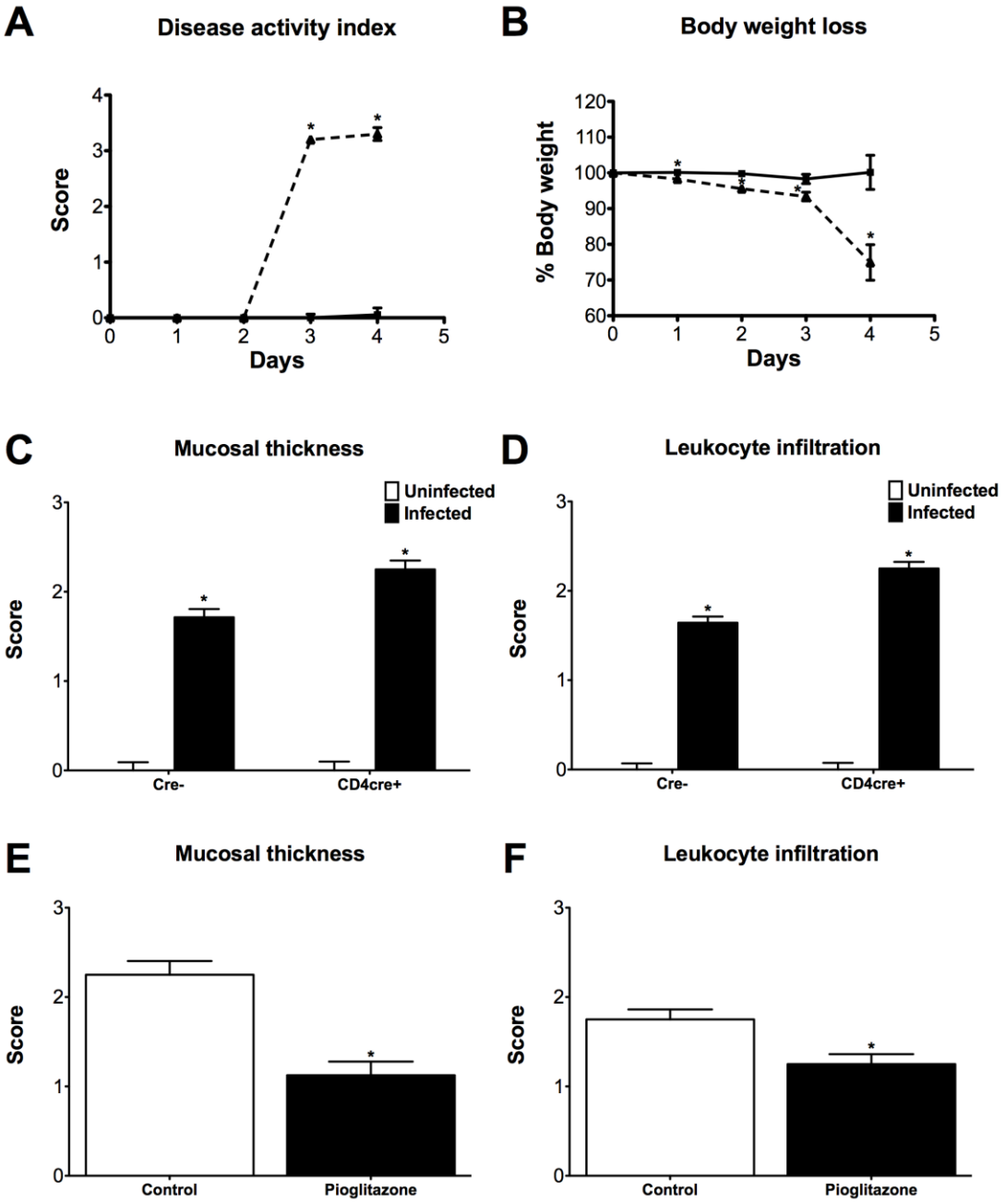

Figure 2.7. Effect of the genotype and treatment on the body weight loss, disease activity index and histologic lesions in the colons of mice infected with *Clostridium difficile* strain VPI 10463. Mice were weighed (A) and scored (B) daily for mortality and morbidity and the presence of diarrhea and other symptoms (n=8). All colonic specimens underwent blinded histological examination and were scored 0-4 on mucosal wall thickening (C&E) and leukocyte infiltration (D&F). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (P<0.05).

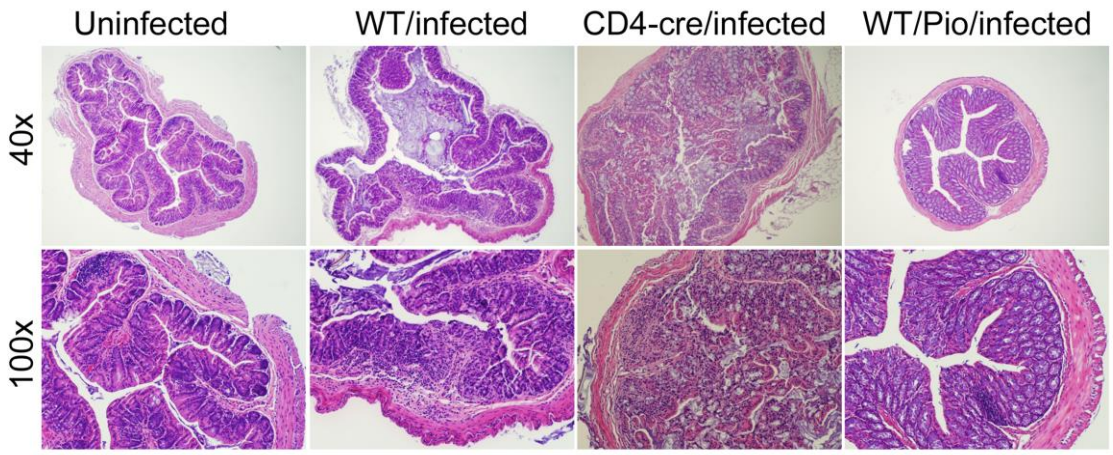

Figure 2.8. **Impact of the loss of PPARγ in T cells and pharmacological activation of PPARγ in colonic inflammatory lesions in *Clostridium difficile*-infected mice.** Representative photomicrographs of colons of uninfected (A and E), infected wild type mice (B and F), infected CD4cre+ mice (C and G) and infected wild-type mice treated orally with pioglitazone (70 mg/kg) (D and H) (n=8). Original magnification at 40× (top panel) and 100× (bottom panel).

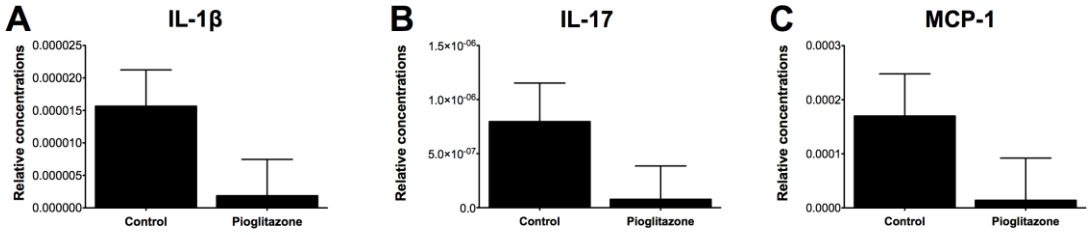

Figure 2.9. Effect of the oral pioglitazone administration on the colon gene expression of mice infected with *Clostridium difficile* strain VPI 10463. Colonic expression of interleukin 1β (IL-1β) (A), monocyte chemoattractant protein 1 (MCP-1) (B) and interleukin 17 (IL-17) (C) were assessed by real-time quantitative RT-PCR in *C. difficile* infected wild type mice treated with pioglitazone (n=8). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (P<0.05).

2.8 The loss of T cell PPARγ causes upregulation of colonic MCP-1 and IL-17 and downregulation of IL-10 in *C. difficile*-infected mice

Colonic IL-10 expression was upregulated in colonic wild type mice when compared to those of T cell-specific PPARγ null (CD4cre+) mice, indicating that the deficiency of

PPARγ in T cells abrogated the ability of *C. difficile* infection to induce IL-10 expression. In addition, the deficiency of PPARγ in T cells caused an upregulation of colonic MCP-1, IL-17 and tumor necrosis factor α (TNF-α) mRNA expression (Figure 2.10). Flow cytometric analyses of immune cell populations indicates an increased percentages of IL-17+ and RORγt+ CD4+ T cells following the infection as well as increased percentages of Th17 cells in spleens of T cell-specific PPARγ null mice (Figure 2.11).

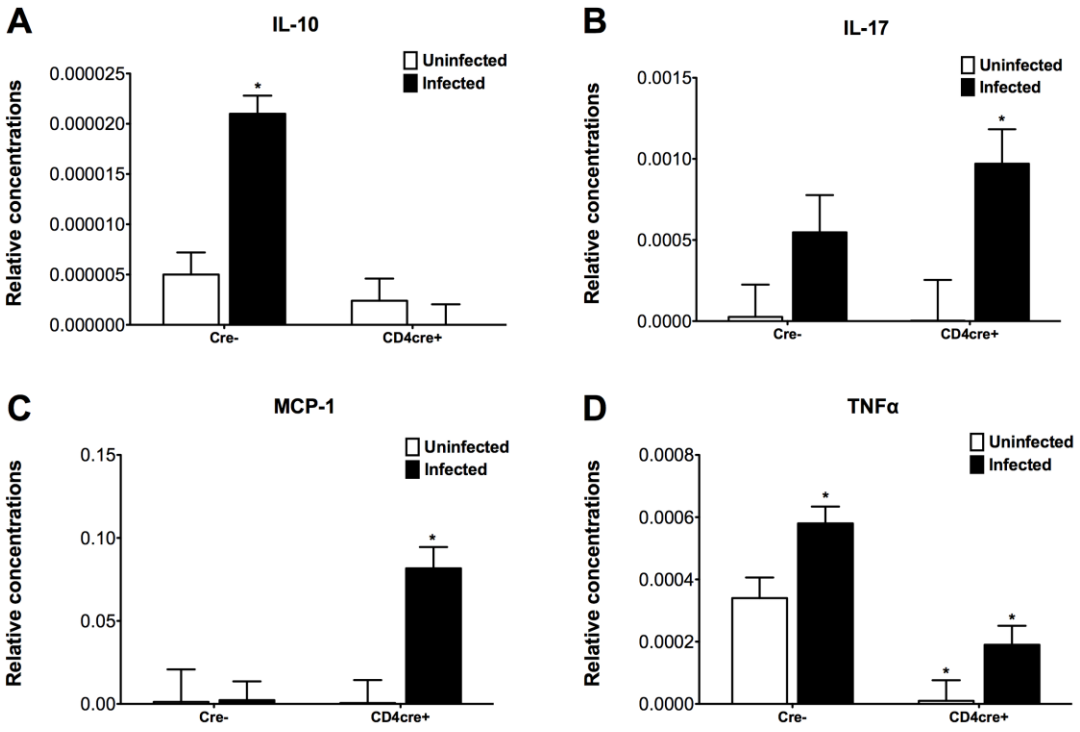

Figure 2.10. The loss of PPARγ in T cells regulates colonic cytokine expression of mice infected with *Clostridium difficile*. Colonic expression of interleukin 10 (IL-10) (A), interleukin 17 (IL-17) (B), monocyte chemoattractant protein 1 (MCP-1) (C) and tumor necrosis factor (TNF-α) (D) were assessed by real-time quantitative RT-PCR in wild type and T cell PPARγ null mice infected with *C. difficile* (n=8). Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the wild type control group (P<0.05).

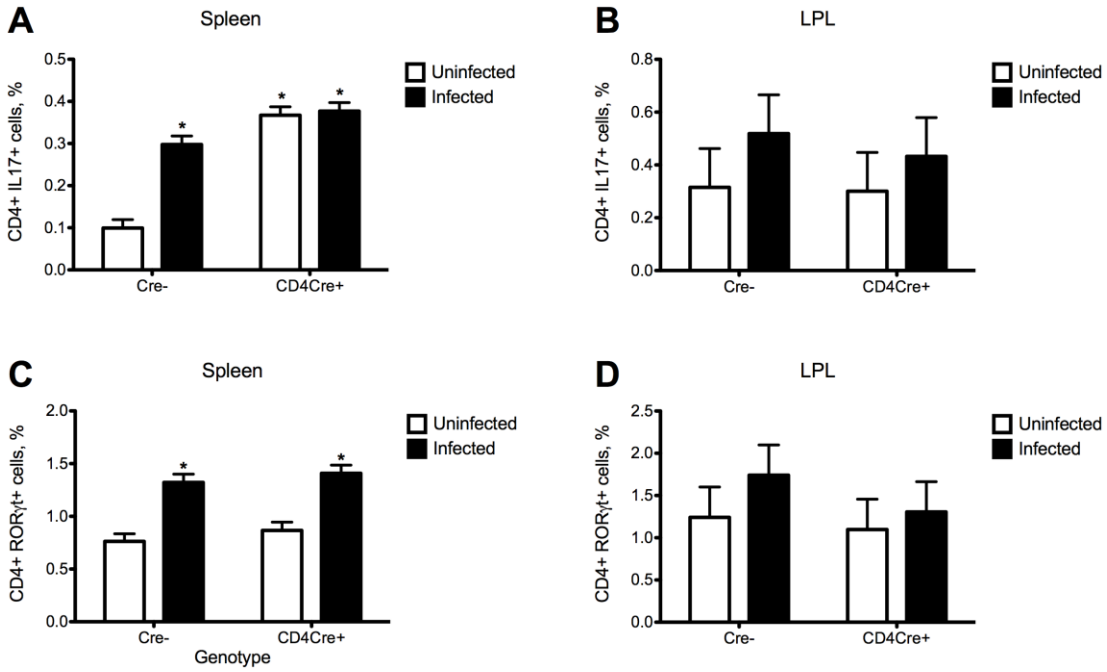

Figure 2.11. The loss of PPARγ in T cells and *Clostridium difficile* infection enhances Th17 responses in spleen and lamina propria of mice. Splenocytes and lamina propria lymphocytes from wild type and T cell PPARγ-null mice infected with *C. difficile* (n=6) were immunophenotyped to identify immune cell subsets by flow cytometry. Data are represented as mean ± standard error. Points with an asterisk are significantly different when compared to the control group (*P*<0.05).

2.9 Discussion and Conclusions

Clostridium difficile is the most common cause of nosocomial infectious diarrhea in the U.S. An increase in the severity and incidence of *C. difficile* infections has been reported in the last few years [71]. Moreover, the increased report of cases in healthy people without traditional risk factors is alarming since it suggests a greater pathogenicity of circulating strains [72, 73]. Hence, elucidating the cellular and molecular basis controlling the host-*C. difficile* interactions is important to discover new broad-based, host-targets therapeutic approaches to control the disease. This report presents fully integrated *in vivo* and computational approaches for studying the mechanisms involved in the regulation of host responses to *C. difficile*. We constructed a network model with five dynamical variables representing miR-146b, NCOA4, PPARγ, IL-17 and IL-10, plus an external input: the infectious dose of *C. difficile*. The computational simulations predicted

an overexpression of miR-146b following *C. difficle* infection, resulting in decreased concentrations of NCOA4, which in turn failed to activate PPARγ. In addition, colonic gene expression analyses demonstrated an upregulation of IL-6 and IL-17 in *C. difficile*-infected mice, suggesting either enhanced Th17 responses or IL-17 production by other mucosal cell types such as paneth cells or γδ T cells. Th17 responses are implicated in immune-mediated diseases and immune responses to some extracellular pathogens. Flow cytometric analyses of immune cell populations indicated increased percentages of IL-17+ and RORγt+ CD4+ T cells in spleens of infected mice, suggesting that *C. difficile* infection enhanced Th17 responses.

PPARγ plays a crucial role during CD4+ T cell differentiation by down-modulating effector and enhancing regulatory responses [55, 74], and thereby contributing to the maintenance of tissue homeostasis. Furthermore, PPARγ agonists have proven therapeutic and prophylactic efficacy in IBD [53, 55, 56]. To determine whether the loss of the PPARγ gene in T cells worsened CDAD, we challenged wild-type and T cell-specific PPARγ null mice with *C. difficile*. The bacterial infection induced a colonic mucosal IL-10-driven anti-inflammatory response as well as an effector response characterized by the production of IL-17 and MCP-1 in wild-type mice. However, the loss of PPARγ in T cells abrogated the ability of *C. difficile* to induce IL-10 expression in the colon, without affecting the overproduction of IL-17, which has been implicated in immune-mediated diseases such as IBD. Flow cytometry analysis confirmed that both *C. difficile* infection and the loss of PPARγ favor Th17 responses. The therapeutic value of PPARγ activation in CDAD was further demonstrated by the significant amelioration of CDAD and suppression of colitis in mice treated with pioglitazone, a full PPAR γ agonist.

Pharmacological manipulation of miRNA has been postulated as a novel and viable therapeutic approach to modulate the host inflammatory response to minimize pathology without negatively affecting pathogen clearance or the ability to mount a protective immune response. Mature miRNAs are incorporated into the RNA-induced silencing complex where they typically recognize and bind to sequences in the 3' untranslated regions, leading to suppression of translation and/or degradation of mRNA. There is accumulating evidence that miRNA play a critical role in immunity and inflammation [67-69]. For instance, distinct miRNA expression profiles have been found in Crohn's disease and ulcerative colitis [67]. By comparing miRNA profiles from *C. difficile*-infected and uninfected mice, we found that miR-146b, miR-1940, and miR-1298 were overexpressed in colons of *C. difficile*-infected mice. We focused our efforts on the

miRNA-146 family (miRNA-146a/b) in this study since we validated its upregulation in the colon of *C. difficile*-infected mice by using RT-PCR. In addition, miRNA-146 is expressed in leukocytes and its function is clearly linked to innate immunity and inflammation [75, 76]. miRNA-146 regulatory circuit improves TLR4 and cytokine signaling in response to microbial components and proinflammatory cytokines [77, 78]. Moreover, it is involved in the regulation of T- and B-cell development [68, 69], differentiation and function [69]. To further understand the role of miRNA-146b during *C. difficile* infection, a list of mRNA potential targets for such miRNA was retrieved from miRBase [79]. Notably, one of the co-activators facilitating the transcriptional activities of the ligand-activated PPARγ, NCOA4, was a predicted target of miRNA-146b. Indeed, gene expression analyses using qRT-PCR demonstrated co-expression of miRNA-146b and NCOA4 in colons of *C. difficile*-infected mice and a negative correlation between expression of miR-146b and its target NCOA4 along with increasing doses of *C. difficile*, suggesting a potential inhibition of NCOA4 by miR-146b resulting in suppressed PPARγ activity, as measured by suppressed expression of PPAR γ-responsive genes (i.e., CD36 and Glut4) in *C. difficile*-infected mice. Thus, miRNAs become promising therapeutic targets once the functional consequences of miRNAs alteration are completely elucidated. Also, future studies should examine more direct therapeutic approaches to prevent overexpression of miRNA-146 during CDAD.

In conclusion, we have used loss-of-function approaches in combination with pharmacological activation of PPARγ and computational modeling to investigate the critical role of PPARγ in regulating immune responses and disease severity following *C. difficile* infection. Our data suggests that overexpression of miRNA-146b in the colon might exacerbate inflammatory responses by suppressing PPARγ activity through a mechanism possibly involving suppression of NCOA4, a co-activator molecule required for activation of PPAR γ (Figure 2.12).

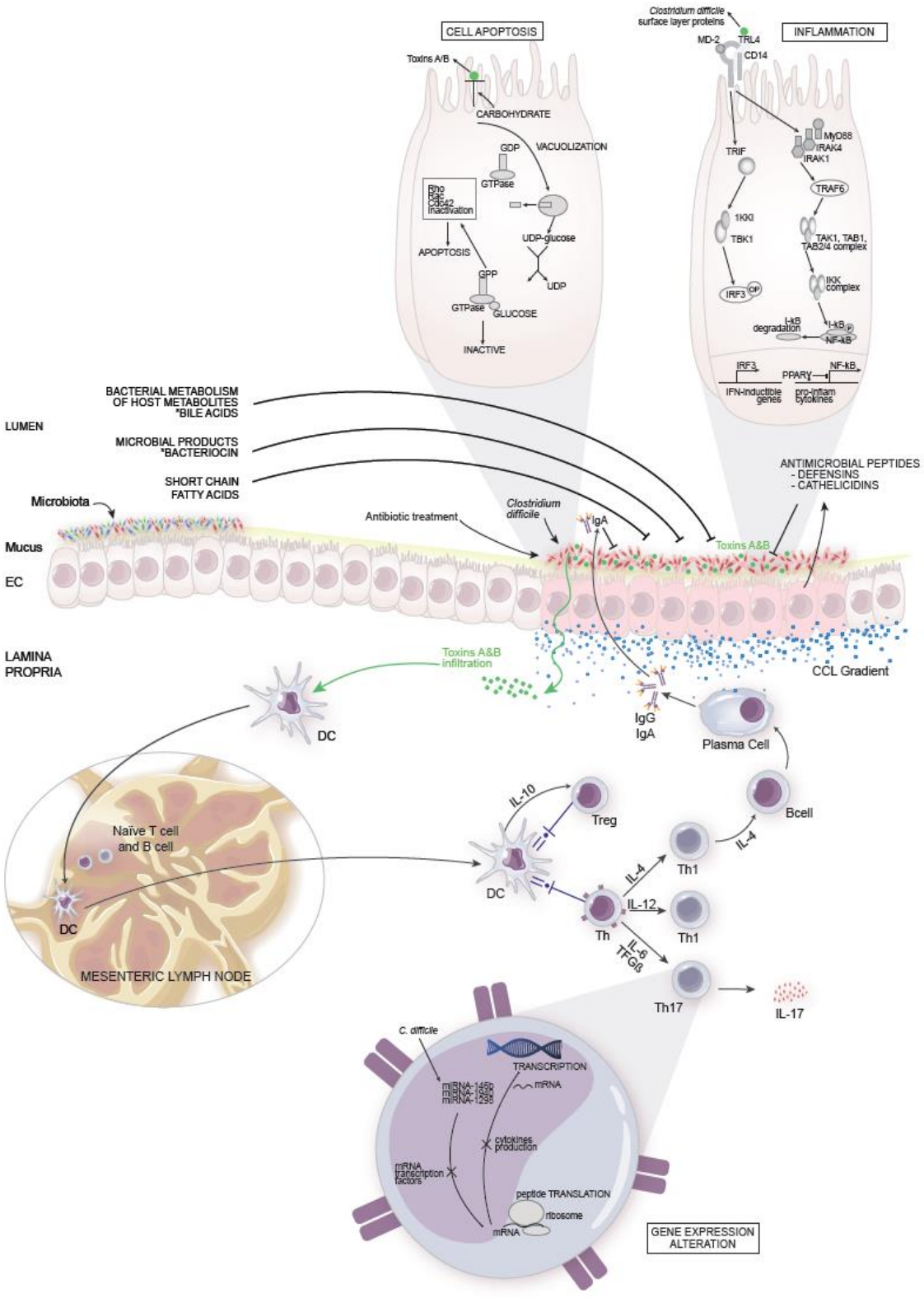

Figure 2.12. *Clostridium difficile* interaction with the gut microbiota and the host immune response. The normal gut microbiota provides the host with colonization resistance against *C. difficile* infection (CDI). Microbiota-derived metabolites such as short chain fatty acids (SCFA) and bacteriocins contribute to preventing CDI, as well as metabolizing host-derived metabolites such

as bile acids. Host epithelial cells also respond CDI by producing several antimicrobial peptides including defensins and cathelidicins. Colonization resistance can be impaired by antibiotic administration through changes in the composition and function of the gut microbiota, thus facilitating *C. difficile* spore germination and subsequent infection. *C. difficile* then produces toxins A and B, which disrupt the epithelial layer allowing toxin infiltration into the lamina propria, where they will be recognized by dendritic cells, T cells and macrophages and elicit a host immune response. Toxins A and B are recognized by carbohydrates in the epithelial cell surface, enter the cell through receptor-mediated endocytosis and disrupt normal signaling pathways by glycosylating Rho, Rac and Cdc42 proteins. Surface layer proteins can be recognized by TLR4 and activate NF-KB but not IRF3, which results in the secretion of pro-inflammatory molecules. *C. difficile* can also alter miRNA expression. Specifically, CDI increases expression of miRNA-146b, miRNA-1940 and miRNA-1298 in the colon, thus altering gene expression and CDI clinical outcome.

2.10 Materials and Methods

Ethics Statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act. The IACUC approval ID for the study was 10-087-VBI.

Animal procedures

C57BL/6J wild type mice were bred in our laboratory animal facilities. Tissue-specific PPARγ null mice were generated as described previously [53, 80, 81]. The tail and colonic genotypes of mice were determined by PCR analysis as described previously [53, 82] . Specifically, we used CD4-Cre+ mice lacking PPARγ in T cells [74] and MMTV-Cre+ mice with a deletion in epithelial and hematopietic cells [53]. Mice were maintained at the experimental facilities at Virginia Tech.

Antibiotic pretreatment prior to the bacterial challenge

Previous studies have demonstrated that three days of pre-treatment with a mixture of antibiotics in the drinking water is sufficient to disrupt the intestinal microflora and allows *C. difficile* infection [83]. The antibiotic mixture consisted of colistin 850 U/mL (which corresponds to 4.2 mg/kg), gentamicin 0.035 mg/mL (which corresponds to 3.5 mg/kg), metronidazole 0.215 mg/mL (which corresponds to 21.5 mg/kg) and vancomycin 0.045 mg/mL (which corresponds to 4.5 mg/kg). The mixture was prepared and added to the

drinking water for a 3-day pre-treatment period. A control group that received no antibiotics was also included. Following the treatment all mice were given regular autoclaved water for 2 days and all mice including the control group received a single dose of clindamycin (32 mg/kg) intraperitoneally 1 day before *C. difficile* challenge.

Clostridium difficile mouse challenge studies

On day 5 of the study mice were challenged intragastrically with *Clostridium difficile* strain VPI 10463 (ATCC 43255). To optimize the infection dose for further studies, we conducted a dose-response experiment using the following *C. difficile* infectious doses: 10^5, 10^6 and 10^7 cfu/mouse. The highest dose was used for the following challenges. In some experiments mice received pioglitazone at 70 mg/kg by oral gavage once daily starting 3 days before the infection date and until the necropsy day. Mice were weighed and scored daily to assess mortality and the presence of diarrhea and other general disease symptoms (e.g., piloerection, hunchback position).

Histopathology

Segments of colon (3 cm of the anatomic middle of the colon) were fixed in 10% buffered neutral formalin. Samples were embedded in paraffin, sectioned (6 μm), and then stained with hematoxylin and eosin for histological examination. Tissue slides were examined with an Olympus microscope (Olympus America). Images were captured using the FlashBus FBG software (Integral Technologies) and processed in Adobe Photoshop Elements 2.0 (Adobe Systems). The different tissue segments were graded with a compounded histological score, including the extent of: 1) crypt damage and regeneration; 2) metaplasia/hyperplasia; 3) lamina propria vascular changes; 4) submucosal changes; and 5) presence of inflammatory infiltrates. The sections were graded with a range from 0 to 4 for each of the previous categories and data were analyzed as a normalized compounded score.

Analyses of miRNA-seq

Next-generation sequencing allows the sequencing of miRNA molecules and simultaneous quantification of their expression levels. We used Illumina deep

sequencing to survey miRNA profiles of colons from uninfected and *C. difficile*-infected mice with most distinct phenotypes. All reads were mapped against the mouse reference genome. To determine putative gene targets of miRNA, the EMBL-EBI Microcosm v5 database was used. Additionally, precursor and mature sequences were retrieved from MirBase v16 [79] and entered into microRNAminer [84]. Following characterization of miRNA expression profiles with Partek Genomics Suite, pair-wise analyses between infected and uninfected colonic samples were conducted to identify the most differentially expressed miRNA. Data was submitted to NCBI's GEO database (Accession Number GSE39235).

Functional correlation between the expression of miR-146b and some of its potential targets.

Potential targets for miR-146b and the regulatory pathways that are expected to be regulated were identified in the literature [85]. The list of candidates mRNA targets was retrieved from the MicroCosm Targets database Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm), formely known as mirBase::Targets [79], that uses the miRanda algorithm [86] to identify potential binding sites for a given miRNA in gene sequences. Among these findings, we focused on mRNAs we expected to be involved in the pathogenesis of *C. difficile* by regulating genes at acute stages of the disease. Knowing that miRNA can induce a significant degradation of its target and assuming that evolution progressively selected inverse regulation of expression of mRNAs and their specific miRNAs, we selected nuclear receptor coactivator 4 (NCOA4), a miR-146b target for differential expression testing using qRT-PCR between mice uninfected or mice infected with 10^7 cfu of *C. difficile*. NCOA4 was selected for further analyses since it is a well-known activator of PPARγ.

Quantitative real-time RT-PCR

Total RNA was isolated from mouse colons using a Qiagen RNA Isolation Mini kit according to the manufacturer's instructions. Total RNA (1 µg) was used to generate a cDNA template using an iScript cDNA Synthesis kit (Bio-Rad). The total reaction volume was 20 µL, with the reaction incubated as follows in an MJ MiniCycler: 5 min at 25°C, 30 min at 52°C, 5 min at 85°C, and hold at 4°C. PCR was performed on the cDNA using

Taq DNA polymerase (Invitrogen) under previously described conditions [87]. Each gene amplicon was purified with the MiniElute PCR Purification kit (Qiagen) and quantified both on an agarose gel by using a DNA mass ladder (Promega) and with a nanodrop. These purified amplicons were used to optimize real-time PCR conditions and to generate standard curves in the real-time PCR assay. Primers were designed using Oligo 6 software. Primer concentrations and annealing temperatures were optimized for the iCycler iQ system (Bio-Rad) for each set of primers using the system's gradient protocol. PCR efficiencies were maintained between 92 and 105% and correlation coefficients > 0.98 for each primer set during optimization and also during the real-time PCR of sample DNA. cDNA concentrations for genes of interest were examined by real-time qPCR using an iCycler IQ System and the iQ SYBR green supermix (Bio-Rad). A standard curve was generated for each gene using 10-fold dilutions of purified amplicons starting at 5 pg of cDNA and used later to calculate the starting amount of target cDNA in the unknown samples. SYBR green I is a general double-stranded DNA intercalating dye and may therefore detect nonspecific products and primer/dimers in addition to the amplicon of interest. To determine the number of products synthesized during the real-time PCR, a melting curve analysis was performed on each product. Real-time PCR was used to measure the starting amount of nucleic acid of each unknown sample of cDNA on the same 96-well plate.

Mmu-miR-146b* expression was analyzed with quantitative RT-PCR using TaqMan MicroRNA Assays from Applied Biosystems. Two small nucleolar RNAs, snoRNA202 and snoRNA234, were used as endogenous normalizers for target miR-146b*. A total of 100 ng/sample of RNA was used for cDNA synthesis using the ABI TaqMan MicroRNA Reverse Transcription Kit and the manufacturer protocol. To test for genomic DNA and reaction contamination, two types of negative controls were used for PCR: reverse transcriptase-omitted products and blanks (DEPC-treated water). No amplification was observed in any of the negative controls. TaqMan Universal Master Mix II, the ABI StepOnePlus RT-PCR System and the instrument default cycling conditions were used for PCR. There were 6-8 biological replicates per group, with each RNA sample assayed twice in separate RT reactions. The threshold cycle (C_T) ratios between the target miRNA and the average endogenous control were calculated, arcsin-transformed, and one-way repeated measures ANOVA in R (v. 2.14.0) was used to test differences between treatments and PCR replicates.

Immunophenotyping

Colon samples were processed for lamina propria lymphocyte (LPL) isolation. Specifically, cells (6×10^5 cells/well) were seeded into 96 well-plates, centrifuged at 4°C at 2000 rpm for 3 minutes, and washed with PBS containing 5% serum and 0,09% sodium azide (FACS buffer). Cells were then incubated for T cell assessment with fluorochrome-conjugated primary antibodies to T cell markers. Cells were first incubated with AF700-labeled anti mouse CD45, PECy5-labeled anti mouse CD3 and PECy7-labeled anti mouse CD4. Cells were then fixed and permeabilized with Cytofix-Cytoperm solution (Pharmingen) and incubated with PerCpCy5.5-labeled anti mouse IL-17 and PE-labeled anti mouse RORγt. Cells were resuspended in 0.2 mL of FACS buffer. Data acquisition was computed with a BD LSR II flow cytometer and analysis performed with FACS Diva software (BD Pharmingen).

Model construction

The network model of colonic gene expression changes during *C. difficile* infection was designed based on the experimental data generated in our laboratory and published in this paper. This is a first step toward a more comprehensive understanding of the role that miRNAs and PPARγ play in the host immune response to *C. difficile* infection.

Clostridium difficile*: C. difficile* is the leading cause of diarrhea in the healthcare setting and becoming an increasingly common cause of diarrhea in the community [6]. Differential expression of miRNAs between uninfected and *C. difficile* infected mice was evaluated by miRNA-sequencing. An increase in the expression of miRNA146b was detected among infection. Results were further validated by RT-PCR.

miRNA146b: There is accumulating evidence that miRNAs orchestrate responses to pathogen infections and play a critical role in immunity and inflammation. For example, miRNAs such as miR-146 and miR-155 are involved in the regulation of T- and B-cell development [68], their differentiation and function (reviewed by Sonkoly [69]). Misregulated miR-146 and miR-155 have been associated with various autoimmune diseases including SLE, RA, and MS. However, the role of miRNAs in CDAD remains poorly understood.

NCOA4: Potential targets for miR-146b were identified in the literature [85]. Knowing that miRNA can induce a significant degradation of its target and assuming that

evolution progressively selected inverse regulation of expression of mRNAs and their specific miRNAs, we selected one miR-146b target, Nuclear Receptor Coactivator 4 (NCOA4), for differential expression testing using qRT-PCR between mice uninfected or mice infected with 10^7 cfu of *C. difficile*. Results showed how upregulation of miRNA146b within infection correlates to decreased expression of its target NCOA4. Interestingly, NCOA4 is a PPAR γ co-activator [88].

PPAR γ: PPAR γ is a nuclear receptor and transcription factor involved in lipid metabolism and glucose homeostasis [89]. Moreover, it antagonizes several pro-inflammatory pathways such as STAT, AP-1 and NF-κB. It is also a critical regulator of CD4+ T cell differentiation since it promotes a regulatory T cell phenotype by activating FoxP3 while it blocks effector phenotypes, such as inhibition of RORγ in Th17 or the inhibition of IFNγ in Th1 [62]. In macrophages, PPAR γ favors a switch from a pro-inflammatory "classically activated" M1 to an M2 "alternatively activated" anti-inflammatory phenotype [90]. Therefore, activation of PPARγ represents a conserved anti-inflammatory mechanism involved in the prevention of cancer, diabetes [91-93], atherosclerosis[94], obesity [95], infectious [96-100] and immune-mediated diseases [53, 55, 87, 101]. The first evidence of the involvement of PPARγ in the regulation of intestinal inflammation came from the use of the PPARγ synthetic agonists thiazolidinediones (TZD) in mice with colitis induced by oral administration of dextran sodium sulfate (DSS) [102]. At the time, there are several publications reporting prophylactic and therapeutic effects of PPARγ in different strains of mice, rats and pigs with acute colitis induced by chemical compounds [102, 103], bacteria [87], and also chronic colitis occurring after the transfer of immunocompetent T cells in SCID mice [53] or spontaneously in IL-10 deficient mice [104]. PPARγ is mainly expressed in the colon by epithelial cells and lamina propria mononuclear cells such as macrophages and T and B cells. Although some studies show the importance of PPARγ in each cell type, additional investigations in animals with cell type specific expression of PPARγ are required to determine the main cellular source responsible for the therapeutic effect of PPARγ [105].

IL-10: Interleukin 10 is a cytokine with broad anti-inflammatory properties. It is released by M2 macrophages as well as T helper type 2 (Th2) and regulatory T cells (Treg). It acts as an immune response mediator by suppressing Th1 differentiation and blocking the activation of cytokine synthesis. In addition, IL-10 contributes to the regulation of

proliferation and differentiation of B cells, mast cells and thymocytes. The activation of PPAR γ induces the expression of IL-10 by these cell types [106].

IL-17: Interleukin 17 is a cytokine secreted by activated Th17 cells responsible for inducing and regulating proinflammatory responses by recruiting monocytes and neutrophils to the site of inflammation in response to the invasion of the immune system by extracellular pathogens [107-109]. PPARγ is a known inhibitor of Th17 differentiation as its activation can inhibit STAT-3 and hence contribute to the down-regulation of IL-17 through the IL-6/STAT-3/RORγt/IL-17 axis [110, 111]. Although TGF-β alone is not capable of inducing IL-17 and hence producing Th17, it is necessary for differentiation into Th17 and its absence induced a shift from a Th17 profile to a Th1-like profile [112, 113]. Moreover, PPARγ is a key negative regulator of human and mouse Th17 differentiation since it reduced RORγt transcription on a single-cell level [62].

In silico simulations of the involvement of miRNA-146b and PPARγ in modulating colonic host responses to C. difficile infection

Based on the results obtained in the mouse challenge studies, an ordinary differential equation-based computational model was developed describing the molecular dynamics of some key cytokines and transcription factors involved in *C. difficile* infection. Although modeling approaches cannot replace traditional experimentation, the construction of such computational model synthesized, organized and integrated all the concepts and mechanisms studied, facilitating a more systematic hypothesis-generation process. Overall, our modeling process involved: 1) creation of a structural network using Cell Designer; 2) parameter estimation based on published or newly generated experimental data using Complex Pathway Simulator (COPASI); and 3) *in silico* experimentation. We constructed a network model with five dynamic variables representing miR-146b, NCOA4, PPARγ, interleukin 17 (IL-17) and IL-10, plus an external input: the infectious dose of *C. difficile*. The network model was constructed by using CellDesigner [114], a software package that enables users to describe molecular interactions using a well-defined and consistent graphical notation. Modeling was performed using COPASI (http://www.modelingimmunity.org/) [115]. Both COPASI and CellDesigner are Systems Biology Markup Language (SBML) compliant, thus, machine and human readable. The CellDesigner-generated network was imported into COPASI where rate laws were adjusted to create the ordinary differential equations (Figure 2.13). The results of the parameter estimation using Particle Swarm showed a good fitting between the

experimental data and predicted values computationally estimated by COPASI (Table 2.1). These values were then implemented in the reactions and rate laws to adjust the dynamics of the model. COPASI was then used to run sensitivity analysis and *in silico* time-course studies.

$$\frac{d(miR146)}{dt} = [pmiR146] \cdot \left(c_{0(miR)} + c_{1(miR)} \cdot \frac{[Cdiff]^n}{[Cdiff]^n + k_{(miR)}{}^n} \right) - vr_{(miR)} \cdot [miR146]$$

$$\frac{d(NCOA4)}{dt} = [pNCOA4] \cdot \left(c_{0(NCOA4)} + c_{1(NCOA4)} \cdot \frac{k^{n(NCOA4)}}{[miR146]^{n(NCOA4)} + k^{n(NCOA4)}} \right) - vr_{(NCOA4)} \cdot [NCOA4]$$

$$\frac{d(PPARg)}{dt} = [pPPARg] \cdot \left(c_{0(PPARg)} + c_{1(PPARg)} \cdot \frac{[NCOA4]^n}{[NCOA4]^n + k_{(PPARg)}{}^n} \right) - vr_{(PPARg)} \cdot [PPARg]$$

$$\frac{d(IL-10)}{dt} = vf_{(IL-10)} \cdot [pIL-10] \cdot \frac{k^{n(IL-10)}}{[Cdiff]^n + k^{n(IL-10)}} \cdot \frac{[PPARg]^{n1(IL-10)}}{[PPARg]^{n1(IL-10)} + k1^{n1(IL-10)}} - vr_{(IL-10)} \cdot [IL-10]$$

$$\frac{d(IL-17)}{dt} = vf_{(IL-17)} \cdot [pIL-17] \cdot \frac{k^{n(IL-17)}}{[PPARg]^n + k^{n(IL-17)}} \cdot \frac{[Cdiff]^{n1(IL-17)}}{[Cdiff]^{n1(IL-17)} + k1^{n1(IL-17)}} - vr_{(IL-17)} \cdot [IL-17]$$

$$NCOA4_{ratio} = \frac{[NCOA4]}{Values[BActin].InitialValue}$$

$$PPARg_{ratio} = \frac{[PPARg]}{Values[BActin].InitialValue}$$

$$IL-10_{ratio} = \frac{[IL-10]}{Values[BActin].InitialValue}$$

$$IL-17_{ratio} = \frac{[IL-17]}{Values[BActin].InitialValue}$$

Figure 2.13. Equations controlling dynamics of the *Clostridium difficile* infection model. Ordinary Differential Equations (ODE) triggering activation and inhibition of the different reactions in the model. Briefly, mass action and Hill functions were used to reproduce reaction behaviors *in silico* based on initial molecule concentrations.

Fitted Value	Objective Value	Root Mean Square	Error Mean	Error Mean Std. Deviation
miR-146b	1.41E-23	2.65E-12	6.73E-13	2.56E-12
NCOA4_ratio	1.19E-19	2.44E-10	-2.42E-10	2.96E-11
PPARγ_ratio	2.95E-20	1.21E-10	-1.17E-10	3.37E-11

IL10_ratio	7.05E-11	5.94E-06	-6.36E-08	5.94E-06
IL17_ratio	0.431748	0.464623	0.328534	0.328542

Table 2.1. Model fitting performed by using COPASI's global parameter estimation. A species is fitted computationally using experimental data and simulation algorithms. The objective value is the value that the modeling software targets based on the experimental data and the computational simulation.

Statistical analyses

We performed Analysis of Variance (ANOVA) to determine the significance of the model by using the general linear model procedure of SAS (SAS Institute) as previously described [53]. Specifically, we examined the main effect of genotype, treatment, and their interaction when necessary. Differences of $P < 0.05$ were considered significant. Data were expressed as the means ± SE of the mean.

Chapter 3

CD64+CX_3CR1+ Mononuclear Phagocytes Facilitate Bacterial Colonization and Promote Regulatory Responses during *Helicobacter pylori* Infection

Monica Viladomiu, Barbara Kronstreiner, Josep Bassaganya-Riera, Casandra W. Philipson, Andrew Leber, Adria Carbo, and Raquel Hontecillas

3.1 Summary

Helicobacter pylori is the dominant member of the human gastric microbiota and has been associated with increased risk of gastritis, peptic ulcer and gastric cancer in adults. Interestingly, persistent colonization might be associated with protection against allergies, diabetes, asthma and chronic inflammatory autoimmune diseases. The immunoregulatory mechanisms of chronic microbial persistence implicated in protective versus pathogenic outcomes are incompletely understood. Computational modeling of mucosal immunity predicted that macrophages are central regulators of chronic microbial persistence and immunoregulation during *H. pylori* infection. To validate this computational hypothesis we shifted the macrophage phenotype into a proinflammatory state characterized by lower IFN-γ and iNOS expression by deleting PPARγ in myeloid cells and found a 10-fold decrease in gastric bacterial loads at the expense of severe gastric pathology, suggesting that mucosal macrophages afford *H. pylori*

colonization persistence and regulate gastric pathology. Increased *H. pylori* colonization in wild-type (WT) mice was associated with the infiltration of CD11b+F4/80hiCD64+CX_3CR1+ macrophages into the gastric lamina propria (LP), a cell population that PPARγ-deficient mice failed to expand and maintain. Furthermore, macrophage depletion studies resulted in a dramatic reduction of gastric *H. pylori* colonization in WT mice similar to the levels observed in myeloid-specific PPARγ-deficient mice. Moreover, both PPARγ-deficient and macrophage-depleted mice presented decreased IL-10-mediated regulatory myeloid and T cell responses in the gastric mucosa early post-infection. In summary, *H. pylori* infection manipulates the gastric microenvironment through immunoregulatory mechanisms mediated by infiltration of CD64+CX_3CR1+ macrophages and induction of regulatory T cells and macrophage responses characterized by the production of IL-10 that afford chronic persistence of this gastric indigenous microbe.

3.2 Introduction

Helicobacter pylori is a microaerophilic, Gram-negative, spiral-shaped bacterium that selectively establishes lifelong colonization of the gastric mucosa in over 50% of the human population [35, 36]. Approximately 10-15% of *H. pylori*-infected individuals eventually develop peptic or duodenal ulcers. Moreover, *H. pylori* carriers have more than two-fold greater risk for developing gastric cancer in the form of B-cell lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) or adenocarcinoma [39, 40]. Overall, gastric cancers are the third leading cause of cancer-related deaths worldwide, and *H. pylori*-related cancers account for 5.5% of all human cancers [116].

H. pylori colonization of the gastric mucosa leads to a mix of effector and regulatory responses. Most carriers develop chronic superficial gastritis characterized by the infiltration of neutrophils, dendritic cells (DCs), macrophages, natural killer (NK) cells, lymphocytes, and plasmocytes [117]. However, the bacteria persist chronically, suggesting that the regulatory response predominate over effector mechanisms [118-126]. In this regard, the role of *H. pylori* as a beneficial member of the human gastric microbiome has emerged more recently and is supported by epidemiological and experimental data showing that *H. pylori* protects from esophageal cancer, asthma, obesity-induced insulin resistance and IBD [42-47]. This dual role of *H. pylori* as commensal or pathogen denotes a very complex interaction with its host.

Our computational models of the immune response to *H. pylori* predicted that macrophages are central regulators of chronic microbial persistence and immunoregulation [127-129]. Previous work by others has demonstrated that loss of key macrophage genes like caveolin, MMP9 or hemoxygenase results in lower rates of colonization and accentuates pathology [130-132]. It has also been proposed that insufficient bacterial clearance by phagocytic cells contributes to poor eradication. Although phagocytosis is considered the most efficient means of eliminating invading microorganisms [133, 134], *H. pylori* has developed mechanisms to evade the attack by phagocytic cells and cause chronic inflammation [135, 136]. Macrophages belong to the mononuclear phagocytic system, which comprises a heterogeneous class of cells that perform functions ranging from tissue development, remodeling and repair, to pathogen recognition and inflammation. Functional separation based on phenotypic traits and establishment of a clear division of labor has been challenging because these cells arise from common progenitors and undergo radical reprogramming in the presence of danger signals. By performing a detailed analysis of MNP in the stomach of *H. pylori*-infected mice, we have identified a subset of F4/80hi CD11b+CD64+CX_3CR1+ MPS that starts accumulating in the gastric lamina propria between days 21 and 24 post-infection. Surprisingly, instead of mediating a pro-inflammatory response, these MPS coordinate a regulatory response mediated through IL-10 that facilitates colonization. With this work, we have identified a novel cellular system that provides new insights in the mechanisms of immunoregulation underlying protective versus pathogenic behaviour of *H. pylori.*

3.3 Loss of PPARγ in myeloid cells results in low colonization phenotype and alterations in the macrophage compartment

In order to assess the impact that macrophages have on the outcome of *H. pylori* infection, we used a tissue specific Peroxisome Proliferator-Activated Receptor γ (PPAR γ) KO mouse with deletion targeted to myeloid cells (PPAR γ fl/fl; Lys-cre). These mice have been used extensively in the past [53, 80, 81] and the deletion has been shown to affect mainly macrophages and partially dendritic cells [137]. While we did not aim to elucidate the role of PPARγ per se during *H. pylori* infection we chose PPAR γ because it regulates pro-inflammatory cytokine expression without directly affecting macrophage differentiation [137]. Wild Type, PPARγ fl/fl;CD4-cre (CD4cre) and PPARγ fl/fl;LysM-cre (LysMcre) mice, all in C57B/6J background, were infected with *H. pylori* SS1 strain and

bacterial loads were obtained weekly for 6 months. The 3 mouse strains were colonized to similar levels based on reisolation data from weeks 1 and 2 post-infection. However, between weeks 2 and 3, there was an abrupt drop in bacterial burden in LysMcre mice that led to a significant 5 to 10- fold lower colonization levels when compared to WT and CD4-cre mice that were sustained overtime. (Figure 3.1A). Similar results were observed when infecting mice with H. pylori strain PMSS1 (Figure 3.3). Based on these findings, our initial hypothesis to explain the phenotypic differences was that the loss of PPAR□ in macrophages leads to a pro-inflammatory cellular state that, that directly or indirectly, would more efficiently eliminate bacteria from the gastric mucosa. We then performed a detailed analysis of myeloid cells infiltrating the gastric lamina propria using broad selection of markers of mononuclear phagocytes, including CD11b, CD11c, MHC-II, CX_3CR1, F4/80, Cd103 and CD64, that would allow a fine dissection of the myeloid compartment. In this follow-up experiment, we focused on time-points around weeks 2 to 3 post-infection since the drop in *H. pylori* loads in LysMcre mice consistently occurs during this time frame. Flow cytometry results revealed increased numbers of CD11b+F4/80^{lo} and CD11b+F4/80^{hi} populations in WT mice (Figures 3.1B and 3.1C). Particularly, a subset of CD45+F4/80^{hi}CD11b+CD64+ cells started accumulating in the stomach mucosa at day 12 post-infection in the WT but not in the LysMcre mice (Figure 3.1D). Differences between the 2 mouse strains regarding such cell population were maintained at least until day 112 post-infection (week 16 post-infection). Moreover, further phenotypic characterization of this subset of macrophage-like (CD11b+F4/80^{hi}CD64+) mononuclear phagocytes (MNPs) confirmed the expression of CX_3CR1 and high levels of MHC-II (Figure 3.1E). Therefore, high levels of bacterial gastric colonization correlated with infiltration of CD45+F4/80^{hi}CD11b+CD64+ macrophages in WT mice. No differences in regards to dendritic cells (F4/80-CD11c+MHCII^{hi}) or neutrophils (CD11b+ Gr1^{hi}) were seen (Figure 3.2).

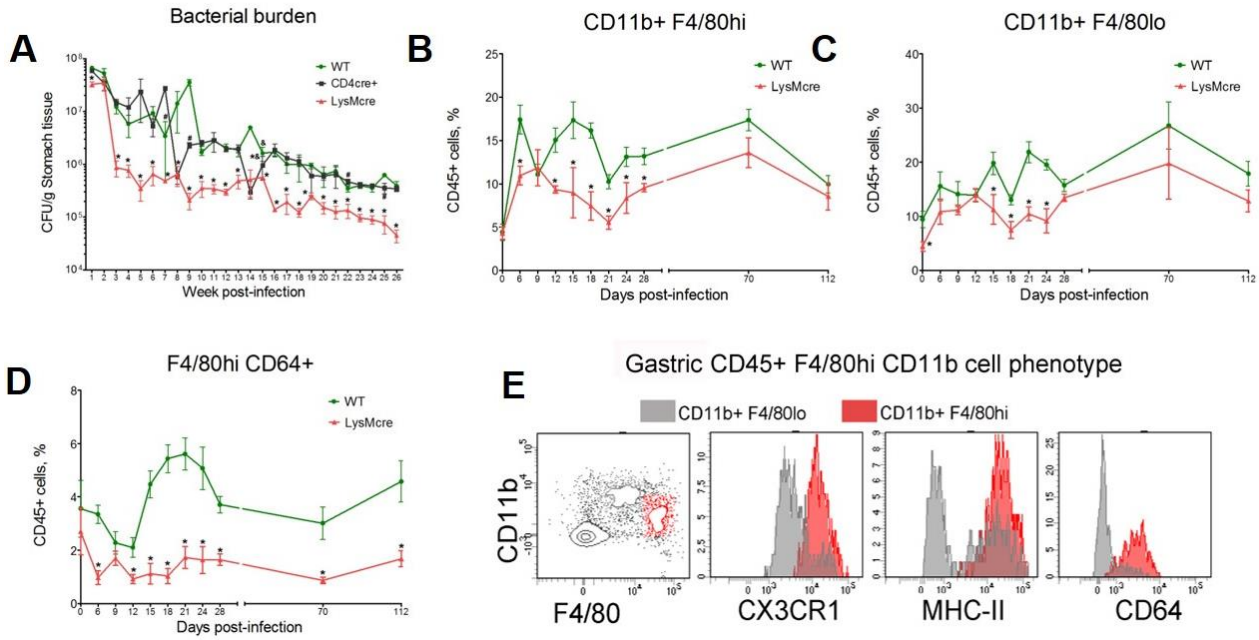

Figure 3.1. High bacterial loads correlate with the expansion of CD11b+ F4/80hi CD64+ CX_3CR1+ macrophages. WT, PPARγ-myeloid cell deficient (LysMcre) or PPARγ-T cell deficient (CD4cre) mice were infected with *H. pylori* SS1. Bacterial burden measured weekly up to 6 months post-infection show a significant reduction due to the loss of PPARγ in myeloid cells (A). Time-course FACS analysis of gastric CD11b+F4/80^{hi} (B), CD11b+F4/80^{lo} (C) and CD11b+F4/80^{hi}CD64+ (D) revealed the expansion of such cell subsets in the stomach of WT mice but not in LysMcre (B). Further phenotypic characterization of infiltrating F4/80hi CD11b+ cells shows expression of CD103, MHC-II+, and CX_3CR1 (E). Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

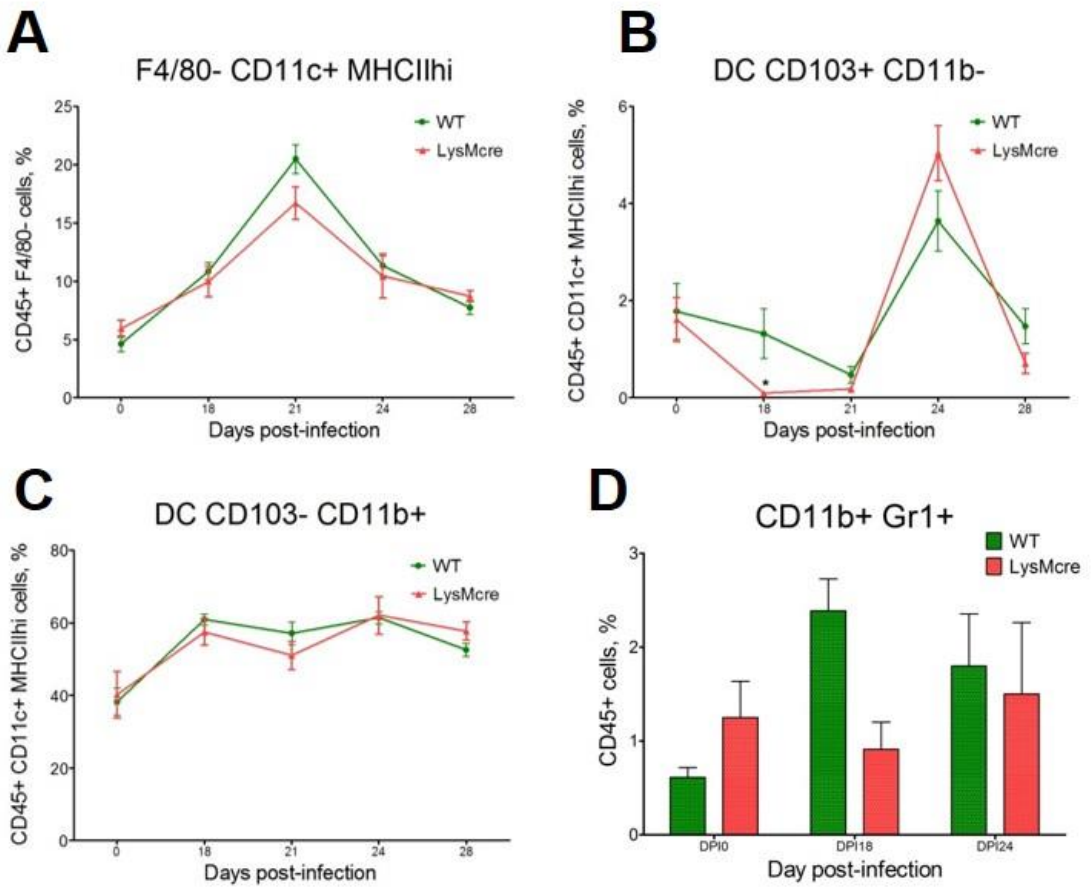

Figure 3.2. Effect of *H. pylori* infection on gastric dendritic cell and neutrophil populations. WT, PPARγ-myeloid cell deficient (LysMcre) or PPARγ-T cell deficient (CD4cre) mice were infected with *H. pylori* SS1. Time-course FACS analysis of gastric lamina propria cells revealed no differences in regards to overall CD11c+MHCII^{hi} dendritic cells (A), CD103+CD11b- DC subset (B), CD103-CD11b+ DC subset (C) and CD11b+Gr1^{hi} neutrophils (D). Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

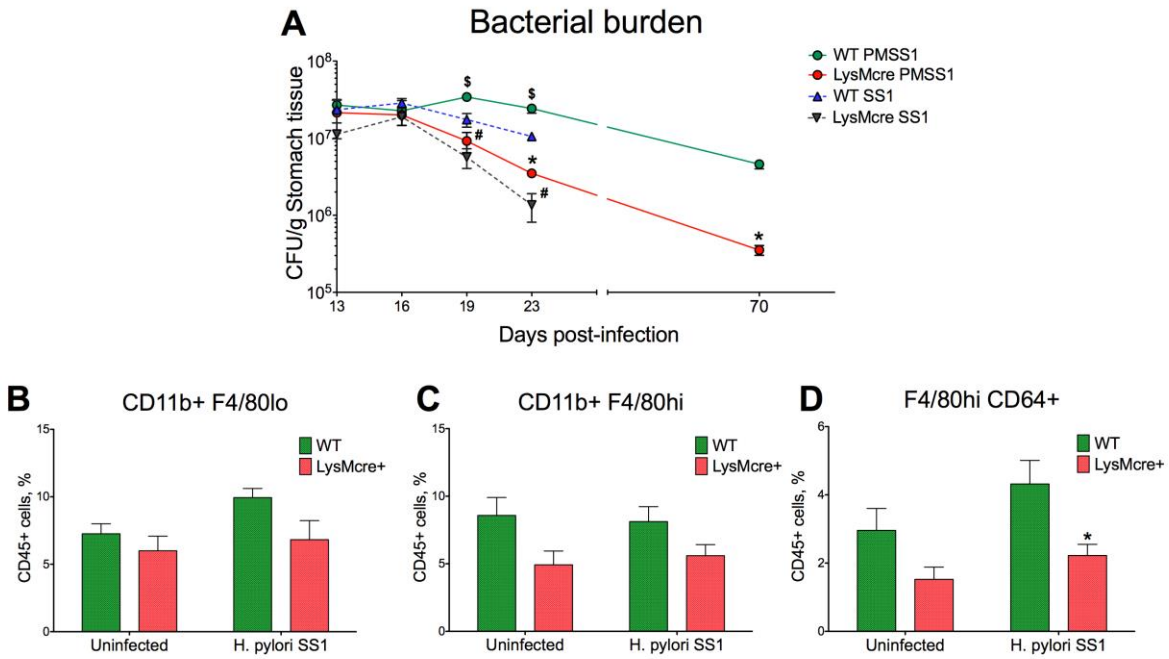

Figure 3.3. Comparison of *Helicobacter pylori* SS1 and PMSS1 strains. WT and PPARγ-myeloid cell deficient (LysMcre) mice were infected with *H. pylori* strain SS1 or PMSS1. Bacterial burden measured at days 13, 16, 19, 23 and 70 post-infection show a significant reduction due to the loss of PPARγ in myeloid cells regardless of the strain used (A). FACS analysis of gastric CD11b+F4/80^{lo} (B), CD11b+F4/80^{hi} (C) and CD11b+F4/80^{hi}CD64+ (D) from uninfected and PMSS1-infected mice revealed increased levels of these cell subsets in the stomach of WT mice but not in LysMcre, similarly to the results obtained after infection with *H. pylori* SS1. Data represents mean ± SEM. Points with different superscripts are significantly different when compared to the control group (P<0.05).

3.4 Macrophage depletion decreases *Helicobacter pylori* levels in the gastric mucosa

In order to determine if CD11b+F4/80^{hi}CD64+ macrophages affect high colonization of WT mice we performed a macrophage depletion study using clodronate liposomes. WT mice were infected with *H. pylori* SS1 and treated with either PBS liposomes (negative control) or clodronate-containing liposomes. A group of non-treated, SS1 infected LysMcre mice was included for comparison. Clodronate or PBS liposomes were administered 4 days before macrophages started to accumulate in the stomach, based on the initial kinetic results (Figure 3.1D**)**. To maintain macrophage depletion, liposomes were administered every 3 days and changes in MNPs populations in the stomach were assessed 24h after each injection (Figure 3.4A). The results showed that clodronate

treatment specifically depleted CD11b+F4/80hiCD64+ macrophages to levels observed in LysMcre mice (Figure 3.4B, 3.4C and 3.E). Interestingly, the most efficient levels of depletion were obtained on day 18 post-infection, after two liposome injections. Numbers of macrophages started to recover quickly which was associated with a significant increase in F4/80loCD11b+ between days 18 and 21 post-infection (Figure 3.4D). These data suggest that the F4/80loCD11b+ contain the precursors of F4/80^{hi}CD11b+CD64+ MNPs.

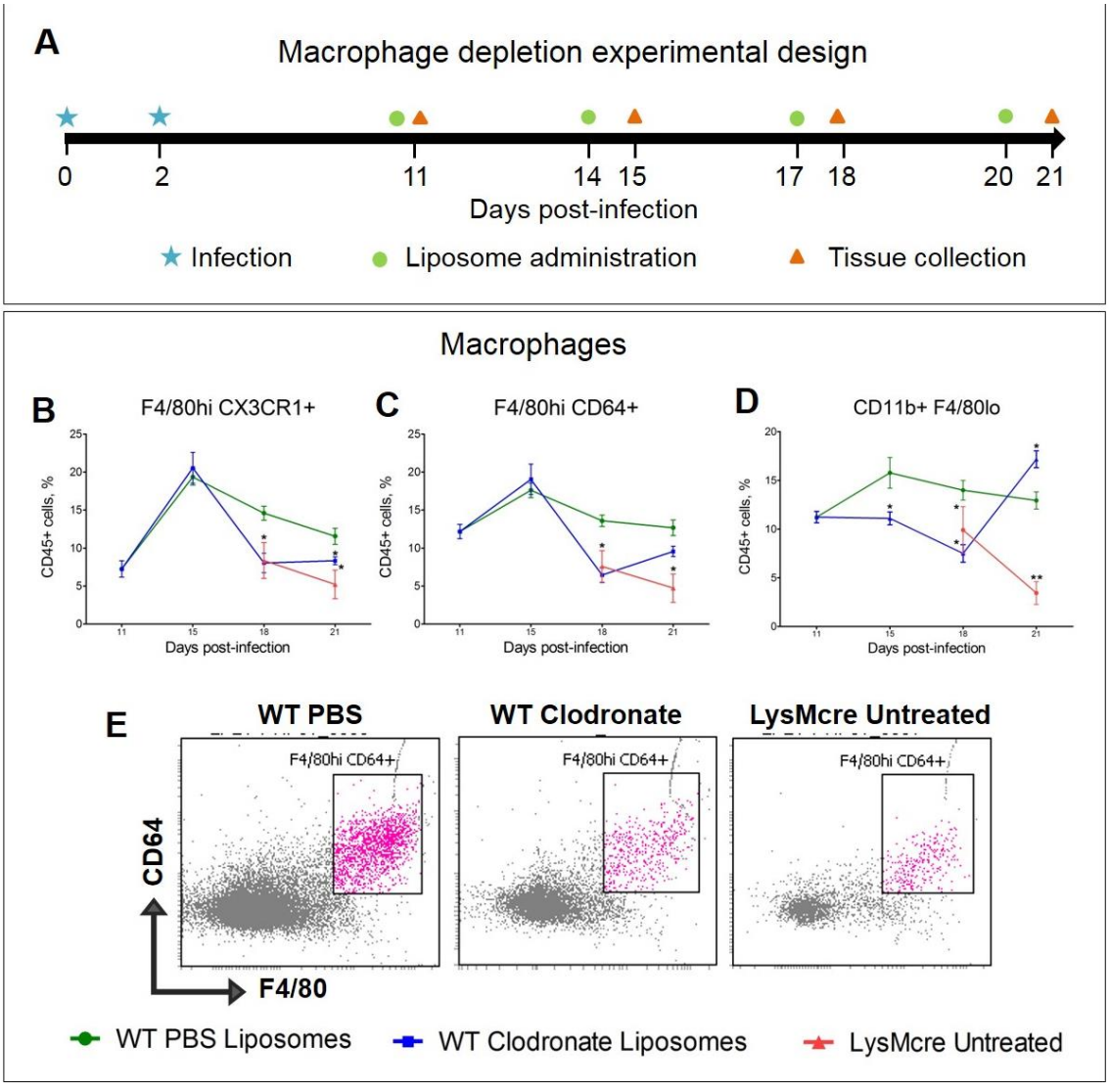

Figure 3.4. Effect of macrophage depletion in gastric macrophage populations. For the macrophage depletion study, WT mice were infected with SS1 and received three doses of either clodronate liposomes or PBS liposomes on days 11, 14, 17, and 20 post-infection. Analyses were performed prior to the first injection, day 11, or one day after each injection. SS1-infected, non-treated LysMcre mice were used for reference (A). Clodronate treatment caused depletion of

F4/80hi CD11b+CX_3CR1+ (B) and F4/80hi CD11b+CD64+ (C,D) cells from the stomach of WT mice. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (*P*<0.05).

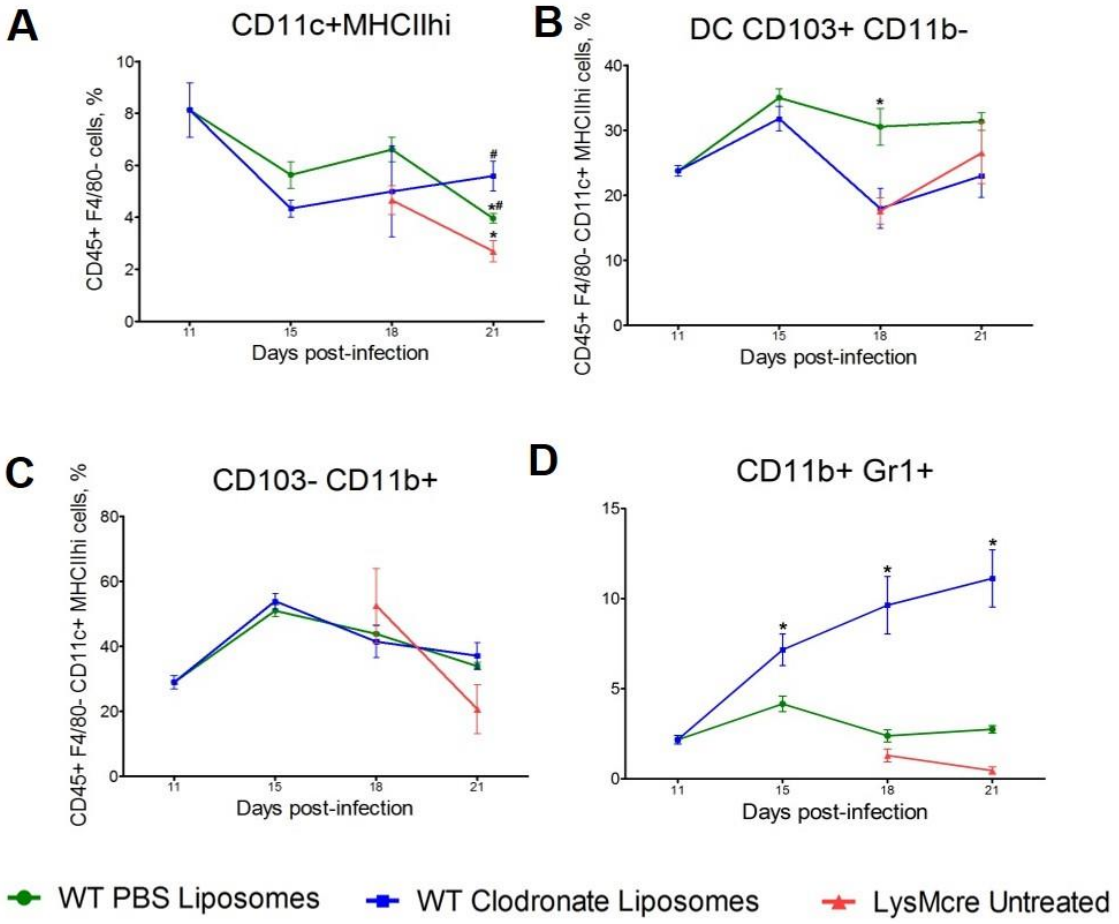

Figure 3.5. Effect of macrophage depletion in gastric dendritic cell and neutrophil populations. For the macrophage depletion study, WT mice were infected with SS1 and received three doses of either clodronate liposomes or PBS liposomes on days 11, 14, 17, and 20 post-infection. Analyses were performed prior to the first injection, day 11, or one day after each injection. Slight or no differences were seen in regards to overall CD11c+MHCII^{hi} dendritic cells (A), CD103+CD11b- DC subset (B), CD103-CD11b+ DC subset (C), whereas CD11b+Gr1^{hi} neutrophils (D) were significantly increased following treatment. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (*P*<0.05).

In contrast, clodronate treatment had no effect on the levels of dendritic cell populations (Figures 3.5A-C). More importantly, clodronate-induced depletion of F4/80^{hi}CD11b+CD64+ MNPs resulted in a decrease in *H. pylori* loads to levels of LysMcre mice (Figures 3.6). Therefore, these results demonstrate that high levels of colonization are achieved on the presence of CD11b+F4/80^{hi}CD64+ MNPS in the gastric

mucosa pointing at these cells as facilitators of infection. Our data so far indicate that the presence or absence of these F4/80^{hi}CD11b+CD64+ MNPs, is more important than whether they have a pro-inflammatory phenotype, as we initially hypothesized in the case of LysM-cre mice, in the level of colonization. A potential explanation is that F4/80^{hi}CD11b+CD64+ promote a regulatory environment at the gastric mucosa that facilitates colonization by *H. pylori*. To assess whether high colonization might be achieved by induction of regulatory responses, we assessed the presence of cells with regulatory phenotypes. While we did not detect any significant increase in the Treg population (CD4+FoxP3+), flow cytometry results revealed an increase in IL-10 producing CD3+CD4+ T cells (Figure 3.7A), CD3+CD4- cells (Figure 3.7B) and CD11b+ cells (Figure 3.7C) in the gastric mucosa of WT mice early post-infection, whereas LysMcre mice presented significantly low levels of IL-10 secreting populations. Interestingly, clodronate-induced depletion of CD11b+F4/80^{hi}CD64+ macrophages correlated with decreased numbers of IL-10 producing cells and a significant increased recruitment of neutrophils (CD11b+Gr1^{hi}) to the stomach mucosa (Figure 3.5D). Overall these results suggest that the production of IL-10 might contribute to high colonization and that CD11b+F4/80^{hi}CD64+ macrophages have a regulatory/suppressor role during infection.

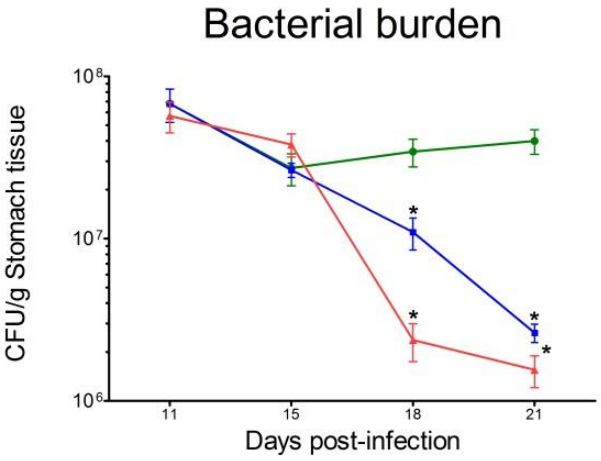

Figure 3.6. Effect of macrophage depletion in gastric *H. pylori* loads. For the macrophage depletion study, WT mice were infected with SS1 and received three doses of either clodronate liposomes or PBS liposomes on days 11, 14, 17, and 20 post-infection. Macrophage depletion suppressed bacterial loads to levels of untreated LysMcre mice. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

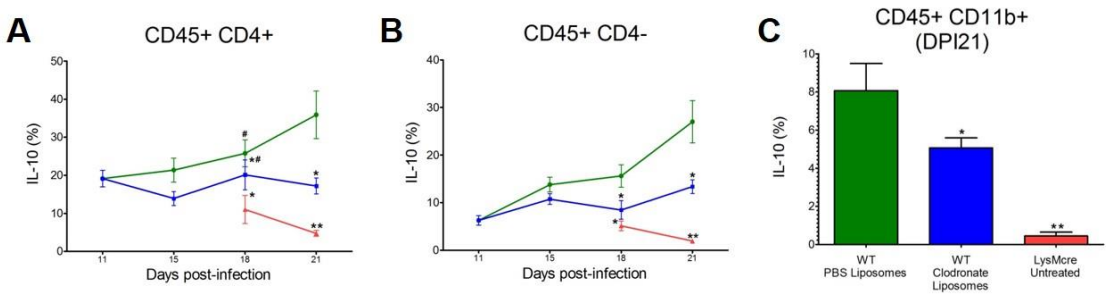

Figure 3.7. Macrophage depletion suppresses IL-10-mediated regulatory responses in the stomach of WT mice. For the macrophage depletion study, WT mice were infected with SS1 and received three doses of either clodronate liposomes or PBS liposomes on days 11, 14, 17, and 20 post-infection. Analyses were performed prior to the first injection, day 11, or one day after each injection. SS1-infected, non-treated LysMcre mice were used for reference. Clodronate treatment diminished IL-10 secreting CD4+ T cells (A), CD4- cells (B) and CD11b+ cells (C) at day 21 post-infection. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

3.5 Phenotypic characterization of IL-10-producing cells during *Helicobacter pylori* infection

WT and LysMcre mice were infected with *H. pylori* SS1 strain and stomachs were collected at days 18 and 24 post-infection to better characterize the source of *H. pylori*-induced IL-10 responses. Consistent with previous infections, *H. pylori* increased the numbers of F4/80^{lo} CD11b+ and F4/80^{hi} CD11b+ populations as well as the F4/80^{hi} CD11b+ CD64+ subset in WT mice (not shown). Interestingly, IL-10 production by all these myeloid cell subsets was significantly increased in WT mice (Figures 3.8A-B). In addition, IL-10 seemed to be constitutively expressed, since it was detected in cells from non-infected mice and infection did not significantly augment the fraction of cells producing this cytokine in WT mice, although IL-10 was suppressed by infection in myeloid cells form LysMcre mice. A plausible scenario is that F4/80^{hi}CD11b+CD64+ MNPs, as source of IL-10, promote T cells with regulatory phenotype in the gastric mucosa. We then assessed changes in IL-10 producing cells, other than F4/80^{hi}CD11b+CD64+. LysMcre mice showed higher levels of both CD4+ T cells (Figure 3.8D) and CD19+ B cells (Figure 3.8F). In both cell types, IL-10 production was significantly induced on day 24 post-infection only in WT mice (Figures 3.8E and 3.8G). Further phenotypic characterization of CD4+ T cells revealed increased Tr1 (CD4+PD1+) and Treg (CD4+FoxP3+) levels due to infection (Figures 3.8H and 3.8J).

Although there was a significant increase in CD4+FoxP3+ Treg cells in WT mice on day 24 post-infection, *H. pylori* induced IL-10 expression was produced by cells from both genotypes to almost same levels. However, only PD-1 cells of WT mice presented upregulated IL-10 production following infection. (Figures 3.8I and 3.8K). Hence, these data further suggest a regulatory role for CD11b+F4/80^{hi}CD64+ macrophages during infection, which are express high levels of IL-10 and induce Tr1 and iTreg regulatory responses.

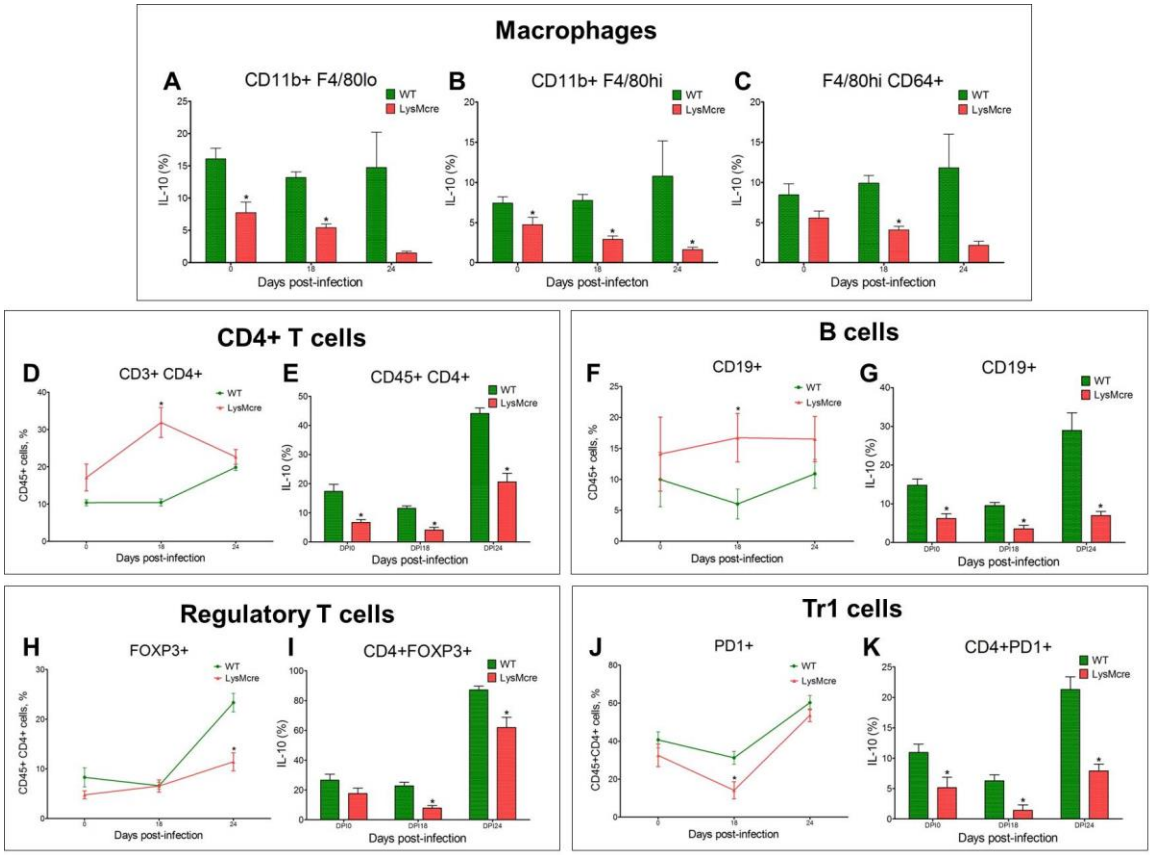

Figure 3.8. Phenotypic characterization of IL-10-producing cells during *Helicobacter pylori* infection. WT and LysMcre mice were infected with *H. pylori* SS1 strain and stomachs were collected at days 18 and 24 post-infection to better characterize the source of *H. pylori*-induced IL-10 responses. FACS analysis of gastric IL-10-producing CD11b+F4/80^{lo} cells (A), IL-10-producing CD11b+F4/80^{hi} cells (B) and IL-10-producing CD11b+F4/80^{hi}CD64+ cells (C), CD4+ T cells (D), CD4+IL10+ T cells (E), Tr1 cells (F), IL-10-producing Tr1 cells (G), iTreg cells (H), IL-10-producing iTreg cells (I), CD19+ B cells (J), and CD19+IL10+ B cells (K). Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

3.6 Discussion and Conclusions

H. pylori is has colonized the stomach since early human evolution, diverged with prehistoric human migrations [138-141], and co-evolved with its human host for over 60,000 years [35, 36]. Infection with this microorganism can lead to different outcomes ranging from asymptomatic histologic gastritis to invasive adenocarcinoma [39, 40]. *H. pylori*-infected individuals generate robust systemic and mucosal inflammatory responses but fail to eradicate bacterial colonization [117, 142]. *H. pylori* chronic persistence was initially attributed to insufficient eradication by phagocytic cells [143]. Functionally, MNPs have been investigated as potential promoters of inflammation that contribute to the induction of mucosal damage and *H. pylori*-related gastric malignancies [39, 40]. However, emerging evidence suggests that the host's failure to clear the bacterium could be associated to *H. pylori*'s ability to induce regulatory responses against T helper cell-mediated effector immunity [118-126]. Previously, it has been proposed that the regulatory effects of *H. pylori* are induced through dendritic cells [124, 125, 144, 145]. However, the role of macrophage subsets in the induction of these responses has not been confirmed.

MNPs are critical immunomodulators of the host response to *H. pylori* [124, 146-148]. *H. pylori*-containing DCs were initially observed in gastric tissue of *H. pylori*-associated MALT lymphoma patients in 1998 [146]. Biopsies of *H. pylori*-infected subjects have identified DCs among epithelial cells and extending into the lumen to contact *H. pylori* located within the gastric glands [147]. Moreover, *H. pylori* has recently been found in the lamina propria associated with CD68+ myeloid cells in humans [149]. Despite the above observations, the mechanisms by which MNPs induce effector versus regulatory responses remain incompletely understood. We developed a computational and mathematical model of effector and regulatory mucosal immune responses to *H. pylori* [127, 128] and through sensitivity analyses predicted that macrophages are critical regulators of *H. pylori* colonization and gastric pathology. Interestingly, *in silico* shifting of macrophage phenotype into pro-inflammatory states resulted in decreased gastric microbial colonization at the expense of enhanced pathology. To validate such computational hypotheses, we run several experimental infections in WT and myeloid-specific PPAR□ null mice to better characterize how macrophage phenotypes shape the outcome of infection, mucosal immune response, and persistence. PPARγ-deficient macrophages present altered control over PAMP-activated transcription factors such as

NFκB, which results in a pro-inflammatory phenotype. Sustained *H. pylori* levels in the gastric mucosa of WT mice were associated with an expansion of a CD11b+F4/80hiCD64+CX_3CR1+ cell subset, while LysMcre mice failed to expand and maintain such population. Based on such immunophenotyping results, we then hypothesized that CD11b+F4/80hiCD64+CX_3CR1+ macrophages are recruited to the gastric mucosa and contribute to the establishment of a fraction of *H. pylori*. However, the mechanisms by which they afford chronic colonization and persistence were not known. We contemplated two potential scenarios: In the first setting, *H. pylori* is phagocytized by infiltrating CD11b+F4/80hiCD64+CX_3CR1+ macrophages, which serve as replication niche and loophole for bacteria to increase colonization and persistence. This hypothesis is based on the demonstration that *H. pylori* can form megasomes and delay killing through manipulation of phagosome maturation pathways [136]. In the second scenario, infiltrating CD11b+F4/80hiCD64+CX_3CR1+ macrophages promote a regulatory environment in the gastric mucosa which indirectly suppresses effector mechanisms required to eliminate *H. pylori*, contributing in this way to baterial colonization. In order to further investigate both hypotheses we run several modeling and *in vivo* challenge studies to compare bacterial loads and induction of regulatory responses during *H. pylori* infection with and without the presence of macrophages in the gastric mucosa. Macrophage depletion in WT mice following *H. pylori* infection reduced the numbers of CD11b+F4/80hiCD64+ macrophages as well as *H. pylori* loads to levels observed in LysMcre mice. Moreover, macrophage depletion was associated with a decreased IL-10 production by CD3+CD4+ T cells, CD3+CD4- cells and CD11b+ cells as well as increased recruitment of neutrophils (CD11b+Gr1hi) in the gastric mucosa of WT mice early post-infection. Similar results have been previously reported by Oertli et al., who observed a significant reduction in bacterial loads in the stomachs of CD11c+ MNPs-depleted mice when compared with WT mice [150]. In this case, reduced *H. pylori* loads were associated with greater gastritis and IFNγ production by gastric T cells, thus providing evidence that CD11c+ MNPs are key suppressors of the host effector immune response during *H. pylori* infection. Taken together, these data indicate that CD11b+F4/80hiCD64+ macrophages contribute to high levels of colonization by inducing IL-10-mediated regulatory responses and creating a tolerant environment that favors *H. pylori* colonization and persistence. Indeed, follow-up infections revealed that IL-10 production by F4/80^{lo} CD11b+, F4/80^{hi} CD11b+ and F4/80^{hi} CD11b+ CD64+ cell populations is significantly increased in WT mice. Notably, IL-10 seems to be

constitutively expressed and its secretion is not augmented in WT mice following infection. However, similarly to the results observed in the macrophage depletion study, *H. pylori* suppressed IL-10 production in myeloid cells from LysMcre mice. Interestingly, WT mice presented increased IL-10 production by gastric CD19+ B cells and CD4+ T cells upon infection, particularly from PD-1+ Tr1 cells and FoxP3+ iTreg cells. Hence, F4/80^{hi}CD11b+CD64+ MNPs, as source of IL-10, promote Tr1 and iTreg IL-10-mediated regulatory responses in the gastric mucosa.

The results observed in this study are in line with the growing consensus that MNPs may play an important role in the induction of regulatory responses that limit effector immunity and promote tolerance during infection. Although early studies evidenced that MNPs induce IFNγ (Th1) and IL-17 (Th17)-mediated effector T cell responses during *H. pylori* infection [126, 127, 151], recent data has shown that the host response to *H. pylori* is also characterized by the induction of IL-10 T cell responses [118-121, 123, 124, 152]. Moreover, prior to the promotion of FoxP3+ Treg cells, *H. pylori* induces the expansion of IL10-producing B cells [124, 153]. While these recent discoveries do not necessarily contradict the previously described and widely accepted effector immune response to *H. pylori*, they do have a significant impact on our knowledge regarding the mechanisms by which *H. pylori* might be able to elicit protective effects against numerous diseases such as obesity, asthma, colitis or MS, among others which is at the core of the dual role of this microbe as a beneficial bacterium or a pathogen.

The increased incidence of autoimmune diseases worldwide over the last decades has been linked to decreased exposure to microbial infections, including *H. pylori [154]*. Several studies have examined the correlation between *H. pylori* infection and several non-gastric diseases such asthma or MS. Arnold et al. demonstrated for the first time how *H. pylori* infection is able to effectively suppress challenge-associated inflammation, thus providing protective effects against allergic asthma [43]. These results were consistent with earlier observations of negative correlation between *H. pylori* infection and asthma prevalence in children [155, 156]. Interestingly, the observed beneficial effects were abrogated by antibiotic *H. pylori* eradication or following systemic Treg cell depletion. Conversely, adoptive transfer of purified Treg cells from *H. pylori*-infected to non-infected but allergen-sensitized mice was sufficient to transfer protection. Most recently, another group has demonstrated that the intrinsic IL-10 production by CD103+CD11b- DCs in the lungs is required for *H. pylori*-induced protection against allergic asthma [47], thus pointing at DCs as critical players for the immunomodulatory

properties of *H. pylori* once again. Furthermore, several epidemiological and clinical studies have reported a negative correlation between *H. pylori* infection and MS, as *H. pylori* is less prevalent in MS patients than healthy individuals [157-160]. Moreover, *H. pylori* provided protection against experimental autoimmune encephalomyelitis (EAE) by suppressing Th1 and Th17 while not altering circulatory FoxP3+ Treg levels [46]. In spite of the mounting evidence demonstrating a protective role of *H. pylori* against several non-gastric diseases, the exact mechanisms by which such non-invasive pathogen is able to induce systemic protective responses is not fully understood. This study identified a CD11b+ F4/80hi CD64+ CX_3CR1+ macrophage population required for the induction of *H. pylori* immunomodulatory functions. Such population promotes an optimal regulatory environment in the gastric mucosa that favors *H. pylori* survival and chronic colonization. Moreover, CD11b+ F4/80hi CD64+ CX_3CR1+ macrophages are required for the induction of systemic IL-10-mediated responses, thus representing potential key players for the modulation of extra-gastric protective mechanisms elicited upon persistent *H. pylori* infection (Figure 3.9).

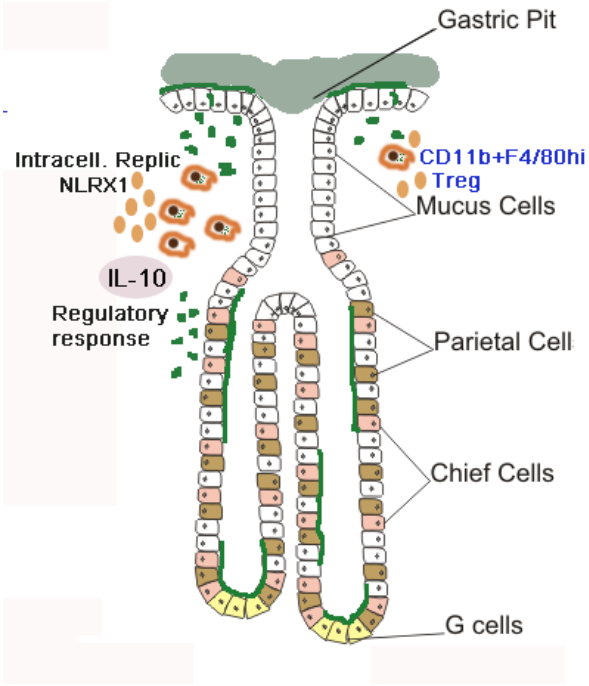

Figure 3.9. Immunological mechanisms regulating *H. pylori* gastric persistence

We present the first identification and characterization of a CD64+CX_3CR1+ macrophage population in the gastric mucosa of *H. pylori*-infected mice. The recruitment

and expansion of such cell subset by *H. pylori* enables the induction of potent local and systemic regulatory responses that create a favorable microenvironment for the survival, replication and consequently chronic colonization of *H. pylori* by limiting effector inflammatory responses required for bacterial clearance, thus favoring tolerance over immunity. CD64+CX_3CR1+ macrophages seem to be essential to ensure long-term *H. pylori* survival as well as to promote tolerogenic responses during infection both locally and systemically. Therefore, CD64+CX_3CR1+ macrophages are critical contributors to the immunomodulatory properties of *H. pylori* and modulating their function could be exploited as tolerization and inflammation regulation strategies aiming to ameliorate not only *H. pylori* infection but also other extra-gastric disease conditions.

3.7 Materials and Methods

Animal Procedures

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act. C57BL/6J wild type mice were bred in Virginia Tech's laboratory animal facilities. Tissue-specific PPARγ null mice were generated as previously described [53, 80, 81]. The tail and gastrointestinal genotypes of mice were determined by PCR analysis as previously described [53]. Specifically, mice lacking PPARγ in T cells (PPARγ fl/fl; CD4 Cre+) [74] and mice with a PPARγ deletion in myeloid cells (PPARγ fl/fl; Lysozyme M Cre-) [60] were used in this study.

Mice were weaned at 21 days of age and transferred to an ABSL2 facility when they reached 8 weeks of age. Following a 6-hour fasting period, *Helicobacter pylori* infection was performed by orogastric gavage. Briefly, a freshly prepared aliquot (500 µl, $5x10^7$ colony forming units (CFU)/mouse) of *H. pylori* strain SS1 in sterile 1X PBS was administered to mice on days 0 and 2 of the study through a gavage needle. A negative control, non-infected group which received the same volume of sterile, plain 1X PBS without any bacteria was included for each genotype. Mice were scored for clinical signs of disease daily. Mice were euthanized at different time-points between 12 days and up to 25 weeks post-infection depending on the experiment. Stomach was collected for the quantification of bacterial loads, assessment of gastric histopathology and

characterization of immune cell populations and phenotypes. In some experiments, spleen, gastric lymph nodes (GLNs), mesenteric lymph nodes (MLNs) and colon were also collected for immunophenotyping purposes.

Helicobacter pylori culture and inoculum preparation

The European mouse-adapted CagA positive strain *H. pylori* SS1 (kindly provided by Dr. Richard Peek, Vanderbilt University) was used in this study. *H. pylori* was grown on plates prepared with Difco Columbia agar base (BD Biosciences) supplemented with 7% of lacked horse blood (Lampire) and *Helicobacter pylori* selective supplement (containing 10 mg/L vancomycin, 5 mg/L trimethoprim, 5 mg/L amphotericin, and 5 mg/L polymyxin from Oxoid) at 37°C under microaerophilic conditions. The challenge inoculum was prepared by harvesting bacteria into sterile 1X PBS and adjusting to Optical Density (OD)=1.0 at 600nm which was estimated as a concentration of $1x10^8$ CFU/ml as previously determined by a growth curve correlating OD measurements with colony counts on blood agar plates.

In vivo macrophage depletion

Macrophages were depleted by intraperitoneal injection of clodronate-containing liposomes (Anionic Clophosome, FormulaMax) 11 days after first *H. pylori* infection (correlating with the observed upregulation of macrophages in the gastric lamina propria after infection). Briefly, WT mice received an initial dose of 200µL of clodronate liposomes. Macrophage depletion was maintained by injecting lower doses (100µL) of clodronate liposomes every 3 days (days 14, 17 and 20 post-infection). Control mice received PBS-containing liposomes. A subset of mice was sacrificed a day after each injection (days 15, 18 and 21 post-infection) and stomachs were collected for the quantification of bacterial loads and the identification of immune populations populations by flow cytometry.

Bacterial reisolation from murine gastric tissue

Following euthanasia, stomach tissue was opened along the longer coverture, thoroughly rinsed in sterile 1X PBS and total CFU were determined by plate counting [127]. Briefly, weighted gastric specimens were homogenized in Brucella broth using a grinder. Homogenates and serial dilutions (1:10, 1:100, 1:1,000 and 1:10,000) were plated onto Difco Columbia agar base plates supplemented with 7% of lacked horse

blood and *Helicobacter pylori* selective antibiotic supplement (containing 10 mg/L vancomycin, 5 mg/L trimethoprim, 5 mg/L amphotericin, and 5 mg/L polymyxin). Plates were incubated for 4 days at 37°C under microaerophilic conditions [127, 161]. Bacterial numbers are reported as the mean and SD of the number of CFU/g of stomach tissue.

Isolation of gastric lamina propria leukocytes

Following euthanasia, the stomach was opened along the longer curvature, thoroughly rinsed in sterile 1X PBS and divided into 2 small pieces. Samples were initially incubated for the removal of epithelial cells in 50mL tubes containing 7mL of Cell Recovery solution (CORNING) for 1h at 4°C under gentle stirring conditions. After filtration and washing, the remaining tissue was then further digested for 45min twice at 37°C under stirring conditions in RPMI media containing 10% FBS, 2.5% Hepes, and 0.3mM CaCl2 along with collagenase at 300U/mL (Sigma) and DNAase (Sigma) at 50U/mL. After filtration, a 44%/67% Percoll gradient (Sigma) was performed to purify leukocytes. Final cell suspensions were washed, count and adjusted to a concentration of $3x10^6$ cells/mL.

Macrophage and lymphocyte cell characterization using flow cytometry

600,000 cells were seeded onto 96-well plates, centrifuged at 2,000 rpm at 4°C for 3 minutes, and washed with PBS containing 5% fetal bovine serum and 0.09% sodium azide (FACS buffer). In order to assess macrophage populations, cells were then incubated in the dark at 4°C for 10 minutes in FcBlock (BD Pharmingen), and then for an additional 20 minutes with fluorochrome-conjugated primary antibodies. For macrophage flow cytometric assessment, cells were incubated with anti-CD45-APCCy7 (BD Pharmingen), anti-F4/80-PECy5 (eBioscience), anti-CD11b-AlexaFluor700 (BD Pharmingen), anti-MHCII-biotin (eBiosciences) followed by streptavidin-PETexasRed (BD Pharmingen), anti-CD64-PE (BD Pharmingen), anti-CX_3CR1-unconjugated (AbD Serotec) followed by anti-IgG(H+L)-FITC (Southern Biotech), and anti-IL10-APC (eBiosciences). For lymphocyte flow cytometric assessment, cells were incubated with different combinations of anti-CD45-APCCy7 (BD Pharmingen), anti-CD3-PECy5 (eBiosciences), anti-CD4-PECy7 (eBiosciences), anti-CD19-PE (eBiosciences), anti-FoxP3-PE (eBiosciences), anti-PD1-PE (eBiosciences) and anti-IL10-APC (eBiosciences). For intracellular staining, cells were fixed and permeabilized with Cytofix-Cytoperm solution (eBiosciences). Flow results were computed with a BD LSR II

flow cytometer and data analyses was performed by using the FACS Diva software (BD).

Statistics

Data are expressed as mean and standard error of the mean. Parametric data were analyzed by using the Analysis of Variance (ANOVA) followed by Scheffe's multiple comparison test as previously described [53]. Analysis of variance (ANOVA) was performed by using the general linear model procedure of SAS, release 9.2 (SAS Institute Inc., Cary, NC). A 2x2 factorial arrangement comparing genotype and infection treatment was employed. Statistical significant was determined at $P \leq 0.05$.

Chapter 4

Mononuclear Phagocytes as Loophole for Bacterial Persistence

Monica Viladomiu, Barbara Kronstreiner, Josep Bassaganya-Riera, Casandra W. Philipson, Pawel Michalak, and Raquel Hontecillas.

4.1 Summary

Although traditionally thought to be an exclusively extracellular bacterium, recent *in vivo* and *in vitro* findings demonstrate the presence of *H. pylori* in the intracellular environment of epithelial and myeloid cells. Similarly to other intracellular microbes, *H. pylori* infection of macrophages and dendritic cells can alter autophagy, phagosome maturation and membrane trafficking, thus interfering with antigen processing and presentation pathways and facilitating bacterial survival within the intracellular compartment of these immune cells. In this regard, we developed a gentamycin protection co-culture assay to explore

whether *H. pylori* is able to replicate in macrophages and the type of response induced by such intracellular infection. *In vitro* infection of bone marrow-derived macrophages obtained from WT and PPARγ-deficient mice revealed differences in their ability to kill intracellular *H. pylori*. Specifically, PPARγ deficiency resulted in diminished bacterial re-isolation similar to the results obtained *in vivo*, which correlated with increased iNOS production and NF-KB activity. Moreover, RNA-seq analyses identified two genes, *chit1* and *iigp1*, that were upregulated in *H. pylori*-infected PPARγ-deficient BMDM. Inhibition of these genes by siRNA significantly increased bacterial survival in PPARγ-deficient macrophages reaching levels similar to the WT control, thus suggesting that the decrease in bacterial loads observed in PPARγ-deficient BMDM when compared to WT BMDM might be due to an enhanced anti-microbial response. Thus, macrophages emerge as absolutely critical contributors to bacterial persistence. Studying the immune responses to intracellular *H. pylori* has the potential to be paradigm-shifting in terms of understanding the mechanisms of host response during infection. In addition, it may result in novel therapeutics that modulate the immune response and prevent *H. pylori* disease but allow the beneficial effects of this bacterium.

4.2 Introduction

H. pylori has been traditionally considered an extracellular bacterium found free-swimming in the mucus lining of the stomach or overlaying the apical side of gastric epithelial cells. However, recent *in vitro* and *in vivo* evidence indicates that *H. pylori* can also multiply and survive within intracellular niches in gastric cells [162]. *H. pylori* has been found inside metaplastic, dysplastic, and neoplastic gastric epithelial cells [163]. Several studies have demonstrated that *H. pylori* can persist in hepatocytes [164] and replicate in macrophages [165], bone marrow-derived dendritic cells [166] as well as gastric epithelial cells *in vitro,* thus providing evidence for its role as facultative intracellular organism with the ability to survive, replicate and successfully evade antibiotic therapy within host cells [167]. Recent findings have further strengthened the role of *H. pylori* as an intracellular pathogen in mice, pigs and humans. *H. pylori* has

been localized to murine gastric epithelial progenitor cells [168] and found to be intracellular in lymphoid aggregates and epithelial cells after infection in pigs [169]. Moreover, it has also been identified in human tissue specifically residing within gastric epithelial cells, parietal cells and lamina propria macrophages [170, 171]. In macrophages, some *H. pylori* strains have the ability to prevent phagosome maturation allowing the bacterium to survive and replicate by escaping phagocytic killing [172, 173]. Furthermore, *H. pylori* have been found in gastric lymph nodes suggesting lymphatic dissemination [170]. One report even described the systemic presence of *H. pylori* in peripheral blood of an *H. pylori* seropositive breast cancer patient with bacteremia [174] providing further *in vivo* evidence that *H. pylori* can spread beyond the gastric mucosa invading other organs and tissues, most likely within migratory phagocytic cells.

In order to better understand the role of *H. pylori* as an intracellular bacterium, we optimized an *in vitro* infection of bone marrow-derived macrophages which allowed us to investigate the replication and survival of *H. pylori* in the intracellular environment and the antigen processing and presentation during *H. pylori* infection. These aspects of the host-bacteria interaction derived from our previous results showing that *H. pylori* infection induces effector Th1 and cytotoxic CD8 responses [151] and *in silico* preliminary data pointing towards macrophages/APCs as critical regulators of colonization levels and pathology [127], as discussed in the previous chapter.

4.3 Replication of *H. pylori* in phagocytic cells

In order to understand the mechanisms by which macrophages recognize, respond to and eliminate intracellular *H. pylori*, we optimized an *in vitro* gentamycin protection assay using BMDM. WT and PPARγ-null BMDM were co-cultured with *H. pylori*, washed and cultured in a gentamycin medium that eliminates non-internalized bacteria, and then harvested at different time-points to assess bacterial loads. Data obtained from several independent time-course studies shows that *H. pylori* replicates more efficiently in BMDM from WT mice compared with PPARγ-deficient cells. *H. pylori* reached significant levels of replication though these were only transiently maintained. The maximum burden occurred at two hours post-infection but numbers of live bacteria dropped to initial levels at 7 hours post-exposure. PPARγ deficiency (either in PPARγ-null macrophages or WT macrophages pre-treated with the antagonist GW9662) consistently

resulted in diminished bacterial reisolation similar to the results obtained *in vivo* (Figure 4.1). These results corroborate that macrophage phenotype is one of the host factors contributing to elimination of *H. pylori* and regulation of the local immune response in the gastric mucosa, as previously predicted by *in silico* simulations [127].

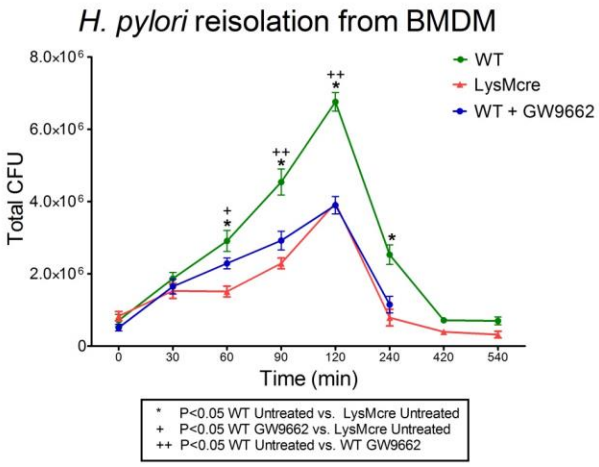

Figure 4.1. Assessment of bacterial loads following gentamicin protection assay in WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1. Cells were exposed to *H. pylori* at MOI of 10 for 15 min followed by extensive washing and addition of gentamicin for the removal of extracellular, non-phagocytized bacteria. Cells were harvested at the indicated time-points to measure intracellular loads by reisolation.

4.4 Inhibition of PPARγ in RAW macrophages enhances NFκB activity upon *H. pylori* infection

To assess if the observed phenotype was due to altered regulation of bacterial recognition, we measured NF-kB activity in a reporter cell line (Invivogen). Indeed, co-culture of *H. pylori* with RAW-Blue™ cells activated NF-kB as early as 2 hours after extracellular bacteria removal, an effect was further enhanced by chemical PPARγ inhibition (Figure 4.2)

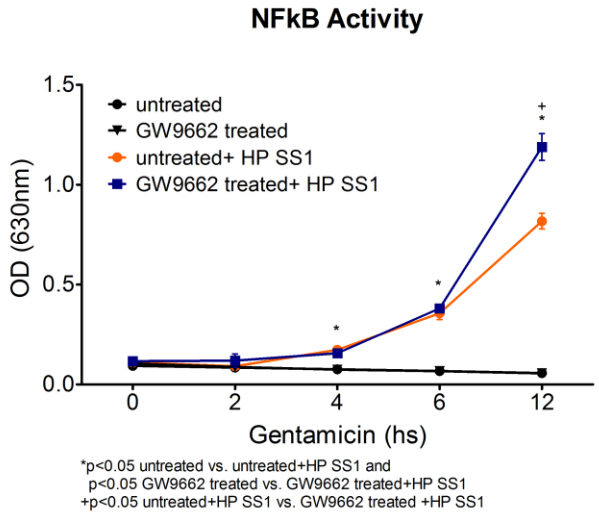

Figure 4.2. Assessment of NF-KB activity following gentamicin protection assay in RAW macrophages co-cultured with *H. pylori* SS1. Cells were exposed to *H. pylori* at MOI of 10 for 15 min followed by extensive washing and addition of gentamicin for the removal of extracellular, non-phagocytized bacteria. Infection of RAW macrophages with *H. pylori* SS1 induced NFκB activity starting after 2 hours of culture. Inhibition of PPARγ by its antagonist GW9662 resulted in significantly higher NFκB activity compared to untreated macrophages at 12 hours.

4.5 Increased iNOS production is partly involved in bacterial clearance

Gene expression results obtained from a time-course experiment showed that *H. pylori* infection upregulates iNOS mRNA levels in both WT and PPARγ-deficient macrophages (Figure 4.3A), although iNOS expression was higher in cells lacking PPARγ. iNOS expression peaked at 4h post-infection in both cell types. However, PPARγ-deficient macrophages consistently showed significantly higher iNOS levels as soon as 1h after the removal of extracellular bacteria when compared to WT macrophages. Interestingly, the increase in iNOS production at 2h post-infection in PPARγ-deficient macrophages correlated with the drastic decrease of *H. pylori* loads observed in these cells.

To further characterize the role of iNOS in bacterial clearance, we performed inhibition studies by pre-treating cells for 30min with 100μM of the selective iNOS inhibitor L-N6-(1-Iminoethyl)lysine dihydrochloride (L-Nil) before infecting them with *H. pylori* at MOI 10 for 15min. L-Nil was present throughout the duration of the experiment. Bacteria were re-isolated and RNA was isolated from cells harvested at 0, 1, 2 and 4 hs after the elimination of non-internalized bacteria. iNOS inhibition increased bacterial re-isolation in both WT and PPARγ-deficient macrophages (Figure 4.3B). However, the effect of iNOS inhibition was stronger in WT macrophages, as the CFU numbers obtained from PPARγ-deficient macrophages treated with L-Nil did not increase to levels comparable to those observed in WT macrophages.

Regarding the actual expression/inhibition of iNOS, SS1 and LPS-stimulated PPARγ-deficient macrophages showed a 2-fold increase in iNOS expression when compared to WT macrophages. L-Nil drastically decreased iNOS expression in PPARγ-deficient macrophages by more than half compared to the untreated control, thus reaching levels similar to the WT untreated control (Figure 4.3C). However, only a marginal decrease in iNOS transcripts in treated WT macrophages was observed. Taken together, these data suggest the involvement of other factors in the survival and replication of *H. pylori* in the absence of PPARγ.

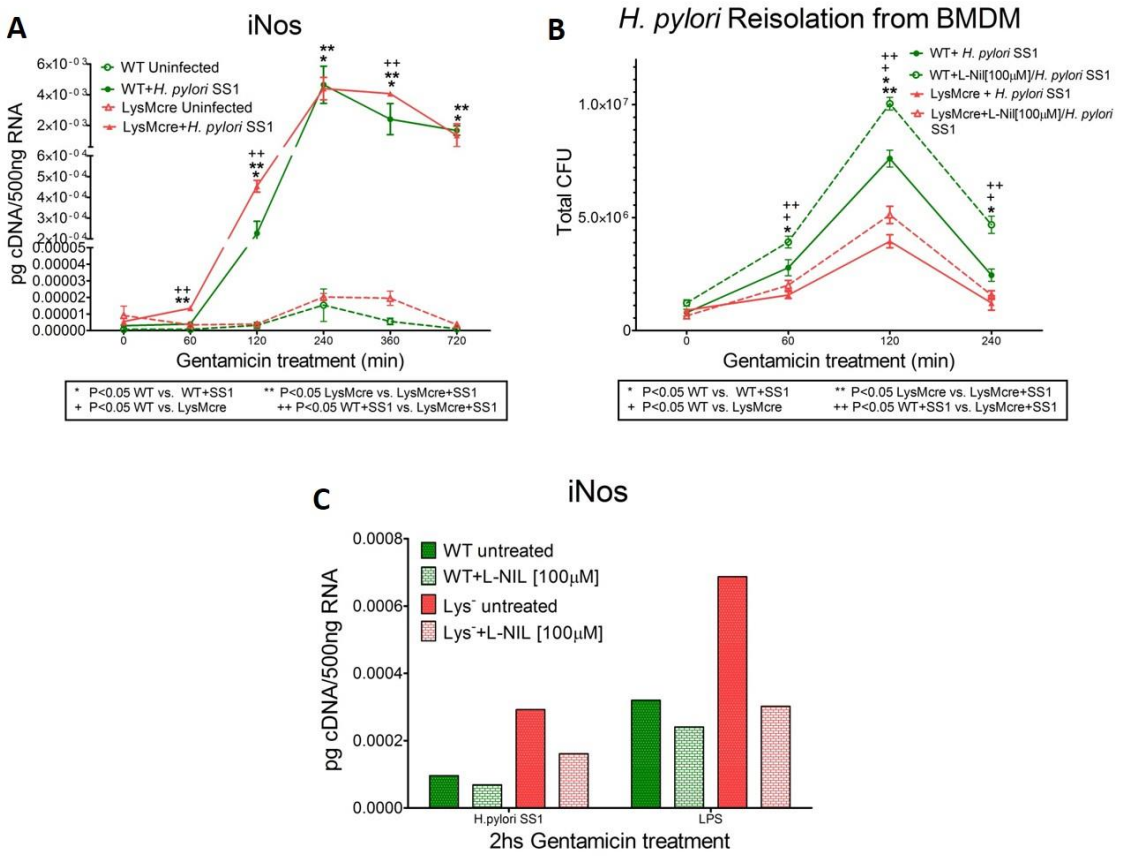

Figure 4.3. Characterizing the role of iNOS during gentamicin protection assay in WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1. Cells were exposed to *H. pylori* at MOI of 10 for 15 min followed by extensive washing and addition of gentamicin for the removal of extracellular, non-phagocytized bacteria. *H. pylori* infection upregulates iNOS mRNA levels in both WT and PPARγ-deficient macrophages. iNOS inhibition increases bacterial re-isolation in both WT and PPARγ-deficient macrophages. Data represents mean ± SEM. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

4.6 Gene expression alteration following *H. pylori* infection

In order to identify other novel factors/genes potentially involved in antibacterial response as well as characterize the impact of *H. pylori* intracellular infection on the modulation of innate immune pathways in macrophages, we performed whole transcriptome analyses of WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1 at time-points 0, 60, 120, 240, 360 and 720 min post-exposure. Fold-changes (Infected WT average expression/Uninfected WT average expression) for each time-point were uploaded into DAVID and BinGO for the identification of over and under-represented

functional gene clusters or GO categories in our dataset. GO enrichment revealed a consistent upregulation of GO Terms involved in regulatory and inflammatory immune responses as well as cell development upon infection, whereas metabolism, response to stress and stimuli, cell morphology, organization, development and differentiation were reported as consistently downregulated up until 6 hours post-co-culture (Figure 4.4). Metabolic pathways related to cholesterol and sterol biosynthesis seem to be upregulated at 0 minutes and 360 min post-co-culture, while time-points 60, 120 and 240 min are characterized by an upregulation of cell death and apoptosis regulation as well as cytokine and chemokine production. Interestingly, there is an initial downregulation of metabolic and ion transport processes early after *H. pylori* removal (0 and 60 min post co-culture), whereas later time-points present a downregulation of cellular processes related to DNA repair/transcription, chromatin and proteasome assembly, as well as cell cycle-related GO categories.

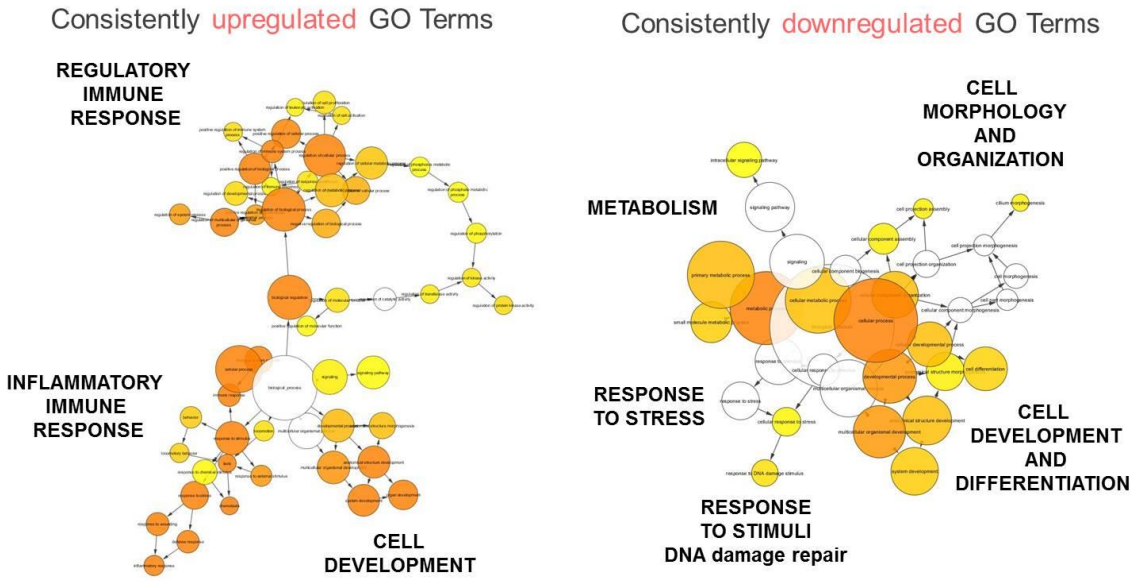

Figure 4.4. Enrichment analyses of differentially expressed genes at a significance *p=0.0001*, using GO terms from GO Slim. These BinGO graphs visualize the GO categories that were found significantly over (on the left) or under (on the right) represented in the context of GO Functional Process hierarchy. According to BINGO documentation, the size of each node is proportional to the number of genes in our gene dataset which are annotated to that node. The color of the node represents the (corrected) p-value. White nodes are not significantly over or under-represented, the other ones are (hypergeometric test, Benjamini & Hochberg False Discovery Rate (FDR) correction), with a color scale ranging from yellow (*p-value* = significance level, here *0.0001*) to dark orange (*p-value* = *5* orders of magnitude smaller than significance level). The color saturates at dark orange for p-values which are more than *5* orders of magnitude smaller than the chosen significance level.

Fold-changes were then imported into IPA, where we run core and comparison analyses between the different time-points for each genotype. The Top1 upregulated pathway at time 0 minutes for WT macrophages was the “Superpathway of cholesterol biosynthesis”, which is then downregulated at times 60 and 120 minutes and it starts slowly increasing again until 720 minutes post-co-culture. Interestingly, the upregulation of such pathway at 720 min is in line with the increased FXR/RXR and LXR/RXR activation. Moreover, the downregulation of cholesterol metabolism at 120 min post-co-culture correlates with the initiation of bacterial clearance in WT macrophages (Figure 4.5). *H. pylori* is a known cholesterol autotroph. It extracts this essential nutrient from epithelial and antigen presenting cells and incorporates it into the bacterial membrane. Although cholesterol is not an essential nutrient for *H. pylori*, it has been shown that it promotes bacterial growth in serum-free media. Moreover, *H. pylori* also uniquely form cholesterol α-glycoside, a metabolite that can be further modified by acylation or phosphatidylation and then abrogate immune responses towards the pathogen by suppressing phagocytosis and T cell activation. On the other hand, cholesterol has been shown to induce APCs’ phagocytic activity and enhance antigen-specific-T-cell responses. Therefore, the positive correlation between decreased cholesterol biosynthesis and lower bacterial loads seen in our dataset is in line with these previously published observations.

Decreased loads of *Helicobacter pylori* also correlate with increased production of nitric oxide and reactive oxygen species by macrophages, as well as the canonical pathway of “Macrophage Migration Inhibitory Factor (MIF) Regulation of Innate Immunity” (Figure 4.5), a proinflammatory cytokine of the innate immune system that has been linked to several infectious diseases including *Salmonella typhimurium* [175] and that positively correlates with increased iNOS production [176].

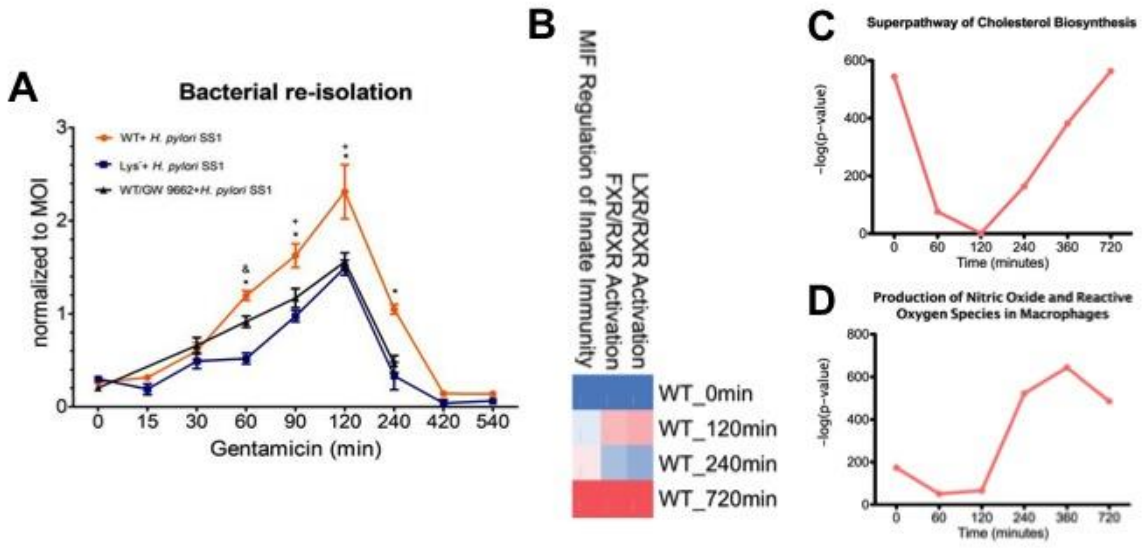

Figure 4.5. Bacterial clearance in wild type macrophages correlates with decreased cholesterol biosynthesis and increased ROS production. WT and PPARγ-deficient (Lys) BMDM were co-cultured with *H. pylori* SS1 for 15 min at 37ºC. After extensive washing, cells were incubated with media supplemented with 100ug/ml Gentamicin for up to 12 hours. Bacterial loads within cells were assessed by culturing lysates on blood agar plates for 4 days (A). RNA-seq data was analyzed using the Ingenuity Pathway Analyzer software (Ingenuity® Systems, www.ingenuity.com). The significance value associated with a function in Global Analysis is a measure for how likely it is that genes from the dataset file under investigation participate in that canonical pathway. The significance is expressed as a *p*-value, which is calculated using the right-tailed Fisher's Exact Test (B, C and D).

The initiation of decreased bacterial loads seen between 120 and 240 minutes post-co-culture also correlates with the activation of several other canonical pathways. The signaling pathway of several transcription factors including PPARs, NF-ΚB and AhR are strongly upregulated 240 minutes after bacteria removal (Figure 4.6). The signaling of cytokines IL-6, IL-8, IL-10 and IL-12 is upregulated at 120 minutes post-co-culture, whereas IL-17 and interferon responses are not seen until 12 hours post-co-culture. In line with the novel discoveries presenting *H. pylori* as an intracellular bacterium, the Global Canonical Pathway analyses reported changes in several intracellular pathogen-related pathways such as the “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses”, “Toll-like Receptor Signaling”, as well as “Virus Entry Via Endocytic Pathways” at 120 and 240 minutes. This resulted in the activation of IRF by cytosolic Pattern Recognition Receptors at 720 minutes, which is line with the late interferon response (Figure 4.6).

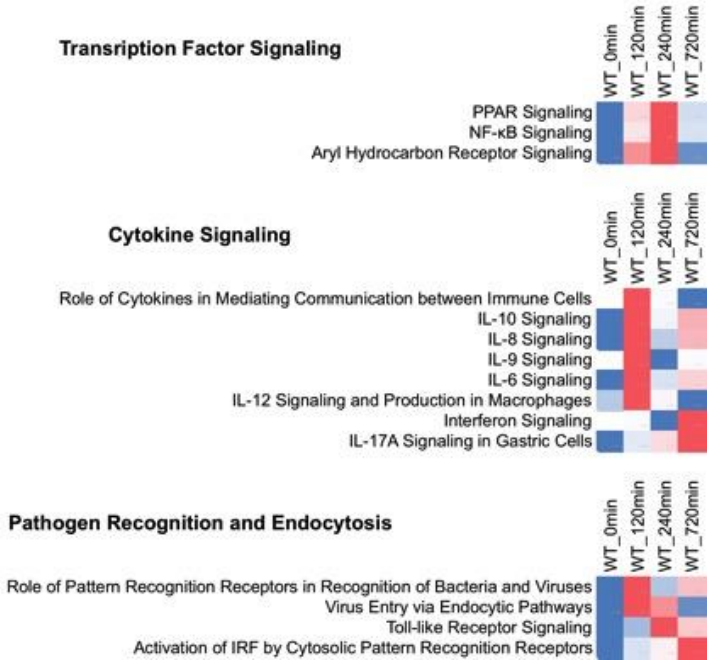

Figure 4.6 Global Canonical Pathway analysis (up-, down-regulated gene datasets). RNA-seq data was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The significance is expressed as a *p*-value, which is calculated using the **right-tailed Fisher's Exact Test**. The color indicates the expression level of the canonical pathways (red indicating up-regulated pathways and blue indicating down-regulated pathways).

We then compared gene expression between uninfected WT and PPARγ-deficient macrophages with the aim to identify whether macrophages lacking PPARγ present some kind of predisposition to infection that would partly explain the differences in bacterial loads consistently seen in our *in vitro* and *in vivo* experiments. We calculated the fold-change based on genotype (Uninfected PPARγ-deficient/Uninfected WT) and imported them into IPA, where we run core and comparison analyses to identify changes in gene expression, molecular functions and canonical pathways. IPA revealed a significant differential cytokine expression characterized by an increased production of IL-1β, IL-2, IL-4, IL-6, IFNγ, TNFα and TGFβ, as well as a significantly increased expression of the NF-κB complex. Consistent with the upregulation of cytokine production, the “Canonical Pathways” module of IPA also reported an upregulation of several canonical cytokine signaling pathways including IL-10, IL-17, IL-12, IL-6 and IL-1β. IPA also revealed an upregulation of several other pathways such as “VDR/RXR activation”, “LXR/RXR activation”, “Aryl Hydrocarbon Receptor Signaling”, “iNOS signaling” and “NF-κB signaling”. Some metabolic changes were also detected, as seen by the upregulation of “Calcium transport”, “Calcium Signaling” and “Tryptophan degradation”. When looking at the “Disorders and function” module of IPA, we saw a dysregulation of several molecular functions, most of them related to metabolism. In particular, PPARγ-deficient macrophages showed an upregulation of vitamin and mineral

metabolism, Ca^{2+} flux, mobilization, influx, release and quantity in particular. They also showed changes in lipid metabolism (Figure 4.7), as seen by the accumulation of eicosanoids. Finally, an increased production and release of nitric oxide was also detected, which is in line with the results observed in the “Canonical pathways” module.

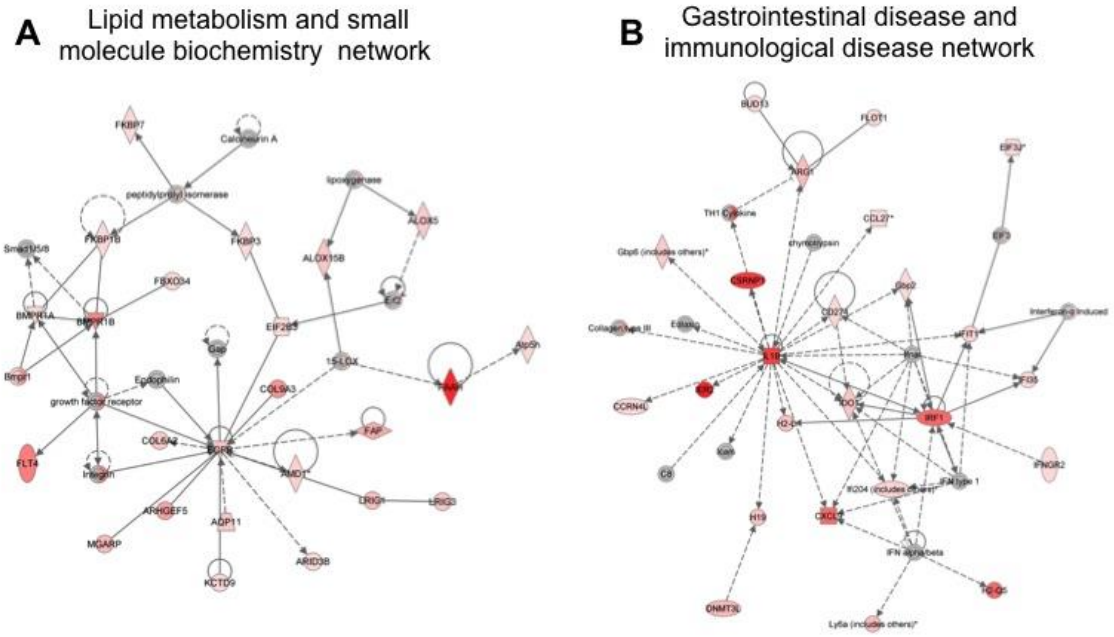

Figure 4.7. Differentially expressed networks in uninfected PPARγ-deficient mice. RNA-seq data was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The color indicates the expression level of the canonical pathways (red indicating up-regulated pathways and blue indicating down-regulated pathways).

Similarly to the results observed when comparing uninfected WT and PPARγ-deficient macrophages, we saw that the lack of PPARγ also results in increased expression of several cytokines such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and TNFα early after co-culture (0 to 240 min post-co-culture). These results are consistent with the upregulation of several canonical pathways including “IL-1 signaling”, “IL-6 signaling”, “IL-8 signaling”, “IL-10 signaling”, “Aryl Hydrcarbon Signaling”, “PPARα/RXRa activation” and “LXR/RXR activation”. As soon as 120 min post-co-culture, and correlating with the heavy decrease in bacterial loads seen in PPARγ-null macrophages, a dysregulation of several metabolic pathways can be detected. These changes are maintained up until 720 min post-bacterial removal. Interestingly, at time 360 min the most detectable dysregulations belong to the “mitochondrial dysfunction”, “Oxidative phospholyration”, “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses” pathways.

4.7 Intracellular *H. pylori* recognition

Nucleotide-binding oligomerization domain (NOD)-like Receptors (NLRs) are pivotal intracellular sensors of infection and stress in intracellular compartments [177]. We wanted to assess changes in the expression of the NLR family during *Helicobacter pylori* infection since no information regarding the role of such receptors has been reported in such context to date. As seen in Figure 4.8, some NLRs seem to be upregulated during the first time-points post-co-culture and down-regulated in later timepoints. Such NLRs include Nlrc3, Nlrp10, Nlrp1 and Nlrx1. On the other hand, other NLRs seem to be initially downregulated and highly expressed later on. Some of these are Nlrc5, Nlrp5 and Nlrp3, as well as the firstly described Nod1 and Nod2.

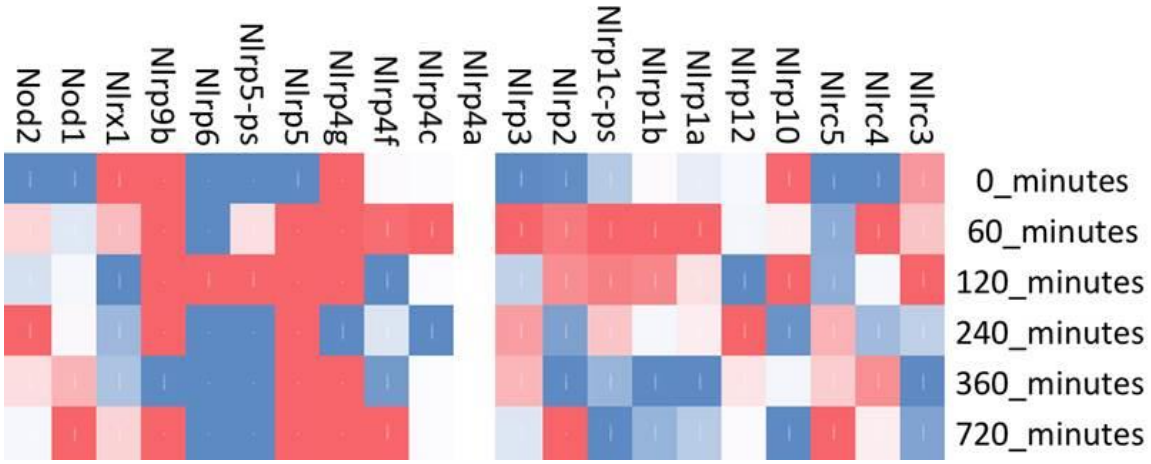

Figure 4.8. Effect of *Helicobacter pylori* infection in NLR family expression. RNA-seq data was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The significance is expressed as a *p*-value, which is calculated using the right-tailed Fisher's Exact Test. The color indicates the expression level of the canonical pathways (red indicating up-regulated pathways and blue indicating down-regulated pathways).

The activation of some of these NLRs has been previously reported in the context of viral or intracellular bacterial infection. One example is Nlrc3, a regulatory NLR known to inhibit Toll-like receptor (TLR)-dependent activation of the transcription factor NF-κB by interacting with the TLR signaling adaptor TRAF6 to attenuate Lys63 (K63)-linked ubiquitination of TRAF6 and activation of NF-κB [178]. Another NLR of interest is NLRX1, which is known to affect innate immunity to viruses and intracellular bacteria by interfering with the mitochondrial antiviral signaling protein (MAVS)/retinoic-acid-inducible gene I (RIG-I) mitochondrial antiviral pathway [179, 180]. Finally, NLRC5 is

linked to innate immunity to viruses and intracellular bacteria potentially by regulating interferon activity and inducing MHC-I [181]. Interestingly, the upregulated expression of NLRC5 at later time-points post-co-culture correlate with increased antigen presentation through the MHC-I complex as seen in Figures 4.9 and 4.10.

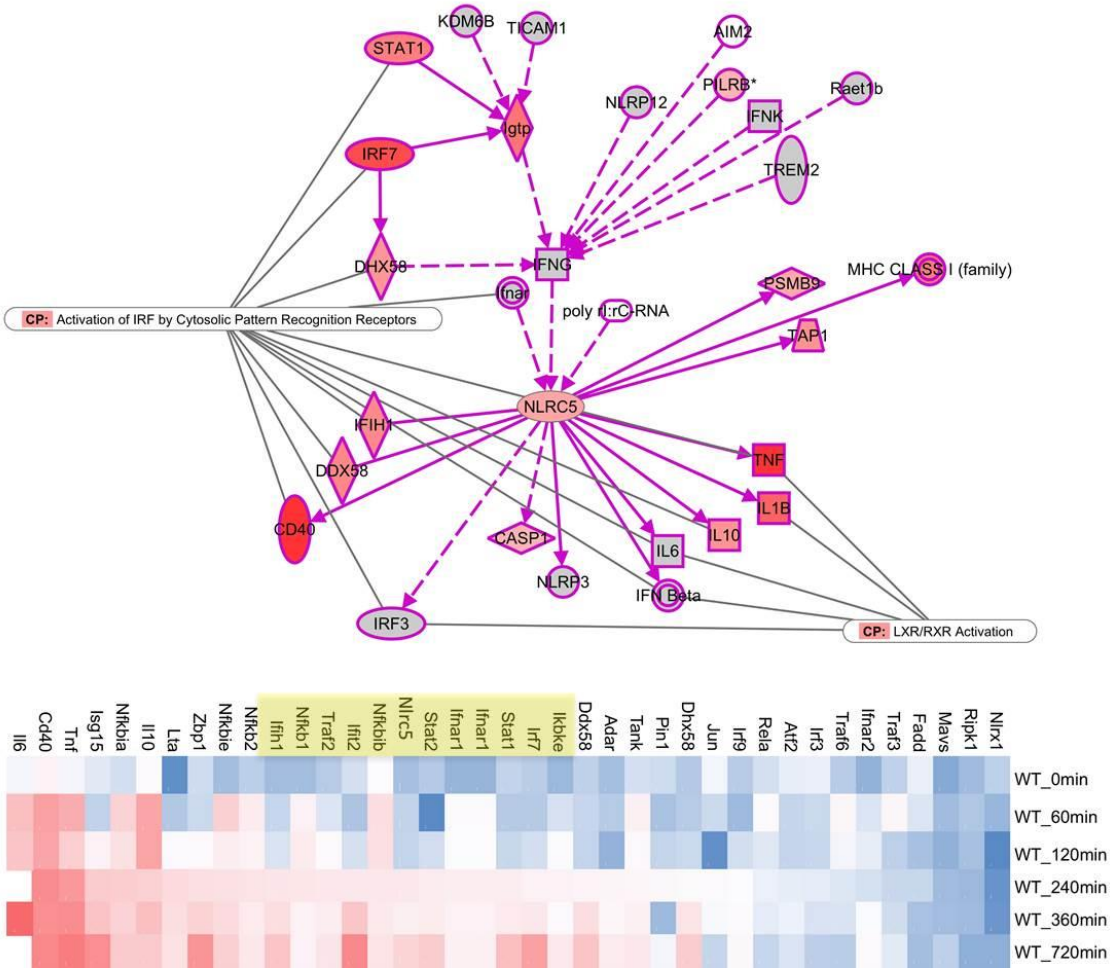

Figure 4.9. *Helicobacter pylori* infection induces NLRC5 expression, which results in MHC-I antigen presentation. RNA-seq data was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The network was manually created in IPA using data at time-point 360 min post-co-culture. In the heatmap, the significance is expressed as a *p*-value, which is calculated using the right-tailed Fisher's Exact Test. The color indicates the expression level of the canonical pathways (red indicating up-regulated pathways and blue indicating down-regulated pathways).

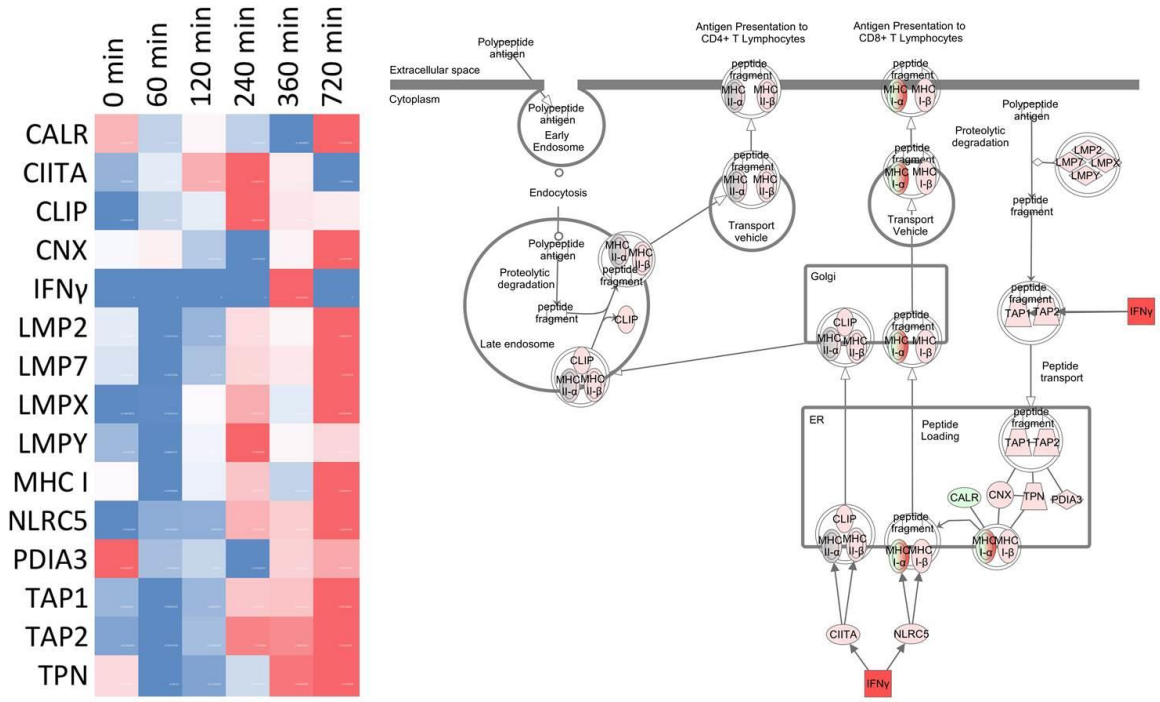

Figure 4.10. *Helicobacter pylori* infection induces NLRC5 expression, which results in MHC-I antigen presentation. RNA-seq data was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The "Antigen Presentation" canonical pathway" shown on the right side of the figure was overlapped with the expression data at time 720 min post-co-culture. In the heatmap, the significance is expressed as a *p*-value, which is calculated using the right-tailed Fisher's Exact Test. The color indicates the expression level of the canonical pathways (red indicating up-regulated pathways and blue indicating down-regulated pathways).

4.8 Whole transcriptome analyses revealed the differential expression of 8 genes in macrophages during *Helicobacter pylori* infection: *chit1, etv5, iigp1, ptger4, sqle, osm, hspa2* and *rptoros*

In order to identify other novel factors/genes potentially involved in antibacterial response as well as characterize the impact of *H. pylori* intracellular infection on the modulation of innate immune pathways in macrophages, we performed whole transcriptome analyses of WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1 at time-points 0, 60, 120, 240, 360 and 720 min post-exposure. Three-way ANOVA revealed the differential expression of 8 genes ($P < 0.05$) when considering the interaction between genotype (WT versus PPARγ-deficient), infection status and

collection time-point: *chit1, etv5, iigp1, ptger4, sqle, osm, hspa2* and *rptoros* (Figure 4.11). Results were validated by qRT-PCR (Figure 4.12A-B).

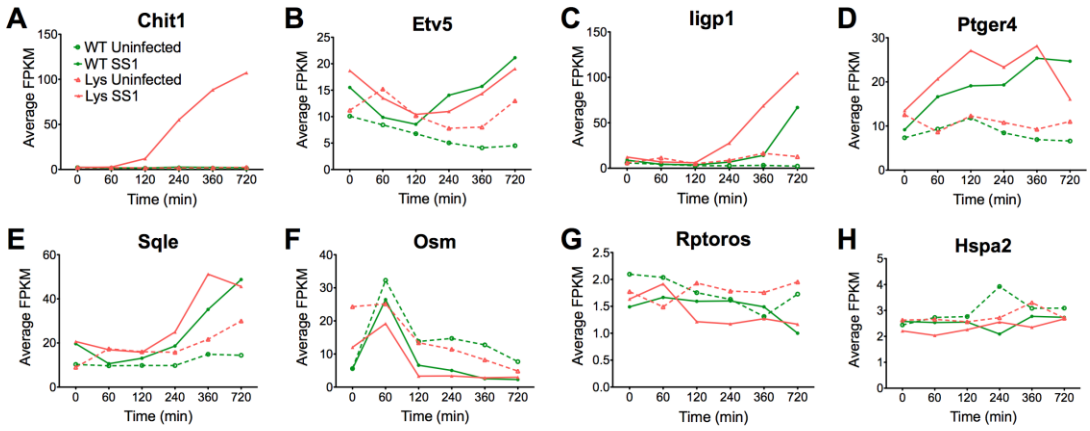

Figure 4.11. Expression of differential expressed genes based on a 3-way (genotype, infection and time) ANOVA analyses. FPKM (fragments per kilobase per million sequenced reads) was used as the measurement of expression level. All genes with median expression level in all samples greater than 0 were included in a 3-way ANOVA analysis. Normal quantile transformation (qqnorm from R [182]) was used to normalize the FPKM to fit the normality assumption of ANOVA (tested with Kolmogorov-Smirnov test). The 3-way ANOVA analysis was carried in R [182], FDR [183] and Bonferroni were used to calculate the adjusted *P*-values.

4.9 *chit1* and *iigp1*, but not *sqle*, are directly involved in bacterial clearance

Out of the 8 differentially expressed genes revealed by whole transcriptome analyses, we further validated the contribution of *chil1*, *iigp1* and *sqle* to intracellular *H. pylori* clearance and their role in modulating macrophage anti-bacterial responses. To do so, we performed siRNA knockdown studies for these 3 genes. Specifically, WT and PPARγ-null BMDM were transfected with siRNA (either siRNA for sqle, iigp1, chil1 or scramble) 24h before infection. Cells were infected following the gentamycin assay described above, and harvested at 2h post-infection to assess intracellular bacterial loads (2h – corresponding to the peak of *H. pylori* intracellular loads as seen in previous time-course studies). The knockdown of *iigp1* and *chil1* resulted in increased *H. pylori* numbers when compared to the scramble regardless of the genotype (Figure 4.12D). Both *iigp1* and *chil1* are highly expressed following infection, especially in PPARγ-deficient BMDM. Thus, these results suggest that the lower bacterial loads observed in untreated PPARγ-deficient BMDM when compared to WT BMDM is directly linked to an

enhanced anti-microbial response. No differences in bacterial numbers were seen during *sqle* knockdown when compared to the negative control. Specific gene knockdown was confirmed by qRT-PCR (Figure 4.13).

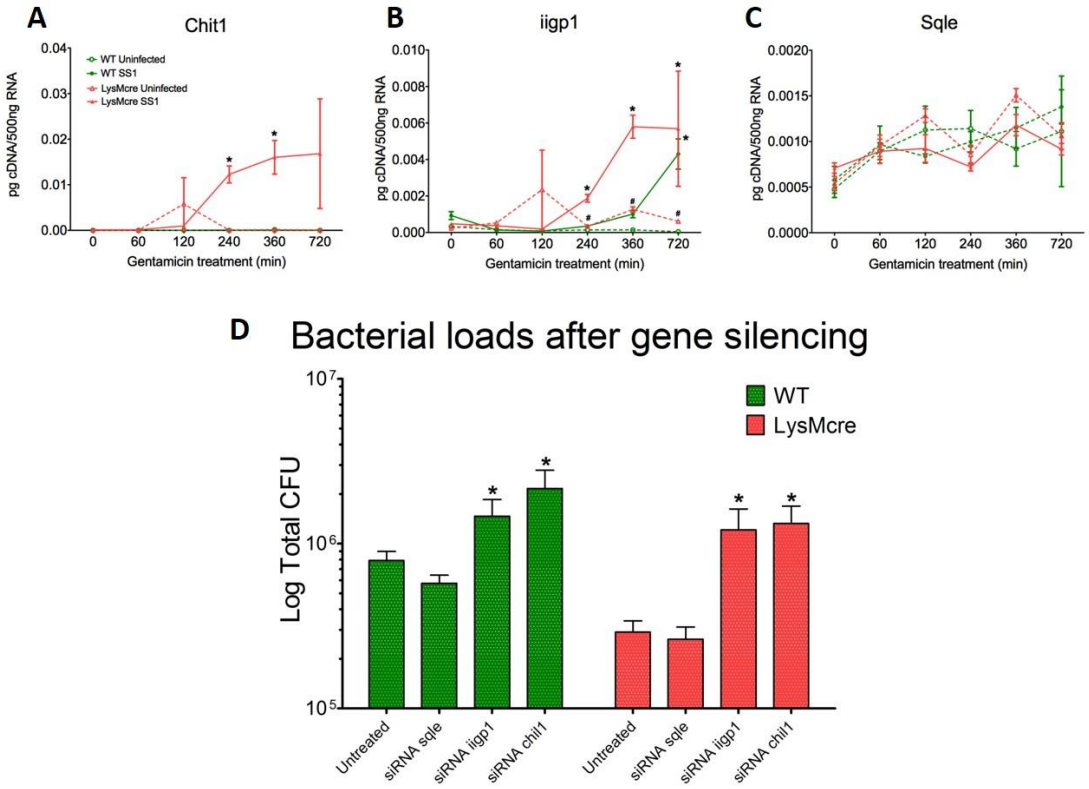

Figure 4.12. Gentamicin protection assay in WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1 following gene knock down. Cells were exposed to *H. pylori* at MOI of 10 for 15 min followed by extensive washing and addition of gentamicin for the removal of extracellular, non-phagocytized bacteria. Cells were then harvested at time-points 0, 60, 120, 240, 360 and 720 min post-infection for whole transcriptome analysis, which revealed a significant upregulation of *sqle*, *iigp1*, and *chit1* expression upon infection, which were validated by qRT-PCR (A-C). BMDM were transfected with siRNA (either siRNA for sqle, iigp1, chit1 or scramble) 24h post-infection. Cells were harvested at 2h post-infection to measure intracellular loads by reisolation (D).

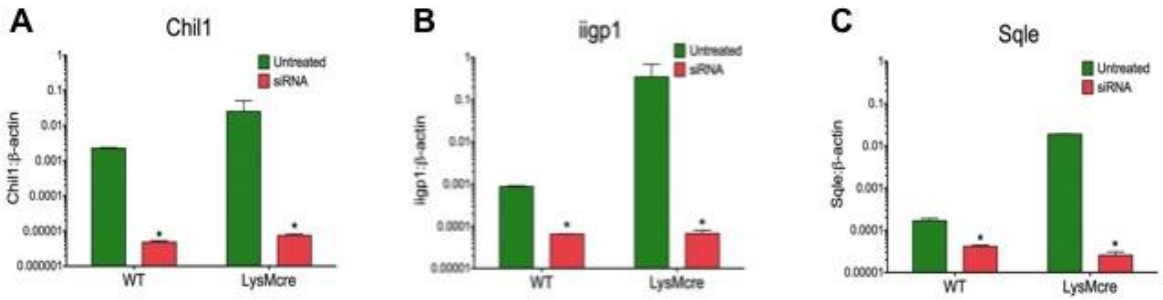

Figure 4.13. Gene knock-down validation by quantitative RT-PCR. Bone marrow-derived macrophages obtained from and WT and and PPARγ-myeloid cell deficient (LysMcre) mice were transfected with 10nM siRNA (either siRNA for *sqle*, *iigp1, chit1* or scramble) using Lipofectamine 2000 24h before infection. Gene knock-down was validated by qRT-PCR at 2h post-infection.

4.10 IFNγ-mediated responses significantly contribute to bacterial clearance

The expression of both *iigp1* and *chil1* are known to be induced by IFNγ. Although IFNγ did not appear in the top list of differentially expressed genes in the RNAseq dataset, we measured IFNγ levels by qRT-PCR and found that, although IFNγ is upregulated upon infection in both macrophage genotypes, infected PPARγ-deficient BMDM showed significantly increased levels of IFNγ as soon as 60min post-infection when compared to infected WT BMDM. Such differences were maintained throughout all the time-points (60 to 720min post-infection), and correlated with the increased levels of *iigp1* and *chil1* seen in PPARγ-deficient macrophages (Figure 4.14A). Therefore, one can speculate that the pro-inflammatory macrophage phenotype due to lack of PPARγ results in a stronger IFNγ production following *H. pylori* infection, which in turn induces the activation of *chil1* and *iigp1* and enhances antimicrobial responses. Indeed, and as described below, macrophage stimulation with IFNγ and LPS resulted in decreased bacterial loads in both WT and LysMcre cells (Figure 4.14B).

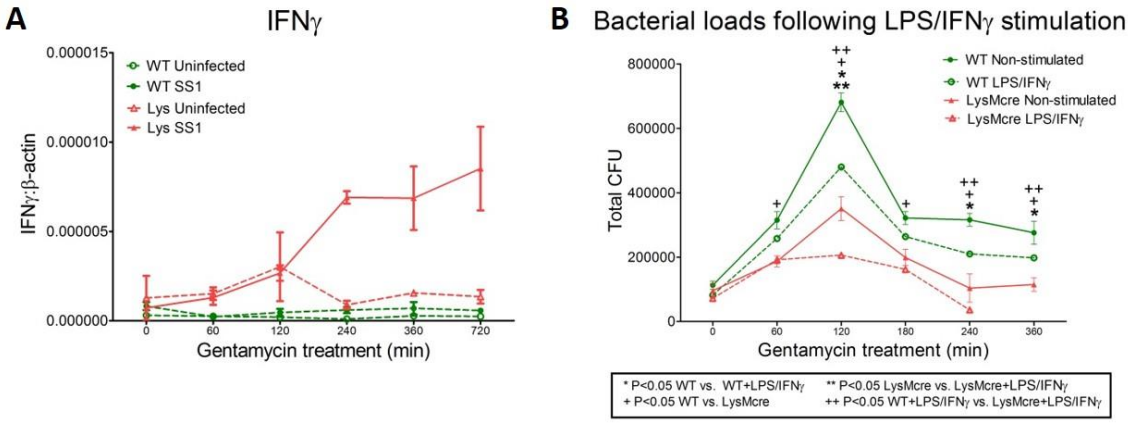

Figure 4.14. Gentamicin protection assay in WT and PPARγ-null BMDM co-cultured with *H. pylori* SS1 following macrophage M1 polarization. Differentiated BMDM were stimulated with LPS and IFNγ overnight before infection. *H. pylori* infection induced IFNγ production, especially in PPARγ-null macrophages (A). Moreover, LPS/IFNγ pre-treatment enhanced bacteria elimination

(B). Data represents mean ± SEM. Data represents mean ± SEM. Points with an asterisk are significantly different when compared to the control group (P<0.05).

4.11 M1 polarization enhances *H. pylori* elimination

Based on the predictions from our previously published computational model of *H. pylori* infection, the differences in *H. pylori* elimination between WT and PPARγ-deficient macrophages should be due to the induction of an inflammation-prone phenotype in PPARγ-deficient BMDM. To determine the effect of pro-inflammatory M1 differentiation on survival of *H. pylori*, we polarized WT and PPARγ-deficient macrophages towards an M1 phenotype by stimulating cells with LPS and rIFN-γ overnight prior to bacterial co-culture. The ability of *H. pylori* to proliferate was impaired in M1 compared to non-polarized macrophages (M0). Proliferation of *H. pylori* was almost completely abrogated in PPARγ-deficient M1, as *H. pylori* was cleared after 6 hours of extracellular bacteria removal. In contrast, bacteria was not eliminated from WT M1 and WT and PPARγ-deficient M0 until 12 hours post-infection (Figure 4.14B).

4.12 Discussion and Conclusions

An important aspect to consider in the host-pathogen interaction with regards to *H. pylori* is that the bacteria can be found at different locations in the stomach. *H. pylori* interacts with various host molecules and cell types depending on its location within the gastric mucosa and ultimately this interaction determines the type of immune response elicited. CD11b+F4/80hiCD64+ cells control a fraction of *H. pylori* (as discussed in Chapter 3), however we do not know where this control takes place.

H. pylori has long been considered an extracellular bacterium found mainly in the gastric mucus layer and in lesser proportions, in close association with the cell surface of epithelial cells. More recently, the role of *H. pylori* as a facultative intracellular pathogen has re-emerged. *In vitro* studies have demonstrated that *H. pylori* can infect macrophages and replicate within the cellular niche. The bacterium can avoid cellular mechanisms of killing by manipulating membrane trafficking processes and delaying

phagosomal maturation and lysosome killing [172, 173, 184]. Two recent papers demonstrate that the loss of matrix metalloproteinase 7 [185] and protease activated receptor-1 [186] by macrophages results in the acquisition of a more pro-inflammatory phenotype and suppressed *H. pylori* burden, which is consistent with the results obtained when infecting mice lacking PPARγ in macrophages.

In line with our findings showing higher colonization in WT mice when compared to LysM-cre, we investigated the possibility of *H. pylori* replication within macrophages. We have used an *in vitro* gentamycin protection assay with BMDM from WT and LysMcre mice. In our system, BMDM are co-cultured with *H. pylori* in antibiotic free medium. After 15 min, cells are thoroughly washed to remove extracellular bacteria, and gentamycin is added to the culture. Our data from several time-course studies shows that, in line with the *in vivo* findings, *H. pylori* replicates more efficiently in BMDM from WT mice compared with PPARγ-deficient cells. Hence, macrophages emerge as absolutely critical contributors to bacterial persistence. However, the intracellular role of *H. pylori* replicating in macrophages has not been addressed to date. In this regard, we combined bioinformatics approaches and immunology experimentation to elucidate some of the immune mechanisms triggered by intracellular *H. pylori* infection.

RNA-seq analyses identified two genes, *chit1* and *iigp1*, that were upregulated in *H. pylori*-infected PPARγ-deficient BMDM. Inhibition of these genes by siRNA significantly increased bacterial survival in PPARγ-deficient macrophages reaching levels similar to the WT control, thus suggesting that the decrease in bacterial loads observed in PPARγ-deficient BMDM when compared to WT BMDM might be due to an enhanced anti-microbial response. Chitinase-like 1 (*chit1*) and interferon-γ-inducible 47-kDa GTPase (*iigp1*) are promising host response targets during intracellular infection of macrophages with *H. pylori*. The induction of *chit1* during *Streptococcus pneumoniae* infection promotes bacterial clearance while simultaneously augmenting host tolerance [187]. Moreover, the activation of *iigp1*, widely known as Irga6, mediates resistance to *Toxoplasma gondii* infection in mice [188]. We propose that *H. pylori* infection induces a stronger activation of *chit1* and *iigp1* in PPARγ-deficient macrophages, thus resulting in decreased bacterial replication and faster clearance after infection. However, failure to completely eliminate the microorganism suggests the involvement of other factors in the survival and replication of *H. pylori* in the absence of PPARγ.

Studying the immune responses to intracellular *H. pylori* has the potential to be paradigm-shifting in terms of understanding the mechanisms of host response during infection. A better understanding of the immunoregulatory mechanisms underlying immune responses to *H. pylori* has the potential to open a new host-targeted therapeutics-based era that limits the pathogenic effects of the bacterium while maintaining the protective effects of this indigenous gastric organism.

4.13 Materials and Methods

Generation of Bone Marrow Derived Macrophages

Macrophages were differentiated from bone marrow (BM) progenitor cells of WT and LysMcre mice. Briefly, femur and tibia were excised, washed in 70% EtOH and cold 1xPBS. BM progenitors were flushed out with cRPMI_M (RPMI 1640, 10% FBS, 2.5% Hepes, 1% Sodium pyruvate, 1% Pen/Strep, 1% L-Glutamine, 50uM 2-mercaptoethanol), filtered through a 100µm cell strainer and centrifuged for 8 min at 1,200 rpm at 4°C. Red blood cells were eliminated by osmotic lysis and cell suspensions were filtered and centrifuged again as described above. BM progenitors were adjusted to $1x10^6$ cells/mL with cRPMI_M containing 35ng/mL recombinant mouse M-CSF (Peprotech). Cells were plated in 100mm petri dishes and incubated at 37°C, 95% humidity and 5% CO_2. Fresh media containing M-CSF was added on day 3. On day 6, non-adherent cells were removed and bone marrow derived macrophages (BMDM) were detached by incubation in ice cold 1xPBS at 4°-8°C for 10 minutes. Cells were carefully removed from the dish by flushing them off the culture dish. BMDM were centrifuged, resuspended in fresh cRPMI_M without antibiotics and used for the Gentamicin infiltration assay.

Gentamicin protection assay

BMDM cell suspensions were resuspended in cRPMI without antibiotics and seeded in triplicate in 12-well plates ($2.5x10^5$ for re-isolation experiments, $1x10^6$ cells for RNA isolation). Cells were left to adhere overnight at 37°C, 95% humidity and 5% CO_2. The following day, cells were infected with *H. pylori* SS1 at a MOI 10 and synchronized by quick spun to ensure immediate contact. For iNOS inhibition experiments, cells were incubated with the selective iNOS inhibitor L-N6-(1-Iminoethyl)lysine dihydrochloride (L-NIL) at 100µM for 30 min. In some experiments, WT BMDM were treated with the

PPARγ antagonist GW9662 (10µM) for 2 hours prior to bacterial co-culture. In the case of M1 induction BMDM were pre-treated with LPS (100ng/ml) and rIFN-γ (100ng/ml) overnight.

After incubation for 15 minutes at 37°C, 95% humidity, and 5% CO_2, non-internalized bacteria were killed by washing the cells three times with PBS/5%FBS containing 100ng/ml Gentamicin. Cells for time-point 0 were washed with 1xPBS and immediately collected for downstream assays. The cells allocated for the remaining time points (15 min to 24 hours) were covered with culture media supplemented with 100ng/ml Gentamicin to kill extracellular *H. pylori*. Cells were incubated for the indicated time-points and then collected as described below.

Bacterial re-isolation from cells

For re-isolation of *H. pylori* from cells, BMDM were washed three times with 1xPBS and collected in 100µl of sterile Brucella broth. Cells were scraped off the well and sonicated for 5 sec to release intracellular bacteria and serial dilutions were plated as described above.

NFκB Reporter Assay

NFκB activity was assessed using RAW-Blue™ cells (Invivogen) which stably express the secreted embryonic alkaline phosphatase (SEAP) gene inducible by NFκB and AP-1 transcription factors through binding of TLR2, TLR4 and NOD. Cells were pre-treated with GW9662 (10µM) for 2 hours or remained untreated and were subsequently co-cultured with *H. pylori* as described in the Gentamicin infiltration assay. Some cells were treated with 10µM of the PPARγ agonist rosiglitazone (ROS) at the same time of bacterial infection. NFκB activity was assessed by colorimetric detection of SEAP levels. Culture supernatants from different time-points after the start of gentamicin treatment were incubated with QuantiBlue detection solution. The optical density at 630nm was measured on a Modulus Microplate Reader.

Assessment of differential gene expression by qRT-PCR

Cells for RNA isolation were washed with ice cold 1xPBS and collected in 350µl of RLT buffer supplemented with 1% β-mercaptoethanol. RNA was isolated from BMDM using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Total mRNA concentrations were quantified by optical density at 260nm with a Nanodrop (Invitrogen).

500ng of RNA per sample were used to synthesize cDNA using iSCRIPT cDNA Synthesis Kit (Bio-Rad) and stored at -20ºC. qRT-PCR was performed to assess the absolute expression of iNOS, IFNγ, sqle, iigp1 and chit1. Standard curves were created using diluted gene amplicon at known concentrations ranging from 5 to $5x10^{-6}$ pg per reaction. RT-PCR was performed using a CFX96 Real Time System (BioRad). Target gene expression was normalized to the housekeeping gene GAPDH.

Whole transcriptome gene expression assessment

RNA isolated from WT and PPARγ-deficient BMDM collected at time-points 0, 30, 60, 120, 240, 360 and 720 minutes post-infection was submitted for whole transcriptome gene expression assessment using Illumina Hiseq (Virginia Bioinformatics Institute Core Lab Facilities). Once fastq files containing 100bp-long pair-end reads were received, poor quality reads (>40% of bases with PHRED score <10; percentage of N greater than 5%; and polyA reads) were filtered out. The remaining reads were mapped to RefSeq (mm10 from http://genome.ucsc.edu/) using Bowtie [189] (version: 1.0.0) with parameters set to '-l 25 -I 1 -X 1000 -a -m 200'. To calculate gene expression levels, RSEM [190] was used, a program based on expectation-maximization algorithm. FPKM [191] (fragments per kilobase per million sequenced reads) was used as the measurement of expression level. All genes with median expression level in all samples greater than 0 were included in a 3-way (genotype, treatment and time) ANOVA analysis. Normal quantile transformation (qqnorm from R [182]) was used to normalize the FPKM to fit the normality assumption of ANOVA (tested with Kolmogorov-Smirnov test). The 3-way ANOVA analysis was carried in R [182], FDR [183] and Bonferroni were used to calculate the adjusted *P*-values. Data was submitted to NCBI's GEO database (Accession Number GSE67270).

mRNAseq validation by in vitro gene silencing

WT and PPARγ-null BMDM were transfected with 10nM siRNA (either siRNA for sqle, iigp1, chit1 or scramble) using Lipofectamine 2000 (Invivogen) 24h before infection. Cells were infected following the gentamycin assay described above, and harvested at 2h post-infection to assess intracellular bacterial loads (2h – corresponding to the peak of *H. pylori* intracellular loads as seen in previous time-course studies). Gene knock-down was validated by qRT-PCR.

Data analyses

***Ingenuity Pathway Analysis*:** IPA, version 7 (Ingenuity Systems, USA; www.analysis.ingenuity.com) was performed to identify the molecular pathways and functional groupings based on published literature for the significantly DE mRNAs. Expression data was uploaded into IPA and overlaid onto a global molecular network developed from information contained in the application. Networks of these genes were generated by IPA based on their connectivity, each ranked by a score. This score is based on the hypergeometric distribution, calculated with the right-tailed Fisher's Exact Test, and corresponds to the negative log of this p-value. Functional analysis in IPA identified the published biological functions and canonical pathways that were most significantly associated with the genes in the network. Genes or gene products are represented as nodes, where shape indicates functional groups, and the biological relationship between two nodes is represented as an edge (line). All lines are supported by at least one reference in literature, textbook, or from canonical information stored in the Ingenuity Pathways knowledge database.

Database for Annotation, Visualization and Integrated Discovery: DAVID [192, 193], version 6.7, is an open-source integrated biological knowledgebase and analytic tool that was used to perform Gene Ontology enrichment in our dataset and cluster the differentially expressed genes based on GO Terms, or in other words, molecular function.

***BinGO*:** BinGO [194] is an open-source bioinformatics software platform implemented as a Cytoscape [195] plugin used to determine statistically overrepresented GO categories in our dataset and to visualize and integrate molecular interaction networks.

Statistics

Data are expressed as mean and standard error of the mean. Parametric data were analyzed by using the Analysis of Variance (ANOVA) followed by Scheffe's multiple comparison test as previously described [53]. Analysis of variance (ANOVA) was performed by using the general linear model procedure of SAS, release 9.2 (SAS Institute Inc., Cary, NC). A 2x2 factorial arrangement comparing genotype and infection treatment was employed. Statistical significant was determined at $P \leq 0.05$.

Chapter 5

Concluding Remarks

The culmination of this work can be summed in several separate accomplishments. The first chapter provides an overview of mucosal immunology and highlights the need to develop novel therapeutic strategies that limit the pathogenic effects of invading bacteria while maintaining their protective functions. The mucosal immune system has the daunting task of maintaining immune homeostasis by eliminating potentially harmful microorganisms and limiting tissue injury while inducing tolerogenic responses to luminal antigens such as innocuous food, commensal microbiota and self-antigens. The gastrointestinal tract houses the largest reservoir of immune cells in the body, which carefully orchestrate and balance down-regulatory mechanisms of tolerance under normal conditions and proper inflammation-mediated defensive responses during disease. Changes in such delicate balance are linked to the development of gastrointestinal pathology as well as systemic disease states. Despite its critical role in human health and disease, there is still an insufficient mechanistic understanding on how enteric pathogens are able to modulate mucosal immune responses and how such responses influence disease outcome and contribute to gastrointestinal and systemic pathogenesis. Moreover, there is a lack of successful treatments for the management of several acute and chronic inflammatory gastrointestinal infections and immune-mediated disorders. In this regard, this dissertation presents a systematic effort to generate novel mechanistic hypothesis based on computational predictions and experimentally elucidate the mechanisms of action underlying mucosal immune responses and

pathology in the gut. By using model enteric microorganisms *Helicobacter pylori* and *Clostridium difficile*, this thesis aims to build-on and contribute to our understanding of immunoregulatory mechanisms underlying immune responses to gastroenteric infections and introduce a potential new host-targeted therapeutics-based era that limits the pathogenic effects of the invading bacteria and reduces disease incidence.

In Chapter 2, I discuss the mechanisms underlying PPARγ modulation of mucosal immune responses to *C. difficile*, including a possible relationship between nuclear receptors and miRNAs. Specifically, we applied mathematical and computational modeling approaches in combination with mouse challenge studies to study the mechanisms underlying the interactions between PPARγ activity and miRNA-146b to regulate colitis during *C. difficile* infection. Next, we investigated how either T cell-specific deletion or pharmacological activation of PPARγ modulate colonic inflammatory cytokines and effector Th17 responses to *C. difficile* infection in mice. These data indicate that T cell PPARγ prevents colitis and down-modulates effector T cell responses in mice with CDAD and suggest a potential crosstalk between miRNAs and the PPARγ pathway. Although it was only 10 years ago that the first human miRNA was described, changes in their expression have already been correlated to the pathogenesis of several diseases including viral infections [196], cancer [197], immune-mediated diseases [198, 199] and enteric infections [200]. There have been plenty of studies correlating miRNAs and enteric disease pathogenesis during the last years, as illustrated by the 50-fold increase in the number of miRNA publications within the last decade. However, this is the first report describing the modulation of CDI by miRNAs and the disruption of regulatory TFs such as PPARs by miR-146b in mice [201]. Understanding the role of miRNAs in antibacterial immune and inflammatory responses holds promise of new molecular diagnostic markers as well as novel gene therapy strategies for treating bacterial infections and associated immunopathologies.

Chapter 3 describes the mechanisms by which some invading bacteria are able to modulate immune responses and successfully establish chronic infection. Based on in-house *in silico* predictions pointing at macrophages as central regulators of chronic microbial persistence and immunoregulation, we experimentally investigated the role of the gastric myeloid compartment during infection. By using a murine model of *H. pylori* enteric infection, we identified and characterized a CD64+CX_3CR1+ macrophage subset

in the gastric mucosa of *H. pylori*-infected mice. The recruitment and expansion of such cell subset by *H. pylori* enables the induction of potent local and systemic IL-10-mediated regulatory responses that create a favorable microenvironment for the survival, replication and consequently chronic colonization of *H. pylori* by limiting effector inflammatory responses required for bacterial clearance, thus favoring tolerance over immunity. CD64+CX_3CR1+ macrophages seem to be essential to ensure long-term *H. pylori* survival as well as to promote tolerogenic responses during infection. Therefore, CD64+CX_3CR1+ macrophages are critical contributors to the immunomodulatory properties of *H. pylori* and modulating their function could be exploited as tolerization and inflammation regulation strategies aiming to ameliorate not only *H. pylori* infection but also other extra-gastric disease conditions.

Finally, and simultaneously advancing along with the studies described in Chapter 3, Chapter 4 investigates the potential role of *H. pylori* as an intracellular facultative pathogen. Specifically, we have developed a gentamycin protection co-culture assay to explore whether *H. pylori* is able to replicate in macrophages and the type of response induced by such intracellular infection. Interestingly, macrophage phenotype shift to a pro-inflammatory state by deleting the regulatory transcription factor PPARγ diminished intracellular bacterial levels, which correlated with increased iNOS production and NF-KB activity. Moreover, by using RNAseq analyses and gene-targeted knock-down studies, we were able to identify two genes, *chit1* and *iigp1,* that represent promising host response targets during intracellular *H.pylori* infection in macrophages. Overall, macrophages emerge as absolutely critical contributors to intracellular bacterial survival and replication. However, and before making any conclusions, such observations should be translated into the *in vivo* gastric environment. Nonetheless, studying the immune responses to intracellular *H. pylori* has the potential to be paradigm-shifting in terms of understanding the mechanisms of host response during infection. Additionally, it may result in the development of novel therapeutic strategies that modulate the immune response and prevent *H. pylori*-associated malignancies but preserve the beneficial effects of this fascinating bacterium.

I believe that the knowledge generated throughout this dissertation exemplifies how a combination of computational modeling, immunoinformatics and experimental immunology holds enormous potential for discovering unforeseen host targets and

developing novel vaccines and cures for infectious, allergic and immune-mediated diseases.

References

1. Xavier RJ, Podolsky DK: **Unravelling the pathogenesis of inflammatory bowel disease**. *Nature* 2007, **448**(7152):427-434.
2. Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT: **Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota**. *Immunol Rev* 2005, **206**:260-276.
3. Cho JH: **The genetics and immunopathogenesis of inflammatory bowel disease**. *Nat Rev Immunol* 2008, **8**(6):458-466.
4. Hall IC OTE: **Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, Bacillus difficilis.** *Am J Dis Child* 1935, **49**:390-402.
5. Bartlett JG: **Clostridium difficile: history of its role as an enteric pathogen and the current state of knowledge about the organism**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1994, **18 Suppl 4**:S265-272.
6. Lo Vecchio A, Zacur GM: **Clostridium difficile infection: an update on epidemiology, risk factors, and therapeutic options**. *Curr Opin Gastroenterol* 2012, **28**(1):1-9.
7. Barbut F, Jones G, Eckert C: **Epidemiology and control of Clostridium difficile infections in healthcare settings: an update**. *Current opinion in infectious diseases* 2011, **24**(4):370-376.
8. Rupnik M, Wilcox MH, Gerding DN: **Clostridium difficile infection: new developments in epidemiology and pathogenesis**. *Nature reviews Microbiology* 2009, **7**(7):526-536.
9. Urban E, Terhes G, Markotics A, Soki J, Nagy E: **Rare extraintestinal infection caused by toxin-producing Clostridium difficile**. *Anaerobe* 2010, **16**(3):301-303.
10. Lavallee C, Laufer B, Pepin J, Mitchell A, Dube S, Labbe AC: **Fatal Clostridium difficile enteritis caused by the BI/NAP1/027 strain: a case series of ileal C. difficile infections**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2009, **15**(12):1093-1099.

11. Boice JL: **Reactive arthritis induced by Clostridium difficile**. *The Western journal of medicine* 1994, **160**(2):171-172.
12. Croagh DG, Bach SP, Keck J: **A rare cause of acute abdomen after proctocolectomy**. *American journal of surgery* 2009, **197**(4):e41-42.
13. Libby DB, Bearman G: **Bacteremia due to Clostridium difficile--review of the literature**. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* 2009, **13**(5):e305-309.
14. Fluit AC, Wolfhagen MJ, Verdonk GP, Jansze M, Torensma R, Verhoef J: **Nontoxigenic strains of Clostridium difficile lack the genes for both toxin A and toxin B**. *Journal of clinical microbiology* 1991, **29**(11):2666-2667.
15. Bartlett JG, Onderdonk AB, Cisneros RL, Kasper DL: **Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters**. *The Journal of infectious diseases* 1977, **136**(5):701-705.
16. George RH, Symonds JM, Dimock F, Brown JD, Arabi Y, Shinagawa N, Keighley MR, Alexander-Williams J, Burdon DW: **Identification of Clostridium difficile as a cause of pseudomembranous colitis**. *British medical journal* 1978, **1**(6114):695.
17. Kim JW, Lee KL, Jeong JB, Kim BG, Shin S, Kim JS, Jung HC, Song IS: **Proton pump inhibitors as a risk factor for recurrence of Clostridium-difficile-associated diarrhea**. *World J Gastroenterol* 2010, **16**(28):3573-3577.
18. Hookman P, Barkin JS: **Clostridium difficile associated infection, diarrhea and colitis**. *World journal of gastroenterology : WJG* 2009, **15**(13):1554-1580.
19. Vaishnavi C: **Established and potential risk factors for Clostridum difficile infection**. *Indian journal of medical microbiology* 2009, **27**(4):289-300.
20. Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St Sauver JL, Harmsen WS, Zinsmeister AR: **The epidemiology of community-acquired Clostridium difficile infection: a population-based study**. *The American journal of gastroenterology* 2012, **107**(1):89-95.
21. Kuehn BM: **Scientists seek strategies to prevent Clostridium difficile infections**. *JAMA* 2011, **306**(17):1849-1850.
22. Lim PL, Barkham TM, Ling LM, Dimatatac F, Alfred T, Ang B: **Increasing incidence of Clostridium difficile-associated disease, Singapore**. *Emerging infectious diseases* 2008, **14**(9):1487-1489.
23. Khanna S, Pardi DS: **The growing incidence and severity of Clostridium difficile infection in inpatient and outpatient settings**. *Expert review of gastroenterology & hepatology* 2010, **4**(4):409-416.
24. Zilberberg MD, Shorr AF, Kollef MH: **Increase in adult Clostridium difficile-related hospitalizations and case-fatality rate, United States, 2000-2005**. *Emerging infectious diseases* 2008, **14**(6):929-931.
25. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM, Nguyen T, Frenette C, Kelly M *et al*: **A predominantly clonal multi-institutional outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality**. *The New England journal of medicine* 2005, **353**(23):2442-2449.
26. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pepin K, Chouinard D: **Clostridium difficile-associated diarrhea in a region of Quebec**

from 1991 to 2003: a changing pattern of disease severity. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* 2004, **171**(5):466-472.

27. McDonald LC, Killgore GE, Thompson A, Owens RC, Jr., Kazakova SV, Sambol SP, Johnson S, Gerding DN: **An epidemic, toxin gene-variant strain of Clostridium difficile**. *The New England journal of medicine* 2005, **353**(23):2433-2441.
28. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, Drudy D, Fitzpatrick F, Wiuff C, Brown DJ *et al*: **Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008**. *Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin* 2008, **13**(31).
29. Borgmann S, Kist M, Jakobiak T, Reil M, Scholz E, von Eichel-Streiber C, Gruber H, Brazier JS, Schulte B: **Increased number of Clostridium difficile infections and prevalence of Clostridium difficile PCR ribotype 001 in southern Germany**. *Euro surveillance : bulletin europeen sur les maladies transmissibles = European communicable disease bulletin* 2008, **13**(49).
30. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, Bergwerff AA, Dekker FW, Kuijper EJ: **Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2008, **47**(9):1162-1170.
31. Pawlowski SW, Archbald-Pannone L, Carman RJ, Alcantara-Warren C, Lyerly D, Genheimer CW, Gerding DN, Guerrant RL: **Elevated levels of intestinal inflammation in Clostridium difficile infection associated with fluoroquinolone-resistant C. difficile**. *J Hosp Infect* 2009, **73**(2):185-187.
32. Weiss K, Boisvert A, Chagnon M, Duchesne C, Habash S, Lepage Y, Letourneau J, Raty J, Savoie M: **Multipronged intervention strategy to control an outbreak of Clostridium difficile infection (CDI) and its impact on the rates of CDI from 2002 to 2007**. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* 2009, **30**(2):156-162.
33. Dubberke ER, Butler AM, Reske KA, Agniel D, Olsen MA, D'Angelo G, McDonald LC, Fraser VJ: **Attributable outcomes of endemic Clostridium difficile-associated disease in nonsurgical patients**. *Emerging infectious diseases* 2008, **14**(7):1031-1038.
34. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC: **Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe**. *Lancet* 2005, **366**(9491):1079-1084.
35. Stolte M: **Helicobacter pylori gastritis and gastric MALT-lymphoma**. *Lancet* 1992, **339**(8795):745-746.
36. Pernitzsch SR, Sharma CM: **Transcriptome complexity and riboregulation in the human pathogen Helicobacter pylori**. *Frontiers in cellular and infection microbiology* 2012, **2**:14.

37. Bruce MG, Maaroos HI: **Epidemiology of Helicobacter pylori infection**. *Helicobacter* 2008, **13 Suppl 1**:1-6.
38. Makola D, Peura DA, Crowe SE: **Helicobacter pylori infection and related gastrointestinal diseases**. *Journal of clinical gastroenterology* 2007, **41**(6):548-558.
39. Amieva MR, El-Omar EM: **Host-bacterial interactions in Helicobacter pylori infection**. *Gastroenterology* 2008, **134**(1):306-323.
40. Suerbaum S, Michetti P: **Helicobacter pylori infection**. *The New England journal of medicine* 2002, **347**(15):1175-1186.
41. Asaka M, Kato M, Sakamoto N: **Roadmap to eliminate gastric cancer with Helicobacter pylori eradication and consecutive surveillance in Japan**. *J Gastroenterol* 2013.
42. Pacifico L, Anania C, Osborn JF, Ferraro F, Chiesa C: **Consequences of Helicobacter pylori infection in children**. *World journal of gastroenterology : WJG* 2010, **16**(41):5181-5194.
43. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, Muller A: **Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells**. *The Journal of clinical investigation* 2011, **121**(8):3088-3093.
44. Selgrad M, Bornschein J, Kandulski A, Hille C, Weigt J, Roessner A, Wex T, Malfertheiner P: **Helicobacter pylori but not gastrin is associated with the development of colonic neoplasms**. *International journal of cancer Journal international du cancer* 2014, **135**(5):1127-1131.
45. Bassaganya-Riera J, Dominguez-Bello MG, Kronsteiner B, Carbo A, Lu P, Viladomiu M, Pedragosa M, Zhang X, Sobral BW, Mane SP *et al*: **Helicobacter pylori colonization ameliorates glucose homeostasis in mice through a PPAR gamma-dependent mechanism**. *PloS one* 2012, **7**(11):e50069.
46. Cook KW, Crooks J, Hussain K, O'Brien K, Braitch M, Kareem H, Constantinescu CS, Robinson K, Gran B: **Helicobacter pylori infection reduces disease severity in an experimental model of multiple sclerosis**. *Frontiers in microbiology* 2015, **6**:52.
47. Engler DB, Reuter S, van Wijck Y, Urban S, Kyburz A, Maxeiner J, Martin H, Yogev N, Waisman A, Gerhard M *et al*: **Effective treatment of allergic airway inflammation with Helicobacter pylori immunomodulators requires BATF3-dependent dendritic cells and IL-10**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**(32):11810-11815.
48. Vaishnavi C: **Clinical spectrum & pathogenesis of Clostridium difficile associated diseases**. *The Indian journal of medical research* 2010, **131**:487-499.
49. Akerlund T, Persson I, Unemo M, Noren T, Svenungsson B, Wullt M, Burman LG: **Increased sporulation rate of epidemic Clostridium difficile Type 027/NAP1**. *Journal of clinical microbiology* 2008, **46**(4):1530-1533.
50. Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G: **Human hypervirulent Clostridium difficile strains exhibit increased sporulation as well as robust toxin production**. *Journal of bacteriology* 2010, **192**(19):4904-4911.

51. McFarland LV: **Alternative treatments for Clostridium difficile disease: what really works?** *Journal of medical microbiology* 2005, **54**(Pt 2):101-111.
52. Ryan A, Lynch M, Smith SM, Amu S, Nel HJ, McCoy CE, Dowling JK, Draper E, O'Reilly V, McCarthy C *et al*: **A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins**. *PLoS pathogens* 2011, **7**(6):e1002076.
53. Bassaganya-Riera J, Reynolds K, Martino-Catt S, Cui Y, Hennighausen L, Gonzalez F, Rohrer J, Benninghoff AU, Hontecillas R: **Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease**. *Gastroenterology* 2004, **127**(3):777-791.
54. Li M, Pascual G, Glass CK: **Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene**. *Molecular and cellular biology* 2000, **20**(13):4699-4707.
55. Hontecillas R, Bassaganya-Riera J: **Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis**. *Journal of immunology* 2007, **178**(5):2940-2949.
56. Bassaganya-Riera J, Hontecillas R: **CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD**. *Clinical nutrition* 2006, **25**(3):454-465.
57. Lewis JD, Lichtenstein GR, Deren JJ, Sands BE, Hanauer SB, Katz JA, Lashner B, Present DH, Chuai S, Ellenberg JH *et al*: **Rosiglitazone for active ulcerative colitis: a randomized placebo-controlled trial**. *Gastroenterology* 2008, **134**(3):688-695.
58. Bassaganya-Riera J, Hontecillas R, Horne WT, Sandridge M, Herfarth HH, Bloomfeld R, Isaacs KL: **Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease**. *Clinical nutrition* 2012.
59. Guri AJ, Mohapatra SK, Horne WT, 2nd, Hontecillas R, Bassaganya-Riera J: **The role of T cell PPAR gamma in mice with experimental inflammatory bowel disease**. *BMC gastroenterology* 2010, **10**:60.
60. Hontecillas R, Horne WT, Climent M, Guri AJ, Evans C, Zhang Y, Sobral BW, Bassaganya-Riera J: **Immunoregulatory mechanisms of macrophage PPAR-gamma in mice with experimental inflammatory bowel disease**. *Mucosal immunology* 2011, **4**(3):304-313.
61. Mohapatra SK, Guri AJ, Climent M, Vives C, Carbo A, Horne WT, Hontecillas R, Bassaganya-Riera J: **Immunoregulatory actions of epithelial cell PPAR gamma at the colonic mucosa of mice with experimental inflammatory bowel disease**. *PloS one* 2010, **5**(4):e10215.
62. Klotz L, Burgdorf S, Dani I, Saijo K, Flossdorf J, Hucke S, Alferink J, Nowak N, Beyer M, Mayer G *et al*: **The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity**. *The Journal of experimental medicine* 2009, **206**(10):2079-2089.
63. Lewis BP, Burge CB, Bartel DP: **Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets**. *Cell* 2005, **120**(1):15-20.

64. Hassan T, McKiernan PJ, McElvaney NG, Cryan SA, Greene CM: **Therapeutic modulation of miRNA for the treatment of proinflammatory lung diseases**. *Expert review of anti-infective therapy* 2012, **10**(3):359-368.
65. Papaioannou MD, Nef S: **microRNAs in the testis: building up male fertility**. *Journal of andrology* 2010, **31**(1):26-33.
66. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function**. *Cell* 2004, **116**(2):281-297.
67. Dalal SR, Kwon JH: **The Role of MicroRNA in Inflammatory Bowel Disease**. *Gastroenterology & hepatology* 2010, **6**(11):714-722.
68. Chen CZ, Li L, Lodish HF, Bartel DP: **MicroRNAs modulate hematopoietic lineage differentiation**. *Science* 2004, **303**(5654):83-86.
69. Sonkoly E, Stahle M, Pivarcsi A: **MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation**. *Seminars in cancer biology* 2008, **18**(2):131-140.
70. Oertli M, Engler DB, Kohler E, Koch M, Meyer TF, Muller A: **MicroRNA-155 is essential for the T cell-mediated control of Helicobacter pylori infection and for the induction of chronic Gastritis and Colitis**. *Journal of immunology* 2011, **187**(7):3578-3586.
71. Kuntz JL, Chrischilles EA, Pendergast JF, Herwaldt LA, Polgreen PM: **Incidence of and risk factors for community-associated Clostridium difficile infection: a nested case-control study**. *BMC infectious diseases* 2011, **11**:194.
72. Blondeau JM: **What have we learned about antimicrobial use and the risks for Clostridium difficile-associated diarrhoea?** *The Journal of antimicrobial chemotherapy* 2009, **63**(2):238-242.
73. Kyne L, Sougioultzis S, McFarland LV, Kelly CP: **Underlying disease severity as a major risk factor for nosocomial Clostridium difficile diarrhea**. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* 2002, **23**(11):653-659.
74. Wohlfert EA, Nichols FC, Nevius E, Clark RB: **Peroxisome proliferator-activated receptor gamma (PPARgamma) and immunoregulation: enhancement of regulatory T cells through PPARgamma-dependent and -independent mechanisms**. *J Immunol* 2007, **178**(7):4129-4135.
75. Moschos SA, Williams AE, Perry MM, Birrell MA, Belvisi MG, Lindsay MA: **Expression profiling in vivo demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids**. *BMC genomics* 2007, **8**:240.
76. Schmidt WM, Spiel AO, Jilma B, Wolzt M, Muller M: **In vivo profile of the human leukocyte microRNA response to endotoxemia**. *Biochemical and biophysical research communications* 2009, **380**(3):437-441.
77. Lederhuber H, Baer K, Altiok I, Sadeghi K, Herkner KR, Kasper DC: **MicroRNA-146: tiny player in neonatal innate immunity?** *Neonatology* 2011, **99**(1):51-56.
78. Taganov KD, Boldin MP, Chang KJ, Baltimore D: **NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(33):12481-12486.

79. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ: **miRBase: tools for microRNA genomics**. *Nucleic acids research* 2008, **36**(Database issue):D154-158.
80. Akiyama TE, Sakai S, Lambert G, Nicol CJ, Matsusue K, Pimprale S, Lee YH, Ricote M, Glass CK, Brewer HB, Jr. *et al*: **Conditional disruption of the peroxisome proliferator-activated receptor gamma gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux**. *Molecular and cellular biology* 2002, **22**(8):2607-2619.
81. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L: **Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice**. *Transgenic research* 2001, **10**(6):545-553.
82. Cui Y, Miyoshi K, Claudio E, Siebenlist UK, Gonzalez FJ, Flaws J, Wagner KU, Hennighausen L: **Loss of the peroxisome proliferation-activated receptor gamma (PPARgamma) does not affect mammary development and propensity for tumor formation but leads to reduced fertility**. *The Journal of biological chemistry* 2002, **277**(20):17830-17835.
83. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP: **A mouse model of Clostridium difficile-associated disease**. *Gastroenterology* 2008, **135**(6):1984-1992.
84. Artzi S, Kiezun A, Shomron N: **miRNAminer: a tool for homologous microRNA gene search**. *BMC bioinformatics* 2008, **9**:39.
85. Garbacki N, Di Valentin E, Huynh-Thu VA, Geurts P, Irrthum A, Crahay C, Arnould T, Deroanne C, Piette J, Cataldo D *et al*: **MicroRNAs profiling in murine models of acute and chronic asthma: a relationship with mRNAs targets**. *PloS one* 2011, **6**(1):e16509.
86. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS: **Human MicroRNA targets**. *PLoS biology* 2004, **2**(11):e363.
87. Hontecillas R, Wannemeulher MJ, Zimmerman DR, Hutto DL, Wilson JH, Ahn DU, Bassaganya-Riera J: **Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid**. *The Journal of nutrition* 2002, **132**(7):2019-2027.
88. Heinlein CA, Ting HJ, Yeh S, Chang C: **Identification of ARA70 as a ligand-enhanced coactivator for the peroxisome proliferator-activated receptor gamma**. *The Journal of biological chemistry* 1999, **274**(23):16147-16152.
89. Jones JR, Barrick C, Kim KA, Lindner J, Blondeau B, Fujimoto Y, Shiota M, Kesterson RA, Kahn BB, Magnuson MA: **Deletion of PPARgamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(17):6207-6212.
90. Hevener AL, Olefsky JM, Reichart D, Nguyen MT, Bandyopadyhay G, Leung HY, Watt MJ, Benner C, Febbraio MA, Nguyen AK *et al*: **Macrophage PPAR gamma is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones**. *The Journal of clinical investigation* 2007, **117**(6):1658-1669.

91. Guri AJ, Hontecillas R, Bassaganya-Riera J: **Peroxisome proliferator-activated receptors: Bridging metabolic syndrome with molecular nutrition**. *Clin Nutr* 2006, **25**(6):871-885.
92. Guri AJ, Hontecillas R, Ferrer G, Casagran O, Wankhade U, Noble AM, Eizirik DL, Ortis F, Cnop M, Liu D *et al*: **Loss of PPAR gamma in immune cells impairs the ability of abscisic acid to improve insulin sensitivity by suppressing monocyte chemoattractant protein-1 expression and macrophage infiltration into white adipose tissue**. *J Nutr Biochem* 2008, **19**(4):216-228.
93. Guri AJ, Hontecillas R, Si H, Liu D, Bassaganya-Riera J: **Dietary abscisic acid ameliorates glucose tolerance and obesity-related inflammation in db/db mice fed high-fat diets**. *Clin Nutr* 2007, **26**(1):107-116.
94. Guri AJ, Misyak SA, Hontecillas R, Hasty A, Liu D, Si H, Bassaganya-Riera J: **Abscisic acid ameliorates atherosclerosis by suppressing macrophage and CD4+ T cell recruitment into the aortic wall**. *J Nutr Biochem* 2010, **21**(12):1178-1185.
95. Bassaganya-Riera J, Misyak S, Guri AJ, Hontecillas R: **PPAR gamma is highly expressed in F4/80(hi) adipose tissue macrophages and dampens adipose-tissue inflammation**. *Cell Immunol* 2009, **258**(2):138-146.
96. Bassaganya-Riera J, Hontecillas R, Zimmerman DR, Wannemuehler MJ: **Dietary conjugated linoleic acid modulates phenotype and effector functions of porcine cd8(+) lymphocytes**. *J Nutr* 2001, **131**(9):2370-2377.
97. Bassaganya-Riera J, Pogranichniy RM, Jobgen SC, Halbur PG, Yoon KJ, O'Shea M, Mohede I, Hontecillas R: **Conjugated linoleic acid ameliorates viral infectivity in a pig model of virally induced immunosuppression**. *J Nutr* 2003, **133**(10):3204-3214.
98. Paranavitana C, Pittman PR, Velauthapillai M, Zelazowska E, Dasilva L: **Transcriptional profiling of Francisella tularensis infected peripheral blood mononuclear cells: a predictive tool for tularemia**. *FEMS Immunol Med Microbiol* 2008, **54**(1):92-103.
99. Aldridge JR, Jr., Moseley CE, Boltz DA, Negovetich NJ, Reynolds C, Franks J, Brown SA, Doherty PC, Webster RG, Thomas PG: **TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection**. *Proc Natl Acad Sci U S A* 2009, **106**(13):5306-5311.
100. Mohapatra SK, Cole LE, Vogel SN, Evans C, Sobral BW, Bassaganya-Riera J, Hontecillas R, Castra OR: **Murine hepatic gene expression changes during LPS-induced protection of host from infection with Francisella tularensis LVS**. *BMC Infectious Diseases* 2010, **In Press**.
101. Bassaganya-Riera J, Hontecillas R: **CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD**. *Clin Nutr* 2006, **25**(3):454-465.
102. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD: **A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response**. *The Journal of clinical investigation* 1999, **104**(4):383-389.

103. Desreumaux P, Dubuquoy L, Nutten S, Peuchmaur M, Englaro W, Schoonjans K, Derijard B, Desvergne B, Wahli W, Chambon P *et al*: **Attenuation of colon inflammation through activators of the retinoid X receptor (RXR)/peroxisome proliferator-activated receptor gamma (PPARgamma) heterodimer. A basis for new therapeutic strategies**. *The Journal of experimental medicine* 2001, **193**(7):827-838.
104. Lytle C, Tod TJ, Vo KT, Lee JW, Atkinson RD, Straus DS: **The peroxisome proliferator-activated receptor gamma ligand rosiglitazone delays the onset of inflammatory bowel disease in mice with interleukin 10 deficiency**. *Inflammatory bowel diseases* 2005, **11**(3):231-243.
105. Dubuquoy L, Rousseaux C, Thuru X, Peyrin-Biroulet L, Romano O, Chavatte P, Chamaillard M, Desreumaux P: **PPARgamma as a new therapeutic target in inflammatory bowel diseases**. *Gut* 2006, **55**(9):1341-1349.
106. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR: **Interleukin-10**. *Annual review of immunology* 1993, **11**:165-190.
107. Miossec P, Korn T, Kuchroo VK: **Interleukin-17 and type 17 helper T cells**. *The New England journal of medicine* 2009, **361**(9):888-898.
108. Korn T, Bettelli E, Oukka M, Kuchroo VK: **IL-17 and Th17 Cells**. *Annual review of immunology* 2009, **27**:485-517.
109. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ: **TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology**. *Nature immunology* 2007, **8**(12):1390-1397.
110. Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T: **Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells**. *Proceedings of the National Academy of Sciences* 2008, **105**(28):9721-9726.
111. Li B, Reynolds JM, Stout RD, Bernlohr DA, Suttles J: **Regulation of Th17 Differentiation by Epidermal Fatty Acid-Binding Protein**. *J Immunol* 2009, **182**(12):7625-7633.
112. Korn T, Bettelli E, Oukka M, Kuchroo VK: **IL-17 and Th17 Cells**. *Annual Review of Immunology* 2009, **27**(1):485-517.
113. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, Soumelis V: **A critical function for transforming growth factor-[beta], interleukin 23 and proinflammatory cytokines in driving and modulating human TH-17 responses**. *Nat Immunol* 2008, **9**(6):650-657.
114. Funahashi A, Jouraku A, Matsuoka Y, Kitano H: **Integration of CellDesigner and SABIO-RK**. *In silico biology* 2007, **7**(2 Suppl):S81-90.
115. Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N, Singhal M, Xu L, Mendes P, Kummer U: **COPASI--a COmplex PAthway SImulator**. *Bioinformatics* 2006, **22**(24):3067-3074.
116. Polk DB, Peek RM, Jr.: **Helicobacter pylori: gastric cancer and beyond**. *Nature reviews Cancer* 2010, **10**(6):403-414.
117. Cherdantseva LA, Potapova OV, Sharkova TV, Belyaeva YY, Shkurupiy VA: **Association of Helicobacter pylori and iNOS production by macrophages and**

lymphocytes in the gastric mucosa in chronic gastritis. *Journal of immunology research* 2014, **2014**:762514.

118. Lundgren A, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS: **Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. pylori in infected individuals**. *Infection and immunity* 2003, **71**(4):1755-1762.
119. Raghavan S, Suri-Payer E, Holmgren J: **Antigen-specific in vitro suppression of murine Helicobacter pylori-reactive immunopathological T cells by CD4CD25 regulatory T cells**. *Scandinavian journal of immunology* 2004, **60**(1-2):82-88.
120. Rad R, Brenner L, Bauer S, Schwendy S, Layland L, da Costa CP, Reindl W, Dossumbekova A, Friedrich M, Saur D *et al*: **CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo**. *Gastroenterology* 2006, **131**(2):525-537.
121. Lundgren A, Stromberg E, Sjoling A, Lindholm C, Enarsson K, Edebo A, Johnsson E, Suri-Payer E, Larsson P, Rudin A *et al*: **Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients**. *Infection and immunity* 2005, **73**(1):523-531.
122. Kandulski A, Wex T, Kuester D, Peitz U, Gebert I, Roessner A, Malfertheiner P: **Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher H. pylori colonization and increased gene expression of TGF-beta1**. *Helicobacter* 2008, **13**(4):295-303.
123. Harris PR, Wright SW, Serrano C, Riera F, Duarte I, Torres J, Pena A, Rollan A, Viviani P, Guiraldes E *et al*: **Helicobacter pylori gastritis in children is associated with a regulatory T-cell response**. *Gastroenterology* 2008, **134**(2):491-499.
124. Kao JY, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, Eaton KA, Zou W, Berndt BE, Cole TS *et al*: **Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice**. *Gastroenterology* 2010, **138**(3):1046-1054.
125. Kao JY, Rathinavelu S, Eaton KA, Bai L, Zavros Y, Takami M, Pierzchala A, Merchant JL: **Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense**. *American journal of physiology Gastrointestinal and liver physiology* 2006, **291**(1):G73-81.
126. Bimczok D, Clements RH, Waites KB, Novak L, Eckhoff DE, Mannon PJ, Smith PD, Smythies LE: **Human primary gastric dendritic cells induce a Th1 response to H. pylori**. *Mucosal immunology* 2010, **3**(3):260-269.
127. Carbo A, Bassaganya-Riera J, Pedragosa M, Viladomiu M, Marathe M, Eubank S, Wendelsdorf K, Bisset K, Hoops S, Deng X *et al*: **Predictive computational modeling of the mucosal immune responses during Helicobacter pylori infection**. *PloS one* 2013, **8**(9):e73365.
128. Carbo A, Olivares-Villagomez D, Hontecillas R, Bassaganya-Riera J, Chaturvedi R, Piazuelo MB, Delgado A, Washington MK, Wilson KT, Algood HM: **Systems modeling of the role of interleukin-21 in the maintenance of effector CD4+ T cell responses during chronic Helicobacter pylori infection**. *mBio* 2014, **5**(4):e01243-01214.

129. Wendelsdorf K, Alam M, Bassaganya-Riera J, Bisset K, Eubank S, Hontecillas R, Marathe M: **ENteric Immunity SImulator: A tool for in silico study of gastroenteric infections**. *IEEE Transactions on NanoBioScience* 2012, **11**:273-288.
130. Hitkova I, Yuan G, Anderl F, Gerhard M, Kirchner T, Reu S, Rocken C, Schafer C, Schmid RM, Vogelmann R *et al*: **Caveolin-1 protects B6129 mice against Helicobacter pylori gastritis**. *PLoS pathogens* 2013, **9**(4):e1003251.
131. Li SL, Zhao JR, Ren XY, Xie JP, Ma QZ, Rong QH: **Increased expression of matrix metalloproteinase-9 associated with gastric ulcer recurrence**. *World journal of gastroenterology : WJG* 2013, **19**(28):4590-4595.
132. Barton SG, Rampton DS, Winrow VR, Domizio P, Feakins RM: **Expression of heat shock protein 32 (hemoxygenase-1) in the normal and inflamed human stomach and colon: an immunohistochemical study**. *Cell stress & chaperones* 2003, **8**(4):329-334.
133. Aderem A, Underhill DM: **Mechanisms of phagocytosis in macrophages**. *Annual review of immunology* 1999, **17**:593-623.
134. Underhill DM, Ozinsky A: **Phagocytosis of microbes: complexity in action**. *Annual review of immunology* 2002, **20**:825-852.
135. Allen LA: **Phagocytosis and persistence of Helicobacter pylori**. *Cellular microbiology* 2007, **9**(4):817-828.
136. Allen LA, Schlesinger LS, Kang B: **Virulent strains of Helicobacter pylori demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages**. *The Journal of experimental medicine* 2000, **191**(1):115-128.
137. Welch JS, Ricote M, Akiyama TE, Gonzalez FJ, Glass CK: **PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(11):6712-6717.
138. Wirth T, Wang X, Linz B, Novick RP, Lum JK, Blaser M, Morelli G, Falush D, Achtman M: **Distinguishing human ethnic groups by means of sequences from *Helicobacter pylori*: lessons from Ladakh**. *Proc Natl Acad Sci U S A* 2004, **101**(14):4746-4751.
139. Falush D, Wirth T, Linz B, Pritchard JK, Stephens M, Kidd M, Blaser MJ, Graham DY, Vacher S, Perez-Perez GI *et al*: **Traces of human migrations in *Helicobacter pylori* populations**. *Science* 2003, **299**(5612):1582-1585.
140. Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW *et al*: **An African origin for the intimate association between humans and Helicobacter pylori**. *Nature* 2007, **445**(7130):915-918.
141. Mane SP, Dominguez-Bello MG, Blaser MJ, Sobral BW, Hontecillas R, Skoneczka J, Mohapatra SK, Crasta OR, Evans C, Modise T *et al*: **Host-interactive genes in Amerindian Helicobacter pylori diverge from their Old World homologs and mediate inflammatory responses**. *Journal of bacteriology* 2010, **192**(12):3078-3092.

142. Lewis ND, Asim M, Barry DP, de Sablet T, Singh K, Piazuelo MB, Gobert AP, Chaturvedi R, Wilson KT: **Immune evasion by Helicobacter pylori is mediated by induction of macrophage arginase II**. *J Immunol* 2011, **186**(6):3632-3641.
143. Du SY, Wang HJ, Cheng HH, Chen SD, Wang LH, Wang WC: **Cholesterol glucosylation by Helicobacter pylori delays internalization and arrests phagosome maturation in macrophages**. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi* 2014.
144. Shiu J, Blanchard TG: **Dendritic cell function in the host response to Helicobacter pylori infection of the gastric mucosa**. *Pathogens and disease* 2013, **67**(1):46-53.
145. Zhang M, Liu M, Luther J, Kao JY: **Helicobacter pylori directs tolerogenic programming of dendritic cells**. *Gut microbes* 2010, **1**(5):325-329.
146. Kobayashi K, Yokota K, Yoshino T, Kawahara Y, Dey A, Hirai Y, Oguma K, Akagi T: **Detection of Helicobacter pylori associated antigen and heat shock protein 60 on follicular dendritic cells in the germinal centres of low grade B cell lymphoma of gastric mucosa associated lymphoid tissue (MALT)**. *Journal of clinical pathology* 1998, **51**(5):396-398.
147. Mueller A, O'Rourke J, Chu P, Chu A, Dixon MF, Bouley DM, Lee A, Falkow S: **The role of antigenic drive and tumor-infiltrating accessory cells in the pathogenesis of helicobacter-induced mucosa-associated lymphoid tissue lymphoma**. *The American journal of pathology* 2005, **167**(3):797-812.
148. Algood HM, Gallo-Romero J, Wilson KT, Peek RM, Jr., Cover TL: **Host response to Helicobacter pylori infection before initiation of the adaptive immune response**. *FEMS immunology and medical microbiology* 2007, **51**(3):577-586.
149. Parlog G, Murarescu D, Ungureanu C, Danciu M, Mihailovici MS: **[Histopathologic and immunohistochemic changes in Helicobacter pylori colonised gastric mucosa]**. *Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi* 2010, **114**(3):813-817.
150. Oertli M, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, Maxeiner J, Hansson M, Taube C, Quiding-Jarbrink M *et al*: **DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection**. *The Journal of clinical investigation* 2012, **122**(3):1082-1096.
151. Kronsteiner B, Bassaganya-Riera J, Philipson C, Viladomiu M, Carbo A, Pedragosa M, Vento S, Hontecillas R: **Helicobacter pylori infection in a pig model is dominated by Th1 and cytotoxic CD8+ T cell responses**. *Infection and immunity* 2013, **81**(10):3803-3813.
152. Kandulski A, Selgrad M, Malfertheiner P: **Helicobacter pylori infection: a clinical overview**. *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver* 2008, **40**(8):619-626.
153. Wei L, Wang J, Liu Y: **Prior to Foxp3(+) regulatory T-cell induction, interleukin-10-producing B cells expand after Helicobacter pylori infection**. *Pathogens and disease* 2014, **72**(1):45-54.

154. Okada H, Kuhn C, Feillet H, Bach JF: **The 'hygiene hypothesis' for autoimmune and allergic diseases: an update**. *Clinical and experimental immunology* 2010, **160**(1):1-9.
155. Chen Y, Blaser MJ: **Helicobacter pylori colonization is inversely associated with childhood asthma**. *The Journal of infectious diseases* 2008, **198**(4):553-560.
156. Blaser MJ, Chen Y, Reibman J: **Does Helicobacter pylori protect against asthma and allergy?** *Gut* 2008, **57**(5):561-567.
157. Wender M: **[Prevalence of Helicobacter pylori infection among patients with multiple sclerosis]**. *Neurologia i neurochirurgia polska* 2003, **37**(1):45-48.
158. Li W, Minohara M, Su JJ, Matsuoka T, Osoegawa M, Ishizu T, Kira J: **Helicobacter pylori infection is a potential protective factor against conventional multiple sclerosis in the Japanese population**. *Journal of neuroimmunology* 2007, **184**(1-2):227-231.
159. Mohebi N, Mamarabadi M, Moghaddasi M: **Relation of helicobacter pylori infection and multiple sclerosis in Iranian patients**. *Neurology international* 2013, **5**(2):31-33.
160. Yoshimura S, Isobe N, Matsushita T, Masaki K, Sato S, Kawano Y, Ochi H, Kira J: **Genetic and infectious profiles influence cerebrospinal fluid IgG abnormality in Japanese multiple sclerosis patients**. *PloS one* 2014, **9**(4):e95367.
161. Hoffman PS, Vats N, Hutchison D, Butler J, Chisholm K, Sisson G, Raudonikiene A, Marshall JS, Veldhuyzen van Zanten SJ: **Development of an interleukin-12-deficient mouse model that is permissive for colonization by a motile KE26695 strain of Helicobacter pylori**. *Infection and immunity* 2003, **71**(5):2534-2541.
162. Terebiznik MR, Vazquez CL, Torbicki K, Banks D, Wang T, Hong W, Blanke SR, Colombo MI, Jones NL: **Helicobacter pylori VacA toxin promotes bacterial intracellular survival in gastric epithelial cells**. *Infection and immunity* 2006, **74**(12):6599-6614.
163. Semino-Mora C, Doi SQ, Marty A, Simko V, Carlstedt I, Dubois A: **Intracellular and interstitial expression of Helicobacter pylori virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma**. *J Infect Dis* 2003, **187**(8):1165-1177.
164. Ito K, Yamaoka Y, Ota H, El-Zimaity H, Graham DY: **Adherence, internalization, and persistence of Helicobacter pylori in hepatocytes**. *Digestive Diseases and Sciences* 2008, **53**(9):2541-2549.
165. Wang YH, Wu JJ, Lei HY: **The autophagic induction in Helicobacter pylori-infected macrophage**. *Experimental biology and medicine* 2009, **234**(2):171-180.
166. Wang YH, Gorvel JP, Chu YT, Wu JJ, Lei HY: **Helicobacter pylori impairs murine dendritic cell responses to infection**. *PLoS One* 2010, **5**(5):e10844.
167. Chu YT, Wang YH, Wu JJ, Lei HY: **Invasion and multiplication of Helicobacter pylori in gastric epithelial cells and implications for antibiotic resistance**. *Infection and Immunity* 2010, **78**(10):4157-4165.

168. Oh JD, Karam SM, Gordon JI: **Intracellular Helicobacter pylori in gastric epithelial progenitors**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(14):5186-5191.
169. Kronsteiner B, Bassaganya-Riera J, Philipson N, Hontecillas R: **Novel insights on the role of CD8+ T cells and cytotoxic responses during Helicobacter pylori infection**. *Gut microbes* 2014, **5**(3):357-362.
170. Ito T, Kobayashi D, Uchida K, Takemura T, Nagaoka S, Kobayashi I, Yokoyama T, Ishige I, Ishige Y, Ishida N *et al*: **Helicobacter pylori invades the gastric mucosa and translocates to the gastric lymph nodes**. *Laboratory Investigation* 2008, **88**(6):664-681.
171. Ozbek A, Ozbek E, Dursun H, Kalkan Y, Demirci T: **Can Helicobacter pylori invade human gastric mucosa?: an in vivo study using electron microscopy, immunohistochemical methods, and real-time polymerase chain reaction**. *Journal of Clinical Gastroenterology* 2010, **44**(6):416-422.
172. Borlace GN, Keep SJ, Prodoehl MJ, Jones HF, Butler RN, Brooks DA: **A role for altered phagosome maturation in the long term persistence of Helicobacter pylori infection**. *American journal of physiology Gastrointestinal and liver physiology* 2012.
173. Borlace GN, Jones HF, Keep SJ, Butler RN, Brooks DA: **Helicobacter pylori phagosome maturation in primary human macrophages**. *Gut Pathog* 2011, **3**(1):3.
174. Han XY, Tarrand JJ, Dickey BF, Esteva FJ: **Helicobacter pylori bacteremia with sepsis syndrome**. *Journal of Clinical Microbiology* 2010, **48**(12):4661-4663.
175. Koebernick H, Grode L, David JR, Rohde W, Rolph MS, Mittrucker HW, Kaufmann SH: **Macrophage migration inhibitory factor (MIF) plays a pivotal role in immunity against Salmonella typhimurium**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(21):13681-13686.
176. Huang XR, Chun Hui CW, Chen YX, Wong BC, Fung PC, Metz C, Cho CH, Hui WM, Bucala R, Lam SK *et al*: **Macrophage migration inhibitory factor is an important mediator in the pathogenesis of gastric inflammation in rats**. *Gastroenterology* 2001, **121**(3):619-630.
177. Shaw PJ, Lamkanfi M, Kanneganti TD: **NOD-like receptor (NLR) signaling beyond the inflammasome**. *European journal of immunology* 2010, **40**(3):624-627.
178. Schneider M, Zimmermann AG, Roberts RA, Zhang L, Swanson KV, Wen H, Davis BK, Allen IC, Holl EK, Ye Z *et al*: **The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB**. *Nature immunology* 2012, **13**(9):823-831.
179. Lei Y, Wen H, Ting JP: **The NLR protein, NLRX1, and its partner, TUFM, reduce type I interferon, and enhance autophagy**. *Autophagy* 2013, **9**(3):432-433.
180. Lei Y, Wen H, Yu Y, Taxman DJ, Zhang L, Widman DG, Swanson KV, Wen KW, Damania B, Moore CB *et al*: **The mitochondrial proteins NLRX1 and**

TUFM form a complex that regulates type I interferon and autophagy. *Immunity* 2012, **36**(6):933-946.
181. Kobayashi KS, van den Elsen PJ: **NLRC5: a key regulator of MHC class I-dependent immune responses**. *Nature reviews Immunology* 2012, **12**(12):813-820.
182. Ihaka R, Gentleman R: **R: A Language for Data Analysis and Graphics**. *Journal of Computational and Graphical Statistics* 1996, **5**(3):299-314.
183. Benjamini Y, Hochberg Y: **Controlling the false discovery rate: a practical and powerful approach to multiple testing**. *Journal of the Royal Statistical Society Series B (Methodological)* 1995:289-300.
184. Borlace GN, Butler RN, Brooks DA: **Monocyte and macrophage killing of helicobacter pylori: relationship to bacterial virulence factors**. *Helicobacter* 2008, **13**(5):380-387.
185. Krakowiak MS, Noto JM, Piazuelo MB, Hardbower DM, Romero-Gallo J, Delgado A, Chaturvedi R, Correa P, Wilson KT, Peek RM, Jr.: **Matrix metalloproteinase 7 restrains Helicobacter pylori-induced gastric inflammation and premalignant lesions in the stomach by altering macrophage polarization**. *Oncogene* 2014.
186. Chionh YT, Ng GZ, Ong L, Arulmuruganar A, Stent A, Saeed MA, Wee JL, Sutton P: **Protease-activated receptor 1 suppresses Helicobacter pylori gastritis via the inhibition of macrophage cytokine secretion and interferon regulatory factor 5**. *Mucosal Immunol* 2014.
187. Dela Cruz CS, Liu W, He CH, Jacoby A, Gornitzky A, Ma B, Flavell R, Lee CG, Elias JA: **Chitinase 3-like-1 promotes Streptococcus pneumoniae killing and augments host tolerance to lung antibacterial responses**. *Cell host & microbe* 2012, **12**(1):34-46.
188. Pawlowski N, Khaminets A, Hunn JP, Papic N, Schmidt A, Uthaiah RC, Lange R, Vopper G, Martens S, Wolf E *et al*: **The activation mechanism of Irga6, an interferon-inducible GTPase contributing to mouse resistance against Toxoplasma gondii**. *BMC biology* 2011, **9**:7.
189. Langmead B, Trapnell C, Pop M, Salzberg SL: **Ultrafast and memory-efficient alignment of short DNA sequences to the human genome**. *Genome biology* 2009, **10**(3):R25.
190. Li B, Dewey CN: **RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome**. *BMC bioinformatics* 2011, **12**:323.
191. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation**. *Nature biotechnology* 2010, **28**(5):511-515.
192. Huang da W, Sherman BT, Lempicki RA: **Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources**. *Nature protocols* 2009, **4**(1):44-57.
193. Huang da W, Sherman BT, Lempicki RA: **Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists**. *Nucleic acids research* 2009, **37**(1):1-13.

194. Maere S, Heymans K, Kuiper M: **BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks**. *Bioinformatics* 2005, **21**(16):3448-3449.
195. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: **Cytoscape: a software environment for integrated models of biomolecular interaction networks**. *Genome research* 2003, **13**(11):2498-2504.
196. Sullivan CS, Ganem D: **MicroRNAs and viral infection**. *Molecular cell* 2005, **20**(1):3-7.
197. Yoshino H, Seki N, Itesako T, Chiyomaru T, Nakagawa M, Enokida H: **Aberrant expression of microRNAs in bladder cancer**. *Nature reviews Urology* 2013.
198. Iborra M, Bernuzzi F, Correale C, Vetrano S, Fiorino G, Beltran B, Marabita F, Locati M, Spinelli A, Nos P *et al*: **Identification of serum and tissue micro-RNA expression profiles in different stages of the inflammatory bowel disease**. *Clinical and experimental immunology* 2013.
199. Coskun M, Bjerrum JT, Seidelin JB, Nielsen OH: **MicroRNAs in inflammatory bowel disease--pathogenesis, diagnostics and therapeutics**. *World journal of gastroenterology : WJG* 2012, **18**(34):4629-4634.
200. Lawless N, Foroushani AB, McCabe MS, O'Farrelly C, Lynn DJ: **Next generation sequencing reveals the expression of a unique miRNA profile in response to a gram-positive bacterial infection**. *PloS one* 2013, **8**(3):e57543.
201. Viladomiu M, Hontecillas R, Pedragosa M, Carbo A, Hoops S, Michalak P, Michalak K, Guerrant RL, Roche JK, Warren CA *et al*: **Modeling the Role of Peroxisome Proliferator-Activated Receptor gamma and MicroRNA-146 in Mucosal Immune Responses to Clostridium difficile**. *PLoS One* 2012, **7**(10):e47525.