Effects of Dietary Vitamin D in Mediating Protection Against Colitis and Colitis-Associated Colon Cancer in *Smad3*$^{-/-}$ Mice

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Abstract

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Epidemiologic studies suggest that low serum vitamin D levels are associated with an increased risk of colon cancer and inflammatory diseases such as inflammatory bowel disease (IBD). We have utilized 129-*Smad3*^{tm1Par}/J (*Smad3*$^{-/-}$) mice, which have dysregulated transforming growth factor β signaling and are a model of colitis and inflammation-associated colon cancer to evaluate the effects of modulated dietary vitamin D on colitis and colon cancer. Through these studies, we have determined that increased dietary vitamin D reduces typhlocolitis and colon cancer using both bacterially-induced colitis through infection with *Helicobacter bilis* and chemically-induced colitis using dextran sodium sulfate. Our data suggest that increased dietary vitamin D is

beneficial in preventing inflammation-associated colon cancer at least in part through suppression of inflammatory responses during tumor initiation or early stage carcinogenesis. Importantly, the protective effects afforded by dietary vitamin D are dependent in part upon the presence of $CD4^+$ cells. In addition to evaluating the effects of increased dietary vitamin D, we have utilized a model of chronic vitamin D deficiency to evaluate the effects of vitamin D deficiency on colitis and colitis associated colon cancer. Interestingly, while vitamin D deficiency did not affect the disease severity or progression in *H. bilis*-infected *Smad3*$^{-/-}$ mice, vitamin D-deficient *Smad3*$^{-/-}$ mice were protected against DSS-induced colon cancer due to increased cellular proliferation during the healing phase following DSS-treatment. Finally, we have utilized our model to demonstrate that the alteration of vitamin D concentrations in the diet is sufficient to significantly alter the gut microbiome. Together, these studies provide important insights into the mechanisms through which dietary vitamin D modulates inflammation and tumor development in a genetically susceptible host.

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DEDICATION

This work is dedicated to my wonderful family for their encouragement, love, and constant support that has allowed me to accomplish so much in my endless career as a student.

Chapter 1. INTRODUCTION AND BACKGROUND

INTRODUCTION:

Vitamin D is primarily known for its role in regulation of bone metabolism by controlling intestinal calcium absorption [1-3] and bone remodeling [4], the importance of which is demonstrated by prolonged vitamin D deficiency resulting in delayed growth and rickets in children [1] as well as osteoporosis and osteopenia in adults [5]. In addition to its role in bone metabolism, it has become increasingly apparent that vitamin D participates in a variety of other physiological functions, including immune responses. Consequently, vitamin D deficiency is also associated with a wide range of diseases including: asthma [6], multiple sclerosis [7], rheumatoid arthritis [8], type 1 diabetes [9, 10], heart disease [11, 12], depression [13, 14], and tuberculosis [15]. For our interests, epidemiologic studies link low serum vitamin D levels with an increased risk of developing inflammatory bowel disease (IBD) and colon cancer [16-23]. As vitamin D deficiency or insufficiency is increasingly prevalent around the world, with estimates of 30-50% of children and adults at risk of vitamin D deficiency worldwide [3], it could significantly influence incidence and progression of IBD and colon cancer. Here, we will review current evidence linking vitamin D, IBD, and colitis-associated colon cancer (CAC) and summarize the potential benefits and risks of using vitamin D to prevent or treat these diseases. Several reviews have been published recently concerning vitamin D and the immune system [18, 24-27] and cancer [19, 28, 29].

OVERVIEW OF IBD, CAC, AND VITAMIN D:

IBD is a group of diseases characterized as chronic remittent or progressive inflammation of the gastrointestinal tract. The two primary conditions in humans are Crohn's Disease (CD) and ulcerative colitis (UC), and the prevalence of both conditions have been increasing in western countries over the past 50 years with CD affecting 50-200/100,000 people and UC affecting 120-200/100,000 people per year [30]. Though the precise etiology underlying IBD remains unclear, dysregulation of the mucosal immune system in response to enteric antigens is

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believed to be one of the initiators of the chronic inflammation [31]. Patients diagnosed with IBD are at increased risk for developing CAC compared to the general population, especially if the colitis is not well controlled [32-34]. Inflammation is believed to play a role in the development of CAC through promotion of angiogenesis, tumor-promoting cytokine production, tumor cell invasive behavior, cellular proliferation and alterations in immune responses [35]. Treatments to limit inflammation may be beneficial in reducing the incidence of CAC in these high-risk populations [36]. Because vitamin D has been shown to alter many of the pathways involved in inflammation as well as tumorigenesis, the potential to use vitamin D supplementation as an adjunct therapy in IBD patients is being explored [37-40].

We have recently shown that dietary vitamin D supplementation provides protection against IBD and subsequent inflammation-induced colon cancer in the *Smad3*$^{-/-}$ mouse model of CAC [41]. Our data suggest that the chemoprotective effect of vitamin D is likely due to decreased colitis prior to tumor development. Our findings are consistent with other studies demonstrating association between vitamin D supplementation and decreased colitis, disease activity and/or alleviated symptoms of IBD in both mice [41-44] and humans [22, 38, 39]. Additionally, both our work and others have demonstrated that vitamin D decreases the incidence of dysplasia, a precursor to cancer, in models of IBD-associated colon cancer [44-46]. These data support the notion that vitamin D may be a beneficial adjunct therapy for IBD and CAC. Because there is currently no cure for IBD, patients typically require long-term immunomodulatory drug therapy to manage their symptoms with substantial economic costs over their lifetime [47]. The use of vitamin D as a treatment in patients with IBD would be simple and inexpensive to implement. However, the specific mechanisms through which vitamin D ameliorates colitis remain unknown though vitamin D has been shown to regulate immune cell trafficking and differentiation, gut barrier function, and antimicrobial peptide synthesis, all of which may play a role in mediating protection from disease [48-50]. Thus, additional studies are needed to determine characteristics of patient population that might benefit from vitamin D therapy.

VITAMIN D METABOLISM AND SIGNALING:

To explain mechanisms by which vitamin D might influence IBD and colon cancer, we will briefly review the metabolism and actions of vitamin D. More thorough reviews on this topic can be found in recent publications [3, 51].

Humans obtain the majority of their vitamin D from exposure to sunlight as UVB radiation is able to convert 7-dehydrocholesterol to vitamin D_3 (cholecalciferol) in the skin. Diet, however, becomes an important means of obtaining vitamin D in individuals with limited exposure to sunlight, such as might occur due to insufficient exposure to natural sunlight because of residing at higher latitudes or limited amounts of time spent outdoors, or increased protection against sun exposure through clothing and/or sunscreen. Therefore, inadequate intake of foods rich in vitamin D such as oily fish, beef liver, and fortified milk products can significantly impact vitamin D status in some individuals [2, 52].

Vitamin D, synthesized in the skin or taken up from the diet, undergoes two hydroxylation steps to become biologically active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). The first hydroxylation step occurs in the liver where a 25-hydroxylase (Cyp2R1 and possibly other enzymes) produces 25-hydroxyvitamin D (25(OH)D) [53, 54]. This is the main circulating form of vitamin D and is most often used as an indicator of nutritional vitamin D status, both in humans and animals, as it is highly stable and has a long half-life [3]. The majority of 25-hydroxyvitamin D circulates in blood bound to either vitamin D binding protein (DBP; 85-90% bound) or albumin (10-15% bound) with <1% unbound as free vitamin D [48]. The second hydroxylation-step occurs primarily in the kidney by 1α-hydroxylase (Cyp27b1) to produce active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). The active form of vitamin D can also be measured in the serum, although its concentrations do not reflect nutritional vitamin D status as the half-life is relatively short (4-20hr) and its production is highly regulated [3] by serum levels of calcium and phosphorus as well as activities of parathyroid hormone (PTH) and fibroblast-like growth factor-23 [3]. Both 25(OH)D and $1,25(OH)_2D$ can also be inactivated through further hydroxylation by 24-hydroxylase (Cyp24a1), which is induced by vitamin D signaling in almost all target tissues of vitamin D [3]. More detailed information on the synthesis, uptake, and metabolism of vitamin D has been reviewed [3, 55]. Although the majority of vitamin D hydroxylation occurs in the liver (25-hydroxylation) and kidney (1α -hydroxylation), other

tissues, including intestinal epithelial cells and immune cells (T and B cells, macrophages, dendritic cells), express vitamin D hydroxylase enzymes (Cyp27b1, Cyp24a1) [56, 57], suggesting that these cells can regulate levels of the active hormone locally.

$1,25(OH)_2D$, elicits its effects through binding to the vitamin D receptor (VDR), a transcription factor for genes with promoters containing vitamin D response elements (VDREs) [58]. VDR bound by $1,25(OH)_2D$ can recruit transcription machinery to activate a plethora of down-stream genes. Through chromatin precipitation parallel DNA sequencing studies (CHIP-seq) more than 2,776 VDR binding sites have been identified throughout the human genome [59]. Interestingly, this study noted that VDR binding sites were significantly enriched near genes known to be associated with autoimmune disease and cancer, including *PTPN2,* a gene that has been implicated in CD and UC due to its important role in maintaining epithelial barrier function [59]. This suggests that expression of these genes may be decreased in people with inadequate levels of vitamin D due to reduced VDR ligand. More in depth reviews on VDR and gene expression have been previously published [60-62].

Multiple VDR polymorphisms have been reported in humans and are associated with various diseases including IBD, cancer, asthma and obesity [63-65]. However, many of these polymorphisms occur in non-coding regions of the VDR gene and appear to have no direct consequence to the VDR protein itself, except for one polymorphism where the transcription start site is changed resulting in a shorter and more active VDR [63]. More frequently, VDR gene polymorphisms affect VDR transcription due to changes in the promoter region or 3'UTR but the functional consequence(s) of these changes is not well defined [63]. Loss of function mutations in the VDR result in vitamin D deficiency symptoms including rickets [66]. When mutations affect VDR's binding to its ligand, $1,25(OH)_2D$, symptoms are similar to vitamin D nutritional deficiency. However, when mutations affect VDR's binding to DNA (via VDREs), alopecia can occur in addition to symptoms of vitamin D deficiency, suggesting that VDR has ligand-independent functions [66]. These types of mutations have been replicated in murine models [67, 68], and studies using these animal models revealed that VDR can actively suppress down-stream genes when bound to VDREs without its ligand. Thus, mice with VDR mutations affecting ligand binding but that maintain DNA binding ability have more severe skeletal defects than mice with vitamin D deficiency or mice with VDR null mutations [67]. The mutated VDR, VDR_{gem}, does not respond to endogenous VDR ligand 1,25(OH)2D but can be activated by

synthetic analogs, gemini ligands. Hence, the VDR_{gem} model may be useful in deciphering ligand-dependent and -independent roles of VDR [67]. For our interests, this mouse model could be used to determine if ligand-independent VDR function influences immune responses and thus the development of IBD/CAC and to develop drugs to target these specific functions [67].

HUMAN EPIDEMIOLOGIC DATA ASSOCIATING SERUM VITAMIN D LEVELS, IBD, AND CAC:

It has been observed that the incidence of IBD is higher in people residing at northern latitudes compared to those at more southern latitudes both in the United States [69] and in Europe [70-72], suggesting that limited exposure to UVB and resultant decreased vitamin D levels may influence risk of IBD. In addition, lower UVB exposure and vitamin D deficiency has been associated with onset of IBD [16], increased clinical disease activity [73-75], greater rates of hospitalization, prolonged hospitalization and an increased need for bowel surgery in patients with IBD [76, 77], and risk of malignant transformation [23]. Along similar lines, results from a questionnaire assessing the overall quality of life of IBD patients (141 CD and 79 UC), noted that increased serum vitamin D levels correlated with increased health-related quality of life in both CD and UC patients during winter/spring months [78]. Interestingly, VDR expression was significantly decreased in intestinal epithelial cells from IBD patients compared to healthy controls. In addition, intestinal VDR expression was lower in patients with active CD compared to those with quiescent disease despite one cohort of patients studied having “adequate” serum levels of vitamin D [79], suggesting that epithelial responses to vitamin D may be diminished during active IBD and this may not be altered by increasing serum vitamin D alone.

Similar to the findings in IBD patients, epidemiologic data demonstrate a significant north/south gradient associated with colon cancer with increasing incidence and mortality associated with reduced exposure to natural light [20, 21, 80]. Prospective cohort studies have demonstrated an inverse association between serum vitamin D levels (25(OH)D) and cancer development (colon, breast and prostate) [29, 81, 82]. Interestingly, Ananthakrishnan *et al.* demonstrated that within a cohort of 2,809 IBD patients almost a third of the patients were vitamin D deficient [23]. Over an eleven-year follow up period, 7% of those patients went on to develop cancer. Strikingly, the patients that went on to develop cancer had significantly lower

vitamin D levels than those who did not develop cancer. It was estimated that for every 1 ng/ml increase in plasma 25(OH)D concentration, there was a 6% reduction in colorectal cancer risk [23].

Although there is a strong inverse association between serum levels of 25(OH)D and the risk for IBD/CAC, there is no consensus about what the adequate serum vitamin D should be to prevent these diseases. Historically, adequate levels of vitamin D were defined based on maintenance of calcium homeostasis without causing bone disease. However, as our knowledge of the impacts of vitamin D on human health and disease become broader, experts have sought to re-define the levels of vitamin D to provide the most protective health benefits. Unfortunately, this is much more difficult to determine as "health benefit" is hard to define. According to the Institute of Medicine, vitamin D sufficiency was determined to be ≥ 50 nM serum 25(OH)D, while serum vitamin D levels of 25-50 nM were classified as insufficient and <25 nM deficient [55]. In contrast, the Endocrine society defined vitamin D sufficiency as ≥ 75 nM serum 25(OH)D and deficiency as < 50 nM [83]. At this time, further research investigating how varied serum levels of vitamin D are related to health and disease are needed in order to better define "adequate" serum vitamin D levels.

Though the epidemiologic data suggest a link between circulating vitamin D levels and disease outcome, these studies cannot address the question as to whether vitamin D deficiency is a cause of or sequela of the disease. For these questions, animal models have been useful to address mechanisms through which vitamin D influences inflammation and cancer development.

POTENTIAL MECHANISMS OF ACTION OF VITAMIN D IN IBD DEVELOPMENT AND PROGRESSION:

Animal models are an important and necessary tool for studying the effects of vitamin D deficiency and/or supplementation on IBD and cancer. Mouse models permit modulation of vitamin D levels while controlling for other environmental and genetic factors that may influence disease development. **Tables 1.1** & **1.2** provide summaries of animal models of IBD (**Table 1.1**) and CAC (**Table 1.2**) in which the role of vitamin D with regard to disease has been addressed. Mouse models of IBD and CAC have been categorized into vitamin D supplementation, vitamin D deficiency, and animals with genetically altered VDR. Categories have been further broken

down to highlight studies that show amelioration vs. exacerbation of disease. In the following section, we briefly discuss some of the important mechanisms through which vitamin D impacts IBD and CAC using the models outlined in **Tables 1.1** & **1.2**.

Table 1.1. Vitamin D and Mouse Models of IBD

Strain	Model	Vitamin D metabolite / analog	Dose, Route	Treatment window and duration	Serum Vit D measure	Outcome	Ref
Vitamin D Supplementation							
Protection							
Il10$^{-/-}$	Spontaneous	1,25$(OH)_2D_3$ & D_3	0.005μg 1,25$(OH)_2D_3$/day or 5 μg D_3/day, diet 0.2 μg 1,25$(OH)_2D_3$/day, diet	3 wks of age; maintained throughout study. At the first sign of IBD (diarrhea), lasted for 2 wks	n.d.	Ameliorated IBD Effective at blocking the progression and ameliorating the symptoms in established disease.	[42]
Il10$^{-/-}$	Spontaneous	1,25$(OH)_2D_3$	20ng/day, diet	4 wks of age; maintained throughout study	n.d.	Significantly reduced spontaneous colitis. More effective protection with combined treatment with calcium	[84]
C57BL/6	DSS (0.5-3.5%), 5 days	1,25$(OH)_2D_3$	50ng/day, diet or 10ng, intrarectally	Diet given 1 wk. prior to DSS treatment; maintained throughout study. Intrarectal administration given 1 day prior to DSS; given every other day	n.d.	Decreased DSS-induced colitis. Intrarectal administration more effective than dietary delivery.	[85]
Balb/c	DSS (3%), 7 days	ZK191784 (1,25$(OH)_2D_3$ analog)	100 μg/kg/day, orally	3 days prior or at the start of DSS treatment; given daily.	n.d.	Significantly decreased DSS-induced colitis.	[86]
C57BL/6	DSS (3%), 7 days	1,25$(OH)_2D_3$	0.5 μg/kg, IP	1 day prior to DSS treatment; given daily up to 11 days	n.d.	Decreased inflammation and tissue damage following DSS treatment; Increased weight loss likely due to hypercalcemia.	[43]
C57BL/6	DSS (2%), 7 days	1,25$(OH)_2D_3$	0.2 μg/25g BW/day, oral gavage	After DSS treatment	n.d.	Decreased clinical disease, colonic inflammation and intestinal permeability following DSS treatment.	[87]
Cyp27b1$^{-/-}$ and *Vdr*$^{-/-}$	DSS (3.5%), 5 days	1,25$(OH)_2D_3$	50 ng/day, diet	2 wks prior to DSS treatment; maintained throughout study	n.d.	*Cyp27b1*$^{-/-}$ mice more susceptible to DSS induced colitis; supplementation with 1,25$(OH)_2$ D_3 partially ameliorated disease.	[88]
C57BL/6	DSS (3%), 7 days	NA	Adoptive transfer of CYP27B1 over-expressing monocytes	Adoptive transfer on 5th day of DSS treatment	n.d.	CD11b+/Gr1+ monocytes overexpressing CYP27B1 trafficked to the inflamed colon and ameliorated DSS-induced colitis	[89]
Smad3$^{-/-}$	*Helicobacter bilis*	D_3	1000 IU/kg or 5000 IU/kg, diet	2 wks prior to *H. bilis* infection; maintained throughout study	25(OH)D	Higher vitamin D significantly decrease colonic inflammation	[45]
Balb/c	TNBS (100mg/kg)	1,25$(OH)_2D_3$	0.2 μg/kg, IP	Given 2 hrs before and 3 days post (acute) or 3,4, and 5 days post TNBS (interventional)	n.d.	Significantly reduced colitis.; greater protection with concurrent treatment with dexamethasone.	[90]
C57BL/6	TNBS (100mg/kg)	Paracalitriol (1,25$(OH)_2D_3$ analog)	0.5 μg/kg, IP	Given 30 min before and 1, 3, and 5 days post TNBS	n.d.	Ameliorated TNBS colitis and protected against epithelial barrier disruption	[91]

Neutral							
Il10$^{-/-}$	Spontaneous colitis	D_3	25IU/kg or 5000IU/kg, diet	Before conception; maintained throughout study	25(OH)D	No significant difference in inflammation score	[92]
Rag$^{-/-}$	Adoptive transfer of *Il10*$^{-/-}$ T cells + piroxicam (7 days)	D_3	1000 IU/kg or 5000 IU/kg, diet	After peroxicam treatment ends; for 12 days	1,25(OH)$_2$ D, 25(OH)D	Vitamin D supplementation during active colitis did not alter colonic inflammation; increased trabecular bone deterioration.	[93]
Exacerbation							
C57BL/6	*Citrobacter rodentium*	1,25(OH)$_2$D$_3$	0.5 μg/kg, IP	1 day prior to treatment; daily	n.d.	Increased colonic ulceration and bacterial burdens compared to controls.	[43]
Vitamin D Deficiency							
Il10$^{-/-}$	Spontaneous	NA	Vit D deficient diet	Maintain throughout study	n.d.	Increased mortality and more rapid disease development compared to vitamin D sufficient animals.	[42]
C57BL/6	DSS (2.5%), 6 days	NA	Vit D deficient diet	6 wks prior to DSS treatment; maintained throughout study	25(OH)D	Exacerbated DSS colitis and increased bacterial loads within the colonic tissue.	[94]
Il10$^{-/-}$	AOM + DSS (2%), 7 days	NA	Vit D deficient diet	3 wks of age; maintained throughout study	25(OH)D	Increased mortality and disease severity in AOM/DSS colitis.	[95]
Smad3$^{-/-}$	*Helicobacter bilis*	NA	Vit D deficient diet	2 wks prior to infection; maintain throughout study	25(OH)D	Did not alter IBD development or severity	[45]
Il10$^{-/-}$	DSS (2%), 9 days or *E. coli*	NA	Vit D deficient diet	Maintain throughout experiment	1,25OH)$_2$D & 25(OH)D	Decreased survival and increased intestinal permeability following DSS treatment; increased susceptibility to infection with invasive *E. coli*.	[96]
Other (genetically altered VDR)							
hVDR Tg	TNBS, DSS, and adoptive T cell transfer	NA	NA	NA	n.d.	Overexpression of VDR in the intestinal epithelial cells protected against TNBS, DSS and adoptive T cell transfer - induced colitis.	[97]
Vdr$^{-/-}$	DSS (0.5-3.5%), 5 days	NA	NA	NA	n.d.	Increased sensitivity, mortality and disease severity in response to DSS	[98]
Vdr$^{-/-}$	DSS (2-2.5%), 7 days	NA	NA	NA	n.d.	More sensitive to DSS inducted colitis resulting in severe mucosal ulcerations, impaired mucosal wound healing, decreased epithelial barrier function and increased mortality.	[99]
VDR$^{\Delta IEC}$	DSS (5%), 7 days	NA	NA	NA	n.d.	Loss of intestinal epithelial VDR exacerbated DSS colitis, altered paneth cell development and changed autophagy gene expression.	[100]
Vdr$^{-/-}$and *Il10*$^{-/-}$/ *Vdr*$^{-/-}$	Adoptive T Cell Transfer and Spontaneous	NA	NA	NA	n.d.	*Il10*$^{-/-}$/ *Vdr*$^{-/-}$ mice developed more severe IBD and increased mortality compared to *Il10*$^{-/-}$ mice. Adoptive transfer of *Vdr*$^{-/-}$ CD4 T cells into *Rag*$^{-/-}$ mice induced severe IBD.	[101]

Il10$^{-/-}$/ *Vdr*$^{-/-}$	Spontaneous	NA	NA	NA	n.d.	*Il10*$^{-/-}$/ *Vdr*$^{-/-}$ mice developed more severe IBD compared to *IL10*$^{-/-}$ mice	[85]
Il10$^{-/-}$/ *Vdr*$^{-/-}$	Spontaneous and Adoptive T cell Transfer	NA	NA	NA	n.d.	Adoptive transfer of *Il10*$^{-/-}$/ *Vdr*$^{-/-}$ CD4 T cells into *Rag*$^{-/-}$ mice induced severe colitis. Loss of VDR results in decreased T cell homing to the gut and decreased CD4/CD8αα intraepithelial lymphocytes	[102]
Vdr$^{-/-}$ *Il10*$^{-/-}$/ *Vdr*$^{-/-}$, & *Rag*$^{-/-}$	Adoptive T Cell Transfer	NA	NA	NA	n.d.	Adoptive transfer of *Il10*$^{-/-}$/ *Vdr*$^{-/-}$ CD8 T cells into *Rag*$^{-/-}$ mice induces severe colitis. *Vdr*$^{-/-}$ CD8 T cells exacerbate CD4/CD45RB^{high} cell-induced colitis.	[103]

n.d. not done

Table 1.2. Vitamin D and Mouse Models of CAC

Strain	Model	Vitamin D metabolite / analog	Dose, Route	Treatment window and duration	Serum Vit D measure	Outcome	Ref
Vitamin D Supplementation							
A/J	AOM + DSS (3%), 7 days	Ro26-2198 (analog)	0.01μg/kg/day, subcutaneous pump	1 wk. prior to AOM	n.d.	Delayed onset of clinical colitis, decreased cellular proliferation and decreased dysplasia following AOM/DSS.	[46]
CF1	AOM + DSS (2.5%) x 7 days	D_3, 25(OH)D, $1,25(OH)_2D_3$, & $1,25(OH)_2D_5$	500μg D_3/kg diet , - 500μg 25(OH)D/kg diet, 2.5μg $1,25(OH)_2D_3$/kg diet , and 25μg $1,25(OH)_2D_5$/kg diet	1 wk. prior to AOM+DSS treatment and maintained throughout study	n.d.	D_3, 25(OH)D, and $1,25(OH)_2D_5$ significantly decreased tumor incidence. $1,25(OH)_2D_3$ induced significant weight loss compared to other treatments; not included in final analysis.	[104]
C57BL/6	AOM + DSS (2%), 4 days x 3 cycles	D_3	100, 400, 1000, 2500, or 5000 IU/kg, diet	2.5 wks prior to AOM+DSS treatment.	25(OH)D	Vitamin D supplementation significantly decreased dysplasia in a dose dependent manner	[44]
Smad3$^{-/-}$	*Helicobacter bilis*	D_3	1000 IU/kg or 5000 IU/kg, diet	1 wk. prior to infection; maintain throughout study	25(OH)D	Higher vitamin D significantly decreased tumor incidence	[45]
Vitamin D Deficiency							
Smad3$^{-/-}$	*Helicobacter bilis*	NA	Vit D deficient diet	2 wks prior to infection; maintain throughout study	25(OH)D	No change in dysplasia score or tumor incidence.	[45]
Other (VDR Knockout)							
Vdr$^{-/-}$	AOM + DSS (1.5%), 5 days x 3 cycles	NA	NA	NA	n.d.	Increased inflammation and tumor burdens; increased activation of EGFR and ErbB2 signaling	[105]

n.d. not done

Vitamin D – Impact on immune responses. One mechanism through which vitamin D may impact IBD development and progression is through altering immune responses [18, 27, 106, 107]. Multiple studies have demonstrated that T cells are both direct and indirect targets of vitamin D [108-111]. $CD4^+$ effector T cells that lack VDR or CYP27B1 express higher concentrations of the inflammatory cytokines IL-17 and IFNγ, proliferate more rapidly, and induce more severe colitis following adoptive transfer into naïve recipient mice, compared to WT cells. In addition, *Vdr*$^{-/-}$ mice fail to develop iNKT and CD8αα T cell populations, both of which are important regulatory T cell subsets necessary in the resolution of inflammation [102, 112, 113]. Treatment of T cells or mice with active vitamin D both *in vitro* and *in vivo* results in decreased proliferation of both $CD4^+$ and $CD8^+$ subsets as well as decreased secretion of IL-2 and IFNγ, resulting in decreased Th1 and Th17 responses and an overall dampening of inflammation [109, 112]. In addition to affecting T cells directly, vitamin D alters inflammation by affecting antigen-presenting cells. Macrophages and dendritic cells treated with vitamin D have decreased secretion of proinflammatory cytokines following activation [88, 114]. In addition, vitamin D treatment modulates dendritic cell differentiation and maturation: vitamin D-treated dendritic cells express fewer maturation markers including CD80, CD86, and MHCII and secrete less IL-12. This results in decreased dendritic cell-mediated activation and proliferation of T cells and an increase in T cells with regulatory phenotypes [114-116]. The effects of vitamin D on NK cell responses are understudied considering the role these cells have been shown to have in autoimmune disease [117] as well as their role in interacting and modulating dendritic and T cell responses [118]. While Vitamin D has been shown to enhance cytotoxic function of human NK cell lines, [119, 120], vitamin D may also impair NK cell development and inflammatory cytokine expression in the presence of vitamin D [121].

Vitamin D - Effects on intestinal barrier function. Another mechanism through which vitamin D may protect against IBD is by improving intestinal epithelial barrier function. Patients with CD have increased intestinal barrier permeability which has been associated with inflammation and dysbiosis [122] as it results in increased exposure of the immune system to intestinal microbiota. *In vitro* studies demonstrate that vitamin D signaling is imperative for the maintenance of epithelial barrier integrity by increasing the expression of tight junction proteins including Occludin, Zo-1, Zo-2, vinculin, and Claudin, and altering transepithelial resistance [96,

99, 123, 124]. Interestingly, *Kong et al.* have demonstrated that mice lacking VDR have decreased transepithelial electric resistance (TER) and disruption of the epithelial cell tight junctions resulting in an increased susceptibility to DSS-induced colitis [99]. Conversely, *Liu et al* have demonstrated that overexpression of VDR within the colonic epithelial cells is protective in several different mouse models of colitis, including DSS and the adoptive transfer of T cells into naïve mice, by preserving epithelial tight junctions and attenuating epithelial cell apoptosis thus preserving the gut epithelial barrier function [79].

VITAMIN D DEFICIENCY:

In agreement with the epidemiologic human data, vitamin D deficiency exacerbates IBD in mouse models. Mice fed a vitamin D deficient diet are more susceptible to DSS-induced colitis compared to those fed a vitamin D sufficient diet [94]. Similarly, vitamin D deficient *Il10*$^{-/-}$ mice, which develop colitis in response to enteric flora, develop more severe disease compared to vitamin D sufficient animals [85]. In addition, mice lacking VDR or CYP27B1 develop more severe IBD following treatment with DSS compared to wild type controls [88, 99, 108], again supporting the epidemiologic evidence that vitamin D deficiency may play a role in IBD development and progression. Conversely, vitamin D supplementation ameliorates symptoms of colitis in vitamin D deficient animals [79, 98]. However, in mice, vitamin D deficiency does not always worsen IBD and CAC. While increased dietary vitamin D protected *Smad3*$^{-/-}$ mice from developing IBD and CAC induced by *Helicobacter*, a vitamin D deficient diet did not increase colitis and CAC in these mice compared to a control group fed normal levels of dietary vitamin D [45]. Mice fed a vitamin D deficient diet had significantly lower levels of serum 25(OH)D compared to controls. Interestingly, while high vitamin D diet suppressed early inflammation, the vitamin D deficient diet did not cause more severe inflammation compared to controls, suggesting that high levels of vitamin D may inhibit inflammation but lack of vitamin D does not accelerate or exacerbate the process. Another possible explanation for this observation is that the severity of the deficiency may be important to disease susceptibility in this mouse model: in this study, although serum levels of vitamin D were significantly decreased at the time of disease initiation, mice were not completely vitamin D deficient as they had only been on diet for two weeks prior to infection with *Helicobacter*.

EFFICACY OF VITAMIN D TREATMENT IN IBD:

Despite the majority of studies showing strong inverse association between vitamin D levels and IBD, studies that use vitamin D to treat IBD have as of yet, yielded variable results (**Table 1.1**). *Larmonier et al*. used supplemental dietary vitamin D to ameliorate IBD in an adoptive T cell transfer model of colitis [93]. In this study, animals were placed on increased dietary vitamin D supplementation after the onset of colitis but there was no difference in colitis or expression in proinflammatory cytokines in vitamin D-supplemented animals [93]. In a study performed by *Glenn et al., Il10*$^{-/-}$ mice were fed either 25IU/kg or 5000IU/kg vitamin D in the diet throughout life until 3 months of age, when the animals were necropsied. While animals fed low levels of dietary vitamin D had decreased VDR expression in proximal colon, there were no differences in incidence or severity of IBD nor were there differences in gene expression by microarray between the groups [92]. *Ryz et al*. demonstrated that while daily treatment with active vitamin D was sufficient to protect against DSS-induced colitis, it exacerbated colitis induced by infection with *Citrobacter rodentium [43]*. Our group showed protection with supplemental vitamin D using a bacterially-induced colitis model. In *Smad3*$^{-/-}$ mice, increased dietary vitamin D resulted in decreased inflammation, dysplasia, and colon cancer incidence [45]. We also demonstrated dietary supplementation of vitamin D was a convenient and effective way to improve vitamin D nutritional status as evidenced by a significant increase in serum vitamin D in mice fed increased vitamin D compared to those fed maintenance diet. Importantly, we showed that dietary vitamin D levels that were 5 – 10 times that of control diet levels did not alter serum calcium levels. These studies as well as others are summarized in **Table 1.1**.

One of the conundrums of vitamin D therapy has been how to achieve effective concentrations of the active form of vitamin D within the target tissues without inducing hypercalcemia and vitamin D toxicity. Although effective as an anti-inflammatory or anti-tumor agent, administering metabolically active $1,25(OH)_2D_3$ to animals and humans has resulted in hypercalcemia [42, 84, 125-127]. Interestingly, *Li et al*. attempted to avoid vitamin D toxicity by genetically engineering intestinal macrophages to upregulate expression of *Cyp27b1* (1α-hydroxylase) specifically in the intestinal tissue following an inflammatory trigger. Using this strategy, they were able to protect mice against DSS-induced colitis and decrease the expression of proinflammatory cytokines without altering serum calcium levels by localizing the active

vitamin D to the colon [89]. Other investigators including *Strauch et al.* and *Zhu et al.* have administered 1,25$(OH)_2$D analogs and shown protection against IBD in mouse models while avoiding hypercalcemic effects of active vitamin D [86, 91].

VITAMIN D AND INFLAMMATION-ASSOCIATED COLON CANCER:

Chronic inflammation is believed to play a key role in carcinogenesis [35]. Links between inflammation and cancer have been observed in colon cancer in human patients with IBD [31] as well as in liver, pancreatic, stomach, esophageal, and prostate cancers [35]. As discussed above, both human and animal studies suggest that vitamin D can be beneficial in preventing or ameliorating inflammation and clinical disease in IBD [42, 43, 87]; however, few of these studies further evaluate how these changes in inflammation impact subsequent tumor development. Our laboratory has demonstrated that increased dietary vitamin D significantly decreases the incidence and severity of IBD in *Smad3*$^{-/-}$ mice, which resulted in significant decreases in resultant tumor formation [45]. Consistent with these results, other investigators have demonstrated that increased concentrations of dietary vitamin D are protective against preneoplasic lesions in another model of inflammation-associated cancer, DSS/AOM, in a dose-dependent manner [44, 104]. Results of animal studies performed evaluating the effects of vitamin D on CAC are summarized in **Table 1.2**. Based on our data and that of others, we believe that vitamin D is protective against inflammation-associated cancer at least in part by altering key inflammatory pathways and dampening inflammation that *precedes cancer development*. However, we have not yet determined whether vitamin D is protective against CAC after the onset of inflammation in *Smad3*$^{-/-}$ mice, an important question to address, and one that can be addressed, using this animal model. While these studies suggest that vitamin D supplementation may be a useful therapy in a subset of patients with IBD and/or CAC, further research to understand the mechanisms through which vitamin D is working in order to better predict the timing of when patients should be treated with supplemental vitamin D and which populations would benefit the most. In addition, research of vitamin D analogs that do not cause hypercalcemia would be useful in the treatment of IBD/CAC without significant vitamin D toxicity. *Lu et al.* and *Klampfer et al.* provide additional comprehensive reviews of vitamin D signaling pathways and how they might be involved in carcinogenesis [128, 129].

GUT MICROBIOTA, IBD, AND VITAMIN D:

Aberrant immune responses to intestinal microbiota have been implicated in the etiology of IBD in humans [130]. Similarly, the role of gut bacteria in IBD development is well documented in animal models of IBD [131, 132]. While no specific bacteria have been identified as a causative pathogen for IBD, general dysbiosis [128, 133-135] as well as an overall decrease in microbial diversity [23, 136] has been observed in IBD patients compared to healthy controls. In addition, UC patients had lower microbial diversity during active disease periods compared to periods of disease remission [50, 136], demonstrating the fluidity of the microbial composition even within the same individual. Studies using twin pairs discordant for IBD also show that microbial diversity is reduced in twins with IBD compared to healthy counterparts [137]. Interestingly, the twin pairs discordant for UC had not only lower microbial diversity but also reduced host gene transcripts in colonic mucosa compared to healthy twin pairs, suggesting that cross-talk between host and microbiota might also be reduced in UC patients [137]. Antibiotics are part of the treatment armamentarium for IBD patients with active and/or fistulizing disease [38], indicating that manipulation of the microbiome can affect the disease process. In addition, studies are underway to examine the benefit of fecal transplantation for therapy and/or cure of IBD [39, 138-141].

The gut microbiome is not only strongly linked to IBD, but also linked to the development of colon cancer in humans and mice [142]. The microbiota of colorectal cancer patients has been found to be enriched in bacteria that reflect an overall pro-inflammatory configuration [143]. Although the association between an altered gut microbiome and IBD appears to be strong, it is currently unknown whether this is a cause or a consequence of the disease. Recently, the bacterial driver-passenger model has been proposed by *Tjalsma et al*. as a hypothesis that could explain microbial changes observed during colon tumorigenesis [144]. In this model, specific bacterial populations with pro-carcinogenic features (driver bacteria) contribute to tumor initiation through the induction of epithelial cell damage, DNA damage, and inciting of inflammation. Then as the tumor progresses, other bacterial populations (passenger bacteria) gain growth advantage with changes in the tumor microenvironment, slowly replacing the “driver” bacteria. *Candela et al.* delve further into the potential role of gut microbiota in colon cancer carcinogenesis in their recent review [143].

Recent studies indicate that diet plays a role in regulating the microbiome [137] and that the interaction between the gut microbiota and diet may significantly affect IBD development [145, 146]. However, mechanistic studies evaluating the impact of individual dietary nutrients on disease and microbiota are not feasible to perform in human populations and therefore, animal models are crucial for answering these questions.

As vitamin D plays a significant role in host immune responses, it has the potential to influence the gut microbiome. Although the role of vitamin D in shaping the microbiome has not been explored, studies indicate several mechanisms through which vitamin D may influence microbiota. Vitamin D can affect both immune cells and colonic epithelial cells; via modulating immune cell differentiation and maturation, colon barrier function, and the secretion of antimicrobial peptides, mucins, and cytokines, all of which have the potential to modulate the gut bacteria [88, 123, 147]. Vitamin D deficient mice have decreased expression of Angiogenin-4 (an antimicrobial peptide) within the colon and consequently have an increased bacterial load within the colon, suggesting that vitamin D deficiency may impair microbial homeostasis [94]. Similarly, mice lacking VDR in the colonic epithelial cells have increased bacterial loads within the colonic mucosa, an increased concentration of *Bacteroides fragilis,* a bacterial species that has been associated with IBD in humans, and an increased susceptibility to colitis [100]. In addition, mice lacking the ability to respond to vitamin D (due to lack of VDR or CYP27B1) have distinct differences in their gut microbiome compared to wild-type littermates with intact vitamin D signaling [88, 100], demonstrating that host responses to vitamin D impact the microbiome. Interestingly, when the VDR mutant animals are co-housed with wild type animals, the wild type mice exhibit an increase in susceptibility to DSS colitis, indicating that the susceptibility-inducing microbiota is transferrable and directly influences clinical disease [100].

While these studies suggest that vitamin D signaling can influence the microbiome and vice versa, further studies are needed to better understand specifically how vitamin D is altering the composition of the microbiome and what role this could play in disease development. Recent technical advancement in germ-free animal research and microbiome research provide opportunities to address whether microbiota play a causative role on IBD/CAC as well as whether nutrients such as vitamin D can influence the microbiome.

CONCLUSIONS:

Both human epidemiologic and animal studies suggest that vitamin D plays a role in the development of inflammatory bowel disease as well as colon cancer [16-23]. While vitamin D has been shown to regulate immune cell trafficking and differentiation, cellular proliferation, gut barrier function and antimicrobial peptide synthesis[48-50], the specific mechanisms through which vitamin D ameliorates colitis remain unknown. The majority of evidence demonstrates protective links between vitamin D, colitis, and colitis-associated colon cancer, although studies attempting to demonstrate the usefulness of vitamin D as a therapy for these diseases in animal models have yielded mixed results, likely due to our lack of complete understanding of the model systems as well as the role of vitamin D in inflammation and gut homeostasis. As the use of vitamin D as a treatment in patients with IBD would be simple and inexpensive to implement, it is important that we continue to gain additional insights into its mechanisms of action to determine which patient populations may best benefit from this potential adjunctive therapy.

Chapter 2. CHEMOPROTECTIVE EFFECTS OF INCREASED DIETARY VITAMIN D AGAINST COLITIS-ASSOCIATED COLON CANCER IN HELICOBACTER INFECETED *SMAD3*$^{-/-}$ MICE

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in both men and women [148]. Studies indicate that diet and lifestyle choices play a significant role in the development of and prognosis of colon cancer [149]. Vitamin D status of an individual is also influenced by both diet and lifestyle [19]. The link between vitamin D and colon cancer was first suggested by Garland *et al.* who observed that populations residing in the northeastern United States had an increased incidence of colon cancer-related mortality compared to those living in the southern United States [20]. Since then, adequate serum vitamin D levels have been associated with decreased incidence of colon cancer and decreased mortality in patients diagnosed with colon cancer [21, 150]. Adequate serum vitamin D levels also correlate with reduced risk for developing inflammatory diseases, such as Crohn's disease, a risk factor for colon cancer [16, 19].

The risk of colon cancer is increased in patients diagnosed with IBD (Crohn's disease or ulcerative colitis) compared to the general population, supporting the notion that colonic inflammation impacts cancer development [31]. The chronic inflammation seen in IBD, is likely due in part to dysregulated mucosal immune responses to enteric antigens [31] and is believed to progress to cancer through the promotion of angiogenesis, tumor-promoting cytokine production, tumor cell invasive behavior and cellular proliferation [35].

Vitamin D has a protective effect against colon cancer in various mouse models [46, 125]. However, these cancer models, such as *Apc*$^{Min/+}$ mice or mice given a chemical mutagen such as azoxymethane, are not driven by inflammation [126, 127, 151, 152]. Currently, the protective effect of vitamin D on inflammation-associated colon cancer is not known.

In order to investigate the potential chemopreventative effects of elevated dietary vitamin

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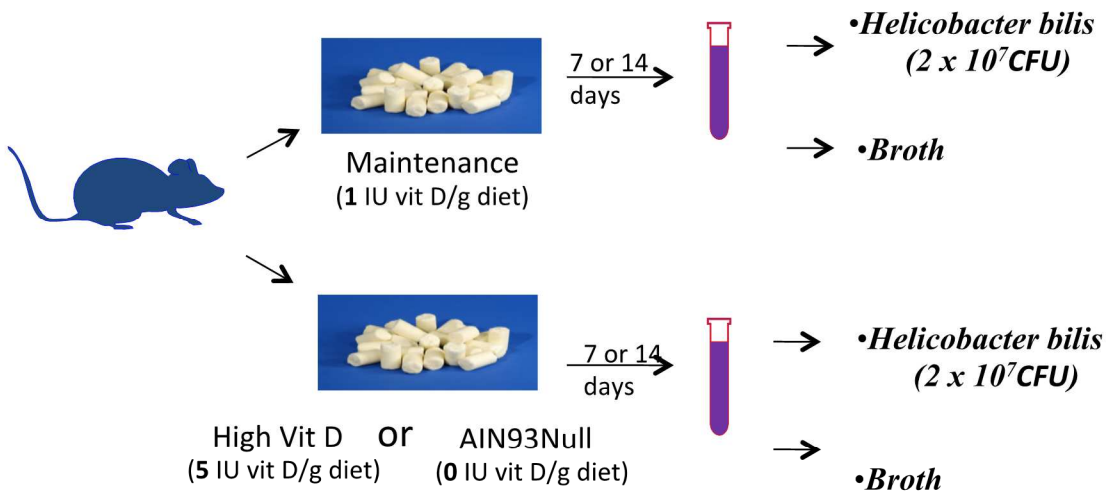
D on inflammation-associated colon cancer, we utilized *Smad3*$^{-/-}$ (*Smad3*^{tm1Par}/J) mice, which have defective TGFβ signaling due to the absence of the transcription factor, Smad3 [153]. In humans, the TGFβ signaling pathway is commonly mutated in colon cancer including colitis-associated colorectal cancer [154]. After being infected with an enteric microorganism, *Helicobacter bilis* [153], *Smad3*$^{-/-}$ mice develop transient colitis followed months later by colon cancer making them a useful model for studying inflammation-associated colon cancer. Using this model, we demonstrated that elevated dietary vitamin D increases serum vitamin D and protects *H. bilis (Hb)*-infected *Smad3*$^{-/-}$ mice from developing colon cancer. These protective effects are mediated through interactions between vitamin D and proinflammatory signaling pathways during the early stages of disease development.

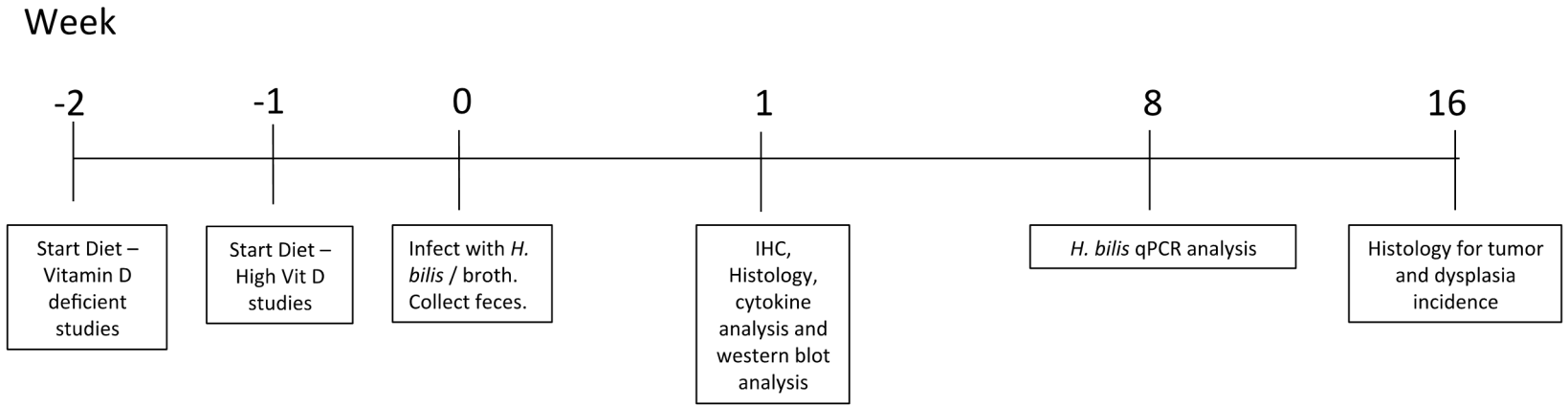
MATERIAL AND METHODS:

Mice and diets. Study mice were colony-bred 129-*Smad3*^{tm1Par}/J (*Smad3*$^{-/-}$) mice (age 6-14 weeks) housed in a specific pathogen-free facility. Mice were screened for rodent pathogens as previously described [155] except that sentinels were collected three times yearly rather than quarterly. In addition to referenced pathogens, annual screens were performed for Minute Virus of Mice, Lymphocytic Choriomeningitis Virus, and Ectromelia virus. Mice were maintained in a *Helicobacter* and Mouse Norovirus - free colony as previously described [156]. The mice were group-housed in ventilated cages and fed a purified, irradiated diet with 1 IU vitamin D (5SRH, maintenance), 5 IU vitamin D (5AAA, high vitamin D) or 0 IU vitamin D per gram (5AV4, AIN93Null). All diets were manufactured by PMI Nutrition International (Lab Diet/Test Diet, St. Louis, MO) based on AIN93M diet that is formulated for the maintenance of rodents' health. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Experimental design. While no age-associated alterations in endpoint disease have been noted in our experience with *Smad3*$^{-/-}$ mice, care was taken to evenly distribute mice across treatment groups with regard to age and sex. Independent studies were performed to evaluate the effects of high and low dietary vitamin D levels on cancer. For high vitamin D studies, mice were started on either maintenance or high vitamin D diets one week prior to infection. For

vitamin D deficient studies, maintenance or AIN93Null diets were initiated two weeks prior to infection (**Fig 2.1**). For each diet, mice were infected with either ~2 x 10^7 CFU *Hb* in Brucella broth or Brucella broth alone (controls) by oral gavage as previously described [153] (**Fig 2.1**). *Helicobacter* infection status was monitored by fecal polymerase chain reaction (PCR) using previously published primer sequences [155]. Fecal samples were collected at 3, 6, and 14 days post infection for subjective fecal scoring and at 7 days post infection for fecal cytokine analysis. Mice were weighed weekly and monitored at least three times weekly for dehydration, diarrhea, lethargy, or weight loss. Animals were euthanized by CO_2 asphyxiation at the designated end points.





*Fecal scores obtained at 3, 6, and 14 days post infection
**Feces collected for fecal cytokine analysis at 1, 2, and 3 weeks post infection

Figure 2.1. Study design.

Serum vitamin D and calcium determination and tissue collection. Following euthanasia, blood was obtained via cardiac puncture. Serum samples were submitted to Heartland Assays (Ames, IA) for quantification of 25-hydroxyvitamin D (radioimmunoassay) and calcium levels. Mesenteric lymph nodes, cecum, colon and rectum were fixed in 10% phosphate buffered formalin and processed for routine histologic examination. For study of the early inflammatory phase, cross-sections of proximal colon (5mm) were prepared for immunohistochemistry (IHC) and histology, and cross-sections of proximal colon (5mm) and cecum (3mm) were stored in RNA later (Qiagen, Valencia, CA) for cytokine analysis by qRT-PCR. For evaluation of *Hb* colonization, 3 cm sections of mid-jejunum, proximal colon, distal colon and whole cecum were harvested, rinsed gently in sterile PBS to remove fecal material and stored at -20°C until DNA extraction. For fecal cytokine analysis, individual fecal samples were collected, homogenized in 250 µl RNAlater stabilization solution (Qiagen) and stored at -80°C until RNA extraction. At the time of collection, a subjective fecal score was assigned to each animal, ranking presence of diarrhea and blood in the stool as a clinical measure of IBD [157].

Histopathology and immunohistochemistry. Whole colon and cecum were evaluated by a board certified veterinary pathologist (PT) blinded to experimental groups to assess the severity of colitis and incidence of neoplasia. An overall IBD score was determined as described [158] with the exceptions that scores were summed from cecum, proximal, mid and distal colons and none were weighted. Analysis of the rectum was included with the distal colon. IBD scores incorporate the severity of mucosal epithelial changes, degree of inflammation, and extent of lesions. A dysplasia score was also generated by determining the degree of dysplasia present in each of four segments as described [159]: cecum, proximal colon, mid colon, and distal colon. For each segment, a score ranging from 0-4 was assigned: 0= none, 1= indefinite, 2= low grade, 3=high grade, and 4= high grade with frank invasion beyond tunica muscularis and distinguished from mucosal herniation. Cancers were classified as adenocarcinomas and mucinous adenocarcinomas [153, 159]. The four individual segmental scores were summed to generate the overall dysplasia score for each animal.

For study of the early inflammatory phase, colonic expression of CD3, F4/80, cleaved caspase 3, and MHCII were evaluated on transverse cross-sections of proximal colon in animals euthanized 1week post-infection. IHC staining within the mucosa, excluding any gastrointestinal

lymphoid tissue, was scored by a pathologist blinded to groups (PT), using a range of 0-4: 0= no positive cells, 1= few single positive cells, 2= few (3 or less) scattered small clusters of positive cells, 3= many (>3) small clusters of positive cells or larger clusters of positive cells, 4= large or coalescing clusters of positive cells. For early time points, in addition to IHC staining, cecum and serial transverse cross-sections of proximal colon were evaluated histologically for evidence of IBD.

All IHC staining was performed by Experimental Histopathology Services at Fred Hutchinson Cancer Research Center (Seattle, WA). Rat monoclonal antibodies were used to detect MHC class II (I-A/I-E, BD Pharmingen), CD3 (MCA1477, Serotec) and F4/80 (MCA497, Serotec). Signals were detected using biotinylated goat anti-rat (Jackson ImmunoResearch) followed by streptavidin HRP (Jackson ImmunoResearch). Cleaved caspase-3 antibody (Biocare Medical CP229B) was followed by Mach 2 anti-rabbit HRP-labeled polymer (Biocare Medical RHRP520L). Staining was visualized with 3,3'-diaminobenzidine (DAB, Dako) and counter-stained with hematoxylin (Dako). Concentration-matched isotype-control slides were run for each tissue sample (Jackson ImmunoResearch).

Cytokine analysis and *Helicobacter* quantification by quantitative real-time PCR analysis. For cytokine analysis, RNA was extracted from cecum, colon, and feces using the RNeasy kit (Qiagen). Fecal RNA samples were further concentrated using the Qiagen mini-elution/cleanup kit. RNA was converted to cDNA using SuperScript First Strand Synthesis System (Invitrogen, Grand Island, NY) followed by qRT-PCR using Power Sybr Green Master Mix (Applied Biosystems, Carlsbad, CA) and a Stratagene Mx3005P analyzer (Agilent Technologies, Santa Clara, CA). Samples were run in duplicate. Cytokine levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed relative to a control sample (RNA from cecum or proximal colon) that was run on every plate as a calibrator. For *Helicobacter* quantification, DNA was extracted from mid-jejunal, cecal, proximal colon and distal colon samples using a previously published protocol [160]. Primer sequences used are outlined in **Table 2.1**. Data were analyzed using Stratagene's MxPro v4.10 software (Agilent Technologies).

Table 2.1. Primer Sequences

RT-PCR Primer	Sequence	Ref.
Cyp24a	F-5'-CTGCCCCATTGACAAAAGGC-3'	Primer Bank
	R-5'-CTCACCGTCGGTCATCAGC-3'	Primer Bank
Cyp27b1	F-5'-TCCTGGCTGAACTCTTCTGC-3'	Primer Bank
	R-5'-GGCAACGTAAACTGTGCGAA -3'	Primer Bank
HPRT	F-5'-GTAATGATCAGTCAACGGGGGAC-3'	[161]
	R-5'-CCAGCAAGCTTGCAACCTTAACCA-3'	[161]
IL-10	F-5'-TGCACTACCAAAGCCACAAGGCAG–3'	[163]
	R-5'-TCAGTAAGAGCAGGCAGCATAGCA-3'	[163]
Il-6	F-5'-AGAGTTGTGCAATGGCAATTCTGA-3'	NA
	R-5'-TGGTACTCCAGAAGACCAGAGGAA-3'	NA
IL-1β	F-5'-GCAGCTGGAGAGTGTGGATCCCAA-3'	[161]
	R-5'-GGCCGAGGACTAAGGAGTCCCC-3'	[161]
IFNγ	F-5'-GGATGCATTCATGAGTATTGC-3'	NA
	R-5'-CCTTTTCCGCTTCCTGAGG-3'	NA

RT-PCR Primer	Sequence	Ref.
MIP-1α	F-5'-GCTCAA ATCATGAAGGTCTCC-3'	[161]
	R-5'-TGCCGGTTTCTC TTAGTCAGG-3'	[161]
TLR-4	F-5'-AGT GGG TCA AGG AAC AGA AGC A-3'	[162]
	R-5'-CTT TAC CAG CTC ATT TCT CAC C-3'	[162]
TNFα	F-5' AGCCGATGGGTTGTACCTTGTCTA-3'	NA
	R-5' TGAGATAGCAAATCGGCTGACGGT-3'	NA
VDR	F-5'-ATCTGCATTGTCTCCCCAGACCGA – 3'	[164]
	R-5'-GATCATCTTGGCGTAGAGCTGGTG-3'	[164]

qPCR Primer	Sequence	
Helicobacter	F-5'-GATCAGCCTATGTCCTATCAGCTT G-3'	[165]
	R-5'-AGTTTACAATC TAAAACCTTCA CCTC-3'	[165]
18S rRNA	F-5'-GAACGTCTGCCCTATCAAC-3'	[160]
	R-5'-CCTCGAAAGAGTCCTGTATTG-3'	[160]

Epithelial and lamina propria cell preparations. Proximal colon (4 cm sections) samples were rinsed in PBS to remove fecal material, and epithelial and lamina propria leukocyte (LPL) populations were isolated using the Lamina Propria Dissociation Kit and gentleMACS Dissociator according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cells were pelleted, snap-frozen in liquid nitrogen, and stored at -80°C.

Western blotting. Protein was extracted by homogenizing cell pellets in RIPA buffer (Thermo Fisher Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Western blot analysis was performed according to a previously published protocol [156] with the following modifications. Proteins (40 μg/lane) were run on gradient (4-15%) Tris-HCl polyacrylamide gels (Biorad, Hercules, CA) and transferred to polyvinylidene fluoride membranes using 100 mM *N*-cyclohexyl-3-aminopropanesulfonic acid buffer with 10% methanol. Primary antibodies used were vitamin D receptor (VDR, SC-1008, Santa Cruz, Santa Cruz, CA), NFκB signaling (Phospho-NF-κB p65 (Ser536), and IκBα, L35A5, Cell Signaling, Danvers, MA), MAPK signaling (Phospho-MAPK Family, 9910, and MAPK Family, 9926, Antibody sampler Kits Cell Signaling), Bcl-xl (54H6, Cell Signaling), and proliferating cell nuclear antigen (PCNA, PC10, Cell Signaling). Beta actin (A5441, Sigma-Aldrich, St. Louis, MO) was used as a loading control.

Statistical analysis. Prior to statistical analysis, the distribution of each dataset was assessed for normality. If data were not normally distributed, transformation was attempted; if transformation did not normalize the distribution, non-parametric tests were performed. Serum vitamin D, serum calcium, histologic scoring, fecal scoring, and densitometries were analyzed using either Unpaired or Mann-Whitney t test. QRT-PCR data were analyzed using the Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test to adjust for multiple comparisons. Cancer and dysplasia incidence significance was determined by Fisher's exact test. All data are presented as mean ± SEM. Differences with a p-value of 0.05 or less were considered significant. All statistical analyses were performed using GraphPad Prism software (Version 5.04, GraphPad Software Inc, La Jolla, CA).

RESULTS:

Increased dietary vitamin D significantly increases serum 25-hydroxyvitamin D without altering serum calcium levels. To determine if high dietary vitamin D increases serum vitamin D status without causing toxicity in *Smad3*$^{-/-}$ mice, we measured serum 25-hydroxyvitamin D and serum calcium in mice fed high vitamin D diet or maintenance diet (control) for one week. The high vitamin D diet significantly increased serum 25-hydroxyvitamin D levels without altering serum calcium (25-hydroxyvitamin D mean: 37.6 vs. 17.6 ng/ml, p=0.016; calcium mean: 9.6 vs. 10.6 mg/dl, p=0.1), demonstrating that the dietary regimen rapidly elevated serum vitamin D levels without causing hypercalcemia. Similarly, after 16 weeks on diet, *Smad3*$^{-/-}$ mice fed high vitamin D diet had serum vitamin D levels that were roughly double that of mice fed maintenance diet (mean: 38.4 vs. 14.4 ng/ml, p<0.0001) (**Fig 2.2A**) while serum calcium levels remained unchanged (**Fig 2.2B**).

Treatment with increased dietary vitamin D significantly reduces cancer incidence in *Hb*-infected *Smad3*$^{-/-}$ mice. *Hb*-infected mice fed increased dietary vitamin D had a significantly reduced incidence of invasive colon cancer compared to *Hb*-infected mice fed maintenance diet (11% vs. 41%, p=0.0121) (**Fig 2.2C**). No neoplastic lesions developed in uninfected mice on either diet (**Fig 2.2C**). Mucinous adenocarcinomas located in the proximal colon were the primary neoplasm diagnosed, as previously noted in this model [153] (**Fig 2.2D**). Well-differentiated mucinous adenocarcinomas were characterized by expansile mucin-filled, epithelial-lined cysts that disrupt the muscular tunics, serosa and expand into mesentery (**Fig 2.2D**). Consistent with the decreased incidence of invasive adenocarcinoma, *Hb*-infected mice fed high vitamin D diet had an average four-fold decrease in dysplasia scores compared to mice fed maintenance diet (mean: 0.71 vs. 2.85, p<0.001, score range 0-16) (**Fig 2.2E**) with a significantly higher percentage of animals with no evidence of dysplasia (83% vs. 40%, p=0.0005). Dysplasia was primarily observed in the cecum and proximal colon. Minimal dysplasia was observed in the mid colon of mice fed maintenance diet but not in high vitamin D fed mice. No dysplasia was observed in the distal colon regardless of diet.

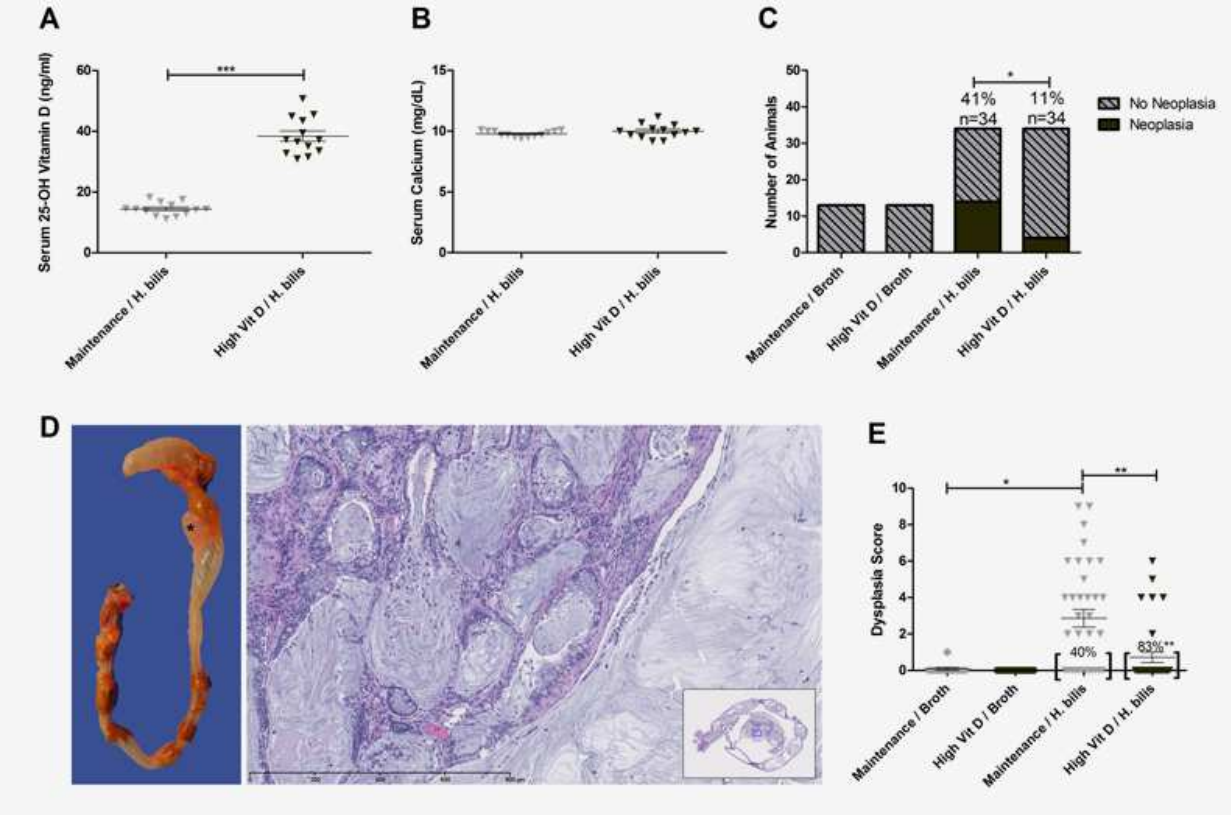


Figure 2.2. Increased dietary vitamin D increases serum vitamin D and decreases dysplasia and cancer in *Hb*-infected *Smad3*$^{-/-}$ mice.

Serum 25-OH D (**A**) and serum calcium (**B**) were measured 16 weeks post *Hb*-infection in *Hb*-infected/ maintenance (n=13) vs. high vitamin D (n=13) diet. ***p<0.0001, Mann-Whitney t test. Colon and cecum were analyzed at 16 weeks post *Hb*-infection for histopathologic evidence of invasive adenocarcinoma and dysplasia (**C-E**). (**C**) Cancer incidence is reduced in mice fed high vitamin D (*p=0.0121, Fisher's exact test). (**D**) *Hb*-infected mice typically develop grossly visible tumors in the cecum or proximal colon as represented by the pale, multilobulated mass in the proximal colon (*). Note the mucin lakes and neoplastic epithelial cells penetrating the colonic wall and proliferating within the muscularis and serosa. H&E staining. Original magnification 20X. Inset: Subgross of the whole colon section; blue box indicates magnified region.

Clinical disease and colonic inflammatory cell infiltrates are reduced during the inflammatory phase in *Hb*-infected *Smad3*$^{-/-}$ mice fed increased dietary vitamin D. *Smad3*$^{-/-}$ develop acute inflammation approximately 3-7 days following *Hb* infection which is characterized by diarrhea, frank blood in the stool, dehydration, lethargy and loss of body condition. Clinical signs typically resolve within 7-14 days until the time that cancers develop

([153], unpublished observations). In order to determine the effects of elevated dietary vitamin D on early disease stages we assessed clinical disease parameters and alterations in inflammatory infiltrates in the colons of *Hb*-infected mice fed high vitamin D diet compared to *Hb*-infected mice fed maintenance diet. During the initial inflammatory period, mice were assigned a subjective fecal score in order to assess clinical evidence of IBD. Animals fed high vitamin D diet had significantly decreased fecal scores at both 3 and 6 days post infection (mean 0.1 vs. 0.8, p=0.0015 and 0.2 vs. 1.0 p=0.0003 respectively, **Fig 2.3A-B**) compared to animals fed maintenance diet. Broth-treated animals showed no evidence of diarrhea, as expected, and by 14 days post-infection all animals had minimal evidence of diarrhea regardless of diet (**Fig 2.3C**). There was no significant difference in body weight change associated with diet following *Hb* infection (data not shown).

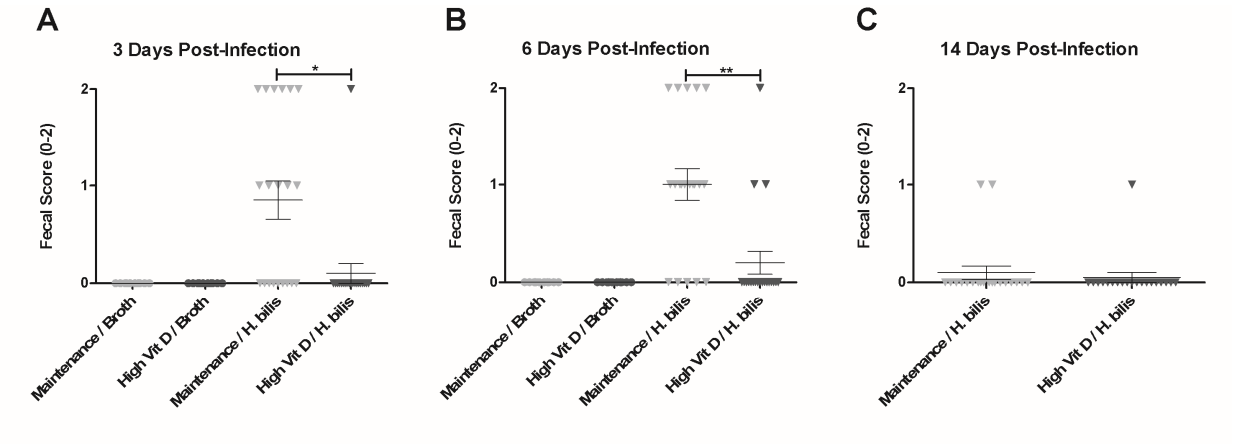


Figure 2.3. High dietary vitamin D decreases diarrhea in *H. bilis*-infected *Smad3*$^{-/-}$ mice. Fecal pellets from individual mice were collected and subjectively scored three (**A**), six (**B**), and fourteen (**C**) days post *H. bilis*-infection. Only *H. bilis*-infected animals were scored at the fourteen day time point. *H. bilis*-infected on maintenance diet (n=20), *H. bilis*-infected on high vitamin D diet (n=20), broth-treated on maintenance diet (n=10), and broth-treated on high vitamin D diet (n=10). Statistical comparison was via Mann-Whitney t test. *p<0.05, **p<0.001.

To determine if improved clinical signs associated with increased dietary vitamin D correlate with decreased colonic inflammation during early disease, proximal colon and cecum of mice fed high vitamin D or maintenance diet were analyzed for inflammation one week post *Hb* infection. *Hb*-infected animals fed high vitamin D diet had significantly reduced colitis compared to infected animals fed maintenance diet (mean 2.7 vs. 15.5, p<0.0001, **Fig 2.4 and Fig 2.5A**). Immunohistochemistry studies further demonstrated that infected mice fed high vitamin D diet had decreased inflammatory infiltrates compared to those fed maintenance diet ($F4/80^+$ cells {mean ± SEM: 2.7 ± 0.19 vs. 3.7± 0.11, p=0.0003}, $CD3^+$ T cells {mean ± SEM: 2.1 ± 0.15 vs. 2.6 ± 0.17, p=0.04}, and MHC II^+ cells {mean ± SEM: 2.05 ± 0.20 vs. 2.85 ± 0.13, p=0.001}) (**Fig 2.4 and Fig 2.5B, 2.5C, and 2.5D**). Broth-treated controls had minimal inflammation regardless of diet.

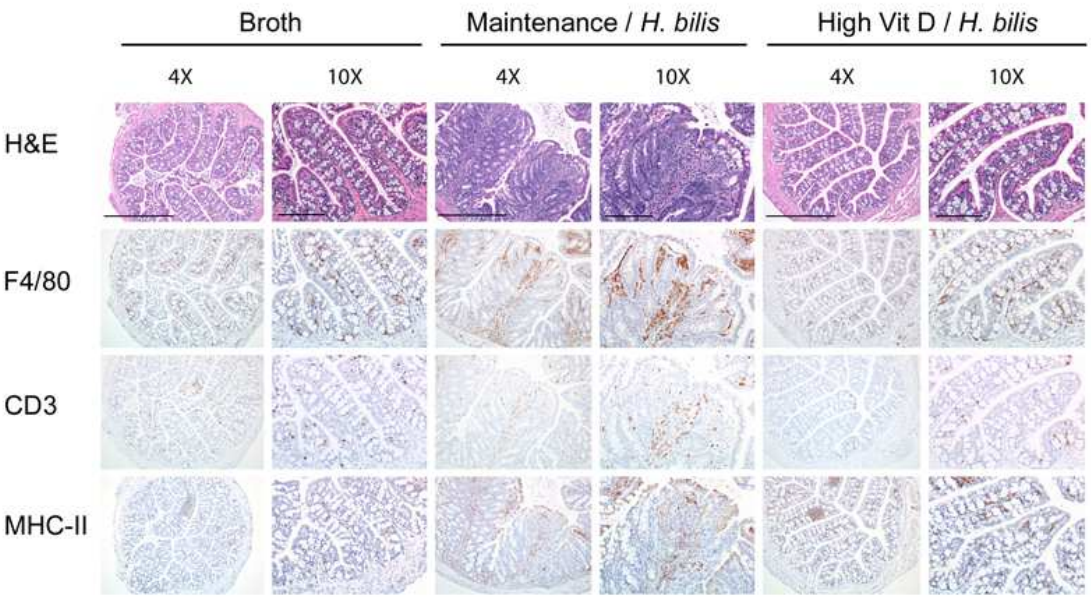


Figure 2.4. Increased dietary vitamin D decreases proinflammatory infiltrates 1 week post *Hb*-infection.

Serial sections of paraffin-embedded proximal colon were stained with H&E for orientation and immunohistochemically for F4/80, CD3, and MHCII antigen. Images from representative samples were captured at the same low (4X – scale bars indicated on top row are 500μm) and high (10X – scale bars indicated on top row are 200μm, all lower rows same magnification) original magnifications. Broth-treated controls fed maintenance diet are shown. No significant differences were noted between maintenance and high vitamin D diet groups treated with broth.

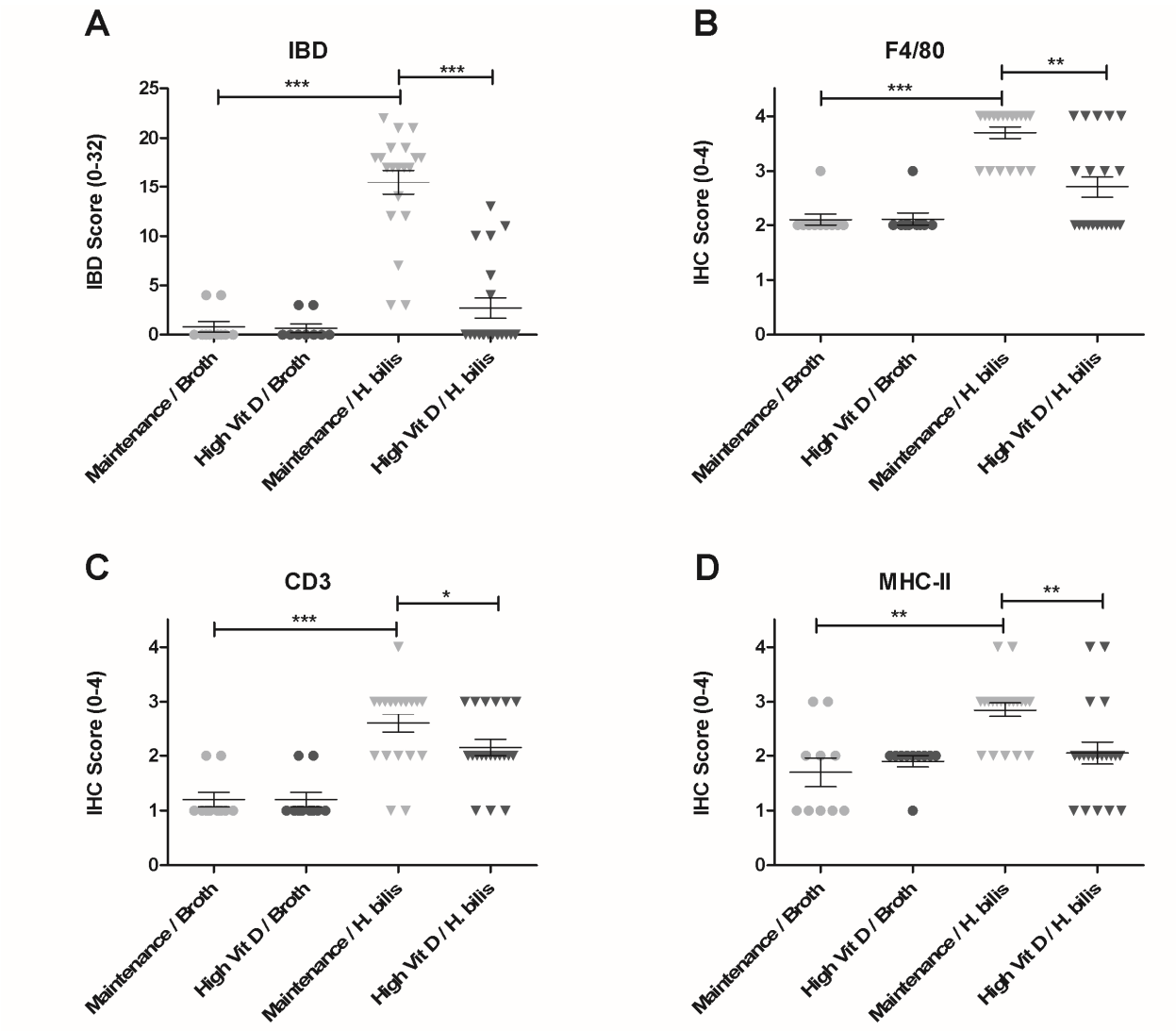
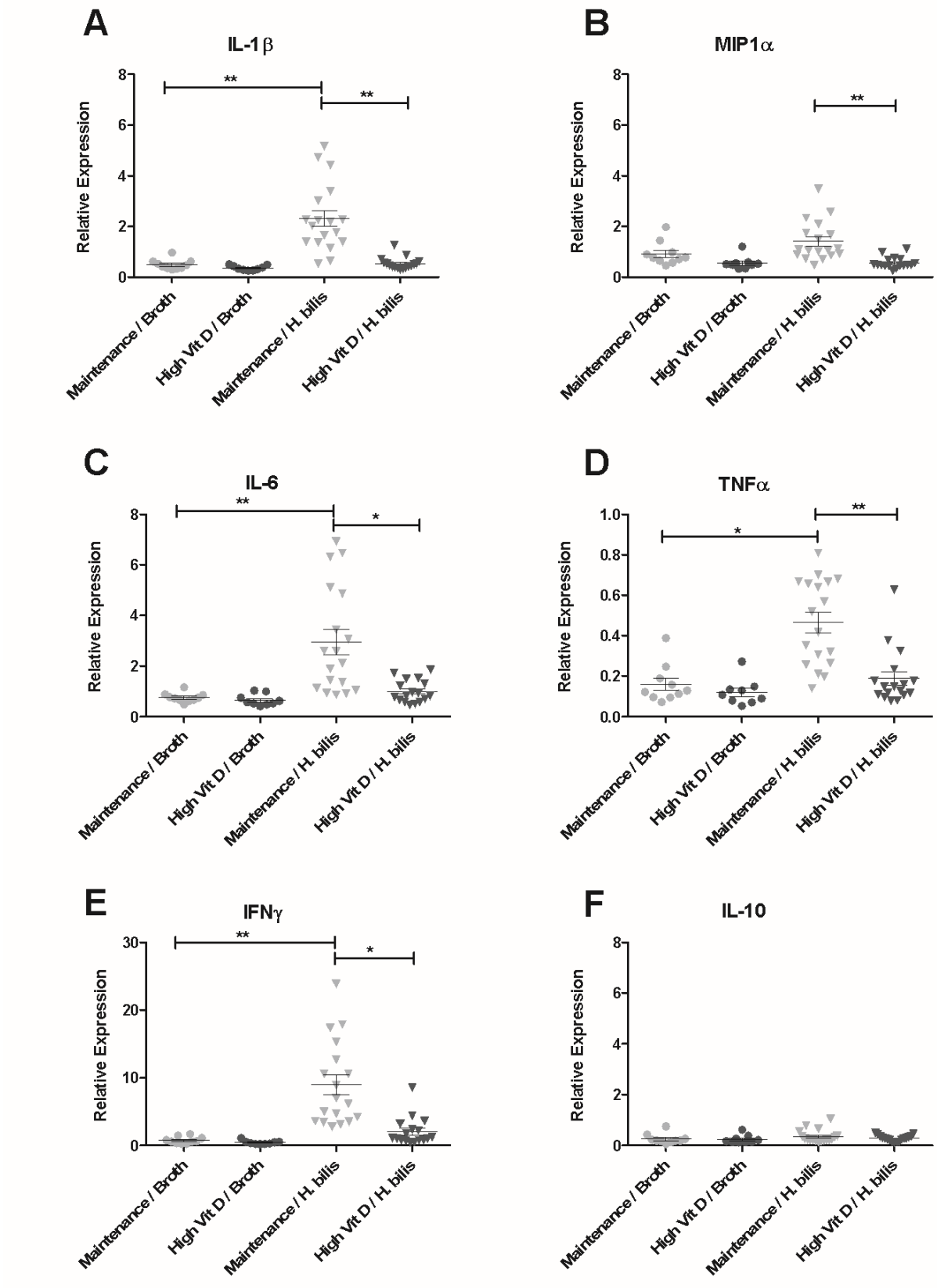


Figure 2.5. Increased dietary vitamin D decreases inflammation in *H. bilis*-infected *Smad3*$^{-/-}$ mice.

Serial sections of paraffin-embedded proximal colon, harvested 1-week post *H. bilis*-infection, were stained with H&E for orientation and immunohistochemically for F4/80, CD3, and MHCII antigen. Cecum and proximal colon were scored by a pathologist blinded to treatment groups for overall evidence of IBD (**A**). Proximal colon was scored for degree of positive cellular antigen (**B** F4/80; **C** CD3; **D** MHCII). *H. bilis* infection was associated with significant increases in inflammatory infiltrates. *H. bilis*-infected on maintenance diet (n=20), *H. bilis*-infected on high vitamin D diet (n=20), broth-treated on maintenance diet (n=10), and broth-treated on high vitamin D diet (n=10). Mann-Whitney t test *p<0.05, **p<0.001, ***p <0.0001.

Because inflammation is often associated with increased cell proliferation, we evaluated if increased dietary vitamin D was associated with changes in cell proliferation in either lamina propria or epithelial cell populations within the colon one-week post *Hb*-treatment. Proliferating Cell Nuclear Antigen (PCNA) was used as a marker of cellular proliferation by Western blot. Analysis of PCNA showed no significant differences associated with diet in either cell population (LPL mean 0.73 vs. 0.67, epithelial cell mean 0.99 vs. 0.85, high vitamin D diet vs. maintenance diet respectively, data not shown).

High dietary vitamin D decreases cecal, proximal colon and fecal proinflammatory cytokines 1-week post *Hb*-infection. Because we previously observed elevated inflammatory cytokines in this model associated with *Hb*-infection [153], proinflammatory cytokine expression in cecal and proximal colon tissues one-week post *Hb*-infection were evaluated to determine whether increased vitamin D would dampen the inflammatory response induced by infection. Increased expression of IL1β, macrophage chemotaxis factor 1α (MIP1α), IL6, TNFα, and IFNγ were noted in cecal tissue from *Hb*-infected animals fed maintenance diet compared to broth-treated controls (**Fig 2.6**) as seen previously with this model [153]. However, there was a significant reduction in expression of those same cytokines in *Hb*-infected mice fed increased dietary vitamin D compared with those fed maintenance diet (**Fig 2.6A-E**). Interestingly, there were no changes in expression of the anti-inflammatory cytokine, IL-10 in response to diet or *Hb*-infection (**Fig 2.6F**). Expression patterns of proinflammatory cytokines in the proximal colon were similar to those observed in cecal tissue (**Fig 2.7A-F).**


A
IL-1β
Relative Expression
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Maintenance / Broth
High Vit D / Broth
Maintenance / H. bilis
High Vit D / H. bilis
B
MIP1α
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C
IL-6
Relative Expression
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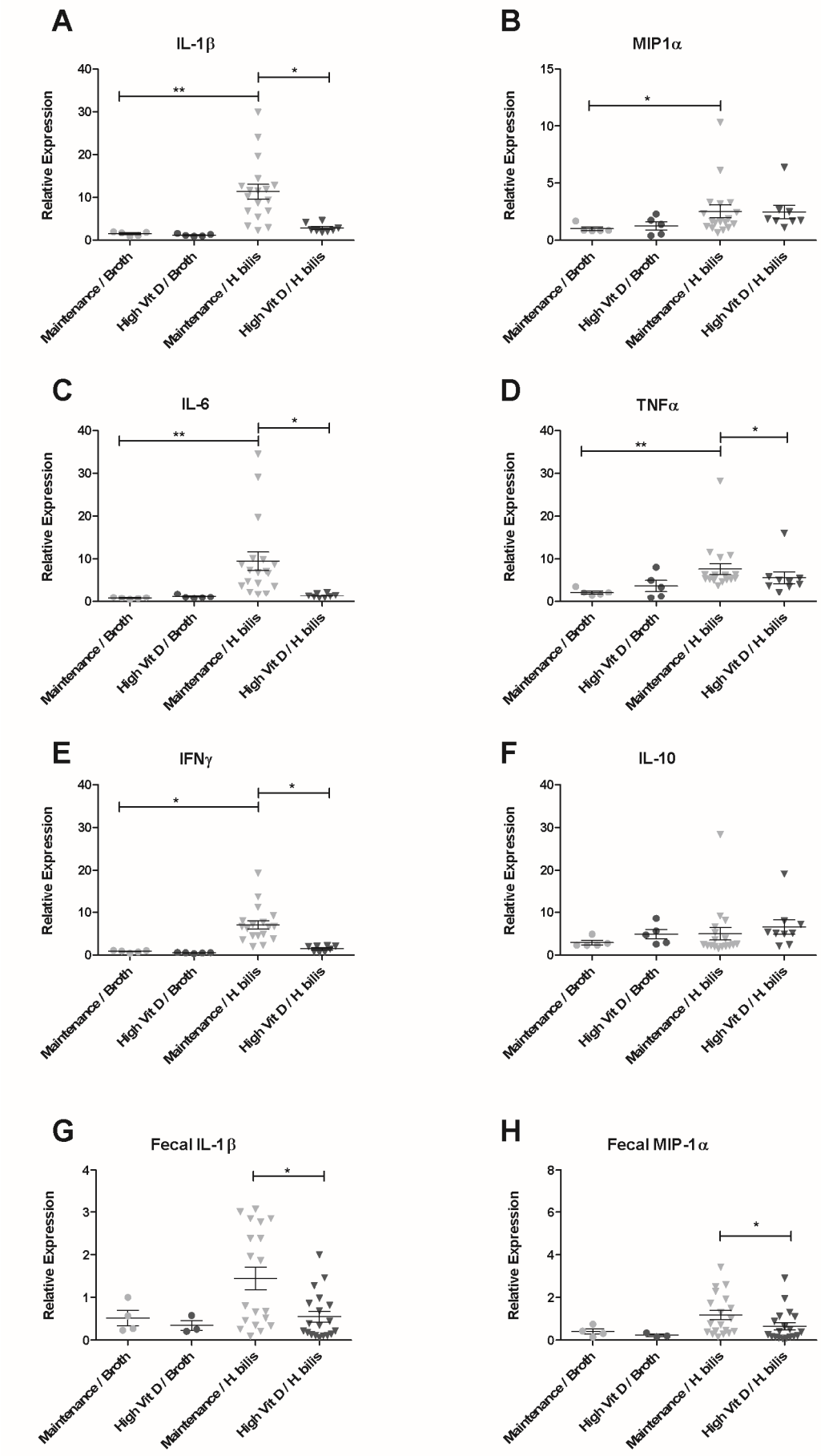
*
D
TNFα
Relative Expression
*
**
E
IFNγ
Relative Expression
**
*
F
IL-10
Relative Expression

Figure 2.6. High dietary vitamin D decreases cecal tissue expression of proinflammatory and chemotactic cytokines 1 week post *Hb*-infection.

Expression of IL-1β (**A**), MIP-1α (**B**), IL-6 (**C**), TNFα (**D**), IFNγ (**E**), and IL-10 (**F**) in cecal tissue from *Smad3*$^{-/-}$ mice fed high vitamin D or maintenance diet with or without *Hb*-infection was determined by real-time PCR. *Hb*-infected/ maintenance diet (n=20), *Hb*-infected/ high vitamin D diet (n=20), broth-treated/ maintenance diet (n=10), and broth-treated/ high vitamin D diet (n=10). Note differences in scale in (D) and (E). Kruskal-Wallis non-parametric test and Dunn's post-hoc test to control for multiple comparisons. *p<0.05, **p<0.001.

Fecal cytokines have been used in the *Hb*-infected *Smad3*$^{-/-}$ mouse model to characterize the inflammatory response and predict development of cancers [161]. To determine if fecal cytokine expression correlated with tissue cytokine expression, expression of IL1β and MIP1α was evaluated in fecal pellets collected from animals 1, 2, and 3 weeks post *Hb*-infection. Similar to the expression pattern of IL1β and MIP1α observed in cecal and proximal colon tissues at 1 week post-infection (**Fig 2.6A and 2.6B, Fig 2.7A and 2.7B),** expression of IL1β and MIP1α in feces were significantly increased in *Hb*-infected animals fed maintenance diet compared with broth-treated animals (**Fig 2.7G-H**). Accordingly, *Hb*-infected mice fed high vitamin D diet had an average 3-fold decrease in fecal IL1β and 1.5-fold decrease in fecal MIP1α expression compared with mice fed the maintenance diet. However, these fecal cytokine changes were transient as there were no significant differences in IL1β or MIP1α expression between any treatment group at 2 or 3 weeks post-infection (data not shown).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Relative Expression
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High Vit D / Broth
Maintenance / H. bilis
High Vit D / H. bilis
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Fecal IL-1β
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Maintenance / H. bilis
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Relative Expression
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High Vit D / Broth
Maintenance / H. bilis
High Vit D / H. bilis

Figure 2.7. High dietary vitamin D decreases expression of proinflammatory and chemotactic cytokines in proximal colon tissue and feces 1 week post *H. bilis*-infection.

Real-time PCR was performed to determine expression levels of IL-1β (**A**), MIP1α (**B**), IL-6 (**C**), TNFα (**D**), IFNγ (**E**), and IL-10 (**F**) in proximal colon tissue from *Smad3*$^{-/-}$ mice fed high vitamin D or maintenance diet with or without *H. bilis*-infection. *H. bilis*-infected on maintenance diet (n=20), *H. bilis*-infected on high vitamin D diet (n=10), broth-treated on maintenance diet (n=5), and broth-treated on high vitamin D diet (n=5). *H. bilis*- infected animals fed high vitamin D had significantly reduced expression of proinflammatory cytokines as compared to *H. bilis*-infected maintenance-fed controls. No significant difference in IL-10 expression was observed in response to infection status or diet treatment. Fecal pellets were collected 1 week post *H. bilis*-infection from mice fed either high or maintenance diet. Real-time PCR was performed to determine expression levels of IL-1β (**G**) and MIP-1α (**H**). *H. bilis*-infected on maintenance diet (n=20), *H. bilis*-infected on high vitamin D diet (n=19), broth-treated on maintenance diet (n=4), and broth-treated on high vitamin D diet (n=3). Kruskal-Wallis non-parametric test and Dunn's post-hoc test to control for multiple comparisons. *p<0.05, **p<0.001, ***p <0.0001.

Increased dietary vitamin D decreases p-P38 MAPK in the colon. *Helicobacter* species have been shown to elicit proinflammatory cytokine production through TLR-4 dependent activation of the MAPK and NFκB pathways [166]. Thus, we determined whether decreased colonic inflammation in *Smad3*$^{-/-}$ mice in response to increased dietary vitamin D was associated with altered MAPK and NFkB signaling pathways during early disease (1 week post *Hb* infection).

For changes in the MAPK pathway, relative levels of protein expression of activated forms of p38 (p-p38), JNK (p-JNK), and Erk1/2 (p-Erk) in LPL and colonic epithelial cell populations were determined. LPLs of *Hb*-infected animals fed increased dietary vitamin D had a 7-fold decrease in p-p38 compared to maintenance diet-fed animals (p=0.012, **Fig 2.8A and Fig 2.9**). Interestingly, similar changes were not detected in colonic epithelial cells (**Fig 2.8A and Fig 2.9**). There was a trend toward decreased p-JNK expression in colonic tissue from animals fed increased dietary vitamin D, however, these differences were not statistically significant (LPL: 4.5-fold decrease p= 0.14 and epithelial cells: 2-fold decrease p=0.052, **Fig 2.8B and Fig 2.9**. There were no notable differences in p-Erk1/2, total P38 or total JNK in either cell population (data not shown).

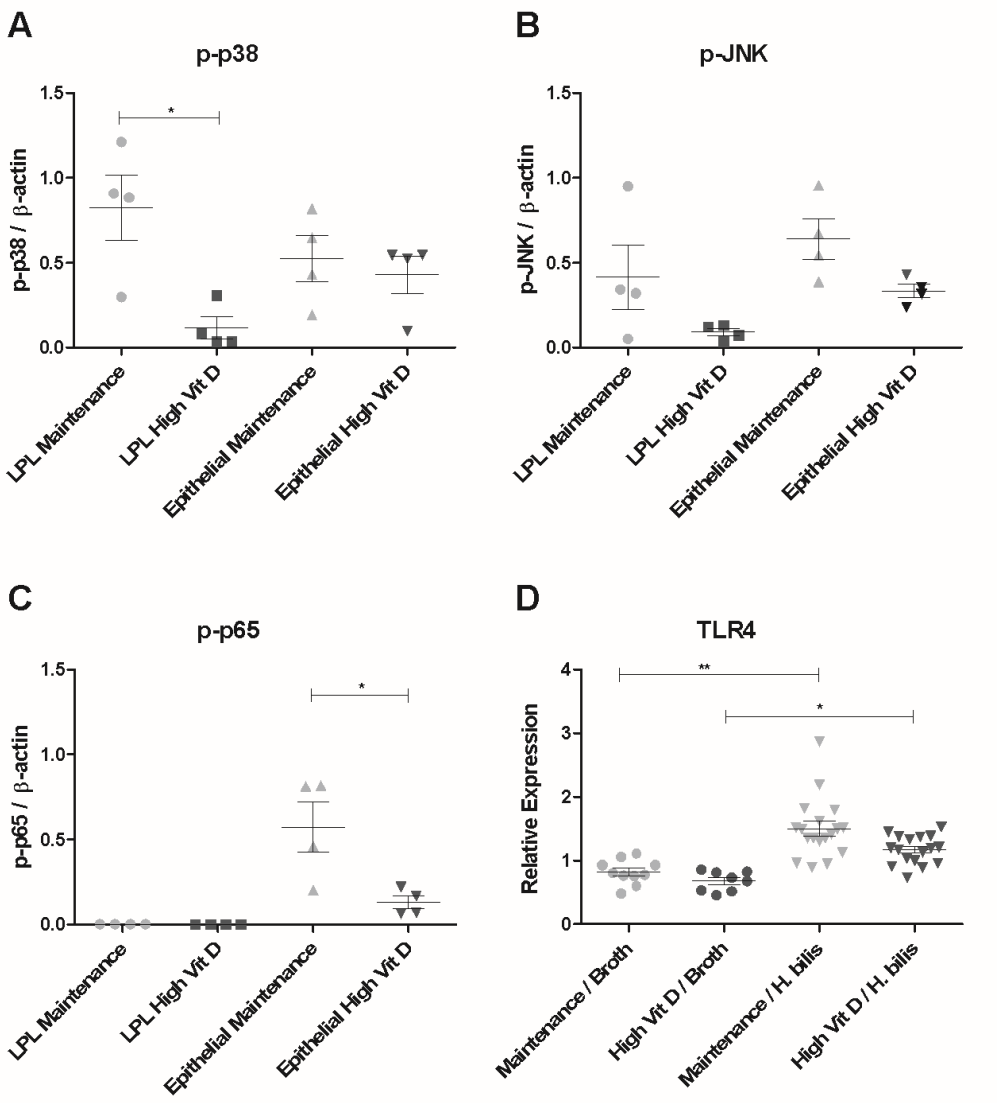


Figure 2.8. Increased dietary vitamin D decreases MAPK and NFκB signaling in *Hb*-infected *Smad3*$^{-/-}$ mice.

Whole cell lysates were isolated from proximal colon LPL and epithelial cells harvested 1 week post *Hb* infection. Expression levels of p-p38 (**A**), p-JNK (**B**), and p-p65 (**C)** were determined by western blot and densitometry. Densitometry results of each protein level were normalized to β-actin. Unpaired t test *p<0.05, **p<0.001. *Hb*-infected on maintenance diet (n=4) and *Hb*-infected on high vitamin D diet (n=4). (**D**) Colonic expression of TLR4 was determined by qRT-PCR from mice fed high vitamin D or maintenance diet and infected with and without *Hb. Hb*-infected/ maintenance diet (n=20), *Hb*-infected/ high vitamin D diet (n=20), broth-treated/ maintenance diet (n=10), and broth-treated/ high vitamin D diet (n=10). Kruskal-Wallis non-parametric test with Dunn's post-hoc test (*p<0.05 and **P<0.001).

For alterations in the NFκB pathway, phosphorylated p65 (p-p65) was evaluated by western blot analysis in LPL and epithelial cell populations. Mice fed high vitamin D diet had a 4.5-fold decrease in p-p65 in colonic epithelial cells compared to mice fed maintenance diet ($p=0.028$, **Fig 2.8C and Fig 2.9**). We did not detect p-p65 in LPL regardless of diet while IκBα, an inhibitor of NFκB activation, was present in both LPL and colonic epithelial cells (**Fig 2.9**).

To determine if the changes in p-P38 or p-P65 were associated with decreased TLR4 expression, qRT-PCR was performed on cecal tissues collected from mice 1week post *Hb*-infection. Average TLR4 expression in *Hb*-infected animals was modestly increased (1.3-fold) compared to broth-treated controls ($p<0.01$, **Fig 2.8D**). Although TLR4 expression in *Hb*-infected mice was lower when fed high vitamin D diet, the difference was not statistically significant.

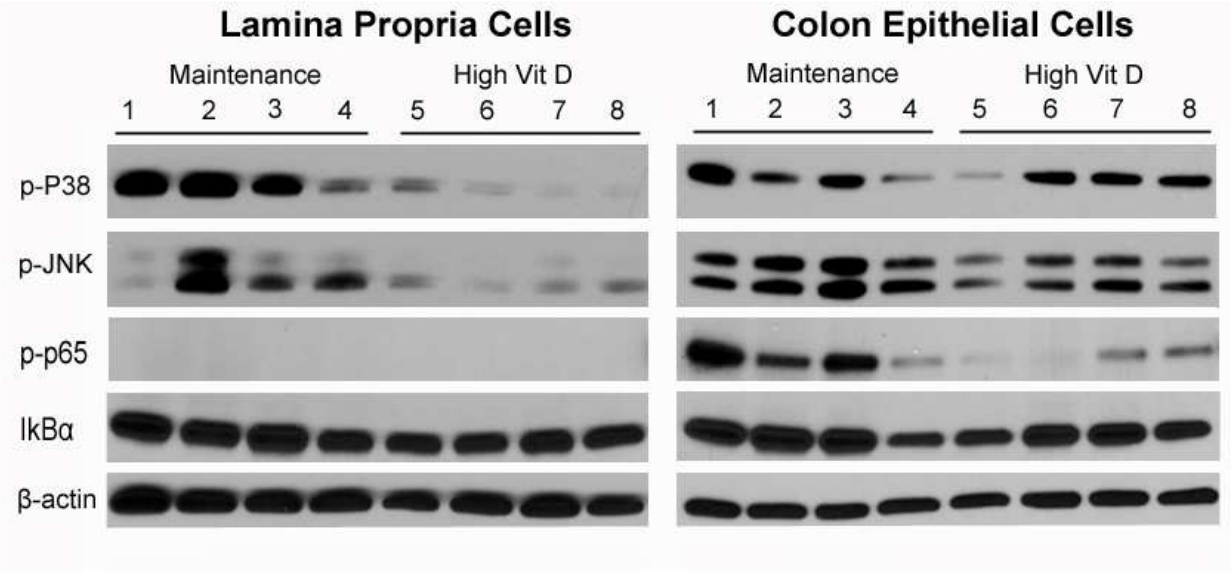


Figure 2.9. High dietary vitamin D decreases p-P38 and p-P65 expression.

Mice were fed either high vitamin D or maintenance diet (n=4/diet) and infected with *H. bilis.* One-week post *H. bilis*-infection, LPL and epithelial cells were isolated from proximal colon, and total proteins were analyzed for MAPK and NFκB signaling pathways by western blot. High vitamin D diet was associated with decreased expression p-p38 in lamina propria cells and decreased levels of p-p65 in epithelial cells. Multiple blots were run and figure represents representative blots for each protein.

High dietary vitamin D does not alter *Hb* colonization in *Smad3*$^{-/-}$ mice. Changes in the gut microbiome or changes in bacterial load can influence disease severity in both human patients [167] and animal models of IBD [131, 167]. As TLR4 expression levels tend to be lower in high vitamin D fed *Smad3*$^{-/-}$ mice following *Hb* infection, we determined whether vitamin D alters *H. bilis* colonization in cecum and colon where they preferentially reside [153]. Quantitative RT-PCR was used to compare the relative amount of *Helicobacter* organisms in cecal, proximal and distal colonic tissues collected 8-weeks post *Hb*-infection from mice fed either increased or maintenance levels of vitamin D. While cecal tissues had the highest concentration of *Helicobacter* as previously reported [153], no significant differences in *Helicobacter* colonization were associated with diet (**Fig 2.10)**.

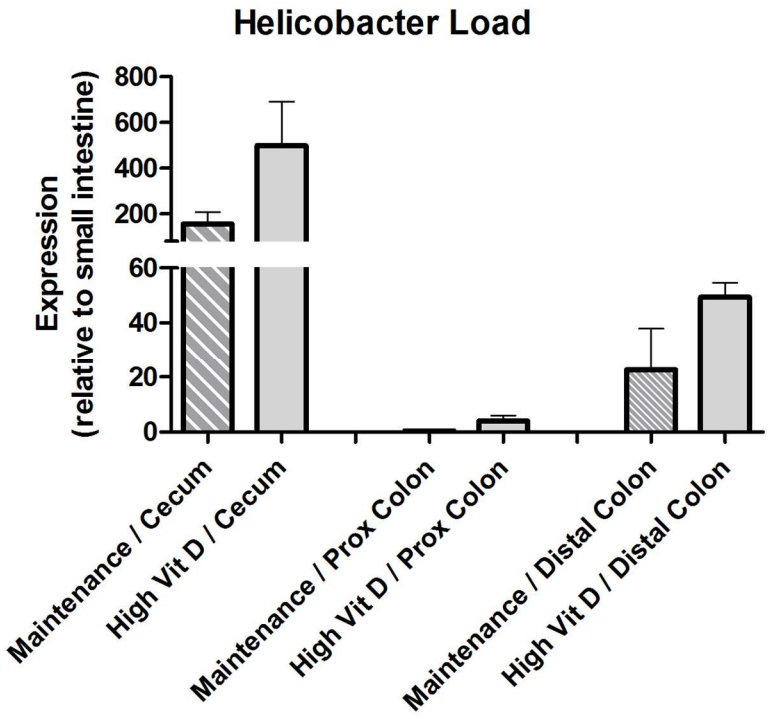


Figure 2.10. *Helicobacter* colonization is not altered by increased dietary vitamin D.

Real-time PCR for *H. bilis* was performed using DNA from mid-jejunum, proximal colon, distal colon and whole cecum with 18S DNA as a loading control. (n=5 per diet, tissues collected 8 weeks post-infection). *Helicobacter* DNA levels in colon or cecum were calculated relative to those in small intestine.

High dietary vitamin D does not alter cecal expression of vitamin D receptor or enzymes involved in vitamin D metabolism. To determine whether the protective effect of increased dietary vitamin D was associated with changes in proteins involved in vitamin D signaling and/or metabolism, quantitative RT-PCR was used to evaluate RNA expression of vitamin D receptor (VDR) as well as two enzymes involved in conversion of vitamin D into its active and inactive forms, $25(OH)D_3$-1α-hydroxylase (Cyp27b1), and $1,25(OH)_2D_3$ 24 hydroxylase (Cyp24a1) in cecal tissue at one-week post *Hb*-infection. A small yet significant increase in VDR expression was detected in mice fed high vitamin D diet following *Hb*-infection (**Fig 2.11A**). However, there were no changes in mRNA levels of Cyp27b1 and Cyp24a1 associated with either diet or infection-status (**Fig 2.11B-C**). Although VDR mRNA was altered with diet, differences in VDR protein expression were not detected using western blot analysis of proximal colon tissue (data not shown).

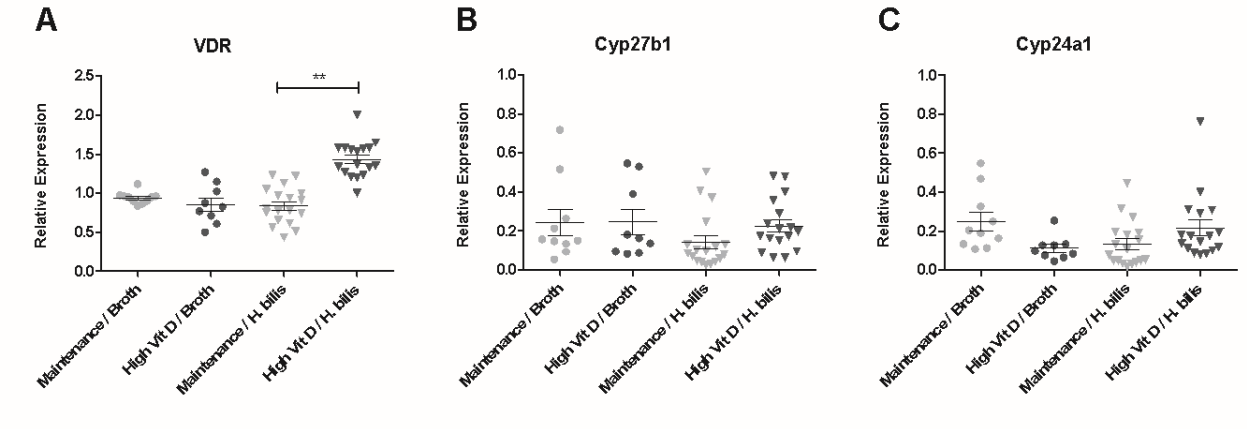


Figure 2.11. Increased dietary vitamin D increases VDR mRNA expression but does not alter enzymes involved in vitamin D metabolism (Cyp27b1 and Cyp24a1).

Expression levels of VDR (**A**), Cyp27b1 (**B**) and (**C**) Cyp24a1 were determined with qRT-PCR using RNA from proximal cecum (3mm piece) isolated from mice 1 week post *Hb*-infection. *Hb*-infected/ maintenance diet (n=20), *Hb*-infected/ high vitamin D diet (n=20), broth-treated/ maintenance diet (n=10), and broth-treated/ high vitamin D diet (n=10). Kruskal-Wallis non-parametric test and Dunn's post-hoc test to control for multiple comparisons. ** $p<0.001$.

A vitamin D deficient diet did not exacerbate colitis or colitis-associated colon cancer in *Hb*-infected *Smad3*$^{-/-}$ mice. Because decreased serum vitamin D is associated with an increased risk for developing IBD as well as colon cancer in humans [16, 20, 21, 150], we hypothesized that a diet deficient in vitamin D would exacerbate inflammation and potentially increase the incidence of colon tumors in *Hb*-infected *Smad3*$^{-/-}$ mice. We tested this hypothesis by feeding mice maintenance diet or diet devoid of vitamin D (AIN93Null) and induced inflammation by *Hb* infection. Serum 25-hydroxyvitamin D levels were significantly decreased in AIN93Null-fed mice compared to mice fed maintenance diet (mean: 5.7 vs. 12.2 ng/ml, p=0.01) after two weeks on the diet and were below the limit of detection at the end of the 16 week study (**Fig 2.12A-B**). Despite decreased serum vitamin D levels, mice maintained on AIN93Null diet showed no differences in serum calcium compared to maintenance diet fed controls (**Fig 2.12C**). Clinical disease during the early inflammatory phase was assessed by monitoring subjective fecal scores and body weight change following *Hb* infection. Mice maintained on AIN93Null diet showed no differences in fecal scores or body weight change compared to maintenance diet fed controls (data not shown). To determine if decreased dietary vitamin D exacerbated IBD at early disease stages, proximal colon and cecal tissue of mice fed maintenance diet or AIN93Null were analyzed for inflammation one-week post *Hb* infection. There were no differences in IBD associated with diet (**Fig 2.12D)**. To further evaluate any effect of AIN93Null diet, proinflammatory cytokine expression in proximal colon tissues was measured to determine if decreased dietary vitamin D would exacerbate the inflammatory response induced by *Hb*. In correlation with the lack of effect on IBD scores, no differences were noted in cytokine gene expression associated with diet (**Fig 2.13**). Consistent with the lack of altered inflammation early in disease, there were no differences in cancer incidence or dysplasia noted between AIN93Null fed mice compared with maintenance fed animals (**Fig 2.12E-F**) when necropsied after 16 weeks of diet.

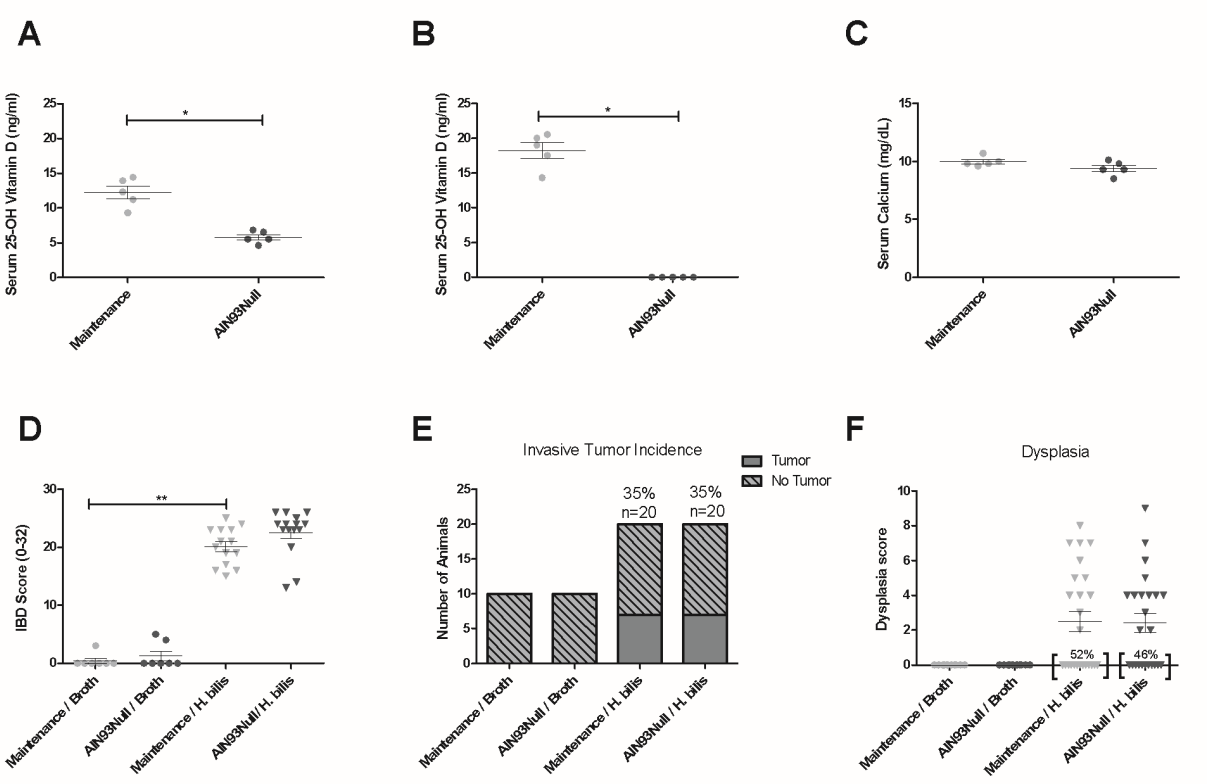


Figure 2.12. AIN93Null diet does not exacerbate *Hb*-induced disease in *Smad3*$^{-/-}$ mice.

Serum 25-hydroxyvitamin D was measured after (**A**) two weeks or (**B**) 18 weeks on diet. Serum calcium levels (**C**) were determined at 18 weeks after diet initiation. Mann-Whitney t test. *p<0.05, **p<0.001. One week post *Hb*-infection, cecum and proximal colon were histologically scored for inflammation (**D**). *Hb*-infected/ maintenance diet (n=14), *Hb*-infected/ AIN93Null diet (n=15), broth-treated/ maintenance diet (n=7), and broth-treated/ AIN93Null diet (n=7). Mann-Whitney t test. **p<0.001. 16 weeks post infection, colon and cecum were analyzed for cancer incidence (**E**) and dysplasia (**F**)**.** Incidence of no dyplasia is indicated by percent near brackets (**F**). *Hb*-infected/ maintenance diet (n=20), *Hb*-infected/ AIN93Null diet (n=20), broth-treated/ maintenance diet (n=10), and broth-treated/ AIN93Null diet (n=10).

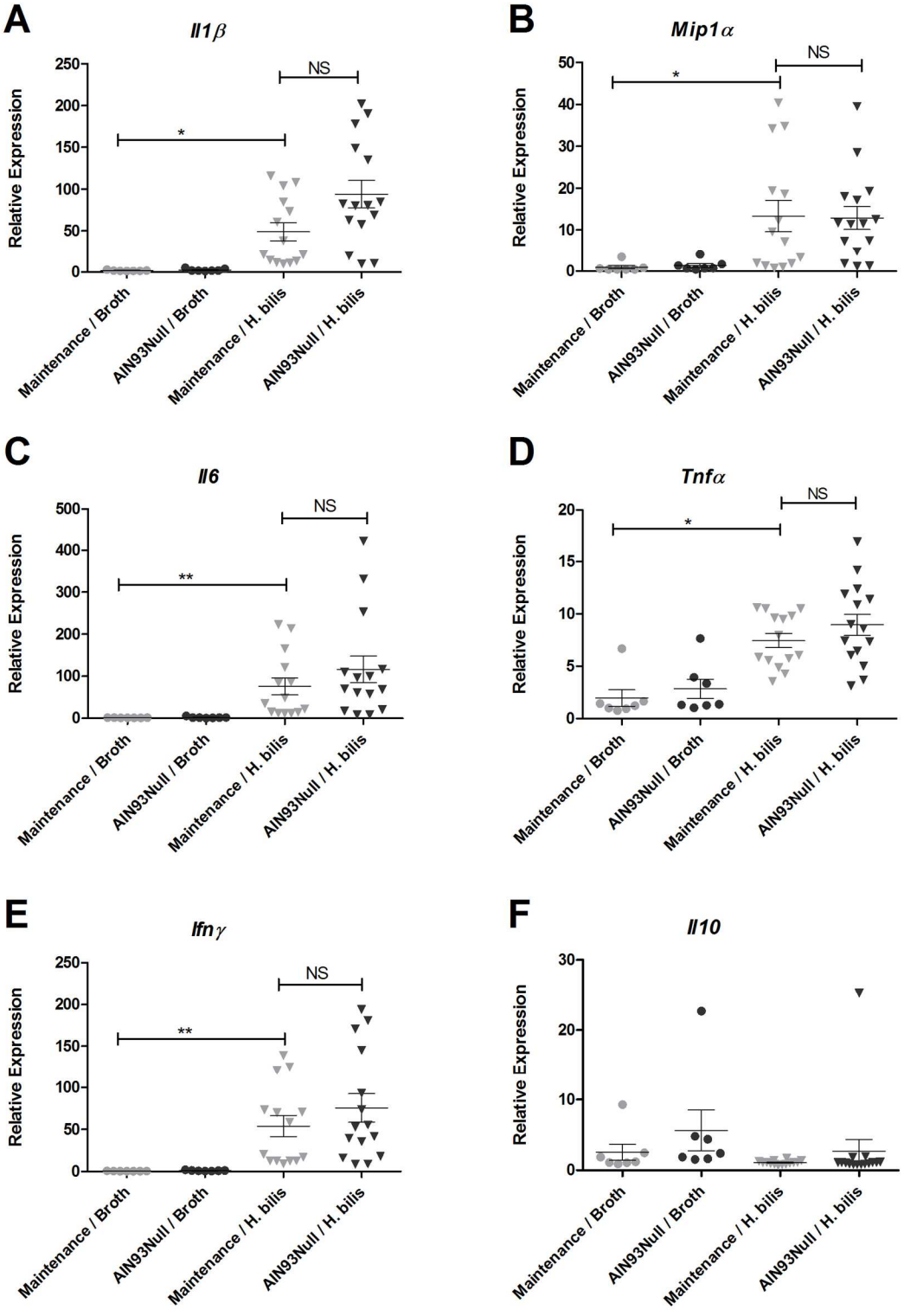


Figure 2.13. AIN93Null diet does not alter expression of proinflammatory and chemotactic cytokines in proximal colon tissue 1 week post *H. bilis*-infection.

Real-time PCR was performed to determine expression levels of IL-1β (**A**), MIP1α (**B**), IL-6 (**C**), TNFα (**D**), IFNγ (**E**), and IL-10 (**F**) in proximal colon tissue from *Smad3*$^{-/-}$ mice fed maintenance diet or AIN93Null with or without *H. bilis*-infection. *H. bilis*-infected on maintenance diet (n=14), *H. bilis*-infected on high vitamin D diet (n=15), broth-treated on maintenance diet (n=10), and broth-treated on high vitamin D diet (n=10). *H. bilis*- infected animals had significantly increased expression of proinflammatory cytokines as compared to broth inoculated controls regardless of diet. No significant differences in inflammatory cytokine levels were found in *H. bilis* infected animals in response to diet. No significant difference in IL-10 expression was observed in response to infection status or diet treatment. Kruskal-Wallis non-parametric test and Dunn's post-hoc test to control for multiple comparisons. *p<0.05, **p<0.001.

DISCUSSION:

Using *Smad3*$^{-/-}$ mice, we have shown that increased dietary vitamin D affords protection against the development of colon cancer. In this model, we have demonstrated that increased dietary vitamin D a) induces elevated serum 25-hydroxyvitamin D without causing hypercalcemia, b) significantly decreases inflammation, dysplasia and tumor incidence following infection with *Hb,* and c) is associated with decreased p- p38 (MAPK) and p-P65 (NFκB) expression during the acute inflammatory stage of disease. These studies provide evidence that the protective effect(s) of elevated dietary vitamin D supplementation in a model of inflammation-associated colon cancer are mediated through suppression of the inflammatory responses triggered following infection with colitogenic bacteria.

Animals fed increased concentrations of dietary vitamin D demonstrated significant protection against inflammation and tumor formation. It should be noted that though serum vitamin D levels are increased in mice fed high vitamin D diet compared to those fed maintenance diet, levels remain within the comparable recommended range for humans without reaching super-physiologic levels [168]. Many animal and human studies that have shown anti-tumor effects of vitamin D administer metabolically active $1,25(OH)_2D_3$ which results in hypercalcemia and vitamin D toxicities [125-127]. Our studies show that dietary vitamin D supplementation offers a way to improve vitamin D status and provide protection from inflammation-associated colon cancer while avoiding vitamin D toxicity.

Vitamin D supplementation decreases cancer incidence in several rodent models of colon cancer [126, 127, 151, 152]. These models rely on either genetic predisposition for the development of gastrointestinal neoplasias as is the case with *APC*$^{Min/+}$ mice or treatment with a chemical mutagen such as azoxymethane (AOM) to simulate the adenoma to carcinoma progression that occurs during the development of sporadic colon cancer [31, 169]. However, the molecular changes, disease progression, and pathology of inflammation-associated cancer are distinct from those changes observed in sporadic or familial colon cancers and therefore may influence the efficacy of vitamin D as well as mechanisms through which it promotes protection from tumor formation.

Chronic inflammation is believed to frequently play a key role in carcinogenesis [35]. Links between inflammation and cancer have not only been observed in colon cancer in human patients with IBD [31] but also in liver, pancreatic, stomach, esophageal, and prostate cancers [35]. Several studies utilizing mouse models of colitis have shown that vitamin D can be beneficial in preventing or ameliorating inflammation and clinical disease [42, 43, 87]; however, these models do not typically progress to neoplasia. Recently, the DSS/AOM model, another model of inflammation associated cancer which does progress to dysplasia and tumor formation, was used to demonstrate that increasing concentrations of dietary vitamin D are protective against preneoplasic lesions in a dose-dependent manner [44]. Consistent with these findings, we have shown that increased dietary vitamin D is effective at not only preventing inflammation and dysplasia, but subsequent invasive tumor formation as well.

Chronic inflammation is associated with increased production of proinflammatory cytokines including TNFα, IL1β, and IL-6 which contribute to carcinogenesis through influences on cell proliferation, apoptosis, differentiation, and angiogenesis [170]. Dietary vitamin D supplementation significantly lowered inflammatory cytokines induced in response to *Hb* in *Smad3*$^{-/-}$ mice. In human patients with colon cancer, these proinflammatory cytokines are positively associated with increased cancer growth, higher neoplastic grade, and increased risk of mortality [170]. Proinflammatory cytokines are also upregulated in IBD patients, even before the onset and progression to dysplasia or neoplasia [171], and vitamin D supplementation has been linked to decreased circulating proinflammatory cytokines in patients with colorectal adenomas [172]. Epidemiologic evidence suggests that treatments which limit inflammation may be beneficial in reducing the incidence of inflammation-associated colon cancer in high-risk

populations [36]. Together, these data suggest that dietary vitamin D may be effective at decreasing the proinflammatory milieu in IBD patients and serve as a useful adjunct treatment in certain populations.

The mechanism by which vitamin D suppresses colon cancer in *Smad3*$^{-/-}$ mice is not completely clear, although our data suggests the anti-inflammatory effects of vitamin D are important. *Helicobacter* species are Gram negative, microaerophilic bacteria that can induce local production of proinflammatory cytokines and chemokines through TLR4 signaling and subsequent activation of the MAPK and NFκB pathways [166]. Both of these pathways have been shown to be upregulated in human patients with IBD [173] and are thought to be important links between inflammation and cancer [173-175]. *In vitro* evidence suggests that vitamin D is able to suppress MAPK activity and subsequent proinflammatory cytokine production through the upregulation of MAPK phosphatase-1[176] and NFκB signaling through the upregulation of IκBα, an inhibitor of NFκB activation [177], or through decreased expression of the NFκB component RelB which can lead to inhibition of dendritic cell differentiation and maturation [178]. During the early inflammatory disease phase in *Hb*-infected *Smad3*$^{-/-}$ mice, vitamin D high diet was associated with dramatic decreases in p-p38 in the lamina propria cells, decreased NFκB activation in epithelial cell populations, and suppressed proinflammatory cytokine expression compared to that observed in infected mice on maintenance diet. Based on these data, we propose a model where vitamin D suppresses inflammation by decreasing p38 MAPK activation in lamina propria cells, resulting in decreased proinflammatory cytokine production by those cells, which in turn decreases NFκB activation in colonic epithelial cells.

We have shown that while increased dietary vitamin D affords protection against the development of colon cancer, decreased dietary vitamin D was not sufficient to exacerbate disease in *Smad3*$^{-/-}$ mice. Because epidemiologic studies [16, 21, 42] as well as studies utilizing mouse models of colitis suggest that vitamin D deficiency or lack of vitamin D signaling can exacerbate IBD [94, 101, 179], we hypothesized that decreased dietary vitamin D would exacerbate disease in the *Smad3*$^{-/-}$ mouse model. Interestingly, although the AIN93Null diet significantly depleted circulating serum 25-hydroxyvitamin D levels, we did not see exacerbation of *Hb*- induced inflammation or subsequent inflammation-associated colon cancer. These findings are consistent with the idea that modulation of inflammation is likely responsible for the protection afforded by increased dietary vitamin D.

In conclusion, increased dietary vitamin D suppresses acute inflammation and consequently neoplastic development in a mouse model of bacterial-driven colon cancer. While additional studies are needed to elucidate the molecular mechanisms through which vitamin D and TGFβ interact to afford protection in this model, these findings suggest that vitamin D supplementation may prove useful in the treatment of IBD or potentially the prevention of inflammation-associated cancer by limiting inflammation early in disease development.

Chapter 3. PROTECTIVE EFFECTS OF VITAMIN D ARE DEPENDENT UPON $CD4^+$ T CELLS

INTRODUCTION:

Though the precise etiology of inflammatory bowel disease (IBD) remains unclear, it is commonly believed that pathogenesis is multifactorial with a combination of genetic, immunologic, microbial and environmental triggers. The end result presumably involves dysregulated immune responses to enteric flora resulting in chronic, poorly controlled intestinal inflammation [180]. Both adaptive and innate immune responses play a role in the development and progression of IBD. Dendritic cells play a key role in promoting the differentiation and proliferation of effector T cells (Th1 and Th17 cells in Crohn's disease and Th2 cells in ulcerative colitis) resulting in an overall increase in inflammatory cytokine secretion and inflammatory infiltrates in the gastrointestinal tract [181]. *Helicobacter*-infection in *Smad3*$^{-/-}$ mice results in an overall increase in Th1/Th17 pro-inflammatory cytokines and infiltrates as is seen in patients with Crohn's disease [45, 153]

Vitamin D and its active metabolite, $1,25(OH)_2D$, have been shown to be important modulators of immune responses [182], and many populations of immune cells have been shown to express VDR [183]. *In vitro* and *in vivo* data has demonstrated that vitamin D affects both innate and adaptive immune responses resulting in decreased cell differentiation, cellular proliferation, and secretion of pro-inflammatory cytokines [18]. Vitamin D deficiency has been associated with exacerbation and progression of several immune-mediated diseases including multiple sclerosis [7], type 1 diabetes [9, 10], and inflammatory bowel disease [48, 184]. Interestingly treatment with $1,25(OH)_2D$ ameliorates clinical symptoms in mouse models of these diseases [42, 185, 186], further suggesting that vitamin D can alter disease development through modulation of the immune system.

Our previously published data suggests that vitamin D protects against colitis-associated tumor development at least in part by decreasing early stage inflammation ([45], Chapter 2). In these studies, our data indicated that vitamin D inducing a universal decrease in lamina propria cellularity rather than altering a specific immune cell populations within the colonic lamina propria. We demonstrated that increased dietary vitamin D is associated with significantly

decreased proinflammatory infiltrates (T cells, antigen presenting cells (APCs), and macrophages) within the colon tissue early during disease development following infection with *H. bilis* along with a generalized decrease in effector and regulatory T cell subsets Additionally, we determined specifically which immune cell subsets were necessary for vitamin D mediated-protection against *H. bilis*-induced colitis and colon cancer in *Smad3*$^{-/-}$ mice.

MATERIALS AND METHODS:

Mice and diets. Experiments were performed using *129-Smad3*^{tm1Par}*/J (Smad3*$^{-/-}$*)* or *Smad3/Rag2* double knock out (DKO) mice which were created by crossing *Smad3*$^{-/-}$ mice with 129S6/SvEvTac- *Rag2* ^{tm1Fwa} mice to produce *Smad3*$^{-/-}$ mice deficient in T and B cells. Age-matched male and female colony-bred mice (age 4-14 weeks) were housed in a specific pathogen-free facility, maintained free of *Helicobacter* and Mouse norovirus and screened for rodent pathogens as previously described [45]. The mice were group-housed in individually ventilated cages with autoclaved acidified water delivered in water bottles, and fed a purified, irradiated diet with 1 IU vitamin D (5SRH, maintenance) or 15 IU vitamin D per gram of diet (5BTC, high vitamin D). All diets were manufactured by PMI Nutrition International (Lab Diet/Test Diet, St. Louis, MO) based on AIN93M diet that is formulated for the maintenance of rodents' health. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Experimental design. In order to determine how increased dietary vitamin D affected lamina propria leukocyte populations, age matched male and female *Smad3*$^{-/-}$ mice were placed on either maintenance or high vitamin D diet for one week prior to *H. bilis* infection. Mice were then infected with ~2 x 10^7 CFU *H. bilis* in Brucella broth by oral gavage as previously described [153] and euthanized during the peak inflammatory period,1-week following infection. Studies were performed twice with n=4-5 mice per diet per study. Data from both studies are combined for analysis..

To determine if vitamin D mediated protection was dependent upon the presence of adaptive immune cells (T and B cells) *Smad3/Rag2* DKO mice were placed on either maintenance or high vitamin D diets and infected as described above. Mice were randomized so

that ages and genders were evenly distributed among the treatment groups. Animals were weighed weekly and monitored at least three times weekly for dehydration, diarrhea, lethargy, and weight loss. Animals were euthanized 16-18 weeks following *H. bilis* infection to evaluate for tumor development or earlier if they experienced signs of severe disease including >20% loss in body weight, severe dehydration and diarrhea, ulcerated rectal prolapse or lethargy. The experiment was carried out twice with n=8-9 mice per diet per study.

In order to determine if vitamin D mediated protection was dependent upon the presence of T cells ($CD4^+$), age matched male and female *Smad3*$^{-/-}$ mice (n=9-10 per group) were placed on either maintenance or high vitamin D diets for one week prior to *H. bilis* infection. Animals received intraperitoneal injections of 200μg anti-CD4 monoclonal antibody (Clone GK1.5, BioXCell, Lebanon, New Hampshire) or Rat IgG (Sigma Aldrich, St. Louis, Missouri) two days prior to and 3 days following infection with *H. bilis*. Animals were euthanized 6-7 days following infection or earlier if they experienced signs of severe colitis (as defined above).

For all studies, *Helicobacter* infection status was monitored by fecal PCR using previously published primer sequences [155]. Animals were euthanized by CO_2 asphyxiation at the designated end points or earlier if necessary as indicated above.

Serum vitamin D and calcium determination and tissue collection. Following euthanasia, blood was obtained via cardiac puncture. Serum samples were submitted to Heartland Assays (Ames, IA) for quantification of 25-hydroxyvitamin D (radioimmunoassay) and calcium levels. As previously demonstrated [45], a high vitamin D diet was effective at significantly increasing serum vitamin D levels without altering serum calcium levels (data not shown). Mesenteric lymph nodes, cecum, colon and rectum were fixed in 10% phosphate buffered formalin and processed for routine histologic examination. For studies to characterize the lamina propria leukocyte populations, cross-sections of proximal colon (5mm), along with the distal half of the colon were prepared for histology while the remaining proximal and mid colon were used to isolate lamina propria leukocytes as described below. For all other studies, the entire colon was processed for histology using the "Swiss roll" technique. For all studies 3mm sections of cecal tissue and cecal contents were collected into RNA later (Qiagen, Valencia, CA) and stored at -80°C for RNA or DNA extraction.

Histopathology. Colon, cecum and mesenteric lymph node were evaluated by a board certified veterinary pathologist (PT) blinded to experimental groups to assess the severity of colitis and incidence of neoplasia. An overall IBD score was determined as described [45]. IBD scores incorporate the severity of mucosal epithelial changes, degree of inflammation, and extent of lesions. For long-term studies, a dysplasia was scored as previously described [45]. Cancers were classified as adenocarcinomas and mucinous adenocarcinomas [153, 159]. Cancer incidence was assessed in animals that survived the acute inflammatory phase of disease and had sufficient time to develop adenocarcinoma. Animals surviving longer than 8 weeks were included in the analysis for neoplasia incidence.

***Helicobacter* quantification by quantitative real-time PCR analysis.** *Helicobacter* quantification was performed on cecal tissue from *Smad3*$^{-/-}$ mice fed maintenance or high vitamin D diet and euthanized 1-week post *H. bilis*-infection. DNA was extracted and analyzed by qRT-PCR using Power Sybr Green Master Mix (Applied Biosystems, Carlsbad, CA) and a Stratagene Mx3005P analyzer (Agilent Technologies, Santa Clara, CA) as previously described [45]. Samples were run in duplicate. Helicobacter levels were normalized to total expression of the 16S RNA gene. Data were analyzed using Stratagene's MxPro v4.10 software (Agilent Technologies).

Lamina propria cell preparations. Proximal colon (proximal half of the entire colon length) samples were rinsed in PBS to remove fecal material, and epithelial and lamina propria leukocyte (LPL) populations were isolated using the Lamina Propria Dissociation Kit and gentleMACS Dissociator according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cells were suspended in RPMI media (Life Technologies, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 100 units/ml Penicillin / Streptomycin (Life Technologies). Cell counts were manually determined using a hemocytometer after staining with Trypan Blue. One million cells per sample were used for each stain. All samples were incubated with anti-mouse CD16/CD32 (clone 2.4G2, Biosciences, San Jose, CA) antibody for 10 minutes at room temperature to block Fc receptors prior to staining. For intracellular cytokine staining, cells were first stimulated *in vitro* by incubating at 37 °C for 4 hours in RPMI containing 10% FBS, 100 units/ml Penicillin / Streptomycin, 100ng/ml Phorbol

12-myristate 13-acetate (PMA, Sigma-Aldrich), 1µg/ml Ionomycin (EMD Millipore), and 0.66ul/ml Golgistop (BD Bioscience, San Jose, CA). Cells were then permeablized and stained using Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions using anti-CD4-PerCPcy5.5 (eBioscience), anti-IL-4 PE (BD Biosciences), anti-IL-17 AF647 (BioLegend), and anti-IFNϒ BV421 (BioLegend) antibodies. For analysis of $FoxP3^+$ regulatory T cells, samples were permeablized and stained using a FoxP3 / Transcription Factor Staining Buffer Set (eBioscience, San Diego CA). Staining was performed in a 96 well plate with one million cells per well. Following surface staining with anti-TCRβ PE (BD Biosciences), anti-CD4 PerCPCy5.5 (eBioscience), and anti-CD25 BV421 (BioLegend) antibodies, cells were permeablized for 1 hour at 4°C, then stained with anti-FoxP3 APC (eBioscience) antibody for 20 minutes at 4°C using buffers prepared according to manufacturer's instructions. Two additional surface stain combinations were performed to further characterize the immune cell populations present within the lamina propria. Surface staining was performed by incubating samples for 20 minutes at room temperature in PBS with 5% FBS with the following antibodies. Stain 1: anti-TCRβ APC (eBioscience), anti-CD4 PerCPCy5.5 (eBioscience), anti-CD8 PE (BD Bioscience), anti-CD49b FITC (BioLegend), anti-CD25 BV421 (BioLegend), and anti-CD44 PECy7 (eBioscience). Stain 2: anti-CD19 PE-CF594 (BD Biosciences), anti-TCRγδ FITC (BioLegend), anti-GR-1 APC-Cy7 (BioLegend), anti-CD11c PE (BD Bioscience), anti-CD11b BV421 (BD Bioscience), and anti-CD103 AF647 (BioLegend).

Flow cytometric analysis. All samples were resuspended in 200-300µl Phosflow Fix Buffer (BD Bioscience) and stored overnight at 4°C prior to analysis. Samples were run on an LSRII cytometer (BD Bioscience) at the University of Washington Immunology Flow Cytometry Core Facility. At least 500,000 reads per sample were collected and analyzed using FlowJo Single Cell Analysis software (FlowJo LLC, Ashland OR). For all populations, total live cells (for granulocytes, NK cells, and APCs) or lymphocytes were gated based on FSC-A and SCC-A. Single cells were then selected based on FSC-A X FSC-W, and autofluorescent cells were excluded based upon fluorescence detected in unstained/empty channels. T cells were defined as $TCR\beta^+$ then further gated as $CD4^+$ or $CD8^+$ populations. NKT cells were $TCR\beta^+$, $CD49b^+$ while NK cells were gated as $TCR\beta^-$, $CD49b^+$. Gamma Delta (γδ) T cells were $\gamma\delta^+$. Granulocytes were gated as $Gr\text{-}1^+$ cells. Dendritic cell and macrophage subsets were gated as

follows: $CD11c^+$, $CD11b^-$; $CD11b^+$, $CD11c^-$; and $CD11b^+$, $CD11c^+$ for analysis (**Figure 3.1**). In addition these cell types were evaluated as total $CD11c^+$ and total $CD11b^+$ populations. B cells were gated as $CD11c^-$, $CD19^+$ lymphocytes. T regulatory cells were $TCR\beta^+$, $CD4^+$, $FoxP3^+$ cells. Intracellular cytokines (IFNγ, IL-4, and IL-17) were evaluated in $CD4^+$ lymphocytes.

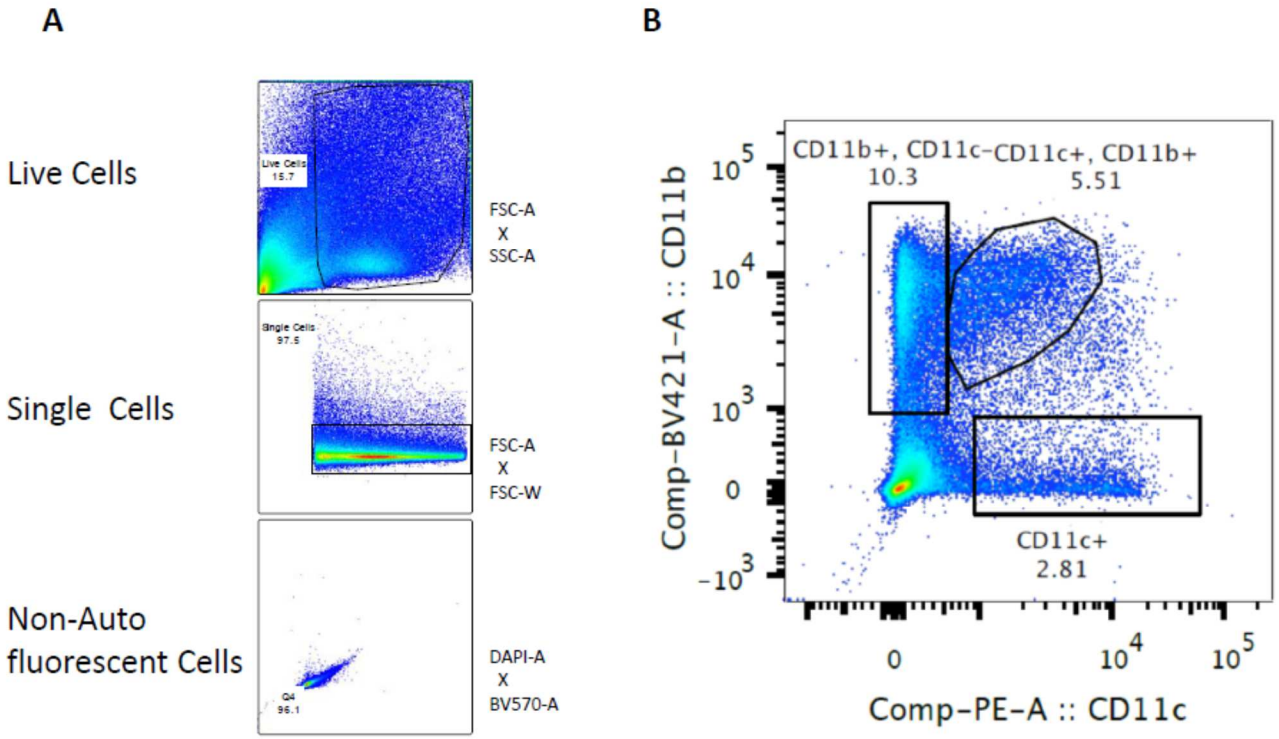


Figure 3.1. Representative gating for $CD11b^+$ and $CD11c^+$ populations.

A) Gating strategy for live cells, single cells and autofluorescent cells were based on forward and side scatter, forward scatter area and width and fluorescence in empty channels, respectively. B) Gating strategy showing classification of macrophage and dendritic cells into $CD11b^+$, $CD11c^-$; $CD11b^+$, $CD11c^+$; or $CD11b^-$, $CD11c^+$ subsets..

Statistical analysis. The distribution of each dataset was assessed for normality and transformation of the data was attempted if the data were not normally distributed. If transformation did not normalize the distribution of the data or variance was unequal, non-parametric tests were performed. Data were analyzed using either unpaired t test, Mann-Whitney t test, or one way ANOVA with Tukey's Multiple Comparison post hoc test as indicated in the figure legends with the exception of cancer and dysplasia incidence, which was analyzed by

Fisher's exact test. Survival was assessed by Log Rank Test. All data are presented as mean ± SEM. Differences with a p-value of 0.05 or less were considered significant. All statistical analyses were performed using GraphPad Prism software (Version 5.04, GraphPad Software Inc., La Jolla, CA).

RESULTS:

Increased dietary vitamin D decreases the number of colonic lamina propria leukocytes following Helicobacter-infection. Vitamin D has been shown to directly and indirectly modulate many of the immune cell subsets important in the development and progression of inflammatory bowel disease including macrophages, dendritic cells, and T cells [88, 109, 112, 114-116]. We have previously shown that increased dietary vitamin D decreases the incidence of colitis-associated colon cancer in Helicobacter-infected *Smad3*$^{-/-}$ mice and that this protection is in part due to decreases in early stage inflammation characterized by decreased colonic inflammatory infiltrates and decreased pro-inflammatory cytokine expression in colonic tissues (**Chapter 2**, [45]). This observation could be explained if increased dietary vitamin D resulted in a universal dampening of colonic lamina propria immune cell subsets or decreased specific populations while increasing or maintaining others (such as regulatory immune cell subsets). To test this hypothesis, we sought to characterize the lamina propria cell populations from *H. bilis*-infected mice on either maintenance or high vitamin D diets early in disease (1 week post infection) using flow cytometric analysis. Animals fed increased dietary vitamin D had a significant decrease in LPL cellularity compared to the animals fed maintenance levels of vitamin D (**Fig 3.2**), which is consistent with our previous histologic and immunohistochemical findings outlined in **Chapter 2** [45]. Interestingly, while increased vitamin D resulted in a significant decrease in lamina propria cellularity (**Fig 3.2**) correlating with a decrease in colonic inflammation, there were no changes in the percentage of any cellular subsets evaluated, including T cells (**Fig. 3.3**), antigen presenting cells (**Fig. 3.4 B and D**), or granulocytes (**Fig. 3.4 A and C**). However, in concurrence with the overall decrease in LPL cell number, increased dietary vitamin D was associated with a significant decrease in the absolute cell numbers within each population (**Fig 3.3 and 3.4**).

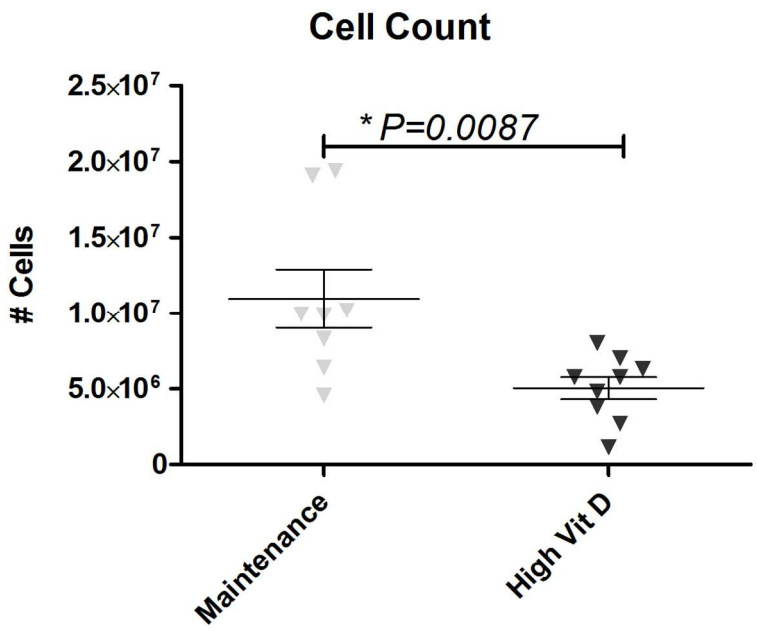
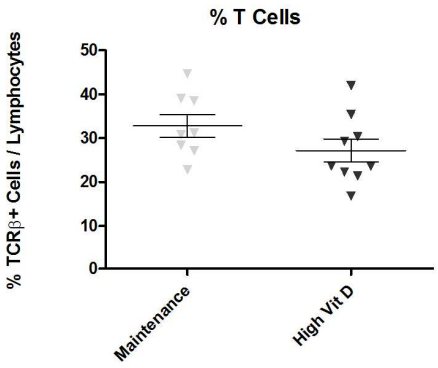
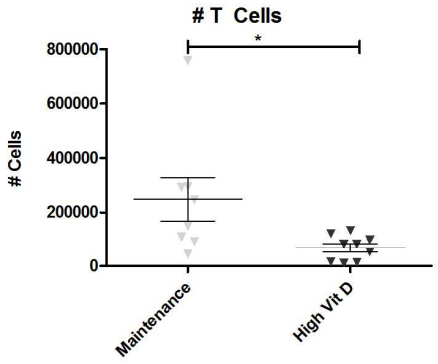


Figure 3.2. Lamina propria cellularity is significantly decreased in *H. bilis*-infected *Smad3*$^{-/-}$ mice fed increased dietary vitamin D.

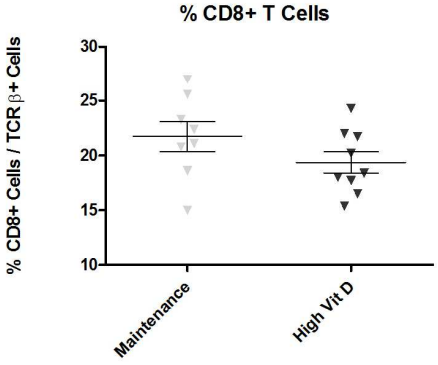
Cells were isolated from the proximal half of the colon 1 week following *H. bilis*-infection, stained with trypan blue and counted using a hemocytometer. Data are combined from two separate experiments with n=4-5 mice per group per experiment. * Unpaired T test.

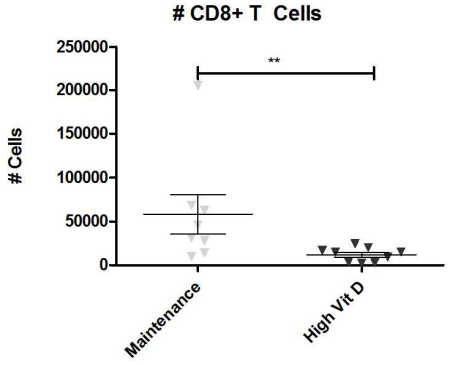
A


% T Cells
% TCRβ+ Cells / Lymphocytes
50
40
30
20
10
0
Maintenance
High Vit D

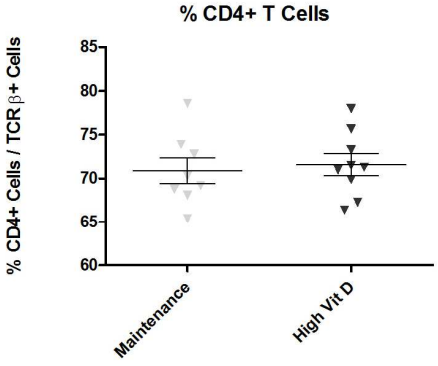

T Cells
*
Cells
800000
600000
400000
200000
0
Maintenance
High Vit D

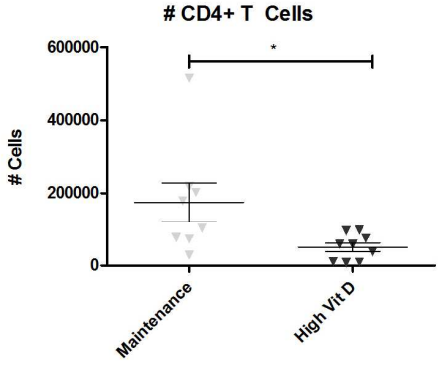
B


% CD8+ T Cells
% CD8+ Cells / TCRβ+ Cells
30
25
20
15
10
Maintenance
High Vit D

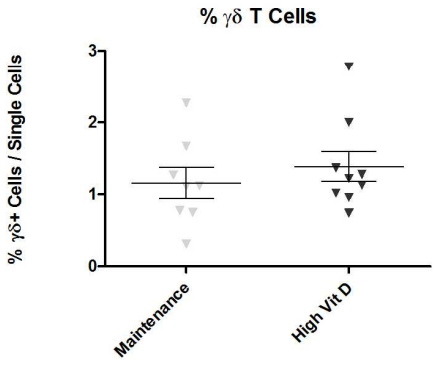

CD8+ T Cells
**
Cells
250000
200000
150000
100000
50000
0
Maintenance
High Vit D

C


% CD4+ T Cells
% CD4+ Cells / TCRβ+ Cells
85
80
75
70
65
60
Maintenance
High Vit D


CD4+ T Cells
*
Cells
600000
400000
200000
0
Maintenance
High Vit D

D


% γδ T Cells
% γδ+ Cells / Single Cells
3
2
1
0
Maintenance
High Vit D

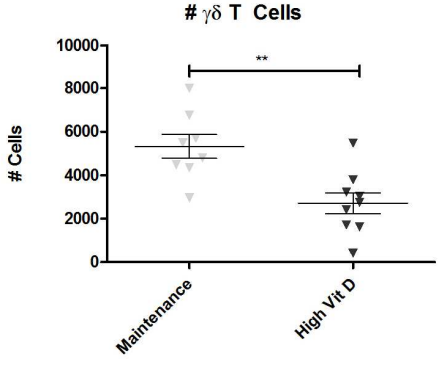

γδ T Cells
**
Cells
10000
8000
6000
4000
2000
0
Maintenance
High Vit D

Figure 3.3. Increased dietary vitamin D significantly decreases lamina propria T cell numbers.

Cells were gated as described in the materials and methods. Percentage of each cell population (left) and corresponding absolute cell numbers (right) are shown. Data are combined from two separate experiments with n=4-5 mice per group per experiment. Populations are indicated on the graph and graph axes. Unpaired T Test, *<0.05, **<0.01.

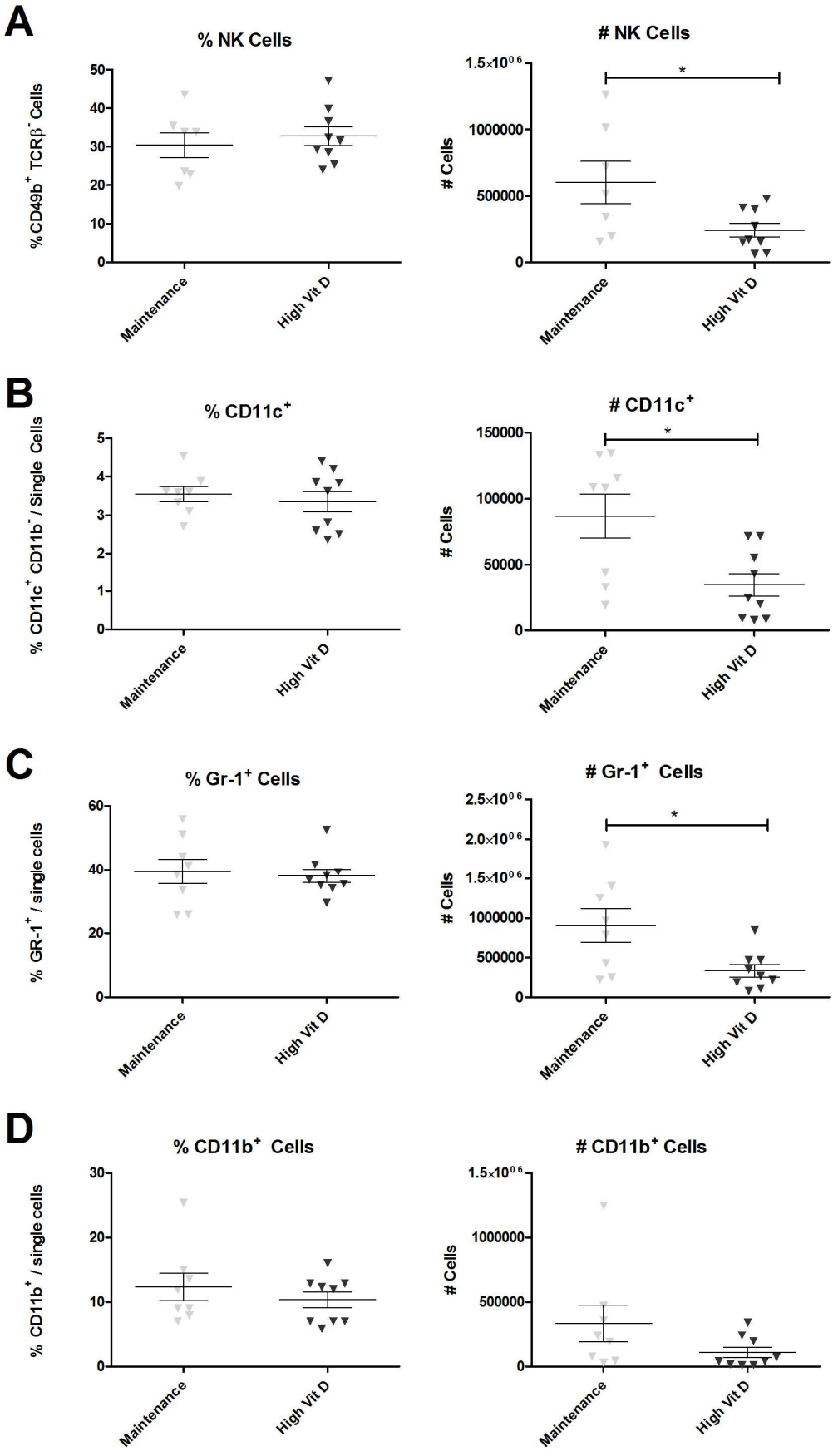

A
% NK Cells
% CD49b+ TCRβ- Cells
50
40
30
20
10
0
Maintenance
High Vit D
NK Cells
Cells
1.5x10^06
1000000
500000
0
*
Maintenance
High Vit D
B
% CD11c+
% CD11c+ CD11b- / Single Cells
5
4
3
2
1
0
Maintenance
High Vit D
CD11c+
Cells
150000
100000
50000
0
*
Maintenance
High Vit D
C
% Gr-1+ Cells
% GR-1+ / single cells
60
40
20
0
Maintenance
High Vit D
Gr-1+ Cells
Cells
2.5x10^06
2.0x10^06
1.5x10^06
1000000
500000
0
*
Maintenance
High Vit D
D
% CD11b+ Cells
% CD11b+ / single cells
30
20
10
0
Maintenance
High Vit D
CD11b+ Cells
Cells
1.5x10^06
1000000
500000
0
Maintenance
High Vit D

Figure 3.4. Increased dietary vitamin D decreases lamina propria APC and granulocyte cell numbers.

Cells were gated as described in the materials and methods. Percent cell population (left) and corresponding absolute cell numbers (right) are shown. Data are combined from two separate experiments with n=4-5 mice per group per experiment. A) $TCR\beta^{-}CD49b^{+}$ Cells, B) $CD11c^{+}CD11b^{-}$ Cells C) $Gr\text{-}1^{+}$ Cells D) $CD11b^{+}CD11c^{-}$ Cells. Unpaired T Test, *<0.05.

.

Vitamin D has been shown to both directly and indirectly modulate effector and regulatory T cell phenotypes [109, 112, 114-116] resulting in shifts among the Th1/Th2/Th17 and regulatory T cell populations. To determine if increased dietary vitamin D was associated with changes in T effector or regulatory cell subsets, lamina propria cells were isolated from *H. bilis*-infected *Smad3*$^{-/-}$ mice fed either maintenance or high vitamin D diet. T cell phenotype was assessed using flow cytometric analysis of intracellular cytokine expression of IFNγ (Th1), IL-4 (Th2), and IL-17 (Th17) along with co-staining for CD4. There was a general decrease in cell numbers within all T cell populations that were evaluated in mice fed a high vitamin D diet. However, there was no difference in the percentage of specific population of cells between the two dietary treatment groups (**Fig 3.5)**. These findings suggest that increased dietary vitamin D results in an overall decrease in all inflammatory cell subsets rather than shifting specific cell populations.

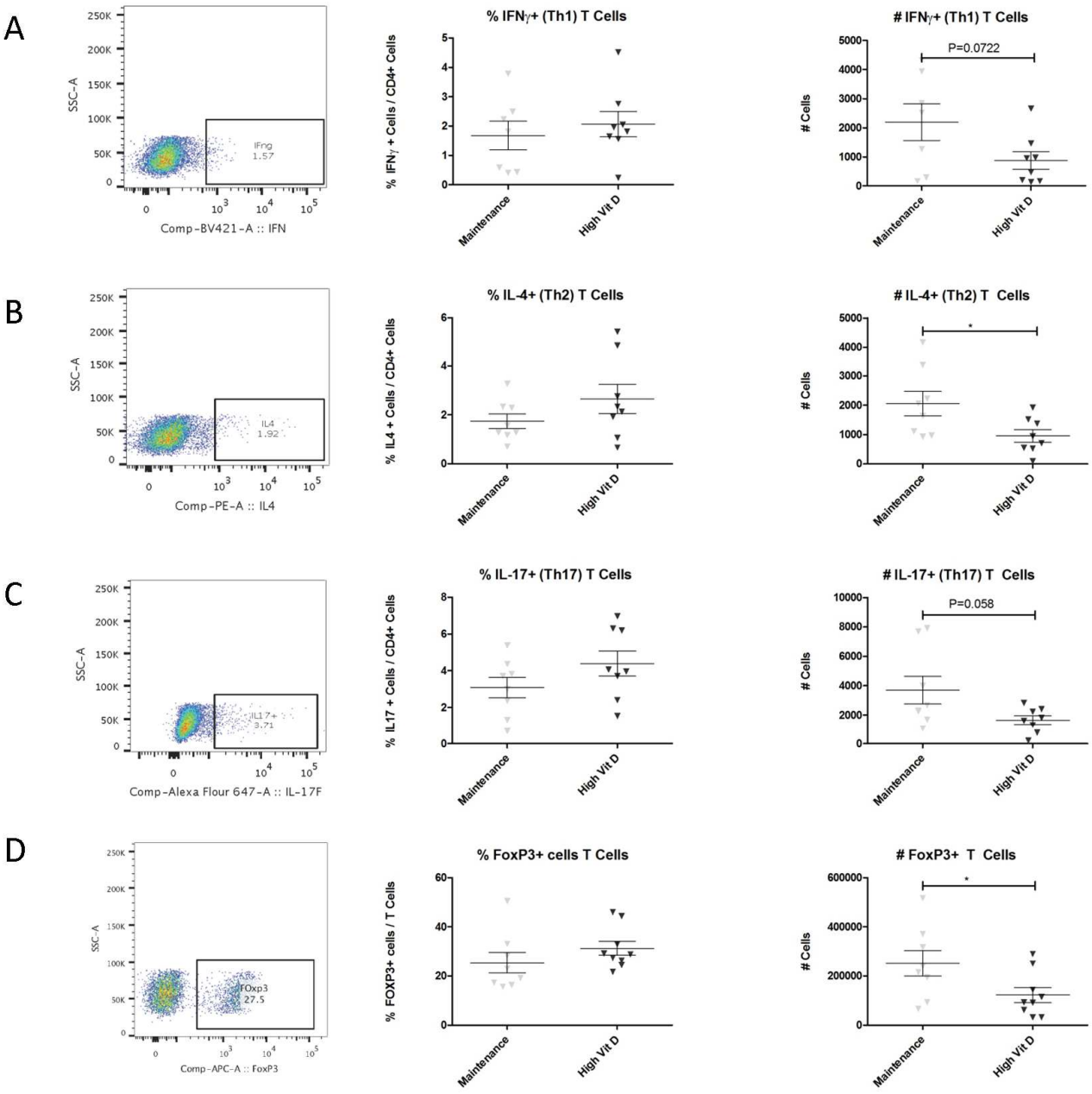


Figure 3.5. Increased dietary vitamin D significantly decreases the number of effector and regulatory lamina propria T cell subsets.

Cells were gated as described in the materials and methods. Representative gating (left), percent cell population (center) and the absolute cell number (right) are shown for each subset. Data are combined from two separate experiments with n=4-5 mice per group per experiment. A) IFNγ$^+$CD4$^+$ cells (Th1 cells), B) IL-4$^+$CD4$^+$ cells (Th2 cells) C) IL-17$^+$CD4$^+$ cells (Th17 cells) D) FoxP3$^+$CD4$^+$ cells (T regs). Unpaired T Test, *<0.05.

Vitamin D-mediated protection is dependent upon adaptive immune cells. Vitamin D has been shown to modulate both the innate (macrophages and dendritic cells) and adaptive immune cell populations [32-34], and both populations have been shown to be important in the development and progression of IBD [180]. In order to determine if the protective effects of increased dietary vitamin D were dependent upon the presence of adaptive immune cells, we tested the efficacy of increased dietary vitamin D on colon cancer development in *Smad3/Rag2* DKO mice that lack T and B cells. *Smad3/Rag2* DKO mice develop colitis and colon cancer following *H. bilis*-infection with a higher incidence than is seen in *Smad3*$^{-/-}$ mice [156]. *Smad3/Rag2* DKO mice were fed maintenance or increased dietary vitamin D for one week prior to *H. bilis* infection to trigger inflammation and cancer. Mice were followed for 16 weeks post infection and monitored closely for clinical evidence of severe IBD using signs such as weight loss, diarrhea, dehydration and lethargy. There was no difference in clinical disease presentation or weight change (data not shown) as well as overall survival associated between the treatment groups (**Fig 3.6 A**). At the end of the study period, cecum and colon tissues were evaluated histologically for inflammation, dysplasia, and invasive tumor incidence. We found no differences in tumor incidence in mice fed increased dietary vitamin D compared to maintenance diet (**Fig 3.6 B),** suggesting that the presence of adaptive immune cells is necessary for vitamin D-mediated protection.

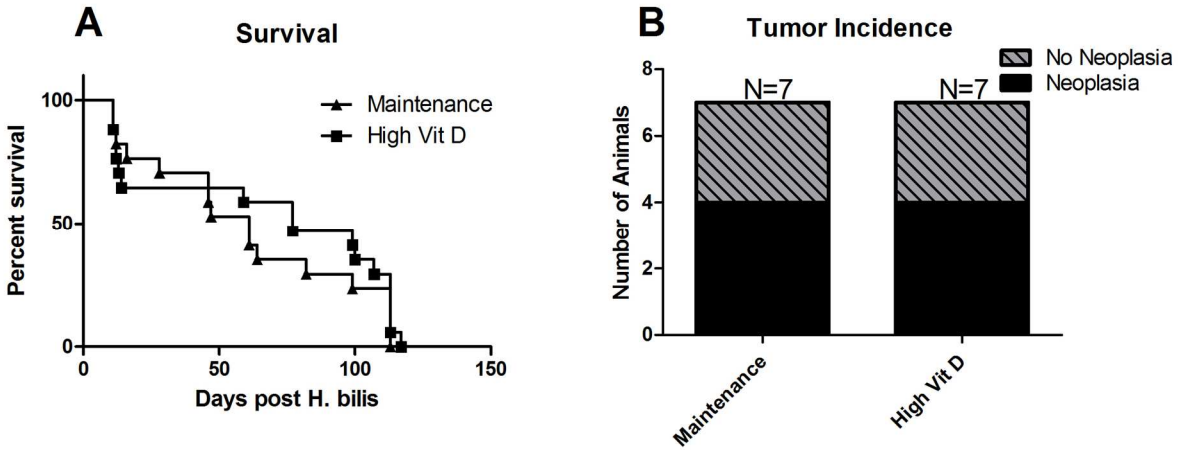


Figure 3.6. Increased dietary vitamin D does not protect against colitis-associated colon cancer in the absence of T and B cells.

Smad3/Rag2 DKO mice were fed maintenance or increased dietary vitamin D and infected with *H. bilis* to induce colitis and colon cancer. Mice were euthanized 16 weeks following *H. bilis*-infection or earlier if they showed evidence of severe disease. Data are compiled from two studies n=8-9 mice per group per study. A) Kaplan-Meier survival curves demonstrating that increased dietary vitamin D did not influence survival B) Animals that were euthanized >8 weeks post-infection were evaluated for evidence of colonic neoplasia. 20/34 animals (10 animals from each treatment group) developed severe disease prior to tumor development and were excluded in the tumor analysis.

Vitamin D-mediated protection is dependent upon the presence of $CD4^+$ cells. Our findings suggest that vitamin D may be mediating its protective effects through adaptive immune cells. Because vitamin D has been shown to have both direct and indirect effects on T cells *in vivo,* resulting in decreased T cell proliferation and secretion of pro-inflammatory cytokines including IFNγ and IL-17, and increased proliferation and function of regulatory T cell subsets [187], we sought to determine if the protective effects of increased dietary vitamin D are dependent upon the presence of $CD4^+$ effector T cells. Monoclonal antibodies were used to deplete $CD4^+$ cells in *Smad3*$^{-/-}$ mice fed the maintenance diet or high vitamin D diet. Animals were then infected with *H. bilis* and IBD was assessed histologically 1-week post infection during acute disease development. As we have previously described [45], increased dietary vitamin D significantly decreased colonic inflammation in control animals (IgG-treated) following infection with *H. bilis* (**Fig 3.7**). However, this protective effect was abrogated following antibody-mediated depletion of $CD4^+$ cells (**Fig 3.7**) suggesting that $CD4^+$ cells are necessary for vitamin D-mediated protection. Interestingly, $CD4^+$ cell depletion was associated with more severe clinical disease as demonstrated by increased diarrhea, dehydration and lethargy in the Maintenance/IgG group compared to either $CD4^+$ depleted group. In addition, $CD4^+$ cell depleted mice tend to show higher IBD scores compared to maintenance-fed/IgG treated mice although this did not reach statistical significance (P=0.06).

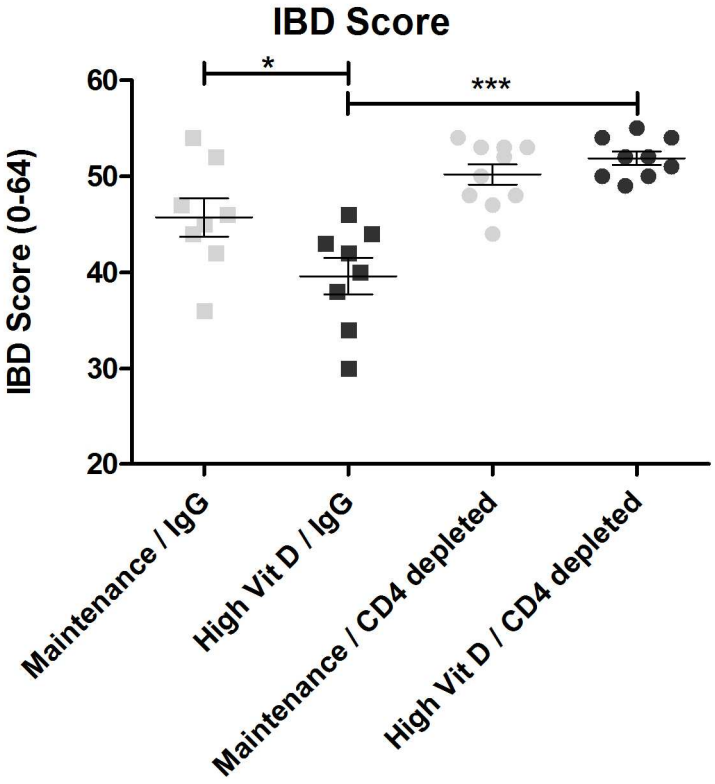


Figure 3.7. Vitamin D-mediated protection in *H. bilis*-infected *Smad3*$^{-/-}$ mice is dependent upon the presence of CD4^{+} T cells.

Monoclonal anti-CD4 antibody or rat IgG (control) was administered IP to selectively deplete CD4^{+} cells in *Smad3*$^{-/-}$ mice fed maintenance or high vitamin D diet. Animals were infected with *H. bilis.* Tissues were collected 1-week post infection during the acute inflammatory disease and evaluated histologically for evidence of IBD. One way ANOVA and Tukey's Multiple Comparison post hoc test. *P<0.05.***P<0.001

Increased dietary vitamin D does not alter *Helicobacter* colonization in *Smad3*$^{-/-}$ mice during the acute phase of IBD. We have previously shown that *H. bilis* persistently colonizes the gastrointestinal tract of *Smad3*$^{-/-}$ mice [153] and chronic colonization is not altered by increased dietary vitamin D (**Chapter 2,** [45]). In order to determine if increased dietary vitamin D altered *Helicobacter* colonization during the acute inflammatory phase of disease development, we quantified *Helicobacter* in cecal tissue of *Smad3*$^{-/-}$ mice fed maintenance or increased dietary vitamin D at 1-week post *H. bilis*-infection. Dietary vitamin D had no effect on

Helicobacter colonization at 1-week post infection (**Fig 3.8**), demonstrating that vitamin D is not altering disease by decreasing initial colonization of *H. bilis* organisms.

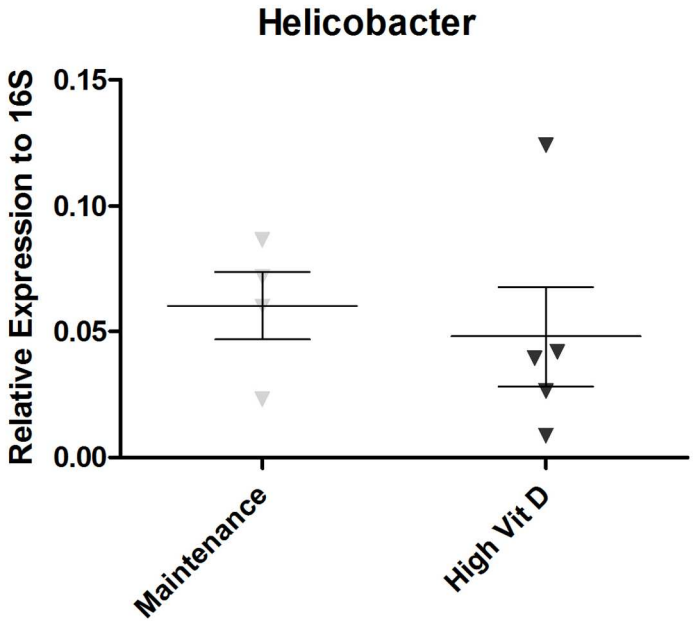


Figure 3.8. Increased dietary vitamin D does not alter *Helicobacter* colonization during acute disease development in *Smad3*$^{-/-}$ mice.

Cecal tissue was collected 1-week post *H. bilis*-infection (n=4-5 mice per diet) and analyzed by qRT-PCR. Samples were normalized to total 16S rRNA gene expression.

DISCUSSION:

The effects of vitamin D on the immune system are broad-reaching. Studies performed in *Vdr*$^{-/-}$ mice have demonstrated that though VDR is not necessary for the development of most immune cell subsets, vitamin D signaling plays a crucial role in regulating the inflammatory response [102, 107]. Loss of VDR-signaling leads to an over-exuberant proinflammatory phenotype by affecting both the innate and adaptive immune cells [101, 107]. *Vdr*$^{-/-}$ T cells overproduce IFNγ and IL-17 and induce more severe colitis when adoptively transferred into immunocompromised hosts compared to *Vdr* sufficient T cells [101, 107]. In addition, there is a

significant reduction in the number of tolerogenic dendritic cells present in *Vdr*$^{-/-}$ animals compared to controls [107]. Many immune cell subsets including T cells, macrophages and dendritic cells not only express VDR but also express vitamin D hydroxylase enzymes [23, 24], suggesting that these cells may be able to synthesize and respond to vitamin D locally affecting the inflammatory response at the tissue level. Both *in vitro* and *in vivo* studies have demonstrated that vitamin D treatment directly decreases proliferation of effector T cells subsets [109, 112]. In addition, vitamin D has been shown to affect antigen-presenting cells including macrophages and dendritic cells resulting in decreased secretion of proinflammatory cytokines and decreased dendritic cell-mediated activation and proliferation of T cells [88, 114-116]. Together, these results suggest that vitamin D signaling via VDR is playing a crucial role in the immunological control of inflammation affecting both innate and adaptive immunity.

Based on the diverse and complex actions of vitamin D, it is difficult to determine the mechanisms through which vitamin D mediates the protective effects observed in the *Smad3*$^{-/-}$ mouse model. Our flow cytometric phenotypic analysis of the colonic lamina propria cells in *H. bilis*-infected *Smad3*$^{-/-}$ mice suggests that vitamin D broadly suppresses colonic inflammation but does not correlate a specific cell population with disease-mediation as percentages of cell subsets remained similar between treatment groups while there was a decrease in total cell number of almost all subets examined associated with increased dietary vitamin D. Focusing on T cells, others have shown that treatment of T cells *in vitro* with $1,25(OH)_2D$ results in decreased production of Th1 cytokines (IFNγ and TNFα) and increased production of Th2 cytokines (IL4) [188, 189]. These findings have been validated *in vivo* using a mouse model of IBD in which colitis is induced using trinitrobenzene sulfonic acid (TNBS). Mice treated with $1,25(OH)_2D$ were protected against colitis and had significantly decreased expression of Th1-associated cytokines and a corresponding increase in Th2-associated cytokines [90]. In *Smad3*$^{-/-}$ mice, increased dietary vitamin D is associated with significantly decreased expression of Th1-associated cytokines within the colon and cecal tissues during early stage colitis, however, we did not appreciate a compensatory increase in Th2-associated cytokines [45]. Our flow cytometric analysis of LPLs demonstrated an overall decrease in both Th1 and Th2 cell populations. Although these data did not point to any particular cell subset being associated with disease protection, it is possible that a more detailed phenotyping analysis of certain immune subsets (such as APC's), analyzing activation markers and expression of cytokines and

chemokines, or perhaps an analysis of the role of colonic epithelium in the immune response would help identify a more specific cell type associated with disease prevention.

In another approach to identify cell types necessary for or associated with vitamin D-mediated protection against *H. bilis*-induced colitis and cancer, we selectively removed specific cell subsets during disease studies. We utilized *Smad3/Rag2* DKO mice, which are deficient in adaptive (T and B) immune cells and demonstrated that vitamin D-mediated protection was completely abrogated in the absence of these cells. Furthermore, depletion of $CD4^+$ T cells via anti-CD4 antibody treatment in *Smad3*$^{-/-}$ mice also rendered dietary vitamin D treatment ineffective early after infection with Helicobacter, suggesting these cells are necessary in vitamin D-mediated protection from colitis-associated cancer. Our findings are consistent with those of *Mayne et al.* who demonstrated that VDR expression specifically on $CD4^+$ cells is necessary for vitamin D-mediated protection against experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis [190]. Further studies are needed to determine if vitamin D is influencing regulatory T cells subsets, effector cells, or both.

Our immunohistochemical staining of colonic tissue as well as our lamina propria cellular characterization during acute colitis in the *Smad3*$^{-/-}$ mouse model show a significant decrease in T cell infiltrates in colonic tissue and a corresponding decrease in expression of proinflammatory cytokines within cecal and colonic tissues [45] associated with increased dietary vitamin D, suggesting that vitamin D is at least modulating effector T cells but this could be via direct or indirect mechanisms. Vitamin D has been shown by others to directly affect T cells and modify their response to activation. To date, more than 100 genes have been identified in T cells that are directly regulated by vitamin D [188]. One mechanism through which vitamin D has been shown to directly modulate T cell proliferation is via VDR-mediated inhibition of cytokines, including interleukin 2 (IL-2) [191, 192]. Increased availability of vitamin D interferes with the transcription factor NFAT and results in increased suppression of IL-2 and a resultant decrease in T cell proliferation. Vitamin D has been shown to suppress other crucial proinflammatory cytokines including GM-CSF, IL-17 and IFNγ through interfering with NFAT in a similar manner [192] [112]. Further studies will be needed to determine what mechanisms are at play in vitamin D-mediated protection in *Smad3*$^{-/-}$ mice.

Our data contribute to existing the data showing the potential effects of vitamin D on T cells and demonstrate a critical role for $CD4^+$ cells in vitamin D-mediated protection against *H.*

bilis-induced colitis and colon cancer in *Smad3*$^{-/-}$ mice. Though further investigation is needed to determine if vitamin D is acting directly on the CD4^{+} T cells to induce these effects or indirectly through other cell populations such as dendritic cells, our results enhance the current literature by demonstrating that a specific cell population is necessary in vitamin D-mediated protection in an inflammation-associated colon cancer model.

Chapter 4. EFFECTS OF DIETARY VITAMIN D MODULATION IN DSS-TREATED *SMAD3*$^{-/-}$ MICE

INTRODUCTION:

Vitamin D deficiency is increasingly prevalent around the world, both in industrialized as well as third world countries. Epidemiologic studies suggest that as many as 30-50% of children and adults are at risk of vitamin D deficiency worldwide [193]. Humans obtain a majority of their vitamin D from exposure to sunlight through UVB radiation; however, diet becomes an important means of maintaining adequate serum vitamin D levels in individuals who have limited exposure to sunlight. There are many factors that may contribute to widespread vitamin D deficiency including inadequate intake of foods rich in vitamin D, insufficient exposure to natural sunlight, residing at higher latitudes where the UVB exposure is limited, or increased protection against sun exposure through clothing and/or sunscreen [2, 52].

Prolonged vitamin D deficiency historically has been associated with delayed growth and rickets in children [1] as well as osteoporosis and osteopenia in adults [5] due to decreased intestinal calcium absorption [1-3]. Recently, it has become apparent that vitamin D deficiency is associated with a wide range of diseases including: asthma [6], multiple sclerosis [7], rheumatoid arthritis [8], type 1 diabetes [9, 10], heart disease [11, 12], inflammatory bowel disease [48, 184], depression [13, 14], and tuberculosis [15]. Epidemiologic studies also suggest that vitamin D deficiency increases the risk of developing and dying from a number of different cancers including colorectal, breast, and prostate cancers [29, 194].

Animal models are an important and necessary tool for studying the effects of vitamin D deficiency on disease progression and outcome. It has been shown that mice lacking vitamin D receptor (VDR) or Cyp27a1, an important enzyme in the vitamin D metabolism pathway, develop more severe IBD compared to wild type controls [42, 88, 101]. Interestingly, vitamin D supplementation ameliorates symptoms of colitis in vitamin D deficient *IL-10*$^{-/-}$ animals [42].

In addition to influencing colitis, vitamin D deficiency has been associated with an increased incidence in and mortality from colon cancer in human patients [16, 29, 82]. The risk of colon cancer is significantly higher in patients diagnosed with IBD (Crohn's disease or ulcerative colitis) compared to the general population, supporting the notion that colonic

inflammation impacts cancer development [31]. The pathogenesis of inflammation-associated cancer is distinct from that of sporadic or familial colon cancers and therefore may influence the efficacy of vitamin D supplementation as well as mechanisms through which it influences disease progression and tumor formation. Currently, the effects of vitamin D deficiency specifically on inflammation-associated colon cancer are not known.

Our laboratory has evaluated the effects of dietary vitamin D on colitis and tumor formation in the *Smad3*$^{-/-}$ (*Smad3*^{tm1Par}/J) mouse model of inflammation-associated colon cancer. *Smad3*$^{-/-}$ mice [195] lack the transcription factor SMAD3 resulting in defective TGFβ signaling and develop colitis and subsequent colon adenocarcinomas when infected with Helicobacter [153] or treated with dextran sodium sulfate (DSS) [196].We hypothesized that elevated dietary vitamin D would protect and deficient dietary vitamin D would exacerbate colitis-associated cancer in *Smad3*$^{-/-}$ mice. The experiments described here in DSS-induced colitis induced cancer in *Smad3*$^{-/-}$ mice demonstrated that increased dietary vitamin D resulted in decreased inflammation, decreased colonic dysplasia, and improved survival following DSS-induced disease, albeit these improvements were relatively smaller in magnitude compared to similar experiments in *Smad3*$^{-/-}$ mice when disease was induced via Helicobacter infection [45]. Surprisingly, we also found that vitamin D-deficient mice were markedly protected against DSS-induced colon tumors. Our data suggest that the protective effects of vitamin D deficiency are mediated through changes in cellular proliferation and healing following epithelial cell damage induced by DSS treatment.

METHODS:

Mice and diets. *Smad3*$^{+/-}$ (*Smad3*^{tm1Par}/J) were obtained from The Jackson Laboratory (Bar Harbor ME) and bred at the University of Washington to generate *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ (wild type) mice that were subsequently maintained as separate lines via homozygote breeding [153]. All mice were housed in a specific pathogen-free colony free of Helicobacter and Murine Norovirus and were screened for pathogens as previously described [41]. The mice were group-housed in ventilated cages. Animals were provided with autoclaved acidified bottled water and were fed a purified rodent diet with 5IU (5AAA, AIN93H, high vitamin D), 1 IU (5SRH, AIN93M, maintenance), 0.2 IU (5ACE, AIN93L, low vitamin D) or <0.2 IU vitamin D_3 per

gram diet (5AV4, AIN93Null). All diets were formulated by Test Diet (St. Louis, MO). Vitamin D levels in the diet were confirmed by Liquid Chromatography-Mass Spectrometry (LCMS) (Covance Inc. Madison, WI). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee. The outlined experiments used 3-14 week old male and female mice. Care was taken to evenly distribute animals among treatment groups based on age and gender.

Induction of colitis. Animals were fed experimental diets as described in the section "Mice and diets" for at least one week prior to induction of colitis. Colitis was triggered using dextran sodium sulfate (DSS) (36,000-50,000 MW, MP Biomedical, Solon, OH), which was prepared as a stock solution (40%) in autoclaved distilled water and then diluted further to 1.5% solution in autoclaved, acidified water and placed in autoclaved water bottles. Animals were placed on 1.5% DSS in the drinking water for 3 days then returned to regular autoclaved acidified bottled water. For studies evaluating effects of increased dietary vitamin D on IBD and colon cancer, animals were exposed to a second round of DSS-treatment (3 days of 1.5% DSS 14 days after the first treatment). A single round of 1.5% DSS for 3 days was used for studies to determine the effects of vitamin D deficiency on IBD and cancer because of the concern that vitamin D deficiency may exacerbate colitis [85, 94]. Animals were weighed weekly, and health checks were performed at least three times weekly as described in Chapter 2. Mice were euthanized by CO_2 asphyxiation at designated endpoints or when they developed signs of severe disease including 30% body weight loss, ulcerated rectal prolapse, diarrhea, hunched posture, and dehydration.

Serum vitamin D, calcium and tissue collection. For repeated blood sampling, submandibular puncture was performed no more than every 2 weeks to collect a maximum total volume of 200µl per collection. At the termination of the each study, blood was obtained via cardiac puncture immediately following euthanasia via CO_2 asphyxiation. Serum was stored at -80°C until samples were submitted to Heartland Assays (Ames, IA) for quantification of 25-hydroxyvitamin D_3 (radioimmunoassay), calcium, and phosphorus levels. Mesenteric lymph nodes, cecum, colon and rectum were fixed in 10% phosphate buffered formalin and processed for routine histologic examination. For 9 and 16 day acute time points and studies evaluating the

effects of increased dietary vitamin D, the colon was opened longitudinally, feces were removed by gently flushing with PBS, the colon was cut into thirds (proximal, mid and distal) so as to fit into tissue cassettes, and fixed flat in 10% neutral buffered formalin. After fixation, the sections of colon were further sectioned longitudinally into strips and were embedded on edge to get longitudinal sections along the length of the colon. For long term studies evaluating vitamin D deficiency, the colon was prepared in a "Swiss roll" technique [197]. Hind limbs were fixed in 10% phosphate buffered formalin, decalcified and processed for routine histologic examination**.** All tissues were stained with hematoxylin and eosin for evaluation.

Radiography and bone mineral density. Bone density was assessed by radiography and bone mineral density scanning in *Smad3*$^{-/-}$ and wild type mice after 18 weeks of being fed either maintenance or AIN93Null diet. Following euthanasia and abdominal tissue harvest, animals were radiographed using either Model MX-20 laboratory radiography system (Faxitron X-Ray Corp., Wheeling, IL) or Canon CXDI-60G digital radiography system (Cannon Medical Systems, Melville, NY), and bone mineral density (BMD) was measured using a PIXImus Lunar densitometer (GE Healthcare, Waukesha, WI).

Pathology. Cecum and colon were evaluated for inflammation, dysplasia, and neoplasia by a pathologist blinded to treatment groups. IBD scores were assigned by scoring tissue on severity of mucosal loss, mucosal epithelial changes, degree of inflammation and extent of pathology as previously described [196]. Dysplasia was classified according to Boivin and colleagues [159, 196] and only frankly invasive adenocarcinoma was scored as neoplasia [156, 196]. Cortical and medullary regions of the femur and tibia were evaluated by a board certified veterinary pathologist blinded to experimental groups for evidence of bone remodeling and/or osteomalacia.

Bromodeoxyuridine staining. In order to assess cellular proliferation following DSS administration, mice were injected with bromodeoxyuridine (BrdU) labeling reagent (0.2ml per animal, Invitrogen, Eugene Oregon) intraperitoneally twenty-four hours prior to euthanasia. Following euthanasia, tissues were collected and processed as described above. Paraffin embedded tissues were submitted to the Histology and Imaging Core at the University of

Washington (Seattle, Washington) for immunohistochemical staining of BrdU. Colonic epithelial cell proliferation was assessed by dividing the colon roughly into thirds (proximal, middle, and distal) and counting BrdU positive and negative epithelial cells in 8-10 crypts in full plane of section within each segment of colon (i.e. proximal, middle, and distal).

Statistical analysis. Prior to statistical analysis, distribution of data was assessed for normality. If data were not normally distributed, transformation was attempted; if transformation did not normalize the distribution, non-parametric tests were performed. Serum vitamin D, serum calcium, histologic scoring, and bone mineral densities were analyzed using either unpaired or Mann-Whitney t test. Cancer and dysplasia incidence was analyzed by Fisher's exact test. Survival was assessed by Log Rank Test. All data are presented as mean ± SEM. Differences with a P value of 0.05 or less were considered significant. All statistical analyses were performed using GraphPad Prism software (Version 5.04, GraphPad Software Inc, La Jolla, CA).

RESULTS:

Increased dietary vitamin D protects against dysplasia and improves survival in DSS-treated *Smad3*$^{-/-}$ mice. Colitis and colon cancer can be triggered in *Smad3*$^{-/-}$ mice using either a bacterial driver such as *H. bilis,* as was described in **Chapters 2 and 3** or using a chemical agent such as dextran sodium sulfate (DSS) [196]. In order to determine if elevated vitamin D would prevent or reduce DSS-induced colitis and cancer similar to what was seen in *H. bilis*-induced disease (**Chapter 2**), *Smad3*$^{-/-}$ mice were fed either maintenance or high vitamin D diet. Colitis and colon cancer was induced by administering DSS via the drinking water Mice were euthanized at 16 weeks post DSS treatment unless they met early euthanasia criteria. Survival, IBD scores, summed total dysplasia and incidence of invasive carcinoma (**Fig 4.1**) were evaluated and compared between animals fed maintenance or high vitamin D diet.

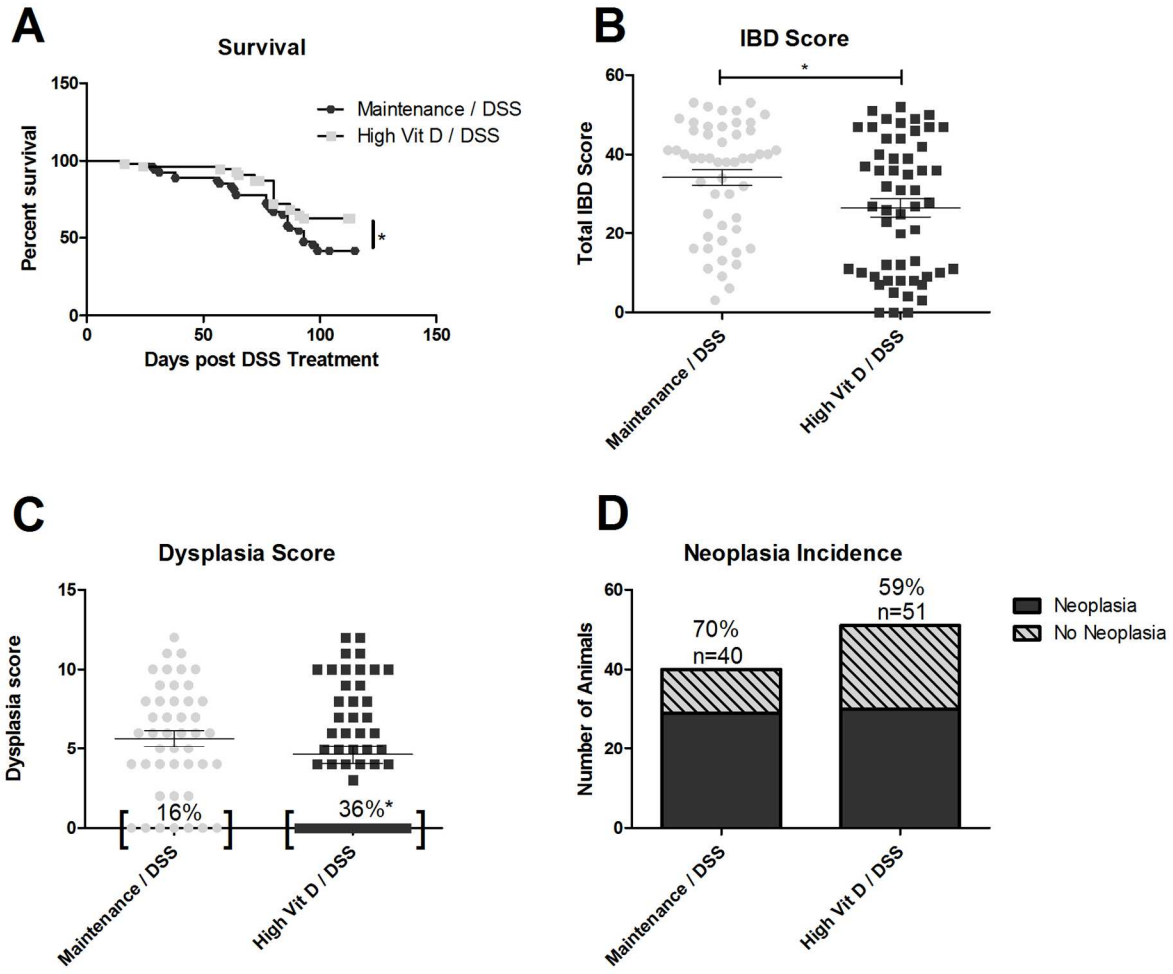


Figure 4.1. Increased dietary vitamin D improves survival and decreases dysplasia incidence in DSS-treated *Smad3*$^{-/-}$ mice.

Smad3$^{-/-}$ mice were fed high vitamin D or maintenance diet and treated with DSS (N=55-57 mice per group). Survival (**A**) was compared by Log Rank Test. Colonic IBD (**B**) and dysplasia (**C**) were assessed histologically at the study end point. (**D**) Animals surviving greater than 8 weeks were assessed histologically for evidence of colonic neoplasia IBD score and dysplasia scores were compared by Mann-Whitney t test and dysplasia incidence and neoplasia incidence was compared by Fisher's exact test. *p<0.05

As hypothesized, mice fed increased dietary vitamin D were protected against DSS-induced disease compared to maintenance diet fed mice. Mice fed increased dietary vitamin D had increased survival compared to mice on maintenance diet (P=0.0255, **Fig 4.1A**). Severity of IBD was also significantly reduced in mice given high vitamin D diet versus maintenance diet (mean IBD score =26.6 vs.34.2, **Fig 4.1B**), though the magnitude of the effect in these studies were less impressive as those demonstrated in *H. bilis*-infected mice (**Chapter 2**). While there was no significant difference in the severity of dysplasia between the two treatment groups (mean score = 4.6 vs. 5.6, **Fig 4.1C**), interestingly, the overall incidence of dysplasia was significantly decreased in mice fed high vitamin D diet with 36% of animals showing no evidence of dysplasia compared to only 16% of animals fed maintenance diet demonstrating no evidence of dysplasia (**Fig 4.1C**). In addition, mice fed high vitamin D diet showed decreased incidence of invasive neoplasia compared to mice fed the maintenance diet (59% vs. 70%), though this decrease was not statistically significant (**Fig 4.1D**). Together, these data indicate that elevated dietary vitamin D is only mildly protective in this relatively severe model of DSS-induced IBD and cancer in *Smad3*$^{-/-}$ mice as compared to the robust protection against colitis and colitis associated colon cancer observed in *H. bilis*-infected *Smad3*$^{-/-}$ mice (**Chapter 2** [45]). We believe that this may in part be due to differences in the mechanisms through which colitis and colon cancer are triggered in the two models, though further studies are needed to validate these hypotheses.

Vitamin D-null diet decreases serum 25-hydroxyvitamin D_3 without altering serum calcium levels in *Smad3*$^{-/-}$ and wild type mice. In order to determine effects of vitamin D deficiency on colon cancer, we first determined the level of dietary vitamin D required to cause vitamin D deficiency without inducing clinical morbidity that would limit the ability to perform long-term studies using this model. Since chronic vitamin D deficiency can result in calcium imbalances and bone weakness [1, 2, 198, 199], *Smad3*$^{-/-}$ mice were first fed a diet low in vitamin D, to produce a measurable decrease in serum vitamin D status without causing other significant health problems relating to vitamin D deficiency. Serum 25-hydroxyvitamin D and serum calcium were measured in mice weaned onto either AIN93L (vitamin D low) or maintenance diet six weeks post diet initiation. No changes were noted in either serum vitamin D

or serum calcium levels in association with decreased vitamin D levels in the AIN93L diet (**Fig 4.2A and B**).

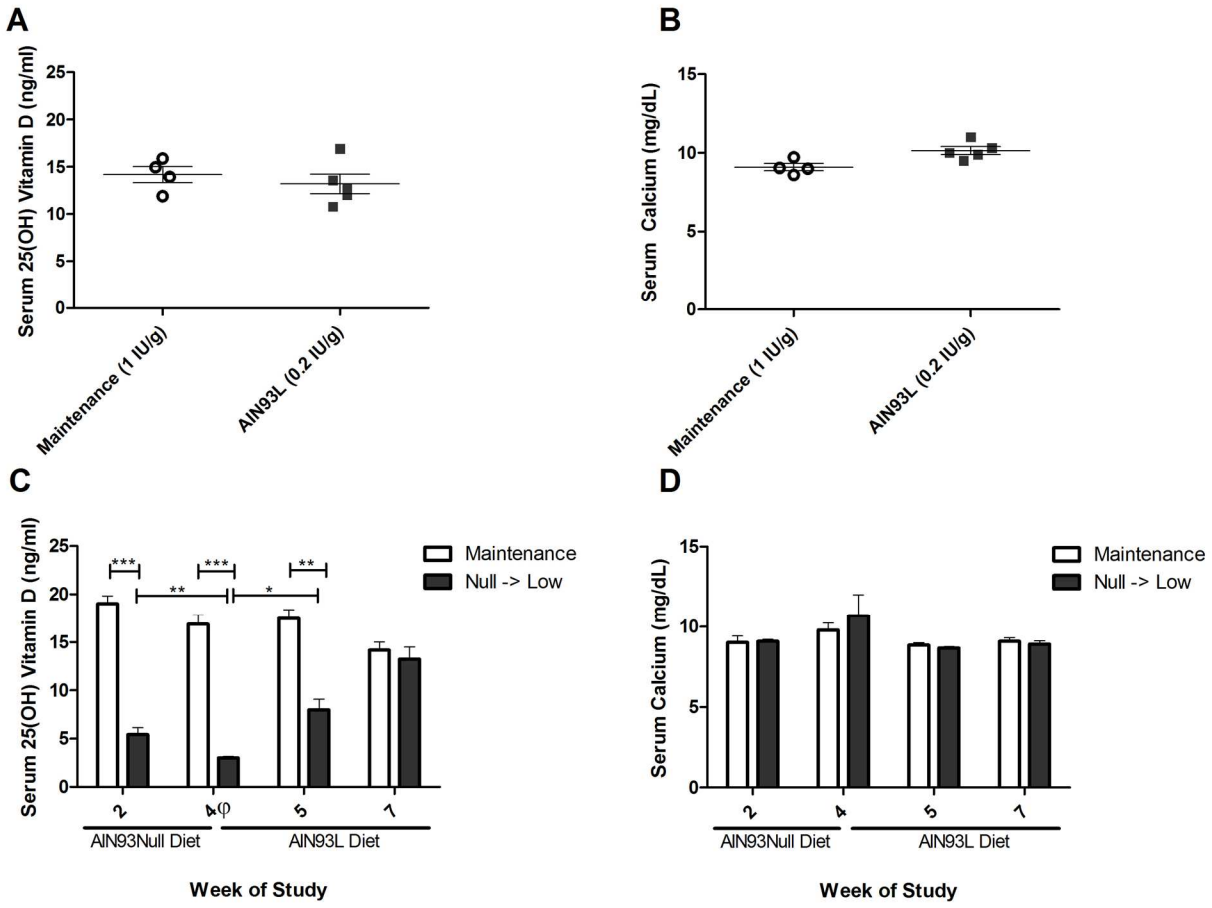


Figure 4.2. AIN93L diet does not alter serum vitamin D or serum calcium levels in *Smad3*$^{-/-}$ mice.

Serum 25-OH D (**A**) and serum calcium (**B**) was measured after animals were fed maintenance (n=4) or AIN93L (n=5) diet for 6 weeks following weaning. In a subsequent study, serum 25-OH D (**C**) and serum calcium (**D**) was measured at weeks 2, 4, 5 and 7 from study initiation in mice that received AIN93Null for 4 weeks and then AIN93L versus those on maintenance diet for the duration of the study. ϕ indicates the point where mice on AIN93Null were switched to AIN93L (vitamin D low) diet. **p*<0.05, ***p*<0.001, ***p<0.0001, student's t test. Error bars represent standard error of the mean.

Since AIN93L, containing 80% less vitamin D than provided in maintenance diet, did not result in decreased circulating serum vitamin D levels; *Smad3*$^{-/-}$ mice were fed a diet devoid of vitamin D (AIN93Null). After two weeks on the diet, animals fed AIN93Null had significantly decreased serum 25-hydroxyvitamin D compared to mice fed maintenance diet (25-hydroxyvitamin D mean: 6.0 vs. 19.0 ng/ml, $p<0.0001$) (**Fig 4.2C**). Serum vitamin D levels continued to decrease from week 2 to week 4 on this diet, demonstrating that a vitamin D-null diet is required to significantly decrease serum vitamin D levels in *Smad3*$^{-/-}$ mice (**Fig 4.2C**). Since it was necessary to induce vitamin D deficiency without causing complications due to hypocalcemia for an extended period of time we next tested a dietary regimen that include 4 weeks of AIN93Null diet followed by a switch to AIN93L diet. We reasoned that the rapid decrease in serum vitamin D levels by the AIN93Null diet would be maintained by feeding AIN93L in animals that had been depleted of vitamin D stores, and could potentially help to avoid severe vitamin D deficiency that might occur if AIN93Null diet was continuously administered long term. Surprisingly, serum 25-hydroxyvitamin D levels of mice fed AIN93L diet rebounded and reached similar levels to those of mice fed a control diet within 3 weeks of switching to AIN93L (**Fig 4.2C**). Serum calcium values remained unchanged on AIN93Null or AIN93L diet (**Fig 4.2D**). These data demonstrate that a 0.2 IU vitamin D/g diet is sufficient to maintain similar serum vitamin D levels as a diet containing 1 IU vitamin D/g and does not induce "mild" vitamin D deficiency in this mouse model.

***Smad3*$^{-/-}$ mice maintained on a vitamin D-null diet for 18 weeks had no evidence of clinical vitamin D deficiency.** Because decreased levels of dietary vitamin D did not cause "mild" vitamin D deficiency based on serum vitamin D, we next tested whether AIN93Null diet can be used for a long-term study without causing severe vitamin D deficiency in mice. *Smad3*$^{-/-}$ mice were fed AIN93Null for 18 weeks and used to determine whether significant vitamin D deficiency-related issues developed. Most published studies utilizing vitamin D-deficient diets in mice are either short term [94, 199] or multigenerational studies with vitamin D deficient diets intended to produce animals with severe calcium imbalances [199]. 3-week- (weanling) and 6-week-old (adult) *Smad3*$^{-/-}$ mice were fed AIN93Null diet for 18 weeks. We hypothesized that weanling and young adult animals, still experiencing rapid phases of growth, would be most impacted by low dietary vitamin D and thus, would likely show more severe vitamin D

deficiency symptoms when fed a vitamin D-null diet. Blood samples were collected at 2 - 3 week intervals to measure serum 25-hydroxyvitamin D and serum calcium. Serum 25-hydroxyvitamin D levels in AIN93Null-fed mice were significantly decreased compared to mice fed maintenance diet after two weeks on the diet and dropped below the limit of detection after 9 weeks on the diet (**Fig 4.3A**). However, AIN93Null diet did not cause changes in serum calcium (**Fig 4.3B**), serum phosphorus (data not shown) and body weight (**Fig 4.3C**) compared to maintenance diet-fed controls throughout the 18-week experimental period.

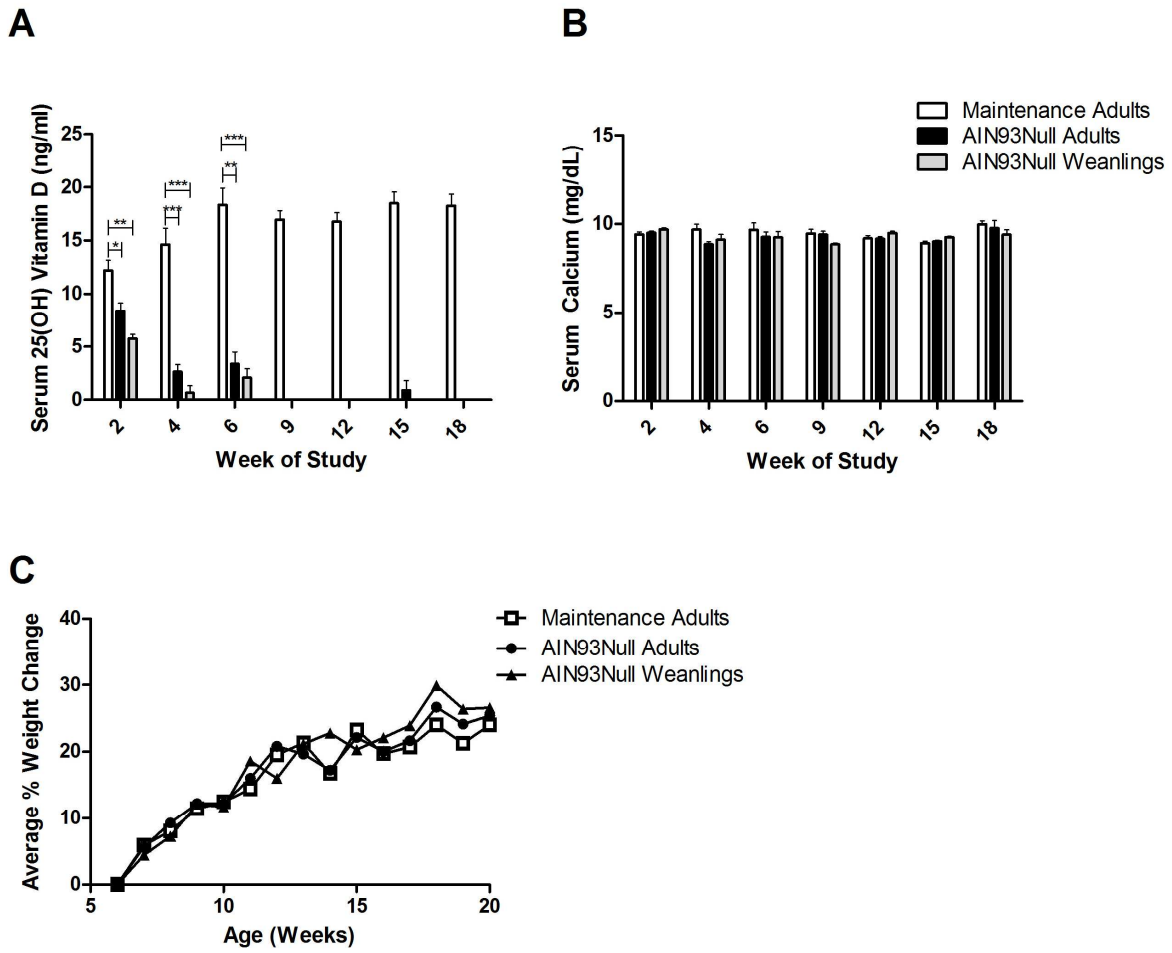


Figure 4.3. AIN93Null diet significantly decreases serum vitamin D levels without altering serum calcium or weight gain.

Serum 25-OH D (**A**) and serum calcium (**B**) from mice fed maintenance (n=5, 6 wk old mice) or AIN93Null (n=5, 3wk old mice (weanling) and n=5, 6 wk old mice (adult)) diets. Average percent weight change (**C**) for each group at each time point. **p*<0.05, ***p*<0.001, ***p<0.0001, student's t test. Error bars represent standard error of the mean.

***Smad3*$^{-/-}$ mice maintained on an AIN93Null diet for 18 weeks had no evidence of bone changes.** Because vitamin D deficiency can significantly increase bone resorption, animals were evaluated for evidence of bone disease through radiography, bone mineral density scanning, and histology. After 18 weeks on AIN93Null diet, *Smad3*$^{-/-}$ mice showed no evidence of cortical bone loss or bone remodeling based on radiography (**Fig 4.4A**), bone mineral density (**Fig 4.4B**), or histology (data not shown). These data are in accordance with the finding that serum calcium levels do not change in *Smad3*$^{-/-}$ fed AIN93Null diet compared to those fed maintenance diet, indicating that calcium homeostasis can be maintained with AIN93Null diet for a prolonged period (18 weeks).

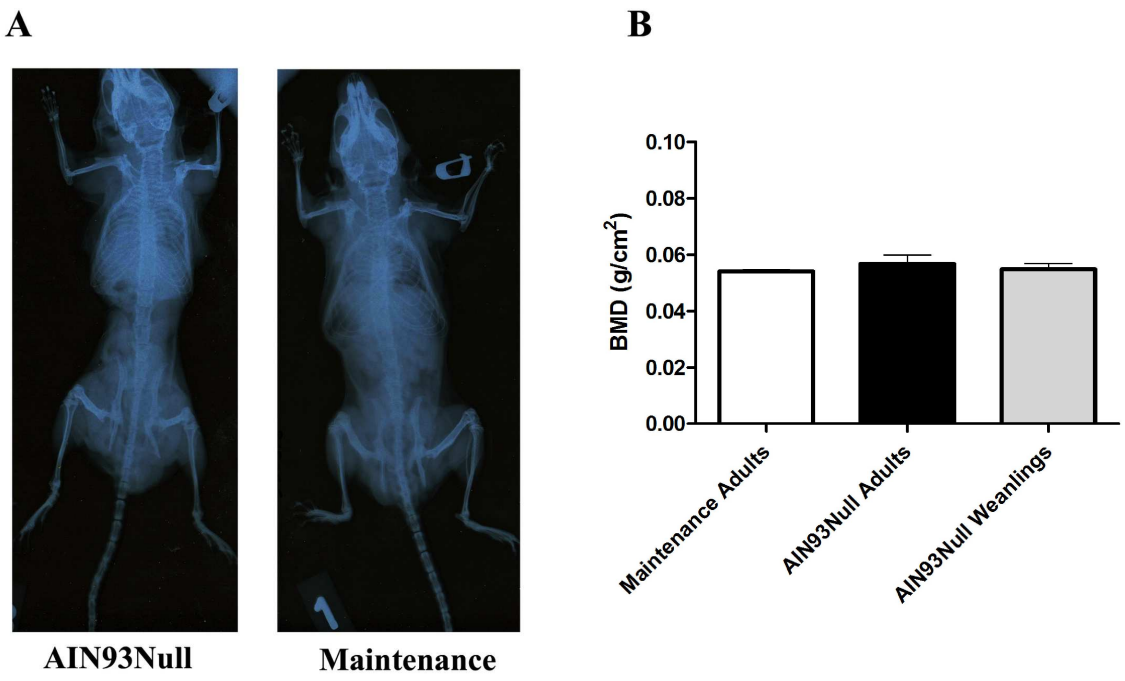


Figure 4.4. AIN93Null diet does not alter bone density in *Smad3*$^{-/-}$ mice.

Mice fed maintenance (n=5, 6wk old mice) or AIN93Null diet (n=5, 3wk old mice (weanling) and n=5, 6wk old mice (adult)) were euthanized 18 weeks after diet initiation and radiographed. Images (**A**) depicted are representative of animals on each diet. Total body bone mineral density scanning (**B**) was performed following euthanasia. Error bars represent standard error of the mean.

Wild type mice maintained on a vitamin D-null diet for 18 weeks have no evidence of clinical vitamin D deficiency or bone changes. SMAD3 is a transcription factor involved in TGFβ and activin signaling, and both pathways are involved in regulation of bone formation [200]. Mice lacking SMAD3 have been shown to have decreased bone formation and increased osteopenia compared to wild type controls despite having normal serum vitamin D and calcium levels [201]. SMAD3 interacts with PTH to induce bone formation in osteoblasts [202, 203]. Thus, it is possible that the lack of bone changes observed in response to AIN93Null diet is a phenomenon specific to the *Smad3*$^{-/-}$ mouse. In order to determine if the results obtained from feeding mice AIN93Null diet were specific to the *Smad3*$^{-/-}$ mouse or could be applied to a broader population of mice, 6-week-old wild type mice were fed AIN93Null diet for 18 weeks. Six weeks post diet initiation, serum 25-hydroxyvitamin D levels in AIN93Null fed mice were significantly decreased compared to mice fed maintenance diet (**Fig 4.5A**) similar to what had been observed in *Smad3*$^{-/-}$ mice. Serum 25-hydroxyvitamin D levels of wild type mice were below the limit of detection after 18 weeks on AIN93Null diet (**Fig 4.5A**). Wild type mice showed no differences in serum calcium (**Fig 4.5B**) or body weight (**Fig 4.5C**) compared to maintenance diet-fed controls throughout the experiment.

Because serum calcium levels can be maintained through bone resorption, animals were assessed for bone remodeling and osteomalacia through bone mineral density scanning and radiography. Again, similar to what was observed in *Smad3*$^{-/-}$ mice, there was no evidence of cortical bone loss or bone remodeling based on bone mineral density (**Fig 4.5D**) or radiography (**Fig 4.5E**). These data suggest that AIN93Null diet induces mild vitamin D deficiency in both *Smad3*$^{-/-}$ and wild type mice and can be used to study effects of chronic vitamin D deficiency in these mice without inducing bone disease or calcium imbalances for up to 18 weeks.

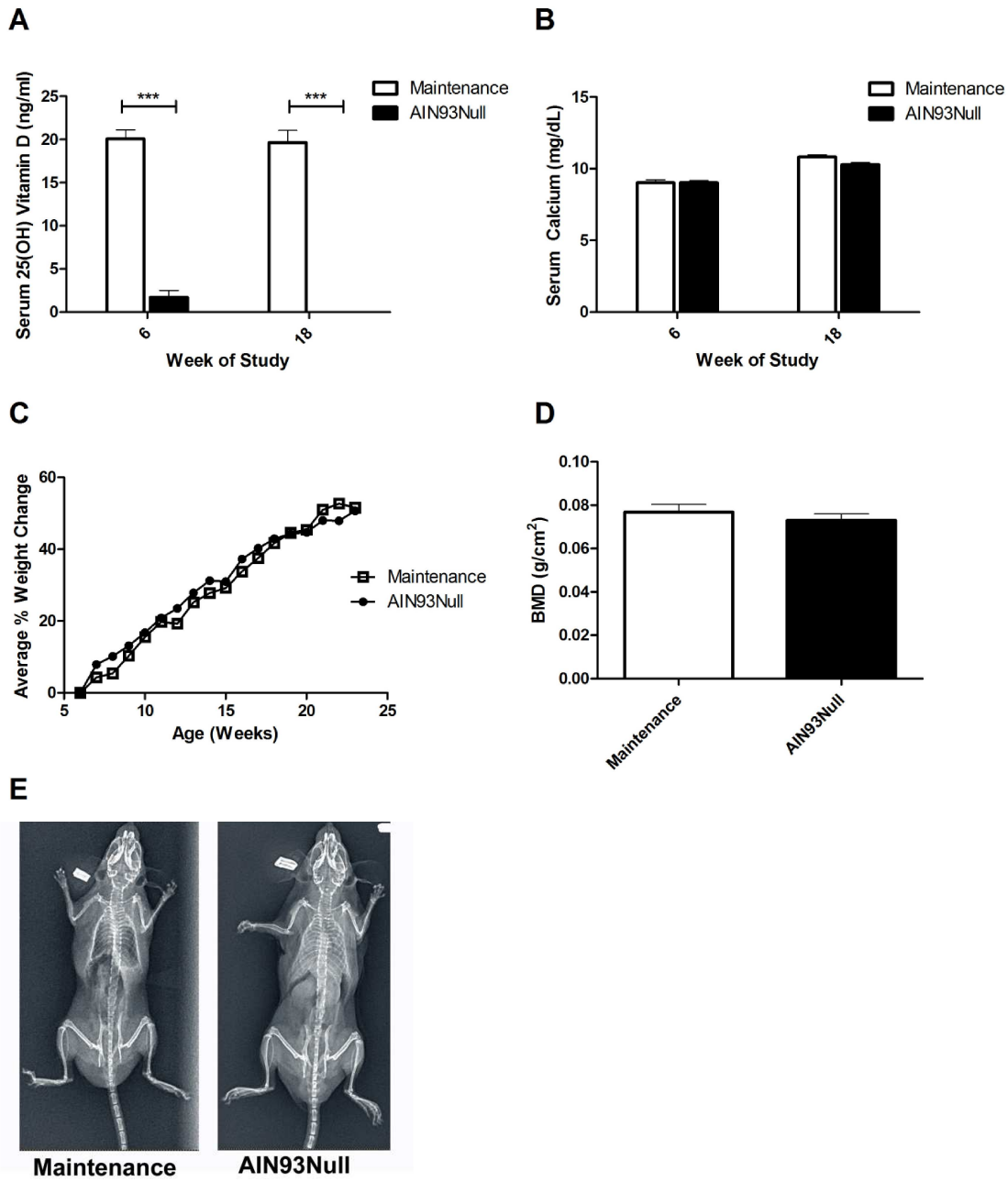


Figure 4.5. AIN93Null diet significantly decreases serum vitamin D levels without altering serum calcium, weight gain, or bone mineral density in wild type mice.

Serum 25-OH D (**A**) and serum calcium (**B**) from wild type mice fed maintenance (n=5) or AIN93Null diet (n=5). Average percent weight change (**C**) for each group at each time point. Mice were euthanized 18 weeks after diet initiation and total body bone mineral density scanning (**D**) and radiographs were performed. Images (**E**) depicted are representative of animals on each diet. *p<0.05, **p<0.001, ***p<0.0001, student's t test. Error bars represent standard error of the mean.

Vitamin D_3 deficiency is protective against DSS-induced IBD and cancer in *Smad3*$^{-/-}$ mice. Once we established that AIN93Null diet could induce chronic vitamin D deficiency without affecting calcium homeostasis in our mouse model, we next sought to determine whether vitamin D deficiency would exacerbate DSS-induced disease in *Smad3*$^{-/-}$ mice. For these studies, mice were placed on either maintenance or AIN93Null diet for two weeks prior to insult with DSS (1.5% DSS for 3 days). Mice were followed for 16 weeks post DSS-treatment and survival, IBD, summed total dysplasia and incidence of invasive carcinoma were assessed. Unexpectedly, vitamin D-deficient mice exhibited less disease as indicated by survival, dysplasia and tumor incidence compared to maintenance diet-fed mice. Survival was significantly increased in animals fed AIN93Null diet ($p=0.009$, **Fig 4.6A**) and was not significantly different from vitamin D deficient *Smad3*$^{-/-}$ animals given regular water instead of DSS ($p=0.2337$). IBD scores were significantly reduced in DSS-treated vitamin D deficient animals compared to DSS-treated maintenance diet-fed animals ($p<0.0001$, **Fig 4.6B**) as was the degree of colonic dysplasia (**Fig 4.6C**). In mice fed AIN93Null diet, 69% of animals had no evidence of dysplasia compared to only 38% in DSS-treated mice fed maintenance diet ($p=0.0014$, **Fig. 4.6C**). Finally, incidence of invasive colon carcinoma was significantly reduced (4.3 fold, $p=0.0007$, **Fig 4.6D**) in animals fed AIN93Null diet compared to those fed maintenance diet.

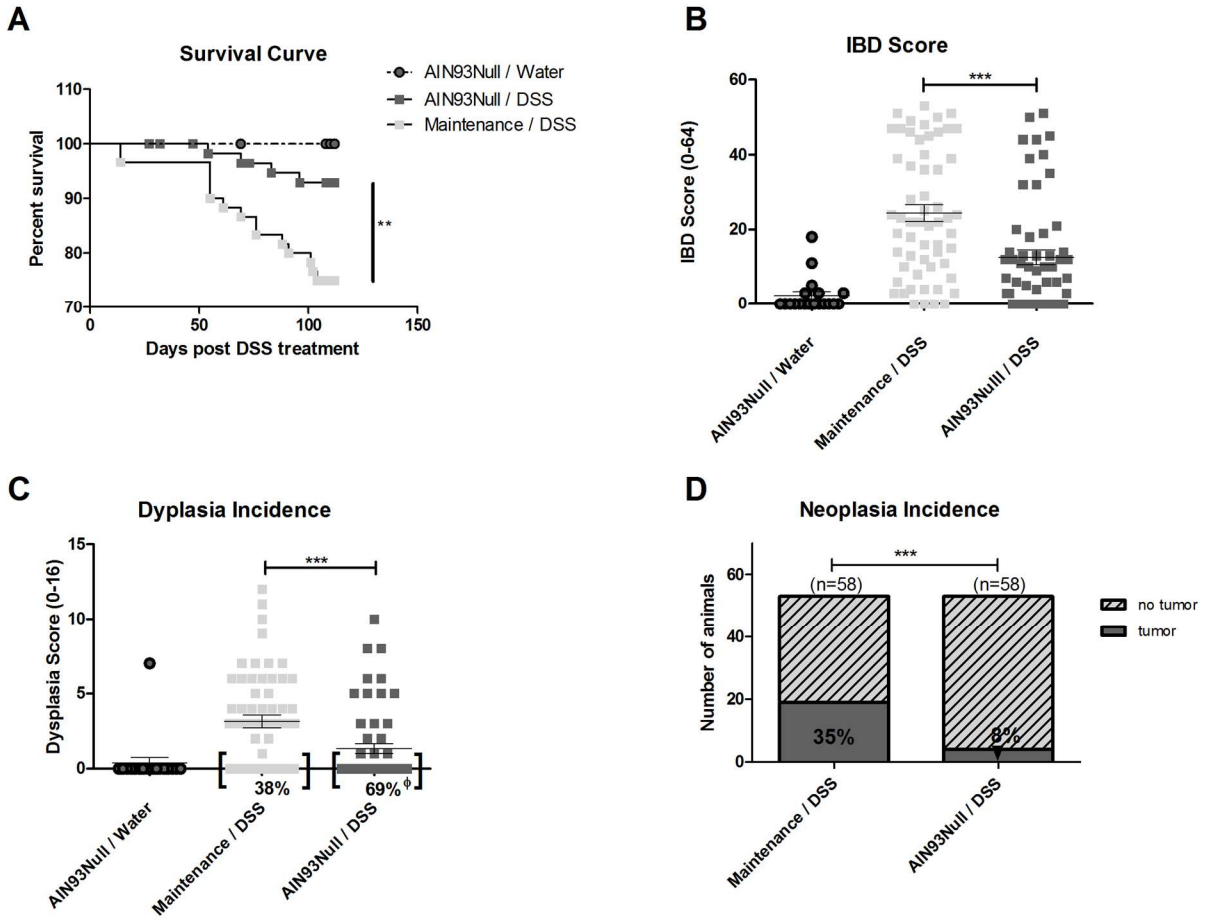


Figure 4.6. AIN93Null diet protects against DSS-induced colitis and colon cancer in *Smad3*$^{-/-}$ mice.

Smad3$^{-/-}$ mice were fed AIN93Null or maintenance diet and treated with DSS (N=58 mice per group) or regular water (N=20). Survival (**A**) was compared by Log Rank Test. Colonic IBD (**B**) and dysplasia (**C**) was assessed histologically at the study end point. Cecum and colon were assessed histologically (**D**) for evidence of neoplasia **p*<0.05, **p<0.01, ***P<0.001 ϕP=0.0014. IBD score and dysplasia scores were compared by Mann-Whitney t test and dysplasia incidence and neoplasia incidence was compared by Fisher's exact test.

Vitamin D deficiency is associated with increased cellular proliferation during acute inflammation following DSS-treatment. In order to determine why vitamin D deficiency would be associated with less DSS-induced disease, we evaluated tissue inflammation and cell proliferation. Vitamin D has been shown to directly regulate genes involved in cell cycle regulation and cell proliferation including p21waf1, p27, and p53 [204]. *Vdr*$^{-/-}$ mice have been

shown to have increased colonic epithelial cell proliferation [205]. Conversely, *in vitro* assays have demonstrated that increased concentrations of $1,25(OH)_2D$ are effective at decreasing proliferation of multiple cell types including epithelial cells, fibroblasts keratinocytes, and prostate cells [206, 207]. In order to determine if the protective effects of vitamin D deficiency against DSS-induced colitis and colitis-associated colon cancer were associated with changes in cell proliferation during the injury or healing phase acutely following DSS insult, we examined colonic inflammation and epithelial cell proliferation in DSS-treated *Smad3*$^{-/-}$ mice during the early inflammatory phase following DSS insult. Two specific time points were chosen for evaluation of IBD; 9 days post DSS-treatment, which has previously been shown to be associated with the highest degree of damage following DSS-treatment and 16 days post DSS-treatment as a means of evaluating disease and cellular proliferation during the healing phase of disease [196, 208]. As expected, 9 days post DSS-treatment, DSS-treated mice demonstrated increased IBD scores compared to animals maintained on regular water (**Fig 4.7A**) and there were no differences in IBD score between the two diet groups. Interestingly, IBD scores were significantly decreased at day 16 compared to day 9 post DSS treatment in animals fed AIN93Null diet (**Fig 4.7A & B**), while IBD scores were similar at day 9 and day 16 post DSS-treatment in maintenance diet-fed animals (**Fig 4.7A & B**). This data suggests that regardless of diet, mice develop a similar degree of colitis initially following DSS treatment but the IBD resolves more quickly in animals fed AIN93Null diet. In addition to having decreased IBD scores, mice fed AIN93Null diet had increased epithelial cell proliferation, demonstrated by an increased uptake of BrdU staining (**Fig 4.7C**). DSS is believe to induce colitis in mice at least in part through its direct cytotoxic effects on the colonic epithelium, leading to epithelial cell death and loss of epithelial cell barrier integrity against enteric bacteria [208]. Our results suggest that in the face of epithelial cell damage, vitamin D deficiency results in increased epithelial cell proliferation, which in this model may result in faster mucosal healing following DSS treatment. Thus, we propose that the alleviation of early stage IBD due to faster mucosal healing is partly responsible for the decrease in subsequent tumor incidence seen in *Smad3*$^{-/-}$ mice fed AIN93Null diet.

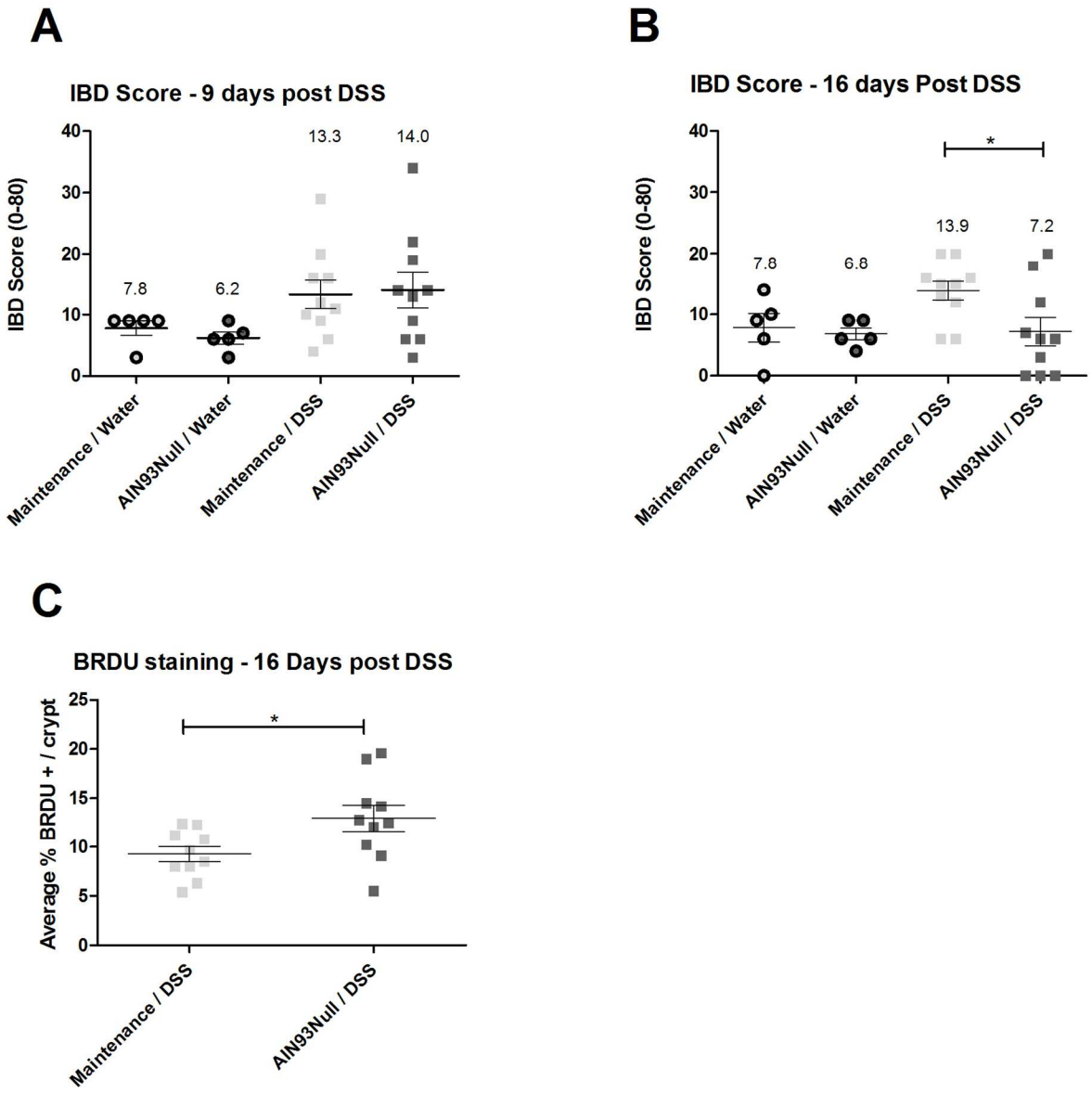


Figure 4.7. AIN93Null diet is associated with increased cell proliferation following DSS-treatment in *Smad3*$^{-/-}$ mice.

Smad3$^{-/-}$ mice were fed AIN93Null or maintenance diet and treated with DSS (N=10 mice per group per time point) or regular water (N= 5 mice per group per time point). Animals were euthanized 9 (**A**) and 16 (**B**) days post initiation of DSS-treatment and IBD scores were generated based on histologic examination of cecum and colon tissue. BrdU staining (**C**) was used to quantify actively proliferating colonic epithelial cells during the healing phase of disease following DSS treatment (16 days post DSS)*p<0.05 Mann-Whitney t test.

DISCUSSION:

Animal models offer a unique opportunity to further dissect the association between individual dietary components and disease initiation and progression by allowing for the controlled manipulation of specific nutrients. Our laboratory has previously demonstrated that vitamin D supplementation is protective against colitis and colitis-associated colon cancer development in a bacterial driven model of IBD-associated colon cancer [45]. To gain further insight into the mechanisms through which vitamin D might be working to afford protection, we evaluated whether vitamin D was protective where IBD and subsequent colitis-associated colon cancer is driven in *Smad3*$^{-/-}$ mice by DSS [196]. Our findings demonstrate that vitamin D supplementation is also associated with an improved survival and a decreased incidence of dysplasia, a precursor to cancer, in this model of chemically induced IBD and colon cancer, although the protective effects are not as robust as those seen in the bacterial driven model.

Because vitamin D protects *Smad3*$^{-/-}$ mice from developing IBD and colon cancer, we hypothesized that vitamin D deficiency would exacerbate IBD and increase the incidence of colon cancer. To study the effects of vitamin D deficiency on IBD and colon cancer, we first determined the amount of dietary vitamin D needed in a diet to induce chronic vitamin D deficiency without causing calcium imbalance and bone diseases. Unexpectedly, diets containing 5 times less vitamin D (0.2 IU/g diet) than the level recommended for maintenance of health for rodents (1 IU vitamin D /g diet) did not alter serum vitamin D levels suggesting that dietary vitamin D requirements in laboratory housed rodents may be lower than expected. We therefore determined if a vitamin D null diet (AIN93Null) could be used to modulate serum vitamin D levels for chronic studies. We found that AIN93Null diet can be used effectively to induce vitamin D deficiency for up to 18 weeks without causing calcium imbalances or bone density depletion. These data suggest that this dietary regimen may be useful for studying the effects of mild vitamin D deficiency on chronic diseases. In addition, we demonstrated similar results in wild type mice suggesting that mice may be more resistant to diet-induced severe vitamin D deficiency. Thus, AIN93Null diet may be a useful tool to study effects of mild vitamin D deficiency on chronic diseases in mouse models of human diseases.

Of interesting note in these studies were the findings that mice could maintain normal serum 25-hydroxyvitamin D levels when very small quantities of vitamin D were provided in the diet. Our initial two studies utilized a diet containing 80% less vitamin D than is provided in our standard rodent diet yet we were unable to alter serum 25-hydroxyvitamin D levels. In fact, even after depleting serum 25-hydroxyvitamin D levels through the use of AIN93Null diet, serum levels quickly rebounded when animals were placed back on the low vitamin D diet. These data emphasize the importance of verifying serum vitamin D status in animals to ensure that appropriate levels are obtained especially when working with novel diets. It addition it suggests that standard levels of vitamin D provided in commercial rodent chows are markedly higher than levels required to maintain serum vitamin D.

We used a vitamin D-Null diet to evaluate whether vitamin D deficiency would exacerbate colitis-associated colon cancer in *Smad3*$^{-/-}$ mice. Unexpectedly, we found that vitamin D deficient animals had improved survival, decreased dysplasia, and significantly fewer colon tumors compared to maintenance diet-fed animals. DSS is commonly used to model IBD as it can effectively induce colitis in a variety of mouse strains [209] by directly damaging the colonic epithelium resulting in mucosal erosions and loss of epithelial cell barrier function [196, 210]. Vitamin D has been shown to play a role in cell cycle regulation and cell proliferation [204]. Increased concentrations of vitamin D decrease cellular proliferation in a number of different cell types *in vitro* including epithelial cells. Conversely, *Vdr*$^{-/-}$ mice have been shown to have increased colonic epithelial cell proliferation [205]. Our data have demonstrated that similar to *Vdr*$^{-/-}$ mice, vitamin D deficient *Smad3*$^{-/-}$ mice have increased epithelial cell proliferation during the acute healing stages following DSS treatment. We believe that this increase in cellular proliferation as a result of vitamin D deficiency allowed these animals to repair the epithelial barrier damage induced by DSS faster than vitamin D sufficient animals. This hypothesis is supported by the differences in IBD progression between the groups. IBD scores from animals euthanized on day 9, corresponding to the peak period of damage, indicate that there were no differences in initial disease associated with the diet. However, vitamin D deficient animals euthanized 16 days post DSS treatment, during the healing and regeneration phase, had significantly lower IBD scores compared to vitamin D sufficient animals. Also of note, the IBD scores from the vitamin D deficient animals euthanized on day 16 post DSS were decreased from those of vitamin D deficient animals on day 9 post DSS, while vitamin D sufficient animals had

similar IBD scores on both days. We believe that increased colonic epithelial proliferation allowed vitamin D deficient animals to restore epithelial barrier damage induced by DSS faster than maintenance diet fed animals resulting in a decrease in exposure to luminal bacteria and an overall decreased inflammatory response triggered by this barrier disruption. It would be of interest to determine if AIN93Null diet would be similarly protective when multiple rounds of DSS are delivered. Under conditions of chronic damage, disease might have become exacerbated.

Previous studies have demonstrated that vitamin D deficiency exacerbates DSS colitis in C57BL/6 [94] and *Il10*$^{-/-}$ mice [96]. In addition, *Vdr*$^{-/-}$ mice are more susceptible to DSS-induced colitis [98, 99]. These results are contrary to what we observed in our studies. One potential difference between these studies and ours is that our study used a lower concentration and shorter duration of DSS treatment, likely resulting in a milder disease event. An additional difference is the use of *Smad3*$^{-/-}$ mice, which have defective TGFβ signaling due to loss of the transcription factor, SMAD3. TGFβ signaling is important in wound healing and epithelial regeneration, and our previous work with this model has demonstrated that *Smad3*$^{-/-}$ mice are more susceptible to DSS-induced colitis compared to wild type mice, likely due at least in part to impaired mucosal healing and abnormal epithelial proliferation [196, 211]. This abnormal epithelial proliferation and impaired mucosal healing following DSS treatment is postulated to play a role in predisposing these animals to develop subsequent inflammation-associated colon cancer [196]. Due to impaired mucosal healing, *Smad3*$^{-/-}$ mice may be exposed to luminal antigens leading to an exuberant inflammatory response. It is plausible that vitamin D deficiency leads to an increase in cellular proliferation following DSS injury that compensates for the impaired mucosal healing present in the *Smad3*$^{-/-}$ mice and provides protection in this setting. While these results may be specific to this mouse model, it does raise intriguing questions as to the mechanism of action behind these effects given that the TGFβ pathway is commonly mutated in patients with IBD as well as colon cancer [154]. Although the mechanisms for protection against colitis and colon cancer afforded by vitamin D deficiency are as yet unclear, these findings suggest that targeted or localized vitamin D deficiency may offer an opportunity for prevention or treatment of inflammation-associated colon cancer.

Chapter 5. EFFECTS OF DIETARY VITAMIN D ON MODULATING THE MICROBIOME

INTRODUCTION:

Recent studies indicate that diet plays a role in regulating the microbiome [29] and that the interaction between the gut microbiota and diet may significantly affect IBD development in part by altering the microbiota [30, 31]. In addition, recent reports have suggested that the microbial composition can influence tumor formation independent of inflammation [132]. While the composition of the diet can influence IBD at least in part through changes in the microbiome [30], the role of micronutrients in shaping the gut microbiota has not been studied. Since mechanistic studies evaluating the impact of nutrients on disease and microbiota are difficult to perform in human populations, mouse models offer a unique solution. By using mouse models, investigators can modulate individual nutrients or specific microbial populations while closely controlling environmental and genetic influences. The effects of these modulations can then be monitored over the period of disease progression. In addition, the increasing availability of gnotobiotic mice as well as the continued advancement in microbial sequencing technology is providing opportunities for further study of interactions between gut microbiota, host genetics, and environmental factors.

It is commonly accepted that intestinal commensal bacteria can play important roles in the health and disease of both humans and mice. Commensal enteric influence nutrient metabolism, immune system development, and immune responses to invading organisms [133, 212-216]. Recent studies evaluating differences in human gut microbiota during health and disease suggest that microbial diversity or structure of microbial community may be associated with specific diseases, such as IBD and obesity [133, 215, 217-220]. However, it is still not understood whether these differences are a cause of or an effect of the disease. Because studies evaluating cause and effect relationships are difficult to perform in humans, animal models offer the opportunity to answer this type of question.

Increased serum vitamin D levels are associated with a decreased risk of developing IBD and colon cancer in both humans and animals [1-8, 12-15]. While it is clear that the composition of the diet can influence IBD at least in part through changes in the microbiome [30] the role of

vitamin D, a micronutrient, in shaping the microbiome has not been explored. Vitamin D has the potential to modulate the microbiome directly by acting as a substrate for specific bacterial populations supporting growth of these communities, or indirectly through modulating the mucosal immune system. Increased serum vitamin D levels can influence both immune cells and colon epithelial cells. Studies suggest its effect may be via modulating immune cell differentiation and maturation, colon barrier function, and the secretion of antimicrobial peptides, mucins, and cytokines, all of which have the potential to modulate the gut bacteria and could also play an important role in the modulation of colonic inflammation [67-69]. Thus for our initial studies, we simply determined whether dietary vitamin D could alter the microbiome regardless of mechanism. For these studies, we used both specific pathogen free and germ-free mice and we demonstrated that modulating the vitamin D levels in the diet were sufficient to alter the gut microbial community. We also determined that the gut microbial community established by feeding increased dietary vitamin D, could be transplanted into germ-free mice.

MATERIALS AND METHODS:

SPF animal studies. Six-week-old female 129-Smad3^{tm1Par}/J (Smad3$^{-/-}$) were placed on a purified, irradiated diet with either 1 IU vitamin D (5SRH, maintenance) or 5IU (5AAA, AIN93H, high vit D) manufactured by PMI Nutrition International (Lab Diet/Test Diet, St. Louis, MO) for one week. Mice were group-housed in individually ventilated cages with autoclaved acidified water delivered in water bottles and maintained specific pathogen-free and screened for rodent pathogens as previously described [45]. One week following diet initiation; mice were euthanized by CO_2 asphyxiation. Cecal contents were collected and homogenized in RNALater. Cecal tissue was rinses in sterile PBS and placed in RNALater. Samples were stored at -80°C until processing. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Gnotobiotic studies. Five-week-old male and female colony-bred germ-free Swiss Webster mice were obtained from the University of Washington Gnotobiotic Animal Core (GNAC) facility. Mice were bred and maintained in flexible-film isolators (CBC, Madison, WI). Within the isolators, mice were housed in open-top cages with autoclaved Enrich-n' Pure

bedding (Andersons, Maumee, Ohio), fed autoclaved rodent chow (Laboratory autoclavable Rodent Diet 5010 or 5021, Lab Diet, St. Louis, MO) *ad lib.* and provided with autoclaved water in water bottles. Isolator sterility was monitored weekly by aerobic and anaerobic culture of fresh feces collected from animals housed in each isolator. In addition, 16S rRNA PCR was performed using DNA extracted from pooled fecal samples collected from each isolator monthly. Prior to study, animals were transferred from the flexible-film isolator to individually ventilated Isopositive cages (Tecniplast, West Chester, PA). Mice were housed 3 per cage. Animals were transferred using a modified sterile technique prior to working with the mice [221]. Animals were handled in a biosafety cabinet prepared by thorough spraying with Clidox solution (1:3:1, base:water:activator, v:v:v) three hours prior to using it [221].

Germ free Swiss Webster mice were gavaged with a fecal slurry prepared from pooled fecal samples from 12-week-old male *Smad3*$^{-/-}$ mice fed an irradiated purified maintenance diet (5SRH, AIN93M, 2 cages, n=3/cage, 1 cage each males and females) or an irradiated purified diet containing 15 IU vitamin D (5BTC, high vitamin D diet, 2 cages, n=3/cage, 1 cage each males and females). Diets were manufactured by PMI Nutrition International (Lab Diet/Test Diet, St. Louis, MO) based on AIN93M diet. Diets were nutritionally identical except for the vitamin D content. All recipient mice were fed autoclavable rodent chow (5010, Lab diet). Frozen fecal samples were used to prepare the fecal slurry for transplantation. Feces were mashed in PBS with a sterile wooden dowel (1g feces in 5 ml PBS) and centrifuged at 800g for 3 min to obtain the supernatant. Mice were gavaged with 100µl of supernatant. Fecal samples were collected from each cage just before the gavage to confirm germ-free status by 16S rRNA PCR. Individual fecal samples were collected 1, 2 and 4-week post fecal transfer for DNA extraction.

DNA extraction methods. All samples were stored at -80°C prior to extraction. For mouse cecal content preparation (CC), samples were thawed on ice and the contents were transferred to a sterile tube. One milliliter of sterile PBS was added and the sample was homogenized using an OMNI tissue homogenizer 115 (OMNI Inc., Marietta, GA). A sub-sample of 300µl was used for DNA extraction. Bacterial genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) with 1 minute of bead beating as previously described [222]. For cecal tissue (CM) preparation, samples were thawed on ice then using sterile technique each sample was placed in a separate sterile petri dish. The cecal tissue was

chopped thoroughly with a clean razor and then transferred to a sterile microfuge tube. One milliliter of PBS was added to each tube and the sample was homogenized as above. Genomic DNA was extracted from 0.3 ml aliquots following *Li et al.* [223]. Fecal samples were lysed using Lysing Matrix E (MP Biomedical, Santa Ana, CA), and DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) or Zymo ZR Fecal DNA MicroPrep kit (Zymo Research, Irvine, CA) following the manufacturers' instructions.

Pyrosequencing and bioinformatics analysis of SPF animals. Bacterial genomic DNA was sent to Molecular Research and Testing Laboratory (Lubbock, TX) for 16S rRNA pyrosequencing. Cecal content and cecal tissue sequences were sent to Fred Hutchinson Cancer Research Center for bioinformatics analyses by Dr. Meredith Hullar. Briefly, a threshold of 25 was used for average quality score of any 50 base pair window to trim off the unreliable part of the sequences after the initial quality check. The cleaned QC checked sequences were used for bioinformatics analysis performed in the QIIME pipeline [224]. Operational taxonomic units (OTU's) were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). OTU's were taxonomically classified using BLASTn against a curated GreenGenes database [225]. The OTU's for this analysis include sequences that are between 77-80% similar for Phylum level classification and between 90 and 95% similar for Genera. If a sequence could not be placed at the genera level, it was included at the most resolved taxon possible. A data file with the number of sequences per taxon per sample was then checked for entries less than 20 sequences to adjust for the empirically determined detection limit for this pyrosequencing set-up and relative percent of the sequences per taxon was compiled. These data were then arcsin-transformed and used to generate distance matrices for multivariate analyses. A factorial design perMANOVA [226, 227] on the Euclidean distance matrix was used to test whether there was a significant difference in the bacterial community composition (Phylum) between the sample prep (CC vs. CM) within the two experimental treatments (maintenance vs. high vitamin D). T-tests were used to test for differences in the abundance of each bacterial phyla between treatments. Principle co-ordinate analysis, on the Euclidean distance matrix, was used to illustrate whether the community composition, measured as phyla, clustered by treatment [228]. Multiple response permutation procedure (MRPP) was used to test for significant differences in the gut microbial community genera between treatments. Indicator species analysis (ISA) [229] was used to test

whether the distribution of a genera was different between the two treatments. A bi-plot was used to visualize the distribution of the genera across replicates and treatments.

Fecal microbiome data were analyzed by Dr. David Beck at the University of Washington. Briefly, the UPARSE method was used for amplicon sequence handling and OTU clustering with USEARCH version 7.0.1001 [230]. During trimming, the maximum expected error was set to 0.5 (-fastq_maxee 0.5). Clustering was performed at 97%. Chimeras were identified by validation with the ChimeraSlayer reference database in the Broad Microbiome Utilities version r20110519 as obtained from the UCHIME distribution [231]. Initial taxonomic assignments were made using the RDP Classifier from the Ribosomal Database Project, downloaded on October 22, 2013 [232]. The resulting reads per OTU were scaled so that the number of reads in each sample was equal and used for all subsequent analyses. Hierarchical clustering of samples and OTUs was performed using the percentage of reads per OTU for the 35 most abundant taxa. Persistent OTUs were identified as those present as more than 2.5% of the total population in at least one sample. Bray-Curtis distances, Shannon indices, ordination plots and multivariate analyses were calculated using the *vegan* library version 2.2-1 [233] in R version 3.1.1 (http://www.R-project.org/).

RESULTS:

The microbiome does not differ between cecal tissue and cecal contents. It has been demonstrated that the microbial community can differ in different regions of the gastrointestinal tract [234]. There is also a stratification of bacteria within a given section of the bowel based on oxygen availability and the presence of mucosal sections with more anaerobic bacteria being present in the central luminal contents and microaerophilic organisms residing closer to the mucosal border [234]. In the future, we hope to use the *Smad3*$^{-/-}$ mouse model described in **Chapters 2-4**, to study the interactions between the microbiome, diet, and disease development. In the *Helicobacter*-driven model of colitis and colon cancer, *Helicobacter bilis* preferentially resides in the cecum and colon [153] and consequently these tissues typically develop the highest incidence of tumors. We therefore focused our initial gut microbiome studies on the cecal tissue. We sought to determine if the microbiota obtained from cecal tissue sections differed from that of the luminal contents collected from the cecum. Analysis of the 16S rRNA gene showed that

the composition of the microbiome is similar between the cecal contents and cecal tissue at both the phyla (**Fig 5.1A**, r^2=0.91) and genera (**Fig 5.1B**, r^2 0.99) levels. These data suggest that either cecal tissue or cecal contents can be used for analysis of the microbial community.

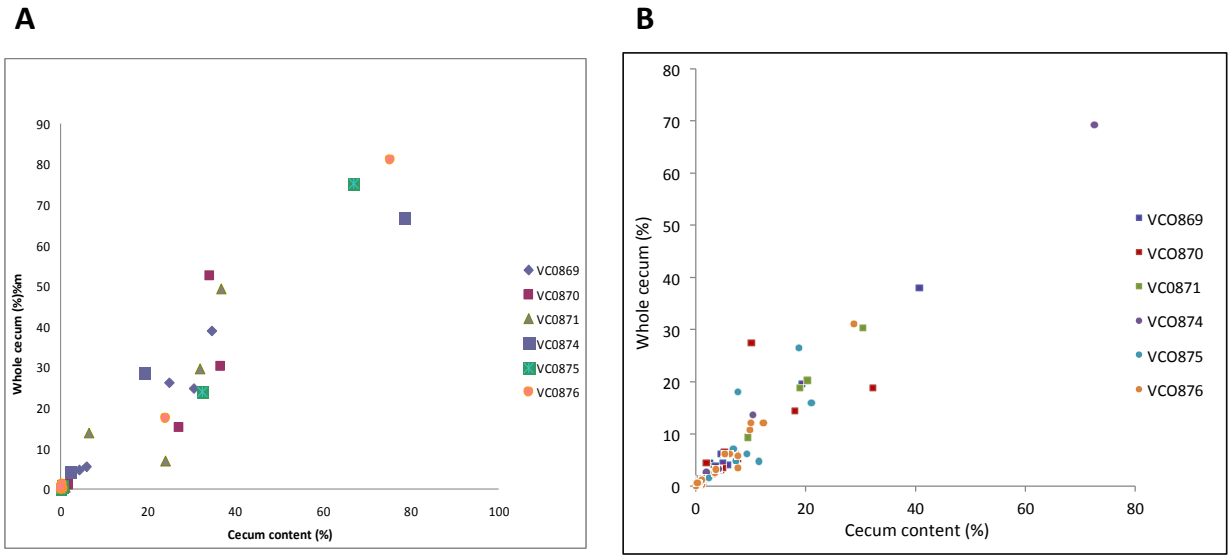


Figure 5.1. Comparison between sample preparation across the distribution of bacterial phyla and genera.

A factorial design perMANOVA [226, 227] on the Euclidean distance matrix was used to test whether there was a significant difference in the bacterial community composition between the sample prep (CC vs CM) at the phyla (A; y=0. 97x + 0.005;r^2=0.91) or genera (B; y=0.95x+ 0.14, r^2=0.99) levels. Samples VC0784-VC0786 were fed maintenance diet and samples VC0869-VC0871 were fed high vitamin D diet. The composition of bacterial phyla (A) and genera (B) directly correlate between the cecal content and cecal tissue from a specific animal after 1 week on diet.

Increased dietary vitamin D alters the composition of the gut microbiome: Because various dietary components play a role in regulating the microbiome [29], we evaluated whether increased dietary vitamin D could alter the microbiota in *Smad3*$^{-/-}$ mice. Cecal contents and cecal tissues of uninfected *Smad3*$^{-/-}$ mice fed either maintenance diet or high vitamin D diet were collected for 16S rRNA microbial analysis after 1 week on diet. Multivariate analysis of the gut microbial community (GMC) showed clustering by diet treatment (**Fig 5.2A**). Significant differences at the phyla level (*Tenericutes* and *Firmicutes*) were associated with the diets (**Fig 5.2B**), suggesting that dietary concentrations of vitamin D can significantly influence the GMC.

There was a significant difference in the composition of the microbial community at the genera level between the two diet groups as well (MRPP, A=0.13, p=0.024). Indicator species analysis (ISA) showed that the abundance of genera in the family Erysipelotrichaceae ($p<0.0008$) and Parasutterella ($p< 0.0008$) were significantly different between treatments. The genera Oscillobacter (0.002), and Allistipes ($p<0.003$) differed between treatments as well, though this difference did not reach statistical significance based on our Bonferroni cut-off $p<0.001$. The bi-plot shows the distribution of the genera across treatments (**Fig. 5.3**).

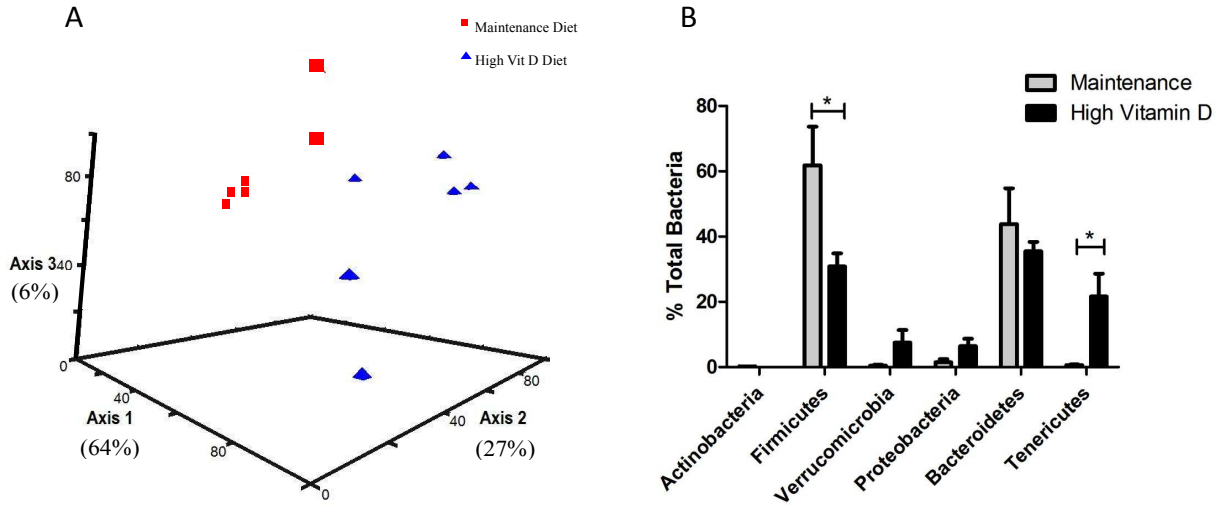


Figure 5.2. Increased dietary vitamin D shifts gut microbial populations.

(A) Microbial composition differentially clusters by diet. Principle co-ordinate analysis, on the Euclidean distance matrix, represents the community composition (phyla). B) Analysis of bacterial phyla from cecal tissue demonstrate significant differences in *Firmicutes* and *Tenericutes* (* $p<0.05$) associated with diet.

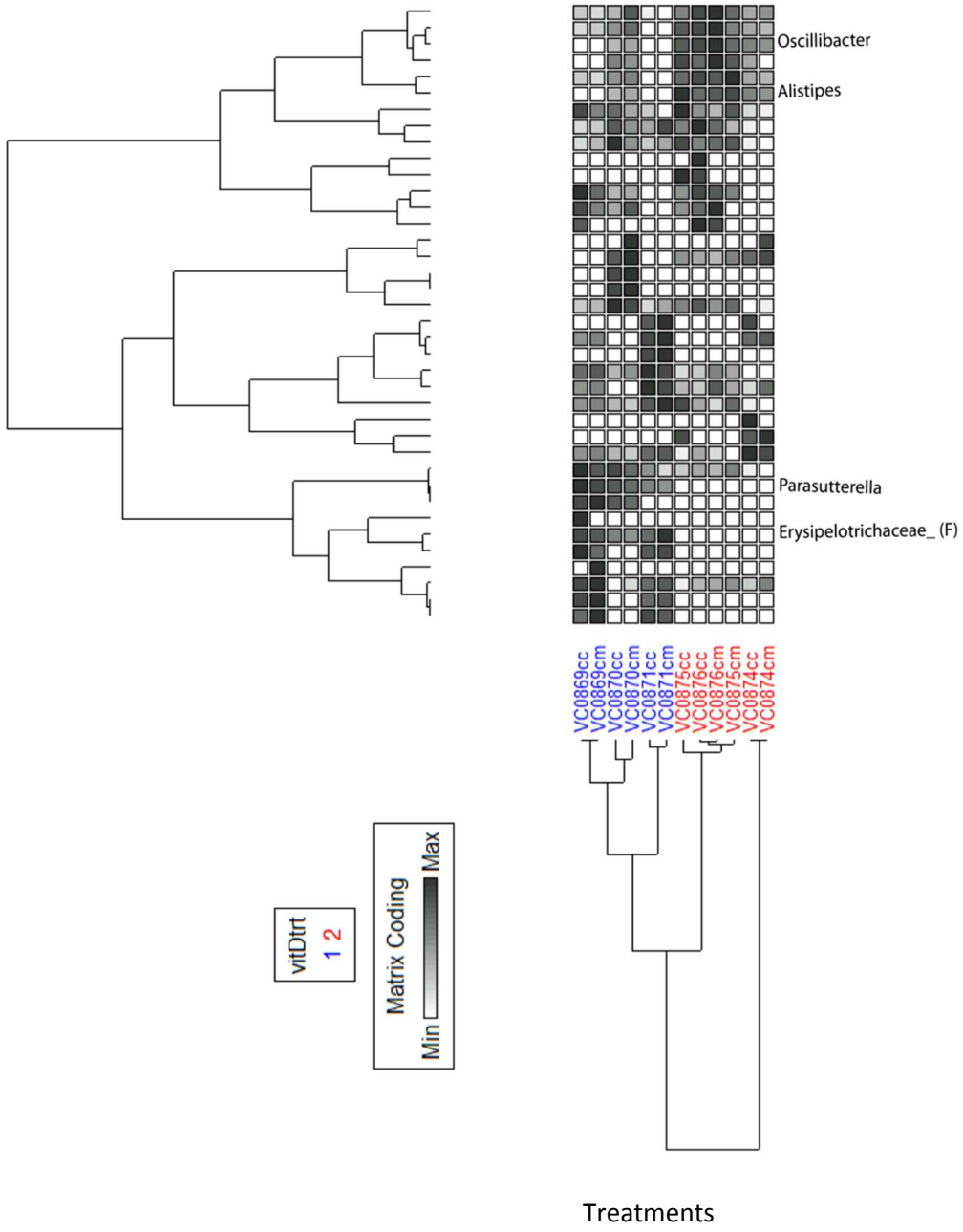


Figure 5.3. Biplot between the experimental treatments and genera.

Treatment groups are clustered on the X axis and organism abundance is clustered on the y axis. Animals fed maintenance diet are indicated in red (VC0874-VC0875) and high vitamin D-fed animals are indicated in blue (VC0869-VC0871). The microbial profile of animals distinctly clusters by diet. The abundance of genera in the families Erysipelotrichaceae ($p<0.0008$) and Parasutterella ($p< 0.0008$) were significantly different between treatments. Organisms in the genera Oscillobacter (0.002), and

Allistipes ($p<0.003$) differed in abundance as well, though did not reach statistical significance. cc=cecal content and cm=whole cecum.

Vitamin D-associated microbiota can be transplanted into germ-free mice. We showed that increased dietary vitamin D levels were sufficient to alter the gut microbial community in *Smad3*$^{-/-}$ mice. Ultimately, we want to determine whether the gut microbiome changes induced by vitamin D contribute to protection of *Smad3*$^{-/-}$ from colitis. Because we hope to utilize fecal transplantation techniques and colonization of germ-free mice for these studies, we determined whether the dietary-induced changes in microbiota observed with increased vitamin D would persist when transplanted into germ-free mice. For these studies, Germ free Swiss Webster mice were colonized with fecal microbiota from *Smad3*$^{-/-}$ mice fed maintenance or high vitamin D diet. Serial fecal samples were collected from the mice 1, 2, and 4 weeks post fecal transplant to monitor the microbial colonization of the animals. It is important to note, that the Swiss Webster recipient animals were all fed the same autoclaved rodent chow diet, which was different from donor animals' diets.

The community structure of serially collected fecal samples was probed by 16S rRNA profiling. Interestingly, several OTUs were present in the high vitamin D donor samples and the high vitamin D recipients but were not detected in the maintenance diet recipients including Porphyromonadaceae, Bacteroidetes, Alistipes, Tannerella, Porphyromonadaceae, and Clostridales. These OTUs contributed substantially to the separation based on donor diet. The separation in abundance profiles of recipients based on donor diet was statistically significant ($p < 0.01$) and demonstrated by multidimensional scaling (MDS) analysis (**Fig 5.4A**). The relative abundance profiles were shown to be divergent by PERMANOVA ($p < 0.05$). Interestingly, the high vitamin D diet microbiota recipients had a more diverse community structure compared to the recipients of maintenance diet-associated microflora via Shannon index (**Fig. 5.4B**). These data suggest that the gut microbial community can be transplanted into germ free mice and the microbial community structure can be maintained even in the absence of additional dietary vitamin D supplementation.

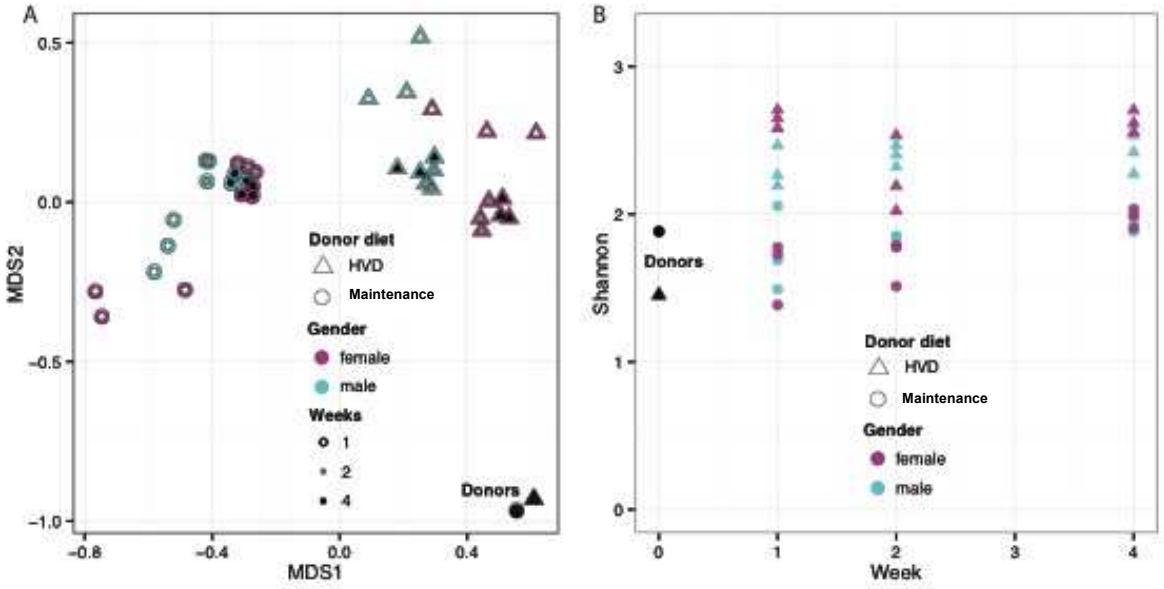


Figure 5.4. Gut microbiota from maintenance and high vitamin D fed mice is transferrable to germ free mice.

(A) Multidimensional scaling of OTU abundances from the fecal transplant experiment with donors fed maintenance and high vitamin D diet. The single black data points (lower right corner) represent the two donor samples for the maintenance and high vitamin D diet. Triangles represent high vitamin D and circles represent maintenance diet. The cyan and magenta indicate gender (male and female, respectively) while the inner color indicates time post fecal transplant in weeks as white, grey and black for one, two and four weeks, respectively. As with the initial fecal transplant study, the samples diverge from the donors rapidly but begin to become more donor-like by week 4. There is a strong division between the two diets in post-transplant. (B) Community complexity for fecal transplant with donors fed maintenance and high vitamin-D diets. The donor samples for each diet are shown at week 0. There is no significant difference by gender, however, the microbiota from high vitamin-D diet appears to establish a more diverse bacterial community. Figure adapted from *Paik et al.* 2015 [221].

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DISCUSSION:

Recent epidemiologic data suggest that diet plays a major role in altering the gut microbiota and may influence colonic inflammation [29, 43, 44]. In addition, changes in the microbiome are associated with inflammation and disease severity in both human patients [45] and animal models of IBD [46, 47]. Though vitamin D deficiency is increasingly prevalent and

vitamin D supplementation is common, little is known about how micronutrients and particularly vitamin D supplementation can impact the microbiome and potentially influence IBD. Our laboratory has previously shown that increased dietary vitamin D can reduce inflammation and subsequent colon cancer [45], and we are interested in pursuing future studies to evaluate interactions between vitamin D-associated microbiome changes and disease development in a genetically predisposed mouse model, *Smad3*$^{-/-}$ mice. In our initial preliminary studies we determined whether (1) dietary vitamin D could alter the microbiota and (2) whether vitamin D-induced changes in the microbiota would persist when transferred into germ-free mice maintained on a standard rodent diet. This has important implications for micronutrient-induced changes in microbiota, the persistence of those changes, and the potential for therapeutic changes in patient microflora.

We have demonstrated that dietary vitamin D can modulate the microbiota (independent of inflammation). Future studies will focus on the mechanisms through which vitamin D works to alter the gut microbial community. One potential mechanism is that the availability of vitamin D within the luminal contents is able to change the gut flora directly. Although prokaryotes do not express VDR, some bacteria do have the ability to metabolize vitamin D to 1,25(OH)D_3 [48]. Therefore it is possible that specific bacteria are able to utilize vitamin D or its metabolites as a substrate thereby fostering the expansion of those populations. Other potential mechanisms through which vitamin D may be working to indirectly modulate the microbiota is through systemic interactions within the host. Increased serum vitamin D levels can influence both immune cells and colon epithelial cells; via modulating immune cell differentiation and maturation, colon barrier function, and the secretion of antimicrobial peptides, mucins, and cytokines, all of which have the potential to modulate the gut bacteria [67-69]. It has been shown that mice lacking the ability to respond to vitamin D (due to lack of VDR or Cyp27b1 (an enzyme involved in vitamin D metabolism)) have distinctly different gut bacteria compared to wild-type littermates with intact vitamin D signaling [67], suggesting that host responses to vitamin D impact the microbiome. However, the specific cell types involved in mediating these effects have not yet been determined. Our studies demonstrate that germ-free mice can be stably colonized with specific defined microbial flora which will allow us the opportunity to directly study how a specific gut microbial community alters disease progression. Future studies utilizing fecal transplantation of high vitamin D or maintenance diet flora into germ-free mice will allow

us to study the impacts of vitamin D mediated microbiota on colon cancer incidence independent of systemic effects of vitamin D.

Together the data presented in this chapter offer evidence that dietary vitamin D can modulate the gut microbiota. Additionally, we have demonstrated that these vitamin D-induced changes in microbiota can be successfully transplanted and persist within germ-free mice. Together these findings offer exciting possibilities for future studies aimed at exploring the complex interactions between diet, genetics, the microbiome and therapeutic interventions potentially altering disease progression.

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