UNIVERSITY of CAPE TOWN

Faculty of Health Sciences

Institute of Infectious and Molecular Medicine

Department of Pathology

Division of Immunology

University of Cape Town

Determining the impact of *Heligmosomoides polygyrus* infection on the development of colitis.

Brunette Katsandegwaza

Thesis Presented for the Degree of

DOCTOR of PHILOSOPHY

University of Cape Town

September 2019

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Declaration

I, *Brunette Katsandegwaza*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: …. Signed by candidate

Date: ……26/09/2019……

For indeed I have tasted, and seen that the Lord is good- Psalm 34:8

To my husband, Gerald, and my daughter, Tadiwanashe, thank you for the support you have given me for the past three years. I hope to be able to repay you for all your kindness and love.

To my family, my dad and my siblings; thank you for your unwavering support.

To my laboratory group mates and the greater Division of Immunology group, where would I have been without all the interesting discussions, the tears, joy and support. Thank you.

To my friends Kanga, Rozwi, Mucha, and Mona. Thank you for obliging my venting sessions.

Eventually, to my supervisors, Katie Smith and Bill Horsnell, thank you. Katie, it was definitely a long journey, sometimes I could not see the light at the end of the tunnel, but we have persevered. Thank you for your guidance, I have really learnt a lot in these past three years, and I am indeed grateful.

This study is dedicated to the memory of my late mother, Flora Mkwende (1962-2001); I know that somewhere out there, she is proud of the woman I have become.

Table Of Contents

Declaration **3**

Abstract **11**

Preface **12**

Abbreviations **14**

INTRODUCTION AND LITERATURE REVIEW **16**

1 INTRODUCTION **17**

2 LITERATURE REVIEW **23**

2.1 IBD immunopathology **23**

2.1.1 Mucus layers 24

2.1.2 Innate cells, adaptive cells and AMPs 25

2.2 Association of the gastrointestinal microbiota and IBD **27**

2.2.1 The gut microbiota, immune regulation and anergy 30

2.2.2 Genome Wide Association Studies (GWAS) associations with IBD 31

2.2.3 Innate signaling and the gut microbiota 33

2.2.4 Secretory IgA, microbiota and IBD 33

2.2.5 The microbiota, diet and inflammation 34

2.3 IBD Interventions **37**

2.3.1 Fecal Stream Diversion 38

2.3.2 Antibiotics 39

2.3.3 Enteral Nutrition Therapy (ENT) 40

2.3.4 Fecal Microbiota Transplantation (FMT) 42

2.3.5 Probiotics 43

2.3.6 Helminth Immunotherapy 45

2.4 Murine IBD models **51**

2.4.1 TNBS-Induced Colitis 51

2.4.2 Iκκ-γ (NEMO) Deficiency Colitis 52

2.4.3 Interleukin-10 (IL-10) Deficiency Colitis 53

2.4.4 T Cell Adoptive Transfer Model 53

2.4.5 Oxazolone Colitis 55

2.4.6 Dextran Sodium Sulfate-Induced Colitis 56

2.4.7 Limitations of using murine models in IBD research 58

STUDY AIM AND OBJECTIVES 60

3 AIMS, RATIONALE & OBJECTIVES 61

MATERIALS AND METHODS 63

4 Materials and Methods 64

4.1 Materials 64

4.1.1 Ethical considerations 64

4.1.2 Animals 64

4.1.3 Diet 64

4.1.4 Helminth propagation and maintenance 65

4.1.5 Media preparation 66

4.1.6 Histology materials 68

4.2 Methods 72

4.2.1 *H.polygyrus* Infection 72

4.2.2 Probiotic administration 72

4.2.3 Oxazolone colitis induction 72

4.2.4 DSS colitis induction 73

4.2.5 Evaluation of colitis 73

4.2.6 Tissue Isolation and cell processing 74

4.2.7 Histology 75

4.2.8 Bacterial isolation, cultivation and identification 79

4.2.9 Microbiome data analyses 81

EXPERIMENTAL RESULTS CHAPTER ONE 84

5 *Heligmosomoides polygyrus* and oxazolone colitis 85

EXPERIMENTAL RESULTS CHAPTER TWO 107

6 *Heligmosomoides polygyrus* and DSS colitis 108

EXPERIMENTAL RESULTS CHAPTER THREE 149

7 Probiotics, *H.polygyrus* and DSS colitis 150

CONCLUSION AND FUTURE WORK 176

8 Conclusion 177

9 Future work 179

APPENDIX 180

10 Appendix 181

10.1 Optimisations 181

10.2 Diet Specifications 190

10.3 Gating Strategies 193

BIBLIOGRAPHY 196

Abstract

The ability of helminths to regulate inflammatory disorders and the mechanisms by which they carry this out are of great scientific interest. Currently, established literature emphasises the protective role of helminth infection in mouse models of inflammatory bowel disease (IBD). Utilising two well-established murine models of human disease, the oxazolone and dextran sulfate sodium (DSS) models, I found that induction of murine IBD is highly sensitive to diet change and mouse gender. Using the gastrointestinal helminth *Heligmosomoides polygyrus* (*H.polygyrus*), I demonstrate that helminth infection exacerbates IBD in both the oxazolone and DSS models of colitis. Underlying helminth infection results in increased inflammation locally in the colon and systemically in the spleen in both models of IBD, as measured by histology and flow cytometry. Exacerbation of DSS colitis is dependent on the dose of *H.polygyrus* but is independent of the phase of *H.polygyrus* infection, with both acute and chronic infections resulting in the same phenotype. Helminth exacerbated DSS colitis is characterised by significant bacterial translocation to the spleen, which is concluded to be due to loss of intestinal epithelial integrity. Helminth infection also resulted in a microbial shift of translocating bacteria following DSS administration, as evidenced by gram staining and bacterial sequencing. Administration of an 8-strain probiotic during acute helminth infection ameliorated helminth exacerbation of DSS colitis, restored epithelial integrity and abrogated splenomegaly. This work uncovers an unexpected and novel role for live helminth infection in exacerbating IBD and suggests that helminth-induced dysbiosis of the microbiota may drive disease. These studies reveal restoration of the microbiota through probiotics or helminth eradication as potential therapies for the treatment of gastrointestinal inflammatory disorders.

Preface

The work presented in this study centers around the influence of helminth infections on gut inflammatory bowel diseases (IBD). I determined the influence of prior *Heligmosomoides polygyrus* infection on the development of IBD using two murine models of human ulcerative colitis, oxazolone and DSS-induced colitis. I then further assessed the impact of microbial modulation on the influence of helminth-exacerbation of DSS colitis.

In that respect, the first chapter (Introduction and Literature Review), introduces what is currently known about IBD, with a subsequent review of the current findings regards IBD pathology and interventions, relevant to this study. I round up this chapter by discussing various murine models of IBD, and their relevance in the scientific field. Chapter 4 (Materials and Methods) gives an outline of the steps I followed to achieve the study goals. This chapter ends in a brief section of sets of experiments I carried out to optimise the murine models, used as a basis to test our hypothesis.

Chapters five to seven report the findings of this thesis, separated in to three individual studies. The experimental results from each chapter are preceded by a focused literature review, putting into context previously published findings inspiring our studies. Finally, I conclude this thesis by summing up all the important findings of this research, and briefly discuss the potential future work pertinent to address some questions arising from this whole study.

Abbreviations

IBD	Inflammatory Bowel Disease
UC	Ulcerative Colitis
CD	Crohn's Disease
TNBS	Trinitrobenzene Sulfonic Acid
DNBS	Dinitrobenzene Sulfonic Acid
DSS	Dextran Sodium Sulfate
Ικκ-γ	Inhibitor of Nuclear Factor kappa-B kinase subunit gamma
NEMO	Nuclear Factor-kappa B Essential Modulator
ENT	Enteral Nutrition Therapy
FMT	Fecal Microbiota Transfer
SCFA	Short Chain Fatty Acids
PUFA	Poly Unsaturated Fatty Acid
CLA	Conjugated Linoleic Acid
AA	Arachidonic Acid
GWAS	Genome Wide Association Studies
MUC2	Mucin 2
AMP	Antimicrobial Peptides
MHC	Major Histocompatibility Complex
NODs	Nucleotide-binding oligomerisation domain
TLR	Toll Like Receptors
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
NFκB	Nuclear Factor Kappa B
TcR	T cell Receptor
NKT	Natural Killer T cell
Tregs	T regulatory cells
AAMs	Alternatively Activated Macrophages
CD	Cluster of Differentiation
IL	Interleukin
TNF	Tumor Necrosis Factor
Ig	Immunoglobulin
LPS	Lipopolysaccharide
WT	Wild Type
DAPI	4',6-diamidino-2-phenylindole
FMO	Fluorescence Minus One
MFI	Mean Flourescense Intenstity

INTRODUCTION AND LITERATURE REVIEW

1 INTRODUCTION

In 2025, the prevalence of Inflammatory Bowel Disease (IBD) in regions such as Alberta, Canada, with a historically high-risk of disease, will be nearly 70% higher than in 2015 (Rezaie *et al.*, 2012). This is due to the compounding influence of low population mortality, as well as the young age of disease onset (Rezaie *et al.*, 2012). Indeed, the number of pediatric IBD cases in Alberta is exponentially increasing, suggesting an underestimation of disease prevalence by the year 2025 (Benchimol *et al.*, 2009). An even more disturbing fact is the apparent increase of IBD incidence and prevalence in newly industrialized developing countries from Africa, Asia and South America (**Fig 1.1**) (Sood *et al.*, 2003; Victoria *et al.*, 2009; Jemal *et al.*, 2012; Ng *et al.*, 2017). A change in diet and living standards to model a more westernized lifestyle appears as the major driving factor behind increased disease incidence in these developing countries (Kaplan, 2015); thereby presenting a complicated health challenge to already weak national health systems. The annual cost of biological therapy per patient in developed countries is estimated to be >US$25,000 (Marchetti and Liberato, 2014), a price beyond the financial grip of many IBD patients from developing countries.

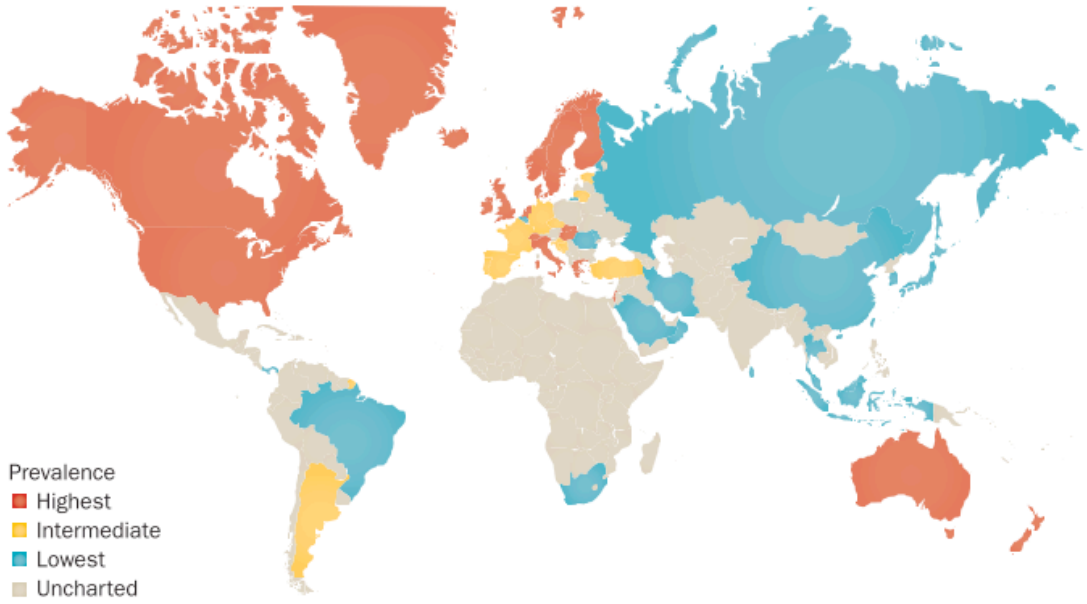


Figure 1-1: The global prevalence of IBD in 2015. Data from (Molodecky *et al.*, 2012),image provided by PresenterMedia.

Unfortunately, chronic untreated IBD increases the risk factor of colorectal cancer (CRC) (Feagins *et al.*, 2009; Saleh and Trinchieri, 2010), with an estimated 12-20% of chronic IBD sufferers (>30 years) developing CRC (Eaden *et al.*, 2001; Lakatos, 2008). The costs, limitations of treatment and potential complications associated with IBD in developing countries support the need for research into disease risk factors and the immuno-pathogenesis associated with disease; in order to determine points and affordable avenues of timely intervention.

In addition to genetic susceptibility (Xavier and Podolsky, 2007), the combination of a number of environmental risk factors poses a serious threat to developing countries. Moreover, they potentially undermine the ability of these nations to either prevent or treat IBD. Besides weak health systems and considerable economic challenges; the adoption of 'westernised' diets, the high incidence of tobacco smoking as well as a high prevalence of infectious diseases increases the vulnerability of developing to IBD onset (Kaplan, 2015). Of the environmental risk factors mentioned, the high burden of chronic infections provides complications not only to IBD therapy, but also to other non-communicable diseases currently on the rise in endemic areas. Chronic infections account for approximately 33% of all cancers in sub-Saharan Africa (de Martel *et al.*, 2012). The human papillomavirus (HPV) is highly correlated to cervical cancer, Kaposi Sarcoma Herpes Virus (KSHV) to Kaposi's Sarcoma, hepatitis virus to liver cancer and *Schistosoma spp.* to CRC and bladder cancer (Yosry, 2006; Fried *et al.*, 2011; Jemal *et al.*, 2012; Oh and Weiderpass, 2014). The ability of infectious diseases to drive a chronic low profile inflammatory response provides a favourable environment for the development and progression of inflammation associated pathologies in endemic regions; emphasising the need for research on the bio-interactions between infections and non-communicable diseases such as IBD.

Although a number of infections are associated with the onset and progression of cancer (Yosry, 2006; Fried *et al.*, 2011; Oh and Weiderpass, 2014), epidemiological findings from the recent past demonstrate an inverse relationship between infectious and inflammatory disease (**Fig 1.2 below in relation to Fig 1.1**), giving birth to the 'hygiene hypothesis'. Simply stated, the hygiene

hypothesis postulates that a high incidence and prevalence of infectious diseases may exert an immunosuppressive pressure on the development of inflammatory disorders (Martinez, 2001). Indeed, a number of scientific publications demonstrate the ability of some protozoan and helminth infections to suppress bystander inflammatory conditions in both human populations and murine models of disease (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005; De Trez *et al.*, 2015).

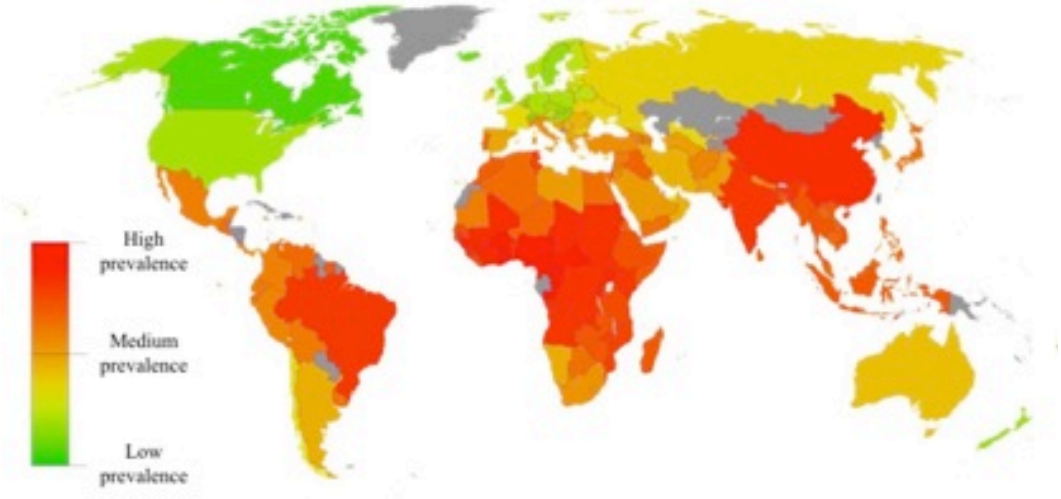


Figure 1-2: Global prevalence of infections. Adapted from (Nikolaev and Salahodjaev, 2017).

Although helminth infections can result in debilitating disease and morbidity, some helminth infections modulate the host's immune system to evade the anti-helminth responses, the modification of which is associated with the 'unexpected' suppression of bystander concurrent inflammatory conditions (Elliott *et al.*, 2004; Sutton *et al.*, 2008; León-Cabrera *et al.*, 2014). Currently, helminth immunotherapy trials for the treatment of human inflammatory disorders are under serious deliberations (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005; Bager *et al.*, 2010; Fleming, 2013; Helmby, 2015). Inconclusive trial results demonstrate helminth immunotherapy to be highly dependent on the condition of the individual study as well as inflammatory condition that is being treated. For instance, although *Summers et al.,* reported alleviated symptoms for 80% of Crohn's disease (CD) and 43% of ulcerative colitis (UC) after *Trichuris suis* ova and *Trichuris suis* infection respectively; *Trichuris suis* ova had no beneficial effect on pollen-induced rhinitis patients (R W Summers *et al.*, 2005; Robert W.

Summers *et al.*, 2005; Bager *et al.*, 2010). Despite contradictory human trial findings, experimental murine research demonstrates the ability of helminth infections to exert immunomodulatory effects through a number of mechanisms. These include the induction of immunosuppressive regulatory T cells (Treg) (Grainger et al., 2010), production of regulatory cytokines e.g. IL-10 (Turner *et al.*, 2008), impairment of mucosal mastocytosis (Kim *et al.*, 2000), exhaustion of protective $CD8^+$ T cell responses (Hernández-Ruiz *et al.*, 2010) and alternative activation of macrophages (Kreider *et al.*, 2007). These experimental findings support the need for further helminth therapy research, as it has great promise as an alternative biological therapy for the treatment of inflammatory conditions.

Interestingly, a recent study demonstrates the increased predisposition of women to the development of autoimmune inflammatory conditions, a fact that may have serious implications on therapy design. A number of inflammatory conditions that include rheumatoid arthritis (RA), systemic lupus erythematous (SLE) and multiple sclerosis (MS) are more prevalent in the female population compared to their male counterparts (Wolfe *et al.*, 1968; Ramagopalan *et al.*, 2010; Crosslin and Wiginton, 2011). A study by the American college of obstetricians and gynecologists (ACOG) reported that women of childbearing age and those experiencing hormonal surges stand a higher risk of developing SLE (*ACOG practice bulletin: Use of hormonal contraception in women with coexisting medical conditions.*, 2006). The international collaborative report of 2001 categorically demonstrated that women on progesterone only contraceptives had increased risk of developing inflammatory conditions that include urticaria, eczema and alopecia (Farley and Sivin, 2001). Although sexual dimorphism is not demonstrated for both CD and UC, small epidemiological studies suggest sexual dimorphism in IBD prevalence and severity (Gupta *et al.*, 2007; Asakura *et al.*, 2009). In the event that IBD follows a pattern sexual diphormism, host directed IBD therapies would have to take into account hormonal differences between male and female patients, in order to deliver efficient drug treatments.

The recent finding of the microbiota as a key player in a number of inflammatory conditions has revolutionized disease therapy research. The term microbiota refers to the microbial flora; which represents symbiotic, commensal and pathogenic

microorganisms (also termed pathobionts) harbored by humans (Turnbaugh *et al.*, 2007). Microbial diversity and composition is subject to change as a function of age, sex, diet, infections, environment and lifestyle **(Fig 1.3)** (Ng *et al.*, 2018; de la Cuesta-Zuluaga *et al.*, 2019). A shift in the balance amongst the major gut microbial clusters is defined as microbial dysbiosis, a phenomenon typically associated with gut and systemic inflammatory pathologies (Mar *et al.*, 2014; Schwab *et al.*, 2014; Houlden *et al.*, 2015; Rajoka *et al.*, 2017).

A need to better understand IBD pathology and its interaction with the gut microbiota as well as gastrointestinal helminth infection would further research into biological therapies tailored for patients with complicated IBD etiology in developing countries. Fortunately, a number of experimental IBD murine models (discussed later) have enabled researchers into making great inroads into human IBD pathology. These include the IL-$10^{-/-}$ and the $TNF^{deltaARE}$ spontaneous models of disease; the chemical-induced trinitrobenzene sulfonic acid (TNBS), oxazolone and dextran sulfate sodium models as well as the $CD4^{+}$ T cell transfer model (introduced fully in the following section) (Okayasu *et al.*, 1990; Kühn *et al.*, 1993; Kontoyiannis *et al.*, 1999; Heller *et al.*, 2002; Powrie and Uhlig, 2004; Antoniou *et al.*, 2016). Although most murine models of disease only partially model human IBD, these models have contributed a tremendous amount of data to research; data that has shaped IBD therapy design.

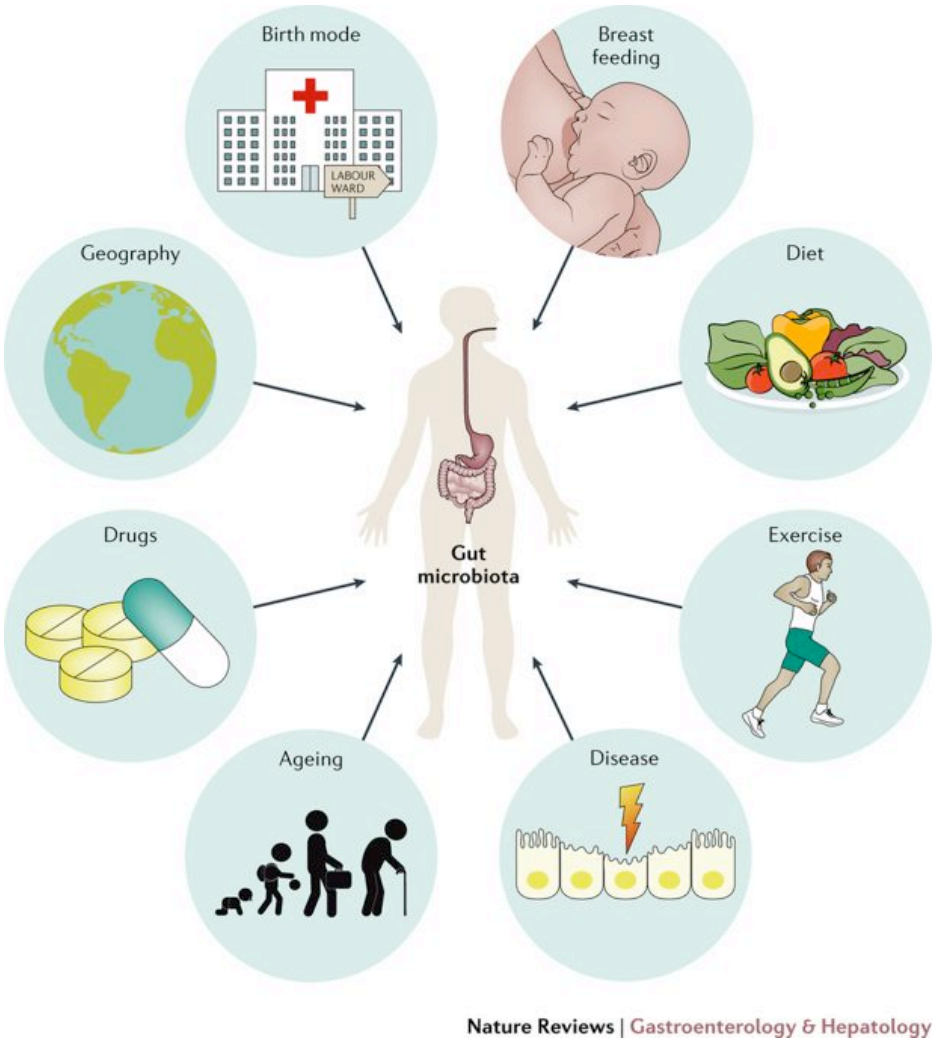


Figure 1-3: The potential impact of the gut microbiota on human physiology is affected by different factors that modulate the pathophysiological mechanisms underlying gut inflammation. From (Quigley, 2017)

2 LITERATURE REVIEW

2.1 IBD immunopathology

Crohn's disease (CD) and Ulcerative Colitis (UC) are both highly inflammatory diseases of the gastrointestinal tract (Haggar and Boushey, 2009), collectively known as IBD. Although the etiopathogenesis of IBD is undetermined, the resultant de-regulation of both the innate and adaptive immune responses towards the gastrointestinal microbiota is proposed to result in an overt inflammatory response that culminates in tissue degradation and systemic inflammation(Strober *et al.*, 2002). CD pathology results in patchy inflammation affecting the ileum, caecum and the colon, while UC pathogenesis is mainly concentrated in the distal colon and rectum (**Fig 2.1**) (Garud and Peppercorn, 2009; Lee *et al.*, 2018).

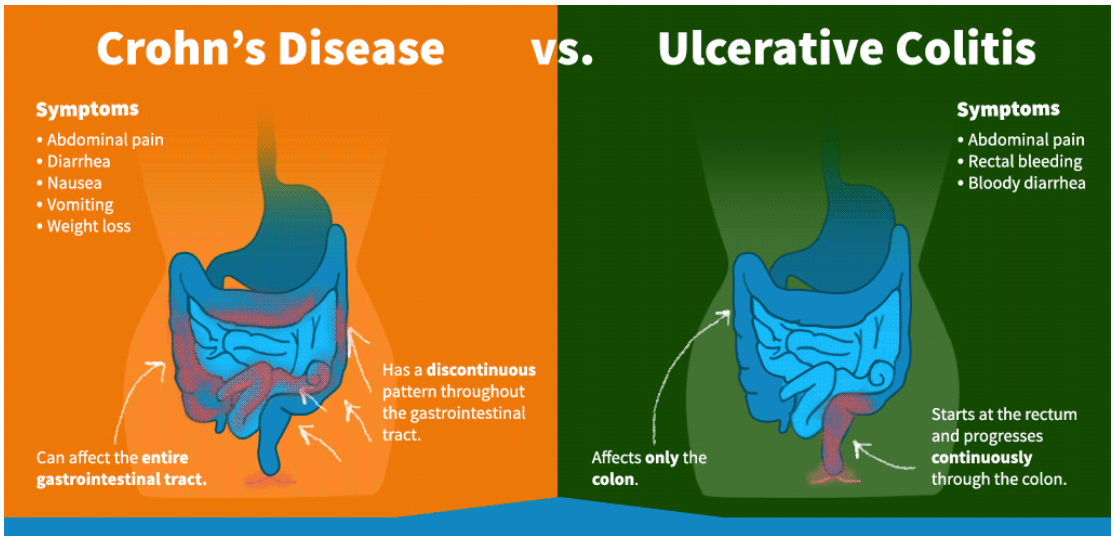


Figure 2-1: Crohn's disease and Ulcerative Colitis symptoms. Adapted from (https://www.celgene.com/ulcerative-colitis-chrons-different-strategies/).

The inflammatory pathology concentrated in the colon is of major importance in this study. The colon has a complex network of innate and adaptive immune mediators maintaining the colon under homeostasis that include:

i. Mucus layers
ii. Innate immune cells e.g. dendritic cells, neutrophils, macrophages
iii. Innate lymphoid cells

iv. Adaptive immune cells e.g. $CD4^+$ T-regulatory cells, $CD4^+$ and $CD8^+$ T cells

v. Anti-microbial proteins (AMPs).

2.1.1 Mucus layers

Mucus layers are the first major protective barriers that shield the intestinal epithelial cells from irritation by lumen antigens that include food particles, commensal microbiota as well as chemical irritants (Shirazi *et al.*, 2000; Johansson *et al.*, 2008; Bergstrom *et al.*, 2010). The colon has two mucus layers, the inner layer that is attached to the epithelial cells and completely sterile of microbes and the outer loose layer that is motile and provides a niche for commensal microbes (Johansson *et al.*, 2008; Kim and Ho, 2010). Mucus consists of carbohydrate rich mucins (e.g. MUC2) produced in large volumes by goblet cells, contributing to the formation of the mucus layers (Johansson *et al.*, 2008). Bacteria in the outer mucus layer, such as *Akkermansia muciniphila* (Derrien *et al.*, 2008; Ganesh *et al.*, 2013), degrades the inner mucus layer constantly, contributing to the maintenance of the outer viscous layer. Mucus produced by the goblet cells fills into the inner layer to maintain its thickness.

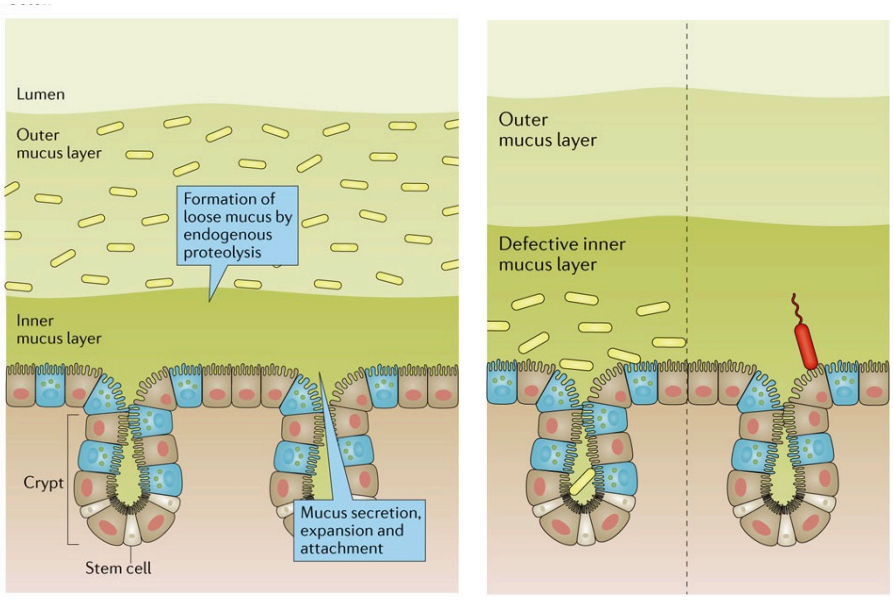


Figure 2-2: Segregation of the commensal and pathogenic microbes by the colon mucus layers, defects of which result in microbe penetrating the layers and inducing inflammation. Adapted from (Johansson and Hansson, 2016).

2.1.2 Innate cells, adaptive cells and AMPs

Under steady state conditions, enterocytes, goblet cells and microfold (M) cells constantly sample gut microbiota for antigen presentation (Snoeck *et al.*, 2005). Intestinal dendritic cells (DCs) are always in constant contact with colonic and mesenteric lymph node Tregs (Coombes *et al.*, 2007), the stimulation of which results in gut microbe and food tolerance (Mann and Li, 2014). Sampling of microbial components also results in the release of AMPs that include defensins (in humans, defensins are also produced by neutrophils) (Karlsson *et al.*, 2008; Wu *et al.*, 2015). AMPS slowly diffuse into the mucus and opsonize pathogenic bacteria to enable microbial clearance using a weep and sweep motion (*reviewed in* Bevins and Salzman, 2011).

Any perturbation to gut homeostasis that may result in epithelial breakdown or the reduction/overexpansion of mucin stimulating/mucin degrading microbiota respectively, contributes to the thinning and weakening of both mucus layers (Bergstrom *et al.*, 2010; Ganesh *et al.*, 2013; Seregin *et al.*, 2017). Loss of mucus layer integrity enables both pathogenic and commensal bacteria to translocate from the outer layer, into the inner layer and eventually to the epithelial cells, resulting in epithelial cell irritation (**Fig 2.2**). An overt overstimulation of Toll-Like Receptors (TLR) by translocating bacteria potentially activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) signaling (Wachi *et al.*, 2014; Wu *et al.*, 2016), resulting in the transcription of pro-inflammatory cytokines and chemokines that include tumor necrosis factor (TNF)-α, interleukin (IL)-17A and IL-6 (Jana *et al.*, 2015; Wu *et al.*, 2016). This pro-inflammatory cytokine milieu induces the recruitment of pro-inflammatory cells, with IL-17A signaling associated with neutrophil recruitment to sites of inflammation (Stark *et al.*, 2005; Ley *et al.*, 2006).

Inflammatory resolving neutrophils are short-lived and undergo natural apoptosis after inflammation resolution, to prevent inflammation driven bystander cell damage. However, persistent assault may result in non-resolving inflammation, leading to an accumulation of inflammatory cells that include long-lived neutrophils, inflammatory monocytes, macrophages and helper T cells **(Fig 2.3)**

(Yen *et al.*, 2006; Soehnlein *et al.*, 2008; Dunay *et al.*, 2010; Nakanishi *et al.*, 2015). Under these events of extreme inflammation, goblet cells practice compound mucin exocytosis in an attempt to flush away assaulting microbes, an action that results in the secretion of more than 90% of packaged mucins (Specian and Neutra, 1980; Gersemann *et al.*, 2009; van der Post *et al.*, 2019). This condition is histologically visualized as goblet cell depletion in colon sections of both human and experimental IBD sufferers (Specian and Neutra, 1980; van der Post *et al.*, 2019). Excessive inflammatory stimulation undermines the ability of goblet cells to synthesize and replenish their MUC2 proteins, preventing a compensatory mechanism that is protective to the host. Unfortunately, this aids in bacterial translocation, giving rise to systemic inflammation that may result in potential organ failure and mortality (Gardiner *et al.*, 1993; Johansson *et al.*, 2008).

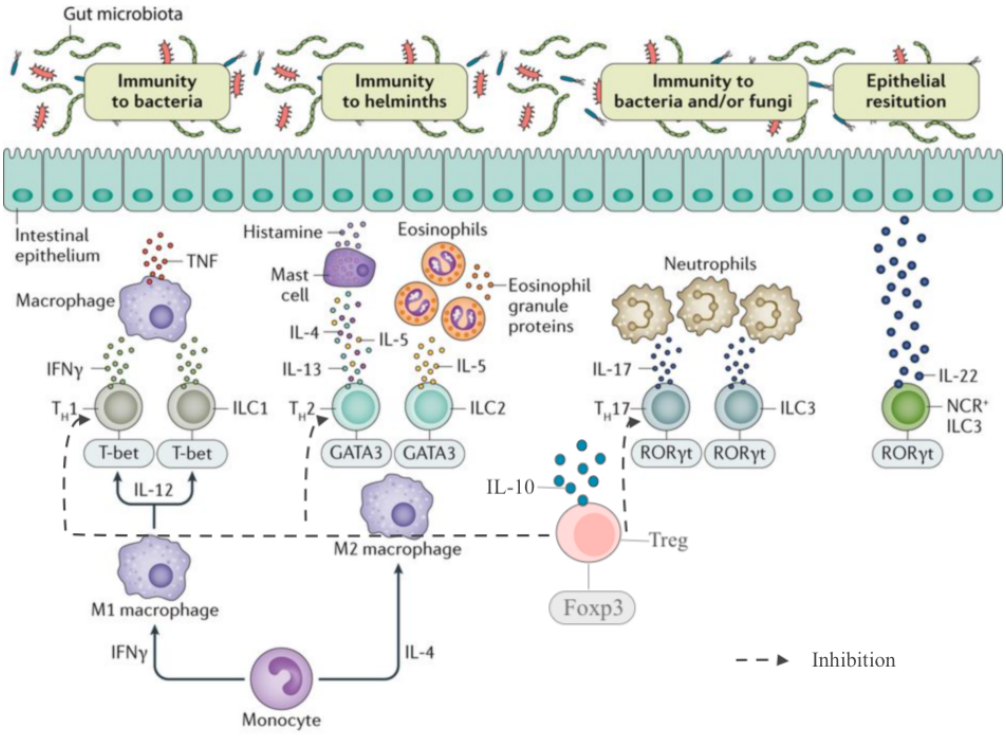


Figure 2-3: Gut mucosal immune responses to different pathogens. Adapted from (Powell *et al.*, 2017).

Neutrophilia, a hallmark of both UC and CD (Rhodes and Jewell, 1983; Bennike *et al.*, 2015), initially begins as a counteractive measure to microbe invasion, expected to quickly subside through neutrophil apoptosis (Fuchs *et al.*, 2007). However, persistent microbial attack results in sustained neutrophilia, culminating

in NETosis (Fuchs *et al.*, 2007; Yipp *et al.*, 2012), a process by which neutrophils self-destruct and release substantial amounts of reactive oxygen species (ROS) capable of bacterial killing (Brinkmann *et al.*, 2004). However, the same ROS results in epithelial tissue damage, leading to persistent inflammation as well as loss of epithelial integrity. Infiltration of inflammatory cells can also result in the redistribution and reduced expression of tight junction proteins (TJP), including e.g e-cadherin and zonal occludin (Ajuebor *et al.*, 1999; Burns *et al.*, 2000), which can enable immune cells to cross into the lamina propria and neutralise offending pathogens. Side effects of such an action include the escape of gut microbes into the periphery, either through the portal vein or through the peritoneal cavity, resulting in systemic inflammation **(Fig 2.4)**.

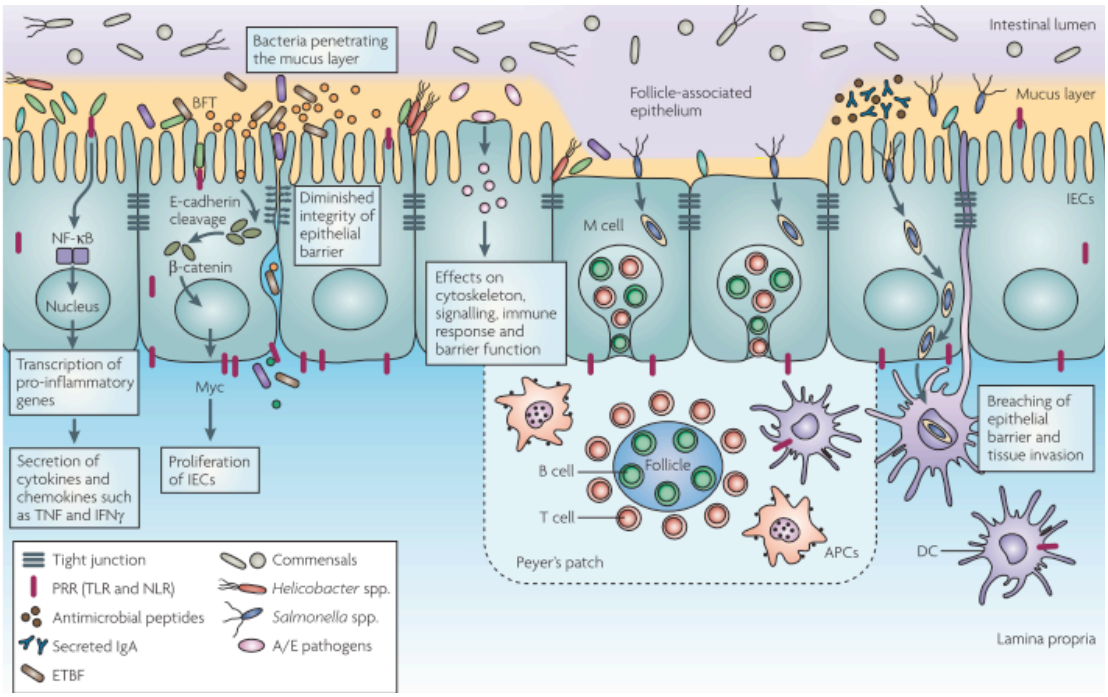


Figure 2-4: The gut microbiota influences the local immune responses associated with epithelial integrity. From (Nell *et al.*, 2010)**.**

2.2 Association of the gastrointestinal microbiota and IBD

The healthy human microbiota is estimated to contain >100 trillion bacteria, a number greater than the number of all cells in a healthy human (Turnbaugh *et al.*, 2007; Boulangé *et al.*, 2016). Under steady state conditions, the gut microbiota forms close complex relations with the mucosal immune system, and plays key

roles in immune modulation, digestion and immune physiology (Maccaferri *et al.*, 2012; Zeng *et al.*, 2016; Luu *et al.*, 2019). Microbial metabolic byproducts and commensal microbe antigens activate and modulate gastrointestinal immune cells, driving protective responses against pathobionts (pathogenic bacteria) (Chang *et al.*, 2014; Venkatesh *et al.*, 2014). However, any perturbation to the complex relationship between the gut microbiota and the host may result in a hyper-inflammatory state as that witnessed in crohn's disease and ulcerative colitis patients (Haberman *et al.*, 2014; Tomasello *et al.*, 2014). This perturbation is termed 'microbial dysbiosis'.

Although a causal-effect relationship between IBD and microbial dysbiosis has been challenging to ascertain in human patients, both epidemiological and experimental research studies strongly associate microbial dysbiosis with both CD and UC (Frank *et al.*, 2007; Tomasello *et al.*, 2014; Ahmed *et al.*, 2016; Rajoka *et al.*, 2017). One study demonstrated a reduced prevalence of the Firmicutes phylum, specifically the Clostridium cluster in CD patients (Frank *et al.*, 2007); while a separate study by the same group reported a decrease in the fecal abundance of *Faecalibacterium spp.* and an increase in the *Enterobacteriaceae spp.* such as *Escherichia coli* in CD patients (Sokol *et al.*, 2008; Palmela *et al.*, 2018). The microbial dysbiosis-IBD relationship is complicated by reports that intestinal inflammation may drive microbial diversity shifts (Kuehl *et al.*, 2005; Lupp *et al.*, 2007), suggesting dysbiosis as a responsive and not a causative phenomenon in disease pathology. However, the efficacy of antibiotic treatment, fecal transfers and fecal diversion in both UC and CD patients attests to the importance of the gut microbiota in IBD pathology (Sartor, 1998; Yamamoto *et al.*, 2000; Dutta *et al.*, 2014; Suskind *et al.*, 2015). Moreover, the disparity in microbial status between discordant monozygotic twins with IBD suggests the dispensability of environmental and genetic risks in IBD pathology, underscores the importance of the microbiota in IBD (Willing *et al.*, 2010).

Interestingly, research findings demonstrate the biological advantage of pathogenic aerotolerant bacteria such as proteobacteria and actinobacteria during inflammation (Lupp *et al.*, 2007; Hartman *et al.*, 2009; Rivera-Chávez *et al.*, 2016; Litvak *et al.*, 2017). Given that inflammation is an oxidative process and that the

colon is mostly hypoxic, IBD would result in the expansion of mostly oxidative resistant microbes. *Citrobacter rodentium*, a mouse pathogen, increases oxygenation and aerobic respiration at mucosal tissues (Lupp *et al.*, 2007; Hartman *et al.*, 2009), giving it a survival advantage over other strict anaerobes that include *Bacteroidetes spp*.. Besides oxygenation, the production of electron charged molecules during inflammation potentially provides terminal electron acceptors for facultative anaerobes such as *Salmonella spp.* (Winter *et al.*, 2010), a species of the *Enterobacteriaceae* family that are associated with gut inflammation (Darfeuille-Michaud *et al.*, 2004; De la Fuente *et al.*, 2014). These findings suggest the importance of inflammation and host driven metabolic processes as modulators of microbial dysbiosis during IBD.

A caveat to microbial dysbiosis-IBD research is the lack of strong evidence linking particular pathobionts to either CD or UC disease in human patients, although a number of bacterial species maybe capable of driving inflammatory pathology in IBD through their ability to infect epithelial cells *in vitro* (Michail and Abernathy, 2002). Nonetheless, the bacterium *Fusobacterium nucleatum* is positively associated with UC and CRC (Strauss *et al.*, 2011; Yang *et al.*, 2017), while the adherent-invasive *Escherichia coli* (AIEC) bacterium is associated with ileitis during CD (Darfeuille-Michaud *et al.*, 2004; De la Fuente *et al.*, 2014). The fungal pathogen, *Candida tropicalis*, increased in abundance during IBD dysbiosis, instigates local and systemic inflammation (Zwolinska-Wcislo *et al.*, 2009), while Caudovirales bacteriophage sequences are abundant in pediatric CD ileal biopsies (Wagner *et al.*, 2013; Norman *et al.*, 2015).

Keeping in line with the previous discussion, the reduced presence of the mucin degrading *Akkermansia muciniphila* bacterium is positively correlated with IBD (Ganesh *et al.*, 2013; Berry *et al.*, 2015). Short chain fatty acid (SCFA)-producing microbes are associated with lower IBD risk (Venkatraman *et al.*, 2003; Vieira *et al.*, 2012; Rivera-Chávez *et al.*, 2016)(discussed later), while *Lactobacilli spp.* are demonstrated to produce an aryl hydrocarbon receptor (AhR) ligand, which can promote antimicrobial peptides (AMPs) expression in the presence of tryptophan catabolites and contribute to the suppression of gut inflammation (Zelante *et al.*, 2013). Interestingly, a number of *Clostridium spp.* can suppress gut inflammation

through their ability to expand colonic $CD4^+$ Treg cells (Atarashi *et al.*, 2013; Rivera-Chávez *et al.*, 2016). Although many microbial species are associated with IBD, it remains inconclusive whether microbial dysbiosis is due to inflammation or dysbiosis drives inflammation.

2.2.1 The gut microbiota, immune regulation and anergy

An impaired Treg compartment in germ free and gnotobiotic mice demonstrates the importance of the gut microbiome in shaping the host's immune system (Cebra, 1999; Rawls *et al.*, 2006; Atarashi *et al.*, 2011), while the indiscriminate use of antibiotics in early childhood is associated with increased risk of autoimmune disease in later life (Rolny *et al.*, 1983; Droste *et al.*, 2000; Horton *et al.*, 2014); underscoring the importance of the gut microbiota in host health. Gut homeostasis and immune tolerance is attributed to the role of regulatory cells that include $CD4^+$ Tregs cells as well as dendritic cells. Two types of Tregs; naturally derived Tregs (thymus derived) and peripherally induced Tregs, contribute to the steady state tolerance evident in healthy hosts. Identified by the Forkhead box P3 transcription factor ($Foxp3^+$) and induced under specific conditions, including Tumor Growth Factor-Beta (TGFβ) signaling (Veldhoen *et al.*, 2006; Gagliani *et al.*, 2015), Tregs accumulate at mucosal surfaces and differentiate under the influence of commensal organisms and environmental signals (Round and Mazmanian, 2010; Atarashi *et al.*, 2013; Smith *et al.*, 2013).

The chronic multiple organ failure (including enteritis) apparent in humans with Foxp3 mutations demonstrates the importance of Tregs in immune regulation (Wan and Flavell, 2007). Genome Wide Associated Sequencing (GWAS) demonstrates the association of human IBD with IL-10 polymorphisms (Glocker *et al.*, 2011), a cytokine positively correlated with the suppressive function of Treg. Indeed, congenital IL-10 deficiency leads to severe childhood colitis (Sydora *et al.*, 2003), while $IL\text{-}10^{-/-}$ mice develop microbiota-driven spontaneous colitis dominated by Thelper (Th)17 cell signaling (Kühn *et al.*, 1993; Daniel J Berg *et al.*, 1996; Sydora *et al.*, 2007). Interestingly, specific microbial species such as *Bacteriodetes fragilis* and some *Clostridia spp.* strains can alleviate inflammation

in several colitis models through their ability to promote and expand Tregs (Round and Mazmanian, 2010; Atarashi *et al.*, 2013). The promiscuity of TGFβ in promoting either the Treg or Th17 compartments demonstrates the importance of the signaling environment in determining regulation and inflammation in the gut **(Fig 2.5)**.

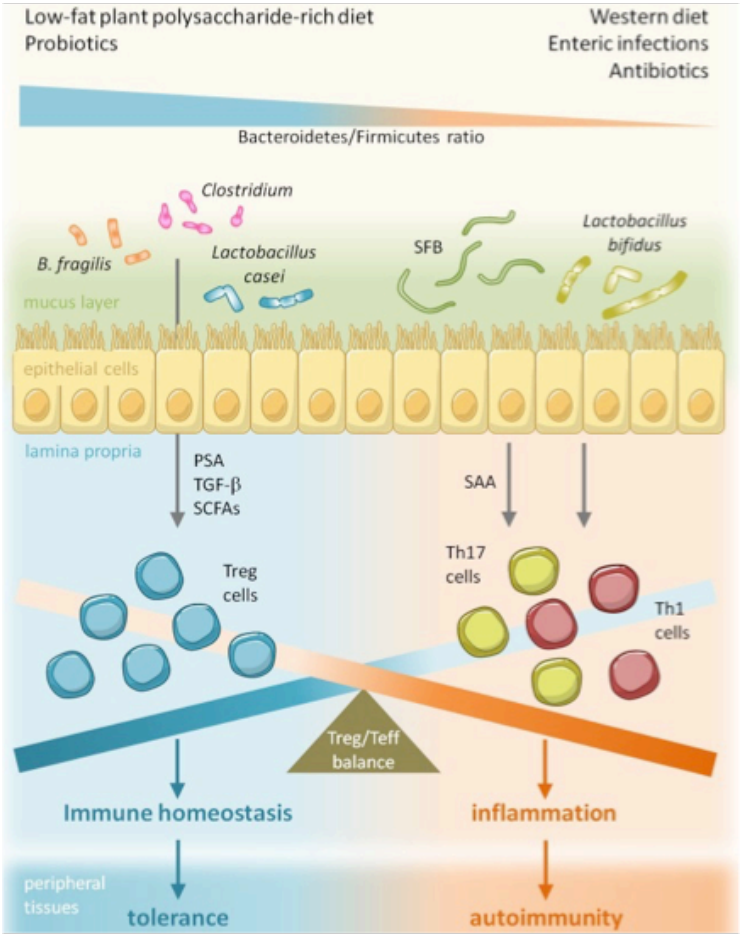


Figure 2-5: Homeostatic balance of the Treg:Th17 in the gut is highly dependent on the environmental factors imprinting on the gut microbiota. From (Sorini and Falcone, 2013)

2.2.2 Genome Wide Association Studies (GWAS) associations with IBD

A number of experimental colitis models have been employed in order to determine the contribution of IL-17 signaling in colitis pathology (Melgar *et al.*, 2005; Yen *et al.*, 2006; Elliott *et al.*, 2008; Wang *et al.*, 2015). Although some authors refute the importance of IL-17 in acute DSS colitis (Kim *et al.*, 2011), IL-17A has been positively associated with human IBD and murine colitis (Ito *et al.*, 2008; Alex *et al.*, 2009; Gelderblom *et al.*, 2012; Hyun *et al.*, 2012; Cătană *et al.*, 2015) . IL-17, a major cytokine secreted by Th17 and type 3 innate lymphoid cells

(ILC3) cells (*reviewed in* Annunziato, Romagnani and Romagnani, 2015), is a major antimicrobial cytokine copiously secreted in response to microbial attack (Korn *et al.*, 2009; Wang *et al.*, 2015). Interestingly, IL-17 signaling is associated with neutrophil accumulation (Stark *et al.*, 2005), supporting the heightened neutrophil infiltration in UC and CD colon biopsies (Rhodes and Jewell, 1983; Bennike *et al.*, 2015).

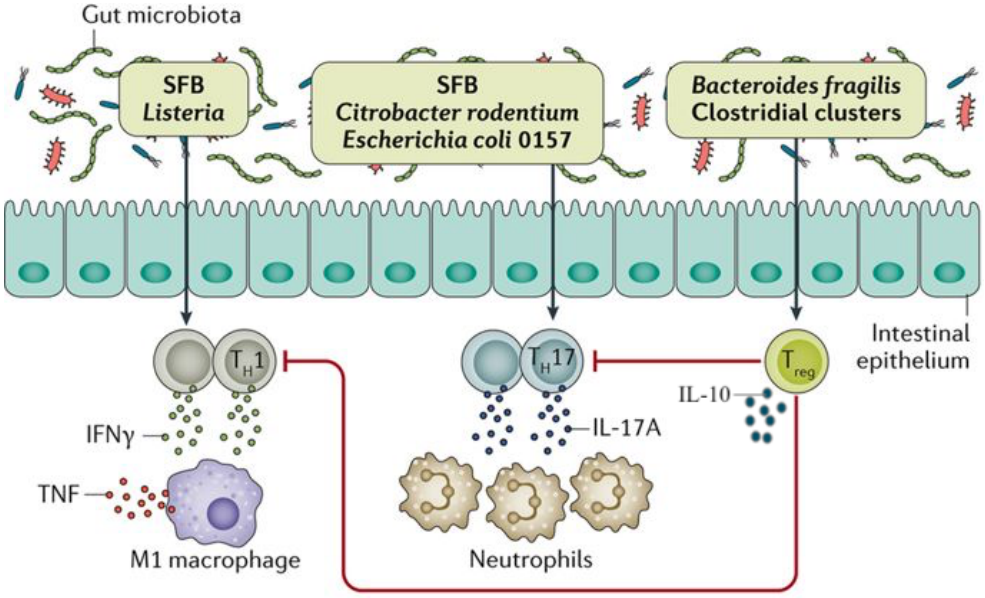


Figure 2-6: Commensal and pathogenic bacteria can both modulate gut immune responses. Adapted from (Powell *et al.*, 2017).

Segmented filamentous bacteria (SFB), a known gut pathobiont, can induce IL-17 expression *in vitro*, suggesting a similar effect of other yet unidentified pathobionts *in vivo* during IBD pathogenesis (**Fig 2.6**) (Ivanov *et al.*, 2009). As stated previously, transforming growth factor (TGF)-β signaling induces the differentiation of both Th17 cells and Tregs depending on the cytokine milieu (Mangan *et al.*, 2006). Besides TGFβ, IL-23 is also demonstrated to expand Th17 and ILC3 cells (Yen *et al.*, 2006; Wang *et al.*, 2015), both potent producers of IL-17. IL-23, primarily secreted by intestinal DCs, plays a key role in the pathogenesis of several immune mediated diseases including IBD (Yen *et al.*, 2006). Blockade of IL-12p40, the gene encoding the β subunit of IL-23 is an effective treatment for CD disease (Elson *et al.*, 2004), emphasising the importance of the Th17 signaling pathway in CD pathology.

2.2.3 Innate signaling and the gut microbiota

The aberrant functioning of some Toll-Like Receptors (TLRs) and their downstream signaling molecules is implicated in intestinal inflammation (Rakoff-Nahoum *et al.*, 2004; Araki *et al.*, 2005; Frantz *et al.*, 2012). TLRs and nucleotide-binding oligomerisation domain (NOD) are important in mediating microbial signaling (Mähler *et al.*, 1998; Nagai *et al.*, 2006). Innate immune cells detect pathogenic and commensal bacteria via a conserved set of pattern recognition receptors that potentially activate NFkB signaling, inflammasome assembly and epithelial repair pathways (Newton and Dixit, 2012; Levy *et al.*, 2015). Defects in the TLR and NOD like receptor (NLR) are associated with spontaneous microbiota driven gut inflammation (Elinav *et al.*, 2011), while the disruption of the myeloid differentiation protein 88 (MyD88) central adapter (TLR signaling) leads to profound immune dysfunction (Araki *et al.*, 2005; Frantz *et al.*, 2012; Natividad *et al.*, 2013). In a DSS murine model of colitis, MyD88 deficiency resulted in severe and lethal colitis compared to their wild-type counterparts (WT mice), although exacerbated colitis was independent of the gut microbiota (Rakoff-Nahoum *et al.*, 2004). However, MyD88$^{-/-}$ mice demonstrate heightened bacteremia when colonized with *Citrobacter rodentium*, resulting in systemic inflammation (Lebeis *et al.*, 2007; Bhinder *et al.*, 2014). This demonstrates that a defect in innate immunity leads to the outgrowth and the systemic translocation of gut pathogens that contribute to the overall disease phenotype, which, interestingly, maybe abrogated by antibiotic treatment in a murine model of type 1 diabetes (Wen *et al.*, 2008).

2.2.4 Secretory IgA, microbiota and IBD

Secretory IgA maintains gut microbiota diversity through it ability to opsonize pathobionts such as *Prevotellaceae*, *Helicobacter spp.* and Segmented filamentous bacteria (SFB) (Birkholz *et al.*, 1998; Suzuki *et al.*, 2004); species all identified as extremely colitogenic (Kuehl *et al.*, 2005; Hrncir *et al.*, 2007; Elinav *et al.*, 2011; Whary *et al.*, 2014). IgA^{+} bacteria isolated from IBD patients, but not from healthy individuals, led to severe DSS induced colitis when transferred to germ-

free mice (Palm *et al.*, 2014). Indeed, colitogenic IgA^+ can penetrate the mucus layer and trigger T cell dependent high affinity IgA production (Palm *et al.*, 2014), while IgA deficiency is positively associated with both UC and CD susceptibility (Palm *et al.*, 2014).

2.2.5 The microbiota, diet and inflammation

A high fiber diet is positively correlated with reduced IBD risk (Walker *et al.*, 1986; De Filippo *et al.*, 2010), while a high fat diet is associated with a number of inflammatory pathologies (Teixeira *et al.*, 2011; Kim *et al.*, 2012; Laroui *et al.*, 2012; Cheng *et al.*, 2016). Diet influence is one of the contributing factors behind the inverse distribution of IBD when comparing high-income countries (HIC) and low-to-middle income countries (LMIC). A change in diet to a purified animal-based diet rather than a plant-based has been associated with an increased risk of IBD in HIC. A complex plant-based diet provides a fermentable base for the gut microbiota, releasing SCFAs that have been shown to support epithelial cell function (Maslowski *et al.*, 2009; Chassaing *et al.*, 2015; Williams *et al.*, 2017). The three most important SCFAs of current scientific interest are butyrate, propionate and acetate; with butyrate being the most studied SCFA in relation to gut homeostasis (Furusawa *et al.*, 2013; Chang *et al.*, 2014; Rivera-Chávez *et al.*, 2016). Butyrate is the preferred energy source for colonial epithelial cells (Gaudier *et al.*, 2004), with reduced availability and absorption associated with increased intestinal inflammation.

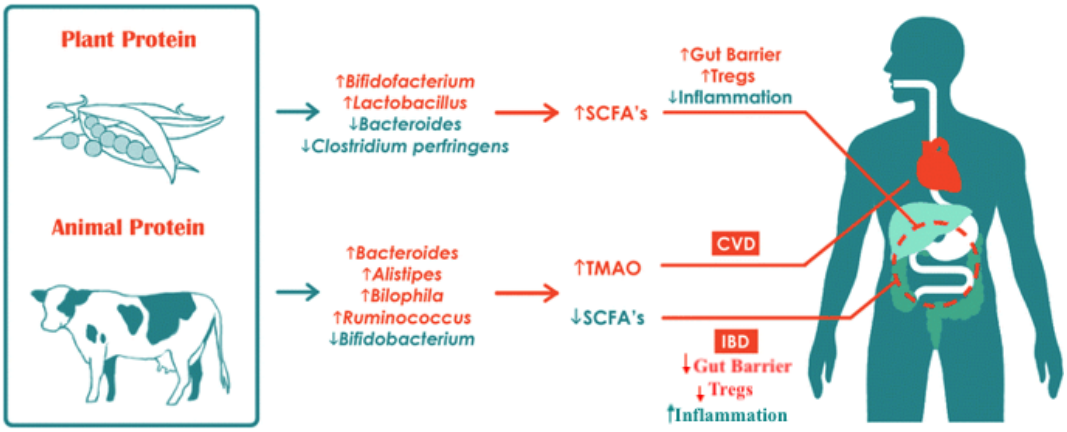


Figure 2-7: Diet influence on gut inflammation, SCFA (short chain fatty acids), TMAO (trimethylamine N-oxide), CVD (Cardiovascular disease), IBD (Inflammatory Bowel Disease). Adapted from (Singh *et al.*, 2017).

Butyrate is thought to play a role in the following functions:

i. Promotes production of MUC2, contributing to mucus layer integrity (Gaudier *et al.*, 2004).
ii. Regulates the production of growth factors and anti-inflammatory cytokines that include IL-10 (Säemann *et al.*, 2000).
iii. Suppresses activation of nuclear factor kappa beta (NFkB) and production of heat-shock protein (Venkatraman *et al.*, 2003).
iv. Regulates the activity of proteins involved in apoptosis such as caspase-3 and caspase-7 (Schwab *et al.*, 2006).
v. Increases the activity of anti-oxidant enzymes such as the glutathione S-transferase (GST) (Ebert *et al.*, 2003).
vi. Promotes the production of anti-microbial peptides (AMPs) (Schauber *et al.*, 2003).
vii. Inhibits the deacetylation of histones, hence controls cell proliferation and apoptosis (Chang *et al.*, 2014).

Interestingly, butyrate has been demonstrated to selectively support the growth of butyrate metabolizing cancer cells *in vivo* (Serpa *et al.*, 2010), while a separate study demonstrated the inflammatory capacity of butyrate *in vitro* at concentrations higher than physiological titers *in vivo* (Kespohl *et al.*, 2017). This may suggest the vacillating nature of butyrate regards immune modulation.

Besides complex polysaccharides, dietary fat content is positively associated with a number of inflammatory pathologies (Pacheco *et al.*, 1987; Kim *et al.*, 2012; Ghosh *et al.*, 2013). Dietary fatty acids fall into the following categories:

i. Short Chain Fatty Acids (SCFAs)
ii. Medium Chain Fatty Acids (MCFAs)
iii. Long Chain Fatty Acids (LCFAs)
iv. Unsaturated Long Chain Fatty Acids (ULCFAs)
v. Poly Unsaturated Fatty Acids (PUFAs)

A diet rich in n-6 PUFA (linoleic acid) has been associated with disease, sepsis and mortality due to the production of proinflammatory arachidonic acid metabolites (de Silva *et al.*, 2010). Researchers report the ability of an n-6 PUFA diet to modify the murine gut microbiome (Ghosh *et al.*, 2013), resulting in intestinal inflammation. Furthermore, linoleic acid oxidized metabolites can activate peroxisome proliferator-activated receptor (PPAR)-γ receptor signaling in colon tumor cell lines, acting as endogenous ligands for PPARγ signaling and resulting in inflammatory responses. Indeed, PPARγ signaling is demonstrated as key in adipocyte differentiation, lipid metabolism and intestinal inflammation regulation (Tontonoz *et al.*, 1994). The relationship between IBD and PPARγ is quite volatile, with contradictory research findings demonstrating either a beneficial or pathogenic role of PPARγ in intestinal inflammation (Shrestha *et al.*, 2010; Hume *et al.*, 2012; Poliska *et al.*, 2012). Despite the contentious discussions on the role of PPARγ in IBD, an important take home message is the existence of a nuclear transcription factor capable of modulating intestinal inflammation through lipid metabolite interaction with innate receptors.

Sphingolipids are another class of lipids that are part of intestinal membranes and play an important role in regulating digestion, absorption and mucosal protection (Serhan *et al.*, 1996). Dietary sphingolipids are sourced from dairy, eggs, meat and soy, while an infant's only source of dietary sphingolipids is breast milk. Abundantly expressed on the apical membrane of intestinal cells, sphingolipids act as bacterial and toxin receptors. Glycosphingolipids, a member of the sphingolipid group, are reported to bind porcine rotavirus and *Escherichia coli*, enabling

infection of enterocytes (Lanne *et al.*, 1995; Rolsma *et al.*, 1998). Abnormal sphingolipid metabolism and composition patterns are positively associated with inflammatory responses and carcinogenesis (Schmelz and Merrill Jr., 1998). Indeed, administration of sphingomyelin in a mouse model of cancer prevented aberrant crypt foci development and tumorigenesis, demonstrating a protective effect of sphingolipids during carcinogenesis (Dillehay *et al.*, 1994). Certain members of the gut microbiota that include *Bacteroides spp.* and *Sphingomonas spp.* can synthesize sphingolipids from the dietary components of their hosts (Olsen and Jantzen, 2001; An *et al.*, 2011), demonstrating a microbiota-sphingolipid-host interaction important in intestinal regulation.

In conclusion, diet can control regulation of intestinal homeostasis in many ways. Bioactive molecules released by commensal bacteria are highly dependent on the substrates available to them within the gut, reinforcing the importance of a balanced diet-microbiome axis in gut inflammatory diseases.

2.3 IBD Interventions

IBD preferentially affects the intestinal regions with the highest abundance of bacteria (Hermiston and Gordon, 1995). The complexity of IBD pathology has limited most interventions to inflammatory symptom treatment, while the latest therapeutic interventions are majoring on host as well as microbiome manipulation to avoid steroid side effects. This section will focus on interventions aimed at microbial manipulation, as there exists abundant evidence supporting the role of the gut microbiota in IBD pathogenesis that includes:

- Association of microbial dysbiosis with CD and UC (Litvak *et al.*, 2017).
- Association of IBD related genetic polymorphisms with host-microbiome interactions as well as the mucosal barrier (Glocker *et al.*, 2011).
- Efficiency of both antibiotic and probiotic administration in disease remission induction (Prantera *et al.*, 2006; Sood *et al.*, 2009; Horton *et al.*, 2014).

- Failure to induce colitis in germ free mice, with the introduction of gut microbiota from conventional mice associated with colitis in germ free mice (Brimnes *et al.*, 2001; Mazmanian *et al.*, 2008).
- Mutations in Dectin-1 signaling receptor is associated with CD (Dectin-1 is a fungi restricted receptor, important in modulating fungal populations) (de Vries *et al.*, 2009).
- Fecal stream diversion is beneficial in CD patients (Yamamoto *et al.*, 2000).
- Gnotobiotic mice have diffuse underdeveloped gastrointestinal lymph node structures, and are prone to spontaneous colitis (Cebra, 1999).

Hence, below, we discuss a couple of interventions directed at microbial modulation during IBD therapy.

2.3.1 Fecal Stream Diversion

Fecal diversion is the process whereby surgeons create an opening between the skin and either the small intestine (ileostomy) or the colon (colostomy) to treat rectal/anal infections, IBD/colon cancer and incontinence **(Fig 2. 8)**.

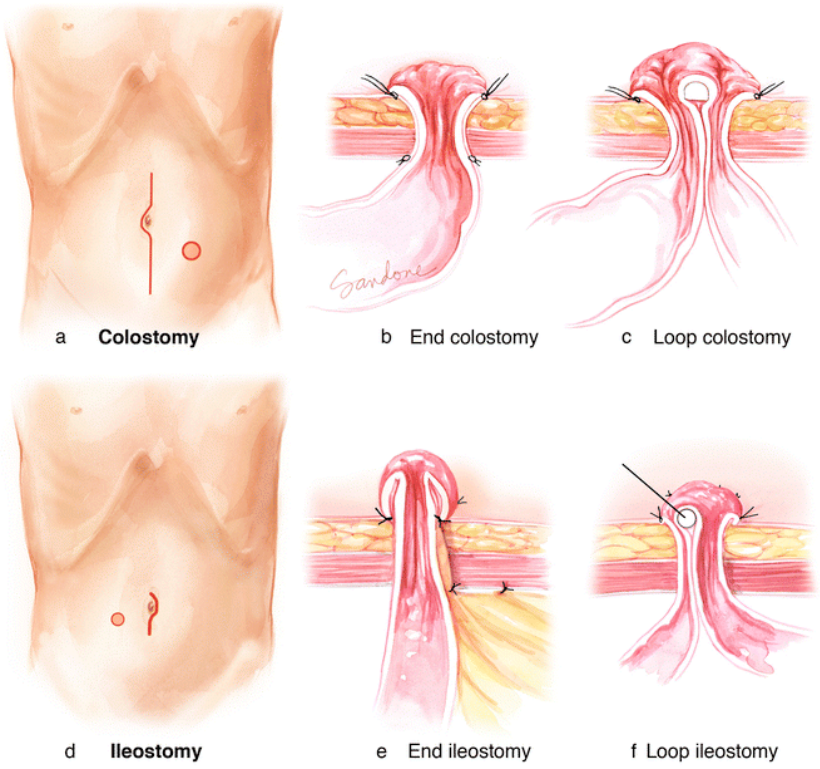


Figure 2-8: Fecal diversion and ostomies procedures. Reprinted with permission from Springer Link, image from (Bennett and Wick, 2017)**.**

Fecal diversion results in the reduced exposure of the colon epithelium to dietary and fecal microbial antigens, resulting in inflammation abrogation. However, fecal diversion is also used to correct for anastomosis during IBD. The risk of developing an anastomotic leak during IBD pathology is associated with some biological therapies that include the use of anti-TNF (infliximab) therapy. The association of infliximab therapy and IBD anastomosis is quite controversial, as some researchers failed to demonstrate a relationship between the treatment and anastomotic leakage in patients, while some noted a positive association between IBD, surgery and infliximab therapy (Selvasekar *et al.*, 2007; Appau *et al.*, 2008; Kunitake *et al.*, 2008; Krane *et al.*, 2013). Meta-data analyses failed to clear the uncertainty between anastomosis and infliximab therapy in IBD, leading to a controversial discussion on the importance of fecal diversion in IBD patients (Selvaggi *et al.*, 2015). However, a number of CD patients have benefitted from this procedure (Bafford *et al.*, 2017), although it is not universally effective due to the potential deprivation of SCFAs to colonocytes, potentially resulting in colitis recursion (Harig *et al.*, 1989).

2.3.2 Antibiotics

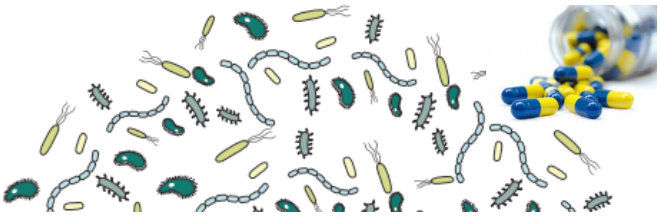


Figure 2-9: Illustration of antibiotic treatment on the gut microbiota. Adapted from (Dickson, 2017) and www.championnh.com.

The effectiveness of antibiotic treatment of human IBD is limited, although antibiotics are reported to alleviate intestinal inflammation in various animal models of colitis (Perencevich and Burakoff, 2006; Reikvam *et al.*, 2011; Horton *et al.*, 2014). Several clinical studies have shown that broad-spectrum antibiotics improved rates of steroid-free remission in CD *reviewed in* (Su *et al.*, 2015), while meta-analyses of randomized controlled trials showed a statistically significant benefit of antibiotics relative to placebo in treatment of both CD and UC (Gilat *et al.*, 1987). Surprisingly, antibiotic therapy did not appear to benefit patients with

severe UC (Chapman *et al.*, 1986; Mantzaris *et al.*, 2001). In separate clinical trials, monotherapy of either the antibiotics ciprofloxacin or metronidazole did not have a beneficial outcome on IBD (Sutherland *et al.*, 1991; Su *et al.*, 2015), demonstrating the need for antibiotic cocktails to maintain remission. However, antibiotic interventions probably result in antibiotic resistance as well extensive physiological damage to the host, as prolonged exposure to metronidazole is associated with neurologic side effects (Gugler *et al.*, 1989; Kuriyama *et al.*, 2011).

Some caveats of antibiotic treatment include the potential rebound of bacterial colonies soon after cessation of antibiotic therapy, as demonstrated in a clinical trial where a dramatic increase of bacteria and inflammation was evident within one week of antibiotic withdrawal (Swidsinski *et al.*, 2008). A recent Chinese study also noted that two thirds of gram-negative bacteria isolated from CD patients were ciprofloxacin resistant (Park *et al.*, 2013), suggesting the possibility of antibiotic resistance in IBD patients. Inconsistent outcomes of antibiotic treatment are probably due to the heterogeneity of IBD as well as the diversity within human populations. As a cautionary tale, indiscriminately targeting the gut microbiota with broad-spectrum antibiotics potentially depletes both pathogens and beneficial bacteria, exposing the host to a number of unpredictable consequences. Identification of specific bacterial species associated with CD or UC in the general populace would help in the therapeutic design of a targeted antibiotic preparation.

2.3.3 Enteral Nutrition Therapy (ENT)

The modification of the dietary constituents of patients has long been used as a therapy intervention for CD *reviewed in* (Griffiths, 2000), with ENT being used as first line therapy for the induction of remission in CD (Griffiths, 2000). Although the mechanism of action of ENT is not well defined, prominent hypothesis include its ability to reduce luminal antigens arising from the patient's food and also the ability of an ENT diet to modulate the gut microbiome as well as its metabolome (O'Morain *et al.*, 1980). The implementation of ENT for CD therapy is quite

controversial, with the only ascertained beneficial results appreciated in pediatric and not adult CD (Griffiths *et al.*, 1995; Dziechciarz *et al.*, 2007). Due to the dietary and growth challenges faced by pediatric CD patients (Griffiths *et al.*, 1993; Haberman *et al.*, 2015), ENT is one of the considered therapies to mitigate against CD as well malnutrition in these patients. Enteral nutrition is preferred to parenteral nutrition, as the latter is rarely able to deliver the calories and nutrients required for the maintenance of the gut immune system, with **Fig 2.10** one of the methods used for enteral feeding.

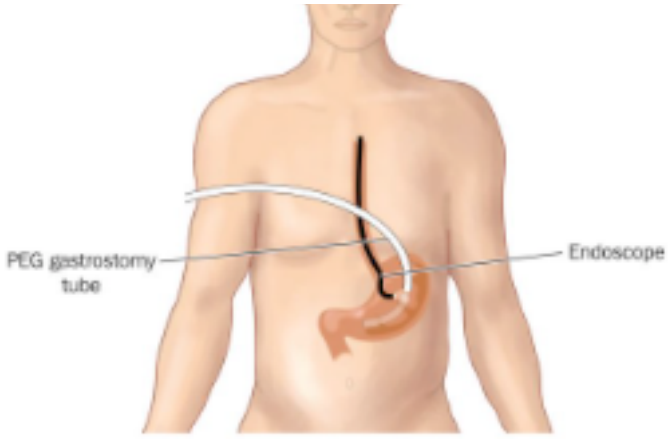


Figure 2-10: Insertion of a percutaneous endoscopic gastronomy (PEG) tube for enteral feeding. From (https://www.acquiredbraininjury-education.scot.nhs.uk/impact-of-abi/nutrition-and-hydration/nutritional-support/enteral-feeding-methods/)

Apart from providing nutritional value, ENT may potentially modulate gut inflammation through availing SCFAs as well as suppressing pro-inflammatory cytokine signaling (Sanderson, 2001). In one study, As part of a polymeric ENT formula, TGFβ administration (as part of a polymeric formula), resulted in a 79% remission rate in pediatric CD patients, accompanied by the suppression of IL-1, IFNγ and IL-8 transcription levels (Fell *et al.*, 2000). In a separate study, ENT resulted in a fecal microbiota composition shift as well some functional metabolome changes that led to increased levels of anti-inflammatory SCFAs in children with CD (Leach *et al.*, 2008). Interestingly, favourable therapeutic outcomes of ENT were associated with an initial shift in microbial composition towards even greater dysbiosis relative to healthy individuals (Shiga *et al.*, 2012); pointing to the complex relationship between dysbiosis and IBD.

2.3.4 Fecal Microbiota Transplantation (FMT)

The concept of FMT was introduced as a measure of reconstituting patient gut microbiota with microbiota from healthy donors (deemed as fit). FMT proponents postulate that children born through vaginal delivery are exposed to maternal vaginal and fecal microbiota (Dominguez-Bello *et al.*, 2010), giving them their first dose of healthy microbiota with long-lasting functions. A caesarean birth is proposed to be too sterile and limits the amount of microbiota exposed to neonates (Dominguez-Bello *et al.*, 2010), eventually having a long lasting immunological disadvantage that increases risks to inflammatory conditions that include gastrointestinal pathologies (van Best *et al.*, 2015).

The successful use of FMT to treat *Clostridium difficile* colitis **(Fig 2.11)** (Di Bella *et al.*, 2015) as well as result in remission of adult UC in a placebo-controlled trial (Uygun *et al.*, 2017) was however dampened by the non-beneficial result of FMT in a recent placebo-controlled CD trial (Gutin *et al.*, 2019). However, a small cohort of CD patients demonstrated rescued pathology after FMT (Suskind *et al.*, 2015), adding to the controversial outcomes of FMT therapy on either CD or UC.

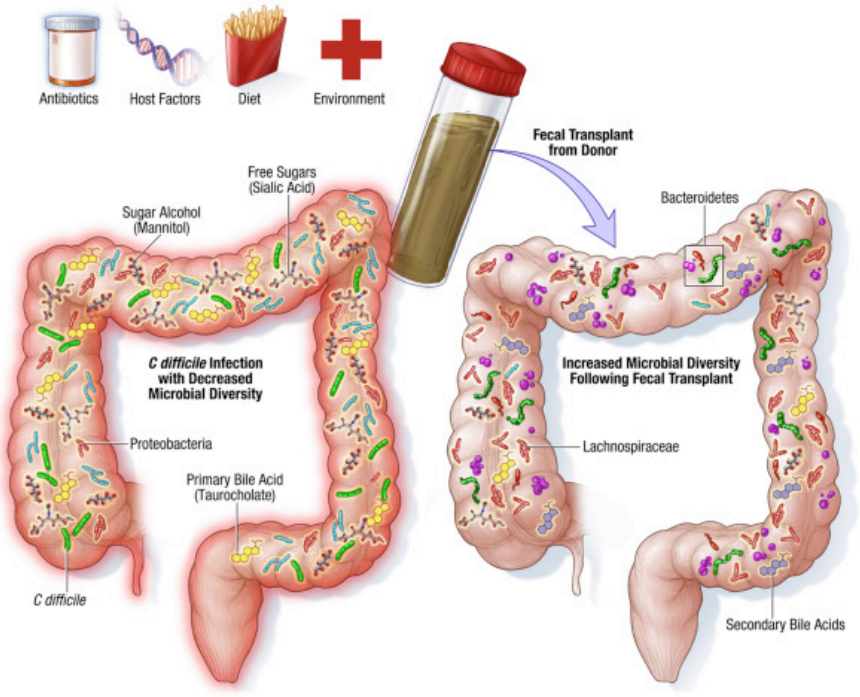


Figure 2-11: Fecal Microbiota Transfer for *Clostridium difficile* colitis. From (A. Kucher *et al.*, 2017)**.**

A recent clinical study that had 81 patients with mild to moderate UC randomized into two groups: one receiving a placebo and the other an intensive FMT treatment (the FOCUS trial), demonstrated the ability of FMT (from pooled donors) to result in UC remission after 8 weeks of FMT (Paramsothy *et al.*, 2017). Remission was confirmed in 27% of the FMT treated patients through a Mayo score as well as endoscopic evaluation compared to only 8% of placebo treated patients (Paramsothy *et al.*, 2017). These success findings are supported by another prospective study of 20 UC patients that demonstrated increased bacterial diversity and clinical disease remission in 15% of the FMT treated patients after 4 weeks of treatment (Ishikawa *et al.*, 2017). However, the low success rates signal yet unknown confounding factors of FMT, which may include but are not limited to previous exposure to biologics, recipient host immune system dynamics and recipient host gut microbiota. Although the use of FMT for IBD therapy is promising, the scarcity of long-term prospective data casts a huge shadow over FMT adoptability in the medical field. Other questions pertinent to FMT research and use include: issues regarding the safety and durability of FMT in immune-compromised patients as well as questions regarding the most appropriate dose, composition, donors and recipients in FMT.

2.3.5 Probiotics

The administration of probiotics as a microbial modulation measure of IBD is a recently appreciated practice with equivocal findings. Probiotic administration is beneficial in preventing antibiotic treatment-dependent post surgical complications in UC patients that have recently undergone an ileal pouch anal anastomosis (Gionchetti *et al.*, 2003).

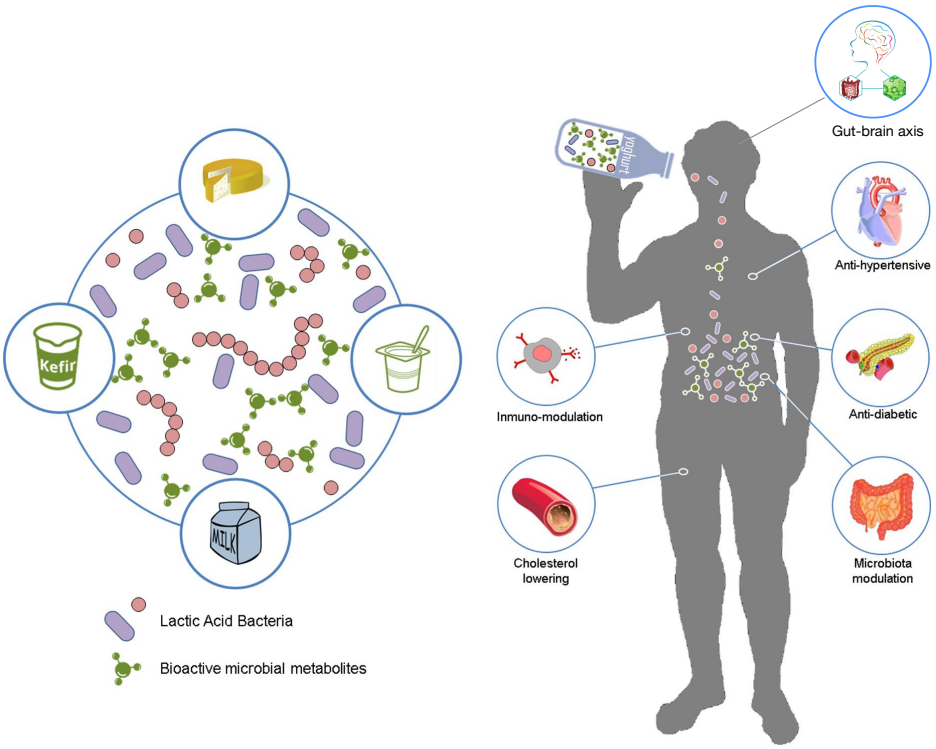


Figure 2-12: Influence of probiotic intake on human physiology exemplified by the influence of dietary derived lactic acid producing bacteria. Adapted from (Linares *et al.*, 2017).

Probiotics are mixtures of bacteria and or yeasts with perceived beneficial health effects, utilized to restore gut microbial balance (**Fig 2.12**). The potential major mechanisms by which probiotics regulate homeostasis in the gut include the following:

i. Modification of intestinal microbiota composition by reducing the prevalence of pathogenic bacteria through competition for nutrients, growth factors and adhesion receptors *reviewed in* (dos Reis *et al.*, 2017). This also leads to changes in the general metabolite composition of the gut microbiome through the reduction of pro-carcinogenic enzymes (e.g B-glucosidase and nitrate reductase) (Rivera-Chávez *et al.*, 2016).

ii. Probiotics can also aid in the production of bioactive compounds with anti-carcinogenic activity. This includes the increased bioavailability of SCFAs (Nath *et al.*, 2018), compounds generally found in higher quantities in healthy individuals.

iii. VSL#3 administration positively correlated with increased colonic conjugated linoleic acid (CLA) (Bassaganya-Riera *et al.*, 2012), an anti-proliferative and pro-apoptotic fatty acid. CLA lends its beneficial properties to

its ability to increase expression of peroxisome proliferation activated gamma receptor (PPARγ) (Bocca *et al.*, 2007; Evans *et al.*, 2010), a molecule involved in the modulation of lipid metabolism, apoptosis and immune system function (Poliska *et al.*, 2012). CLA is also capable of replacing arachidonic acid (AA) in the cell membrane and has been shown to interfere with the activity of cyclooxygenase (COX_2) and lipoxygenase (LOX) (Yeganeh *et al.*, 2016). COX_2 AND LOX are enzymes that catalyze the production of prostaglandins and leukotrienes from arachidonic, which are linked to increased risk of IBD and CRC (The IBD in EPIC Study Investigators, 2009; De Silva *et al.*, 2011).

iv. Probiotics are demonstrated to stimulate the expansion of colon $CD4^+$ $Foxp3^+$ Tregs leading to immune tolerance (Atarashi *et al.*, 2013; Smith *et al.*, 2013).

v. Some probiotics reduce intestinal permeability through their ability to modify three components in the gut:
   a. Intracolonic pH (Kruis *et al.*, 1985; Kamath *et al.*, 1988).
   b. Cellular junction proteins (Corridoni *et al.*, 2012).
   c. Production of mucins (Caballero-Franco *et al.*, 2007).

The presence of mucins ensures efficient peristalsis, hence hindering the adhesion of pathogenic bacteria to attach to the epithelium. However, the optimal composition of probiotics, timing of administration and durability of the response are some of the questions that remain unanswered pertaining adoption of probiotics as the first line of treatment in IBD patients.

2.3.6 Helminth Immunotherapy

Helminth immunotherapy is one of the methods currently being trialled as an intervention IBD. Gastrointestinal helminths share an ecological niche with the intestinal microbiota and the presence of helminths alters the microbiota composition as well as the metabolic signature of the host (Walk *et al.*, 2010; Reynolds *et al.*, 2012; S. C. Lee *et al.*, 2014; Houlden *et al.*, 2015). Helminths can mount chronic infections through their ability to modulate the host's immune

system to avoid expulsion (Finney *et al.*, 2007; Smith *et al.*, 2011). Typically, both humans and mice mount a type-2 immune response characterised by the production of IL-4, IL-5, IL-9 and IL-13 from type-2 innate lymphoid cells (ILC2) and T helper 2 (Th2) cells *reviewed in* (McSorley and Maizels, 2012). A type-2 immune response is also associated with the recruitment of eosinophils, mast cells, basophils and alternatively activated macrophages (AAMs), required to effectively eliminate a helminth infection (Anthony *et al.*, 2007; Wynn, 2015; van Rijt *et al.*, 2016). However, successful *Heligmosomoides polygyrus (H.polygyrus)* infection is noted to modify the host's Th2 immune response into a T-regulatory dominated immune response (Finney *et al.*, 2007; Smith *et al.*, 2016), enabling efficient colonization of the host.

Helminth modification of the host's immune response

Helminth infection is associated with the modulation of bystander inflammatory conditions that include airway hyperactivity, human and experimental colitis, obesity and dermatitis **(Fig 2.13)** (Reardon *et al.*, 2001; R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005; Smith *et al.*, 2007; Elliott *et al.*, 2008; Ledesma-Soto *et al.*, 2015).

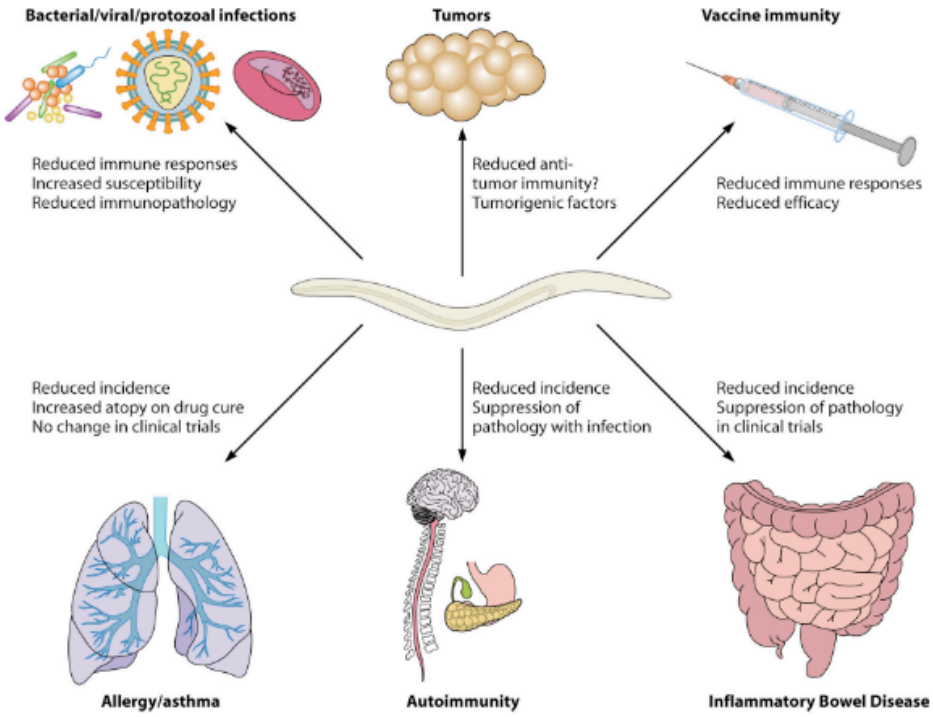


Figure 2-13: Bystander effects of helminth infections. From (McSorley and Maizels, 2012)**.**

The ability of *H.polygyrus* to mount chronic infections in their hosts is positively associated with the expansion of host regulatory $CD4^+Foxp3^+$ Tregs, AAMS, regulatory B cells and dendritic cells (Grainger *et al.*, 2010; McSorley and Maizels, 2012; Rick M. Maizels *et al.*, 2012; Blankenhaus *et al.*, 2014). *H.polygyrus* and *Nippostrongylus brasiliensis* release excretory-secretory (ES) products that include immuno-modulatory proteins derived from glycoproteins and miRNAs (Holland *et al.*, 2000; Grainger *et al.*, 2010; Johnston *et al.*, 2015), with the *H.polygyrus* excretory-secretory (HES) products containing a TGFβ mimic capable of directly inducing $CD4^+Foxp3^+$ Tregs in vitro (Grainger *et al.*, 2010). Interestingly, live *H.polygyrus* infection drives the expansion of mesenteric lymph node (MLN) $CD4^+CD25^+Foxp3^+$ Tregs (Smith *et al.*, 2011; Pastille *et al.*, 2017). The ability of both live *H.polygyrus* infection and HES products to expand the regulatory compartments points to the extremely immunomodulatory capacity of *H.polygyrus*.

Human epidemiological studies in Ethiopia showed a negative association between helminth infection and airway allergy (Scrivener *et al.*, 2001), while a meta-analysis study performed by *Flohr and Yeo* found evidence to support an inverse relationship between helminth infection and atopic dermatitis (Flohr and Yeo, 2011). Interestingly, Correale and colleagues noted that during a five year follow up on multiple sclerosis (MS) patients, a subset of patients who accidentally got infected by helminths experienced an alleviation of MS symptoms, while their 'uninfected' counterparts experienced the 'expected' deteriorating effects of the MS condition (Correale and Farez, 2007). *Summers et al.* reported an improvement of both UC and CD symptoms in patients treated with *Trichuris suis* worms and *Trichuris suis* ova (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005), demonstrating an immunosuppressive role of both *Trichuris suis* and its ova.

In murine models of inflammatory disease, chronic infection with the filarial worm *Litosomoides sigmodontis* was found to protect against airway hyperreactivity (Dittrich *et al.*, 2008), while the tapeworm *Taenia Crassiceps* was able to inhibit colonic inflammation and tumor promotion during colitis-associated cancer (CAC) (León-Cabrera *et al.*, 2014). The murine gastrointestinal helminth,

H.polygyrus, which is closely related to the human hookworms *Necator americanus* and *Ancylostoma duodenale*, modulates a number of bystander conditions *reviewed in* (Rick M. Maizels *et al.*, 2012). *H.polygyrus* and *H.polygyrus* excretory/ secretory products (HES) have the potential of suppressing airway allergy (Wilson *et al.*, 2005), autoimmune type-1 diabetes (Saunders *et al.*, 2007) and colitis in mice (Elliott *et al.*, 2004; Chen *et al.*, 2005; Sutton *et al.*, 2008; Hang *et al.*, 2010). Although some helminth infections are associated with immune suppression, Wang and colleagues reported exacerbation of oxazolone colitis through an IL-5 dependent pathway by the rat tapeworm, *Hymenolepis diminuta* (Hunter *et al.*, 2007; Wang *et al.*, 2010), demonstrating either a condition, helminth or host dependent effect of helminth immunomodulation.

Helminth infections, gut microbiota and host immune responses

Although helminths can disrupt the gut microbial composition (Walk *et al.*, 2010; S. C. Lee *et al.*, 2014; Houlden *et al.*, 2015), helminth-elicited taxonomical shifts vary over the course of infection as well as depending on bystander conditions in mice. Factors that include the lifecycle; size and niche of the helminth within the host; baseline differences in microbial compositions between individuals that maybe affected by the host's diet; the helminth species and any other environmental factors that may impact either the helminth or the host play a key role in helminth-elicited taxonomic shifts. It has been suggested as well as demonstrated that helminths affect the structure of intestinal microbiota communities in the following ways:

i. Possibly through the direct/indirect anti-microbial activity of their excretory-secretory products (Reynolds *et al.*, 2014).
ii. By altering nutrient and niche availability in the intestine, for example, impairment of glucose absorption (Houlden *et al.*, 2015) .
iii. Favoring outgrowth of certain *Clostridiale spp.*, a family highly published for its ability to expand host Tregs (Atarashi *et al.*, 2013; Zaiss *et al.*, 2015).
iv. Modification of both the innate and adaptive host's immune responses that can drive modulation of intestinal microbes (Hang *et al.*, 2014).

Successful *H.polygyrus* infection is positively correlated with an increase of gut Lactobacilliceae (Reynolds *et al.*, 2014), providing evidence of a helminth-gut microbiota interaction. Since helminths alter the microbiota to enable host colonisation, helminths may drive expansion of protective microbiota members leading to inflammatory bacteria inhibition. A helminth-associated bloom of *Clostridiale spp.* blocked expansion of pro-inflammatory *Bacteroides vulgatus* and ameliorated disease in a mouse model of IBD (Ramanan *et al.*, 2016). Transfer of gut microbiota from helminth infected mice to uninfected mice alleviated airway allergy (Zaiss *et al.*, 2015), suggesting that helminths can mediate allergic inflammation through gut microbial modification. However, the ability of a helminth-modified microbiota to protect from intestinal inflammation likely depends on the bacterial species that are driving inflammation. In a mouse model of *Citrobacter rodentium* colitis, transfer of an *H.polygyrus*-modified microbiota to uninfected mice exacerbated colitis (C. Su *et al.*, 2018), while *H.polygyrus* infected mice were unable to suppress *Salmonella typhimurium* invasion of epithelial cells (Su *et al.*, 2014). The inability to suppress bacterial invasion may contribute to the increased intestinal colonisation of *S.typhimurium* seen during helminth infection (Bobat *et al.*, 2014; Reynolds *et al.*, 2017).

Induction of the type-2 immune response following helminth infection may have indirect effects on microbial composition as well as functionality. Both *Nippostrongylus brasiliensis* or *Trichuris muris* infection are associated with changes in the host's expression of AMPs that results in resistance to helminth infection (Datta *et al.*, 2005; D'Elia *et al.*, 2009). Interestingly, a type-2 response, even in the absence of live helminth infection, is sufficient to modulate intestinal microbiota populations *reviewed in* (McCoy *et al.*, 2018). Effects of a type-2 immune response on AMP expression and mucus likely stimulates the host to induce tissue repair pathways as a result of damage caused by the helminth infection. Surprisingly, helminth-elicited shifts in microbial populations are independent of IL-4 and IL-13 signaling (Rausch *et al.*, 2013), as these shifts occur in IL-4Rα deficient *H.polygyrus* infected mice, demonstrating the dispensability of a Th2 anti-helminth immune response in microbial modulation (Rausch *et al.*, 2013). Interestingly, a helminth modified microbiota protected against an a respiratory syncytial virus (RSV) infection in $RAG^{-/-}$ and IL-$4R\alpha^{-/-}$

mice (McFarlane *et al.*, 2017). Surprisingly, protection against lung RSV infection occurred after infection with irradiated *H.polygyrus* L3 larvae (larvae that are incapable of maturing into adults) (McFarlane *et al.*, 2017). The intestinal damage caused by migrating larvae into intestinal tissue and the coincidental translocation of microbiota species into the tissue may induce a systemic innate immune response thus facilitating an anti-viral response in the lung, as protection was also lost in germ-free mice (McFarlane *et al.*, 2017).

Helminth impact on metabolism

Metabolic shifts are likely a combined result of altered host, bacterial and helminth production of metabolites as well as altered patterns of metabolite utilization, excretion and absorption during helminth infection. Helminths produce and utilize a number of host-derived metabolites (Liu *et al.*, 1990; Beall and Pearce, 2002), with some *Ascaris spp.* known to produce acetate (Tielens *et al.*, 2010), hence contributing to the host's metabolite milieu. *H.polygyrus* infection is correlated with increased carbohydrate metabolism and SCFA concentration in mice (Zaiss *et al.*, 2015), while a suppression of carbohydrate metabolic pathways is reported during *Ascaris spp.* infection in humans (S. C. Lee *et al.*, 2014). Helminth-elicited effects on host metabolites may reflect different immune evasion strategies between helminth species; for example, *Trichuris spp.* may have evolved strategies to down-regulate carbohydrate metabolism as expulsion of *T.suis* was associated with elevated SCFAs levels in cecal contents of infected pigs (Thomsen *et al.*, 2005). *Ascaris spp.* vitality is affected by SCFAs, as demonstrated by the ability of an SCFA cocktail to reduce the viability of *Ascaris spp.* eggs at low pH; hence the need to deplete it for efficient colonization (Butkus *et al.*, 2011; S. C. Lee *et al.*, 2014). On the other hand, the positive association between *H.polygyrus* and SCFAs (Zaiss *et al.*, 2015) may be an evolutionary adaptation to enable expansion of Tregs, cells that are pivotal to efficient host colonization. It is however important to identify key microbial metabolites that are affected during helminth infection as this will lead to greater insight into the impact of helminths on metabolism and gut inflammation.

2.4 Murine IBD models

Murine experimental models of human IBD exhibit immune pathological signatures similar to either CD or UC, although none can fully recapitulate human disease. In this section, we will review some experimental IBD models and discuss their beneficial aspects as well as shortcomings.

2.4.1 TNBS-Induced Colitis

Administration of a single dose of the contact sensitizing allergen 2,4,6-trinitrobenzene sulfonic acid (TNBS) intrarectally results in the induction of an IL-12 driven acute T-cell mediated colitis (Morris *et al.*, 1989). TNBS colitis closely models and resembles human CD through the involvement of the NOD2 gene in both pathologies (Watanabe *et al.*, 2008). TNBS colitis is characterized by histopathological features that include mucosal edema, crypt distortion, diffuse colon transmural inflammation and Th1 dominated responses (Elson *et al.*, 1995; Isik *et al.*, 2011). A high infiltration of leukocytes, erythrocytes, neutrophils, macrophages and lymphocytes in the mucosa and submucosa drives colon epithelium pathology (Cheon *et al.*, 2012).

Although the mode of irritation in TNBS is unclear, TNBS is proposed to haptenise the gut microbiota or epithelial cell proteins, resulting in them becoming immunogenic to the host and hence initiating colitis (Little and Eisen, 1966). The clinical importance of the TNBS model was demonstrated by the adoption of anti-IL-12p40 antibody findings from TNBS murine experiments, and translating them to successful human trials (Croxford *et al.*, 2014). The identification of the rVEGF164b protein (Cromer *et al.*, 2013), an angiogenesis inhibitory molecule in IBD, supported the importance of the TNBS model as a suitable model for IBD epithelial barrier as well intestinal inflammation studies. Interestingly, although heightened IL-17 signaling is positively associated with human IBD as well as a number of IBD murine models, IL-17 signaling is protective during TNBS colitis. IL-17$^{-/-}$ deficient mice developed mild TNBS colitis (Zhang *et al.*, 2009), while

IL-23 deficient mice were more resistant to intestinal inflammation (Becker *et al.*, 2006).

Unfortunately, a small percentage of mice per each treatment will either display low or very high symptoms of inflammation, leading to a very high variation in the TNBS readout. Because of these reasons, researchers are advised to have a minimum of 10 mice per group to compensate for the variation. The observed variation may probably be due to inter-mouse variation, even within the same cage and experiment, and may have little to do with TNBS solution preparation. Experimental readout variation may be a negative factor to researchers, as some experimental plans may not accommodate high numbers of mice due to mice and experimental costs.

2.4.2 Ικκ-γ (NEMO) Deficiency Colitis

Ικκ-γ deficiency in intestinal epithelial cells results in spontaneous severe chronic colitis in mice (Nenci et al., 2007). Ικκ-γ (NEMO) is the IKK regulatory subunit important for NFκB activation, and NFkB is important in regulating epithelial integrity as well as the gut microbiota-mucosal immune system relationship (Ghosh *et al.*, 2002). Intestinal inflammation following NFκB inactivation is characterized by extensive bacterial translocation from the lumen into the mucosa due to excessive epithelial cell apoptosis that leads to loss of epithelial integrity (Nenci *et al.*, 2007). Interestingly, Myd88 deficiency protected against inflammation in these NFκB inactivated mice, demonstrating the importance of TLR signaling in disease pathology (Nenci *et al.*, 2007). The resultant heightened sensitivity of epithelial cells to TNF in this model demonstrates the important role of NFκB in the maintenance of epithelial integrity (Nenci *et al.*, 2007; Zaph *et al.*, 2007). A typical feature of these Ικκ-γ (NEMO) deficient mice is the diminished expression of defensin-3, a Paneth cell produced AMP, and this deficiency is associated with IBD risk in humans (Wehkamp *et al.*, 2005).

2.4.3 Interleukin-10 (IL-10) Deficiency Colitis

IL-10, a cytokine positively associated with immune-suppression and produced by a subset of $CD4^+$ Tregs, is important in regulating intestinal immune responses so as to maintain an amenable relationship between commensal microbes and the host's immune response (Shevach, 2009). IL-$10^{-/-}$ mice are highly sensitive to autologous commensal microbiota (Kühn *et al.*, 1993), have poor regulatory responses and are extremely prone to spontaneous colitis as well as colon cancer (Sturlan *et al.*, 2001). The importance of regulatory IL-10 is demonstrated by an inflammatory phenotype in T cell specific IL-$10^{-/-}$ mice (Seiffarta *et al.*, 2015), showing the importance of T-cell derived IL-10 in disease pathology (Erdman *et al.*, 2003). In humans, IL-10 polymorphisms are linked to congenital colitis (Glocker *et al.*, 2011), while GWAS have associated IL-10 gene flanking SNPs with UC (Franke *et al.*, 2008).

IL-$10^{-/-}$ mice develop exaggerated Th1 immune responses when exposed to TLR ligands include lipopolysaccharide (LPS) (Daniel J Berg *et al.*, 1996), suggesting that IL-10 deficiency results in failed immune-regulation and immune tolerance. Indeed, heightened gut inflammation in this model is apparent in regions with abundant gut flora, as demonstrated by *Schaedler* and colleagues (Schaedler *et al.*, 1965), underscoring the role of the gut microbiota in this colitis model. Surprisingly, colitis extent in the IL-$10^{-/-}$ model is influenced by host genetics, as C57BL/6 mice are more resistant to colitis compared to 129/SvEv and BALB/c mice (D J Berg *et al.*, 1996). Unfortunately, research has not yet fully explained the disparities in colitis susceptibility amongst the above mentioned mice strains, although the T cell receptor (TCR) functionality is suggested to play an important role (D J Berg *et al.*, 1996).

2.4.4 T Cell Adoptive Transfer Model

Mouse $CD4^+45RB^{hi}$ cells can induce small bowel inflammation in immune compromised SCID or RAG1/$2^{-/-}$ mice **(Fig 2.14)** (Powrie *et al.*, 1993), within 5-8 weeks following adoptive transfer.



Figure 2-14: $CD4^{+}45RB^{hi}$ cell adoptive transfer scheme. Adapted from (Kiesler *et al.*, 2015).

After transfer, the distal colon exhibits histopathological features that include inflammatory cell infiltration, aberrant crypt formation, transmural inflammation and epithelial cell degradation (Powrie *et al.*, 1993). Adoptively transferred cells home to the intestinal tract, where they are demonstrated to differentiate into Th1 immune cells (Chen *et al.*, 2016). Despite their ability to home to the gut as well as colonize it, $CD4^{+}CD45RB^{lo}$ or total $CD4^{+}$ T cells are incapable of inducing colitis (Powrie *et al.*, 1993), due to the presence of the $CD4^{+}CD25^{+}Foxp3^{+}$ T-regulatory compartment in the aforementioned cell groups (Read et al., 2000). A further optimization advised the transfer of $CD4^{+}CD25^{-}$ T cells to produce a robust colitis model, a population of cells that would exclude the T-regulatory compartment (Kjellev *et al.*, 2006). The ability to induce T-cell adoptive transfer colitis in anti-IL-10 treated SCID mice underscores the important role of IL-10 in abrogating disease pathology in this model (Kjellev *et al.*, 2006).

Interestingly, anti-IFNγ and anti-TNF treatment ameliorates colitis disease, consistent with the findings that this model of disease is associated with exaggerated Th1 cell responses (Powrie *et al.*, 1994). The importance of the gut microbiota in this colitis model is highlighted by the fact that antibiotic treatment ameliorated disease, although it is unclear whether the microbiota drive the gut inflammation or merely exacerbate it (Lindebo Holm *et al.*, 2012). An advantage to this model is the ability to examine early immunological events associated with gut inflammation during the course of disease. The apparent inflammatory response in both the small bowel as well as the colon makes this model ideal for the study of CD (Ostanin *et al.*, 2006). The ability of the Treg compartment to abrogate colitis induction during T-cell transfer makes this an opportune model to also study Treg dynamics and functionality during intestinal inflammation.

2.4.5 Oxazolone Colitis

Oxazolone colitis is established by the intrarectal administration of a haptenating agent oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) solution (Griswold *et al.*, 1974). Surprisingly, unlike its closely related TNBS colitis model, oxazolone induces a Th2 dominated immune response characterised by IL-4 and IL-13 cytokine dominance; with colon inflammation that closely resembles human UC rather than CD like TNBS (Heller *et al.*, 2005; Meroni *et al.*, 2018). A single dose administration of oxazolone results in acute superficial inflammation of the distal colon (Heller *et al.*, 2002), with histopathological features that include goblet cell depletion, infiltration of lymphocytes and neutrophils, lamina propria edema and ulceration (Wang *et al.*, 2004). Long lasting oxazolone colitis is induced through a pre-sensitization subcutaneous application (Heller *et al.*, 2002), followed by a single intrarectal challenge of oxazolone, while weekly administration of oxazolone can also elicit chronic colitis in BALB/c mice (Kojima *et al.*, 2004).

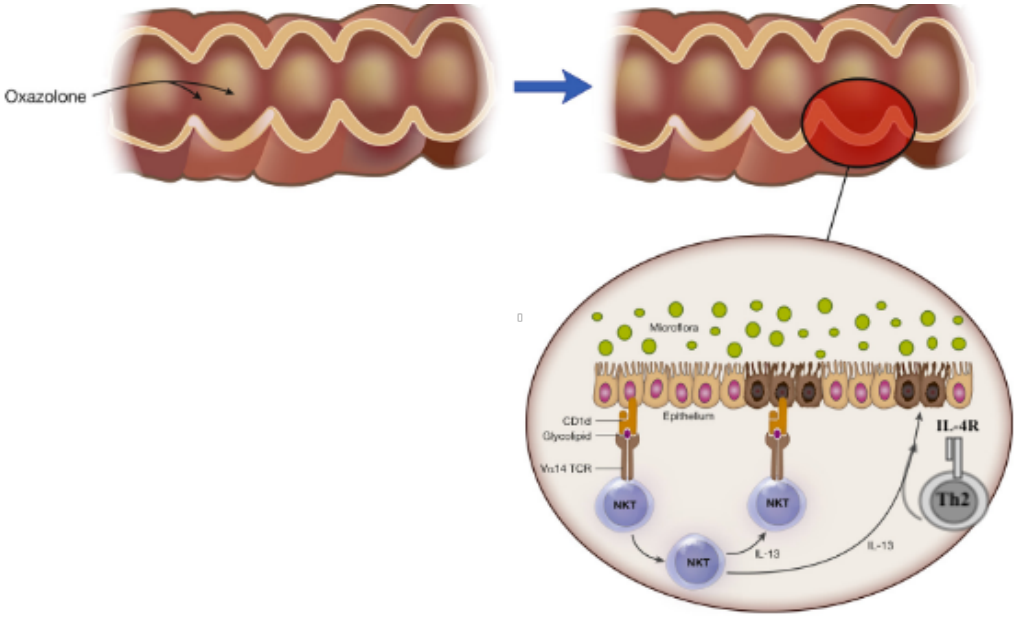


Figure 2-15: The importance of NKT, IL-4 responsive $CD4^+$ T cells and IL-13 signaling in oxazolone colitis. Adapted from (Kiesler *et al.*, 2015; Hoving *et al.*, 2017)**.**

NKT and other IL-13 producing cells are pivotal for the induction and sustenance of oxazolone colitis (**Fig 2.15**) (Heller *et al.*, 2005), as NKT cell deficient mice and IL-13 neutralization protected mice from oxazolone colitis (Heller *et al.*,

2005). NKT cells are cytotoxic to epithelial cells bearing NKT targets (Korsgren *et al.*, 1999), a function augmented by IL-13 signaling (Terabe *et al.*, 2000), leading to epithelial damage. Interestingly, IL-13 can undermine epithelial function through tight junction interference as well as epithelial cell apoptosis (Ahdieh *et al.*, 2001; Sugita *et al.*, 2018). Unfortunately, the initial source of IL-13 in oxazolone colitis is unknown; although epithelial cell-derived IL-33 is assumed to induce IL-13 production from first responders (Rankin *et al.*, 2010). Interestingly, oxazolone colitis is a highly valued murine model, as it closely models human UC in terms of morphology as well as immunopathology dominated by IL-13 signaling (Heller *et al.*, 2002). The major disparity between oxazolone colitis and UC is the difference in the dominating NKT cell, with the former being driven by invariant type I NKT cells, and the latter by sulfatide glycolipid recognizing type II NKT cells *reviewed in* (Liao *et al.*, 2013). The challenging aspects of oxazolone colitis are similar to those of the TNBS colitis model, in that there is a huge variation in the inflammatory response to oxazolone, resulting in some mice with very high distress scores and some with minimal scores. This model also exhibits a high mortality, necessitating the need of higher numbers of mice per research group.

2.4.6 Dextran Sodium Sulfate-Induced Colitis

Dextran Sodium Sulfate (DSS) colitis is the most widely established and robust experimental model of colitis established by Okayasu in 1990 through the administration of a 40-50kDa DSS salt in drinking water (Okayasu *et al.*, 1990). It is assumed to induce inflammation through binding to medium chain fatty acids (MCFA) present on colon epithelial cells, resulting in irritation, disruption of the epithelial barrier (Laroui et al., 2012) and increased permeability to commensal bacteria that results in local and systemic inflammation **(Fig 2.16)** (Chassaing *et al.*, 2014).

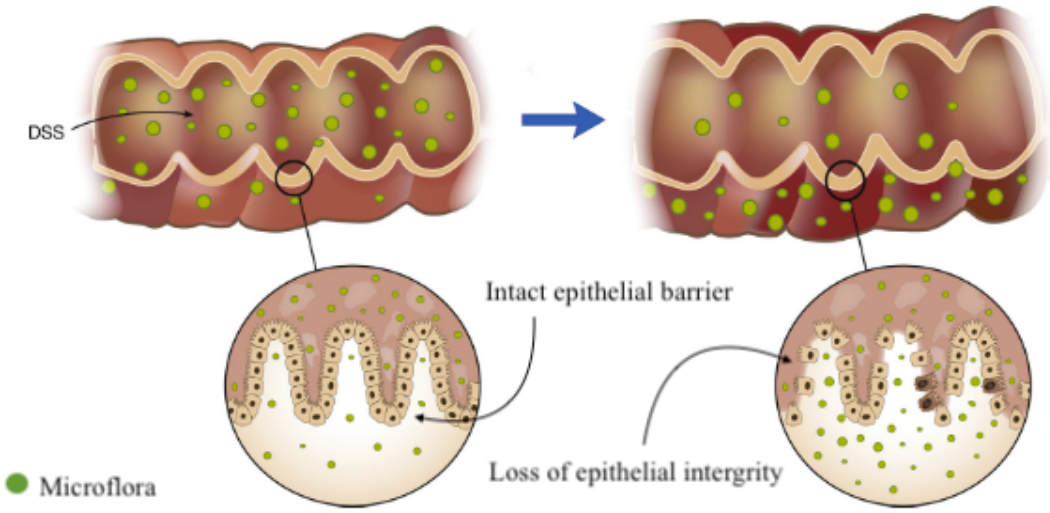


Figure 2-16: Mechanism of DSS irritation on the gut epithelium. Adapted from (Kiesler *et al.*, 2015).

The inflammatory response following epithelial inflammation is characterised by an influx of antimicrobial innate immune cells accompanied by a Th1 dominated immune response during the acute stage (Melgar *et al.*, 2005). A number of cells play key roles in maintaining gut homeostasis and are pivotal during gut pathogenesis in DSS colitis. These cells include inflammatory neutrophils and macrophages, both cell types important for microbial control and epithelial healing (Chassaing *et al.*, 2014). Adaptive cells that include $CD8^+$ cytotoxic cells, $CD4^+$ Th1 cells and $CD4^+$ T-regulatory cells also play important roles in pathogenesis (Melgar *et al.*, 2005; Jones *et al.*, 2018), giving rise to a pathological phenotype that resembles human UC.

DSS colitis exhibits a mixed Th1 immune response of elevated levels of IL-6 and TNF in SCID (severe combined immunodeficient mice) mice mucosal tissue(Dieleman *et al.*, 1994); as well as clinical features such as mucosal ulceration, diarrhea, occult fecal blood, loss of weight and colon shortening(Okayasu *et al.*, 1990). In its chronic state, DSS colitis is typified by a high expression of IL-4, with inhibition of IL-4 ameliorating disease (L Stevceva *et al.*, 2001). *Dieleman et al* demonstrated that in its chronic state, DSS is characterised by both Th1 and Th2 cytokines in Swiss-Webster mice (Dieleman *et al.*, 1998), while BALB/c mice lean towards a Th2 dominated immune response

(Dieleman *et al.*, 1998). Interestingly, DSS colitis can be induced in immune-deficient mice e.g. Recombination-activating gene-1 knock out (RAG-1KO) and SCID mice, highlighting the importance of the innate immune system in driving this disease (Dieleman *et al.*, 1994; Kim *et al.*, 2006). Recent reports support the role of the Th17 immune response in DSS colitis (Ito *et al.*, 2008), similar to human UC (Arisawa *et al.*, 2008), although some studies report the dispensability of the Th17 response during DSS colitis (Kim *et al.*, 2011).

The disruption of the epithelial barrier during DSS colitis is associated with the translocation of bacteria and bacterial antigens from the gut lumen into the submucosa (Axelsson and Midtvedts, 1996). Bacterial translocation is clearly established as a disease mechanism during DSS colitis; as numerous models with mucosal barrier defects develop gut microbiota driven spontaneous colitis or are easily induced of colitis (Berg *et al.*, no date; Kühn *et al.*, 1993; Frantz *et al.*, 2012). A seminal study by Hermiston and Gordon in which they replaced E-cadherin with N-cadherin in intestinal epithelial cells (thereby undermining the ability of epithelial cells to adhere to each other) resulted in gastrointestinal inflammation only on areas adjacent to villi/patches of tissue with the protein substitution (Hermiston and Gordon, 1995). This strongly suggests the entry of commensal bacteria through the defective tight junction areas as inducers of gut inflammation. DSS colitis induction is very sensitive to inter-batch variability (of the DSS chemical). The following reviews may help future researchers optimize their DSS experiments (Perše and Cerar, 2012; Chassaing *et al.*, 2014)

2.4.7 Limitations of using murine models in IBD research

Although a number of murine findings have contributed immensely to IBD research, the translation of mouse work to human applications should be cautionary to all researchers due to a number of reasons mentioned below:

i. Mice and humans differ in their immune responses due to different immune system development, immune activation and immune responses to similar antigens (Mestas and Hughes, 2004).

ii. Mouse experiments tend to not recapitulate the genetic and environmental diversity inherent in human populations.
iii. To determine the importance of certain genes in disease pathology, researchers tend to depend on transgenic knock out mice, whereas in human disease risk is rarely associated with the complete loss of function of all alleles.
iv. Most mouse experiments fail to account for variability in response to a therapeutic intervention that may appear in human trials due to genetic polymorphisms.

Although these limitations exist, the murine model presents a good opportunity to test the impact of specific factors on IBD, if proper and carefully experimental planning takes into account the different caveats associated with murine research.

STUDY AIM AND OBJECTIVES

3 AIMS, RATIONALE & OBJECTIVES

AIM

In this study, I sought to determine the influence of helminth infection in two differing murine models of IBD. I also determined how microbial dsybiosis following helminth infection contributed to colitis development.

RATIONALE

Inhabitants of LMIC are prone to helminth infections in the early stages of their lives, before exposure to environmental factors that increase the risk of developing IBD. Moreover, live helminths and their secretory products are demonstrated to successfully protect against IBD, suggesting the presence of helminth infections may have a suppressive effect on IBD development. Although helminth modification of the host's microbiota has been reported, little is known about how microbial modulation during helminth infection stages can influence subsequent IBD development. I used *Heligmosomoides polygyrus*, a well-characterised helminth that wholly resides in the small intestine and is reported to systemically influence inflammatory conditions at distant locale.

SPECIFIC OBJECTIVES

1. Demonstrating the impact of prior helminth exposure on colitis development.

-The impact of *Heligmosomoides polygyrus* infection on both oxazolone and DSS colitis development was defined, and the phenotypic outcomes of helminth influence were characterised.

2. Defining the impact of helminth infection on innate and adaptive immune responses associated with colitis development.

-Both the innate and adaptive immune responses are pivotal during helminth infection and colitis. *Heligmosomoides polygyrus* infection is reported to expand the host's Treg compartment to enable efficient host colonization, a move that potentially results in bystander modulation of inflammatory conditions. Host

Treg and effector cell immune responses where characterised during both oxazolone and DSS colitis.

3. Defining the helminth-microbiota relationship during DSS colitis, and the influence of microbial modulation on DSS colitis

-Microbial modulation during helminth infection is reported to influence bystander inflammatory conditions. I characterised translocating bacterial dynamics during helminth influenced DSS colitis, and determined the influence of probiotic administration on helminth influenced DSS colitis.

MATERIALS AND METHODS

4 Materials and Methods

4.1 Materials

4.1.1 Ethical considerations

All the animal research work was conducted in accordance with the South African Veterinary and Para-Veterinary Profession Act 9 of 1982. The University of Cape Town Faculty of Health Sciences Animal Ethics Research Committee (UCT-AEC) approved the study protocol, registered under the Ethics code of 015/001. The South African Veterinary Council (SAVC) trained and approved all study personnel for the handling and care of animals (AL16/1578-SAVC registration number).

4.1.2 Animals

Specific pathogen free (SPF) age-matched male and female BALB/c and C57BL/6 mice (6-8 weeks) bred in an SAVC authorized facility at the University of Cape Town Research Animal Facility (UCT-RAF, registration number FR15/14226) were grouped and housed in the UCT animal house facility under controlled temperature and photoperiods. Mice were allowed unrestricted access to food and autoclaved drinking water and allowed to acclimatise to the research unit conditions before inclusion into experiments. Care and experimentation of mice was performed in accordance with the institutional guidelines under protocols approved by the South African Veterinary Council.

4.1.3 Diet

Mice had unrestricted access to either a standard chow diet (#2012, NutritionHub-) or a linoleic acid modified diet (D16083101, Research Diets) *ad libatum* for the duration of the experimental period. The respective diet constituents are elaborated in the **Appendix** chapter. .

4.1.4 Helminth propagation and maintenance

Heligmosomoides polygyrus infective larvae were propagated as previously described (Johnston *et al.*, 2015). In summary:

Life cycle and propagation

- Distilled water-stored *Heligmosomoides polygyrus* L3 larvae were thrice washed with distilled water by centrifuging at 1500rpm/5 min. All but 500ul of water were removed between each wash, taking care not to disturb the pellet. After the last wash, the pellet was re-suspended in distilled water, ready for infection.
- 6-8 week C57BL/6 mice were infected with 400 *H. polygyrus* L3 larvae in 200 µl of distilled water by oral gavage and infection was incubated for 14 days.
- After 14 days, mice were euthanised and the full gastrointestinal tract (from below the stomach to the colon) was sterile excised and drawn out in a petri dish.
- Fecal material removed from the cecum and large intestine was collected in a sterile petri dish for further processing.
- Feces were mixed in a ratio of at least 1:1 with washed granulated charcoal to achieve a consistency damp enough to adhere to filter paper.
- Using a Pasteur pipette, we mildly dampened the filter paper and smeared a thin layer of the charcoal and feces mix (best layered with a forceps) on the center of the filter paper, making sure the mix stuck to the filter paper and other charcoal particles.
- Filter paper was placed into a petri dish that was then placed into a humid box (layered with damp towel paper) and incubated in the dark at room temperature for 12-14 days.
- L3 larvae was collected from day 7 onwards and collected at least twice per week for the next 2 weeks. Larvae form a ring around the edge filter paper in the petri dish (very prone to drying out if not collected in time). Using a Pasteur pipette, we washed the L3 from the edge of the filter paper using sterile water, making an effort not to disturb the charcoal mix.

- L3 larvae was collected into a 50ml falcon tube, and washed three times in distilled water at 1500 rpm for 10 min. Larvae was then stored at 4°C in 50 ml distilled water until required for infection (they remain viable for at least 6 months, after which a new batch needs to be made).

4.1.5 Media preparation

Blood Agar

Pre-made blood agar plates were supplied by the National Health Laboratory Services, Green Point, Cape Town, South Africa. To make our own blood agar plates, we used BD Bacto Agar and horse blood (kindly provided by the Lindi Masson group, Medical Virology, UCT) acquired from Stellenbosch University, South Africa.

- 12g of agar were added to 500ml of warmed sterile Reverse Osmosis (RO) water and agitated to dissolve completely. Solution was topped up to 1000ml with cool sterile RO water.
- The agar was split into two 1 (one) liter bottles (to prevent spill over during autoclaving) and autoclaved at 121°C for 15 min, and left to cool to body temperature.
- 37°C-warmed horse blood was added to the warm agar, carefully agitated to enable full mixing (rolling closed bottle on BSC hood is more efficient) before pouring approximately 20ml per petri dish (leave lids slightly open to prevent condensation).
- Agar plates were stored closed, inverted at 4°C overnight. Plates were opened slightly and left to dry before use.

LB Broth

LB Broth was prepared according to the manufacturer's instructions. In summary:

- To make 1000ml of LB broth, 20g powder was dissolved in 1000 ml of distilled water.

- The 1000 ml of LB solution were split into two 1000ml autoclave bottles for sterilization in the autoclave (liquids increase in volume during autoclaving, so broth will spill over if autoclaved in one 1000ml bottle).
- LB broth was autoclaved at 121°C to sterilize it, left to cool, and stored for further use.

Magnetic-activated cell sorting buffer (MACs)

To make 1000ml of pH 7.5 MACS buffer:

- 2mM EDTA (4ml of 0.5M EDTA) + 5g of Bovine Serum Albumin (0.5% BSA) were added to 976ml of 1X Phosphate Buffered Saline (1X PBS) and left to dissolve on bench-top at room temperature.
- Solution was sterile filtered (0.2µm), and stored at 4°C

2% Phosphate Buffered Saline Triton X-100 (2% PBST)

To make 1000ml 2% PBST:

- 20ml of Triton X-100 was added to 980ml 1X PBS
- Solution was sterile filtered (0.2µm), and stored at room temperature.

Red Cell Lysis Buffer (RCLB)

To make 1000ml RCLB (pH7.3):

- 8.26g (155mM NH_4CL) + 1.19g (10mM $KHCO_3$) + 200 µl (0.5M EDTA) were added to 1 liter of distilled water and left to dissolve on bench-top.
- Solution was pH adjusted to pH7.3
- Solution was sterile filtered (0.2µm), and stored at 4°C.

Complete Media

To make 500ml of complete media:

- 5ml of L-glutamine (100units) + 5mls PenStrep (100units) were added to 500ml of Roswell Park Memorial Institute (RPMI) 1640.
- Solution was stored at 4°C until use.

Citrate Buffer (CB)

To make 1000ml of 10mM CB:

- 1.92g of anhydrous citric acid was dissolved in 1000ml distilled water.
- pH was adjusted to pH 6.0 with NaOH, 0.5ml of Tween 20 were added and mixed well.
- Solution was stored at room temperature.

4.1.6 Histology materials

All histology materials were prepared, stored and used by Lizette Fick, UCT.

Haematoxylin and Eosin (H & E)

Reagant: Mayer's Haemalum

Dissolving one reagent at a time

- 1g of haematoxylin (yellow) pH5-7.2 was added to distilled water (stir-use heat if necessary to dissolve).
- Next, 50g of ammonium alum (aluminium ammonium/potassium sulphate) was added, as well as 0.2g of sodium iodate and 50g of chloral hydrate.
- Mixture was filtered and stored in the dark at room temperature.

Reagant: Eosin

- To make 1% Eosin Yellow: 1g of Eosin Yellow was dissolved in distilled water (thymol was added to prevent bacterial growth).
- To make 1% Phloxine: 1g of Phloxine was dissolved in distilled water.
- 2 parts of 1% Eosin and 1part of 1% phloxine were added to an equal amount of distilled water.
- Mixture was filtered before use.

Periodic Acid Schiff (PAS)

Reagant: Schiff's

- 1g of Pararosaniline was dissolved in 200ml boiling distilled water in stoppered flask and continuously stirred for 5 min.
- Solution was cooled to 50°C, filtered and 20ml of 1M hydrochloric acid (HCl) was added to the filtrate.
- Solution was further cooled to 25°C, 1g of potassium Metabisulphite was added and solution was stored in the dark for 18-24 hours.
- 2g of activated charcoal was added, stirred for a few min and solution was filtered then stored in a dark bottle.

Reagant: 1% Periodic Acid

- 1ml Periodic Acid was added to 100ml distilled water.

Reagant: Scotts Water

- 2g Potassium Bicarbonate and 20g Magnesium Sulfate were dissolved in 1000ml of distilled water.

Table 4.1: List of antibodies and suppliers

ANTIBODY STAIN	**FLOUROCHROME**	**SUPPLIER**
SURFACE STAINS		
CD3 (clone 17A2)	AF700	Biolegend, Sirigen
CD4	PerCP-Cy5.5	eBioscience, San Diego
CD8	V500	BD Bioscience
CD11b (clone M1/70)	BV421	Biolegend, Sirigen
GR-1 (RB6-8C5)	APC-eFlour 780	Affymetrix, San Diego
Ly6G (clone 1A8)	APC	BD Biosciences
F4/80 (clone BM8)	PerCp-Cy5.5	(eBioscience, San

		Diego)
TRANSCRIPTION FACTORS		
FOXP3 (clone FJK-165)	APC	eBioscience, Carlsbad
KI67 (556026)	FITC	BD Biosciences
INTRACELLULAR CYTOKINES		
IL-4	BV605	Biolegend, Sirigen
IL-5	BV421	Biolegend, Sirigen
IL-6 (MP5-20F3)	PE	eBioscience, San Diego
IL-10	BV421	Biolegend, Sirigen
IL-17A (TCII-18H10.1)	FITC	Biolegend, Sirigen
IFNγ (clone XMG.1.2)	APC	Biolegend, Sirigen
TISSUE IMMUNOFLUORESCENCE ANTIBODIES		
CD3 (A0452)		Dako, Agilent
β-catenin (D10A8)Xp		Cell Signaling Technology
E-cadherin (4A2)		Cell Signaling Technology
DAPI		Sigma Aldrich

Table 4.2: List of reagents and suppliers

REAGENT	**SUPPLIER**
BSA fraction V	Roche, Mannheim Germany

Fetal Calf Serum	Gibco, Life Technologies, UK
RPMI 1640 (1X)	Gibco, Life Technologies, UK
DPBS (1X)	Gibco, Life Technologies, UK
Hanks Media (H9394)	Sigma Aldrich
Foxp3 Transcription Buffer Set	Invitrogen, Thermofisher, eBioscience
BD Cytofix/Cytoperm (554722)	BD, Biosciences
Brefeldin A (B7651)	Sigma, St Louis, Missouri, USA
Ionomycin Calcium Salt (13909)	Sigma Life Technologies, USA
Phorbol 12-Myristate 13-Acetate	Sigma, St Louis, Missouri, USA
Penicillin/Streptamicin (10000/10000units)/ml	Lonza, Belgium
L-glutamine (200mM)	Lonza, Belgium
Dextran Sulfate Sodium (DSS-molecular weight 40,000-50,000 MW)	Affymetrix, Thermo Fisher, San Diego
4-ethoxymethylen-2-phenyl-2-oxazolin-5-one (oxazolone)	Sigma Aldrich, St Louis, USA
Vivomixx (trademark VSL#3)	Orphan SA Pharmaceuticals, South Africa
Mouse IL-6 ELISA set	Biolegend, San Diego
Mouse TNF ELISA set	Biolegend, San Diego

Table 4.3: List of buffers and their acronyms

BUFFER	ACRONYM
Magnetic activated cell sorting buffer	MACS
Red Cell Lysis Buffer	RCLB
Phosphate Buffered Solution Triton X-100	PBST
Citrate buffer	CB
RPMI+10% FCS+ L.glutamine (100u)+PenStrep(100u)	Complete Media
4% Paraformaldehyde in 1X PBS	4% PFA

4.2 Methods

Part of the helminth infections, colitis induction and tissue processing steps were kindly assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT; Rodney Lucas, Alisha Chetty, UCT and Matthew Darby, UCT.

4.2.1 *H.polygyrus* Infection

6-8 week old experimental BALB/c mice were infected with 200 L3 *H.polygyrus* larvae in 200µl of distilled water through oral gavage (21-gauge round tip needle) and infection was incubated depending on the experimental plans.

4.2.2 Probiotic administration

VSL#3 (trade name Vivomixx®) is a commercially available 8-strain lactic acid producing probiotic cocktail consisting of *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Streptococcus salivarus* subsp. *Thermophilus* (Simone, 2016). VSL#3 was administered daily during the *H.polygyrus* infection phase only in drinking water *ad libatum*. 1 capsule/day was dissolved in 400ml of UCT-RAF autoclaved water, with one mouse approximately drinking 1.4x10^9 CFU/5 *ml*/day (Arthur *et al.*, 2013).

4.2.3 Oxazolone colitis induction

H.polygyrus infected or uninfected 6-8 week Specific Pathogen Free (SPF1) male BALB/c mice were anaesthetised using Isoflourane before intrarectal administration of ethoxymethylen-2-phenyl-2-oxazolin-5-one (oxazolone). 150µl of 13,3mg/ml oxazolone dissolved in 47,5% ethanol was administered per mouse using an 18-gauge gavage needle (coated with KY gel) (Heller *et al.*, 2002). Mice were held vertically for approximately 1 min by the base of the tail while

under anaesthesia, to prevent expulsion of the oxazolone solution.

4.2.4 DSS colitis induction

H.polygyrus infected or uninfected 6-8 week age matched SPF1 BALB/c mice were exposed to 2% (wt/vol) Dextran sulfate sodium salt (DSS) (molecular weight 40,000-50,000 MW) dissolved in autoclaved drinking water for 7 days followed by 3 days of normal drinking water as previously published (Bábíčková *et al.*, 2015). Control mice received the same drinking water without DSS for the same number of days.

4.2.5 Evaluation of colitis

Animals were monitored (not-blinded) daily for weight-loss, morbidity, stool consistency, pilo-erection and the presence of blood in the faeces. Disease activity index (DAI) was calculated as previously published (Iijima *et al.*, 2004) and illustrated in **Table 4.4** below.

Table 4.4: Disease Activity Index

APPEARANCE		**NATURAL BEHAVIOUR**	
Normal	**0**	Normal	**0**
General lack of growing	**1**	Minor changes	**1**
Coat staring, ocular nasal discharge	**2**	Less mobile and alert, self isolation	**2**
Pilo-erection, hunched up	**3**	Vocalization, restlessness, very still	**3**
CLINICAL SIGNS		**PROVOKED BEHAVIOUR**	
Normal color and movement	**0**	Normal	**0**
Slight change in activity	**1**	Minor depression or exaggerated response	**1**

Moderated change: weight loss, minimal diarrhea, minimal rectal bleeding	**2**	Moderate change in expected behaviour	**2**
Severe change: immobility, lameness, serious diarrhea, heavy rectal bleeding	**3**	Reacts violently or very weak	**3**

4.2.6 Tissue Isolation and cell processing

Animals were euthanised using halothane according to the institutional guidelines. Mice were dissected and the peritoneal lavage (PerC), entire colon, mesenteric lymph nodes (MLN) as well as the spleen were collected into RPMI media complemented with 10% fetal calf serum and 100 units L-glutamine+Penstrep (5mls each). Single cell suspension of solid organs was carried out through the manual homogenizing of organ through a 70µm cell strainer.

T cell staining

For the detection of $CD4^+$, $CD8^+$ and $CD4^+$ $Foxp3^+$ cells, $1x10^{\wedge 6}$ cells were stained for 20 min at 4^oC with an antibody cocktail mixture of anti-CD3, anti-CD4 and anti-CD8 antibodies (as detailed in **Table 4.1**) diluted in MACs buffer according to the concentrations determined by titration. Cells were twice washed in MACS buffer at 1500/pm for 4 min, and then fixed for intra-nuclear Foxp3 staining using the Foxp3 transcription buffer set for 30 min at 4^oC. Fixed cells were further twice washed in Foxp3 permeabilisation buffer at 1500/pm for 4mins before staining in Foxp3 transcription buffer with the anti-Foxp3 antibody. After a 30 min incubation in the dark at 4^oC, cells were centrifuged at 1500/pm for 4 min and resuspended in Foxp3 permeabilisation buffer, before reconstitution in 200µl MACs buffer. Cells were acquired on an LSRII (BD Biosciences).

Intracellular staining for cytokines

$5x10^{\wedge 6}$ cells per sample were stimulated with a cocktail consisting of (0.5µg/ml) phorbol myristate acetate (PMA) + (1µg/ml) ionomycin + (10µg/ml) Brefeldin A for 3½ hours at 37°C. Surface staining was carried out on $1x10^{\wedge 6}$ with an antibody cocktail mixture for anti-CD3, anti-CD4 and anti-CD8 (as detailed in **Table 4.1**) diluted in MACs buffer (as detailed in **Table 4.3**) according to the concentrations determined by titration antibodies. Cells were washed twice at 1500/pm for 4 min with MACs buffer, and fixed for intracellular cytokine staining using the Cytofix/Cytoperm BD kit for 30 min at 4°C in the dark. Fixed cells were further twice washed at 1500/pm for 4min using the Cytoperm buffer before staining with a cytokine antibody cocktail consisting of anti-IL-17A, anti-IL-6 and anti-IFNγ antibodies. After a 30 min incubation at 4°C in the dark, cells were twice washed in Cytoperm buffer and reconstituted in 200µl MACs buffer. Cells were acquired on an LSRII (BD Biosciences)

Innate cell staining

For the detection of neutrophils, inflammatory monocytes and macrophages, $1x10^{\wedge 6}$ cells were stained for 20 min at 4°C in the dark with an antibody cocktail mixture of anti-CD11b, anti-Gr-1 (Ly6c + Ly6g), anti-Ly6G and anti-F4/80 antibodies (as detailed in **Table 4.1**) diluted in MACs buffer (as detailed in **Table 4.3**) according to the titration determined concentrations. Cells were washed twice at 1500pm for 4 min in MACs buffer and reconstituted in 200µl MACS buffer. Cells were acquired on an LSRII (BD Biosciences).

Flow cytometry analysis

All data acquired on the LSRII (BD Biosciences) was analysed in Flow Jo™ 10.4.2 (v3.05481). Gating strategies for T-cells, Intracellular and Innate cell staining are illustrated in the **Appendix** chapter.

4.2.7 Histology

All histological sectioning and staining kindly carried out by Lizette Fick, UCT.

Colons were excised and measured from just below the caecum to the rectum.

5µm segments were fixed in 4% Paraformaldehyde, before sectioning and staining with Haematoxylin and Eosin (H&E), Periodic Acid Shiff (PAS) and anti-CD3, as described below.

Haematoxylin and Eosin (H&E)

- 5µm colon sections were rehydrated in sequential steps (1 min/step) summarised as follows (xylol x3 - 100% alcohol x3 – 96% alcohol x2 – 70% alcohol x1 – tap water).
- Slides were then incubated in Haematoxylin for 9 min before rinsing in tap water and then Scotts water for 3 min.
- Next, sections were re-washed in tap water for 2 min before immersing in 1% Eosin for 2 min.
- Sections were quickly rinsed in tap water and dehydrated through alcohols to xylol (inverse of the first rehydration step).
- Sections were covered in Entellan loaded coverslips.

Periodic Acid Schiff (PAS)

- 5µm colon sections were rehydrated in sequential steps (1 min/step) summarised as follows (xylol x3 - 100% alcohol x3 – 96% alcohol x2 – 70% alcohol x1 – tap water).
- Sections were oxidized for 5-10 min in 1% aqueous periodic acid and washed in running tap water for 5 min before rinsing in distilled water.
- Next, we treated sections with the Schiff reagent for 15 min and washed them in running tap water for 10 min.
- Mayer's Haematoxylin stain was used as a counterstain for 1 min, and then rinsed in Scott's water for 2 min before washing in running tap water for 5 min.
- Sections were dehydrated through alcohols to xylol (inverse of the first rehydration step)
- Sections were covered in Entellan loaded coverslips

Anti-CD3

- 5µm colon sections were rehydrated in sequential steps (1 min/step) summarised as follows (xylol x3 - 100% alcohol x3 – 96% alcohol x2 – 70% alcohol x1 – tap water).
- Samples were blocked for 15min in 3% H_2O_2 (diluted in distilled water) before rinsing in distilled water.
- For antigen retrieval, tissue was incubated for 2min in 10mM Citrate Buffer (pH 6) in a pressure cooker.
- Tissue was cooled in tap water and blocked for 20 min in normal goat serum (1:20), then rinsed with 2% PBST.
- Next, colon sections were stained with the rat anti-mouse CD3 antibody (diluted to 1:100 ratio) for 90 min at room temperature before washing with PBST.
- Sections were left to dry at room temperature before application of Rabbit Envision (Dako K4003) for 30 min incubation.
- Samples were washed again in 2% PBST; the DAB (Dako K3468) substrate was applied for 10 min and rinsed off in tap water for 5 min.
- We next added Mayer's Haematoxylin (to counter stain the nuclei of other cells) for 4 min, rinsed tissue in tap water for 5 min and dehydrated in Zylol before application of a coverslip sealed with Entellan.

Histology imaging

All histology images were captured with either a Nikon DS-Ri2high performance camera, Frank Brombacher Lab,UCT or the Nikon DIC Light Microscope, Confocal and Light Microscope Imaging Facility, UCT.

Histology sections grading

Grading of histopathology scores for colitis development was performed blindly as previously described by (Iijima *et al.*, 2004; Hoving *et al.*, 2012) on each sample as follows:

(1) Presence of mononuclear cells,

(2) Reduced goblet cells,

(3) Epithelial injury,

(4) Granulocyte infiltration, and

(5) Edema.

Each criterion was scored from 0 to 3, resulting in a total cumulative score between 0 (absence of colitis) and 15 (maximal colitis activity).

Immunofluorescence staining

Immunofluorescence imaging kindly assisted by Susan Cooper, UCT

- 5µm colon sections were rehydrated in sequential steps (1 min/step) summarised as follows (xylol x3 - 100% alcohol x3 – 96% alcohol x2 – 70% alcohol x1 – tap water).
- For antigen retrieval, tissue was incubated for 2min in 10mM Citrate Buffer (pH 6) in a pressure cooker.
- Tissue was cooled in tap water and blocked for 20 min in normal goat serum (1:20), then rinsed with PBST.
- Sections were blocked in 1% BSA (bovine serum albumin) for 30 min and rinsed in PBST.
- A cocktail of anti-E-cadherin (1:50) + anti-β-catenin (1:50) (Table. 1) was applied to the sections and incubated overnight at 4°C.
- Next, sections were washed in PBST before incubation with secondary antibodies.
- Sections were incubated for 60 min in a cocktail of Cy3 conjugated rabbit anti-mouse (1:1000) + Alexa-488 conjugated rat anti-rabbit (1:500) and then washed with PBST.
- We next incubated sections in DAPI (0.5µg/ml PBS) for 10 min and then washed with PBST.
- Further incubation in 0.1% Sudan Black (diluted in 70% alcohol) for 10 min and rinsed first in PBST then in distilled water.
- Sections were blotted dry and coverslips were mounted in Fade Fluorescent mounting medium. Slides were stored in the dark at 4°C.
- 3D image stacks were acquired by confocal microscopy (ZEISS LSM 510, Thornwood, NY), and images displayed as 2D maximum intensity projections.

4.2.8 Bacterial isolation, cultivation and identification

All bacterial isolation, cultivation and gram-staining work was carried out under the guidance of Monalisa Manhanzva, Lindi Masson group, UCT.

Isolation

Spleens isolated from euthanized mice were weighed and collected into 2ml cold sterile PBS in order to isolate bacterial counts (Barquero-Calvo *et al.*, 2013).Spleens were homogenized in 2mls cold sterile PBS using the back of a 2ml syringe and a 40µm cell strainer. 200µl of the neat solution of each sample was plated onto 2% blood agar plates, and the plates were left to dry before incubation. Plates were anaerobically incubated in a 37°C culture room for 48 hours (Groß, 2012) and bacterial CFU/g were enumerated manually using the following formula:

Total CFU units were calculated as follows: χ x γ x (ε/ν µl), where

- χ is equal to the number of bacterial colonies you counted
- γ is the dilution factor
- ν volume you plated (200µl)
- ε is the total volume of the sample (2000µl of the neat solution).

To obtain CFU/g of tissue, the total CFU was divided by the weight of the spleen.

Cultivation for bacterial identification

50µl of homogenized spleen suspension prepared above was inoculated into 15ml of Miller's LB broth (Gibco, Life Technologies). Sample tubes were anaerobically incubated in a 37°C culture room for 48 hours. After 48 hours, samples were centrifuged at 1500/pm for 5 minutes before isolating the pellet for DNA extraction.

DNA extraction

Samples from the previous procedure were enzymatically digested and manually digested using the bead beating process and centrifuged at 10000 rpm for 1 min. Bacterial DNA was extracted from bacterial pellets using the PowerSoil®DNA isolation kit (MO BIO Laboratories Inc.) and later quantified using a Qubit® flourometer (ThermoFisher Scientific).

16s rRNA gene library preparation

Gene library preparation was kindly carried out by Enock Hyvarimana, Heather Jaspan Lab, UCT.

Bacterial DNA was prepared for the 16s rRNA library to be sequenced on the Illumina HiSeq 2500 platform. The V6 region was selected because of its taxonomic resolution as well as its short length (110 to 140 bp), which is compatible with the Illumina HiSeq sequencing technology. The Illumina Hiseq paired-end adaptor sequences and a 4-5-nucleotide barcode (enabling multiplexing) were included in the universal V6 primers. In summary, using a two step process, we amplified the hypervariable V6 region of the 16s rRNA gene using primers (**Table 4.5 below**) in PCR (Berry *et al.*, 2012).

1st step: Set 1 barcoded primers (Thermo Scientific™ Phusion™) were used to carry out a high fidelity DNA PCR reaction on 50ng of extracted DNA of each sample. PCR reactions were held at 94°C for 3 min followed by 10 amplification cycles using a touchdown protocol with denaturation at 94°C for 45s, annealing at 61°C for 45s with 1°C drop in each cycle and an extension at 72°C for 45s. Amplification continued subsequently with 15 additional cycles using 51°C as the annealing temperature. The PCR was then terminated with a final elongation at 72°C for 2 min.

2nd step: Set 2 primers (Thermo Scientific™ Phusion™) that are complementary to the flow cell primers at their 5' ends and to the Illumina paired-end sequencing adapter at their 3' end were used in a second PCR reaction. 15µl of the 1st PCR products were used as templates for the 2nd PCR step. Initially, DNA was incubated at 94°C for 3 min, followed by 15 amplification cycles at 94°C for 45s, 65°C for 45s and 72°C for 45s with a last extension step at 72°C for 2 min.

Gel electrophoresis was used to verify PCR amplicons, and the purified libraries were quality assessed using a Bioanalyzer (Agilent). The samples were then sequenced on the Illumina-Hiseq 2500 platform (100 bases paired end) and were verified by gel electrophoresis at The Center for Applied Genomics (TAGC,

Sick Kids, Toronto), generating approximately 500 000 high quality reads/sample.

Table 4.5: V6 set of primers used for 16s sequencing.

Set 1 of primers		
Primer name	**LinkerPrimerSequence (5' to 3')**	**Barcode sequences**
MF1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	ATAGCG
MF2	*AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT*	AGGGT
MF3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	TTCAT
MF4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	GATCGT
MF5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	GCCCGT
MF6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTGTC
MF7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	CACGT
MF8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	CGTACG
MF9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	GGAC
MF10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	TAGA
MF11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	TCAT
MF12	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACTT
MR1	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	ATAGCGA
MR2	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	AGGGTA
MR3	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	TTCATA
MR4	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	GATCGTA
MR5	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	GCCCGTA
MR6	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	CTGTCA
MR7	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	CACGTA
MR8	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	CGTACGA
MR9	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	GGACA
MR10	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	TAGAA
MR11	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	TCATA
MR12	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	ACTTA
	Set 2 of primers	
PCRFWD1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	
PCRRVS1	CAAGCAGAAGACGCCATACGAGATCGGTCTCGGCATTCCGTCTGAACCGCTCTTCCGATCT	

4.2.9 Microbiome data analyses

All microbiome data analysis and graph generation was carried out by Jerome Wendoh, Heather Jaspan Lab, UCT.

Generation of Operational Taxonomic Units (OTUs)

QIIME (Quantitative Insights Into Microbial Ecology) platform version 1.8.0-dev was used for quality filtering of sequencing reads (Caporaso *et al.*, 2010). The QIIME processing pipeline corrects for possible biases arising from different depths of sequences across samples. It splits the raw sequence data into samples by bar codes and filters the low-quality reads, which is done using the QIIME database default parameters. UCLUST, a third party algorithm tool, was used to cluster sequencies into OTUs (Edgar, 2010), which were then blasted

against the GreenGenes database (DeSantis *et al.*, 2006). A closed-reference OTU picking protocol at 97% sequencing identity was used.

Determination of alpha and beta diversity

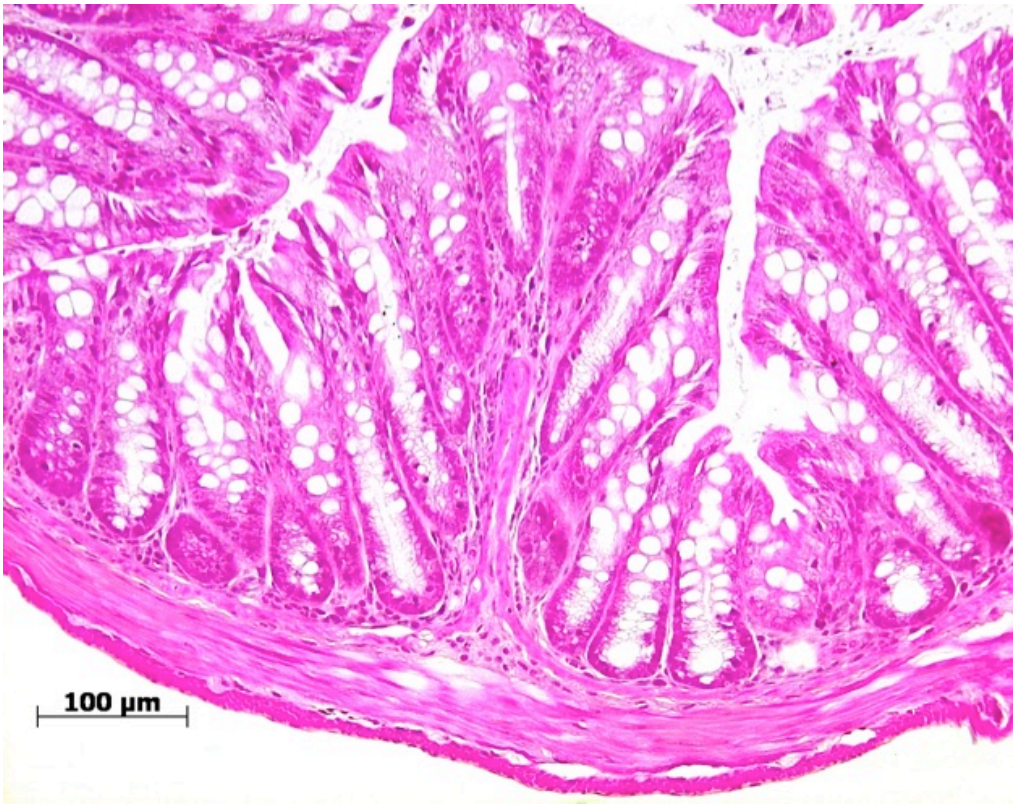
Once we had eliminated singletons and doubletons, we determined the relative abundance of taxa (from phylum to the species level where possible) in QIIME. We selected one representative sequence from each OTU (based on the most abundant sequence) and used it for further downstream analysis. PyNAST was used to align sequences with a minimum length of 150bp and a minimum identity of 75%, and we then assigned taxonomy using the RDP classifier (Cole *et al.*, 2007). Unifrac analysis was then used to build principal coordinate analyses (PCoA) beta-diversity plots (Lozupone and Knight, 2005). β-diversity was estimated using weighted and un-weighted UniFrac distances, the latter of which were then used to generate a hierarchy of cluster and ordinate samples. Distinct community types were defined based on hierarchical clustering and examination of species-level classifications (Zakrzewski *et al.*, 2016). We further calculated α-diversity estimates using R-vegan library, and both the numerical values of alpha and beta diversity were further analysed using the Mann Whitney U test (Morgan and Huttenhower, 2012).

Determining abundant taxa

Differential abundance testing at higher taxonomic levels (to include bacteria not identified by the GreenGenes data base) was carried out to determine the taxa that were significantly different between groups using an adjusted p value of ($p<0.05$) and a False Discovery Rate (FDR) of 0.05 (Zakrzewski *et al.*, 2016). All statistical analyses were carried out using R software (v.3.6.0), and Calypso software (v.8.72) (Zakrzewski *et al.*, 2016).

Statistical Analysis

Statistical analyses were performed with a one-way ANOVA for all samples $n\geq4$, and the non-parametric Kruskall Wallis test for some $n\leq4$. For all experiments, p values are indicated or *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Graph generation and statistical analyses were performed with Prism v.7c software (GraphPad).



Hp + Ivermectin + oxazolone (mouse colon tissue). Inflammatory cell infiltrate (H&E staining). Image credits: *Brunette Katsandegwaza*, 2019, UCT)

EXPERIMENTAL RESULTS CHAPTER ONE

Helminth exacerbation of oxazolone colitis is dependent on live *Heligmosomoides polygyrus* infection.

5 *Heligmosomoides polygyrus* and oxazolone colitis

The immunopathogenesis of Ulcerative Colitis (UC) has baffled scientists for a long time, as it fits neither the Th1 nor Th2 immune paradigm. Although neither IFNγ (Th1) nor IL-4 (Th2) signaling dominates the acute phase of UC, an IL-13 (Th2) response dominates and sustains subsequent chronic UC pathology (Heller *et al.*, 2005; Danese *et al.*, 2015). Due to the uncertainty of UC pathology, researchers have found it quite challenging to design immune specific therapies to counteract UC disease pathology. Fortunately, the oxazolone murine model of colitis highly recapitulates human UC, and enables research into otherwise uncharted waters of human UC research.

Oxazolone colitis is induced by the administration of an oxazolone enema that elicits a Th2 immune response, localised in the distal part of the mouse colon (Kiesler *et al.*, 2015; Wirtz *et al.*, 2017). Two methods of oxazolone colitis induction are currently employed to elicit either a self-resolving acute or a chronic response. The former is achieved by the single administration of an oxazolone enema, while the latter is preceded by a dermal presensitisation five days before administration of an oxazolone enema (Heller *et al.*, 2002; Yang *et al.*, 2009). Both acute and chronic oxazolone colitis are characterised by a superficial inflammation of the colon mucosa, with a high volume of inflammatory infiltrate (neutrophils, macrophages and lymphocytes) and goblet cell depletion (Heller *et al.*, 2002; Wang *et al.*, 2004).

A type-2 immune response dominated by IL-13 signaling is demonstrated as the pathology-driving factor during oxazolone colitis (Heller *et al.*, 2002; Kiesler *et al.*, 2015). IL-13 is reported to reduce epithelial barrier function by increasing epithelial cell apoptosis as well as tight junction permeability (Heller *et al.*, 2002), leading to gut pathology. Promising anti-IL-13 treatments in UC therapy have met with conflicting results that demonstrate inefficiency of an anti-IL-13 intervention on UC pathology (Reinisch *et al.*, 2015; Sandborn *et al.*, 2017; Hoving, 2018). However, a recent seminal study by *Hoving et al* reiterated the importance of IL-13 signaling and therapeutics during a murine model of UC

(Hoving *et al.*, 2017). These authors reported an inability to induce oxazolone colitis in IL-13 deficient mice, supporting the significance of IL-13 signaling in this model of UC and potentially in human UC as well (Hoving *et al.*, 2017).

Although the initial cell source of IL-13 is yet undetermined, both NKT and conventional IL-4 responsive $CD4^+$ T cells are pivotal during oxazolone colitis immunopathogenesis (Heller *et al.*, 2002; Hoving *et al.*, 2017). Failure to induce colitis in Lck^{cre}IL-4R$\alpha^{-/lox}$ (BALB/c mice lacking the IL-4Rα specifically on CD4 T cells) (Hoving *et al.*, 2017) demonstrated the importance of $CD4^+$IL-4 signaling in colitis induction, although IL-4 importance wanes after the initial 4-5 days of colitis development (Fuss and Strober, 2008). Interestingly, although NKT cell deficiency protected mice from colitis induction, *Hoving et al* demonstrated colitis induction in Lck^{cre}IL-4R$\alpha^{-/lox}$ mice that had been reconstituted with NKT depleted $CD4^+$ T cells ($CD4^+/DX5^-/CD1d^-$) before oxazolone administration (Hoving *et al.*, 2017). This demonstrated the dispensability of NKT cells ($DX5^+/CD1d^+$) in oxazolone colitis induction, and points to other IL-13 sources as equally important during oxazolone colitis. Indeed, findings from *Smith et al* suggest IL-13 secretion from innate lymphoid cells (ILC2s) during type-2 immune response initiation (Smith *et al.*, 2012). ILC2 related IL-13 production (and consequent type-2 immune response induction) occurred independently of the Th2 immune response, suggesting that innate cells may be the initial as well as strong IL-13 cell sources in type-2 driven pathologies (Smith *et al.*, 2012).

A number of conditions and diseases that include allergy, airway hyperreactivity and helminth infections are driven by a type-2 immune pathology (Niwa *et al.*, 2004; Chen *et al.*, 2012; Jemal *et al.*, 2012; Smith *et al.*, 2012; Licona-Limón *et al.*, 2013; Mishra *et al.*, 2014; van Rijt *et al.*, 2016). Helminth infections are known to induce a type-2 immune response in both humans and mice (Ramanan *et al.*, 2016). A typical type-2 response is characterized by both innate and adaptive immune cells that include eosinophils, alternatively activated macrophages (AAMs), dendritic cells, innate lymphoid cells 2 (ILC2) and Th2 $CD4^+$ T cells (Allen and Maizels, 2011). The Th2 cytokine storm that includes IL-4, IL-5 and IL-13 signaling probably has downstream effects that include

heightened mastocytosis, B cell class switching and mucosal surface goblet cell hyperplasia amongst other responses (Else *et al.*, 1993; Urban *et al.*, 1998; Meeusen and Balic, 2000; Maizels and Yazdanbakhsh, 2003; Finkelman *et al.*, 2004; Turner *et al.*, 2013). A helminth directed Th2 immune response characterized by IL-4 and IL-13 ensures helminth clearance and resistance in immune-competent hosts (Urban *et al.*, 1991).

Intermediate and resistant hosts mount a strong type-2 immune response to clear out a helminth infection, characterised by mucosal lining production of IL-33 alarmins, IL-4, IL-13, as well as a high activity of alternatively-activated macrophages and Th2 cells (Hepworth and Grencis, 2009; Chen *et al.*, 2012; Elliott and Weinstock, 2012; Reynolds *et al.*, 2012). However, adult *Heligmosomoides polygyrus* (*H.polygyrus*) elicits expansion of the host's $CD4^{+}Foxp3^{+}$ (Tregs) early in infection in both susceptible and intermediate responder mice (C57BL/6 and BALB/c mice), thereby suppressing the host's natural type-2 immune response (Behnke *et al.*, 1987; Robinson *et al.*, 1989; Zhong and Dobson, 1996; Smith *et al.*, 2016). This immunosuppressive environment is characterised by heightened $CD4^{+}$Treg activity and a resultant diminished helminth expulsion ability (Finney *et al.*, 2007). Interestingly, such helminth evasion tactics have a bystander effect on a number of inflammatory pathologies that include the suppression of Th1, Th2 and Th17 driven experimental colitis pathologies (Smith *et al.*, 2007; Ledesma-Soto *et al.*, 2015). Early $CD4^{+}$Treg expansion during *H.polygyrus* infection is significantly associated with efficient larvae colonisation, as early boosting of both thymus derived and peripheral induced $CD4^{+}$Tregs through IL-2C administration resulted in increased worm burdens and suppressed Th2 cell effector functions (Smith *et al.*, 2016).

Surprisingly, unexpected high worm burdens accompanied by mixed adverse inflammatory responses are followed by complete Treg depletion in BALB/c mice, demonstrating immune chaos as well as the need of low level Treg activity to drive anti-helminth responses (Smith *et al.*, 2016). This Treg:Teff balance is probably responsible for the dynamics of *H.polygyrus* infection in mildly resistant BALB/c mice, as these mice are initially susceptible then naturally clear

out the helminth infection by day-28 post infection (Reynolds *et al.*, 2014), preventing a long term chronic phase. BALB/c helminth clearance most probably demonstrates the eventual abrogation of helminth driven Treg activity and an eventual host Th2 response during the chronic phase (>14 days infection). This may suggest a chasm between the host's immune responses during the acute and chronic phase of *H.polygyrus* infection in BALB/c mice, with the former dominated by Treg activity and the latter Th2 dominated.

In this study, oxazolone colitis was initially optimised by exposing male BALB/c mice fed different diets to an oxazolone enema. A linoleic acid rich diet is reported to modify the immune response associated with UC immunology (Pacheco *et al.*, 1987; Wiese *et al.*, 2016); while human and experimental dietary interventions demonstrate contradictory findings regards UC clinical outcomes (Tobias *et al.*, 2003; Ferrucci *et al.*, 2006; Fristche, 2008). After determining the influence of diet on oxazolone colitis induction, male BALB/c mice were exposed to a chronic (28 days before) *H.polygyrus* infection where they exhibited exacerbated weight-loss and distress after oxazolone administration. Interestingly, exacerbation appeared to be associated with a suppressed Th2 immune response, suggesting a diversion from previous similar studies of helminths and oxazolone colitis. Although helminth clearance before oxazolone administration suggested to rescue mouse distress, differences between antihelminthic treated and non-treated colitis induced mice were not statistically significant, despite clear trends of heightened Th2 cytokine titers. I believe this study is the first to suggest helminth exacerbation of oxazolone colitis independent of heightened IL-13 proportions, and is also the first to suggest a beneficial role of antihelminthic treatment on helminth-exacerbated oxazolone colitis. However, further tests are needed to reduce experimental variability as well as confirm the functional role of IL-13 during *H.polygyrus* exacerbation of oxazolone colitis.

STUDY HYPOTHESIS

I proposed that chronic helminth infection would exacerbate oxazolone colitis (experimental model of UC) due to a compounded Th2 immune response.

RESULTS

Increased susceptibility to oxazolone-induced colitis is associated with a low linoleic acid diet in male BALB/c mice.

One prospective human study demonstrates a positive correlation between linoleic acid intake and UC development, while in another experimental dietary intervention trial, n-6 and n-3 PUFA administration have no influence on UC pathology (Tobias *et al.*, 2003; Pearl *et al.*, 2014; Wiese *et al.*, 2016).

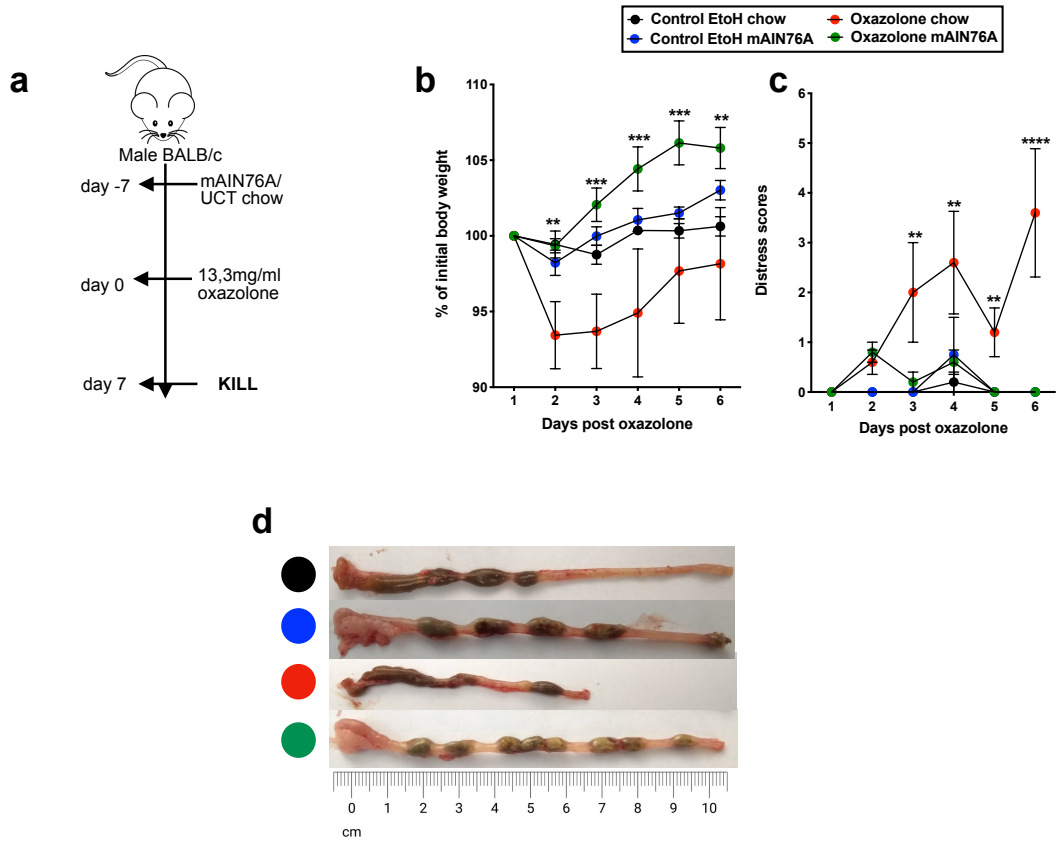


Figure 5-1: A linoleic acid low diet increases susceptibility to oxazolone-induced colitis in male BALB/c mice.
6-8 week male BALB/c mice fed a diet of chow or modified AIN-76A (mAIN76A) *ad libatum* were administered oxazolone as illustrated (**a**). Weight-loss (**b**) and distress (**c**) of vehicle control chow (black symbols), vehicle mAIN76A diet (blue symbols), chow + oxazolone (red symbols) and mAIN76A + oxazolone (green symbols) were monitored over a period of 7 days as a measure of oxazolone colitis induction. At day 7 post oxazolone administration, mice were euthanised and the colons were sterile excised and measured (**d**). Data is presented as mean ± SEM and are representative of two independent repeat experiments, with n≥3 mice per group. Statistical significance was calculated using one-way ANOVA, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Oxazolone mAIN76A and Oxazolone chow groups. Experimental run assisted by Katherine Smith, UCT.

The influence of diet on the induction of oxazolone colitis was determined by exposing male BALB/c mice to either a high linoleic acid modified AIN76A diet (D16083101, Research Diets) or a standard chow diet (#2005, NutritionHub) (**Fig 5.1a**). Surprisingly, increased susceptibility was associated with a low linoleic acid diet compared to a linoleic acid enriched diet, and this was demonstrated by increased weight-loss and mouse distress (**Fig 5.1b & c**). Chronic ulcerative colitis is characterised by colon shortening due to excessive fibrosis, a complication that leads to digestive problems for UC patients (Rieder and Fiocchi, 2008). Colon length was measured at point of kill (**Fig 5.1d**) and male BALB/c mice fed the UCT chow diet demonstrated shorter colons compared to their vehicle controls.

Heightened systemic IFNγ signaling in male mice fed a chow diet was associated with increased susceptibility to oxazolone colitis.

Both human and experimental colitis are characterised by heightened neutrophilia and IL-13 signaling, the latter being a type-2 cytokine synonymous with exaggerated pathology in human UC (Heller *et al.*, 2002, 2005; Fuss and Strober, 2008; Kmieć *et al.*, 2017; Meroni *et al.*, 2018). There was no significant difference in the total proportion of IL-13 expression within live cells of the MLN, PerC and Spleen (**Fig 5.2b**), compared between the standard chow and mAIN76A diet fed mice (**Fig 5.2b**). Surprisingly, susceptibility positively correlated with increased peritoneal (**Fig 5.2cii**) and splenic (**Fig 5.2ciii)** IFNγ proportions. However, although no statistical significant differences were evident in neutrophil proportions between the UCT chow and mAIN76A diet, a trend of increase in the Perc (peritoneal cavity wash) and spleen was apparent with respect to the standard chow fed mice (**Fig 5.2dii & iii**).

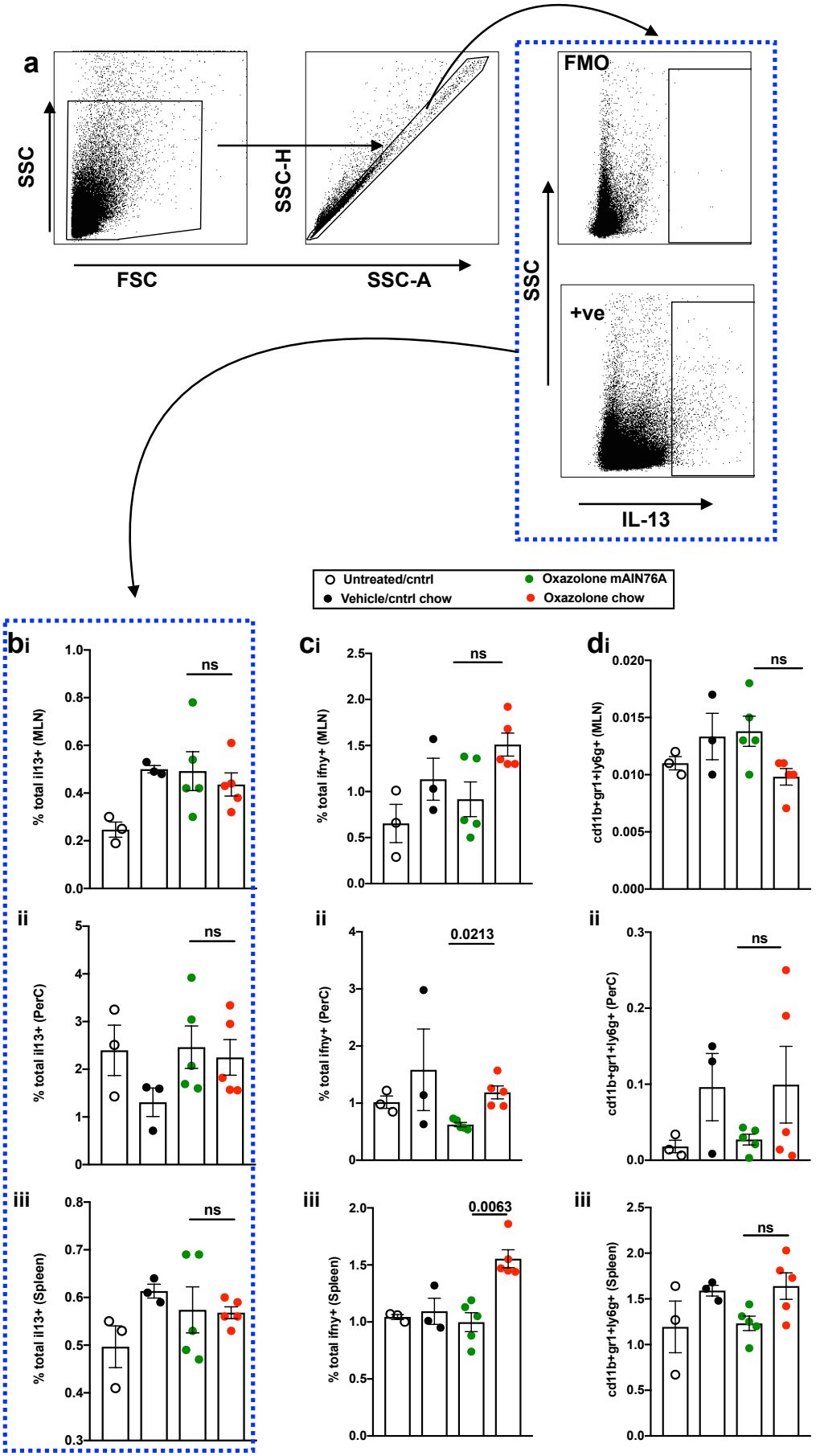

a
SSC
FSC
SSC-H
SSC-A
FMO
+ve
SSC
IL-13
Untreated/cntrl
Vehicle/cntrl chow
Oxazolone mAIN76A
Oxazolone chow
bi
% total il13+ (MLN)
ns
ii
% total il13+ (PerC)
ns
iii
% total il13+ (Spleen)
ns
ci
% total ifny+ (MLN)
ns
ii
% total ifny+ (PerC)
0.0213
iii
% total ifny+ (Spleen)
0.0063
di
cd11b+gr1+ly6g+ (MLN)
ns
ii
cd11b+gr1+ly6g+ (PerC)
ns
iii
cd11b+gr1+ly6g+ (Spleen)
ns

Figure 5-2: Heightened systemic IFNγ signaling in male mice fed a chow diet was associated with increased susceptibility to oxazolone colitis.
6-8 week male BALB/c mice fed a diet of chow or modified AIN-76A (mAIN76A) *ad libatum* were administered oxazolone. At day 3 post oxazolone administration, mesenteric lymph nodes (MLN), peritoneal lavage (PerC) and spleen cells were collected and analysed by flow cytometry (**a**). For intracellular cytokine staining, cells were unspecifically stimulated with PMA+Ionomycin and total cytokine proportions analysed using flow cytometry. The proportion of IL-13 and IFNγ within live total cells acquired was quantified in the MLN, PerC and Spleen (**b and c**) in untreated controls (empty symbols), vehicle control (black symbols), mAIN76A+ oxazolone (green symbols) and chow + oxazolone (red symbols). MLN, PerC and splenic neutrophils ($Gr1^{high}$ + $Ly6G^{high}$) were gated on the $CD11b^{high}$ population. Data is presented as mean rank ± SEM and are representative of two independent repeat experiments, with n≥5 mice per group. Statistical significance was calculated using the non-parametric Kruskall Wallis test, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Oxazolone mAIN76A and Oxazolone chow groups. Experimental run assisted by Katherine Smith, UCT.

A live helminth infection exacerbated oxazolone colitis while antihelminthic treatment suggested disease amelioration.

Helminth infection is demonstrated to suppress a range of Th1/Th2/Th17 driven inflammatory conditions that include allergy, airway sensitivity, colitis as well as colitis-associated cancer through its ability to expand immune suppressive host Tregs (Wilson *et al.*, 2005; Rick M Maizels *et al.*, 2012; León-Cabrera *et al.*, 2014; Ledesma-Soto *et al.*, 2015). However, BALB/c mice are mildly resistant to chronic *H.polygyrus* infection and naturally clear out an *H.polygyrus* infection by day-28 post infection(Reynolds *et al.*, 2014), suggesting a weakening of the helminth-elicited suppressive milieu. Using the modified protocol of oxazolone induction (male BALB/c + standard chow diet), the influence of a prior chronic *H.polygyrus* infection on the development of oxazolone colitis was determined as indicated (**Fig 5.3ai**). *H.polygyrus* infected mice exhibited increased weight-loss compared to their oxazolone and vehicle only controls (**Fig 5.3aii**) and had a higher mortality rate (**Fig 5.3aiii**).

Due to the self-resolving nature of oxazolone colitis as well as the high mortality of chronic infected mice, subsequent experiments were terminated 3 days after colitis induction (**Fig 5.3bi**). Antihelminthic treatment is advised in helminth endemic areas to mitigate against helminth induced malnutrition and morbidity. With the use of Ivermectin, a chronic helminth infection was cleared out one

week before oxazolone colitis induction (**Fig 5.3bi**). Eggs per gram were confirmed before and after antihelminthic treatment (**Fig 5.3bii & iii**). Although differences between the *H.polygyrus* infected and the helminth-cleared groups after oxazolone colitis were not statistically significant with respect to weight-loss, distress scores and colon lengths (**Fig 5.3c**), a trend towards disease amelioration was evident with ivermectin treatment, demonstrated by rescued colon pathology (**Fig 5.3d**).

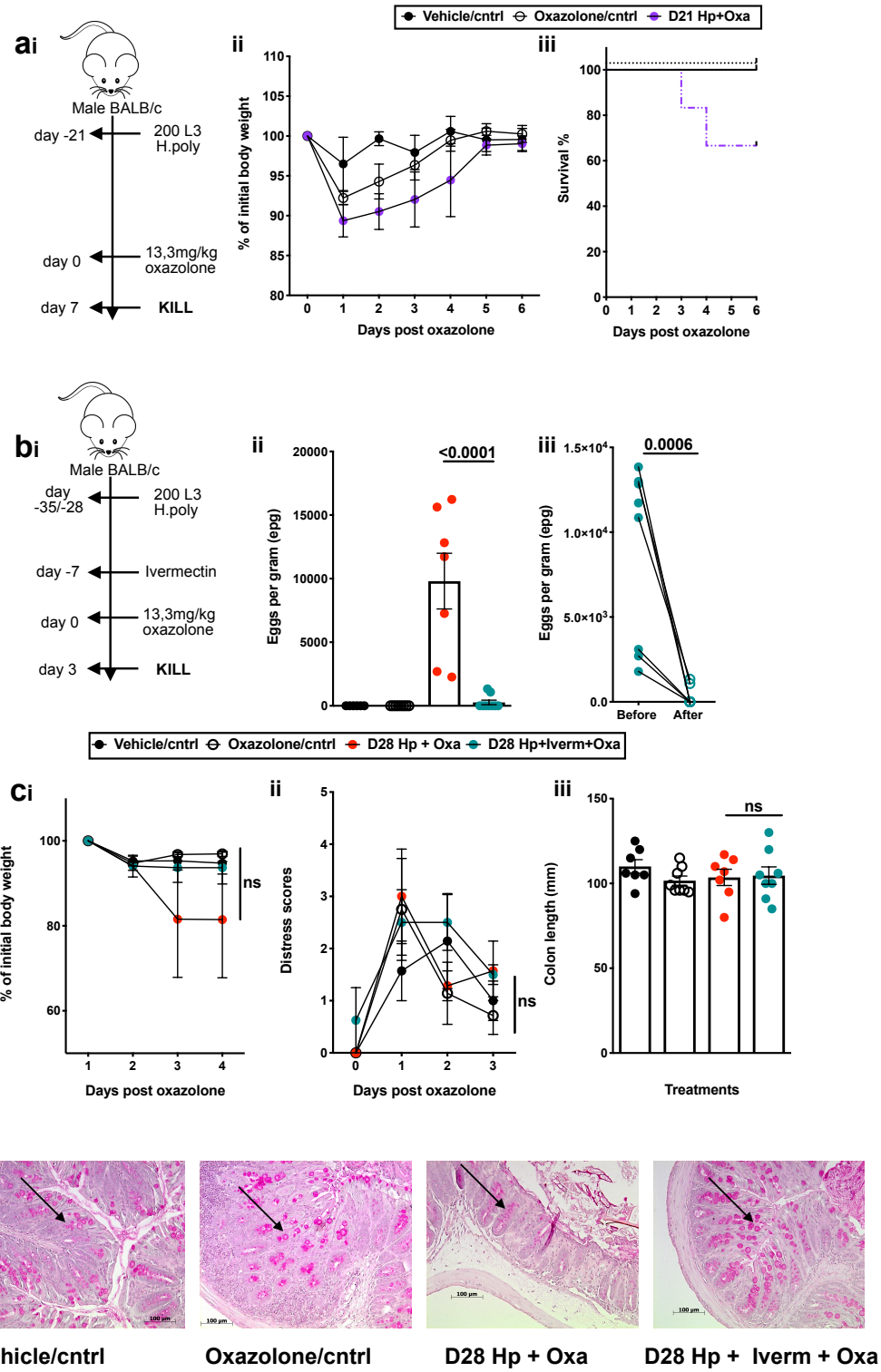


Figure 5-3: A live helminth infection exacerbated oxazolone colitis while antihelminthic treatment correlated with a colon integrity rescue.

6-8 week male BALB/c male mice fed the chow diet were infected with 200 L3 *H.polygyrus* larvae for 21 days before oxazolone administration as illustrated (**ai**) and weight-loss and rate of survival were monitored for a period of 7 days (**aii & aiii**) in vehicle controls (black symbols), oxazolone controls (empty symbols) and helminth infected + oxazolone (purple symbols). In a

follow up experiment (**bi**), chronic helminth infection was cleared out using ivermectin and eggs per gram (**epg**) were measured in vehicle controls (black symbols), oxazolone controls (empty symbols), helminth infected + oxazolone (red symbols) and helminth infected+ ivermectin + oxazolone (teal symbols) before oxazolone colitis induction as illustrated (**b)**. Weight-loss and distress severity were monitored for a period of 3 days (**ci and cii**). Colon length was determined at point of euthanasia (**ciii**), and 1mm distal colon sections were fixed in 4% PFA, sectioned and stained for inflammatory cells (H&E) and goblet cell distribution (PAS). Illustrated are representative PAS (goblet cells) sections (**d**). Data is presented as mean ± SEM and are pooled from two independent repeat experiments, with n≥5 mice per group. Statistical significance was calculated using one-way ANOVA, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the helminth infected + oxazolone and helminth infected + ivermectin + oxazolone groups. Histology staining assisted by Lizette Fick, UCT

Antihelminthic treatment of oxazolone colitis infected mice appeared to rescue mesenteric IL-4, IL-5 and IL-13 cytokine proportions.

Host anti-helminth responses are characterised by type-2 immune responses typified by heightened IL-13 signaling (Th2 immune responses) (Brombacher, 2000; Reynolds *et al.*, 2012). Chronic *H.polygyrus* infected mice demonstrated relatively reduced levels of total IL-4, IL-5 and IL-13 after oxazolone administration (**Fig 5.4a**) compared to the oxazolone only treated controls, suggesting an immunosuppressive effect of helminths on total Th2 cytokine production. Interestingly, ivermectin treated mice demonstrated a bias towards heightened total IL-4, IL-5 and IL-13 proportions compared to the helminth infected group (**Fig 5.4a**). Ivermectin is reported to cause an increase in chloride ion influx into adult nematode worms, resulting in a nervous and cellular signaling breakdown and eventually leading to death and clearance of the worm (Wahid and Conway, 1989). Current research demonstrates the importance of NKT cells as well as IL-4 responsive $CD4^+$ T cells as major IL-13 producers during experimental Ulcerative Colitis (Hoving *et al.*, 2017). $CD4^+$ and NKT (gating strategy in appendix) cells from ivermectin treated mice displayed a bias towards heightened IL-4, IL-5 and IL-13 frequencies (**Fig 5.4b & c**), although these were not statistically significant compared to their non-treated infected

controls.

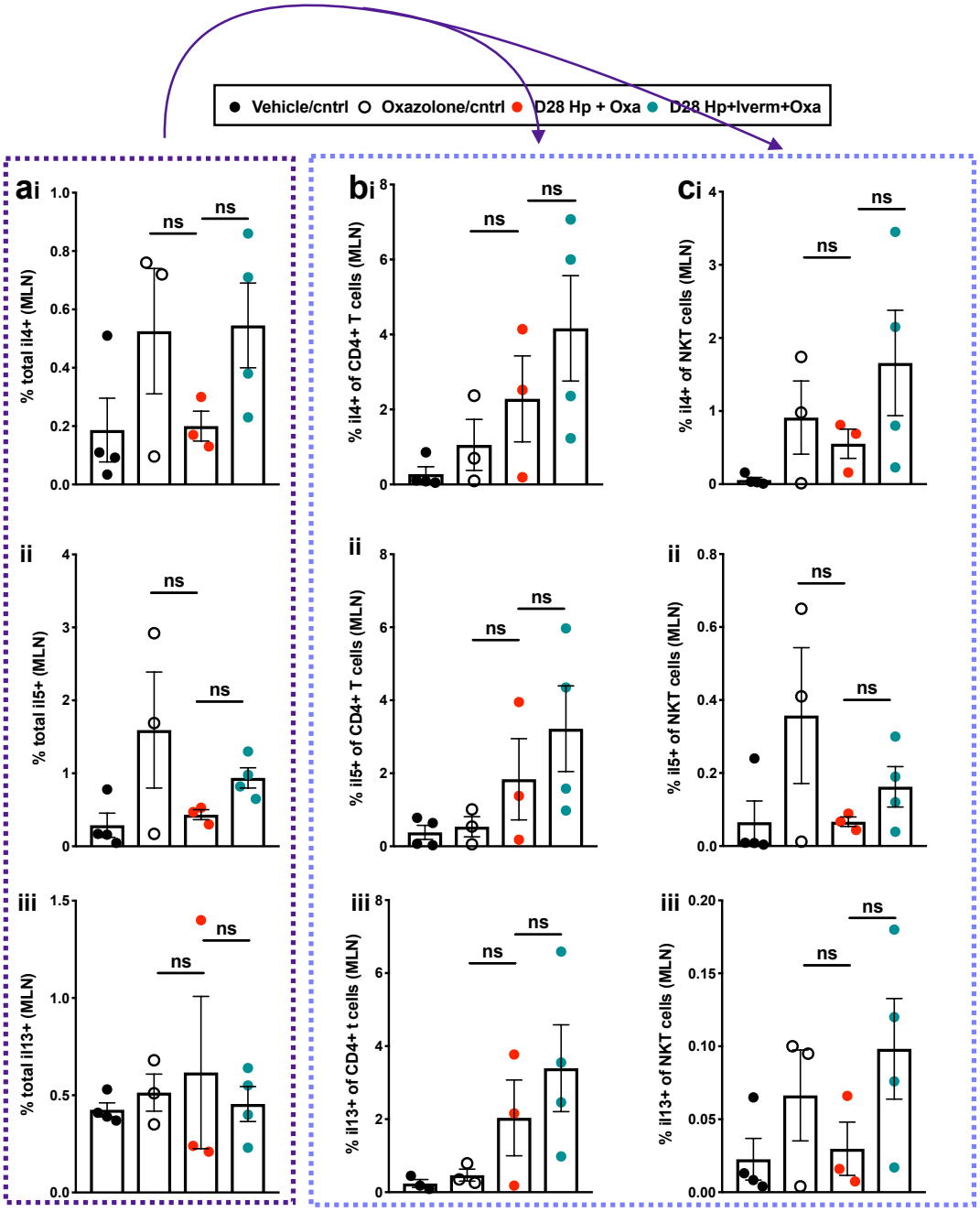


Figure 5-4: Antihelminthic treatment of oxazolone colitis infected mice is associated with a heightened Th2 immune response characterised by a rescue of mesenteric IL-4, IL-5 and IL-13 cytokine proportions.

H.polygyrus chronic infected chow fed mice were treated with an antihelminthic before oxazolone administration. At day 3 post oxazolone administration; mesenteric lymph nodes (MLN) were collected, unspecifically stimulated with PMA+ Ionomycin and analysed by flow cytometry for IL-4, IL-5 and IL-13 cytokines. Total IL-4, IL-5 and IL-13 cytokine proportions were determined from total cells acquired (**a**), with further characterisation of both CD4+ (**b**) and NKT (**c**) IL-4, IL-5 and IL-13 cytokines in vehicle controls (black symbols), oxazolone controls (empty symbols), helminth infected + oxazolone (red symbols) and helminth infected+ ivermectin + oxazolone (teal symbols). Data is presented as mean rank ± SEM and are representative of three independent repeat experiments, with n≥3 mice per group. Statistical

significance was calculated using the non-parametric Kruskall Wallis test, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the helminth infected + oxazolone and helminth infected + ivermectin + oxazolone groups.

Rescued local Th2 immune responses after Ivermectin treatment positively associated with exhaustion of systemic Treg absolute numbers.

The Treg:Teffector relationship regulates homeostatic and inflammatory responses. Although the Treg compartment is redundant during chronic *H.polygyrus* infection immunity, low level Treg activity is required for efficient Th2 anti-helminth immune responses as well as to prevent immune chaos (Smith *et al.*, 2016). After determining the rebound of mesenteric Th2 immune responses after helminth clearance, the Treg and T effector kinetics respective to Ivermectin treatment were determined (**Fig 5.5a**). Although $CD4^{+}Foxp3^{+}$ Treg proportions were quite similar between the helminth infected and helminth cleared groups (**Fig 5.5bi**), $CD4^{+}Foxp3^{+}$ Treg absolute numbers were significantly different between the compared groups (**Fig 5.5bii**). Interestingly, the same profile was mirrored in the $CD4^{+}$ effector proportions and absolute numbers (**Fig 5.5c**), suggesting helminth elicited splenomegaly as a factor behind significantly different absolute numbers. Surprisingly, helminth clearance did not have an impact on typical Th1 (IFNγ) and Th17 (IL-17A) immune response (**Fig 5.5d & e**), pointing to a restricted Thelper activation milieu.

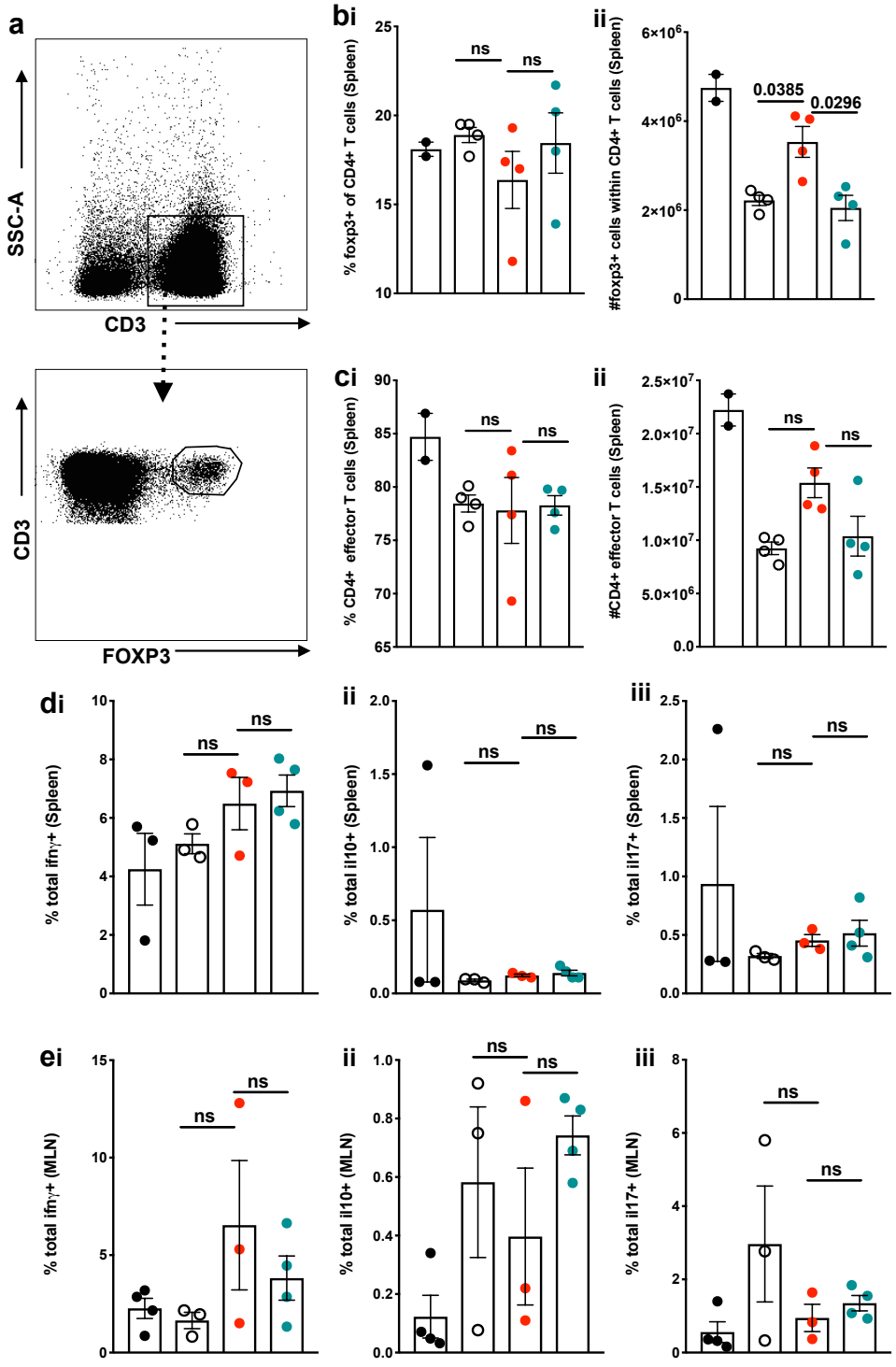


Figure 5-5: Exhaustion of systemic Treg numbers positively correlated with Th2 immune response rescue after ivermectin treatment.

H.polygyrus chronic infected chow fed mice were treated with an antihelminthic drug before oxazolone administration. At day 3 post oxazolone administration mesenteric lymph nodes (MLN), peritoneal lavage (PerC) and Spleen were collected and analysed by flow cytometry for $CD4^{+}Foxp3^{+}$(Tregs) and $CD4^{+}Foxp3^{-}$ (effectors) (**a**). For intracellular cytokine staining, cells were unspecifically stimulated with PMA+Ionomycin and total IFNγ, IL-10 and IL-17A total cytokine proportions analysed in vehicle controls (black symbols), oxazolone controls (empty symbols), helminth infected + oxazolone (red symbols) and helminth infected+ ivermectin + oxazolone (teal symbols) using flow cytometry. Data is presented as mean rank ± SEM and are

representative of three independent repeat experiments, with n≥3 mice per group. Statistical significance was calculated using the non-parametric Kruskall Wallis test, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the helminth infected + oxazolone and helminth infected + ivermectin + oxazolone groups.

Helminth clearance had no impact on systemic neutrophil proportions.

Colon and systemic neutrophilia is one of the hallmark features of chronic human UC as well as being an important readout for experimental colitis (Rudolph *et al.*, 1995; Watanabe *et al.*, 1998; Mizoguchi, 2006). We analysed for systemic neutrophilia to determine extent of inflammatory responses (**Fig 5.6a**). Although helminth infection resulted in splenic neutrophilia, antihelminthic treatment had no influence on both peritoneal and splenic neutrophil proportions (**Fig 5.6b**). *H.polygyrus* infection is associated with increased regulatory alternatively activated macrophages (AAM) activity (Kreider *et al.*, 2007), while infiltrating macrophages are positively associated with UC pathology (Heinsbroek and Gordon, 2009). Surprisingly, helminth infection correlated with suppressed peritoneal and spleen macrophages proportions, suggesting dispensability of macrophages in both helminth exacerbation of oxazolone colitis as well as anti-helminth responses in this model.

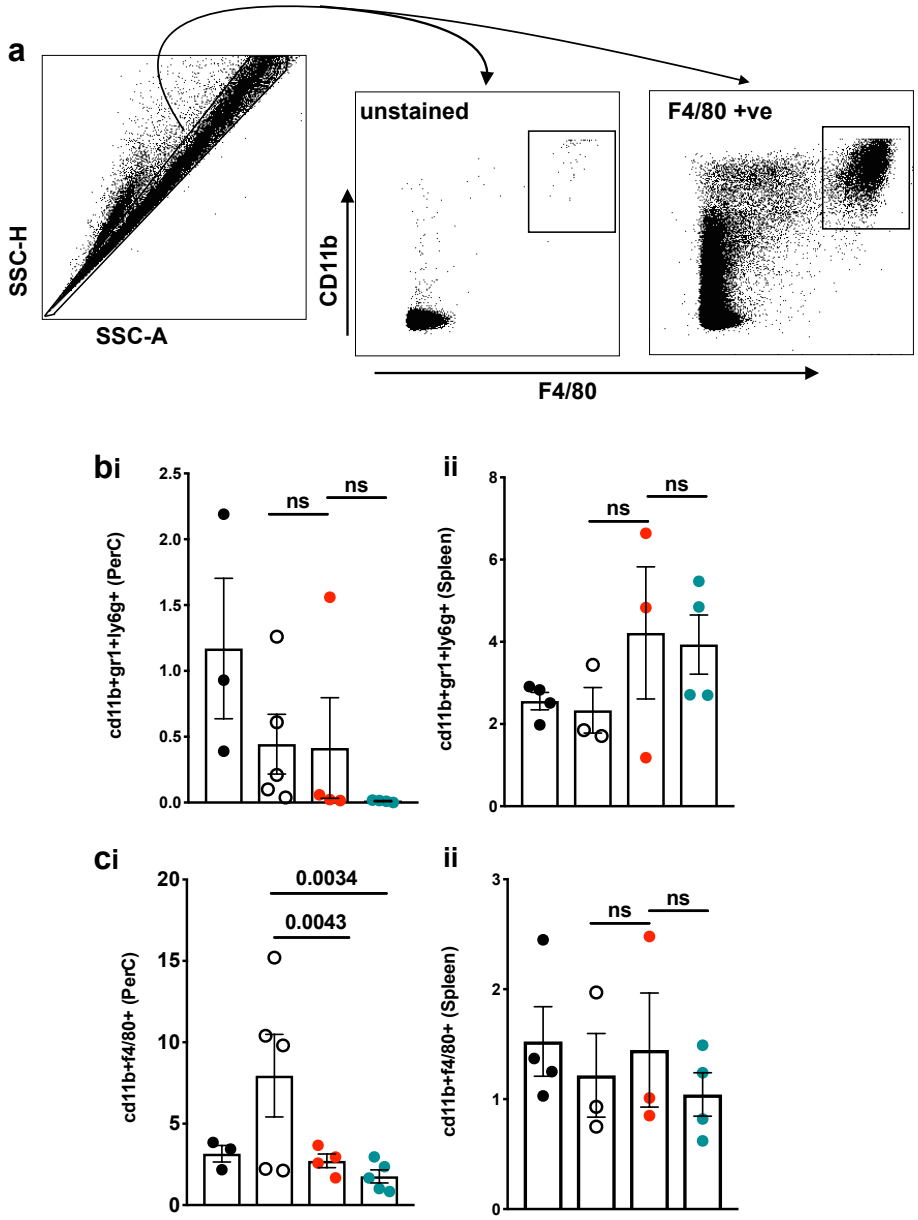


Figure 5-6: Helminth clearance had no impact on systemic neutrophil proportions.
H.polygyrus chronic infected chow fed mice were treated with an antihelminthic drug before oxazolone administration. At day-3 post oxazolone administration, mice were killed and peritoneal lavage (PerC) and Spleen were collected and analysed by flow cytometry for neutrophils ($Gr1^{high}Ly6G^{high}$) and macrophages ($CD11b^{+}F4/80^{+}$) (peritoneal cavity gating illustrated)(**a**). Peritoneal and splenic neutrophil (b) macrophage (c) proportions for vehicle controls (black symbols), oxazolone controls (empty symbols), helminth infected + oxazolone (red symbols) and helminth infected+ ivermectin + oxazolone (teal symbols) are illustrated. Data is presented as mean rank ± SEM and is representative of three independent repeat experiments, with n≥3 mice per group. Statistical significance was calculated using the non-parametric Kruskall Wallis test, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the helminth infected + oxazolone and helminth infected + ivermectin + oxazolone groups.

DISCUSSION

Although there is no guaranteed dietary intervention for Ulcerative Colitis (UC), recent research findings suggest a diet low in linoleic acid to be beneficial to gut health. Two recent 4-year prospective study (EPIC) demonstrated an increased risk of UC in patients with a high linoleic acid intake (The IBD in EPIC Study Investigators, 2009; De Silva *et al.*, 2011), while a retrospective study by *Geerling et al.,* reported increased UC risk with total n-6 PUFA intake (Geerling *et al.*, 2000). While diets rich in n-3 PUFAs instead of n-6 PUFAs (linoleic acid) are mostly advised for and associated with reduced gut inflammation (Stenson *et al.*, 1992; The IBD in EPIC Study Investigators, 2009; De Silva *et al.*, 2011; Pearl *et al.*, 2014), a number of studies demonstrate non influential roles of both n-3 and n-6 PUFAs on UC (Hawthorne *et al.*, 1992; Hart *et al.*, 2008; Turner *et al.*, 2011; Ananthakrishnan *et al.*, 2014), leading to controversial discussions on the efficacy of n-6/n-3 diet influence on UC.

Interestingly, in this study, heightened colitis exacerbation was associated with a standard chow diet (low in linoleic acid) (**Fig 5.1**), contrary to published research findings of linoleic acid driving colitic inflammation (DeMar Jr *et al.*, 2004; Kansal *et al.*, 2014; Pearl *et al.*, 2014). Standard chow diet consists of a low n6:n3 PUFA ratio compared to the high n6:n3 ratio in the linoleic acid modified AIN76A diet (**see Appendix**). Dietary linoleic acid is metabolically converted to arachidonic acid (AA) (Ghosh *et al.*, 2013), and AA, an important component of the epithelial phospholipid layer, is further metabolized into either eicosanoids, prostaglandins, leukotrienes or thromboxanes. High titers of prostaglandins, eicosanoids, COX_2 and leukotriene B4 are reported in human and murine UC mucosal biopsies (Sharon and Stenson, 1985; Isseroff *et al.*, 1987; Nishida *et al.*, 1987; DeMar Jr *et al.*, 2004; Gravaghi *et al.*, 2011; Kansal *et al.*, 2014), suggesting a debilitating role for AA metabolites in UC pathology. However, in this study, increased susceptibility to oxazolone was independent of linoleic acid, demonstrating the possibility of either host determining factors or other diet components playing a role in colitis susceptibility.

Quite surprising, I found that susceptibility to oxazolone colitis positively

associated with increased systemic IFNγ frequencies (**Fig 5.2**), while negatively associating with both local and systemic IL-13 frequencies, contrary to published literature (Heller *et al.*, 2002; Hoving *et al.*, 2012; Meroni *et al.*, 2018). Although a complex set of pro-inflammatory cytokines is apparent in human UC, the importance of IFNγ is only demonstrated in a few studies that include a dextran sulfate sodium (DSS) model of experimental IBD as well as in IFNγ driven human IBD (Ito *et al.*, 2006; Rafa *et al.*, 2010). In the former case, pathology was correlated to heightened gut IFNγ secretion, with $IFN\gamma^{-/-}$ mice refractory to DSS colitis (Ito *et al.*, 2006). Interestingly, type-I interferons can downregulate Th2 (IL-4 and IL-13) immune signaling (McRae *et al.*, 1997; Kaser and Blumberg, 2011; McFarlane *et al.*, 2017). It is possible then that a high IFNγ titer in the oxazolone administered mice might have suppressed IL-13 titers, albeit with the same expected phenotype outcome of exacerbated pathology.

Although I demonstrated that helminth infection exacerbated oxazolone colitis (**Fig 5.3**), it was quite surprising that exacerbation was independent of heightened Th2 cytokine proportions (**Fig 5.4**). Indeed, a closer look at my findings demonstrated a local (MLN) and systemic (spleen) IFNγ bias (**Fig 5.5**) in helminth-infected animals with severe oxazolone colitis. Th2 cytokine titers (**Fig 5.4**) negatively associated with pathology, quite contrary to findings by *Hunter et al.,* who demonstrated helminth exacerbation of oxazolone colitis associating with a heightened Th2 immune response (Hunter *et al.*, 2007). The previous authors made use of an acute *Hymenolepis diminuta (H.diminuta)* infection, while this study used a chronic *H.polygyrus* infection, and this difference, including the fact that *H.diminuta* is an extraintestinal non-murine parasite while *H.polygyrus* is a small intestine parasite, may contribute to the immunological differences between our studies. Despite the experimental differences between these two studies, we appreciate that helminth infection exacerbates oxazolone colitis, irrespective of helminth species and phase of infection (Hunter *et al.*, 2007; Wang *et al.*, 2010). The dominance of IFNγ proportions in both oxazolone only administered mice as well as helminth infected oxazolone administered mice may suggest an IFNγ dependent susceptibility in male BALB/c mice fed a standard chow diet. This proposed mechanism for disease could be tested further through the use of IFNγ blocking

antibodies, microRNA targeting IFNγ expression or IFNγ$^{-/-}$ mice in order to confirm the role of IFNγ in helminth exacerbation of oxazalone colitis.

Given the impact of helminth infection on oxazalone-induction, I then tested whether live, chronic infection was required for exacerbation of disease. I made use of the antihelminthic ivermectin during infection and assessed how helminth clearance would influence colitis induction. Helminth endemic nations carry out herd antihelminthic treatments in an effort to control helminth induced malnutrition and morbidity. Coincidentally, these programs target school going age groups, children who are not yet in the 'high environmental risk' category of UC development. Hence, I induced helminth expulsion before colitis induction, in order to model current kinetics of helminth infection and UC development in human populations. Helminth clearance appeared to rescue pathology (**Fig 5.3**) and was associated with a trend towards a Th2 cytokine rebound (**Fig 5.4**). Although both NKT and CD4$^+$ T cells are reported to be major IL-13 producers (Heller *et al.*, 2002; Hoving *et al.*, 2017), I found that CD4$^+$DX5$^-$T cells (deficient of NKT cells), secreted higher titers of IL-13 compared to NKT cells (**Fig 5.4**). These findings suggest that CD4$^+$ T cells produce higher titers of IL-13 compared to NKT cells, and supports previously published findings of IL-4 responsive CD4$^+$ T cells as an important group in IL-13 driven oxazolone colitis (Hoving *et al.*, 2017). Surprisingly, a minor reduction of colitic symptoms through antihelminthic treatment associated with mesenteric IFNγ ablation, further supporting our premise of IFNγ associated colitis pathology.

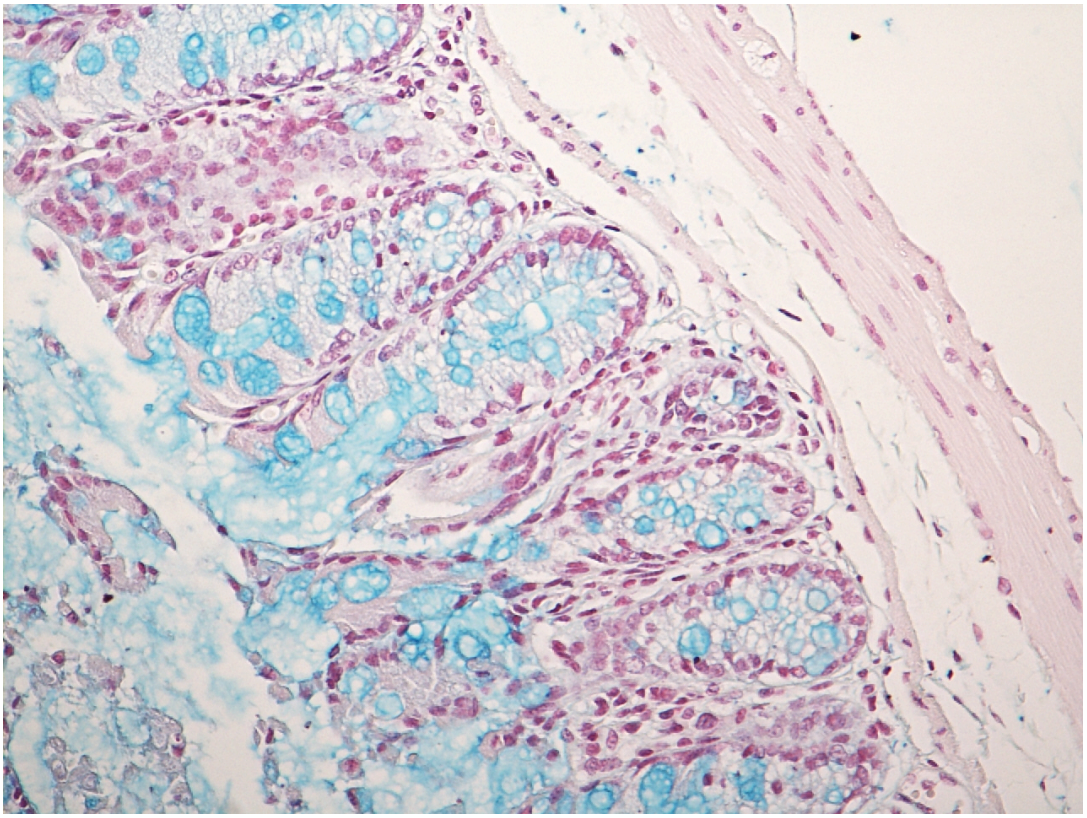
The Treg:Teffector immune milieu determines extent of *H.polygyrus* infection in BALB/c mice. In this study, increased splenic Treg numbers positively correlated with live helminth infection and the suppression of Th2 cytokine titers (**Fig 5.5**). This supports previous publications demonstrating Treg suppression of anti-helminth effector responses and the consequent Th2 dependent responses in helminth cleared mice, both of which are widely published (Grainger *et al.*, 2010; Smith *et al.*, 2016; C. wen Su *et al.*, 2018). Part of the Th2 dependent effector responses includes expansion of IL-4 receptive alternatively activated macrophages (AAMs). AAMs play an important role in larvae-elicited granuloma formation (Kreider *et al.*, 2007) and are also quickly recruited to

damaged or inflamed epithelial sites to enable mucosal healing. Although we did not further characterise macrophage phenotypes in this study, dampened Th2 responses and increased Treg numbers in helminth infected mice correlated with depressed peritoneal and macrophage proportions (**Fig 5.6**), a phenomenon quite similar to the depression of AAMs after Treg boosting in *H.polygyrus* infected BALB/c mice (Smith *et al.*, 2016). Surprisingly, helminth clearance failed to rescue the macrophage proportions, suggesting either the redundancy of macrophages in helminth exacerbation of oxazolone colitis, or the possibility of a phenotype switch. Indeed, parasite specific Th2 activated neutrophils were demonstrated to prime M2-like macrophages leading to efficient helminth clearance (Chen *et al.*, 2014) during a *Nippostrongylus brasiliensis* lung infection, demonstrating the possibility of macrophage phenotype switching during helminth infections. Interestingly, peritoneal and splenic neutrophil proportions paralleled macrophage proportions in the same locale (**Fig 5.6**), suggesting a universal impact of helminth infection as well as ivermectin treatment on innate cell populations.

Some of the major caveats of this study lie in the difficulty to reproduce the model with high precision in a number of repeat experiments. The model solely relies on the intrarectal administration of a haptenating agent that was mostly hindered or affected by fecal matter in the host's gastrointestinal tract, resulting in a lack of reproducibility between one mouse and the next. Once infected, oxazolone administration resulted in high mouse mortality rates, hindering long-term immunological studies. Fortunately, sampling at an earlier time point enabled immunological evaluations of disease pathology, although this also prevented monitoring of pathology over a longer period. Besides researcher-introduced errors, biological variance maybe introduced through *H.polygyrus* infection. Although approximately 200 L3 *H.polygyrus* larvae were oral gavaged into each mouse, we did not determine the sex ratios of male to female L3 larvae. Research reports the high antigenicity of male *H.polygyrus* adult worms in mice, making them more vulnerable to host immune responses (Adams *et al.*, 1988). Hence, inter-mouse variation between mice can result in different immune responses in oxazolone-induced mice. We also carried out predisposition and not therapeutic studies, hence we can only confidently conclude on how prior

helminth infection potentially exacerbates colitis and not how a helminth infection may be used as a treatment for colitis.

Overall, my findings suggests that live chronic helminth infection can influence the host's Treg activity, an effect that probably influences Th2 (effector) cell and innate immune cell functionality leading to host colonisation. However, in this study, host colonisation probably resulted in oxazolone colitis exacerbation, an outcome abrogated through helminth clearance. Helminth clearance appeared to result in the rebound of Th2 responses. In conclusion, we demonstrated a diet dependent susceptibility of male BALB/c mice to oxazolone colitis, which was associated with heightened systemic IFNγ frequencies. Helminth infection exacerbated colitis, and this correlated to suppressed type-2 immune responses. Antihelminthic treatment of infected mice appeared to ameliorate exacerbation of oxazolone colitis through a heightened Th2 immune response, characterised by increased $CD4^+$ T cell and NKT cell secretion of IL-4, IL-5 and IL-13 in the mesenteric lymph nodes. We believe our research is the first to demonstrate the influence of diet on oxazolone colitis as well as the importance of antihelminthic treatment on helminth exacerbated experimental and possibly human ulcerative colitis. We place an argument for further research into the influence of anti-helminthic treatment on helminth infection driven pathologies..



DSS only colon image. Cell nuclei (red) and mucus (Alcian blue staining). (Image credits: *Brunette Katsandegwaza*, 2017, UCT)

EXPERIMENTAL RESULTS CHAPTER TWO

H.polygyrus exacerbation of DSS colitis is associated with dysregulation of local and systemic $CD4^+$ T cell responses.

6 *Heligmosomoides polygyrus* and DSS colitis

The quest for Ulcerative Colitis (UC) biological therapies has opened up interesting fields of research that have contributed vast amounts of knowledge and furthered the understanding of Inflammatory Bowel Disease (IBD) pathology. Of the different potential UC biological therapies, the field of helminth immunotherapy has not only gripped the minds of researchers, but has actually lent great promise to UC therapy. Helminth immunotherapy is the process whereby patients with either autoimmune or inflammatory conditions are exposed to either a helminth infection, helminth ova or helminth products in an attempt to suppress or ameliorate disease pathology. The premise behind this therapy is derived from epidemiological findings of the global inverse relationship between helminth infection prevalence and IBD incidence.

These epidemiological findings are supported by human experimental research demonstrating the ability of helminth infections as well as helminth products in ameliorating both Ulcerative Colitis (UC) and Crohn's disease (CD) symptoms (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005). Clinical trials report the beneficial influence of helminth therapy on inflammatory diseases that include Multiple Sclerosis (Correale and Farez, 2007), asthma (Feary *et al.*, 2010) and UC (Broadhurst *et al.*, 2010). Reported successes appear to be study, disease and patient specific as successful outcomes after *Trichuris suis* infection and *Trichuris suis* ova treatment on both CD and UC respectively are contradicted by the non beneficial effect of *Trichuris suis* ova on grass pollen-induced rhinitis (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005; Bager *et al.*, 2010). The limitations of human experimental research necessitate the heavy dependence of disease pathology research on either cell culture or murine models.

Indeed, murine work emphasises the ability of helminth infection to suppress disease in different models of colitis (Elliott *et al.*, 2003, 2004; Smith *et al.*, 2007; Sutton *et al.*, 2008; Wang *et al.*, 2008; Ruyssers *et al.*, 2009; Hang *et al.*, 2010). However, contradictory findings in mouse models of disease demonstrate

an aggravatory influence of helminths on some bystander conditions. *Hymenolepis diminuta* is known to exacerbate murine experimental bacterial colitis and oxazolone colitis (Hunter *et al.*, 2007; Wang *et al.*, 2010), while *Heligmosomoides polygyrus (H.polygyrus)* infection drives carcinogenesis in a murine model of colon cancer (Pastille *et al.*, 2017). Despite the controversial findings behind experimental helminth therapy, the ability of helminths to chronically establish in their hosts is suggested to be the driving factor behind their immunomodulatory effects on bystander conditions. *H.polygyrus*, a natural murine helminth infection closely related to the human pinworm, *Trichuris spp*. expands the host's immunosuppressive regulatory T cells (Tregs) through the secretion of a TGFβ mimic capable of signaling through the host's TGFβ signaling pathway (Takahashi *et al.*, 1998; Finney *et al.*, 2007; Grainger *et al.*, 2010). Interestingly, expansion of Tregs in host mucosal tissues is a consequence of *H.polygyrus* larval migration and worm establishment (Smith *et al.*, 2016), and the initial Treg:Teffector dynamic plays an important role in the outcome of helminth infection.

The significance of Treg expansion during early helminth infection is demonstrated by heightened *H.polygyrus* worm and egg burdens in BALB/c mice after IL-2C Treg boosting (Smith *et al.*, 2016). Treg boosting immediately before infection also resulted in diminished Th2 cytokine and alternative activated macrophages (AAMs) proportions, leading to impaired anti-helminth responses (Smith *et al.*, 2016). The partial ablation of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs during early *H. polygyrus* infection as well as during *Strongyloides ratti* infection in BALB/c mice resulted in increased helminth expulsion; suggesting that infection drives Treg expansion in order to evade host immunity. This consequentially increases the helminth's chance of survival in the host while suppressing immune responses to bystander conditions (Blankenhaus *et al.*, 2014; Smith *et al.*, 2016). Interestingly, Treg depletion in late stage *H.polygyrus* infection had no impact on worm or egg burden in C57BL/6 mice, implying the dispensability of Treg activity during late stage infection, or a host dependent response (Smith *et al.*, 2016).

Indeed, the *H.polygyrus* larval stage is suggested to be the most antigenic and immunosuppressive compared to the adult worms (Donskow-Łysoniewska *et al.*, 2013). This would suggest that, in partially resistant mouse strains (e.g. BALB/c), the early stages of *H.polygyrus* infection drives efficient immunosuppression through Treg expansion, a phenomenon eventually overridden by the host's Th2 responses in the late stages of infection, resulting in the eventual clearance of adult worms. Because the kinetics of helminth infection (in this case *H.polygyrus* infection) in mice are key determinants of the host's innate and adaptive immune responses during the different phases of infection; I investigated the influence of prior helminth infection on the development of DSS colitis, a murine model of human UC. Making use of either an acute or chronic infection, the influence of different phases of infection on the host's innate, adaptive and regulatory immune responses during dextran sulfate sodium (DSS) colitis was assessed.

The DSS colitis model is an extensively used robust experimental model for human UC (Okayasu *et al.*, 1990), typified by a type-1 (IFNγ) acute immune response that later matures into a type-2 (IL-4 driven) immune response in the chronic phase, similar to human UC (Strober and Fuss, 2011; Chassaing *et al.*, 2014). Recent reports point to the potential role of a microbial driven Th17 immune response during DSS colitis, after findings of the seminal role of the gut microbiota in DSS colitis pathology (Alex *et al.*, 2009; Chassaing *et al.*, 2014). This IL-17/IL-23 axis plays an important role in colitis regulation (Yen *et al.*, 2006), demonstrated by colitis amelioration in IL-17 deficient mice (Ito *et al.*, 2008) as well as high IL-17 titers in human UC tissue (Duerr *et al.*, 2006; Cătană *et al.*, 2015). Paradoxically, *Kim et al.,* demonstrated ablated Th17 cells in DSS administered colon tissue, arguing for a controversial role of the Th17 response during experimental UC (Kim *et al.*, 2011).

Despite the disparities in the immune responses dominating the different DSS phases, both acute and chronic DSS phases are characterized by weight-loss, occult blood, goblet cell depletion, epithelial degradation, changes in cell surface junction proteins and ulceration (Liljana Stevceva *et al.*, 2001; Melgar *et al.*, 2005). The differences between the acute and the chronic phase are the

occasional infiltration of neutrophils in the former and that of mononuclear leukocytes and lymphocytes in the latter (Axelsson and Goldschmidt, 1996; Chassaing *et al.*, 2014). These DSS clinical and histological features model those seen in human UC, making this simple colitis model perfect for UC research.

An interesting limitation of this model, differentiating it from human UC, is the dispensability of both T and B cells in DSS colitis induction; demonstrated by the successful induction of colitis in both SCID and RAG-1 KO mice (mice deficient in both T and B cells) (Dieleman *et al.*, 1994; Kim *et al.*, 2006). Interestingly, although *Kim et al.,* demonstrated colitis induction in RAG-1 KO mice, the resultant mild colitis in these mice compared to their wild-type counterparts insinuates that lymphocytes may be necessary for subsequent colitis progression (Dieleman *et al.*, 1994; Kim *et al.*, 2006). Actually, a different colitis model, the $CD4^+$ T cells adoptive transfer model, highlights the importance of lymphocytes in inducing and promoting gut inflammation (Powrie *et al.*, 1993). The importance of IL-4 receptive B and T cells in driving oxazolone colitis also lends support to the significance of lymphocytes during colitis (Hoving *et al.*, 2012, 2017). Furthermore, *Broadhurst et al*., reported increased mucosal Th17 cells in a patient biopsy with active UC (Broadhurst *et al.*, 2010), while neutralization of the T cell gut homing integrin (CD103) is beneficial in IBD patients (O'Keeffe *et al.*, 2015). More pertinent to this study, is the increased numbers of Tregs in mucosal tissue from UC patients (Holmén *et al.*, 2006), and the 60% loss of immune suppressivity of circulatory Tregs in some IBD patients compared to healthy controls (Ueno *et al.*, 2013). Remarkably, the increased rate of apoptosis in inflamed tissue derived Tregs (Veltkamp *et al.*, 2011) suggests an impairment in Treg functionality during both CD and UC.

Although research has elucidated different parameters of DSS colitis, the etiopathogenesis of DSS colitis remains relatively undetermined up to today. DSS particles are reportedly engulfed by infiltrating macrophages, with *Kitajima et al.,* demonstrating the presence of DSS molecules in Kupffer and spleen cells after DSS administration, suggesting an antigen processing role for macrophages during colitis (Kitajima *et al.*, 1999). Previous research findings also suggest the formation of lipid nanocomplexes between DSS and medium chain fatty acids

(MCFAs) that are capable of fusing with epithelial cell membranes and facilitating the movement of DSS into the cell cytoplasm (Laroui *et al.*, 2012). These nanocomplexes then activate and initiate inflammatory signaling pathways that include the NFkB pathway, consequently leading to loss of epithelial barrier integrity (Laroui *et al.*, 2012), and increased bacterial translocation (Gardiner *et al.*, 1993). As with most fatty acids, MCFAs are found in high concentrations in the colon, with a high fat diet contributing to increased colonic MFCA titers.

Indeed, both experimental and human IBD are influenced by host diet; with a polyunsaturated fat enriched diet positively correlated with aggravated disease outcomes (Ma *et al.*, 2008; Gregor and Hotamisligil, 2011; Teixeira *et al.*, 2011; van der Logt *et al.*, 2013). A high fat diet is positively correlated with inflammatory conditions that include obesity, cardiovascular conditions and experimental colitis (Cani *et al.*, 2007; Kim *et al.*, 2012; Slavin, 2013; Chassaing *et al.*, 2015; Cheng *et al.*, 2016; Miles *et al.*, 2017). Recent research findings demonstrate the importance of regulating the n-6: n-3 polyunsaturated fatty acids (PUFA) ratio as a measure of UC risk prevention. Linoleic acid (n-6 PUFA), a major constituent of most cooking household oils, is positively associated with increased human and experimental UC risk. The european prospective investigation into cancer and nutrition (EPIC) studies demonstrate the high risk of UC development in patients with increased n-6 PUFA intake (Silva *et al.*, 2008; The IBD in EPIC Study Investigators, 2009). The positive association between UC and n-6 PUFA intake was also reported in a retrospective study that compared the associations of different diets with either UC or CD (Geerling *et al.*, 2000). Despite the positive outcomes associated with the n-6: n-3 ratio reduction (Stenson *et al.*, 1992; Tobias *et al.*, 2003; The IBD in EPIC Study Investigators, 2009; De Silva *et al.*, 2011; Pearl *et al.*, 2014), human studies finding the non-beneficial effect of dietary PUFA manipulation add to the controversy surrounding the efficacy of dietary fatty acids manipulation on human UC (Hawthorne *et al.*, 1992; Hart *et al.*, 2008; Turner *et al.*, 2011; Ananthakrishnan *et al.*, 2014; Wiese *et al.*, 2016).

However, experimental research reiterates the increased association between linoleic acid metabolites and exacerbated gut inflammation, as with human UC

(Nishida *et al.*, 1987; Silva *et al.*, 2008; de Silva *et al.*, 2010; Wiese *et al.*, 2016). Linoleic acid (n-6 PUFA) is metabolized into arachidonic acid (AA), the latter a precursor of inflammatory prostaglandins and leukotrienes (Salem *et al.*, 1999). Some prostaglandins and leukotrienes are natural PPARγ ligands, the activation of which results in apoptosis as well as inflammatory gene transcription (Calder, 2008; Shrestha *et al.*, 2010; Kansal *et al.*, 2014). Prostaglandin activity is also linked to the activation of the wnt signaling pathway due to their ability to interfere with the β-catenin complex stability, leading to the transcription of inflammation and tumorigenesis associated T-cell factor (TCF) genes (Kansal *et al.*, 2014). In contrast, dietary n-3 interventions demonstrate inflammatory suppression in both human and experimental UC (T Shimizu *et al.*, 2001; Toshiaki Shimizu *et al.*, 2001; Tobias *et al.*, 2003; Gravaghi *et al.*, 2011; Kansal *et al.*, 2014). Lipoxins and resolvins, both metabolites of n-3 PUFA, are suggested to suppress inflammation by outcompeting n-6 PUFA during lipid metabolism (Schwab *et al.*, 2007; Campbell *et al.*, 2010). In summary, implications of the effect of diet change on gut inflammation are centered on the ability of diet to alter the host's immune responses (Kim *et al.*, 2012; Basson *et al.*, 2016; Rajoka *et al.*, 2017).

Besides diet, other confounding factors determine the incidence and prevalence of UC. Although sexual dimorphism is not clearly apparent in IBD, high female to male ratios of disease incidence are evident in autoimmune diseases that include multiple sclerosis, primary biliary cholangitis, celiac disease and systemic lupus erythematous (SLE) (Mcnally *et al.*, 2003; Amarapurkar and Patel, 2007; Bentzen *et al.*, 2010; Ramagopalan *et al.*, 2010; Chiche *et al.*, 2012; Feldman *et al.*, 2013). An interesting study by *Babickova et al.,* demonstrated a protective role of female estrogens during DSS colitis, with protection closely associated with the suppression of TNF (Bábíčková *et al.*, 2015). The beneficial role of estrogen and its derived metabolites is supported by findings associating these compounds with ameliorated disease in different colitis models that include the acetic model of colitis, the HLA-B27 transgenic model of colitis as well as in the DNBS model (Mähler *et al.*, 1998; Günal *et al.*, 2003; Harnish *et al.*, 2004). Findings of estrogen exacerbating inflammation and colitis have also been reported, contributing to the controversy behind sexual diphormism in colitis and

IBD (Mähler *et al.*, 1998; Verdú *et al.*, 2002). The estrogen-dependent promotion of tumorigenesis during murine colorectal cancer and the delayed onset of puberty in rat experimental models of IBD supports the premise of adverse effects of hormonal dynamics during IBD (Azooz *et al.*, 2001; Deboer and Li, 2011; Heijmans *et al.*, 2014).

In this study, I sought to determine the influence of different helminth infection phases on DSS colitis development, based on the premise that acute infection-elicited Treg expansion would limit the inflammatory response associated with DSS colitis; while the anti-helminth response associated with a chronic infection in BALB/c mice would exacerbate colitis. Initially, I demonstrated sexual diphormism and diet dependence in DSS exposed mice, with female mice fed a linoleic-acid modified diet exhibiting increased susceptibility to disease. Disease exacerbation was dominated by systemic neutrophilia and heightened inflammatory monocyte infiltration. Surprisingly, both prior acute and chronic helminth infection exacerbated DSS colitis, although they exhibited different immune signatures. Acute helminth-infected mice displayed high levels of IL-6 and IL-17A titers after DSS admnistration; both hallmark cytokine responses of experimental and human IBD. Disruption of the $CD4^{+}Foxp3^{+}$ regulatory T cell (Treg) and $CD4^{+}$ effector T cell (Teff) responses positively correlated with increased local and peripheral inflammation in both acute and chronic *H.polygyrus* infected mice after DSS administration. Hence, helminth influence on DSS colitis is dependent on the influence of helminth infection phase, as well as the impact of this phase on the host's innate and adaptive immune responses.

STUDY HYPOTHESIS

I proposed that an acute *H.polygyrus* infection would ameliorate DSS colitis through its ability to induce immune-regulatory cell populations that include $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs), while chronic *H.polygyrus* infection would exacerbate DSS colitis due to heightened anti-helminth directed host immune responses.

RESULTS

Increased susceptibility of female mice to DSS induced colitis under the modified AIN76A diet.

Although sexual diphormism is reported for a number of autoimmune conditions that include multiple sclerosis and systemic lupus erythematous, IBD incidence and prevalence is generally reported not to follow a gender dependent distribution (Mcnally *et al.*, 2003; Bentzen *et al.*, 2010; Ramagopalan *et al.*, 2010; Chiche *et al.*, 2012). However, a number of studies report a key role for gender in IBD, with one study demonstrating the protective role of estrogens in DSS administered mice (Mähler *et al.*, 1998; Azooz *et al.*, 2001; Bábíčková *et al.*, 2015). In addition, other risk factors such as a linoleic acid (n-6) rich diet are associated with aggravated human UC (Hart *et al.*, 2008; De Silva *et al.*, 2011), while n-3 supplementation is correlated with disease remission (Ananthakrishnan *et al.*, 2014).

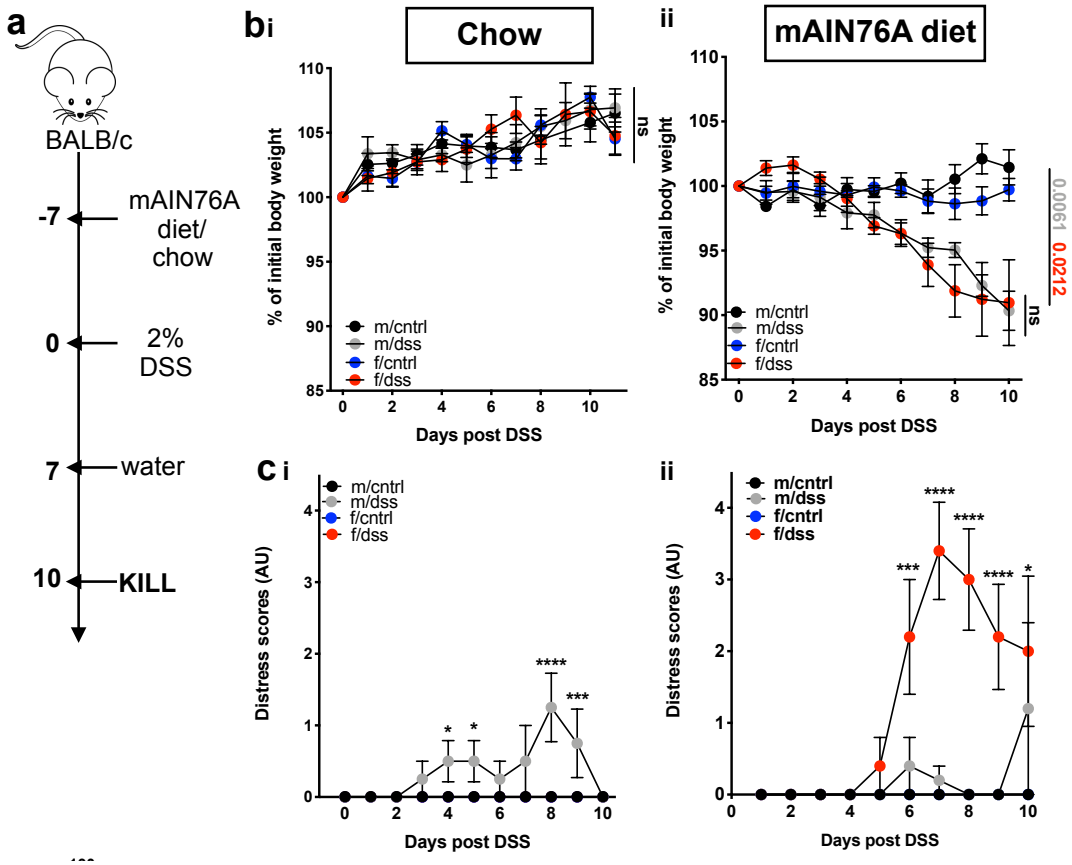


Figure 6-1: Increased sensitivity of females fed a modified AIN76A diet to DSS administration.

Male and female BALB/c mice fed a diet of chow and a modified AIN76A diet (mAIN76A) *ad libatum* were given 2% DSS *ad libatum* in the water from day 0-10 as illustrated (**a**). Weight-loss (**b**) and distress scores (**c**) for male mice fed chow diet (black symbols), male mice fed mAIN76A diet (grey symbols), female mice fed chow diet (blue symbols) and female mice fed mAIN76A diet (red symbols) were monitored over the course of DSS colitis induction. Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, statistical comparisons are between sex-matched treatments.

In order to determine whether gender and diet had an impact on DSS colitis induction, age matched BALB/c mice of different sexes were exposed to either a high linoleic acid modified AIN76A (mAIN76A) diet (D16083101, Research Diets) or a standard chow diet (#2012, NutritionHub) before DSS colitis induction (**Fig 6.1a**). Interestingly, both female and male mice fed the mAIN76A diet were highly susceptible to DSS colitis induction compared to their chow counterparts, and this was demonstrated by their heightened weight-loss (**Fig 6.1b**) and distress scores (**Fig 6.1c**) monitored during colitis development. Although both male and females under the mAIN76A diet had significantly shortened colons compared to their controls (**Fig 6.2a & b**), female mice exhibited severe colon inflammatory infiltrate and epithelial stress (**Fig 6.2c**) as demonstrated by severe histopathological scores (**Fig 6.2d**).

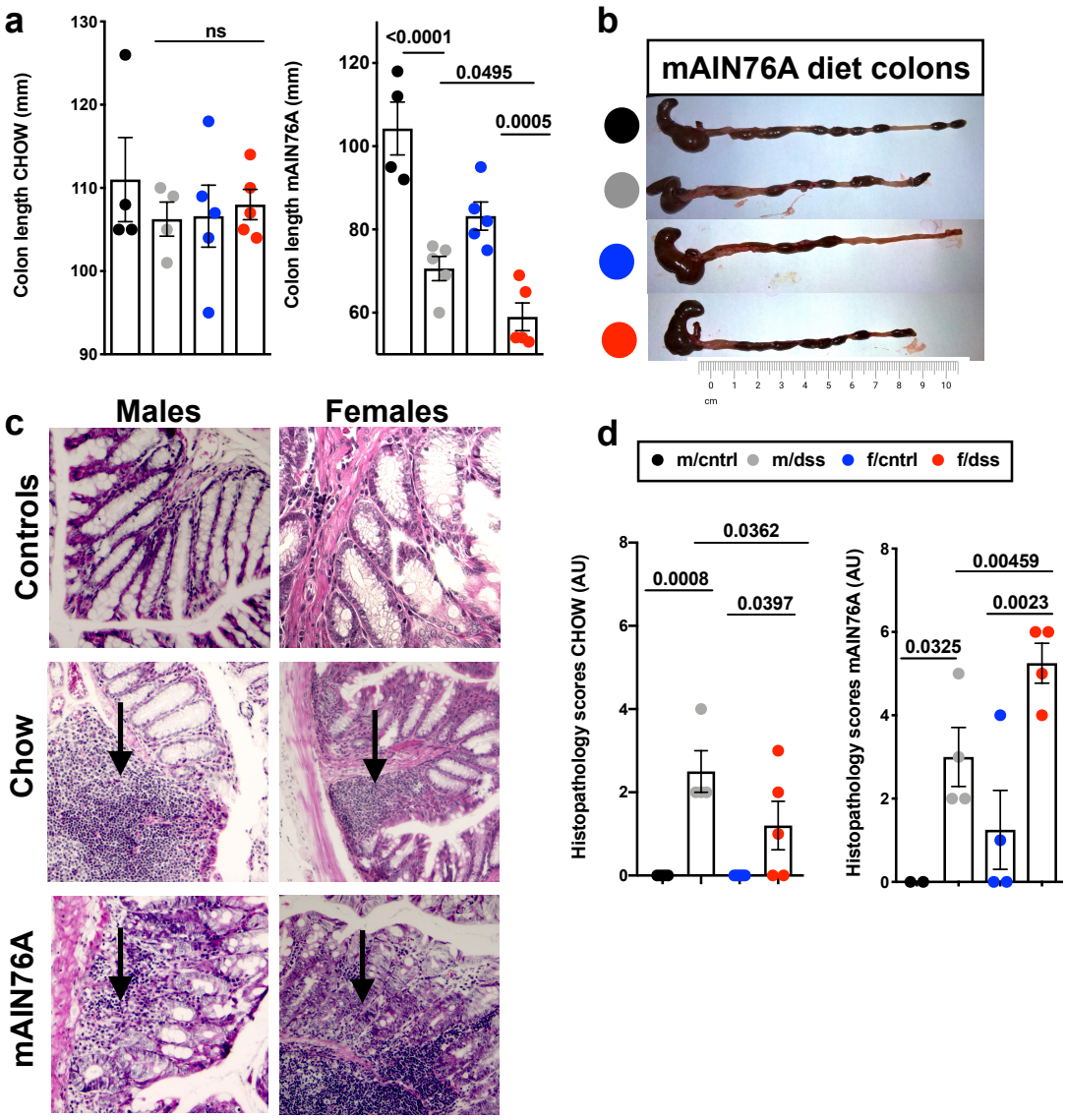


Figure 6-2: Increased DSS sensitivity in mAIN76A fed female mice is accompanied by severe colon pathology.

Male and female BALB/c mice fed a diet of chow and a modified AIN76A diet (mAIN76A) *ad libatum* were given 2% DSS *ad libatum* in the water from day 0-7. At day 10 post-treatment, colons were sterile excised, measured in length (**a, b**) for male mice fed chow diet (black symbols), male mice fed mAIN76A diet (grey symbols), female mice fed chow diet (blue symbols) and female mice fed mAIN76A diet (red symbols). Approximately 1mm distal colon sections were fixed in 4% PFA for histological sectioning and staining. Representative H&E images illustrating inflammatory cell infiltrate (indicated) in colon sections are shown here (**c**). Histological sections were manually scored for disease related pathology to determine extent of disease (**d**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, statistical comparisons are between sex-matched treatments. Histology slides prepared by Lizette Fick, UCT.

Susceptibility to DSS colitis was associated with a minimal change in the adaptive immune responses.

Studies have reported the induction of DSS colitis in SCID and RAG-1KO mice, suggesting the dispensability of the adaptive immune response during DSS colitis induction (Dieleman *et al.*, 1994; Kim *et al.*, 2006). However, the same authors demonstrate a mild colitis phenotype in the absence of adaptive immune responses, underscoring the importance of both B and T cells in colitis progression (Kim *et al.*, 2006). Furthermore, the ability of a heterologous $CD4^+$ T cell adoptive transfer to induce colitis in SCID mice as well as the increased lymphocyte infiltration in human UC biopsies demonstrates the importance of the adaptive immune response during gut inflammation (Powrie *et al.*, 1993; Broadhurst *et al.*, 2010).

In order to determine immune responses associated with susceptibility to DSS colitis, the mesenteric lymph nodes (MLN), peritoneal cavity wash (PerC) and spleen tissue were collected and analysed for immune cells of interest using flow cytometry. In support of the DSS kinetics findings from **Fig 6.1** & **Fig 6.2**, mAIN76A female fed mice demonstrated significant systemic neutrophilia **(Fig 6.3a)** and inflammatory monocyte **(Fig 6.3a)** frequencies (gating strategy illustrated in **Fig 6.3b**) compared to their male counterparts and untreated female mice (N/fem).

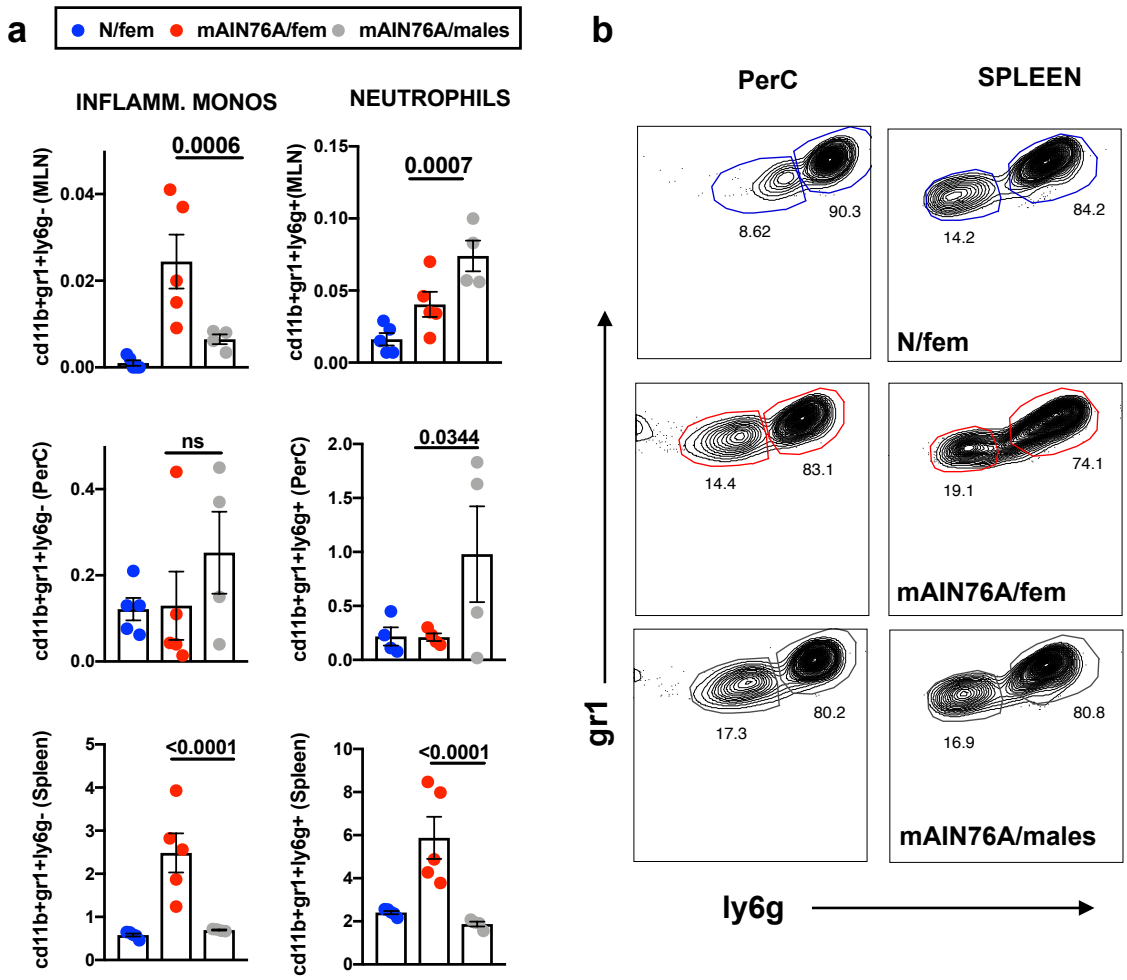


Figure 6-3: Increased disease in females fed a modified AIN76A diet is associated with increased proportions of systemic neutrophils and inflammatory monocytes.
Male and female BALB/c mice fed a chow diet and a modified AIN76A diet (mAIN76A) *ad libatum* were given 2% DSS *ad libatum* in the water from day 0-7. At day 10 post-treatment, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. The proportion of inflammatory monocytes ($Gr1^{high}$, $Ly6G^{low}$) and neutrophils ($Gr1^{high}$, $Ly6G^{high}$) within $CD11b^{+}$ cells were quantified in the MLN, PerC and spleen in female mice fed chow diet (blue symbols), female mice fed mAIN76A diet (red symbols) and male mice fed mAIN76A diet (grey symbols) (**a**). Representative density plots of these populations are shown (**b**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, statistical comparisons are between females mAIN76A (red symbols) and males mAIN76A (grey symbols).

Although DSS administration resulted in shrinkage of the T cell compartment (**Fig 6.4**), susceptibility to DSS was generally not associated with any significant changes in both the $CD4^{+}$ and $CD8^{+}$ T cell compartments isolated from the MLN, PerC and the Spleen (**Fig 6.4a & b**) when comparing the female and male mice fed the mAIN76A diet. Because acute DSS colitis is either a Th1 (IFNγ) or

a mixed Th1/Th17 (IFNγ/IL-17A) dominated pathology, I determined the immune responses accompanying colitis development. Surprisingly, neither IFNγ nor IL-17A total proportions in all three locations we analysed for correlated with either susceptibility or protection to DSS colitis (**Fig 6.4c & d**), although we demonstrated increased total MLN IL-17A proportions in male (mAIN76A/males) compared to the female mice (mAIN76A/fem). Further characterisation of the contribution of T-helper as well as $CD8^+$ T cells to the cytokine milieu demonstrated a non-significant change in the Th1 immune response ($CD4^+IFN\gamma^+$) during DSS colitis (**Fig 6.5**). Interestingly, $CD4^+$ and $CD8^+$ MLN IL-17A proportions in the male mice (mAIN76A) (**Fig 6.5**) mirrored the total MLN IL-17A proportions (**Fig 6.4d**).

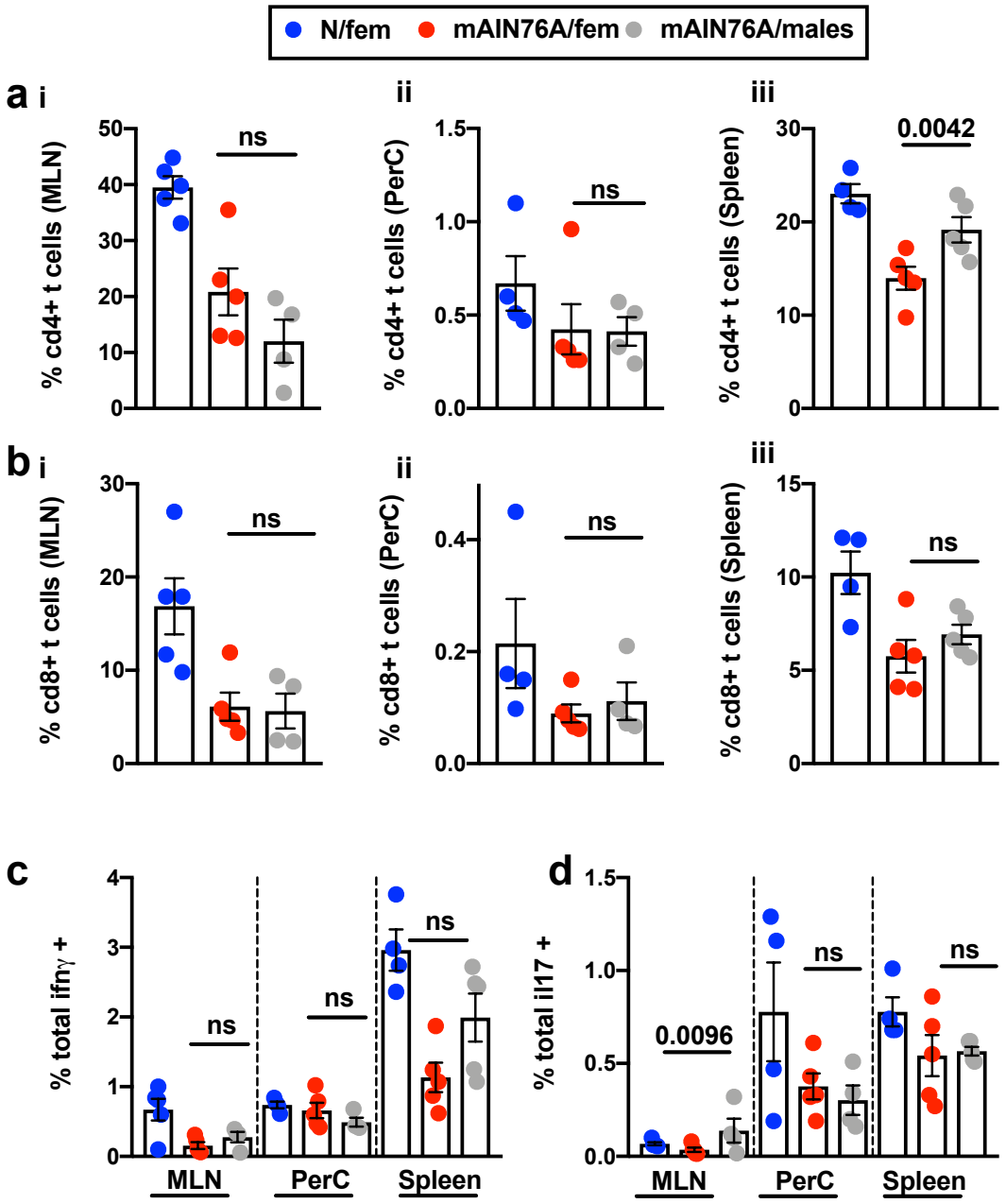


Figure 6-4: Adaptive immune responses did not significantly change in relation to increased disease susceptibility in females fed a modified AIN76A diet.
Male and female BALB/c mice fed a diet of chow and a modified AIN76A diet (mAIN76A) *ad libatum* were given 2% DSS *ad libatum* in the water from day 0-7. At day 10 post-treatment, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. The proportion of $CD4^+$ T cells and $CD8^+$ T cells (gated within $CD3^+$ T cells) were quantified in the MLN, PerC and spleen in female mice fed chow diet (blue symbols), female mice fed mAIN76A diet (red symbols) and male mice fed mAIN76A diet (grey symbols) (**a,b**). The percentage of IFNγ and IL-17A expression by all cells was also quantified in the MLN, PerC and Spleen following intracellular cytokine staining (**c,d**).Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, statistical comparisons are between females mAIN76A (red symbols) and males mAIN76A (grey symbols).

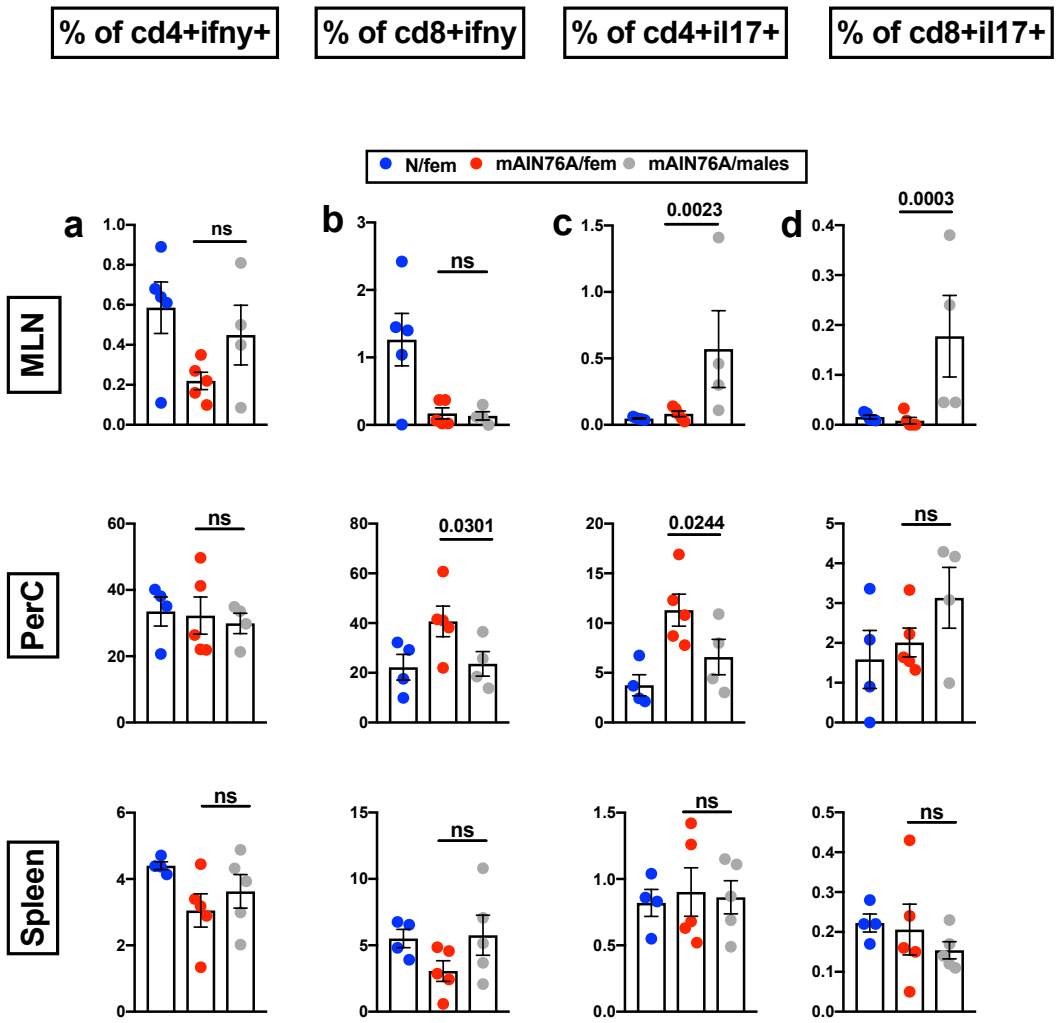


Figure 6-5: T cell IFNγ signaling does not significantly change with heightened susceptibility to DSS colitis development in mAIN76A fed mice
Male and female BALB/c mice fed a diet of chow and a modified AIN76A diet (mAIN76A) *ad libatum* were given 2% DSS in the water from day 0-7. At day 10 post-treatment, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. The percentage of IFNγ and IL-17A expression within $CD4^+$ and $CD8^+$ T cells in the MLN, PerC and spleen of female mice fed chow diet (blue symbols), female mice fed mAIN76A diet (red symbols) and male mice fed mAIN76A diet (grey symbols) was quantified following intracellular cytokine staining (**a-d**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, statistical comparisons are between females mAIN76A (red symbols) and males mAIN76A (grey symbols).

Both acute and chronic *H.polygyrus* infections exacerbate an adapted/optimised model of DSS-induced colitis.

Previous studies have demonstrated that *Heligmosomoides polygyrus* (*H.polygyrus*) infection modulates murine DSS-induced colitis (Pastille *et al.*, 2017), *Citrobacter rodentium*-induced colitis (Chen *et al.*, 2005; Weng *et al.*,

2007; C. Su *et al.*, 2018) as well TNBS colitis (Sutton *et al.*, 2008). Interestingly, it is also reported that the *H.polygyrus* fourth stage larvae (L4) can protect against DSS colitis (Donskow-Łysoniewska *et al.*, 2013), with the early phase of *H.polygyrus* infection suggested to be more immunosuppressive on bystander conditions (Smith *et al.*, 2016). Following optimisation of the DSS model (**Fig 6. 1-6.5**), I aimed to determine the impact of both an acute and chronic helminth infection on colitis in my **adapted model** of DSS colitis.

To determine whether helminth infection can modify colitis induction, naïve, acute (D7) and chronic (D28) *H.polygyrus*-infected mAIN76A fed female mice were exposed to 2% DSS in their drinking water for 7 days (**Fig 6.6a**). As demonstrated (**Fig 6.6b & c**), both *H.polygyrus* infected groups suffered increased weight-loss and distress during the course of DSS administration. Histological analysis of colon sections revealed a significant increase in inflammatory cell infiltrate and goblet cell depletion in the distal colon of acute helminth-infected mice treated with DSS compared to the chronic infected mice and the DSS only controls (**Fig 6.6d**). Although all DSS treated groups demonstrated similar colon shortening as a sign of colon inflammation (**Fig 6.6e**), overall histopathological scores were significantly higher for acute infected mice (D7) compared to the DSS only group (**Fig 6.6f**).

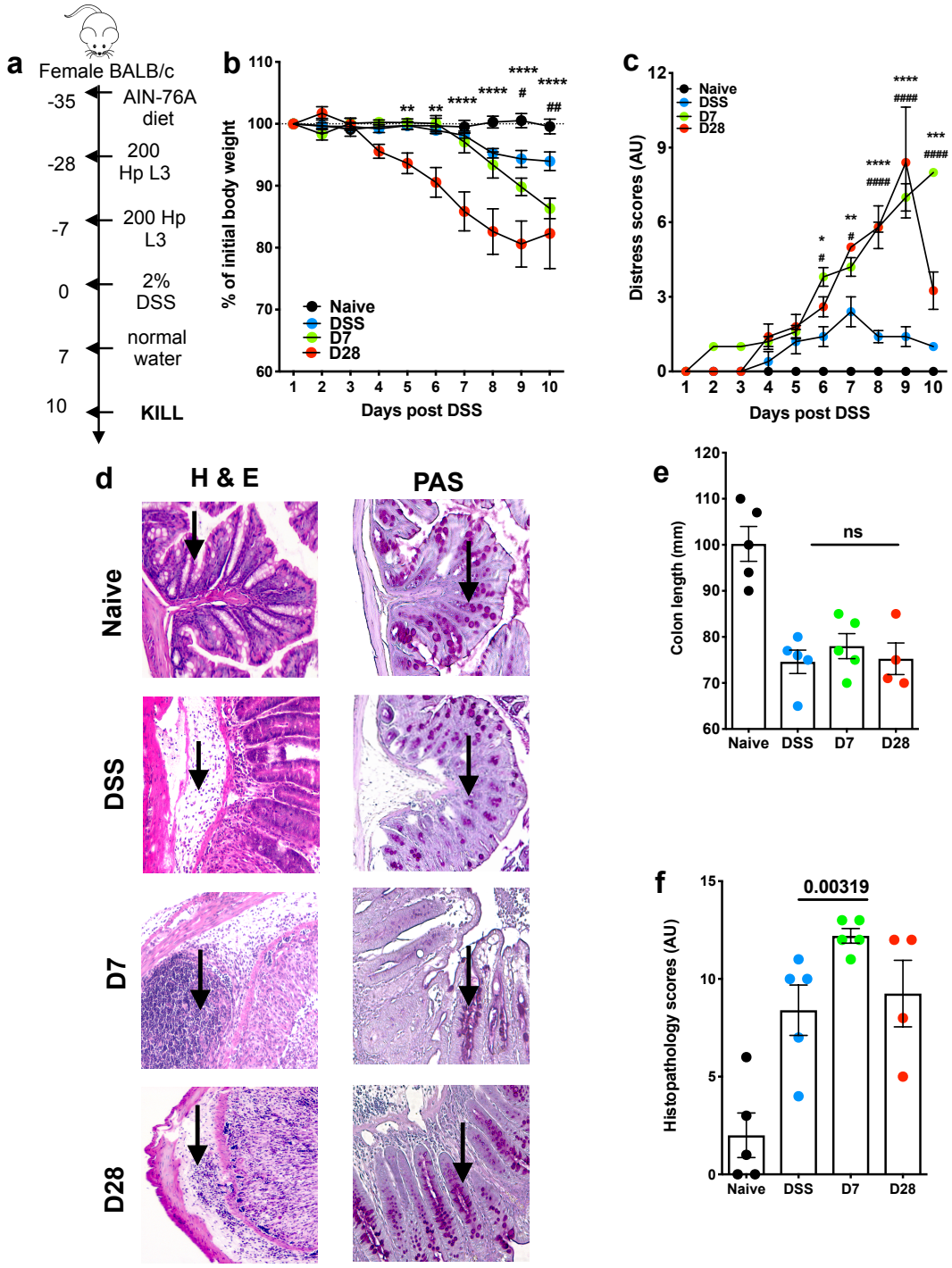


Figure 6-6: Both acute and chronic *H.polygyrus* infections exacerbate DSS induced colitis. Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were exposed to either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS in the water as illustrated (**a**). Weight-loss (**b**) and distress (**c**) were monitored for naïve untreated mice (black symbols), dss only mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols) over the course of colitis development. At day 10 post-treatment, colons were sterile excised and colon length was measured (**e**). Approximately 1mm distal colon sections were fixed in 4% PFA for histological sectioning and staining. Representative H&E and PAS images illustrating inflammatory cell infiltrate and goblet cell depletion respectively in colon sections are shown here (**d**). Furthermore, histological sections were manually scored for disease related pathology to determine extent of disease **(f)**. Data is presented as mean ± SEM and is

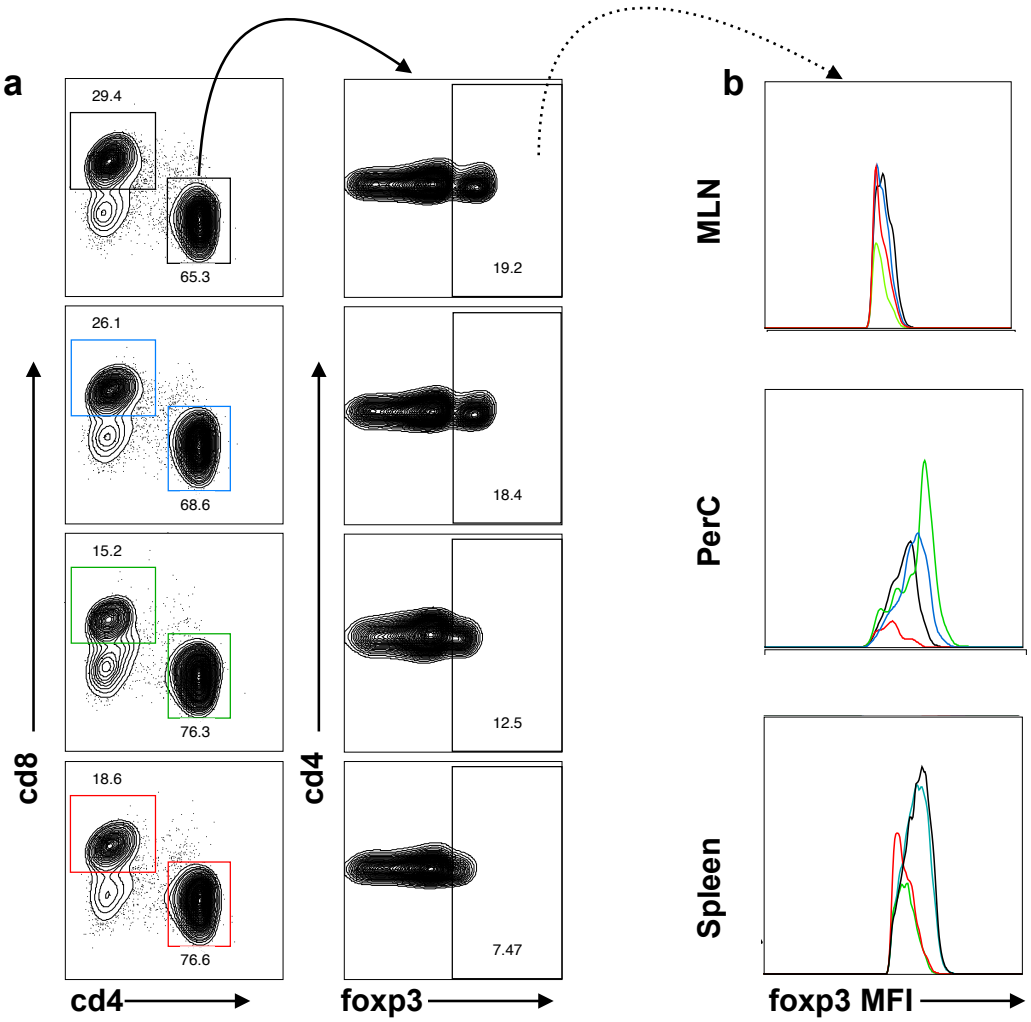
representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*/\#}$ p<0.05, $^{**/\#\#}$ p<0.01, $^{***/\#\#\#}$ p<0.001 and $^{****/\#\#\#\#}$ p<0.0001. (#) comparisons between DSS and D7; (*) comparisons between DSS and D28. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT and Alisha Chetty, UCT. Histology slides prepared by Lizette Fick, UCT.

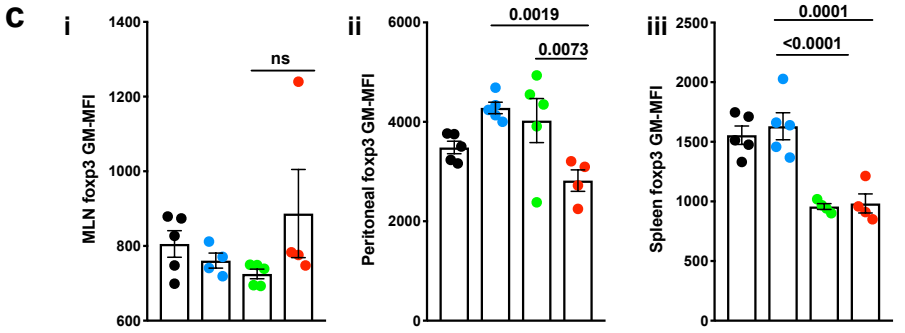
Dysregulated systemic effector T cell and Treg dynamics positively correlated with increased inflammation during helminth exacerbated DSS colitis.

Smith et al., demonstrated the expansion of the host's thymus and induced Treg compartments during the early phase *H.polygyrus* infection (Smith *et al.*, 2016), an immune phenomenon reported to result in the immunesuppression of a number of bystander inflammatory conditions. Surprisingly, helminth exacerbation of DSS colitis was associated with increased lamina propria Treg proportions in a previous study looking at the impact of an acute *H.polygyrus* infection on DSS colitis (Pastille *et al.*, 2017), contributing to the controversy behind helminth immunomodulation. Interestingly, Treg depletion is reported to exacerbate DSS induced colitis, with a Treg associated IL-10 knock-out mouse developing spontaneous colitis (Kühn *et al.*, 1993; Daniel J Berg *et al.*, 1996; Barnett and Fraser, 2011; K.-H. Lee *et al.*, 2014).

The previous observation of increased sensitivity of helminth-infected mice to DSS (**Fig 6.6**) suggested a loss of immunosuppression in the host. In order to determine the Treg: Teffector dynamics during both the acute and chronic infections during DSS colitis, the MLN, peritoneal wash and spleen were collected and analysed for Teffectors and Tregs as illustrated (**Fig 6.7a**). Foxp3 MFI (a metric used to determine the average quantity of Foxp3 protein per cell and is positively correlated with the suppressive capacity of $Foxp3^+$ cells) was determined as illustrated (**Fig 6.7b**). Interestingly, Foxp3 MFI (**Fig 6.7c**) mirrored $CD4^+Foxp3^+$ frequencies (**Fig 6.7d**), with chronic *H.polygyrus* infection resulting in the exhaustion of both peritoneal and splenic $CD4^+Foxp3^+$ (**Fig 6.7d ii & iii**) as well as reduction of the Foxp3 MFI (**Fig 6.7c ii & iii**) after DSS administration. Foxp3 MFI was generally similar amongst all treatments in the MLN and varied within the PerC and spleen depending on helminth infection status (**Fig 6.7c**). Interestingly, $CD4^+Foxp3^+$ Treg proportions and Foxp3 MFI were inversely proportional to the $CD4^+$ effector T (Teff) cell proportions (gated

as illustrated in **Fig 6.8a**) in all three compartments (**Fig 6.8b**), while both acute and chronic helminth infection resulted in increased splenic Teffectors after DSS administration (**Fig 6.8b**). However, a depletion of MLN and splenic $CD8^+$ effector T cells was apparent in both helminth-infected groups compared to the DSS only group (**Fig 6.8c**).


a
29.4
65.3
19.2
26.1
68.6
18.4
15.2
76.3
12.5
18.6
76.6
7.47
cd8
cd4
cd4
foxp3
b
MLN
PerC
Spleen
foxp3 MFI


c
i
MLN foxp3 GM-MFI
ns
ii
Peritoneal foxp3 GM-MFI
0.0019
0.0073
iii
Spleen foxp3 GM-MFI
0.0001
<0.0001

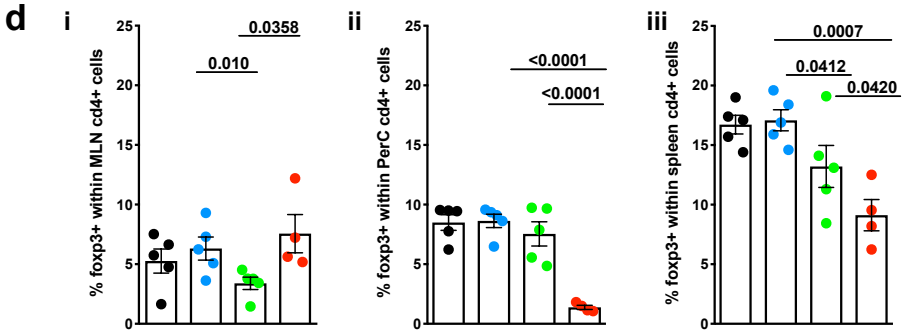

d
i
% foxp3+ within MLN cd4+ cells
0.0358
0.010
ii
% foxp3+ within PerC cd4+ cells
<0.0001
<0.0001
iii
% foxp3+ within spleen cd4+ cells
0.0007
0.0412
0.0420

Figure 6-7: Dysregulated systemic effector T cell and Treg dynamics positively correlated with increased inflammation during helminth exacerbated DSS colitis.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were exposed to either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post DSS administration, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. Representative density plots of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+Foxp3^+$ Tregs are illustrated (**a**). The Foxp3 geometric mean fluorescence intensity (MFI) of MLN, Perc and spleen $CD4^+Foxp3^+$ cells for naïve untreated mice (black symbols), DSS only mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols) was calculated and representative histograms are shown (**b**). Foxp3 MFI (**c**) and $CD4^+Foxp3^+$ Treg proportions (**d**) from the MLN, Perc and spleen were quantified. Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT and Alisha Chetty, UCT.

After determining the changes associated with regulatory and effector T cells during helminth exacerbation of DSS colitis, I next sought to elucidate whether these changes had any impact on the cytokine responses associated with both experimental and human UC by analysing for two cytokines mostly associated with both conditions. The acute infected group (D7) demonstrated heightened splenic total IL-6 and total IL-17A proportions compared to the chronic infected (D28) group and the DSS only control after DSS administration (**Fig 6.8d**). Surprisingly, cytokine responses in the PerC appeared generally ablated in helminth-infected groups after DSS induction, with the exception of the heightened IL-6 proportions in the chronic infected mice (**Fig 6.8d**). Further analysis of the contribution of $CD4^+$ and $CD8^+$ T cells to the cytokine responses demonstrated a significant contribution of both $CD4^+$ and $CD8^+$ T cells from helminth-infected groups to the IL-6 responses in all three tissues we collected (MLN, PerC and Spleen) (**Fig 6.9**). However, a suppressed IL-17A response from both $CD4^+$ and $CD8^+$ T cells was apparent, especially in the PerC of both acute and chronic infected groups (**Fig 6.9**). Intriguingly, the minimal contribution of splenic $CD4^+$ and $CD8^+$ T (**Fig 6.9**) to the total IL-17A titers (**Fig 6.8e**) may suggest another source of IL-17A besides T cells during acute helminth infection exacerbation of DSS colitis.

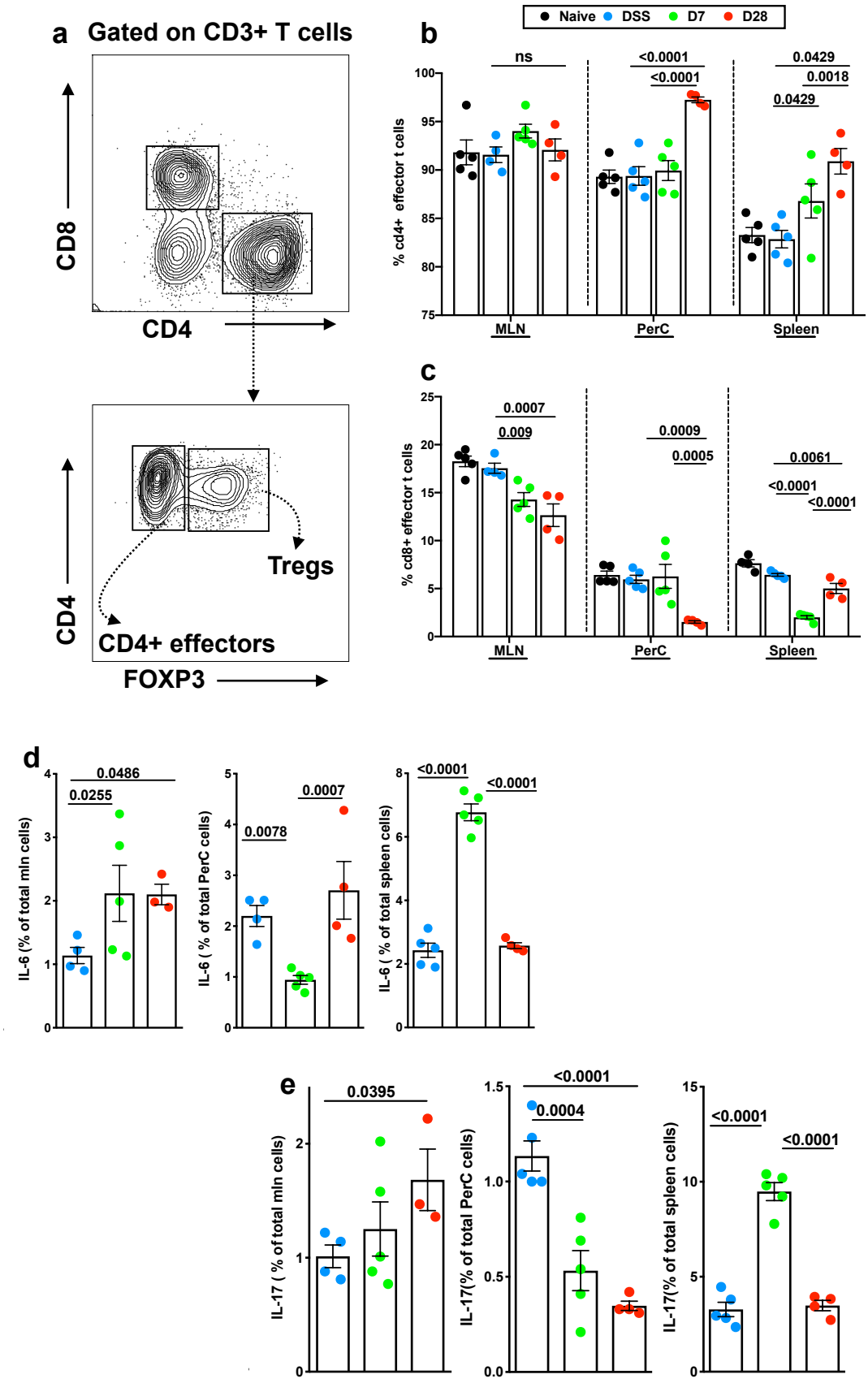

a
Gated on CD3+ T cells
CD8
CD4
CD4
FOXP3
Tregs
CD4+ effectors
b
Naive
DSS
D7
D28
% cd4+ effector t cells
ns
<0.0001
<0.0001
0.0429
0.0018
0.0429
MLN
PerC
Spleen
c
% cd8+ effector t cells
0.0007
0.009
0.0009
0.0005
0.0061
<0.0001
<0.0001
MLN
PerC
Spleen
d
IL-6 (% of total mln cells)
0.0486
0.0255
IL-6 (% of total PerC cells)
0.0007
0.0078
IL-6 (% of total spleen cells)
<0.0001
<0.0001
e
IL-17 (% of total mln cells)
0.0395
IL-17(% of total PerC cells)
<0.0001
0.0004
IL-17(% of total spleen cells)
<0.0001
<0.0001

Figure 6-8: Acute helminth infection-elicited splenic total IL-6 and IL-17A immune responses positively associated with exacerbated disease.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were exposed to either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post DSS administration, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. Representative density plots of $CD4^+$ T effector cells are illustrated (**a**). $CD4^+$ and $CD8^+$ T effector cells from naïve untreated mice (black symbols), DSS only mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols) were quantified demonstrating the deleterious impact of prior helminth infection on DSS colitis induction (**b,c**). The percentage of IL-6 and IL-17A expression within all cells of the MLN, PerC and spleen was quantified following intracellular cytokine staining (**d,e**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT.

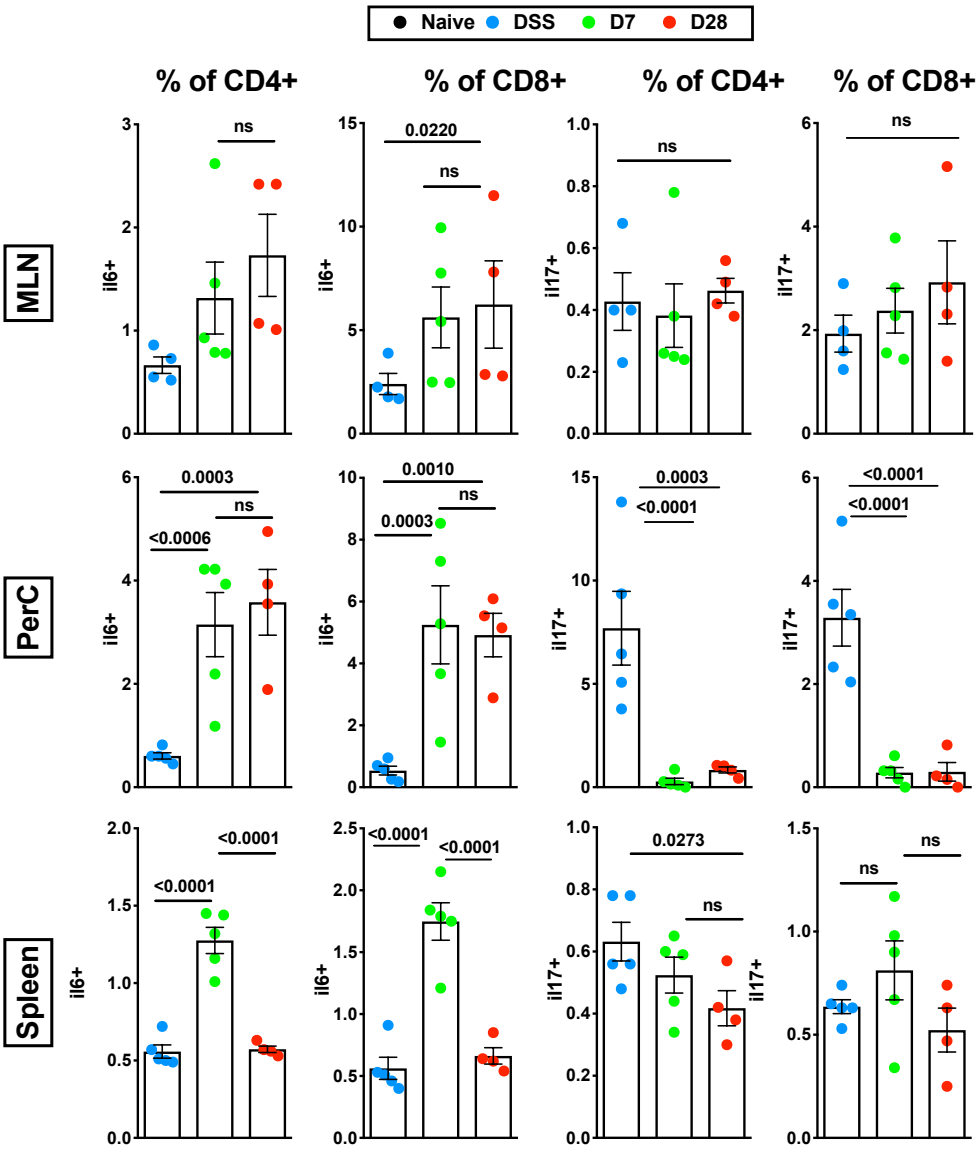


Figure 6-9: Both $CD4^+$ and $CD8^+$ T cells actively contribute to disease pathology in helminth-infected mice.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were exposed to either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post DSS administration, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. Representative density plots of $CD4^+$ T effector cells are illustrated (**a**). $CD4^+$ and $CD8^+$ T effector cells from naïve untreated mice (black symbols), DSS only mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols) were quantified demonstrating the deleterious impact of prior helminth infection on DSS colitis induction (**b,c**). The percentage of IL-6 and IL-17A expression within all cells of the MLN, PerC and spleen was quantified following intracellular cytokine staining (**d,e**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT.

Acute helminth infection exacerbation of DSS colitis is accompanied by potent anti-helminth Th2 immune responses.

Although both humans and mice mount strong Th2 immune responses against helminth infection, *H.polygyrus* is reported to expand the host's Treg compartment early during infection; enabling immune evasion due to the suppression of the host's anti-helminth responses. Surprisingly, although both $CD4^+$ (**Fig 6.10b**) and $CD8^+$ (**Fig 6.10c**) T cells from both helminth-infected groups contributed to the IL-13 responses after DSS administration, total IL-13 frequencies (**Fig 6.10a**) were significantly heightened only in the MLN, PerC and spleen tissues of acute infected mice after DSS administration.

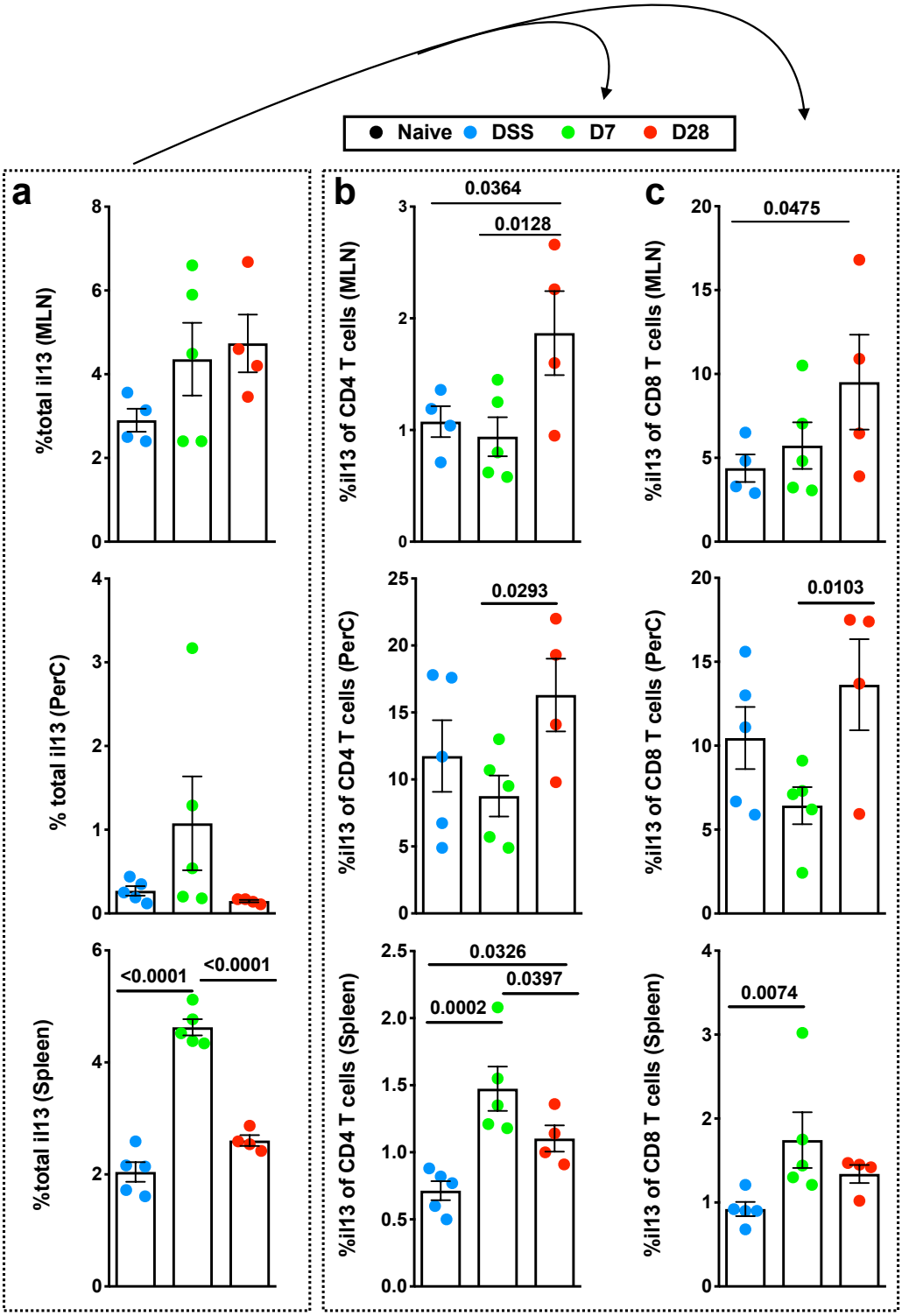


Figure 6-10: Acute helminth infection exacerbation of DSS colitis was positively associated with heightened total IL-13 proportions, while chronic infection resulted in IL-13 ablation. Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were exposed to either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post DSS administration, mesenteric lymph nodes (MLN), peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. The percentage of IL-6 and IL-17A expression within all cells (**a**), $CD4^+$ (**b**) and $CD8^+$ (**c**) T cells of the MLN, PerC and spleen for DSS only

mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols) was quantified following intracellular cytokine staining. Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT

Systemic neutrophilia dominates acute infected-exacerbated DSS induced colitis.

In **Fig 6.3**, I reported a positive correlation between increased susceptibility of female mice fed the mAIN76A diet and increased mesenteric and splenic neutrophilia as well as monocytosis. Under inflammatory conditions, both neutrophils and inflammatory monocytes are recruited to sites of inflammation (Shi and Pamer, 2011; Jones *et al.*, 2018), with neutrophilia being a distinct marker of progressive experimental and human UC (Kolaczkowska and Kubes, 2013). To determine whether inflammatory monocytes and neutrophils also correlated with helminth exacerbation of DSS colitis, we determined the proportion of inflammatory monocytes and neutrophils in acute (D7) and chronic (D28) *H.polygyrus* infected mice and compared this to naïve and DSS treatment alone (**Fig 6.11a**). Helminth infection associated with an increase in the proportion of neutrophils (defined as Gr-1^{high}Ly6G^{high}) within the peritoneum and spleen (**Fig 6.11b**). In particular, acute helminth infected mice resulted in a highly significant increase in the proportion and number of neutrophils in both compartments (**Fig 6.11b**). Although DSS administration resulted in increased splenic inflammatory monocytes (defined as Gr-1^{high}Ly6G^{low}) across all DSS administered groups (**Fig 6.11c**), inflammatory monocyte numbers were only significantly increased in the acute infected mice compared to the chronic infected and DSS treated only mice (**Fig 6.11cii**).

Neutrophil priming, activation and adhesion is dependent on TNF signaling, with variable TNF titers modulating neutrophil susceptibility to apoptosis (Guthrie *et al.*, 1984; Tracey *et al.*, 1988). Acute infection resulted in a significant ablation of splenic $CD4^{+}$ and $CD8^{+}$ TNF (**Fig 6.11d**), contributing to the significantly reduced total splenic TNF in acute infected mice (**Fig 6.11d**). Interestingly, ablation of total splenic TNF inversely correlated with splenic neutrophils in acute infected mice.

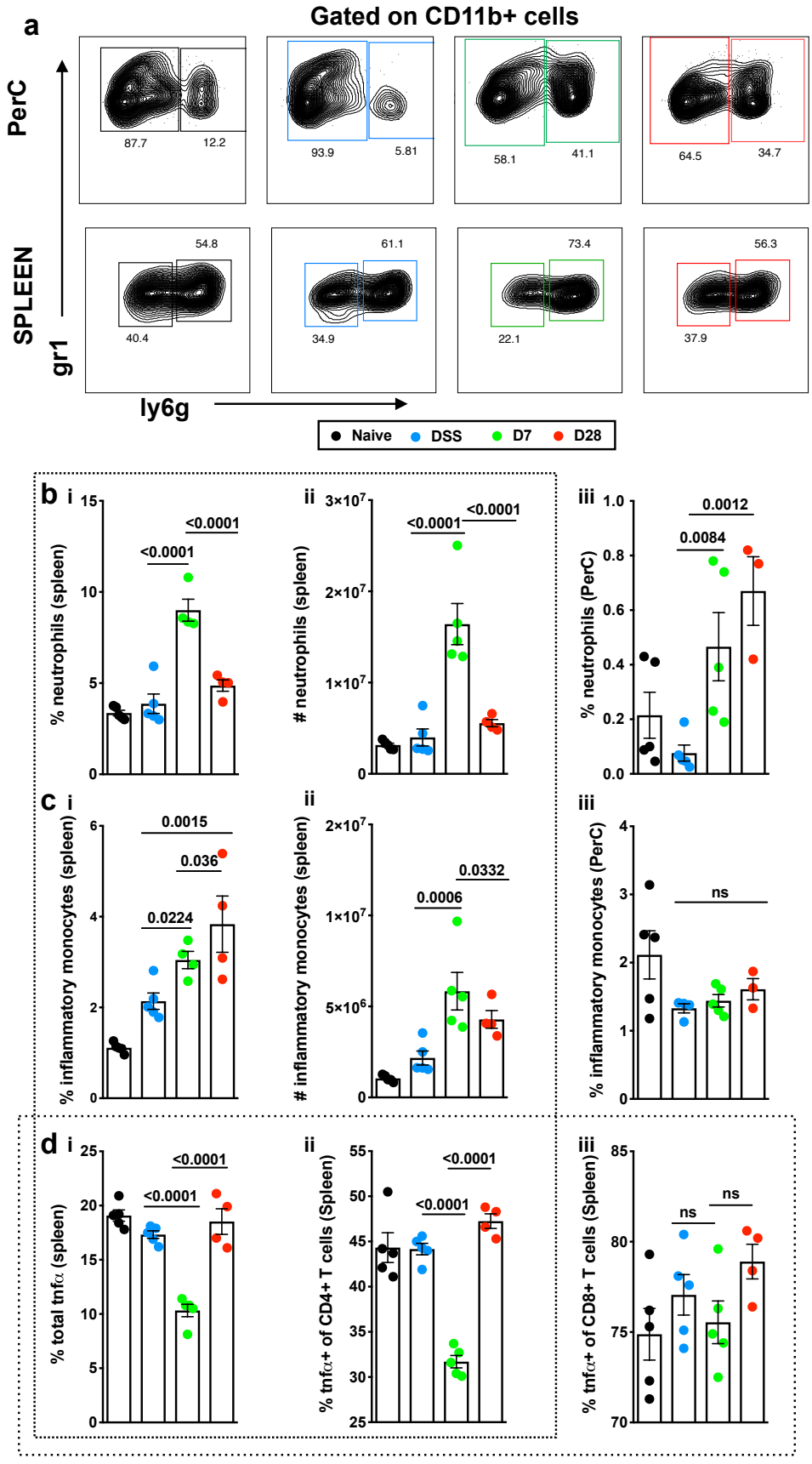

a
Gated on CD11b+ cells
PerC
SPLEEN
gr1
ly6g
87.7
12.2
93.9
5.81
58.1
41.1
64.5
34.7
54.8
40.4
61.1
34.9
73.4
22.1
56.3
37.9
Naive
DSS
D7
D28
b i
% neutrophils (spleen)
<0.0001
<0.0001
ii
neutrophils (spleen)
<0.0001
<0.0001
iii
% neutrophils (PerC)
0.0012
0.0084
c i
% inflammatory monocytes (spleen)
0.0015
0.036
0.0224
ii
inflammatory monocytes (spleen)
0.0332
0.0006
iii
% inflammatory monocytes (PerC)
ns
d i
% total tnfα (spleen)
<0.0001
<0.0001
ii
% tnfα+ of CD4+ T cells (Spleen)
<0.0001
<0.0001
iii
% tnfα+ of CD8+ T cells (Spleen)
ns
ns

Figure 6-11: Heightened systemic neutrophilia and monocytosis in acute infected mice positively associated with worsened disease.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) *ad libatum* were infected with either an acute (D7) or chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post DSS administration, the peritoneal wash (PerC) and spleen were collected and analysed by flow cytometry. Representative plots of inflammatory monocytes ($Gr1^{high}$, $Ly6G^{low}$) and neutrophils ($Gr1^{high}$, $Ly6G^{high}$) within $CD11b^{+}$ cells are shown (**a**). The proportions of inflammatory monocytes and neutrophils were quantified in the PerC and spleen (**b,c**) of naïve untreated mice (black symbols), DSS only mice (blue symbols), acute infected mice D7 (green symbols) and chronic infected mice D28 (red symbols). The proportions of splenic $CD4^{+}$ and $CD8^{+}$ TNF as well as total splenic TNF was quantified following intracellular cytokine staining (**d**). Data is presented as mean ± SEM and is representative of two independent experiments, with n≥4 per group. Statistical significance was calculated using one-way ANOVA, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT.

Chronic helminth infection directly associated with depletion of Tregs and promotion of inflammation in the local draining lymph nodes.

Previous studies demonstrated exacerbation of colitis following helminth infection (Hunter *et al.*, 2007; Pastille *et al.*, 2017; C. Su *et al.*, 2018). However, in many of these studies, the worm dose is fixed at 200 L3 larvae, which may not represent conditions in endemic areas where patients are exposed to differing doses of infectious parasites (Miller, 1983; Hotez *et al.*, 2008; Hotez and Kamath, 2009). In order to determine the relationship between infectious dose and exacerbation of colitis, I used the optimised DSS colitis model where we exposed female mice fed a mAIN76A diet to increasing doses of 100, 200 and 400 *H.polygyrus* L3 larvae, and compared colitis to uninfected mice following administration of DSS (**Fig 6.12**). Similar to previous publications, we quantified worm fecundity using fecal egg burden (Urban *et al.*, 1991; Goodroe *et al.*, 2016; Smith *et al.*, 2016) in mice infected with 100, 200 and 400 *H.polygyrus* larvae at day 28 post-infection (**Fig 6.12a**). Weight-loss (**Fig 6.12b**) and distress (**Fig 6.12c**) extent directly followed the worm burden gradient. Ten days after DSS colitis induction, mice were euthanised and colon shortening and pathology were assessed as measure of colitis development. Although colon shortening was similar across all DSS administered mice (**Fig 6.12d**), colon TNF titers (assessed using ELISA) followed the worm burden gradient, although only the Hp400 titers were statistically different to the DSS only group (**Fig 6.12e).** Interestingly,the same worm burden dependent gradient was also evident in the

colon histopathological sections (**Fig 6.12f**), with an apparent increase in inflammatory infiltrate and loss of epithelial integrity as the worm burden increased from Hp100 to Hp 400 (**Fig 6.12f).**

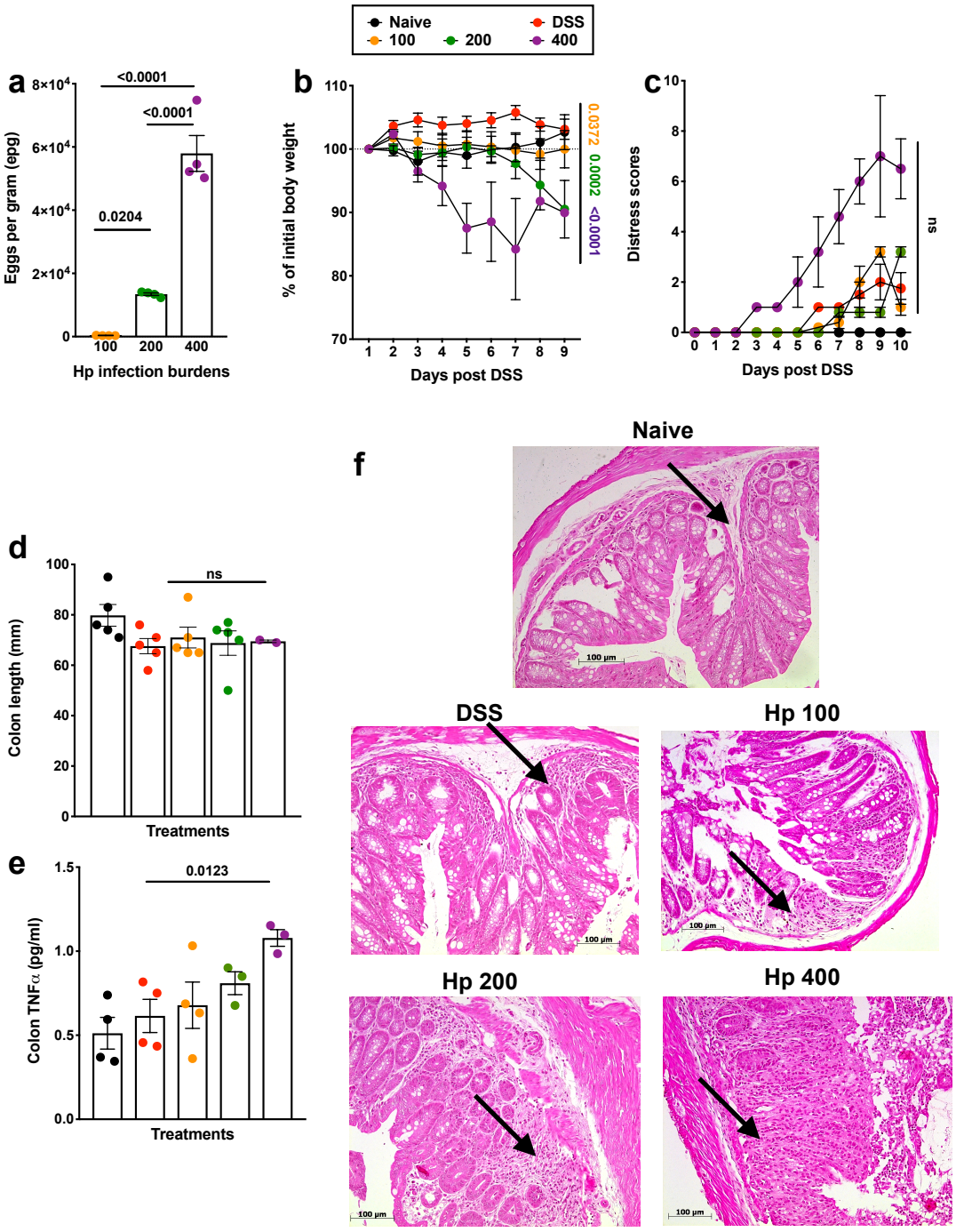


Figure 6-12: Increased disease susceptibility in chronic infected mice is dependent on the worm burden .

Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) were infected with a chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. Worm fecundity was quantified using fecal egg burden (**a**) at day 28 post infection (before DSS administration). Weight-loss (**b**) and

distress (**c**) were monitored for naïve untreated mice (black symbols), DSS only mice (red symbols), 100 L3 *H.polygyrus* larvae infected mice (tangerine), 200 L3 *H.polygyrus* larvae infected mice (green symbols) and 400 L3 *H.polygyrus* larvae (purple symbols) over the course of colitis development. At day 10 post-treatment, colons were sterile excised and colon length was measured (**d**). Two (2) sets of 1mm distal colon sections were isolated. One set was incubated in complete RPMI media for 11 hours at 37°C,and ELISA was used to quantify TNF in the colon supernatant (**e**). The other set was fixed in 4% PFA for histological sectioning and staining. Representative H&E sections illustrating inflammatory cell infiltrate in colon sections are shown here (**f**). Data is presented as mean ± SEM and is collated from two independent experiments, with n≥4 per group (lower numbers demonstrate increased mortality during DSS administration). Statistical significance was calculated using the Kruskall Wallis test, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, all comparisons are between each infected group and the DSS only group. Experimental run assisted by Katherine Smith, UCT; Brittany-Amber Jacobs, UCT. Histology slides prepared by Lizette Fick, UCT.

Next, we elucidated the Treg: Teff kinetics as the worm burden increased. Interestingly, although the $CD4^{+}Foxp3^{+}$ Treg proportions were similar across all the DSS administered mice in the MLN (**Fig 6.13ai**), the absolute numbers of $CD4^{+}Foxp3^{+}$ Tregs declined, following the worm burden gradient (**Fig 6.13aii**). As previously demonstrated in **Fig 6.7 and 6.8**, $CD4^{+}Foxp3^{-}$ (Teff) proportions and absolute numbers were inversely proportional to $CD4^{+}Foxp3^{+}$ Treg proportions (**Fig 6.13b**). Surprisingly, total MLN IL-17A signaling proportions (**Fig 6.13 ci**) also followed the worm burden gradient as they declined across the groups, although the IL-17A MFI (**Fig 6.13cii**) increased as the worm burden increased (**Fig 6.13cii**).

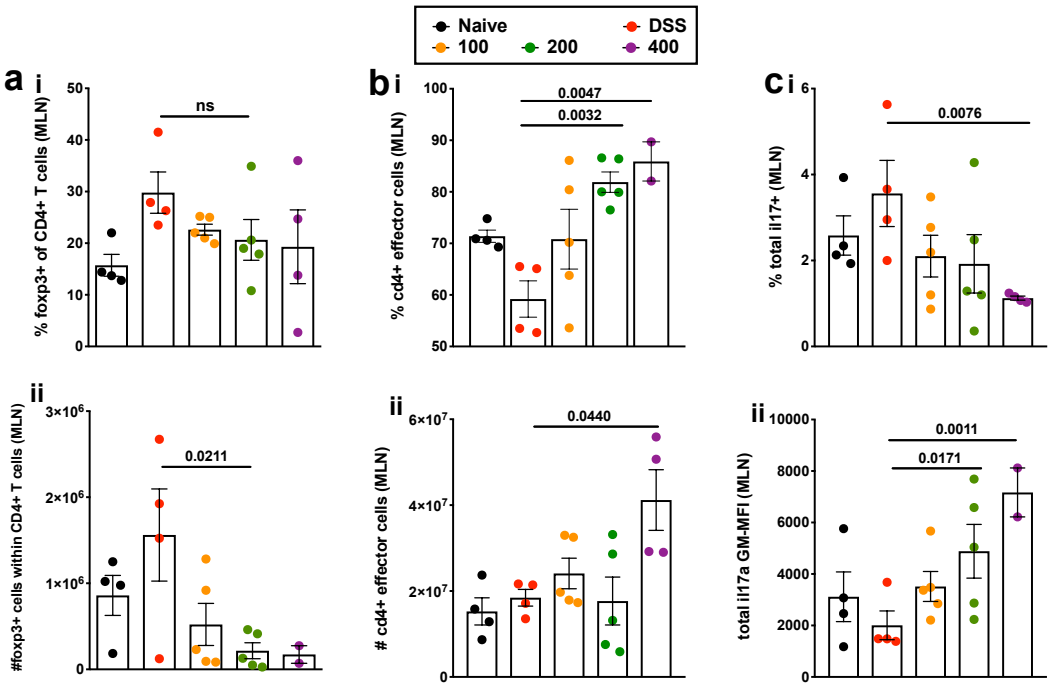


Figure 6-13: Worm burden dependent exacerbation of DSS colitis is positively associated with MLN Treg depletion and increased CD4$^+$ T effector cell numbers.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) were exposed to a chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post-treatment, mesenteric lymph nodes (MLN) were collected and processed using flow cytometry. The proportions and absolute numbers of CD4$^+$Foxp3$^+$ Tregs and CD4$^+$ effector cells for naïve untreated mice (black symbols), DSS only mice (red symbols), 100 L3 *H.polygyrus* larvae infected mice (tangerine), 200 L3 *H.polygyrus* larvae infected mice (green symbols) and 400 L3 *H.polygyrus* larvae (purple symbols) were quantified (**a,b**). The proportion and MFI of total MLN IL-17A was quantified following intracellular cytokine staining. Data is presented as mean ± SEM and is collated from two independent experiments, with n≥4 per group (lower numbers demonstrate increased mortality during DSS administration). Statistical significance was calculated using the Kruskall Wallis test, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Unless indicated, all comparisons are between each infected group and the DSS only group. Experimental run assisted by Brittany-Amber Jacobs, UCT.

Following the demonstration of worm burden dependent exacerbation of DSS colitis (**Fig 6.12**) and the general ablation of Th2 immune responses in chronic infected mice after DSS administration (**Fig 6.10**), I attempted to answer whether exhaustion of Th2 immune responses was a consequence of DSS administration or of helminth infection. Interestingly, ablation of total IL-13, IL-5 and IL-6 MLN titers appeared helminth dependent, as they declined as the worm burden increased (**Fig 6.14a**). Surprisingly, systemic (spleen) anti-helminth responses were completely opposite to local (MLN) anti-helminth responses, as a bias towards increased Th2 cytokine responses was evident in both splenic CD4$^+$ T cells and total spleen cells (**Fig 6.14b & c**).

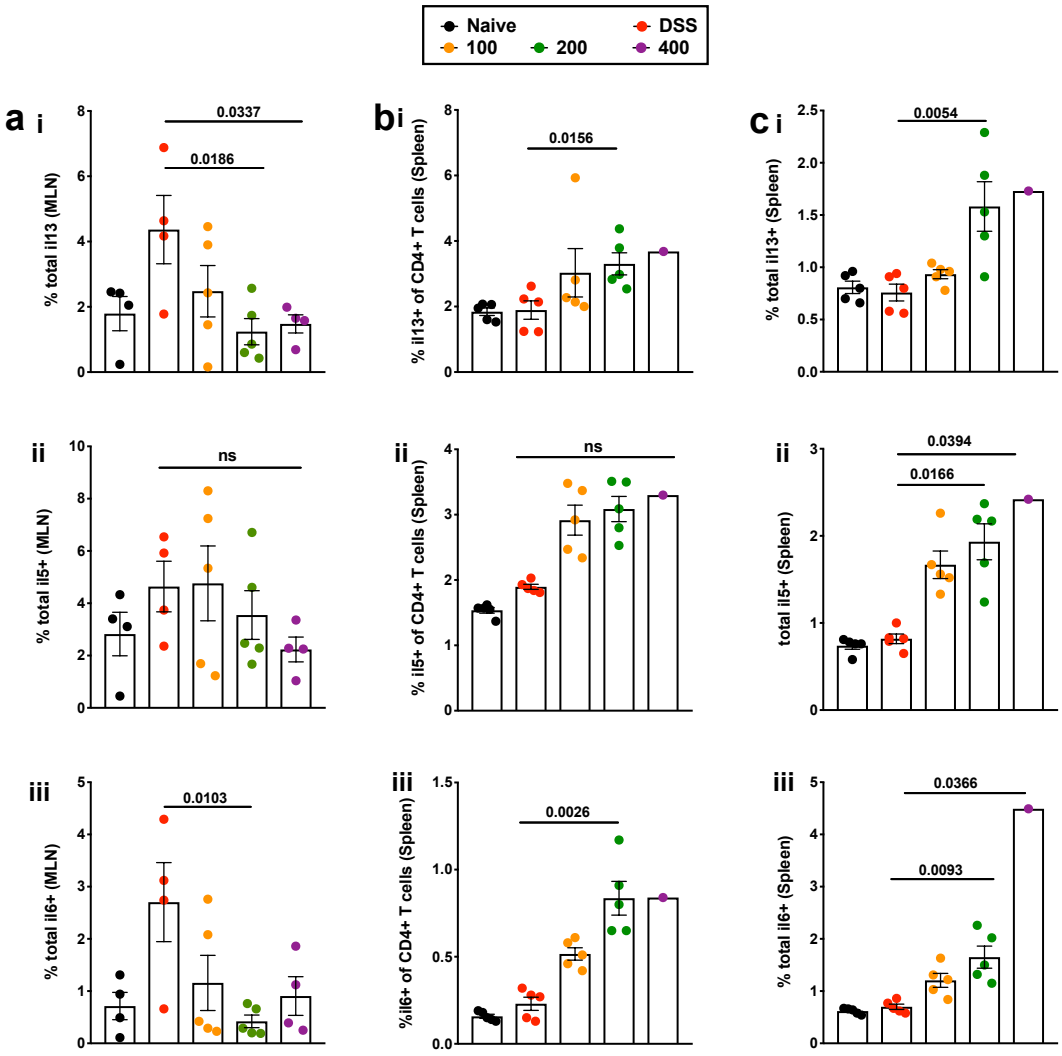


Figure 6-14: Ablation of mesenteric Th2 immune responses after DSS administration in chronically infected mice is highly dependent on helminth infection.
Using the optimised model of DSS colitis induction, female BALB/c mice fed a modified AIN76A diet (mAIN76A) were exposed to a chronic (D28) *H.polygyrus* infection before administration of 2% DSS *ad libatum* in drinking water. At day 10 post-treatment, mesenteric lymph nodes (MLN) and spleens were collected and processed using flow cytometry. The proportions of total MLN IL-13, IL-5 and IL-6 for naïve untreated mice (black symbols), DSS only mice (red symbols), 100 L3 *H.polygyrus* larvae infected mice (tangerine), 200 L3 *H.polygyrus* larvae infected mice (green symbols) and 400 L3 *H.polygyrus* larvae (purple symbols) were quantified following intracellular cytokine staining(**a**). We further quantified the splenic $CD4^+$ T cell and total IL-13, IL-5 and IL-6 after intracellular cytokine staining (**b,c**). Data is presented as mean ± SEM and is collated from two independent experiments, with n≥4 per group (lower numbers demonstrate increased mortality during DSS administration). Statistical significance was calculated using the Kruskall Wallis test, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$. Unless indicated, all comparisons are between each infected group and the DSS only group. Experimental run assisted by Brittany-Amber Jacobs, UCT.

DISCUSSION

The use of biological therapies for both CD and UC have mostly assumed uniformity of disease pathology across patients, leading to the production of universal treatments expected to result in similar remission across the divide. Although the advent of helminth therapy as an alternative biological to previously used biologicals that including anti-TNF, anti-IL-6 and anti-IL-17A treatments has shown promise (Kornbluth, 2011; Khan *et al.*, 2015), it will be remise to assume a general beneficial effect of all different helminth species on colitis. Indeed, experimental findings display a myriad of controversial results regards the influence of helminth therapy on colitis (Hunter *et al.*, 2007; Smith *et al.*, 2007; Wang *et al.*, 2010; León-Cabrera *et al.*, 2014), suggesting a species dependent as well as time of intervention dependent effect. Here, the influence of different phases of prior helminth infection on colitis development was investigated, after initially determining different factors affecting susceptibility to DSS colitis.

In this study, DSS colitis displayed sexual diphormism and increased penetrance in female mice, especially those fed a linoleic acid enriched diet (**Fig 6.1**). These findings of increased female sensitivity to DSS colitis are in contradiction to previous research that demonstrated mild colitis in female mice compared to their male counterparts (Bábíčková *et al.*, 2015). This same study also demonstrated worse disease pathology in ovariectomized mice, which was ameliorated by estradiol administration, underscoring the protective role of estrogen factors in their study (Bábíčková *et al.*, 2015) . Interestingly, disease protection in an HLA-B27 model of colitis was dependent on Estrogen Receptor (ER) signaling, with an ER antagonist exacerbating colitis and resulting in increased neutrophil infiltration in the colon (Harnish *et al.*, 2004). The disparity between our findings and the previously discussed studies may dial down to the species of mice used, as the other studies used either transgenic or C57BL/6 mice, while we used BALB/c mice.

Although mouse species may have contributed to the disparity between our findings and previous research findings, research diet may have had a significant

contribution to heightened sensitivity in female mice. As demonstrated in **Figures 6.1 & 6.2**, a modified linoleic acid rich (mAIN76A) diet increased disease susceptibility in both females and males fed the mAIN76A diet compared to those on the standard chow diet. Severe distress, colon epithelial degradation and increased systemic neutrophilia and monocytosis, all features of enhanced inflammation illustrated increased penetrance in female mice under the mAIN76A diet. Linoleic acid, an n-6 PUFA, is controversially associated with increased risk and susceptibility to both human and experimental UC (Pacheco *et al.*, 1987; The IBD in EPIC Study Investigators, 2009; De Silva *et al.*, 2011), while some human trials report non-influential effects of both n-6 and n-3 dietary interventions on UC (Hart *et al.*, 2008).

The metabolic breakdown of linoleic acid through the arachidonic acid (AA) pathway is implicated in the transduction of inflammatory responses associated with a high linoleic acid diet (Shimizu and Wolfe, 1990; Calder, 2010). AA is the metabolic precursor for eicosanoid synthesis through the activity of cyclooxygenase-2 (COX-2). Prostaglandins and leukotrienes, some of the eicosanoids derived from AA (Isseroff *et al.*, 1987; Salem *et al.*, 1999), are actively increased during active experimental colitis (Gravaghi *et al.*, 2011), where they are involved in the modulation of inflammatory responses(Shimizu, 2009). Indeed, high AA concentrations are reported in both experimental and human UC (Nishida *et al.*, 1987; Pacheco *et al.*, 1987; Ramakers *et al.*, 2007; Silva *et al.*, 2008), with the increased expression of leukotrienes also reported in both rats and IBD patients (Sharon and Stenson, 1985). Interestingly, some non genomic estrogen effects are reported to be mediated by arachidonic acid metabolites in breast cancer (Kato, 1989), suggesting an interaction between estrogen and arachidonic acid during inflammatory processes. This interaction may be the basis of the exacerbated disease observed in female mice fed a linoleic acid rich diet, and needs further research that may potentially influence IBD therapy.

Arachidonic acid is also reported to increase neutrophil chemotaxis, as well expression of tumor necrosis factor receptor (TNFR) on human neutrophils, making them highly sensitive to TNF signaling during inflammation as well as

microbial attacks (Moghaddami *et al.*, 2003). Highly susceptible female mice exhibited heightened systemic neutrophilia and inflammatory monocytosis (**Fig 6.3**), cell types synonymous with colon inflammation in human ulcerative colitis (Banks *et al.*, 2003). Both neutrophils and inflammatory monocytes are recruited to the inflamed colon epithelium as well as any other inflamed tissue for inflammation resolution, executed through either direct microbial attack or intracellular killing of bacteria-infected cells (Guthrie *et al.*, 1984; Narni-Mancinelli *et al.*, 2011; Bennike *et al.*, 2015). The increased neutrophil and monocyte frequencies in the spleen of susceptible mice may therefore represent splenic inflammation in response to DSS induced inflammation, directed through the microbiota.

UC associated inflammatory monocytes are reported to display an inflammatory M1 (classically activated macrophage) phenotype after extravasing into inflamed tissue (Rhodes and Jewell, 1983; Zigmond *et al.*, 2012), resulting in aggravated colon inflammation. Persistent gut inflammation is associated with a continued influx of these inflammatory cells (also referred to as inflammatory infiltrate), with the superoxide activity of neutrophils suggested to drive fibrosis and colon shortening, such as that observed in the DSS sensitive mice (**Fig 6.2**) (Okayasu *et al.*, 1990). Besides the oxidative nature of neutrophils, excessive net trapping by mucosal neutrophils also results in surrounding tissue damage, leading to epithelial tissue damage. The cumulative effects of this disproportionate epithelial inflammatory response are crypt destruction, goblet cell exhaustion (depletion), ulceration and leukocytosis, all features demonstrated in the histological sections of DSS sensitive mice (**Fig 6.2**). My findings support the contribution of the innate immune responses to DSS susceptibility (**Fig 6.3**), as adaptive immune responses were not significantly altered in these mice(Dieleman *et al.*, 1994; Kim *et al.*, 2006). Although neither IFNγ (Th1) nor IL-17 (Th17) (**Fig 6. 4 & 5**) frequencies were highly expressed in the highly DSS sensitive mAIN76A female fed mice, further studies including the use of $RAG^{-/-}$ mice, or cytokine specific depleted mice are needed to determine the dispensability of these cytokine responses in mediating DSS colitis in BALB/c mice.

Surprisingly, contrary to the study hypothesis, both prior acute and chronic *H.polygyrus* infections exacerbated colitis in the optimised model of DSS colitis induction (**Fig 6.6**). Although *H.polygyrus*, especially the larval stages of *H.polygyrus* infection, are associated with the expansion of host $CD4^+Foxp3^+$ Tregs and suppression of bystander conditions (Finney *et al.*, 2007; Sutton *et al.*, 2008; Smith *et al.*, 2016; Pastille *et al.*, 2017); acute infection was associated with the exhaustion of both local (MLN) and systemic (spleen) Treg frequencies, as well as diminished Foxp3 MFI in splenic Tregs, following DSS treatment (**Fig 6.7**). The drop in splenic Foxp3 MFI, a measure of Treg suppressive function, suggests the loss of systemic immunosuppression in acute helminth infected mice after DSS administration. Exhaustion of both $CD4^+Foxp3^+$ Treg frequencies and Foxp3 MFI in the MLN and spleen of acute infected mice (**Fig 6.7**) was inversely proportional to the frequency of $CD4^+$ effector T cells (**Fig 6.8**), demonstrating a compensatory/host driven expansion of effector cells after impairment of $CD4^+$ Treg activity. The rescue of effector cells as well as effector cell immune responses is supported by *Smith et al.,* and *Blankehaus et al.,* who demonstrated a rebound of host effector responses after Treg depletion (Blankenhaus *et al.*, 2014; Smith *et al.*, 2016), supporting the theory of an Treg: Teff dynamic during inflammation.

Indeed, increased MLN and splenic $CD4^+$ T cell effector proportions in acute infected mice also positively correlated with heightened MLN and splenic total IL-6 and IL-17A proportions (**Fig 6.8**), with both $CD4^+$ and $CD8^+$ T cells actively contributing to the total IL-6 titer (**Fig 6.9**). The dismal contribution of both $CD4^+$ and $CD8^+$ T cells to the total IL-17A production suggests a role of either non T cells, such as type 3 innate lymphoid cells (ILC3) in contributing to IL-17A secretion (Annunziato *et al.*, 2015). IL-6, a cytokine noted to modulate the Treg: Th17 balance, is also demonstrated to abrogate Th2 immune responses in *H.polygyrus* infected mice (Smith and Maizels, 2014), enabling the immune evasion of helminths. However, contrary research findings also demonstrate the ability of IL-6 to polarize $CD4^+$ to a Th2 immune response (Rincón *et al.*, 1997), pointing to the promiscuity of IL-6 in immune regulation. Interestingly, heightened MLN and splenic IL-6 titers (**Fig 6.8**) positively correlated with potentiated overall IL-13 proportions in acute infected mice (**Fig 6.10**),

demonstrating heightened Th2 responses. Acute helminth infection exacerbation of DSS colitis was also characterised by enhanced systemic neutrophilia and monocytosis (**Fig 6.11**), implying increased systemic inflammation. Neutrophils, primed and activated by TNF, (Moghaddami *et al.*, 2003; Wright *et al.*, 2010) migrate following an increasing TNF gradient towards inflamed tissue. Interestingly, moderate TNF titers (as observed in acute infected mice, **Fig 6.11**) are reported to prevent neutrophil apoptosis, leading to persistent inflammatory responses (Salamone *et al.*, 2001; Kobayashi *et al.*, 2003). The resultant neutrophilia found in helminth infected mice is also positively associated with IL-17A production (**Fig 6.8 & 11**) (Ley *et al.*, 2006; Li *et al.*, 2012). Both IL-17A and neutrophils are intricately linked with type-2 immunity during helminth infections (Allen *et al.*, 2015), where type-2 associated neutrophils regulate larval migration, albeit at the expense of the host. The pathological effects associated with neutrophil and IL-17A during acute helminth infection may be the aggravatory factors behind acute infection after DSS administration in this study.

Although chronic infection also exacerbated DSS colitis, the immune signature driving exacerbation of disease in this instance was completely different from the influence of acute infection on DSS colitis. DSS exacerbation following chronic helminth infection was characterised by an extremely significant exhaustion of Treg proportions as well as Treg suppressive capacity in both the peritoneum and the spleen (**Fig 6.7**). As with the acute infected mice, Treg exhaustion was associated with an increase in the peritoneal and splenic $CD4^+$ effector T cells. However, unlike the acute infected mice, chronic mice exhibited diminished mesenteric and splenic IL-6 and IL-17A (**Fig 6.8**), up-regulating total peritoneal IL-6 proportions instead (a process most probably enabled by the reduced immunosuppressive activity in this compartment). The ablation of mesenteric and splenic IL-6 and IL-17A in these mice positively correlated with ablated IL-13 (Th2) (**Fig 6.10**) responses as well as diminished neutrophilia and monocytosis (**Fig 6.11**).

However, what sets the chronic infection immune response apart from the acute infection response may be the peritoneal immune response as well as splenic

TNF signaling (**Fig 6.11**). The peritoneum is maintained under a high level of immune suppression, regulated by the stable presence of myeloid derived suppressor cells, alternatively activated macrophages and T regulatory cells (Aufricht *et al.*, 2012; Bain *et al.*, 2016). Peritonitis, which usually results from a microbial attack or infection as expected during intense gut inflammation (translocating bacteria inducing systemic inflammation), is a detrimental condition linked to sepsis and eventual host mortality. Abrogation of peritoneal immune suppression during peritonitis is associated with increased IL-6 signaling, resulting in increased neutrophil infiltration to enable microbial attack resolution. The heightened peritoneal IL-6 and neutrophilia in chronic infected mice (**Fig 6.11**) may therefore be due to a sustained microbial onslaught, with neutrophil infiltration resulting in exacerbated disease instead of the resolution of inflammation. Intriguingly, although the total splenic TNF in chronic infected mice was similar to the TNF titers in the DSS only mice (**Fig 6.11**), loss of Treg suppression and proportions in the former group compared to the latter implies a highly inflammatory systemic immune response in chronic infected mice (**Fig 6.7**). Excessive systemic TNF signaling in the absence of immune regulation is associated with cachexia; a condition characterised by loss of appetite, loss of weight, muscle atrophy, fatigue and weakness in the affected individual (Tracey *et al.*, 1988), all the characteristics witnessed in colitic mice with a chronic *H.polygyrus* infection.

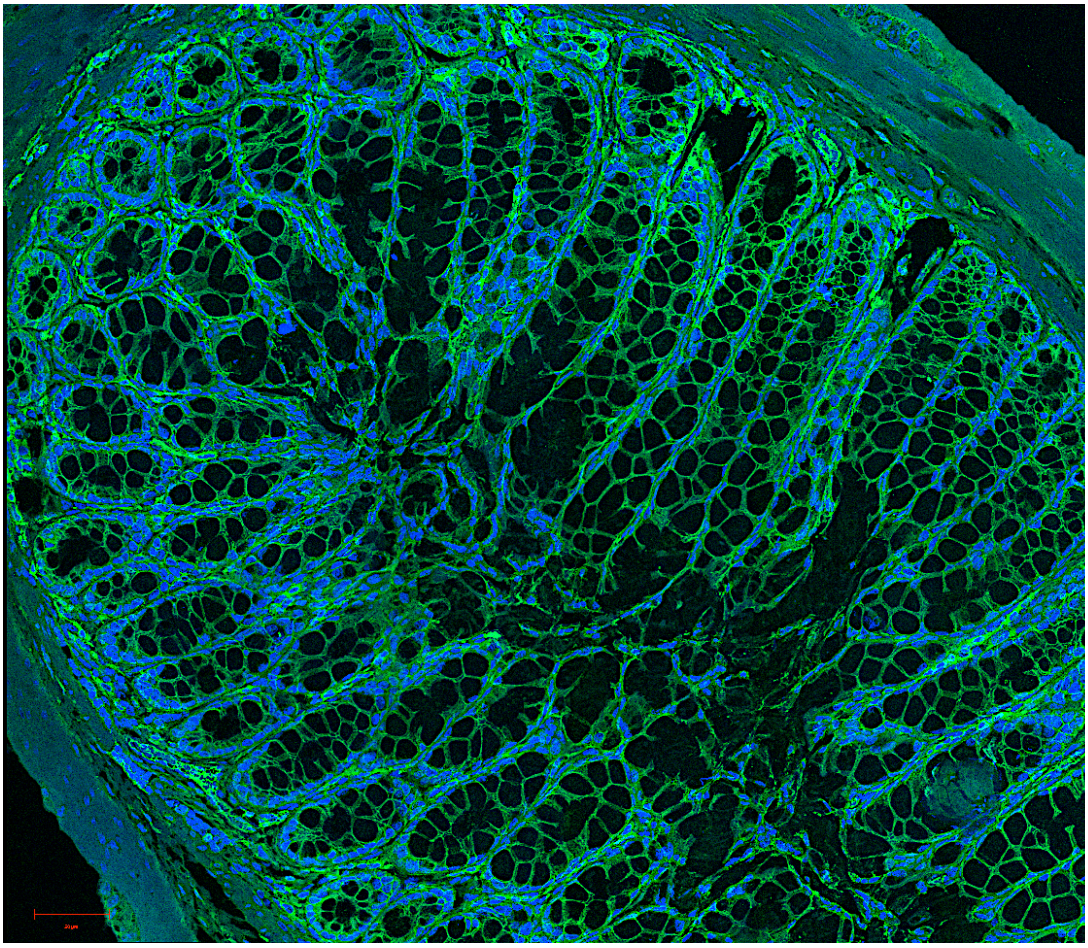
Interestingly, both acute and chronic helminth infected groups exhibited significantly depleted mesenteric lymph node, peritoneal and splenic $CD3^+CD8^+$ T cell proportions following DSS administration (**Fig 6.8**), an occurrence reported by other researchers (Pastille *et al.*, 2014). $CD8^+$ T cell inhibition during helminth infection is reported as a function of the host's Th2 anti-helminth immune response (Marple *et al.*, 2017), which is reported to result in impaired antibacterial and anti-viral immune responses in helminth infected mice (Actor *et al.*, 1993). The biological significance of impaired $CD8^+$ T cells during colitis is still undetermined, although $CD8^+$ T cells are associated with relapsing experimental and human colitis (Nancey *et al.*, 2006; Boschetti *et al.*, 2016), suggesting a key role for $CD8^+$ T cells in IBD.

My findings demonstrate the helminth dependent exacerbation of DSS colitis, and confirm that indeed, modified immunological parameters in helminth-infected mice after DSS administration are a direct function of both helminth infection as well as the worm burden. As previously discussed, in human populations, worm infection is considered in terms of both prevalence and worm burden (Hotez and Kamath, 2009), as the latter factor determines the extent of helminth related pathology. Due to the obvious site of helminth infection (intestine), a higher worm burden would result in extensive damage to the intestinal epithelium, leading to worse pathology. Indeed, DSS exacerbation worsened as worm burden increased (**Fig 6.12**), illustrated by increased weight-loss, distress and colon tissue inflammatory cell infiltrate in the Hp 400 L3 group compared to both the Hp 200 and Hp 100 L3 groups. Similar to the chronic infected mice in the previous set of experiments, helminth infection resulted in depleted MLN $CD4^{+}Foxp3^{+}$ Tregs and a compensatory increase in $CD4^{+}$ T effector cells (**Fig 6.13**), both of which were highly dependent on the worm burden. The surprising disparity between the MLN and splenic Th2 immune responses suggests a separation of immune responses depending on locale (**Fig 6.14**), an occurrence observed across this whole study. However, these worm burden findings are a cautionary tale to helminth, epidemiology and autoimmune disease researchers reiterating the importance of helminth burden, not just prevalence, on host health as well as potential worm burden effect on underlying or subsequent inflammatory disease progression.

Some caveats of this study include the lack of confirmatory effects of the Th2 immune response on helminth viability, especially in the acute infected mice. These confirmatory tests could have included fecal egg counts as well as adult worm burden counts after DSS administration. Another limiting factor in our analysis is the lack of TGFβ data, as well as colon immunology data (e.g lamina propria LP cells). During the course of these experiments, a scientific dilemma existed between collecting colon samples for histology and ELISAs or for LP preparations, with the latter fraught to total loss of sample if mishandled. Hence, to avoid loss of sample, I preferred histology sections compared to LP data, but will seek to include LP data in future studies. TGFβ is reported to polarize TH17 cell production in the presence of IL-6 while limiting induction of peripheral

Tregs (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). It would have been interesting to verify the dynamic of Th17/Treg induction during helminth exacerbation of DSS.

In summary, this study demonstrated the increased sensitivity of female mice fed a linoleic acid enriched diet to DSS colitis, which was characterised by neutrophilia and monocytosis. It also validated helminth exacerbation of DSS colitis irrespective of the phase of helminth infection. Both helminth phases resulted in impaired Treg proportions and Treg suppressive capacity; $CD4^+$ T effector cell increases; mesenteric lymph node $CD8^+$ T cell depletion and systemic neutrophilia. Although both helminth phases exacerbated colitis, they exhibited different immunological signatures demonstrating a difference between the larval and adult worm immunogenic stimulation. Acute helminth infection exacerbation was characterised by heightened systemic IL-6 and IL-17A expression, which positively correlated with neutrophilia and monocytosis. A potentiated Th2 immune response in acute infected mice, combined with neutrophilia and IL-17A signaling suggests that DSS administration abrogates the immunosuppressive environment usually associated with acute *H.polygyrus* infection, resulting in a complex type-2 immune response that unfortunately drives colitic disease. On the other hand, chronic infected mice exhibited abrogated Th2 responses, loss of peritoneum regulation and increased splenic TNF production, setting it apart from the acute helminth infected mice. Disease progression in chronic infected mice was a direct consequence of helminth infection, demonstrated by increased pathology as worm burden increased. This work provides an insight into how different helminth infection phases as well as helminth burden may influence colitis development in immunocompetent hosts.



Naïve mouse colon image. β-catenin (green) and cell nuclei (DAPI) (*Image credits: Brunette Katsandegwaza, 2018, UCT*)

EXPERIMENTAL RESULTS CHAPTER THREE

Oral probiotic administration ameliorates *H.polygyrus*-exacerbation of DSS colitis

7 Probiotics, *H.polygyrus* and DSS colitis

IBD researchers are currently in a great drive to develop novel therapeutics capable of causing disease remission with minimal side effects. The advent of biological therapies in the recent past not only revolutionised IBD therapy, but also improved the lives of a huge percentage of patients who otherwise had endured the debilitating nature of this disease. A number of these biological therapeutics target chemokines, cytokines or signaling pathways that drive inflammation during disease development and progression; with the blockage of such pathways proving beneficial to both Crohn's disease (CD) and Ulcerative Colitis (UC) patients. However, a group of both CD and UC patients has become refractory to most biologicals, resulting in the need of a new line of treatment in the near future.

Previous and current biological therapeutics have included the use of daclizumub (anti-IL-2Ra), infliximab (anti-IL-6), tocilizumab (anti-IL-6R), ustekinumab (anti-IL-12/23) and anti-TNF treatment in the treatment of IBD. Besides biologicals, previous treatments have also included the use of aminosalicylates, corticosteroids and cyclosporine to induce remission of disease in patients (Sutherland, 1998; Van Assche *et al.*, 2002; Hanauer, 2004; Lichtiger, 2006; Gout *et al.*, 2011; Kornbluth, 2011; Rather *et al.*, 2016). However, most of the treatments result in a number of side effects that include hypertension, psychiatric disorders, immunological and physiological disturbances that may predispose to opportunistic infections such as *Mycobacterium tuberculosis* (Shim, 2014; Gîlca *et al.*, 2017). This has led researchers on a path that seeks to harness the body's natural ability to regulate gut inflammation in an attempt to package them as innovative CD and UC therapies.

Both human CD and UC pathologies feature microbial alteration (dysbiosis) (Andoh *et al.*, 2011), a condition characterised by decreased biodiversity and altered proportions of specific colon bacterial species that include the Firmicutes and Bacteriodetes phyla (Roediger *et al.*, 1997; Manichanh *et al.*, 2006; Frank *et al.*, 2007). Indeed, both epidemiological and experimental research studies

strongly associate microbial dysbiosis with both CD and UC (Frank *et al.*, 2007; Tomasello *et al.*, 2014; Ahmed *et al.*, 2016; Rajoka *et al.*, 2017). The fecal dominance of *Faecalibacterium spp.* and colon *Escherichia coli* in CD patients demonstrates the strong association between dysbiosis and disease in CD (Sokol *et al.*, 2008; Palmela *et al.*, 2018); while the reduced prevalence of the Clostridium cluster (of the Firmicutes phyla) in CD supports the findings of altered bacterial diversity during IBD (Frank *et al.*, 2007). Although a causal-effect argument between microbial dysbiosis and IBD exists, the importance of the microbiota in IBD is underscored by the efficacy of fecal transfers, dietary interventions and antibiotic treatment in IBD disease (Sartor, 1998; Yamamoto *et al.*, 2000; Dutta *et al.*, 2014; Suskind *et al.*, 2015).

The use of dietary interventions, fecal microbiota transfers (FMT), fecal diversion and probiotic administration as microbial modulation therapeutics have transformed the lives of IBD patients. However, some dietary interventions that include enteral and parenteral nutrition employed to induce remission in patients for ages have mostly been successful in only a few pediatric cases of CD, nullifying them as efficient therapies for adult CD (Griffiths *et al.*, 1995; Leach *et al.*, 2008). The surgical complications associated with fecal diversion makes this procedure less desirable by a group of IBD patients, a situation complicated by an increased risk of either malnutrition or relapsing colitis due to colonocyte nutrient deprivation (Bafford *et al.*, 2017). Although FMT has been quite successful in the treatment of both experimental and human UC; a number of challenges that include the mode of fecal transfer, the biological safety of donated matter as well as the dose of donated matter still undercuts the acceptance of FMT in both the general and scientific communities (Di Bella *et al.*, 2015; Moayyedi *et al.*, 2015). However, one mode of microbial modulation, oral probiotic administration, has been met with positive enthusiasm, mostly due to its non-invasive nature as well as the positive outcomes witnessed in most IBD patients.

The maintenance of gut bacterial homeostasis through oral probiotic administration is reported to induce remission of IBD in patients (Venturi *et al.*, 1999; Gionchetti *et al.*, 2003; Bibiloni *et al.*, 2005) and prevents or ameliorates

recurrent colitis in mouse models of disease (Madsen *et al.*, 1999; McCarthy *et al.*, 2003; Giacinto *et al.*, 2005). *Lactobacilli* and *Bifidobacteria spp* are the most widely utilised probiotic species demonstrated to significantly modify human health. This is achieved through their ability to increase the concentration of essential byproducts of carbohydrate fermentation, outcompete pathogenic microbes for nutrients and niche occupation, modulation of intraepithelial host's immune cell signaling, xenobiotic detoxification, enhancement of the gut epithelial barrier tight junction proteins as well synthesis of beneficial vitamins such as vitamin K (Bentley and Meganathan, 1982; Mazmanian *et al.*, 2005; Candela *et al.*, 2008; Nilsson *et al.*, 2008; Natividad *et al.*, 2013). Treatment with a probiotic mix of *Lactobacillus acidophilus* and *Bifidobacterium longum* suppressed gut inflammation in a TNBS model of experimental colitis, characterised by increased $CD4^+$ Treg and IL-10 frequencies and a suppression of $CD4^+$ effector and pro-inflammatory cytokines in the colon (Roselli *et al.*, 2009).

However, an independent experiment demonstrated suppressed IL-10 and TGFβ frequencies after *Lactobacillus acidophilus* and *Lactobacillus salivarus* treatment in BALB/c rectums (Petersen *et al.*, 2011), demonstrating variability in the effect of specific microbial species on inflammatory conditions. While preventative administration of the commercially available probiotic cocktail VSL#3 can attenuate ileitis in murine models as well as colon inflammation through the suppression of IL-8 signaling, VSL#3 was demonstrated to have no beneficial effect on murine DSS colitis, or colitis-associated colorectal cancer, despite colonizing the gut and modifying the mucosal microbiome (Elliott *et al.*, 2008; Arthur *et al.*, 2013). This varied outcome of the influence of probiotics on different forms of murine colitis has resulted in a re-focusing on specific microbial species and mechanisms associated with microbial regulation of intestinal inflammation (Hrncir *et al.*, 2007; Ivanov *et al.*, 2009; Atarashi *et al.*, 2013; Sokol *et al.*, 2017) to fully comprehend the interactions between gut microbes and host immune responses.

Interestingly, one of the innovative biological therapies currently in consideration is reported to also result in microbial modulation. Helminth

therapy, demonstrated to modulate a number of bystander inflammatory conditions (R W Summers *et al.*, 2005; Robert W. Summers *et al.*, 2005; Wilson *et al.*, 2005; Fallon and Mangan, 2007; Saunders *et al.*, 2007; Smith *et al.*, 2007), is also reported to result in the modification of the host's gut microbial composition (Reynolds *et al.*, 2014; S. C. Lee *et al.*, 2014; Houlden *et al.*, 2015; Li *et al.*, 2016). This modification is positively associated with the modification of systemic inflammatory disorders, including bacterial-induced colitis and intestinal inflammation (Sutton *et al.*, 2008; Broadhurst *et al.*, 2012; Zaiss *et al.*, 2015a; McFarlane *et al.*, 2017; Su *et al.*, 2018). Successful helminth infection is positively correlated with an increase of gut *Lactobacilliceae* (Reynolds *et al.*, 2014), providing evidence of a helminth-gut microbiota interaction. Since helminths alter the microbiota to enable host colonisation, helminths may drive expansion of protective microbiota members leading to inflammatory bacteria inhibition.

Certainly, a helminth-induced bloom of *Clostridiale spp.* blocked expansion of pro-inflammatory *Bacteroides vulgatus* and ameliorated disease in a mouse model of IBD (Ramanan *et al.*, 2016). Transfer of a helminth-modified microbiota to uninfected mice alleviated airway allergy (Zaiss *et al.*, 2015), suggesting that helminths can mediate allergic inflammation through gut microbial modification. However, the ability of a helminth-modified microbiota to protect from inflammation likely depends on the bacterial species that are driving inflammation. In a mouse model of *Citrobacter rodentium* colitis, transfer of *H.polygyrus*-modified microbiota to uninfected mice exacerbated colitis (C. Su *et al.*, 2018), while *H.polygyrus* infected mice were unable to suppress *Salmonella typhimurium* invasion of epithelial cells (Su *et al.*, 2014). The inability to suppress bacterial invasion may contribute to the increased intestinal colonisation of *S.typhimurium* noticed during helminth infection (Bobat *et al.*, 2014; Reynolds *et al.*, 2017). Overall, the impact of a helminth infection on the host's microbiota plays a key role in the outcome of IBD pathology.

In the previous result chapter, I demonstrated helminth exacerbation of gut inflammation in an optimised model of DSS colitis. I also demonstrated an SPF

dependent helminth exacerbation of DSS colitis (Appendix-Optimisations), suggesting the role of specific members of the gut microbiome in colon inflammation. Interestingly, *H.polygyrus*, our helminth of choice, invades and inhabits the small intestine, but had deleterious effects in the distal colon, an area beyond their migratory niche, suggesting an indirect effect of *H.polygyrus* on the colon immune responses. Following with this understanding and the previously published findings of helminth microbial modifications negatively influencing gut inflammation, I hypothesised that microbial dynamics may play a role in colitic disease exacerbation during helminth infection. Therefore, in this study, I sought to determine the effect of probiotic administration on helminth-exacerbated DSS colitis. Initially, a positive association between helminth infection and gut microbial dysbiosis was demonstrated, characterised by an abundance of the gram-positive *Firmicutes* phyla of the Enterococcus genus translocating to the periphery. Administration of an eight-strain probiotic (VSL#3) during *H. polygyrus* infection ameliorated helminth exacerbation of DSS colitis, and this was associated with suppressed systemic neutrophilia as well as mesenteric IL-17A signaling. Probiotic treatment preserved MLN $CD8^+$ T cells, E-cadherin and β-catenin distribution in the gut epithelium and abrogated $CD3^+$ T cell infiltration, which positively correlated with reduced bacterial translocation to the spleen. We believe this research is the first to demonstrate the beneficial role of probiotic administration on a helminth-modified gut microbiota during colitis, and propose that maintaining gut microbial homeostasis may play an important role in alleviating dysbiosis-associated pathogenic conditions.

STUDY HYPOTHESIS

The previous chapters demonstrated that infection with *H. polygyrus* exacerbates DSS-induced colitis and that different Specific Pathogen Free (SPF) conditions influence colitis outcome (Fig 10.1- Appendix). Therefore, I proposed that the gut microbiota plays an inflammatory role in helminth exacerbation of DSS colitis.

RESULTS

H.polygyrus infection resulted in a dominance of the Firmicutes phyla translocating to the spleen during DSS colitis.

Helminth-induced alterations to the intestinal epithelial barrier have been associated with increased *Citrobacter rodentium*-induced colitis (Chen *et al.*, 2005). In order to determine whether helminth infection exacerbates DSS colitis by increasing the breakdown of the intestinal epithelium, an *H.polygyrus* burden experiment was carried out (**Fig 7.1a**). Spleen weights were measured, and bacterial translocation to the peripheral tissues was confirmed through Gram stains. Although DSS treatment did not have a significant impact on spleen weight, infection with *H.polygyrus* increased spleen weight (**Fig 7.1b**), with splenomegaly showing a strong positive correlation with *H.polygyrus* burden (**Fig 7.1b**). Helminth infection resulted in bacterial translocation to the spleen, with Gram-staining of anaerobically cultured bacteria isolated from the spleen indicating that helminth infection resulted in the dominance of gram-positive bacteria, which exhibited a coccoid appearance, often occurring in pairs (diplococcoid) (**Fig 7.1c**). Interestingly, although helminth infection did not affect the alpha diversity of bacteria translocating to the spleen (**Fig 7.1d**), UniFrac-based analysis of this microbiota indicated that helminth infected and control samples were composed of distinct bacterial communities following DSS treatment (**Fig 7.1e**). Further 16S sequencing and analysis of translocating bacterial species demonstrated an increased dominance of the Firmicutes and decrease of the Bacteriodetes phyla associated with increased *H.polygyrus* burden (**Fig 7.1f**). A broader comparison of the infected and the uninfected groups indicated a heightened frequency of the Enterococcus genus (**Fig 7.1g**) associating with helminth infection after DSS administration.

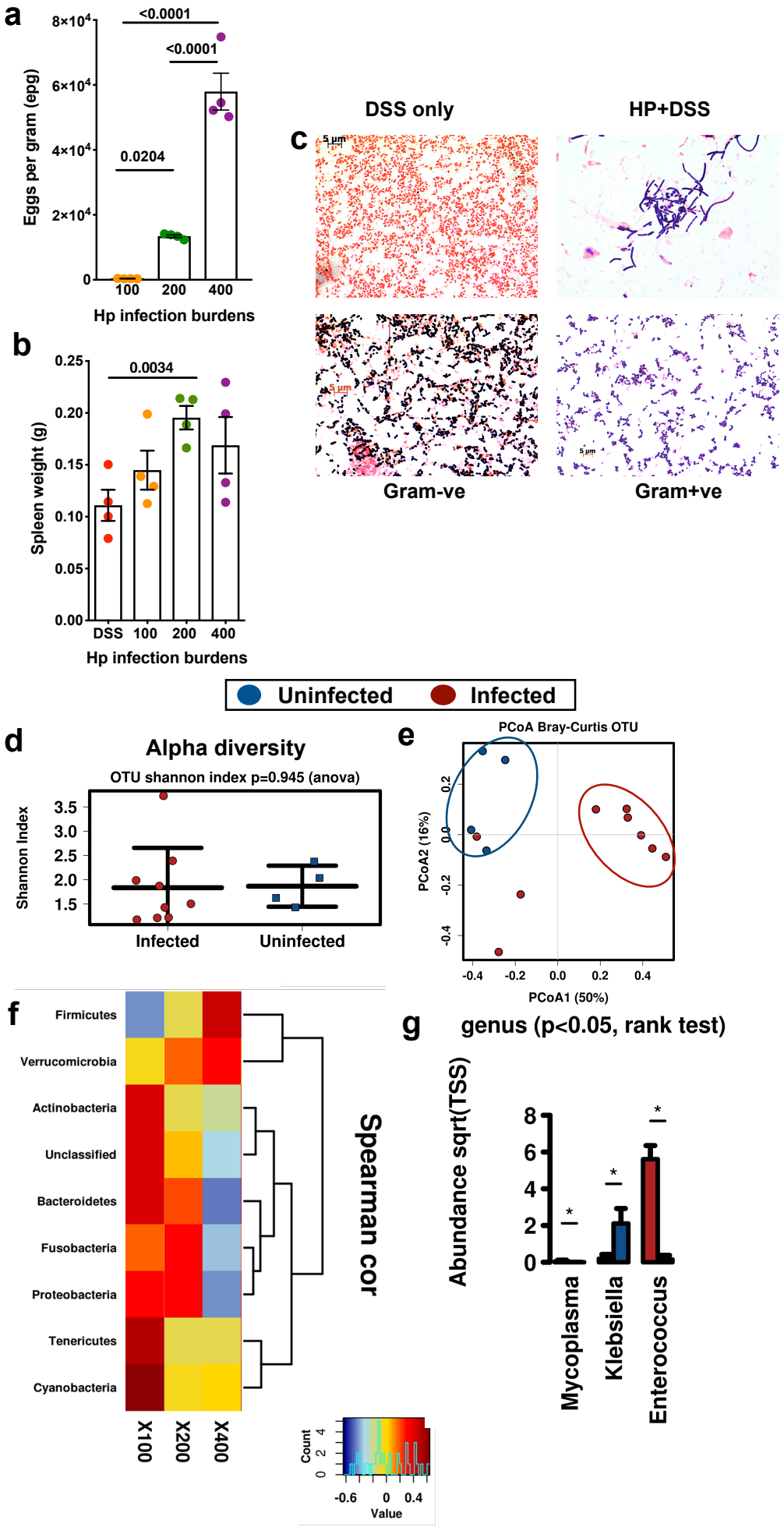

a
Eggs per gram (epg)
8×10^4
6×10^4
4×10^4
2×10^4
0
<0.0001
<0.0001
0.0204
100
200
400
Hp infection burdens
b
Spleen weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
0.0034
DSS
100
200
400
Hp infection burdens
c
DSS only
HP+DSS
Gram-ve
Gram+ve
Uninfected
Infected
d
Alpha diversity
OTU shannon index p=0.945 (anova)
Shannon Index
3.5
3.0
2.5
2.0
1.5
Infected
Uninfected
e
PCoA Bray-Curtis OTU
PCoA2 (16%)
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4
PCoA1 (50%)
f
Firmicutes
Verrucomicrobia
Actinobacteria
Unclassified
Bacteroidetes
Fusobacteria
Proteobacteria
Tenericutes
Cyanobacteria
X100
X200
X400
Spearman cor
Count
0
2
4
-0.6
0
0.4
Value
g
genus (p<0.05, rank test)
Abundance sqrt(TSS)
8
6
4
2
0
*
*
*
Mycoplasma
Klebsiella
Enterococcus

Figure 7-1: Firmicutes dominance in translocating microbiota from helminth infected mice after DSS administration. Using the optimised DSS colitis model, 6-8 week female BALB/c mice (fed the mAIN76A diet) were chronically infected with different *H.polygyrus* burdens (**a**) 28 days before administration of DSS. Spleens were sterile harvested, weighed (**b**) and anaerobic bacterial culture was carried out. Bacterial colonies were isolated, gram stained (**c**) and bacterial DNA was extracted for 16s genetic sequencing to determine microbial species and diversity. The alpha (**d**) and beta diversity (**e**) of the infected and uninfected groups was determined post sequencing using Calypso software. Further worm burden dependent microbial analysis demonstrates variation of bacterial species as worm burden increased (**f**), with (**g**) demonstrating the dominance of the Enterococcus genus in the helminth-infected mice. Data is presented as mean ± SEM and is representative of two independent repeat experiments (worm burden experiment) and one experiment (bacterial sequencing) with n≥4 mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. 16S sequencing and analysis carried out in collaboration with the Heather Jaspan group, genomic graphs generated by Jerome Wendoh from the Heather Jaspan group (Cape Town,SA and Seattle, USA).

Administration of an 8-strain probiotic ameliorates *H.polygyrus* exacerbation of DSS-induced colitis.

Gut microbial alterations play an important role in intestinal inflammation(Roy *et al.*, 2017), with disease development highly dependent on the host's microbial composition (Nagalingam *et al.*, 2011; Berry *et al.*, 2015). Previously, I demonstrated helminth-associated dysbiosis in translocating bacteria, with a particular increase in the gram-positive Enterococcus genus from the Firmicutes phyla. In order to determine whether helminth exacerbation of colitis was due to gut microbial shifts, oral probiotics were administered during the helminth infection phase only, before induction of colitis using the optimised model of DSS colitis (**Fig 7.2a**). Initial determination of individual effects of either probiotics or helminth on the host's immunology demonstrated the significant depletion of MLN Tregs during a helminth infection, while probiotics significantly boosted MLN Tregs to numbers higher than in naïve mice (**Fig 10.2 -appendix**). Therefore, I tested to see probiotics could influence the immunology and outcome of a helminth-exacerbated DSS colitis. Probiotic administration positively associated with abrogated weight-loss and distress (**Fig7.2b & c**). Although probiotics had no effect on colon length (**Fig. 2d**), histological analysis demonstrated a reduction in inflammatory cell infiltrate and the maintenance of normal goblet cell distribution in the probiotic administered group compared to the helminth-only infected group (**Fig 7.2f**). Changes in the colon architecture associated with VSL#3 treatment correlated with an overall reduction in the

production of the inflammatory cytokines IL-6 and TNF by the colon, although the latter did not reach statistical significance (**Fig 7.2g & 2h**).

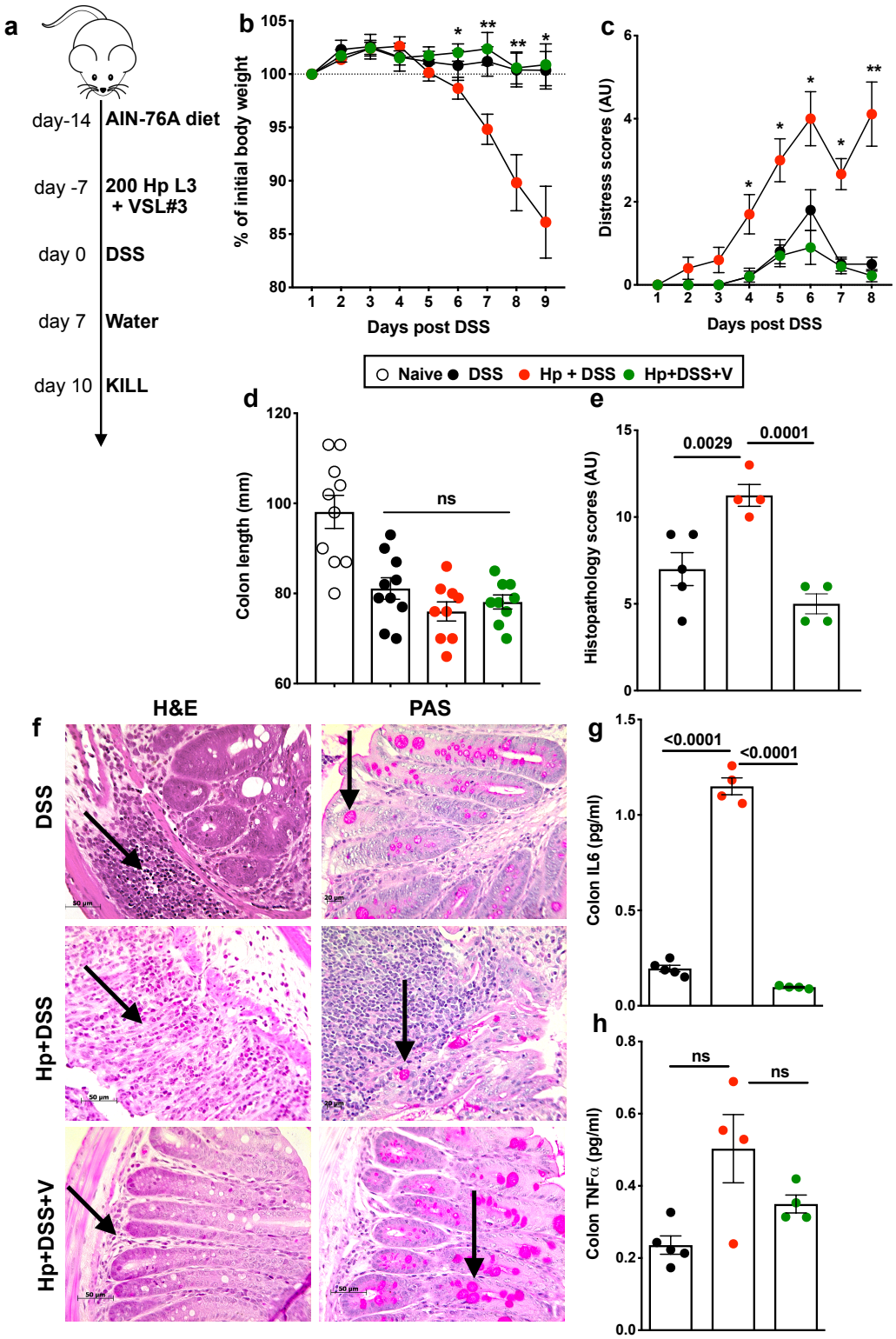


Figure 7-2: Administration of an 8-strain probiotic ameliorates *H.polygyrus* exacerbation of DSS-induced colitis. Using the optimized protocol for DSS colitis induction, 6-8 week old

female BALB/c mice were acutely infected with *H.polygyrus* 7 days before DSS administration (**a**). 10 $^{\wedge 9}$ CFU bacterial probiotics (VSL#3) were administered during the helminth infection phase only and stopped on the day of DSS administration. Weight-loss (**b**) and mouse distress (**c**) for naïve untreated controls (open symbols), DSS only controls (black symbols), Hp+DSS only (helminth infected only-red symbols) and Hp + DSS + V (probiotic treated group-green symbols) were monitored for a period of 7 days before euthanasia. At point of euthanasia, mouse colons were excised, measured (**d**) and sectioned for histological purposes (**f**). Histological colon sections were scored (**e**) from H&E (inflammatory infiltrate-indicated) and PAS (goblet cells-indicated) stained sections (representative PAS sections are shown here). 1mm colon sections were also incubated in complete media for 11 hours, IL-6 and TNF cytokine assays were carried out on the supernatant using ELISA (**g and h**). Data is presented as mean ± SEM and is representative of two independent repeat experiments with n≥ 4 mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Hp+DSS and Hp+DSS+V groups. Colon supernatants ELISAs assisted by Katherine Smith (UCT), colon histology assisted by Lizette Fick (UCT).

Probiotic administration restores gut epithelial integrity through modification of epithelial cell-cell interaction proteins.

Microbial modulation of the wnt signaling pathway results in the translocation of β-catenin into the cell nucleus, where it instigates transcription of inflammatory genes such as TCF through the NFκB pathway, resulting in the induction of Th17 immune responses (Kim *et al.*, 2006, 2012; Wu *et al.*, 2006; Rubinstein *et al.*, 2013). Previously (**Fig 7.2**), I showed a reduction in colon inflammation demonstrated by the rescuing of the colon architecture, a reduction in colon inflammatory infiltrate and suppression of colon IL-6 in the probiotic-treated group after DSS administration. Therefore, I next sought to determine the integrity of colon epithelial cells by looking at cell-cell interaction protein distribution as well as the ability of bacteria to translocate from the lumen into the periphery. Administration of an 8-strain probiotic abrogated the overexpression and apparent re-distribution from the cell membrane to the cytoplasm of E-cadherin and β-catenin that was highly apparent in the DSS only and Hp+DSS mice after DSS administration (**Fig 7.3**). Inflamed tissue with cell-cell interaction protein accumulation was noted to break away from the mucosae, associated with the high infiltration of $CD3^{+}$ T cells as well as epithelial disintegration in the Hp+DSS group compared to the Hp+DSS+V group (**Fig 7.3**). Quantification of both β -catenin and E-cadherin proteins (maximum intensity expression) in IMAGE-J demonstrated an abrogation of protein overexpression by probiotic administration (**Fig 7.4a & b**). In-situ immunofluorescence labeling of LPS in the colon epithelium illustrates an increased presence of bacterial species within the colon epithelium of the

Hp+DSS group compared to the Hp+DSS+V mice after DSS treatment (**Fig 7.4c**), supported by high splenic bacterial CFU/g (**Fig 7. 4d**).

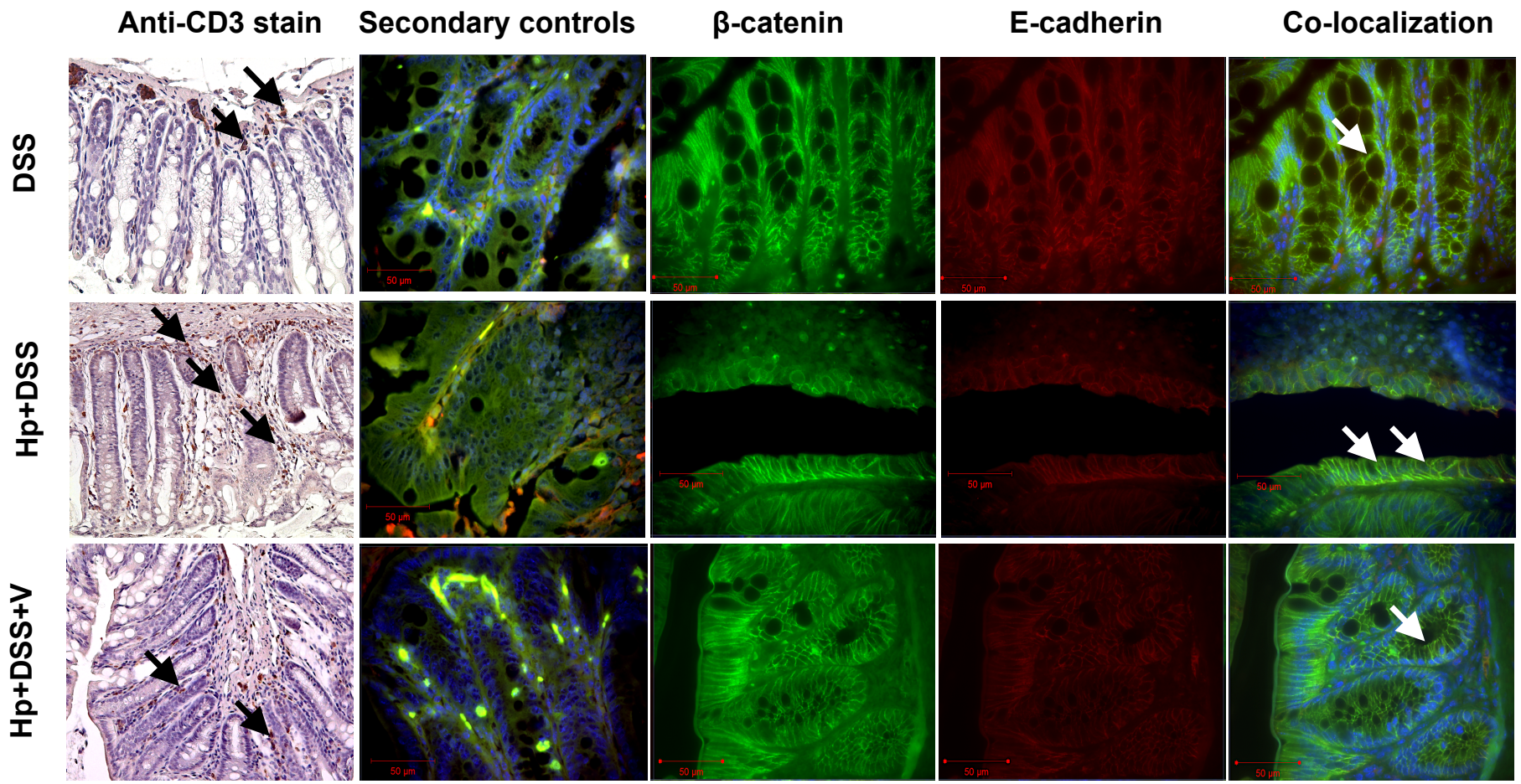

Anti-CD3 stain
Secondary controls
β-catenin
E-cadherin
Co-localization
DSS
Hp+DSS
Hp+DSS+V
50 µm

Figure 7-3: Administration of an 8-strain probiotic abrogates CD3$^+$ cell infiltration as well as cell-cell interaction protein over-expression. Mouse colon tissue from the probiotic treatment experiment was fixed in 4% PFA and stained for CD3$^+$ T cell infiltration (black arrows), β-catenin and E-cadherin proteins before imaging (white arrows show tight junction). Images shown are representative of two-independent repeat experiments with n≥4 mice per group. Confocal microscopy assisted by Susan Cooper (UCT), anti-CD3$^+$ T cell staining by Lizette Fick (UCT).

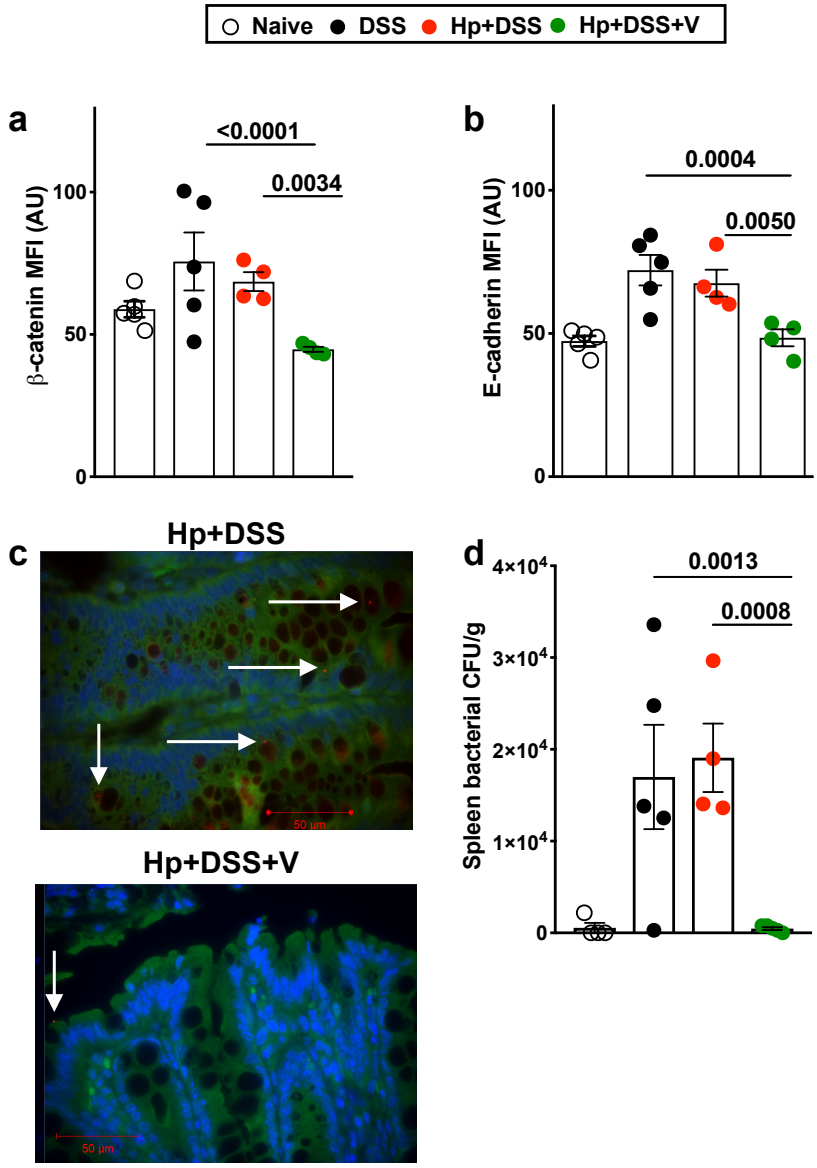


Figure 7-4: Probiotic administration to helminth-infected mice maintains tight junction integrity and dampens bacterial translocation. Mouse colon tissue from the probiotic treatment experiment was fixed in 4% PFA and stained for β-catenin and E-cadherin proteins. Maximum intensity projection images were further analysed in IMAGE-J to quantify the expression of both β-catenin (**a**) and E-cadherin (**b**) for naïve untreated controls (open symbols), DSS only (black symbols), Hp+DSS (red symbols) and Hp+DSS+V (green symbols) and graphs were plotted in Graph pad prism. In-situ immunofluorescence labeling of bacteria specimens (red-indicated by white arrows) (**c**) in the colon tissue was carried out using an anti-LPS antibody. Sterile harvested spleens were anaerobically cultured on blood agar plates and bacterial CFU/g was quantified to determine amount of translocating bacteria (**d**). Data is presented as mean ± SEM and is representative of two independent repeat experiments with n≥4 mice per

group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Hp+DSS and Hp+DSS+V groups. Confocal microscopy assisted by Susan Cooper (UCT),

Administration of an 8-strain probiotic prevents enhanced inflammatory responses associated with helminth-exacerbated DSS colitis.

Host regulatory and effector cell dynamics during helminth infections are pivotal to the etiopathology of bystander conditions (Wilson *et al.*, 2005; Dittrich *et al.*, 2008), while probiotic administration is demonstrated to either suppress or have no influence on host's effector immune responses (Arthur *et al.*, 2013; Chang *et al.*, 2013; Mar *et al.*, 2014; Isidro *et al.*, 2017).

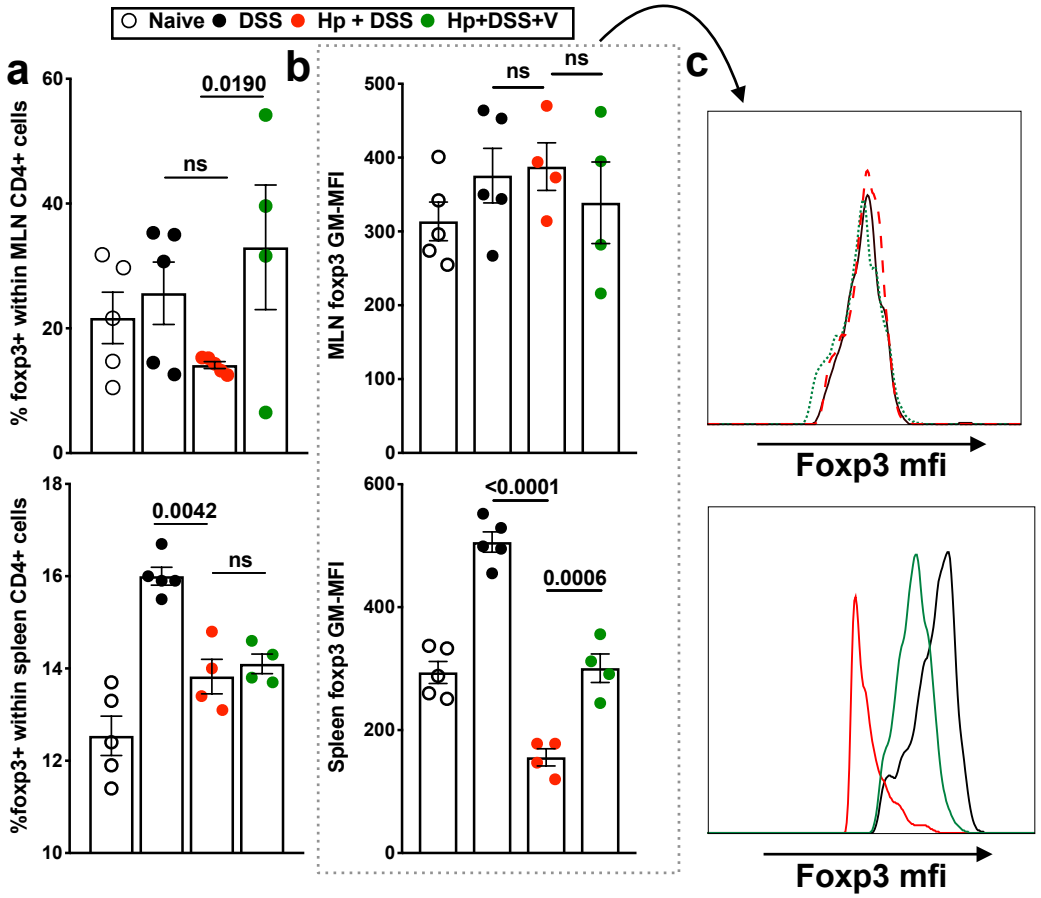


Figure 7-5: Administration of the 8-strain probiotic prevented the depletion of MLN CD4+Foxp3^{+} induced by helminth infection. Using the optimised protocol for DSS colitis induction, 6-8 week old female BALB/c mice were acutely infected with *H.polygyrus* 7 days before DSS administration. 10 $^{\wedge 9}$ CFU bacterial probiotics (VSL#3) were administered during the helminth infection phase only and stopped on the day of DSS administration. Host MLN and spleens were excised and cells of interest were analysed for naïve untreated controls (open symbols), DSS only (black symbols), Hp+DSS (red symbols) and Hp+DSS+V (green symbols) were analysed for using flow cytometry. Foxp3 expression within MLN and spleen cells was quantified (**a**), and their FOXP3 MFI (**b**) was determined as illustrated in (**c**). Data is presented

as mean ± SEM and is representative of two independent repeat experiments with n≥4 mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Hp+DSS and Hp+DSS+V groups. Experimental run and analysis assisted by Rodney Lucas, Alisha Chetty and Brittany-Amber Jacobs, UCT.

$CD4^+Foxp3^+$ regulatory T cells (Tregs) are important regulators of colon inflammation, capable of suppressing excessive anti-microbial pro-inflammatory responses (Kim *et al.*, 2016). The same group of cells is expanded during early *H.polygyrus* infection and are thought to contribute to the regulation of helminth immunity and bystander inflammatory conditions (Finney *et al.*, 2007; Smith *et al.*, 2016). Although helminth infection did not significantly affect mesenteric $CD4^+Foxp3^+$ proportions, probiotic administration resulted in a significant increase of mesenteric $CD4^+Foxp3^+$ Tregs (**Fig 7.5a**). All MLN Tregs from the different treatments exhibited similar Foxp3 MFI (**Fig 7.5b**) as illustrated in **Fig 7.5c**. Interestingly, probiotic administration increased splenic Treg MFI compared to the Tregs from the helminth-infected group only (**Fig 7.5b**) as illustrated in **Fig 7.5c**.

Surprisingly, both the administration of 10^9 CFU of the 8-strain probiotic (trade-name Vivomixx®) as well as helminth infection had no significant impact on $CD4^+$ T cell effector cells from both the MLN and spleen (**Fig 7.6b**). However, probiotic administration rescued the MLN $CD8^+$ T cell compartment, a compartment I previously demonstrated to be depleted in helminth infected groups after DSS administration (Result chapter 2). Further characterisation of the $CD4^+$ T cell compartment from the peritoneal cavity and spleen indicated diminished expression of both IL-17A and IL-6 in the probiotic administered group, following DSS administration (**Fig 7.7a & b**), which mirrored the suppressed expression of total IL-17A and IL-6 in the same compartments (**Fig 7.7c**). IFNγ production by $CD4^+$ T cells within the spleen was significantly increased in helminth-infected mice treated with VSL#3, compared to helminth infection alone after DSS administration (**Fig 7.7a**).

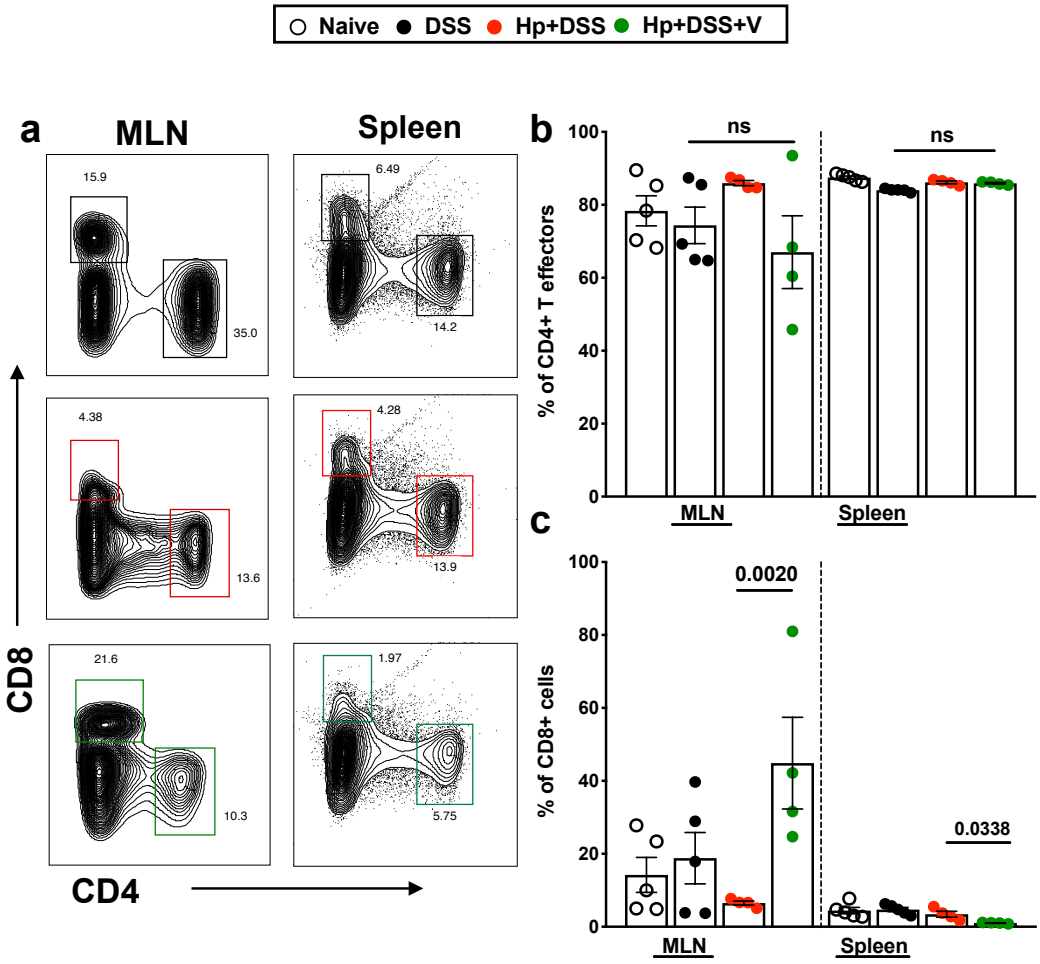


Figure 7-6: Administration of an 8-strain probiotic prevents enhanced inflammatory responses associated with helminth-exacerbated DSS colitis. Mesenteric lymph nodes (MLN), peritoneal lavage and spleen were collected and processed for flow cytometry. $CD4^+$ T effector and $CD8^+$ T cells (gated on single cells) (**a**) for naïve untreated controls (open symbols), DSS only (black symbols), Hp+DSS (red symbols) and Hp+DSS+V (green symbols) from the MLN and the spleen were analysed (**b and c**). Data is presented as mean ± SEM and is representative of two independent repeat experiments with an average of five mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Hp+DSS and Hp+DSS+V groups. Experimental run and analysis assisted by Rodney Lucas, Alisha Chetty and Brittany-Amber Jacobs, UCT.

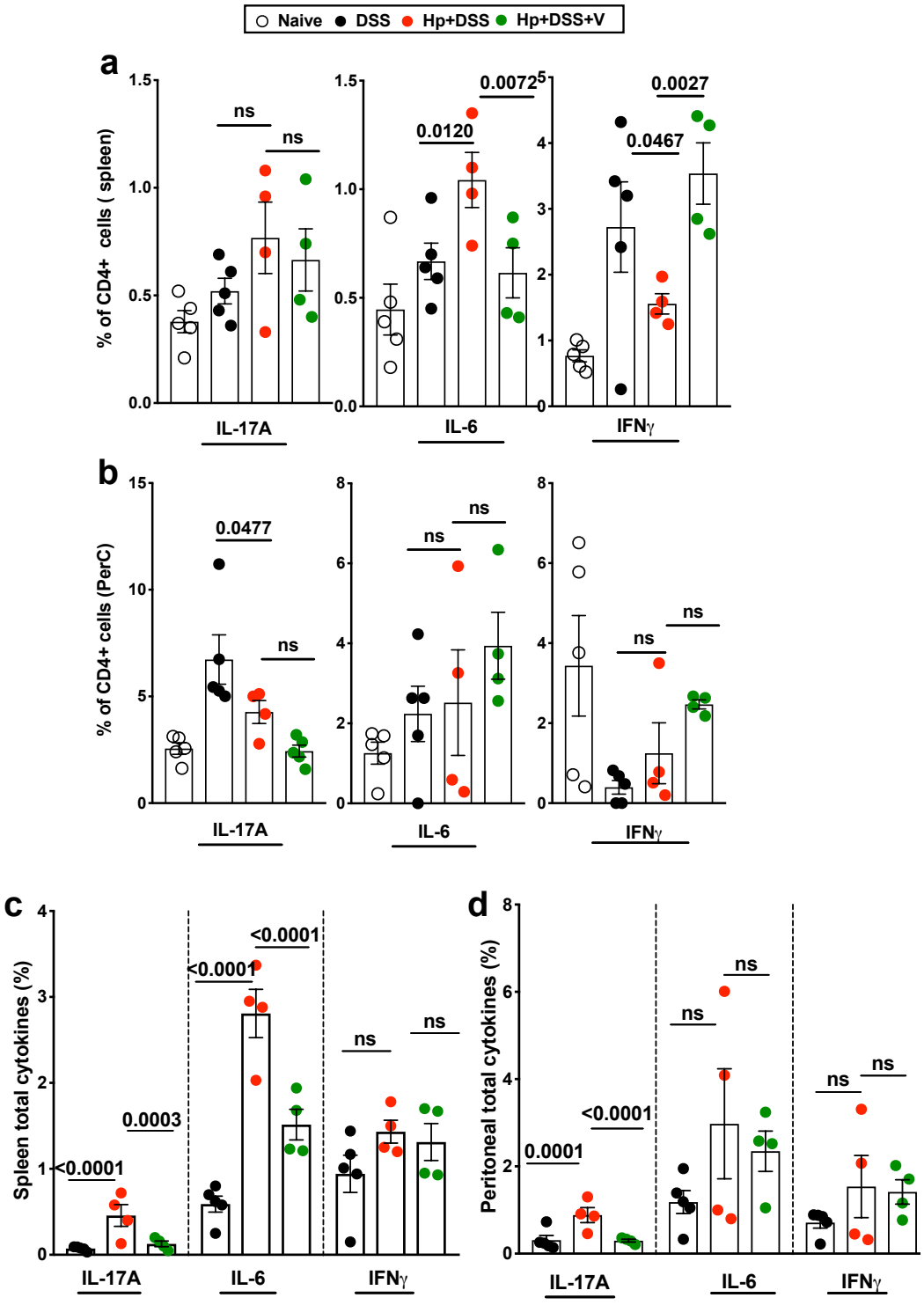


Figure 7-7: Probiotic treatment abrogated proinflammatory responses associated with helminth infection after DSS administration. Peritoneal lavage and spleen were collected and processed for flow cytometry. $CD4^+$ T (**a,b**) cells as well total IL-17A and IL-6 (**c,d**) cytokine expression for naïve untreated controls (open symbols), DSS only (black symbols), Hp+DSS (red symbols) and Hp+DSS+V (green symbols) in the spleen and peritoneal wash were determined after intracellular cytokine staining. Data is presented as mean ± SEM and is representative of two independent repeat experiments with n≥4 mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are

between the Hp+DSS and Hp+DSS+V groups. Experimental run and analysis assisted by Rodney Lucas, Alisha Chetty and Brittany-Amber Jacobs, UCT.

Abrogated splenic and peritoneal IL-17A positively correlated with reduced neutrophilia

In the previous chapter, helminth-associated neutrophilia, which positively correlated to systemic IL-17A production and inflammation was demonstrated. Neutrophilia is an important readout for both experimental and human UC (Talukdar *et al.*, 2012), with neutrophil infiltration positively correlated with colon inflammation (Fournier and Parkos, 2012). Previously, I have shown the suppression of splenic and peritoneal total IL-17A proportions in probiotic treated helminth infected mice (**Fig 7.7**). Interestingly, probiotic administration also resulted in the ablation of spleen neutrophil proportions (**Fig 7.8**)

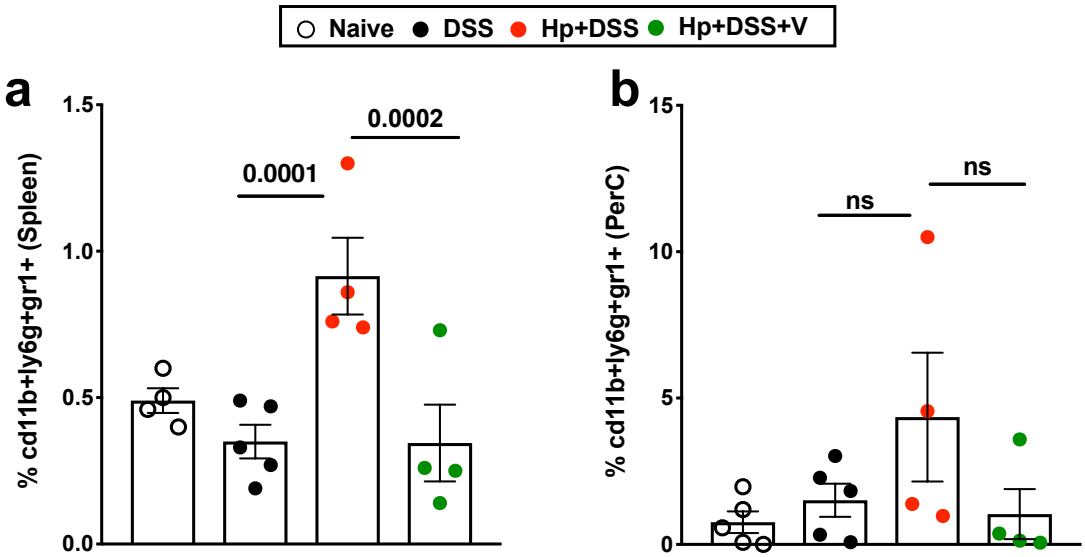


Figure 7-8: Diminished spleen IL-17A immune responses positively associated with abrogated spleen neutrophilia. Spleen and peritoneal wash (PerC) single cells were processed using flow cytometry for neutrophils ($Gr1^{high}$, $Ly6g^{high}$) gated on $CD11b^{+}$ cells. Spleen (**a**) and Perc (**b**) neutrophils ($Gr1^{high}$, $Ly6g^{high}$) proportions were quantified for naïve untreated controls (open symbols), DSS only (black symbols), Hp+DSS (red symbols) and Hp+DSS+V (green symbols) and graphs were plotted in Graphpad Prism. Data is presented as mean ± SEM and is representative of two independent repeat experiments with n≥4 mice per group. Statistical significance was calculated using one-way ANOVA at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless indicated, statistical comparisons are between the Hp+DSS and Hp+DSS+V groups. Experimental run and analysis assisted by Rodney Lucas, Alisha Chetty and Brittany-Amber Jacobs, UCT.

DISCUSSION

The gut microbiota plays an important role in maintaining intestinal health, where perturbations can cause intestinal disease and systemic disease (Aich *et al.*, 2016; Ni *et al.*, 2017). Probiotic intake is generally advised for the non-invasive treatment of human Ulcerative Colitis, where it has been suggested that probiotic administration modifies the diversity of the gut microbiota and promotes the maintenance of the colon mucosal layers. Besides their beneficial effects in the gut, probiotics may also affect the functionality of other visceral organs through immune system modification (de Vrese and Schrezenmeir, 2008). It is however unknown to what extent this microbial modulation influences the host's physiology or immunity in relation to helminth exacerbation of colitis.

In Chapter 2, I demonstrated the exacerbation of DSS colitis by *H.polygyrus* infection. The data presented in this chapter is the first to demonstrate the beneficial role of probiotic administration on helminth-exacerbation of colitis Initially; I found that *H.polygyrus* burden defines modification of the microbiota translocating from the lumen to the periphery (**Fig 7.1**). Research reiterates the importance of the Firmicutes: Bacteriodetes ratio in the gut, with a high ratio associated with obesity, IBD and metabolic syndrome (Frank *et al.*, 2007; Guo *et al.*, 2008; Kim *et al.*, 2012; Miles *et al.*, 2017). Similar microbial modification is reported during a *Trichuris spp.* and *Toxocara spp.* infection (Houlden *et al.*, 2015; Duarte *et al.*, 2016). Interestingly, I demonstrate a significant increase in the gram-positive Enterococcus genus of the Firmicutes phyla with increased worm burden (**Fig 7.1**). *Nakanishi et al.,* reported the enrichment of gram-positive bacteria (dominated by the Firmicutes phyla) in DSS administered mice, which had the ability to recruit proinflammatory monocytes and macrophages to the host's colon (Nakanishi *et al.*, 2015). My data suggest that helminth-dependent enrichment of Firmicutes phyla may result in increased inflammation in the colon through a similar mechanism.

Besides the inflammatory role of gram-positive bacteria during DSS colitis, gram positive *Enterococcus spp.*, a genus significantly increased in the helminth infected groups in this study (**Fig 7.1**), are associated with inflammatory

pathology in both human and experimental IBD (Balish and Warner, 2002; Golińska *et al.*, 2013; Zhou *et al.*, 2016). The Enterococcus and Clostridia genera are reported as opportunistic intestinal pathogens that flourish when the host's microbiome is exposed to antibiotics such as Vancomycin and they are both associated with colitis (Hrncir *et al.*, 2007; Duarte *et al.*, 2016). Expansion of the Firmicutes phyla during helminth infection is also reported in a C57BL/6 mice after *H.polygyrus* infection, where expansion of *Lactobacilli spp.* (members of the Firmicutes phyla) was associated with efficient host colonization (Reynolds *et al.*, 2014), demonstrating a beneficial helminth-host microbiota relationship. Hence, we argue that in this study, *H.polygyrus* infection skewed the host's microbial community to a Firmicutes dominated community, a microbial shift that may have been detrimental to DSS colitis pathology.

The accompanying increase in the Verrucomicrobia phyla as helminth burden increased was surprisingly interesting, as this phylum contains an interesting *Akkermansia muciniphila* species (**Fig 7.1**). *A.muciniphila* is a mucin degrading bacterium, capable of excessively breaking down the colon mucus barriers if left unchecked (Ganesh *et al.*, 2013; Berry *et al.*, 2015; Seregin *et al.*, 2017). Expansion of *A.muciniphila* during helminth infection may be beneficial to helminth colonisation, as the bacterium would constantly breakdown copious amounts of mucus that would normally enable helminth clearance. However, this may have the side effect of weakening the mucus barrier leading to exaggerated contact between lumen bacterial antigens and epithelial tissue during DSS administration.

Although the diversity of translocating bacteria from helminth infected mice clustered separately from the uninfected mice (beta diversity) **(Fig 7.1**), it was quite surprising that helminth infection had no impact on the overall average diversity of the host's microbial species (alpha diversity). Literature reports either a reduction or increase of alpha diversity with helminth infection (Houlden *et al.*, 2015), although these shifts maybe dependent on the host's diet. A carbohydrate rich diet is demonstrated to support expansion of the Bacteriodetes phyla, a community that plays an important role of carbohydrate fermentation in the host's gut, availing bacterial dependent metabolites reported to suppress

inflammation through IL-10 and Treg signaling (Chen *et al.*, 2017; Williams *et al.*, 2017). This phyla is enriched in young children from Burkina Faso who have a carbohydrate rich diet, compared to Italian children who have an animal fat rich diet dominated by a Firmicutes microbiota (De Filippo *et al.*, 2010). In my studies, an n-6 PUFA (linoleic acid) modified diet was used within an optimised model of DSS colitis, potentially providing a suitable canvas for helminth manipulation towards a Firmicutes dominated microbiota.

To determine if the helminth microbial shift played a significant role in helminth exacerbation of DSS colitis, we proposed to administer a lactic acid producing 8-strain oral probiotic to counteract *H.polygyrus* driven dysbiosis, in anticipation of a rescue in DSS colitis exacerbation (**Fig 7.2**). Interestingly, administration of the 8-strain probiotic during helminth infection ameliorated helminth-exacerbation of DSS, as demonstrated by rescued weight-loss and distress compared to helminth infection alone (Hp+DSS) (**Fig 7.2**). Although probiotic administration had no effect on colon shortening, I found that there was a significantly reduced colon infiltrate and colon IL-6 production in probiotic treated mice compared to the probiotic untreated mice (**Fig 7.2**). My finding of heightened IL-6 production during *H.polygyrus* exacerbation of DSS colitis tallies with a recent publication (Pastille *et al.*, 2017). However, my findings are the first to demonstrate a suppression of this important colitic cytokine following probiotic administration. A caveat to this study is the absence of microbial data following probiotic administration, as it would have been interesting to determine whether probiotic administration prevented the helminth driven microbial shift witnessed during exacerbation. It would also have been interesting to note if rescue of the colon architecture, especially goblet cell functionality, would have been associated with a decreased prevalence of the Verrucomicrobiota phyla.

Despite the lack of confirmatory tests of the probiotic effect on gut microbial communities, I was able to demonstrate the beneficial role of probiotics on gut epithelium integrity (**Fig 7.3**). VSL#3 administration is reported to improve gut epithelial barrier through the maintenance of tight junction proteins that include e-cadherin, occludin, zonula occludens-1 and claudins (Mennigen *et al.*, 2009; Corridoni *et al.*, 2012; Isidro *et al.*, 2017). Tight junction conservation is

positively correlated with reduced macrophage infiltrate into inflamed colons during acute colitis, paralleling a significant reduction in typical inflammatory cytokines such as IL-2 and IL-17A (Isidro *et al.*, 2017). In this study, helminth exacerbation of DSS colitis was associated with overexpression and the re-distribution of both β-catenin and E-cadherin, from the epithelium to the cytoplasm (**Fig 7.3**). Surprisingly, colon sections from probiotic treated mice had intact epithelial cell junctions, with the latter group also demonstrating the diminished infiltration of $CD3^+$ T cells compared to the Hp+DSS only group (**Fig 7.3**), suggesting reduced T cell infiltration to the colon following probiotic treatment. Interestingly, microbial driven re-distribution and stabilisation of the β-catenin protein can drive transcription of inflammatory genes through the NFkB pathway (Sears, 2009; Goodwin *et al.*, 2011; Sun and Kato, 2016), contributing to increased epithelial inflammation through the induction of Th17 immune responses including IL-17, IL-23, IL-6 and IL-1β expression, all of which are positively enhanced by TNF signaling (Murch *et al.*, 1993; Wu *et al.*, 2009).

Excessive gut insult (such as continuous administration of DSS in drinking water) incapacitates the regular checks and balances required to heal, maintain and suppress inflammation, leading to an overwhelmed immune response that cannot control bacterial invasion and bacterial translocation into the periphery (Gardiner *et al.*, 1993; Carroll and Maharshak, 2013). Bacterial translocation is highly correlated with tight junction distribution in the colon epithelium. This process is proposed to weaken cell-to-cell adhesion, permitting bacterial escape from the colon lumen into the periphery (Weng *et al.*, 2007; Bergstrom *et al.*, 2010; Kansal *et al.*, 2014). Indeed, helminth–exacerbated colitis was associated with increased bacterial translocation to the spleen (**Fig 7.4**), with *in situ* fluorescence labeling showing higher numbers of LPS labeled bodies within epithelial tissue in the Hp+DSS colons compared to the Hp+DSS+V colons (**Fig 7.4**). The administration of probiotics significantly reduced the amount of translocating bacteria, and diminished the frequency of LPS labeled bodies within epithelial tissue, implying a rescued colon integrity as demonstrated in **Figures 7.2 and 7.3**. Excessive bacterial translocation during both experimental and human colitis is suggested to result in systemic inflammation, with an

increased potential of organ failure and mortality (Zhi-Yong *et al.*, 1992; Swank and Deitch, 1996). This correlates with the measure of distress recorded in helminth-infected mice, following administration of DSS (**Fig 7. 2**).

After demonstrating the abrogation of colon inflammation and pathology in probiotic treated mice, I next determined whether this treatment would have an effect on local and systemic immune responses. Under steady state conditions, systemic and intestinal regulatory cells that include $CD4^+$ Tregs suppress excessive inflammatory responses to gut lumen antigens. Colonic and recruited $CD4^+$ Tregs are reported to shuttle between the colon and the mesenteric lymph nodes to suppress T cell priming by antigen presenting cells and dampen inflammatory responses (Pabst, 2012; Geem *et al.*, 2016; Nakanishi *et al.*, 2017). Similar to my previous findings (**Fig 4.2**), probiotic amelioration of colitis was characterised by increased MLN $CD4^+$ Treg proportions in the Hp+DSS+V group compared to the Hp+DSS group (**Fig 7.5**), although their Foxp3 MFI was quite similar. Foxp3 MFI is a measure of the suppressive capacity of a $CD4^+Foxp3^+$ cell, with a higher MFI associated with suppressive functionality. Although all groups had similar Foxp3 MFI, the Hp+DSS+V group exhibited higher frequencies of MLN $CD4^+$ Tregs, alluding to a more suppressive milieu in the MLN compared to the Hp+DSS only. Surprisingly, the spleen Treg Foxp3 MFI was significantly higher in the probiotic treated group compared to the DSS only as well as Hp+DSS only groups (**Fig 7.5**). This occurrence may be due to suppressed local expansion of lymphocyte populations in the spleen (Lee *et al.*, 2009; Kim, 2010). Hence, the suppressive milieu in the probiotic treated group may have prevented excessive inflammatory responses to any other antigenic stimulation that may have escaped gut epithelial containment, unlike in the Hp+DSS group.

After determining the rescue of MLN Tregs by probiotic treatment, we looked at the $CD4^+$ and $CD8^+$ T cell response following DSS administration in probiotic treated mice. Unexpectedly, probiotic treatment had no significant effect on MLN and splenic $CD4^+$ effector T cells, although this treatment did result in the rescue of the MLN $CD8^+$ T cell compartment (**Fig 7.6**). *H.polygyrus*-related depletion of $CD8^+$ T cells has been published in models where *H.polygyrus*

exacerbated DSS colitis and carcinogenesis (Actor *et al.*, 1993; Filbey *et al.*, 2014; Pastille *et al.*, 2014, 2017), and I also showed a depletion of the MLN $CD8^+$ T cells in helminth infected mice (**Fig 10.1-appendix**). This demonstrates that $CD8^+$ T cell depletion in this study is a function of helminth infection, which may negatively affect DSS colitis induction. Interestingly, early $CD8^+$ T cell researchers demonstrated the ability of IL-4 to drive the differentiation of non-cytolytic $CD8^-$ T cells from cytolytic $CD8^+$ T cells, indicating the possibility of anti-helminth immune responses (IL-4) to influence loss of $CD8^+$ cytotoxic activities (Erard and Le Gros, 1994). Hence, we may be witnessing de-differentiation of cytotoxic $CD8^+$ T cells instead of $CD8^+$ T cell depletion during helminth exacerbation of DSS colitis. IL-4 stimulated CD8 T cells are reported to support Th2 immune responses, and may be important during anti-helminth immune responses. However, such a conversion would severely impair anti-bacterial and anti-viral $CD8^+$ IFNγ responses, leaving the host prone to bacterial infections, a phenomenon similar to the immune-phenotype of Hp+DSS mice in this study.

Further characterization of the immune responses associated with $CD4^+$ T cells showed a highly inflammatory state associated with helminth-exacerbated colitis, dominated by increased spleen $CD4^+$ IL-6 responses and diminished $CD4^+$ IFNγ responses (**Fig 7.7**). These $CD4^+$ T cell cytokine responses were also mirrored in the total cytokine responses, with an additional increased spleen and peritoneal IL-17A responses associated with exacerbated disease. However, probiotic administration associated with increased $CD4^+$ T cell antibacterial responses (IFNγ) and suppressed total IL-17A and IL-6 proportions in both the spleen and the peritoneum (**Fig 7.7**), cytokines published to be positively associated with exacerbated DSS colitis as well as microbial infections/attacks (Melgar *et al.*, 2005; Noguchi *et al.*, 2007; Pastille *et al.*, 2017). Interestingly, persistent IL-17 signaling is associated with increased neutrophil recruitment and inflammatory cell accumulation in an attempt to resolve a microbial attack, the latter feature which is apparent in the Hp+DSS only colon sections and abrogated in the Hp+DSS+V colon sections (**Fig 7.8**). Surprisingly, neutrophilia and IL-17A signaling plays an important role during helminth expulsion in a mouse model of lung inflammation (Chen *et al.*, 2014). Hence, the significant neutrophilia

witnessed in the Hp+DSS mice may be a helminth directed host immune response that is exacerbated by bacterial translocation to the spleen, leading to exacerbated local and systemic inflammation. Unfortunately, one of the other weaknesses of our report is the lack of helminth directed responses data such as worm/egg burden and IL-4 or IL-13 responses after probiotic administration, which would have helped determine if probiotic administration indirectly ameliorated DSS colitis through an impact on helminth infection, or directly influenced DSS colitis through microbiome modification.

In conclusion, I demonstrated how microbial signatures are pivotal during helminth exacerbation of DSS colitis. Consequently, I ameliorated helminth-exacerbated DSS colitis through administration of an 8-strain probiotic. Probiotic administration rescued the gut epithelial architecture demonstrated by maintenance of cell-cell interaction proteins, goblet cell distribution and prevented infiltration of inflammatory cells such as $CD3^+$ T cells into the colon epithelium. Inflammatory suppression was also characterised by abrogation of the IL-17A and IL-6 immune responses as well as boosting of MLN $CD4^+$ Treg suppressive cells in the probiotic treated mice. This work provides an insight of how microbial modifications may influence inflammatory conditions such as colitis. In natural settings where hosts may be infected with a variable number of gastrointestinal parasites, gut microbial dysbiosis may have a detrimental effect on the host immunology and may present complex health implications that may be compounded by host diet. Probiotic treatment of helminth infected individuals may counteract helminth exacerbated inflammatory pathologies.

CONCLUSION AND FUTURE WORK

8 Conclusion

The work presented in this study demonstrates the ability of *H.polygyrus* to exacerbate both oxazolone and DSS colitis, with exacerbation in the latter model independent of helminth phase of infection. Interestingly, *H.polygyrus* wholly resides in the small intestine, and yet demonstrated an influence on colitis, a condition mostly located in the colon. In both the first and second studies, chronic *H.polygyrus* exacerbation of both models of colitis was associated with diminished Th2 cytokine production, suggesting the dispensability of type-2 associated immune responses during helminth associated colitis exacerbation. Findings from the first study also suggest that $CD4^+$ derived IL-13 is equally as important as NKT derived IL-13 during oxazolone colitis, although susceptibility to oxazolone colitis associated with increased IFNγ and not IL-13, as expected. Antihelminthic treatment of infected mice appeared to rescue exacerbation of oxazolone colitis through a heightened Th2 immune response characterised by increased $CD4^+$ T cell and NKT cell secretion of IL-4, IL-5 and IL-13 in the mesenteric lymph nodes.

Findings from the second study not only showed a gender and diet dependence of DSS colitis, but also demonstrated that different phases of helminth infection induce different immune responses that both exacerbate DSS colitis. A mixed neutrophil, IL-17A and Th2 immune cytokines in the acute infected mice suggest a type-2 immune response exacerbating DSS colitis in these mice. However, heightened peritoneal inflammation and spleen TNF in the chronic infected mice correlated with exacerbated disease. Interestingly, both acute and chronic infections positively associated with MLN $CD8^+$ T cell depletion, a compartment that was rescued by probiotic infection in the final study.

Administration of an 8-strain probiotic before DSS administration ameliorated disease in acute *H.polygyrus* infected mice, characterised by the regulation of E-cadherin and β-catenin cell-cell interaction proteins. Overall, my findings demonstrate the deleterious effect of helminth infection on gut inflammation, with helminth exacerbation of colitis possibly enabled by gut microbial shifts. In all three studies, regulatory and effector T-cell dynamics appear to determine the

extent of helminth influence on gastrointestinal and systemic inflammation. Here, I present novel and interesting data that not only adds to the field of helminths and colitis, but also presents a cautionary tale to the adoption of 'non-specific' helminth therapy on inflammatory pathologies. Interestingly, I also demonstrate that not only does a probiotic influence the host's microbial diversity, but also it also has direct effect on the host's immunology, and potentially interacts with other underlying conditions giving rise to unexpected outcomes. This should be taken into account when designing therapeutic probiotic mixtures.

An interesting aspect of the phase of helminth infection brings into focus how experiments are set-up, and output data should be interpreted, throwing a curve ball to already published findings that may have appeared as contradictory when in reality they may just have sampled at different time points. Lastly, the interaction of diet, parasite infection and colitis may explain the current increase in colitis and colitis associated cancer incidence in recently industrialised countries found in parasite endemic areas.

9 Future work

The shortcomings of each study accompanied each result chapter, and here we suggest some potential steps or experiments that may address the challenges arising during the course of research.

Oxazolone Colitis

The major challenge faced in the oxazolone colitis study was increased mouse mortality, leading to reduced sample numbers and affecting the statistical power of the study. Following on from my experience of this model, I would suggest that the impact of helminth infection on colitis be determined using a modified oxazolone colitis model optimized by *Hoving et al.,* where a cutaneous pre-sensitization step is carried out before oxazolone administration to enable the development of mild colitis (Hoving *et al.*, 2012).

Microbial modulation in DSS colitis

Although I found that probiotic administration was able to ameliorate helminth-induced colitis, a proof of concept step would be needed, in order to formalise my findings

- A Fecal Microbiota Transfer (FMT) experiment could be performed, where fecal matter from helminth-infected and probiotic treated mice (donors) is transferred to helminth-infected mice (recipients) before DSS administration.
- Depletion of $CD8^+$ T cells before DSS administration could be performed in helminth-infected mice, in order, to ascertain whether the rescue of MLN $CD8^+$T cells had an influence on amelioration of disease.
- Lastly, confirmatory tests that include faecal egg/adult worm burden and quantification of the anti-parasite immune response would help in determining whether probiotics rescued colitis through a direct effect on worm viability.

APPENDIX

10 Appendix

10.1 Optimisations

Brief

The role of the microbiota in models of inflammatory and autoimmune diseases has been extensively characterised and reviewed (Atarashi *et al.*, 2013; Rooks *et al.*, 2014; Schaubeck *et al.*, 2015; Melhem *et al.*, 2019). However, IBD is a complex disease with multiple risk factors, most of which are still to be determined. Recent findings of the ability of gastrointestinal helminth infections to exacerbate bacterial colitis as well as modify the host's microbial communities suggests the ability of the gut microbiota to determine extent of gut inflammation during helminth infection (Chen *et al.*, 2005; Whary *et al.*, 2014; Houlden *et al.*, 2015; Kreisinger *et al.*, 2015).

1st optimisation: Specific Pathogen Free (SPF) status influences extent of helminth exacerbation of DSS colitis.

To determine if helminth exacerbation of DSS colitis would be influenced by host microbial status, age-matched female SPF1 and SPF2 mice were infected with an acute *H.polygyrus* infection before DSS administration, and murine tissue was processed as previously mentioned in the methods and materials. Findings from this set of experiments were pivotal in the consequent experimental runs of *H.polygyrus* infection and DSS colitis, and they provided the basis for the seminal probiotic experiments and findings discussed in this report.

Findings

Research demonstrates that antibiotic treatment or breeding of IBD mouse models in germ free facilities is positively correlated with amelioration of gut inflammation during experimental inflammatory bowel disease; demonstrating an important role of the gut microbiota in pathogenesis (Dove *et al.*, 1997; Sellon *et al.*, 1998). SPF1 and SPF2 female BALB/c mice fed a modified AIN76A diet

(D16083101, Research Diets) were acutely infected (7 days) with *H.polygyrus* before a 10-day DSS administration course. Weight-loss (**Fig 4.1a**) and mouse distress (**Fig 4.1b**) were monitored as a measure of DSS colitis development and progression. After the 10-day DSS course, mice were euthanized and the spleen was excised and weighed to determine extent of inflammation (**Fig 4.1c**). Next, the frequencies and numbers of splenic $CD4^{+}Foxp3^{+}$ Tregs (**Fig 4.1d**) were compared to the proportions and numbers of effector $CD4^{+}$ (**Fig 4.1e**) and $CD8^{+}$ (**Fig 4.1f**) T-cells to determine if there was a shift in the regulatory: effector balance. The $CD4^{+}$ T-helper cell compartment was further characterised for immune responses (**Fig 4.1g**) important for an anti-helminth response (IL-4) and DSS colitis response (IL-6). Consequently, a broad characterisation of the total splenic type-1, type-2 and type-17 immune responses was carried out.

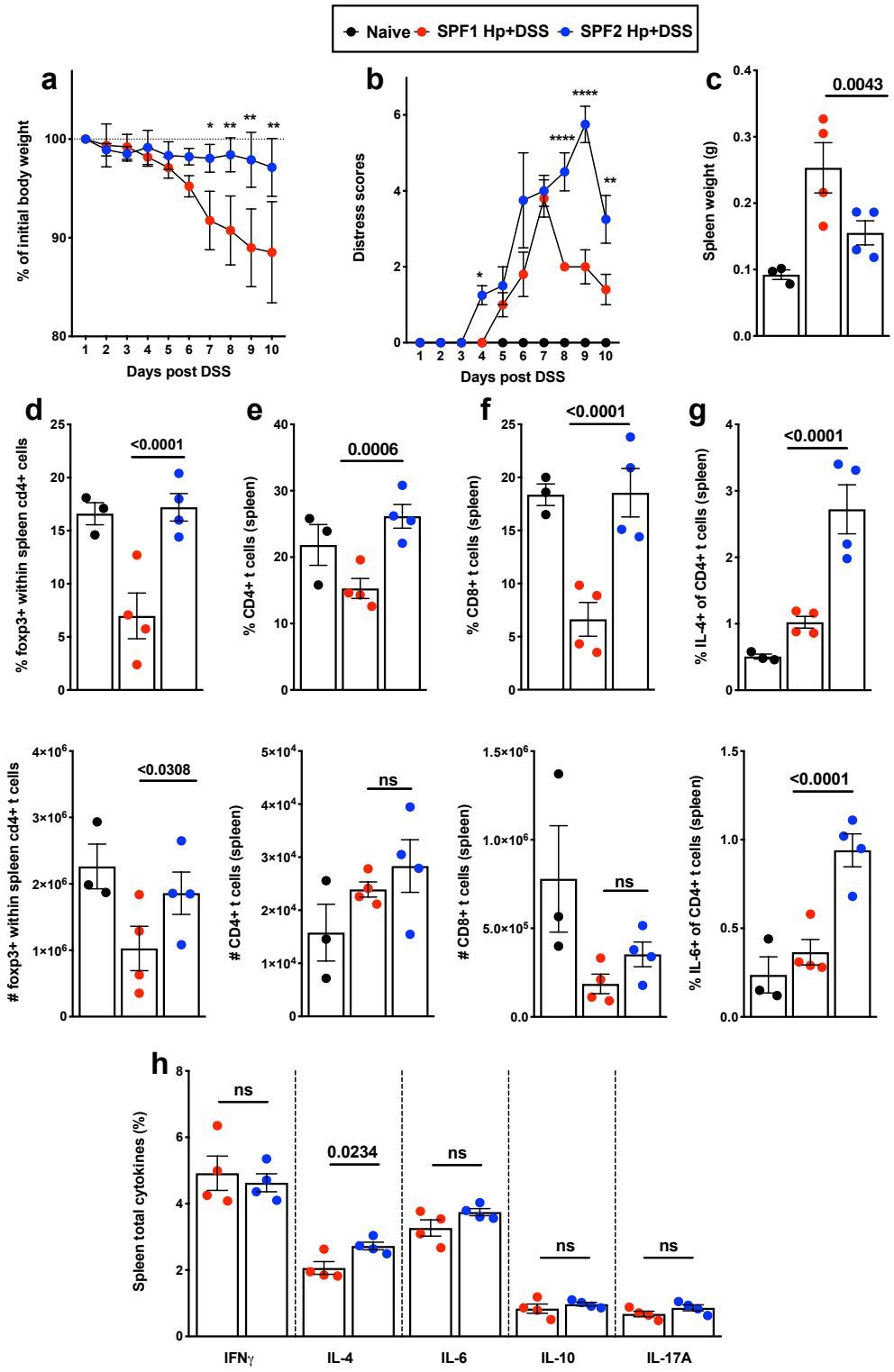


Figure 10-1: Gut microbial status influences the ability of helminth infection to exacerbate DSS colitis. Age-matched female SPF1 and SPF2 mice were acutely infected with *H.polygyrus* before DSS administration. Weight-loss (**a**) and distress scores (**b**) for naïve mice (black symbols), SPF1 *H.polygyrus* infected + DSS administered mice (red symbols) and SPF2 *H.polygyrus* infected + DSS administered mice (blue symbols) was monitored over the course of DSS administration. The spleen was sterile excised and weighed (**c**) at point of euthanasia, and

single cell suspension was carried. The proportions and numbers of $CD4^+Foxp3^+$ Tregs (**d**) and effector $CD4^+$ (**e**) and $CD8^+$ T (**f**) cells were determined in flow cytometry. $CD4^+$ T cell IL-4 and IL-6 (**g**) immune responses were determined and compared to a general screening of type1, type 2 and type17 total cytokine immune responses (**h**). Data is presented as mean ± SEM and is representative of one experiment with n≥4 mice per group. Statistical significance was calculated using an unpaired t-test at 95% significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant. Unless stated, all comparisons were between SPF1 and SPF2 Hp + DSS groups. Experimental monitoring assisted by Brittany-Amber Jacobs, UCT.

2nd optimisation: Administration of an 8-strain probiotic to naïve mice results in significantly increased mesenteric lymph node $CD4^+Foxp3^+$ Treg compartment.

Findings from the first optimization experiments demonstrated the importance of the host microbiome in helminth exacerbation of DSS colitis. Microbial dynamics are pivotal during the pathology of both UC and CD (Frank *et al.*, 2007; Carroll and Maharshak, 2013; Haberman *et al.*, 2014; Walters *et al.*, 2014), with the contact between lumen commensals and gut epithelium strongly contributing to colitis pathogenesis. Maintaining gut microbial homeostasis through probiotic intake is advised to relieve colitis symptoms, a practice with mixed results, although most patients witnessed an improvement in disease due to probiotic intake (Hoermannsperger *et al.*, 2009; Sood *et al.*, 2009; Petersen *et al.*, 2011). In this section, we exposed female BALB/c (fed the modified AIN76A diet) to either an *H.polygyrus* infection or probiotics only for a period of 7 days in order to determine the individual effects of *H.polygyrus* and probiotics on host immune responses. Results from this optimization run helped in defining probiotic-associated host immune responses in *H.polygyrus* infected + probiotics administered mice during DSS colitis.

Findings

Administration of probiotics, especially VSL#3, is reported to prevent or ameliorate gut inflammation in both experimental and human colitis disease; although other publications have demonstrated a non beneficial role of VSL#3 intake on both colitis and colitis associated cancer in murine models of disease (Gionchetti *et al.*, 2003; Bibiloni *et al.*, 2005; Giacinto *et al.*, 2005; Arthur *et al.*, 2013; Sang *et al.*, 2013). Although the effects of both helminth infection or

VSL#3 administration on disease have been well researched (Giacinto *et al.*, 2005; Smith *et al.*, 2007; Hang *et al.*, 2010; Wang *et al.*, 2018), I was interested in determining the individual effects of both these parameters on naïve untreated healthy mice. Age-matched female BALB/c fed a modified AIN76A diet were exposed to either an *H.polygyrus* only infection or to probiotics *ad libatum* in drinking water for a 7-day period. Total cellularity of the mesenteric lymph node (MLN), peritoneal cavity (PerC) and the spleen was determined (**Fig 4.2a**). Next, we decided to look at the $CD4^{+}Foxp3^{+}$ Treg profile (**Fig 4.2b**), as research has reported the ability of both *H.polygyrus* infection and probiotic administration to modify host Treg populations (Giacinto *et al.*, 2005; Grainger *et al.*, 2010; Atarashi *et al.*, 2013). Eventually, the $CD4^{+}$ (**Fig 4.2c**) and $CD8^{+}$ (**Fig 4.2d**) effector cell total numbers in all three tissues we characterised, in order to compare host regulatory: effector dynamics.

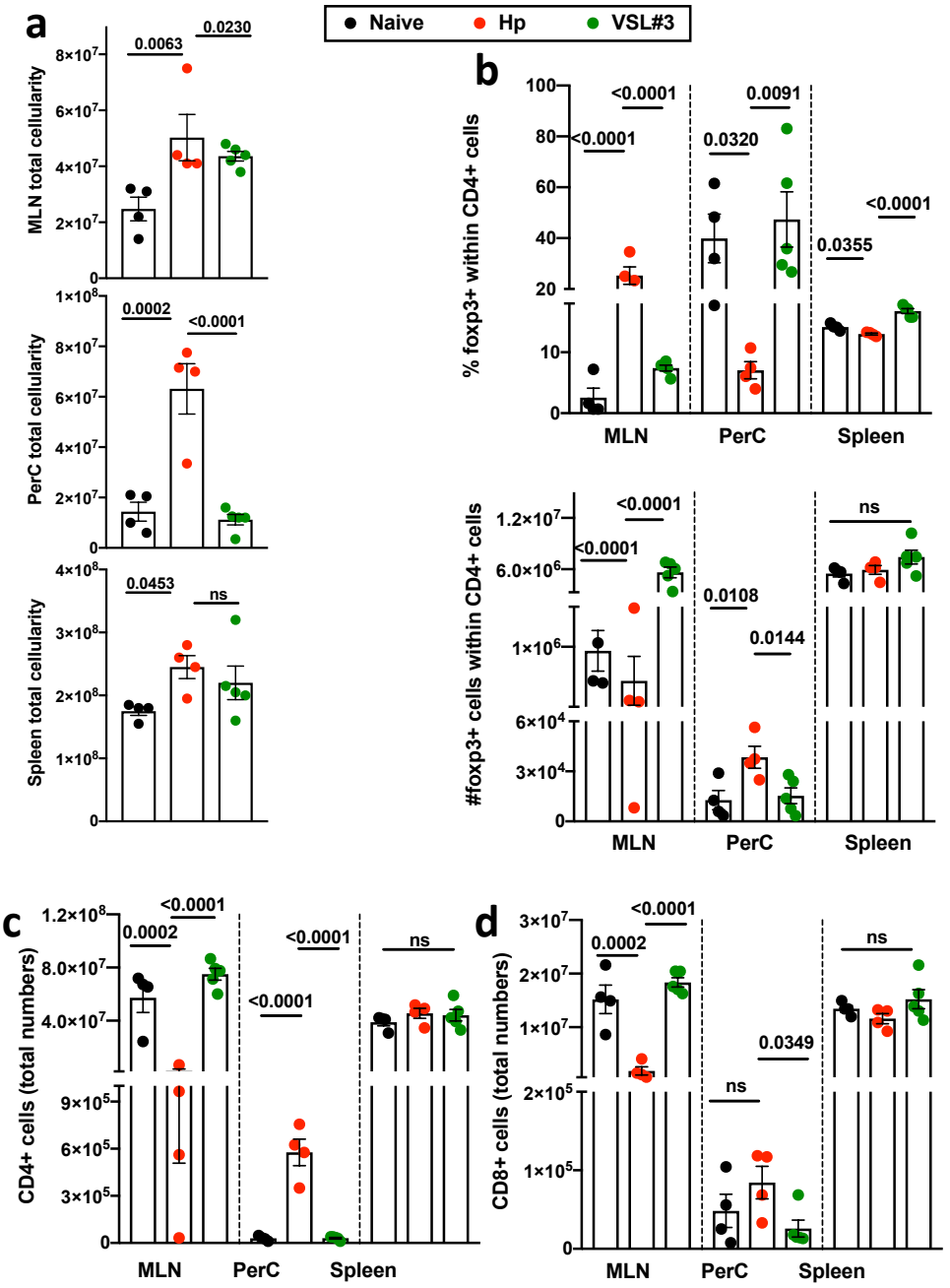


Figure 10-2: VSL#3 administration potentiates mesenteric lymph node CD4$^+$Foxp3$^+$ (Treg) numbers. SPF1 age-matched female BALB/c mice were exposed to either an *H.polygyrus* infection (red symbols) or probiotics (VSL#3-green symbols) for a period of 7 days. Mesenteric lymph node (MLN), Peritoneal Cavity (PerC) and Spleen cells were harvested at point of euthanasia, analysed in flow cytometry and the findings were compared to cell proportions and numbers from untreated mice (naïve-black symbols). Total MLN, PerC and Spleen cellularity was determined (**a**). Next, we determined the CD4$^+$Foxp3$^+$ Treg proportions and numbers (**b**), which were then compared to the total numbers of CD4$^+$ (c) and CD8$^+$ (d) T cell effector numbers. Data is presented as mean ± SEM and is representative of one experiment with n≥4 mice per group. Statistical significance was calculated using one-way ANOVA at 95%

significance level, with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns=not significant.

Optimisation Discussion

The commensal relation between the gut microbiome and the host has a huge impact on the host's metabolism, nutrition and immune responses; the latter of which is not only limited to the gut but has systemic effects on organs at a distance from the gut. Gut microbial modification is associated with increased susceptibility to UC and CD as well as motor neurone disease in humans and colitis associated cancer (CAC) in murine models (Manichanh *et al.*, 2006; Elinav *et al.*, 2011; Blacher *et al.*, 2019). In these optimization studies, the microbial status of SPF1 mice increased susceptibility to helminth exacerbated DSS colitis compared to the SPF2 microbial status (**Fig 4.1**). Both UCT SPF1 and SPF2 mice are completely free of all Federation of European Laboratory Animal Science Association (FELASA) listed organisms (Kunstyr and Nicklas, 2000), with the former having an additional of four more microbial genera, namely, *Pasturella spp.*, *Helicobacter spp*., Murine Norovirus and *Trichomonas spp*.

Microbial status differences between SPF and conventionally housed mice are demonstrated to influence gastrointestinal inflammation in IL-$10^{-/-}$ and $TNF^{deltaARE}$ mice models of spontaneous IBD (Keubler *et al.*, 2015; Schaubeck *et al.*, 2015), with SPF mice exhibiting delayed disease onset compared to conventionally housed mice. This demonstrates the importance of certain groups of bacteria/ microbiota in driving gut inflammation. Specifically, the *Helicobacter spp.* and the *Bacteroides spp.* exacerbated gut inflammation in an IL-10-/- model of colitis (Fox *et al.*, 2010; Bloom *et al.*, 2011). Interestingly, in these studies, worse pathology was evident in *Helicobacter spp. and Pasturella spp.* carrying SPF1 mice, supporting published literature demonstrating the importance of these bacterial species in both experimental and human IBD (Haberman *et al.*, 2014). Surprisingly, *H.polygyrus* infection is reported to suppress *Helicobacter spp.* associated gastritis in transgenic INS-GAS mice, although an earlier publication demonstrated heightened overall gastritis despite suppressed *Helicobacter spp.* inflammation in mice (Fox *et al.*, 2000; Whary *et*

al., 2014); demonstrating the importance of microbial groups and not individual microbial species in studying microbial effects on host responses.

Probiotic intake is usually advised for the treatment of gastritis, with a number of IBD patients professing an amelioration of IBD symptoms during a probiotic course (Kruis *et al.*, 1997, 2004; Rembacken *et al.*, 1999). Research demonstrates the ability of probiotics to colonize the host's gut (Gaudier *et al.*, 2005), modulate the gut's Treg compartment as well as suppress inflammatory conditions such as DSS colitis (Mennigen *et al.*, 2009; Mar *et al.*, 2014), ileitis (Corridoni *et al.*, 2012). Although the VSL#3 probiotic is extensively published as beneficial in ameliorating disease in a number of mouse models (Venturi *et al.*, 1999; Tursi *et al.*, 2004; Sood *et al.*, 2009), contradictory findings demonstrate the ability of this probiotic to only colonize the host's gut without influencing the host's immune system or actually exacerbating disease (Vahabnezhad *et al.*, 2013).

Using SPF1 female BALB/c fed a modified AIN76A diet, I demonstrated the ability of VSL#3 to increase $CD4^{+}Foxp3^{+}$ Treg numbers in the mesenteric draining lymph nodes (MLN) (**Fig 4.2**), suggesting a heightened immunosuppressive milieu in the VSL#3 treated gut compared to *H.polygyrus* infected mice. Overall, VSL#3 administration maintained $CD4^{+}$ and $CD8^{+}$ T cell frequencies and cell numbers similar to naïve mice in all three tissues assessed, unlike the depletion (MLN) or expansion (PerC) of effector cells in *H.polygyrus* infected mice. The heightened cellularity in the MLN, PerC and spleen of *H.polygyrus* infected mice demonstrates increased local and systemic inflammation associated with the infection, a phenomenon not witnessed in the VSL#3 administered mice. Surprisingly, neither of the *H.polygyrus* infected nor VSL#3 administered mice exhibited physical manifestations of the change in their immunology over the course of 7 days they were monitored.

Research findings from these optimization studies demonstrated the importance of the microbial status in helminth exacerbation of DSS, which is supported by findings from *Roy et al.,* who demonstrated the effect of different SPF statuses on gut inflammation (Roy *et al.*, 2017). I further demonstrated how VSL#3

administration modifies the host's immune system resulting in an increase in mesenteric lymph node $CD4^{+}Foxp3^{+}$ Tregs, a phenomenon that may be pivotal in suppression of inflammatory diseases if administered to diseased mice. A weakness of these optimizations is the absence of genomic microbial data that may have helped determine how intervention may have influenced the host's gut microbial communities. Overall, I conclude that the gut microbiome is an important factor in our helminth + DSS colitis. Understanding the dynamics behind host-helminth-microbiome-diet balance may contribute to the therapy of microbiome associated inflammatory conditions.

10.2 Diet Specifications

LabChef
RESEARCH NUTRITION

RODENT MAINTENANCE DIET #1845

LABCHEF RODENT MAINTENANCE DIET is a complete and balance diet that is suitable for feeding rats, mice, hamsters and gerbils. It is formulated and produced to be of premium palatability and digestibility



FEEDING GUIDELINES

Rats 10 - 30g / day
Mice 5 - 8 15g / day
Hamsters 10 – 15g / day



PELLET SIZE

- **Standard: 15 x 10 mm**
- **Maxi: 20 x 40 mm**

NUTRITIONAL VALUE

	"As is" (g kg-1)	DM (g kg^{-1})
Protein (min)	180	200
Moisture (max)	10	-
Oils and fats (min)	45	50
Linoleic acid (min)	*9*	10
Fiber (max)	40	45
Ash (max)	70	75
Ca:P ratio	1.1-2:1	1.1-2:1
Calcium (min)	12	14
Phosphorous (min)	7.0	8.0
Vitamin A (min)	16 000 (IU/kg)	16 000 (IU/kg)
Vitamin D (min)	2000 (IU/kg)	2000 (IU/kg)
Vitamin E (min)	100 (g/kg)	100 (g/kg)

DECLARATION OF INGREDIENTS

Maize, wheat bran, Fish meal, Soybean, soybean protein concentrate, fish meal, molasses, sucrose, calcium carbonate, maize protein concentrate sodium chloride, calcium phosphate, approved vitamins and minerals.

QUALITY & SAFETY

LABCHEF is manufactured in a facility that adheres to regular quality, traceability and hygiene audits to ensure that this products is safe and wholesome.

Developed by animal nutritionists to provide quality nutrition & wellbeing

Pr. Sci. Nat 400046/99

Member of PFI PET FOOD INDUSTRY OF SOUTHERN AFRICA

Nutritionhub (Pty) Ltd # 2012/141960 • 3 Tioga Street, Stellenbosch, SOUTH AFRICA • ph: +27 21 8800432 email: labchef@nutritionhub.co.za

Figure 10-3: #2012, NutritionHub dietary constituents. Diet is referred to as standard chow throughout this study.

D10001 and D16083101

Formulated by:
Research Diets, Inc.

AIN-76A Rodent Diet and Same With Sunflower Oil

Product #	**D10001**		**D16083101**	
	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*
Fat	5	*12*	5	*12*
Total		*100.0*		*100.0*
kcal/gm	3.90		3.90	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*
Corn Starch	150	*600*	150	*600*
Sucrose	500	*2000*	500	*2000*
Cellulose, BW200	50	*0*	50	*0*
Corn Oil	50	*450*	0	*0*
Sunflower Oil, High Linoleic	0	*0*	50	*450*
Mineral Mix S10001	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
Total	**1000**	***3902***	**1000**	***3902***

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

SmithKatie01.for

Figure 10-4: Dietary comparison of the conventional AIN76A diet (D1001) vs the linoleic acid modified diet (D16083101) diet.

OpenSource DIETS

Fatty Acid Composition of D10001 and D16083101

Prepared by: Sara Sturgess
Research Diets, Inc.
September 2017

	D10001 (with Corn Oil)	D16083101 (with Sunflower Oil)
Total Fat (gm)	50	50
C2, Acetic	0.0	0.0
C4, Butyric	0.0	0.0
C6, Caproic	0.0	0.0
C8, Caprylic	0.0	0.0
C10, Capric	0.0	0.0
C12, Lauric	0.0	0.0
C14, Myristic	0.0	0.0
C14:1, Myristoleic, n-9	0.0	0.0
C15	0.0	0.0
C16, Palmitic	5.5	3.1
C16:1, Palmitoleic, n-9	0.0	0.3
C16:2, n-4	0.0	0.0
C16:3, n-9	0.0	0.0
C16:4, n-4	0.0	0.0
C17	0.0	0.0
C17:1	0.0	0.0
C18, Stearic	1.0	2.4
C18:1, Oleic, n-9	12.5	11.0
C18:1, Elaidic, Trans	0.0	0.0
C18:2, Linoleic	30.1	30.1
C18:2, Trans	0.0	0.0
C18:3, Linolenic	0.7	0.3
C18:3, n-6	0.0	0.0
C18:3, Trans	0.0	0.0
C18:4, Stearidonic	0.0	0.0
C20, Arachidic	0.0	0.3
C20:1	0.0	0.0
C20:2	0.0	0.0
C20:3, n-6	0.0	0.0
C20:3, n-3	0.0	0.0
C20:4, Arachidonic, n-6	0.0	0.0
C20:4, n-3	0.0	0.0
C20:5, Eicosapentaenoic, n-3	0.0	0.0
C21:5, n-3	0.0	0.0
C22, Behenic	0.0	0.0
C22:1, Erucic	0.0	0.0
C22:4, Clupanodonic, n-6	0.0	0.0
C22:5, Docosapentaenoic, n-3	0.0	0.0
C22:6, Docosahexaenoic, n-3	0.0	0.0
C24, Lignoceric	0.0	0.0
C24:1	0.0	0.0
Total	49.6	47.3
Saturated (g)	6.4	5.8
Monounsaturated (g)	12.5	11.2
Polyunsaturated (g)	30.8	30.4
Saturated (%)	12.9	12.2
Monounsaturated (%)	25.1	23.7
Polyunsaturated (%)	62.0	64.2
n6	30.1	30.1
n3	0.7	0.3
n6:n3 ratio	42.9	120.4
trans fat (gm)	**0.0**	**0.0**

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

RESEARCH DIETS INC. www.ResearchDiets.com

Figure 10-5: Fatty acid composition, comparing conventional AIN76A diet (D1001) vs the linoleic acid modified diet (D16083101) diet.

10.3 Gating Strategies

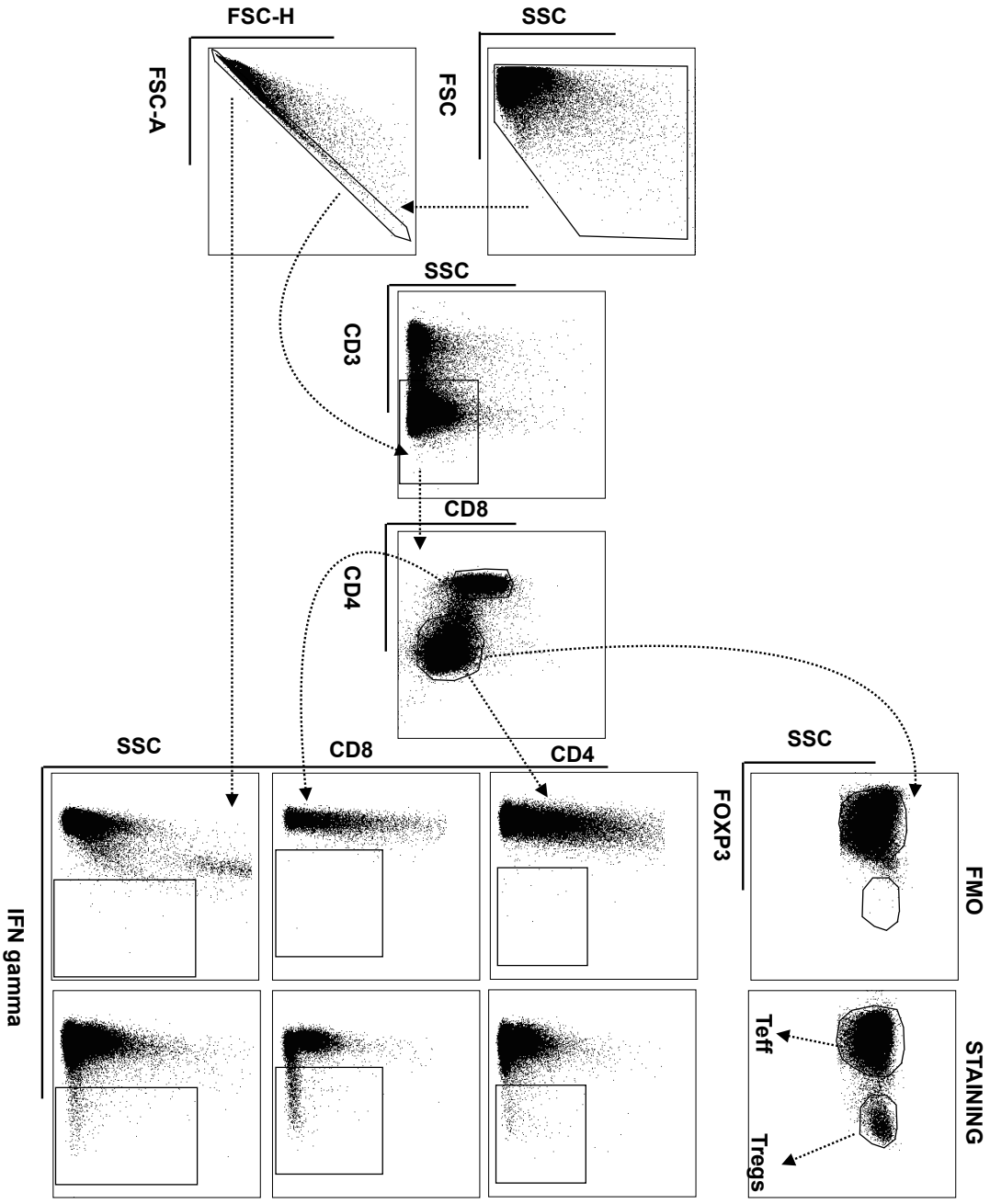


Figure 10-6: The gating strategy for adaptive immune cells and their cytokine responses. MLN gating strategy is illustrated.

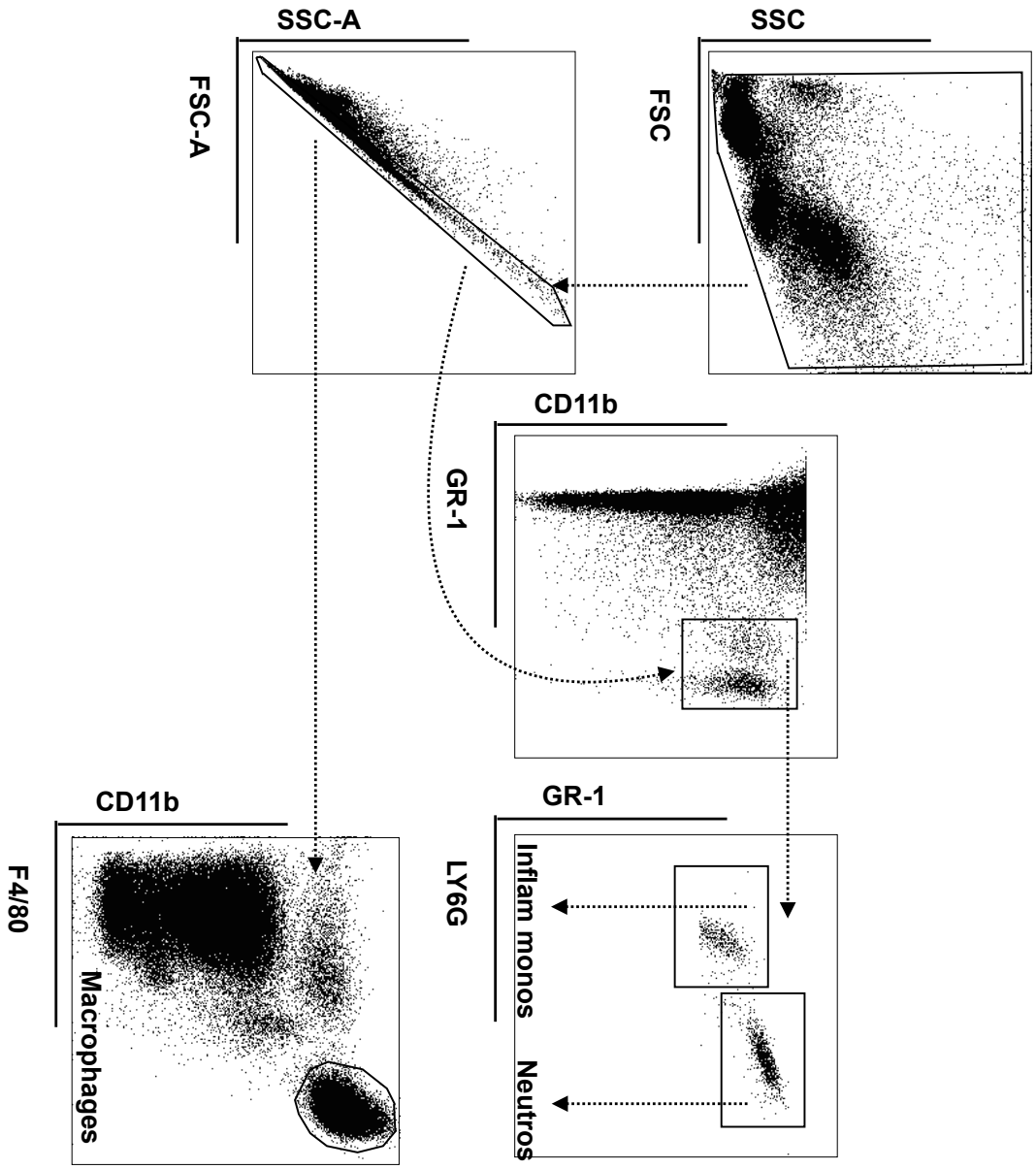


Figure 10-7: The gating strategy for innate cells. Peritoneal cavity gating strategy is illustrated**.**

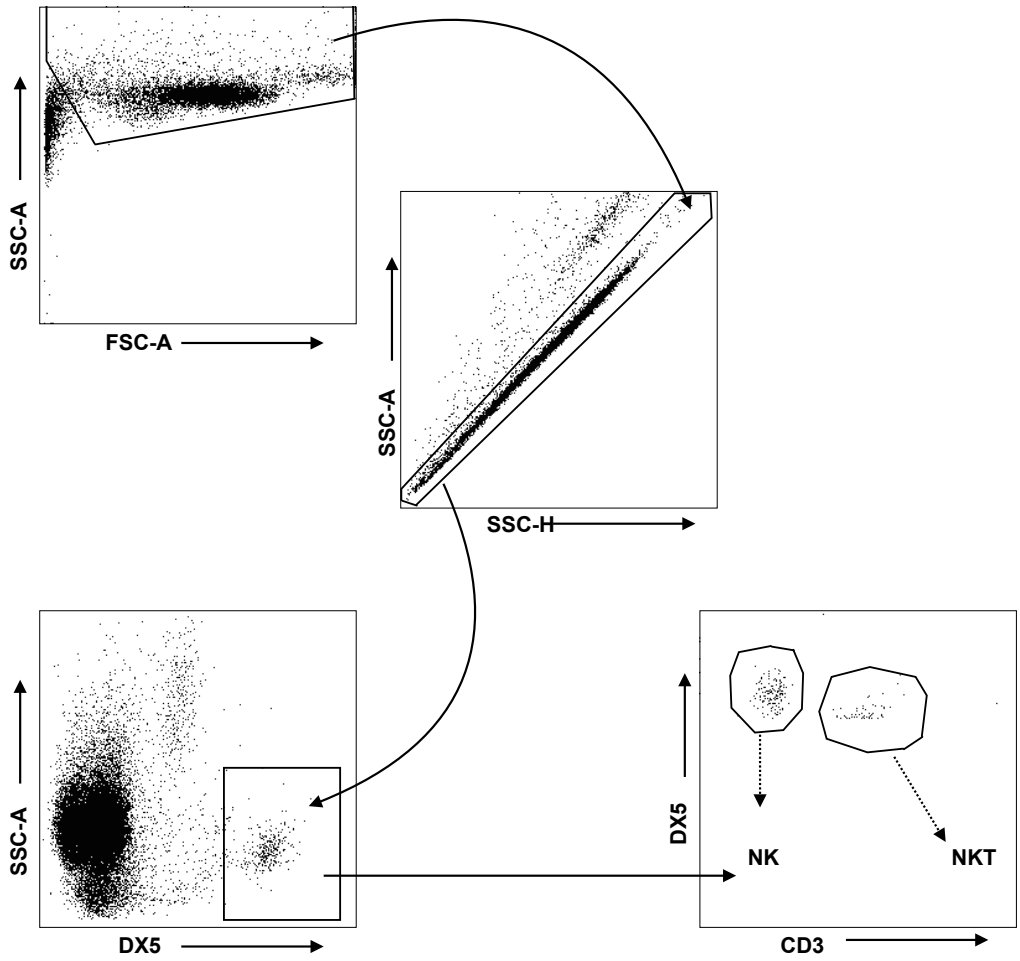


Figure 10-8: The gating strategy for NKT cells. Mesenteric lymph node gating strategy is illustrated here.

BIBLIOGRAPHY

A. Kucher, M. *et al.* (2017) 'Fecal microbiota transplantation as a method to treat complications after hematopoietic stem cell transplantation', *Cellular Therapy and Transplantation*, 6(March), pp. 20–29.

ACOG practice bulletin: Use of hormonal contraception in women with coexisting medical conditions. (2006) *Obstetrics and Gynecology*.

Actor, J. K. *et al.* (1993) 'Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance', *Proc Natl Acad Sci U S A*, 90(3), pp. 948–952.

Adams, J. H. *et al.* (1988) 'Sex-specific antigens on the surface and in the secretions of Nematospiroides dubius', *International Journal for Parasitology*, 18(7), pp. 999–1001.

Ahdieh, M. *et al.* (2001) 'Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-γ', *American Journal of Physiology-Cell Physiology*. American Physiological Society, 281(6), pp. C2029–C2038.

Ahmed, I. *et al.* (2016) 'Microbiome, Metabolome and Inflammatory Bowel Disease', *Microorganisms*, 4(2), pp. 1–19.

Aich, P. *et al.* (2016) 'Gut Microbiota and Health: a Review With Focus on Metabolic and Immunological Disorders and Microbial Remediation', *Biomedical Reviews*, 27(November), pp. 1–17.

Ajuebor, M. N. *et al.* (1999) 'Role of Resident Peritoneal Macrophages and Mast Cells in Chemokine Production and Neutrophil Migration in Acute Inflammation: Evidence for an Inhibitory Loop Involving Endogenous IL-10', *The Journal of Immunology*, 162(3), pp. 1685 LP – 1691.

Alex, P. *et al.* (2009) 'Distinct Cytokine Patterns Identified from Multiplex Profiles of Murine DSS and TNBS-Induced Colitis', *Inflammatory Bowel Diseases*, 15(3), pp. 341–352.

Allen, J. E. *et al.* (2015) 'IL-17 and neutrophils : unexpected players in the type 2 immune response', *Current Opinion in Immunology*, 34, pp. 99–106.

Allen, J. E. and **Maizels, R. M.** (2011) 'Diversity and dialogue in immunity to helminths', *Nature Reviews Immunology*. Nature Publishing Group, 11(6), pp. 375–388.

Amarapurkar, D. N. and **Patel, N. D.** (2007) 'Spectrum of autoimmune liver diseases in western India', *Hepatology*, 22, pp. 2112–2117.

An, D. *et al.* (2011) 'Membrane sphingolipids as essential molecular signals for Bacteroides survival in the intestine', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 108(Suppl 1), pp. 4666–4671.

Ananthakrishnan, A. N. *et al.* (2014) 'Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease', *Gut*. 2013/07/04, 63(5), pp. 776–784.

Andoh, A. *et al.* (2011) 'Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis', *Journal of Gastroenterology*, 46(4), pp. 479–486.

Annunziato, F. *et al.* (2015) 'The 3 major types of innate and adaptive cell-

mediated effector immunity', *Journal of Allergy and Clinical Immunology*. Elsevier Ltd, 135(3), pp. 626–635.

Anthony, R. M. *et al.* (2007) 'Protective immune mechanisms in helminth infection.', *Nature Reviews Immunology*, 7(12), pp. 975–987.

Antoniou, E. *et al.* (2016) 'The TNBS-induced colitis animal model : An overview', *Annals of Medicine and Surgery*. Elsevier Ltd, 11, pp. 9–15.

Appau, K. A. *et al.* (2008) 'Use of infliximab within 3 months of ileocolonic resection is associated with adverse postoperative outcomes in Crohn's patients', *Journal of Gastrointestinal Surgery*. 2008/08/15. United States, 12(10), pp. 1738–1744.

Araki, A. *et al.* (2005) 'MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis', *Journal of Gastroenterology*, 40(1), pp. 16–23.

Arisawa, T. *et al.* (2008) 'The Influence of Polymorphisms of Interleukin-17A and Interleukin-17F Genes on the Susceptibility to Ulcerative Colitis', *Journal of Clinical Immunology*, 28(1), pp. 44–49.

Arthur, J. C. *et al.* (2013) 'VSL#3 probiotic modifies mucosal microbial composition but does not reduce colitis-associated colorectal cancer', *Scientific reports*. Nature Publishing Group, 3(2868), pp. 1–9.

Asakura, K. *et al.* (2009) 'Prevalence of Ulcerative colitis and Crohn's disease in Japan', *J Gastroenterology*, 44, pp. 659–665.

Van Assche, G. *et al.* (2002) *The role of aminosalicylates in the treatment of ulcerative colitis*, *Acta gastro-enterologica Belgica*.

Atarashi, K. *et al.* (2011) 'Induction of colonic regulatory T cells by indigenous Clostridium species', *Science (New York, N.Y.)*. 2010/12/23, 331(6015), pp. 337–341.

Atarashi, K. *et al.* (2013) 'Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited, 500(7461), pp. 232–236.

Aufricht, C. *et al.* (2012) 'Peritoneal infection and inflammation.', *Mediators of inflammation*, 2012, pp. 1–2.

Axelsson, L. and **Goldschmidt, T. J.** (1996) 'Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice : Effects in CD4 + -cell depleted , athymic and NK-cell depleted SCID mice', *Inflamm Res*, 45, pp. 181–191.

Axelsson, L. and **Midtvedts, T.** (1996) 'The Role of Intestinal Bacteria, Bacterial Translocation and Endotoxin in Dextran Sodium Sulphate-Induced Colitis in the Mouse', *Microbial Ecology in Health and Disease*, 9, pp. 225–237.

Azooz, O. G. *et al.* (2001) 'Delayed puberty and response to testosterone in a rat model of colitis', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. American Physiological Society, 281(5), pp. R1483–R1491.

Bábíčková, J. *et al.* (2015) 'Sex Differences in Experimentally Induced Colitis in Mice: a Role for Estrogens', *Inflammation*, 38(5), pp. 1996–2006.

Bafford, A. C. *et al.* (2017) 'The Use of Temporary Fecal Diversion in Colonic and Perianal Crohn's Disease Does Not Improve Outcomes', *Digestive Diseases and Sciences*, 62(8), pp. 2079–2086.

Bager, P. *et al.* (2010) 'Trichuris suis ova therapy for allergic rhinitis: A

randomized, double-blind, placebo-controlled clinical trial', *Journal of Allergy and Clinical Immunology*, 125(1–3), pp. 123–130.

Bain, C. C. ***et al.*** (2016) 'Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities', *Nature Communications*. Nature Publishing Group, 7(May), pp. 1–14.

Balish, E. and **Warner, T.** (2002) 'Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice', *The American journal of pathology*. American Society for Investigative Pathology, 160(6), pp. 2253–2257.

Banks, C. ***et al.*** (2003) *Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease*, *The Journal of pathology*.

Barnett, M. and **Fraser, A.** (2011) 'Animal Models of Colitis : Lessons Learned , and Their Relevance to the Clinic, Ulcerative Colitis - Treaments, Special Populations and the Future', *InTech*, pp. 161–178.

Barquero-Calvo, E. ***et al.*** (2013) 'Bacterial Counts in Spleen', *Bio-protocol*, 3(21), pp. 1–6.

Bassaganya-Riera, J. ***et al.*** (2012) 'Probiotic bacteria produce conjugated linoleic acid locally in the gut that targets macrophage PPAR γ to suppress colitis', *PloS one*. 2012/02/21. Public Library of Science, 7(2), pp. e31238–e31238.

Basson, A. ***et al.*** (2016) 'Mucosal interactions between genetics, diet, and microbiome in inflammatory bowel disease', *Frontiers in Immunology*, 7(August), pp. 1–34.

Beall, M. J. and **Pearce, E. J.** (2002) 'Transforming growth factor-beta and insulin-like signalling pathways in parasitic helminths', *International Journal for Parasitology*, 32(4), pp. 399–404.

Becker, C. ***et al.*** (2006) 'Cutting Edge: IL-23 Cross-Regulates IL-12 Production in T Cell-Dependent Experimental Colitis', *The Journal of Immunology*, 177(5), pp. 2760 LP – 2764.

Behnke, J. M. ***et al.*** (1987) 'Immunological relationships during primary infection with Heligmosomoides polygyrus (Nematospiroides dubius): The capacity of adult worms to survive following transplantation to recipient mice', *Parasitology*, 95(3), pp. 569–581.

Di Bella, S. ***et al.*** (2015) 'Fecal microbiota transplantation (FMT) for Clostridium difficile infection: Focus on immunocompromised patients', *Journal of Infection and Chemotherapy*, 21(4), pp. 230–237.

Benchimol, E. I. ***et al.*** (2009) 'Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data', *Gut*, 58(11), pp. 1490 LP – 1497.

Bennett, J. L. and **Wick, E. C.** (2017) 'Fecal Diversion and Ostomies BT - Pelvic Floor Dysfunction and Pelvic Surgery in the Elderly: An Integrated Approach', in Gordon, D. A. and Katlic, M. R. (eds). New York, NY: Springer New York, pp. 349–360.

Bennike, T. B. ***et al.*** (2015) 'Neutrophil Extracellular Traps in Ulcerative Colitis : A Proteome Analysis of Intestinal Biopsies', *Inflamm*, 21(9), pp. 2052–2067.

Bentley, R. and **Meganathan, R.** (1982) 'Biosynthesis of vitamin K (menaquinone) in bacteria', *Microbiological reviews*, 46(3), pp. 241–280.

Bentzen, J. ***et al.*** (2010) 'Prevalence of multiple sclerosis in Denmark 1950 –

2005', 16(5), pp. 520–525.

Berg, Daniel J *et al.* (1996) 'Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses', *The Journal of clinical investigation*, 98(4), pp. 1010–1020.

Bergstrom, K. S. B. *et al.* (2010) 'Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa.', *PLoS pathogens*. Edited by C. R. Roy, 6(5), p. e1000902.

Berry, D. *et al.* (2012) 'Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis', *The ISME journal*. 2012/05/10. Nature Publishing Group, 6(11), pp. 2091–2106.

Berry, D. *et al.* (2015) 'Intestinal Microbiota Signatures Associated with Inflammation History in Mice Experiencing Recurring Colitis', *Frontiers in Microbiology*, 6(December), pp. 1–11.

van Best, N. *et al.* (2015) 'On the origin of species: Factors shaping the establishment of infant's gut microbiota', *Birth Defects Research Part C: Embryo Today: Reviews*. John Wiley & Sons, Ltd, 105(4), pp. 240–251.

Bettelli, E. *et al.* (2006) 'Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells', *Nature*, 441(7090), pp. 235–238.

Bevins, C. L. and **Salzman, N. H.** (2011) 'Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis', *Nature Reviews Microbiology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 9, p. 356.

Bhinder, G. *et al.* (2014) 'Intestinal epithelium-specific MyD88 signaling impacts host susceptibility to infectious colitis by promoting protective goblet cell and antimicrobial responses', *Infection and immunity*. 2014/06/23. American Society for Microbiology, 82(9), pp. 3753–3763.

Bibiloni, R. *et al.* (2005) 'VSL # 3 Probiotic-Mixture Induces Remission in Patients with Active Ulcerative Colitis', *American Journal of Gastroenterology*, 100, pp. 1539–1546.

Birkholz, S. *et al.* (1998) 'Decreased *Helicobacter pylori*- Specific Gastric Secretory IgA Antibodies in Infected Patients', *Digestion*, 59(6), pp. 638–645.

Blacher, E. *et al.* (2019) 'Potential roles of gut microbiome and metabolites in modulating ALS in mice', *Nature*.

Blankenhaus, B. *et al.* (2014) 'Foxp3+Regulatory T Cells Delay Expulsion of Intestinal Nematodes by Suppression of IL-9-Driven Mast Cell Activation in BALB/c but Not in C57BL/6 Mice', *PLoS Pathogens*, 10(2), pp. 1–9.

Bloom, S. M. *et al.* (2011) 'Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease', *Cell host & microbe*, 9(5), pp. 390–403.

Bobat, S. *et al.* (2014) 'Natural and Vaccine-Mediated Immunity to Salmonella Typhimurium is Impaired by the Helminth Nippostrongylus brasiliensis', *PLOS Neglected Tropical Diseases*. Public Library of Science, 8(12), p. e3341.

Bocca, C. *et al.* (2007) 'Involvement of PPARγ and E-cadherin/β-catenin pathway in the antiproliferative effect of conjugated linoleic acid in MCF-7 cells', *International Journal of Cancer*, 121(2), pp. 248–256.

Boschetti, G. *et al.* (2016) 'Enrichment of Circulating and Mucosal Cytotoxic

CD8+ T Cells Is Associated with Postoperative Endoscopic Recurrence in Patients with Crohn's Disease', *Journal of Crohn's & colitis*. 2015/11/19. Oxford University Press, 10(3), pp. 338–345.

Boulangé, C. L. *et al.* (2016) 'Impact of the gut microbiota on inflammation, obesity, and metabolic disease', *Genome Medicine*. Genome Medicine, 8(1), pp. 1–12.

Brimnes, J. *et al.* (2001) 'Enteric bacterial antigens activate CD4+ T cells from scid mice with inflammatory bowel disease', *European Journal of Immunology*, 31, pp. 23–31.

Brinkmann, V. *et al.* (2004) 'Neutrophil Extracellular Traps Kill Bacteria', *Science*, 303(5663), pp. 1532 LP – 1535.

Broadhurst, M. J. *et al.* (2010) 'IL-22+ CD4+ T Cells Are Associated with Therapeutic Trichuris trichiura Infection in an Ulcerative Colitis Patient', *Science Translational Medicine*, 2(60), pp. 60ra88 LP-60ra88.

Broadhurst, M. J. *et al.* (2012) 'Upregulation of Retinal Dehydrogenase 2 in Alternatively Activated Macrophages during Retinoid-dependent Type-2 Immunity to Helminth Infection in Mice', *PLoS Pathogens*. Edited by P. J. Murray, 8(8), p. e1002883.

Brombacher, F. (2000) 'The role of interleukin-13 in infectious diseases and allergy', *BioEssays*, 22(7), pp. 646–656.

Burns, A. R. *et al.* (2000) 'Analysis of tight junctions during neutrophil transendothelial migration', *Journal of Cell Science*, 113(1), pp. 45 LP – 57.

Butkus, M. A. *et al.* (2011) 'Inactivation of Ascaris suum by short-chain fatty acids', *Applied and environmental microbiology*. 2010/11/05. American Society for Microbiology (ASM), 77(1), pp. 363–366.

Caballero-Franco, C. *et al.* (2007) 'The VSL # 3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells', *American Journal of Physiology. Gastrointestinal and liver physiology*, 292, pp. 315–322.

Calder, P. C. (2008) 'The relationship between the fatty acid composition of immune cells and their function', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 79(3), pp. 101–108.

Calder, P. C. (2010) 'Omega-3 fatty acids and inflammatory processes', *Nutrients*, 2(3), pp. 355–374.

Campbell, E. L. *et al.* (2010) 'Resolvin E1-induced intestinal alkaline phosphatase promotes resolution of inflammation through LPS detoxification', *Proc Natl Acad Sci USA*, 107.

Candela, M. *et al.* (2008) 'Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production', *International Journal of Food Microbiology*, 125(3), pp. 286–292.

Cani, P. D. *et al.* (2007) 'Metabolic Endotoxemia Initiates Obesity and Insulin Resistance', *Diabetes*, 56(7), pp. 1761 LP – 1772.

Caporaso, J. G. *et al.* (2010) 'QIIME allows analysis of high-throughput community sequencing data', *Nature methods*. 2010/04/11, 7(5), pp. 335–336.

Carroll, I. M. and **Maharshak, N.** (2013) 'Enteric bacterial proteases in inflammatory bowel diseasepathophysiology and clinical implications', *World Journal of Gastroenterology*, 19(43), pp. 7531–7543.

Cătană, C.-S. *et al.* (2015) 'Contribution of the IL-17/IL-23 axis to the pathogenesis of inflammatory bowel disease', *World journal of gastroenterology*. Baishideng Publishing Group Inc, 21(19), pp. 5823–5830.

Cebra, J. J. (1999) 'Influences of microbiota on intestinal immune system development', *The American Journal of Clinical Nutrition*, 69(5), pp. 1046s-1051s.

Chang, B. *et al.* (2013) 'The protective effect of VSL#3 on intestinal permeability in a rat model of alcoholic intestinal injury', *BMC Gastroenterology*, 13(1), pp. 151–159.

Chang, P. V *et al.* (2014) 'The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition', *Proceedings of the National Academy of Sciences*, 111(6), pp. 2247 LP – 2252.

Chapman, R. W. *et al.* (1986) 'Controlled trial of intravenous metronidazole as an adjunct to corticosteroids in severe ulcerative colitis', *Gut*, 27(10), pp. 1210–1212.

Chassaing, B. *et al.* (2014) 'Dextran sulfate sodium (DSS)-induced colitis in mice', *Current Protocols in Immunology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., 18(SUPPL.104), pp. 1–14.

Chassaing, B. *et al.* (2015) 'Lack of soluble fiber drives diet-induced adiposity in mice', *American journal of physiology. Gastrointestinal and liver physiology*. 2015/07/16. American Physiological Society, 309(7), pp. G528–G541.

Chen, C.-C. *et al.* (2005) 'Concurrent Infection with an Intestinal Helminth Parasite Impairs Host Resistance to Enteric Citrobacter rodentium and Enhances Citrobacter-Induced Colitis in Mice', *Infection and Immunity*, 73(9), pp. 5468 LP – 5481.

Chen, F. *et al.* (2012) 'An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection.', *Nature medicine*. Nature Publishing Group, 18(2), pp. 260–6.

Chen, F. *et al.* (2014) 'Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion', *Nature immunology*. 2014/08/31, 15(10), pp. 938–946.

Chen, T. *et al.* (2017) 'Fiber-utilizing capacity varies in Prevotella- versus Bacteroides-dominated gut microbiota', *Scientific Reports*, 7, pp. 1–7.

Chen, Y.-L. *et al.* (2016) 'Early Detection of T cell Transfer-induced Autoimmune Colitis by In Vivo Imaging System', *Scientific Reports*. The Author(s), 6, pp. 1–13.

Cheng, L. *et al.* (2016) 'High fat diet exacerbates dextran sulfate sodium induced colitis through disturbing mucosal dendritic cell homeostasis', *International Immunopharmacology*. Elsevier B.V., 40, pp. 1–10.

Cheon, G. J. *et al.* (2012) 'Mechanisms of motility change on trinitrobenzenesulfonic Acid-induced colonic inflammation in mice', *The Korean journal of physiology & pharmacology*. The Korean Physiological Society and The Korean Society of Pharmacology, 16(6), pp. 437–446.

Chiche, L. *et al.* (2012) 'Seasonal variations of systemic lupus erythematosus flares in southern France', *European Journal of Internal Medicine*, 23(3), pp. 250–254.

Cole, J. R. *et al.* (2007) 'The ribosomal database project (RDP-II): Introducing myRDP space and quality controlled public data', *Nucleic Acids Research*, 35(SUPPL. 1), pp. 169–172.

Coombes, J. L. *et al.* (2007) 'A functionally speciliased population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF beta and a retinoic acid-dependent mechanism', *J Exp Med*, 204, pp. 1757–64.

Correale, J. and **Farez, M.** (2007) 'Association between parasite infection and immune responses in multiple sclerosis', *Annals of Neurology*, 61(2), pp. 97–108.

Corridoni, D. *et al.* (2012) 'Probiotic Bacteria Regulate Intestinal Epithelial Permeability in Experimental Ileitis by a TNF-Dependent Mechanism', *PLOS ONE*. Public Library of Science, 7(7), pp. 1–10.

Cromer, W. E. *et al.* (2013) 'VEGF-A isoform modulation in an preclinical TNBS model of ulcerative colitis : protective effects of a VEGF164 b therapy', *Journal of Translational Medicine*. Journal of Translational Medicine, 11(207), pp. 1–12.

Crosslin, K. L. and **Wiginton, K. L.** (2011) 'Sex Differences in Disease Severity Among Patients With Systemic Lupus Erythematosus', *Gender Medicine*, 8(6), pp. 365–371.

Croxford, A. L. *et al.* (2014) 'IL-12-and IL-23 in health and disease', *Cytokine & Growth Factor Reviews*, 25(4), pp. 415–421.

D'Elia, R. *et al.* (2009) 'Expulsion of Trichuris muris is associated with increased expression of angiogenin 4 in the gut and increased acidity of mucins within the goblet cell', *BMC genomics*. BioMed Central, 10(492), pp. 1–14.

Danese, S. *et al.* (2015) 'Tralokinumab for moderate-to-severe UC: a randomised, double-blind, placebo-controlled, phase IIa study', *Gut*, 64(2), pp. 243 LP – 249.

Darfeuille-Michaud, A. *et al.* (2004) 'High Prevalence of Adherent-Invasive Escherichia coli Associated With Ileal Mucosa in Crohn's Disease', *Gastroenterology*, 127, pp. 412–421.

Datta, R. *et al.* (2005) 'Identification of novel genes in intestinal tissue that are regulated after infection with an intestinal nematode parasite', *Infection and immunity*. American Society for Microbiology, 73(7), pp. 4025–4033.

Deboer, M. D. and **Li, Y.** (2011) 'Puberty Is Delayed in Male Mice With Dextran Sodium Sulfate Colitis Out of Proportion to Changes in Food Intake , Body Weight , and Serum Levels of Leptin', *Pediatric Research*. International Pediatrics Research Foundation, Inc., 69(1), pp. 34–39.

DeMar Jr, J. C. *et al.* (2004) 'Half-lives of docosahexaenoic acid in rat brain phospholipids are prolonged by 15 weeks of nutritional deprivation of n-3 polyunsaturated fatty acids', *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 91(5), pp. 1125–1137.

Derrien, M. *et al.* (2008) 'The Mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract', *Applied and environmental microbiology*. 2007/12/14. American Society for Microbiology (ASM), 74(5), pp. 1646–1648.

DeSantis, T. Z. *et al.* (2006) 'Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB', *Applied and Environmental Microbiology*, 72(7), pp. 5069–5072.

Dickson, I. (2017) 'Gut microbiota: Diagnosing IBD with the gut microbiome', *Nature Reviews Gastroenterology and Hepatology*, 14(4), p. 195.

Dieleman, L. A. *et al.* (1998) 'Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines', *Clinical*

and Experimental Immunology, 114(3), pp. 385–391.

Dieleman, L. a *et al.* (1994) 'Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice.', *Gastroenterology*. Elsevier Inc., 107(6), pp. 1643–52.

Dillehay, D. L. *et al.* (1994) 'Dietary Sphingomyelin Inhibits 1,2-Dimethylhydrazine-Induced Colon Cancer in CF1 Mice', *The Journal of Nutrition*, 124(5), pp. 615–620.

Dittrich, A. M. *et al.* (2008) 'Helminth Infection with Litomosoides sigmodontis Induces Regulatory T Cells and Inhibits Allergic Sensitization, Airway Inflammation, and Hyperreactivity in a Murine Asthma Model', *The Journal of Immunology*, 180(3), pp. 1792–1799.

Dominguez-Bello, M. G. *et al.* (2010) 'Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns', *Proceedings of the National Academy of Sciences*, 107(26), pp. 11971 LP – 11975.

Donskow-Łysoniewska, K. *et al.* (2013) 'Colitis Promotes Adaptation of an Intestinal Nematode: A Heligmosomoides Polygyrus Mouse Model System', *PLoS ONE*, 8(10), pp. 1–15.

Dove, W. F. *et al.* (1997) 'Intestinal Neoplasia in the ApcMin Mouse: Independence from the Microbial and Natural Killer (beige locus) Status', *Cancer Research*, 57(5), pp. 812 LP – 814.

Droste, J. H. *et al.* (2000) 'Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease?', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*, 30(11), pp. 1547–53.

Duarte, A. M. *et al.* (2016) 'Helminth infections and gut microbiota - A feline perspective', *Parasites and Vectors*. Parasites & Vectors, 9(1), pp. 1–9.

Duerr, R. H. *et al.* (2006) 'A Genome-Wide Association Study Identifies IL23R as an Inflammatory Bowel Disease Gene', *Science*, 314(5804), pp. 1461 LP – 1463.

Dunay, I. R. *et al.* (2010) 'Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice', *Infection and Immunity*, 78(4), pp. 1564–1570.

Dutta, S. K. *et al.* (2014) 'Efficacy of Combined Jejunal and Colonic Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection', *Clinical Gastroenterology and Hepatology*, 12(9), pp. 1572–1576.

Dziechciarz, P. *et al.* (2007) 'Meta-analysis : enteral nutrition in active Crohn ' s disease in children', *Alimentary Pharmacology & Therapeutics*, 26(July), pp. 795–806.

Eaden, J. A. *et al.* (2001) 'The risk of colorectal cancer in ulcerative colitis: a meta-analysis', *Gut*, 48(4), pp. 526–535.

Ebert, M. N. *et al.* (2003) 'Expression of glutathione S -transferases (GSTs) in human colon cells and inducibility of GSTM2 by butyrate', *Carcinogenesis*, 24(10), pp. 1637–1644.

Edgar, R. C. (2010) 'Search and clustering orders of magnitude faster than BLAST', *Bioinformatics*, 26(19), pp. 2460–2461.

Elinav, E. *et al.* (2011) 'NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis', *Cell*. Elsevier Inc., 145(5), pp. 745–757.

Elliott, D. E. *et al.* (2003) 'Exposure to schistosome eggs protects mice from TNBS-induced colitis.', *American journal of physiology. Gastrointestinal*

and liver physiology, 284(3), pp. G385-91.

Elliott, D. E. *et al.* (2004) 'Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice', *European Journal of Immunology*, 34(10), pp. 2690–2698.

Elliott, D. E. *et al.* (2008) 'Colonization with Heligmosomoides polygyrus Suppresses Mucosal IL-17 Production', *The Journal of Immunology*, 181, pp. 2414–2419.

Elliott, D. E. and **Weinstock, J. V.** (2012) 'Helminth-host immunological interactions: Prevention and control of immune-mediated diseases', *Annals of the New York Academy of Sciences*, 1247(1), pp. 83–96.

Else, K. J. *et al.* (1993) 'Correlations between worm burden and markers of Th1 and Th2 cell subset induction in an inbred strain of mouse infected with Trichuris muris', *Parasite Immunology*, 15(10), pp. 595–600.

Elson, C. O. *et al.* (1995) 'Experimental Models of Inflammatory Bowel Disease', *Gastroenterology*, 109, pp. 1344–1367.

Elson, C. O. *et al.* (2004) 'Anti–Interleukin-12 Antibody for Active Crohn's Disease', *The New England Journal Of Medicine*, 351, pp. 2069–2079.

Erard, F. and **Le Gros, G.** (1994) 'Th2-like CD8 T cells: their role in protection against infectious diseases.', *Parasitology today (Personal ed.)*, 10(8), pp. 313–5.

Erdman, S. E. *et al.* (2003) 'CD4 + CD25 + Regulatory Lymphocytes Require Interleukin 10 to Interrupt Colon Carcinogenesis in Mice Carcinogenesis in Mice 1', *Cancer Research*, pp. 6042–6050.

Evans, N. P. *et al.* (2010) 'Conjugated linoleic acid ameliorates inflammation-induced colorectal cancer in mice through activation of PPARg 1 – 3', *Cellulose*, 140, pp. 515–521.

Fallon, P. G. and **Mangan, N. E.** (2007) 'Suppression of TH2-type allergic reactions by helminth infection.', *Nature reviews. Immunology*, 7(3), pp. 220–30.

Farley, T. M. M. and **Sivin, I.** (2001) 'International Collaboration Post Marketing Surveillance of Norplant.', *Contraception*, 63, pp. 187–20.

Feagins, L. A. *et al.* (2009) 'Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer', *Nature Reviews Gastroenterology and Hepatology*. Nature Publishing Group, 6(5), pp. 297–305.

Feary, J. R. *et al.* (2010) 'Experimental hookworm infection: a randomized placebo-controlled trial in asthma', *Clinical & Experimental Allergy*. John Wiley & Sons, Ltd (10.1111), 40(2), pp. 299–306.

Feldman, C. H. *et al.* (2013) 'Epidemiology and Sociodemographics of Systemic Lupus Erythematosus and Lupus Nephritis Among US Adults With Medicaid Coverage , 2000 – 2004', *Arthritis and Rheumatism*, 65(3), pp. 753–763.

Fell, J. M. *et al.* (2000) 'Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease', *Alimentary pharmacology & therapeutics*. England, 14(3), pp. 281–289.

Ferrucci, L. *et al.* (2006) 'Relationship of Plasma Polyunsaturated Fatty Acids to Circulating Inflammatory Markers', *The Journal of Clinical Endocrinology & Metabolism*, 91(2), pp. 439–446.

Filbey, K. J. *et al.* (2014) 'Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection',

Immunology and Cell Biology. Nature Publishing Group, 92(5), pp. 436–448.

De Filippo, C. *et al.* (2010) 'Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa', *Proceedings of the National Academy of Sciences*, 107(33), pp. 14691–14696.

Finkelman, F. D. *et al.* (2004) 'Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites', *Immunological Reviews*, 201, pp. 139–155.

Finney, C. A. M. *et al.* (2007) 'Expansion and activation of CD4+CD25+ regulatory T cells in Heligmosomoides polygyrus infection', *European Journal of Immunology*, 37(7), pp. 1874–1886.

Fleming, J. O. (2013) 'Helminth therapy and multiple sclerosis', *International Journal for Parasitology*, 43(3–4), pp. 259–274.

Flohr, C. and **Yeo, L.** (2011) 'Atopic dermatitis and the hygiene hypothesis revisited.', *Current Problems in Dermatology, Volume 41*, 41, pp. 1–34.

Fournier, B. M. and **Parkos, C. A.** (2012) 'The role of neutrophils during intestinal inflammation', *Mucosal Immunology*. Society for Mucosal Immunology, 5(4), pp. 354–66.

Fox, J. G. *et al.* (2000) 'Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy', *Nature Medicine*, 6(5), pp. 536–542.

Fox, J. G. *et al.* (2010) 'Helicobacter hepaticus infection in mice: models for understanding lower bowel inflammation and cancer', *Mucosal Immunology*. Society for Mucosal Immunology, 4(1), pp. 1–22.

Frank, D. N. *et al.* (2007) 'Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases', *Proceedings of the National Academy of Sciences*, 104(34), pp. 13780 LP – 13785.

Franke, A. *et al.* (2008) 'Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility', *Nature Genetics*. Nature Publishing Group, 40(11), pp. 1319–23.

Frantz, A. L. *et al.* (2012) 'Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides', *Mucosal immunology*. 2012/04/11. Nature Publishing Group, 5(5), pp. 501–512.

Fried, B. *et al.* (2011) 'Helminths in human carcinogenesis', *Cancer Letters*. Elsevier Ireland Ltd, 305(2), pp. 239–249.

Fristche, K. L. (2008) 'Too much linoleic acid promotes inflammation — doesn ' t it ?', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 79, pp. 173–175.

Fuchs, T. A. *et al.* (2007) 'Novel cell death program leads to neutrophil extracellular traps', *The Journal of cell biology*. 2007/01/08. The Rockefeller University Press, 176(2), pp. 231–241.

Furusawa, Y. *et al.* (2013) 'Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 504, pp. 446–450.

Fuss, I. J. and **Strober, W.** (2008) 'The role of IL-13 and NK T cells in

experimental and human ulcerative colitis', *Mucosal Immunology*, 1(November), pp. 31–33.

Gagliani, N. *et al.* (2015) 'TH17 cells transdifferentiate into regulatory T cells uring resolution of inflammation', *Nature*, 523(7559), pp. 221–225.

Ganesh, B. P. *et al.* (2013) 'Commensal Akkermansia muciniphila Exacerbates Gut Inflammation in Salmonella Typhimurium-Infected Gnotobiotic Mice', *PLOS ONE*. Public Library of Science, 8(9), p. e74963.

Gardiner, K. R. *et al.* (1993) 'Colonic bacteria and bacterial translocation in experimental colitis', *British Journal of Surgery*, 80(4), pp. 512–516.

Garud, S. and **Peppercorn, M. A.** (2009) 'Ulcerative colitis: current treatment strategies and future prospects', *Therapeutic advances in gastroenterology*. SAGE Publications, 2(2), pp. 99–108.

Gaudier, E. *et al.* (2004) 'Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 287(6), pp. G1168–G1174.

Gaudier, E. *et al.* (2005) 'The VSL # 3 Probiotic Mixture Modifies Microflora but Does Not Heal Chronic Dextran-Sodium Sulfate – Induced Colitis or Reinforce the Mucus Barrier in Mice', *American Society for Nutrition*, (August), pp. 2753–2761.

Geem, D. *et al.* (2016) 'Contribution of Mesenteric Lymph Nodes and GALT to the', *Cellular and Molecular Gastroenterology and Hepatology*. Elsevier Inc, 2(3), pp. 274-280.e3.

Geerling, B. J. *et al.* (2000) 'Diet as a Risk Factor for the Development of Ulcerative Colitis', *The American Journal of Gastroenterology*, 95(4), pp. 2–7.

Gelderblom, M. *et al.* (2012) 'Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke', *The American Society of Hematology*, 120(18), pp. 3793–3803.

Gersemann, M. *et al.* (2009) 'Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis', *Differentiation; research in biological diversity*. 2008/10/22. England, 77(1), pp. 84–94.

Ghosh, S. *et al.* (2002) 'Missing Pieces in the NF-kB Puzzle', *Cell*, 109, pp. 81–96.

Ghosh, S. *et al.* (2013) 'Diets rich in n-6 PUFA induce intestinal microbial dysbiosis in aged mice', *British Journal of Nutrition*. 2013/01/08. Cambridge University Press, 110(3), pp. 515–523.

Giacinto, C. Di *et al.* (2005) 'Probiotics Ameliorate Recurrent Th1-Mediated Murine Colitis by Inducing IL-10 and IL-10-Dependent TGF-β-Bearing Regulatory Cells', *The Journal of Immunology*, 174(6), pp. 3237 LP – 3246.

Gilat, T. *et al.* (1987) 'A comparative study of metronidazole and sulfasalazine in active, not severe, ulcerative colitis. An Israeli multicenter trial', *Journal of clinical gastroenterology*. United States, 9(4), pp. 415–417.

Gîlca, G.-E. *et al.* (2017) 'Sarcoidosis associated with infliximab therapy in ulcerative colitis: A case report', *Medicine*, 96(10), pp. 1–6.

Gionchetti, P. *et al.* (2003) 'Prophylaxis of pouchitis onset with probiotic therapy: A double-blind, placebo-controlled trial', *Gastroenterology*, 124(5), pp. 1202–1209.

Glocker, E.-O. *et al.* (2011) 'IL-10 and IL-10 receptor defects in humans', *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd

(10.1111), 1246(1), pp. 102–107.

Golińska, E. *et al.* (2013) 'Virulence factors of Enterococcus strains isolated from patients with inflammatory bowel disease', *World Journal of Gastroenterology*, 19(23), pp. 3562–3572.

Goodroe, A. E. *et al.* (2016) 'Guidance regarding sample collection and refinement of fecal flotation exam for the isolation of Aspiculuris tetraptera.', *Journal of the American Association for Laboratory Animal Science*, 55(5), pp. 541–547.

Goodwin, A. C. *et al.* (2011) 'Polyamine catabolism contributes to enterotoxigenic Bacteroides fragilis-induced colon tumorigenesis', *Proceedings of the National Academy of Sciences*, 108(37), pp. 15354–15359.

Gout, T. *et al.* (2011) 'Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: A systematic literature review', *Clinical Rheumatology*, 30(11), pp. 1471–1474.

Grainger, J. R. *et al.* (2010) 'Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway.', *The Journal of experimental medicine*, 207(11), pp. 2331–41.

Gravaghi, C. *et al.* (2011) 'Cox-2 expression , PGE 2 and cytokines production are inhibited by endogenously synthesized n -3 PUFAs in inflamed colon of fat-1 mice', *The Journal of Nutritional Biochemistry*. Elsevier Inc., 22(4), pp. 360–365.

Gregor, M. F. and **Hotamisligil, G. S.** (2011) 'Inflammatory Mechanisms in Obesity', *Annual Review of Immunology*. Annual Reviews, 29(1), pp. 415–445.

Griffiths, A. M. *et al.* (1993) 'Growth and clinical course of children with Crohn's disease', *Gut*, 34(7), pp. 939–943.

Griffiths, A. M. *et al.* (1995) 'Meta-analysis of enteral nutrition as a primary treatment of active Crohn's disease', *Gastroenterology*. York (UK): Centre for Reviews and Dissemination (UK), 108(4), pp. 1056–1067.

Griffiths, A. M. (2000) 'Enteral Nutrition: The Neglected Primary Therapy of Active Crohn's Disease', *Journal of Pediatric Gastroenterology and Nutrition*, 31(1), pp. 3–5.

Griswold, D. E. *et al.* (1974) 'Quantification and pharmacological dissection of oxazolone-induced contact sensitivity in the mouse', *Cellular Immunology*, 11(1–3), pp. 198–204.

Groß, P. (no date) '# 9530 DRAFT Sharing ideas , sharing success Analysis of colonic bacteria translocation in colitic mice using the gentleMACS ™ Dissociator', pp. 1–2.

Gugler, R. *et al.* (1989) 'The course of Crohn disease and side effect profile with long-term treatment using metronidazole TT', *Zeitschrift fur Gastroenterologie*. Germany, 27(11), pp. 676–682.

Günal, Ö. *et al.* (2003) 'Estradiol Treatment Ameliorates Acetic Acid-Induced Gastric and Colonic Injuries in Rats', *Inflammation*, 27(6), pp. 351–359.

Guo, X. *et al.* (2008) 'Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs', *Letters in Applied Microbiology*, 47, pp. 367–373.

Gupta, N. *et al.* (2007) 'Gender Differences in Presentation and Course of Disease in Pediatric Patients With Crohn Disease', *Pedriatics*, 120, pp. 1–

10.

Guthrie, L. A. *et al.* (1984) 'Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme', *The Journal of experimental medicine*. The Rockefeller University Press, 160(6), pp. 1656–1671.

Gutin, L. *et al.* (2019) 'Fecal microbiota transplant for Crohn disease : A study evaluating safety , efficacy , and microbiome profile', *United European Gastroenterology Journal*, 7(6), pp. 807–814.

Haberman, Y. *et al.* (2014) 'Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature', *The Journal of clinical investigation*. 2014/07/08. American Society for Clinical Investigation, 124(8), pp. 3617–3633.

Haberman, Y. *et al.* (2015) 'Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature Find the latest version : Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature', (March).

Haggar, F. and **Boushey, R.** (2009) 'Colorectal Cancer Epidemiology: Incidence, Mortality, Survival, and Risk Factors', *Clinics in Colon and Rectal Surgery*, 22(04), pp. 191–197.

Hanauer, S. (2004) 'Review article : aminosalicylates in inflammatory bowel disease', *Ailment Pharmacol Ther*, 20(Suppl.4), pp. 60–65.

Hang, L. *et al.* (2010) 'Heligmosomoides polygyrus Infection Can Inhibit Colitis through Direct Interaction with Innate Immunity', *The Journal of Immunology*, 185(6), pp. 3184–3189.

Hang, L. *et al.* (2014) 'Heligmosomoides polygyrus baker infection activates colonic Foxp3+ T cells enhancing their capacity to prevent colitis', *J Immunol*, 191(4), pp. 617–636.

Harig, J. M. *et al.* (1989) 'Treatment of Diversion Colitis with Short-Chain-Fatty Acid Irrigation', *New England Journal of Medicine*. Massachusetts Medical Society, 320(1), pp. 23–28.

Harnish, D. C. *et al.* (2004) 'Beneficial effects of estrogen treatment in the HLA-B27 transgenic rat model of inflammatory bowel disease', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 286(1), pp. G118–G125.

Hart, A. R. *et al.* (2008) 'Diet in the Aetiology of Ulcerative Colitis: A European Prospective Cohort Study', *Digestion*, 77(1), pp. 57–64.

Hartman, A. L. *et al.* (2009) 'Human gut microbiome adopts an alternative state following small bowel transplantation', *Proceedings of the National Academy of Sciences of the United States of America*. 2009/09/17. National Academy of Sciences, 106(40), pp. 17187–17192.

Hawthorne, A. B. *et al.* (1992) 'Treatment of ulcerative colitis with fish oil supplementation : a prospective 12 month randomised controlled trial', *Gut*, 33, pp. 922–928.

Heijmans, J. *et al.* (2014) 'Oestrogens promote tumorigenesis in a mouse model for colitis-associated cancer', *Gut*, 63(2), pp. 310 LP – 316.

Heinsbroek, S. E. M. and **Gordon, S.** (2009) 'The role of macrophages in inflammatory bowel diseases', *Expert Reviews*, 11(May), pp. 1–19.

Heller, F. *et al.* (2002) 'Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells', *Immunity*, 17(5), pp. 629–638.

Heller, F. *et al.* (2005) 'Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution', *Gastroenterology*, 129(2), pp. 550–564.

Helmby, H. (2015) 'Human helminth therapy to treat inflammatory disorders-where do we stand?', *BMC Immunology*, 16(1), p. 12.

Hepworth, M. R. and **Grencis, R. K.** (2009) 'Disruption of Th2 immunity results in a gender-specific expansion of IL-13 producing accessory NK cells during helminth infection.', *Journal of immunology (Baltimore, Md. : 1950)*, 183(6), pp. 3906–14.

Hermiston, M. L. and **Gordon, J. I.** (1995) 'Inflammatory Bowel Disease and Adenomas in Mice Expressing a Dominant Negative N-Cadherin', *Science*, 270(5239), pp. 1203 LP – 1207.

Hernández-Ruiz, J. *et al.* (2010) 'CD8 cells of patients with diffuse cutaneous leishmaniasis display functional exhaustion: the latter is reversed, in vitro, by TLR2 agonists.', *PLoS neglected tropical diseases*. Edited by G. Milon, 4(11), p. e871.

Hoermannsperger, G. *et al.* (2009) 'Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation', *PloS one*. 2009/02/06. Public Library of Science, 4(2), pp. e4365–e4365.

Holland, M. J. *et al.* (2000) 'Proteins secreted by the parasitic nematode Nippostrongylus brasiliensis act as adjuvants for Th2 responses', *European Journal of Clinical Microbiology and Infectious Diseases*, 30, pp. 1977–1987.

Holmén, N. *et al.* (2006) 'Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity', *Inflammatory Bowel Diseases*, 12(6), pp. 447–456.

Horton, H. A. *et al.* (2014) 'Antibiotics for Treatment of Clostridium difficile Infection in Hospitalized Patients with Inflammatory Bowel Disease', *Antimicrobial Agents and Chemotherapy*, 58(9), pp. 5054–5059.

Hotez, P. J. *et al.* (2008) 'Helminth infections: the great neglected tropical diseases', *The Journal of clinical investigation*, 118(4), pp. 1311–1321.

Hotez, P. J. and **Kamath, A.** (2009) 'Neglected tropical diseases in sub-Saharan Africa: Review of their prevalence, distribution, and disease burden', *PLoS Neglected Tropical Diseases*, 3(8), pp. 2–11.

Houlden, A. *et al.* (2015) 'Chronic Trichuris muris Infection in C57BL/6 Mice Causes Significant Changes in Host Microbiota and Metabolome: Effects Reversed by Pathogen Clearance', *PLOS ONE*. Public Library of Science, 10(5), p. e0125945.

Hoving, J. C. *et al.* (2012) 'B Cells That Produce Immunoglobulin E Mediate Colitis in BALB/c Mice', *Gastroenterology*. Elsevier Inc., 142(1), pp. 96–108.

Hoving, J. C. *et al.* (2017) 'Interleukin 13-mediated colitis in the absence of IL-4R α signalling', *Gut*, 0, pp. 2016–2017.

Hoving, J. C. (2018) 'Targeting IL-13 as a Host-Directed Therapy Against Ulcerative Colitis', *Frontiers in Cellular and Infection Microbiology*, 8(November), pp. 6–11.

Hrncir, T. *et al.* (2007) 'Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells', *Inflammatory Bowel Diseases*, 13(10), pp.

1202–1211.

Hume, G. E. *et al.* (2012) 'Common PPARγ variants C161T and Pro12Ala are not associated with inflammatory bowel disease in an Australian cohort', *Journal of gastrointestinal and liver diseases : JGLD*. Romania, 21(4), pp. 349–355.

Hunter, M. M. *et al.* (2007) 'Helminth Infection Enhances Disease in a Murine TH2 Model of Colitis', *Gastroenterology*, 132(4), pp. 1320–1330.

Hyun, Y. S. *et al.* (2012) 'Role of IL-17A in the development of colitis-associated cancer', *Carcinogenesis*, 33(4), pp. 931–936.

Iijima, H. *et al.* (2004) 'Specific regulation of T helper cell 1-mediated murine colitis by CEACAM1.', *The Journal of experimental medicine*, 199(4), pp. 471–482.

Ishikawa, D. *et al.* (2017) 'Changes in Intestinal Microbiota Following Combination Therapy with Fecal Microbial Transplantation and Antibiotics for Ulcerative Colitis', *Inflammatory bowel diseases*. England, 23(1), pp. 116–125.

Isidro, R. A. *et al.* (2017) 'The Probiotic VSL#3 Modulates Colonic Macrophages, Inflammation, and Microflora in Acute Trinitrobenzene Sulfonic Acid Colitis', *Journal of Histochemistry & Cytochemistry*. Journal of Histochemistry & Cytochemistry, 65(8), pp. 445–461.

Isik, F. *et al.* (2011) 'Protective Effects of Black Cumin (Nigella sativa) Oil on TNBS-Induced Experimental Colitis in Rats', *Digestive Diseases and Sciences*, 56(3), pp. 721–730.

Isseroff, R. R. *et al.* (1987) 'Conversion of linoleic acid into arachidonic acid by cultured murine and human keratinocytes.', *Journal of Lipid Research* , 28(11), pp. 1342–1349.

Ito, R. *et al.* (2006) 'Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice', *Clinical and experimental immunology*. Blackwell Science Inc, 146(2), pp. 330–338.

Ito, R. *et al.* (2008) 'Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice', *Biochemical and Biophysical Research Communications*, 377(1), pp. 12–16.

Ivanov, I. I. *et al.* (2009) 'Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria', *Cell*. Elsevier Ltd, 139(3), pp. 485–498.

Jana, Š. *et al.* (2015) 'Co-administration of a probiotic strain Lactobacillus plantarum LS / 07 CCM7766 with prebiotic inulin alleviates the intestinal in fl ammation in rats exposed to N , N-dimethylhydrazine', *International Immunopharmacology*, 24, pp. 361–368.

Jemal, A. *et al.* (2012) 'Cancer burden in Africa and opportunities for prevention', *Cancer*. Nature Publishing Group, 118(18), pp. 4372–4384.

Johansson, M. E. V. and **Hansson, G. C.** (2016) 'Immunological aspects of intestinal mucus and mucins', *Nature Reviews Immunology*. Nature Publishing Group, pp. 639–649.

Johansson, M. E. V *et al.* (2008) 'The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.', *Proceedings of the National Academy of Sciences*, 105(39), pp. 15064–15069.

Johnston, C. J. C. *et al.* (2015) 'Cultivation of Heligmosomoides Polygyrus: An Immunomodulatory Nematode Parasite and its Secreted Products', *Journal of Visualized Experiments*, (98), pp. 1–10.

Jones, G.-R. *et al.* (2018) 'Dynamics of Colon Monocyte and Macrophage

Activation During Colitis', *Frontiers in Immunology*, pp. 1–14.

Kamath, P. S. *et al.* (1988) 'Short-Chain Fatty Acids Stimulate Ileal Motility in Humans', *Gastroenterology*. American Gastroenterological Association, 95(6), pp. 1496–1502.

Kansal, S. *et al.* (2014) 'Alterations in lipid mediated signaling and Wnt/β-catenin signaling in DMH induced colon cancer on supplementation of fish oil', *BioMed Research International*. Hindawi Publishing Corporation, 2014.

Kaplan, G. G. (2015) 'The global burden of IBD: from 2015 to 2025', *Nature Publishing Group*. Nature Publishing Group, 12(12), pp. 720–727.

Karlsson, J. *et al.* (2008) 'Regional variations in Paneth cell antimicrobial peptide expression along the mouse intestinal tract', *BMC immunology*. BioMed Central, 9, p. 37.

Kaser, A. and **Blumberg, R. S.** (2011) 'Lessons from type I interferons in ulcerative colitis', *Gut*. 2011/01/25, 60(4), pp. 430–431.

Kato, J. (1989) 'Arachidonic acid as a possible modulator of estrogen, progestin, androgen and glucocorticod receptors in the central and peripheral tissues', *Journal of steroid Biochem*, 34, pp. 219–227.

Kespohl, M. *et al.* (2017) 'The Microbial Metabolite Butyrate Induces expression of Th1- Sssociated Factors in CD4 + T cells', *Frontiers in Immunology*, 8(August), pp. 1–12.

Keubler, L. M. *et al.* (2015) 'A Multihit Model: Colitis Lessons from the Interleukin-10-deficient Mouse', *Inflammatory bowel diseases*. Lippincott Williams & Wilkins, 21(8), pp. 1967–1975.

Khan, H. M. W. *et al.* (2015) 'Optimal management of steroid-dependent ulcerative colitis', *Clinical and Experimental Gastroenterology*, 8, pp. 293–302.

Kiesler, P. *et al.* (2015) 'Experimental Models of Inflammatory Bowel Diseases', *CMGH Cellular and Molecular Gastroenterology and Hepatology*, 1(2), pp. 154–170.

Kim, C. H. (2010) 'Homeostatic and pathogenic extramedullary hematopoiesis', *Journal of Blood Medicine*, 1, pp. 13–19.

Kim, I. *et al.* (2000) 'Mucosal mast cell responses in the small intestine of rats infected with Echinostoma hortense', *The Korean Journal of Parasitology*, 38(3), pp. 139–143.

Kim, K.-A. *et al.* (2012) 'High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway', *PloS one*. Public Library of Science, 7(10), p. e47713.

Kim, T. W. *et al.* (2006) 'Involvement of lymphocytes in dextran sulfate sodium- induced experimental colitis', *World Journal of Gastroenterology*, 12(2), pp. 302–305.

Kim, Y.-I. *et al.* (2016) 'Compensatory roles of CD8+ T cells and plasmacytoid dendritic cells in gut immune regulation for reduced function of CD4+ Tregs', *Oncotarget*. Impact Journals LLC, 7(10), pp. 10947–10961.

Kim, Y. S. *et al.* (2011) 'Th17 responses are not induced in dextran sodium sulfate model of acute colitis', *Immune Netw*, 11(6), pp. 416–419.

Kim, Y. S. and **Ho, S. B.** (2010) 'Intestinal goblet cells and mucins in health and disease: Recent insights and progress', *Current Gastroenterology Reports*, 12(5), pp. 319–330.

Kitajima, S. *et al.* (1999) 'Tissue Distribution of Dextran Sulfate Sodium (DSS)

in the Acute Phase of Murine DSS-Induced Colitis', *Journal of Veterinary Medical Science*, 61(1), pp. 67–70.

Kjellev, S. *et al.* (2006) 'Reconstitution of Scid mice with CD4+CD25− T cells leads to rapid colitis: An improved model for pharmacologic testing', *International Immunopharmacology*, 6(8), pp. 1341–1354.

Kmieć, Z. *et al.* (2017) 'Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease', *Advances in Medical Sciences*, 62(1), pp. 1–16.

Kobayashi, S. D. *et al.* (2003) 'Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils', *Proceedings of the National Academy of Sciences*, 100(19), pp. 10948 LP – 10953.

Kojima, R. *et al.* (2004) 'Oxazolone-Induced Colitis in BALB / C Mice : a New Method to Evaluate the Efficacy of Therapeutic Agents for Ulcerative Colitis', *J Pharmacol Sci*, 313, pp. 307–313.

Kolaczkowska, E. and **Kubes, P.** (2013) 'Neutrophil recruitment and function in health and inflammation', *Nature Reviews Immunology*. Nature Publishing Group, 13(3), pp. 159–175.

Kontoyiannis, D. *et al.* (1999) 'Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies', *Immunity*. Elsevier, 10(3), pp. 387–398.

Korn, T. *et al.* (2009) 'IL-17 and Th17 Cells', *Annual Review of Immunology*, 27(1), pp. 485–517.

Kornbluth, A. (2011) 'Cyclosporine versus infliximab for the treatment of severe ulcerative colitis', *Gastroenterology & hepatology*. Millennium Medical Publishing, 7(10), pp. 677–679.

Korsgren, M. *et al.* (1999) 'Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice.', *The Journal of Experimental Medicine*, 189(3), pp. 553–62.

Krane, M. K. *et al.* (2013) 'Preoperative infliximab therapy does not increase morbidity and mortality after laparoscopic resection for inflammatory bowel disease', *Diseases of the colon and rectum*. United States, 56(4), pp. 449–457.

Kreider, T. *et al.* (2007) 'Alternatively activated macrophages in helminth infections', *Current Opinion in Immunology*, 19(4), pp. 448–453.

Kreisinger, J. *et al.* (2015) 'Interactions between multiple helminths and the gut microbiota in wild rodents', *Philosophical Transactions of the Royal Society B*, 370(1675), pp. 1–13.

Kruis, W. *et al.* (1985) 'Contractile patterns and transit of fluid in canine terminal ileum', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 249(2), pp. G264–G270.

Kruis, W. *et al.* (1997) 'Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis', *Alimentary Pharmacology and Therapeutics*, 11, pp. 853–858.

Kruis, W. *et al.* (2004) 'Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine', *Gut*, 53, pp. 1617–1623.

Kuehl, C. J. *et al.* (2005) 'Colonization of the cecal mucosa by Helicobacter hepaticus impacts the diversity of the indigenous microbiota', *Infection and immunity*. American Society for Microbiology, 73(10), pp. 6952–6961.

Kühn, R. *et al.* (1993) 'Interleukin-10-deficient mice develop chronic enterocolitis', *Cell*, 75(2), pp. 263–274.

Kunitake, H. *et al.* (2008) 'Perioperative treatment with infliximab in patients with Crohn's disease and ulcerative colitis is not associated with an increased rate of postoperative complications', *Journal of gastrointestinal surgery*. 2008/08/16. United States, 12(10), pp. 1730–1737.

Kunstyr, I. and **Nicklas, W.** (2000) 'Chapter 8: Control of SPF Conditions: FELASA Standards', in Krinke, G. J. B. T.-T. L. R. (ed.) *Handbook of Experimental Animals*. London: Academic Press, pp. 133–142.

Kuriyama, A. *et al.* (2011) 'Metronidazole-Induced Central Nervous System Toxicity: A Systematic Review', *Clinical neuropharmacology*, 34, pp. 241–247.

de la Cuesta-Zuluaga, J. *et al.* (2019) 'Age- and Sex-Dependent Patterns of Gut Microbial Diversity in Human Adults', *mSystems*. Edited by S. Baranzini, 4(4), pp. e00261-19.

De la Fuente, M. *et al.* (2014) 'Escherichia coli isolates from inflammatory bowel diseases patients survive in macrophages and activate NLRP3 inflammasome', *International journal of medical microbiology : IJMM*. 2014/02/06, 304(3–4), pp. 384–392.

Lakatos, P.-L. (2008) 'Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies', *World Journal of Gastroenterology*, 14(25), p. 3937.

Lanne, B. *et al.* (1995) 'Enhanced binding of enterotoxigenic Escherichia coli K99 to amide derivatives of the receptor ganglioside NeuGc-GM3', *Biochemistry*. United States, 34(6), pp. 1845–1850.

Laroui, H. *et al.* (2012) 'Dextran sodium sulfate (dss) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon', *PloS one*. Public Library of Science, 7(3), pp. e32084–e32084.

Leach, S. T. *et al.* (2008) 'Sustained modulation of intestinal bacteria by exclusive enteral nutrition used to treat children with Crohn's disease', *Alimentary Pharmacology & Therapeutics*. John Wiley & Sons, Ltd (10.1111), 28(6), pp. 724–733.

Lebeis, S. L. *et al.* (2007) 'TLR Signaling Mediated by MyD88 Is Required for a Protective Innate Immune Response by Neutrophils to Citrobacter rodentium', *The Journal of Immunology*, 179(1), pp. 566 LP – 577.

Ledesma-Soto, Y. *et al.* (2015) 'Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins', *BioMed Research International*, 2015, pp. 1–17.

Lee, J. H. *et al.* (2009) 'FoxP3+ Regulatory T Cells Restrain Splenic Extramedullary Myelopoiesis via Suppression of Hemopoietic Cytokine-Producing T Cells', *The Journal of Immunology*, 183(10), pp. 6377–6386.

Lee, K.-H. *et al.* (2014) 'Bacterial β-(1,3)-glucan prevents DSS-induced IBD by restoring the reduced population of regulatory T cells', *Immunobiology*, 219(10), pp. 802–812.

Lee, S. C. *et al.* (2014) 'Helminth Colonization Is Associated with Increased Diversity of the Gut Microbiota', *PLOS Neglected Tropical Diseases*. Public Library of Science, 8(5), p. e2880.

Lee, S. H. *et al.* (2018) 'Immunological pathogenesis of inflammatory bowel disease', *Intestinal research*. 2018/01/18. Korean Association for the Study

of Intestinal Diseases, 16(1), pp. 26–42.

León-Cabrera, S. *et al.* (2014) 'Extraintestinal Helminth Infection Reduces the Development of Colitis-Associated Tumorigenesis', *International Journal of Biological Sciences*, 10(9), pp. 948–956.

Levy, M. *et al.* (2015) 'Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling', *Cell*, 163(6), pp. 1428–1443.

Ley, K. *et al.* (2006) 'IL-17A-producing neutrophil-regulatory Tn lymphocytes', *Immunologic Research*, 34(3), pp. 229–242.

Li, J. *et al.* (2012) 'Neutralisation of Peritoneal IL-17A Markedly Improves the Prognosis of Severe Septic Mice by Decreasing Neutrophil Infiltration and Proinflammatory Cytokines', *PLoS ONE*, 7(10).

Li, R. W. *et al.* (2016) 'The effect of helminth infection on the microbial composition and structure of the caprine abomasal microbiome', *Scientific Reports*. Nature Publishing Group, 6, pp. 1–10.

Liao, C.-M. *et al.* (2013) 'The functions of type I and type II natural killer T cells in inflammatory bowel diseases', *Inflammatory bowel diseases*, 19(6), pp. 1330–1338.

Lichtiger, S. (2006) 'The Role of Cyclosporine Therapy in Ulcerative Colitis Treatment', *Gastroenterology & hepatology*. Millennium Medical Publishing, 2(9), pp. 624–626.

Licona-Limón, P. *et al.* (2013) 'TH2, allergy and group 2 innate lymphoid cells.', *Nature immunology*. Nature Publishing Group, 14(6), pp. 536–42.

Linares, D. M. *et al.* (2017) 'Lactic Acid Bacteria and Bifidobacteria with Potential to Design Natural Biofunctional Health-Promoting Dairy Foods ', *Frontiers in Microbiology* , p. 846.

Lindebo Holm, T. *et al.* (2012) 'Pharmacological Evaluation of the SCID T Cell Transfer Model of Colitis: As a Model of Crohn's Disease', *International journal of inflammation*. 2012/02/19. Hindawi Publishing Corporation, 2012, pp. 1–11.

Little, J. R. and **Eisen, H. N.** (1966) 'Preparation and characterization of antibodies specific for the 2,4,6-trinitrophenyl group', *Biochemistry*. United States, 5(11), pp. 3385–3395.

Litvak, Y. *et al.* (2017) 'Dysbiotic Proteobacteria expansion: a microbial signature of epithelial dysfunction', *Current Opinion in Microbiology*, 39, pp. 1–6.

Liu, L. X. *et al.* (1990) 'Intravascular filarial parasites elaborate cyclooxygenase-derived eicosanoids.', *The Journal of Experimental Medicine*, 172(3), pp. 993 LP – 996.

van der Logt, E. M. J. *et al.* (2013) 'Westernized high-fat diet accelerates weight loss in dextran sulfate sodium-induced colitis in mice, which is further aggravated by supplementation of heme', *The Journal of Nutritional Biochemistry*, 24(6), pp. 1159–1165.

Lozupone, C. and **Knight, R.** (2005) 'UniFrac : a New Phylogenetic Method for Comparing Microbial Communities UniFrac : a New Phylogenetic Method for Comparing Microbial Communities', *Applied and environmental microbiology*, 71(12), pp. 8228–8235.

Lund, J. M. *et al.* (2008) 'Coordination of Early Protective Immunity to Viral Infection by Regulatory T Cells', *Science*, 320(5880), pp. 1220 LP – 1224.

Lupp, C. *et al.* (2007) 'Host-Mediated Inflammation Disrupts the Intestinal

Microbiota and Promotes the Overgrowth of Enterobacteriaceae', *Cell Host & Microbe*, 2(2), pp. 119–129.

Luu, M. *et al.* (2019) 'The short-chain fatty acid pentanoate suppresses autoimmunity by modulating the metabolic-epigenetic crosstalk in lymphocytes', *Nature Communications*. Springer US, 10(1), p. 760.

Ma, X. *et al.* (2008) 'High-fat diet modulates non-CD1d-restricted natural killer T cells and regulatory T cells in mouse colon and exacerbates experimental colitis', *Clinical and experimental immunology*. Blackwell Science Inc, 151(1), pp. 130–138.

Maccaferri, S. *et al.* (2012) 'In vitro fermentation of potential prebiotic flours from natural sources: Impact on the human colonic microbiota and metabolome', *Molecular Nutrition and Food Research*, 56(8), pp. 1342–1352.

Madsen, K. L. *et al.* (1999) 'Species Prevents Colitis in Interleukin 10 Gene–Deficient Mice', pp. 1107–1114.

Mähler, M. *et al.* (1998) 'Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis function in mice Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis', *American Journal Physiology Gastrointestinal Liver Physiology*, 274, pp. G544–G551.

Maizels, Rick M *et al.* (2012) 'Experimental Parasitology Immune modulation and modulators in Heligmosomoides polygyrus infection', *Experimental Parasitology*. Elsevier Inc., 132(1), pp. 76–89.

Maizels, Rick M. *et al.* (2012) 'Immune modulation and modulators in Heligmosomoides polygyrus infection', *Experimental Parasitology*. Elsevier Inc., 132(1), pp. 76–89.

Maizels, R. M. and **Yazdanbakhsh, M.** (2003) 'Immune Regulation by helminth parasites: cellular and molecular mechanisms', *Nature Reviews Immunology*, 3(9), pp. 733–744.

Mangan, P. R. *et al.* (2006) 'Transforming growth factor-β induces development of the TH17 lineage', *Nature*, 441(7090), pp. 231–234.

Manichanh, C. *et al.* (2006) 'Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach', *Gut*, 55(2), pp. 205–211.

Mann, E. R. and **Li, X.** (2014) 'Intestinal antigen-presenting cells in mucosal immune homeostasis: crosstalk between dendritic cells, macrophages and B-cells', *World journal of gastroenterology*. Baishideng Publishing Group Inc, 20(29), pp. 9653–9664.

Mantzaris, G. J. *et al.* (2001) 'A prospective randomized controlled trial of intravenous ciprofloxacin as an adjunct to corticosteroids in acute, severe ulcerative colitis', *Scandinavian journal of gastroenterology*. England, 36(9), pp. 971–974.

Mar, J. S. *et al.* (2014) 'Amelioration of DSS-induced murine colitis by VSL#3 supplementation is primarily associated with changes in ileal microbiota composition.', *Gut Microbes*. Taylor & Francis, 5(4), pp. 494–503.

Marchetti, M. and **Liberato, N. L.** (2014) 'Biological therapies in Crohn's disease: are they cost-effective? A critical appraisal of model-based analyses', *Expert Review of Pharmacoeconomics & Outcomes Research*. Taylor & Francis, 14(6), pp. 815–824.

Marple, A. *et al.* (2017) 'Cutting Edge: Helminth Coinfection Blocks Effector Differentiation of CD8 T Cells through Alternate Host Th2- and IL-10–

Mediated Responses', *The Journal of Immunology*, 198(2), pp. 634–639.

de Martel, C. *et al.* (2012) 'Global burden of cancers attributable to infections in 2008: a review and synthetic analysis', *The Lancet Oncology*. Elsevier Ltd, 13(6), pp. 607–615.

Martinez, F. D. (2001) 'The coming-of-age of the hygiene hypothesis.', *Respiratory research*, 2(3), pp. 129–132.

Maslowski, K. M. *et al.* (2009) 'Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43', *Nature*, 461(7268), pp. 1282–1286.

Mazmanian, S. K. *et al.* (2005) 'An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System', *Cell*. Elsevier, 122(1), pp. 107–118.

Mazmanian, S. K. *et al.* (2008) 'A microbial symbiosis factor prevents intestinal inflammatory disease', *Nature*, 453(7195), pp. 620–625.

McCarthy, J. *et al.* (2003) 'Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance', *Gut*. Copyright 2003 by Gut, 52(7), pp. 975–980.

McCoy, K. D. *et al.* (2018) 'Microbiota and Type 2 immune responses', *Current Opinion in Immunology*, 54, pp. 20–27.

McFarlane, A. J. *et al.* (2017) 'Enteric helminth-induced type I interferon signaling protects against pulmonary virus infection through interaction with the microbiota', *Journal of Allergy and Clinical Immunology*, 140(4), pp. 1068-1078.e6.

Mcnally, R. J. Q. *et al.* (2003) 'Seasonal variation in the patient diagnosis of Primary Biliary Cirrhosis: Further evidence for an environmental component to etiology.', *Hepatology*, 54(6), pp. 2099–2103.

McRae, B. L. *et al.* (1997) 'Human recombinant interferon-β influences T helper subset differentiation by regulating cytokine secretion pattern and expression of homing receptors', *European Journal of Immunology*. John Wiley & Sons, Ltd, 27(10), pp. 2650–2656.

McSorley, H. J. and **Maizels, R. M.** (2012) 'Helminth infections and host immune regulation', *Clinical Microbiology Reviews*, 25(4), pp. 585–608.

Meeusen, E. N. T. and **Balic, A.** (2000) 'Do eosinophils have a role in the killing of helminth parasites?', *Parasitology Today*, 16(3), pp. 95–101.

Melgar, S. *et al.* (2005) 'Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation', *American journal of physiology. Gastrointestinal and liver physiology*, 288(6), pp. G1328-38.

Melhem, H. *et al.* (2019) 'Metabolite-Sensing G Protein-Coupled Receptors Connect the Diet-Microbiota-Metabolites Axis to Inflammatory Bowel Disease', *Cells*, 8(450), pp. 1–23.

Mennigen, R. *et al.* (2009) 'Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 296(5), pp. G1140–G1149.

Meroni, E. *et al.* (2018) 'Functional characterization of oxazolone- induced colitis and survival improvement by vagus nerve stimulation', *PLOS ONE*, 13(5), pp. 1–19.

Mestas, J. and **Hughes, C. C. W.** (2004) 'Of Mice and Not Men: Differences

between Mouse and Human Immunology', *The Journal of Immunology*, 172(5), pp. 2731 LP – 2738.

Michail, S. and **Abernathy, F.** (2002) 'Lactobacillus plantarum Reduces the In Vitro Secretory Response of Intestinal Epithelial Cells to Enteropathogenic Escherichia coli Infection', *Journal of Pediatric Gastroenterology and Nutrition*, 35(3).

Miles, J. P. *et al.* (2017) 'Supplementation of Low- and High-fat Diets with Fermentable Fiber Exacerbates Severity of DSS-induced Acute Colitis', *Inflammatory bowel diseases*, 23(7), pp. 1133–1143.

Miller, E. (1983) 'Population Dynamics of Infectious Diseases: Theory and Applications', *Immunology*, 50(4), p. 677.

Mishra, P. K. *et al.* (2014) 'Systemic impact of intestinal helminth infections', *Mucosal Immunology*. Nature Publishing Group, 7(4), pp. 753–762.

Mizoguchi, E. (2006) 'Chitinase 3-Like-1 Exacerbates Intestinal Inflammation by Enhancing Bacterial Adhesion and Invasion in Colonic Epithelial Cells', *Gastroenterology*. Elsevier, 130(2), pp. 398–411.

Moayyedi, P. *et al.* (2015) 'Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial', *Gastroenterology*. 2015/04/07. United States, 149(1), pp. 102-109.e6.

Moghaddami, N. *et al.* (2003) 'Unique Effect of Arachidonic Acid on Human Neutrophil TNF Receptor Expression: Up-Regulation Involving Protein Kinase C, Extracellular Signal-Regulated Kinase, and Phospholipase A2', *The Journal of Immunology*, 171(5), pp. 2616 LP – 2624.

Molodecky, N. A. *et al.* (2012) 'Increasing Incidence and Prevalence of the Inflammatory Bowel Diseases With Time, Based on Systematic Review', *Gastroenterology*. Elsevier, 142(1), pp. 46-54.e42.

Morgan, X. C. and **Huttenhower, C.** (2012) 'Chapter 12: Human Microbiome Analysis', in *PLoS Computational Biology*.

Morris, G. P. *et al.* (1989) 'Hapten-Induced Model of Chronic Inflammation and Ulceration in the Rat Colon', *Gastroenterology*, 96, pp. 795–803.

Murch, S. H. *et al.* (1993) 'Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease.', *Gut*, 34(12), pp. 1705 LP – 1709.

Nagai, Y. *et al.* (2006) 'Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment.', *Immunity*, 24(6), pp. 801–12.

Nagalingam, N. A. *et al.* (2011) 'Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis', *Inflammatory bowel diseases*. 2010/11/08, 17(4), pp. 917–926.

Nakanishi, Y. *et al.* (2015) 'Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization', *Mucosal Immunology*. Nature Publishing Group, 8(1), pp. 152–160.

Nakanishi, Y. *et al.* (2017) 'Regulatory T cells with superior immunosuppressive capacity emigrate from the inflamed colon to draining lymph nodes', *Mucosal Immunology*. Society for Mucosal Immunology, 11(2), pp. 437–448.

Nancey, S. *et al.* (2006) 'CD8+ Cytotoxic T Cells Induce Relapsing Colitis in Normal Mice', *Gastroenterology*, 131, pp. 485–496.

Narni-Mancinelli, E. *et al.* (2011) 'Inflammatory Monocytes and Neutrophils

Are Licensed to Kill during Memory Responses In Vivo', *PLoS Pathogens*. Edited by D. S. Schneider, 7(12), p. e1002457.

Nath, A. *et al.* (2018) 'Biological Activities of Lactose-Derived Prebiotics and Symbiotic with Probiotics on Gastrointestinal System', *Medicina*, 54(18), pp. 1–28.

Natividad, J. M. M. *et al.* (2013) 'Differential induction of antimicrobial REGIII by the intestinal microbiota and Bifidobacterium breve NCC2950', *Applied and Environmental Microbiology*, 79(24), pp. 7745–7754.

Nell, S. *et al.* (2010) 'The impact of the microbiota on the pathogenesis of IBD: Lessons from mouse infection models', *Nature Reviews Microbiology*. Nature Publishing Group, 8(8), pp. 564–577.

Nenci, A. *et al.* (2007) 'Epithelial NEMO links innate immunity to chronic intestinal inflammation', *Nature*. Nature Publishing Group, 446(7135), pp. 557–61.

Newton, K. and **Dixit, V. M.** (2012) 'Signaling in innate immunity and inflammation', *Cold Spring Harbor Perspectives in Biology*, 4, pp. 1–19.

Ng, S. C. *et al.* (2017) 'Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies', *The Lancet*. Elsevier, 390(10114), pp. 2769–2778.

Ng, S. H. *et al.* (2018) 'The influence of diet and environment on the gut microbial community of field crickets', *Ecology and evolution*. John Wiley and Sons Inc., 8(9), pp. 4704–4720.

Ni, J. *et al.* (2017) 'Gut microbiota and IBD: Causation or correlation?', *Nature Reviews Gastroenterology and Hepatology*. Nature Publishing Group, 14(10), pp. 573–584.

Nikolaev, B. and **Salahodjaev, R.** (2017) 'Historical Prevalence of Infectious Diseases, Cultural Values, and the Origins of Economic Institutions', *Kyklos*. John Wiley & Sons, Ltd (10.1111), 70(1), pp. 97–128.

Nilsson, A. C. *et al.* (2008) 'Including Indigestible Carbohydrates in the Evening Meal of Healthy Subjects Improves Glucose Tolerance, Lowers Inflammatory Markers, and Increases Satiety after a Subsequent Standardized Breakfast', *The Journal of Nutrition*, 138(4), pp. 732–739.

Nishida, T. *et al.* (1987) 'Increased arachidonic acid composition of phospholipids in colonic mucosa from patients with active ulcerative colitis', *Gut*, 28, pp. 1002–1007.

Niwa, Y. *et al.* (2004) 'An Association Between Ulcerative Colitis and Atopic Dermatitis: Diseases of Impaired Superficial Barriers', *The Journal of investigative dermatology*, 123, pp. 999–1000.

Noguchi, D. *et al.* (2007) 'Blocking of IL-6 signaling pathway prevents CD4+T cell-mediated colitis in a Th17-independent manner', *International Immunology*, 19(12), pp. 1431–1440.

Norman, J. M. *et al.* (2015) 'Disease-specific alterations in the enteric virome in inflammatory bowel disease', *Cell*. 2015/01/22, 160(3), pp. 447–460.

O'Keeffe, M. *et al.* (2015) 'Human dendritic cell subsets and function in health and disease', *Cellular and Molecular Life Sciences*, 72(22), pp. 4309–4325.

O'Morain, C. *et al.* (1980) 'Elemental diets in treatment of acute Crohn's disease', *British medical journal*, 281(6249), pp. 1173–1175.

Oh, J. and **Weiderpass, E.** (2014) 'Infection and Cancer : Global Distribution and Burden of Diseases', *Annals of Global Health*. Elsevier Inc, 80(5), pp. 384–392.

Okayasu, I. ***et al.*** (1990) 'A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice', *Gastroenterology*, 98(3), pp. 694–702.

Olsen, I. and **Jantzen, E.** (2001) 'Sphingolipids in Bacteria and Fungi', *Anaerobe*, 7(2), pp. 103–112.

Ostanin, D. V ***et al.*** (2006) 'T cell-induced inflammation of the small and large intestine in immunodeficient mice', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 290(1), pp. G109–G119.

Pabst, O. (2012) 'Trafficking of regulatory T cells in the intestinal immune system', *International Immunology*, 25(3), pp. 139–143.

Pacheco, S. ***et al.*** (1987) 'Increased arachidonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease', *Clinical Science*, 73(4), pp. 361 LP – 364.

Palm, N. W. ***et al.*** (2014) 'Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease', *Cell*, 158(5), pp. 1000–1010.

Palmela, C. ***et al.*** (2018) 'Adherent-invasive Escherichia coli in inflammatory bowel disease', *Gut*, 67(3), pp. 574 LP – 587.

Paramsothy, S. ***et al.*** (2017) 'Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial', *Lancet (London, England)*. 2017/02/15. England, 389(10075), pp. 1218–1228.

Park, S.-K. ***et al.*** (2013) 'Ciprofloxacin Usage and Bacterial Resistance Patterns in Crohn's Disease Patients With Abscesses', *Journal of clinical gastroenterology*, 48.

Parkin, D. M. ***et al.*** (2002) 'Global cancer statistics, 2002', *CA: a cancer journal for clinicians*, 55(2), pp. 74–108.

Pastille, E. ***et al.*** (2014) 'Transient Ablation of Regulatory T cells Improves Antitumor Immunity in Colitis-Associated Colon Cancer', *Cancer Research*, 74(16), pp. 4258–4269.

Pastille, E. ***et al.*** (2017) 'Intestinal helminth infection drives carcinogenesis in colitis-associated colon cancer.', *PLoS pathogens*, 13(9), pp. 1–22.

Pearl, D. S. ***et al.*** (2014) 'Altered colonic mucosal availability of n-3 and n-6 polyunsaturated fatty acids in ulcerative colitis and the relationship to disease activity.', *Journal of Crohn's and Colitis*. European Crohn's and Colitis Organisation, 8(1), pp. 70–79.

Perencevich, M. and **Burakoff, R.** (2006) 'Use of antibiotics in the treatment of inflammatory bowel disease', *Inflammatory Bowel Diseases*, 12(7), pp. 651–664.

Perše, M. and **Cerar, A.** (2012) 'Dextran sodium sulphate colitis mouse model: Traps and tricks', *Journal of Biomedicine and Biotechnology*, 2012, pp. 1–13.

Petersen, E. R. ***et al.*** (2011) 'Consumption of Probiotics Increases the Effect of Regulatory T Cells in Transfer Colitis', *Inflammatory Bowel Diseases*, 18(1), pp. 131–142.

Poliska, S. ***et al.*** (2012) 'Association of peroxisome proliferator-activated receptor gamma polymorphisms with inflammatory bowel disease in a Hungarian cohort', *Inflammatory bowel diseases*. 2011/06/24. England, 18(3), pp. 472–479.

van der Post, S. ***et al.*** (2019) 'Structural weakening of the colonic mucus barrier

is an early event in ulcerative colitis pathogenesis', *Gut*, p. gutjnl-2018-317571.

Powell, N. *et al.* (2017) 'The mucosal immune system: master regulator of bidirectional gut–brain communications', *Nature Reviews Gastroenterology & Hepatology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 14(3), pp. 143–159.

Powrie, F. *et al.* (1993) 'Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice', *International Immunology*, 5(11), pp. 1461–1471.

Powrie, F. *et al.* (1994) 'Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with $CD45RB^{hi}$ $CD4^{+}$ T cells', *Immunity*. Elsevier, 1(7), pp. 553–562.

Powrie, F. and **Uhlig, H.** (2004) 'Animal models of intestinal inflammation: clues to the pathogenesis of inflammatory bowel disease', *Novartis Foundation symposium*. England, 263, pp. 164–218.

Prantera, C. *et al.* (2006) 'Antibiotic treatment of Crohn ' s disease : results of a multicentre , double blind , randomized , placebo-controlled trial with rifaximin', *Alimentary Pharmacology & Therapeutics*, 23, pp. 1117–1125.

Quigley, E. M. M. (2017) 'Gut microbiome as a clinical tool in gastrointestinal disease management: are we there yet?', *Nature Reviews Gastroenterology &Amp; Hepatology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 14, p. 315.

Rafa, H. *et al.* (2010) 'Involvement of Interferon-γ in Bowel Disease Pathogenesis by Nitric Oxide Pathway: A Study in Algerian Patients', *Journal of Interferon & Cytokine Research*. Mary Ann Liebert, Inc., publishers, 30(9), pp. 691–697.

Rajoka, M. S. R. *et al.* (2017) 'Interaction between diet composition and gut microbiota and its impact on gastrointestinal tract health', *Food Science and Human Wellness*, 6, pp. 121–130.

Rakoff-Nahoum, S. *et al.* (2004) 'Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis', *Cell*. Elsevier, 118(2), pp. 229–241.

Ramagopalan, S. V *et al.* (2010) 'Sex ratio of multiple sclerosis and clinical phenotype', pp. 634–637.

Ramakers, J. D. *et al.* (2007) 'Arachidonic acid but not eicosapentaenoic acid (EPA) and oleic acid activates NF-kappaB and elevates ICAM-1 expression in Caco-2 cells', *Lipids*, 42(8), p. 687—698.

Ramanan, D. *et al.* (2016) 'Helminth infection promotes colonization resistance via type 2 immunity', *Science*, 352(6285), pp. 608–612.

Rankin, A. L. *et al.* (2010) 'IL-33 Induces IL-13–Dependent Cutaneous Fibrosis', *The Journal of Immunology*, 184(3), pp. 1526 LP – 1535.

Rather, I. A. *et al.* (2016) 'Ulcerative colitis and probiotics: An overview', *Pakistan journal of pharmaceutical sciences*, 29(5), pp. 1877–1880.

Rausch, S. *et al.* (2013) 'Small Intestinal Nematode Infection of Mice Is Associated with Increased Enterobacterial Loads alongside the Intestinal Tract', *PLOS ONE*, 8(9), pp. 1–13.

Rawls, J. F. *et al.* (2006) 'Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection', *Cell*, 127(2), pp. 423–433.

Reardon, C. *et al.* (2001) 'Tapeworm Infection Reduces Epithelial Ion Transport

Abnormalities in Murine Dextran Sulfate Sodium-Induced Colitis', *Infection and Immunity*, 69(7), pp. 4417–4423.

Reikvam, D. H. *et al.* (2011) 'Depletion of murine intestinal microbiota: Effects on gut mucosa and epithelial gene expression', *PLoS ONE*, 6(3), pp. 1–13.

Reinisch, W. *et al.* (2015) 'Anrukinzumab, an anti-interleukin 13 monoclonal antibody, in active UC: efficacy and safety from a phase IIa randomised multicentre study', *Gut*, 64(6), pp. 894 LP – 900.

dos Reis, S. A. *et al.* (2017) 'Review of the mechanisms of probiotic actions in the prevention of colorectal cancer', *Nutrition Research*. Elsevier B.V., 37, pp. 1–19.

Rembacken, B. J. *et al.* (1999) 'Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial', *The Lancet*. Elsevier, 354(9179), pp. 635–639.

Reynolds, L. A. *et al.* (2012) 'Immunity to the model intestinal helminth parasite Heligmosomoides polygyrus', *Seminars in Immunopathology*, 34(6), pp. 829–846.

Reynolds, L. A. *et al.* (2014) 'Commensal-pathogen interactions in the intestinal tract lactobacilli promote infection with, and are promoted by, helminth parasites', *Gut Microbes*, 5(4), pp. 522–532.

Reynolds, L. A. *et al.* (2017) 'Enteric Helminths Promote Salmonella Coinfection by Altering the Intestinal Metabolome', *The Journal of Infectious Diseases*, 215(8), pp. 1245–1254.

Rezaie, A. *et al.* (2012) 'Time trends in prevalence and incidene of inflammatory bowel disease in Alberta: a population based study', *Can J Gastroenterol*, 26(A133).

Rhodes, J. M. and **Jewell, D. P.** (1983) 'Motility of neutrophils and monocytes in Crohn's disease and ulcerative colitis', *Gut*, (24), pp. 73–77.

Rieder, F. and **Fiocchi, C.** (2008) 'Intestinal fibrosis in inflammatory bowel disease: progress in basic and clinical science.', *Current opinion in gastroenterology*. European Crohn's and Colitis Organisation, 24(4), pp. 462–8.

van Rijt, L. *et al.* (2016) 'Type 2 innate lymphoid cells: at the cross-roads in allergic asthma', *Seminars in Immunopathology*, 38(4), pp. 483–496.

Rincón, B. M. *et al.* (1997) 'Interleukin (IL)-6 Directs the Differentiation of IL-4–producing CD4+ T Cells', *The Journal of Experimental Medicine*, 185(3), pp. 461–469.

Rivera-Chávez, F. *et al.* (2016) 'Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella', *Cell host & microbe*, 19(4), pp. 443–454.

Robinson, M. *et al.* (1989) 'Immunological relationships during primary infection with Heligmosomoides polygyrus (Nematospiroides dubius): Dose-dependent expulsion of adult worms', *Parasitology*, 98(1), pp. 115–124.

Roediger, W. E. W. *et al.* (1997) 'Colonic Sulfide in Pathogenesis and Treatment of Ulcerative Colitis', *Digestive Diseases and Sciences*, 42(8), pp. 1571–1579.

Rolny, P. *et al.* (1983) 'Occurrence of Clostridium difficile toxin in inflammatory bowel disease', *Scandinavian journal of gastroenterology*. England, 18(1), pp. 61–64.

Rolsma, M. D. *et al.* (1998) 'Structure and Function of a Ganglioside Receptor

for Porcine Rotavirus', *J virol*, 72(11), pp. 9079–9091.

Rooks, M. G. *et al.* (2014) 'Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission', *ISME Journal*, 8(7), pp. 1403–1417.

Roselli, M. *et al.* (2009) 'Prevention of TNBS-induced colitis by different Lactobacillus and Bifidobacterium strains is associated with an expansion of γδT and regulatory T cells of intestinal intraepithelial lymphocytes', *Inflammatory Bowel Diseases*, 15(10), pp. 1526–1536.

Round, J. L. and **Mazmanian, S. K.** (2010) 'Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota', *Proceedings of the National Academy of Sciences*, 107(27), pp. 12204 LP – 12209.

Roy, U. *et al.* (2017) 'Distinct Microbial Communities Trigger Colitis Development upon Intestinal Barrier Damage via Innate or Adaptive Immune Cells', *Cell Reports*, 21, pp. 994–1008.

Rubinstein, M. R. *et al.* (2013) 'Fusobacterium nucleatum Promotes Colorectal Carcinogenesis by Modulating E-Cadherin/β-Catenin Signaling via its FadA Adhesin', *Cell Host and Microbe*. Elsevier Inc., 14(2), pp. 195–206.

Rudolph, U. *et al.* (1995) 'Ulcerative colitis and adenocarcinoma of the colon in Gαi2-deficient mice', *Nature Genetics*, 10(2), pp. 143–150.

Ruyssers, N. E. *et al.* (2009) 'Therapeutic potential of helminth soluble proteins in TNBS induced colitis in mice', *Inflammatory Bowel Diseases*, 15(4), pp. 491–500.

Saemann, M. D. *et al.* (2000) 'Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production', *The FASEB Journal*. Federation of American Societies for Experimental Biology, 14(15), pp. 2380–2382.

Salamone, G. *et al.* (2001) 'Promotion of Neutrophil Apoptosis by TNF-α', *The Journal of Immunology*, 166(5), pp. 3476 LP – 3483.

Saleh, M. and **Trinchieri, G.** (2010) 'Innate immune mechanisms of colitis and colitis-associated colorectal cancer', *Nature Reviews Immunology*. Nature Publishing Group, 11(1), pp. 9–20.

Salem, N. J. *et al.* (1999) 'In vivo conversion of linoleic acid to arachidonic acid in human adults', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 60, pp. 407–410.

Sandborn, W. J. *et al.* (2017) 'Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis', *New England Journal of Medicine*. Massachusetts Medical Society, 376(18), pp. 1723–1736.

Sanderson, I. R. (2001) 'Nutritional factors and immune functions of gut epithelium', *The Proceedings of the Nutrition Society*. England, 60(4), pp. 443–447.

Sang, L. X. *et al.* (2013) 'Heat-killed VSL#3 ameliorates dextran sulfate sodium (DSS)-induced acute experimental colitis in rats', *International Journal of Molecular Sciences*, 15(1), pp. 15–28.

Sartor, R. B. (1998) 'Postoperative recurrence of Crohn's disease: The enemy is within the fecal stream', *Gastroenterology*, 114(2), pp. 398–400.

Saunders, K. A. *et al.* (2007) 'Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection', *Infection and Immunity*, 75(1), pp. 397–407.

Schaedler, R. W. *et al.* (1965) 'The development of the bacterial flora in the

gastrointestinal tract of mice.', *The Journal of experimental medicine*. The Rockefeller University Press, 122(1), pp. 59–66.

Schaubeck, M. *et al.* (2015) 'Dysbiotic gut microbiota causes transmissible Crohn ' s disease-like ileitis independent of failure in antimicrobial defence', *Gut*, 0, pp. 1–13.

Schauber, J. *et al.* (2003) 'Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways', *Gut*. Copyright 2003 by Gut, 52(5), pp. 735–741.

Schmelz, E. M. and **Merrill Jr., A. H.** (1998) 'Ceramides and ceramide metabolites in cell regulation: evidence for dietary sphingolipids as inhibitors of colon carcinogenesis', *Nutrition*, 14(9), pp. 717–719.

Schwab, C. *et al.* (2014) 'Longitudinal study of murine microbiota activity and interactions with the host during acute inflammation and recovery', *The ISME journal*. 2014/01/09. Nature Publishing Group, 8(5), pp. 1101–1114.

Schwab, J. M. *et al.* (2007) 'Resolvin E1 and protectin D1 activate inflammation-resolution programmes', *Nature*, 447(7146), pp. 869–74.

Schwab, M. *et al.* (2006) 'PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2', *Apoptosis*, 11(10), pp. 1801–1811.

Scrivener, S. *et al.* (2001) 'Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study', *The Lancet*, 358(9292), pp. 1493–1499.

Sears, C. L. (2009) 'Enterotoxigenic Bacteroides fragilis: A rogue among symbiotes', *Clinical Microbiology Reviews*, 22(2), pp. 349–369.

Seiffarta, V. *et al.* (2015) 'IL10 - Deficiency in CD4 + T Cells Exacerbates the IFNγ and IL17 Response During Bacteria Induced Colitis', *Cell Physiol Biochem*, 36, pp. 1259–1273.

Sellon, R. K. *et al.* (1998) 'Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice', *Infection and immunity*. American Society for Microbiology, 66(11), pp. 5224–5231.

Selvaggi, F. *et al.* (2015) 'Effect of preoperative biologic drugs on complications and function after restorative proctocolectomy with primary ileal pouch formation: systematic review and meta-analysis', *Inflammatory bowel diseases*. England, 21(1), pp. 79–92.

Selvasekar, C. R. *et al.* (2007) 'Effect of infliximab on short-term complications in patients undergoing operation for chronic ulcerative colitis', *Journal of the American College of Surgeons*. United States, 204(5), pp. 956–963.

Seregin, S. S. *et al.* (2017) 'NLRP6 Protects Il10 À / À Mice from Colitis by Limiting Colonization of Akkermansia muciniphila', *CellReports*. ElsevierCompany., 19(4), pp. 733–745.

Serhan, C. N. *et al.* (1996) 'Lipid mediator networks in cell signaling: update and impact of cytokines.', *The FASEB Journal*. Federation of American Societies for Experimental Biology, 10(10), pp. 1147–1158.

Serpa, J. *et al.* (2010) 'Butyrate-rich colonic microenvironment is a relevant selection factor for metabolically adapted tumor cells', *Journal of Biological Chemistry*, 285(50), pp. 39211–39223.

Sharon, P. and **Stenson, W. F.** (1985) 'Metabolism of Arachidonic Acid in Acetic Acid Colitis in Rats Similarity to Human Inflammatory Bowel Disease', *Gastroenterology*. American Gastroenterological Association,

88(1), pp. 55–63.

Shevach, E. M. (2009) 'Mechanisms of Foxp3+T Regulatory Cell-Mediated Suppression', *Immunity*. Elsevier Inc., 30(5), pp. 636–645.

Shi, C. and **Pamer, E. G.** (2011) 'Monocyte recruitment during infection and inflammation', *Nature Reviews Immunology*. Nature Publishing Group, 11(11), pp. 762–774.

Shiga, H. *et al.* (2012) 'Changes of faecal microbiota in patients with Crohn's disease treated with an elemental diet and total parenteral nutrition', *Digestive and Liver Disease*, 44(9), pp. 736–742.

Shim, T. S. (2014) 'Diagnosis and Treatment of Latent Tuberculosis Infection in Patients with Inflammatory Bowel Diseases due to Initiation of Anti-Tumor Necrosis Factor Therapy', *Intest Res*. Korean Association for the Study of Intestinal Diseases, 12(1), pp. 12–19.

Shimizu, Toshiaki *et al.* (2001) 'Colon Effects of n – 3 Polyunsaturated Fatty Acids and Vitamin E on Colonic Mucosal Leukotriene Generation , Lipid Peroxidation , and Microcirculation in Rats with Experimental', 0033, pp. 49–54.

Shimizu, T *et al.* (2001) 'Effects of n–3 Polyunsaturated Fatty Acids and Vitamin E on Colonic Mucosal Leukotriene Generation, Lipid Peroxidation, and Microcirculation in Rats with Experimental Colitis', *Digestion*, 63(1), pp. 49–54.

Shimizu, T. (2009) 'Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation', *Annu Rev Pharmacol Toxicol*, 49.

Shimizu, T. and **Wolfe, L. S.** (1990) 'Arachidonic Acid Cascade and Signal Transduction', *Journal of Neurochemistry*. John Wiley & Sons, Ltd (10.1111), 55(1), pp. 1–15.

Shirazi, T. *et al.* (2000) 'Mucins and inflammatory bowel disease', *Postgraduate Medical Journal*, 76(898), pp. 473–478.

Shrestha, U. K. *et al.* (2010) 'Distribution of peroxisome proliferator-activated receptor-gamma polymorphisms in Chinese and Dutch patients with inflammatory bowel disease', *Inflammatory bowel diseases*. England, 16(2), pp. 312–319.

De Silva, P. *et al.* (2011) 'Dietary arachidonic acid and the aetiology of ulcerative colitis: data from a UK prospective cohort study, using 7-day food diaries', *Gut*, 60(Suppl 1), p. A140 LP-A140.

de Silva, P. S. A. *et al.* (2010) 'An Association Between Dietary Arachidonic Acid, Measured in Adipose Tissue, and Ulcerative Colitis', *Gastroenterology*. Elsevier, 139(6), pp. 1912–1917.

Silva, P. S. De *et al.* (2008) 'Dietary Arachidonic Acid and the Aetiology of Ulcerative Colitis - Data From a UK Prospective Cohort Study, Using 7-Day Food Diaries', *Gastroenterology*. AGA, 140(5), p. S-114.

Simone, C. D. E. (2016) 'Case 8:15-cv-01356-TDC Document 155-3 Filed 03/23/16'.

Singh, R. K. *et al.* (2017) 'Influence of diet on the gut microbiome and implications for human health', *Journal of Translational Medicine*, 15(1), p. 73.

Slavin, J. (2013) 'Fiber and prebiotics: mechanisms and health benefits', *Nutrients*. MDPI, 5(4), pp. 1417–1435.

Smith, K. A. *et al.* (2011) 'Chronic Helminth Infection Promotes Immune

Regulation In Vivo through Dominance of CD11cloCD103- Dendritic Cells', *The Journal of Immunology*, 186(12), pp. 7098–7109.

Smith, K. A. *et al.* (2012) 'Type 2 Innate Immunity in Helminth Infection Is Induced Redundantly and Acts Autonomously following CD11c+ Cell Depletion', *Infection and Immunity*, 80(10), pp. 3481–3489.

Smith, K. A. *et al.* (2016) 'Low-level regulatory T-cell activity is essential for functional type-2 effector immunity to expel gastrointestinal helminths', *Mucosal Immunology*, 9(2), pp. 428–443.

Smith, K. A. and **Maizels, R. M.** (2014) 'IL-6 controls susceptibility to helminth infection by impeding Th2 responsiveness and altering the Treg phenotype in vivo', *European Journal of Immunology*, 44(1), pp. 150–161.

Smith, P. *et al.* (2007) 'Infection with a Helminth Parasite Prevents Experimental Colitis via a Macrophage-Mediated Mechanism', *The Journal of Immunology*, 178(7), pp. 4557–4566.

Smith, P. M. *et al.* (2013) 'The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic Treg Cell Homeostasis', *Science*, 341(6145), pp. 569 LP – 573.

Snoeck, V. *et al.* (2005) 'The role of enterocytes in the intestinal barrier function and antigen uptake', *Microbes Infect*, 7, pp. 997–1004.

Soehnlein, O. *et al.* (2008) 'Neutrophil secretion products pave the way for inflammatory monocytes', *Blood*. 2008/05/19. American Society of Hematology, 112(4), pp. 1461–1471.

Sokol, H. *et al.* (2008) 'Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients', *Proceedings of the National Academy of Sciences*, 105(43), pp. 16731 LP – 16736.

Sokol, H. *et al.* (2017) 'Fungal microbiota dysbiosis in IBD', *Gut*, 66(6), pp. 1039–1048.

Sood, A. *et al.* (2003) 'Incidence and prevalence of ulcerative colitis in Punjab, North India', *Gut*, 52, pp. 1587–1590.

Sood, A. *et al.* (2009) 'The Probiotic Preparation, VSL#3 Induces Remission in Patients With Mild-to-Moderately Active Ulcerative Colitis', *Clinical Gastroenterology and Hepatology*. Elsevier, 7(11), pp. 1202-1209.e1.

Sorini, C. and **Falcone, M.** (2013) 'Shaping the (auto)immune response in the gut: the role of intestinal immune regulation in the prevention of type 1 diabetes.', *American journal of clinical and experimental immunology*, 2(2), pp. 156–71.

Specian, R. D. and **Neutra, M. R.** (1980) 'Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine', *The Journal of cell biology*. The Rockefeller University Press, 85(3), pp. 626–640.

Stark, M. A. *et al.* (2005) 'Phagocytosis of Apoptotic Neutrophils Regulates Granulopoiesis via IL-23 and IL-17', *Immunity*. Elsevier, 22(3), pp. 285–294.

Stenson, W. F. *et al.* (1992) 'Dietary Supplementation with Fish Oil in Ulcerative Colitis', *Annals of Internal Medicine*, 116(8), pp. 609–614.

Stevceva, L *et al.* (2001) 'Dextran sulphate sodium-induced colitis is ameliorated in interleukin 4 deficient mice', *Genes and immunity*. Nature Publishing Group, 2(6), p. 309.

Stevceva, Liljana *et al.* (2001) 'The inflammatory infiltrate in the acute stage of the dextran sulphate sodium induced colitis : B cell response differs

depending on the percentage of DSS used to induce it', *BMC Clinical Pathology*, 1(3), pp. 1–11.

Strauss, J. *et al.* (2011) 'Invasive potential of gut mucosa-derived fusobacterium nucleatum positively correlates with IBD status of the host', *Inflammatory Bowel Diseases*, 17(9), pp. 1971–1978.

Strober, W. *et al.* (2002) 'The Immunology Of Mucosal Models Of Inflammation', *Annual Review of Immunology*, 20(1), pp. 495–549.

Strober, W. and **Fuss, I. J.** (2011) 'Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases', *Gastroenterology*. Elsevier Inc., 140(6), pp. 1756–1767.

Sturlan, S. *et al.* (2001) 'Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development ', *Carcinogenesis*, 22(4), pp. 665–671.

Su, C. *et al.* (2018) 'Helminth-induced alterations of the gut microbiota exacerbate bacterial colitis', *Mucosal Immunology*, 11(1), pp. 144–157.

Su, C. wen *et al.* (2018) 'Helminth infection protects against high fat diet-induced obesity via induction of alternatively activated macrophages', *Scientific Reports*, 8(1), p. 4607.

Su, J. W. *et al.* (2015) 'Use of antibiotics in patients with Crohn's disease: A systematic review and meta-analysis', *Journal of Digestive Diseases*. John Wiley & Sons, Ltd (10.1111), 16(2), pp. 58–66.

Su, L. *et al.* (2014) 'Coinfection with an Intestinal Helminth Impairs Host Innate Immunity against Salmonella enterica Serovar Typhimurium and Exacerbates Intestinal Inflammation in Mice', *Infection and Immunity*. Edited by J. A. Appleton, 82(9), pp. 3855 LP – 3866.

Sugita, K. *et al.* (2018) 'Type 2 innate lymphoid cells disrupt bronchial epithelial barrier integrity by targeting tight junctions through IL-13 in asthmatic patients', *Journal of Allergy and Clinical Immunology*, 141(1), pp. 300-310.e11.

Summers, Robert W. *et al.* (2005) 'Trichuris suis therapy for active ulcerative colitis: A randomized controlled trial', *Gastroenterology*, 128(4), pp. 825–832.

Summers, R W *et al.* (2005) 'Trichuris suis therapy in Crohn's disease.', *Gut*, 54(1), pp. 87–90.

Sun, J. and **Kato, I.** (2016) 'Gut microbiota, inflammation and colorectal cancer', *Genes and Diseases*. Elsevier Ltd, 3(2), pp. 130–143.

Suskind, D. L. *et al.* (2015) 'Fecal Microbial Transplant Effect on Clinical Outcomes and Fecal Microbiome in Active Crohn's Disease', *Inflammatory Bowel Diseases*, 21(3), pp. 556–563.

Sutherland, L. *et al.* (1991) 'Double blind , placebo controlled trial of metronidazole in Crohn ' s disease', pp. 1071–1075.

Sutherland, L. R. (1998) 'Aminosalicylates in the Treatment of Inflammatory Bowel Disease Aminosalicylates in the Treatment of Children with Inflammatory Bowel Disease', (May), p. 1998.

Sutton, T. L. *et al.* (2008) 'Anti-inflammatory mechanisms of enteric Heligmosomoides polygyrus infection against trinitrobenzene sulfonic acid-induced colitis in a murine model', *Infection and Immunity*, 76(10), pp. 4772–4782.

Suzuki, K. *et al.* (2004) 'Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut', *Proceedings of the National Academy of Sciences*,

101(7), pp. 1981 LP – 1986.

Swank, G. M. and **Deitch, E. A.** (1996) 'Role of the Gut in Multiple Organ Failure: Bacterial Translocation and Permeability Changes', *World Journal of Surgery*, 20(4), pp. 411–417.

Swidsinski, A. *et al.* (2008) 'Bacterial Biofilm Suppression with Antibiotics for Ulcerative and Indeterminate Colitis: Consequences of Aggressive Treatment', *Archives of Medical Research*, 39(2), pp. 198–204.

Sydora, B. C. *et al.* (2003) 'Lack of Interleukin-10 Leads to Intestinal Inflammation, Independent of the Time at Which Luminal Microbial Colonization Occurs', *Inflammatory Bowel Diseases*, 9(2), pp. 87–97.

Sydora, B. C. *et al.* (2007) 'Epithelial barrier disruption allows nondisease-causing bacteria to initiate and sustain IBD in the IL-10 gene-deficient mouse', *Inflammatory Bowel Diseases*, 13(8), pp. 947–954.

Takahashi, T. *et al.* (1998) 'Immunologic self-tolerance maintained by CD25+CD4+naturally anergic and suppressive T cells: Induction of autoimmune disease by breaking their anergic/suppressive state', *International Immunology*, 10(12), pp. 1969–1980.

Talukdar, S. *et al.* (2012) 'Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase', *Nature medicine*, 18(9), pp. 1407–1412.

Teixeira, L. G. *et al.* (2011) 'The combination of high-fat diet-induced obesity and chronic ulcerative colitis reciprocally exacerbates adipose tissue and colon inflammation', *Lipids in Health and Disease*, 10(204), pp. 1–15.

Terabe, M. *et al.* (2000) 'NKT cell–mediated repression of tumor immunosurveillance by IL-13 and the IL-4R–STAT6 pathway', *Nature Immunology*, 1(6), pp. 515–520.

The IBD in EPIC Study Investigators (2009) 'Linoleic acid , a dietary n-6 polyunsaturated fatty acid , and the aetiology of ulcerative colitis : a nested case – control study within a European prospective cohort study', *Gut*, 58(12), pp. 1606–1611.

Thomsen, L. E. *et al.* (2005) 'The influence of dietary carbohydrates on experimental infection with Trichuris suis in pigs', *Parasitology*. 2005/09/13. Cambridge University Press, 132(6), pp. 857–865.

Tielens, A. G. M. *et al.* (2010) 'Acetate formation in the energy metabolism of parasitic helminths and protists', *International Journal for Parasitology*, 40(4), pp. 387–397.

Tobias, P. *et al.* (2003) 'Habitual Dietary Intake of n-3 and n-6 Fatty Acids in Relation to Inflammatory Markers Among US Men and Women', *Circulation*. American Heart Association, 108(2), pp. 155–160.

Tomasello, G. *et al.* (2014) 'Dismicrobism in inflammatory bowel disease and colorectal cancer: Changes in response of colocytes', *World Journal of Gastroenterology*, 20(48), pp. 18121–18130.

Tontonoz, P. *et al.* (1994) 'mPPAR /2: tissue-specific regulator of an adipocyte enhancer', *Genes and Development*, 4(8), pp. 1224–1234.

Tracey, K. J. *et al.* (1988) 'Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation.', *The Journal of experimental medicine*, 167(3), pp. 1211–27.

De Trez, C. *et al.* (2015) 'Experimental African trypanosome infection by needle passage or natural tsetse fly challenge thwarts the development of collagen-induced arthritis in DBA/1 prone mice via an impairment of

antigen specific B cell autoantibody titers', *PLoS ONE*, 10(6), pp. 1–10.

Turnbaugh, P. J. *et al.* (2007) 'The Human Microbiome Project', *Nature*. Nature Publishing Group, 449(7164), pp. 804–810.

Turner, D. *et al.* (2011) 'Maintenance of Remission in Inflammatory Bowel Disease Using Omega-3 Fatty Acids (Fish Oil): A Systematic Review and Meta-analyses', *Inflamm Bowel Dis*, 17(1), pp. 336–345.

Turner, J. *et al.* (2013) 'IL-22 Mediates Goblet Cell Hyperplasia and Worm Expulsion in Intestinal Helminth Infection', *PLoS Pathogens*, 9(10), pp. 1–7.

Turner, J. D. *et al.* (2008) 'Intensity of intestinal infection with multiple worm species is related to regulatory cytokine output and immune hyporesponsiveness.', *The Journal of infectious diseases*, 197(8), pp. 1204–1212.

Tursi, A. *et al.* (2004) 'Low-dose balsalazide plus a high-potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acute mild-to-moderate ulcerative colitis', *Med Sci Monit*, 10(11), pp. 126–131.

Ueno, A. *et al.* (2013) 'Increased Prevalence of Circulating Novel IL-17 Secreting Foxp3 Expressing CD4+ T Cells and Defective Suppressive Function of Circulating Foxp3+ Regulatory Cells Support Plasticity Between Th17 and Regulatory T Cells in Inflammatory Bowel Disease Patients', *Inflammatory Bowel Diseases*, 19(12), pp. 2522–2534.

Urban, J. F. *et al.* (1991) 'Heligmosomoides polygyrus: CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice', *Experimental Parasitology*, 73(4), pp. 500–511.

Urban, J. F. *et al.* (1998) 'IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis', *Immunity*, 8(2), pp. 255–264.

Uygun, A. *et al.* (2017) 'Fecal microbiota transplantation is a rescue treatment modality for refractory ulcerative colitis', *Medicine*, 96(16), p. e6479.

Vahabnezhad, E. *et al.* (2013) 'Lactobacillus Bacteremia Associated With Probiotic Use in a Pediatric Patient With Ulcerative Colitis', *J Clin Gastroenterol*, 47(5), pp. 437–439.

Veldhoen, M. *et al.* (2006) 'TGFβ in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells', *Immunity*, 24(2), pp. 179–189.

Veltkamp, C. *et al.* (2011) 'Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNF treatment', *Gut*, 60(10), pp. 1345 LP – 1353.

Venkatesh, M. *et al.* (2014) 'Symbiotic Bacterial Metabolites Regulate Gastrointestinal Barrier Function via the Xenobiotic Sensor PXR and Toll-like Receptor 4', *Immunity*. Elsevier Inc., 41(2), pp. 296–310.

Venkatraman, A. *et al.* (2003) 'Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-κB', *American Journal of Physiology-Gastrointestinal and Liver Physiology*. American Physiological Society, 285(1), pp. G177–G184.

Venturi *et al.* (1999) 'Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis', *Alimentary Pharmacology and Therapeutics*, 13(8), pp. 1103–1108.

Verdú, E. F. ***et al.*** (2002) 'Modulatory effects of estrogen in two murine models of experimental colitis', *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 283(1), pp. G27–G36.

Victoria, C. R. ***et al.*** (2009) 'Incidence and prevalence rates of Inflammatory Bowel Disease, in midwestern of sao Paulo state, Brazil', *Arq Gastroenterol*, 46(1), pp. 20–25.

Vieira, E. L. M. ***et al.*** (2012) 'Oral administration of sodium butyrate attenuates inflammation and mucosal lesion in experimental acute ulcerative colitis', *The Journal of Nutritional Biochemistry*, 23(5), pp. 430–436.

de Vrese, M. and **Schrezenmeir, J.** (2008) 'Probiotics, Prebiotics, and Synbiotics', in *Journal of Clinical Gastroenterology*, pp. 1–66.

de Vries, H. S. ***et al.*** (2009) 'Genetic association analysis of the functional c.714T>G polymorphism and mucosal expression of dectin-1 in inflammatory bowel disease', *PloS one*. Public Library of Science, 4(11), pp. e7818–e7818.

Wachi, S. ***et al.*** (2014) 'Lactobacillus delbrueckii TUA4408L and its extracellular polysaccharides attenuate enterotoxigenic Escherichia coli-induced inflammatory response in porcine intestinal epitheliocytes via Toll-like receptor-2 and 4', *Molecular Nutrition & Food Research*. John Wiley & Sons, Ltd, 58(10), pp. 2080–2093.

Wagner, J. ***et al.*** (2013) 'Bacteriophages in gut samples from pediatric Crohn's disease patients: metagenomic analysis using 454 pyrosequencing', *Inflammatory bowel diseases*. England, 19(8), pp. 1598–1608.

Wahid, F. N. and **Conway, D. J.** (1989) 'Factors Affecting the Efficacy of Ivermectin against Heligmosomoides polygyrus (Nematospiroides dubius) in mice', *Veterinary Parasitology*, 32(1989), pp. 325–340.

Walk, S. T. ***et al.*** (2010) 'Alteration of the murine gut microbiota during infection with the parasitic helminth Heligmosomoides polygyrus', *Inflammatory bowel diseases*, 16(11), pp. 1841–1849.

Walker, A. R. P. ***et al.*** (1986) 'Faecal pH, dietary fibre intake, and proneness to colon cancer in four South African populations', *Br. J. Cancer*, 53, pp. 489–495.

Walters, W. A. ***et al.*** (2014) 'Meta-analyses of human gut microbes associated with obesity and IBD', *FEBS Letters*. John Wiley & Sons, Ltd, 588(22), pp. 4223–4233.

Wan, Y. Y. and **Flavell, R. A.** (2007) 'Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression', *Nature*, 445(7129), pp. 766–770.

Wang, A. ***et al.*** (2010) 'Exacerbation of Oxazolone Colitis by Infection with the Helminth Hymenolepis diminuta', *The American Journal of Pathology*. American Society for Investigative Pathology, 177(6), pp. 2850–2859.

Wang, J. ***et al.*** (2018) 'Larval Echinococcus multilocularis infection reduces dextran sulphate sodium-induced colitis in mice by attenuating T helper type 1/type 17-mediated immune reactions', *Immunology*, 154(1), pp. 76–88.

Wang, R. ***et al.*** (2015) 'Neutralizing IL-23 Is Superior to Blocking IL-17 in Suppressing Intestinal Inflammation in a Spontaneous Murine Colitis Model', *Inflammatory Bowel Diseases*, 21(5), pp. 973–984.

Wang, S. ***et al.*** (2008) 'Infant intestinal Enterococcus faecalis down-regulates inflammatory responses in human intestinal cell lines.', *World journal of*

gastroenterology, 14(7), pp. 1067–1076.

Wang, X. *et al.* (2004) 'Oxazolone-induced murine model of ulcerative colitis', *Chinese Journal of Digestive Diseases*, 5(4), pp. 165–168.

Wang, Z. *et al.* (2014) 'Regulatory T cells promote a protective Th17-associated immune response to intestinal bacterial infection with C. rodentium', *Mucosal Immunology*. Society for Mucosal Immunology, 7, p. 1290.

Watanabe, M. *et al.* (1998) 'Interleukin 7 transgenic mice develop chronic colitis with decreased interleukin 7 protein accumulation in the colonic mucosa', *The Journal of experimental medicine*. The Rockefeller University Press, 187(3), pp. 389–402.

Watanabe, T. *et al.* (2008) 'Muramyl dipeptide activation of nucleotide- binding oligomerization domain 2 protects mice from experimental colitis', *The Journal of Clinical Investigation*, 118(2), pp. 545–559.

Wehkamp, J. *et al.* (2005) 'Reduced Paneth cell alpha-defensins in ileal Crohn's disease', *Proceedings of the National Academy of Sciences of the United States of America*. 2005/12/05. National Academy of Sciences, 102(50), pp. 18129–18134.

Wen, L. *et al.* (2008) 'Innate immunity and intestinal microbiota in the development of Type 1 diabetes', *Nature*, 455(7216), pp. 1109–1113.

Weng, M. *et al.* (2007) 'Alternatively Activated Macrophages in Intestinal Helminth Infection: Effects on Concurrent Bacterial Colitis', *The Journal of Immunology*, 179(7), pp. 4721–4731.

Whary, M. T. *et al.* (2014) 'Helminth co-infection in Helicobacter pylori infected INS-GAS mice attenuates gastric premalignant lesions of epithelial dysplasia and glandular atrophy and preserves colonization resistance of the stomach to lower bowel microbiota', *Microbes and Infection*. Elsevier Masson SAS, 16(4), pp. 345–355.

Wiese, D. M. *et al.* (2016) 'Serum fatty acids are correlated with inflammatory cytokines in ulcerative colitis', *PLoS ONE*. Public Library of Science, 11(5), pp. 1–15.

Williams, B. A. *et al.* (2017) 'Gut fermentation of dietary fibres: Physico-chemistry of plant cell walls and implications for health', *International Journal of Molecular Sciences*, 18(10).

Willing, B. P. *et al.* (2010) 'A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes', *Gastroenterology*, 139(6), pp. 1844-1854.e1.

Wilson, M. S. M. S. *et al.* (2005) 'Suppression of allergic airway inflammation by helminth-induced regulatory T cells.', *The Journal of experimental medicine*, 202(9), pp. 1199–212.

Winter, S. E. *et al.* (2010) 'Gut inflammation provides a respiratory electron acceptor for Salmonella', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 467(7314), pp. 426–9.

Wirtz, S. *et al.* (2017) 'Chemically induced mouse models of acute and chronic intestinal inflammation', *Nature Protocols*, 12(7), pp. 1295–1309.

Wolfe, A. M. *et al.* (1968) 'The epidemiology of rheumatoid arthritis: a review.II. Incidence and diagnostic criteria', *Bull Rheum Dis*, 19(3), pp. 524–529.

Wright, H. L. *et al.* (2010) 'Neutrophil function in inflammation and inflammatory diseases', *Rheumatology*, 49(9), pp. 1618–1631.

Wu, S. *et al.* (2006) 'The Bacteroides fragilis toxin binds to a specific intestinal

epithelial cell receptor', *Infection and Immunity*, 74(9), pp. 5382–5390.

Wu, S. *et al.* (2009) 'A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses', *Nature Medicine*. Nature Publishing Group, 15(9), pp. 1016–1022.

Wu, S. *et al.* (2015) 'Vitamin D receptor pathway is required for probiotic protection in colitis', *American journal of physiology. Gastrointestinal and liver physiology*. 2015/07/09. American Physiological Society, 309(5), pp. G341–G349.

Wu, Y. *et al.* (2016) 'Protective effects of Lactobacillus plantarum on epithelial barrier disruption caused by enterotoxigenic Escherichia coli in intestinal porcine epithelial cells', *Veterinary Immunology and Immunopathology*, 172, pp. 55–63.

Wynn, T. A. (2015) 'Type 2 cytokines: mechanisms and therapeutic strategies', *Nature Reviews Immunology*. Nature Publishing Group, 15(5), pp. 271–282.

Xavier, R. J. and **Podolsky, D. K.** (2007) 'Unravelling the pathogenesis of inflammatory bowel disease', *Nature*. Nature Publishing Group, 448(7152), pp. 427–34.

Yamamoto, T. *et al.* (2000) 'Effect of Fecal Diversion Alone on Perianal Crohn's Disease', *World Journal of Surgery*, 24(10), pp. 1258–1263.

Yang, J. *et al.* (2009) 'Biomechanical changes in oxazolone-induced colitis in BALB/C mice', *Journal of Biomechanics*, 42(7), pp. 811–817.

Yang, Y. *et al.* (2017) 'Fusobacterium nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor−κB, and Up-regulating Expression of MicroRNA-21', *Gastroenterology*. Elsevier Ltd, 152(4), pp. 851-866.e24.

Yeganeh, A. *et al.* (2016) 'Trans10, cis12 conjugated linoleic acid inhibits 3T3-L1 adipocyte adipogenesis by elevating β-catenin levels', *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. Elsevier B.V., 1861(4), pp. 363–370.

Yen, D. *et al.* (2006) 'IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6', *The Journal of clinical investigation*. American Society for Clinical Investigation, 116(5), pp. 1310–1316.

Yipp, B. G. *et al.* (2012) 'Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo', *Nature medicine*, 18(9), pp. 1386–1393.

Yosry, A. (2006) 'Schistosomiasis and Neoplasia', *Infection and Inflammation: Impacts on Oncogenesis*, 13, pp. 81–100.

Zaiss, M. M. *et al.* (2015) 'The Intestinal Microbiota Contributes to the Ability of Helminths to Modulate Allergic Inflammation', *Immunity*. The Authors, 43(5), pp. 998–1010.

Zakrzewski, M. *et al.* (2016) 'Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions', *Bioinformatics*, 9(December 2016), pp. 2261–2274.

Zaph, C. *et al.* (2007) 'Epithelial-cell-intrinsic IKK-β expression regulates intestinal immune homeostasis', *Nature*. Nature Publishing Group, 446(7136), pp. 552–6.

Zelante, T. *et al.* (2013) 'Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22', *Immunity*. Elsevier, 39(2), pp. 372–385.

Zeng, M. Y. *et al.* (2016) 'Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens', *Immunity*, 44(3), pp. 647–658.

Zhang, H.-Q. *et al.* (2009) 'Therapeutic effects of Clostridium butyricum on experimental colitis induced by oxazolone in rats', *World journal of gastroenterology*. The WJG Press, 15(15), pp. 1821–1828.

Zhi-Yong, S. *et al.* (1992) 'Bacterial translocation and multiple system organ failure in bowel ischemia and reperfusion', *The Journal of trauma*, 32(2), p. 148—153.

Zhong, S. and **Dobson, C.** (1996) 'Heligmosomoides polygyrus: Resistance in inbred, outbred, and selected mice', *Experimental Parasitology*, 82(2), pp. 122–131.

Zhou, Youlian *et al.* (2016) 'Increased Enterococcus faecalis infection is associated with clinically active Crohn disease', *Medicine*. Wolters Kluwer Health, 95(39), pp. e5019–e5019.

Zigmond, E. *et al.* (2012) 'Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells', *Immunity*, 37(6), pp. 1076–1090.

Zwolinska-Wcislo, M. *et al.* (2009) 'Effect of Candida colonization on human ulcerative colitis and the healing of inflammatory changes of the colon in the experimental model of Colitis ulcerosa', *Journal of Physiology and Pharmacology*, 60(1), pp. 107–118.