DIET AND ITS MODULATORY EFFECTS ON INFLAMMATORY BOWEL DISEASE:
A FOCUS ON VITAMIN D AND INFECTIOUS COLITIS

by

Natasha Ronda Ryz

B.Sc., The University of Manitoba, 2003
M.Sc., The University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2015

© Natasha Ryz, 2015
Abstract

Vitamin D deficiency increases the risk of developing inflammatory bowel disease (IBD), a disease characterized by exaggerated immune responses to luminal bacteria. While it is unclear how vitamin D impacts IBD development, it is recognized that vitamin D plays an important role in host defense against pathogenic microbes. However, the mechanisms underlying vitamin D’s ability to affect a host’s susceptibility to infection is poorly understood.

*Escherichia coli* is a pathobiont associated with IBD. Intestinal mucosa associated *E. coli* have been observed in greater numbers in patients with IBD compared to healthy controls, and these bacteria have been shown to play a role in driving intestinal inflammation. Since clinically important strains of *E. coli* generally do not colonize mice, researchers often rely on the related but mouse-specific attaching and effacing bacterial pathogen *Citrobacter rodentium*. This thesis explores the impact of vitamin D in modulating host defenses and intestinal homeostasis during infection with *C. rodentium*.

In chapter 2, I describe how treatment with active vitamin D, calcitriol worsens colitis during *C. rodentium* infection. Surprisingly, calcitriol treatment of infected mice led to increased pathogen burdens, exaggerated tissue pathology and mucosal erosions. In association with their increased susceptibility, calcitriol-treated mice had substantially reduced numbers of Th17 T-cells within their infected colons and defects in their production of the antimicrobial peptide RegIIIγ.
In chapter 3, I describe how dietary induced vitamin D3 deficiency also increases susceptibility to *C. rodentium* infection. Vitamin D3 deficient mice carried higher *C. rodentium* burdens, developed worsened histological damage and had higher inflammatory tone. Notably, these exaggerated inflammatory responses accelerated the loss of commensal microbes and were associated with an impaired ability to detoxify bacterial lipopolysaccharide.

Together, these studies show that vitamin D plays an important role in regulating host response during enteric infection. Vitamin D deficiency impairs host defense, yet treatment with the active vitamin D also suppresses Th17 T-cell responses *in vivo*, and may impair mucosal host defense against bacteria. These findings have important implications for patients with IBD who suffer from overactive immune responses, yet are also have a high risk of enteric infection.
Preface

Chapter 3

A version of this chapter has been published in the American Journal of Physiology-Gastrointestinal and Liver Physiology. I designed and conducted the majority of the studies described in the chapter, analyzed all the data and wrote the manuscript under the supervision of Dr. Bruce A. Vallance and Dr. Kevan Jacobson. Dr. Scott J. Patterson, Dr. Y. Zhang, Ms. Tina Huang (laboratory technician), Dr. Jan Dutz and Dr. Megan Levings helped with FACS analysis, shown in Figure 3.11. Ms. Caixia Ma (laboratory technician) assisted with serum collection for FITC-dextran analysis, as shown in Figure 3.8. Ms. Tina Huang (laboratory technician) performed the TUNEL stain. Dr. Andy Sham, Ms. Ganive Bhinder (graduate student) and Mr. Justin Chan performed the blinded histology pathology scoring shown in Figure 3.1D, Figure 3.5B and Figure 3.5D. Ms. Alexa Glesby, an undergraduate student under my supervision, performed the blinded TUNEL +ve cell counting for Figure 3.7A. The American Journal of Physiology- Gastrointestinal and Liver Physiology granted me permission to reproduce the manuscript in full, as part of this thesis.

Chapter 4

A version of this chapter has been submitted for publication, and accepted with revisions by the American Journal of Physiology- Gastrointestinal and Liver Physiology. I designed and conducted the majority of the studies described in the chapter, analyzed all the data and wrote the manuscript under the supervision of Dr. Bruce A. Vallance and Dr. Kevan Jacobson. Ms. Kirandeep Bhullar (graduate student) assisted with the LPS dephosphorylation assay shown in Figure 4.10A. Ms. Caixia Ma (laboratory technician) assisted with serum collection for FITC-dextran analysis, in Figure 4.2C. Ms. Tina Huang (laboratory technician) performed the F4/80 (macrophage) staining and Ms. Else Bosman (graduate student) counted the F4/80 +ve cells. Mr. Arion Lochner, an undergraduate student under my supervision, and Ms. Ganive Bhinder (graduate student) performed the blinded pathology scoring in Figure 4.4D. Dr. Xiujuan Wu helped with the real time quantitative polymerase chain reaction analysis of IL-17A and IL-17F in Figure 4.7. Dr. Sheila Innis and her laboratory manager Roger Dyer assisted with the HPLC analysis of serum 25(OH)D3.


Ethics approval: Animal work was performed with the approval of the UBC Animal Care and Use Committee (UBC protocol numbers: A07-0084, A07-0089, A09-0604, A11-0290, A11-0253)
# Table of Contents

Abstract.................................................................................................................................................. ii

Preface................................................................................................................................................... iv

Table of Contents ................................................................................................................................... vi

List of Tables .......................................................................................................................................... xii

List of Figures ......................................................................................................................................... xiii

List of Symbols and Abbreviations ....................................................................................................... xviii

Acknowledgements .................................................................................................................................. xxiv

Dedication ............................................................................................................................................... xxvi

Chapter 1: Literature Review.................................................................................................................. 1

1.1 Introduction....................................................................................................................................... 1

1.2 The intestinal epithelial barrier ...................................................................................................... 4

1.2.1 Overview of the gastrointestinal tract ...................................................................................... 5

1.2.2 Intestinal epithelial lineages ..................................................................................................... 7

1.2.3 Intestinal epithelial cell turnover .............................................................................................. 9

1.2.4 Tight junctions and the intestinal epithelial barrier ................................................................. 10

1.3 Immune defense in the gut ............................................................................................................. 11

1.3.1 Gut associated lymphoid tissue .............................................................................................. 12

1.3.2 Innate defense in the gastrointestinal tract .............................................................................. 13

1.3.3 Th1/Th17 immune responses in the gastrointestinal tract ....................................................... 17

1.4 Commensal microbes ..................................................................................................................... 19

1.4.1 Attaching and effacing (A/E) bacterial pathogens ................................................................. 22

1.4.2 Lactobacillus ............................................................................................................................ 22

1.4.3 Other commensal bacteria........................................................................................................ 23

1.5 Gut microbiota and obesity ............................................................................................................ 23

1.6 Summary .......................................................................................................................................... 25
1.4.2 *Citrobacter rodentium*: mouse model of infectious colitis ........................................ 25

1.5 Inflammatory bowel disease ..................................................................................... 27

1.5.1 Mouse models of colitis ....................................................................................... 29

1.5.2 Pathobionts associated with inflammatory bowel disease .................................. 32

1.5.3 Inflammatory bowel disease and diet ..................................................................... 34

1.5.4 Inflammatory bowel disease and vitamin D ......................................................... 36

1.6 Vitamin D overview ............................................................................................... 38

1.6.1 Vitamin D metabolism ....................................................................................... 41

1.6.2 Vitamin D and the intestinal epithelial barrier ..................................................... 43

1.6.3 Vitamin D and innate defense ............................................................................. 44

1.6.4 Vitamin D and Th1/Th17 immune responses ....................................................... 46

1.6.5 Vitamin D and bacteria ....................................................................................... 47

1.7 Research hypothesis and objectives ....................................................................... 48

Chapter 2: Experimental Methods ............................................................................. 50

2.1 Mice and experimental diets ................................................................................... 50

2.2 Calcitriol treatment ............................................................................................... 50

2.3 Dextran sodium sulfate colitis ............................................................................... 51

2.4 Bacteria strains and infection of mice .................................................................... 51

2.5 FITC-dextran intestinal permeability assay ............................................................. 51

2.6 Tissue collection .................................................................................................... 52

2.7 Serum vitamin D analysis ...................................................................................... 52

2.8 Serum calcium analysis ......................................................................................... 54

2.9 *Citrobacter rodentium* enumeration .................................................................... 54
2.10 Assessment of total microbes via DAPI staining ........................................... 55
2.11 Commensal microbe analysis ........................................................................... 55
2.12 Immunofluorescence staining ......................................................................... 56
2.13 Fluorescence intensity measurements .............................................................. 57
2.14 Epithelial cell apoptosis .................................................................................. 57
2.15 Histopathological scoring ................................................................................ 58
2.16 RNA extraction and quantitative RT-PCR ....................................................... 58
2.17 Lamina propria cell isolation ........................................................................... 61
2.18 Antibody staining and flow cytometry ............................................................. 61
2.19 Serum CD14 analysis ...................................................................................... 62
2.20 Lipopolysaccharide dephosphorylation assay .................................................. 62
2.21 Statistical analysis .......................................................................................... 63

Chapter 3: Active Vitamin D (Calcitriol) Increases Host Susceptibility to Citrobacter rodentium by Suppressing Mucosal Th17 Responses ................................................. 64

3.1 Introduction ....................................................................................................... 64
3.2 Results ............................................................................................................. 66

3.2.1 Calcitriol administered intraperitoneally protects mice against acute dextran sodium sulfate-induced colitis .................................................................................. 66
3.2.2 Protective effects of calcitriol during acute dextran sodium sulfate-induced colitis is dependent on dose and route of administration .............................................. 69
3.2.3 Calcitriol-treated mice develop macroscopic erosions in the colon and cecum at day 10-post infection with Citrobacter rodentium ...................................................... 70
3.2.4 Calcitriol-treated mice carry higher *Citrobacter rodentium* burdens at day 6 and day 10-post infection ................................................................. 73

3.2.5 Calcitriol-treated mice have worsened histological damage at day 10-post infection with *Citrobacter rodentium* ................................................................. 76

3.2.6 Calcitriol-treated mice show altered epithelial responses at day 10-post infection with *Citrobacter rodentium* infection ................................................................. 78

3.2.7 Calcitriol-treated mice carry higher bacterial burdens and ulceration at day 10-post infection with attenuated *Citrobacter rodentium* ................................................................. 82

3.2.8 Calcitriol treatment suppresses colonic cytokine mRNA levels at day 10-post infection with *Citrobacter rodentium* ................................................................. 85

3.2.9 Calcitriol-treated mice have fewer CD4+IL-17A+ cells in the colon at day 10-post infection with *Citrobacter rodentium* ................................................................. 86

3.2.10 Calcitriol-treated mice have reduced expression of the antimicrobial peptide RegIIIγ at day 10-post infection with *Citrobacter rodentium* ................................................................. 88

3.3 Discussion .............................................................................................................. 89

Chapter 4: Dietary vitamin D3 alters intestinal mucosal defense and increases susceptibility to an enteric bacterial pathogen ..................................................................... 96

4.1 Introduction ........................................................................................................... 96

4.2 Results .................................................................................................................. 98

4.2.1 Vitamin D3 deficient mice are more susceptible to *Citrobacter rodentium* infection, carrying higher cecal and extra-intestinal pathogen burdens ........................................... 98

4.2.2 Vitamin D3 deficient mice carry heavier *Citrobacter rodentium* burdens in their cecal contents at day 10-post infection ................................................................. 102
4.2.3 Vitamin D3 deficient mice display exaggerated cecitis at day 10-post infection with *Citrobacter rodentium* ................................................................. 104

4.2.4 Vitamin D3 deficient mice develop an elevated inflammatory tone in the cecum under both uninfected and infected conditions ............................................. 106

4.2.5 Vitamin D3 deficiency alters commensal bacteria at baseline and during infection with *Citrobacter rodentium* ........................................................................... 110

4.2.6 Vitamin D3 deficient mice have higher expression of the antimicrobial peptide RegIIIγ at day 10-post infection with *Citrobacter rodentium* ......................... 112

4.2.7 Bacterial lipopolysaccharide dephosphorylation is impaired in vitamin D3 deficient mice at day 10-post infection with *Citrobacter rodentium* ......................... 114

4.2.8 Vitamin D3 deficient mice have higher levels of serum CD14 at day 10-post infection with *Citrobacter rodentium* ................................................................. 114

4.3 Discussion ............................................................................................................. 116

Chapter 5: Discussion .................................................................................................. 123

5.1 Vitamin D and pathogenic *Escherichia coli* .................................................. 124

5.2 Vitamin D, bacterial translocation and sepsis .................................................. 126

5.3 How does vitamin D affect commensal microbes? .......................................... 128

5.4 How do bacteria affect vitamin D metabolism? ............................................. 130

5.5 Future directions .................................................................................................. 131

5.5.1 Can vitamin D3 supplementation protect against enteric infection? ........ 132

5.5.2 Does vitamin D suppress IL-22 expression during infection with *Citrobacter rodentium*? ........................................................................................................ 135
5.5.3 Further examine the effects of dietary vitamin D3 levels on the ecology of the intestinal microbiota........................................................................................................... 137

5.5.4 Identify the vitamin D receptor expression cell types involved in controlling gut inflammatory tone........................................................................................................... 138

5.5.5 Explore vitamin D and iron interactions during enteric infection ......................... 139

5.5.6 How do dietary factors modulate vitamin D activation? ....................................... 140

5.5.7 Can topical vitamin D therapy protect against colitis? ........................................ 143

5.6 Final remarks ........................................................................................................ 144

References.................................................................................................................. 147

Appendices.................................................................................................................. 170

Appendix A Addendum to Chapter 3 ......................................................................... 170

Appendix B Addendum to Chapter 4.......................................................................... 184
List of Tables

Table 2.1 Primer sets for quantitative RT-qPCR .......................................................... 60
List of Figures

Figure 3.1 Calcitriol-treated mice have less histological damage when given 3% dextran sodium sulfate... 68
Figure 3.2 Calcitriol-treated mice are protected against barrier disruption during 3% dextran sodium sulfate-induced colitis ... 69
Figure 3.3 Calcitriol-treated mice develop macroscopic erosions in the colon at day 10-post infection with Citrobacter rodentium ... 71
Figure 3.4 Calcitriol-treated mice have greater pathogen burdens at day 6 and day 10-post infection with Citrobacter rodentium ... 74
Figure 3.5 Calcitriol-treated mice have worsened histological damage at day 10-post infection with Citrobacter rodentium ... 76
Figure 3.6 Calcitriol-treated mice have less cell proliferation in the colon at day 10-post infection with Citrobacter rodentium ... 79
Figure 3.7 Calcitriol-treated mice have more cell death in the mid colon at day 10-post infection with Citrobacter rodentium ... 81
Figure 3.8 Calcitriol-treated mice have worsened barrier integrity at day 6-post infection with Citrobacter rodentium ... 82
Figure 3.9 Calcitriol-treated mice have worsened barrier integrity at day 6-post infection with Citrobacter rodentium ... 83
Figure 3.10 Calcitriol-treated mice have suppressed colonic cytokine mRNA levels at day 10-post infection with Citrobacter rodentium ... 85
Figure 3.11 Calcitriol-treated have fewer CD4+IL-17A+ cells in the lamina propria at day 10-post infection with *Citrobacter rodentium* ................................................................. 87

Figure 3.12 Calcitriol-treated have reduced expression of the antimicrobial peptide RegIIIγ..... 89

Figure 4.1 Vitamin D3 deficient mice lose more body weight during infection with *Citrobacter rodentium* and have thickened colon and shrunken ceca at day 10-post infection ....................... 99

Figure 4.2 Vitamin D3 deficient mice are more susceptible to *Citrobacter rodentium* infection and carry higher bacteria burdens in ceca and extra-intestinal tissues at day 10-post infection 101

Figure 4.3 Vitamin D3 deficient mice carry higher *Citrobacter rodentium* burdens in the cecal contents at infection at day 10-post infection and show delayed clearance of pathogen .......... 103

Figure 4.4 Vitamin D3 deficient mice suffer worsened histological damage with increased cell proliferation in the ceca at day 10-post infection with *Citrobacter rodentium* ...................... 105

Figure 4.5 Vitamin D3 deficient mice have more macrophages in ceca at day 10-post infection with *Citrobacter rodentium* ........................................................................................................... 107

Figure 4.6 Vitamin D3 deficient mice have higher cecal expression of tumor necrosis factor-α, interleukin-1β and, interleukin-6 .................................................................................................. 108

Figure 4.7 Vitamin D3 deficient mice have higher cecal expression of interleukin-17A, interleukin-17F and, transforming growth factor-β ......................................................................................... 109

Figure 4.8 Vitamin D3 deficient mice have more segmented filamentous bacteria in stool at baseline conditions and have a more dramatic drop in commensal bacteria at day 6-post infection with *Citrobacter rodentium* ........................................................................................................... 111

Figure 4.9 Vitamin D3 deficient mice have higher cecal expression of RegIIIγ at day 10-post infection with *Citrobacter rodentium* ........................................................................................................... 113
Figure 4.10 Vitamin D3 deficient mice have impaired dephosphorylation of bacterial lipopolysaccharide and higher serum levels of CD14 at day 10-post infection with *Citrobacter rodentium* ................................................................. 115

Figure 5.1 Vitamin D3 deficient supplemented mice are more susceptible to *Citrobacter rodentium* infection and carry higher bacterial burdens at day 10-post infection .................. 133

Figure 5.2 Vitamin D3 supplemented mice have worsened histological damage in distal colon at day 10-post infection with *Citrobacter rodentium* ................................................................. 134

Figure 5.3 Calcitriol-treated mice have no change in colonic IL-22 mRNA expression at day 10-post infection with *Citrobacter rodentium* ................................................................. 136

Supplemental Figure A.1 Mice treated daily with 10 ng calcitriol lose more body weight compared to vehicle-treated mice ................................................................. 170

Supplemental Figure A.2 Low dose 5 ng calcitriol is not protective against 3% dextran sodium sulfate induced colitis ................................................................. 172

Supplemental Figure A.3 Calcitriol given by intraperitoneal injection is more protective against 3% dextran sodium induced damage in colon, compared to intra-rectal administration........... 172

Supplemental Figure A.4 Calcitriol given by intraperitoneal injection is more protective against 3% dextran sodium sulfate induced damage in ceca, compared to intra-rectal administration.. 173

Supplemental Figure A.5 Calcitriol-treated mice have more damage in colon when administered 4% dextran sodium sulfate................................................................. 174

Supplemental Figure A.6 Calcitriol-treated mice have more damage in ceca when administered 4% dextran sodium sulfate................................................................. 175

Supplemental Figure A.7 Calcitriol-treated mice have cecal erosions at day 6-post infection with *Citrobacter rodentium* ................................................................. 176
Supplemental Figure A.8 Low dose calcitriol-treated mice have cecal erosions at day 10-post infection with *Citrobacter rodentium* ................................................................. 177

Supplemental Figure A.9 Calcitriol-treated mice have higher *Citrobacter rodentium* burdens at day 1-post infection .............................................................................................................. 178

Supplemental Figure A.10 Low dose calcitriol-treated mice have higher *Citrobacter rodentium* burdens at day 10-post infection .................................................................................. 179

Supplemental Figure A.11 Calcitriol-treated mice have trend for more histological damage in distal colon and ceca at day 10-post infection with *Citrobacter rodentium* burdens ............. 180

Supplemental Figure A.12 No differences in colonic expression of tumor necrosis factor-α or inducible nitric oxide synthase between calcitriol-treated or vehicle-treated mice at day 10-post infection .............................................................................................................. 180

Supplemental Figure A.13 No differences in colonic expression of cytokines between calcitriol-treated or vehicle-treated mice at day 10-post infection with *Citrobacter rodentium* .............. 181

Supplemental Figure A.14 No difference in CD4+IL-17A+ cells in spleen between calcitriol-treated or vehicle-treated mice .............................................................................................................. 182

Supplemental Figure A.15 No differences in colonic expression of RegIIIβ, S100A8 or S100A9 between calcitriol-treated or vehicle-treated mice at day 10-post infection with *Citrobacter rodentium* ................................................................. 182

Supplemental Figure B.1 No differences in approximate feed intake or body weight between vitamin D3 deficient and sufficient mice during 5-week feeding trial ........................................ 184

Supplemental Figure B.2 No change in barrier integrity between vitamin D3 deficient and sufficient mice at day 6-post infection with *Citrobacter rodentium* ........................................ 184
Supplemental Figure B.3 Vitamin D3 deficient mice have trend for more histological damage in distal colon at day 10-post infection with \textit{Citrobacter rodentium} ........................................ 185

Supplemental Figure B.4 No differences in cecal expression of mCramp or \( \beta \)-defensins between vitamin D3 deficient or sufficient mice at day 10-post infection with \textit{Citrobacter rodentium} .. 186
List of Symbols and Abbreviations

° Degree
=
Equal
< Less Than
> Greater Than
≥ Greater Than or Equal To
± Plus-Minus Symbol
-/- Knock-out
% Percent
α Alpha
β Beta
κ Kappa
γ Gamma
µ Micro
µl Microliter
µM Micromolar
Ab Antibody
AIEC Adherent-Invasive Escherichia coli
A/E Attaching and Effacing
APC Adenomatous Polyposis Coli
BSA Bovine Serum Albumin
C Celsius
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CK</td>
<td>Casein Kinase</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CRAMP</td>
<td>Cathelicidin-Related Antimicrobial Peptide</td>
</tr>
<tr>
<td>DAEC</td>
<td>Diffusely Adherent <em>E. coli</em></td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>dL</td>
<td>Deciliter</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Minimal Essential Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sodium Sulfate</td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>E. coli</em></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>Esp</td>
<td><em>E. coli</em> Secreted Protein</td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>E. coli</em></td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> Hybridization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead Box P3</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen Synthase Kinase</td>
</tr>
<tr>
<td>HIP</td>
<td>Hepatocarcinoma-Intestine-Pancreas</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic Uremic Syndrome</td>
</tr>
<tr>
<td>IAP</td>
<td>Intestinal Alkaline Phosphatase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial Lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNKT</td>
<td>invariant Natural Killer T-cell</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional Adhesion Molecule</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LEE</td>
<td>Locus of Enterocyte Effacement</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancing Factor</td>
</tr>
<tr>
<td>LPMC</td>
<td>Lamina Propria Mononuclear Cell</td>
</tr>
</tbody>
</table>
LPS  Lipopolysaccharide
LRP  Low-density Lipoprotein Receptor Related Protein
m  Meter
M  Microfold
M1  Classically Activated Macrophage
M2  Alternatively Activated Macrophage
MAP  Mycobacterium avium subspecies paratuberculosis
MAPK  Mitogen-Activated Protein Kinase
mCRAMP  murine cathelicidin-related antimicrobial peptide
mg  Milligram
ml  Milliliter
MLN  Mesenteric Lymph Node
mM  Millimolar
MS  Mass Spectrometry
MyD  Myeloid Differentiation
NF  Nuclear Factor
NFAT  Nuclear Factor of Activated T-cells
ng  Nanogram
NLR  NOD-like Receptor
nm  Nanometer
nM  Nanomolar
NO  Nitric Oxide
NOD  Nucleotide-Binding Oligomerization Domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSAIDs</td>
<td>Non Steroidal Anti-inflammatory Drugs</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerise Chain Reaction</td>
</tr>
<tr>
<td>pi</td>
<td>Post Infection</td>
</tr>
<tr>
<td>PPRs</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>REG</td>
<td>Regenerating Islet-Derived</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROR</td>
<td>RAR-related orphan receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Real Time</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X Receptor</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acid</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented Filamentous Bacteria</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single Immunoglobulin IL-1R-Related Molecule</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell Factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tir</td>
<td>Translocated Intimin Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T-regulatory cell</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labelling</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 Secretion System</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra-High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D Response Element</td>
</tr>
<tr>
<td>VTEC</td>
<td>Verocytotoxin-Producing <em>E. coli</em></td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per Volume</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula Occluden</td>
</tr>
</tbody>
</table>
Acknowledgements

I am very fortunate to have received unreserved support from many exceptional individuals during my PhD studies. I am forever grateful to my graduate co-supervisors, Dr. Bruce Vallance and Dr. Kevan Jacobson for their patience, kindness and guidance throughout my PhD tenure. Thank you both for giving me the freedom to pursue my own research interests and for providing me with opportunities to learn different laboratory techniques and experimental models. Bruce-
Thank you for teaching me how to “tell a story” through research and scientific writing. Thank you Kevan for teaching me about the clinical side of gut disorders and supporting my early nutrition ideas about vitamin D and gut health. I thank my graduate supervisory committee Dr. Laura Sly and Dr. Jan Dutz, for inspiring me with their own research and for providing me with guidance and valuable insights.

To all the past and current members of the Vallance and Jacobson laboratories, I thank all of them for creating a great working environment that encourages collaboration and supports new ideas. To my summer students Arion Lochner and Alexa Glesby – Thank you for all the hard work and friendship during long experiments. I thank Ms. Caixia Ma, Ms. Tina Huang and Dr. Shelley Wu for teaching me many laboratory techniques and always being ready to lend a helping hand. Thank you to Dr. Scott Patterson and Dr. Yiqun Zhang for helping me with FACS experiments. To Else Bosman, thank you for taking over my vitamin D projects with enthusiasm, curiosity and hard work. I feel happy leaving my vitamin D ideas with you and am excited to see where you take the work. Thank you to my colleagues who have provided friendship, encouragement and thoughtful suggestions through all the ups and downs of grad school,
especially Dr. Kirk Bergstrom, Dr. Andy Sham, Justin Chan, Ganive Bhinder, Shauna Crowley, Kirandeep Bhullar, Dr. Vijay Morampudi and Dr. Martin Stahl. Thank you to new laboratory members Franzi Gräf, Else Bosman, Dr. Hongbing Yu for sharing new ideas and keeping me involved with their research.

Thank you to my husband Dave for endless support, lots of love, “big picture” discussions, and for always scheming and dreaming about new ideas. And finally, thank you to my parents Ron and Marie and to my family and friends for always encouraging me, supporting me and believing me when I said I was almost done school…

I would like to acknowledge Vanier Canada Graduate Scholarships and the University of British Columbia Four Year Fellowship for funding.
To patients who suffer from gastrointestinal disease
Chapter 1: Literature Review

1.1 Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal (GI) tract that includes both Crohn’s disease and ulcerative colitis. IBD is a complex, multi-factorial disease thought to occur in genetically susceptible individuals who suffer from a weakened intestinal barrier or a “leaky gut”, maladaptive immune responses and microbial dysbiosis. Normally, the epithelial barrier and overlying mucus layer function as a semi-permeable barrier to separate the host from the intestinal luminal contents, while also allowing the absorption of nutrients. However, if the gut barrier is damaged, food antigens, commensal bacteria and their products can leak across the intestinal wall and activate underlying gut immune cells, leading to chronic inflammation and tissue damage. Specific bacterial pathogens or pathobionts, including adherent-invasive Escherichia coli (AIEC) have also been implicated (to varying degrees) in the pathophysiology of IBD (1). While the exact cause of IBD is unknown, evidence suggests that environmental factors, including diet and sunlight exposure may affect the development of IBD. The incidence of both Crohn’s disease and ulcerative colitis has gradually increased since the 1950’s, with the highest rates occurring in northern Europe and North America (2), where sunlight exposure is limited, which first suggested a link to vitamin D. Canada has among the highest prevalence and incidence rates of IBD in the world (3). However, the epidemiology of IBD is changing globally, as rates continue to rise in previously low-incidence areas such as Eastern Europe, Asia and Africa (4). Furthermore, individuals who migrate from low prevalence areas (e.g. South Asia) to high prevalence countries (e.g. Canada) are at increased risk for developing IBD, particularly among first- and second- generation
immigrants (5). The recent rise in IBD rates cannot be readily explained by changes in the genome, but rather provide evidence for the importance of environmental factors in the pathogenesis of IBD (4).

It is estimated that ~1 billion people worldwide are vitamin D deficient or insufficient (6), which has been linked to the pathophysiology of several autoimmune and inflammation-mediated diseases, including IBD (7). However, it is not known if low vitamin D status is a contributing factor to IBD, or merely a consequence of the disease, since patients with IBD are at greater risk of vitamin D deficiency due to inadequate absorption resulting from diarrhea, tissue damage and/or intestinal surgery. The role of vitamin D during IBD pathophysiology is intriguing, since the active form of vitamin D (1,25(OH)2D3 or calcitriol) is a hormone involved in diverse cellular functions including cell proliferation, expression of tight junctions, secretion of antimicrobial peptides and T-cell mediated defense (8, 9). As such, vitamin D appears to play a critical role in maintaining intestinal homeostasis by promoting epithelial barrier integrity as well as regulating host immunity and defense. Vitamin D also plays an important role in regulating host defense against pathogenic microbes. Vitamin D deficiency has been linked to an increased risk of lung infections, including those caused by Pseudomonas aeruginosa (10, 11) and Mycobacterium tuberculosis, the causative agent of most cases of tuberculosis (TB) (12, 13). Vitamin D status has also been shown to influence the risk and severity of Clostridium difficile infection, which is the most common cause of hospital-acquired infectious diarrhea (14, 15). Despite its role in host defense against mucosal bacterial pathogens, exactly how vitamin D affects a host’s susceptibility to infection is unknown. In addition, the role of vitamin D during infection with a non-invasive enteric bacterial pathogen is unknown.
Pathogenic strains of *Escherichia coli*, including enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are classified as non-invasive attaching and effacing (A/E) pathogens, based on their ability to intimately attach to the intestinal epithelium, rearrange actin into pedestal-like formations and collapse absorptive microvilli (16). EPEC causes watery diarrhea, particularly in young children in developing countries (16). EHEC is one of the more common causes of infectious colitis in Western Countries, leading to bloody diarrhea, and potentially fatal hemolytic uremic syndrome (17). Interestingly, mucosa associated *E. coli* (pathobionts) has been observed in greater numbers in patients with IBD compared to healthy controls, and these bacteria have been shown to play a role in driving inflammation in the intestine (18). Since EPEC and EHEC do not colonize mice, many researchers rely on the related, but mouse specific A/E pathogen *Citrobacter rodentium*. *C. rodentium* is transmitted via the fecal–oral route and infects the epithelial cells lining the colon and cecum, causing both mild diarrhea and colitis – as well as pathology known as transmissible murine crypt hyperplasia (19).

More specifically, *C. rodentium* infection, and the resulting host response results in barrier disruption, dysbiosis and mucosal infiltration with inflammatory/immune cells and a strong T-helper (Th)1/Th17 mediated immune response (20). *C. rodentium* infection can also be used to model aspects of several important human intestinal disorders, including IBD and colon tumorigenesis (21). This thesis explores the role of vitamin D during infection with *C. rodentium*. The goal of my studies is to increase our understanding of the interactions between vitamin D and the host immune system during infection with an enteric bacterial pathogen. This work is important as a means to help define basic mechanisms of mucosal integrity and intestinal health, with implications for both gut infections, as well as the maladaptive responses to bacteria that develop in IBD.
1.2 The intestinal epithelial barrier

The following section will give a brief overview of the GI tract and the intestinal epithelial barrier. The total mucosal surface area of the adult human GI tract extends to 200-300 m² and represents the largest area of the body in contact with an external environment (22). A single layer of epithelial cells, covered by a thick secreted mucus layer, is all that separates the host from the luminal environment - a potentially toxic milieu of bacteria, fungi, food particles, chemicals, environmental pollutants and other antigens. This dynamic barrier is constantly renewed and functions to keep the host in a state of mucosal homeostasis, through communication with both luminal antigens (e.g. bacteria) via innate receptors (e.g. Toll-like receptor) and host immune cells through secreted chemokines and other factors. Maintaining epithelial barrier integrity is very important for normal health and a “leaky gut” has been shown to play a role in the pathophysiology of various GI disorders, including IBD, celiac disease and bacterial infections with C. difficile and EPEC (23-26). Additionally, a disrupted epithelial barrier can allow bacteria and their products to enter systemic circulation causing sepsis and potential organ damage/failure and death (27). Several factors have been shown to weaken the integrity of the intestinal barrier, including alcohol use (28, 29), prolonged strenuous exercise (30), psychological stress (31) and nonsteroidal anti-inflammatory drugs (NSAIDs) (32). By using capsule endoscopy, researchers have reported that ~ 70% of patients using NSAIDs for long periods exhibit some degree of mucosal damage in the small intestine (33). There may also be cumulative effects, since NSAIDs (e.g. Ibuprofen) have been found to aggravate exercise-induced small intestinal injury and induce gut barrier dysfunction in healthy individuals (34). Understanding how the host regulates epithelial barrier integrity is crucial for understanding the underlying mechanism of gut disorders including IBD.
1.2.1 Overview of the gastrointestinal tract

The following section will provide an overview of the GI tract, intestinal epithelial cells and tight junctions to help understand how the epithelial barrier is regulated in a normal healthy state. The GI tract is the organ system responsible for consuming and digesting food, absorbing nutrients and expelling waste. The GI tract consists of a long hollow muscular tube starting from the oral cavity, where food enters the mouth, continuing to the stomach and small intestine where food is digested and absorbed, through to the colon, rectum and anus, where feces is formed and waste products are expelled. A thorough description of the entire GI tract is outside the scope of this literature review, so the following discussion will focus on the small intestine and colon, which are directly relevant to this thesis. The small intestine is approximately 6-7 m in length and is made up of three sections, starting with the duodenum (closest to the stomach), followed by the jejunum and the ileum, which extends to the ileocecal valve and empties into the colon or large intestine. The duodenum plays an important role in the digestion of food and receives digestive enzymes from the pancreas, as well as alkaline buffer, which aids in the neutralization of acidic chyme from the stomach. Bile from the liver is also secreted into the duodenum to help emulsify dietary fat for absorption. Intestinal alkaline phosphatase (IAP) is a small intestinal brush border enzyme that is luminally secreted in the duodenum by enterocytes and plays several important roles in digestion, including regulation of bicarbonate secretion and absorption of dietary fat. IAP also plays an important role in host defense, by dephosphorylating and thus detoxifying bacterial lipopolysaccharide (LPS), thereby reducing LPS’s ability to trigger intestinal inflammation as well as limiting bacterial translocation into the systemic circulation (35). Following the digestion of food, the absorption of nutrients takes place throughout the jejunum and ileum. Nutrients may be absorbed into enterocytes by diffusion, facilitated diffusion, active
transport, and endocytosis or by the paracellular route (between cells) (36). The small intestine is structured to maximize surface area; large folds of mucosa protrude into the lumen and the mucosal surface is covered with villi, long fingerlike projections lined with enterocytes, which are also coated with microscopic projections called microvilli (36). In between the villi, there are numerous invaginations that form intestinal glands called crypts of Lieberkuhn, which are also found throughout the colon. The colon is wider in diameter and much shorter (~1.5 m) than the small intestine (37). The cecum is the first part of the colon, followed by the ascending colon, which travels up the right side of the abdomen, to the transverse colon, which runs across the abdomen, to the descending colon, which travels down the left side of body into the sigmoid colon and finally to the rectum. The appendix is an appendage found off the cecum, and is thought to be a reservoir or “safe-house” for normal gut bacteria (38). The large intestine hosts a major population of bacteria that play an important role in food digestion and metabolism. Nutrients released by gut bacteria can be absorbed into the body or used locally by epithelial cells lining the gut. The large intestine absorbs the remaining water from the lumen and stores the dried condensed fecal matter in the rectum and sigmoid colon until it can be eliminated by defecation.

There are four main layers found in a cross section of the GI tract, the mucosa (innermost layer), the submucosa, the muscularis externa and the serosa (outer layer). The mucosa is made up of three sublayers: the epithelium or epithelial lining, the underlying lamina propria and the muscularis mucosae. The intestinal epithelium lines the lumen of the GI tract and is the surface that is in direct contact with the external environment- i.e. the food we eat, commensal bacteria and invading microorganisms. The lamina propria lies below the epithelium and consists of
connective tissue, small blood vessels, lymphatic vessels, and lymphoid tissue, and is thus rich in immune cells. The muscularis mucosa consists of a thin layer of smooth muscle, which separates the lamina propria from the underlying submucosa. The second layer of the GI tract is the submucosa and is made up of connective tissue and more lymphoid tissue and contains a network of nerves called the submucosal plexus or plexus of Meissner, which helps control secretions from the mucosal glands and regulates mucosal movements and blood flow. The muscularis externa, the third layer of the GI tract, contains both circular and longitudinal smooth muscle (important for peristalsis), as well as the myenteric plexus or plexus of Auerbach, which controls the frequency and strength of contractions of the muscularis to modulate GI motility. The serosa is the outermost layer and consists of connective tissue and the visceral peritoneum, which is a membrane that surrounds the organs of the abdominal and pelvic cavities (36).

1.2.2 Intestinal epithelial lineages

One of the hallmark characteristics of the intestinal epithelium is its self-regenerative capacity. The intestinal epithelium is composed of at least seven differentiated cell types, which all originate from multipotent stem cells located at the base of the crypts. These cells, which were previously described as diminutive, constantly cycling crypt base columnar (CBC) cells, have been shown to express the Wnt target gene, Lgr5, in both the small intestine and colon (39). The self-renewing capacity of these cells enables expansion and proliferation of the intestinal crypts. Interestingly, small intestinal Lgr5+ stem cells cycle much more rapidly than colonic Lgr5+ cells reflecting differences in the rate of epithelial turnover between the small intestine and the colon. These rare stem cells give rise to a pool of transit-amplifying cells which differentiate into one of several epithelial cell types as they migrate up the crypt (40). There are four major types of epithelial cells that comprise the
intestinal epithelium: enterocytes, goblet cells, Paneth cells (only found in the small intestine) and enteroendocrine cells. The most abundant cells in the intestinal epithelium are the enterocytes, which play a key role in nutrient absorption, but they also provide defense against luminal microbes by forming a physical border and producing antimicrobial peptides, including β-defensins and cathelicidins. Enterocytes exhibit apical-basal polarity where the apical membrane faces the intestinal lumen and the basal membrane is oriented away from the lumen (41). The apical side of the polarized enterocyte forms finger-like projections (microvilli) increasing the surface area of the cell to maximize absorption of water and other nutrients (42). Goblet cells are the second most abundant type of epithelial cell in the intestine and they increase in numbers toward the distal portion of the GI tract, with the highest numbers found in the colon. Goblet cells produce heavily glycosylated mucins that, once secreted apically, hydrate to form a mucus matrix that covers the lumen-facing surface of the intestinal epithelium. The mucus gel layer physically separates the intestinal epithelial surface from luminal contents and also provides a matrix rich in antimicrobial peptides to further prevent bacterial penetration. The mucus gel layer also acts as a semi-permeable barrier that prevents large molecules from reaching the epithelial surface, while allowing small molecules such as short-chain fatty acids (SCFA) to pass through to the underlying epithelial cells, which can use SCFA as a nutrient source (43, 44). Interestingly, some gut bacteria can also forage on glycans provided by the mucus layer (45). In the small intestine, another type of secretory cell, Paneth cells, are present at the base of crypts and secrete antimicrobial peptides such as α and β defensins and lysozyme, which help maintain and defend the mucosal barrier (46). Enteroendocrine cells are the fourth type of epithelial cell and constitute less than 1% of the epithelium (47). Enteroendocrine cells produce hormones and neuropeptides, including serotonin, substance P and vasoactive intestinal
peptide that control vital GI functions, including motility, secretion, nutrient uptake, transport of fluid and electrolytes, barrier integrity and local immune regulation (48). There are additional specialized cells in the intestine, including tuft cells, which are the main source of endogenous intestinal opioids and express cyclooxygenase enzymes (49). Furthermore, the Microfold or M-cell are found directly overlying immune structures called Peyer’s Patches or isolated lymphoid follicles, and play a role in the immunological sampling of luminal contents (50).

1.2.3 **Intestinal epithelial cell turnover**

Homeostasis of the intestinal epithelium is maintained through a dynamic process of epithelial cell proliferation, differentiation, migration and programmed cell death. The intestinal surface is lined by a single layer of epithelial cells, which all originate from multipotent stem cells located at the base of the crypts (51). Epithelial stem cells undergo self-renewal and generate a population of transit amplifying cells, which differentiate into one of several epithelial cell types as they migrate up the crypt. Transit-amplifying cells are controlled by the Wnt/β-catenin signalling pathway— the primary signalling component of epithelial cell proliferation in intestinal crypts. In normal cells, β-catenin is sequestered in a large cytoplasmic protein complex, called the β-catenin destruction box (Axin, Apc and the GSK3β and CK1 kinases), which promotes β-catenin phosphorylation and its subsequent degradation. However, Wnt activation of its receptors, Frizzled and LRP5/6, inhibits the destruction complex and results in accumulation of β-catenin, which then acts as a co-activator of LEF/TCF and regulates the expression of a variety of genes, including c-myc and cyclin D, which promote cell proliferation (8). Intestinal epithelial homeostasis requires the expansion of specific cell types to maintain functional balances within the GI tract. The transit-amplifying cells undergo terminal differentiation into one of the four
epithelial cell types depending on the signals they receive via the Notch pathway. The Notch pathway functions as a hierarchical signalling pathway that pushes cells towards either an absorptive lineage (enterocyte) or a secretory lineage (goblet, enteroendocrine or Paneth cells). As the epithelial cells reach the top of the crypts they undergo anoikis, a type of programmed cell death, and are sloughed off into the intestinal lumen (52). Under normal condition, intestinal epithelial cell turnover is rapid—the epithelial lining of the crypts and of the mucosal surface is replaced every three to five days (53). Although the intestinal epithelium constantly undergoes cellular turnover, the mechanisms underlying this process are poorly understood.

1.2.4 Tight junctions and the intestinal epithelial barrier

The epithelial barrier is regulated in large part by the apical junctional complex, which is composed of the apically located tight junctions, and the underlying adherens junctions and desmosomes. Tight junctions function as a semipermeable gate permitting the passive entry of luminal nutrients, ions, and water, and at the same time restricting microbial entry and thus regulating the barrier function of the epithelium. Tight junctions are multi-protein complexes (over 50 proteins) made of transmembrane proteins and cytoplasmic plaque proteins. Transmembrane proteins mediate cell-to-cell adhesion and seal the paracellular space between epithelial cells. They can be divided into tetra-span and single-span proteins. The tetra-span proteins include the claudin family of proteins, tricellulin and occludin. Single span transmembrane proteins are mostly junctional adhesion molecules (JAM). The claudins are considered to be the structural backbone of tight junctions and play an important role in regulating paracellular selectivity to small ions. In the intestine, claudin-1, -3, -4, -5, and -8 tighten tight junctions (decreasing paracellular permeability); whereas claudin-2 forms charge-
selective paracellular pores (54). While claudins can regulate the space between two adjacent cells, tricellulin (also known as MARVELD2) is found at cell-to-cell contact points among three adjacent cells. Occludin has also been linked to the regulation of intermembrane diffusion and paracellular diffusion of small molecules (55). The JAM family has been shown to regulate epithelial barrier function and is also implicated in tight junction assembly (27). The adherens junctions are located in the apical region of the epithelial cell, just below the tight junctions. The adherens junctions are made up of cadherin and β-catenin and help to maintain cell surface polarity and keep neighboring cells in close contact, thus allowing tight junction assembly (23). In contrast with tight junctions, which are crucial for maintaining barrier function, adherens junctions are responsible for cell–cell recognition, initiating and maintaining cell to cell contacts and are important sites of intercellular communication (56). Desmosomes are located below adherens junctions and are connected to intermediate filaments in the cytoplasm. The major function of the desmosome is to anchor adjacent cells and to facilitate cell-to-cell communication and contact. Together, tight junctions, adherens junctions and desmosomes form the apical junction complex that allows for an effective and highly regulated epithelial barrier.

1.3 Immune defense in the gut

The GI tract hosts the largest number of immune cells of any tissue in the body, which is often referred to as the gut associated lymphoid tissue (GALT), which defends the body against invading organisms and also regulates tissue homeostasis. An appropriate immune response is critical to a healthy host, since a poor or weak immune response can increase susceptibility to infection, while an overactive immune response can cause chronic inflammation and tissue
damage. The following section will provide an overview of the GALT and briefly describe the innate and acquired arms of the immune response in the gut.

1.3.1 Gut associated lymphoid tissue

The GALT is composed of aggregated lymphoid tissue, which includes cryptopatches, isolated lymphoid follicles and Peyer’s patches as well as non-aggregated tissue, including intraepithelial (IEL) lymphocytes and lymphoid cells found in the lamina propria (50). Cryptopatches are small structures present at birth and distributed throughout the small intestine and colon, consisting of innate lymphoid cells and dendritic cells. Cryptopatches can mature into isolated lymphoid follicles, which also contain B-cells, which preferentially differentiate into immunoglobulin A (IgA) plasma cells and facilitate local intestinal immunity. Interestingly, cryptopatches are present in germ-free mice but colonization with commensal bacteria causes cryptopatches to shift towards mature isolated lymphoid follicles (50). Peyer’s patches are aggregated lymphoid follicles found in the mucosa and submucosa of the small intestine, with the highest number found in the ileum. Peyer’s patches are rich in T-helper (CD4+) and effector T-cells (CD8+), B-cells, macrophages and dendritic cells. The GALT-associated epithelium possesses relatively few goblet cells and therefore the mucus overlying it is not as thick (50). Overlying the Peyer’s patches and interspersed along the overlying epithelium are specialized M cells (microfold cells), that can endocytose and transport antigens to the underlying follicles. In the Peyer’s patches, antigens are processed and presented to activate B and T cells. The activated B cells differentiate into plasma cells and produce IgA (57). Activated immune cells leave the Peyer’s patches and populate the lamina propria and intraepithelial regions of the intestine. The lamina propria is rich in CD4+ T cells and IgA secreting plasma cells. IEL lymphocytes are located in
the interstitial spaces of the mucosal epithelium and are predominantly CD8+ T-cells (50). Mesenteric lymph nodes (MLN) are not situated within the intestinal mucosa but rather in the mesenteric ligaments, but are still considered part of the GALT. When CD4+ and CD8+ cells mature in the Peyer’s patches, they leave via the MLN to enter systemic circulation, and can return via the MLN to the lamina propria regions of the gut. Thus, MLN are rich in CD4+ and CD8+ cells (50). Overall, the GALT is a complex immune system that is capable of mounting an immune response against invading pathogenic antigens, while also maintaining tolerance.

1.3.2 Innate defense in the gastrointestinal tract

The innate immune system is considered the first line of host defense and is induced by recognition of microbial components, known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Cells of the innate immune system, including intestinal epithelial cells, express PRRs, which are germline encoded receptors and include toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), both of which recognize bacterial products, including peptidoglycan (TLR2, NOD1, NOD2), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5) and bacterial DNA (TLR9). RIG-1-like receptors are a type of intracellular PRR involved in the recognition of viruses by the innate immune system. Most TLR signalling involves the adaptor protein MyD88 and results in the activation of the transcription factor nuclear factor (NF)-kB, which in turn controls the expression of an array of inflammatory and immunoregulatory genes, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6, which elicit innate responses against pathogens and direct the development of adaptive immunity (58). CD14 is another pattern recognition receptor that aids in the detection of several bacterial products including LPS (59). Soluble CD14
(sCD14) is secreted into bodily fluids and in combination with TLR4, detection of LPS by CD14 results in a pro-inflammatory immune response (60). Interestingly, studies show that activation of PRRs by commensal bacterial products play a role in promoting intestinal epithelial growth, barrier function and tissue repair under baseline conditions (61, 62). As such, mutations in pattern recognition receptors may impair host immune defenses leading to increased susceptibility to pathogens resulting in chronic inflammation of the gut.

Secreted bactericidal peptides or defensins produced by the intestinal epithelia represent another crucial element of innate mucosal defense, including cathelicidin, β-defensins and regenerating islet-derived protein 3 gamma (RegIIIγ). The cathelicidin proform is processed to the mature bioactive peptide, which is called LL-37 in humans and mCRAMP (murine cathelicidin-related antimicrobial peptide) in mice. Cathelicidin (LL-37 and mCramp) is expressed within epithelial cells and macrophages found in the healthy human and mouse colon (63-65). Cathelicidin secretion is induced by intestinal microbiota and has broad-spectrum antimicrobial activity against bacteria, viruses, and fungi (66). Beta-defensins are expressed by enterocytes of the small and large intestine. Human β-defensin 1 is expressed constitutively by enterocytes while the expression levels of human β-defensin 2 and human β-defensin 3 are upregulated following exposure of intestinal epithelial cells to microbial products and inflammatory cytokines, including IL-1β, TNF-α and IL-17. The defensive mechanisms of β-defensins include binding to negatively charged microbial membranes, thereby causing microbial cell death and the chemoattraction of immune cells (66). Expression of the antimicrobial C-type lectin RegIIIγ (hepatocarcinoma-intestine-pancreas/pancreatic associated protein (HIP/PAP) in humans) is upregulated in the small intestine and colon after bacterial reconstitution of germ-free mice (67,
RegIIIγ can be expressed by several epithelial cell lineages, namely enterocytes, Paneth cells, and goblet cells (69). RegIIIγ restricts bacterial colonization of the intestinal epithelial surface and consequently limits activation of adaptive immune responses by the microbiota (70).

Effector inflammatory cells, including innate T cells, neutrophils, and macrophages, are the cellular part of the innate immune system and play an important role in identifying and eliminating pathogens. Innate T cells are a heterogeneous group of αβ and γδ T cells that respond rapidly (< 2 h) upon activation. Three major populations within the innate T cell group are the invariant natural killer T cells, mucosal associated invariant T cells, and gamma delta T cells. These cells recognize foreign/self-lipid presented by non-classical MHC molecules, such as CD1d, MR1, and CD1a. They are activated during the early stages of bacterial infection and act as a bridge between the innate and adaptive immune systems (71). Neutrophils (polymorphonuclear leukocytes) are the most abundant leukocyte population in the blood, comprising 50 – 60% of the circulating leukocytes (72). As part of the normal gut inflammatory response, neutrophils are recruited by chemotaxis to sites of injury or infection within minutes, with a peak response within 24-48 hours and are considered the hallmark of acute inflammation (73). Resident cells, including macrophages, epithelial cells and T-cells contribute to the recruitment of neutrophils through the production of chemokines, forming a chemoattractant gradient that allows neutrophils to transverse the vascular endothelium to reach the intestinal lamina propria. The main function of neutrophils in the intestine is to kill luminal microbes that translocate across the epithelium and invade the mucosa (73). Neutrophils have three strategies for directly attacking microorganisms, including phagocytosis (ingestion), release of soluble antimicrobials and generation of neutrophil extracellular traps. Upon contact with invading
microbes, neutrophils form massive amounts of reactive oxygen species that can effectively destroy pathogens. Neutrophils also contain granules filled with myeloperoxidase, lysozyme and antimicrobial peptides, including α-defensins and cathelicidins that are released upon contact with microbes (73). Neutrophils also play an important role in the resolution of inflammation, by cleaning up cellular debris, releasing growth factors as well as pro-resolution lipid mediators, including lipoxins and resolvins (74).

Macrophages provide a first line of defense against intracellular pathogens by generating an inflammatory and respiratory burst and initiating antigen presentation to activate adaptive immunity. Upon tissue damage or infection, monocytes are rapidly recruited to the tissue, where they differentiate into tissue macrophages. Macrophages are remarkably plastic and can change their functional phenotype depending on the environmental cues they receive. Stimulation with the Th1 cytokine interferon (IFN)-γ and microbial byproducts, such as LPS, promotes maturation of classically activated macrophages (M1), whose microbicidal actions include the respiratory burst, nitric oxide (NO), and reactive oxygen species (ROS), and the secretion of proinflammatory cytokines, such as TNFα and IL-12 to enhance cell-mediated immunity (75). In contrast, exposure of macrophages to the Th2 cytokine IL-4 produces an alternatively activated M2 macrophage phenotype, which secretes relatively more IL-10. M2 macrophages help with parasite clearance, suppress inflammation and promote tissue remodeling. Macrophages also play a major role in the phagocytosis of debris and pathogens, and are highly specialized in the removal of dying or dead cells and cellular debris (75). Through their ability to clear pathogens and instruct other immune cells, macrophages and neutrophils have a central role in protecting the host but also contribute to the pathophysiology of inflammatory and degenerative diseases.
1.3.3 Th1/Th17 immune responses in the gastrointestinal tract

Acquired immunity is a form of target specific host defense, which is mediated by B and T lymphocytes. This type of defense exhibits immunological memory and can recognize self from non-self. The basic steps of the acquired immune response are antigen processing and presentation, activation of T helper cells (CD4+), which can then activate cell-mediated immunity and/or humoral immunity. Cell-mediated immunity involves effector T cells (CD8+) and acts mainly on cancer cells or infected cells. Humoral immunity refers to immune responses that involve antibodies produced by B-cells. Interspersed throughout the GI mucosa are resident CD4+ T-helper cells that play an important role in orchestrating mucosal integrity and preventing the systemic spread of bacteria (i.e. sepsis) (76). Naïve CD4+ T helper cells (Th) can differentiate into various subsets, including Th1, Th2 and Th17 cells depending on the type of antigen and cytokines encountered in the environment. In turn, Th1, Th2 and Th17 cells each make their own distinct cytokines. Intracellular bacteria and viruses activate macrophages, dendritic cells and other antigen presenting cells to release the cytokine IL-12, which influences naïve T cells to become Th1 cells (77). Th1 cells express the transcription factor T-bet and secrete the cytokines IFN-\(\gamma\) and TNF-\(\alpha\), and play an essential role in inhibiting replication of intracellular pathogens (77). Interestingly, IFN-\(\gamma\) can induce expression of the vitamin D activating enzyme CYP27B1 in human monocytes and macrophages, thereby controlling local production of calcitriol (78).

The presence of IL-4, released by mast cells and eosinophils in response to allergens and parasites favors the Th2 pathway. Th2 cells express the transcription factor GATA-3 and secrete cytokines IL-4, IL-5 and IL-13, which regulate humoral immunity against parasite infections,
including helminths (77). In the presence of IL-6 and transforming growth factor (TGF)-β, naïve CD4+ T cells differentiate into Th17 cells. Th17 cells express the transcription factor ROR-γt and are characterized by the production of the cytokines IL-17A, IL-17F and IL-22. Th17 cells are abundant at most mucosal surfaces and play an important role in providing protection against extracellular bacteria and fungal infections (79). However, Th17 responses have also been shown to play a critical role in driving inflammatory and autoimmune diseases, including rheumatoid arthritis, psoriasis, multiple sclerosis, and IBD (80). Receptors for IL-17A and IL-17F (Il-17Ra and IL-17Rc) are expressed by several cell types, including antigen presenting cells and epithelial cells, whereas, receptors for IL-22 (IL-22R) appear to be localized to the epithelium (81). IL-17A has been shown to induce the production of pro-inflammatory cytokines (IL-6 and TNF-α) and induce granulopoietic factors and chemokines to recruit neutrophils in response to infection (82). Neutrophil recruitment induced by Th17 cells is necessary for host defense and pathogen clearance in several infections including *Mycoplasma pneumoniae*, *Bordetella pertussis*, *Candida albicans*, *Staphylococcus aureus*, and *C. rodentium* (83). The cytokines IL-17A and IL-17F also have been shown to induce antimicrobial peptides, including β-defensin 1, 3 and 4 in the colon during infection with *C. rodentium* (84). Expression of the S100A8 and S100A9 subunits of calprotectin – an antimicrobial peptide that chelates the essential nutrients zinc and manganese (85) - is also induced in response to IL-17 and IL-22. IL-22 signals through the IL-22 receptor to induce STAT3-dependent innate epithelial defense mechanisms, including stimulating the production of antimicrobials such as RegIIIγ, lipocalin-2 and β-defensins, along with reinforcing tight junctions between enterocytes, and enhancing epithelial cell proliferation (86). In addition to Th17 cells, a number of other RORγt-expressing cell types can secrete IL-17A and IL-22, including CD8+ αβ T cells, γδ T cells, innate lymphoid cells and invariant
Natural Killer T (iNKT) cells (87). Unlike Th17 cells, antigen priming is not required for activation of innate lymphoid cells; IL-23 stimulation is often sufficient for inducing IL-17A and IL-22 secretion by these cell types (87).

CD4+ T cells can also develop into regulatory cells (Treg) that express forkhead box P3 (FoxP3) and produce transforming growth factor (TGF)-β and IL-10. Tregs play a key role in suppressing inflammatory responses and inducing tolerance and tissue homeostasis. There are four basic mechanisms that Tregs use to suppress immune responses; modulation of antigen presenting cell maturation and function, disruption of metabolic pathways, production of anti-inflammatory cytokines (including IL-10), and the killing of target cells- i.e. Tregs express Granzymes A and B, and perforin, which can trigger the apoptosis of effector T cells (88). Th17 cells and Treg cells can both develop in response to TGF-β (Th17 cells also require the presence of IL-6) (89). Interestingly, it has been demonstrated that an increase in Th17 cells in the gut is linked to a reciprocal decrease in Treg cells (90). It is thought that control of inflammation in the gut relies in part on the balance between Th17 cells and Treg cells (89).

### 1.4 Commensal microbes

The GI tract hosts a major population of commensal bacteria, and the resident microbial communities becomes larger and more diverse as one moves down the GI tract. In the stomach, only a few bacterial species are able to colonize, due to the low pH from hydrochloric acid and the activity of proteolytic enzymes (91). In the small intestine, approximately 10^7 bacteria/gram of luminal material can be detected (92). Within the large intestine, the slower transit times and the high supply of nutrients from undigested food particles allow for the highest density and
abundance of microorganisms (93). The healthy adult colon contains more than 100 trillion ($10^{14}$) bacteria that comprise many different strains (94) and the total number of genes derived from this diverse microbiome exceeds that of the entire human genome by at least 100-fold (95).

Commensal microbes play a critical role in maintaining human health, through the synthesis of vitamins, nutrient digestion, energy recovery, maintenance of intestinal barrier integrity, immune education and host defense. The host, in turn, provides a stable environment for the microbes to live along with a steady nutrient supply. Vitamins are essential for human health and must be provided from exogenous sources, including diet and supplementation. The microbiota of the human colon is known to produce vitamin K (menaquinones) as well as most of the water-soluble vitamins of group B, including biotin, nicotinic acid, folates, riboflavin, thiamine, pyridoxine, panthotenic acid, and cobalamin (vitamin B12) (96). Unlike dietary vitamins, which are mainly absorbed in the proximal part of the small intestine, the uptake of microbial vitamins predominantly occurs in the colon (97). Colonocytes appear to be able to absorb biotin, thiamin, folates, riboflavin, panthotenic acid, and menaquinones, indicating that the microbiota-produced vitamins may contribute to systemic vitamin levels (97, 98). The gut microbiota also produces enzymes that the host lacks, which are involved in the breakdown of complex molecules, such as plant polysaccharides and fibers. The fermentation of dietary components results in the production of SCFAs (e.g., acetic, lactic, propionic, and butyric acids), branched chain fatty acids (e.g., isobutyric, isovaleric, and 2-methylbutyric acids), carbon monoxide, ammonia, amines and several other end products. These fermentation products affect the gut environment and the host’s health, acting as energy sources, regulators of gene expression and cell differentiation, as well as anti-inflammatory agents. Commensal bacteria and probiotics have
been shown to promote intestinal barrier integrity both \textit{in vitro} and \textit{in vivo} by targeting the expression and distribution of tight junction proteins (27). The gut microbiota can also effectively defend the host against enteric bacterial infections by competing with pathogens for habitats and nutrients (99). As such, host-microbe interactions are essential for host resistance to pathogenic infections, gut development, and epithelial homeostasis (100).

The composition of the gut microbiome is largely determined by environmental factors, for instance vaginal birth vs. caesarian section, breast milk vs. formula feeding and eating an animal vs. plant-based diet have all been shown to alter microbial composition (101). In human adults, the colon is characterized by the predominance of two phyla, \textit{Bacteroidetes} and \textit{Firmicutes} (which include \textit{Clostridium} and \textit{Lactobacillus} microbes), with minor contributions from the \textit{Proteobacteria} and \textit{Actinobacteria} (including \textit{Bifidobacteria}) phyla (102). The small intestine contains a higher relative abundance of members of the \textit{Actinobacteria} and \textit{Streptococcaceae} phyla (103). The small intestine of infants has also been shown to contain segmented filamentous bacteria (SFB), gram-positive, spore-forming bacteria that can specifically induce Th17 cells (104). It is now appreciated that some symbiotic microorganisms in the GI tract can induce pathology under certain conditions, including states of inflammation, and the term 'pathobionts' has been used to describe resident microbes with pathogenic potential. Pathobionts are innocuous to the host under normal conditions, distinct from traditional pathogens, which may cause disease even in healthy hosts (105).
1.4.1 Attaching and effacing (A/E) bacterial pathogens

*Escherichia coli* are Gram-negative, facultative anaerobes, within the family *Enterobacteriaceae*, class *Gammaproteobacteria* and phylum *Proteobacteria*. *E. coli* is normally a commensal bacterium that co-exists in the intestines of its human host in a mutually beneficial relationship (106). However, through the acquisition of pathogenicity islands, prophages, and plasmids, several highly adapted *E. coli* clones have evolved the ability to efficiently colonize specific anatomical sites in their hosts and cause disease. Pathogenic *E. coli* can cause a variety of diarrheal diseases and some (i.e., ExPEC) are capable of infecting extra-intestinal sites such as the urinary tract and kidneys (uropathogenic *E. coli* – UPEC) or the bloodstream and/or the central nervous system, particularly in the neonate (107). There are six main categories of pathogenic *E. coli* that affect the intestines of humans: Shiga-toxin-producing *E. coli* (STEC; also called verocytotoxin-producing *E. coli* or VTEC), of which enterohemorrhagic *E. coli* (EHEC) are a pathogenic sub-group; enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC); enteroinvasive *E. coli* (EIEC); and diffusely adherent *E. coli* (DAEC).

Of the *E. coli* pathotypes, EPEC and EHEC are unique in colonizing the human intestinal mucosa via ‘attaching and effacing’ (A/E) lesions, characterized by their ability to intimately attach to the intestinal epithelium, rearrange actin into pedestal-like formations and flatten/destroy absorptive microvilli (16). A/E pathogens share a chromosomally located pathogenicity island called the locus of enterocyte effacement (LEE), which encodes the outer membrane adhesin (intimin), a type 3 secretion system (T3SS), chaperones, translocators and effector proteins, including the translocated intimin receptor (Tir) (108). Among the A/E
pathogens, the pathogenesis of EPEC is the best described. Following ingestion, and passage through the upper GI tract, EPEC adheres to enterocytes and Tir is inserted into the host-cell membrane by EPEC’s T3SS, which acts as a molecular syringe to translocate bacterial effector proteins into host cells. Tir then acts as a receptor for the intimin protein, which is located on the outer membrane surface of EPEC. Upon binding to the intimin protein, the intracellular region of Tir initiates a complex signalling cascade within the host cell to mediate actin rearrangements and pedestal formation (108). A/E lesion-forming pathogens do not, in general, invade deeper layers of the mucosa or spread systemically, making them predominantly non-invasive mucosal (luminal) pathogens.

Diarrhea remains the second leading cause of death in children younger than 5 years globally, accounting for 1.3 million deaths annually (109). EPEC is among the most important diarrheal pathogens infecting children worldwide due to its high prevalence in both community and hospital settings (110), and because it is one of the main causes of persistent diarrhea (111). EPEC causes watery diarrhea, particularly in young children in developing countries (16). In contrast, EHEC is one of the common causes of infectious colitis in Western Countries, leading to bloody diarrhea, and potentially fatal haemolytic uremic syndrome (HUS) (17). EHEC O157:H7 and common non-O157 EHEC serotypes reside in the GI tracts of cattle and other ruminants (e.g., deer, goats, and sheep) and can survive and grow in both the environment and in food (112). Recent outbreaks of EHEC have been linked to the ingestion of undercooked beef, various types of processed, prepackaged, and frozen foods, and also fresh produce -including spinach and sprouts (113). It should be noted that EHEC and EPEC do not normally infect plants, however under certain conditions they can survive on, penetrate into, and colonize
internal plant tissues causing serious food-borne disease outbreaks. Contaminated irrigation water, farm workers with limited means of proper sanitation, and fecal contamination in the farm by animals can expose plants to human pathogens before harvest of the edible parts (114). After harvest, contamination can occur during unclean modes of transportation, processing, and bagging (114).

EHEC O157:H7 was first identified as a new diarrheagenic pathogen in 1982 during an investigation of an outbreak of GI illness, which was traced to the consumption of contaminated hamburgers (115). For EHEC O157:H7 infection, the incubation period between ingestion of bacteria and onset of diarrhea is approximately 3 days, although this can vary between 2 and 12 days (116). Although not all patients with EHEC O157:H7 in their stool are symptomatic, severe abdominal cramping, fever and watery diarrhea typically occur during the first 1 to 3 days of illness, and may be accompanied by vomiting (117). In approximately 90% of patients, hemorrhagic colitis (bloody diarrhea) follows the initial phase of illness (113). HUS occurs in 5-15% of infected patients and is the most severe complication of EHEC infection. HUS is characterized by microangiopathic hemolytic anemia (damage of small blood vessels with destruction of red blood cells), thrombocytopenia (decrease of platelets) and acute kidney failure (113). In the absence of HUS, there is spontaneous resolution of symptoms in most patients. Because EHEC is extremely virulent, even a low infectious dose (100–1000 organisms) can result in a clinically significant infection (118, 119).
1.4.2 *Citrobacter rodentium*: mouse model of infectious colitis

Until recently, the study of A/E bacterial pathogens has largely been restricted to *in vitro* experiments, as EPEC does not effectively colonize/infect the intestines of mice or other laboratory animals (120). Our laboratory has been one of the leading groups using the murine pathogen *Citrobacter rodentium* as a model of EPEC and EHEC infection. *C. rodentium*, formally called *Citrobacter freundii* biotype 480 is a mouse specific, Gram-negative pathogen that shares 67% of its genes with both EPEC and EHEC, including the LEE pathogenicity island (121). Most research studies involve inoculating mice by oral gavage with laboratory-cultured *C. rodentium*, which results in a highly reproducible infection cycle. Infection by *C. rodentium* lasts approximately three weeks, with the peak of infection seen at 8-10 days and clearance by day 28, causing only modest morbidity and low rates of mortality in most mouse strains (C57BL/6, NIH Swiss and Balb/c) except C3H/HeJ and C3HOu/J mice, which are extremely susceptible to infection and suffer high mortality rates (122). Following oral gavage, *C. rodentium* initially colonizes the cecal patch and spreads to surrounding cecal epithelial cells over the first 2-3 days and then progresses to the large intestine, colonizing the distal colon and rectum within 4-6 days after infection (19, 123). *C. rodentium* intimately attaches to epithelial cells lining the cecum and colon, resulting in a host response involving barrier disruption, crypt hyperplasia, goblet cell depletion as well as a strong Th1/Th17 response, resulting in immune cell infiltration of the intestinal mucosa (20). Th1/Th17 responses play a protective role during *C. rodentium* infection. As such, mice lacking IFN-γ (Th1) or IL-17A (Th17) are more susceptible to *C. rodentium*, with increased bacterial burdens and worsened histological damage in the colon, compared to wildtype mice (84, 124, 125). *C. rodentium* induces an IL-17A/F response in the cecum and colon at early time points (days 4–7 post infection), followed by a robust adaptive IL-17
response at 10–14 days post infection (85). Several β-defensins are induced in the gut in response to *C. rodentium* infections, with induction of β-defensins 1, 3, 4 dependent on IL-17A and IL-17F (84). IL-22 expression in the cecum and colon is also induced very early during *C. rodentium* infection, at day 4-post infection, and IL-22-deficient mice succumb to disease by 10 days post infection (85). Notably, IL-22-dependent RegIIIγ secretion is crucial for mediating protection against *C. rodentium*, since exogenously added RegIIIγ has been shown to rescue IL-22-deficient mice from mortality and morbidity (85). Moreover, mice deficient for IL-6 (126) or IL-23 (85), two inflammatory cytokines that induce IL-17A and IL-22 responses, also fail to control *C. rodentium* infection and display enhanced mortality.

Gram-negative bacteria, such as *C. rodentium* contain LPS as a major component of their bacterial cell wall. Bacterial LPS is recognized by the host innate receptor Toll-like receptor 4 (TLR4), in combination with CD14, MD-2 and the LPS binding protein, found on the surface of monocytes, macrophages and intestinal epithelial cells. Activation of TLR4 and its co-factors by bacterial LPS causes a signalling cascade that leads to the recruitment of inflammatory cells and the production of inflammatory mediators. It has previously been shown that TNF-α, IL-1β and IL-6 play an important role in controlling *C. rodentium* burdens in the gut and preventing tissue injury during infection (126-128). However, excess TNF-α and IL-1β are also known for contributing to tissue injury during infection with *C. rodentium* (127, 128). Ultimately the host immune/inflammatory response helps control these infections, but it also causes significant pathology and pathophysiology. Similarly while the induction of antimicrobial genes at the mucosal surface help to eventually clear *C. rodentium* from its hosts, these responses also remove competing commensal microbes, reducing colonization resistance in the intestine (129).
The *C. rodentium* colitis model is very robust and can be used to study several aspects of disease that are relevant to IBD, including barrier disruption, Th1/Th17 mediated inflammatory responses and bacteria-host interactions at the mucosal surface.

1.5 **Inflammatory bowel disease**

IBD generally describes a heterogeneous group of idiopathic conditions that share the characteristic of chronic inflammation of the GI tract. The two most common conditions in this category are Crohn’s disease and ulcerative colitis. Crohn’s disease can involve any part of the GI tract- from mouth to anus, however the distal ileum and cecum are the most commonly affected regions, whereas ulcerative colitis only involves the colon, and has a continuous distribution starting in rectum. Crohn’s disease manifests in a patchy/non-continuous fashion and is transmural- i.e. all the layers of the intestinal wall may be involved (130). In contrast, ulcerative colitis is characterized by inflammation that is limited to the superficial (mucosal) layers of the colon. In general, Crohn’s disease is characterized by increased production of Th1/Th17 related cytokines, while ulcerative colitis seems to be a Th2 cytokine-associated disease (131). Macrophages and dendritic cells in the lamina propria are increased in absolute number and have an activated phenotype in both forms of IBD and the production of proinflammatory cytokines, including TNF-α, IL-1β and IL-6 is enhanced (58). Symptoms of IBD include severe abdominal cramps and pain, bloody diarrhea, loss of bowel control, weight loss, fever, fatigue, swelling of joints, body aches and pain. Extraintestinal manifestations of IBD may also involve inflammatory-mediated damage to the eyes, skin, bones, liver and biliary tract, kidneys, lungs and the vascular system (132). IBD can severely impact one’s psychological and social well being, with high rates of stress, anxiety and depression (133), as well as concerns
related to body image, loss of control, isolation, fear of not reaching ‘full potential’ and disease stigma (134, 135).

Family history is a risk factor for developing IBD and numerous genes have been implicated in the pathogenesis of IBD (2, 130). Indeed, over 160 genes have been implicated in controlling susceptibility to IBD, and they encompass multiple physiological processes, including intestinal epithelial defense, lymphocyte activation and microbe recognition (130). Although the genetic basis of IBD continues to be characterized, most of the risk alleles involved with IBD as determined through Genome-Wide Association Studies only increase the odds of disease development by a relatively small amount (136). In most cases, environmental risk factors likely increase the probability of disease or trigger the disease (137). Medications including NSAIDS (138), birth-control pills (estrogens) (139) and childhood antibiotic exposure, as well as repeated use of antibiotics (140-143), have all been associated with an increased risk of developing IBD. Furthermore, evidence suggests that lack of sunlight exposure and dietary changes, including a shift to a Western style diet can contribute to the development and progression of inflammatory mediated GI disorders, including colon cancer and IBD (130).

Current therapies for IBD include aminosalicylates, corticosteroids, immune modulators and biologics, which are directed at controlling inflammation and preventing complications. Exclusive enteral nutrition “tube feeding” is also used in the management of Crohn’s disease. Remarkably, enteral nutrition has been shown to be effective for inducing remission and mucosal healing in patients with Crohn’s disease, especially children (144), although its mode of action remains unknown. Most non-biological drug therapies provide symptomatic improvement, but
do not stop the underlying inflammatory processes (145). Furthermore there are numerous adverse events of certain therapies. For instance corticosteroid therapy can cause mood changes, undesirable weight gain and bloating, severe acne, opportunistic infections, and long-term use can increase the risk of developing diabetes, atherosclerosis, osteoporosis and cause growth delay in children (146). Biologics are the newest class of drugs used in treating IBD and include the anti-TNF-α agents infliximab, adalimumab, certolizumab, and golimumab which have dramatically changed the course of IBD- including steroid sparing, less hospitalizations, fewer surgeries, and achieving deep remission and better quality of life (147). Despite the benefits of anti-TNF therapy, there are some limitations, including the lack of primary response and the loss of response to treatment in some patients. For instance, loss of response has been reported in 25%–40% of Crohn’s disease patients in randomized controlled trials, with an estimated annual loss of response rate of about 13% per patient-year under scheduled treatment with infliximab (148). Approximately 70% of people with Crohn’s disease and 40% of those with ulcerative colitis will require surgery at least once during the course of disease (149). There is currently no cure for Crohn’s disease, whereas ulcerative colitis may be ‘cured’ by surgically removing the colon, however this is not an ideal or a desired result. These findings underscore the need for new IBD therapies that can complement or replace existing treatments.

1.5.1 Mouse models of colitis

There are several important mouse models that can be used to study different aspects IBD. For instance, the CD45RBhigh T-cell transfer model of colitis is the most widely used mouse model to understand how T-cells mediate the initiation, induction and regulation of chronic colitis. Administering CD4+CD45RBhigh T-cells to immunodeficient mice initiates the development of
T-cell mediated chronic enterocolitis, similar to human Crohn’s disease. Another model, the II-
I0-/- mouse develops spontaneous chronic enterocolitis similar to human UC, due to immune
responses against resident bacteria in conventional animal facilities. There are also several
chemically induced models of colitis, including trinitrobenzene sulfonic acid (TNBS) and DSS.
The remainder of this section will focus on DSS colitis, since it is directly relevant to this thesis.

The DSS colitis model is the most widely used colitis model in the world. There is some
evidence that chronic DSS colitis is T-cell mediated, while acute DSS colitis specifically
involves the innate immune system, since T- and B- cell deficient SCID mice are fully capable of
developing severe intestinal inflammation when exposed to DSS for short periods (150). The
acute DSS model is therefore useful to study the role of the innate immune system in colitis.
Chemically, DSS is a sulfated polysaccharide, with a sulfur content of ~17%, which corresponds
to ~2 sulfate groups per glucosyl residue of the dextran molecule (position C2 and C4). One
cycle of 3-5% DSS in drinking water for 5-7 days results in an acute colitis, characterized by
loose stools/diarrhea, weight loss and rectal bleeding, as originally reported by Okayasu et al.
(151). Histologically, the distal colon shows crypt and epithelial cell damage, infiltration of
granulocytes and mononuclear cells, and tissue edema and ulceration during DSS exposure
(152). It is thought that DSS is directly toxic to colonic epithelial cells, as demonstrated by
increased apoptosis and decreased epithelial proliferation, leading to a breakdown of the
epithelial barrier (150). Indeed, it has been shown that colonic mucosal permeability increases in
mice post DSS administration, prior to erosion or ulcer formation, indicating that dysfunction of
the mucosal barrier may play an initial role in the initiation of DSS colitis (150). The mucosal
barrier is regulated in large part by tight junction proteins, which help maintain cell surface
polarity and keep neighboring cells in close contact. The TJs are located apically on the epithelial cells, and contain transmembrane proteins [occludin, claudin, and junctional adhesion molecule (JAM)] and the underlying cytoplasmic plaque proteins (the zonula occludens ZO-1). Mice given DSS have a loss of epithelial JAM-A expression (153). Furthermore, mice lose colonic expression of ZO-1 protein after 1 day of DSS treatment, suggesting that alterations in the tight junction complex precede intestinal inflammation in the DSS colitis model and do not occur as a consequence of it (154). DSS has also been shown to rapidly cause alterations to the normally sterile inner mucus layer, making it permeable to bacteria within 12 hours of administration (155).

Luminal bacteria have been shown to be altered by, as well as play a role in DSS colitis. Okayasu et al. (151) first observed changes in bacterial populations in the colon of mice given DSS, with increases in the bacterial species Bacteroides, Enterobacter and Clostridium, and decreases in Eubacterium and Enterococcus. Axelsson et al. (156) first reported that acute colitis can be induced in germ-free mice given DSS and the resulting intestinal inflammation was histologically similar to that of conventional mice, indicating a direct toxic effect of DSS. However, the same group also reported that germ-free mice given 5% DSS for up to 6 days developed severe signs of colitis with a 75% mortality rate, compared to no deaths in the conventional control group (157). The authors concluded that the bacterial microbiota is not critical for the induction of inflammation by DSS, but may actually play a protective role. Later groups also found that germ-free mice show significant aggravated colonic inflammation as compared with specific pathogen-free mice after DSS treatment (158). In agreement, mice
treated with broad-spectrum antibiotics (vancomycin, neomycin, metronidazole and ampicillin for 4 weeks) and depleted of all detectable commensals show severe mortality and morbidity when given DSS (159). In contrast, mice given select antibiotics to deplete certain classes of bacteria all show 100% survival, with minimal morbidity and colonic bleeding similar to mice not given antibiotics (159). These results suggest that it may not be any particular group of commensal bacteria that provides protection, but merely the presence of bacteria that is important. Interestingly, reports also indicate that monocolonization with probiotics (Escherichia coli strain Nissle 1917, Lactobacillus, and Bifidobacterium lactis) or symbiotics (B. fragilis and B. vulgatus) could reduce colitis severity in germ-free mice (160-163). Furthermore, treatment with various probiotic strains have also been shown to ameliorate DSS-induced colitis, improving disease activity index and histological scores (150). Interestingly, lysed bacteria, heat killed bacteria or bacterial DNA have all been shown to ameliorate DSS colitis, indicating that bacterial viability is not necessary for protective effects (150). However, other studies have shown little to no beneficial effect of probiotics in DSS colitis (150). Overall, the role of bacteria during DSS colitis is complex, yet intriguing. The DSS colitis model can be used to study several aspects of IBD, including barrier disruption, mucosal healing and bacteria-host interactions at the mucosal surface.

1.5.2 Pathobionts associated with inflammatory bowel disease

The intestinal microbiota has been implicated in the pathophysiology of IBD. A consistent finding across the majority of studies is that patients with Crohn’s disease have reduced gut bacterial diversity and a decreased abundance of Firmicutes (including Clostridium, Bacillus and
Lactobacilli species), and an increase of Proteobacteria (including Escherichia and Campylobacter species), compared to healthy patients (1). In patients with ulcerative colitis, bacterial diversity within the colonic microbiota is generally decreased, with drops in the abundance of Bacteroidetes (particularly Prevotellaceae), whereas Actinobacteria and Proteobacteria are more abundant (164, 165). Although dysbiosis is one of several explanations for the development of IBD, it is also suggested that specific pathogenic bacterial species may be sufficient to initiate a cascade of events, leading to IBD. Bacteria suggested to play such a role include Mycobacterium avium subspecies paratuberculosis (MAP), Campylobacter species, Clostridium difficile and adherent-invasive Escherichia coli (AIEC) (1).

A number of culture-based and molecular-based studies support E. coli as a microbiological factor implicated in Crohn’s disease, but controversy exists regarding its role in ulcerative colitis (166). E. coli have been found in the mucus layer, close to the intestinal epithelial cells and in ulcers of both Crohn’s disease and ulcerative colitis patients (166). Of note, a higher abundance of E. coli is observed in active Crohn’s disease patients compared to that seen in patients in remission (167, 168) and high numbers of E. coli are correlated with a reduced amount of time before disease relapse (169). A study using Fluorescent In Situ Hybridization (FISH) also found higher E. coli numbers in the epithelium and within the lamina propria in the tissues of active Crohn’s disease patients compared to inactive Crohn’s disease patients (170). Translocation across the intestinal mucosa has been primarily observed in Crohn’s disease (167) and higher numbers of intracellular E. coli were detected in inflamed compared to the non-inflamed mucosa. Interestingly, E. coli isolated from IBD patients carry different sets of virulence genes that are characteristic of extraintestinal E. coli (ExPEC) strains, whereas intestinal pathogenic E. coli are
rare or absent, leading to the classification of a new pathobiont called adherent-invasive *Escherichia coli* (AIEC), that can adhere to, and invade epithelial cells and can survive and replicate within immune cells, including macrophages and neutrophils (166). In the last decade, several independent laboratories have reported a higher prevalence of AIEC in Crohn’s disease patients compared to healthy subjects (166). Furthermore, virulence properties of AIEC described to date can explain several features of Crohn’s disease pathophysiology such as inflammation, mucosal bacterial translocation and granuloma formation (166). Although AIEC bacteria may colonize the intestinal mucosa of non-IBD patients, these bacteria usually do not translocate across a healthy mucosal barrier, as bacterial invasion of the mucosa has not been frequently observed in control patients (170).

### 1.5.3 Inflammatory bowel disease and diet

The transition from a hunter-gatherer type of lifestyle to modern Western society with its technological advances in food processing led to significant changes in food intake and composition. The Western-style diet, also called the meat-sweet diet or standard American diet, is characterized by an over availability of food, with high intakes of highly processed and refined foods, high-fat foods, high-sugar desserts and drinks, as well as high intakes of red meat, refined grains, and high-fat dairy products (171). The Western-style eating pattern has been recognized as the major contributor to metabolic disturbances and the development of obesity-related diseases including type 2 diabetes, hypertension, cardiovascular disease and kidney disease (171). Food can be a significant source of pathogens and certain dietary styles may promote gut dysbiosis. The Western-style diet is characterized by high intakes of red meat, poultry and dairy products, which can be a source of *Mycobacterium avium subspecies paratuberculosis* (MAP),
Campylobacter species, and adherent-invasive Escherichia coli (AIEC) – all of which have been implicated (albeit not proven) in IBD pathogenesis (166, 172). A systematic review found that Western-style or meat-based diets are positively associated with low-grade inflammation, including higher serum levels of the IL-6 and C-reactive protein, whereas plant-based diets or fish-based diets, which have lower microbial loads tend to be inversely correlated with inflammatory markers (173). The Western-style diet is also characterized by the high intake of processed carbohydrates, refined sugars and food additives that can cause intestinal microbial dysbiosis and promote inflammation (174-176). Food additives may also change the response of bacteria in the gut- for instance; maltodextrin has been shown to markedly enhance E. coli and AIEC biofilm formation and adhesion to intestinal epithelial cells and macrophages by inducing type 1 pili (176). Several studies have examined the associations between dietary patterns and the incidence of IBD. A systematic review concluded that diets with high levels of total fats, omega-6 fatty acids, and meat are associated with an increased risk of Crohn’s disease and ulcerative colitis; high fiber and high fruit intake are associated with a decreased risk of Crohn’s disease, and high vegetable intake is associated with a decreased risk of ulcerative colitis (177). In a prospective study, patients who reported consuming higher amounts of meat, eggs, protein, and alcohol were more likely to experience a relapse of ulcerative colitis (178). Interestingly, a semi-vegetarian diet was shown to be highly protective against relapse of Crohn’s disease in a 2-year clinical trial with 16 patients (179), indicating that plant-based diet therapy is potentially important for IBD management. The Western style diet is also typically low in fruits, vegetables and dietary fibers that are necessary for the growth of beneficial bacteria in the colon. Furthermore, The Western-style diet is low in beans and legumes, nuts and seeds, fatty fish and fermented foods, and thus missing many key nutrients that are critical for gut health, including
fiber, plant polyphenols, anti-inflammatory n-3 fatty acids, as well as beneficial microorganisms. The Western-Style diet is also typically low or deficient in vitamin D, a key nutrient required for the proper functioning of immune responses. Overall, eating a Western-style diet may enhance the development and progression of IBD.

1.5.4 Inflammatory bowel disease and vitamin D

Vitamin D deficiency is common in patients with IBD and has been associated with higher disease activity, longer relapse duration and an increased risk of surgery and hospitalization (180-183). In agreement, low ultraviolet exposure (presumably yielding lower vitamin D levels) is associated with greater rates of hospitalization, prolonged hospitalization and an increased need for bowel surgery in patients with IBD (184), whereas high sunlight exposure has been associated with decreased incidence of Crohn’s disease, but not ulcerative colitis in woman (185). Polymorphisms in the VDR gene have also been associated with increased risk of IBD in East Asian and European patients (186). In addition, patients with ulcerative colitis (n=112) showed significantly reduced expression of the VDR by their colonic epithelial cells (187) as compared to healthy controls, a finding also seen in patients with colon cancer (187-190), indicating that altered vitamin D signalling may contribute to these colonic diseases. Animal models of colitis, including the Il-10/- mouse model, the DSS model and the TNBS model, demonstrate that vitamin D deficiency exacerbates disease, while treatment with the active form of vitamin D- calcitriol ameliorates colitis (191-196). Furthermore, Crohn’s disease patients supplemented with vitamin D3 had lower relapse rates compared to placebo treated patients (197), while a pilot study in Crohn’s disease patients demonstrated a short-term beneficial effect
of taking an oral calcitriol analogue on disease activity over a one-year course (198), indicating that vitamin D plays a role (albeit poorly defined) in the pathophysiology of IBD.

To test the effectiveness of vitamin D3 treatment in Crohn’s disease, Jorgensen et al. (197) performed a double-blind clinical trial with 108 patients with Crohn’s disease in remission. Patients were randomized to receive either 1200 IU vitamin D3 (n = 46) or placebo (n = 48) once daily for 12 months. The primary endpoint was clinical relapse. Oral intake of 1200 IU vitamin D3 once daily was well tolerated and significantly increased serum 25(OH)D3 from mean 69 nmol/L to 96 nmol/L after 3 months and this increase was sustained throughout the 12-month treatment period. There was also a trend for a lower relapse rate of 29% to 13% (P = 0.06) in vitamin D3 treated patients over the 1-year period (197). To determine the role of active vitamin D analogues on Crohn’s disease activity, Miheller et al (197) performed a preliminary study with 37 patients with Crohn’s disease in remission. Patients were treated orally with either 0.25 µg alfacalcidiol or 1000 IU vitamin D3 for 12 months. At week 6, the Crohn’s Disease Activity Index (CDAI) scores and concentration of C-reactive protein decreased significantly by the third month (CDAI score 69.44 vs. 57.0 and 15.8 mmol/L versus 7.81 mmol/L, respectively, P < 0.05), however, these changes disappeared by the 12th month (197).

The potential for vitamin D to ameliorate inflammation in patients with IBD is intriguing. Several studies have found that vitamin D supplementation can improve several inflammatory markers. For instance, a randomized clinical trial found that vitamin D deficient Iranian IBD patients (n = 108) treated with 50,000 IU vitamin D3 or control once weekly for 12 weeks had higher serum levels of 25(OH)D3, the major marker of vitamin D (67.89 nmol/L vs. 23.9
nmol/L) and lower serum levels of TNF-α (p = 0.07), compared to placebo-treated IBD patients (199). Children and adolescents with IBD (n = 63) supplemented with 2000 IU of vitamin D2 over the course of 1 year had lower serum levels of C-reactive protein and IL-6, compared to patients that received 400 IU vitamin D2 (200). Furthermore, it has recently been shown that (otherwise healthy) vitamin D-deficient individuals (n = 25) administered vitamin D3 in a dose-escalation from 2000 up to 8000 IU for 12 weeks had less circulating IL-17+ CD4+ and IFN-γ CD4+ T-cells, compared to the control group (201). However, other studies have found little to no effect of vitamin D supplementation on inflammatory markers. For instance, a recent randomized, double blind, placebo-controlled trial of supplemental oral vitamin D (placebo, 1,000, 2,000, or 4,000 IU/day of vitamin D3 orally for 3 months) in 328 healthy African Americans found no significant changes in CRP, IL-6 or TNF-α after the vitamin D supplementation period (202). These results indicate that supplemental vitamin D may reduce inflammatory markers in patients with IBD; however further research is necessary before high dose vitamin D can be recommended to patients.

1.6 Vitamin D overview

The following section will provide some background about vitamin D and how the nutrient plays an important role in maintaining barrier integrity and immune defense. Finally, the potential role of vitamin D during enteric infection will be discussed. For decades, vitamin D “the sunshine vitamin” has been known to be essential for the development, function, and maintenance of healthy bones through the regulation of calcium and phosphorus homeostasis (203). Bone diseases including rickets and osteomalacia were traditionally treated with the administration of cod liver oil and sunlight exposure, both rich sources of vitamin D (204). Rickets became a
major concern for children in the 1800’s, during the industrial revolution in Northern Europe, due to lack of sunlight exposure thought to be caused by housing conditions and coal burning causing major air pollution (204). By the turn of the 20th century it was estimated that upwards of 80–90% of children living in Northern Europe and in northeastern United States had evidence of rickets (204). However, in the 1930s, fortification of milk with vitamin D2 in the United States and in most industrialized countries including Great Britain and other European countries essentially eradicated rickets within a few years (204). Interestingly, it was recognized that young children with rickets had a much higher risk of developing pneumonia and upper respiratory tract infections and were more likely to die from lung diseases (205). In the early 1900s Finsen observed that exposure to sunlight dramatically improved skin lesions caused by tuberculosis (TB) infection (Lupus vulgaris) and he received the Nobel Prize in 1903 for these observations. This led to the use of solariums, where sunlight therapy was prescribed to treat and prevent TB and upper respiratory tract infections (204). However, sunlight therapy was largely abandoned during the 1940s and 1950s after the introduction of effective anti-TB medications (204). In 1945, GB Dowling et al. (206) reported successfully treating skin lesions on several patients with Lupus vulgaris using high dose (± 50,000 IU) oral vitamin D2 (ergocalciferol). These early observations established vitamin D as an essential vitamin to maintain normal health. Together with the recent discovery that the vitamin D receptor (VDR) is present in most tissues and cells of the body including immune cells provides new insight into the non-calcemic functions of vitamin D.

Current dietary reference intakes for vitamin D by the U.S. Institute of Medicine (jointly funded by the U.S. and Canadian governments) are based on maintaining skeletal health and have been
set using the assumption that sun exposure is minimal. For infants (0 - 1 year), vitamin D recommendations are 400 IU per day; for children and adults, including pregnant and lactating women (1 - 70 years) = 600 IU per day; for elderly adults (> 70 years) = 800 IU per day. The tolerable upper limit is set at 1000 IU for infants (0 - 6 months) and up to 4000 IU for adults. Serum concentration of 25(OH)D3 is the best indicator of vitamin D status, as it reflects total vitamin D input- from food, supplements and sun exposure. Serum 25(OH)D3 is also the major circulating form of vitamin D with a half-life of approximately 2-3 weeks. Although calcitriol is the biologically active form of vitamin D, it is not the best measure for vitamin D status. The circulating half-life of calcitriol is short- only 4-6 hours. Furthermore, calcitriol levels may not necessarily reflect changes in vitamin D status – for instance as one becomes vitamin D deficient, a corresponding increase in parathyroid hormone levels can result in normal or even elevated levels of calcitriol. This makes the calcitriol assay unreliable as a measure of vitamin D status (207). There is considerable debate surrounding the serum concentrations of 25(OH)D3 associated with optimal health, and cut points have not been developed by a scientific consensus process. However, the Institute of Medicine expert committee stated that its review of the data suggests that, relative to bone health: People are at risk of vitamin D deficiency (rickets or osteomalacia) at serum 25(OH)D3 concentrations < 30 nmol/L. Some are potentially at risk for inadequacy at levels ranging from 30 – 50 nmol/L. Practically all people are sufficient at levels ≥ 50 nmol/L. There may be reason for concern of hypercalcemia at serum concentrations > 125 nmol/L (208).
1.6.1 Vitamin D metabolism

Vitamin D is a group of fat-soluble pro-hormones including the two physiologically relevant forms vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D2 is made from ergosterol in response to ultraviolet B (UVB) radiation in invertebrates, fungus and plants, whereas vitamin D3 is obtained through UVB irradiation of the skin of humans and animals (36), as well as the leaves of certain plants, such as waxy leaf nightshade, tomato and bell pepper (209). Vitamin D is an interesting nutrient, because it is not actually a vitamin in the traditional sense. Unlike true vitamins - it is not an essential dietary factor, but can instead be synthesized photochemically in the skin from 7-dehydrocholesterol, a cholesterol derivative, during exposure to UVB radiation. However, it should be noted that for certain species- including domestic cats, vitamin D is a true vitamin, since 7-dehydrocholesterol is virtually undetectable in their skin (210). In most animals the highest concentrations of 7-dehydrocholesterol are found in the epidermal layer of skin, specifically in the stratum basale and stratum spinosum. During exposure to sunlight, UVB photons (wavelength of 280-320 nanometers) penetrate the skin and cause 7-dehydrocholesterol to ultimately form vitamin D3, which diffuses from the skin into the blood. This process is very efficient (15 min exposure of 40% exposed skin is enough for a daily production of vitamin D) and highly regulated to prevent over production. However, melanin, the pigment granules that gives skin its color, can also efficiently absorb UVB radiation. Therefore, the high melanin content in darker skin reduces the skin’s ability to produce vitamin D from sunlight (211). Studies of people in Northern latitudes, such as Canada also demonstrate that cutaneous synthesis of vitamin D is minimal from October to March due to the solar zenith angle – less UVB photons reach the earth and are therefore not strong enough for the conversion of 7-dehydrocholesterol to vitamin D3 (212). Furthermore, usage of sunscreen can dramatically
reduce the levels of vitamin D produced in the skin (212). So the amount of vitamin D that is produced is a multifactorial process where skin type, latitude, time of the day and season, amount of skin exposed and the use of sunscreen determine a person’s vitamin D production. Therefore, dietary and supplemental forms of vitamin D are becoming more important sources of this vitamin at times when sufficient sun exposure is not available.

Dietary sources of vitamin D2 include UV exposed mushrooms, while the best sources of vitamin D3 are fatty fish, including salmon and sardines. Because dietary sources are limited, milk in the United States and Canada is fortified with vitamin D. However, a study found that 49% of 173 milk samples collected in the US and British Columbia, Canada, contained less than 80% of the vitamin D content on the label and 14% did not contain any detectable vitamin D, raising serious questions about the role of milk in providing consumers with their vitamin D requirements (213). Dietary and supplemental vitamin D is absorbed from a micelle in association with fat and bile salts via passive diffusion into the epithelial cells lining the small intestine. Vitamin D is then incorporated into chylomicrons, which enter the lymphatic system and are then transported through the blood for delivery to extrahepatic tissues (36). Vitamin D is transported in the blood by a plasma protein called the vitamin D-binding protein. In classical vitamin D endocrinology, vitamin D is metabolized in the liver by 25-hydroxylase to form 25(OH)D3, the major circulating form of vitamin D. In the kidney, 25(OH)D3 is further metabolized by 1α-hydroxylase (CYP27B1) to yield the active calcitriol (36). CYP27B1 activity in the kidney is controlled by calcium-regulating hormones, including parathyroid hormone and calcitriol, which suppresses its own production (9). Calcitriol also induces its own catabolism, by stimulating the expression of 24-hydroxylase (CYP24A1), leading to calcitroic acid and other
breakdown products (36). Calcitriol signals through the vitamin D receptor (VDR), a nuclear receptor that is expressed on almost all nucleated cells in the body, including immune cells and colonic epithelial cells (9). Calcitriol binding to the VDR activates the VDR to translocate to the nucleus and heterodimerize with the retinoid X receptor (RXR). The VDR-RXR complex binds to specific DNA sequences, known as vitamin D-responsive elements (VDRE) to induce transcription (9). The classic role of calcitriol is to regulate serum calcium concentration. Low serum calcium induces the secretion of parathyroid hormone by the parathyroid gland, which in turn increases renal CYP27B1 activity and subsequent calcitriol production, which then stimulates calcium release from the bone, calcium resorption in the kidney and calcium absorption in the intestine, leading to an increase in serum calcium (36). However, calcitriol directly or indirectly regulates > 200 different genes through the VDR and is involved in diverse cellular functions including cell proliferation, apoptosis, secretion of antimicrobial peptides and T-cell mediated defense (9), all of which play an important role in maintaining intestinal homeostasis.

1.6.2 Vitamin D and the intestinal epithelial barrier

Almost every nucleated cell-type in the GI tract expresses the VDR, including intestinal epithelial cells (9). As such, the VDR is highly expressed throughout the small intestine and colon, with the highest expression found in the cecum and proximal colon (214, 215). While the specific impact of vitamin D on GI physiology is poorly understood, studies suggest that vitamin D may play a role in intestinal homeostasis. For instance, calcitriol has been shown to induce cell differentiation, inhibit cell proliferation and induce apoptosis in human colonic epithelial cells in vitro (216-218). Interestingly, vitamin D can directly suppress cell proliferation by modulating
the Wnt signalling pathway. In addition, calcitriol has been shown to antagonize Wnt signalling in a VDR-dependent manner through a variety of mechanisms, including sequestration of β-catenin through a direct VDR/β-catenin interaction and the induction of nuclear export of β-catenin. Calcitriol also enhances the expression of DKK1, which is an endogenous inhibitor of Wnt signalling (8). Studies suggest that calcitriol induces the expression of the tight junction proteins claudin-1, occludin, ZO-1, ZO-2, as well as adherence junction protein E-cadherin in human colonic cells, *in vitro* (219, 220), demonstrating that vitamin D signalling may play a role in promoting barrier integrity. Indeed, several studies have found that vitamin D can help maintain the intestinal epithelial barrier (193, 219, 221, 222). Recently, calcitriol has been shown to alter *E. coli* O157:H7-induced impairment of transepithelial electrical resistance, as well as reducing permeability, and preserving barrier integrity, *in vitro* (221). However, the role of vitamin D may be more complex, since calcitriol has also been shown to induce expression of the pore-forming tight junction proteins claudin-2 and claudin-12 *in vitro*, which are suggested to form paracellular calcium channels (219, 223).

### 1.6.3 Vitamin D and innate defense

Epithelial cells and immune cells, including monocytes, macrophages and dendritic cells constitutively express the VDR as well as the calcitriol activating and deactivating enzymes CYP27B1 and CYP24, respectively (189, 190, 215), thereby regulating local metabolism of vitamin D. Studies have shown that calcitriol signalling through the VDR can alter inflammatory responses in the host through a number of mechanisms, including suppressing TLR expression (224), blocking NF-κB signalling (225), targeting mitogen-activated protein kinase (MAPK) phosphatase-1 (226), and directly modulating dendritic cell and macrophage behavior (227).
calcitriol has been shown to down-regulate expression of TLR2, TLR4 and TLR9 in human monocytes and colonic cells (HT29) in vitro (224, 228, 229). Furthermore, challenge of calcitriol treated human monocytes with either a TLR2 or TLR4 ligand resulted in impaired NF-κB/RelA translocation to the nucleus and less TNF-α production compared to cells not exposed to calcitriol. The down-regulation of TLR2 and TLR4 levels was shown to be VDR-dependent, since treatment with the VDR antagonist ZK 159222 reversed calcitriol induced TLR down-regulation (224). In agreement, mouse colonic cells lacking the VDR showed increased translocation of NF-κB to the nucleus, compared to controls (215). In fact, the VDR has been shown to physically interact with the NF-κB subunit p65 (215, 230, 231) thereby decreasing translocation of p65 to the nucleus and reducing activation of NF-κB in mouse colonic cells in vitro (215). However, the effect of vitamin D on TLR signalling in vivo is currently unknown. Paradoxically, innate defenses including autophagy, phagocytosis and antimicrobial peptide production (cathelicidin and β-defensins) are increased after treatment with calcitriol in vitro (232-234). It should be noted that the human cathelicidin gene contains a VDRE in the promoter region, however the mouse gene does not (235). Interestingly, calcitriol can induce cathelicidin in human monocytes and keratinocytes, but not in colonic epithelial cells (HT-29) under baseline conditions (236). However, colonic cells (BBe clone of Caco-2) treated with calcitriol and then stimulated with bacterial ligands can induce cathelicidin. In contrast, calcitriol suppressed the TLR4 induced expression of the antimicrobial peptide β-defensin 4 (194). These results indicate that colonic responses to bacteria induced TLR signalling may be regulated/modulated by vitamin D. In summary, the relationship of vitamin D and the innate immune system in the gut is
complex and vitamin D may stimulate or suppress innate responses depending on the type of antigen encountered.

### 1.6.4 Vitamin D and Th1/Th17 immune responses

Resting T cells express low levels of the VDR, but levels of the receptor increase upon activation. *In vitro*, calcitriol acts directly on T cells to inhibit proliferation and production of cytokines, including IL-2, IFN-γ and TNF-α (237). Recent studies have also shown that calcitriol can suppress Th17 cells and their associated cytokines IL-6, IL-17, IL-22 and IL-23, *in vitro* (238-241), while induction of regulatory T cells has been demonstrated both *in vitro* and *in vivo* (9, 237-239, 241). CD4+ cells from *Vdr/-* mice respond more readily in a mixed lymphocyte reaction (pro-inflammatory) and CD4+/CD45RBhigh T-cells from *Vdr/-* mice induced a more severe colitis compared to their wildtype counterparts in the CD45RB transfer model (193). Recently it was demonstrated that CD4+ T cells from *Vdr/-* mice make more IFN-γ and IL-17 when stimulated *in vitro*, compared to cells from wildtype mice (89, 193). Furthermore, calcitriol and its analogues reduced the severity of TNBS-induced colitis in wildtype mice, by down-regulating Th1 (IL-12, T-bet) and Th17 (IL-6, IL-17) related parameters in the colon (192, 242). In addition, calcitriol treatment promoted regulatory T cells, as evident by a marked increase in colonic protein expression of IL-10, TGF-β and FoxP3 (192).

Vitamin D has also been shown to inhibit IL-17 through a number of mechanisms: 1) the VDR can bind with the transcription factor nuclear factor of activated T-cells (NFAT) and recruit histone deacetylase to the human IL-17 promoter, thus inhibiting its activation, 2) calcitriol and VDR can inhibit binding of the transcription factor Runx1 to the mouse IL-17A promoter, 3)
calcitriol can induce Foxp3, which inhibits IL-17, and 4) calcitriol can induce the expression of C/EBP homologous protein (CHOP), a molecule involved in endoplasmic reticulum stress and translational inhibition (243). Interestingly, UVB therapy is also linked to suppression of proinflammatory Th1 (IFN-γ) and Th17 (TNF-α, IL-17, IL-22 and IL-23) immune responses in psoriasis patients (244) presumably mediated through vitamin D. Overall, these studies suggest that vitamin D may be beneficial in preventing Th1/Th17 mediated inflammatory diseases. However, the immunosuppressive actions of vitamin D could potentially impair host defense, since Th17 responses are also critical for the clearance of many bacterial infections. The full impact of vitamin D levels on Th17 responses and colitis development needs to be further researched.

1.6.5 Vitamin D and bacteria

It is increasingly being recognized that vitamin D also plays an important role in regulating host defense against pathogenic microbes. Vitamin D deficiency has been linked to an increased risk of lung infections, including those caused by *Pseudomonas aeruginosa* (10, 245) and *Mycobacterium tuberculosis*, the causative agent of most cases of tuberculosis (TB) (12, 13). Innate defenses including autophagy, phagocytosis and the induction of antimicrobial peptides, including cathelicidin and β-defensins are increased after treatment with calcitriol *in vitro*, which has been shown to kill *Mycobacterium tuberculosis* (232, 234). Recently, a study investigating supplementation with high doses of vitamin D (2 doses of 600,000 international units (IU) of intramuscular vitamin D3) accelerated clinical, radiographic improvement in all TB patients and increased host immune activation (246). However, two vitamin D oral supplementation trials failed to demonstrate any benefit during TB infection (247, 248) possibly due to the low doses
used. Vitamin D status has also been shown to influence the risk and severity of *Clostridium difficile* infection (14, 15). It has recently been shown that patients with IBD who have higher vitamin D plasma levels have a reduced risk of *C. difficile* infection (15). Furthermore, vitamin D deficiency is associated with increased incidence of GI infections and higher rates of diarrhea and vomiting in school age children (249). However, a recent study found that high dose vitamin D (6 quarterly doses of 100,000 IU calcitriol over 18 months) had no effect on diarrheal diseases in infants (250). Vitamin D receptor knockout mice also show increased susceptibility to infection and carry higher bacterial burdens during challenge with *Salmonella typhimurium* (215). Surprisingly, despite its broad linkage to host defense against predominantly mucosal bacterial pathogens, exactly how vitamin D affects a host’s susceptibility to these infections is poorly understood. Furthermore, the role of vitamin D during infection with a non-invasive enteric pathogen is currently unclear.

### 1.7 Research hypothesis and objectives

Taken together, these studies suggest that vitamin D may be beneficial in preventing idiopathic or immune-mediated inflammatory diseases such as IBD. However, the impact of vitamin D levels on colitis development need to be further researched and the mechanisms involved need to be defined. Moreover, we wondered whether the immunosuppressive actions of vitamin D could potentially impair host defense, since Th1/Th17 responses are also critical for the clearance of many bacterial infections. Therefore the goal of my PhD thesis was to determine how vitamin D regulates inflammatory responses in the GI tract during bacteria induced colitis, focusing on intestinal epithelial pathology and the Th17 immune response. *C. rodentium* was chosen as an infectious model because it replicates A/E bacteria pathogenesis as well as certain aspects of
IBD, especially the epithelial barrier disruption, dysbiosis, Th1/Th17-mediated inflammation, and GI damage. Furthermore, the *C. rodentium* induced colitis mouse model is very robust and allows us to study how a nutrient, such as vitamin D can influence host-bacteria interactions during infection, *in vivo*. It is hypothesized that vitamin D beneficially modulates the host colitic response against *Citrobacter rodentium*. The objectives of my thesis are 1) To examine the role of calcitriol treatment during infection with *C. rodentium*, and 2) To determine how vitamin D deficiency impacts colonic homeostasis and susceptibility to infection with *C. rodentium*. Findings from these studies will increase our understanding of the interactions between vitamin D and the immune system and help define basic mechanisms of mucosal integrity and intestinal health.
Chapter 2: Experimental Methods

2.1 Mice and experimental diets

All mice were maintained in sterilized, filter-topped cages, handled in tissue culture hoods and given free-access to water under specific pathogen-free conditions in the animal facility at the Child and Family Research Institute. For calcitriol studies in Chapter 2, 6 to 8-week-old male C57BL/6 mice were obtained from the Centre for Disease Modeling (CDM) at the University of British Columbia. Mice were fed autoclaved food (PicoLab Rodent Diet 20 #5053, Laboratory Diet, Brentwood, MO). For dietary vitamin D3 studies in Chapter 3: weanling (3-week-old) female C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada). Mice were fed either a vitamin D3 deficient diet (0 IU) or a vitamin D3 sufficient diet (1000 IU) for 6 weeks, as previously described (194). All diets were procured from Research Diets, Inc. (New Brunswick, NJ, Canada). Sentinel animals were routinely tested for common pathogens. The protocols used were approved by the University of British Columbia’s Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

2.2 Calcitriol treatment

Crystalline calcitriol (1,25(OH)2D3) (SAFC Pharma) was dissolved in RNA-grade ethanol at 0.025 mg/ml and stored at -20°C. The calcitriol stock was diluted in sterile phosphate buffered saline (PBS) immediately prior to injection. Mice were administered either vehicle (PBS with 0.12% ethanol) or 10 ng calcitriol (0.5 µg/kg) via intraperitoneal injection daily for up to 11
days. The calcitriol dose was chosen based on previous in vivo studies with mice (251). For additional dose-response studies, mice were administered 5 ng or 15 ng calcitriol via daily intraperitoneal injection.

2.3 Dextran sodium sulfate colitis
For DSS studies, colitis was induced by adding dextran sodium sulfate (36,000-55,000 Da, MP Biomedicals #160110, Solon, Ohio, USA) to sterile drinking water at a concentration of 3% (w/v). Animals were treated with DSS for 7 days and then allowed to recover by removing the DSS from their drinking water for an additional 2 days (9 days total). Mice were weighed daily and monitored for signs of distress, including stool consistency and rectal bleeding.

2.4 Bacteria strains and infection of mice
Mice were infected by oral gavage with 0.1 ml of an overnight culture of Luria broth (LB) containing approximately 2.5 x 10^8 CFU of wildtype or ΔespF streptomycin resistant C. rodentium (formerly C. freundii biotype 4280, strain DBS100). Mice were weighed daily and monitored for signs of distress.

2.5 FITC-dextran intestinal permeability assay
Mice were orally gavaged with 150 µl of 80 mg/ml 4 kDa FITC-dextran (Sigma; FD4) in PBS 4 h prior to sacrifice. In subsequent studies, mice were given 100 µl of 80 mg/ml 4 kDa FITC-dextran intra-rectally 2 h prior to sacrifice. The same FITC-dextran solution administered to mice for experiments was kept and later diluted and used to create a standard curve for analysis. Mice were anaesthetized and blood was collected by cardiac puncture. Blood was then
immediately added to a final concentration of 3% acid-citrate dextrose (20 mM citric acid, 100 nM sodium citrate, 5 mM dextrose) (Harald Schulze, Shivdasani Laboratory, DFCI). Plasma was collected and fluorescence was quantified using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) at excitation 485 nm, emission 530 nm for 0.1 s.

2.6 Tissue collection

Mice were anesthetized with halothane, killed by cervical dislocation, and tissues were collected for further analysis. The large intestine was resected and divided into 4 sections, i.e. cecum, proximal colon, mid colon and distal colon. Tissues were immediately placed in 10% neutral buffered formalin (Fisher) (48 hrs, 4°C) for histological studies, or placed in RNAlater (Qiagen) and stored at -80°C for subsequent RNA extraction. For certain experiments, the colon was opened longitudinally, stool was gently removed, and the tissue was rolled (Swiss roll) from distal to proximal end, fixed with a needle and placed in 10% neutral buffered formalin (Fisher) (48 hrs, 4°C) for histological processing.

2.7 Serum vitamin D analysis

Mice were anaesthetized with halothane and blood was collected by cardiac puncture. Blood was allowed to clot naturally at room temperature and cells were removed by centrifugation. Serum was collected and stored at -80°C until analysis. Serum samples were processed and 25(OH)D3 was assessed using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). To extract samples: 75 µl of plasma were added into a 1.7 ml Eppendorf tube with 10 µl of internal standard (IS) 25(OH)D3, vortexed and brought to room
temperature. 150 µl of methanol was then added to precipitate proteins. Samples were vortexed and centrifuged at 13,000 rpm for 10 minutes. Supernatant was removed and placed into 12 X 75 mm glass test tube, to which the following was added: 1ml 0.9 % saline, then 1 ml ethyl acetate. Samples were vortexed and centrifuged at 2000 rpm for 5 minutes to separate the solvent layers. The ethyl acetate (top layer) was removed and placed into a new HPLC auto sample vial and the solvent was completely evaporated under a nitrogen flow. The derivatization of 25(OH)D3 was performed using Amplifex diene reagent. The derivatized sample was injected into the Waters Acquity UHPLC. Separation was achieved with a binary solvent system (A = deionized water containing 15 mM ammonium formate and 0.1 % formic acid, B = methanol containing 0.1 % formic acid) and a Waters BEH-C8 column (2.1 X 50 mm, 1.7 um particle size) at a flow rate of 0.25 ml/min and a gradient separation using starting conditions of 40/60 (A/B), held for 2 minutes, then a linear gradient to 22/78 (A/B) from 2.0 to 2.1 minutes, held until 4 minutes, then the column was flushed with 100% methanol before equilibration to starting conditions. Peak elution was at 3.1 minutes. The injection cycle was 7 minutes. Detection was attained using a Waters Quattro micro triple quadrupole mass spectrometer controlled by MassLynx 4.1 software in MRM mode. Quantification of peak areas used QuanLynx 4.1 software. Area ratios of samples were compared to standard curves created by dilution of purified standards purchased from Sigma Aldrich. The intra-assay coefficient of variation for the assay was 4.4% and the inter-assay cv was 8.9%. 
2.8 Serum calcium analysis

Mice were anaesthetized with halothane and blood was collected by cardiac puncture. Blood was allowed to clot naturally at room temperature and cells were removed by centrifugation. Serum was collected and stored at -80°C until analysis with the calcium colorimetric assay kit (BioVision Research, Mountain View, CA; Cat #K380-250). This assay utilizes the chromogenic complex, which forms between calcium ions and 0-cresolphthalein. Samples were quantified at OD 575 nm using a Wallac Victor fluorimeter (Perkin-Elmer Life Sciences, Boston, MA) and compared to the calcium standard provided with the kit.

2.9 Citrobacter rodentium enumeration

For enumeration of bacteria within the tissue and luminal compartments, whole mouse ceca or colons were sliced open longitudinally, and their luminal contents were collected in a pre-weighed 2.0 ml microtube containing 1.0 ml of phosphor buffered saline (PBS) and a 5.0 mm steel bead (Qiagen). Cecal and colonic tissues were washed vigorously in PBS (pH 7.4), cut into several pieces, and also placed in a tube as above. Tissue and lumen contents were weighed, and then homogenized in a MixerMill 301 bead miller (Retch) for a total of 6 mins at 30 Hz at room temperature. Tissue homogenates were serially diluted in PBS and plated onto luria broth (LB) agar plates containing 100 mg/ml streptomycin, incubated overnight at 37°C, and bacterial colonies were enumerated the following day, normalizing them to the tissue or stool weight (per gram). For fecal bacterial burden analysis, stool pellets were collected from live mice at different time points post infection and processed as described above.
2.10 Assessment of total microbes via DAPI staining

Enumerating total microbes was performed as previously described (129). Briefly, 2 fecal pellets were collected from each animal. After homogenization, samples were placed in 10% Neutral Buffered Formalin diluted to a final concentration of 3%. Samples were further diluted 1:10 in PBS, vortexed briefly, and stored at 4°C. Next, 2–5 ml of the 1:10 diluted sample was further diluted in 1 ml PBS and filtered onto Anodisc 25 filters (Whatman International Ltd) with a pore size of 0.2 mM and 2.5 cm diameter. The samples were thoroughly dried and then mounted on glass slides with ProLong Gold Antifade reagent containing DAPI (Molecular Probes) and sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software. The mean number of DAPI positive microbes was counted in 3 to 6 randomly chosen fields per disc (1000x). The total number of commensal microbes was calculated based on the mean numbers of all the counted fields and the dilution factor. The total number of commensal microbes was presented as the percentage of uninfected controls.

2.11 Commensal microbe analysis

Microbial composition analysis was performed by quantitative PCR (qPCR) as previously described (252). DNA was extracted from at least two fecal pellets from each animal using the Qiagen DNA stool extraction kit. Extracted DNA with 50 ng/reaction was used for qPCR. Group-specific primers for 16S rRNA were used to determine the relative abundance of the selected bacterial phyla: *Actinobacteria* (Fwd: 5’-TAC GGC CGC AAG GCTA-3’; Rev: 5’-CGT CAT CCC CAC CTT CCT CCG-3’), *Bifidobacterium* (Fwd: 5’-GGG TGG TAA TGC GCTA-3’), *Bacteroides* (Fwd: 5’-GAT GAC CGT CTT GCT CCA-3’; Rev: 5’-CGT CAT CCC CAC CTT CCT CCG-3’), *Clostridium* (Fwd: 5’-GAT GAC CGT CTT GCT CCA-3’; Rev: 5’-CGT CAT CCC CAC CTT CCT CCG-3’), and *Escherichia coli* (Fwd: 5’-GAT GAC CGT CTT GCT CCA-3’; Rev: 5’-CGT CAT CCC CAC CTT CCT CCG-3’).
ATG-3\'; Rev: 5′-CCA CCG TTA CAC CGG GAA-3′), *Lactobacillus* (Fwd: 5′-AGC AGT AGG GAA TCT TCC A-3′; Rev: 5′- CAC CGC TAC ACA TGG AG-3′), Segmented filamentous bacteria (*Candidatus savagella*) (Fwd: 5′- CGG AGC ATG TGG TTT AAT TC; Rev: 5′- GCT GTC TCG CTA AAG TGC TC-3′), alpha-Proteobacteria (Fwd: 5′- CTA GTG TAG AGG TGA AATT-3′; Rev: 5′- CCCCCGATCCAATTCAGTTTGGAGTTTT-3′), *Bacteroidetes* (Fwd: 5′-GAG AGG AAG GTC CCC A-3′; Rev: 5′-CGC TAC TTG GCT GGT TCA G-3′), *Firmicutes* (Fwd: 5′-GGA GYA TGT GGT TTA ATT CGA AGC A-3′; Rev: 5′-AGC TGA CGA CAA CCA TGC AC-3′), and *Gammaproteobacteria* (Fwd: 5′-TCG TCA GCT CGT GTY GTG A-3′; Rev: 5′-CGT AAG GGC CAT GAT G-3′). Universal Eubacteria primers (5′=ACTCCT ACG GGA GGC AGC AGT-3′ and 5′=ATT ACC GCG GCT GCT GGC3′) were used to determine total bacterial 16S rRNA in each sample, and the relative abundance of each taxonomic group was determined by calculating the average threshold cycle (CT) value relative to this number, normalized to each primer’s determined efficiency.

### 2.12 Immunofluorescence staining

Immunofluorescence staining of colonic tissues for Ki67, F4/80 and Tir were performed as previously described (62, 253). Briefly, paraffin-embedded sections were deparaffinized and then rehydrated, followed by antigen retrieval using 0.1 M citric acid monohydrate (Sigma) with 0.05% Tween 20 (pH 6.0) and steam for 45 min. Slides were blocked in PBS with 2% normal goat serum, 1% BSA, 0.1% Triton X-100 and 0.05% Tween 20. Primary antibodies used were rabbit antisera generated against Ki67 (1:200; Abcam), F4/80 (1:8K; Serotec) or rat antisera generated against *C. rodentium* specific Tir (1:5K; gift from W. Deng). This was followed by
secondary Alexa568-conjugated goat anti-rabbit or anti-rat IgG antibodies (Molecular Probes) and Prolong® Gold antifade reagent containing 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL; Roche, Toronto, Ontario, Canada) was performed as described by the manufacturer (Roche Diagnostics, Germany). Sections were viewed at 350 and 594 nm on a Zeiss AxioImager microscope. Images were obtained using a Zeiss AxioImager microscope equipped with an AxioCam HRm camera operating through AxioVision software (Version 4.4).

2.13 Fluorescence intensity measurements

The fluorescence intensity of immunostained samples was assessed using ImageJ software to determine the ratio of F480 relative to DAPI staining. This was done using the integrated density measurement tool. Integrated density for each image was assessed on separate channels to determine the pixel intensity of DAPI and F480 for each section. The fluorescence intensity then was represented as the integrated density value of F480 relative to total DAPI integrated density values.

2.14 Epithelial cell apoptosis

Terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) staining was employed using the in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.
2.15 Histopathological scoring

To assess tissue pathology, paraffin-embedded colonic tissue sections (5 µm) were stained with haematoxylin and eosin, and were examined by two blinded observers. For DSS colitis, tissue sections were assessed for inflammatory cell infiltration (0 = none; 1 = mild; 2 = moderate; 3 = severe) and overall tissue damage (0 = none; 1 = up to 50% loss of crypts; 2 = more than 50% loss of crypts; 3 = total disruption of crypts and loss of epithelial cells). The maximum score that could be obtained with this system is 6 points. For *Citrobacter rodentium*, tissue sections were assessed for submucosal edema (0 = no change; 1 = mild; 2 = moderate; 3 = profound), epithelial hyperplasia (scored based on percentage above the height of the control where 0 = no change; 1 = 1–50%; 2 = 51–100%; 3 = > 100%), epithelial integrity (0 = no change; 1 = < 10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; 4 = epithelial ulceration with severe crypt destruction) and neutrophil and mononuclear cell infiltration (0 = none; 1 = mild; 2 = moderate; 3 = severe), as previously described (253). The maximum score that could be obtained with this system is 13 points.

2.16 RNA extraction and quantitative RT-PCR

Colon and cecal tissues stored in RNALater (Qiagen) at −86°C were thawed, weighed, and total RNA extracted using the Qiagen RNeasy kit following the manufacturer's instructions. Tissues were homogenized in a 2.0 ml microtube containing 0.6 ml of Buffer RLT (supplied in Qiagen RNeasy kit) and a 5.0 mm steel bead (Qiagen), and homogenized in a MixerMill 301 bead miller (Retche) for 4 minutes at 30 Hz at room temperature. Total RNA was quantified using a NanoDrop Spectrophotometer (ND1000). 1–2 ug of RNA was reverse-transcribed using a Qiagen Omniscript RT kit (Qiagen), according to manufacturer's instructions. For quantitative
PCR, cDNA was diluted 1:5 in RNase/DNase free H₂O and 5 µl was added to 15 µl PCR reaction mix. The final reaction volume was 20 uL, containing BioRad Supermix used at a 1:2 dilution, and primers at a final concentration of 0.6 uM each. qPCR was carried out using a BioRad Miniopticon or Opticon2. Melting point analysis confirmed the specificity for each of the PCR reactions. Quantitation was performed using GeneEx Macro OM 3.0 software. Primer sequences and annealing temperatures are provided below in Table 2.1.
<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>PRIMER SETS</th>
<th>Annealing Temperature</th>
</tr>
</thead>
</table>
| β-actin    | Fwd: 5'-CAGCTTTCTTTGCAGCTCCTT-3'  
Rev: 5'-CTTCTCCCATGTCGTCCAGGT-3' | 55°C |
| TNF-α      | Fwd: 5'-CATCTTTCTAAAATTTGAGTGACAA-3'  
Rev: 5'-TGGGAGTAGCAAGGTACAACCC-3' | 55°C |
| mCramp     | Fwd: 5'-CTTCAACCAGGCTCCAGGACAGGA-3'  
Rev: 5'-TCCAGGTCAGGACAGGTA-3' | 55°C |
| iNOS       | Fwd: 5'-TGGGAATGGACTGGTCCCAG-3'  
Rev: 5'-GGGATCTGAATGTGATGTTTG-3' | 60°C |
| IFN-γ      | Fwd: 5'-TCAAGTGCGATAGTGGAAGAA-3'  
Rev: 5'-TGCTCTGCAAGGTTTTTCCATG-3' | 60°C |
| IL-17A     | Fwd: 5'-GCTCCAGAAGGCTCAGA-3'  
Rev: 5'-CCTTCCTCCGCCATTGACA-3' | 60°C |
| IL-17F     | Fwd: 5'-TGCTACTGTAGTTGGCCAC-3'  
Rev: 5'-AATGCCCTGTTTGGTTGAA-3' | 55°C |
| IL-22      | Fwd: 5'-ACCTTTTCTGACCAACCTCA-3'  
Rev: 5'-AGCTCTTTCTCCGTCAGAGC-3' | 58°C |
| IL-23p19   | Fwd: 5'-TGCTGTGCTAGGTAGGACAG-3'  
Rev: 5'-TTCATGCTCTCCTTCTTAGATT-3' | 60°C |
| RegIIIγ    | Fwd: 5'-TGCTATGCTCCTATTGCT-3'  
Rev: 5'-CACTCCATCCACCTGT-3' | 58°C |
| RegIIIβ    | Fwd: 5'-CTGGTTGAATGTCAGAAG-3'  
Rev: 5'-TGACTGGCGAGACAGAAG-3' | 58°C |
| TGF-β      | Fwd: 5'-GACTCTTCCACCTGCAAGACCAT-3'  
Rev: 5'-GGGACTGGCGAGCCTAGTT-3' | 59°C |
| IL-10      | Fwd: 5'-GTTGCAAGCCTCTCGGAA-3'  
Rev: 5'-CCAGGGAATTCAATGCTCCT-3' | 55°C |
| β-defensin 1| Fwd: 5'-CTCTCTGACTCTGGGAA-3'  
Rev: 5'-ATCGCTCGGTCTTATGTCCT-3' | 60°C |
| β-defensin 3| Fwd: 5'-CTCCACCTGAGCTTTAGC-3'  
Rev: 5'-GCTAGGGAGAATTGTGCTC-3' | 60°C |
| S100A8     | Fwd: 5'-GCGGTCTGAACTGGAGAAAGGCC-3'  
Rev: 5'-TCAACATCCGAAGGACTTCCTCG-3' | 55°C |
| S100A9     | Fwd: 5'-AGGAAGGAGACACCTGACACCC-3'  
Rev: 5'-ACGTGGTTGTTCTCATGCA-3' | 55°C |
2.17 Lamina propria cell isolation

For lamina propria mononuclear cells (LPMC) colons and ceca were collected, washed and placed in a shaking incubator at 37°C in RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS) and 5 mM EDTA. Tissues were then cut into 1 mm pieces followed by digestion with 0.2 mg/mL type VIII collagenase (Sigma Aldrich, Oakville, ON). Samples were then layered on a Percoll gradient (Amersham Biosciences, Uppsala, Sweden), and the LPMC collected from the 40% to 75% Percoll interface were used in subsequent assays.

2.18 Antibody staining and flow cytometry

Surface Ab staining was performed at 4°C in PBS/2% FCS with 0.1% sodium azide (FACS buffer) with fluorescently labeled CD4, CD25, and FoxP3 Ab (BD Pharma and eBiosciences). For intracellular cytokine staining, 0.5 x10^6 cells were resuspended in culture medium (RPMI1640 containing 10% heat inactivated FCS, 100 U/mL penicillin, 100 lg/mL streptomycin, 2 mM L-glutamine, and 5 lM b-mercaptoethanol) and stimulated with 50 ng/mL phorbol myristate acetate (Calbiochem, Mississauga, ON) and 1 lg/mL ionomycin (Sigma Aldrich) for 6 hours, with the addition of 10 lg/mL brefeldin A (Sigma Aldrich) after 1 hour. Cells were subsequently fixed with 4% formaldehyde in FACS buffer (fixation buffer) for 10 minutes and incubated with fluorescently labeled IFN-γ and IL-17 Abs (BD Biosciences) in FACS buffer containing 1% saponin (permeabilization buffer). Unbound Abs were washed away using the permeabilization buffer and cells were resuspended in FACS buffer. Samples were read on a FACSCanto (BD Biosciences) and analyzed using FlowJo Software Version 8.7.
2.19 Serum CD14 analysis

Mice were anaesthetized with halothane and blood was collected by cardiac puncture. Blood was allowed to clot naturally at room temperature and cells were removed by centrifugation. Serum was collected and stored at -80°C until analysis. CD14 was measured using the Quantikine ELISA Mouse CD14 Immunoassay Kit (R&D Systems Inc, Minneapolis, MN; Cat # MC140), and the assays were performed according to directions provided by manufacturer. This assay employs the quantitative sandwich enzyme immunoassay technique. Samples were quantified at OD 450 nm using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) and compared to the standard provided with the kit.

2.20 Lipopolysaccharide dephosphorylation assay

LPS-dephosphorylating activity was measured by the malachite green assay, which measure free phosphate release (254). Intestinal tissues were homogenized in 500 mL of homogenization buffer and then centrifuged at 11,000 rpm for 3 min to remove insoluble material. To determine the protein concentrations, a Bradford assay (Bio-Rad) was performed on the tissue samples according to the manufacturers instructions. A standard curve was created using a stock solution of 1 mg/mL BSA (Sigma) in triplicates with multiple concentrations. 40 mL of a 5 mg/mL solution of *Escherichia coli* 055:B5 LPS (Sigma L2880) was then added to 15 mL lysate and left for 2 h at room temperature. 40 mL of a solution composed of 0.01% malachite green (Sigma), 16% sulfuric acid (Fisher), 1.5% ammonium molybdate (Sigma) and 0.18% Tween-20 (Sigma) was incubated with the lysate for 10 min. LPS-dephosphorylating activity was determined from colorimetric measurements taken at an absorbance of 620 nm.
2.21 Statistical analysis

Statistical significance was calculated by using either a two-tailed Student’s t-test or the Mann-Whitney test unless otherwise indicated, with assistance from GraphPad Prism Software Version 4.00 (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)). A $P$ value of $\leq 0.05$ was considered significant. The results are expressed as the mean value with standard error of the mean (SEM).
Chapter 3: Active Vitamin D (Calcitriol) Increases Host Susceptibility to *Citrobacter rodentium* by Suppressing Mucosal Th17 Responses

3.1 Introduction

The active form of vitamin D, calcitriol (1,25(OH)2D3) is a hormone that plays a critical role in many different cellular processes, including cell proliferation, apoptosis and immune modulation (6). In fact, epidemiological studies have implicated vitamin D insufficiency in the pathophysiology of T-cell mediated, inflammation-driven diseases, such as inflammatory bowel diseases (IBD), including Crohn’s disease and ulcerative colitis (7). At present it is unclear exactly how vitamin D levels impact on the development of IBD; however, research has shown that vitamin D deficiency is associated with higher disease activity and longer disease duration in IBD patients (180-183). Furthermore, a pilot study in Crohn’s disease patients demonstrated a short-term beneficial effect of a calcitriol analogue on disease activity over a one-year course (198). Animal models of colitis, including the dextran sodium sulfate (DSS) model, demonstrate that vitamin D deficiency exacerbates disease, while calcitriol supplementation ameliorates colitis (191-196), indicating that vitamin D plays a role (albeit poorly defined) in the pathophysiology of IBD.

Although the basis for the beneficial actions of vitamin D during colitis are unclear, studies have suggested that calcitriol signalling through the vitamin D receptor (VDR) can alter inflammatory responses in the host through a number of mechanisms, including skewing T-cell responses toward a regulatory phenotype (240, 255). As such, calcitriol can be considered a potent
immunosuppressive agent, and we therefore wondered if the immunosuppressive actions of calcitriol treatment could potentially impair host defenses against enteric microbes, since Th1/Th17 responses are critical for the clearance of many bacterial infections (76). Hence, vitamin D therapy may be a novel treatment option for patients with IBD, however further research is required to understand the underlying mechanisms and potential limitations of treatment with the active compound calcitriol.

Pathogenic strains of *Escherichia coli* including EHEC and EPEC are important causes of infectious diarrhea, with EPEC contributing to as many as 1 million infant deaths per year in developing nations (16). Mucosa associated *E. coli* has been observed in greater numbers in patients with IBD compared to healthy controls, and these microbes have been shown to play a role in driving inflammation in the intestine (18). Since pathogenic strains of *E. coli* do not colonize mice, researchers rely on the related but mouse-specific attaching and effacing bacterial pathogen *Citrobacter rodentium*. Following infection, *C. rodentium* intimately attaches to epithelial cells lining the cecum and colon, resulting in barrier disruption, crypt hyperplasia, loss of goblet cells, mucosal infiltration with immune cells and a strong Th1/Th17 response (20). The effect of vitamin D levels on host responses to *C. rodentium* infection have not yet been addressed. To test this, we treated mice undergoing either DSS or *C. rodentium*-induced colitis with calcitriol or with vehicle. Interestingly, while calcitriol-treated mice were strongly protected against chemically induced colitis, they were significantly more susceptible to bacterial induced colitis, and suffered widespread mucosal ulceration and increased pathogen burdens. Through assessment of the host immune response, we found that calcitriol treatment led to a selective suppression of Th17 T-cells and the associated antimicrobial peptide RegIIIγ. These studies thus
report, for the first time, that calcitriol treatment suppresses mucosal Th17 responses, thereby increasing host susceptibility to an enteric bacterial pathogen.

3.2 Results

Supplemental Figures for Chapter 3 can be found in Appendix A.

3.2.1 Calcitriol administered intraperitoneally protects mice against acute dextran sodium sulfate-induced colitis

Prior to testing the potential effect of calcitriol on the host response to C. rodentium infection, we first sought to verify previously published studies showing that calcitriol and its analogues can ameliorate DSS-induced colitis in C57BL/6 mice. These earlier studies administered calcitriol either orally or intra-rectally at high doses (~50 ng per day) (193, 195). Although the luminal delivery of calcitriol was protective, the high doses used, as well as the variation in the results obtained, led us to test the effect of calcitriol using lower doses and an alternative route of delivery that offers less variation. Mice were given either vehicle or 10 ng of calcitriol via intraperitoneal injection 1 day prior to being exposed to 3% DSS in their drinking water, and then everyday thereafter until sacrifice. Mice treated with calcitriol lost more body weight during DSS challenge compared to vehicle-treated mice (20% vs. 12% of initial body weight, respectively) (Figure 3.1A). However, control mice treated daily with calcitriol lost 10% of their initial body weight compared to vehicle-treated mice (Supplemental Figure A.1A). This result suggests that the heightened weight loss seen in mice given both DSS and calcitriol reflects weight loss triggered by calcitriol rather than increased susceptibility to DSS colitis. calcitriol induced weight loss may be a sign of hypercalcemia, which is defined as serum calcium levels
higher than 12 mg/dL (256). As expected, calcitriol-treated control mice had higher serum calcium levels compared to vehicle-treated control mice (10.89 ± 0.55 mg/dL vs. 7.54 ± 0.66 mg/dL (p < 0.05; n=8 per group), respectively) following 10 days of treatment, however values were all within the normal range of 7-12 mg/dL (256). Despite their increased weight loss, calcitriol-treated mice showed no clinical signs of morbidity (i.e. hunched posture, ruffled fur) and appeared active and healthy. Upon sacrifice, the ceca and colons of calcitriol-treated mice appeared healthy, whereas the ceca of vehicle-treated mice were shrunken and their colons contained no formed stool (Figure 3.1B). Histologically, vehicle-treated mice had severe mucosal damage in the distal colon, characterized by a loss of crypts, severe ulceration and infiltration of inflammatory cells (Figure 3.1C). In contrast, the distal colons of calcitriol-treated mice showed minimal pathology with histopathological scoring revealing significantly less tissue damage than vehicle-treated mice, scoring 1.0 ± 0.5 versus 2.5 ± 0.3 (p < 0.05; n=6-7 per group) (Figure 3.1D). There was no difference in histological damage between control mice treated with vehicle or calcitriol (Supplemental Figure A.1B and Supplemental Figure A.1C). Intestinal barrier integrity was assessed in vivo at various time points by FITC-dextran assay. Mice were orally gavaged with FITC-dextran and their plasma was assessed for levels of translocated FITC-dextran. There was no difference in FITC-dextran levels at day 1, day 3 or day 5 post 3% DSS between vehicle or calcitriol-treated mice (Figure 3.2). However, at day 7 post DSS and day 9 post DSS, calcitriol-treated mice had significantly lower levels of FITC-dextran in their plasma, compared to vehicle-treated mice, indicating that calcitriol treatment protects barrier integrity during challenge with DSS (Figure 3.2). Taken together, our assessments confirm that calcitriol dramatically protects the mammalian GI tract during DSS colitis.
Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to 3% dextran sodium sulfate (DSS). DSS was given in drinking water for 7 days, followed by tap water for 2 days. A) Body weight. Each data point represents the average body weight pooled from 8 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 2 independent experiments, n = 8 per group, Student’s t-test was conducted at each time point, ** p < 0.01, *** p < 0.001, Mann-Whitney Test. B) Representative digital macroscopic image of the lower gastrointestinal (GI) tract. C) Formalin fixed “Swiss rolled” tissues were stained with H & E. Focus is on distal colon. Top panel is vehicle, bottom panel is calcitriol. Original magnification = 200X, Scale bar = 200 µm. D) Distal colon was assessed for histological damage by scoring system described in Methods. Results are representative of 2 independent experiments, n = 6 - 7 per group, * p < 0.05, Mann-Whitney test.
Figure 3.2 Calcitriol-treated mice are protected against barrier disruption during 3% dextran sodium sulfate-induced colitis

Male 8-week-old C57BI/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to 3% dextran sodium sulfate (DSS). DSS was given in drinking water for 7 days, followed by tap water for 3 days. To assess intestinal barrier integrity, mice were orally gavaged with FITC-dextran and their plasma was assessed for levels of translocated FITC-dextran at various time points. Day 1, n = 4 per group; Day 3, n = 4 per group; Day 5, n = 4 per group; Day 7, n = 4 per group; Day 9, n = 7 - 10 per group. * p < 0.05, Mann-Whitney test. Veh = Vehicle; Cal = Calcitriol.

3.2.2 Protective effects of calcitriol during acute dextran sodium sulfate-induced colitis is dependent on dose and route of administration

During preliminary studies, mice were administered a lower dose of 5 ng calcitriol per day during 3% DSS challenge. There was no difference in weight loss between vehicle and 5 ng calcitriol-treated mice throughout the study or at day 7 post-DSS (22.75 g ± 0.85 SEM vs. 23.00 g ± 0.66 SEM, respectively). However, 5 ng calcitriol did not protect against DSS-induced histological damage, compared to 10 ng calcitriol (Supplemental Figure A.2). Furthermore,
when compared dose for dose, 10 ng calcitriol was more protective against DSS-induced damage when given via intraperitoneal injection, compared to intra-rectal administration (Supplemental Figure A.3 and Supplemental Figure A.4). Previous studies have shown that larger doses of 50 ng calcitriol or analogues administered intra-rectally were protective against DSS induced damage (193, 195). Intriguingly, when mice were given 10 ng calcitriol and challenged with higher doses of 4% DSS, mice were no longer protected against DSS-induced colonic damage and suffered worsened histological damage compared to vehicle-treated mice challenged with 4% DSS (Supplemental Figure A.5 and Supplemental Figure A.6). These findings demonstrate that calcitriol treatment may have a narrow window of protection during colitis and may actually worsen colitis if the host suffers from exaggerated damage.

3.2.3 Calcitriol-treated mice develop macroscopic erosions in the colon and cecum at day 10-post infection with *Citrobacter rodentium*

To determine the role of calcitriol during infection with *C. rodentium*, mice were administered 10 ng of calcitriol via intraperitoneal injection 1-day-prior to infection and then every day thereafter for the duration of the study. As shown in Figure 3.3A, infected vehicle-treated mice displayed a slight drop in body weight at day 2 pi, followed by full recovery by day 10 pi. In contrast, infected calcitriol-treated mice steadily lost weight as their infection progressed and by day 10 pi, they had lost 18% of their initial body weight. Serum calcium levels were higher in calcitriol-treated mice compared to vehicle-treated mice at day 10 pi. There was however no difference in serum calcium levels between *C. rodentium* infected vs. non-infected mice (Figure 3.3B). There were no observed differences in stool consistency or rectal bleeding between infected mice given vehicle and calcitriol-treated mice. Surprisingly the calcitriol-treated mice
had shortened colons, compared to vehicle-treated mice (Figure 3.3C and Figure 3.3D), and 70% of these mice displayed macroscopic mucosal damage and small ulcers in their mid/distal colons at day 10 pi, whereas similar pathology was only seen in 20% of vehicle-treated mice (Figure 3.3E and Figure 3.3F). At earlier time points (day 6 pi), calcitriol-treated mice developed macroscopic erosions in the cecum, whereas vehicle-treated ceca appeared normal (Supplemental Figure A.7). Dose-response studies also demonstrated that mice administered a lower daily dose of 5 ng calcitriol during infection developed macroscopic ulceration in the cecum at day 10 pi, whereas vehicle-treated ceca appeared normal (Supplemental Figure A.8).

Figure 3.3 Calcitriol-treated mice develop macroscopic erosions in the colon at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. A) Body weight. Each data point represents the average body weight pooled from 8 - 16 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 2 - 4 independent experiments. B) Serum calcium. Serum was collected by cardiac puncture and analyzed for calcium by calorimetric assay. Results are representative of 2 independent experiments, n = 8 per group, * p < 0.05, ** p < 0.01, Mann-Whitney test). C) Top: Representative digital image of the lower gastrointestinal (GI) tract from mice at day 10 pi. Vehicle-treated mice (left) appeared normal, compared to calcitriol-treated mice (right), which had shortened, inflamed colons nearly devoid of contents. D) Colon length (measured from cecal base/proximal region to distal tip) at day 10 pi. Results are representative of 5 independent experiments, n = 18 per group, ** p< 0.01, Mann-Whitney test. E) Representative digital image of the lower GI tract, opened longitudinally from proximal (top) to distal (bottom) from calcitriol-treated mice at day 10 pi. Arrows are pointing to erosion/ulcerated regions in mid/distal colon. F) The percentage of mice with macroscopic erosions in the colon at day 10 pi. Results are representative of 4 independent experiments, n = 16 per group, ** p < 0.01, Mann-Whitney test. Veh = Vehicle; Cal = Calcitriol; CR = *C. rodentium*; pi = post infection; D10 = day 10
3.2.4 Calcitriol-treated mice carry higher *Citrobacter rodentium* burdens at day 6 and day 10-post infection

To address whether the exaggerated mucosal damage suffered by the calcitriol-treated mice was associated with increased *C. rodentium* burdens, tissues were homogenized, plated and bacteria was quantified. At both day 6 and day 10 pi, calcitriol-treated mice carried significantly higher *C. rodentium* burdens in their colons and ceca, as compared to vehicle-treated mice (Figure 3.4). *C. rodentium* is a non-invasive pathogen, and bacterial loads are primarily limited to the intestinal lumen and mucosal surface of wildtype mice. Interestingly, calcitriol-treated mice carried more culturable *C. rodentium* from extra-intestinal tissues, including MLN, spleen and liver at day 6 pi and day 10 pi, compared to vehicle-treated mice, indicating greater bacterial translocation to these systemic sites (Figure 3.4). To localize the *C. rodentium*, colons were Swiss-rolled and immunostained for *C. rodentium*-derived translocated intimin receptor (Tir) (Deng et al. 2003). As expected, *C. rodentium* was localized to the distal colon in vehicle-treated mice at day 10 pi. In contrast, calcitriol-treated mice had increased Tir staining throughout the entire colon with significant staining found in the mid colon and even reaching the proximal colon (Figure 3.4C). To better define the time course of the heightened *C. rodentium* colonization, stool was collected and analyzed over the first 10 days of infection. At day 1 pi, calcitriol-treated mice carried higher bacterial burdens in their stool, as compared to vehicle-treated mice, however there were no significant differences between groups at day 2, 3, 4 or 5 pi (Supplemental Figure A.9). Mice treated with lower dose of 5 ng calcitriol also had higher bacterial burdens in the colon and ceca, compared to vehicle-treated mice at day 10 pi (Supplemental Figure A.10A). However, at the lower dose, there was no difference in bacterial translocation to the MLN, liver or spleen between vehicle and calcitriol-treated mice.
(Supplemental Figure A.10B). These data show that calcitriol treatment increases pathogen burdens during the course of infection.

**Figure 3.4 Calcitriol-treated mice have greater pathogen burdens at day 6 and day 10-post infection with *Citrobacter rodentium***

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Whole tissues were homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens. A) Day 6 pi. For colon and ceca, n = 20 per group; For MLN, n = 20 per group; For Spleen, n = 20 per group; For Liver, n = 12 per group. Results are representative of 3 - 5 independent experiments, * p < 0.05, *** p < 0.001, Mann-Whitney test. B) Day 10 pi. For colon and ceca, n = 12 per group; For MLN, n = 19 per group; For Spleen, n = 12 per group; For Liver, n = 8 per group. Results are representative of 3 - 5 independent experiments, * p < 0.05, ** p < 0.01, *** p < 0.001, Mann-Whitney test. C) To localize *C. rodentium*, colons were “Swiss-rolled” and immunostained for *C. rodentium*-derived translocated intimin receptor (Tir) Representative images of colon tissues at day 10 pi. Blue = DAPI; Red = Tir. Original magnification = 50X, Scale bar = 200 µm. CR = *C. rodentium*; MLN = mesenteric lymph nodes; pi = post infection
3.2.5 Calcitriol-treated mice have worsened histological damage at day 10-post infection with *Citrobacter rodentium*

At day 10 pi, calcitriol-treated mice had significantly increased submucosal edema and a higher number of mucosal ulcers throughout the entire colon, as determined by examining Swiss-rolled sections (Figure 3.5A, Figure 3.5B). Tissue cross-sections were also examined and there was a trend for more damage in the calcitriol-treated mice in the distal colon and cecum between groups at day 10 pi (Supplemental Figure A.11). In contrast, the mid colon of calcitriol-treated mice was the site of the greatest damage with significantly elevated scores for edema, goblet cell depletion, hyperplasia and infiltrating inflammatory cells, compared to vehicle-treated mice (Figure 3.5C, Figure 3.5D). Note, that calcitriol had no effect on histology scores in uninfected mice (Supplemental Figure A.1).

**Figure 3.5 Calcitriol-treated mice have worsened histological damage at day 10-post infection with *Citrobacter rodentium***

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. A) Representative images of formalin fixed “Swiss rolled” tissues stained with H&E. Original magnification = 50X. B) Swiss rolled colon tissues were assessed for submucosal edema (scoring system: 0 = no change; 1 = mild; 2 = moderate; 3 = profound) and histological ulcers (number of ulcers per tissue) at day 10 pi. Results are representative of 2 independent experiments, n = 6-7 per group. ** p < 0.01, Mann-Whitney test. C) Representative image of cross section of the mid colon at day 10 pi. Original magnification = 200X. D) The Mid colon was assessed for histological damage by scoring system for *C. rodentium* described in Methods. Results are representative of 2 independent experiments, n = 7 per group, ** p < 0.01, Mann-Whitney test. Representative image of cross section of the mid colon at day 10 pi. Original magnification = 200X. CR = *Citrobacter rodentium*; pi = post infection
A

Vehicle + CR D10
Whole Colon

Calcitriol + CR D10
Whole Colon

B

Day 10 pi

Submucosal Edema Score

Vehicle + CR
Calcitriol + CR

# Histological Ulcers

Vehicle + CR
Calcitriol + CR

C

Vehicle + CR D10
Mid Colon

Calcitriol + CR D10
Mid Colon

D

Day 10

Pathology Score

Vehicle + CR
Calcitriol + CR

Inflammatory Cell Infiltrate
Epithelial Integrity
Hyperplasia
GC Depletion
Submucosal Edema
3.2.6 Calcitriol-treated mice show altered epithelial responses at day 10-post infection with *Citrobacter rodentium* infection

To address the mechanisms underlying the mucosal damage that developed in the infected calcitriol-treated mice, intestinal epithelial cell proliferation and cell death were determined by Ki67 staining and the TUNEL assay, respectively. Infected mice had more Ki67+ve cells in their colons, compared to uninfected mice at day 10 pi (Figure 3.6). However, infected calcitriol-treated mice had fewer Ki67+ve cells in the mid and distal colon, compared to vehicle-treated infected mice, indicating impaired proliferation at day 10 pi (Figure 3.6). Infected mice also had more TUNEL +ve cells in their colons, compared to uninfected mice at day 10 pi (Figure 3.7A). However, calcitriol-treated mice also had more TUNEL+ve epithelial cells in the mid colon, compared to vehicle-treated mice at day 10 pi (Figure 3.7). Furthermore, calcitriol-treated mice had large numbers of TUNEL+ve cells in both the cecal and colonic lumen at day 10 pi, indicating greater epithelial cell sloughing within these mice (Figure 3.7B). These results are in agreement with previous work indicating that calcitriol can inhibit cell proliferation and induce apoptosis in human colonic epithelial cells *in vitro* (218). However, these changes were only seen during infection, as there were no obvious effects of calcitriol on cell proliferation or apoptosis between uninfected groups (Figure 3.6; Figure 3.7A). To determine if the observed changes in cell proliferation and apoptosis coincided with altered barrier integrity *in vivo*, mice were orally gavaged with FITC-dextran at day 6 pi and plasma was assessed for levels of translocated FITC-dextran. Vehicle-treated mice infected with *C. rodentium* had higher levels of detectable FITC-dextran in their plasma, compared to control mice at day 6 pi (Figure 3.8). However, calcitriol-treated mice had significantly higher levels of FITC-dextran in their plasma,
compared to vehicle-treated mice, indicating that calcitriol treatment leads to exaggerated barrier disruption during infection with *C. rodentium* (Figure 3.8).

**Figure 3.6** Calcitriol-treated mice have less cell proliferation in the colon at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. A) Distal colon and mid colon were assessed for number of Ki67+ve cells in the lumen (percent of +ve Ki67 per +ve DAPI per area measured). Results are representative of 2 independent experiments, n = 4 per group (uninfected); n = 6 per group at day 10 pi, ** p < 0.01, Mann-Whitney test. B) Representative images of formalin fixed “Swiss rolled” colon tissues from uninfected mice. Immunofluorescence staining: Blue = DAPI; Red = Ki67. Original magnification = 50X. C) Representative image of cross section of the mid colon at day 10 pi. Immunofluorescence staining: Blue = DAPI; Red = Ki67. Original magnification = 200X. D) Representative image of cross section of the distal colon at day 10 pi. Immunofluorescence staining: Blue = DAPI; Red =Ki67. Original magnification = 200X. D) CR = *Citrobacter rodentium*; D10 = Day 10; pi = post infection.
B

Vehicle Uninfected Whole Colon
Calcitriol Uninfected Whole Colon

C

Vehicle + CR D10 Mid Colon
Calcitriol + CR D10 Mid Colon

D

Vehicle + CR D10 Distal Colon
Calcitriol + CR D10 Distal Colon
Figure 3.7 Calcitriol-treated mice have more cell death in the mid colon at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. A) Colon was assessed for number of TUNEL+ve cells (per 10 intact crypts). Uninfected, n = 4; Day 10 pi. Results are representative of 2 independent experiments, n = 7-8 per group, ** p < 0.01, Mann-Whitney test. B) Representative image of cross section of the mid colon at day 10 pi. Immunofluorescence staining: Blue = DAPI; Green = TUNEL. Original magnification = 200X. CR = *Citrobacter rodentium*; D10 = Day 10; pi = post infection
Figure 3.8 Calcitriol-treated mice have worsened barrier integrity at day 6-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 7 days, starting one day prior to oral infection with *C. rodentium*. To assess intestinal barrier integrity, mice were orally gavaged with FITC-dextran at day 6 pi and their plasma was collected via cardiac puncture and assessed for levels of translocated FITC-dextran. Uninfected vehicle-treated n = 3; uninfected calcitriol-treated n = 4; Vehicle and calcitriol-treated at day 6 pi n = 12 per group. Results are representative of 3 independent experiments, * p < 0.05, *** p < 0.001, Mann-Whitney test. CR = *Citrobacter rodentium*; pi = post infection

![Graph showing FITC/dextran levels](image)

3.2.7 Calcitriol-treated mice carry higher bacterial burdens and ulceration at day 10-post infection with attenuated *Citrobacter rodentium*

To determine if the observed epithelial pathology that develops in infected mice during calcitriol treatment reflected exaggerated bacterial driven pathology, or alternatively, an abnormal host response to *C. rodentium*, we challenged mice with a *C. rodentium* mutant lacking the translocated bacterial effector protein EspF (257). Previous studies have shown that the EspF
protein plays a critical role in the disruption of tight junctions and the induction of apoptosis in infected colonic epithelial cells (258, 259). Furthermore, this effector has been shown to play a critical role in causing mucosal damage and ulcerations in susceptible mouse strains (62). As expected, vehicle-treated mice challenged with the ΔespF mutant suffered little in the way of weight loss or mucosal damage. In contrast, infected calcitriol-treated mice steadily lost weight as their infection progressed and by day 10 pi, they had lost ~18% of their initial body weight (Figure 3.9A). Furthermore, calcitriol-treated mice developed macroscopic erosions in their mid/distal colons at day 10 pi. Histologically, the mid colon of calcitriol-treated mice showed significant edema and inflammatory cell infiltrate, with areas of ulceration (Figure 3.9B). In agreement with this increased damage, calcitriol-treated mice had 20-fold higher pathogen burdens in their colons, compared to vehicle-treated mice, indicating greater colonization. calcitriol-treated mice also carried more culturable C. rodentium mutant from extra-intestinal tissues at day 10 pi, compared to vehicle-treated mice, indicating greater bacterial translocation to these systemic sites (Figure 3.9C).

Figure 3.9 Calcitriol-treated mice have worsened barrier integrity at day 6-post infection with Citrobacter rodentium

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with ΔespF C. rodentium. A) Body weight. Mice were treated with vehicle or calcitriol intraperitoneally daily throughout infection. Each data point represents the average body weight pooled from 8 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 2 independent experiments, n = 8 per group, Student’s t-test was conducted at each time point, ** p < 0.01, *** p < 0.001, Mann-Whitney Test. B) Representative image of cross section of the mid colon at day 10 pi. Original magnification = 200X. C) ΔespF C. rodentium burden day 10 pi. Results are representative of 3-5 independent experiments, n = 8 per group, * p < 0.05, ** p < 0.01, *** p < 0.001, Mann-Whitney test. Veh = Vehicle; Cal = Calcitriol; CR = Citrobacter rodentium, pi = post infection
A

% Initial Body Weight

Vehicle

10 ng Calcitriol

Day 0
Day 2 pi
Day 4 pi
Day 6 pi
Day 8 pi
Day 10 pi

B

Vehicle + ΔespF CR
Mid Colon

Calcitriol + ΔespF CR
Mid Colon

C

Colonic CFU/g of tissue

Day 10 pi

Colon

Cecum

MLN

Spleen

Liver

10^10

10^9

10^8

10^7

10^6

10^5

10^4

10^3

Veh + ΔespF

Cal + ΔespF

Veh + ΔespF

Cal + ΔespF

Veh + ΔespF

Cal + ΔespF

Veh + ΔespF

Cal + ΔespF

Veh + ΔespF

Cal + ΔespF

Veh + ΔespF

Cal + ΔespF
3.2.8 Calcitriol treatment suppresses colonic cytokine mRNA levels at day 10-post infection with *Citrobacter rodentium*

To determine if the increased susceptibility of calcitriol-treated mice to *C. rodentium* infection could reflect modulation of the host immune response, we assessed the distal colon for expression of genes encoding cytokines that have been shown to influence susceptibility to *C. rodentium* infection. The distal colon was chosen for analysis since there was comparable histological damage between both groups at this site. The inflammatory mediators TNF-α and iNOS have previously been shown to play an important role in controlling *C. rodentium* pathogen load (128, 260). Surprisingly, there were no changes in the mRNA levels of these genes between vehicle and calcitriol-treated mice at day 10 pi (Supplemental Figure A.12). As expected, the expression of Th1 (IFN-γ) and Th17-related cytokines (IL-6, IL-17A) were upregulated in the distal colon of mice challenged with *C. rodentium* compared to uninfected controls, as previously shown (85, 126, 261). However, at day 10 pi, calcitriol-treatment strikingly suppressed the elevated expression of IFN-γ, IL-6 and IL-17A as compared to vehicle-treated mice (Figure 3.10). We also measured gene expression of other cytokines and growth factors and found no differences in the colonic expression of the anti-inflammatory cytokines IL-10 or TGF-β or other Th17-related cytokines (IL-22, IL-23 or IL-17F) between vehicle and calcitriol-treated mice at day 10 pi (Supplemental Figure A.13).

**Figure 3.10** Calcitriol-treated mice have suppressed colonic cytokine mRNA levels at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Expression of IFN-γ, IL-17A and IL-6 in
distal colon at baseline and day 10 pi. as assessed by RT qPCR. Results are representative of 3 independent experiments, n= 8 per group, * p < 0.05, Mann-Whitney test. CR = Citrobacter rodentium, pi = post infection

3.2.9 Calcitriol-treated mice have fewer CD4+IL-17A+ cells in the colon at day 10-post infection with Citrobacter rodentium

Based on the reduced expression of IFN-γ and IL-17A, we next asked whether calcitriol treatment led to alterations in immune cell populations in the colon and in the spleen. We therefore isolated lymphocytes from the spleen and colonic lamina propria and assessed intracellular cytokine expression by FACS analysis. Effector CD4+ T cells can be classified based on the signature cytokines that they produce. For instance, Th1 cells produce IFN-γ, while Th17 cells are characterized by IL-17A (82). CD4+ T cells can also develop into regulatory cells (Treg) that express FoxP3. As expected, there were more CD4+IFN-γ+ and CD4+IL-17A+ cells in the lamina propria of C. rodentium challenged mice at day 10 pi, compared to uninfected mice (Figure 3.11). Although calcitriol treatment had no overt impact on CD4+IFN-γ+ cell numbers in the colons of C. rodentium infected mice, it did lead to significantly fewer colonic CD4+IL-
17A+ cells compared to vehicle-treated mice at day 10 pi. This suppression seemed to be specific to the intestine, since there was no difference in CD4+IL-17A+ populations in the spleens of calcitriol-treated vs. vehicle-treated mice at day 10 pi (Supplemental Figure 14.A). The lamina propria of *C. rodentium* infected mice contained fewer CD4+CD25+FoxP3+ cells at day 10 pi compared to uninfected mice (Figure 3.11). Although there was a trend for calcitriol-treated mice to have more CD4+CD25+FoxP3+ cells in their colonic lamina propria at day 10 pi, compared to vehicle-treated mice, these changes did not reach significance.

Figure 3.11 Calcitriol-treated have fewer CD4+IL-17A+ cells in the lamina propria at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. A) FACS analysis. Lamina propria (colon + cecum with cecal patch removed) from 2-4 mice was pooled together for each data point. Results are representative of 3 independent experiments, * p < 0.05, Mann-Whitney test. B) Representative FACS plot of proportion of CD4+IL-17+ cells in lamina propria at Day 10 pi. (vehicle + CR 14.4% vs. calcitriol + CR 8.82%). CR = *Citrobacter rodentium*; pi = post infection
3.2.10 Calcitriol-treated mice have reduced expression of the antimicrobial peptide RegIIIγ at day 10-post infection with Citrobacter rodentium

Th17 associated cytokines exert strong host protective roles during *C. rodentium* infection although the basis for their protective effects are poorly understood. Considering that a major feature of the calcitriol-treated mice was increased *C. rodentium* burdens, we decided to assess gene expression for several antimicrobial factors previously linked to host defense against *C. rodentium*, since these could be the effector molecules through which Th17 cells exert their effects. As previously shown, the expression of RegIIIγ was upregulated in the distal colon of mice challenged with *C. rodentium* compared to uninfected controls (85). Interestingly, calcitriol-treated mice had reduced expression of RegIIIγ in the distal colon at day 10 pi,
compared to vehicle-treated mice (Figure 3.12). There were no differences in the expression of RegIIIβ, S100A8 or S100A9 between vehicle and calcitriol-treated mice at day 10 pi, indicating a selective effect of calcitriol on RegIIIγ (Supplemental Figure 15A).

**Figure 3.12 Calcitriol-treated have reduced expression of the antimicrobial peptide RegIIIγ**

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Expression of RegIIIγ in distal colon at baseline and day 10 pi as assessed by RT qPCR. Results are representative of 3 independent experiments, n= 8 per group, * p < 0.05, Mann-Whitney test. CR = *Citrobacter rodentium*, pi = post infection

3.3 Discussion

Our work confirms that calcitriol-treatment can protect mice against DSS-induced colitis, however this is the first study to show that providing exogenous and active vitamin D (i.e. calcitriol) increases host susceptibility to an enteric bacterial pathogen. It has previously been shown that mice treated with high dose calcitriol (75 ng, 3 times per week) developed
exaggerated crypt hyperplasia at day 12-post infection with *C. rodentium*, however other aspects of disease were not reported (262). We found that calcitriol-treated infected mice suffered from increased *C. rodentium* burdens and exaggerated colonic tissue damage, compared to vehicle-treated mice. In agreement with these findings, calcitriol-treated mice showed defects in their expression of colonic IFN-$\gamma$, IL-17A and IL-6 at day 10 pi. These cytokines have been shown to play important roles in regulating pathogen burdens as well as mucosal damage during *C. rodentium* infection (124-126). In association with their increased susceptibility, calcitriol-treated infected mice showed substantially reduced numbers of Th17 T-cells in their colons during *C. rodentium* infection, whereas only modest changes were noted in Th1 and Treg T-cell numbers. In agreement with a suppression of Th17 responses, calcitriol-treated mice showed defects in their production of the Th17 associated antimicrobial peptide RegIII$\gamma$, which has been shown to play a role in mucosal repair and host defense during infection with *C. rodentium* (85).

As previously shown, mice treated with calcitriol were protected against DSS-induced colitis (193, 195). Vehicle-treated mice developed severe mucosal damage in the colon post-DSS, characterized by a loss of crypts and infiltration of inflammatory cells, whereas the colons of calcitriol-treated mice appeared relatively undamaged. It is currently unclear how calcitriol ameliorates DSS-induced colitis. Previous studies have indicated that calcitriol treatment can suppress several inflammatory mediators in mice challenged with DSS, including TNF-$\alpha$ and IFN-$\gamma$ (222), however the underlying mechanism is unknown. We have not investigated the Th17 response in DSS challenged mice; since the effect of calcitriol treatment is so dramatic, it is likely that all host inflammatory/immune markers are suppressed. It is also unknown whether
Th17 responses play a role during DSS challenge, since T and B cells are dispensable in the acute DSS colitis model (263). However, Alex et al. (264) showed that mice administered 3% DSS for 7 days had increased colonic expression of IL-12 and IL-17, while il-17a/- mice were protected against DSS-challenge, indicating the IL-17A may play an important role in the pathophysiology of DSS colitis (265).

The newly described Th17 cells are abundant at mucosal surfaces and characterized by the production of IL-17A, a pro-inflammatory cytokine associated with increased severity of various inflammatory diseases, including type-1 diabetes, multiple sclerosis and IBD (266). As such, strategies to block and/or regulate Th17 responses are currently being investigated, including calcitriol treatment. Indeed, it was recently shown that calcitriol can directly suppress IL-17A by dissociation of histone acetylase activity from the IL-17A promoter and recruitment of histone deacetylase and VDR/RXR binding to NFAT sites (267). Furthermore, recent studies have found that calcitriol can suppress the development of Th17 cells and production of associated cytokines such as IL-6, IL-17, IL-22 and IL-23, in vitro (238-241, 267). However, most of these studies have focused on in vitro regulation, whereas in vivo studies are needed to better clarify the potential therapeutic use of modifying vitamin D levels to alter Th17 responses. Our findings indicate that calcitriol does indeed suppress Th17 responses in vivo, and while such suppression may prove protective against autoimmune diseases, it can also impair antimicrobial defenses in the GI tract. In fact, IL-17A has been shown to induce the expression of antimicrobial peptides, stimulate the production of pro-inflammatory cytokines and induce granulopoietic factors and chemokines to recruit neutrophils in response to infection (82). As such, Th17 responses are
critical for the clearance of extracellular bacterial infections (76). In agreement with the suppressed colonic Th17 response, we found that calcitriol-treated mice carried significantly higher *C. rodentium* burdens in their colons and ceca compared to vehicle-treated mice at day 10 pi. Furthermore, calcitriol-treated mice suffered *C. rodentium* colonization throughout their entire colon, as determined by Tir staining on infected epithelial cells, including the mid and proximal regions of the colon- areas not normally colonized by *C. rodentium* in wildtype mice. It is currently unknown why calcitriol treatment altered the typical infection pattern, however, it can be assumed that calcitriol treatment modified the host response to *C. rodentium*, rather than having a direct effect on the bacteria, since previous work has shown that calcitriol does not directly alter bacterial growth or activity, *in vitro* (235).

Interestingly, we found that calcitriol-treated mice developed worsened histological damage, particularly in the mid colon, an area not typically damaged by *C. rodentium* in wildtype mice. In association with the mucosal damage, we found that calcitriol-treatment selectively suppressed IL-6 mRNA levels within the colon. IL-6 is a cytokine shown to play a key role in mucosal protection during challenge with *C. rodentium* (126). Furthermore, our group has found that mice deficient in TLR2 develop ulceration in the mid colon during *C. rodentium* challenge, which was attributed to a defect in IL-6 production within the colonic mucosa (62). Normally, *C. rodentium* colonizes the cecum and then spreads to the distal colon of wildtype mice, resulting in histological damage to these regions. At first thought, it seems that the exaggerated damage in the mid colon of calcitriol-treated mice was simply caused by higher bacterial burdens in the colon- or perhaps, the immunosuppressive effects of calcitriol allowed the pathogenic bacteria to
colonize areas of the gut it does not normally inhabit. However, we also found similar results when calcitriol-treated mice were challenged with a *C. rodentium* strain lacking the translocated effector espF (*ΔespF*), a strain that does not typically cause mucosal damage in wildtype mice. These findings indicate that the observed erosions and epithelial damage in calcitriol-treated mice are most likely due to host driven changes in the epithelial response to *C. rodentium* challenge, since *C. rodentium* typically requires EspF to induce mucosal pathology.

The intestinal surface is lined by a single layer of epithelial cells, which function as a barrier to separate the bacteria rich lumen from underlying host cells. Previous work has shown that vitamin D plays an important role in maintaining barrier integrity in the intestine (193, 214, 219, 222), however the role of calcitriol on barrier integrity is less clear. Our results show that calcitriol-treated mice develop worsened intestinal epithelial barrier dysfunction, compared to vehicle-treated mice during infection with *C. rodentium*, as determined by the FITC-dextran assay. Correspondingly, calcitriol-treated mice suffered from increased bacterial translocation out of the GI tract and to the MLN, spleen and liver at day 6 and day 10 pi, compared to vehicle-treated mice. We also found that calcitriol-treated mice had altered epithelial responses at day 10 pi, including impaired cellular proliferation and increased cell death, as determined by Ki67 and TUNEL staining respectively. Our results are in agreement with previous work demonstrating that calcitriol can inhibit cell proliferation and induce apoptosis in human colonic epithelial cells *in vitro* (218). However, it is currently unknown if the defects in barrier integrity are primary or secondary to the immune-mediated effects of calcitriol.
Our current understanding of the potential for vitamin D levels to impact on host susceptibility to pathogens is limited. There is an established link between vitamin D deficiency and tuberculosis (12, 13) and viral respiratory infections (268, 269). Moreover, low dietary vitamin D intake (77 IU per day vs. 166 IU per day) has also been correlated with differences in fecal microbial composition— including elevated levels of *Bacteroides* in vitamin D deficient volunteers (270). However, the role of vitamin D during enteric infections is currently unclear. Recently, Edrington et al. (271) found that supplementing cattle with vitamin D3 had no effect of fecal shedding of *E. coli* 0157:H7, however the researchers also looked at seasonal variation and found that the proportion of cattle shedding *E. coli* 0157:H7 was higher in the summer months (16.7% in the summer vs. 6.7% in the winter, p = 0.08), which correlated with higher serum vitamin D levels. Together with our results, these findings raise the question of whether there may be an unexpected benefit to vitamin D deficiency, i.e. potentially the development of stronger Th17 responses against specific bacterial pathogens. Correspondingly, while vitamin D supplementation has been shown to protect against autoimmune /inflammatory conditions (89), it is currently unknown whether such immune suppression could increase the susceptibility of a host to specific types of infection. It is tempting to speculate that human migration away from the equator and to more northerly climes and its associated risk for vitamin D insufficiency may have yielded both beneficial as well as detrimental effects on our immune system, leaving individuals at greater risk of autoimmune disease, but more resistant to enteric infections. Future research is necessary to answer these questions and to help clarify the role of vitamin D in maintaining mucosal homeostasis during enteric infections and other inflammatory challenges. Together, these studies show that calcitriol is a potent yet selective immunosuppressive agent, and as such, calcitriol treatment may protect against Th17 T-cell driven damage during
experimental and potentially, clinical colitis. However caution should be advised, since Th17 responses are also critical in providing host defense against extracellular bacteria, including those pathogenic strains of *E. coli* implicated in the pathophysiology of IBD.
Chapter 4: Dietary vitamin D3 alters intestinal mucosal defense and increases susceptibility to an enteric bacterial pathogen

4.1 Introduction

It is being increasingly recognized that vitamin D plays an important role in host defense against pathogenic microbes. Vitamin D deficiency affects more than 1 billion people worldwide and is associated with an increased risk of bacterial infection, including *Mycobacterium tuberculosis* (12, 13), *Pseudomonas aeruginosa* (10, 245) and *Clostridium difficile* (14, 15). Furthermore, vitamin D deficiency is associated with an increased risk of systemic bacterial infection leading to sepsis (272). Animal models of vitamin D deficiency, including the *Vdr*-/− mice also have increased susceptibility to infection and carry higher bacterial burdens during challenge with *Salmonella typhimurium* (215). However, the mechanisms and pathways through which vitamin D affects a host’s susceptibility to these infections are poorly understood.

The impact of vitamin D on host defense against *C. rodentium* is also poorly understood. We previously found that treatment with the active form of vitamin D; calcitriol can increase the susceptibility of mice to *C. rodentium* by suppressing key inflammatory factors required for bacterial clearance (273). This is in keeping with several studies showing that calcitriol can inhibit inflammatory responses in the host through a number of mechanisms, including skewing T-cell responses toward a regulatory phenotype (240, 255). Moreover calcitriol acts directly on T-cells to inhibit proliferation and production of inflammatory cytokines, including IL-2, IFN-γ,
TNF-α and IL-17 (89). As such, calcitriol can be considered a potent immunosuppressive agent, and may inadvertently impair host defenses against enteric microbes.

Correspondingly, studies suggest that loss of vitamin D signalling promotes exaggerated intestinal inflammatory responses. Mice fed a vitamin D3 deficient diet and mice deficient in the VDR gene have both been shown to exhibit a higher baseline colonic inflammatory tone, as well as elevated serum levels of IL-6 (215, 221). Published studies that have examined the impact of vitamin D deficiency during _C. rodentium_ infection have shown an exaggerated colitic phenotype (221, 274). Chen et al. (274) showed that _Vdr^-/-_ mice are resistant to colonization with _C. rodentium_, but their resistance appeared to depend on a dysbiotic microbiota rather than a direct effect of VDR deficiency. Assa et al. (221) did not directly examine pathogen burdens but focused on the impact of a vitamin D3 deficient diet on epithelial barrier function. To complement these studies, we sought to test whether a vitamin D3 deficient diet would be beneficial or detrimental to intestinal host defense, as well as host specific inflammatory/anti-microbial factors that during _C. rodentium_ infection are regulated by vitamin D.

We fed C57Bl/6 mice either a vitamin D3 deficient or sufficient diet for 5 weeks and challenged them with _C. rodentium_. Interestingly, despite having a higher baseline inflammatory tone in their intestines, vitamin D3 deficient mice carried significantly higher pathogen burdens in the ceca and in the mesenteric lymph nodes (MLN), spleen and liver at day 10-post infection, indicating greater susceptibility to pathogen translocation. In accordance with their inflammatory immune responses, intestinal tissues of infected vitamin D3 deficient mice showed significantly higher gene transcription levels of inflammatory mediators as well as the antimicrobial peptide
Moreover, the vitamin D3 deficient mice showed defects in the ability to detoxify bacterial LPS and carried higher serum levels of CD14. Overall, these findings show that dietary-induced vitamin D deficiency alters host mucosal defense and increases susceptibility to an enteric bacterial pathogen.

4.2 Results

Supplemental Figures for Chapter 4 can be found in Appendix B.

4.2.1 Vitamin D3 deficient mice are more susceptible to *Citrobacter rodentium* infection, carrying higher cecal and extra-intestinal pathogen burdens

To determine the role of vitamin D3 during enteric infection, we first fed weanling mice either a vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks. There was no difference in food intake or body weight between the two groups during the 5-week feeding trial (Supplemental Figure B.1). Dietary vitamin D3 is converted in the liver into 25(OH)D3, which is the major circulating form of vitamin D in the body and used to assess vitamin D status. After the 5-weeks, vitamin D3 deficient mice had significantly lower levels of serum 25(OH)D3, compared to vitamin D3 sufficient mice (Figure 4.1A), in agreement with previous studies (194, 221). Vitamin D also plays an important role in regulating calcium metabolism in the body, and supplemental dietary calcium has previously been shown to protect against *C. rodentium* infection (262, 275), but we found no difference in serum calcium between the diet groups (Figure 4.1B), similar to findings by Lagishetty et al. (194), whose feeding protocol (including diet manufacturer) we replicated. After challenge with *C. rodentium*, vitamin D3 deficient mice lost 5-8% of their body weight by day 2 pi, significantly greater than vitamin D3 sufficient mice,
with this greater weight loss maintained until the mice were euthanized at day 10 pi (Figure 4.1C). Vitamin D3 deficient mice had thicker colons and shrunken ceca, which were often devoid of stool contents when compared to infected vitamin D3 sufficient mice (Figure 4.1D).

**Figure 4.1 Vitamin D3 deficient mice lose more body weight during infection with *Citrobacter rodentium* and have thickened colon and shrunken ceca at day 10-post infection**

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium* for 10 days. A) Serum 25(OH)D3. Results are representative of 3 independent experiments, n = 5 - 6 per group, * p < 0.05, Mann-Whitney test. B) Serum Calcium. Results are representative of 3 independent experiments, n = 6 - 8 per group. C) Body weight. Each data point represents the average body weight pooled from 8 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 3 independent experiments, n = 8 per group, Student’s t-test was conducted at each time point, * p < 0.05, Mann-Whitney test. D) Macroscopic images of lower gastrointestinal tract (cecum + colon) taken at day 10 pi are representative of group phenotype. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection
To determine pathogen burdens, tissues were homogenized and plated to quantify *C. rodentium*. While no significant differences were found regarding pathogen burdens in the colon between the groups at day 10 pi, vitamin D3 deficient mice were found to carry 5-10 fold higher *C. rodentium* burdens in their ceca (cecal tissue + contents) than the vitamin D3 sufficient mice (Figure 4.2A). Interestingly, vitamin D3 deficient mice also carried significantly more culturable *C. rodentium* from extra-intestinal tissues, including MLN, spleen and liver compared to vitamin
D3 sufficient mice at day 10 pi, indicating greater bacterial translocation to these systemic sites with vitamin D deficiency (Figure 4.2B).

To determine if the exaggerated pathogen translocation in vitamin D deficiency reflected increased disruption of intestinal barrier integrity, mice were administered FITC-dextran through oral or intra-rectal routes as measures of proximal or distal intestine, respectively, and their plasma was collected and assessed for translocated FITC-dextran. We found no significant differences in translocated FITC-dextran levels between vitamin D3 deficient and sufficient mice at day 6 pi (Supplemental Figure B.2) or day 10 pi (Figure 4.2C), indicating the exaggerated bacterial translocation may not reflect an overt epithelial barrier defect. Overall these findings indicate that vitamin D3 deficient mice are more susceptible to infection with *C. rodentium*.

**Figure 4.2 Vitamin D3 deficient mice are more susceptible to *Citrobacter rodentium* infection and carry higher bacteria burdens in ceca and extra-intestinal tissues at day 10-post infection**

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium* for 10 days. Whole tissues were homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens. A) Cecum and Colon at Day 10 pi. Results are representative of 3 independent experiments, n = 7-8 per group, **p < 0.01, Mann-Whitney test. B) Spleen, Liver and MLN at Day 10 pi. Results are representative of 3 independent experiments, n = 8 per group, *p < 0.05, Mann-Whitney test. C) To assess intestinal barrier integrity, FITC-dextran was administered orally or intra-rectally and plasma was assessed for levels of translocated FITC-dextran at day 10 pi. Results are representative of 2 independent experiments, n = 7-9 per group. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection; NS = non significant
4.2.2 Vitamin D3 deficient mice carry heavier Citrobacter rodentium burdens in their cecal contents at day 10-post infection

To explore the basis for the greater *C. rodentium* burdens seen in vitamin D3 deficient mice, we carefully separated colon and cecal tissues from their luminal contents and quantified the...
pathogen burdens. Vitamin D3 deficient mice carried significantly more *C. rodentium* in their cecal contents, whereas there was no significant difference in cecal tissue burdens between groups (Figure 4.3A). These findings indicated that the increased *C. rodentium* burdens seen in the ceca of vitamin D3 mice were not adherent to the tissue, but rather residing in either cecal crypts or in the lumen. No differences were found in *C. rodentium* numbers in the colon tissue or colon contents between diet groups at day 10 pi (Figure 4.3B). To determine if vitamin D3 deficient mice showed any impairment in clearing *C. rodentium* infection, we assessed bacterial burdens at later time points. At day 18 pi, vitamin D3 deficient mice showed a trend for higher *C. rodentium* burdens in both the colon and cecum compared to vitamin D3 sufficient mice (Figure 4.3C), however, the burdens in both groups were still 3-fold lower than that seen at day 10 pi (height of infection), suggesting that pathogen clearance was still occurring.

**Figure 4.3** Vitamin D3 deficient mice carry higher *Citrobacter rodentium* burdens in the cecal contents at infection at day 10-post infection and show delayed clearance of pathogen

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Cecal tissues were separated from their contents, homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens at day 10 pi. Results are representative of 3 independent experiments, n = 7 - 9 per group, * p < 0.05, Mann-Whitney test. B) Colonic tissues were separated from their contents, homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens at day 10 pi. Results are representative of 3 independent experiments, n = 7 - 9 per group. C) Whole cecal and colonic tissues were homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens at day 18 pi. Results are from 1 experiment, n = 4 per group. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection
4.2.3 Vitamin D3 deficient mice display exaggerated cecitis at day 10-post infection with *Citrobacter rodentium*

At day 10 pi, the ceca of vitamin D3 deficient mice displayed worsened histological damage, with significantly more submucosal edema and crypt hyperplasia compared to vitamin D3 sufficient mice, whose ceca had modest damage, as typical for a wildtype C57Bl/6 mouse at day 10 pi (Figure 4.4A, Figure 4.4B). We also examined Swiss-rolled sections of the colon for histological damage. Although we found a trend for worsened *C. rodentium*-induced damage in
the distal colon of vitamin D3 deficient mice at day 10 pi, the differences between dietary groups did not reach statistical significance (Supplemental Figure B.3A). Furthermore, there was no difference in histological scores between dietary groups under uninfected conditions in the colon or cecum (Supplemental Figure B.3B). Since we found higher pathogen burdens and greater tissue damage in the ceca of vitamin D3 deficient mice, we focused our additional analysis on cecal tissues.

To further characterize the mucosal pathology and responses to infection, epithelial cell proliferation in the cecum was determined by Ki67 staining. At day 10 pi, vitamin D3 deficiency mice displayed significantly more Ki67+ve intestinal epithelial cells when compared to vitamin D3 sufficient infected mice (Figure 4.4C and Figure 4.4D). These results are in agreement with previous work showing that active vitamin D (calcitriol) can inhibit cell proliferation in human colonic epithelial cells in vitro (218). However, these changes were only seen during infection, as there were no overt differences in epithelial cell proliferation between uninfected groups (Figure 4.4D).

**Figure 4.4** Vitamin D3 deficient mice suffer worsened histological damage with increased cell proliferation in the ceca at day 10-post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Representative image of cross section of the cecum at day 10 pi. Original magnification = 50X for top panels, 200X for lower panels, Scale bar = 200 µm. B) Cecum was assessed for histological damage by scoring system for *C. rodentium* described in Methods. Results are representative of 3 independent experiments, n = 8 per group, * p < 0.05, Mann-Whitney test. C) Representative images of formalin fixed cross section of cecum at day 10 pi. Immunofluorescence stained: Blue = DAPI; Red = Ki67. Original magnification = 50X, Scale bar = 200 µm. D) Cecum was assessed for number of Ki67+ve cells in the lumen (percent of +ve Ki67 per +ve DAPI per area measured). Results are representative of 3 independent experiments, n = 4 per group (uninfected) and n = 11 per group at Day 10 pi, ** p < 0.01, Mann-Whitney test.
4.2.4 Vitamin D3 deficient mice develop an elevated inflammatory tone in the cecum under both uninfected and infected conditions

To determine if the increased susceptibility of vitamin D3 deficient mice to *C. rodentium* infection could reflect an altered host immune response, we assessed cecal tissues for inflammatory cells and mediators. While no differences in macrophage numbers were noted in the cecum of vitamin D3 deficient or sufficient groups under uninfected conditions, at day 10 pi,
vitamin D3 deficient mice showed more infiltrating macrophages in their cecal tissues, particularly in the submucosal regions, compared to vitamin D3 sufficient mice (Figure 4.5).

Figure 4.5 Vitamin D3 deficient mice have more macrophages in ceca at day 10-post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium* for 10 days. A) Representative image of cross section of the cecum during uninfected condition and day 10 pi. Immunofluorescence stained: Blue = DAPI; Red = F4/80. Original magnification = 200X. B) Cecum was assessed for number of F480+ve cells in the lumen (fluorescence intensity of F480 relative to DAPI was measured). Results are representative of 2 independent experiments, n = 4 - 7 per group, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection

We next assessed cecal tissues for the transcription of genes encoding cytokines that influence susceptibility to *C. rodentium* infection. Interestingly, vitamin D3 deficient mice showed higher transcript levels for the acute inflammatory cytokines TNF-α, IL-1β and IL-6 under both
uninfected and day 10 pi conditions (Figure 4.6), indicating a higher baseline inflammatory tone as well as a stronger inflammatory response to *C. rodentium* infection. As expected, infection also induced an increase in transcript levels for Th17-related cytokines (IL-17A, IL-17F) in the ceca, similar to findings previously described in the infected colon (85, 126). However the infection induced increase in IL-17A and IL-17F transcripts was significantly greater in vitamin D3 deficient mice as compared to vitamin D3 sufficient mice (Figure 4.7A). Interestingly, the vitamin D3 deficient mice also showed higher transcript levels of the anti-inflammatory cytokines IL-10 and TGF-β (Figure 4.7B), suggesting an attempt to counteract the increased inflammatory tone.

**Figure 4.6 Vitamin D3 deficient mice have higher cecal expression of tumor necrosis factor-α, interleukin-1β and, interleukin-6**

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. Expression of TNF-α, IL-1β and IL-6 in cecum during uninfected conditions and day 10 pi as assessed by RT qPCR. Results are representative of 3 independent experiments, n = 4 - 12 per group, * p < 0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection
Figure 4.7 Vitamin D3 deficient mice have higher cecal expression of interleukin-17A, interleukin-17F and, transforming growth factor-β

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with C. rodentium. Expression of IL-17A, IL-17F, IL-10 and TGF-β in cecum during uninfected conditions and day 10 pi as assessed by RT qPCR. Results are representative of 3 independent experiments, n = 4 - 10 per group, * p < 0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = C. rodentium; D10 = Day 10; pi = post infection
4.2.5 Vitamin D3 deficiency alters commensal bacteria at baseline and during infection with *Citrobacter rodentium*

Considering the higher luminal and systemic pathogen burdens carried by infected vitamin D3 deficient mice, we next examined whether their increased susceptibility could be attributed to differences in their commensal intestinal microbiota. It has recently been shown that vitamin D3 deficient mice have an altered fecal microbiome composition (221), while mice with altered vitamin D signalling; including *Vdr*-/- mice and *Cyp27b1*-/- mice have been described as suffering intestinal microbial dysbiosis (276). Assessing the microbiota within the stool (using Sybr Green stain) of our two dietary groups under uninfected and infected conditions, we found no difference in the number of commensal bacteria/gram between the dietary groups under uninfected conditions (Figure 4.8A). Interestingly, vitamin D3 deficient mice carried significantly more segmented filamentous bacteria (SFB) in their stool at day 0, compared to vitamin D3 sufficient mice (Figure 4.8B), as determined by qPCR. In contrast, we found no significant differences in *Bacteroidetes* or *γ-Proteobacteria* in the stool between dietary groups during uninfected conditions (Figure 4.8B), and although there was a trend (*p* = 0.0850) for vitamin D3 deficient mice to carry fewer *Firmicutes*, it did not reach statistical significance.

Previous studies have shown that *C. rodentium* infection is associated with a host driven depletion of commensal microbes that potentially aids pathogen colonization by reducing colonization resistance (129). We assessed commensal microbe numbers over the course of infection and found a modest but significant acceleration in commensal loss in the vitamin D3 deficient mice by day 6 pi, in keeping with the increased inflammatory response seen in these
mice (Figure 4.8C). The makeup of the cecal microbiota at day 10 pi was also assessed, (Figure 4.8D) with vitamin D3 deficient mice found to carry significantly higher levels of *Actinobacteria* and *Bifidobacteria*, compared to vitamin D3 sufficient mice (Figure 4.8E). There was also a trend for higher levels of γ-proteobacteria and SFB in the cecal contents of vitamin D3 deficient mice at day 10 pi (Figure 4.8E), but no significant differences in the major bacteria phyla *Firmicutes* or *Bacteroides* were found between dietary groups at day 10 pi (Figure 4.8D, Figure 4.8E).

**Figure 4.8** Vitamin D3 deficient mice have more segmented filamentous bacteria in stool at baseline conditions and have a more dramatic drop in commensal bacteria at day 6-post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Total commensals were assessed in stool during uninfected conditions by DAPI Stain. Results are representative of 2 independent experiments, n = 8 per group. B) Microbial composition of stool was analyzed by qPCR. Results are representative of 2 independent experiments, n = 6 - 7 per group, * p < 0.05, Mann-Whitney test. C) % Commensal bacteria in stool relative to baseline levels were assessed at day 2 pi and day 6 pi. Results are representative of 2 independent experiments, n = 6 - 7 per group, *** p < 0.0001, Mann-Whitney test. D) Makeup of cecal microbiota at day 10 pi was assessed by qPCR. Each bar represents 1 sample. E) Makeup of cecal microbiota at day 10 pi was assessed by qPCR. Results are representative of 2 independent experiments, n = 5 - 9 per group, * p < 0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection; SFB = segmented filamentous bacteria
4.2.6 Vitamin D3 deficient mice have higher expression of the antimicrobial peptide RegIIIγ at day 10-post infection with *Citrobacter rodentium*

To understand a potential basis for the altered commensal microbes seen in the vitamin D3 deficient group, as well as explore why higher numbers of *C. rodentium* would be only found within their cecal lumen as opposed to being tissue adherent, we assessed gene transcripts for
several antimicrobial factors including RegIIIγ, which has been shown to play a role in mucosal repair and host defense during infection with *C. rodentium* (85). Moreover in our previous studies, supplementation with the active form of vitamin D, calcitriol suppressed transcription of RegIIIγ, thereby impacting on the susceptibility of mice to *C. rodentium* infection (273). Notably, RegIIIγ transcription was dramatically elevated in vitamin D3 deficient mice (Figure 4.9), supporting the belief that it is regulated by vitamin D3 and suggesting it may be protecting the cecal mucosa from exaggerated *C. rodentium* colonization. In contrast, no difference between diet groups was detected for transcript levels of other antimicrobial genes that encode beta-defensin 1, beta-defensin 3 or mcramp at day 10 pi (Supplemental Figure B.4).

**Figure 4.9 Vitamin D3 deficient mice have higher cecal expression of RegIIIγ at day 10-post infection with *Citrobacter rodentium***

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. Expression of RegIIIγ in cecum during uninfected conditions and day 10 pi as assessed by RT qPCR. Results are representative of 2 independent experiments, n = 4 - 7 per group, * p<0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection
4.2.7 Bacterial lipopolysaccharide dephosphorylation is impaired in vitamin D3 deficient mice at day 10-post infection with Citrobacter rodentium

Lastly, we explored why the intestinal inflammatory response was exaggerated in vitamin D3 deficient mice. We previously showed that the inflammatory response during C. rodentium infection is largely LPS dependent, as it is significantly reduced in TLR4 deficient mice (277). We therefore examined if vitamin D3 deficient mice have altered responses to bacterial LPS. Many of the immune activating abilities of LPS can be attributed to the lipid A unit, which contains two phosphate groups coupled to glucosamines. Removal of one of the phosphate groups generates a monophosphoryl lipid A that is a 100-fold less toxic than the unmodified lipid A (278). Using the malachite green assay to measure LPS dephosphorylation, we found LPS activity significantly increased in vitamin D3 sufficient mice during infection both in the duodenum and ileum (Figure 3.8A), likely as a means to limit inflammatory responses against C. rodentium. In contrast, this increase in LPS activity did not occur in vitamin D3 deficient mice during challenge with C. rodentium (Figure 3.8B), indicating that vitamin D3 deficient mice may possess reduced LPS dephosphorylating activity during infection.

4.2.8 Vitamin D3 deficient mice have higher levels of serum CD14 at day 10-post infection with Citrobacter rodentium

In keeping with the assessment of responses against bacterial LPS, we examined serum levels of cluster of differentiation 14 (CD14), a pattern recognition receptor responsible for the detection of several bacterial products including LPS (59). CD14 is found in two forms, membrane bound CD14 (mCD14) on the surface of monocytes, macrophages and neutrophils (59), and soluble
CD14 (sCD14), which is secreted into bodily fluids including tears, blood and breast milk (60). While we noted no difference in serum CD14 levels between uninfected groups, at day 10 pi however, vitamin D3 deficient mice carried significantly higher levels of serum CD14, compared to vitamin D3 sufficient mice (Figure 3.8B), confirming that in the absence of vitamin D3, responses against bacterial LPS are exaggerated.

**Figure 4.10 Vitamin D3 deficient mice have impaired dephosphorylation of bacterial lipopolysaccharide and higher serum levels of CD14 at day 10-post infection with *Citrobacter rodentium***

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Relative LPS dephosphorylating activity in duodenum and ileum at Day 10 pi as determined by the malachite green assay, which measures free phosphate release. Results are representative of 2 independent experiments, n = 6 per group, ** p<0.0043, Mann-Whitney test. B) Serum CD14 during uninfected conditions and at day 10 pi. Results are representative of 3 independent experiments, n = 7 - 8 per group, * p < 0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection; LPS = lipopolysaccharide
4.3 Discussion

Vitamin D has been shown to modulate a wide variety of immune responses; however its potential to impact host defense against pathogens within the GI tract is poorly understood. The most significant impact of vitamin D3 in our study was its effect on the host inflammatory response within the intestine. Even under uninfected conditions, vitamin D3 deficient mice showed a higher intestinal inflammatory tone, with elevated cecal expression of TNF-α, IL-1β, IL-6 and IL17A gene transcripts. Similarly, Assa et al. (221) has shown that vitamin D3 deficient mice had elevated expression of IL-17A and IL-17F in the distal colon compared to vitamin D3 sufficient mice. Moreover infection with *C. rodentium* was associated with an appropriate, but exaggerated response with a further rise in cecal transcript levels of TNF-α, IL-1β, IL-6, IL17A and IL17F with levels that were significantly elevated above vitamin D3 sufficient infected mice. It has previously been shown that TNF-α, IL-1β and IL-6 play an important role in controlling *C. rodentium* burdens in the gut and preventing tissue injury during infection (126-128). However, excess TNF-α and IL-1β are also known to promote tissue injury during infection with *C. rodentium* (127, 128). Indeed, along with the heightened inflammatory response, vitamin D3 deficient mice also suffered worsened cecal histological damage at day 10 pi, compared to vitamin D3 sufficient mice. Overall, our results demonstrate that vitamin D3 deficiency results in dysregulated inflammatory responses in the ceca during bacteria challenge.

The basis for this exaggerated inflammatory response was uncertain however the elevated levels of TNF-α, IL-1β and IL-6, even under baseline conditions suggested the involvement of innate signalling. Bacterial LPS a major component of the cell wall of Gram-negative bacteria is
recognized by the host innate receptor TLR4, in combination with CD14, MD-2 and the LPS binding protein, found on the surface of monocytes, macrophages and intestinal epithelial cells. Activation of TLR4 and its co-factors by bacterial LPS induce a signalling cascade that leads to the production of inflammatory mediators and localized recruitment of inflammatory cells. Considering that the active form of vitamin D, calcitriol has previously been shown to inhibit LPS-induced inflammatory responses in the host through a number of mechanisms, including suppressing TLR4 expression, blocking NF-κB signalling and targeting MAPK phosphatase-1 (224, 226), we decided to examine whether vitamin D3 deficient mice show exaggerated responses to LPS.

In the current study, we found that vitamin D3 deficient mice showed defects in their ability to dephosphorylate bacterial LPS, compared to vitamin D3 sufficient mice, at day 10 pi, as determined by the malachite green assay. LPS dephosphorylation can occur by intestinal alkaline phosphatase (IAP)- a small intestinal brush border enzyme that plays a critical role in host defense and maintaining intestinal homeostasis. IAP has been shown to reduce intestinal inflammation and limit bacterial translocation into systemic sites (35). Interestingly, although IAP is secreted mainly in the duodenum, it has been shown to retain its activity throughout the small intestine and colon (279) and increased IAP activity has been observed in the colon during infection with \textit{C. rodentium} (254). A previous study showed that vitamin D deficient rats exhibited lower total alkaline phosphatase activity in their duodenum, compared to vitamin D sufficient rats (280). Furthermore, calcitriol has been shown to stimulate the activity of IAP in
the duodenum of chicks (281). Overall these findings indicate that vitamin D may play a role in detoxification of LPS and future studies should investigate IAP.

Along with impaired LPS dephosphorylation, we also noted an increase in levels of CD14, a pattern recognition receptor responsible for the detection of several bacterial products including LPS (59). Soluble CD14 (sCD14) is secreted into bodily fluids and in combination with TLR4; detection of LPS by CD14 can result in a pro-inflammatory immune response (60). However, soluble CD14 may also decrease immune responses to LPS by binding LPS and keeping it from mCD14-expressing cells and providing clearance of LPS through the liver (60). Interestingly, the active form of vitamin D, calcitriol has been shown to increase the expression of CD14 in human monocytes, while suppressing the expression of TLR4 (224, 228). Furthermore, a vitamin D derivative 1α,25-dihydroxy-22-oxavitamin D3 (Oxa-D3) has been shown to increase the release of soluble CD14 from intestinal HT-29 cells through ERK1/2 activation, in vitro (282). While we noted no difference in serum CD14 levels between uninfected groups, at day 10 pi vitamin D3 deficient mice carried significantly higher levels of serum CD14, compared to vitamin D3 sufficient mice, confirming that in the absence of vitamin D3, responses against bacterial LPS are exaggerated.

Despite their heightened baseline inflammatory tone, vitamin D3 deficient mice proved more susceptible to *C. rodentium* infection, carrying higher bacterial burdens both in the cecal lumen and at systemic sites. Interestingly, although several previous studies have found that vitamin D can help maintain the intestinal epithelial barrier (193, 219, 221, 222), we did not find any
difference in barrier integrity (FITC-dextran assay) between dietary groups at either day 6 pi or day 10 pi, suggesting the translocation to systemic sites was not due to an overtly weaker gut barrier. Although an overt alteration in epithelial barrier integrity was not observed in the vitamin D3 deficient mice, bacteria can enter the systemic circulation through several routes including through intestinal-epithelial microfold cells (M-cells), which are permeable to bacteria as well as macromolecules (283). More likely the increased systemic spread of C. rodentium reflects the impaired ability of vitamin D3 deficient mice to dephosphorylate LPS, permitting greater C. rodentium translocation out of the gut. With respect to their increased luminal burdens, previous studies showed that Vdr-/- mice carried higher pathogen burdens in their ceca during infection with Salmonella typhimurium (215), although the basis for this was not defined. A potential explanation reflects the concept that pathogens such as C. rodentium actually benefit from intestinal inflammation, since it helps deplete competing commensal bacteria, creating a niche within the intestine where C. rodentium can colonize and proliferate. Furthermore, studies have shown that enteric bacterial pathogens can utilize nutrients and metabolites released within the inflamed intestine that are not used by commensal species (99, 284). We recently demonstrated that mice deficient in SIGIRR, a negative regulator of innate signalling not only developed greater inflammatory/antimicrobial responses during C. rodentium infection, but they also proved more susceptible to infection, carrying much higher C. rodentium burdens than wildtype mice (129). Notably, in the current study, we found no difference in the total number of commensal bacteria/gram between the dietary groups under uninfected conditions or at day 2 pi. However, by day 6 pi, vitamin D3 deficient mice showed a significant drop in commensal microbe populations, in keeping with the increased inflammatory response seen in these mice. Moreover we found an exaggerated induction of the gene encoding the antimicrobial factor
RegIIIγ in the vitamin D3 deficient mice. However, we have also shown in previous studies that calcitriol treatment in vivo can increase susceptibility to C. rodentium infection by suppressing Th17-mediated immune responses, as well as RegIIIγ transcription, leading to increased tissue adherent pathogen burdens (273). Thus our studies show RegIIIγ is clearly dependent on vitamin D3 and its upregulated expression in vitamin D3 deficient mice might explain the accelerated loss of commensal microbes in these mice, as well as selective increase in C. rodentium burdens in the cecal lumen, rather than at the mucosal surface where RegIIIγ is expressed.

While the increased inflammatory tone seen in the uninfected intestines of vitamin D3 deficient mice did not affect total commensal numbers, vitamin D3 deficient mice were found to carry more segmented filamentous bacteria (SFB) in their gut contents under both uninfected and infected conditions, compared to vitamin D3 sufficient mice. SFB can induce the differentiation of Th17 cells in the small intestine and can also potentially protect hosts against extracellular bacterial infections, or promote inflammatory diseases (104). It is notable that by simply altering dietary vitamin D levels, we were able to alter SFB populations in the gut and change Th17 response. As for other commensals, we found no significant differences in Bacteroidetes or γ-Proteobacteria in the stool between dietary groups during uninfected conditions, and although there was a trend for vitamin D3 deficient mice to carry fewer Firmicutes, it did not reach statistical significance. At day 10 pi, vitamin D3 deficient mice had significantly higher levels of Actinobacteria as well as γ-Proteobacteria in the stool, in agreement with findings from Assa et al. (221). Interestingly, infected vitamin D3 deficient mice also carried significantly higher levels
of *Bifidobacterium*, compared to vitamin D3 sufficient mice, however we found no differences in the major bacteria phyla- *Firmicutes* or *Bacteroides* between dietary groups at day 10 pi, similar to findings by Assa et al. (221).

There is an established link between vitamin D deficiency and respiratory infections (12) and systemic infections (272). However our understanding of the potential for vitamin D levels to impact host susceptibility to GI infections is limited. Our current study demonstrates that a dietary induced vitamin D3 deficiency increases bacterial overgrowth during infection with the enteric pathogen *C. rodentium* by altering host factors required for bacterial detoxification, indicating that vitamin D plays a protective role during enteric infection. However, these results should be interpreted with caution, since we have previously shown that treating *C. rodentium* infected mice with active vitamin D, calcitriol led to increased pathogen burdens and exaggerated tissue pathology. In association with their increased susceptibility, calcitriol-treated mice showed less expression of IL-6 and IL-17A, and substantially reduced numbers of Th17 T cells within their infected colons. Th17 responses play a protective role during *C. rodentium* infection (84, 124, 125). Therefore, too much active vitamin D can suppress Th17-mediated inflammatory responses, which can impair host defense against *C. rodentium*. To summarize- during calcitriol supplementation- there is suppression of Th17 mediated immune responses that are normally important for clearing *C. rodentium* infection. In contrast- during vitamin D deficiency, there is a higher baseline inflammatory tone and higher antimicrobial peptides, which leads to a faster loss in commensal flora, reducing commensal microbial competition with *C. rodentium*, and allowing the pathogen to overgrow and cause increased intestinal damage.
Overall these results suggest that to promote gut health, there is likely an optimal range of vitamin D and that too little or too much of this vitamin may promote gut inflammation and/or increase susceptibility to enteric infections.
Chapter 5: Discussion

It is well established that vitamin D deficiency is associated with an increased risk of inflammatory mediated diseases, including multiple sclerosis, rheumatoid arthritis, diabetes, colon cancer, bacterial infections, sepsis and IBD (7). In agreement, we found that dietary induced vitamin D3 deficient mice had a higher intestinal inflammatory tone and were significantly more susceptible to infection with *C. rodentium* -showing higher pathogen burdens and worsened histological damage, compared to vitamin D3 sufficient mice. The VDR is expressed on most immune cells throughout the body, including dendritic cells, macrophages and activated T-cells. Calcitriol signalling through the VDR can inhibit inflammatory responses through a number of mechanisms, including blocking NF-κB signalling (225) and directly suppressing Th1/Th17 responses, including production of the inflammatory cytokines IL-2, IFN-γ, TNF-α and IL-17 (89, 240, 255). However, the impact of vitamin D supplementation in ameliorating inflammation and modulating host responses to infection is uncertain. We were the first to show that treating mice with active vitamin D, calcitriol can increase susceptibility to an enteric bacterial infection, which was associated with suppressed Th17 mediated immune responses. These findings raise important concerns for the therapeutic use of vitamin D for patients with IBD, who suffer from overactive inflammation, but are also at increased risk for bacterial infections. Furthermore, several questions arise from this work; including the role that vitamin D plays during infection with pathogenic strains of *E. coli*, how does vitamin D status affect barrier integrity and how does vitamin D metabolism alter commensal bacteria? These questions will be discussed in the following sections.
5.1 Vitamin D and pathogenic *Escherichia coli*

The effect of vitamin D deficiency on susceptibility to enteric infection with the A/E pathogens EPEC or EHEC is currently unknown. Our data, as well as recent findings from Assa et al. (221) indicate that dietary induced vitamin D deficiency can increase susceptibility to infection with the A/E pathogen *C. rodentium*. During infection, vitamin D3 deficient mice lost significantly more body weight, carried higher *C. rodentium* burdens, and developed worsened histological damage. Intestinal tissues of infected vitamin D3 deficient mice displayed increased inflammatory cell infiltrates as well as significantly higher gene transcript levels of the inflammatory mediators TNF-α, IL-1β, IL-6, IL-17A, IL-17F and the antimicrobial peptide RegIIIγ. Furthermore, our data found that these exaggerated inflammatory responses occurred in concert with the accelerated loss of commensal microbes and were associated with an impaired ability to detoxify bacterial LPS. Surprisingly, Chen et al. (274) found that *Vdr*-/- mice were resistant to infection with *C. rodentium*, with lower pathogen burdens in stool, reduced inflammatory cell infiltrates in the lamina propria, including fewer monocytes, neutrophils and CD3+ T-cells, and impaired colonic expression of inflammatory cytokines IFN-γ, IL-6 and IL-17 at day 10 pi, compared to wildtype mice (274). However, disruption of commensal microflora with the antibiotic vancomycin reversed this phenotype, and the *Vdr*-/- mice became extremely susceptible to *C. rodentium*, suffering higher pathogen burdens and higher rates of mortality (274). Recently it was shown that vitamin D-deficient C57BL/6 mice given a course of 2% DSS exhibited pronounced epithelial barrier dysfunction and were more susceptible to AIEC colonization, and showed exacerbated colonic injury, compared to vitamin D sufficient mice (221). These findings may have important implications for patients with Crohn’s disease, since they suffer from barrier dysfunction and often carry high numbers of AIEC (166).
Vitamin D may play a role during infection with the *E. coli* pathotype uropathogenic *Escherichia coli* (UPEC), the primary cause of urinary tract infections (UTIs), which are among the most common infectious diseases of humans (285). It is estimated that 40-50% of women and 5% of men will develop a UTI in their lifetime (285). While UTIs are typically considered extracellular infections, UPEC can invade and replicate within epithelial cells, suggesting that this bacterial pathogen may occupy an intracellular niche within the host (286). In a retrospective study, vitamin D deficiency was associated with recurrent UTIs in premenopausal women (287). The mechanism(s) that link vitamin D deficiency with recurrent UTIs are unknown. Infections of the urinary tract induce epithelial cells to produce cathelicidin LL-37, which is known to protect against bacterial infection (288). Vitamin D is a potent stimulator of antimicrobial peptides including cathelicidin LL-37 (289). Recently, Hertting et al. (290) observed a significant increase in cathelicidin in response to vitamin D in biopsy samples taken from the bladders of patients infected by UPEC. These results indicate the vitamin D deficiency can increase susceptibility to pathogenic strains of *E. coli*; however further research is required to determine the role of vitamin D during EHEC/EPEC infections.

The role of vitamin D supplementation during enteric infection with EPEC or EHEC is also unknown. Recently, Edrington et al. (271) found that supplementing cattle with vitamin D3 had no effect on their level of fecal shedding of *E. coli 0157:H7*. However, EHEC infections in both cattle and humans follow a consistent seasonal pattern and occur much more commonly in the spring and summer months (271, 291), when sunlight exposure is highest. In southern Alberta, cattle carcasses were shown to have higher levels of *E. coli* in the summer months (n=591) compared to winter months (n = 686), although detection of EHEC O157:H7 did not differ by
season (292). Furthermore, sheep flocks were shown to shed higher amounts of EHEC 0157 in fecal samples during the summer months (36.8% farm prevalence), compared to the winter (10.4% farm prevalence) and spring (0% farm prevalence) over a 3-year study period (293). In the US, pediatric diarrhea rates also vary seasonally, with enteroaggregative *E. coli* (EAEC) infections seen more commonly during the spring (294). In Mexico, the rate of ETEC infection has been shown to increase by 7% for each degree centigrade increase in weekly ambient temperature, which is important, considering up to 60% of US visitors to Mexico develop travelers’ diarrhea from ETEC infection (295). In contrast, EPEC was identified in similar proportions during the winter and summer seasons in Mexico (295). The warmer and wetter summer months are generally associated with an increased occurrence of diarrhea (296). However, warmer climates may encourage propagation of enteric bacterial pathogens in food (297) and water (298) potentially explaining the increase in bacterial diarrhea during the summertime. We found that treating *C. rodentium* infected mice with active vitamin D; calcitriol led to increased pathogen burdens and exaggerated tissue pathology. In association with their increased susceptibility, calcitriol-treated mice showed substantially reduced numbers of Th17 T cells within their infected colons and defects in their production of the antimicrobial peptide RegIIIγ. These studies indicate that too much vitamin D may suppress inflammatory responses and increase the risk of enteric infection. Overall, research indicates that vitamin D balance is important for host defense against A/E pathogens.

5.2 Vitamin D, bacterial translocation and sepsis

Our results show that dietary induced vitamin D deficient mice had higher pathogen numbers recovered from their extraintestinal tissues, including MLN, spleen and liver at day 10 post *C.
rodentium infection, compared to vitamin D sufficient mice, indicating that vitamin D deficiency may increase susceptibility to events resembling sepsis. C. rodentium is typically a non-invasive luminal pathogen, however we found that vitamin D deficiency altered host defense, leading to defects in the ability to detoxify bacterial LPS, allowing the exaggerated and systemic spread of infection. Vitamin D deficiency is associated with an increased risk of systemic bacterial infection leading to sepsis in critically ill patients (272). A recent meta-analysis of observational cohort studies on vitamin D deficiency in the intensive care unit following 9,715 critically ill patients found that levels of 25(OH)D3 less than 50 nmol/L were associated with increased rates of infection, sepsis and mortality (299). Lower vitamin D levels are also associated with increased risk of early-onset neonatal sepsis in term infants (300). However, it has also been shown that children with sepsis showed significantly higher serum levels of 25(OH)D3, compared to control group (185 nmol/L vs. 70 nmol/L) (301). In North America, approximately 700,000 cases of sepsis occur each year, with mortality rates ranging between 30% and 50% (302). Sepsis is a deadly immunological disorder and its pathophysiology is still poorly understood. In response to infection, pro-inflammatory cytokines, including IL-6, IL-8, IL-18 and TNF-α are increased to help eliminate pathogens, however excessive production of these factors can lead to tissue and organ damage (303). There is emerging evidence that IL-17 may also play an adverse role in sepsis by causing exaggerated inflammatory responses (302). The most common underlying causes of sepsis are lung infections, intra-abdominal infections, and urinary tract infections. Etiologic organisms cover the spectrum of pathogens, with bacteria and fungi being predominant. However, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus species, Streptococcus pneumoniae, Pseudomonas aeruginosa, Klebsiella species, Candida species and E. coli account for most of the pathogens described (304, 305). E. coli is the
second leading cause of early-onset sepsis (EOS) in neonates, accounting for about 24% of all EOS episodes, with 81% of such cases occurring in preterm infants (306). When very low birth-weight infants are considered alone, \textit{E. coli} is the most frequent cause of EOS, accounting for 33.4% of episodes in a large, multicenter study (307). Coliforms, including \textit{E. coli}, are frequently colonizers of the maternal vaginal canal, and infants acquire them at or just before delivery. EOS secondary to \textit{E. coli} often presents with bacteremia with or without meningitis at the time of delivery. Septic shock with clinical features associated with endotoxemia may be present and mortality is high within the first 72 hours of infection (11). Recently it was shown, in critically ill patients with sepsis, that a single dose of calcitriol (2 µg intravenously) had no effect on plasma cathelicidin protein levels or plasma cytokine levels (IL-10, IL-6, tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IL-2) 24 h post administration (308). We found that calcitriol-treated mice had increased bacterial translocation out of the GI tract and into the MLN, spleen and liver at day 6 and day 10 pi, compared to vehicle-treated mice during infection with \textit{C. rodentium}, which was associated with suppressed Th17 mediated immune responses, indicating that too much vitamin D may also increase susceptibility to sepsis by suppressing immune responses. Overall these results show that the role of vitamin D during sepsis is complicated and more research is required.

5.3 How does vitamin D affect commensal microbes?

The role of vitamin D on commensal microflora is just beginning to be explored. Although we found that vitamin D deficient mice had different commensal bacteria, compared to control mice, this is most likely caused indirectly by vitamin D mediated immune changes rather than a direct effect of vitamin D on bacteria. Vitamin D on its own does not appear to have antibacterial activity (235), yet media from calcitriol treated human cells has been shown to kill bacteria in
vitro, including *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, by modulating host defense, including secretion of inflammatory mediators and antimicrobial peptides (232-236). Vitamin D deficiency has been shown to contribute to higher baseline inflammatory tone- indeed, we found that vitamin D deficient mice had higher transcript levels for the acute inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17A in their cecal tissues during uninfected conditions. Similarly, Assa et al. (221) found that vitamin D3 deficient mice had elevated expression of IL-17A and IL-17F in their distal colons compared to vitamin D3 sufficient mice. Intestinal inflammation has been shown to deplete commensal bacteria, creating a niche where pathogens can colonize and proliferate- since certain pathogens can utilize nutrients and metabolites released within the inflamed intestine that are not used by commensal species (99, 284). Indeed, previous work from our group has shown that *C. rodentium* infection is associated with a host inflammation driven depletion of commensal microbes that potentially aids pathogen colonization (129). In agreement, we found that the exaggerated inflammatory responses in vitamin D deficient mice accelerated the loss of commensal microbes during challenge with *C. rodentium* infection and were associated with an impaired ability to detoxify bacterial LPS. Although we did not find any difference in antimicrobial peptides between vitamin D deficient and sufficient groups during uninfected conditions, we did note significantly higher expression of RegIIIγ in vitamin D3 deficient mice at day 10 pi with *C. rodentium*. In contrast, no difference between diet groups was detected for transcript levels of other antimicrobial genes that encode beta-defensin 1, beta-defensin 3 or mcramp at day 10 pi (Supplemental Figure B.4). Overall these findings indicate that vitamin D deficiency can alter commensal microflora, however further research is required to determine if the commensal changes are caused by changes in host defense pathways.
5.4 How do bacteria affect vitamin D metabolism?

While it is clear that vitamin D can impact gut microbes, perhaps a better question to ask is how are luminal bacteria influencing vitamin D metabolism in the GI tract? Since calcitriol has such a profound effect on host responses in the GI tract, including suppressing Th1/Th17 mediated inflammation, inducing secretion of antimicrobial peptides and regulating epithelial cell responses, bacterial influence over vitamin D signalling would be very advantageous for gut bacteria in terms of controlling the luminal environment they exist in. Interestingly, pathogenic bacteria have been shown to induce VDR expression as well as the vitamin D activating and deactivating enzymes CYP27B1 and CYP24, respectively, thereby modulating local calcitriol metabolism. For instance, stimulation of human monocytes with TLR2/1 ligand to mimic M. tuberculosis increased expression of CYP27B1 and VDR (232). Conversely, stimulation with TLR2 and TLR4 ligands (mimicking exposure to bacteria) did not induce CYP27B1 or CYP24 in human colonic cells (Caco-2) (194), indicating that local metabolism of vitamin D may also be cell type specific. However, upon infection of human Caco-2 cells with pathogenic Salmonella, expression of CYP24 was induced (215), indicating that pathogenic bacteria may promote the breakdown of calcitriol. Bacteria have also been shown to play a role in regulating VDR expression in vivo; Germ-free wildtype mice showed less staining for VDR in their colons, which was localized to the top of colonic crypts, however when mice were administered commensal bacteria, strong VDR staining was induced in the middle and bottom of the crypts (215). Furthermore, mice infected with pathogenic Salmonella showed increased VDR expression by their IEC, whereas commensal E. coli F18 did not alter VDR expression, indicating that IEC responses to pathogenic bacteria may be determined in part through VDR signalling (215). Recently, the expression of VDR in the gastric mucosa was also found to be
significantly upregulated in patients infected with *Helicobacter pylori*, which was also positively correlated with chronic inflammation scores (309).

Intriguingly, Jones et al. (310) have recently shown that patients supplemented with probiotic bacteria had higher serum levels of vitamin D. In this double blind, placebo-controlled, randomized, parallel-arm, multicenter trial, 127 adults with high serum cholesterol were administered either placebo or probiotic *Lactobacillus reuteri* NCIMB 30242, a strain with previously demonstrated cholesterol-lowering activity. After 9 weeks, patients supplemented with *L. reuteri* had increased serum levels of 25(OH)D3 by 14.9 nmol/L, or 25.5%, over the intervention period, which was a significant mean change relative to placebo of 17.1 nmol/L, or 22.4%, respectively (P = .003) (310). Although the mechanism is unknown, studies show that bacteria, including certain *Lactobacillus* strains and commensal *E. coli* strains can induce the activity of cytochrome P450 enzymes *in vitro* (311-313), which may play a role in vitamin D metabolism.

5.5 Future directions

The work presented in this thesis answers some fundamental questions about the role of vitamin D during enteric infection. However, it also raises many new questions about the safety of vitamin D therapy for controlling pathogen-host interactions. Furthermore, it raises the question of what is the best way to administer vitamin D? For instance, is it best to give vitamin D through diet, supplements or topical? The following section of my thesis discusses these issues and how they may be addressed.
5.5.1 Can vitamin D3 supplementation protect against enteric infection?

Our research demonstrates that a short 5-week dietary vitamin D3 induced deficiency increases susceptibility to infection with *C. rodentium* by altering host defense. However, treating mice with the active form of vitamin D also increased susceptibility to *C. rodentium* infection by suppressing Th1/Th17 mediated immune responses. We therefore wondered whether supplemental vitamin D3 was protective or harmful during enteric infection. To determine the role of vitamin D3 supplementation during enteric infection, the following preliminary study was conducted. Weanling mice were fed either vitamin D3 sufficient (1000 IU) or vitamin D3 supplemented (20,000 IU) diets for 5 weeks, and then infected with *C. rodentium* and sacrificed at day 10 pi. Dietary vitamin D3 is converted into 25(OH)D3 in the liver, which is the major circulating form of vitamin D in the body and used to assess vitamin D status. After the 5-weeks, vitamin D3 supplemented mice had significantly higher levels of serum 25(OH)D3, compared to vitamin D3 sufficient mice (Figure 5.1A). Upon challenge with *C. rodentium*, vitamin D3 supplemented mice lost 10% more of their body weight by day 2 pi as compared to vitamin D3 sufficient mice (Figure 5.1B) and this significantly greater weight loss was maintained until the mice were euthanized at day 10 pi. To determine pathogen burdens, tissues were homogenized and plated to quantify *C. rodentium*. While no significant differences were found regarding pathogen burdens in the colon samples between groups at day 10 pi, vitamin D3 supplemented mice were found to carry 40-fold higher *C. rodentium* burdens in their ceca (cecal tissue + contents), as compared to vitamin D3 sufficient mice (Figure 5.1C). Interestingly, vitamin D3 supplemented mice also carried significantly more culturable *C. rodentium* in their extra-intestinal tissues, including their spleen and liver at day 10 pi, compared to vitamin D3 sufficient mice, indicating greater and more frequent bacterial translocation to these systemic sites (Figure
5.1D). We also examined Swiss-rolled sections of the colon for histological damage. At day 10 pi, the distal colon of vitamin D3 supplemented mice displayed worsened histological damage, with more crypt hyperplasia and epithelial damage compared to vitamin D3 sufficient mice at day 10 pi (Figure 5.2). Furthermore, vitamin D3 supplemented mice showed large numbers of lymphoid follicles within their colonic mucosa at day 10 pi (Figure 5.2). Future work should investigate the lymphoid follicles in the vitamin D3 supplemented mice to determine which cells are accumulating/proliferating during *C. rodentium* infection. Further studies should also explore lower levels of supplementation to determine the optimal level of dietary vitamin D to protect against enteric infection.

**Figure 5.1 Vitamin D3 deficient supplemented mice are more susceptible to *Citrobacter rodentium* infection and carry higher bacterial burdens at day 10-post infection**

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Serum 25(OH)D3: results are representative of 3 independent experiments, n = 5 -6 per group, * p < 0.05, ** p < 0.001, Mann-Whitney test B) Body weight. Each data point represents the average body weight pooled from 4 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 2 independent experiments, n = 6 per group, * p < 0.05, ** p < 0.001. Student’s t-test was conducted at each time point, Mann-Whitney test. C) Whole tissues were homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens. Colon and Cecum at Day 10 pi. Results are representative of 3 independent experiments, n = 5 -10 per group, ** p < 0.001, Mann-Whitney test. D) MLN, Spleen and Liver at Day 10 pi. Results are representative of 3 independent experiments, n = 6 -12 per group, * p < 0.05, Mann-Whitney test. Abbreviations: MLN = mesenteric lymph nodes; VD3 = Vitamin D3; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection
Figure 5.2 Vitamin D3 supplemented mice have worsened histological damage in distal colon at day 10-post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 sufficient (1000 IU) or vitamin D3 supplemented (20,000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. Top Panel: Representative images of formalin fixed “Swiss rolled” colon and cecal tissues after day 10 pi (distal colon is at centre of roll and cecum is at the outside end). Original magnification = 50X. Bottom Panel: Representative image of cross section of distal colon. Original magnification = 200X. Abbreviations: VD3 = Vitamin D3; CR = *C. rodentium*
5.5.2 Does vitamin D suppress IL-22 expression during infection with *Citrobacter rodentium*?

IL-22 has important functions in host defense at mucosal surfaces as well as in tissue repair. It is unique as a cytokine, in that it is produced by immune cells, including Th17 cells and innate lymphocytes, but acts only on non-hematopoietic stromal cells, in particular epithelial cells and keratinocytes (314). IL-22 has been shown to induce epithelial cells to produce antimicrobial peptides, including RegIIIγ, lipocalin-2 and β-defensins (86). In the gut, innate lymphoid cells (ILCs) that produce IL-22 and IL-17 (ILC3) are critical for early protection against *C.*
rodentium infection (87). It has recently been shown that Vdr-/− mice carry a higher number of IL-22 producing ILC3 cells in their small intestine compared to wildtype mice, however there was no difference in numbers in their colons (274). Furthermore, calcipotriol, a synthetic derivative of calcitriol, has been shown to suppress the expression of the IL-22 receptor in keratinocytes (315), however the mechanism underlying this is unknown. We assessed the expression of IL-22 in the distal colon and found no difference between vehicle and calcitriol-treated mice at day 10 pi (Figure 5.3). However, IL-22 expression in the cecum and colon is induced very early during C. rodentium infection, with a peak at day 4-post infection (85). Therefore, earlier time points need to be assessed to determine if vitamin D can alter IL-22 levels in the intestine during infection with C. rodentium.

Figure 5.3 Calcitriol-treated mice have no change in colonic IL-22 mRNA expression at day 10-post infection with Citrobacter rodentium

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with C. rodentium. Expression of IL-22 in distal colon at day 10 pi as assessed by RT qPCR. Results are representative of 2 independent experiments, = 8 per group. CR = C. rodentium; D10 = Day 10; pi = post infection
5.5.3 Further examine the effects of dietary vitamin D3 levels on the ecology of the intestinal microbiota

To date, analyzing the potential impact of vitamin D levels on the makeup of the gut microbiota has been limited, and has not utilized modern forms of analysis, such as 454 sequencing. Performing such analysis may help clarify the effects of vitamin D3 on the gut and provide clues to how changes in the microbiota could in turn aggravate changes in intestinal epithelial function and immunity. To further explore the impact of vitamin D on gut microbes, we could repeat our studies from Chapter 4, breeding C57BL/6 mice and immediately after weaning, placing the female pups (3 weeks of age) into three groups. Each group would receive a different diet, including a vitamin D deficient group (0 IU), a vitamin D sufficient group (1000 IU) and a vitamin D supplemented group (20,000 IU). After 5 weeks on these diets, we would euthanize the mice and collect their cecal and colonic tissues, separating mucosal adherent versus luminal microbes, and analyze the microbiota. We would preferentially use 454 pyrosequencing to explore much higher resolution of differences in microbes and their abundance amongst the groups. Among the microbes studied, we would specifically assess SFB, since they are known to drive Th17 responses.

Assuming we did note differences, we would explore the location of any divergent microbes by staining intact tissue sections using FISH staining with probes designed specifically for the microbes of interest. If we did identify specific differences in the vitamin D3 deficient group, we could assess whether similar microbiota changes are seen in Vdr-/- mice, as compared to co-housed heterozygous sibling mice.
5.5.4 Identify the vitamin D receptor expression cell types involved in controlling gut inflammatory tone

Most cells in the GI tract express VDR, and while $Vdr^{-/-}$ mice show increased CD4+ T cell responses, increased production of macrophage associated cytokines, and intestinal epithelial cell dysfunction; it is unclear if these effects reflect the loss of VDR in multiple cell types, or if they might reflect loss of VDR in one key cell type, such as CD4+ T cells. Defining how loss of VDR in specific cell types impacts on gut responses would greatly help define the mechanisms by which VD3 modulates intestinal function. We already have a small breeding colony of VDR flox/flox mice, on a C57BL/6 genetic background. We would cross these mice with mice expressing the cre enzyme in specific cell types until we generate $Vdr^{-/-}$ flox/flox mice missing VDR in these specific cell types. We will focus on villin cre mice (specific to IEC), CD4 cre mice (specific to CD4+ T cells) and LysM cre mice (specific to macrophages and neutrophils). Once we generated the VDR cell specific knockout mouse strains, we would then test them for inflammatory and intestinal epithelial cell responses. Specifically we will study inflammatory readouts such as the presence of neutrophils and macrophages in the colons of these mice, as well as measure gene transcripts for cytokines IL-1β and TNF-α, as well as oxidative stress markers. To assess Th17 responses, we will also measure gene transcripts for IL-17A and IL-22 by RT qPCR, and perform FACs analysis to measure Th17 CD4+ T cells. We will assess barrier integrity by FITC-dextran oral gavage and measure intestinal epithelial responses by staining tissues for the proliferation marker Ki67, and use TUNEL stain to measure cell death. We expect through these studies that one or more of these mouse strains would display intestinal epithelial cell dysfunction and heightened intestinal inflammatory tone, indicating the importance of VDR signalling in that cell type.
5.5.5 Explore vitamin D and iron interactions during enteric infection

It has recently been shown that vitamin D may play a role in iron metabolism, by suppressing hepcidin (316, 317). Hepcidin is an antimicrobial peptide that controls plasma iron concentrations and tissue distribution of iron by inhibiting intestinal iron absorption, iron recycling by macrophages, and iron mobilization from hepatic stores (318). Hepcidin is elevated during infections and inflammation, causing a decrease in serum iron levels and contributing to the development of anemia during inflammation, probably as a host defense mechanism to limit the availability of iron to invading microorganisms (318). In a recent pilot study with healthy volunteers, supplementation with a single oral dose of vitamin D (100,000 IU vitamin D2) increased serum levels of 25D-hydroxyvitamin D from 27±2 ng/ml before supplementation to 44±3 ng/ml after supplementation (P<0.001). This response was associated with a 34% decrease in circulating levels of hepcidin within 24 hours of vitamin D supplementation (P<0.05) (316).

Furthermore, calcitriol, the hormonally active form of vitamin D, is associated with decreased hepcidin expression in LPS stimulated THP-1 cells, and treatment with calcitriol resulted in a dose-dependent decrease in the release of the pro-hepcidin cytokines, IL-6 and IL-1β in vitro (317). These findings indicate that supplemental vitamin D may be contraindicated during bacterial infections, since suppressed levels of hepcidin may result in higher serum and tissue levels of iron, resulting in more free iron available to support pathogen growth. Indeed, my preliminary studies show that mice supplemented with high levels of vitamin D (20,000 IU) carry higher bacterial burdens during infection with C. rodentium, however the mechanism behind this increase is currently unknown. Future studies should investigate if vitamin D alters serum hepcidin and iron levels during enteric infection. Furthermore, siderophores and other iron acquisition strategies should be investigated in C. rodentium.
5.5.6 How do dietary factors modulate vitamin D activation?

There are likely significant variations in how IBD patients can absorb and metabolize dietary sources of vitamin D. Proper absorption of oral vitamin D requires dietary fat and bile salts in order for its metabolites to be incorporated into micelles and absorbed as chylomicrons into the lymphatic system (36). Bile acid malabsorption is a common but underestimated pathophysiology of IBD, especially in patients with distal ileum involvement (319). As a consequence of bile acid malabsorption, patients with IBD also have problems with fat digestion and develop clinical steatorrhea (fatty stool) (319). As such, patients with IBD may have problems absorbing dietary and supplemental sources of vitamin D. Furthermore, vitamin D must be converted to its active form calcitriol by 2 separate hydroxylation steps that occur mainly in the liver and kidney. Patients who have liver or kidney damage, which is common in patients with IBD (132) may also have dysregulated vitamin D metabolism. Finally, the CYP27B1 enzyme, required to convert 25(OH)D3 into its active hormonal form calcitriol can be suppressed by various dietary factors, including phosphate (320) and uric acid- a breakdown product of protein metabolism (321). Therefore, dietary sources of vitamin D may not be adequately converted into active forms of vitamin D.

Various dietary factors have been shown to modulate vitamin D activation by targeting CYP27B1. Dietary phosphorus is essential for bone growth and mineralization. However, elevated serum phosphate has an inhibitory effect on the renal activation of 25(OH)D3 into the active metabolite calcitriol (320). Since phosphorus exists in virtually all-living organisms, phosphorus is abundantly supplied in the diet. Excess phosphate intake can significantly disrupt
hormonal regulation of vitamin D and calcium and contribute to disordered mineral metabolism, vascular calcification, impaired kidney function, increased risk of cardiovascular disease and bone loss (320). In fact, dietary phosphorus restriction is often a key strategy for the management of chronic kidney disease (322) and may be useful for the treatment of cardiovascular disease (323) and other inflammatory conditions. Experimental studies have also shown that vascular mineralization can be suppressed by reducing serum phosphate levels, even in the presence of extremely high serum calcitriol and calcium levels (324). Phosphate restriction may be an important dietary strategy for patients with Crohn’s disease, tuberculosis and sarcoidosis, who develop granulomas- nodules filled with large amounts of inflammatory cells that secrete massive amounts of calcitriol and are at risk of systemic hypercalcemia and soft tissue calcification (78, 325-327). Interestingly, it has recently been shown that high dietary phosphate intake can induce systemic inflammation in rats after 8 week feeding trials, including higher serum and tissue levels of TNF-α (328) - although the mechanism is unknown, these findings indicate that dietary phosphate restriction may also play a role in controlling inflammation.

High serum levels of uric acid have also been shown to suppress CYP27B1 leading to lower calcitriol levels in rats (321). Uric acid is the end product of purine metabolism, generated from the breakdown of DNA, RNA and ATP. High circulating concentrations of uric acid can lead to gout, a common form of arthritis and has also been linked to chronic kidney disease, cardiovascular disease and cancer (329). Interestingly, patients with gout had lower serum concentrations of calcitriol, compared to control subjects, whereas no differences were observed for serum 25(OH)D3 (330). Vitamin D insufficiency has also been associated with
elevated serum uric acid levels in postmenopausal Chinese Han women (331). Certain dietary components are thought to affect concentrations of uric acid. For instance, meat and fish may increase the concentration of uric acid because of the high purine content of these foods, whereas dairy products may lower uric acid concentrations by increasing excretion of uric acid and its precursor xanthine (329). Furthermore, several studies have observed a lower concentration of uric acid in vegetarians compared to meat eaters (329, 332-334).

Vitamin D supplementation on its own may not be sufficient for vitamin D metabolism to function properly. Vitamin D metabolism requires several essential cofactors, including magnesium to function properly in the body (335-337). Magnesium is an essential mineral and cofactor for over 300 enzymes that regulate diverse biochemical reactions in the body, including protein synthesis, energy production, nerve and muscle contraction, blood glucose control, blood pressure regulation and bone development (338). Recent studies have also shown that magnesium plays a key role in regulating inflammation in the body (339-341). The richest sources of magnesium include whole and unrefined grains, seeds, cocoa, nuts, especially almonds and green leafy vegetables (342). With almost half of Americans not meeting the daily requirement of magnesium dietary intake (343), hypomagnesemia is a real concern, especially for patients with gut disorders. Indeed, IBD patients appear to be at increased risk of magnesium deficiency, with low magnesium intakes reported in 13%–88% of patients (344, 345). Previous studies have shown that the activities of three major enzymes determining 25(OH)D3 levels (336, 337, 346, 347) and vitamin D binding protein (337) are magnesium dependent. Magnesium deficiency, which leads to reduced calcitriol (337), has been implicated in
‘magnesium-dependent vitamin-D-resistant rickets’ (335). Magnesium supplementation substantially reversed the resistance to vitamin D treatment (335). Overall, these findings suggest that achieving ideal vitamin D status may be influenced by various dietary and supplemental factors. Patients and clinicians should be aware of these factors in order to optimally boost vitamin D levels.

5.5.7 Can topical vitamin D therapy protect against colitis?

The efficacy of dietary supplementation with vitamin D may be limited for patients with GI disorders, since absorption of this fat-soluble nutrient requires the presence of fatty acids, bile and a properly functioning digestive tract. Fortunately, there are other ways of acquiring vitamin D, including cutaneous synthesis during exposure to UVB. UV radiation suppresses immunity in both humans and animal models such as mice. Keratinocytes, circulating and cutaneous T lymphocytes, monocytes, Langerhans cell, mast cells and fibroblasts are all targeted by narrowband ultraviolet-B (NB-UVB) light (348). NB-UVB phototherapy is an effective treatment for psoriasis, however the molecular mechanism underlying its effectiveness is not completely understood. Recent studies have shown that UVB therapy is linked to suppression of proinflammatory Th1 (IFN-γ) and Th17 immune responses (244). Specifically, psoriasis patients treated with NB-UVB rays had significantly less serum TNF-α, IL-17, IL-22 and IL-23, compared to baseline (349). Despite the role of vitamin D in modulating the immune response, the role of UVB therapy in IBD patients is largely unexplored. Crohn’s disease patients with vitamin D malabsorption (n=6) were found to have normal levels of 7-dehydrocholesterol in their skin (350), indicating that vitamin D synthesis through UVB exposure should be possible.
Indeed, it has been reported that vitamin D deficiency was successfully treated over 4 weeks with UVB therapy in a patient with Crohn’s disease (351). Recently, artificial light phototherapy has been shown to ameliorate suppressed DSS-induced colitis in mice by suppressing serum levels of pro-inflammatory cytokines, including TNF-α, IL-17 and IL-6 and promotion of anti-inflammatory cytokines (352, 353). We are currently investigating the role of UVB therapy during DSS induced colitis and during enteric infection with bacterial pathogens.

5.6 Final remarks
Achieving optimal vitamin D status is important for maintaining gut barrier integrity, controlling inflammation and modulating host-bacteria interactions. The findings from my thesis suggest that there is likely an optimal range of vitamin D for a healthy gut and that too little or too much can promote inflammation and/or increase susceptibility to enteric infections. Many factors can influence vitamin D intake and status, including darker skin pigmentation, obesity, sunlight and UVB exposure, sunscreen use and even clothing choices. Absorption of vitamin D also requires a proper functioning gut- including proper bile acid secretion. Patients with IBD should be encouraged to supplement with vitamin D3 and eat vitamin D rich foods such as fatty fish (wild salmon), egg yolks and UVB-exposed mushrooms. If IBD patients cannot properly absorb dietary or supplemental vitamin D, then topical forms of vitamin D and narrow-band UVB therapy should be discussed. Clinicians and health providers should be encouraged to determine the vitamin D status of IBD patients and work together to determine appropriate solutions for improving vitamin D intake.
Despite the promise of vitamin D, this single nutrient alone is not enough to help patients with IBD strengthen the gut barrier, ameliorate inflammation and balance commensal bacteria. Patients with IBD are at greater risk of multiple nutrient deficiencies due to dietary restrictions, inadequate absorption resulting from tissue damage (ulceration/fibrosis), surgery (small-bowel resection), chronic diarrhea, steatorrhea, and dysbiosis. As such, many nutrient deficiencies, including vitamins (vitamin C, folate, vitamin B6, vitamin B12, vitamin A, vitamin K and vitamin D) and minerals (iron, calcium, magnesium and zinc) are common in patients with IBD, particularly in Crohn’s disease patients with active small bowel disease who have had multiple resections (354). These essential nutrients play key roles in regulating energy levels, controlling inflammation and promoting mucosal healing. Many nutrients can affect immune responses, for instance the active forms of vitamin D and vitamin A (retinoic acid) can both directly regulate T-cell responses, by binding to their respective nuclear receptors expressed on lymphoid cells and acting as transcription factors to induce or suppress inflammation (355). Zinc is also necessary for the normal function of the immune system, and dysfunction is observed, even in mild zinc deficiency (356). Vitamin C (ascorbic acid) is well known as a water-soluble antioxidant compound, but it also plays a critical role in all phases of wound healing through the synthesis and degradation of collagen (357). Several nutrients, including vitamin D, vitamin A and zinc also play important roles regulating epithelial cell proliferation and differentiation and promoting intestinal barrier integrity and mucosal healing (216, 358, 359). As such, improving the diet and nutrient intakes of patients with IBD can help suppress overactive inflammation, strengthen the gut barrier and improve mucosal healing.
In conclusion, diet therapy; including adequate vitamin D should be encouraged as a complementary treatment for patients with IBD. Although diet can be very individualistic, especially for patients with IBD- there is currently enough evidence to suggest several foods to emphasize and foods to limit- especially during active stages of disease. To promote a strong gut barrier, patients with IBD could be advised to limit or restrict alcohol intake and NSAIDs use, take stress-management seriously and be encouraged to eat soluble fibers that encourage the production of butyrate- a key nutrient for epithelial cell health. Adequate intake of several nutrients, including vitamin A, zinc and vitamin D should be advocated to promote the growth of healthy epithelial cells lining the gut. To control inflammation, a plant-based diet could be encouraged, along with fatty fish and adequate vitamin D. To reduce the exposure to pathogenic bacteria, proper food handling skills should be emphasized and animal products, including red meat should be limited or avoided. Furthermore, limiting the intake of food additives, such as maltodextrin should be considered since they can contribute to dysbiosis. To promote the growth of beneficial bacteria- probiotic bacteria, naturally fermented foods and prebiotic fibers should be introduced gradually to reduce GI side effects. Future work should focus on designing a dietary protocol for patients with IBD that included options such as safe UVB exposure for improving vitamin D status.
References


149. Canada CsaCFo. 2012. The Imact of Inflammatory Bowel Disease in Canada Final Report and Recommendations. 96.


invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. ISME J 1:403-418.


IL-17A in the pathogenesis of DSS-induced colitis in mice. Biochem Biophys Res Commun 377:12-16.


Supplemental Figure A.1 Mice treated daily with 10 ng calcitriol lose more body weight compared to vehicle-treated mice

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 10 days. A) Body weight. Each data point represents the average body weight pooled from 4 mice and is expressed as the percentage of the initial body weight with SEM, n = 4 per group, * p < 0.05, Student’s t-test was conducted at each time point, Mann-Whitney test. B. No difference in histological appearance. Representative images of formalin fixed “Swiss rolled” colon and cecal tissues after 10 day IP treatment with Vehicle or Calcitriol (distal colon is at centre of roll and cecum is at the outside end). Original magnification = 50X. C) No difference in histological appearance. Representative image of cross section of distal colon. Original magnification = 200X. IP = Intraperitoneal
A

![Graph showing % Initial Body Weight over Days post IP injections.](image)

- **Vehicle IP**
- **10 ng Calcitriol IP**

B

**Vehicle (control) Whole Colon**

**10 ng Calcitriol (control) Whole Colon**

C

**Vehicle (control) Distal Colon**

**10 ng Calcitriol (control) Distal Colon**
Supplemental Figure A.2 Low dose 5 ng calcitriol is not protective against 3% dextran sodium sulfate induced colitis

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle, 5 ng calcitriol, or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to 3% dextran sodium sulfate (DSS) challenge. DSS was administered in drinking water for 7 days, followed by tap water for 3 days. Mice treated with vehicle (Panel A and D) and 5 ng calcitriol (Panel B and E) developed histological damage throughout the colon and cecum, whereas mice treated with 10 ng calcitriol treatment (Panel C and F) were protected against DSS induced damage and had minimal histological damage. Top Panels (A, B, C): Representative images of formalin fixed “Swiss rolled” colon and cecal tissues after 10 day IP treatment with Vehicle or Calcitriol (distal colon is at centre of roll and cecum is at the outside end). Original magnification = 50X. Bottom Panels (D, E and F): Representative image of cross section of Cecum. Original magnification = 50X. IP = Intraperitoneal

Supplemental Figure A.3 Calcitriol given by intraperitoneal injection is more protective against 3% dextran sodium induced damage in colon, compared to intra-rectal administration

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection or intra-rectally every day for 11 days, starting one day prior to 3% dextran sodium sulfate (DSS) challenge. DSS was administered in drinking water for 7 days, followed by tap water for 3 days. Representative images of formalin fixed “Swiss rolled” colon and cecal tissues after 10 day (distal colon is at centre of roll and cecum is at the outside end). Original magnification = 50X. Mice treated with vehicle IP injection (Panel A) or intra-rectally (Panel B) had histological damage throughout the colon (distal and proximal areas), whereas mice treated
with 10 ng calcitriol by IP (Panel C) injection were protected against DSS induced damage and had minimal histological damage. However, calcitriol given intra-rectally were not protective against 3% DSS colitis (Panel D). IP = Intraperitoneal

Supplemental Figure A.4 Calcitriol given by intraperitoneal injection is more protective against 3% dextran sodium sulfate induced damage in ceca, compared to intra-rectal administration

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection or intra-rectally every day for 11 days, starting one day prior to 3% dextran sodium sulfate (DSS) challenge. DSS was administered in drinking water for 7 days, followed by tap water for 3 days. Representative images of formalin fixed cross section of ceca after treatment. Original magnification = 50X. Mice treated with vehicle via IP injection or intra-rectally developed histological damage throughout the ceca (Panel A, B), whereas mice treated with 10 ng calcitriol treatment by IP injection were protected against DSS induced damage and had
minimal histological damage (Panel C). However, calcitriol given intra-rectally were not protective against 3% DSS colitis (Panel D). IP = Intraperitoneal

Supplemental Figure A.5 Calcitriol-treated mice have more damage in colon when administered 4% dextran sodium sulfate

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to administering either 3% or 4% dextran sodium sulfate (DSS). DSS was administered in drinking water for 7 days, followed by tap water for 3 days. Representative images of formalin fixed “Swiss rolled” colon and cecal tissues after 10 day (distal colon is at centre of roll and cecum is at the outside end). Original magnification = 50X. Mice treated with vehicle IP had histological damage throughout the ceca (panel A and B), whereas mice treated with 10 ng calcitriol treatment by IP injection were protected against 3% DSS and had minimal histological damage (panel C). However, 10 ng calcitriol IP was not protective against 4% DSS colitis (panel D). IP = Intraperitoneal

174
Supplemental Figure A.6 Calcitriol-treated mice have more damage in ceca when administered 4% dextran sodium sulfate

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to administering either 3% or 4% dextran sodium sulfate (DSS). DSS was administered in drinking water for 7 days, followed by tap water for 3 days. Representative images of formalin fixed cross section of ceca after treatment. Original magnification = 50X. Mice treated with vehicle IP had histological damage throughout the ceca (Panel A and B), whereas mice treated with 10 ng calcitriol treatment by IP injection were protected against 3% DSS and had minimal histological damage (Panel C). However, 10 ng calcitriol IP was not protective against 4% DSS colitis (Panel D). IP = Intraperitoneal
Supplemental Figure A.7 Calcitriol-treated mice have cecal erosions at day 6-post infection with *Citrobacter rodentium*

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection every day for 7 days, starting one day prior to oral infection with *C. rodentium*. Representative digital image of the lower gastrointestinal (GI) tract from mice at day 6 pi. Vehicle-treated mice (Panel A) appeared normal, compared to calcitriol-treated mice (Panel B), which had shrunken ceca nearly devoid of contents. Calcitriol-treated mice also had cecal erosions at day 6 pi (Panel C). Arrows are pointing to erosion/ulcerated regions in ceca. CR = *Citrobacter rodentium*; D6 = Day 6
Supplemental Figure A.8 Low dose calcitriol-treated mice have cecal erosions at day 10-post infection with *Citrobacter rodentium*

Preliminary Study. Male 8-week-old C57Bl/6 mice were given vehicle or 5 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Representative digital image of the lower gastrointestinal (GI) tract from mice at day 10 pi. Vehicle-treated mice (Panel A), compared to calcitriol-treated mice (Panel B). Calcitriol-treated mice had cecal erosions at day 6 pi (Panel C). Arrows are pointing to erosion/ulcerated regions in ceca. CR = *Citrobacter rodentium*; D6 = Day 6
Supplemental Figure A.9 Calcitriol-treated mice have higher *Citrobacter rodentium* burdens at day 1-post infection

Male 8-week-old C57Bl/6 mice were given vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Stool was collected at various time points throughout infection, homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens. Day 1 pi: n = 8 - 16 per group. Results are representative of 2 - 3 independent experiments, * p < 0.05, Mann-Whitney test. pi = post infection
Supplemental Figure A.10 Low dose calcitriol-treated mice have higher *Citrobacter rodentium* burdens at day 10-post infection

Male 8-week-old C57Bl/6 mice were given vehicle or 5 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Tissues were collected, homogenized and plated on LB/strep-treated plates to enumerate *C. rodentium* burdens. A) Colon and Cecal content: n = 4 per group. B) MLN, Spleen, and Liver: n = 4 per group, * p < 0.05, Mann-Whitney test. Cont = contents; MLN = mesenteric lymph nodes; CR = *Citrobacter rodentium*, pi = post infection
Supplemental Figure A.11 Calcitriol-treated mice have trend for more histological damage in distal colon and ceca at day 10-post infection with *Citrobacter rodentium* burdens

Male 8-week-old C57Bl/6 mice were given vehicle or 5 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Tissue cross sections of the distal colon and cecal was assessed for histological damage by scoring system for *C. rodentium* described in Methods. n = 7 per group. CR = *Citrobacter rodentium*

![Pathology scores for distal colon and cecum](image)

Supplemental Figure A.12 No differences in colonic expression of tumor necrosis factor-α or inducible nitric oxide synthase between calcitriol-treated or vehicle-treated mice at day 10-post infection

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Expression of TNF-α and iNOS in distal colon at day 10 pi as assessed by RT qPCR. n = 4 per group. CR = *Citrobacter rodentium*, pi = post infection
Supplemental Figure A.13 No differences in colonic expression of cytokines between calcitriol-treated or vehicle-treated mice at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Expression of cytokines in distal colon at day 10 pi as assessed by RT qPCR. TGF-β, n = 7 - 8 per group; IL-10, n = 4 per group; IL-23, n = 4 per group; IL-22, n = 8 per group; IL-17F, n = 7 - 8 per group. IL = Interleukin; CR = *Citrobacter rodentium*; pi = post infection
Supplemental Figure A.14 No difference in CD4+IL-17A+ cells in spleen between calcitriol-treated or vehicle-treated mice

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. FACS analysis. n = 4 - 5 per group. Results are representative of 2 independent experiments, * p < 0.05, Mann-Whitney test. CR = *Citrobacter rodentium*; pi = post infection

![% CD4+ IL-17A+ Cells in Spleen](image)

Supplemental Figure A.15 No differences in colonic expression of RegIIIβ, S100A8 or S100A9 between calcitriol-treated or vehicle-treated mice at day 10-post infection with *Citrobacter rodentium*

Male 8-week-old C57Bl/6 mice were given either vehicle or 10 ng calcitriol via intraperitoneal injection every day for 11 days, starting one day prior to oral infection with *C. rodentium*. Expression of RegIIIβ, S100A8 or S100A9 in distal colon at day 10 pi as assessed by RT qPCR. n = 4 per group. CR = *Citrobacter rodentium*; pi = post infection
Appendix B  Addendum to Chapter 4

Supplemental Figure B.1 No differences in approximate feed intake or body weight between vitamin D3 deficient and sufficient mice during 5-week feeding trial

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks. A) Estimated Total Feed intake per cage over 5 week feeding trial (n = 4 mice per cage). Each point represents 1 cage. Total diet pellets per cage were weighed at week 0, week 3 and week 5 for a total sum value. Results are from 4 independent experiments. B) Body Weight: n = 8 per group. Results are from two independent experiments. VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient

![Graph A: Total Feed Intake per Cage](image1)

![Graph B: Body Weight](image2)

Supplemental Figure B.2 No change in barrier integrity between vitamin D3 deficient and sufficient mice at day 6-post infection with *Citrobacter rodentium*

Weanling (3-week-old) C57B/6 female mice were fed either vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then challenged orally with *C. rodentium*. At day 6 pi, mice were orally gavaged with FITC-dextran probe and fasted for 4 hours. Blood was collected at termination and plasma was assessed for translocated FITC-dextran levels to measure gut barrier integrity, *in vivo*. n = 5 per group. VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; pi = post infection
Supplemental Figure B.3 Vitamin D3 deficient mice have trend for more histological damage in distal colon at day 10-post infection with *Citrobacter rodentium*

Weanling (3-week-old) C57B/6 female mice were fed either vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then challenged orally with *C. rodentium*. Tissue cross sections of the distal colon and cecal was assessed for histological damage by scoring system for *C. rodentium* described in Methods. n = 4 - 7 per group. VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; pi = post infection
Supplemental Figure B.4 No differences in cecal expression of mCramp or β-defensins between vitamin D3 deficient or sufficient mice at day 10-post infection with *Citrobacter rodentium*

Weanling (3-week-old) C57B/6 female mice were fed either vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then challenged orally with *C. rodentium*. Expression of antimicrobial peptides in ceca at day 10 pi as assessed by RT qPCR. β-defensin 1, n = 4 per group; β-defensin 3, n = 4 per group; mCramp, n= 10 - 11 per group; VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; D6 = Day 6; CR = *Citrobacter rodentium*; pi = post infection