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EFFECTS OF VITAMIN A AND RETINOID SIGNALING ON HOST ENTERIC IMMUNITY

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ABSTRACT

Vitamin A deficiency (A-) affects millions of children in resource limited countries, and is associated with increased childhood morbidity and mortality due to severe enteric infections. Similarly, it was observed that A- mice were susceptible to the murine enteric pathogen, *Citrobacter rodentium*. In humans and mice, vitamin A supplementation and retinoic acid (RA) dosing (respectively) were protective and promoted host survival. The first objective was to determine the mechanism of RA mediated protection in A- mice and the importance of retinoid signaling in T cells during C. *rodentium* infection. First, I characterized immunological changes in the colons of vitamin A sufficient (A+), A-, and RA treated A- mice (A- RA). A+ mice had significantly more IL-17 secreting cells, a majority of which were CD4+ T cells. A- mice had fewer colonic IL17+ cells, but RA dosing induced CD11b+ cell mediated production of IL17, which was associated with host survival and resolution of infection. Mice lacking retinoid signaling in T cells (T-dnRAR mice) did not succumb to C. *rodentium* infection; however, most became chronically infected and neither early nor late RA dosing induced clearance of the infection. Additionally, I showed that A+, A-, and A- RA had different intestinal immune populations and serum and liver metabolic profiles. These findings indicated that being A- can have long term immunological and metabolic effects that cannot be corrected through RA supplementation. These data also demonstrated that RA regulates CD11b+ mediated production of IL-17a in A- mice and that retinoid signaling in T cells was required for host resistance to C. *rodentium*. The second objective was to inhibit retinoid signaling in intestinal epithelial cells (IECs, villin dnRAR mice) and determine the effects on intestinal immunity and barrier function. IECs are critical for intestinal

immunity and homeostasis: IECs absorb nutrients and water, provide a physical barrier between the host and luminal contents, and engage in signaling cross-talk with intraepithelial lymphocytes. RA signaling in IECs was required to maintain intestinal TCRαβ CD8αα populations. In A+ mice, loss of retinoid signaling in IECs had no effect on host susceptibility to C. *rodentium* infection. After exposure to dextran sodium sulfate (DSS), A+ villin dnRAR mice had mild increases in intestinal permeability compared to A+ WT littermates. In A- mice however, loss of RA signaling in IECs resulted in extremely high mortality rates in response to C. *rodentium* infection and significantly increased weight loss and intestinal permeability after DSS exposure. Together, the work presented in this dissertation demonstrates that retinoid signaling is more important for regulating innate and adaptive immune cell function, not IECs, that is required for host defense against enteric infection and resolution of chemically-induced colonic damage. I also show that A+, A-, and A- RA hosts are phenotypically distinct, and that early vitamin A deficiency may have long term, deleterious effects on immunity and metabolism that cannot be resolved with RA supplementation.

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ABBREVIATIONS

A-: vitamin A deficient

A- RA: RA treated A- hosts

A+: vitamin A sufficient

cDNA: complementary DNA

CFU: colony forming units

DC: dendritic cell

DN: CD4- CD8- double negative thymocyte

dnRAR: dominant negative retinoic acid receptor

DP: CD4+ CD8+ double positive thymocyte

EPEC: enteropathogenic *Esherichia coli*

FMO: fluorescence minus one

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HPRT: hypoxanthine- guanine phosphoribosyltransferase

FOXP3: forkhead box P3

IBD: inflammatory bowel disease

IEC: intestinal epithelial cell

IEL: intraepithelial lymphocytes

IFN: interferon

Ig: immunoglobulin

IL: interleukin

ILC: innate lymphoid cell

IBD: inflammatory bowel disease

KO: knock out

LB: Luria- Bertani medium

LPL: lamina propria lymphocytes

LPS: lipopolysaccharide

MLN: mesenteric lymph nodes

NFκB: nuclear factor κB

NMR: nuclear magnetic resonance spectroscopy

PCA: principle component analysis

RA: retinoic acid

atRA: all trans retinoic acid

RAR: retinoic acid receptor

RARE: retinoic acid response element

RBP: retinol binding protein

CRBP: cellular retinol binding protein

RORγt: RAR-related orphan receptor gamma

RT-PCR: real time quantitative polymerase chain reaction

RXR: retinoid X receptor

SI: small intestine

TCR: T cell receptor

Th: T helper

TLA: thymus leukemia antigen

TLR: toll-like receptor

TNF: tumor necrosis factor

TP: CD4+ CD8αβ+ CD8αα+ triple positive thymocyte

Treg: regulatory T cell

UPLC: ultra pressure liquid chromatography

WT: wild type

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Chapter 1

Introduction

Vitamin A Deficiency: A Public Health Concern

Vitamin A is a dietary micronutrient essential for healthy embryonic development, vision, and immune function. Although most populations in the United States are vitamin A sufficient (A+), vitamin A deficiency (A-) continues to be a global health concern. The World Health Organization estimates that over 250 million preschool age children are A-, making them highly prone to night blindness and increased incidence/severity of diarrheal and lung infections (1, 2). Populations in resource limited countries primarily in sub-Saharan Africa and southeastern Asia, experience the highest rates of vitamin A deficiency. Malnutrition is a driving force of vitamin A deficiency and multiple approaches have been taken to supplement deficient populations. A long term program implemented by UNICEF aims to provide vitamin A supplementation to children (age 6 months to 5 years) who are at high risk for vitamin A deficiency. From 1999 to 2007, coverage (% of children receiving both of the recommended doses of vitamin A) increased from 16% to 58% in 103 high priority countries (3). Vitamin A dosing was associated with raised serum retinol status, reduced severity of diarrheal disease, and enhanced childhood survival (4). In addition, a meta-analysis of 43 trials and 215,633 children found that vitamin A supplementation resulted in a 24% reduction in all cause mortality and a reduction in the incidence of both diarrhea and measles-related morbidity (5). Supplementation programs highlight the global prevalence and health consequences of childhood vitamin A deficiency. Vitamin A deficiency is an ongoing problem worldwide and supplementation is associated with reduced rates of enteric infection and enhanced childhood survival in resource limited countries.

Vitamin A Metabolism and Cell Signaling

Vitamin A is obtained exclusively via the diet in one of two forms, pro-retinoid carotenoids or pre-formed retinoids. Leafy greens and brightly colored fruits and vegetables including sweet potatoes, carrots, and broccoli contain large amounts of carotenoids. Carotenoids are emulsified in fatty acids and bile salts and taken up into enterocytes via scavenger receptor class B protein. Within the enterocytes, carotenoids can be cleaved symmetrically by β-carotene-15,15'-monooxygenase to form retinal (6). The conversion of retinal into retinol is then mediated by retinal reductase. Pre-formed retinoids are obtained from animal products including liver, eggs, and dairy products. Pre-formed retinoids, including retinyl esters, are hydrolyzed into retinol in the intestinal lumen or at the brush border by pancreatic triglyceride lipase and retinyl ester hydrolase respectively (6). Retinol is the stable, circulating form of vitamin A that is measured to determine host vitamin A status. It can be measured by ultra pressure liquid chromatography (UPLC). Serum retinol concentrations below 0.7 μM (20μg/dL) are indicative of vitamin A deficiency (7). Retinol binds to cellular retinol binding protein (CRBPI/II, RBP) and is esterified by lecithin retinol acyltransferase, acyl-CoA: retinol acyltransferase, or diacylglycerol acyltransferase (6, 8). The resulting retinyl esters are packaged with dietary lipids and cholesterol to form chylomicrons, which can be secreted into lymph. Most of the dietary retinoids are taken up into liver hepatocytes and hydrolyzed into retinol. Hepatocyte retinol is then bound by RBP/CRBP and transferred to hepatic stellate cells (8). The retinol is then re-esterified by lecithin retinol acyltransferase and stored within lipid droplets inside hepatic stellate cells. Mobilization of these stored retinyl esters can occur during periods of inadequate vitamin A intake.

Retinyl esters are hydrolyzed to retinol and the RBP-retinol complex is transported back to hepatocytes where they can be secreted into the bloodstream. In the peripheral tissues, binding and uptake of retinol is facilitated by the STRA6 receptor (9). Retinol is oxidized into retinaldehyde by retinol and alcohol dehydrogenases. Retinaldehyde can then be oxidized (via retinaldehyde dehydrogenases) into retinoic acid (RA) - the bioactive vitamin A metabolite that binds RA receptors (RARs) and induces transcriptional changes (6). Given RA's potent ability to regulate gene expression, RA production is tightly regulated and has a half life of 30- 120 minutes (10). In addition, RA signaling induces CYP26 enzymes that rapidly degrade RA into polar metabolites that can be excreted. Under homeostatic conditions, human serum RA levels are tightly regulated and between 2-6 ng/mL (11). Vitamin A compounds undergo several metabolic steps to form RA, the high affinity ligand and bioactive metabolite that binds RARs.

Retinoic acid receptors (RARs) are nuclear hormone receptors that form heterodimers with the retinoid X receptor (RXR) and act as transcription factors (12). There are three RAR and RXR isoforms (α, β, and γ) and each isoform has a differential tissue expression pattern (13, 14). All-trans RA (atRA) and 9- *cis* RA, both bind the RARs; however, only 9-*cis* RA binds RXRs. The RAR-RXR heterodimers bind RA response elements and regulate the transcription of RA responsive genes (12). RA ligand binding triggers a conformational change that causes the release of transcriptional co-repressors (histone deacetylase, nuclear receptor co-repressor 1/2) and the recruitment of co-activating factors (histone acetylase, nuclear receptor co-activator 1/2)(6). Together, the released repression allows for active transcription of RA responsive genes. Vitamin A

and RA are critical regulators of embryonic development, vision, and immune function by signaling through nuclear receptors that regulate gene transcription.

Models of vitamin A deficiency and tissue specific repression of retinoid signaling.

Two methods were used to model the effects of vitamin A. Vitamin A sufficient and deficient mice were generated by feeding mice identical purified diets with or without vitamin A. WT breeding mice were fed the A+ or A- mice as described (15). The resulting pups are maintained on the A+ and A- diets, and serum retinol levels were quantified to verify host vitamin A status. Vitamin A deficiency develops slowly in the mice and by 7 wks of age the serum retinol levels are significantly different between A+ and A- mice. Oral dosing with RA was used to treat A- mice. The half-life of RA is very short *in vivo* and RA cannot be metabolized into retinol; thus, RA dosing is sufficient to induce spikes of retinoid signaling that do not alter the vitamin A status of experimental animals.

Tissue and/or cell specific suppression of retinoid signaling was done using a dominant negative (dn) form of the RAR-α (dnRAR). Damm et al. developed the dnRAR mouse by truncating 59 amino acids from the carboxyl terminus of the human RAR-α (16). The dnRAR is a 403 amino acid peptide that has the amino terminus, DNA binding domain, and a portion of the RA binding domain (RARα403)(16). The dnRAR binds to DNA, heterodimerizes with RXR, and binds RA, but fails to release transcriptional repression of retinoid responsive genes (16, 17). A floxed stop codon upstream of the dnRAR prevents expression in cells unless cleaved by Cre recombinase expression (18). Cre is expressed in a tissue specific manner to induce dnRAR expression in T cells, innate cells or intestinal epithelial cells.

Citrobacter rodentium is a model of human enterophatogenic _Escherichia coli_ infection.

Enteropathogenic *E. coli* (EPEC) is a common cause of persistent diarrhea/enteric infection in humans. EPEC was the estimated cause of 79,000 diarrheal deaths in children in 2011 (19). C. *rodentium* is a natural murine pathogen that causes acute colonic inflammation and pathology very similar to EPEC infection in humans (20). C. *rodentium* colonizes the cecum and colon and forms attaching and effacing lesions on intestinal epithelial cells (21). Colonization in mice is dependent upon C. *rodentium Ler* expression. *Ler* regulated the expression of virulence factors within the locus of enterocyte effacement (LEE)(22). Some critical virulence factors within the LEE locus include intimin, translocated intimin receptor (Tir), a type 3 secretion system and translocator/effector proteins (20). Colonization is dependent upon expression of the type 3 secretion system, which facilitates the injection of host cells with Tir and intimin, induces actin re-organization, and creates pedestal formations characteristic of attaching and effacing lesions. These pedestal lesions are characteristic of both *C. rodentium* infection in mice and EPEC infection in humans (23). Colonization of mice resulted in symptoms that included diarrhea, weight loss, acute colonic inflammation, and colonic hyperplasia (24). In A+ wild type (WT) mice, C. *rodentium* is a self-limiting infection that is cleared within three to four weeks (25). C. *rodentium* is a well-established murine model of human EPEC infection.

Mucosal immunity: Intestinal epithelial cells

The intestinal epithelium is comprised of various types of cells that all contribute to intestinal immunity; acting as a physical barrier to prevent luminal contents from escaping the intestinal tract and supporting homeostatic immune function (26). Enterocytes are the most prevalent type of intestinal epithelial cell (IEC) in both the small intestine (SI) and colon (27). The primary role of enterocytes is to facilitate nutrient catabolism and absorption (27). SI enterocytes are characterized by small finger-like projections called microvilli that increase the amount of surface area available for digestion and absorption (28). Paneth cells are a specialized secretory IEC found only in the SI (28). Paneth cells secrete antimicrobial peptides, which are important for regulating microbial populations in the gut lumen (27, 29). Microfold cells sample antigens from the intestinal lumen (30, 31). Microfold cells are found throughout the intestinal tract but are associated with Peyer's patches (small intestinal lymphoid follicles), caecel and colonic patches (28). Goblet cells are specialized secretory cells responsible for secreting mucins that make up the protective mucus layer of the SI and colonic epithelium (32). The colon has a higher frequency of goblet cells and a thicker mucus layer compared to the SI (28). Given that the colon also contains the highest number of microbes, it is thought that the thicker colonic mucus layer provides an enhanced physical barrier to protect the host from pathogens (28). IECs act together to regulate the intestinal microbiome and provide a physical barrier between luminal contents and the host.

Intestinal epithelial lymphocytes

Intestinal tissue is home to unique immune cell populations that function to protect the host from pathogens, promote tolerance to commensals, and support barrier function (33, 34). Intraepithelial lymphocytes (IEL) are found within the epithelial layer and are in immediate contact with luminal antigens. The IEL contain very few B cells and myeloid cells, but a large number of T cells (33). Conventional αβ CD4+ and CD8+ T cells arise from CD4+ CD8+ double positive thymocytes (33). Within the IEL are a number of specialized non-conventional lymphocytes including T cells (αβ and γδ) that express the CD8αα homodimer and arise from triple positive (CD4+ CD8αβ+ CD8αα+) thymocytes (33, 35, 36). αβ CD8αα T cells are thought to be critical in suppressing inflammation and maintaining intestinal homeostasis (34, 36). In the CD4+ CD45RB^{hi} transfer model of colitis, co-transfer of intestinal αβ CD8αα T cells into T and B cell deficient recipients ameliorated disease in an IL-10 dependent manner (34). γδ T cells also play a critical role in maintaining intestinal homeostasis. Intestinal γδ T cells support IEC turnover, IEC MHCII expression, and serve as cellular sources of IL-17 and IL-22 (37, 38). The intestinal IEL contains many non-conventional lymphocytes that are important for promoting homeostatic conditions and for limiting inflammatory T cell responses in the small intestine and colon.

Intestinal lamina propria immune cells

The lamina propria (LP) is the compartment directly below the IEC monolayer and is rich in conventional lymphocytes and infiltrating myeloid cells including dendritic cells (DCs), macrophages, neutrophils, and innate lymphoid cells (ILC). The LP immune population consists primarily of TCRαβ+ CD4+ and CD8+ T cells (39). Unlike the IEL, about 20% of LP lymphocytes are B cells (40). DCs are abundant in the LP and a large proportion of the DCs are also CD103+ (41). CD103 expression is associated with a tolerogenic phenotype and the ability of DCs to metabolize retinol into RA, which regulates local immune function and induces regulatory T cell differentiation (42). CD11b+ CD11c- F4/80+ macrophage that produce IL-10 have also been identified in the intestinal LP (41). IL-10 secreting populations of DCs and macrophage are critical for maintaining intestinal homeostasis and presenting antigens to T and B cells. There are three classes of ILC in the LP: ILC1 includes NK cells, express the transcription factor T-bet and produce IFNγ (43), ILC2 express the transcription factor GATA3 and produce IL4, IL5, and IL-9 (43, 44), ILC3 express the transcription factor RORγt and produce IL-22 and IL-17 (43, 45, 46). Unlike the IEL, the LP is comprised of conventional αβ T and B cell populations, ILC, and myeloid lineage cells including macrophages, neutrophils, and DC.

IEC signals are required for IEL development and function

IEC communicate with lymphocytes in direct contact with the intestinal epithelium (intestinal epithelial lymphocytes). IECs support the differentiation and function of intraepithelial lymphocytes (IEL) (47) including expression of CD8αα (48, 49). IEC express thymus leukemia antigen (TLA), an MHC-like protein (50). The CD8αα homodimer binds with high affinity to TLA and this interaction has been shown to support the ability of CD8αα T cells to suppress inflammatory responses (49). $TLA^{-/-}$ IEL stimulated with αCD3 produced more IFN-γ compared to $TLA^{+/+}$ IEL (49). In addition, in a model of spontaneous inflammatory bowel disease, $TLA^{-/-}$ mice were more prone to developing colitis than TLA expressing mice (49). IEC also produce IL-15, a critical signal for differentiation, survival, and proliferation of CD8αα T cells (36, 48). IL-15$R\alpha^{-/-}$ mice had fewer CD8αα T cells in the SI IEL (48). Transgenic expression of the human IL-15Rα on IEC (driven by villin Cre promoter) restored CD8αα T cell populations in the IEL (48). IEC are a dynamic population of cells that sustain barrier function and engage in cross talk with the IEL that promotes their function, survival, and proliferation.

Immunity to *C. rodentium*

Host immunity to *C. rodentium* requires both innate and adaptive immune responses to control and clear the infection. Innate immune responses, including leukocytes, ILC, and early sensing of pathogen associate molecular patterns are critical for host survival. Within 2 to 3 days of infection, macrophage and neutrophils infiltrate the colonic LP to engulf and destroy pathogens and secrete pro-inflammatory cytokines including tumor necrosis factor-α and IL-6 (51). Toll like receptor (TLR) signaling

dependent upon the MyD88 adaptor protein was indispensible for these processes to occur (52). In two independent studies, C. *rodentium* infection was lethal in 70-100% of MyD88 -/- mice by day 12 post-infection (52, 53). Infected MyD88$^{-/-}$ animals had exacerbated colonic pathology (d8 post-infection) and reduced serum and colonic levels of TNF-α and IL-6 (52, 53). Inhibiting TLR signaling through MyD88 also resulted in decreased numbers of infiltrating phagocytes and a concomitant increase in tissue and fecal C. *rodentium* burdens (53). These data indicate that early infiltration of phagocytes and their ability to sense pathogenic bacteria are critical for host survival and limiting early C. *rodentium* infection. IL-22 produced by ILC3 cells is also an important cytokine that mediates anti- C. *rodentium* immune responses (54). Intestinal IL-22 stimulates the production of anti-microbial peptides including RegIIIγ and RegIIIβ (54, 55). C. *rodentium* infection is lethal in IL22$^{-/-}$ and ILC3$^{-/-}$ mice and this phenotype was associated with decreased antimicrobial peptide production (56, 57). Early phagocyte infiltration, MyD88- dependent TLR signaling, and ILC3-mediated production of IL-22 are critical innate immune responses that promote host survival during the acute phase of C. *rodentium* infection.

Adaptive immune responses also play an important role in resistance to C. *rodentium*. Rag1$^{-/-}$ mice lacking T and B cells became chronically infected and failed to clear the infection by day 56 (58). B cell deficient mice (μMT$^{-/-}$) mice became chronically infected but did not succumb to infection (59). Antibody mediated depletion of CD4 T cells resulted in chronic infection, while CD8 T cell depletion had no effect on the host's ability to survive or clear *C. rodentium* infection (58). Infection with C. *rodentium* induced a robust colonic Th17 response, which was required for the clearance of the

pathogen (55, 60, 61). Furthermore, IL-17$^{-/-}$ mice had higher bacterial burdens and exacerbated colonic pathology compared to infected WT mice (62). Adaptive CD4 and B cell responses, including a robust Th17 response, are required for clearance of C. *rodentium*.

RA Regulation of Immunity

RA regulates the differentiation and function of innate and adaptive immune responses. RA treatment, coupled with traditional chemotherapy, is used to treat human promyelocytic leukemia, a blood cancer characterized by fusion of the promyelocytic gene with the RARα chromosome that blocks differentiation of blast cells and causes severe coagulopathy (63). RA treatments induced differentiation of leukemia cells into functionally mature granulocytes thereby alleviating blood clotting issues (64). RA treatment successfully induced neutrophil and granulocyte differentiation and reduced the number of atypical cells in patient blood marrow samples within 60 days of treatment (65, 66). RA may also regulate myeloid cell function. One study showed that murine peritoneal macrophages stimulated with LPS and IFN-γ produced TNF-α and nitric oxide (67). Treating peritoneal-derived macrophage cultures with RA reduced TNF-α and nitric oxide production (67). Another study utilized murine bone marrow derived macrophages and treated them for 48 hours with IL-4 and RA to determine the effects of RA on M2 macrophage polarization (68). M2, or alternatively activated macrophages, are cellular sources of IL-4 and are important for wound healing (69). Culturing the bone marrow derived macrophages with IL-4 alone resulted in limited arginase production (functional marker of M2 macrophages); however, the addition of RA induced a robust increase in arginase protein (68). Further studies are required to study the *in vivo* effects of RA on

both macrophage and neutrophil function. RA and RAR signaling regulate neutrophil and myeloid differentiation from promyelocytes and induce macrophage to a more regulatory/ anti-inflammatory phenotype.

Host vitamin A status and RA regulate NK cell and ILC populations. NK cell lytic activity and IFN-γ production was reduced in A- rats (70). Retinol supplementation of the A- rats restored normal NK cell lytic function and IFN-γ production (70). A- mice had fewer ILC3 and decreased production of IL-22, IL-17, and antimicrobial peptides in the SI (45) (46). ILC2 cell frequencies were higher in the A- SI (47). Due to the skewing in ILC frequencies, A- mice were better able to control a parasitic infection (*Trichuris muris*) that relies upon IL-13 and IL-4 (ILC2) immune responses (46). RA dosing of A- mice restored intestinal ILC3 frequencies and reduced ILC2 frequencies to frequencies found in A+ mice (46). Vitamin A is important for NK cells and maintaining ILC2 and ILC3 intestinal populations required for host immunity to enteric bacterial pathogens and parasites.

RA signaling is important for intestinal lymphocyte homing and T cell differentiation and function. RA induced α4β7 and CCR9 expression on T and B cells (71, 72). α4β7 and CCR9 receptors bind MAdCAM-1 and CCL10 respectively, both of which are expressed on IEC and required to recruit lymphocytes to the intestine (73, 74). RA signaling mediates lymphocyte homing to the intestine and studies have shown that A- mice had fewer SI and colonic lymphocytes than A+ mice (71, 75). *In vitro*, RA was a potent inhibitor of Th1 and Th17 function (IFN-γ and IL-17 production). RA inhibited IL23R, IL6R, and *rorc* (RORγt) expression and promoted the conversion of Th17 cells to FoxP3+ regulatory T cells (Treg) (76). A+ mice treated with RA had reduced colonic

inflammation in two different models of acute intestinal inflammation (37). RA treated A+ mice exposed to dextran sodium sulfate or infected with *C. rodentium* infection had reduced colonic inflammation and pathology (37). RA treatment *in vitro* resulted in elevated expression of FoxP3 and reduced IL-17 expression, indicating a shift in the Th17/Treg populations (77). Hall et al reported that A- mice had elevated T. *gondii* burdens, which was associated with diminished Th1 and Th17 responses (78). RA dosing was protective and improved Th1 function in the infected A- mice (78). However, Carman et al reported that A- mice took longer to clear *Trichinella spiralis*, and that the phenotype was associated with elevated systemic Th1 response (79). Mice with the dnRAR expressed in T cells had more Th17 cells and fewer Th1 cells compared to WT littermates (80). Infecting T-dnRAR mice with *Listeria monocytogenes* resulted in more antigen specific Th17 cells and fewer Th1 cells (80). Vitamin A and RA are important for lymphocyte homing to the intestine and for regulating immune responses that are critical for protection from gastrointestinal infection.

The effects of vitamin A supplementation and retinoid signaling on immunity in the gastrointestinal tract are unclear. The purpose of this dissertation was to investigate the effects of RA and RA signaling on enteric immunity in the A- host. Our lab's previous findings indicated that A- mice were more susceptible to C. *rodentium* infection and that RA supplementation of A- mice resulted in 100% host survival and clearance of the pathogen. In chapter 2, I determined the protective immunological effects of RA dosing in infected A- mice and the role of retinoid signaling on T cell development and function during C. *rodentium* infection. In chapter 3, I studied the effects of RAR signaling on IEC barrier function, and the ability of IEC to engage in crosstalk with the

IEL. The final chapter summarizes i) the protective effects of RA in infected A- mice and differential regulation of colonic IL-17 in A+ and A- hosts ii) the dysregulated metabolic and immunological phenotypes of A- mice that cannot be restored with RA dosing iii) the requirement for retinoid signaling in IEC to maintain intestinal TCRαβ+ CDαα+ T cell populations iv) the role of retinoid signaling in IEC in A- mice that protects against acute colonic injury (microbial and chemical) and promotes intestinal barrier function and discusses these major findings in the context of existing literature.

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Chapter 2

Retinoid induction of CD11b+ cell produced IL17 and retinoid responsive T cells are critical for control of *Citrobacter rodentium* infection.

Abstract

Vitamin A deficiency affects over 250 million preschool-age children worldwide and is associated with increased childhood mortality and risk of developing enteric infections. In a murine model of enteric infection, vitamin A deficient (A-) mice developed chronic infections with *Citrobacter rodentium*. A single oral dose of retinoic acid (RA) at day 7 post-infection was sufficient to induce clearance of the pathogen in A- mice. Mice that express a dominant negative (dn) retinoic acid receptor (RAR) in T cells (T-dnRAR) also developed chronic *C. rodentium* infections. RA treatment of the T-dnRAR mice was ineffective for eliminating the *C. rodentium*. T-dnRAR mice had fewer thymic CD4+CD8+ double positive T cells and reduced αβ T cell numbers in the gut. RA treatment of A- mice induced *il17a* expression in the colon. The IL-17 in the A- infected host was produced by CD11b+ cells, while IL-17 production in the infected A+ host was from T cells. The data show that retinoid signaling in T cells is required for *C. rodentium* clearing and that RA treatment of A- mice results in the induction of IL-17 that is associated with the clearance of *C. rodentium* in the A- mice.

Introduction

Vitamin A deficiency (A-) is an important public health problem in resource limited countries including areas in sub-Saharan Africa and southeastern Asia. The most recent World Health Organization studies estimate that 250 million pre-school age children worldwide are vitamin A deficient and these children have increased rates of enteric infection (2, 3). Vitamin A supplementation programs have been shown to effectively raise host vitamin A status and reduce the incidence and severity of enteric infections (4). A- mice infected with the murine enteric pathogen, *Citrobacter rodentium*, developed a severe infection, including premature mortality of 40% of the A- mice (5). Surviving A- mice failed to clear the infection and became chronic carriers of *C. rodentium*. Vitamin A deficiency is associated with severe enteric infections in both humans and mice (5).

C. rodentium is a gram negative, naturally occurring murine pathogen that models human enteropathogenic *Esherichia coli* infections and acute Th1/Th17 driven intestinal inflammation (6). Wild type (WT) mice clear *C. rodentium* within 4 wks of infection. Protection from early infection is provided by innate lymphoid cells (ILC) that produce IL-22 and IL-17 (ILC3) and clearance of infection requires robust Th17 cell responses (7, 8). ILC3 deficient mice succumbed to *C. rodentium* infection prior to d10 post-infection (9). ILC3 cells were the primary producers of IL-22 during early infection and loss of this cell subset was associated with increased fecal burdens and mortality prior to peak infection (9). IL17 knockout (KO) mice had exacerbated colon pathology at peak infection and higher bacterial burdens at d7, d14, and d21 post-infection (10). IL-17 expression levels in the colon were highest during peak and late infection, which corresponded with clearance of *C. rodentium* in WT mice (11). Host resistance to

C. rodentium requires early ILC derived IL-22 and late T cell derived IL-17 for clearance of the infection.

Vitamin A is a well described regulator of immune cell function. Specifically, RA has been reported to suppress IFN-γ and IL-17 production from T cells *in vitro* (12). Inhibition of IL-17 by RA treatments resulted in the induction of FoxP3 and IL-10 secreting regulatory T cells (13). RA induced homing of T and B cells to the gut by up-regulating α4β7 and CCR9 surface expression (14, 15). Treating vitamin A sufficient (A+) mice with RA reduced colonic inflammation caused by dextran sodium sulfate or *C. rodentium* infection (16-18). The protective effect of RA treatment in these models was associated with increased IL-22 production, enhanced antimicrobial peptide production, and suppressed IL-17 production (16-18). A- mice infected with *Trichinella spiralis* overproduced IFN-γ. RA treatments were protective and associated with reduced IFN-γ production *(19)*. A- mice infected with *Toxoplasma gondii* had lower IFN-γ and IL-17 responses and RA treatments in this model enhanced T cell mediated production of IFN-γ and IL-17 and reduced parasite burdens (20).The mechanisms to explain the effects of RA on immunity may depend on the type of infection and/or the vitamin A status of the host.

The effects of RA on gastrointestinal immune responses to *C. rodentium* were examined in the A- host. As little as one dose of RA was enough to prevent early lethality and clearance of *C. rodentium* in A- mice. The effect of RA required functional retinoid signaling in T cells since 60% of mice that expressed a dominant negative (dn) retinoic acid receptor (RAR) in T cells (T-dnRAR) developed a chronic *C. rodentium* infection. RA treatment of T-dnRAR mice failed to induce clearance of *C. rodentium* infection. RA

treatment of A- mice induced colonic *il17a* expression. IL-17 production in the A- host was from CD11b+ cells and not T cells, while most of the IL-17 produced in *C. rodentium* infected A+ mice was T cell derived. RA repletion of the A- mice failed to completely reconstitute mucosal immune populations in A- mice. ^{1}H NMR-based analysis of serum and liver metabolites also indicated that A+, A-, and A- RA mice were three metabolically discrete groups. The effects of RA in A- mice are distinct from the effects of RA in A+ mice.

Materials and Methods

Animals. C57BL/6J WT and Lck- Cre mice were obtained from Jackson Laboratories (Bar Harbor, ME). The dnRAR fl/fl mice (21) were a gift from Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). The dnRAR blocks retinoid signaling through all 3 RAR isoforms (22). dnRAR fl/fl Lck cre -/- (WT littermates) and dnRAR fl/fl Lck cre +/- (TdnRAR) were used for experiments. All mice were bred at the Pennsylvania State University (University Park, PA) according to university and IACUC guidelines. A+ and A- mice were generated by breeding C57BL/6J WT animals on lab prepared purified diets as described previously with and without retinyl acetate (25 μg/d) (23). Serum retinol status was quantified to verify vitamin A status of experimental animals (Fig. 2-1).

***Citrobacter rodentium*.** *C. rodentium* strain ICC169 (nalidixic acid resistant) was a kind gift from Gad Frankel (London School of Medicine and Dentistry, London, UK). Bacteria were grown and cultured in Difco Luria-Bertani broth and agar (LB; Becton, Dickinson, & Co, Franklin Lakes, NJ) containing 50 μg/mL nalidixic acid (Sigma Aldrich, St. Louis, MO, USA). Overnight cultures containing log phase bacteria were used to prepare inoculums. Individually housed mice were fasted overnight and then orally gavaged with 5 x 10^9 CFU *C. rodentium* in 100 μl of sterile saline. Feces and organs were collected, homogenized, and plated in serial dilutions on LB agar plates containing nalidixic acid to quantify bacterial burdens.

Flow Cytometry. Intracellular staining for IL-17 was done by stimulating 10^6 colonic lymphocytes for 4h with phorbol 12-myristate 13-acetate, (50 ng/mL, Sigma, St. Louis, MO) and ionomycin (6 μg/mL) in the presence of brefeldin A (10 μg/mL, Sigma) St. Louis, MO). Cells were stained with fluorescein isothiocyanate (FITC) CD8β, FITC GL3 (γδ TCR), phycoerythrin PE GL3, PE-CF594 CD4, PE-CF594 GR-1, PE-Cy 5 T cell receptor β (TCRβ) (BD Biosciences, San Jose, CA), PE-Cy5 CD11b (eBioscience, San Diego, CA), PE-Cy7 F4/80, PE- PE-Cy7 CCR9, or PE-Cy7 CD8α (BioLegend, San Diego, CA). Intracellular cytokine staining was performed using PE IL17a and the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience). Single positive and fluorescence minus one (FMO) controls were used for gating purposes. Cells were analyzed on an FC500 benchtop cytometer (Beckman Coulter, Brea, CA), and data was analyzed using FlowJo 7.6.5 software (Tree Star, Ashland, OR).

Real Time PCR: Whole tissues were snap frozen in liquid nitrogen and stored until processing. RNA was isolated using TriZOL (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. Complementary DNA (cDNA) was created by reverse transcribing 4-5 ug of RNA using the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA). RT-PCR was performed using SYBR green mix (BioRad, Hercules, CA) and the MyiQ Single-Color Real Time PCR machine (BioRad). Standards were prepared by serially diluting DNA products for each gene of interest. Gene expression levels were then normalized to one or more housekeeping genes (HPRT and/or GAPDH,). Fold change values were reported relative to untreated or un-infected control tissues. Primers for RT-PCR are listed Table 2-1.

NMR. The liver and serum samples were prepared for NMR as previously described (24). ^{1}H NMR spectra were recorded at 298 K on a Bruker Avance III 600 MHz spectrometer equipped with an inverse cryogenic probe (Bruker Biospin, Germany). NMR spectra of liver samples were acquired for each employing the first increment of NOESY pulse sequence (NOESYPR1D) with the recycle delay (RD) of 2 s and mixing time (t_m) of 100 ms. NMR spectra of serum samples were acquired using the Carr-Purcell-Meiboom-Gill sequence (RD-90°-(τ-180°-τ)n-acquisition). The 90° pulse length was adjusted to about 10 μs for each sample and 64 transients were collected into 32 k data points for each spectrum with spectral width of 20 ppm. The chemical shifts of ^{1}H NMR spectra were referenced to TSP (liver) or D-glucose (serum). Each bucketed region (0.004 ppm) was then normalized to the total sum of the spectral integrals prior to statistical data analysis. Principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (OPLS-DA) were carried out using the SIMCA-P+ software (Version 13.0, Umetrics, Sweden).

Statistical Analyses. Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Two-tailed Student *t* tests were used to compare two groups including systemic bacterial burdens, immune cell frequencies, and IL17 producing cell populations. Two-way ANOVA with Bonferroni's *post hoc* test was used to compare fecal shedding curves and mRNA abundance through time and by treatment. A P value of $\leq$0.05 was used as the cut off for a significant change.

Results

A single RA dose clears *C. rodentium* infection in A- mice.

A- mice were infected with *C. rodentium* and left untreated (A-), dosed with RA starting on the day of infection (RA d0), or dosed with RA at day 7 post-infection (RA d7). RA dosing at either d0 or d7 induced clearance of the *C. rodentium* infection with kinetics of clearance similar to those observed in A+ WT mice (Fig. 2-2A). The untreated A- mice continued to shed *C. rodentium* out to d37 post-infection as reported previously (5). A- mice were treated with RA orally once (RA 1x), twice (RA 2x), or three times (RA 3x) beginning on d7 post-infection (Fig. 2-2A). The RA 3x mice cleared the infection by d25 post-infection, while the RA 2x and RA 1x treated mice took 30-32d to clear the *C. rodentium* infection (Fig. 2-2B). RA 3x was more effective than RA 2x or RA 1x for the clearance of *C. rodentium*. As little as one dose of RA given on d7 post-infection to A- mice resulted in clearance of *C. rodentium*.

Mice with T cells that have blocked retinoid signaling fail to clear *C. rodentium*.

To determine the role of vitamin A mediated regulation of T cells in host resistance to *C. rodentium*, mice were generated in which retinoid signaling was blocked in T cells (T-dnRAR). The shedding kinetics of *C. rodentium* in the WT littermates matched that of A+ WT mice (Fig. 2-3A)(5). Forty percent of the T-dnRAR mice cleared the *C. rodentium* infection with the same kinetics as their WT littermates (Fig. 2-3A, 2B)(5). The other sixty percent of the T-dnRAR mice developed a very high and persistent infection that was not cleared by d37 (Fig. 2-3B). Although the fecal shedding of *C. rodentium* was similar at d10 post-infection, the livers and spleens of T-dnRAR mice had significantly higher *C. rodentium* burdens than WT littermates (Fig. 2-3C). Several

chronically infected T-dnRAR mice were treated with RA for two weeks starting at d31 post-infection (Fig. 2-3D). The RA treatments failed to eliminate *C. rodentium* fecal shedding in the chronically infected T-dnRAR mice and pathogen burdens remained high (Log 4-6 CFUs) out to d48 post-infection (Fig. 2-3D). Beginning RA dosing at d7 post-infection also failed to induce clearance of the *C. rodentium* in T-dnRAR mice (Fig. 2-3E). Mice lacking T cells that are responsive to retinoids developed chronic *C. rodentium* infection that was refractory to RA treatment.

Fewer T cells and altered distribution of CD4, and CD8 cells in T-dnRAR mice. There were fewer thymocytes and small intestinal (SI) intraepithelial lymphocytes (IEL) isolated from T-dnRAR mice than from the WT littermates (Fig. 2-4A). Total immune cell numbers were the same in the spleen and mesenteric lymph nodes (MLN) of WT and T-dnRAR mice (Fig. 2-4A). CD4+/CD8+ double positive (DP) thymocytes are the most frequent cell type in the thymus and 83% of the cells in the WT thymus were DP (Fig. 2-4B). T-dnRAR mice had decreased frequencies of DP thymocytes (67%) in the thymus as compared to their WT littermates (Fig. 2-4B). The decrease in the DP thymic population in T-dnRAR was reported previously (25). The frequencies of CD4 and CD8 double negative (DN) and CD4+ cells were the same in WT and T-dnRAR mice (Fig. 2-4B). There was a significant increase in CD8+ single positive frequencies and cell numbers in T-dnRAR thymuses as compared to WT (CD8+ cell numbers: WT 2 x 10^6 vs T-dnRAR 4.6 x 10^6, $p<0.01$). The frequencies of T cell receptor (TCR)αβ cells were lower in T-dnRAR spleens (Fig. 2-4C). The spleen and MLN of T-dnRAR mice had higher CD4 frequencies and lower CD8 frequencies as compared to WT littermates (Fig. 2-4C, D). The frequencies of TCRαβ T cells were lower in the SI IEL compartment of T-dnRAR

mice as compared to WT (Fig. 2-4E). There was no effect of dnRAR expression on the frequencies of TCRγδ, CD8αα, CD8αβ, and CD4 cell in the colon IEL (Fig. 2-4E). In addition, the frequencies of CCR9+ CD4+ and CD8+ T cells in the periphery (spleen) of T-dnRAR mice was significantly less than in WT littermates (Fig. 2-4G). Although reduced CCR9 expression on T-dnRAR splenic T cells did not correspond to altered frequencies of CD4+ or CD8αβ+ cells in the SI or colon, it was associated with an overall decrease in T cell numbers (Fig. 2-4E, F). Retinoid signaling in T cells is required for CCR9 expression and homing of TCRαβ T cells to the GI tract.

RA 2x treatment induced IL17 mRNA in the colons of A- mice.

C. rodentium infected A- mice were left untreated or dosed with RA 1x or RA 2x and sacrificed on d8 or d10 post-infection (Fig. 2-5A). The expression of *rorc*, *foxp3*, *il6*, *il17a*, *il22* and *regIII* mRNA was significantly lower in the duodenum than the ileum and colon of the *C. rodentium* infected mice (Fig. 2-5). There was no effect of the RA treatments on *rorc, foxp3, il6, or il22* mRNA expression (Fig. 2-5B-D, F). RA treatment significantly enhanced *RegIIIy* expression across the GI tract (Fig. 2-5G). RA treatment also enhanced *il17a* expression in the colon but not the duodenum or ileum (Fig. 2-5E). The effect of RA treatment on *il17a* expression in the colon was not evident after RA 1x but was significantly higher after RA 2x treatments in the infected colon (Fig. 2-6). RA treatments of A- mice induced colonic *il17a* expression and *regIII* expression across the SI and colon.

The source of IL-17 in the A- host is myeloid cells and not T cells.

Consistent with the increase in *il17* mRNA expression (Fig. 2-6), the frequency of colonic IL-17 secreting cells increased following RA 2x treatments in *C. rodentium* infected A- mice (Fig. 2-7A). Colonic IL-17 in A- and A- RA 2x treated mice was primarily produced by a large, granular CD4- population (including CD11b+ cells) and not by Th17 cells (Fig. 2-7B, C). Infected A+ mice had significantly more IL-17 producing colonic cells compared to A- mice (Fig. 2-7D). Additionally, CD4+ T cells, not CD11b+ cells, were the predominant producers of colonic IL-17 in the A+ host (Fig. 2-7E). In A- mice CD4+ T cells accounted for only 12% of the colonic IL17 producing cells (Fig. 2-7B). In the A- host, neutrophils (F480-) and macrophages (F480+) within the CD11b+ gate were analyzed to determine the cellular sources of IL-17 (Fig. 2-7C). RA treated A- mice had significantly more IL17 secreting CD11b+ cells compared to untreated A- mice (Fig. 2-7F). The RA treatments had no effect on the numbers of CD11b+, CD11b+/F480- (neutrophils) or CD11b+/F480+ (macrophage) cells in the colon (data not shown). The frequency of neutrophils ($P=0.08$) and macrophages ($P<0.05$) that produced IL-17 increased in the RA 2x treated mice compared to the A- controls (Fig. 2-7G, H). There was no effect of RA 2x treatment on IL-17 production in the colons of infected T-dnRAR mice (Fig. 2-8A-D). WT littermates and T-dnRAR mice had comparable T cell derived IL17 and IFN-γ responses at d10 post-infection (Fig. 2-8E, F). The induction of IL-17 by the RA 2x treatments was associated with the clearance of *C. rodentium* in A- mice.

A+ and A- hosts have distinct metabolic profiles and RA dosing did not recapitulate the A+ phenotype.

RA dosing induced colonic innate cells to produce IL-17 in *C. rodentium* A- mice. This in contrast to other studies that showed that RA dosing inhibited Th1 and/or Th17 responses in A+ hosts (16-18). These observations led to the hypothesis that VA deficiency creates a unique setting in the host and that RA effects in the A- host may differ from those in an A+ host. A+, A-, and A- RA male mice were euthanized at 12 weeks of age and serum and livers were collected for NMR based metabolomics studies. Principle component analysis (PCA) of the metabolite data revealed that A+ and A- mice had unique metabolic profiles that clustered separately from one another (Fig. 2-9A). RA dosed A- mice did not cluster with A+ or A- mice. Instead, RA dosing created a third group with unique serum and liver metabolic profiles (Fig. 2-9A). SI IEL populations were also characterized in A+, A-, and A- RA mice (Fig. 2-9B-F). A- mice had fewer SI IEL lymphocytes than A+ mice and RA dosing restored numbers to A+ levels (Fig. 2-9B). A+ and A- mice had similar TCRβ frequencies, but RA dosing increased TCRβ frequencies significantly (Fig. 2-9C). RA dosed A- mice increased CD8αα frequencies similar to those of A+ mice (Fig. 2-9D); however, RA dosing A- mice did not restore CD8αβ or γδ T cell frequencies (Fig. 2-9E, F). These results indicate that VA deficiency alters host metabolism and that RA dosing does not recapitulate the A+ metabolic profile. Additionally, A- mice have altered SI IEL populations that were not rescued by RA dosing. A- hosts had altered metabolic and immunological systems and it is likely that RA affects A+ and A- hosts differently.

Discussion

RA treatment of infected A- mice induced colonic IL17 production by CD11b+ cells, which was associated with clearance of *C. rodentium* infection. A robust IL-17 response is critical for the clearance of several mucosal pathogens including *C. rodentium* (10, 26-28). In A+ mice T cells provided most of the IL-17 in the infected colon. Conversely the A- host had reduced T cell responses and the RA treatments induced IL-17 production by CD11b+ cells. It is well established that RA inhibits Th17 differentiation and function *in vitro* and it has been published that RA treatment reduced T cell derived IL-17 and improved disease outcomes in several intestinal inflammation models that used A+ animals (16-18). A- mice have fewer intestinal lymphocytes due to defects in intestinal homing (decreased α4β, CCR9 expression), diminished antigen specific IgG_1 production and non specific IgA secretion, and altered intestinal pathophysiology including goblet cell hyperplasia and a reduction in Paneth cells and antimicrobial secretion (5, 29-32). The metabolic phenotype of A- RA mice, in both serum and liver, is distinct and significantly different than either A- or A+ mice. In addition, 2 week RA treatment of A- mice failed to recover SI TCRαβ, CD8αβ, and γδ T cell frequencies to A+ levels. These data suggest that A+ and A- hosts are immunologically and metabolically distinct, RA dosing failed to recapitulate the phenotype of an A+ host, and it is likely that RA mediated effects on IL-17 are dependent upon host VA status. RA has been shown to suppress T cell mediated IL-17 production; however, very little has been published on leukocyte mediated production of IL-17 or the effect(s) RA may have on innate cells producing IL-17. In CD4 T cells, RA regulates the expression of genes associated with Th17 differentiation. RA inhibited the expression of the receptors for IL-6 and IL23 that

are critical in the differentiation of naïve T cells to Th17 (33, 34). A population of RORγt+ IL-17 producing neutrophils have been identified and their function was determined to be RORγt and IL-6 dependent (33). The enhanced colonic IL-17 production in RA treated A- mice could be due to 1) RA enhancing IL-6 and IL-23 production by other cells that indirectly induces IL-17 in myeloid cells or 2) RA directly regulating IL-17 production in myeloid cells. RA dosing enhanced *in vivo* colonic CD11b+ cell production of IL-17 in A- mice infected with *C. rodentium*. The effects of RA on intestinal IL-17 production depend on host vitamin A status.

Retinoid signaling in T cells is required for clearance of *C. rodentium*. Over 60% of T-dnRAR mice were unable to clear the infection and RA treatments failed to induce clearance. T-dnRAR mice also had an overall reduction in intestinal αβ T cells while γδ T cell numbers were similar to WT littermates. Both WT and T-dnRAR mice had robust production of IL-17 in the GI tract and RA treatment of the T-dnRAR mice had no effect on IL-17 production in the colon. Since most, but not all T-dnRAR mice became chronically infected, it may be that there is an immunological threshold requiring T cell responsiveness to vitamin A to clear *C. rodentium.* In the complete absence of dietary vitamin A (A- mice), αβ T cells numbers were reduced, IL-17 production was diminished, and all surviving mice developed a chronic infection. In vitamin A sufficient T-dnRAR mice, T cells and CD11b+ cells were robust producers of IL-17 in response to infection; however, intestinal αβ T cell frequencies were diminished compared to WT littermates. Retinoid signaling in T cells is required for clearance of *C. rodentium*.

Millions of children in resource limited countries are vitamin A deficient and at increased risk of developing severe enteric infections (2, 3). This study demonstrates that RA and retinoid receptor signaling in T cells is important for host resistance to enteric infections. The data show that 5-8 wk RA treatment of A- mice failed to completely restore the mucosal immune responses and/or the metabolic phenotypes to those observed in A+ mice. RA treatment of A-mice induced colonic CD11b+ cells to produce IL-17, a response that was associated with survival and clearance of *C. rodentium* infection. In resource limited countries, RA treatment interventions could prove useful for treating vitamin A deficient children and adults with enteric infections, inducing sterilizing clearance, and minimizing the number of asymptomatic carriers within susceptible populations. Vitamin A deficiency continues to be an important public health problem in the developing world. The effects of retinoid repletion of the previously vitamin A deficient host are likely completely different than the effects of retinoid therapies in the A+ host.

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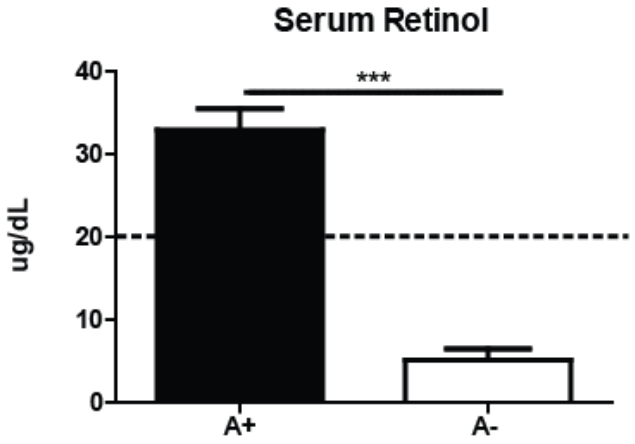

Figure 2-1: Serum retinol was quantified to verify host VA status. Blood was collected at 10 to 12 weeks of age and serum was used to quantify retinol levels by UPLC. Graphical data is representative of animals from several different experiments (A+ n=8, A- n=18). Dotted line indicates deficient/sufficient cutoff (20 ug/dL) Unpaired student's T test with Welch's correction. ***P<0.0001.

Table 2-1: RT-PCR Primer Sequences

HPRT:	F: 3'-GTAATGTTATCGAGAAGTCAGAC-5' R: 3'-CACCTTCTATATTAACTGTGACC-5'
GAPDH:	F: 3'-TAGCTTCCACCTTCTACACCAAA;-5' R: 3'-GATGTCGTTGTCCCACCACC-5'
FOXP3:	F: 3'-GACGTATCGAGGGTCGAAGA-5' R: 3'-CGAGAACGACGTAGCATCG-5'
RORC:	F: 3'-AGGAGGGCACTTTTCTCCA-5' R: 3'-GTTACACCCTCTACACCCT-5'
IL-17A:	F: 3'-TGTGTCTACTTCGAGAGGGAC-5' R: 3'-TAGTAGGGAGTTTCGACTCG-5'
IL-22:	F: 3'-AGACCTACAAGACCAGCAGT-5' R: 3'-CACCTCTCTAGTTCCGCTAA-5'
IL-6:	F: 3'-AGGACTAATATAGGTCAAACCCATCG-5' R: 3'-AAGACCTCATGGTATCGATGGACC-5'
REGIIIγ	F: 3'-AAAACTAGTACCTCCTGTCCTT-5' R: 3'-ACTTGGGTTGTCTCCACCTAC-5'

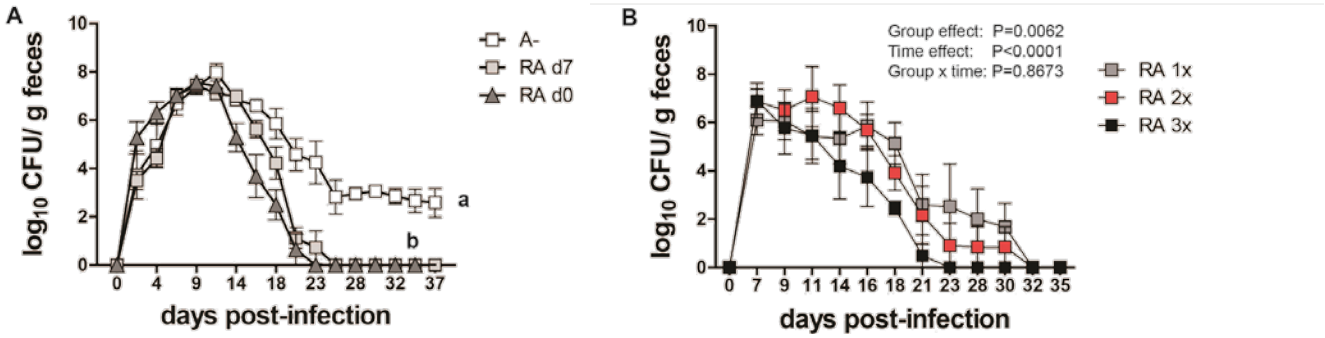

Figure 2-2: **RA treatment clears *C. rodentium* infection in A- mice**. A- WT mice were infected with *C. rodentium* and treated continuously with RA beginning at d0 or d7 (n=3/group) A) post-infection. B) At d7 post-infection A- mice were dosed with RA once (RA 1x), twice (RA 2x), or three times (RA 3x) (n=4/ group). Values are the means ± SEM. Two-way ANOVA with Bonferroni post-hoc test. Groups with different letters are significantly different from each other, P<0.01. *Data generated by K. McDaniel 2015*

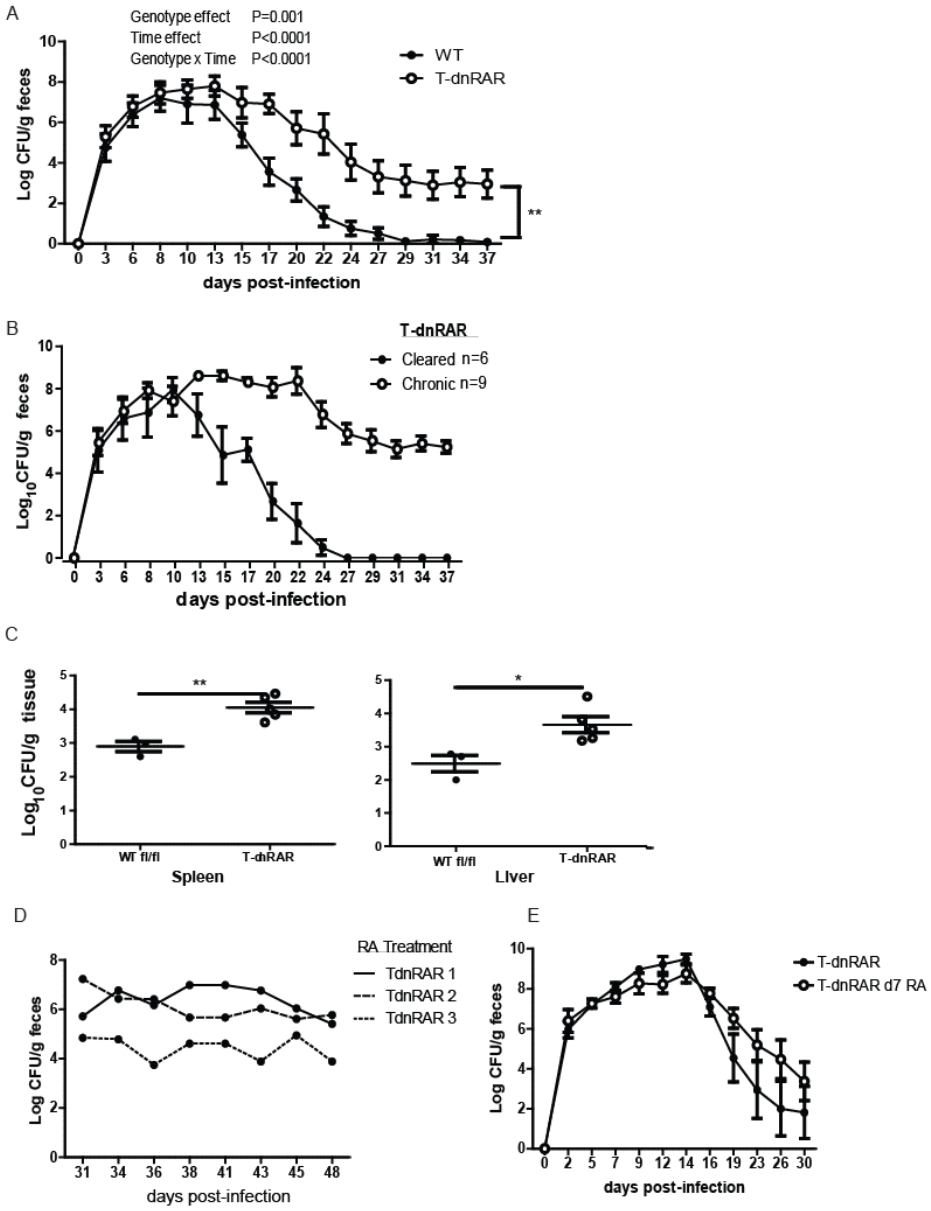

Figure 2-3: T-dnRAR mice fail to clear *C. rodentium* infection. A) Fecal shedding kinetics of T-dnRAR and WT littermates infected with *C. rodentium* (WT n=15 T-dnRAR n=16). B) Fecal shedding in T-dnRAR that either cleared the infection (n=6) or developed chronic infection (n=9). C) Spleen and liver CFU at d10 post-infection (WT n=3, T-dnRAR n=5). D) Chronically infected T-dnRAR mice were began RA treatment on d31 post-infection (Control n=6 RA n=9). Values are the means ± SEM of two to three independent experiments. Unpaired student's T test and Two Way ANOVA analysis *P<0.05, **P<0.01, ***P<0.0001.

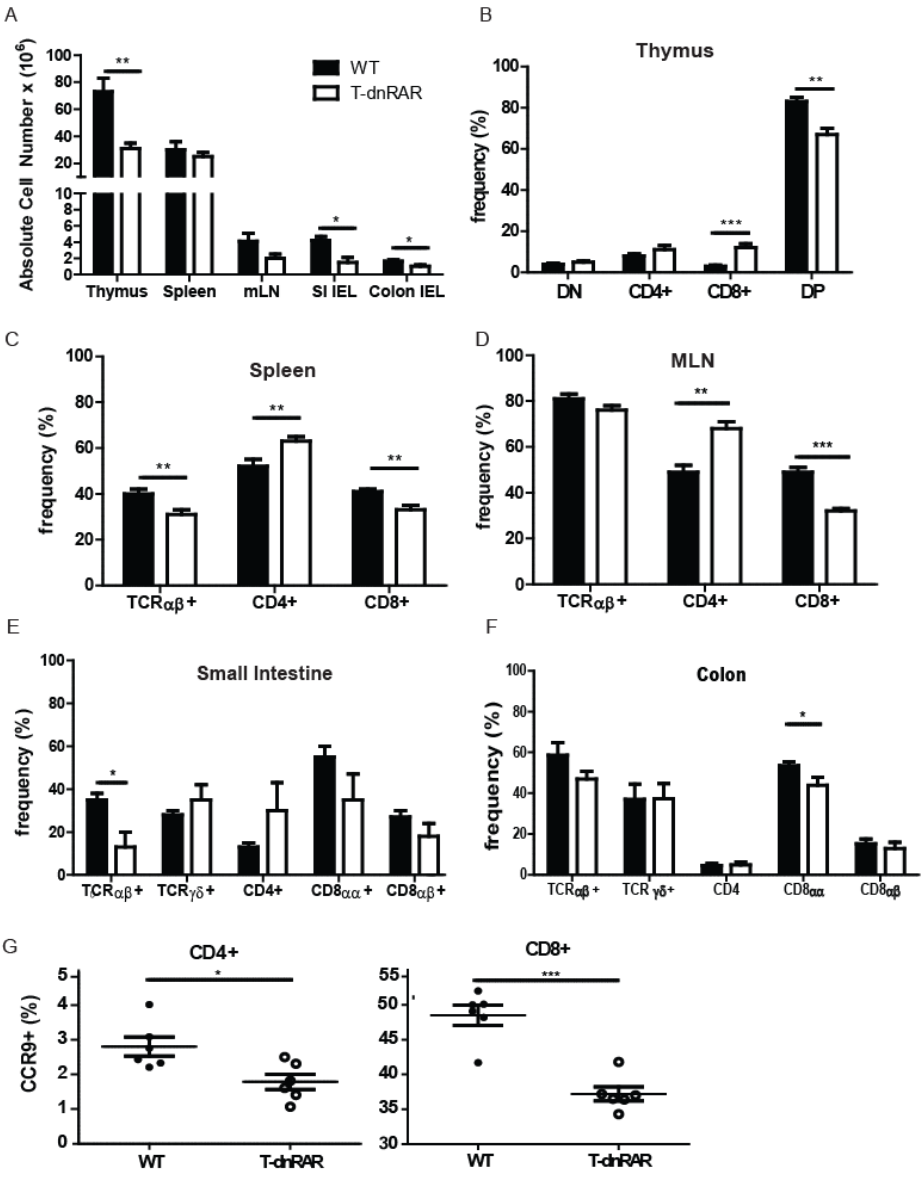

Figure 2-4: T-dnRAR mice had altered thymic and systemic T cell populations. T cell populations were quantified in naïve T-dnRAR and their WT littermates. A) Total cell counts, T cell frequencies in the B) thymus (WT n=7, T-dnRAR n=8), C) spleen (WT n=7, T-dnRAR n=8), D) mesenteric lymph node (WT n=4, T-dnRAR n=5), E) SI IEL (WT n =10, T-dnRAR n=3), and F) colon IEL (WT n=7, T-dnRAR n=5) and G) CCR9 expression on CD4+ and CD8+ splenic T cells (n=6/ group). Values are the means± SEM of two independent experiments. Unpaired student's T test or unpaired student's T test with Welch's correction *P<0.05, **P<0.01, ***P<0.0001.

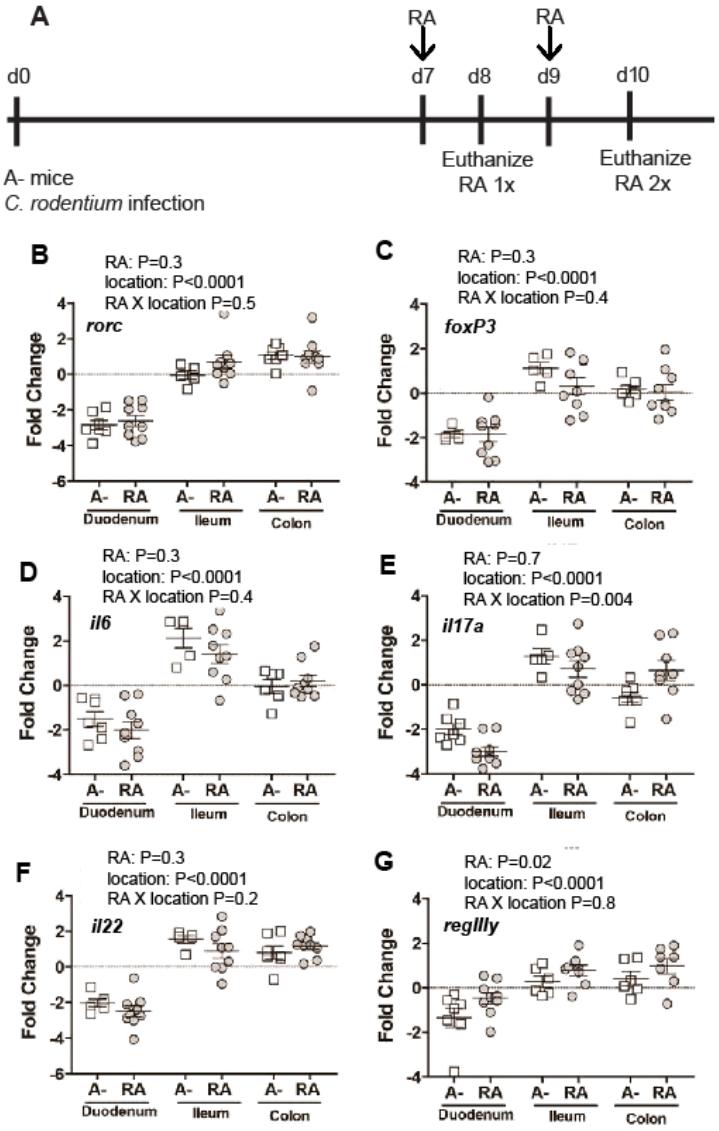

Figure 2-5: mRNA expression in the colon of A- mice treated with RA. *Rorc,Foxp3, Il6, Il17a, Il22, and RegIIIγ* expression was quantified in tissue from RA treated and untreated A- mice infected with *C. rodentium* (A-F). Values were log transformed and are reported as means± SEM from two independent experiments with A- n=6, A- RA n= 9. Two-way ANOVA with Bonferroni post-hoc tests. *Data generated by K. McDaniel 2015*

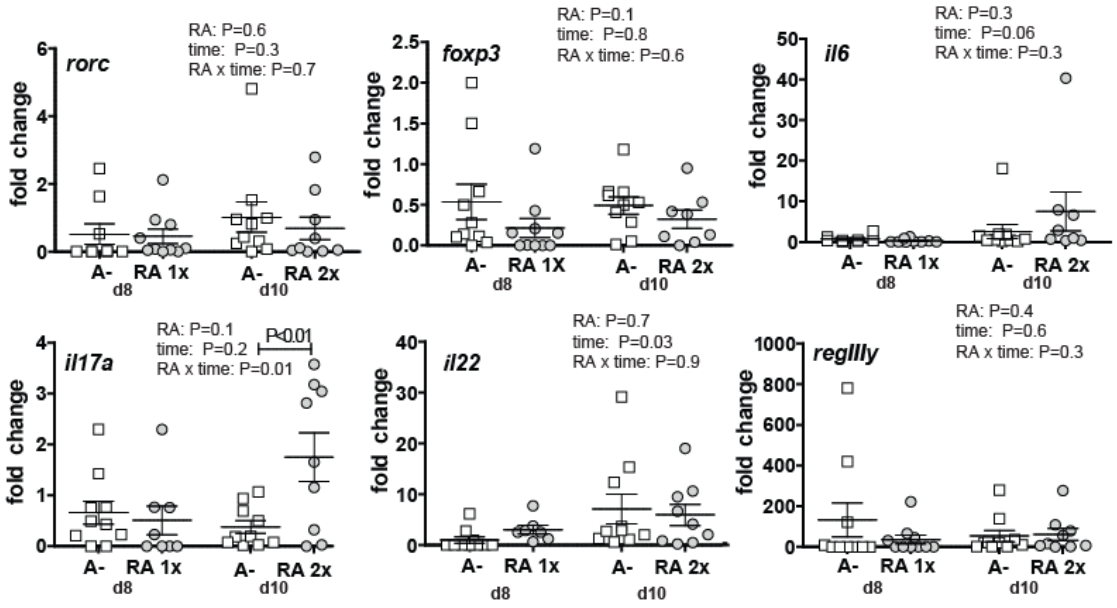

Figure 2-6: RA induced il17a mRNA in the colon of A- mice. A) Infected A- mice were dosed with retinoic acid once (RA 1x) or twice (RA 2x). B) *Rorc, Foxp3, Il6, Il17a, Il22, and RegIIIγ* expression was measured in the colon at d8 and d10 post-infection. Values are mean ± SEM of two combined experiments and A- n=10, RA 1x n=10, and RA 2x n=8. Two-way ANOVA with Bonferroni post- hoc tests. *Data generated by K. McDaniel 2015*

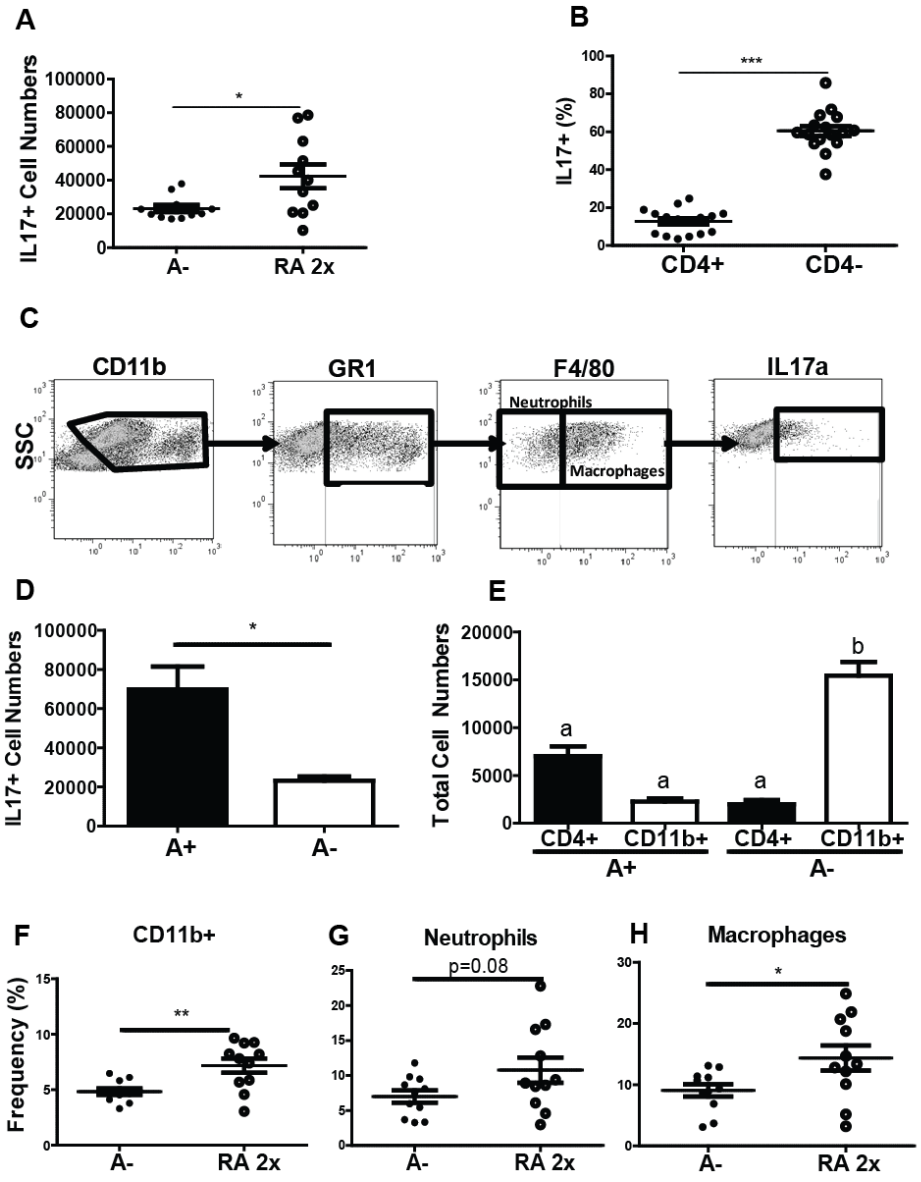

Figure 2-7: RA induced innate CD11b+ mediated IL17a production in the colon of A- mice. d10 colonic lamina propria cells were isolated to determine (A) the absolute number of IL17a+ cells and (B) CD4+ and CD4- cell IL17a+ frequencies in A- and A- 2X RA mice. (C) Flow gating strategy for CD11b+ cells including neutrophils and macrophages (D-) The absolute number of IL17a+ cells and (E) the number of IL-17 secreting CD11b+ and CD4+ T cells in A+ and A- mice (F-H) The frequencies of IL17a producing CD11b+, neutrophil (CD11b+/F480-), and macrophage (CD11b+/F480+) cells. Values are means ± SEM of two combined experiments and A+ n=4, A- n=11, RA 2x n=11. Unpaired student's T test (D),unpaired student's T test with Welch's correction (A, B, F-H) *P<0.05, **P<0.01, ***P<0.001. Kruskal-Wallis one way ANOVA (E) Values with different letters are significantly different from each other (P<0.05).

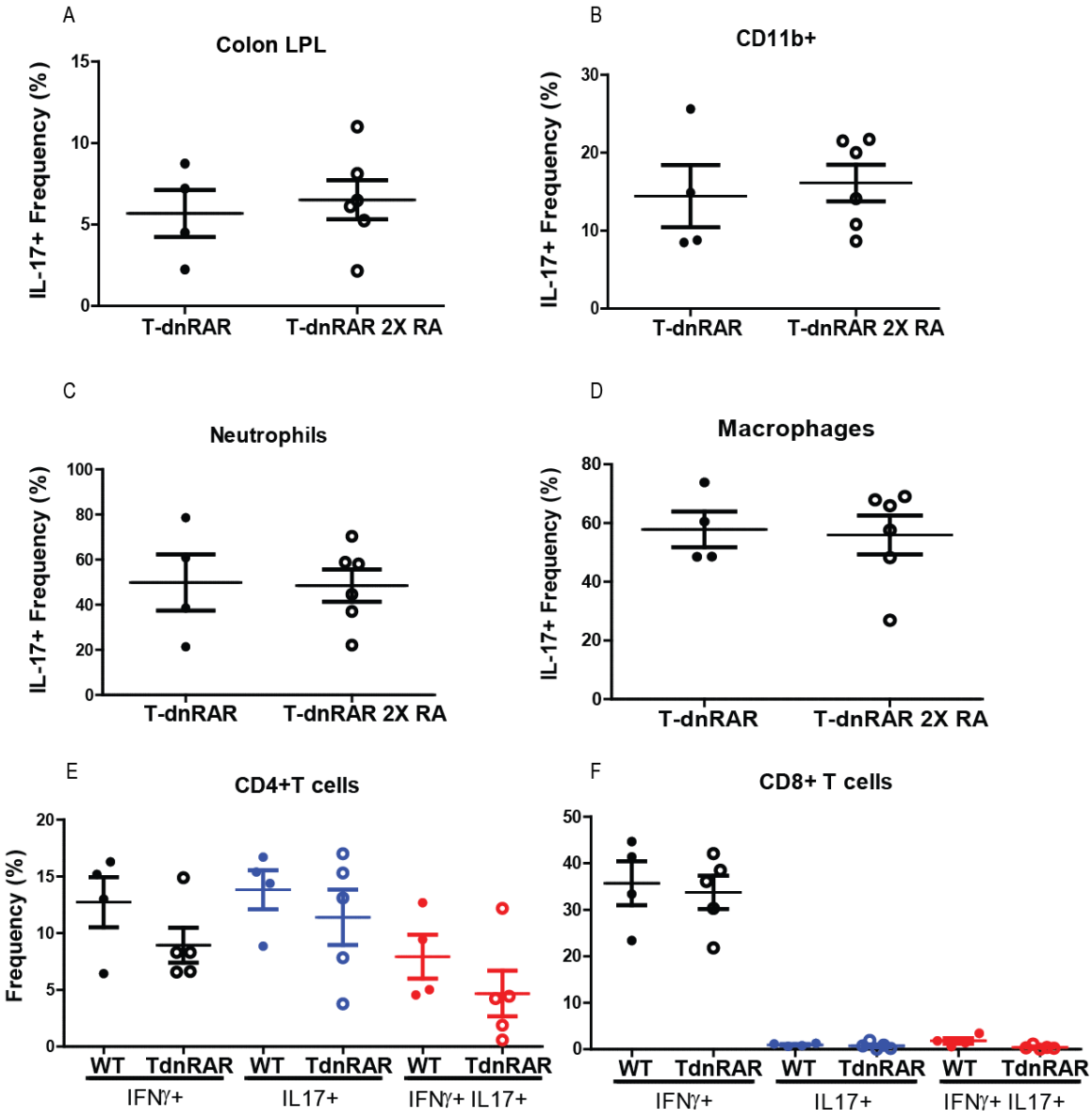

Figure 2-8: Infected T-dnRAR mice had robust innate and adaptive IL-17 responses. IL17a producing populations in the colon lamina propria of d10 infected T-dnRAR and T-dnRAR 2x RA mice were quantified including A) total IL17a+, B) CD11b+, C) neutrophil, and D) macrophage frequencies (T-dnRAR n=4, T-dnRAR 2x RA n=6). E) Colonic Th1/Th17 and F) CD8 responses in WT littermate and T-dnRAR mice were also characterized at peak infection (n=4/ group). Values are means ± SEM of one (E, F) or two combined experiments (A-D). Unpaired student's T test *P<0.05, **P<0.01, ***P<0.0001.

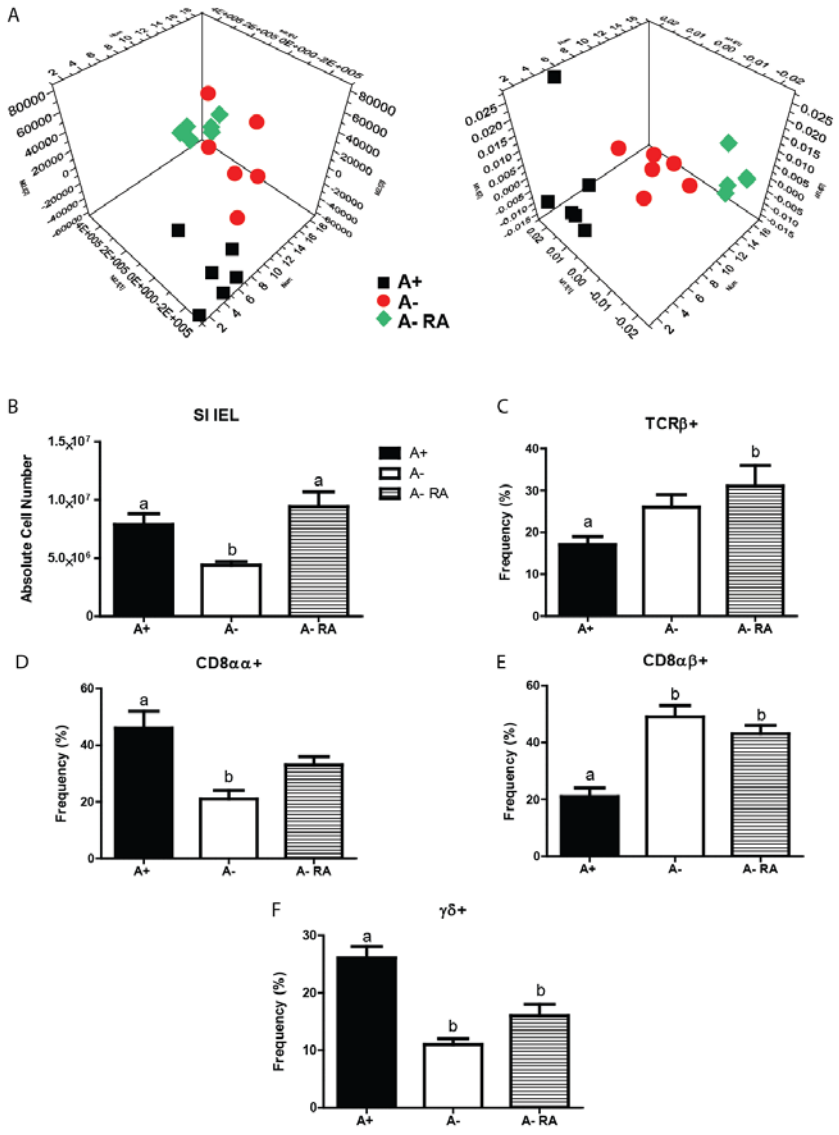

Figure 2-9: A+, A-, and A- RA mice have distinct serum and liver metabolite profiles and mucosal immune populations. A) At 4 weeks of age, male mice (n=6/ group) were placed on A+ and A- diets. A- RA mice were dosed three times per week until all groups were euthanized at 12 weeks of age. Metabolic PCA score plots were generated using serum and liver NMR data. B-F) Naïve A+, A-, and A- RA mice were euthanized at 8-10 weeks of age to characterize SI IEL T cell populations by flow (A+ n=8,A- n=9, A- RA n=8). Values are the means ± SEM of two to three independent experiments One way ANOVA *<0.05, **<0.01, ***<0.0001 with Bonferroni post test, groups with different letters are significantly different from each other, P<0.05. *Data generated by Y. Tian 2017 and K. McDaniel 2015*

Chapter 3

Inhibiting retinoid signaling in intestinal epithelial cells had minor effects on gastrointestinal immunity and barrier function

Abstract

Vitamin A deficiency (A-) is prevalent in resource limited countries and it is estimated that over 250 million preschool-age children are affected. A- children are at an increased risk of mortality and morbidity due to mucosal infections and vitamin A deficiency has been associated with reduced intestinal barrier function. Mice that express a dominant negative (dn) retinoic acid receptor (RAR) in intestinal epithelial cells (villin dnRAR) were generated to assess the role of RA on IEC function and host gastrointestinal immunity. Vitamin A sufficient (A+) and A- WT and villin dnRAR mice were comparable in size and appearance. A+ WT and villin dnRAR IECs were phenotypically similar. Surprisingly, A+ villin dnRAR mice had reduced intraepithelial TCRαβ+ CD8αα frequencies in the small intestine starting at 5 weeks of age. Peripheral splenic and thymic T cell populations were unaffected. Expression of the dnRAR in the IECs of A+ animals did not affect host immunity to *Citrobacter rodentium* infection. After exposure to dextran sodium sulfate (DSS), A+ villin dnRAR mice had a small increase in intestinal permeability compared to A+ WT littermates. Following exposure to DSS, A- villin dnRAR mice lost more weight and had increased intestinal permeability compared to A- WT mice. A- villin dnRAR mice were also extremely susceptible to C. *rodentium* infection and were unable to resolve colonic hyperplasia. Retinoid signaling in IECs is critical for survival and maintenance of intestinal TCRαβ CD8αα T cell populations. Expression of the dnRAR in IECs resulted in a mild defect in intestinal permeability after colonic injury in A+ hosts. Inhibiting retinoid signaling in IEC in A- hosts exacerbated susceptibility to C. *rodentium* and DSS-induced colitis. Retinoid signaling in epithelial cells plays a minor role in maintaining homeostasis in the gastrointestinal tract.

Introduction

Vitamin A is a micronutrient important for embryonic development, vision, and immune function. Vitamin A deficiency (A-) has been a persistent problem in resource limited countries worldwide. It is estimated that over 250 million preschool-age children are A- (1, 2). A- children develop night blindness and have increased susceptibility to infections in the gastrointestinal tract and lungs (2). Longitudinal studies have shown that supplementing at-risk populations in Africa and southeastern Asia with one or two bolus doses of vitamin A reduced infection rates and lessened the severity of enteric infection (3). In addition, vitamin A supplementation significantly reduced the number of Giardia spp. parasitic infections (especially new infections)(4). Supplementation was also associated with improved gastrointestinal barrier function (4). Vitamin A deficiency continues to affect children in resource limited countries worldwide and is associated with an impaired ability to fight infection.

Intestinal epithelial cells (IEC) make up the lining of the intestine and the intestinal epithelium is important for nutrient and water absorption and acts as a barrier between the host and the intestinal microbiota (5). If the barrier becomes compromised, leakage of food antigens and/or bacteria can elicit systemic inflammation. It has been hypothesized that compromised intestinal barrier function contributes to the risk of developing autoimmune diseases (6). Antigens that escape from the gut may exacerbate intestinal inflammation and activate immune cells that also recognize self-antigen(6). Leaky gastrointestinal tracts have been associated with celiac and Crohn's disease (7). IEC express tight junction proteins that are important for maintaining intestinal integrity (8, 9). Treating IEC with retinoic acid (RA), the bioactive VA metabolite, induced the

expression of tight junction structures including ZO-1, occludin, claudin-6, and claudin-7 and the effects were dependent on IEC intrinsic RA receptor signaling (10, 11). Lima et al demonstrated that supplementing A- children with retinyl esters decreased urine lactulose/ mannitol levels, suggesting that vitamin A supplementation in A- human populations can enhance intestinal integrity and (4, 12). IEC play a critical role in intestinal barrier function and vitamin A is an important regulator of gastrointestinal barrier function.

To determine the role of vitamin A in IEC function, mice were generated in which retinoid signaling was knocked down by expressing a dominant negative retinoic acid receptor (dnRAR) in villin expressing IEC. Inhibiting RA signaling in IEC had no effect on weight gain, or the development of vitamin A deficiency (serum retinol) compared to WT littermates. The total cell numbers in the spleen, thymus, mesenteric lymph node (MLN), small intestine (SI) and colon were similar in WT and villin dnRAR mice. Expression of several IEC receptors and genes including *madcam1*, *ccl25*, *occludin*, *claudin7*, *claudin6*, *tla*, and *il15rα* were unchanged between WT and villin dnRAR mice. The frequencies of intraepithelial lymphocytes (IEL) were affected by villin dnRAR expression. Although total IEL numbers were unchanged, CD8αβ+ T cell frequencies increased and TCRαβ+ CD8αα T cell frequencies were lower in villin dnRAR mice compared to WT littermates. The MLN from the villin dnRAR mice had decreased frequencies of TCRβ+ and CD4+ T cells as compared to the MLN from WT mice. Disrupting retinoid signaling in IEC had no effect on susceptibility to C. *rodentium*; however, A+ villin dnRAR mice had a small increase in intestinal permeability after exposure to dextran sodium sulfate (DSS). A- villin dnRAR mice lost more weight and

had enhanced intestinal permeability after exposure to DSS compared to A- WT littermates. A- villin dnRAR mice were also extremely susceptible to C. *rodentium* infection (72% vs. 33%). Surviving A- WT and villin dnRAR mice became chronically infected reservoirs. Unlike A- WT mice, A- villin dnRAR mice were unable to resolve colonic hyperplasia. The effects of dnRAR expression in IEC on host immunity and intestinal permeability were secondary to the effects of vitamin A deficiency. The data suggests that retinoid signaling in other immune cells is more important for enteric immunity, homeostasis, and barrier function than direct regulation of IEC by vitamin A.

Materials and Methods

Animals. C57BL/6J villin- Cre mice from Jackson Laboratories (Bar Harbor, ME) were bred and maintained at the Pennsylvania State University (University Park, PA) according to IACUC and university guidelines. Mice expressing a floxed dnRAR were generously provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). Villin- Cre mice were crossed with dnRAR fl/fl mice to generate villin dnRAR expressing mice that blocked retinoid signaling through all 3 RAR isoforms in intestinal epithelial cells (13). dnRAR fl/fl (WT) littermates were used as controls. A+ and A- mice were generated by maintaining breeders and subsequent litters on purified diets with and without 25 ug/d retinyl acetate as previously described (14). VA status was determined through quantification of serum retinol levels by ultra-high pressure liquid chromatography (UPLC). Vitamin A deficiency was defined as serum retinol concentrations less than 0.7 uM or 20 ug/dL.

Flow Cytometry. IEC and IEL were isolated as described previously(15). Briefly, SI and colon tissues were cut longitudinally to increase surface area, and incubated twice (20 minutes at 37 C) with 1mM 1,4 dithiothreitol (DTT, Sigma Aldrich) and 10mM EDTA to release IEC and IEL. A 25/40% discontinuous Percoll (Sigma Aldrich) gradient was used to purify IEC while a 40/80% discontinuous gradient was used to purify IEL. 1-2 million cells were stained with fluorescein isothiocyanate (FITC) CD8β, FITC GL3 (γδ TCR), phycoerythrin (PE) GL3, PE-CF594 CD4, PE-Cy 5 T cell receptor β (TCRβ), Brilliant violet (BV) 421 TLA (BD Biosciences, San Jose, CA), PE IL15Rα (Thermo Fisher Scientific, Waltham, MA), PE-Cy7 TLR4, or PE-Cy7 CD8α (BioLegend, San Diego, CA). CD8αα was detected in the thymus with PE-labeled TL-tetramer (T3b)(16). The

tetramers were a gift from Dr. Hilde Cheroutre (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Single positive and fluorescence minus one (FMO) controls were used to set gates. Cells were analyzed on an FC500 benchtop cytometer (Beckman Coulter, Brea, CA), and data was analyzed using FlowJo 7.6.5 software (Tree Star, Ashland, OR).

FITC dextran permeability assay. 4000 kDa FITC dextran (Sigma Aldrich, St. Louis, MO) was performed as previously described (17). Briefly, mice were fasted for 4 hours and then gavaged with an 80 mg/kg dose. 4 hours later, mice were bled to obtain serum and a FITC dextran standard curve was prepared by diluting the stock solution with PBS. The standard and serum samples were transferred to black bottom 96 well plates and fluorescence was read at 525 nm on a Perkin Elmer Wallac Microplate Reader (GMI, Ramsey, MN). A linear curve was fitted to the standard and used to quantify serum FITC dextran levels.

Dextran sodium sulfate induced colitis. Mature 10-12 week-old mice were treated with DSS (Sigma Aldrich) to induce acute colonic injury and inflammation. DSS was administered orally in drinking water for 5 days followed by 5 days on water for recovery. Given the differences in susceptibility and based on previous experiments with A+ versus A- males and females the amount of DSS was varied by sex and diet. A+ male mice were treated with 4.5% DSS and A+ females with 4% DSS. A- male mice were treated with 4.25% DSS and A- females with 3.75% DSS. This amount of DSS was enough to cause disease without increasing mortality of the mice. Body weight changes were monitored for 10 days and mice were euthanized at day 10 post DSS exposure.

Colon lengths were measured, colon blood scores were assigned, and distal colon tissue was collected for histological analysis exactly as described (18).

***Citrobacter rodentium*.** The naladixic acid resistant C. *rodentium* strain was kindly provided by Gad Frankel (London School of Medicine and Dentistry, London, UK). Bacteria were cultured in Difco Luria-Bertani broth or agar (LB; Becton, Dickinson, & Co, Franklin Lakes, NJ) for 18-24 hours at 37C. Overnight log phase bacterial cultures in LB broth were used to prepare inoculums. Adult mice 8-10 weeks of age were individually housed, fasted overnight, and then orally gavaged with 100 ul of sterile phosphate buffered saline (PBS) containing 5 x 10^9 CFU C. *rodentium*. Fecal pellets, spleens, and livers were collected and plated in serial dilutions on LB agar plates containing nalidixic acid to quantify bacterial burdens and track infection kinetics.

Histology. Distal colons from naïve and C. *rodentium* infected mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin at the Pennsylvania State University Animal Diagnostics Laboratory. C. *rodentium* tissue sections were coded and scored by a board-certified laboratory animal veterinarian with pathology training. Tissue samples were scored for inflammatory cell infiltrate cell infiltrates and severity, mucosal damage, edema, and crypt loss (19, 20). The five longest crypts were identified visually from each samples and measured to determine an average crypt length.

RT-PCR. Tissues were snap frozen and stored at -80C until RNA isolation with TriZOL (Invitrogen, Carlsbad, CA). Cells (2 x 10^6) were suspended in 0.5 mL TriZOL and stored at -80C until processing. RNA isolation was performed according to TriZOL manufacturer's protocol. Complementary DNA (cDNA) was created by reverse

transcribing 2-4 ug RNA using TaqMan reverse transcription kit (Applied Biosystems, Carlsbad, CA). qPCR was performed using SYBR green mix (BioRad, Hercules, CA) and the MyiQ Single-Color Real Time PCR machine (BioRad). Relative standards were prepared by serially diluting DNA products of the genes of interest. A standard curve was generated to quantify relative expression levels in samples. Relative expression levels were normalized to a housekeeping gene (HPRT) and fold change values were reported relative to WT or untreated control tissues. Primers for RT-PCR are listed in Table 3-1.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Two-tailed student *t* tests were used to compare gene expression fold change, histological scores, and DSS parameters. One-way analysis of variance (ANOVA) with Bonferroni's *post* hoc test was used to analyze histology scores. Two-way ANOVA with Bonferroni's *post hoc* test was used to analyze IEL/ thymocyte populations through time and fecal shedding and weight loss curves. Log rank Mantel-Cox test was used to assess survival rates between genotypes and treatments. A P value of ≤ 0.05 was used as the threshold to determine statistically significant changes.

Results

Expression of the dnRAR did not alter IEC phenotypes.

A+ WT and villin dnRAR mice weighed the same at 10-12 weeks of age (Fig. 3-1A). A- villin dnRAR males weighed less than age matched A- WT mice, however there was no difference in the weights of the A- WT and villin dnRAR females (Fig. 3-1B). The numbers of immune cells isolated from the spleen, thymus, or MLN of villin dnRAR and WT mice were the same (Fig. 3-1C). Serum retinol levels reflected the A+ and A- status of the WT and villin dnRAR mice, and expression of the dnRAR did not affect serum retinol concentrations at 12-14 wks of age (Figure 3-1D). Inhibiting retinoid signaling in IEC did not affect growth, serum retinol levels, or peripheral immune cell numbers.

A+ WT and villin dnRAR SI and colon IEC were isolated and analyzed by flow cytometry for toll-like receptor (TLR)4, IL-15Rα, and TLA expression. Fluorescence minus one (FMO) controls were used to set gates (Fig. 3-2A). SI and colon IEC cell counts were unchanged between WT and villin dnRAR mice (Fig. 3-2B). TLR4+ mean fluorescence intensities (MFI) were slightly elevated on villin dnRAR IEC, but the change was not significant (Fig. 3-2C). No differences were observed in IL15R-α, TLA, or TL4+ IEC frequencies in the SI or colon (Fig. 3-2D, E). MFI for MHCI, IL15R-α, and TLA were not different on the WT and villin dnRAR IEC (data not shown). mRNA expression for *il15rα*, *tla*, *madcam1*, *ccl25*, and several tight junction proteins was quantified (Fig. 3-3B-H). Conversely, *il15* expression was significantly elevated in the villin dnRAR SI tissue (Fig. 3-3A). Expression of the dnRAR in IEC increased *il15* transcript in whole tissue but had no effect on the abundance of tight junction molecules

and lymphocyte homing ligands or IEC surface receptor expression, including TLA, TLR4, and IL15Rα.

Fewer CD8αα T cell frequencies in the IEL of the villin dnRAR mice.

A+ WT and villin dnRAR littermates were euthanized at 3, 5, 8, and 12 weeks of age to characterize the SI IEL. The total number of IEL cells isolated changed significantly as both WT and villin dnRAR mice aged. IEL numbers increased from 3-5wks of age and then plateaued (Fig. 3-4A). TCRαβ+ SI IEL frequencies increased as WT and villin dnRAR mice aged (Fig. 3-4B). At 3 weeks of age, villin dnRAR mice had higher frequencies of CD4+ T cells and lower frequencies of CD8αβ T cells in the SI IEL compared to WT littermates (Fig. 3-4C, D). The frequency of TCRαβ+ CD8αα+ cells increased with age in the SI IEL of both WT and villin dnRAR mice (Fig. 3-4E). WT mice from 5-12 wks old had significantly higher frequencies of TCRαβ+ CD8αα+ T than villin dnRAR mice in the SI IEL (Fig. 4E). There were no differences in the frequencies of TCRαβ+ CD8αα+ T cells in the colonic IEL of WT and villin dnRAR mice (data not shown). The frequencies of TCRγδ+ and TCRγδ+ CD8αα T cells in the SI IEL were not different in WT and villin dnRAR (Fig. 3-4F, G). Thymic CD4-/CD8- double negative (DN), CD4+/CD8+ double positive (DP), and CD4+/CD8+/CD8αα+ triple positive (TP) cell frequencies were not different in WT and villin dnRAR mice at 3, 5, or 12 weeks of age (Fig. 3-5A-C). In the spleen of WT and villin dnRAR mice the frequencies of TCRαβ+, TCRγδ+, CD8+, and CD4+ T cells were the same (Fig. 3-6). In the MLN the frequencies of TCRαβ+ and CD4+ T cells were higher in WT than the villin dnRAR mice (Fig.3-6A, B). Expression of the dnRAR in IEC resulted in a reduction of CD4 and

TCR αβ T cells in the MLN and reduced TCR αβ+ CD8αα+T cell frequencies in the SI IEL.

Increased damage following chemical injury of villin dnRAR mice.

A+ and A- WT and villin dnRAR mice were exposed to DSS in their drinking water to induce chemical colonic injury. The colons of WT and villin dnRAR were shorter after 10 days of DSS than in untreated mice (Fig. 3-7). A+ WT and villin dnRAR mice lost similar amounts of weight after DSS exposure and both groups had 100% survival (Fig. 3-7A). There was no blood observed in the intestines of the mice and the colon lengths were similar at d10 post-DSS in A+ WT and villin dnRAR mice (Fig. 3-7B). Intestinal permeability was slightly higher in A+ villin dnRAR mice, although not significantly (Fig 3-7C). Survival was 100% for A- WT and villin dnRAR mice. A- villin dnRAR mice lost more weight after DSS exposure compared to their WT counterparts (Fig. 3-7D). Blood scores were all 0 and colon lengths were the similar in the A- WT and villin dnRAR mice at day 10 post DSS exposure (Fig. 3-7E). A- villin dnRAR mice had significantly increased intestinal permeability compared to A- WT littermates (Fig. 3-7F). The effect of DSS on weight loss, colon length, and intestinal permeability was not different in male and female mice of either genotype in A+ or A- groups (Fig. 3-8A-B). There was no effect of vitamin A status on susceptibility to DSS (A+ WT versus A- WT), intestinal permeability, or colon length. Blocking retinoid signaling in IEC increased host susceptibility to DSS colitis in A- mice, but had minimal effects on intestinal permeability in A+ mice.

Villin dnRAR mice developed chronic colonic hyperplasia following enteric infection.

C. *rodentium* infection results in acute colonic inflammation and hyperplasia that resolved in 3 to 4 weeks in A+ WT mice (21). A+ villin dnRAR mice survived and were able to clear the *C. rodentium* infection (Fig. 3-9A, B) with kinetics similar to that of an A+ WT mouse (22). Infecting A- villin dnRAR mice resulted in 72% mortality rates with most deaths occurring prior to d12 post infection (Fig. 3-9A). A- villin dnRAR mice who survived past d15 developed chronic infections and shed log 7.9 CFU/g of the pathogen in their feces (Fig. 3-9C). At d30 post-infection, the chronically infected A- villin dnRAR mice had thick, shortened colons, a phenotype that was not observed in chronically infected A- WT mice. RA dosing A- villin dnRAR mice starting at d0 (RA d0) improved survival rates from 28% to 57%, although RA treatment failed to significantly affect fecal shedding kinetics (Fig. 3-9A). Early RA treatment induced \ resolution of macroscopic colonic thickening in the surviving A- villin dnRAR mice (Fig. 3-9D). Histological analysis of distal colon tissue revealed no differences in pathology scores or crypt lengths in uninfected A+/A- WT and A+/A- villin dnRAR mice (Fig. 3-10A). At peak infection (d10), A+ and A- villin dnRAR inflammation scores were similar, but crypt lengths were longer indicative of colonic hyperplasia (Fig. 3-10B). During late infection (d30), A+, A-, and A- RA d0 pathology scores were not significantly different (Fig. 3-10C). A- villin dnRAR crypts were longer than A+ villin dnRAR crypts, and RA d0 treatment reduced crypt lengths similar to those of A+ villin dnRAR mice (Fig. 3-10C). Expression of the dnRAR in IEC increased susceptibility to *C. rodentium* in A-, but not A+ mice.

Discussion

A+ villin dnRAR mice had reduced TCRαβ+ CD8αα T cell frequencies beginning at 5 weeks of age. Thymic CD8αα+/TCRαβ+ precursors (TP cells) were unaffected and CD8αα frequencies were similar between WT and villin dnRAR weanling mice, suggesting that villin dnRAR did not have a CD8αα T cell developmental defect. Retinoid signaling in IEC is required to expand and maintain SI TCRαβ+ CD8αα T cell populations as mice mature. IEC are known to express IL-15 and trans-presentation of IL-15 is required for CD8αα maturation and survival (23). IL15R knockout (KO) mice had reduced intestinal TCRαβ+ CD8αα frequencies and restoration of IL15-Rα expression on IEC restored TCRαβ+ CD8αα populations (23, 24). No changes in IL15-Rα expression were observed on WT and villin dnRAR IEC; however, *il15* expression was higher and not lower in villin dnRAR proximal SI tissue. Defects in IL-15 trans-presentation to the IEL were not ruled out. Other cytokines and ligands derived from IEC may be retinoid signaling dependent signals critical for expansion or survival of TCRαβ+ CD8αα T cell populations. TCRαβ+ CD8αα T cells are important for resolving intestinal inflammation and dampening inflammatory T cell responses (25). In the CD4+ $CD45RB^{hi}$ transfer model of colitis, co-transfer of intestinal TCRαβ+ CD8αα T cells into T and B cell deficient recipients ameliorated disease in an IL-10 dependent manner (25). IL-10 has also been associated with maintenance of intestinal integrity. IL10 deficient mice developed increased intestinal permeability that preceded any observable histological injury in the SI or colon (26). The decrease in SI TCRαβ+ CD8αα T cell frequencies in villin dnRAR mice may, via the production of IL-10, be related to the increased gastrointestinal permeability seen in the villin dnRAR mice. Retinoid signaling in IEC is required for maintaining SI TCRαβ+ CD8αα T cell populations.

Expression of the dnRAR in IEC had mild effects on enteric immunity and intestinal barrier function that were exacerbated by vitamin A deficiency. After DSS treatment, A+ villin dnRAR mice had a small increase in intestinal permeability compared to WT littermates. Intestinal permeability was significantly higher in A- villin dnRAR mice compared to A- WT mice after DSS exposure. dnRAR expression in the IEC of A+ mice did not affect host susceptibility to C. r*odentium*. A- villin dnRAR mice were extremely susceptible to C. *rodentium* (72% mortality rate) and unable to resolve colonic hyperplasia induced by the infection. In an A+ host, retinoid signaling in IEC is not required for host immune responses against an enteric pathogen or for resolving acute colonic damage and inflammation. Inhibiting RA signaling in IEC in an A- mice however, exacerbated host susceptibility to C. *rodentium* and DSS induced colitis. RA has been shown to promote tight junction structure formation and transepithelial resistance (a proxy for barrier function) in IEC (10, 11). DSS induces physical damage that enhances the permeability of the colonic epithelium and C. *rodentium* has been shown to enhance IEC apoptosis, disrupt tight junction expression, and enhance intestinal permeability (27, 28). Both disease models affect the integrity and permeability of the intestinal epithelium. In an A+ villin dnRAR mouse, intestinal integrity was slightly diminished, but innate and adaptive intestinal immune responses likely compensated and promoted clearance of the infection and resolution of inflammation. The effects of inhibiting retinoid signaling in IEC were exacerbated by changes in intestinal immunity associated with vitamin A deficiency. Vitamin A mediated regulation of innate and adaptive immune cells compensated for disrupted retinoid signaling in IEC and promoted gastrointestinal homeostasis and host resistance to *C. rodentium* infection.

Vitamin A deficiency remains a public health concern in resource limited countries and has been associated with decreased intestinal integrity and increased rates of *Giardia* spp. parasite infections (4). Additionally, there is evidence that a chronic leaky gut can contribute to the development of colon cancer and food allergies (29, 30). Gastrointestinal homeostasis has far reaching effects on health and disease and relies heavily upon proper function of the intestinal epithelium. Expression of the dnRAR in IEC led to dysregulated crosstalk with the IEL and reduced frequencies of TCRαβ+ CD8αα T cells. Inhibiting IEC retinoid signaling in A+ hosts had little effect on resistance to *C. rodentium* infection or DSS induced colitis in an A+ host. Conversely, A- villin dnRAR mice were more susceptible to *C. rodentium* infection and DSS colitis than A- WT littermates. Vitamin A deficiency, and the immunological defects associated with it, exacerbated the effects of dnRAR expression in IEC. Together, these observations indicate that although RA regulates IEC function, retinoid signaling may be more important for regulating innate and adaptive responses required for maintaining homeostatic conditions in the intestinal tract. Intestinal integrity and homeostasis have broad implications in health and disease and it is important to continue to studying how these physiological functions are regulated.

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Table 3-1: RT-PCR Primers

hprt:
F: 5’- CAGACTGAAGAGCTATTGTAATG-3’
R: 5’- CCAGTGTCAATTATATCTTCCAC-3’

il15
5’-CAG AGG CCA ACT GGA TAG ATG-3’
5’-ACT GTC AGT GTA TAA AGT GGT GTC AAT-3’

il15rα
5’-TTG GGA GAG AAA GCT TCT GG-3’
5’-CCA GTG CCA ACA GTA GTG ACA-3’

tla
5’-AAA AAG ACA CAG GAG TGC ACA G-3’
5’-TGA TGT CAG CAG GGT AGA AGC-3’

madcam1
5’-GGG CAG GTG ACC AAT CTG TA-3’
5’-ATA GGA CGA CGG TGG AGG A-3’

ccl25
5’-GAG TGC CAC CCT AGG TCA TC-3’
5’-CCA GCT GGT GCT TAC TCT GA-3’

occludin
5’-GTC CGT GAG GCC TTT TGA-3’
5’-GGT GCA TAA TGA TTG GGT TTG-3’

claudin7
5’-GAC GCC CAT GAA CGT TAA GTA-3’
5’-GGA CAG GAG CAA GAG AGC A-3’

claudin6
5’-TAT CCT GTC CCA GTC CCA AG-3’
5’-GTG CGT CTG TCC TGT GAG TTA C-3’

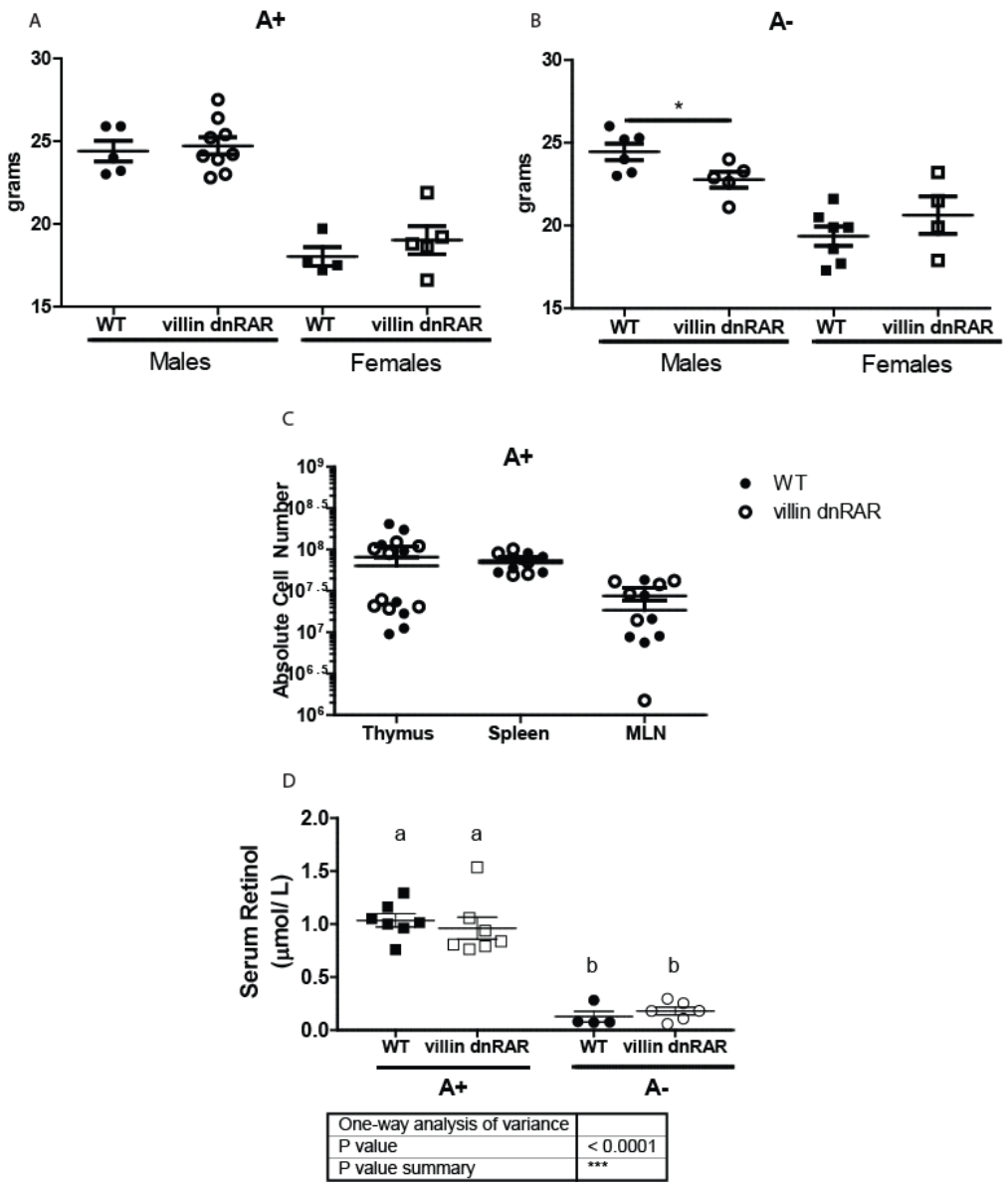


Figure 3-1: Villin dnRAR mice had no defects in growth, peripheral immune cell numbers, or serum retinol levels. A, B) Age matched WT and villin dnRAR mice were weighed (WT n=4-5 (males-females), villin dnRAR n=9-5 (males-females)). C) Thymuses, spleens, and MLNs were processed to enumerate immune cells (WT n=6, villin dnRAR n=7) and D) serum retinol was measure from littermates aged 12 to 14 weeks (A+ n=7/ group, A- n=4-6 (WT-villindnRAR)). Values are mean ± SEM of two to three experiments. Unpaired student's T test (A-C) and one way ANOVA with Bonferroni post-test (D). Groups with different letters are significantly different from each other, *P<0.05.

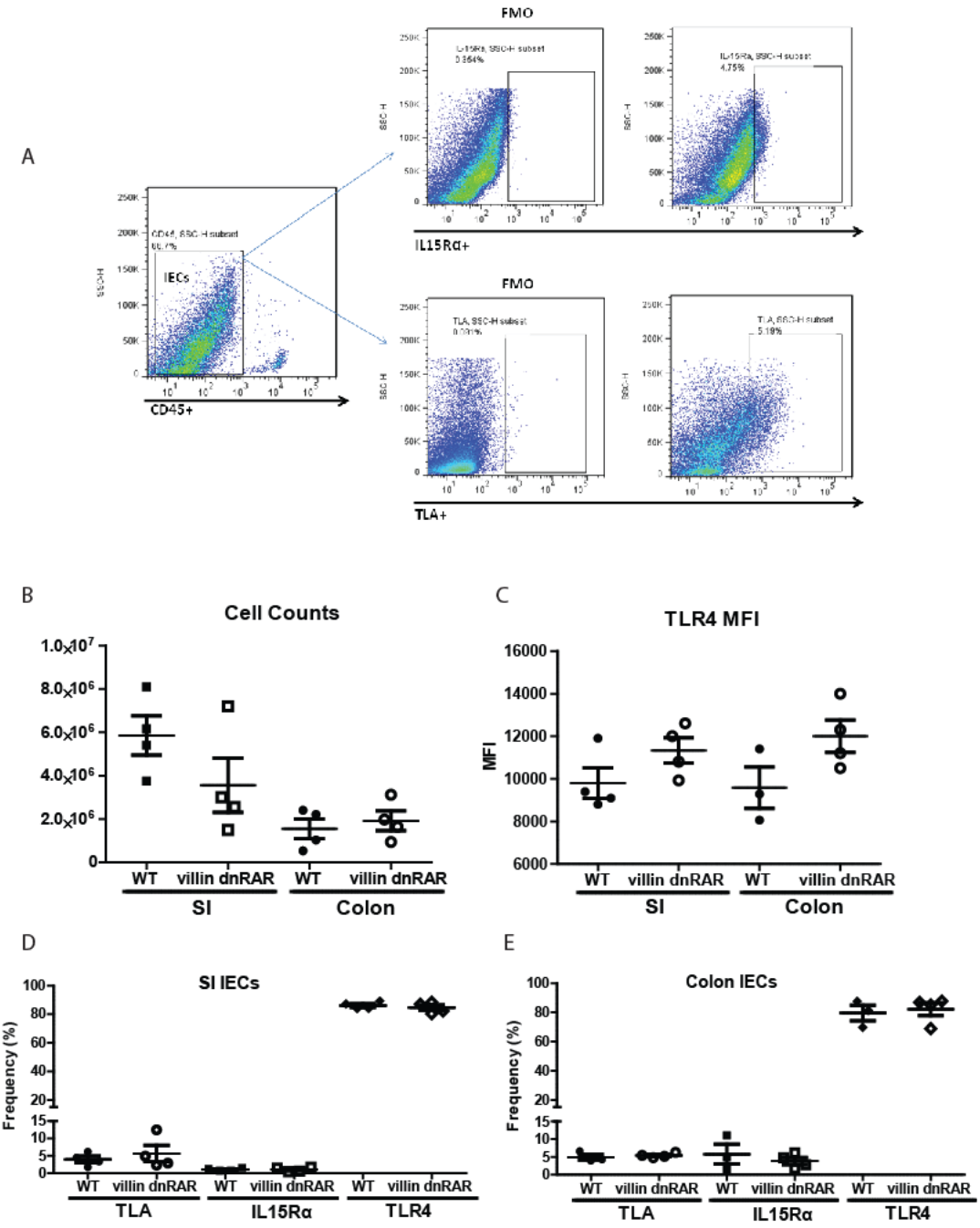


Figure 3-2: dnRAR expression did not alter IEC phenotypes. SI and colonic IECs from mature A+ WT and villin dnRAR mice were analyzed by flow. A) FMO controls were used in the gating strategy. B) SI and colonic IEC counts C) IEC TLR 4 mean fluorescence intensity, and D, E) TLA, IL15Rα, and TLR4+ IEC frequencies were quantified. Values are mean ± SEM of one experiment and n=4/ group. Mann Whitney test (B-E)

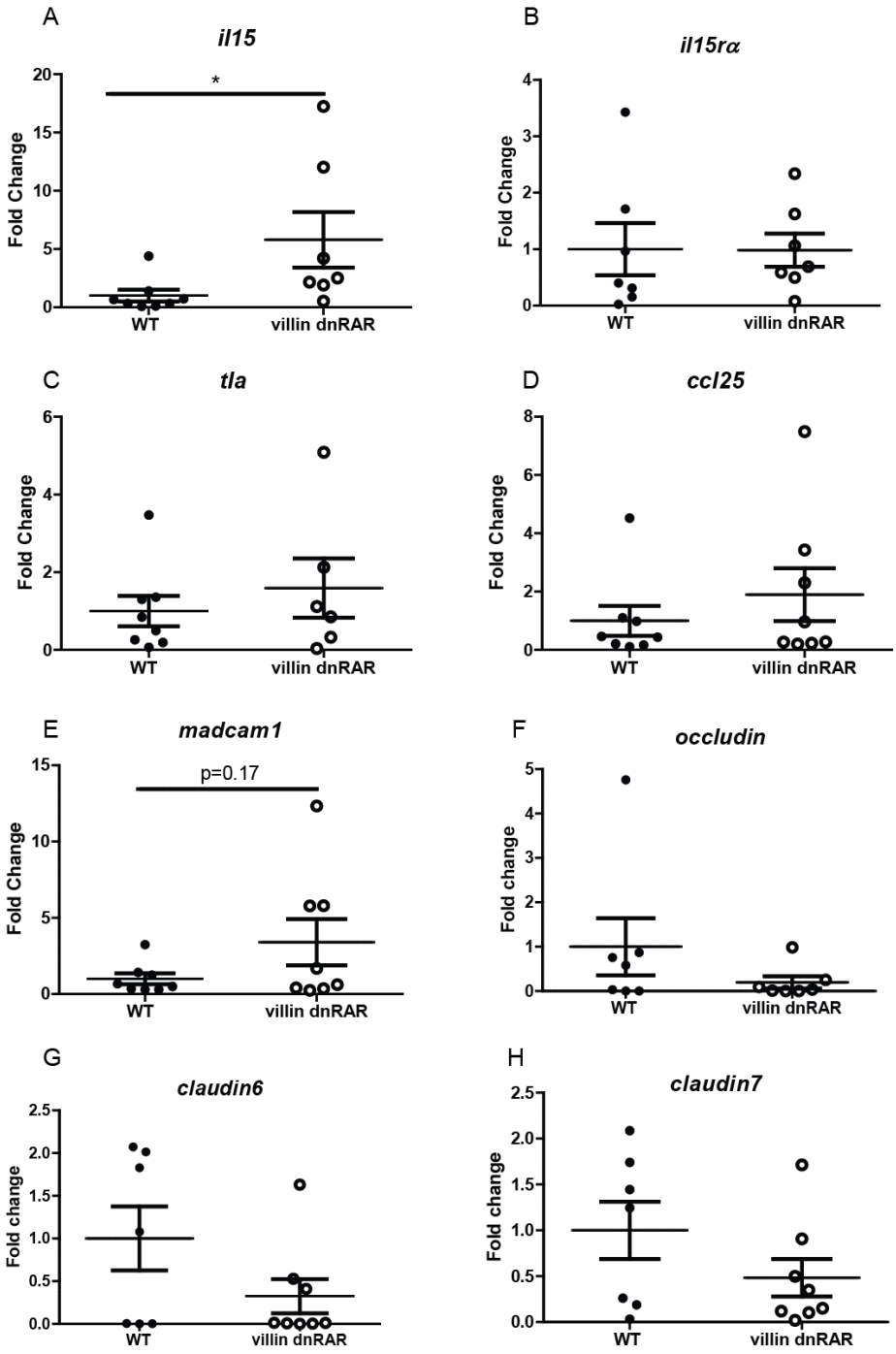


Figure 3-3: Effects of dnRAR expression in IECs on gene expression in the duodenum. Naive A+ WT and villin dnRAR animals 8 to 12 weeks of age were euthanized to collect duodenum tissue. A-E) The abundance of *il15*, *il15rα*, *tla*, *madcam1*, *ccl25*, *occludin*, *claudin6*, and *claudin7* was quantified by RT-PCR. Values are mean ± SEM of two combined experiments and n=8/ group. Mann Whitney test (A, F-H), unpaired student *t* test (B-D), or unpaired student *t* test with Welch's correction (E) *P<0.05

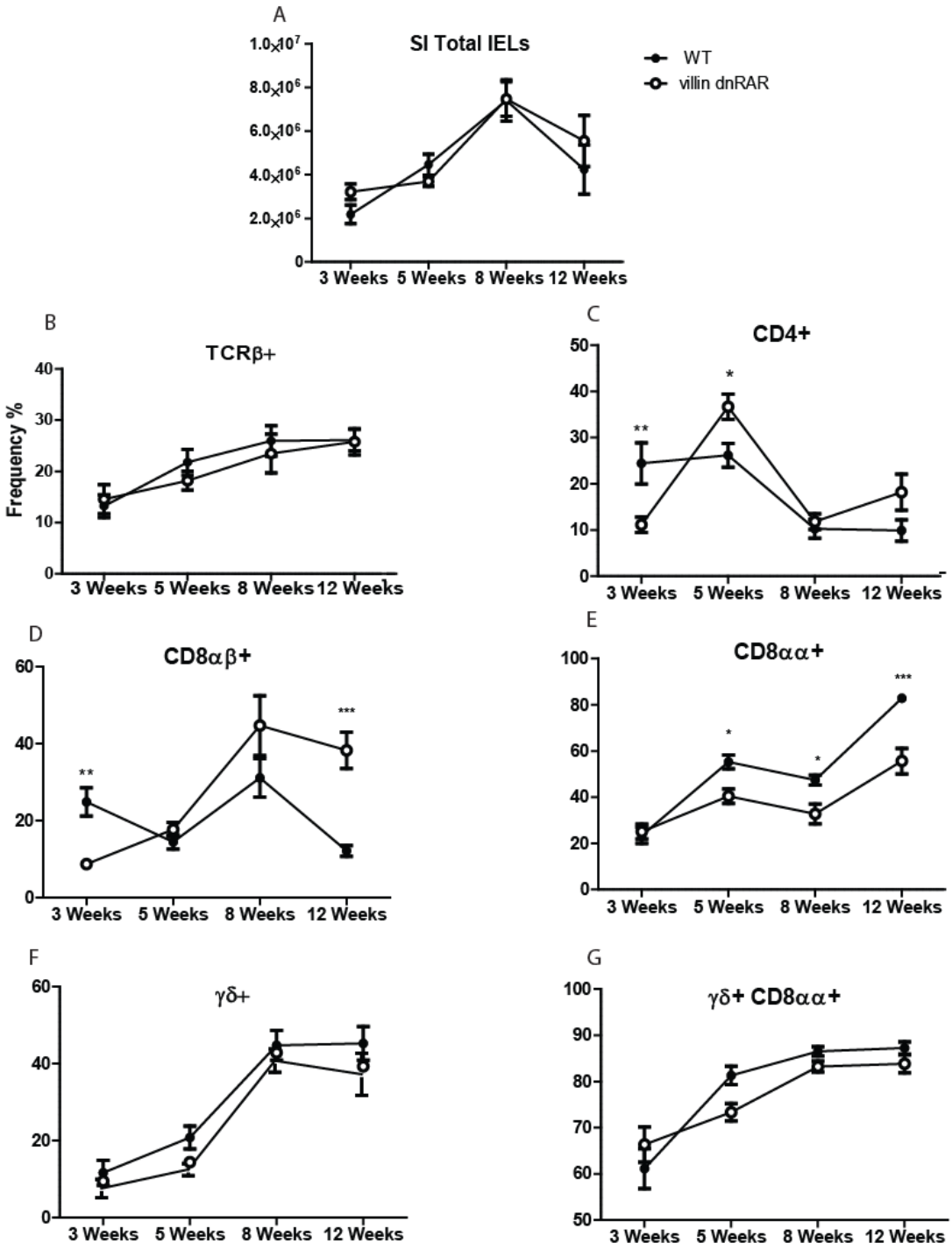


Figure 3-4: Reduced SI TCRαβ+ CD8αα T cell frequencies in villin dnRAR mice. A+ WT and villin dnRAR SI IEL populations were characterized at 3, 5, 8, and 12 weeks. A) Total IEL cells counts, B) TCRβ+, C) TCRβ-, D) CD4+ T cell, E) TCRαβ CD8αα+ T cell, F) TCRαβ CD8αβ T cell, G) γδ+ T cell, and H) γδ+ CD8αα+ frequencies were determined by flow cytometry. Values are mean ± SEM of two combined experiments, n=7-11/genotype at each time point. Two way ANOVA with Bonferroni post- test *P<0.05, **P<0.01, ***P<0.001

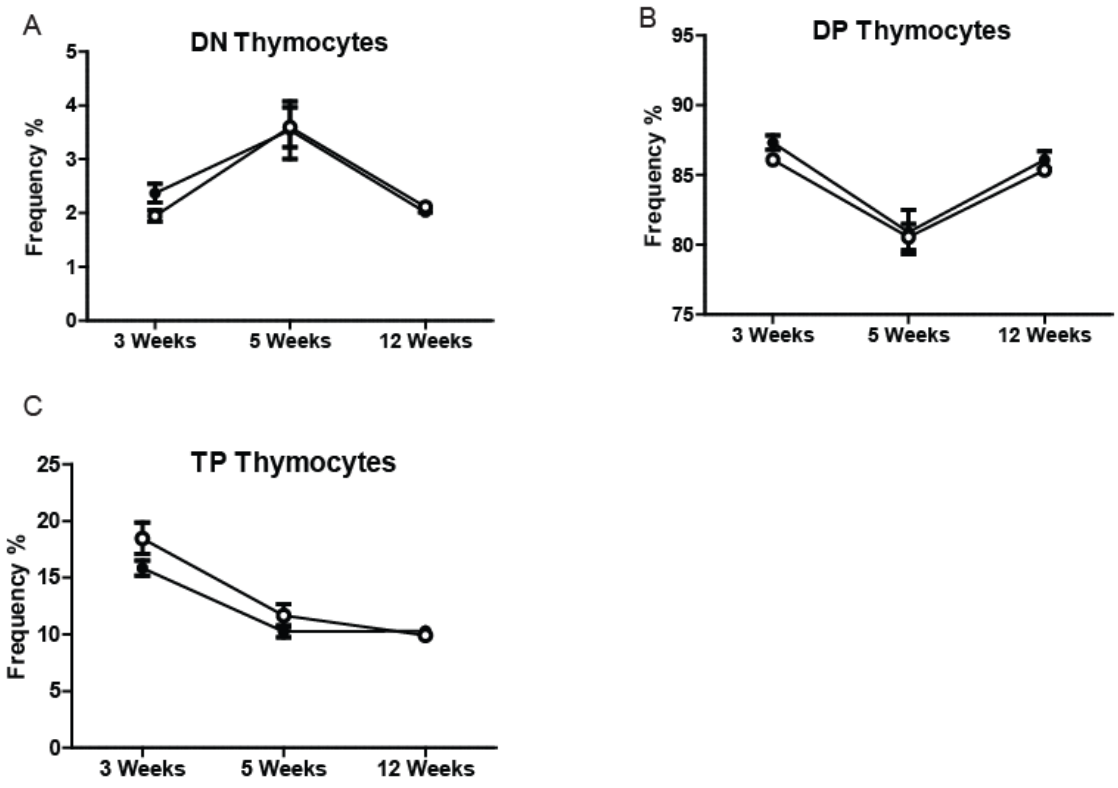


Figure 3-5: Thymocyte development was normal in villin dnRAR mice. A+ WT and villin dnRAR thymocytes were analyzed by flow at 3, 8, and 12 weeks of age. A) Thymocyte cell counts, B) CD4- CD8- DN, C) CD4+ CD8+ DP, and D) CD4+ CD8αα+, CD8αβ+ TP frequencies were determined. Values are mean ± SEM of two combined experiments, n=7-10/genotype at each time point. Two way ANOVA with Bonferroni post- test ***P<0.001

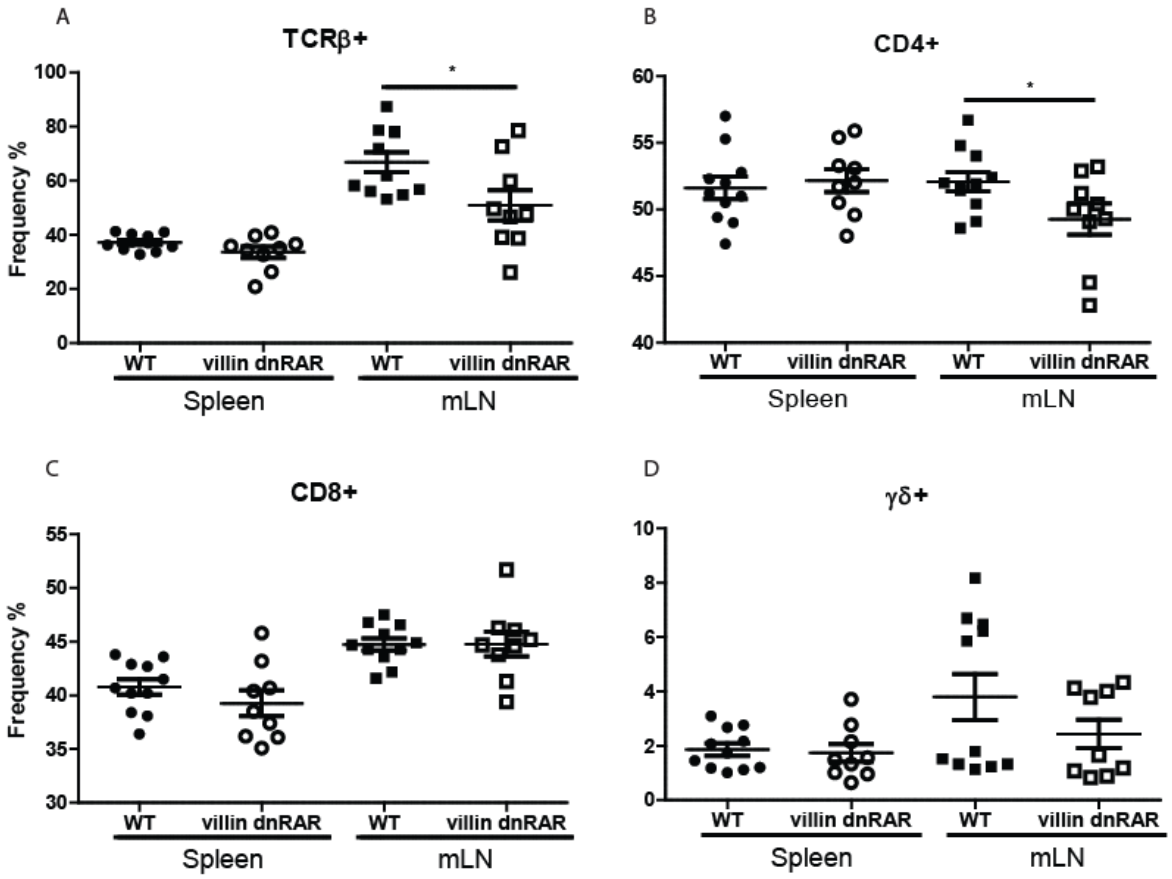
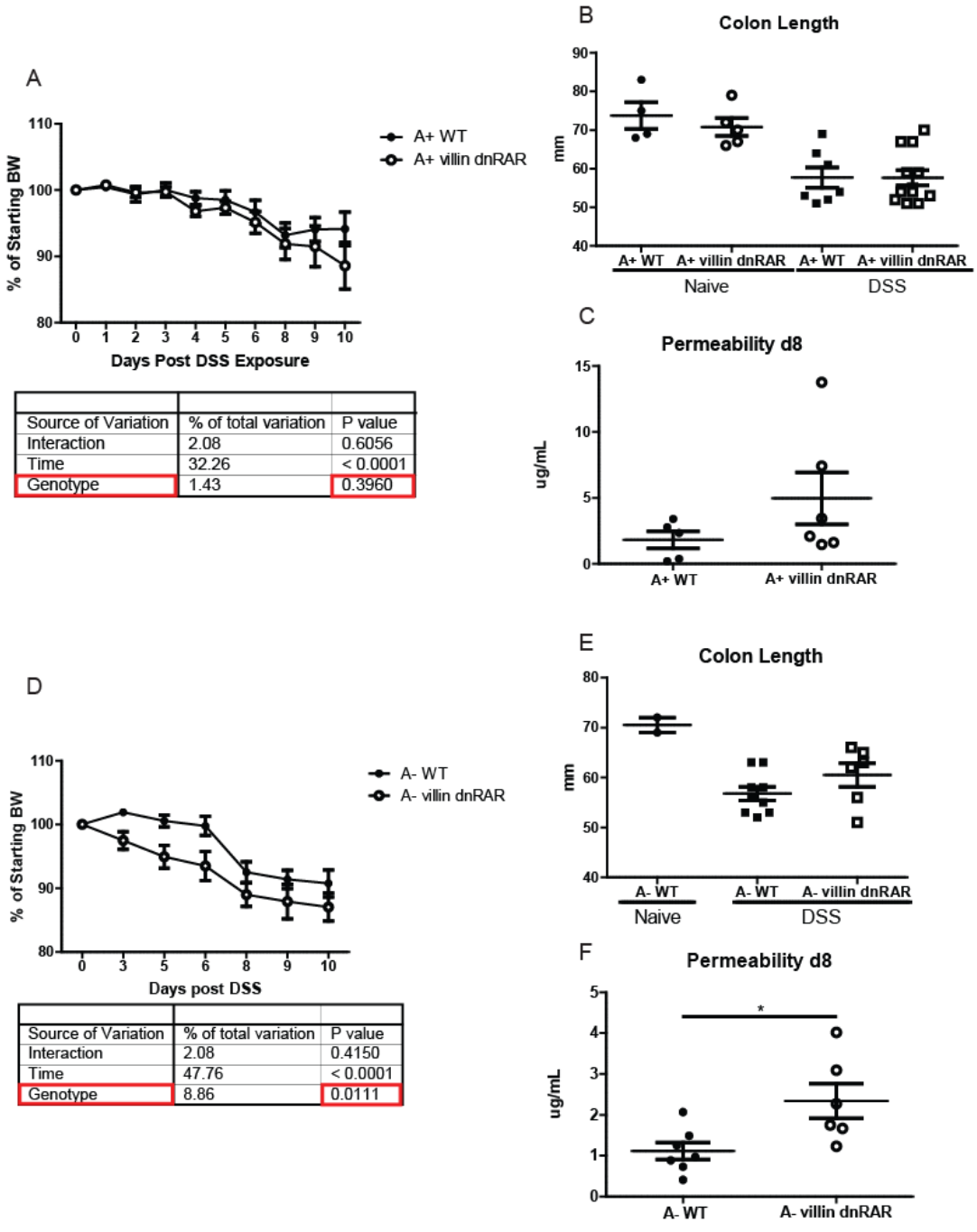


Figure 3-6: TCRβ+ and CD4+ frequencies were reduced in the villin dnRAR MLN. WT and villin dnRAR spleen and MLN single cell suspensions were obtained and T cell populations quantified by flow. A) TCRβ+, B) CD4+, C) CD8+, and D) γδ+ T cell frequencies were quantified. Values are mean ± SEM of two combined experiments, WT n=11, villin dnRAR n=9. Unpaired student's T test (A-D). *P<0.05



Source of Variation	% of total variation	P value
Interaction	2.08	0.6056
Time	32.26	< 0.0001
Genotype	1.43	0.3960

Source of Variation	% of total variation	P value
Interaction	2.08	0.4150
Time	47.76	< 0.0001
Genotype	8.86	0.0111

Figure 3-7: Vitamin A deficiency enhanced villin dnRAR susceptibility to DSS. A+ WT and villin dnRAR mice were exposed to DSS (n=7-12/group). A) Weight loss was monitored and B, C) colon lengths and intestinal permeability were measured (WT n=7, villin dnRAR n=11). D-F) The same parameters were measured in A- WT and villin dnRAR mice exposed to DSS (WT n=9, villin dnRAR n=6). Values are mean ± SEM of two experiments. Two way ANOVA with Bonferroni post-test (A, D) and unpaired student *t* test (B, E, F) and unpaired student *t* test with Welch's correction (C) *P-<0.05

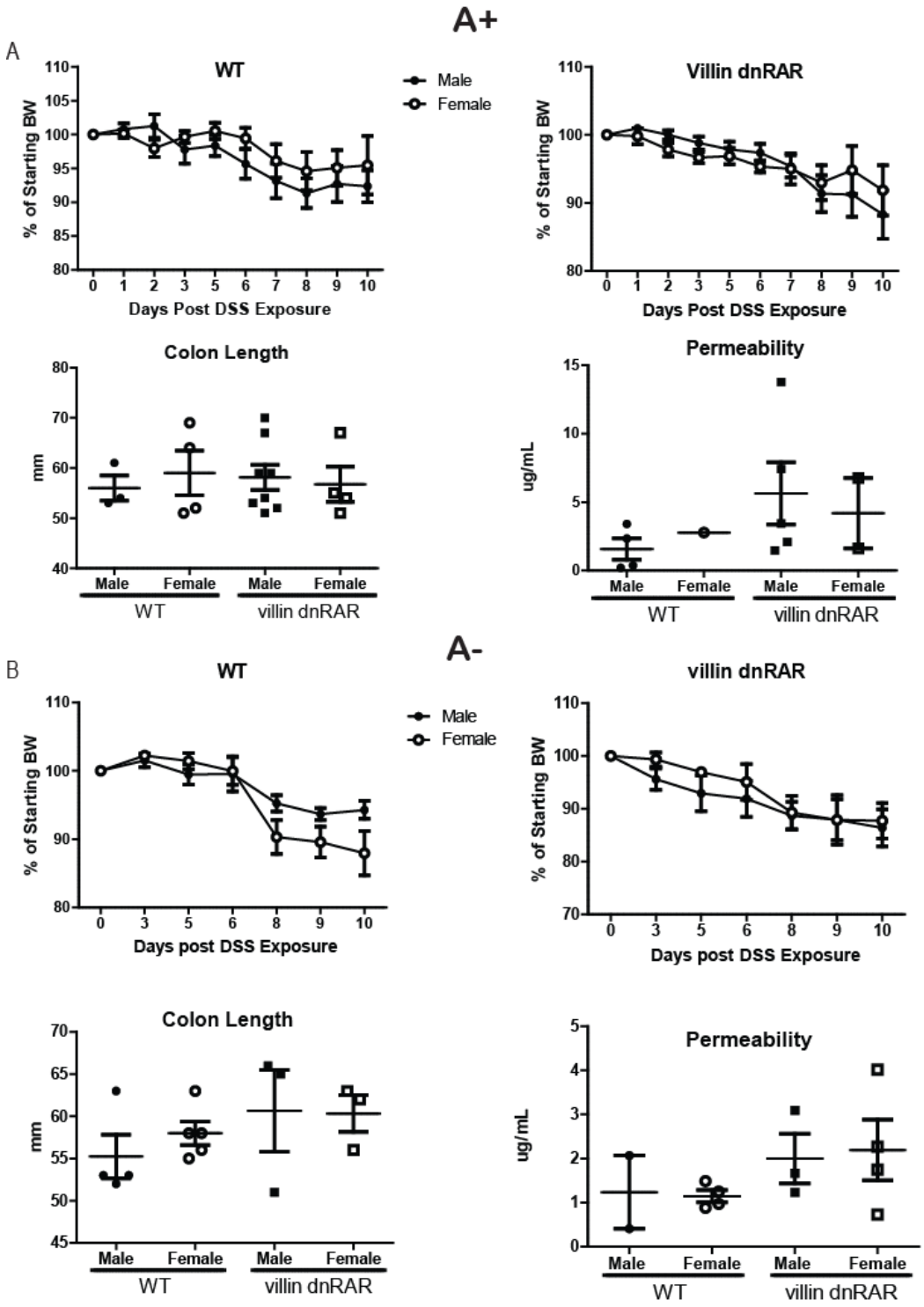
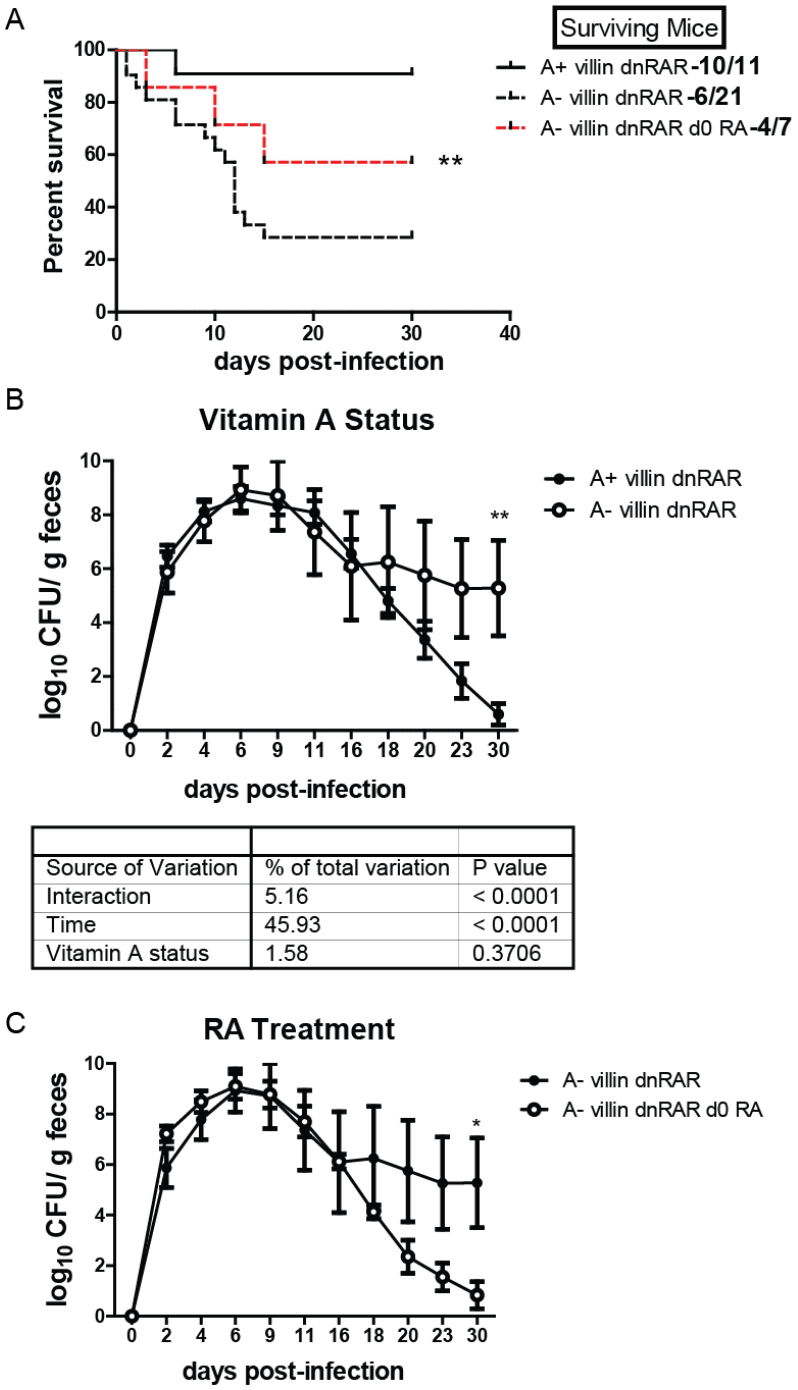


Figure 3-8: Sex did not affect host responses to DSS-induced colitis. DSS parameters including weight loss, colon length, and intestinal permeability were analyzed comparing male and female mice in A) A+ WT and villin dnRAR groups and B) A- WT and villin dnRAR groups. Two way ANOVA with Bonferroni post-test or unpaired student's T test with or without Welch's correction.



Source of Variation	% of total variation	P value
Interaction	5.16	< 0.0001
Time	45.93	< 0.0001
Vitamin A status	1.58	0.3706

Figure 3-9: A- villin dnRAR mice were highly susceptible to C. *rodentium* infection. 8 week old A+ and A- villin dnRAR mice were infected with C. *rodentium*. A) Survival and B-C) fecal shedding were monitored out to day 30 post-infection. Values are mean ± SEM of multiple experiments (A+ villin dnRAR n=10, A- villin dnRAR n=6, A- villin dnRAR RAd0 n=4). Log rank Mantel-Cox test to assess survival (A) and two way ANOVA with Bonferroni post-test (B, C) *P<0.05

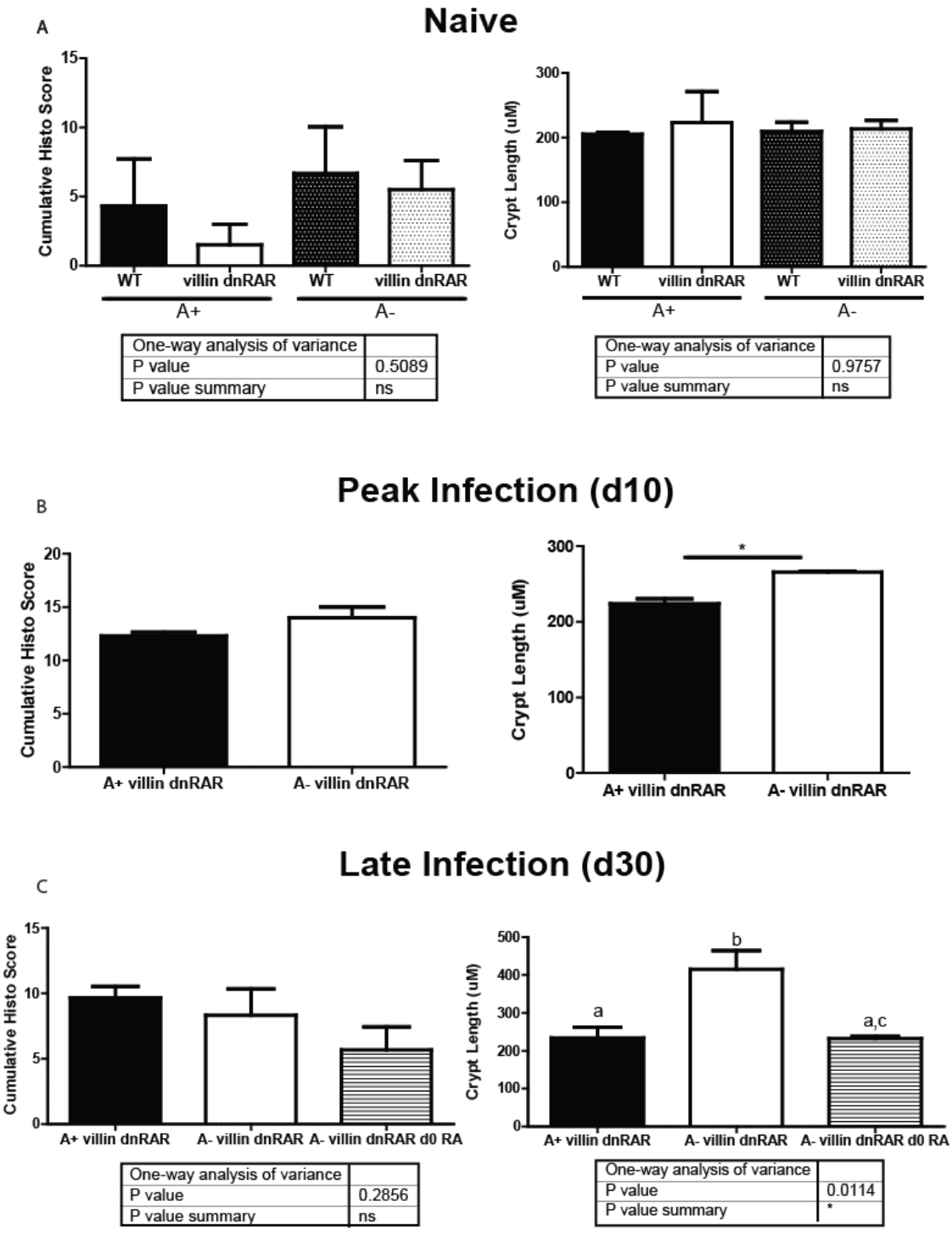


Figure 3-10: A- villin dnRAR mice were unable to resolve colonic hyperplasia following C. *rodentium* infection. A) Distal colons were collected from naïve A+ and A- WT and villin dnRAR mice for histological analysis (WT n=3, villin dnRAR n=4). Tissue was also collected from C. *rodentium* infected mice at B) peak infection (WT n=3, villin dnRAR n=2) and C) late infection (n=3/group). One way ANOVA with Bonferroni post-test (A, C) and unpaired student *t* test (B). Groups with different letters are significantly different from each other, $P<0.05$.

Chapter 4

Summary and Conclusions

Vitamin A deficiency (A-) is prevalent worldwide and is associated with increased morbidity and mortality due to severe enteric infections (1, 2). Similarly, A- mice were more susceptible to *C. rodentium* infection than vitamin A sufficient (A+) mice (3). Although retinoic acid (RA) protected A- mice from succumbing to *Citrobacter rodentium* infection, the mechanism was unclear (3). The role of vitamin A and retinoid signaling in intestinal immunity was investigated in this thesis. In chapter 2, A- and T-dnRAR mice were infected with *Citrobacter rodentium* and the protective effects of RA dosing in A- hosts and retinoid signaling in T cells were evaluated. Here, I show that a single dose of RA was sufficient to promote survival and clearance in A- mice, and RA treatments induced IL-17 production in the colon. This is in contrast to other work showing that RA inhibited Th17 function and IL-17 production *in vitro* and in A+ hosts (4, 5). Compared to A+ mice, A- mice had significantly fewer colonic IL17 secreting cells and CD4+ T cells were the primary producers of IL-17 in A+ mice. In A- mice, innate CD11b+ cells were the primary producers of IL-17 and RA treatment induced colonic CD11b+ cell mediated production of IL-17. Host derived IL-17 production is important for controlling *C. rodentium* infection (6). In an A- host, intestinal T cell homing and T cell numbers are diminished. It is possible that CD11b+ cell derived IL-17 is a compensatory response to infection in the gastrointestinal tract that does not occur in the A+ gut. RA induced IL-17 production by CD11b+ cells represents a novel pathway associated with protection of the A- host from *C. rodentium* infection.

Retinoid signaling in T cells is important for homing and maintenance of intestinal T cell populations and the host's ability to resolve C. *rodentium* infection. T-dnRAR mice had dysregulated thymic development and fewer intestinal αβ T cells compared to WT littermates. T-dnRAR mice survived initial infection and had robust colonic Th1/Th17 and CD11b+ cell responses to *C. rodentium* infection, but 60% of T-dnRAR mice became chronically infected reservoirs. T-dnRAR mice continued to shed high levels of the pathogen at d37 post-infection and persistent shedding was not eliminated with late RA treatment. Early RA dosing of T-dnRAR mice also had no effect on the development of chronically infected A- carriers of *C. rodentium* infection. Interestingly, chronically infected T-dnRAR mice had no symptoms of infection after d37. In 40% of T-dnRAR mice, immune responses were sufficient to induce resolution of the infection. A majority of T-dnRAR mice became chronically infected with *C. rodentium*, regardless of both functional T cell responses and production of IL-17 by CD4+ T cells and CD11b+ cells. Retinoid signaling is required for homing and maintenance of intestinal T cell populations, which is associated with host defense against *C. rodentium*.

Although RA treatment protected infected A- hosts, it was uncertain if RA treatment restored A- mice to an A+ phenotype. This question was addressed by characterizing small intestinal (SI) intraepithelial lymphocyte (IEL) populations and the serum and liver metabolite profiles of A+, A-, and A- RA mice. TCRαβ+ and γδ+ T cell frequencies were reduced in A- mice compared to A+ mice. RA treatment for 8 wks restored TCRαβ+ cells, but not γδ+ T cell frequencies. Furthermore, proton NMR-based metabolomics showed that A- serum and liver metabolites were distinct from those of A+

mice. RA dosing of A- mice resulted in a third metabolic profile that was different from both the A+ and A- mice. I show here that A+ and A- mice are metabolically and immunologically distinct, and that long term RA dosing does not restore A- mice to an A+ phenotype. Overall, chapter 2 provided evidence that the effects of RA are highly dependent on the vitamin A status of the host and that vitamin A deficiency has long term physiological effects that cannot be reversed through RA supplementation.

Chapter 3 utilized villin dnRAR transgenic mice to determine the role(s) of retinoid signaling in IEC. IEC are critical players in intestinal immunity. They serve as a physical barrier between the host and the luminal contents of the intestine; specialized IEC secrete mucins and anti-microbial peptides that shape the gut microbiota; and IEC engage in signaling crosstalk with intraepithelial lymphocytes to promote gastrointestinal homeostasis (7-10). Inhibiting retinoid signaling in IEC had no effect on growth, immune cell populations outside of the gut, or serum retinol concentrations in A+ and A- mice. The IEC from WT and villin dnRAR mice expressed similar levels of several important crosstalk/signaling molecules: TLA, IL15Rα, and TLR4+. Surprisingly, villin dnRAR mice had significantly reduced SI TCRαβ+ CD8αα+ T cell frequencies in the IEL. It has been established that CD8αα+ expression in the gut relies upon trans-presentation of IL-15 by IEC (11). The data in this chapter provides new evidence that signaling between IEC and the IEL is regulated by retinoid signaling. TCRαβ+ CD8αα+ T cells are important for dampening Th responses and inflammation in the intestinal tract (12). Diminished frequencies of these regulatory T cells could leave hosts more susceptible to chronic disease, enhanced intestinal permeability, and an inability to resolve intestinal inflammation.

To determine the effects of retinoid signaling in IEC on intestinal immunity and inflammation, WT and villin dnRAR mice were challenged with DSS and C. *rodentium*. A+ villin dnRAR mice had a small increase in intestinal permeability following DSS exposure. Expression of the dnRAR in IEC of A- mice resulted in enhanced susceptibility to *C. rodentium* infection and DSS colitis. After DSS exposure, A- villin dnRAR mice lost more weight and had increased intestinal permeability compared to A- WT mice. Additionally, ~70% of A- villin dnRAR mice succumbed to C. *rodentium* infection compared to ~30% of A- WT mice. Unlike A- WT littermates, chronically infected A- villin dnRAR mice had visibly thickened colons and significantly increased crypt lengths during peak and late infection. RA dosing of the A- villin dnRAR mice promoted resolution of the chronic colonic hyperplasia induced by *C. rodentium* infection, but RA treatment did not significantly improve survival rates or pathogen clearance. Retinoid signaling in IEC is not required for intestinal immunity and resolution of inflammation in an A+ host. In A- hosts however, disrupting retinoid signaling in IEC exacerbated host susceptibility to microbial and chemical induced colonic damage and caused dysregulated crypt proliferation in response to intestinal damage. Chapters 2 and 3 both demonstrate that retinoids are critical for regulating innate and adaptive immune cell function required to maintain intestinal immunity and homeostasis.

The findings in this dissertation have implications regarding vitamin A deficiency and the treatment of enteric infections and intestinal inflammation. The work presented here suggests that A+ and A- hosts are immunologically and metabolically distinct and that the effects of vitamin A deficiency are not reversed by RA supplementation (Fig 4-1). RA has been reported to inhibit IL-17 and inflammatory T cell responses *in vitro* and

in A+ mice (4, 5). Here, I show that in A- mice, RA induced colonic IL-17 production by CD11b+ cells, a response that was associated with host survival and resolution of the infection (Fig. 4-1). CD11b+ cells are novel retinoid targets and further research is necessary to determine the effects of RAR signaling on CD11b+ differentiation and function. My work demonstrated that retinoid signaling in IEC was required for maintenance of intestinal TCRαβ+ CD8αα T cell population. Expression of the dnRAR in IEC in A- hosts resulted in exacerbation of the effects of vitamin A deficiency on susceptibility to C. *rodentium* and DSS induced colitis. In summary, this work suggests that the effects of RA supplementation are variable in A+ and A- hosts and that supplementing A- mice with RA does not recover the metabolic and immunological phenotype of A+ mice. Supplementing mice with RA induced rapid, acute periods of retinoid signaling, independent of vitamin A metabolism. Conversely, retinyl esters are typically utilized to supplement humans. Retinyl esters must undergo several metabolic processes to be converted to RA and retinol. Retinol is an intermediate metabolite that can be stored for later use in the host. Childhood vitamin A deficiency may have long term, deleterious effects on metabolism and enteric immunity that cannot be corrected with RA supplementation; however, supplementation with retinyl esters may have different results as it can induce more long term, stable retinoid signaling. If vitamin A deficiency is not corrected in children, they may be at increased risk of developing chronic inflammatory gastrointestinal disease. Longitudinal studies should be performed to better understand the long term effects of childhood vitamin A deficiency and retinyl ester supplementation on immune function, metabolism, and risk of developing chronic

diseases. Retinoid signaling regulates innate and adaptive immune responses that are critical for host defense against enteric pathogens and maintaining intestinal homeostasis.

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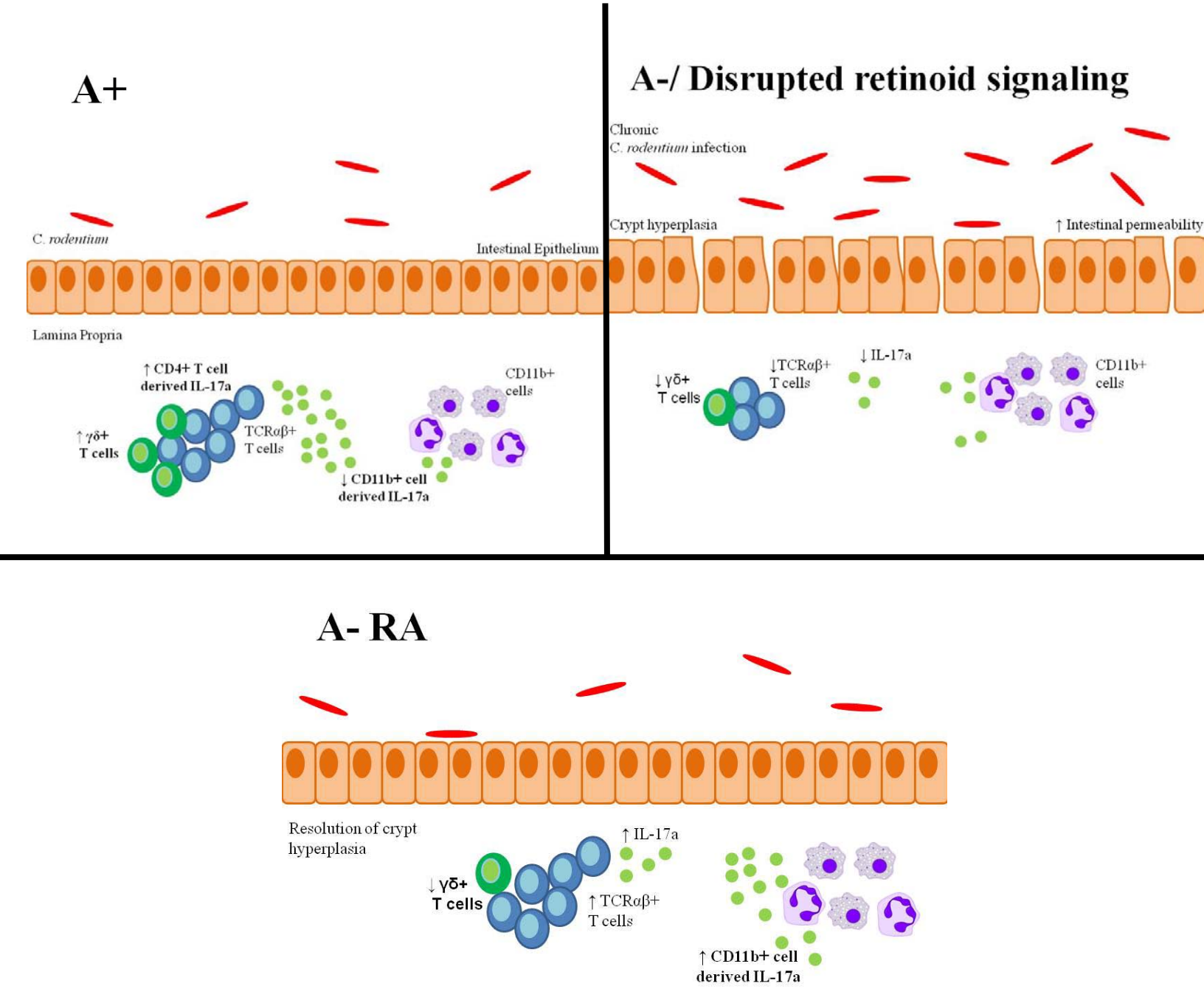

Figure 4-1: Retinoic acid treatment regulates innate and adaptive immune responses and protects A- mice from enteric infection. In a vitamin A sufficient (A+) host, TCRαβ+ and TCRγδ+ T cells home to the gut, T cells produce IL-17 in response to C. *rodentium* infection, epithelial integrity remains intact, and *C. rodentium* infection is resolved. In a vitamin A deficient (A-) host or in the absence of retinoid signaling, T cells are unable to home to the intestine, colonic IL17a production in response to infection is diminished, the intestinal epithelium is leaky and prone to crypt hyperplasia, and mice become chronically infected reservoirs of *C. rodentium*. In RA treated A- mice (A- RA), TCRαβ frequencies are recovered, treatment induces colonic CD11b+ cells to produce IL-17(associated with protection from C. *rodentium* infection), and crypt hyperplasia is resolved; however, TCRγδ + populations remain dysregulated.

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Publications and Professional Presentations

Snyder, L.M., K.L. McDaniel, Y. Tian, C-H. Wei, M.J. Kennett, A.C. Ross, M.T. Cantorna. CD11b+ and T cells are critical retinoid targets for control of *Citrobacter rodentium* infection. (Submitted)

● Dey, A., J. Allen, J. Frasier, **L. Snyder,** Y. Tian, L. Zhang, R.F. Paulson, A. Patterson, M.T. Cantorna, and P. Hankey- Giblin. 2018. Neuroprotective role of the Ron Tyrosine Kinase underlying CNS inflammation in health and disease. *Front. Immunol.* (accepted for publication)

● **Snyder, L.,** V. Weaver, A.C. Ross, M.T. Cantorna. 2017. Retinoic acid induces CD11b+ leukocyte production of IL-17 and T cell responses that protect the vitamin A deficient (A-) host from gastrointestinal infection. International Congress of Mucosal Immunology (oral presentation/poster)

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● **Snyder, L.**, M. Kamat, S. Vasudevan, J. Hester, A. Gernand, F. Diaz, T. Ott, M.T. Cantorna. 2015. Vitamin D and vitamin D receptor signaling effects on implantation and maternal T cells and NK cells during murine pregnancy. American Society for Reproductive Immunology (poster)

● Gernand, A., M.T. Cantorna, F. Diaz, **L. Snyder**, J. Hester, S. Vasudevan, M. Kamat, and T. Ott. 2015. Pre-pregnancy, vitamin D deficiency, and placental development in mice. Experimental Biology (poster)

Awards

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