THE EFFECTS OF VITAMIN A DEFICIENCY ON HOST DEFENSE
DURING CITROBACTER RODENTIUM INFECTION

A Dissertation in
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by
Kaitlin L. McDaniel

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The dissertation of Kaitlin L. McDaniel was reviewed and approved* by the following:

Margherita T. Cantorna  
Distinguished Professor of Nutrition and Immunology  
Dissertation Advisor  
Co-Chair of Committee

A. Catharine Ross  
Huck Chair and Professor of Nutrition  
Co-Chair of Committee

Na Xiong  
Associate Professor of Veterinary Science and Biomedical Sciences

Mary K. Kennett  
Professor of Veterinary Science and Biomedical Sciences

Connie J. Rogers  
Assistant Professor of Nutritional Science

Anthony Schmitt  
Associate Professor of Molecular Virology  
Director, Pathobiology Graduate Program

*Signatures are on file in the Graduate School
ABSTRACT

Vitamin A deficiency is still a prevalent problem in resource limited countries, affecting more than 250 million preschool aged children. Vitamin A deficiency is associated with increased severity of respiratory and diarrheal diseases, a major cause of childhood mortality. Vitamin A supplementation is associated with increased vitamin A status and decreased disease severity, indicating a link between vitamin A status and immune function. Using the murine enteric pathogen, *Citrobacter rodentium*, I explored the relationship between host vitamin A status, intestinal infection and immune response.

The first objective was to determine the effect of vitamin A deficiency on *C. rodentium* infection. My study showed that vitamin A deficient (A-) hosts did not clear the infection and 40% of the A- animals developed a lethal infection. Additionally, retinoic acid treatment of A- hosts promoted infection clearance and survival. The findings from this study suggest that A- hosts may harbor enteric pathogens but retinoic acid treatment was effective during *C. rodentium* infection. The second objective was to determine how retinoic acid promotes *C. rodentium* clearance in the A- host. My data shows that 1 dose of retinoic acid in an A- host was effective at clearing infection. Two doses of retinoic acid treatment promoted *il17a* expression in the colon of A- mice yet, retinoic acid treatment of splenocytes *in vitro* inhibited IL-17 secretion. Vitamin A host status was found to have no effect on the microbiota and B cell numbers in the gut, indicating that other acquired immune cells may be targets of RA. To understand the effects of retinoid signaling on T cells, T-dnRAR mice were generated. T-dnRAR mice had significantly more CD8+ thymocytes but significantly fewer CD8+ T cells in the spleen and mesenteric lymph node. Together, the work presented in this dissertation indicates that
vitamin A deficient hosts may be asymptomatic carriers of enteric infections and that vitamin A treatment during infection promotes a local IL-17a response that is associated with clearance of *C. rodentium*.
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ABBREVIATIONS

A-: vitamin A deficient hosts
A+: vitamin A sufficient hosts
CCL: chemokine ligand
CCR: CC-chemokine receptor
CD: cluster of differentiation
CFU: colony forming units
CTLA-4: cytotoxic T lymphocyte antigen 4
DBD: DNA binding domain
DC: dendritic cell
DN: double negative
dn-RAR: dominant negative retinoic acid receptor
DP: double positive
EtOH: ethanol
FOXP3: Forkhead box P3
GALT: gut associated lymphoid tissues
GI: gastrointestinal tract
IEL: intraepithelial lymphocytes
IFN: interferon
Ig: immunoglobulin
IL: interleukin
ip: intra-peritoneal
iv: intra-venous
KO: knock out
LBD: ligand binding domain
LCK: lymphocyte specific protein tyrosine kinase
LPL: lamina propria lymphocytes
LPS: lipopolysaccharide
MAdCAM-1: mucosal addressin cell adhesion molecule-1
MLN: mesenteric lymph nodes
NK: natural killer
NKT: natural killer T
NTD: NH₂ terminal domain
PP: Peyer’s patches
RA: retinoic acid
RAR: retinoic acid receptor
RARE: retinoic acid response elements
RBP: retinol binding protein
ROR-γt: RAR orphan receptor γt
RXR: retinoid X receptor
SI: small intestine
STRA6: stimulated by retinoic acid gene 6
TCR: T cell receptor
T-dnRAR: T cell specific dn-RAR
TFG: transforming growth factor
**Th:** T helper

**TNF:** tumor necrosis factor

**Treg:** regulatory T cell

**TTR:** transthyretin

**VA:** vitamin A

**VAD:** vitamin A deficiency

**WT:** wild type
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Chapter 1

Introduction
**Vitamin A: Sources and metabolism**

Vitamin A (VA) is a fat-soluble essential micronutrient that humans obtain through their diet. VA is absorbed from the diet as previtamin A retinyl esters present in dairy products and liver and provitamin A carotenoids (from leafy green vegetables and bright colored fruits) (1). The most common form of dietary vitamin A is β-carotene which is found in vegetables and fruits such as sweet potatoes, kale, carrots and cantaloupe. Proper vitamin A intake is associated with eye health, cell development and immune system health, making it an important nutrient.

Dietary vitamin A needs to be converted into an active form so that the body can use it. The metabolism of vitamin A has been summarized in Figure 1-1. Briefly, once vitamin A has been absorbed into intestinal epithelial cells, the associated proteins and fatty acids are removed. This frees the retinyl esters and carotenoids so they can be processed by enzymes in the epithelial cells which convert retinyl esters and carotenoids to retinol (2). Retinol becomes esterified into retinyl palmitate and incorporated into chylomicrons where it enters the lymphatic system and travels to the liver (3). The liver is the major storage organ of vitamin A and also produces retinol binding protein (RBP) (4). Once the chylomicron enters the liver, the retinyl palmitate is hydrolyzed into retinol where it is stored in the liver stellate cells (5, 6). Retinol can associate with RBP which binds with transthyretin (TTR) forming a traveling complex in the serum that can be transported to extrahepatic tissues (1, 7). The RBP:TTR complex delivers retinol to the plasma membrane receptor, STRA6 (stimulated by retinoic acid gene 6), on target cells. The binding to the transport complex to STRA6 releases retinol from its binding proteins and retinol
enters the cell where it is converted to retinoic acid (RA) (Fig. 1-1A and 1-1B). Dietary vitamin A must be converted into RA for the use of cells.

RA is the active metabolite of vitamin A. The effects of RA are exerted when RA binds the retinoic acid receptor (RAR), which is found inside the nucleus (8). The RAR is a member of the superfamily of nuclear receptors and has three isoforms, RAR α, β and γ (8, 9). RARs are expressed in every cell of the body, but RARα is the most common form (10). The RARs are divided into three domains that are necessary for its function, including the COOH-terminal ligand binding domain (LBD) which binds RA, the central DNA binding domain (DBD) which binds to DNA sequences and the NH2 terminal domain (NTD) whose mechanism of action is yet to be determined (11-13). The RAR heterodimerizes with the retinoid X receptor (RXR) to form a complex that, in the presence of ligand, binds to retinoic acid response elements (RARE) in the promoter regions of RA-inducible genes (11) (Fig. 1-1B). All-trans retinoic acid binds to the RAR, inducing conformational changes in the ligand-binding domain which releases co-repressor proteins and recruits activator proteins. The combination of RA, RAR:RXR heterodimer and activator proteins make up a complex that induces gene transcription (11, 12). RAR signaling can also inhibit gene transcription, but the mechanisms of inhibition are largely unknown. RA largely regulates gene expression through nuclear receptor binding and co-activator protein recruitment to gene promoter sites.

Vitamin A Deficiency

Vitamin A deficiency (14) remains a public health issue in resource-limited
countries, such as India and most of Africa (15). VAD is a critical concern in young children and pregnant women (16, 17). It is estimated that over 20% of preschool aged children are clinically vitamin A deficient (15, 17). Vitamin A deficiency in infants occurs during breastfeeding because of the vitamin A deficient status of the mothers. VAD causes xerophthalmia (night blindness) and is associated with increased severity of infectious disease (18, 19). Vitamin A supplementation resulted in a 26-30% reduction of mortality from respiratory and diarrheal diseases by reducing disease severity in populations studied in Africa and India (18, 20). The World Health Organization (WHO) reports vitamin A supplementation reduced measles related mortality by 50% and diarrheal related mortality by 33% (21). Additionally, studies showed that high dose vitamin A supplementation reduced childhood mortality and morbidity in children without VAD (14, 22). Based on these studies the WHO recommends that preschool aged children in counties where VAD is common receive vitamin A supplementation (11, 15). The collective reports indicate that there is a strong link between vitamin A status and resistance to infectious disease.

**Retinoic acid regulation of peripheral immune responses**

The immune system is a network of cells, tissues and systems that protect the host from disease. This defense system is composed of innate immune defenses (barrier defenses and mechanisms that distinguish self from non-self) and adaptive immune defenses (mechanisms that have immunological memory). The innate immune response occurs quickly and initiates the adaptive immune response which
takes over once the initial innate response ends. Fully functional innate and adaptive immunity are required for the host to fight infections.

Retinoic acid is a potent immune cell regulator. It has been shown to affect both the innate and adaptive branches of the immune system. The effects of RA on the innate immune system have been reviewed in detail elsewhere (23). The focus of this dissertation is to elaborate on the effects of RA on the adaptive immune system, mainly T cells.

T cells are a major part of the adaptive immune system. These cells mature in the thymus and express T cell receptors, CD3 and either CD4 or CD8 on their surface. CD4+ T cells are T helper (Th) cells that mediate immune responses primarily though secretion of cytokines which regulate other immune cells such as B cells and macrophages (24). Th cells are able to respond to a wide variety of pathogens, regulate immune responses to control the magnitude of the immune system’s response and protect against autoimmunity (24). Th cells are further categorized based on the cytokines they secrete. Th1 cells are regulated by the transcription factor, T-bet, and are potent cytokine producers, particularly interferon (IFN) -γ that aids in the response to intracellular pathogens (25). Th2 cells secrete interleukin (IL) -4, IL-5 and IL-10 and are regulated by the transcription factor, GATA-3 (26). Th2 cells are important for host responses to helminthes and promote B cell antibody production, the other half of the adaptive immune system (25). IFN-γ secreted from Th1 cells inhibits Th2 cell function and IL-4 produced from Th2 cells inhibit Th1 functions, indicating that Th1 and Th2 cells antagonize each other (27).
Another type of CD4+ T cells are Th17 cells. Th17 cells produce IL-17A, IL17-F, IL-21 and IL-22 to control bacterial infections (28). Th17 cells are induced in the presence of transforming growth factor (TGF)-β, IL-6 and IL-23 (29) and are important for mucosal barrier function (24, 26, 28, 30). These cells are regulated by the transcription factor, ROR-γt. The opposing cell lineage is regulatory T cells (Tregs). Tregs express the transcription factor, FoxP3, and suppress the functions of target T cells to inhibit immune responses (27). Naïve CD4+ T cells express both FOXP3 and ROR-γt and when RA, TFG-β and low concentrations of IL-6 are present, FOXP3 will inhibit Th17 cell development by inhibiting ROR-γt expression (31, 32). Humans and mice with mutations in the foxp3 gene have severe immune impairments indicating that Treg cells are vital to maintaining the balance between inflammatory and anti-inflammatory responses (24, 25). Treg cells secrete anti-inflammatory cytokines such as IL-10 and TGF-β to inhibit effector cell function (27). They also express the surface marker CTLA-4 (cytotoxic T lymphocyte antigen 4) which competes for co-stimulatory receptors on effector cells (25). When CTLA-4 binds to effector cells, an inhibitory signal is sent to the effector cell and inflammatory responses recede (25). Additionally, IFN-γ and IL-17 has been implicated in autoimmune disease pathology while IL-4 and IL-10 have been associated with inflammation resolution (22). Maintaining the balance between Th1/Th17 and Th2/Treg cells is required for inflammatory homeostasis in the host.

The balance between inflammatory and regulatory responses is mediated in part by RA. RA promoted Th2 responses (IL-4, IL-5, IL-10) and inhibited Th1 responses (IFN- γ) in vitro and in vivo (33, 34). T cells treated in vitro with RA
produced less IFN-γ (34) while T cells from A- mice overproduced IFN-γ (35) and under-produced regulatory cytokines (IL-4, IL-10) (36). Additionally, RA treatment of A- T cells resulted in decreased IFN-γ transcription (36). However, a recent study showed that RA signaling was needed for T-bet expression in memory CD4+ T cells (37). Expression of a dominant negative RAR (dnRAR) in T cells (i.e. inhibition of RA signaling in T cells) caused T cells to express high levels of IL-17 and express both IL-17 and IFN-γ (37). Additionally, when dnRAR T cells were transferred into Rag knockout (Rag KO) mice (mice that do not have T or B cells), the RagKO mice that received the dnRAR T cells developed more severe colitis due to the dnRAR T cells overproducing IL-17 and IL-17/IFN-γ (37). This new report is consistent with RA inhibiting IL-17 and IFN-γ. Overall, the literature suggests a regulatory role for RA in maintaining a balance between inflammatory and regulatory cytokine production.

In addition to maintaining cytokine balance, RA can regulate the balance between Th17 and Treg cells. RA, in combination with TGF-β, inhibited Th17 cell induction from naïve CD4+ T cells *in vitro* and *in vivo* while promoting FoxP3 expression and IL-10 production from Treg cells (38, 39). Furthermore, RA inhibited IL-6 mediated Th17 conversion, further promoting FoxP3+ Treg cell differentiation (40). RA regulation of FoxP3 expression is largely mediated through RAR/RXR binding at the FoxP3 promoter region (41). Maintaining balance between Th17 and Treg cell responses is critical for ensuring that the immune system responds correctly to stimuli and RA is a mediator of that balance (42-44). The literature suggests that RA is an important mediator of immune response balance.
The mucosal immune system

The intestine is a complex environment that is constantly exposed to dietary and bacterial antigens. The local immune system must distinguish between commensals and pathogens by reacting effectively against pathogens but benignly to commensals. The gut epithelial cells, microbiota and innate and adaptive immune systems must work in conjunction to protect the host from breaches in the gastrointestinal barrier.

The physical barrier created in the gut is the absolute first line of host defense in the intestine. A thick layer of mucus secreted by goblet cells (specialized epithelial cells) separates the microbiota and gut lumen from the host epithelial cells. Some epithelial cells produce anti-microbial peptides, further bolstering the physical defense between host and microbe. Furthermore, innate and innate-like immune cells secrete cytokines that promote gut epithelial cell production of anti-microbial peptides. Maintaining the physical barrier between host and intestinal lumen is vital for host survival.

The immune compartments associated with intestinal immunity have been termed gut associated lymphoid tissue (GALT). T cells in the gut reside in the mesenteric lymph nodes (MLN), Peyers patches (45), and within the intraepithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) compartments of the small intestine (SI) and colon (46). Additionally, other immune cells such as dendritic cells reside in the GALT. Dendritic cells (DCs) in the MLN, PP and LPL bridge the gap between innate and adaptive immunity by initiating T cell responses (47). The DC presents antigen to the T cell (47). In addition, the DC also provides co-
stimulation and cytokine signals that regulate T cell development into the effector T cell types (47). The relationship between T cells and DCs is an important mediator of immunity in the gut because these cells within the GALT are the first immune cells to interact with pathogens that breach the host through the breaching the intestinal epithelial barrier (48). The adaptive immune cells contained within these GALT compartments are essential for maintaining a healthy host.

Retinoic acid and immune cell trafficking

One of the major functions of RA is the promotion of immune cell trafficking to the mucosa. In a pivotal study, Iwata and colleagues determined that RA treatment of T cells induced expression of the gut homing receptors, CCR9 and α4β7, on CD4+ T cells (44). CCR9 interacts with CCL25 on intestinal epithelial cells while α4β7 mediates cell trafficking to the intestinal endothelium though interactions with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (11). Additionally, RA induced CCR9 and α4β7 on other immune cells (49, 50). In support of the observation that RA is important for immune homing to the gut, multiple groups have reported that A- animals had fewer effector T cells in the gut and decreased gut homing receptor expression (49, 51-53). Surprisingly, RA signaling was not needed for homing since dnRAR T cells homed effectively to the gut of RagKO mice (37). RA is an important metabolite for host defense.
**Retinoic acid and barrier function**

RA affects gut barrier function by supporting intestinal barrier integrity. VAD in humans was associated with increased gut permeability (54) and vitamin A supplementation was associated with improved gut integrity (55-57). Additionally, studies done in animals models have shown that A- status resulted in increased gut permeability and decreased goblet cell function (58-63). RA is important for maintaining epithelial barrier in the GI tract.

When the epithelial barrier is breached, RA can promote epithelial repair. During colitis, RA was bound to RAR/RXR and the IL-22 promoter inducing IL-22 production in innate lymphoid cells and γδ T cells (64). IL-22 is an important cytokine for barrier function that largely targets epithelial cell to promotes tissue regeneration (65) and is required for survival during enteric infection (66). These studies suggest that RA plays a role in gut epithelial repair.

Once the epithelial barrier has been breached, immune cells respond. GALT DCs are one of the first responders. These cells express CD103+ and have been shown to express RA producing enzymes unlike their splenic counterparts (51, 67). The RA produced by these DCs induced expression of the gut homing receptors, CCR9 and α4β7, on T cells (51, 68, 69). Local production of RA, in conjunction with the presence of TFG-β resulted in an environment that promoted Treg cell development and IL-10 secretion (67, 70). Furthermore, DCs isolated from the GALT of A- mice promoted the differentiation of Th17 cells in vitro (71). The SI has a relatively high concentration of RA as determined by liquid chromatography–mass
spectrometry (11, 72). These findings support a role for RA in the promotion of barrier functions.

**Retinoic acid and the balance of mucosal Th17 and Treg cells**

The balance between Th17 and Treg cells is highly important in the gut because both are needed for host defense. If the Th17 response dominates over the Treg response, excess inflammation and damage to the host can occur. However if there is too much inhibition of inflammation, then pathogens can escape the gut and become systemic, leading to sepsis. RA plays a role in maintaining the balance between Th17 and Treg cells.

The effects of RA on Th17 cells are still not completely understood. Many *in vitro* studies have shown that RA inhibited Th17 cell induction from naïve CD4+ T cells (reviewed in (8)). Concentrations used in cell culture studies range from 10nM to 1uM and result in suppressed Th17 cell induction and reduced IFN-\(\gamma\) and IL-17 secretion *in vitro* (11, 73, 74). Paradoxically, multiple groups have shown that A-mice have almost no Th17 cells (73, 75, 76). In support of this observation, two studies reported that 1-30nM RA *in vitro* is needed for IL-17 production from T cells (73, 74) and upregulation of CCR9 and \(\alpha4\beta7\) expression on IL-17 producing T cells (73), indicating that these concentrations of RA may promote Th17 cell induction. Additionally, species of the gut microbiota can induce Th17 cell generation, indicating that the intestinal environment may be a factor regarding the induction of Th17 cells (77, 78). While there is no evidence that shows that RA directly affects the microbiota (as bacteria do not have RARs or RXRs), the combination of local RA
in low concentrations (<30nM) and the presence of the microbiota may result in different regulation of effector T cell differentiation in vivo. These data indicate that the local environment and the concentration of RA may have different effects on Th17 cells in vitro and in vivo.

Genetic mouse models have led to insight as to how RA signaling regulates Th17 and Treg cells. Reports showed that Th17 cell differentiation is impaired in RARα-deficient and A- CD4+ T cells, indicating that RA is needed for the development of Th17 cells (75, 76). Conversely, when T cells expressed the dnRAR (inhibited RA signaling in only T cells), they had a propensity to secrete more IL-17 than IFN-γ (37). dnRAR CD4+ T cells trafficked to the gut and caused more intestinal inflammation when transferred into RagKO mice compared to WT cells (37). More work is needed to fully understand how RA-RAR signaling affect Th17 cells in vivo.

T reg cell are critical to dampen the immune response so that the host is not damaged by overactive inflammation. Treg cells have suppressive function, such as inhibition of effector cell proliferation, secretion of regulatory cytokines and the ability to traffic to the gut through CCR9 and α4β7 (79). CD103+ DCs can promote the conversion of naïve CD4+ T cells to Treg cells in the gut (67). RA induced Tregs are more suppressive during antigen specific CD8+ T cell driven acute intestinal inflammation than Tregs induced without RA (80). However suppression following CD4+ T cell transfer was no different between Treg cells induced with or without RA in vitro (80). Interestingly, FoxP3+ T cell frequencies were not different in WT or dnRAR T cells (37). Overall, RA promotes Treg cell induction in vitro however, the
role of RA in the promotion of Treg cell differentiation *in vivo* is less clear. Currently, the effects of RA and RA signaling on Th17 and Treg cells are not completely understood.

**Vitamin A and disease outcomes**

Due to its immune-regulatory functions, vitamin A is sometimes termed “the anti-infective vitamin” (81, 82). In humans, VA supplementation was associated with improved recovery from a wide variety of diseases, including malaria and measles (83). Many studies looking at the interaction between VAD and human infections have shown that VAD was associated with exacerbated disease (84). In HIV-1 infected humans, vitamin A supplementation decreased vertical transmission of HIV-1 from mother to child (83, 85, 86) and decreased HIV-1 associated mortality in children (83) (87). High dose vitamin A supplementation promoted recovery from measles and decreased severity of malaria infection in young children (83, 88). However, the effects of vitamin A supplementation in respiratory diseases were conflicting as to whether vitamin A supplementation was beneficial, detrimental or not different (83). Laboratory models of vitamin A deficiency indicated that multiple immune responses were impaired in A- hosts. A- rodents had impaired antibody responses and more Th1 cells and fewer Th2 cells (89-92). Conversely, A- mice had decreased Th1 and Th17 responses following infection with *Toxoplasma gondii* (75). RA treatment *in vivo* restored IFN-y production in A- *T. gondii* infected mice (75). RA treatment *in vitro* was able to inhibit effector and memory T cell cytokine production (93). The data suggest a role for vitamin A in host resistance to infection.
Vitamin A and gastrointestinal inflammation

Gastrointestinal infections are also regulated by vitamin A. A- mice developed a more severe enterohemorrhagic *E. coli* infection compared to A+ mice (94). Furthermore, RA treatment reduced colonic inflammation caused by dextran sodium sulfate (DSS) and *Citrobacter rodentium* infection (64). RA treatment during chemical and bacterial inflammation inhibited production of IL-17 and IFN-γ (64) (76). In addition to inhibiting inflammatory cytokine production, RA treatment increased anti-microbial peptide production and IL-22 production which corresponded with decreased inflammation (64, 76). During *C. rodentium* infection, A+ mice cleared the infection more quickly than A- mice (95, 96). VA supplementation shortened the duration of enterotoxigenic *E. coli* infection in children (97). These data suggest that vitamin A is a critical factor in limiting intestinal inflammation.

*Citrobacter rodentium*: disease and pathogenesis

*C. rodentium* is a mouse pathogen that models human infections with enteropathogenic *Escherichia coli* by causing attaching and effacing lesions of the cecum and colon in mice (98). *C. rodentium* is transmitted via the fecal-oral route (as mice are known to be coprophagic) and causes inflammation of the colon. The severity of *C. rodentium* infection can range from a self-limiting colitis to severe inflammation and death, usually due to dehydration (99-102). This mouse model of infection is widely used to study host-pathogen interactions in the gastrointestinal tract.
C. rodentium infection with laboratory grown bacteria is a highly reproducible infection model. Following colonization, C. rodentium becomes more virulent, adapting to the host and becoming more infectious to naïve animals (102, 103). This adaptation allows C. rodentium to colonize the colon and rectum. By day 3 post infection, the bacteria moves to the colon (100). Fecal shedding of C. rodentium peaks between day 7 and 14 post-infection. Crypt hyperplasia develops and effector immune cell infiltration occurs during this time period as well. During the peak of infection, mice can exhibit weight loss and diarrhea (98, 100). Resistant mouse strains clear the infection by day 30 post infection, while susceptible strains succumb to persistent infections without clearing the pathogen (98). C. rodentium is a useful model for studying host/pathogen interactions and mucosal immunity.

**Immunity to Citrobacter rodentium**

The innate immune system is required for early protection as demonstrated in RagKO mice lacking T and B cells; these mice were unable to clear the bacteria and eventually succumbed to the infection (104). Macrophages and innate lymphoid cells produce IL-22 early during infection which promoted epithelial repair (100, 105, 106). IL-22 KO mice developed fatal infections within the first two weeks of C. rodentium infection (66). Furthermore, IL-22 production induced an essential adaptive Th17 responses, leading to clearance of C. rodentium (100, 105).

T cells and B cells are essential for resolution of C. rodentium infection since mice without T cells (CD4 KO) or B cells (Igμ KO) developed fatal infections (100). Furthermore, T cell dependent antibody, Th1 and Th17 responses were required for
bacterial clearance (99) (107). IFN-γ KO and IL-17 KO mice succumbed to lethal C. 
*rodentium* infections (38, 108, 109). Together, the innate and adaptive immune 
responses are required for protection from *C. rodentium*.

**Objectives**

In this dissertation, the effects of host vitamin A status and RA 
supplementation on host defense to a bacterial infection with *C. rodentium* was 
investigated. Some reports have shown that RA inhibits IFN-γ and IL-17 production 
in *vitro* and *in vivo* from T cells however, other studies indicate that RA is critical for 
Th1 and Th17 cell responses *in vivo*. It was unknown how the vitamin A deficient 
host would respond to a *C. rodentium* infection, which requires Th1/Th17 immune 
responses for clearance. Based on experiments that used A- mice and RA dosing in 
models of gut inflammation, we hypothesized that the A- mice might be resistant to 
*C. rodentium* infection. In Chapter 2, we tested the susceptibility of A- mice to *C. 
rodentium* infection. In Chapter 3, we determined some of the RA targets in the gut 
mucosa that would mediate host resistance to *C. rodentium* infection. The last 
Chapter summarizes the major findings and puts the work in the context of the 
growing literature on the role of vitamin A in regulating mucosal T cells in the gut.
REFERENCES:


Figure 1-1. Vitamin A metabolism.
A) Vitamin A is absorbed from the diet and converted into a storable, stable form of vitamin A, retinol. Retinol is transported to and stored in the liver. It can be mobilized and released into the bloodstream for cell utilization.
Adapted from Perusek and Maeda. Nutrients 2013, 5, 2646-2666.

B) When retinol enters the target cell, it is converted into the active form, retinoic acid (RA). RA translocates to the nucleus where it binds the retinoid receptors which regulate gene transcription.
Adapted from Maden, M. Nat Rev Neurosci 2002. 3: 843-853.
Chapter 2

Vitamin A deficient hosts become non-symptomatic reservoirs of *Escherichia coli*-like enteric infections.

Chapter adapted from the manuscript entitled:

“Vitamin A deficient hosts become non-symptomatic reservoirs of *Escherichia coli*-like enteric infections.”

Authors: **Kaitlin L. McDaniel**, Katherine H. Restori, Jeffery W. Dodds, Mary J. Kennett¹, A. Catharine Ross and Margherita T. Cantorna

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ABSTRACT

Vitamin A deficiency (A-) remains a public health concern in developing countries and is associated with increased susceptibility to infection. Citrobacter rodentium was used to model human Escherichia coli infections. A- mice developed a severe and lethal (40%) infection. Vitamin A sufficient (A+) mice survived and cleared the infection by d25. Retinoic acid treatment of A- mice at the peak of the infection eliminated C. rodentium within 16d. Inflammation was not different in A+ and A- colons although the A- mice were still infected at d37. Increased mortality of A- mice was not due to systemic cytokine production, an inability to clear systemic C. rodentium or increased virulence. Instead A- mice developed a severe gut infection with most of the A- mice surviving and resolving inflammation but not eliminating the infection.

Improvements in vitamin A status might decrease susceptibility to enteric pathogens and eliminate potential carriers from spreading infection to susceptible populations.
INTRODUCTION

Vitamin A deficiency is a significant problem in developing countries where inadequate micronutrient intake remains a public health concern (1). The World Health Organization estimates that over 20% of preschool aged children are clinically vitamin A deficient (1, 2). Vitamin A deficiency contributes to the higher prevalence of respiratory and diarrheal diseases as well as increased childhood mortality. Conversely, vitamin A supplementation is practiced as a means to reduce mortality in preschool-aged children by reducing severity of infectious diseases (3).

Vitamin A and its active metabolite, retinoic acid (RA) are important regulators of T cell responses. RA inhibited IFN-γ production from T cells in vitro (4). In addition, T cells from vitamin A deficient (A-) mice overproduced IFN-γ (5). RA also inhibited Th17 cells in vitro and in vivo (6). RA increased the expression of the gut homing receptors, α4β7 and CCR9, on T cells (7), which recruits T cells to the gut mucosa. In vitro, RA inhibited IL-17 and induced expression of the transcription factor FoxP3, associated with regulatory T cells, and IL-10 production (8). Vitamin A and RA are key regulators of T cell cytokine production and gut homing.

Several lines of experimental evidence support a beneficial effect of vitamin A and RA on the host response to infection (9, 10). In the gut, the mechanisms that account for the anti-infective effects of vitamin A include support of B cell function and T cell-dependent B-cell antibody responses (11). Gut infection of A- mice with Trichinella spiralis resulted in T cells that produced IFN-γ but not IL-4, and as a result, reduced the rate of parasite clearance (9). Furthermore, RA treatment reduced colonic inflammation caused by dextran sodium sulfate and infection (12). The reduction in gastrointestinal
(GI) inflammation with RA treatment was attributed to the inhibition of IL-17 and IFN-γ (12, 13). These data suggest that vitamin A and RA regulate T cell function to limit inflammation following chemical and infectious injury in the gut.

*Citrobacter rodentium* is a mouse pathogen that models human infections with enteropathogenic *Escherichia coli* and causes attaching and effacing lesions of the cecum and colon in mice (14). The natural route of *C. rodentium* transmission is fecal-oral. Resistant mouse strains including C57BL/6 mice are infected transiently with *C. rodentium* and clear the infection within 2-3 weeks (14). The acquired immune system was required for early protection from *C. rodentium*, as demonstrated in recombination-activating gene (Rag) knockout (KO) mice lacking T and B cells that were unable to clear the infection (15). Robust IL-22 production from innate lymphoid cells and macrophages has been shown to induce protective Th17 responses that resulted in clearance (16, 17). T cells and B cells were essential for resolution of *C. rodentium* infection since mice without T cells (CD4 KO) or B cells (Igμ KO) developed fatal infections (17). Host resistance to *C. rodentium* depends on IL-22 production from innate cells, T cells, B cells and Th17 cells.

Here, we determined the effect of vitamin A deficiency on host resistance to *C. rodentium* infection in C57BL/6 mice; a normally resistant mouse strain. Because of the well-demonstrated inhibitory effects of RA on the differentiation of Th1 and Th17 cells, we predicted that a bacterial infection that required Th17 cell responses for resistance might be less severe in A- mice, and exacerbated in RA-treated mice. Interestingly and contrary to expectations, A- mice developed a chronic infection with *C. rodentium*. The
C. rodentium infection was lethal for 40% of A- mice while none of the vitamin A sufficient (A+) or RA treated A- mice died prematurely from infection.
MATERIALS AND METHODS

Mice. C57BL6 mice were originally from Jackson Laboratories (Bar Harbor, MN) and bred at the Pennsylvania State University (University Park, PA) for experiments. Vitamin A deficient (A-) and vitamin A sufficient (A+) mice generated as previously described (5, 18). Briefly, mice were fed a purified diet that did not contain any vitamin A (A-) or that contained 25 µg of retinyl acetate (vitamin A) per day (A+). At weaning, mice were continuously fed the A- or A+ diet until the end of the study. Serum retinol status was determined by ultra pressure liquid chromatography at 6-7 weeks of age in pooled samples. For some experiments, A- mice were treated with 37.5 µg of all-trans RA (Sigma Aldrich, St Louis, MO) administered orally in 10 µl corn oil three times per week (19). For some experiments, mice were injected i.p. with E. coli O111:B4 LPS (6 mg/kg, Sigma-Aldrich). Experimental procedures were approved by the Office of Research Protection Institutional Animal Care and Use Committee of the Pennsylvania State University, University Park, PA.

C. rodentium infection. C. rodentium strain ICC169 (nalidixic acid resistant) and bioluminescent strain ICC180 (kanamycin resistant) were kind gifts of Gad Frankel (London School of Medicine and Dentistry, London UK). C. rodentium ICC169 was cultured in Luria-Bertani (LB, EMD Chemicals, Inc., Gibbstown, NJ) broth containing 50 µg/ml nalidixic acid (Sigma-Aldrich) while, C. rodentium ICC180 was cultured in LB broth containing 100 µg/ml kanamycin (Sigma-Aldrich). Mice 8-10 weeks of age were infected by oral gavage with 5×10⁹ CFU in 200 µl of C. rodentium strain unless otherwise noted. For studies looking at in vivo infection kinetic, 5×10⁹ CFU C. rodentium ICC180 was used. Animals were imaged every other day using the IVIS50
small animal imaging system (Xenogen Corp., Alameda, CA, USA). Images were analyzed by Living Image software (PerkinElmer, Waltham, MA). Additional groups of mice were injected i.v. with 10⁴-10⁸ CFU of *C. rodentium* strain ICC169. Feces and other tissues were collected, homogenized and plated in serial dilutions on LB agar plates containing naladixic acid.

For most experiments mice were housed 1 per cage from the time of infection to prevent transmission from mouse to mouse. Natural transmission experiments were performed as previously described (20). Briefly, A-, RA d0, and A+ mice were infected via oral gavage with 5×10⁹ CFU. Three days post-infection, each infected “seed” mouse was co-housed with two naive A+ mice.

**Histology**: Distal colon sections were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin (Pennsylvania State University Animal Diagnostic Laboratory, University Park, PA). Specimens were coded and evaluated in a blinded fashion by a board certified laboratory animal veterinarian with training in pathology. Crypt measurements were taken at 100X using the cellSens software (Olympus Corp., Center Valley, PA, USA). Sections were scored on a scale from 0 to 4 as follows: severity of inflammation (0 = none, 1= minimal, 2= mild, 3= moderate, 4= extensive); epithelial sloughing (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4= extensive); distention of muscularis (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = extensive); edema (0 = none, 1 = minimal, 2= mild, 3 = moderate, 4 = extensive). Total histology scores were generated by adding the scores for each category together, generating a value from 0-16 for each sample.

**Flow cytometry**: Colonic IELs were isolated as previously described and stained for
flow cytometry (21). Cells were counted and stained with PE-Cy5 TCR β (BD Pharmingen, San Jose, CA USA), FITC CD8 β (eBioscience San Diego, CA, USA), PE-Cy7 CD8 α (BioLegend, San Diego, CA, USA) or PE Texas Red CD4 (Invitrogen, Carlsbad, CA, USA). Cells were analyzed on a FC500 Bench top cytometer (Beckman Coulter, Brea, CA, USA) and data was analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA).

**ELISA:** Cytokine production in the serum was measured for TNF-α, IL-1β and IFN-γ levels by ELISA and following the manufacturer’s instructions (BD Biosciences, Minneapolis, MN).

**Statistical Analysis:** Statistical analyses were performed using GraphPad Prism software (Graphpad, La Jolla, CA, USA). Two-tailed Student’s *t*-tests were used for serum retinol analysis. One-way ANOVA with Tukey’s post-hoc test were used to compare the systemic *C. rodentium* loads, bioluminescence quantification, and cell population analysis. Two-way ANOVA with Bonferonni’s post-hoc test were used to compare CFU, histology scores and crypt lengths. Log-rank tests were used for the survival curves and ratios of serum cytokine producers. For all analyses, *P*<0.05 was used as the limit for significance.
RESULTS

A- mice are more susceptible to C. rodentium infection than A+ mice. A+ and A- mice were generated as previously described (5, 18). As expected (22), 6-7 week old A-mice had significantly lower serum retinol than A+ mice (Fig. 2-1A). Bacterial fecal shedding in A+ mice peaked around d10 and cleared within 25 days post-infection (Fig. 2-1B). The infection in A- mice followed the same kinetics as A+ mice until d14. A+ and A- mice had similar numbers of C. rodentium in the cecum and feces at d10 post-infection (cecum data not shown and Fig. 2-1A). After d14 the A- mice continued to shed high numbers of C. rodentium in their feces, whereas the A+ mice began to clear the infection (Fig. 2-1B). All of the infected mice showed a small amount of weight loss (5% of starting weight) within the first 2-4 days of infection but all of the A+ mice recovered and no A+ mice died following C. rodentium infection (Fig. 1C). Conversely, some of the A- mice failed to recover and instead lost significantly more of their initial body weight (10-20%), which resulted in the premature lethality of 40% of the A- mice (Fig. 2-1C). The A- mice that survived did not lose any more weight than the A+ mice, had normal exploratory behaviors (data not shown) and otherwise were undistinguishable from their A+ infected counterparts. A subset of A- mice die following weight loss and the remaining mice resume normal behaviors although they have not cleared C. rodentium.

To visualize the intestinal passage of C. rodentium in vivo, we made use of a bioluminescent C. rodentium strain, and live animal imaging. Between d2 and d6 post-infection, low levels of C. rodentium bioluminescence were detected in the upper parts of the gastrointestinal (GI) tract of A+ mice (Fig. 2-1D). Just before the peak in fecal
sheding (Fig. 2-1B) the intensity of bioluminescence increased and moved into the lower GI tract of A+ mice (Fig. 2-1D). After d10 of infection, the intensity decreased in A+ mice and by d14, when fecal shedding of \textit{C. rodentium} was on the decline in A+ mice, less bioluminescence was detected in the intact A+ animals (Fig. 2-1D). The kinetics of \textit{C. rodentium} transit in the A+ mice was similar to that reported previously in wild-type mice (23). The bioluminescence in the A- mice matched that of the A+ mice at d2 (Fig. 2-1D). However, as early as d4, the A- mice showed bioluminescence throughout the GI tract, which persisted in intensity through d14 (Fig. 2-1D). Unlike the A+ mice, the intensity of bioluminescence in A- mice between d4 and d14 was high throughout the GI tract and significantly higher than in A+ mice (Fig. 2-1D and Fig. 2-2). Thus, the transit of \textit{C. rodentium} through the GI tract occurred more rapidly and persisted for longer in A- than A+ mice and reflected the fecal shedding results.

\textbf{T cell frequencies are lower in the vitamin A deficient gut.} To determine if vitamin A status causes differences in gut mucosal immune cell populations, the intraepithelial lymphocyte (IEL) populations of the colon were characterized. IELs are in direct contact with the intestinal epithelium and a source of T cells from the colon (24). In addition, the T cells in the IEL are in close contact with the microbiota and enteric pathogens in the colon. Fewer total IEL cells were isolated from the colons of uninfected A- versus A+ mice (Fig. 2-3A). \textit{C. rodentium} infection resulted in a significant increase in the number of cells in the colon of both A- and A+ mice (Fig. 2-3A). Interestingly, although the A+ mice resolved the infection by d37, as shown in Fig. 2-1B, the numbers of T cells (TCRβ+) did not return to baseline by d37 pi (Fig. 2-3A and 2-3B). The total number of IELs and TCRβ+ T cells in the colon of A- mice increased following infection but never
reached the levels present in infected A+ mice (Fig. 2-3A). The numbers of TCRβ+, TCRβ+/CD8α+, and TCRβ+/CD8αβ+ T cells in the colon IEL compartment were lower in A- than in A+ mice, both before and after infection (Fig. 2-3A-E). Therefore, although the increases in total cell numbers and T cell subpopulations were similar in A+ and A- mice, the A- mice consistently had fewer T cells in the colonic IEL compartment compared to A+ mice. Differences in T cell numbers and populations between A+ and A- mice were not associated with clearance of *C. rodentium* in A+ mice and persistence of *C. rodentium* in A- mice.

**Exacerbated inflammation and epithelial hyperplasia in A- mice following *C. rodentium* infection.** Histopathology sections of the colon were evaluated before and after infection for signs of inflammation, tissue damage and hyperplasia, including measurements of crypt length. Before infection, A+ and A- mice had low histopathology scores that did not differ with vitamin A status (Fig. 2-3F and Fig. 2-4). Crypt lengths also did not differ between uninfected A+ and A- mice (Fig. 2-3G). After infection, the histopathology scores of A+ colons did not change significantly, either at peak infection (d10) or after resolution of infection (d37) (Fig. 2-3F and 2-4). In A- mice, histopathology scores were significantly higher at d10 post-infection, both compared with baseline A- scores and scores for A+ mice at d10 (Fig. 2-3F and 2-4). By d37, the histopathology scores of A+ and A- mice were the same in spite of the fact that A+ mice had cleared the infection, while A- mice had not (Fig. 2-3F and 2-4). Crypt length in the A+ mice was not affected by infection (Fig. 2-3G). Crypt length in the A- mice increased significantly after infection and were significantly longer on both d10 and d37 than at baseline and as compared to the A+ mice at all time points (Fig. 2-3G). Overall, although
A- mice exhibited more inflammation than A+ mice at d10 post-infection the effect was not present at d37 even though A- mice continued to harbor *C. rodentium* and A+ mice did not.

**RA treatment of A- mice results in clearance of *C. rodentium***. To determine if RA could rescue the severe infection in A- mice, A- mice were orally dosed with RA. RA dosing began either 2 wks before infection (RA -d14), or on the day of infection (RA d0) (Fig. 3). Once started, RA dosing continued 3 times weekly throughout the remainder of the experiment. Treatment of A- mice with RA, either 2 wks before (RA -d14) or on the day of infection (RA d0), resulted in fecal shedding of *C. rodentium* similar to that of A+ mice (Fig. 1B and Fig. 2-5). In a separate experiment, the RA d0 mice were also infected with bioluminescent *C. rodentium*. The bioluminescence in the RA d0 mice was of a high intensity and resembled that in A- rather than A+ mice (Fig. 2-1D and 2-2). Interestingly, by d14 the intensity of bioluminescence in the RA d0 group was lower and resembled the A+ rather than the A- mice (Fig. 2-1D and 2-2).

To determine whether RA could be used therapeutically in A- mice with an established *C. rodentium* infection, the RA treatment of A- mice was started on d14 post-infection (RA +d14). Day 14 of infection occurs just after the peak of fecal shedding and before A+ mice show a rapid decline in *C. rodentium* shedding (Fig. 2-1B). Treatment of A- mice with RA, three times weekly, beginning on d14 resulted in a gradual decline in fecal shedding of *C. rodentium* (Fig. 2-5), which was significant as early as 4 days following the start of RA treatment, as compared to untreated A- mice (d18 post-infection, Fig. 2-5). As observed in earlier experiments, untreated A- mice maintained a persistent infection while the RA +d14 treated mice cleared the infection 18d after the start of the
RA treatment (d32 post-infection, Fig. 2-5). Thus, RA treatment of A- mice cleared the *C. rodentium* infection, which otherwise was persistent.

**Increased *C. rodentium* CFU in the liver and spleen of A- mice.** At d14 post-infection mice that had been infected with the bioluminescence strain of *C. rodentium* were euthanized to determine which organs harbored *C. rodentium*. In the A+ group, bioluminescence was detected in the colon but not in other parts of the GI tract (Fig. 2-6A). In addition, A+ mice did not have visible bioluminescence in their spleen or liver at d14 post-infection (Fig. 2-6B). A- mice had high levels of bioluminescence in the upper portions of the GI tract that were significantly higher than in A+ mice (Fig. 2-6C). A- mice also had visible bioluminescence at d14 in both the spleen and liver (Fig. 2-7A and Fig. 2-6B). The bioluminescence in the spleen and liver of RA d0 mice was more similar to A+ mice than to A- mice at d14 post-infection (Fig. 2-7A and Fig. 2-6B).

Quantification of the bioluminescence showed that A- mice had higher bioluminescence in the spleen, liver and SI than either the A+ or the RA d0 mice (Fig. 2-7A and Fig. 2-6B).

Next we determined whether viable *C. rodentium* was detectable in the spleen and liver of our mice following gastric infection with *C. rodentium*. Cultures of the spleen and liver at d10 and d14 post-infection showed that A+ mice had detectable *C. rodentium* in their internal organs (Fig. 2-7B, and d14 data not shown). A- mice had significantly more *C. rodentium* in the spleen and liver than A+ mice (Fig. 2-7B), while the spleen and liver of RA d0 mice did not differ from those of A+ mice but were significantly different than those of A- mice (Fig. 2-7B). At d37 of infection the A- mice no longer had *C.
rodentium present in the spleen and liver even though they continued to shed C. rodentium in the feces (data not shown).

**Mortality of A- mice following infection with C. rodentium is not due to over-production of systemic cytokines.** To determine whether A- mice died due to cytokine over-production, serum cytokine levels were determined at before (d0) and at the peak (d10) of infection in A+ and A- mice (Table 2-1). None of the A+ mice had detectable levels of any cytokines before infection. At d0, several of the A- mice had detectable levels of TNF-α, IL-1β or IFN-γ in their serum (Table 2-1). Infection resulted in detectable TNF-α, IL-1β and IFN-γ in the serum of both A+ and A- mice. There was significantly more A- mice with IL-1β in the serum (10 of 19) than A+ (1 of 13) mice at d10 post-infection (Table 2-1). Serum IFN-γ was higher but not significantly different in A- mice compared to A+ mice at d10 post-infection (P=0.0817). To measure the capacity for cytokine production, LPS was injected ip into A- and A+ mice to measure serum cytokine response. A- mice produced significantly more TNF-α than A+ mice following LPS injection, while the IL-1β response was the same in A+ and A- mice (Fig. 2-8A and IL-1β data not shown). Overall A- mice were more likely to have IL-1β detectable in the serum after C. rodentium infection and produced more TNF-α after LPS than A+ mice.

We hypothesized that A- mice were dying following oral challenge with C. rodentium because of systemic spread. We attempted to determine the LD50 of i.v. injected C. rodentium in A+ mice. All of the A+ mice survived an i.v. dose of $10^8$ C. rodentium and readily cleared the bacteria. A- mice also survived an i.v. injection of $10^8$ C. rodentium. A+ mice had the highest number of organisms in the spleen and liver 1d post-i.v.
infection with $10^8$ CFU, which declined at d3 and again at d5 and was completely cleared by d10 post-infection (Fig. 2-8B and 2-8C). The group x time interaction showed no differences in the clearance of i.v. injected *C. rodentium* between A+ and A- mice (Fig. 2-8B and 2-8C). In addition, i.v. injection was not lethal and did not induce a systemic cytokine response in either A+ or A- mice (data not shown).

**Host vitamin A status does not alter *C. rodentium* infectivity.** Previous reports have demonstrated that natural infection of *C. rodentium* to co-housed uninfected mice occurs with several fewer log units of bacteria than with laboratory grown *C. rodentium* (20). We hypothesized that vitamin A and RA were attenuating the infectivity (virulence) of *C. rodentium* and that *C. rodentium* that has been passaged through A- mice might be more virulent. A+, RA d0, and A- seed mice were infected by gavage using laboratory grown *C. rodentium* and 3d later each was co-housed with naïve A+ mice, housed in groups of 2 with the seed mice. As described previously (20), all seed mice, despite their vitamin A status, infected all co-housed naïve A+ mice within 3 days of exposure (data not shown). Culturing of the feces, spleen and liver from the cage mates of A+, A- and RA d0 seed mice showed that there were no differences in the CFU of *C. rodentium* recovered in the spleen, liver and feces via natural transmission from either A+, A- or RA d0 seed mice (Fig. 2-9). Thus the data do not show an effect of vitamin A status and or RA treatment on *C. rodentium* infectivity.
DISCUSSION

A- mice were found to be significantly more susceptible to *C. rodentium* infection compared to A+ mice. The increased susceptibility of A- mice included lethality of 40% of the A- mice by d14. The kinetics of shedding of *C. rodentium* in A+ and A- mice were not different before d14, after which the A+ mice proceeded to clear the infection while the A- mice either died or developed a persistent infection. The data suggest that early innate immune responses are adequate in A- mice during the initial phase of the infection, when fecal bacterial counts are increasing, and instead suggest that A- mice failed to generate an acquired immune response necessary to clear the infection. Our results unexpectedly showed that A- mice became chronic carriers of *C. rodentium*, while no longer showing symptoms (inflammation of the colon, diarrhea etc.). Correcting vitamin A status or treating A- mice with RA effectively eliminated the infection and improved survival of A- mice, suggesting an added benefit not previously recognized for vitamin A and/or RA interventions.

The increased mortality of A- mice following GI infection was not due to the systemic spread of *C. rodentium*, increased systemic cytokine production or the virulence of *C. rodentium*. Others have reported mortality following gavage of *C. rodentium* in mice and have attributed that mortality to systemic spread and poly-microbial sepsis (15, 25, 26). Susceptible strains of mice (C3H/HeJ, C3H/HeOuJ and C3H/HeN) develop a severe infection with 100% lethality due to bacterial translocation from the gut, cytokines in the serum and crypt cell apoptosis (25). By comparison, C57BL/6 mice are relatively resistant to *C. rodentium* (15, 17, 25). For the first time, our data clearly show that *C. rodentium* does not grow following i.v. injection of large numbers of bacteria in either
A+ or A- mice, indicating that vitamin A deficiency does not affect the ability to survive a systemic infection with *C. rodentium*. However, A- mice were more susceptible to oral infection with *C. rodentium*. We necropsied one recently deceased and one moribund A- mouse. Very little food was found in the stomach of either mouse, suggesting that the mice had stopped eating. The necropsy of A- mice failed to identify evidence of systemic infection (data not shown). It has been shown that LPS-induced proinflammatory cytokines such as TNF-α and IL-1β can result in anorexia (27). Our data are consistent with TNF-α and IL-1β induced anorexia, since the A- mice that died had lost significant amounts of weight (10-20% of original body weight). In addition, A- mice had higher TNF-α levels after LPS challenge and were more likely to have detectable IL-1β in their serum after infection than A+ mice. Surviving A- mice may have been just below the threshold of the response and therefore survived the infection-induced anorexia. Based on the inability of *C. rodentium* to grow following an iv injection and the necropsies of moribund and dead A- mice, we concluded that the mortality in our A- mice was not due to sepsis and/or the systemic spread of the infection.

A- mice had reduced numbers of total colonic IELs and all IEL T cell subsets in the colon before infection. Infection induced homing and expansion of T cells in the gut of both A+ and A- mice. Although the A- mice were persistently infected with *C. rodentium*, colonic inflammation as determined by histological score resolved and was not different at d37 from A+ mice. This same phenomenon occurred in germfree mice mono-associated with *C. rodentium* (28). In germfree mice *C. rodentium* infection was not cleared but the inflammatory response in the colon was nevertheless resolved (28). It therefore seems that vitamin A is not required to resolve inflammation in the colon and
that in the absence of vitamin A, T cells are able to arrive and respond to the infection. However, the acquired immune response in the A- mice was ineffective at eliminating the infection. The data suggest a requirement for vitamin A/RA to mount effective protective immunity after the initial infection. Since RA treatments effectively reduced *C. rodentium* numbers in already infected A- mice within 4 days of treatment, it will be of interest to identify the targets of RA in the GI tract for *C. rodentium* clearance.

In our study, A- mice developed persistent and sometimes fatal enteric infections. The cause of the premature lethality of A- mice was not due to sepsis and/or systemic spread of *C. rodentium*. Instead it seems that a high load of *C. rodentium* in the gut of A- mice results in infection-induced anorexia in 40% of the mice. When given early, vitamin A and/or RA interventions protected A- mice from the lethality following *C. rodentium* infection and when administered later RA cleared the persistent infections that occurred in surviving A- mice. Our work has important implications for the developing world where vitamin A deficiency is prevalent. In particular, vitamin A-deficient humans and animals could be reservoirs for *E. coli*-like enteric pathogens. Vitamin A and RA treatments might be useful interventions to decrease morbidity and mortality from enteric infections. We show here a novel and unappreciated role for vitamin A/RA for eliminating persistent enteric infections and show added benefits to improving vitamin A status in the developing world.
REFERENCES:


Figure 2-1. A- mice are more susceptible to *C. rodentium* infection than A+ mice. A) Serum retinol analysis from groups of 6-7 week old A+ and A- mice. Each data point represents the serum retinol values in pooled samples from a different litter of mice (n=10-13 liters). B) *C. rodentium* CFU in the feces. Data is shown as mean ± SEM and 1 representative of 5 independent experiments with n=3-5/group. C) Survival of A- and A+ mice during *C. rodentium* infection with data combined from 5 experiments n=9-15/group. D) Whole body imaging of A+, A-, and RA d0 treated mice infected with bioluminescent *C. rodentium* and imaged. The image shows 1 mouse from a total of 3-4 mice/treatment group. Means of the bioluminescence from all the mice are shown in SFig.1. Two-tailed Student t-test (A), two way ANOVA with Bonferroni post-hoc tests (B), log-rank test (C), *P<0.05, **P<0.01, ***P<0.001
Figure 2-2: Quantification of whole body imaging during *C. rodentium* infection. Quantification of bioluminescence from whole body imaging of A+ A-, and RA d0 imaged at d2-14 post-infection. Data is representative from one set of images with n=3-4/treatment group. Two way ANOVA with Bonferroni post-hoc tests, data marked by “a” is significantly different from data marked by “b” with a P values of P<0.001.
Figure 2-3: T cells in the IEL of the colon from A+ and A- mice before and after infection. A) Immune cell numbers in the colon IEL. B) TCRβ+ T cell numbers in the colon IEL. C) CD8α+ T cell numbers in the colon IEL. D) CD8αβ+ T cell numbers in the colon IEL. E) CD4+ T cells numbers in the colon IEL. Values are the mean ± SEM combined data from 2-3 independent experiments with n=6-15/group. Two way ANOVA with Bonferroni post-hoc tests. Asterisks indicate significant differences between groups at specific days by post test, *P<0.05, **P<0.01, ***P<0.001. F) Histology scores and G) crypt length of colons during infection. Values with different letters are significantly different from each other, P<0.05. A) Two way ANOVA with Bonferroni post-hoc tests, *P<0.05, ***P<0.001.
Figure 2-4: Colon histology from *C. rodentium* infected animals. Representative H&E stained distal colon sections from A+ mice (A-D) and A- mice (E-H). A+ mice: A) d0, score of 4; B) d10, score of 5; C) Magnification of d10; D) d37, score of 3; A- mice: E) d0, score of 5; F) d10, score of 9; G) Magnification of d10; H) d37 (score of 3). Lines for scale = 200um for A, B, D-F, and H. Lines for scale = 100um for C and G. Photographs were taken with an Olympus BX61 camera.
Figure 2-5: RA treatment of A- mice eliminates *C. rodentium* infection. A) CFU in the feces of A+ mice A- mice, A- mice treated with RA for 2wks before infection (RA – d14), A- mice treated with RA on the day of infection (RA d0) and A- mice treated with RA starting on d14 of infection (RA d+14). Values are the mean ± SEM of one representative of 2 independent experiments and n=3-5/group. Two-way ANOVA with Bonferroni post-hoc tests. Values with different letters are significantly different from each other, P<0.01-0.0001.
Figure 2-6: Systemic *C. rodentium* in tissues. Images of (A) GI tract and (B) spleen and liver. (C) Quantification of bioluminescence from the small intestine. One way ANOVA with Tukey post-hoc tests, * P<0.05. n.s. - not significant.
Figure 2-7: Systemic spread of *C. rodentium* following GI infection. Mice were sacrificed at d14 post-infection with the bioluminescent strain of *C. rodentium* and the A) GI tract and B) spleen and liver were imaged and quantitated. An example image is shown in Fig. 2-6. Values are from the images of n=3-4 mice/treatment group. CFU of *C. rodentium* recovered in the B) spleen and liver of d10 infected mice. Values in B) are the mean ± SEM combined from three independent experiments with n=9-15/group. One-way ANOVA with Tukey post-tests.
Table 2-1

TABLE 1 Serum cytokines in A+ and A- mice

<table>
<thead>
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<th>TNF-α A-</th>
<th>IL-1β A+</th>
<th>IL-1β A-</th>
<th>IFN-γ A+</th>
<th>IFN-γ A-</th>
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<td>1/13</td>
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</table>

*a A- value is significantly different than A+ value (P < 0.05).
**Figure 2-8**

The effect of vitamin A status on LPS response and i.v challenge with *C. rodentium*. A) Serum TNF-α in A- and A+ mice treated with LPS. CFU in the B) spleen and C) liver of mice following i.v. injection of *C. rodentium*. Values are the mean ± SEM combined from two-three independent experiments with n=8-9/group/time-point. Two-tailed Student t-test (A), Two-way ANOVA with Bonferroni post-hoc tests (B and C). Asterisks indicate significant differences between groups at specific days by post test.; *P<0.05.
Figure 2-9: Natural transmission of *C. rodentium* is not affected by the host vitamin A status. CFU in the feces A), spleen B), and liver C) of A+ cage mates of A-, RA d0 and A+ laboratory inoculated mice. N=4-6/group. Values are the mean ± SEM combined for two independent experiments. One-way ANOVA with Tukey post-tests.
Chapter 3

Short term retinoic acid treatment promotes mucosal IL-17 expression leading to clearance of enteric infection
ABSTRACT

Retinoic acid (RA), the active metabolite of vitamin A, is an important factor in *Citrobacter rodentium* clearance. One dose of RA at d7 post infection was effective at clearing *C. rodentium* infection in vitamin A deficient (A-) mice. The early effects of RA included induction of *il17a* mRNA expression in the colon and *regIIIy* expression in the GI tract that occurred after 4 days or 2 doses of RA treatment. One important source of IL-17a in the gut are CD4+ T cells. Paradoxically, *in vitro* RA treatment of A- CD4+ T cells inhibited IL-17a secretion. To study the specific effects of RA on T cells, mice expressing a dominant negative form of the retinoic acid receptor (dnRAR) were crossed with Lck-cre mice to generate T-dnRAR mice. T-dnRAR mice had significantly more CD8+ single positive thymocytes and significantly fewer CD8+ T cells in the spleen and mesenteric lymph nodes than WT mice. The targets of RA include T cells, IL-17a production and CD8 T cell development and/or differentiation. In the gut induction of mRNA for IL-17A was associated with the ability of RA treated mice to clear *C. rodentium*. 
INTRODUCTION

Vitamin A deficiency remains a significant problem in the human population, particularly in resource limited countries where nutritional intake is a public health concern (1). Vitamin A deficiency is suggested to contribute to higher prevalence of diarrheal and respiratory diseases which leads to increased childhood mortality and vitamin A supplementation effectively decreased mortality in young children by reducing infection severity (2). Vitamin A deficient (A-) mice treated with retinoic acid (RA), the active form of vitamin A, promoted clearance of an enteric bacterial infection (3). Vitamin A is important for protection against infection but the mechanisms of protection are unclear.

Vitamin A regulates immune cell development, phenotype, function and homing. RA has a wide variety of effects on the immune system including upregulating gut homing receptors, α4β7 and CCR9, on T and B cells (4-6) and balancing inflammatory and regulatory immune responses (7). The balance between inflammatory and regulatory responses are particularly important in the gut because the mucosal immune system has to constantly discriminate pathogen from commensal and self from non-self. Dendritic cells in the gut associated lymphoid tissue (GALT) have the capability to produce RA (4). RA, in conjunction with transforming growth factor-β (TGF-β), promoted FoxP3+ expression in naïve CD4+ T cells (8, 9). Conversely, RA suppressed-6 and TGF-β mediated Th17 cell induction (10, 11). These studies suggest that RA is an important mediator of Th17 and Treg immune responses in vitro. Interestingly, in vivo A- mice had fewer total T cells in the gut and decreased Th17 responses and increased Th1 responses
(12-16). How RA regulates T cells differently in vitro compared to in vivo is not completely understood.

*C. rodentium* is a murine pathogen that models human enteropathogenic *E. coli* infection and colonizes the intestinal epithelium of the colon through attaching and effacing lesions (17). *C. rodentium* must compete with the host microbiota to first establish colonization (17). Following colonization with *C. rodentium*, a protective immune response is mounted, which requires IL-22 production from innate lymphoid cells, macrophages and γδ T cells to promote intestinal epithelial barrier protection and induce an adaptive Th17 cell response (18-22). Additionally, T cells and B cells are required for clearance *C. rodentium* as mice deficient in T cells, B cells, or both develop fatal infections within 20 days of inoculation (21, 23).

We have previously reported the effects of host vitamin A status on *C. rodentium* infection. A+ mice cleared the bacteria but A- mice failed to clear the infection and ~40% of the A- mice died between d7 and d14 post-infection (3). RA treatment before, at the time of infection and during infection promoted *C. rodentium* clearance in the A-host (3). There was no difference in fecal shedding in A+, A-, or RA dosed animals before day 10, which suggested that early innate immunity in the A- mice was adequate. A- mice had significantly fewer T cells in the gut before infection, and although the numbers of T cells increased with infection, A- mice had fewer T cells in the gut throughout the course of infection (3). Other studies have shown that animals with acquired immune defects succumb to *C. rodentium* infection between d7 and d14 post-infection (23). Our studies indicate that A- mice are more susceptible to *C. rodentium*
infection, possibly due to an inability to mount an adequate mucosal acquired immune response.

Our previous study suggested that inadequate acquired T cell responses in A-mice might be the cause of increased susceptibility. Experiments were done to determine the targets of RA that promote clearance of *C. rodentium*. RA treatment starting as early as d7 post-infection was effective for clearance of *C. rodentium*. One dose, two doses and three doses of RA starting at d7 post-infection induced bacterial clearance as well. RA induced *il17a* mRNA expression in the colon within 4 days of treatment that was associated with the successful clearance of *C. rodentium*. Two doses of RA also induced *regIIIy* expression in the GI tract. Interestingly, RA treatment *in vitro* inhibited IFN-γ and IL-17a secretion from A- CD4+ T cells. Expression of the dominant negative RAR (dnRAR) in T cells using an Lck promoter resulted in increased CD8+ single positive cells in the thymus but decreased CD8+ cells in the spleen and mesenteric lymph node (MLN). Other possible targets of RA were B cells and the microbiota however no differences in B cell function or microbiota were detected as a function of vitamin A status and/or RA treatments. Our data indicates that *in vivo* RA treatment promoted IL-17a production in the colon and RA signaling was important in CD8+ T cell development.
MATERIALS AND METHODS

Mice: C57BL6 mice from Jackson Laboratories (Bar Harbor, MN) were bred at the Pennsylvania State University (University Park, PA) for experiments. Vitamin A deficient (A-) and vitamin A sufficient (A+) mice generated as previously described (15, 24). Briefly, mice were fed a purified diet that did not contain any vitamin A (A-) or that contained 25 µg of retinyl acetate (vitamin A) per day (A+). At weaning, mice were continuously fed the A- or A+ diet until the end of the study. Vitamin A status was determined by using ultra pressure liquid chromatography to examine pooled serum retinol levels in 6-7 week old mice. For some experiments, A- mice were treated with 37.5 µg of all-trans RA (Sigma Aldrich, St Louis, MO) administered orally in 10 µl corn oil (25). Wild type (WT) C57Bl6 mice fed standard chow diets were used as controls for microbiota studies.

T-dnRAR mice were generated by crossing Lck-cre mice from Jackson Laboratories (Bar Harbor, MI) and dominant negative RARα mice, a kind gift from Dr. Randolph Noelle (Dartmouth University, Lebanon, NH). Experimental procedures were approved by the Office of Research Protection Institutional Animal Care and Use Committee of the Pennsylvania State University, University Park, PA.

C. rodentium infection: C. rodentium strain ICC169 (nalidixic acid resistant) was a kind gifts of Gad Frankel (London School of Medicine and Dentistry, London UK). C. rodentium ICC169 was cultured in Luria-Bertani (LB, EMD Chemicals, Inc., Gibbstown, NJ) broth containing 50 µg/ml nalidixic acid (Sigma-Aldrich) and mice 6-8 weeks of age were infected by oral gavage with 5×10⁹ CFU in 200 µl. Feces and other tissues were
collected, homogenized and plated in serial dilutions on LB agar plates containing naladixic acid.

**Cell isolation and culture:** Spleens, thymus and MLNs were homogenized and lysed with red blood cell lysis buffer to obtain single-cell suspensions. SI LPLs and colonic LPLs were isolated at previously described and stained for flow cytometry (26). For some experiments, CD4+ T cells from the spleen were purified using mouse CD4 cell recovery column kits following the manufacturer’s instructions (Cedarlane Laboratories Ltd, Burlington, NC). The purity of the CD4+ cells after enrichment was between 75-82% CD4+ cells as determined by flow cytometry.

Cultures included the following concentrations of antibodies: 0.5 μg/ml anti-CD3 alone or 0.5 μg/ml anti-CD3 with 5 μg/ml anti-CD28 (BD Pharmingen, San Diego, CA) in RPMI 1640-C containing 10% FBS (Equitech-Bio, Inc, Kerrville, TX), 2 mM L-glutamine, 5 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA), and 10 μg/ml gentamycin (Teknova, Hollister, CA). Some cultures contained 10nM of all-trans RA.

**ELISAs:** Supernatants were collected for cytokine detection and production of IFN-γ, IL-4, IL-10 and IL-17 (BD Biosciences) were measured by ELISAs following the manufacturer’s instructions. Serum was collected for antibody titer determination. Total IgA production was measured by the Mouse IgA ELISA Quantitation Set (Bethyl Laboratories, Inc, Montgomery, TX). *C. rodentium* specific antibody titers were determined by coating ELISAs plates with 30ng/ml sonicated *C. rodentium*. Horseradish peroxidase conjugated anti-mouse IgG1 and IgG2 (BD Pharmagen) were used as detection antibodies and serially diluted pooled serum samples were used as standards to determine relative values.
Flow cytometry: SI LPLs, colonic LPLs, splenocytes and thymocytes were collected and processed according to standard protocol. Cells were counted and stained with FITC B220, PE CD4, PE-CD19, PE-Cy5 TCR β (BD Pharmingen, San Jose, CA USA) and PE-Cy7 CD8 α (BioLegend, San Diego, CA, USA). Cells were analyzed on a FC500 Bench top cytometer (Beckman Coulter, Brea, CA, USA) and data was analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA).

RT-PCR: Whole tissues were snap frozen and RNA was isolated with TRiZOL (Invitrogen). cDNA was synthesized using TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA). RT-PCR was performed using SYBR green mix (BioRad) and MyiQ Single-Color Real-Time PCR machine (BioRad). Serially diluted standards for each gene of interest were run to determine relative copy number. Expression levels were normalized to the expression of the average of HPRT and GAPDH and values from A+ uninfected tissue samples were set at 1.

For bacterial DNA isolation, feces were collected from A- cagemates or A+ cagemates, or from A- cagemates before and after two weeks of RA treatment (37.5 ug/10ul orally three times a week). DNA was isolated from fecal samples using QIAamp DNA stool minikit (Qiagen, Valeriea, CA). 10 ng/sample was amplified using SYBR green mix (BioRad) and MyiQ Single-Color Real-Time PCR machine (BioRad). Data was analyzed using the ΔΔCT method using 16S as the reference gene and expressed as relative expression of specific bacterial phyla (27) compared to bacterial DNA isolated from mice on standards chow diet set as 1. Primers for all RT-PCRs are in Table 3-1.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism software (Graphpad, La Jolla, CA, USA). Two-way ANOVA with Bonferonni’s post-
tests were used to compare CFUs, systemic bacterial loads and tissue RT-PCR across different dietary groups. Student’s t-tests or nonparametric Mann Whitney tests were used to compare bacterial DNA analysis, cytokine production and immune cell populations between dietary groups or genotypes. For all analyses, $P<0.05$ was used as the limit for significance.
RESULTS

RA treatment of A- mice at d7 promoted clearance.

A- mice failed to clear a *C. rodentium* infection (Figure 3-1A) and previously published (3). RA treatment of A- mice at the time of infection (RA d0) resulted in bacterial clearance (Figure 3-1A) (3). To determine whether RA treatment at the peak of infection was as effective as at the day of infection, A- mice were treated continuously beginning at day 7 post-infection (RA d7). RA d7 treatment was as effective as RA d0 treatment for eliminating *C. rodentium* (Figure 3-1A). To determine how many RA doses were needed to protect A- mice from *C. rodentium*, mice were infected for 7 days and received one dose of RA (RA 1x) at d7, two doses of RA (RA 2x) at d7 and d9, or three doses of RA (RA 3x) on d7, d9 and d11 post-infection. All three dosing schemes resulted in clearance of *C. rodentium* from the A- host (Figure 3-1a and 31B). In one of 2 experiments, the RA 3x treated A- mice cleared the infection by d23 and the 3X RA treatment was as effective as continuous dosing with RA beginning at d0 or d7 (Figure 3-1A and 3-1B). In the second experiment, the RA 3x group was not cleared more quickly than the other groups (Figure 3-1C) and therefore the 1X, 2X and 3X RA treatment groups were equally effective at clearing *C. rodentium* in A- mice. The average day of clearance from the combined experiments was not significantly different between the RA 3x and the RA 1x groups (d22 ±1 vs. d26 ±2, P=0.0892 by Mann-Whitney test). However, both experiments showed that 1 dose of RA given at d7 post-infection effectively cleared *C. rodentium* in the A- host.
RA treatment induced *il17a* expression in the colon.

To determine the short term effects of RA during *C. rodentium* infection, A- mice were infected with *C. rodentium* for 7 days. At d7, A- mice received one oral dose of RA or were left untreated. Half of the mice were sacrificed at d9 post-infection while the remaining were dosed a second time with RA (RA 2X) or remained untreated and then sacrificed at d11 post-infection (Figure 3-2A). The *C. rodentium* CFU in the spleen and liver were not different at d9 or d11 post-infection in the RA 1x, RA 2x and A- mice (Figure 3-2A). Short term RA treatment had no effect on the CFU in the spleen and liver.

To identify potential targets of RA in the colon, mRNA analysis of colon tissues were performed. There were no differences in mRNA for *il17a*, *il22*, *regIIIy*, *foxp3* or *rorc* in the d9 and RA 1x treated mice compared to the A- d9 mRNA values (Figure 3-2B). There were no differences in mRNA for *il22*, *regIIIy*, *foxp3* or *rorc* mRNA expression in the colons of d11 post-infection A- and 2x RA mice (Figure 3-2A). *il17a* mRNA expression was significantly increased in the colon of RA 2x dosed mice compared to A- mice at the same time point (P<0.01) (Figure 3-2A). Two doses of RA induced expression of *il-17a* in the colon of A- mice. In addition, there was a significant interaction effect on *il17a* between the VA groups and time (P=0.0105), suggesting that the group effect differed over time. There was a significant time effect on *il22* expression (P=0.0344), indicating that IL-22 production increased during *C. rodentium* infection but was not induced by oral RA treatment.

mRNA levels were measured in the duodenum and ileum of the small intestine (SI) and colon after RA 2x and at d11 post *C. rodentium* infection. The duodenum contains the highest relative concentration of host produced RA in the GI tract (28) and the ileum is
still colonized with *C. rodentium* at d10 post-infection (3). Expression of *rorc, foxp3, il6, il17a, il22* and *regIIIy* were significantly different based on location in the gut (P<0.0001). We observed a significant increase in *il17a* expression in the colon of RA treated mice (P<0.0286) and a significant interaction effect (P=0.0044) suggesting that the group effect differs over time. Average expression was highest in the ileum and lowest in the duodenum for all studied genes, indicating that gene expression can differ in the GI tract based on location (Figure 3-3). A group effect was observed for *regIIIy* (P=0.0169), indicating that RA treatment induced *regIIIy* expression throughout the GI tract (Figure 3-3).

**Vitamin A and the gut microbiota.**

Previous reports have suggested that the microbiota regulated the ability to produce IL-17 (29). Additionally, one report showed that A- mice had fewer gut microbes in the SI and feces as well as fewer mucosal Th17 cells (16). If the microbiota was different between A+ and A-mice, this could explain the differences in IL-17 production and the inability of the A- mice to clear *C. rodentium*. There were no differences in the bacterial 16S ribosomal content of the feces from the mice as a consequence of vitamin A status (Figure 3-4A). In addition, the feces from A+ and A- mice contained similar ratios of Bacteriodies, Firmicutes, Actinobacter and γ-Proteobacter phyla DNA (Figure 3-4B). Our results indicate that vitamin A status did not affect total bacteria in the feces or the representation of 4 of the common phyla of bacteria found in mice.
Serum antibody levels and B cell numbers were not affected by vitamin A deficiency.

Vitamin A regulates B cell responses (5, 7, 25). We hypothesized that A- mice failed to mount a robust *C. rodentium* specific antibody response that might explain the inability to clear an infection. There was no difference in total serum IgA or *C. rodentium* IgA levels between A- and A+ mice but there was a significant interaction effect (P=0.0400) in total IgA levels, indicating that the group effect differed over time (Figure 3-6A). *C. rodentium* specific IgG1 and IgG2 titers were not affected by host vitamin A status (Figure 3-6B). There was an observed time effect for *C. rodentium* specific IgA (P=0.0005) and IgG2 (P=0.0025), indicating that antigen specific antibody levels increased during infection. Additionally, there was no difference in B cell numbers in the SI LPL (Figure 3-6C) or colonic LPL (Figure 3-6 D). B cell numbers and *C. rodentium* specific antibody responses were not affected by vitamin A.

*In vitro* RA treatment inhibited Th1/Th17 cytokine production from T cells.

As previously published, 10nM RA inhibited IFN-γ secretion from A- splenocytes (Figure 3-6A) (30). IL-17a production from A- and RA treated splenocytes was below the limit of detection. RA treatment of enriched CD4+ T cells from mice significantly inhibited IFN-γ and IL-17a production (Figure 3-6B). IL-4 and IL-10 levels were undetectable in splenocyte and CD4+ T cell cultures (data not shown). Our data confirms data in the literature demonstrating an inhibitory effect of RA on IFN-γ and IL-17 production from T cells *in vitro*.
Thymic development was impaired in mice that express a dnRAR receptor in T cells.

T-dnRAR mice were generated to study the effects of retinoid signaling in T cells. To confirm that the dnRAR was functioning in T cells, splenocytes were stimulated with anti-CD3 antibody and treated with 10nM RA or ethanol as a control. RA treatment significantly inhibited IFN-γ production in WT cells (Figure 3-7). RA treatment had no effect on IFN-γ secretion from anti-CD3 stimulated T-dnRAR splenocytes (Figure 3-7). There was no effect of RA on IL-17a in either the WT or T-dnRAR cultures for these assays (Figure 3-7). These data indicated that T cells from the T-dnRAR mice were unable to respond to RA treatment in vitro.

In the thymus, T cells develop through a double negative (DN) to a double positive (DP) stage and then to single positive either CD4 or CD8 T cells. Frequencies of DN, DP, CD4+ and CD8+ cells from the WT littermates were consistent with other reports (31). WT mice had significantly more total thymocytes than T-dnRAR littermates (Table 3-2, Figure 3-8 A). There were more Tcrβ+ T cells in the WT thymus compared to T-dnRAR mice (Table 3-2). WT mice had more DP and less CD8+ single positive thymocytes than the T-dnRAR mice (Table 3-2, Figure 3-8A). In the periphery, WT mice had significantly increased Tcrβ+ cells and CD8+ T cells in the spleen (Table 3-2, Figure 3-8B). WT mice had more total cells and fewer CD8+ T cells in MLN than the T-dnRAR mice (Table 3-2, Figure 3-8C). There were not differences in B cells (B220+) in the spleen and MLN between WT and T-dnRAR mice (data not shown). Overall, the T-dnRAR mice had fewer T cells in the thymus as well as in the periphery than their WT
counterparts, specifically decreased CD8+ T cells in the spleen and MLN. These data indicate that RA signaling is important for CD8+ T cell development.
DISCUSSION

Here, for the first time, we show that short term treatment with RA during enteric infection promoted bacterial clearance. Additionally, RA treatment of A- mice starting at d7 post-infection was as effective as at the time of infection for clearance of \textit{C. rodentium}. Only 1 dose of RA was required for A- mice to clear a \textit{C. rodentium} infection. Induction of IL-17a mRNA expression in the colon of RA treated mice corresponded with the elimination of \textit{C. rodentium} in the RA treated A- mice. IL-17a was induced in the colon following RA treatment. RA treatments of A- hosts could be effective for eliminating carriers of \textit{Escherichia coli}-like infections in the A- host.

The effect of RA \textit{in vitro} and the effects of vitamin A \textit{in vivo} on IL-17 and Th17 cells is paradoxical. Our data confirms that RA treatment \textit{in vitro} inhibited IL-17a production from A- CD4+ T cells \textit{in vitro}. Previously published \textit{in vitro} studies have shown that RA inhibited Th17 induction when IL-6 was absent (7, 32). Other \textit{in vitro} experiments also showed that 10nM to 1uM RA can inhibited Th17 cell induction and reduced IFN-\gamma and IL-17a secretion (12, 33, 34). Two independent studies have shown that \textit{in vitro} treatment of naive T cells with 1nM RA was needed for IL-17a production (12, 34). All referenced \textit{in vitro} assays used naïve CD4+ T cells but used a variety of mouse strains (C57BL/6 and BALB/c (12), C57BL6 OT-II (34), B6.SJL (13), or AKR/J (12)), indicating that different mouse strains may respond to RA or metabolize vitamin A differently (35). Our data show that RA \textit{in vivo} induced IL-17a expression in the colon of C57BL/6 mice. At this time, we do not know the source of the IL-17a mRNA in the colon of RA treated mice. Several different populations of cells produce IL-17a
including T cells, innate lymphoid cells and monocytes (36). The effects of vitamin A in vivo are not completely understood.

One explanation for the increased IL-17a expression as a result of RA treatment may be the recruitment of Th17 cells from the periphery to the colon. We have observed that A- mice had fewer colonic T cells than A+ mice (3) and it is known that RA upregulates gut homing receptors on T cells in vitro (4). A- mice and retinoic acid receptor (RAR)α deficient mice had very few Th17 cells in the gut (12-14, 16). The decreased Th17 cells in the gut of A- mice was also observed in a genetic mouse model of Crohn’s disease (SAMP1/YP mouse model) however this seemed to have no effect on the model (37). IL-17+ T cells generated in vitro in the presence of 1nM RA expressed high levels of CCR9 and α4β7 (12). Furthermore, when these IL-17+ T cells were transferred into RagKO mice, they trafficked to the gut and caused inflammation (12). If RA was required to get Th17 cells to home to the gut during infection, this could account for the observed increased expression of IL-17a in the RA treated animals compared to the untreated mice.

To further determine possible targets of vitamin A in host resistance to C. rodentium, the microbiota and B cells were examined. Because C. rodentium competes with the commensal microbiota for colonization of the host, we measured 16S DNA and bacteria phyla in the feces of A+ and A- mice. We found no differences in total bacterial DNA. Additionally, we found no effect of vitamin A status on Bacteroidetes, Firmicutes, Actinobacteria or γ-Proteobacteria. Others have reported that A- mice have fewer gut bacteria in the ileum and feces by RT-PCR and bacterial cultures (16). A wide range of factors affect gut microbiota composition (i.e. diet, animal facility, vendors) and these
factors along with experimental differences could account for the different results (38-40). In addition, we found no differences in serum IgA, antigen specific IgA or antigen specific IgG1 and IgG2 antibody levels in A- and A+ mice throughout infection or in B cell numbers in the LP of the GI tract. Currently, our data does not support an effect of vitamin A on the microbiota or B cells in the gut, however the relationship between RA and gut microbiota or RA and mucosal B cells has not yet been thoroughly studied. These data indicate that other acquired immune system components, like T cells, may be a main target of RA during *C. rodentium* infection.

To examine the effects of RA on T cells, we generated T-dnRAR mice. Others have used the dnRAR mouse to inhibit RA signaling in T cells using a CD4-cre mouse (31, 41). Reports using the CD4 cre-dnRAR mice showed that, like in our study, the CD8+ single positive thymocytes frequencies were increased compared to WT littermates (31). However, there were no differences in the CD8+ T cell populations in the periphery compared to WT mice in the CD4 cre-dnRAR mice and we showed that there were fewer CD8+ in the periphery using the Lckcre-dnRAR mouse (31). Memory CD4+ T cell numbers were not different between WT and CD4 cre-dnRAR mice although the CD4cre dnRAR T cells produced more IL-17 and less IFN-γ than the WT T cells (41). By using the Lck cre mouse, we have inhibited RA signaling at the DN stage of development while the groups that use the CD4 cre mouse inhibited retinoid signaling at the DP stage (31, 41). Our data showed that when RA signaling is inhibited in T cells, double positive (CD8+CD4+) thymocyte numbers were significantly decreased but CD8+ thymocyte numbers were significantly increased. The Lck cre dnRAR CD8+ T cells may have a defect in survival, maturation or proliferation. Defects in CD8+ T cells could result in
the inability of host to survive viral or intracellular bacterial infections. Thymic organ culture and flow cytometry showed that in vitro RA treatment of human thymocytes promoted CD4+ thymocyte development and inhibited CD8+ thymocyte development from the DP stage, indicating that RA may affect CD8+ T cell development (42). The data suggest that RA signaling is important for CD8+ T cell development and for maintaining CD8+ T cells periphery.

From this study, we determined that RA treatment of A- mice at d7 post infection was just as effective as RA treatment starting at d0 in clearing C. rodentium infection. Additionally, we found that one dose of RA at d7 post infection eliminated C. rodentium infection from the A- host. One target of RA in the colon that corresponded with C. rodentium clearance was IL-17a mRNA levels. However, we do not know what cells were making IL-17a. Determining which cells were producing IL-17 would be critical to understanding the mechanisms underlying the effects of RA on host resistance in A- mice.. The work shows that RA is a critical regulator of T cell development and the local IL-17 response during C. rodentium infection.
REFERENCES:


Figure 3-1: RA treatment starting at d7 results in similar clearance when RA is given at d0. A) CFU in the feces of A- mice, A- mice treated with RA starting at d7 post infection (RA d7) and A- mice treated with RA on the day of infection (RA d0). Values are the mean ± SEM of one experiment and n=4-5/group. B and C) CFU in the feces of A- mice given one dose of RA at d7 post-infection (RA 1x), one dose of RA at d7 and 9 post-infection (RA 2x), or one dose of RA on d7, 9 and 11 post-infection (RA 3x). Values are the mean ± SEM of one experiment and n=3-4/group. Two-way ANOVA with Bonferroni post-hoc tests. (A)Values with different letters are significantly different from each other, P<0.01-0.0001.
Figure 3-2: Short term RA treatment results in increased *il17a* mRNA. 

A) Experimental timeline B) Spleen and liver CFU and C) RT-PCR of whole colon after RA 1x or RA 2x in A- mice. The RA treatments were started at d7 post-infection. Mice were sacrificed at d9 and d11 post infection. Values are the mean ± SEM of two combined experiments and n=8-10/group. Two-way ANOVA with Bonferroni post-hoc tests.
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<td>3'-GGTAAAGGTTCCTCGATCTAT-5'</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>3'-TGAAACTYAAAGGAATGTGACG-5'</td>
<td>3'-ACCATGACCACCTGTG-5'</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>3'-TACGGCCGCAAGGCTA-5'</td>
<td>3'-TCRCTCCACCTCTCCCGT-5'</td>
</tr>
<tr>
<td>y-Proteobacteria</td>
<td>3'-TCGTGACGCTCCTGTYGTGTA-5'</td>
<td>3'-CGTAAGGGCCATGATG-5'</td>
</tr>
</tbody>
</table>
Figure 3-3: The effects of RA treatment on mRNA expression in duodenum, ileum and colon at d11 post-infection and 2 doses of RA. Whole tissue mRNA was collected from the duodenum, ileum and colon after two RA doses begun at d7 post-infection. Mice were scarified at d11 post-infection. Data is log transformed to account for distribution and combined from two independent experiments with n=5-9/group. Two-way ANOVA with Bonferroni post-hoc tests.
Figure 3-4: The microbiota is not affected by host VA status. RT-PCR analysis of A) fecal 16S bacterial DNA from A+ and A- mice and (B) bacterial phyla from A+ and A- mice. Values are the mean ± SEM of three experiments and n=8-9/group. Mann Whitney test.
Figure 3-5: RA inhibition of IFN-γ and IL-17a in A- whole splenocytes and CD4+ T cells in vitro A) Anti-CD3 stimulated IFN-γ production from A+ and A- splenocytes after 72 hours. A- + RA samples were treated with 10nM RA. B) Anti-CD3/CD28 CD3 stimulated IFN-γ and IL-17a production from A+ and A- CD4+ T cells after 72 hours. A- + RA samples were treated with 10nM RA. Values are the mean + SEM of two combined experiments and n=5-7/group. Student’s t test or Mann Whitney test.
Figure 3-6: Serum antibody titers and GI B cell numbers are not affected by host vitamin A status: A) Total and *C. rodentium* specific IgA titers and B) *C. rodentium* specific IgG1 and IgG2 titers in the serum of A+ and A- mice during infection. Samples were combined from 1-3 experiments, n=4-12/time point/VA group. Two way ANOVA. C) Total SI LPL and B cell number and D) total colonic LPL and B cell numbers. Data are combined from two independent experiments, n=8-10/group. Student’s t-test.
Figure 3-7: RA treatment \textit{in vitro} does not affect T-dnRAR splenocyte response to stimulation. Anti-CD3 stimulated IFN-\( \gamma \) and IL-17a production from WT and T-dnRAR mice after 72 hours with or without 10nM RA treatment. Values are the mean ± SEM from one experiment and \( n=3-4/\text{group} \). Mann Whitney test.
Table 3-2: RA signaling inhibition in T cells leads to immune cell population imbalance in the periphery.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>WT</th>
<th>T-dnRAR</th>
<th>Cell Number x (10^6)</th>
<th>WT</th>
<th>T-dnRAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>8 ± 1</td>
<td>11 ± 2</td>
<td></td>
<td>56 ± 1</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>CD8+</td>
<td>3 ± 0.5</td>
<td>12 ± 2 ***</td>
<td></td>
<td>2 ± 0.5</td>
<td>4.6 ± 0.8 **</td>
</tr>
<tr>
<td>DN (CD8-CD4-)</td>
<td>4 ± 0.5</td>
<td>5 ± 0.5</td>
<td></td>
<td>23 ± 0.5</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>DP (CD8+CD4+)</td>
<td>83 ± 2</td>
<td>67 ± 3 **</td>
<td></td>
<td>60 ± 8</td>
<td>26 ± 3 **</td>
</tr>
<tr>
<td>Tcrβ+</td>
<td>8 ± 1</td>
<td>90 ± 1 *</td>
<td></td>
<td>62 ± 8</td>
<td>34 ± 4 **</td>
</tr>
<tr>
<td>Total cell number</td>
<td></td>
<td></td>
<td></td>
<td>73 ± 10</td>
<td>31 ± 4 **</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcrβ+</td>
<td>40 ± 1</td>
<td>31 ± 12 **</td>
<td></td>
<td>12 ± 2</td>
<td>7.8 ± 0.9 *</td>
</tr>
<tr>
<td>CD4+</td>
<td>52 ± 3</td>
<td>63 ± 2 **</td>
<td></td>
<td>6.5 ± 1</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>CD8+</td>
<td>41 ± 1</td>
<td>33 ± 2 **</td>
<td></td>
<td>5.1 ± 0.7</td>
<td>2.6 ± 0.3 **</td>
</tr>
<tr>
<td>Total cell number</td>
<td></td>
<td></td>
<td></td>
<td>30 ± 6</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Mesenteric Lymph Node</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tcrβ+</td>
<td>84 ± 2</td>
<td>77 ± 1 *</td>
<td></td>
<td>2.5 ± 0.6</td>
<td>1.1 ± 0.2 *</td>
</tr>
<tr>
<td>CD4+</td>
<td>51 ± 3</td>
<td>65 ± 2 **</td>
<td></td>
<td>1.3 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>CD8+</td>
<td>48 ± 3</td>
<td>35 ± 2 **</td>
<td></td>
<td>1.2 ± 0.3</td>
<td>0.4 ± 0.07 **</td>
</tr>
<tr>
<td>Total cell number</td>
<td></td>
<td></td>
<td></td>
<td>3.2 ± 0.7</td>
<td>1.5 ± 0.3 *</td>
</tr>
</tbody>
</table>

Values are the frequencies as percentiles. Frequency and cell numbers are expressed as means + SEM for 5-10 mice/group. Data is combined from two independent experiments. CD4 and CD8 frequencies are expressed as percentages of Tcrβ+ populations. Gating strategy can be found in Figure 3-9. * P<0.05, ** P<0.01, *** P<0.001 by Student’s t-test or Mann Whitney test.
Figure 3-8: RA signaling inhibition in T cells leads to increased CD8+ T cells in the thymus and decreased CD8+ T cells in the periphery. Graphical data from Table 3-2. A) Total thymocyte cell number, DP cell number and CD8+ T cell numbers from the thymus. B) Total cell and CD8+ T cell numbers from the spleen. C) Total cell and CD8+ T cell numbers from the MLN.
Figure 3-9: Gating strategy for flow cytometry analysis. A) shows gating strategy for analysis of thymocytes. B) shows gating strategy for analysis of spleen and MLN.
Chapter 4

Summary and conclusions
The role of vitamin A in enteric infection was investigated in this thesis. In Chapter 2, the effect of host vitamin A status during *Citrobacter rodentium* infection was evaluated. While it has been known that vitamin A deficiency is associated with respiratory and diarrheal diseases in children (1, 2), the effects of vitamin A during *C. rodentium* infection were unknown. I show here that A- mice failed to clear *C. rodentium* infection. T cell numbers were decreased in the colon of A- mice compared to A+ mice. Additionally, my work showed that RA treatment before infection, at the time of infection and during infection eliminated the pathogen from the host. I observed 40% mortality in A- mice following *C. rodentium* infection. Death following *C. rodentium* infection had been attributed to sepsis (3-5), however I did not find elevated serum cytokines in the A- mice compared to A+ mice. Furthermore, necropsies of moribund mice did not show signs of sepsis but did find indicators of severe gut damage. Together the data suggests that the *C. rodentium* related mortality in the A- host was not caused by sepsis. However, this manuscript did not determine why the A- mice did not clear *C. rodentium* or how RA treatment promoted infection clearance in A- mice. The major conclusions from the first manuscript were that A- mice are susceptible to *C. rodentium* infection and RA can be given before, at the time of or during infection to promote survival and clearance of the infection. The work demonstrated a new role for vitamin A for eliminating possible reservoirs of enteric bacteria in A- hosts (6).

The timing of RA treatment and the mechanisms by which RA regulated host resistance to enteric infection were probed in Chapter 3. RA treatment starting at d7 post- infection was as effective as at the time of infection for *C. rodentium* clearance.
Furthermore, it was determined that one 37.5 ug dose of RA starting at d7 post infection was enough to clear \textit{C. rodentium} from the A- host. The data showed that RA treatment during infection induced IL-17a production which was associated with clearance of \textit{C. rodentium}. Other reports indicated that A- mice have inadequate Th17 immune responses and fewer Th17 cells in the gut (7) and our data showed that A- mice were unable to clear an infection. Our previous study also showed that A- mice had fewer total T cells in the colon compared to A+ mice (6) (7-10). However, \textit{in vitro} RA inhibited IL-17a production from A- CD4+ T cells. In addition to CD4+ T cells, IL-17a is produced by many other cell types including CD8+ T cells, \(\gamma\delta T\) cells, innate lymphoid cells, natural killer (NK) T cells, NK cells and neutrophils (11, 12). There are at least three possibilities that could explain the induction of RA mRNA in the colon of A- mice infected with \textit{C. rodentium} and directly inhibiting IL-17a from CD4+ T cells: 1) RA treatment promoted IL-17a production from other (CD8, \(\gamma\delta T\), ILC etc.) cells in the gut 2) RA treatment promoted IL-17 producing immune cells to home to the gut or 3) RA indirectly resulted in CD4+ T cells making more IL-17a in the gut via innate cell production of IL-22. These three possibilities are not mutually exclusive. Further work needs to be done to determine which of the mechanisms account for increased IL-17a in the colon following RA treatment of \textit{C. rodentium} infected mice.

Because the early shedding kinetics (before day 10-11) of \textit{C. rodentium} were not different in A-, A+ and RA treated mice, we hypothesized that the adaptive immune response and not the early innate immune responses was the target of vitamin A in the gut. In addition, it has been shown that both T cell and B cell responses are required for the host to clear a \textit{C. rodentium} infection (3). Our data showed that vitamin A status had
no effect on antibody responses during *C. rodentium* infection or B cell numbers in the lamina propria of the SI and colon. T cells are well-described vitamin A targets. To study the role of vitamin A in T cells we generated T-dnRAR mice. These mice expressed the dnRAR in T cells under the Lck promoter and were therefore non-responsive to RA treatment. Preliminary characterizations of the immune cell populations in the T-dnRAR mice indicated that RA signaling was important for T cell development and in particular the CD8+ T cells from the T-dnRAR mice were lower in the periphery. The role of CD8+ T cells in host resistance is unclear since CD8 KO mice survived and presumably cleared *C. rodentium* infection (3). It would be interesting to see if IL-17a production from T-dnRAR CD8+ T cells is impaired compared to WT CD8+ T cells.

The findings from this dissertation have implications in the treatment of enteric pathogens, particularly in vitamin A deficient hosts. The work presented here highlights a potential role for vitamin A supplements to eliminate reservoirs of enteric pathogens. Vitamin A deficiency has been implicated as a factor important in the severity of diarrheal disease and as a contributor to childhood mortality. I show that supplementation with RA, the active form of vitamin A, reduced infection severity and promoted survival of A- mice, indicating that vitamin A supplementation during enteric infections in humans may be beneficial. Additionally, my work showed that RA treatment may regulate IL-17a which is needed to clear *C. rodentium* (Figure 5-1). Further experiments are needed to determine other targets of RA during *C. rodentium* infection such as intestinal epithelial cells and other cytokines. It remains to be
determined whether vitamin A deficient humans become asymptomatic carriers of *E. coli* or other enteric bacteria that have the potential to cause disease. A vitamin A supplement that could clear bacterial infections and decrease carriers would be an effective way to limit mortality and disease from enteric pathogens.
Figure 5-1. Retinoic acid treatment during enteric infection promotes protective mucosal immune responses in the vitamin A deficient host.

A) In a vitamin A sufficient or RA supplemented host, immune cells home to the gut and during *C. rodentium* infection produce adequate amounts of IL-17A.

B) In a vitamin A deficient host, immune cells fail to home to the gut and contain *C. rodentium* in the gut. IL-17A production is reduced and the immune response is not robust enough to clear the infection.
REFERENCES

Vita
Kaitlin L. McDaniel

Education
2010-present  The Pennsylvania State University
          Ph.D. in Pathobiology
          Advised by Dr. Margherita Cantorna, GPA: 3.8/4.0

2008-2010  University of Wyoming
          B.S. in Microbiology/B.S. in Molecular Biology
          Advised by Dr. E. Lee Belden, GPA 3.7/4.0

2006-2008  Northwest College
          A.S. in Biology, GPA 3.8/4.0

Publications

Professional Presentations
- Vitamin A deficient mice have a decreased ability to clear the enteric pathogen, Citrobacter rodentium and increased mortality during infection. American Association of Immunologist Annual Meeting. Pittsburgh, PA. 2014. Oral presentation and poster.
- Retinoic acid supplementation of vitamin A deficient mice promotes clearance of an infection with Citrobacter rodentium. 16th Annual Upstate New York Immunology Conference. Albany, NY. 2013. Poster

Awards and Honors
American Association of Immunologists Trainee Abstract Award 2014
PSU College of Agriculture Graduate Student Grant 2014
NIH Training Grant: Animal Models of Inflammation 2013
PSU College of Agriculture Mr. and Mrs. E. Bowsworth Grier Scholarship 2013
Pennsylvania State University Graduate School FEGR Scholarship Award 2010