THE EFFECTS OF THE MICROBIOME IN ENDOCRINE VITAMIN D METABOLISM

A Dissertation in
Immunology and Infectious Disease

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

August 2017
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ABSTRACT

Vitamin D insufficiency is associated with bone diseases like osteoporosis and immune-mediated disease like inflammatory bowel disease. Endocrine vitamin D metabolism is thought to be controlled by calcium intake and other hormones that interact to regulate mineral homeostasis, such as parathyroid hormone (PTH) from the parathyroid gland and fibroblast growth factor (FGF23) from bone. The gut microbiome consists of trillions of cells that have coevolved with their hosts to influence nutrition, metabolism, and immune function. There is evidence the microbiome regulates bone health, indicating a link between the gut microbiome and endocrine vitamin D metabolism. Using antibiotic treatments and colonization of germ free mice, I examined the effects of host-microbial interactions on regulation of endocrine vitamin D metabolism. My research showed disrupting the microbiome with antibiotics increased FGF23 and 25-hydroxyvitamin D, a stable vitamin D metabolite used as a marker of vitamin D status. These changes were dependent on signaling through toll-like receptors. Colonization of germ free mice decreased host FGF23, leading to increased levels of hormonal vitamin D and circulating calcium. In both antibiotics treated mice and colonized mice, changes in FGF23 and vitamin D metabolites were associated with changes in TNF-α. Together, the work presented in this dissertation suggests host sensing of the gut microbiome regulates FGF23 and endocrine vitamin D metabolism, both of which are critical for mineral homeostasis and bone health.
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ABBREVIATIONS

25D: 25 hydroxyvitamin D₃
1,25D: 1,25 dihydroxyvitamin D₃
24,25D: 24,25 dihydroxyvitamin D₃
1,24,25D: 1,24,25 trihydroxyvitamin D₃
-D: vitamin D deficient
+D: vitamin D supplemented
Abx: antibiotics
ASF: altered Schaedler’s flora
BM: bone marrow
C/EBPβ: CCAAT enhancer binding protein β
CN: conventionalized
CFU: colony forming units
Ctl: control
DC: dendritic cell
FGF23: fibroblast growth hormone
cFGF23: cleaved FGF23
iFGF23: intact FGF23
FOXP3: Forkhead box P3
GF: germ free
IFN: interferon
IL: interleukin
IBD: inflammatory bowel disease
KO: knock out
LPS: lipopolysaccharide
NFκβ: nuclear factor κβ

PTH: parathyroid hormone

RANK: receptor activator of nuclear factor κβ

RANKL: receptor activator of nuclear factor κβ ligand

RXR: retinoid X receptor

STAT1: Signal transducer and activator of transcription 1

TCR: T cell receptor

Th: T helper

TLR: toll-like receptor

TNF: tumor necrosis factor

Treg: regulatory T cell

TRPV5/6: transient potential vanilloid type 5/6

VDR: vitamin D receptor

VDRE: vitamin D response element

WT: wild type
ACKNOWLEDGEMENTS

There are so many people I want to thank for their help and support through my PhD program at Penn State. First, I would like to thank my advisor, Dr. Margherita Cantorna for her instruction and patience. While it sometimes felt like working with Q because I was never quite sure what she had in store, she gives her students freedom to try new experiments and doesn’t fault their mistakes, provided a lesson is learned. Dr. Cantorna gave valuable guidance as I developed the critical thinking necessary for science, but also for so many other things, and I hope to never lose that perspective. I would also like to thank the rest of my committee members: Dr. Andrew Patterson, Dr. Sandeep Prabhu, and Dr. Connie Rogers. I never doubted their office doors would be open to me when I had questions or needed help.

I would like to thank my lab members, Lindsay Snyder, Yang-Ding Lin, and Veronika Weaver for their support, advice, and friendship. I do not exaggerate when I say the lab would cease to function without Veronika. Nor am I exaggerating when I say Lindsay is a fabulous baker, and it has been a pleasure to be in the same lab with her based on her cupcakes alone. Lindsay and Veronika have been such good friends in and out of the lab, and I look forward to long friendships with them wherever our paths take us. I would like to thank past Cantorna lab members, the Huck Institute and Veterinary and Biomedical Sciences program, and the Metabolomics Core for their help during my time at Penn State. Special thanks go to Dr. Philip Smith whose expertise in LCMS was critical for my experiments, and whose sense of humor made my many hours in the Metabolomics Core more enjoyable.
Finally, my family and friends deserve my deepest thanks. I do not have the words to express my gratitude to Sean Bodley for his unwavering support and encouragement (especially when “support” meant doing all of the dishes), except to say that he is a rare and beautiful unicorn amongst men. My sister, Ruthie Bora, is my rock. She pushed me to broaden my thinking as far back as I can remember, and I will always look up to my big sister. My Mom and Dad taught me strength, resilience, and work ethic. My parents were the first to encourage my curiosity about the world around me which formed the basis of my interest in science. My thanks also go to the Greenwald and Bodley family – I am lucky to count them as my family. I want to thank my friends and my peers in the science policy community: both helped me realize science does not exist in a vacuum, and it is not enough to just do your work and hope the rest of world does not affect you. I would also like to thank Pippa E. Pup for all the joy she brings, despite the fact that she will never read this because she is in fact a pup. My family and friends made me who I am today, and without their support I would not be here. A very sincere and heartfelt thanks to everyone who has supported me through grad school!
Chapter 1

Introduction
Vitamin D deficiency and health

Vitamin D is a fat-soluble vitamin that is a critical regulator of calcium and phosphate homeostasis. According to guidelines established by the National Institutes of Health (NIH), serum 25-hydroxyvitamin D (25D) concentrations of less than 12 ng/mL is considered deficient, 12-20 ng/mL is considered insufficient, and 20-50 ng/mL is considered sufficient (1). Vitamin D deficiency causes rickets in children and osteomalacia in adults, which are characterized by defects in bone mineralization, and vitamin D insufficiency results in poor bone health and osteoporosis (1, 2). Vitamin D deficiency and insufficiency are correlated with low sun exposure; therefore, vitamin D insufficiency is more common in northern and southern regions than equatorial regions (3). Fortifying foods such as dairy products, juices, and cereals with vitamin D has made rickets rare, but vitamin D insufficiency is common and increasing. In the United States, one third of the population was vitamin D insufficient or deficient, according to the most recent National Health and Nutrition Examination Survey (NHANES), and this frequency increased from previous surveys (4). The Endocrine Society argues 25D levels below 30 ng/mL should be considered insufficient, and by this standard about 40% of men and 50% of women are vitamin D insufficient (5, 6). However, the functions of vitamin D go beyond bone health. Vitamin D insufficiency is linked to incidence of immune-mediated diseases (3). Inflammatory bowel diseases (IBD) are chronic inflammatory diseases that affect about 1.3 million people in the United States. There is no cure for IBD, and in the US the direct cost of living with IBD is $5,000-8,000 per year (7). Insufficient vitamin D is associated with increased incidence and severity of IBD. Understanding how vitamin D
is metabolized provides insight into vitamin D’s important role in bone development and immune function.

**Endocrine metabolism of vitamin D**

Vitamin D is produced in the skin from UVB radiation of 7-dehydrocholesterol to form pre-vitamin D, which is isomerized by heat in the skin to form vitamin D (Fig. 1-1A) (8). Vitamin D can also be obtained through diet and supplements. Once obtained through diet or synthesized in the skin, vitamin D must be metabolized into its active, hormonal form. Vitamin D circulates to the liver, where it is hydroxylated to 25(OH) vitamin D (25D) by 25-hydroxylases. While multiple P450 cytochromes can catalyze the conversion of vitamin D to 25D, Cyp2R1 is the primary producer of 25D (Fig. 1-1A) (9, 10). 25D has a half-life of about 15 days, which is why it is a useful measure of vitamin D status. 25D can bind to the vitamin D receptor (VDR), though it requires concentrations 100-fold higher than the active form of vitamin D, 1,25dihydroxyvitamin D (1,25D) (11). In order to form active vitamin D, 25D is hydroxylated again in the kidney by 1α-hydroxylase (Cyp27B1) to form 1,25(OH)\(_2\)D. Excess 25D and 1,25D are hydroxylated in the kidney by the 24-hydroxylase (Cyp24A1) to form 24,25(OH)\(_2\)D (24,25D), and 1,24,25(OH)\(_3\)D (1,24,25D), which are degradation products of vitamin D and lack the hormonal activity of 1,25D (Fig. 1-1A) (12-15). Metabolized vitamin D is then excreted into the feces via bile (16).
Regulation of calcium and phosphate homeostasis by 1,25D

Biological actions of hormonal vitamin D are carried out via the vitamin D receptor (VDR), which is a nuclear receptor. As mentioned above, 25D can bind the VDR, but 1,25D has much greater affinity for the VDR (11, 17, 18). Binding of 1,25D to the VDR causes a conformational change that allows it to dimerize with the retinoid X receptor (RXR) in the nucleus. VDR/RXR binds vitamin D response elements (VDRE) in the promoter region of target genes, and can induce or repress transcription (19, 20). The functions of 1,25D are carried out through the VDR.

The major function of 1,25D signaling is to increase calcium mobilization by increasing calcium uptake from the intestine and kidney. 1,25D increases expression of a cation channel protein, transient potential vanilloid type 6 (TRPV6), and a calcium binding protein (Calbindin D9k) to increase calcium by facilitated diffusion in the epithelium of the gut (21-23). In the kidney 1,25D acts on similar proteins as the intestine, TRPV5 and Calbindin D28k, to increase calcium absorption (24). 1,25D also induces absorption of phosphate from the intestine by increasing expression of a sodium-phosphate transporter (25, 26). The effects of 1,25D on kidney phosphate are not as well understood as the effects in the intestine, and it is not clear if there is a direct effect of 1,25D on the sodium-phosphate transporters in the kidney (20). 1,25D acts on two other hormones that both cause phosphate to be excreted, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) (27-31). However, because of 1,25D-induced absorption of phosphate in the intestine, the net effect of 1,25D is to increase both serum calcium and phosphate.
1,25D causes bone resorption to raise circulating calcium levels. The balance of bone formation and bone resorption is mediated by two types of cells: osteoblasts and osteoclasts. Osteoblasts form new bone by depositing calcium phosphate. Osteoblasts mature into osteocytes, which make up cells of the bone matrix. Osteoclasts dissolve bone by secreting proteinases to degrade the bone matrix, and acid to dissolve the minerals deposited in the matrix (32). Osteoclasts are derived from a myeloid cell lineage and, similar to macrophages, can engulf osteocytes (33-35). 1,25D stimulates bone resorption indirectly by acting on osteoblasts which increase differentiation and activity of osteoclasts (36, 37). Osteoclasts are activated through receptor activator of nuclear factor κβ (RANK), and the ligand (RANKL) is produced by osteoblasts and osteocytes (38-41). 1,25D induces RANKL production in osteoblasts to promote development of bone resorbing osteoclasts, allowing calcium and phosphate mobilization from the bone.

Defects in 1,25D/VDR signaling highlight the importance of vitamin D in calcium and phosphate homeostasis. Inactivating mutations in Cyp27B1 causes vitamin D dependent rickets type I, and VDR mutations cause vitamin D dependent rickets type II (42). Both types of rickets are characterized by hypocalcemia and hypophosphatemia, bone and muscle pain, bone deformity, growth defects, weakness, and seizures (42). Cyp27B1 and VDR knock out (KO) mice display the same symptoms as humans. Supplying 1,25D treats rickets caused by Cyp27B1 mutations. Rickets caused by VDR mutations is vitamin D resistant, and is treated with calcium injections and/or high oral doses of calcium. In both types of rickets, reversing hypocalcemia eliminates the muscular and skeletal problems in mice and humans. In VDR KO mice, if VDR expression is restored just in the intestines the rickets phenotype does not occur (43).
Normalizing calcium levels in vitamin D dependent rickets also rescues hypophosphatemia, indicating vitamin D is not necessary for the body to use these minerals (42). The main function of vitamin D signaling is absorption of calcium and phosphate necessary for the normal development of bones, muscles, and neurological function.

Defects in 1,25D catabolism also result in poor bone health, as excess 1,25D tilts the balance of bone remodeling towards bone resorption and increases circulating calcium (20). Mutations in Cyp24A1, which causes idiopathic infantile hypercalcemia, are characterized by hypercalcemia, hypercalciuria, weight loss, muscle weakness, and kidney calcification and kidney stones (44). Patients with Cyp24A1 mutations typically have elevated 1,25D and low 24,25D, though 25D levels may be normal (44, 45). Genetic testing for Cyp24A1 mutations is a recent advance, and it has been suggested that defects in vitamin D catabolism may play a greater role in kidney stone formation than previously thought, especially if there is a family history of kidney stones (45, 46). Cyp24A1 KO mice also accumulate 1,25D, and have impaired bone mineralization. About half of pups die before weaning, indicating another pathway exists to breakdown 1,25D though it is not known why this compensatory mechanism is induced sporadically (47). Creating Cyp24A1/VDR double KO rescues the bone phenotype of Cyp24A1 KO, which suggests excess 1,25D, and not the lack of 24,25D, is the cause of health problems in the mice (47). Catabolism of 1,25D by Cyp24A1 is crucial for normal bone remodeling and maintaining calcium homeostasis.
**Positive regulation of 1,25D by PTH**

1,25D production is induced by PTH when calcium is low to prevent hypocalcemia. PTH is a hormone produced by the parathyroid gland, and like 1,25D, the main function of PTH is to increase calcium levels. PTH actions in the bone may be vitamin D independent, and both PTH and 1,25D stimulate RANKL production through their own respective receptors to free calcium from the bones (36, 48). PTH induces Cyp27B1 in the kidney to produce 1,25D (Fig. 1-1B) (49). Mice lacking the parathyroid gland are unable to produce 1,25D, and PTH injections restore 1,25D levels suggesting PTH is necessary to produce 1,25D (50). *In vitro*, PTH increased luciferase activity in a cyp27b1 promoter-reporter construct indicating PTH regulates Cyp27B1 at the gene level (29, 49, 51). PTH also decreases catabolism of vitamin D by decreasing the half-life of cyp24a1 mRNA (52-54). Increased PTH in response to low calcium is the primary inducer of Cyp27B1, resulting in increased 1,25D/VDR mediated intestinal calcium absorption.

**FGF23 and negative regulation of 1,25D**

FGF23 is a hormone produced in the bone by osteoblasts and osteocytes that acts on the kidney to inhibit phosphate uptake (55). FGF23 binds to multiple FGF receptors (FGFR), but the affinity of FGF23 for FGFR is low in the absence of the protein cofactor, αKlotho (56, 57). αKlotho is primarily expressed in the kidney indicating the kidney is the major site of action for FGF23, though αKlotho has been found in other tissues (58, 59). FGF23 inhibits sodium phosphate transporters, NaPi-2a and NaPi2c, in the kidney to reduce phosphate uptake (30, 60, 61). FGF23 is cleaved post-translationally to inhibit
excess phosphate excretion, and mutations that render FGF23 resistant to cleavage cause hypophosphatemic rickets/osteomalacia suggesting post-translational cleavage is an important mechanism in regulating phosphate (62, 63). iFGF23 is biologically active and cleaved FGF23 (cFGF23) is considered inactive (62, 63). It has been suggested that the C-terminus cleaved fragments inhibit iFGF23 by competitively binding FGFR/αKlotho to reduce phosphate wasting, but another study showed no effect of cFGF23 on phosphate levels (62, 64). Furthermore, in chronic kidney disease, elevated cFGF23 does not alleviate phosphate imbalances caused by elevated iFGF23, suggesting cFGF23’s role in signaling, if there is any, is not comparable to the role of iFGF23 (65). iFGF23 decreases phosphate absorption in the kidney, and defects in regulation of FGF23 causes hypophosphatemia.

FGF23 expression is regulated by 1,25D. 1,25D administration increased FGF23, and the FGF23 promoter has a VDRE (Fig. 1-1B) (30, 31). VDR KO mice produce very low levels of FGF23, indicating VDR signaling is necessary for FGF23 expression. However, if VDR KO mice are placed on high calcium, high phosphate “rescue diet,” FGF23 levels normalize (66). The exact mechanisms of vitamin D-independent FGF23 actions are not clear, but it is possible FGF23 responds directly to dietary phosphate. Dietary phosphate loading increased FGF23 in mice, but this did not occur in human males (67-70). FGF23 is induced by 1,25D/VDR signaling, but other mechanisms in FGF23 regulation are yet to be determined.

FGF23 negatively regulates 1,25D. FGF23 decreased expression of PTH in the parathyroid and decreased PTH levels in serum, resulting in decreased 1,25D (Fig. 1-1B) (59). In vitro cultures with cyp27b1 promoter-reporter constructs showed Cyp27B1 is
also directly inhibited by FGF23 (Fig. 1-1B), and FGF23 deletion in vivo upregulated expression of cyp27b1 in the kidney, increased 1,25D, and caused hypercalcemia and hyperphosphatemia (71, 72). Furthermore, FGF23 injections induced cyp24a1 expression, and decreased cyp27b1 expression in the kidney (30, 59). However, it is not clear if FGF23 acts directly on Cyp24A1, or if Cyp24A1 increases because suppression by PTH is lessened. FGF23 is an important negative regulator of 1,25D, and inhibits 1,25D directly and indirectly to prevent hypercalcemia and hyperphosphatemia.

Regulation of Cyp27B1 and Cyp24A1 by 1,25D.

In addition to negative regulation by FGF23, 1,25D also acts directly on Cyp27B1 and Cyp24A1 to limit excess 1,25D levels (73, 74). 1,25D decreases expression of Cyp27B1 to inhibit its own synthesis (73, 75, 76). Like other targets of vitamin D signaling, inhibition of Cyp27B1 by 1,25D is dependent on the VDR. 1,25D administration decreased Cyp27B1 expression in WT but had no effect on Cyp27B1 in VDR KO, and VDR KO over express Cyp27B1 in the kidney resulting in elevated 1,25D (77). The Cyp27B1 gene has VDREs, providing evidence that 1,25D/VDR directly inhibits Cyp27B1 expression to limit 1,25D production (73). 1,25D/VDR also limits 1,25D production by inducing Cyp24A1 to catabolize 1,25D and 25D (Fig. 1-1B) (78-81). 1,25D induced expression of Cyp24A1 both in the kidneys and intestine which suggests catabolism of 1,25D in extra-renal sites (52). Functional VDREs were identified in the cyp24a1 promoter, indicating direct induction of Cyp24A1 by 1,25D/VDR signaling (74, 80, 82). The PTH gene is also negatively regulated by VDR binding to VDRE, which not only inhibits Cyp27B1 induction by PTH but removes repression of
Cyp24A1 (83-85). 1,25D induces multiple negative feedback loops to maintain 1,25D levels critical for calcium and phosphate homeostasis.

**Non-classical actions of vitamin D and inflammatory bowel disease**

Correlations between low vitamin status and incidence of several immune-mediated diseases suggests vitamin D functions are not limited to bone health (86). The relationship between vitamin D and IBD is a good illustration of the role of vitamin D outside of calcium/phosphate homeostasis. IBD, which includes ulcerative colitis and Crohn’s disease, are chronic inflammatory diseases affecting the gastrointestinal tract. There is no cure for IBD and the causes are unknown, though genetic and environmental factors contribute to disease development (87). Genetic factors often include mutations in genes that regulate immune responses, leading to overactive Th1/Th17 responses and insufficient immune suppressive/Treg responses (88-91). This contributes to inappropriate immune responses to commensal microbes in the gastrointestinal tract (90, 92). Prospective studies showed a significant inverse risk between 25D levels and Crohn’s disease incidence and severity of disease (93-95). There are stronger associations between vitamin D and Crohn’s disease than ulcerative colitis, but one study showed that low 25(OH)D levels (<30ng/ml) resulted in more morbidity and treatment escalation over the 5 years for ulcerative colitis and Crohn’s disease (96). Few clinical interventions have been done using vitamin D supplementation and Crohn’s disease. Two open-label trials improved symptoms and disease index in participants, and one double-blind trial resulted in a statistically insignificant relapse rate with vitamin D treatment (97-100). Vitamin D status is strongly inversely associated with IBD and especially Crohn’s disease, but the
small number and varying designs of human interventions make it difficult to determine the effects of vitamin D in IBD.

Animal models of IBD provide more conclusive evidence that vitamin D regulates inflammation in colitis. VDR KO mice develop more severe disease in several different models of experimental IBD, and transfer of VDR KO T cells into wild-type (WT) mice increased colitis severity (101). Treating mice with 1,25D also ameliorated colitis in multiple models of disease (101). Furthermore, transfer of WT bone marrow into Cyp27B1 KO significantly reduced colitis severity (102). This suggests the increased colitis severity that occurs in Cyp27B1 and VDR KO is not a consequence of dysregulated mineral homeostasis. These results also suggest 1,25D/VDR signaling, specifically by immune cells, regulates inflammation in colitis.

One mechanism by which vitamin D regulates colitis is by promoting barrier function in the gut. The gut barrier is “leaky” in patients with IBD and in animal models of colitis, which drives inflammation and dysbiosis. Colons of VDR KO mice had less tight junction protein (ZO-1, occludin-1, and claudin-1, E-cadherin) on epithelial cells during dextran sodium sulfate colitis (103, 104). VDR KO also had poor barrier integrity in the absence of colitis, indicating they are predisposed to inflammation in the gut (104). 1,25D administration both in vivo and in vitro was protective against chemically-induced barrier disruption, and mice fed a vitamin D deficient diet had decreased tight junction protein levels in response to ethanol-induced colitis compared to vitamin D sufficient mice (103, 105, 106). Vitamin D promotes an effective barrier, which allows the host to remain tolerant to the microbiota.
The discovery of the VDR in immune cells introduced the possibility that 1,25D directly regulates immune function (107). Since then, 1,25D has been found to regulate monocytes, macrophages, dendritic cells (DC), and T cells (87). 1,25D skews macrophage and DC towards immune suppression (108, 109). Macrophage and DC that were pre-treated with 1,25D before stimulation with lipopolysaccharide (LPS) secreted less pro-inflammatory cytokines such as TNF-α, IL-6, and IFN-γ (108, 110). 1,25D treated macrophage and DC were also less effective at presenting antigen to T cells, had lower expression of T cell chemokines, and decreased expression of T cell costimulatory markers (111-114). 1,25D in DC also promoted more IL-10 production, and suppressed proliferation of T cells (109, 112, 113). 1,25D acts on T cells directly, in addition to indirect effects through macrophage and DC. In purified T cell cultures 1,25D inhibited expression of genes involved in T helper 17 (Th17) differentiation, and decreased expression of the pro-inflammatory cytokine, IL-17, produced by Th17 (115, 116). The VDR was found to directly bind the promoter of the il-17a gene and inhibit its activation, and promote Treg development via a VDRE in the foxp3 promoter (115, 117). 1,25D also skewed T cells from a Th1 phenotype towards a Th2 phenotype in vitro (118). The addition of 1,25D inhibited production of IFN-γ and stimulated production of IL-4 from T cells (118). The role of 1,25D in immune cells is to limit Th17/Th1 responses and promote Treg development and IL-10 production.
Extra-renal vitamin D metabolism

1,25D has robust immunosuppressive effects in vitro at nanomolar concentrations, but picomolar levels of 1,25D in circulation suggests endocrine vitamin D metabolism does not produce enough 1,25D to effect local immune responses (119). Studies into vitamin D metabolism and sarcoidosis first provided evidence of 1,25D production at local sites of inflammation. Systemic hypercalcemia caused by increased 1,25D occurs in about 10% of cases of chronic granulomatous diseases like tuberculosis and sarcoidosis, and is alleviated with anti-inflammatory steroids (120, 121). While this suggests immune regulation of 1,25D, an anephric sarcoidosis patient with high 1,25D levels proved extra-renal vitamin D metabolism occurs (121). Cultured pulmonary monocytes and macrophages from granuloma patients were able to convert 25D into 1,25D in response to pro-inflammatory cytokines, which confirmed immune cells could metabolize vitamin D (122, 123). Sarcoidosis studies introduced the possibility that in some rare instances, chronic production of immune stimuli can affect mineral homeostasis at the endocrine level.

Early research into vitamin D metabolism by immune cells indicated extra-renal vitamin D metabolism is regulated by inflammatory signaling, not calcium/phosphate homeostasis. Human DC, monocytes, and macrophages (already shown to produce 1,25D) expressed cyp27b1 and cyp24a1 (124-126). Murine macrophages, dendritic cells, and T cells also expressed cyp27b1 (102, 127). Cyp27B1 is induced by pro-inflammatory signals in extra-renal sites. Only stimulated T cells showed gene expression of cyp27b1 and cyp24a1, and were able to produce 1,25D (102, 128, 129). Bacterial signatures like LPS are sensed by receptors on host immune cells, such as toll-like receptors (TLR),
resulting in activation of those cells (130). Cytokines also provide further stimulus, such as IFN-γ from T cells (131). In monocytes/macrophage, LPS and IFN-γ both increased cyp27b1 gene expression via NF-κβ and STAT1 signaling (132, 133). Inhibition of either NF-κβ or STAT1 decreased cyp27b1 expression (132, 133). Though direct binding of NF-κβ or STAT1 to the cyp27b1 promoter was not observed, NF-κβ and STAT1 both induced a downstream transcription factor, C/EBPβ, which was found to bind directly to the cyp27b1 promoter (132, 133). Inflammatory stimuli increased expression of cyp27b1 in cultured monocytes, even in the presence of 1,25D, though 1,25D inhibits Cyp27B1 in renal cells (76, 132). Furthermore, treatment with IFN-γ and 1,25D decreased cyp24a1 expression compared to 1,25D treatment alone (124). 1,25D positively regulates Cyp24A1 in immune cells and renal cells (80, 81). But unlike renal regulation, IFN-γ inhibited expression of cyp24a1 in immune cells despite the presence of 1,25D, a stimulator of Cyp24A1 (124). Inflammatory signaling dictates autocrine/paracrine vitamin D metabolism to limit inflammatory responses.

**Vitamin D regulation of the microbiome**

Balanced populations of gut bacteria are important for gut health and proper immune development, whereas IBD is often characterized by dysbiosis of the gut microbiota and inflammation in the gastrointestinal tract (87). Cyp27B1 KO and VDR KO mice have altered microbial populations in the gut compared to WT mice, and have severe inflammation in multiple models of colitis (87, 104). Sequencing of fecal DNA from both Cyp27B1 KO and VDR KO mice showed increased frequencies of *Proteobacteria* and decreased frequencies of *Firmicutes*, a change that is mirrored in IBD.
patients (104, 134-137). Within the *Firmicutes* phylum there was a lower frequency of butyrate producing families, *Ruminococcaceae* and *Lachnospiraceae* (104). As the primary energy source for the gut epithelium, butyrate is important for barrier function. Butyrate increased tight junction protein expression and decreased permeability of epithelial cells *in vitro* (138, 139). Butyrate also inhibits Th17 and induces Treg to promote tolerance in the gut (104, 140, 141). Vitamin D supplementation in humans and mice decreased *Proteobacteria* and increased *Firmicutes*, suggesting vitamin D helps maintain the microbiome (142). 1,25D regulates host-microbial interactions to promote tolerogenic immune responses and gut barrier integrity, resulting in a beneficial gut microbiome.

**Antibiotics and vitamin D**

Antibiotics (Abx) are known to decrease the composition and the complexity of the microbiota and, therefore, alter immune sensing by the host of the microbiota (139). Short course ampicillin and neomycin treatment decreased TLR2 expression in the gut, and treatment with ampicillin, gentamycin, metronidazole, neomycin, and vancomycin increased permeability of the gut (139, 143). Two drugs used to treat tuberculosis, rifampicin and isoniazid, increased gene expression in the liver of two 25-hydroxylases (144). In the kidney rifampicin and isoniazid decreased Cyp27B1 and increased Cyp24A1 gene expression and protein levels (144). Three weeks of rifampicin and isoniazid treatment increased serum 25D and 24,25D, but no change in serum 1,25D was detected (144). Rifampicin also induced mRNA for *cyp3a4*, which is a major drug metabolizing enzyme that 24-hydroxylates vitamin D but does not use 25D as a substrate.
The microbiome was also found to regulate many enzymes in the Cyp1, Cyp2, and Cyp3 families, involved in drug metabolism, and some in the Cyp4 family, involved in fatty acid oxidation (148-151). Abx and the microbiome regulate host immune function and several enzymes in the P450 Cyp family.

**Objectives**

This dissertation explores the effects of Abx and the microbiome on endocrine metabolism of vitamin D. The microbiome has recently generated a lot of interest among scientists and the general population because of its broad impacts on host metabolism and health. Recently, some reports showed vitamin D and 1,25D/VDR signaling contribute to host regulation of the microbiome. Whether the microbiome regulates vitamin D metabolism has not been explored. It is well accepted that endocrine vitamin D metabolism is regulated by mineral homeostasis and paracrine/autocrine vitamin D metabolism is regulated by the immune system, without much crosstalk between the two. Based on experiments comparing metabolism and expression of P450 cytochrome enzymes in germ free and conventional mice, we hypothesized that the Cyp enzymes involved in vitamin D metabolism might be regulated by the microbiome. The data presented also determines whether changes to the vitamin D metabolism occur because of renal or extra-renal vitamin D metabolism. Chapter 2 tested indirect and direct effects of Abx on vitamin D metabolism in mice. Chapter 3 determined the microbiome regulates a bone-derived hormone that suppresses 1,25D levels, establishing a link between the microbiome and endocrine vitamin D metabolism. Chapter 4 summarizes the major
findings of this work and discusses its relevance in the context of the existing literature on regulation of vitamin D metabolism.
REFERENCES


1,25-dihydroxyvitamin D3 and parathyroid hormone in AOK-B50 cells. *Arch Biochem Biophys* 381: 323-327.


Figure 1-1. Vitamin D metabolism and regulation. A) Vitamin D is produced in the skin from UV-B radiation and 7-dehydrocholesterol, or is obtained through diet. Once in circulation, vitamin D is hydroxylated in the liver into 25(OH)D (25D), primarily by Cyp2R1. 25D is hydroxylated a second time in the kidney to form 1,25(OH)2D (1,25D) by Cyp27B1 to form the active form of vitamin D. 1,24D is also catabolized in the kidney by Cyp24A1 to form 1,24,25(OH)3D. Cyp24A1 also degrades 25D to form 24,25(OH)2D (24,25D).

B) PTH induces Cyp27B1 to produce 1,25D, 1,25D binds the VDR and allows it to dimerize with RXR and form a transcription factor. 1,25D/VDR induces FGF23. FGF23 then inhibits both PTH and Cyp27B1 to decrease 1,25D. Both Cyp27B1 and FGF23 induce Cyp24A1 to catabolize 1,25D and 25D, further limiting 1,25D production.
Chapter 2

Antibiotics regulate endocrine vitamin D metabolism through toll-like receptors.
ABSTRACT

Vitamin D deficiency changes the microbiota, but the role of the microbiota in vitamin D metabolism is unknown. The microbiota was depleted with antibiotics (Abx) to determine the effect on vitamin D metabolism. Vitamin D, 25D, and 24,25D were measured before and after Abx treatment for 2 wks. Abx increased 25D and 24,25D suggesting that the microbiota or Abx were altering vitamin D metabolism. Abx increased FGF23. Increased 25D, but not 24,25D, following Abx was found to be dependent on MyD88 signaling. This suggests toll like receptors (TLR) regulate systemic 25D levels, but not 24,25D. Abx treatments of VDR KO mice with WT hematopoietic reconstitution failed to induce 24,25D levels in VDR KO mice. The effects of Abx on 24,25D levels required that the VDR be expressed in tissues outside of the hematopoietic system (kidney) and demonstrate that the Abx effect is likely due to alterations to endocrine production of 1,25D acting through the VDR. The regulation of the 25D levels by MyD88 and the immune system is novel and suggests that the microbiota may be regulating 25D levels independently of vitamin D absorption.
INTRODUCTION

Vitamin D is a fat-soluble vitamin that is a critical regulator of calcium and phosphate homeostasis. Classically, vitamin D is an important regulator of bone health by regulating calcium absorption and resorption, and regulates immune function to promote anti-inflammatory immune responses (1, 2). The vitamin D that is either produced in the skin or obtained through diet is inactive. In the liver vitamin D is hydroxylated by the vitamin D 25-hydroxylases (Cyp2R1 and Cyp271A), to 25hydroxyvitamin D (25D) (3). The 25D that is produced has a relatively long half-life, making it a useful marker of vitamin D status (4). 25D does bind to the vitamin D receptor (VDR) at concentrations that are 100-fold higher than the active form of vitamin D, 1,25dihydroxyvitamin D (1,25D) (5). 25D is hydroxylated a second time in the kidney by the 1α-hydroxylase (Cyp27B1) to produce 1,25D. Vitamin D is metabolized into 1,25D, which binds the VDR to carry out the physiological functions of vitamin D.

Endocrine production of 1,25D is tightly regulated to prevent hypercalcemia associated with high amounts of 1,25D in circulation. The 24-hydroxylase (Cyp24A1) breaks down 1,25D and 25D to produce 1,24,25(OH)3D (1,24,25D), and 24,25dihydroxyvitamin D (24,25D), which are degradation products of vitamin D and lack the hormonal activity of 1,25D or 25D (6, 7). 1,25D production is induced by parathyroid hormone (PTH) in response to low calcium. 1,25D, via the VDR, induced calcium uptake in the intestine, and fibroblast growth factor (FGF)23 (8-10). 1,25D inhibits Cyp27B1 and PTH while inducing Cy24A1 expression, resulting in a feedback loop to suppress further 1,25D production (11, 12). 1,25D is controlled by multiple
positive and negative regulators that together control calcium and phosphorous homeostasis through 1,25D, PTH, and FGF23 production.

Cells outside of the kidney under some conditions express cyp24a1 and cyp27b1. Immune cells, especially human macrophage, dendritic cells, and monocytes express cyp24a1 and cyp27b1, and produce 1,25D (13, 14). Other immune cells including T cells express cyp27b1 when activated (7, 15, 16). In vitro, monocytes and macrophages required stimulation by toll like receptors (TLR) or cytokines for induction of Cyp27B1 (14). TLR ligation with lipopolysaccharide (LPS), and subsequent production of cytokines like interleukin (IL)-15 and IFN-γ induced Cyp27B1 in human macrophage to produce 1,25D (17-19). Activation of T cells was also required to induce cyp27b1 expression in human and mouse T cells in vitro (16, 20). Many of the signals that induce extra-renal vitamin D metabolism are present in the gut. Endotoxins like LPS are produced by the microbiota, and manipulation of the gut microbiota alters expression of TLRs that respond to the microbiota (24, 25). Conversely, 1,25D inhibited production of IFN-γ that would reduce the expression of Cyp27B1 by macrophage (21-23). Production of 1,25D by immune cells is regulated by cytokines and TLR ligands.

Antibiotics (Abx) alter the community structure of the microbiota in the gut, especially when delivered orally. Abx have also been shown to directly regulate expression of several of the cytochrome P450 enzymes that include the vitamin D metabolizing enzymes. Two Abx used to treat tuberculosis, rifampicin and isoniazid, decreased cyp27b1 expression in the kidney, and rifampicin alone increased expression of cyp2r1 and cyp27a1 in the liver of uninfected mice (28). Rifampicin treatment led to increased serum 25D and 24,25D (28). Isoniazid specifically targets Mycobacteria which
may limit its effects on the host microbiome, but rifampicin is a broad-spectrum antibiotic that would likely alter the composition of the gut microbiota (29, 30).

This study assessed the effect of an Abx cocktail on vitamin D metabolism in mice. Vitamin D, 25D, and 24,25D were measured before and after Abx treatment. Abx treatment increased 25D levels, and this was dependent on TLR signaling and therefore the microbiota. 24,25D levels also increased following Abx treatment, but 24,25D increases were not due to the increase in 25D substrate. Abx treatments of germfree (GF) mice did not result in elevation of 25D or 24,25D suggesting that the presence of the microbiota is required for the Abx mediated increases in 25D and 24,25D. The increase in 24,25D following Abx treatment was associated with an increase in FGF23 and lower PTH. 24,25D increases following Abx treatment required the VDR while 25D increases did not. The data suggest that changes in 24,25D are regulated by changes in 1,25D/FGF23 and PTH and therefore endocrine regulation of 1,25D levels. Conversely 25D levels were regulated by microbial signals independent of the VDR and 1,25D production. Together the data demonstrate that the broad spectrum Abx mediated increases in 25D and 24,25D depend on anti-microbial effects of the Abx, and are regulated directly by the microbiota shifts (25D) and by endocrine VDR/1,25D signals (24,25D).
MATERIALS AND METHODS

Mice. C57BL/6 VDR KO, CD45.1, and WT breeders were from Jackson Laboratories (Bar Harbor, ME). C57BL/6 MyD88 KO that have a defect in an adapter protein critical for microbial signaling through TLR were a gift from Dr. Matam Vijay-Kumar (The Pennsylvania State University, University Park, PA). C57BL/6 Cyp27B1 KO were a gift from Dr. Hector DeLuca (University of Wisconsin, Madison, WI). Gnotobiotic mice were bred and maintained by The Pennsylvania State University Gnotobiotic Animal Research Facility. Mice were orally supplemented with 5µg vitamin D₃ in corn oil, or given an equal volume of corn oil as a vehicle control, three times weekly throughout the experiment. The Abx treatment cocktail contained 4 Abx: ampicillin (1g/L), metronidazole (1g/L), neomycin (1g/L), and vancomycin (0.5g/L) in drinking water, ad libitum, for 2wks. Mice were fed purified diets without any added vitamin D (D-) that were made in the lab, contained agar and prevented dehydration due to the Abx treatment (26). GF mice were fed autoclavable diets in order to maintain the GF status of the mice. GF mice received the Abx cocktail with 5% sucrose to prevent dehydration. All experimental procedures were approved by the Office of Research Protection’s Institutional Animal Care and Use Committee (Pennsylvania State University, University Park, PA).

Sample preparation for LC-MS/MS. Serum preparation was done as described by Kaufmann et al. (31). 100µL of pooled C₃-vitamin D₃ (100 ng/mL), d₃-25D₃ (100 ng/mL), and d₆-24,25D₃ (50ng/mL) (Isosciences, King of Prussia, PA) was added to 50 µL of each serum sample. Standards were also from Isosciences (synthetic vitamin D₃, 25D₃ and 24,25D₃). 50 µL of 0.1M hydrochloric acid, 50 µL 0.2M zinc sulfate, and 225
µL of 100% methanol were added to precipitate protein. Organic extraction was done by adding 350 µL hexanes, 350 µL MTBE (methyl tertiary butyl ether, Acros Organics, Geel, Belgium) and collecting the upper organic phase. Derivatization was done by dissolving the dried residue in 30 µL DMEQ-TAD (0.1 mg/mL in ethyl acetate, Santa Cruz Biotechnology, Santa Cruz, CA), incubating for 1 h, drying, and the residue was dissolved in 30 µL 50/50 acetonitrile/water. All other LC-MS/MS solvents and reagents were Optima LC-MS grade (Fisher Scientific, Pittsburgh, PA). The limit of detection was 1 ng/mL for vitamin D, 25D, and 24,25D.

**LC-MS/MS.** Samples (5 µl) were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH Phenyl column (100mm x 2.1mm 1.7 µm particle size). Solvents used were HPLC grade water with 0.1% formic acid and HPLC grade acetonitrile with 0.1% formic acid. The initial conditions were 70% water and 30% acetonitrile, increasing to 50% acetonitrile at 10 min, 90% acetonitrile at 12 min where it was held at 90% acetonitrile until 13 min before returning to the initial conditions. The eluate was delivered into a 5600 (QTOF) TripleTOF using a Duospray™ ion source (AB Sciex, Framingham, MA). The capillary voltage was set at 5.5 kV in positive ion mode with a declustering potential of 80V. The mass spectrometer was operated with a 250 ms TOF scan from 50 to 950 m/z, and 7 100 ms MS/MS product ion scans (m/z 730.5, 733.5, 746.5, 749.5, 762.5, 765.5, 768.5) from 50 to 950 per duty cycle using a collision energy of 45V with a 30V spread. Chromatograms and ion spectra, and structure and fragmentation of DMEQ-TAD adducts of vitamin D, 25D, and 24,25D used for detection, are shown in Fig 2-1.
**Bone marrow (BM) transplantation.** Donor BM cells were from WT CD45.1 donors and were transferred into sub-lethally irradiated CD45.2 WT or VDR KO recipients as described (32). Mice were allowed to recover for 4 wks and reconstitution was evaluated in the blood by flow cytometry using antibodies to CD45.1 (A20, BD Pharmingen, San Diego CA) and CD45.2 (104, BioLegend, San Diego, CA), and analyzed on an Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).

**Serum calcium measurements.** Serum calcium levels were measured by colorimetric assay using QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA), according to manufacturer’s instructions.

**ELISA.** Parathyroid hormone (PTH) (1-84) and intact FGF23 levels in serum were measured by ELISA, according to manufacturer’s instructions (Immutopics, San Clemente, CA). Limits of detection were 32 pg/mL PTH, and 25 pg/mL iFGF23.

**RNA isolation and RT-PCR.** RNA from kidney, liver, and colon was extracted using TRIzol reagent using manufacturer’s instructions. 4 μg/sample RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Promega, Madison, WI). Mouse hprt, cyprR1, cyp27a1, cyp24a1, cyp27b1, vdr, tnf-α, il-1β, il-6, and ifn-γ mRNA were quantified by real-time PCR using the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Rockford IL) with StepOnePlus software and BioRad SYBR Green Master Mix (Hercules, CA). Gene expression was determined as relative expression on a linear curve based on a gel-extracted standard and was normalized to hprt amplified from the same cDNA mix. Primer sequences can be found in Table 1.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA). Two-way ANOVA with Bonferroni’s post hoc test was used to
compare levels of vitamin D metabolites, PTH, and FGF23 in mice. Two-tailed Student $t$ test or Mann-Whitney test was used for some vitamin D metabolite, FGF23, and calcium measurements where only two groups were compared. For all analyses, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.0001$. 
RESULTS

*Abx treatments induced vitamin D metabolism.* Broad spectrum Abx treatment was used to disrupt the microbiota in mice (26). Mice were fed –D diets and given corn oil (control, Ctl) or 5 μg vitamin D3 (+D) orally 3 times weekly for 4wks and blood was collected at 2 wks of +D/Ctl and before Abx treatment or at 4wks of +D/Ctl and after 2 wk Abx treatment (Fig. 2-2A). The +D mice had significantly higher levels of vitamin D, 25D and 24,25D than the Ctl mice (Fig. 2-2B-D). In the absence of Abx treatment, +D supplementation of WT mice for 4wks did not further raise vitamin D, 25D or 24,25D levels over the 2wk +D levels ruling out simple accumulation of vitamin D and vitamin D metabolites in the +D groups (Fig 2-3A). Vitamin D levels in the blood of Ctl mice were very low, and did not change significantly after Abx (Fig. 2-2B). +D mice had significantly higher serum vitamin D than Ctl mice and vitamin D went up in the +D mice after Abx (P<0.0001, Fig. 2-2B). 25D levels in the Ctl group were less than 12 ng/mL and therefore vitamin D deficient (Fig. 2-2C). 25D was significantly higher in +D mice compared to Ctl mice (P<0.0001), and increased significantly in +D mice after Abx (P<0.0001, Fig. 2-2C). +D mice had significantly higher 24,25D than Ctl mice (P<0.0001), which also increased significantly after Abx (P<0.01, Fig. 2-2D). There were significant interaction effects between +D and Abx treatments on the vitamin D, 25D and 24,25D levels (Fig. 2-2B-D). +D mice were sacrificed before and after Abx treatments (Fig. 2-4A) for mRNA analysis of *vdr, cyp2r1, cyp27a1, cyp24a1,* and *cyp27b1.* Expression of mRNA for the *vdr* was not affected by Abx treatment in the kidney or colon (Fig. 2-4A, B). There were no statistically significant effects of Abx treatments on *cyp2r1* and *cyp27a1* mRNA expression in the liver (Fig. 2-4C), and no significant effects
of Abx on cyp27b1 mRNA in either the kidney or colon (Fig. 2-4A, B). However, Abx significantly increased cyp24a1 expression in the colon (P=0.0008). Abx treatments increased 25D and 24,25D levels in the serum, though only expression of cyp24a1 in the colon changed significantly in Abx treated mice.

The data in Fig. 2-2 used mice of either sex. In order to determine if there was a sex effect in our results, +D males and females were analyzed separately (Fig. 2-5). Females had higher levels of vitamin D (P=0.0008) and 25D (P=0.0004) compared to males. There was also a significant interaction between sex and Abx treatment for vitamin D (P=0.01) and 25D (P=0.002, Fig. 2-5A, B). Sex was not a factor on 24,25D levels (P=0.3) and there was no interaction between sex and Abx treatment for this metabolite (P=0.1, Fig. 2-5C). Abx treatment of +D mice resulted in increased vitamin D, 25D and 24,25D levels. In addition, there was an effect of sex on vitamin D and 25D levels and females had higher vitamin D and 25D levels than males. The measurement of 24,25D was not affected by sex in our +D mice.

Differential effects of single Abx on vitamin D metabolites. The Abx cocktail increased 25D and 24,25D metabolites in +D mice. The effect of two of the individual Abx metronidazole (M) and vancomycin (V) were tested alone. Mice were all +D since the effects of Abx was only seen following +D supplementation (Fig. 2-2). Vitamin D and 25D levels were significantly increased following treatment with either M or V alone (Fig. 2-6). Neither M nor V treatment alone had an effect on serum 24,25D levels (Fig. 2-6). The increased production of 24,25D with broad-spectrum Abx was not due to either M or V treatment alone. Interestingly, even though 25D levels increased with M or V treatment alone, there was no subsequent increase in 24,25D. The data suggest that the
increased substrate (25D) for Cyp24A1 following Abx treatment is not the cause of the increased 24,25D levels.

The VDR is necessary for Abx-induced production of 24,25D. Abx increased levels of vitamin D, 25D, and 24,25D in +D WT mice (Fig. 2-2). Production of 24,25D is regulated by the VDR (33, 34). +D WT and VDR KO mice were treated as described in Fig. 2-2A. The serum vitamin D levels did not change with Abx treatment in +D WT mice in this experiment (Fig. 2-7A). Overall the effect of Abx on vitamin D levels in the +D WT mice was variable, and vitamin D levels increased significantly in half of our experiments (Fig. 2-2, 2-6) but not in the WT controls for this experiment (Fig. 2-7). +D VDR KO mice in both experiments and overall had increased vitamin D levels following Abx treatment (P=0.0002, Fig. 2-7A). There were significant interactions between genotype and Abx on serum vitamin D levels (P=0.04, Fig. 2-7A). There was a significant increase in serum 25D levels in +D WT mice treated with Abx even though the serum vitamin D levels were not higher in these mice following Abx treatment (Fig. 2-7A), suggesting 25D levels are not simply increasing because of increased vitamin D absorption. The effect of Abx treatment to increase 25D levels occurred in +D VDR KO mice (Fig. 2-7A). There was no effect of genotype on 25D levels and no interaction between genotype and Abx on 25D levels (Fig. 2-7A). As in Fig. 1D, there was a significant effect of Abx on serum 24,25D levels in +D WT (P=0.0009, Fig. 2-7A) mice. Very little 24,25D was detectable in VDR KO mice regardless of whether they received Abx (Fig. 2-7A). Genotype was a significant factor in determining the 24,25D levels (P<0.0001) and there was a significant interaction between genotype and Abx on serum
24,25D levels (P<0.0001, Fig. 2-7A). Abx treatment had no effect on 24,25D in VDR KO mice suggesting that the effect of Abx on 24,25D levels required the VDR.

The VDR is required for the production of 1,25D and the effects of 1,25D in the endocrine regulation of vitamin D metabolism. In order to determine whether the changes in 25D and 24,25D could be as a result of extra-renal production of 1,25D, BM chimeras were generated. The immune cells in the blood of the BM chimeras were 90% of donor origin (WT, CD45.1) in both the WT and VDR KO recipients. Serum vitamin D levels increased significantly in both WT recipients receiving WT BM (WT-WT), and VDR KO recipients receiving WT BM (WT-VDR KO) after Abx (P=0.003), and there was no recipient genotype effect (Fig. 2-7B). There was no significant interaction between recipient genotype and Abx on serum vitamin D levels (P=0.4, Fig. 2-7B). There was an effect of Abx on 25D levels (P=0.005), and a significant increase in 25D levels in WT-WT mice treated with Abx (Fig. 2-7B). However, 25D levels in WT-VDR KO mice were not affected by Abx (Fig. 2-7B). There was no effect of recipient genotype on 25D levels, or a significant interaction between recipient genotype and Abx. As in normal WT, there was a significant effect of Abx on serum 24,25D levels in WT-WT (P=0.02, Fig. 2-7B). 24,25D production in WT-VDR KO was very low and was not affected by Abx treatment, similar to VDR KO (Fig. 5A). Recipient genotype was a significant factor in determining the 24,25D levels (P<0.0001) and there was a significant interaction between recipient genotype and Abx on serum 24,25D levels (P<0.04, Fig. 2-7B). Interestingly, Abx had no effect on 25D levels in WT-VDR KO mice even though there was an increase in vitamin D following Abx treatment of the WT-VDR KO mice. 24,25D were
low in WT-VDR KO mice, indicating that the transplanted WT BM was not able to
induce Cyp24A1 and not responsible for the Abx induced production of 24,25D.

**WT levels of 24,25D in Cyp27B1 KO mice following Abx treatment.** 1,25D binds to the
VDR to induce Cyp24A1 and production of 24,25D. Cyp27B1 KO mice are deficient for
production of 1,25D, when they are fed +D diets, 25D accumulates in the serum and can
bind to the VDR (5, 35). Vitamin D levels did not change after Abx in either the WT or
Cyp27B1 KO, and there were no effects of Abx, genotype or the interaction on vitamin D
levels (Fig. 2-8A). The effect of Abx treatment on 25D levels in WT mice was, as
expected, a significant increase (Fig. 2-8B). The +D treatment of Cyp27B1 KO mice
resulted in extremely high levels of 25D in the mice demonstrating accumulation of this
metabolite in the Cyp27B1 KO mice similar to what has been reported previously (Fig. 2-
8B and (5, 35)). Abx treatment suppressed the production of 25D in the Cyp27B1 KO
mice significantly (Fig. 2-8B) and resulted in 25D levels similar to those in the WT mice
after Abx. The 24,25D levels were higher in WT mice following Abx but unlike all other
experiments in WT mice there was not a significant effect of Abx on WT levels of
24,25D in these experiments (Fig. 2-8C). The levels of 24,25D in the +D Cyp27B1 KO
mice matched those in the WT mice and there was no effect of Abx on 24,25D levels in
Cyp27B1 KO mice (Fig. 2-8C). The effect of Abx on 25D in Cyp27B1 KO mice was
opposite the effect of Abx in WT mice perhaps because of the extremely high levels of
25D present in the Cyp27B1 KO mice (Fig. 2-8B).

**Microbial regulation of 25D but not 24,25D following Abx treatment.** MyD88 is an
adaptor protein necessary for signaling in most TLRs (37, 38). Vitamin D levels were not
affected by Abx in WT mice, but in MyD88 KO mice Abx treatment significantly
reduced vitamin D levels (Fig. 2-9A). As expected and consistent with previous results, 25D increased significantly in WT mice after Abx, treatment (P=0.02). Treatment of +D MyD88 KO with Abx had no effect on 25D levels (Fig. 2-9B). There was a significant effect of MyD88 genotype (P=0.05) on 25D levels and a significant interaction between genotype and Abx on 25D levels (P=0.02, Fig. 2-9B). Abx treatment of WT and MyD88 KO mice resulted in a significant increase in 24,25D levels for a significant effect of Abx on 24,25D levels (P=0.004, Fig. 2-9C). There was no effect of MyD88 KO genotype on 24,25D levels and no interaction between Abx and genotype on 24,25D levels in the mice (Fig. 2-9C). The data demonstrate that the increase in 24,25D following Abx treatment does not require MyD88 signals. Conversely the increase in 25D levels following Abx did require MyD88.

*Vitamin D and 24,25D levels are directly regulated by Abx.* Abx treatments have been shown to have direct effects on host metabolism (39). In order to determine whether there are direct effects of Abx on vitamin D metabolism, experiments were done in +D GF WT mice treated with Abx as described in Fig. 2-2A. Vitamin D levels increased significantly in GF mice after Abx treatments (P=0.007, Fig. 2-10A). There was no effect of Abx treatment on 25D levels in GF mice (P=0.2, Fig. 2-10B). Abx treatment in GF mice resulted in a significant decrease in 24,25D levels (P=0.02, Fig. 2-10C). The effect of Abx on 24,25D levels in conventional mice (Fig. 2-2) was the opposite of the effect of Abx in GF mice (Fig. 2-10C). Overall the direct effects of Abx on the host are to increase vitamin D levels and to decrease 24,25D production. Even though the amount of vitamin D was higher in the Abx treated +D GF mice there was no effect of Abx on 25D levels. The effects of Abx on 25D and 24,25D are different in GF and conventional mice.
Abx treatments affect PTH, FGF23 and TNF-α but not serum calcium. 1,25D together with PTH, and FGF23 regulate calcium homeostasis. There was no change in serum Ca in WT mice as a result of Abx treatments (Fig. 2-11A). Abx treatment resulted in an insignificant (P=0.08) decrease in PTH and a significant increase in FGF23 in WT mice (Fig. 2-10B, Table 2-2). In Cyp27B1 KO mice that had highly elevated 25D levels (Fig. 2-8B), PTH was also very high and FGF23 was lower than in WT (Fig. 2-11B, Table 2-2). Abx treatments of Cyp27B1 KO mice inhibited PTH and induced FGF23 significantly (Fig. 2-11B, Table 2-2). As expected, PTH was high in VDR KO mice and FGF23 was low (Fig. 2-11B). Abx treatment of VDR KO mice induced PTH to even higher levels, while having no effect on FGF23 levels (Fig. 2-11B, Table 2-2). There were effects of Abx on 25D (Fig. 2-7 and 2-8) and PTH (Fig. 2-11B) levels in VDR KO and Cyp27B1 KO mice. There were no effects of Abx on 24,25D levels in VDR KO or Cyp27B1 KO mice and no effect of Abx on FGF23 levels in VDR KO mice (Table 2-2).

Abx treatments increased FGF23 in WT mice, and the FGF23 increase was also evident in Abx treated WT-WT BM recipients (Fig. 2-11C). WT-VDR BM recipients had very low FGF23 levels that resembled those in VDR KO mice (Fig. 2-10C). Abx treatment of the WT-VDR mice had no effect on FGF23 levels (Fig. 2-11C). MyD88 KO mice had significantly higher FGF23 levels than WT mice (P=0.03) that were not changed by Abx treatments (Fig. 2-11C). The effects of Abx on 24,25D and FGF23, but not 25D, required the VDR (Table 2-2).

Colonic inflammation after 2 wks of Abx treatment was evaluated by measuring mRNA for several cytokines. mRNA for TNF-α significantly decreased in the colon of mice treated with Abx for 2 wks (Fig 2-11D). IL-1β decreased (P=0.07) and IL-6 increased
(P=0.06) with Abx treatment although the change did not reach significance for either IL-1β or IL-6 (Fig. 2-11D). There was no change in IFN-γ expression with Abx treatment (Fig. 2-11D). Abx treatments reduced mRNA for TNF-α and IL-1β and increased mRNA for IL-6 in the colon of WT mice.
DISCUSSION

Broad spectrum Abx increased 25D levels in part by decreasing the complexity and overall community structure of the microbiota. Prior to this study the production of 25D was thought to be controlled by vitamin D intake and not regulation of the 25 hydroxylase enzymes (41). Cyp2R1 is the major producer of 25D \textit{in vivo} (42). Mice with targeted deletions and humans with mutations in Cyp2R1 had reduced 25D levels without an effect on circulating 1,25D levels which probably reflects adequate 25D levels in the absence of Cyp2R1 (3, 43). A second 25 hydroxylase (Cyp27A1) is primarily involved in bile acid synthesis but may also contribute to 25D levels \textit{in vivo} (42). In mice, \textit{cyp27a1} deletion disrupted bile acid synthesis and cholesterol metabolism, but also increased 25D, probably by increasing \textit{cyp2r1} expression, suggesting Cyp2R1 may compensate for the lack of Cyp27A1 (3, 44). Together Cyp2R1 and Cyp27A1 account for most of the 25D produced \textit{in vitro} and \textit{in vivo} (42). Functional TLR signaling was required for Abx-induced increases in 25D. The microbiome played a role in regulating 25D levels, since Abx treatment of MyD88 KO mice was ineffective at raising 25D levels. The effect of the microbiome on 25D is not due to an indirect effect mediated by increased vitamin D absorption following Abx treatment since there were several experiments where 25D levels increased even when vitamin D levels were unaffected. The requirement of MyD88 for the Abx mediated effect on 25D suggest that TLR signals regulate 25D levels. Broad spectrum Abx disruption of the microbiota increased the amount of 25D available to the host.

The Abx mediated increase in 24,25D was not mediated by TLR signaling but was likely due to alterations in endocrine regulation of 1,25D. 24,25D is directly induced
by 1,25D/VDR, and is also regulated by PTH and FGF23 (48). FGF23 induced Cyp24A1, possibly by inhibiting PTH, while PTH inhibited Cyp24A1 to increase 1,25D (8, 9, 49). Increased 24,25D was not due to more of the 25D substrate, as there was no direct correlation between 25D and 24,25D levels in multiple experiments (Fig.2-6, 2-8, 2-9, 2-10). 24,25D only increased in mice that did not have defects in 1,25D or VDR. In the Cyp27B1 KO mice, Abx treatment reduced 25D levels but there was no effect on 24,25D. This could be due to increased FGF23 and decreased PTH, which increased Cyp24A1 to catabolize excess 25D. Abx treatment of Cyp27B1 KO mice restored PTH and FGF23 to WT levels, which provides evidence that dysregulated mineral homeostasis may be driven in part by the microbiome. Cyp27B1 KO mice have altered gut microbiota compared to WT mice (26). Cyp27B1 KO mice are a model of human vitamin D-dependent rickets, and based on the data, humans with Cyp27B1 mutations should have dysregulated mineral homeostasis driven by microbial and Abx mediated shifts in PTH and FGF23 (50). The effects of Abx on FGF23 and 24,25D required the VDR and in VDR KO mice FGF23 and 24,25D levels were unaffected by Abx treatments suggesting that endocrine control of 1,25D/VDR control of 24,25D probably through FGF23.

The immune system is the initial sensor of the microbiome, and disrupting the microbiota with Abx may affect host-microbial interactions. Abx treatments have been shown to increase LPS, translocation of commensals into host tissue, and production of TNF-α, IL-17, and IFN-γ in peripheral sites (51, 52). Using a cocktail of four Abx decreased tnf-α in the colon significantly. It has been shown that TNF-α increased FGF23 expression in vitro (53). The data show that following Abx treatment, FGF23 increased as TNF-α decreased in vivo. Whether or not the decrease in TNF-α is related to
the increase in FGF23 is not known. Regulation of FGF23 by TNF-α and/or other inflammatory stimuli has not been studied in vivo. The Abx treatment decreased tnf-α, and increased FGF23. What is not known is whether the shifts in tnf-α and FGF23 caused the changes to 24,25D levels in vivo.

The microbiome is important for digestion and nutrient absorption, and it is possible Abx treatments improved absorption of vitamin D. It is difficult to draw conclusions on Abx and vitamin D absorption as the increase in vitamin D following Abx treatment since did not increase in all experiments. Vitamin D increased in GF mice after Abx treatment, suggesting that in those controlled experiments there was a direct effect of Abx to increase vitamin D levels. The best evidence that the increase in vitamin D was not the cause of the increase in 25D levels was that 25D levels in GF mice treated with Abx did not increase even though vitamin D levels were significantly higher. In conventional mice treated with Abx, 25D increased which suggests the Abx mediated effect on 25D requires the microbiota. The Abx treatment effect on vitamin D levels does not require the microbiota and suggest direct regulation of signals in the gut that increase absorption of the vitamin D in GF mice.

Diseases in which vitamin D metabolism is already dysregulated, such as in chronic kidney disease, Abx-induced increases in FGF23 may contribute to disease development. Chronic kidney disease is characterized by poor renal function, and elevated FGF23 and suppressed 1,25D leading to hyperparathyroidism (56). Use of Abx that increase FGF23 could exacerbate the imbalance in 1,25D and FGF23, leading to worsening kidney function. Conversely, increased 25D as a result of Abx in diseases in which vitamin D absorption is poor, such as inflammatory bowel disease, may be useful
in raising vitamin D status. It is uncommon for people to take four Abx at once, but the
data shows M or V alone increased vitamin D and 25D, which is an unexpected benefit of
these two Abx. These results suggest the gut microbiome contributes to homeostatic
control of vitamin D metabolism, and altering the community of microbes in the gut with
short term Abx treatment may improve vitamin D status.
REFERENCES


Figure 2-1

A

B

Vitamin D$_3$  
24,25(OH)$_2$D$_3$  
25(OH)D$_3$  

Time, min

Intensity

Mass/Charge, Da

247.1  
488.2  
730.5
Figure 2-1. Chromatogram and fragmentation of DMEQ-TAD adducts of vitamin D, 25D, and 24,25D. A) Composite chromatogram of multiple reaction monitoring for vitamin D, 25D, and 24,25D. B) Mass spectra for DMEQ-TAD adducts of vitamin D (730.5->468.2), 245D (746.5->468.2), and 24,25D (762.5->468.2), indicating that m/z=468.2 is the major fragment of vitamin D, 25D, and 24,25D ions, and m/z=247.1 is the fragment of DMEQ-TAD.
Figure 2-2. Abx treatments induced vitamin D metabolism. A) Experimental design of Abx treatment and Ctl or +D supplementation. B) Vitamin D, C) 25D, and D) 24,25D levels from serum of control (Ctl) and vitamin D supplemented (+D) mice, before and after 2 wks of Abx. The results are from one representative of 3 independent experiments. Values are the mean ± SEM of n=4 per group. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-3. Vitamin D status with and without Abx. A) Vitamin D, 25D, and 24,25D at 2 and 4 weeks of vitamin D supplementation. Values are the mean ± SEM of n=5 mice per group, mixed sex. Student’s t-test was used to test significance. B) Ratio of 25D over vitamin D, 24,25D over vitamin D, and 24,25D over 25D from serum of control (Ctl) and vitamin D supplemented (+D) mice, before and after 2 weeks of Abx. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-4. Abx did not significantly affect expression of vitamin D metabolizing genes. +D mice were sacrificed before and after 2wk Abx treatments. A) and B) Expression of Cyp24A1, Cyp27B1, and VDR in the kidney and colon, respectively. C) Expression of Cyp2R1 and Cyp27A1 in the liver. Values are expressed as fold change of the water group. Values are the mean ± SEM of n=5 mice in the water group, and n=13-14 and mixed sexes in the Abx group and pooled data from 3 independent experiments. Mann-Whitney test was used to test significance.
Figure 2-5. Sex effects of +D supplementation and Abx. A) Vitamin D, B) 25D, and C) 24,25D levels from serum of +D male and female mice, before and after 2 weeks of Abx. Values are the mean ± SEM of a total of n= 8 males or n=5 females, and data from 3 independent experiments. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-6. Differential effects of single Abx on vitamin D metabolites. Vitamin D, 25D, 24,25D levels from serum of +D mice, before and after 2 weeks of A) metronidazole (M) or B) vancomycin (V). Values are the mean ± SEM of n=8 mice per group, 4 males and 4 females. Student’s t-test was used to test significance.
Figure 2-7. The VDR is necessary for Abx-induced production of 24,25D. A) Vitamin D, 25D, and 24,25D levels, and ration of 25D/vitamin D from serum of WT and VDR KO mice. Values are the mean ± SEM of n=11-14 mice per group, 15 males and 10 females, and 2 pooled independent experiments. B) Vitamin D, 25D, and 24,25D levels from serum of WT/WT and WT/VDR KO. Values are the mean ± SEM of n=4 recipient male mice per group. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-8. Normal 24,25D in Cyp27B1 KO regardless of Abx treatment.
A) Vitamin D, B) 25D, and C) 24,25D levels from serum of Cyp27B1 KO and WT.
Values are the mean ± SEM of n=12-14 mice per group, 16 males and 10 females combined from 3 independent experiments. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-9

Microbial regulation of 25D but not 24,25D following Abx treatment. A) Vitamin D, B) 25D, and C) 24,25D levels from serum of +D WT and MyD88 KO mice. Values are the mean ± SEM of n=5-6 mice per group, 5 males and 6 females. 2-way ANOVA with Bonferroni post-hoc test was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 2-10. Vitamin D and 24,25D levels are directly regulated by Abx. A) Vitamin D, B) 25D, and C) 24,25D levels from serum of +D WT GF mice. Values are the mean ± SEM of n=12 mice per group, and includes n=9 males and n=3 females, and pooled data from two independent experiments. Student’s t-test was used to test significance.
Figure 2-11. Abx treatments effect PTH, FGF23, and TNF-α but not serum calcium. A) Ca and B) PTH or iFGF23 levels. Values are the mean ± SEM of n=5-10 mice per group and data from 2 independent experiments. C) PTH and FGF23 values from WT/WT, WT/VDR KO and MyD88 KO mice. Values are mean ± SEM n=5-6 per group. D) Expression of TNF-α, IL-1β, IL-6, and IFN-γ in the colon of H2O or Abx treated mice, n=5-8 per group, and data from 2 independent experiments. Student’s t-test was used to test significance.
<table>
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<th>Gene</th>
<th>Forward Primer (5' to 3')</th>
<th>Reverse Primer (5' to 3')</th>
</tr>
</thead>
<tbody>
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<td>5'-CCA GTG TCA ATT ATA TCT TCC AC-3'</td>
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<tr>
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<tr>
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<tr>
<td>IL-6</td>
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<td>5'-CCA GGT AGC TAT GGT ACT CCA GAA-3'</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>5'-GGG TTG ACC TCA AAC TTG GCA-3'</td>
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Table 2-2. Summary of vitamin D metabolites and hormones measured in response to Abx.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Metabolite/Hormone</th>
<th>Effect of Abx</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Vitamin D</td>
<td>variable</td>
</tr>
<tr>
<td></td>
<td>25D</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>24,25D</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>FGF23</td>
<td>↑</td>
</tr>
<tr>
<td>Cyp27B1 KO</td>
<td>Vitamin D</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>25D</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>24,25D</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>PTH</td>
<td>↓</td>
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<tr>
<td></td>
<td>FGF23</td>
<td>↑</td>
</tr>
<tr>
<td>VDR KO</td>
<td>Vitamin D</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>25D</td>
<td>↑</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>PTH</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>FGF23</td>
<td>−</td>
</tr>
</tbody>
</table>

Change in vitamin D; 25D; 24,25D; FGF23, and/or PTH in response to Abx across different genotypes tested. ↑ indicates an increase, ↓ indicates no change, – indicates no significant change from water.
Chapter 3

The gut microbiome promotes endocrine production of hormonally active vitamin D by decreasing FGF23
ABSTRACT

The microbiome regulates metabolism, immune function, and bone health in the host. Germfree (GF) mice had hypocalcemia that was accompanied by low 1,25dihydroxyvitamin D (1,25D) levels and high fibroblast growth factor (FGF)23 levels. Conventionalization (CN) of the GF mice suppressed FGF23 at 3d which was followed by higher 1,25D at 7d and normalization of serum calcium. GF mice had lower levels of 25hydroxyvitamin D (25D) and 24,25dihydroxyvitamin D (24,25D) that increased following 7d CN of the mice. Introducing a limited number of commensals (8 commensals) increased 25D and 24,25D to the same extent as CN. Monocolonization with the enteric pathogen *Citrobacter rodentium* increased 25D and 24,25D, but the values only increased after 4 wks of *C. rodentium* colononization when inflammation resolved. CN resulted in an increase in TNF-α expression in the colon 2d after CN which is indicative of an ongoing immune response. The microbiota regulate calcium homeostasis by regulating endocrine production of 1,25D probably through suppression of FGF23 to reinstate homeostasis to the conventionalized, previously GF host.
INTRODUCTION

The microbiome is a community of microorganisms that have coevolved with their hosts to regulate host metabolism (1, 2). The development of germfree (GF) and gnotobiotic mice have been useful for studying the ways the microbiome affects the host. GF mice are resistant to obesity, and have altered bile acid metabolism and expression of drug metabolizing CYP genes (3-6). GF mice also have underdeveloped mucosal immune compartments, and peripheral immunity is skewed towards Th2 responses, while Th1 immunity is suppressed (7-11). Colonization of GF mice decreased Th2 responses and increased peripheral Th1 immunity, and induced changes in bone density driven by T cells (12-15). The microbiome interacts with the host to regulate metabolism and immune function.

GF mice had increased bone mass, including denser trabecular bone, and decreased bone-resorbing osteoclasts (14). Trabecular bone is formed primarily by osteoblasts, which deposit minerals in the bone matrix. As the matrix forms, osteoblasts become trapped and mature into osteocytes, where they signal to osteoclasts to regulate bone remodeling (16-18). Osteocytes are the main source of fibroblast growth factor (FGF)23 (19). Conventionalization (CN) of GF mice increased osteoclasts and decreased trabecular bone density (14, 17). The cause of the problems with bone remodeling in GF mice is not known.

Vitamin D is a fat-soluble vitamin that is either produced in the skin following UVB exposure, or consumed in the diet. Vitamin D is hydroxylated twice to form the active form of vitamin D, 1,25D. The first vitamin D hydroxalase is in the liver, where Cyp2R1 and possibly Cyp27A1 catalyze the production of 25hydroxyvitamin D (25D).
25D is a relatively stable molecule, has a relatively long half-life, and reflects vitamin D intake, which makes 25D a useful marker of vitamin D status. While 25D can bind the vitamin D receptor (VDR), it does so only at concentrations 100-fold higher than the active form of vitamin D, 1,25D. 1,25D is produced in the kidney by the 1α-hydroxylase (Cyp27B1) from 25D, and binds to the VDR to transcriptionally regulate target genes with vitamin D response elements in their promoters. Endocrine regulation of the production of 1,25D occurs in the liver and kidney.

FGF23 expression is regulated by 1,25D/VDR, and is part of the bone/kidney/parathyroid axis that regulates mineral homeostasis. In response to low calcium, the parathyroid hormone (PTH) stimulates Cyp27B1 to produce 1,25D. 1,25D production regulates bone resorption and increases mineral uptake from the small intestine to regulate phosphate and calcium levels. In order to limit excess bone resorption and mineral uptake, multiple negative feedback loops decrease 1,25D and PTH signaling. 1,25D inhibits PTH expression to limit Cyp27B1, and induces Cyp24A1. Cyp24A1 is a catabolic enzyme that is directly induced by 1,25D/VDR, and inhibited by PTH. Cyp24A1 hydroxylates both 25D and 1,25D to form 24,25-dihydroxyvitamin D (24,25D) and 1,24,25-trihydroxyvitamin D, which leads to the clearance of 1,25D and a decreased pool of available 25D for further Cyp27B1 mediated hydroxylation. 1,25D also induces FGF23 production in bone. FGF23 inhibits PTH and induces Cyp24A1, resulting in further inhibition of 1,25D production. FGF23, PTH and 1,25D form a series of feedback loops that together regulate 1,25D production and mineral homeostasis.
The effect of the microbiota on vitamin D, 25D, 24,25D, and 1,25D levels were determined before and after conventionalization (CN). GF mice had elevated FGF23, low 1,25D and 24,25D, and hypocalcemia at baseline. FGF23 was reduced 3d after CN, followed by increased levels of 1,25D, 24,25D, and serum calcium 7d after CN. CN also induced mild colonic hyperplasia and increased expression of TNF-α in the colon 2d after CN, indicating an immune response to CN. CN of GF mice demonstrated that the microbiota regulate endocrine 1,25D production probably through FGF23 production.
MATERIALS AND METHODS

Mice. GF C57BL/6 wildtype (WT) mice were bred and maintained at The Pennsylvania State University gnotobiotic animal research facility. Mice were orally supplemented with 5 µg vitamin D3 in corn oil, or vehicle treated with corn oil, three times weekly. Microbial transplantation was done using the cecal contents from WT mice or feces from Altered Schaedler’s flora (ASF, Taconic Biosciences, Hudson, NY) colonized mice. Infection with *Citrobacter rodentium* used strain ICC169 (gift of Gad Frankel, London School of Medicine and Dentistry, London, United Kingdom, nalidixic acid resistant) cultured in Luria-Bertani (EMD Chemicals, Inc., Gibbstown, NJ) broth containing 50 g/ml nalidixic acid (Sigma-Aldrich). Mice were infected by oral gavage with 1x10⁹ colony forming units (CFU) of *C. rodentium*. All experimental procedures were approved by the Office of Research Protection’s Institutional Animal Care and Use Committee (The Pennsylvania State University, University Park, PA).

Sample preparation for LC-MS/MS. Sample preparation was done as described by Kaufmann et al. (35). 100µL of pooled C₃-vitamin D₃ (100 ng/mL), d₃-25D₃ (100 ng/mL), and d₆-24,25D₃ (50ng/mL) (Isosciences, King of Prussia, PA) internal standard was added to 50 µL of sample. 50 µL of 0.1M hydrochloric acid, 50 µL 0.2M zinc sulfate, and 225 µL of 100% methanol were added to precipitate protein as described (35). Organic extraction was done by adding 350 µL hexanes, 350 µL MTBE (methyl tertiary butyl ether, Acros Organics, Geel, Belgium) and collecting the upper organic phase. Derivatization was done by redissolving the dried residue in 30 µL DMEQ-TAD (0.1 mg/mL in ethyl acetate Santa Cruz Biotechnology, Santa Cruz, CA), drying, and the residue was dissolved in 30 µL 50/50 acetonitrile/water. All other LC-MS/MS solvents
and reagents were Optima LC-MS grade (Fisher Scientific, Pittsburgh, PA). The limit of detection was 1 ng/mL each of vitamin D, 25D, and 24,25D.

**Measurement of 1,25D.** 1,25D levels were quantified by Dr. Claudia Zeirol using the LIASON XL chemiluminescent assay (DiaSorin, Stillwater MN) (36). The limit of detection was 5 pg/mL. Relative 1,25D was measured by LCMS using a 1,25D purifying kit and the instructions provided (Immundiagnostick, Bensheim, Germany). Briefly 300 µL pooled plasma or standards were used and samples/standards (authentic 1,25D₃, Isosciences) were spiked with 200 µL internal standard (d₃-1,25D₃, Isosciences). After elution from the columns, samples were dried and derivatized with DMEQ-TAD as described above.

**LC-MS/MS.** Samples (5µl) were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH Phenyl column (100mm x 2.1mm 1.7 µm particle size). Solvents used were HPLC grade water with 0.1% formic acid and HPLC grade acetonitrile with 0.1% formic acid. The initial conditions were 70% water and 30% acetonitrile, increasing to 50% acetonitrile at 10 min, 90% acetonitrile at 12 min where it was held at 90% acetonitrile until 13 min before returning to the initial conditions. The eluate was delivered into a 5600 (QTOF) TripleTOF using a Duospray™ ion source (AB Sciex, Framingham, MA). The capillary voltage was set at 5.5 kV in positive ion mode with a declustering potential of 80V. The mass spectrometer was operated with a 250 ms TOF scan from 50 to 950 m/z, and 7 100 ms MS/MS product ion scans (m/z 730.5, 733.5, 746.5, 749.5, 762.5, 768.5) from 50 to 950 per duty cycle using a collision energy of 45V with a 30V spread. 1,25D samples (10 µL) were separated as described above with a Waters (Milford, MA) BEH Phenyl
column (150mm x 1.0 mm 1.7 um particle size). The TOF scan was done as described above, with two 100 ms MS/MS product ion scans (m/z 762.5, 765.5) from 50 to 950 per duty cycle using a collision energy of 37V. Chromatograms, ion spectra, structure and fragmentation of DMEQ-TAD adducts of vitamin D, 25D, 24,25D, and 1,25D examples are shown in Fig 3-1.

**ELISA.** Parathyroid hormone (PTH) (1-84), intact FGF23 were measured by ELISA, according to manufacturer’s instructions (Immutopics, San Clemente, CA). Limits of detection were 32 pg/mL PTH, and 25 pg/mL FGF23.

**Serum calcium measurements.** Serum calcium levels were measured by colorimetric assay using QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA), according to manufacturer’s instructions.

**RNA isolation and RT-PCR.** RNA from kidney, liver, and colon was extracted using TRIzol reagent using manufacturer’s instructions. 4 μg/sample RNA was reverse transcribed into cDNA using AMV reverse transcriptase (Promega, Madison, WI). Mouse HPRT, Cyp2R1, Cyp27A1, Cyp24A1, Cyp27B1, VDR, TNF-α, IL-1β, IL-6, and IFN-γ mRNA were quantified by real-time PCR using the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Rockford IL) with StepOnePlus software and BioRad SYBR Green Master Mix (Hercules, CA). Gene expression was determined as relative expression compared to a linear curve based on a gel-extracted standard. Values were normalized to hprt, and expressed as a fold change over GF. Primer sequences can be found in Table 1.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA). One way-ANOVA with Tukey’s post-hoc, and Two-way
ANOVA with Bonferroni’s post hoc test was used to compare levels of vitamin D metabolites, PTH, and FGF23 in mice. Two-tailed Student’s $t$ test was used for some vitamin D metabolite, FGF23, and calcium measurements. For all analyses, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.0001$. 
RESULTS

25D and 24,25D increases following CN of GF mice. GF mice were supplemented with vitamin D for 2 and 4wks and vitamin D, 25D and 24,25D levels were measured in the +D GF mice (Fig. 3-2A). Vitamin D levels (P=0.1) and 24,25D levels (P=0.4) were not different after 2 or 4wks of +D supplementation of GF mice (Fig. 3-2A). There was a significant increase in 25D levels when comparing 25D levels following 2 and 4wks (P=0.02, Fig. 3-2A). There was an effect of 4wk vitamin D supplementation on 25D (compared to 2wk levels) but not 24,25D levels in GF mice.

GF mice were vehicle treated or supplemented for 2wks (+D) and then CN, while maintaining the vehicle and +D treatments (Fig. 3-2B). Vitamin D levels in the blood were not affected by either the vitamin D supplementation or CN (Fig. 3-2C). The levels of 25D increased significantly in both vehicle and +D treated mice after CN (P<0.0001, Fig. 3-2C). The increase in 25D seen following CN of vehicle treated mice suggests that the microbiota have additional effects on 25D levels outside of any accumulation seen following the 4wk +D treatment (Fig. 3-2A and 3-2C). +D mice had higher serum 25D compared to mice receiving vehicle (P=0.0005), though the combination of +D and CN resulted in the greatest increase in 25D (Fig. 3-2C). There was not a significant interaction between +D and CN on serum 25D levels (P=0.07, Fig. 3-2C). There was no change in 24,25D levels between vehicle (12 ng/mL) and +D (13 ng/mL) treated GF mice. CN caused an overall significant increase in 24,25D in vehicle and +D mice (P<0.0001). There was an effect of +D supplementation on serum 24,25D levels (P=0.009, Fig. 3-1C). There was a significant interaction between +D and CN on 24,25D levels in the serum of mice (P=0.002, Fig. 3-2C). The ratio of 25D/vitamin D and the
ratio of 24,25D/25D was significantly higher in +D mice following CN (Fig. 3-1D) suggesting that an increase in substrate following CN was not the cause of increased 25D and/or 24,25D in the +D mice. +D mice had the highest 25D and 24,25D levels following CN and therefore the rest of the experiments were done using +D treated mice.

The experiments shown in Fig. 3-2 used both male and female mice. Sex was also evaluated separately for effects on vitamin D metabolism (Fig. 3-3). +D males had higher vitamin D levels following CN than females (Fig. 3-3A). Overall, female mice produced more 25D than male mice (Fig. 3-3B). In addition, female mice had the biggest increase in both 25D and 24,25D following CN (Fig. 3-3B, C). There were significant effects of CN in both +D male and +D female mice for 25D and 24,25D levels (Fig. 3-3B, C). Females produced more 25D and 24,25D than males (Fig. 3-3). Since sex is an important variable affecting the measurements of vitamin D metabolites, experiments used equal numbers of males and females when possible, or only one sex as indicated in each figure.

*The effects of CN on expression of mRNA for Cyp metabolizing enzymes.* CN following WT microbial transplants increased 25D and 24,25D levels (Fig. 3-2). mRNA for *cyp2R1, cyp27A1, cyp24A1, cyp27B1,* and the *vdr* were measured in GF mice and 2wk CN mice. mRNA for *cyp2r1* (P=0.004) and *cyp27a1* (P=0.04) increased in the liver following CN (Fig. 3-4A). In the kidney *cyp24a1* was significantly inhibited (P=0.009, Fig. 3-4B) and *cyp27b1* was significantly increased following CN (P=0.03, Fig. 3-4B). There was no change in kidney expression of *vdr* mRNA following CN of GF mice (Fig. 3-4B). CN of the colon resulted in an insignificant induction of *cyp24a1* (P=0.06), no effect on *cyp27b1*, and significant inhibition of *vdr* expression (Fig. 3-4C). CN resulted in increased mRNA in the liver for both 25hydroxylating enzymes Cyp2R1 and Cyp27A1.
that corresponded with the increase in 25D following CN. In the kidney expression of mRNA for cyp24a1 went down at 2 wks post-CN even though 24,25D levels were higher in the serum of CN mice at this time point. Perhaps the higher 24,25D levels in the serum at 2 wks post-CN reflect the induction of mRNA for cyp27b1 that was higher in CN mice.

Kinetics of increased 24,25D following CN of GF mice. +D mice had increased 25D and 24,25D levels after 2 wk of CN (Fig.3-2). +D GF mice were dosed and sacrificed at several different time points following CN to determine the kinetics of the increases in 25D and 24,25D. Early following CN, vitamin D levels decreased and remained low 48h post-CN (Fig. 3-5A). The reduced vitamin D was accompanied by reduced levels of 25D at 12h and 24h post-CN (Fig. 3-5A). 25D levels increased to baseline GF values at 48h (Fig. 3-5A). 24,25D levels went up early post-CN compared to GF mice, but did not reach significance at 48h post-CN (Fig. 3-5A). Experiments were done to look at d3-14 post-CN, using all female mice (Fig. 3-5B) and male and female mice (Fig. 3-5C). Vitamin D levels increased significantly at 3d and were higher at 14d post-CN than in GF female mice (P<0.0001, Fig. 3-5B). In mixed male and female mice vitamin D was not affected by CN at 3-14d (Fig. 3-5C). 25D levels in females spiked at 3d and remained higher than GF mice at 14d post-CN (Fig. 3-5B). In mixed sexes 25D was not affected by CN even 14d after CN (Fig. 3-5C). 24,25D levels increased in female and mixed sexes following CN (Fig. 3-4B, C) and the increase in 24,25D was significantly higher by 7d of CN (Fig. 3-5). The more frequent collection of blood needed for the kinetic experiments shown in Fig. 3-4 required sacrificing mice at different time points. The data shown in Fig. 3-2 utilized the same mice before and after CN. This may account for the
discrepancies between the effects of CN on vitamin D and 25D measurements between experiments. Nonetheless, the data are consistent for the increased 24,25D levels as a result of CN. The effects of the microbiota on 24,25D levels were evident by d7 of CN.

Colonization with only commensals increased 24,25D. CN of GF mice increased 24,25D levels significantly. ASF, which contains eight commensal organisms, was used to colonize GF mice (37, 38). Colonization of +D GF mice with ASF significantly increased vitamin D, 25D, and 24,25D levels in the serum (Fig. 3-6). Vitamin D increased with ASF colonization at 2wks, from a mean of 76 ng/mL to 126 ng/mL (P=0.0006 Fig. 3-6A). Increased vitamin D levels with CN have been seen in some of experiments (Fig. 3-5B). ASF colonization for 2wks increased 25D levels significantly (P<0.0001, Fig. 3-6B), and increased 24,25D levels significantly (P=0.003, Fig. 3-6C).

+D GF mice were infected with C. rodentium, a murine enteric pathogen. C. rodentium infection of WT mice is cleared within 4 wks of colonization, while in GF mice the infection is not cleared although the mice no longer exhibit symptoms (inflammation/diarrhea) (39). Vitamin D levels went down in GF mice monoclonized with C. rodentium for 2wks and then back up after 4wks of monocolonization (Fig. 3-7A). The changes in vitamin D over time following monocolonization with C. rodentium were not significant (P=0.08, Fig. 3-7A). There was a decrease in 25D at 2wks post-colonization with C. rodentium that mirrored the decline in vitamin D at this time point (P<0.0001, Fig. 3-7B). 25D levels increased significantly at 4wks of monocolonization compared to the levels in the GF mice before C. rodentium infection (P<0.0001, Fig. 3-7B). There was an insignificant increase in 24,25D at 2wks and a significant increase at 4wks of monocolonization with C. rodentium (P<0.001, Fig. 3-7C). Monocolonization
with *C. rodentium* for 2wks decreased 25D levels and had no effect on 24,25D levels. The effects of *C. rodentium* monocolonization for 4wks were to increase 25D and 24,25D levels significantly compared to GF mice before infection (Fig. 3-7).

*Effects of CN on calcium, FGF23 and 1,25D levels.* GF mice were hypocalcemic and had high levels of FGF23 and low levels of 1,25D (Fig. 3-8). Calcium levels were still low in the serum of mice 3d post-CN and then went up at 1wk post-CN (P<0.0001, Fig. 3-8A). FGF23 levels went down 3d post-CN and remained reduced at 1wk and 2wk post-CN (P=0.007, Fig. 3-8B). 1,25D was extremely low in GF mice and in some experiments remained below or at detection levels (<5 pg/mL) at 0 and 3d post-CN (Fig. 3-8C, D). 1,25D levels were detectable at 7d after CN (Fig. 3-8D). At 2wks post-CN mice had significantly higher 1,25D levels than GF mice (P=0.007, Fig. 3-8C). The earliest effects of CN were on serum FGF23 levels, which decreased by d3 post-CN. CN increased serum calcium and 1,25D at 1wk, and continued to increase at 2 wk post-CN.

*The effect of CN on colonic inflammation.* The colon length of GF mice decreased at 3d of CN, and did not change significantly thereafter (P=0.005, Fig. 3-9A). mRNA for *tnf-α* increased significantly after 1d and was highest at 2d of CN (P<0.0001, Fig. 3-9B). By 3d of CN *tnf-α* mRNA went down to the same low level present in GF mice (Fig. 3-9B). mRNA in the colon for *il-1β, ifn-γ* and *il-6* did not change significantly with CN (Fig. 3-9B). *ifn-γ* mRNA trended towards being higher from 2d-14d post-CN, (insignificant increase, P=0.08). CN resulted in early (1-2d post-CN) expression of *tnf-α* mRNA in the colon and shortening of the colon compared to GF mice.

*Endotoxemia reduced 24,25D.* Sterile inflammation was induced in +D GF mice following injection with LPS. There was no significant change in vitamin D after LPS
injection, though there was a statistically insignificant decrease after 6h. (Fig. 3-10A)

There was no effect of LPS injection on 25D levels (Fig. 3-10B). 24,25D decreased significantly after LPS injection, with the largest decrease 6h after injection (P=0.03, Fig. 3-10C). Sterile inflammation reduced 24,25D levels in GF mice.
DISCUSSION

The data show CN of GF mice for 3d resulted in lower FGF23 levels, while the effects on 25D, 24,25D and 1,25D took 7d. The delay in the effects of CN on vitamin D metabolites relative to FGF23 suggest that FGF23 reductions by the microbiota may then regulate vitamin D metabolism. GF mice had significantly elevated FGF23, hypocalcemia and low 1,25D and 24,25D levels. Colonic shortening occurred early following CN, and TNF-α increased by d2 post-CN and resolved thereafter. TNF-α has been shown to increase RANKL expression, which induces osteoclastogenesis (40, 41). Reports show GF mice had fewer osteoclasts and less expression of TNF-α in bone, and perhaps as a result abnormally dense trabecular bone (14, 42). CN reduced the density of trabecular bone by increasing osteoclasts perhaps because of increased TNF-α and RANKL (14). However, the study found no differences in 25D or serum calcium between GF and CN mice (14), which is different than what the data show here. It has been suggested that the gut microbiome regulates bone remodeling by increasing peripheral CD4 T cells, and altering TNF-α and IL-1β (43). The data show that GF mice have abnormally high FGF23, low calcium and 1,25D levels. CN reinstates homeostasis by reducing FGF23 that likely re-establishes calcium homeostasis by inducing endocrine production of 1,25D.

24,25D levels were low in GF mice and increased following CN. However, gene expression for Cyp24A1 in the kidney of GF mice was high and decreased after CN, while 24,25D metabolite levels increased after CN. Others have shown increased cyp24a1 mRNA expression but low 24,25D levels (44). In a mouse model of chronic kidney disease, elevated FGF23 correlated with high expression of Cyp24A1 and low
expression of Cyp27B1, but 24,25D and 1,25D levels were both low (44). Deletion of FGF23 decreased cyp24a1 mRNA suggesting that FGF23 induces 24,25D production (44). Cyp24A1 is induced directly by 1,25D/VDR but 1,25D levels in GF mice were low. Instead increased expression of the cyp24a1 mRNA in GF mice may have been a result of the elevated FGF23 in GF mice. However, as elevated cyp24a1 transcript did not result in more 24,25D in GF mice, it is likely post-transcriptional modifications regulate the Cyp24A1 enzyme translation of function and resulted in low 24,25D. CN inhibited mRNA for cyp24a1 but resulted in higher 24,25D levels. 25D is not the only substrate for the Cyp24A1 enzyme, but since germfree mice had low 1,25D it seems unlikely that the rationale for low 24,25D and high mRNA for cyp24a1 is because of 1,24,25trihydroxyvitamin D. Measurements of mRNA for cyp24a1 do not correspond to the levels of 24,25D metabolite measured.

CN with whole cecal contents and colonization with only 8 commensals (ASF) both resulted in increased 25D and 24,25D levels. The lack of differentiation between the more limited commensals and complex microbial signals suggest that the host was responding to general bacterial signals, and not that a specific microbe is directing host vitamin D metabolism (42). Similar effects of CN on vitamin D metabolism using limited commensals and whole cecal contents could also indicate the 8 commensals are present in whole cecal contents, and sufficient to effect vitamin D metabolism. Monocolonization with the murine pathogen C. rodentium resulted in a slightly different response, whereby vitamin D and 25D levels decreased 2 wk after infection, with no effect on 24,25D levels at 2wks. C. rodentium induces acute colitis in both GF and CN mice, with peak inflammation occurring about 1-2wks post-infection (39). Both GF and CN mice resolve
inflammation by 4wks of infection, but GF mice never clear the bacteria (39). By 4 wks after infection, 25D and 24,25D were significantly higher than GF mice, suggesting that after inflammation resolves monocolonization with *C. rodentium* has the same effects as CN on vitamin D metabolism. Sterile and transient acute systemic inflammation following injection of GF mice with LPS significantly decreased 24,25D after 6h. LPS injection and sterile inflammation results in decreased production of 24,25D levels, which is different than the effect of *C. rodentium*, ASF, or CN of GF mice. The early suppression of 24,25D following LPS injection suggests that either Cyp24A1 is directly suppressed via TLR or cytokine (TNF-α) signals, or that rapid production of 1,25D suppresses Cyp24A1 and 24,25D levels following LPS injection.

The data also describe sex differences in 25D and 24,25D levels, both in vehicle and +D mice. CN increased 24,25D in both females and males, but females had a 2-2.5-fold higher increase following CN of GF mice compared to males in both +D and vehicle groups. It is well known that sex hormones have effects on the microbiome in females versus males (45-47). However, shifts in the composition of the microbiota in females versus males is probably not the cause of higher 25D and 24,25D levels in females, since ASF and CN had the same effect on 25D and 24,25D levels. Instead the higher 25D and 24,25D might reflect more robust immune responses in females versus males, and the well described effects of estrogen on adaptive immunity (48). Estrogen and the effect on the immune response in turn affect the composition of the microbiota in females (42, 49). Rapid loss of estrogen induces bone loss in female mice, and this is mediated by TNF-α, IL-17, and RANKL via effects on osteoclasts (49). In addition, there are interactions between 1,25D/VDR and estradiol in females (50). Estradiol increased expression of the
VDR and decreased expression of Cyp24A1 (50) suggesting direct regulation of vitamin D metabolism by estrogen. Increased 25D and 24,25D levels in female versus male mice is likely due to the increased adaptive immune response in females and possibly direct regulation of 25D and 24,25D levels by sex hormones.

The microbiome inhibited FGF23 and induced 1,25D. 24,25D, a downstream target of 1,25D signaling, was also increased. Acute inflammation following injection with LPS, CN, or infection with *C. rodentium* transiently suppressed 25D and 24,25D. Following the initial inhibition of 25D and 24,25D, the levels of these metabolites went up, and were higher by 7d post-CN. By 7 days after CN, inflammation was resolved and homeostasis in the GI tract was reinstated. The increases in 25D and 24,25D are therefore a result of reinstatement of homeostasis rather than direct regulation of 25D or 24,25D by the microbiota. Inflammatory bowel disease (IBD) patients often have dysbiosis, inflammation, and low 25D levels during active disease (51). The data suggest that decreased 25D following acute inflammation caused by dysbiosis may be a contributing factor to low vitamin D status in IBD patients. Furthermore, re-establishment of GI homeostasis (resolution of active inflammation) restored 25D levels. GF mice have impaired immunity, high FGF23, low 1,25D, and hypocalcemia. The microbiota serve to restore immune function, re-establish 1,25D levels and reinstate calcium and GI homeostasis. The microbiota are important and previously unappreciated regulators of vitamin D metabolism, FGF23, and calcium homeostasis that have implications for health and diseases like IBD.
REFERENCES


hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha,25(OH)2D3 in intact animals. *Endocrinology* 140: 2224-2231.


Figure 3-1

A

B
Figure 3-1, continued
Figure 3-1. Chromatogram and fragmentation of DMEQ-TAD adducts of vitamin D, 25D, 24,25D, and 1,25D. A) Composite chromatogram of multiple reaction monitoring for vitamin D, 25D, and 24,25D. B) Mass spectra for DMEQ-TAD adducts of vitamin D (730.5->468.2), 245D (746.5->468.2), and 24,25D (762.5->468.2), indicating that m/z=468.2 is the major fragment of vitamin D, 25D, and 24,25D ions. C) Chromatogram of multiple reaction monitoring for 1,25D, and mass spectra for DMEQ-TAD adduct of 1,25D (762.5->484.2), indicating m/z=484.2 is the major fragment of 1,25D ion. m/z=247.1 is the fragment of DMEQ-TAD.
Figure 3-2. Vitamin D metabolism is induced by the colonization of GF mice with microbiota. A) Vitamin D, 25D, and 24,25D at 2 and 4wks of +D supplementation. Values are ± SEM of n=10 mice per group, 5M and 5F. Student’s t-test was used to test significance. B) Experimental design of CN and vehicle or +D supplementation. C) Vitamin D, 25D, and 24,25D levels from plasma of GF before and after 2 wk CN. D) Ratio of 25D over vitamin D levels and ratio of 24,25D over 25D from vehicle treated or +D, GF males and females before and after 2 wk CN. Values are the mean ± SEM of n=9 mice per group, and two independent experiments that used 4 males or 5 females. Two-way ANOVA with Bonferroni post-hoc tests was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 3-3. Females produce more 25D and 24,25D than males. A) Vitamin D B) 25D, and C) 24,25D in vehicle and +D, male and female mice, before and after 2wks CN. Values are the mean ± SEM of n=9 mice per group, and two independent experiments that used 4 males or 5 females. Two-way ANOVA with Bonferroni post-hoc tests was used to test significance. *P<0.05, **P<0.01, ***P<0.001
Figure 3-4. The effects of CN on expression of mRNA for Cyp metabolizing enzymes
A) Kidney and b) colon expression of cyp24a1, cyp27b1, and vdr. C) Expression of cyp2r1 and cyp27a1 in the liver. Data is expressed as fold change over GF expression levels. Values are the mean ± SEM of n=13 mice per group, 20 females and 6 males, and 3 independent experiments. Student’s t-test was used to test significance.
Figure 3-5. Kinetics of increased 24,25D following CN of GF. A) Vitamin D, 25D, and 24,25D from 0-48h after CN. Values are the mean ± SEM of n=3-4 males per group. Two experiments were done that B) utilized only female mice values are the mean ± SEM of n=5-9 mice per group, or C) utilized male and female mice, 7 males and 17 females, values are the mean ± SEM of n=6/group. Vitamin D, 25D, and 24,25D levels were measured at d0, d3, d7, and d14 following CN. One-way ANOVA with Tukey’s post hoc tests was used to test significance. Bars without a letter in common indicate a significant difference.
**Figure 3-6.** Effect of colonization with 8 commensals (ASF). A) Vitamin D B) 25D and C) 24,25D before and after 2-week colonization with ASF. Values are the mean ± SEM of n=10-15 mice per group, 5 males and 10 females, and two independent experiments. Student’s t-test was used to test significance.
Figure 3-7. Monoclonization with *C. rodentium* increased 24,25D after 4wks. A) Vitamin D B) 25D and C) 24,25D at 0, 2, and 4wks after colonization with 1x10⁹ CFU *C. rodentium*. Values are the mean ± SEM of n=7-16 mice per group, 6 males and 10 females, and two independent experiments. One-way ANOVA was used to test significance. One-way ANOVA with Tukey’s post hoc tests was used to test significance. Bars without a letter in common indicate a significant difference.
Figure 3-8. Effects of CN on calcium, iFGF23, and 1,25D levels. A) Ca from mice 0, 3, 7 and 14d after CN. Values are the mean ± SEM of n=3-21 mice per group, and 1-3 independent experiments. B) iFGF23 levels from GF 0, 3, 7, and 14d after CN. Values are the mean ± SEM of n=3-6 female mice per group, and 1-2 experiments. One-way ANOVA with Tukey’s post hoc tests was used to test significance. Bars without a letter in common indicate a significant difference. C) 1,25D from GF before and after 2 wks CN. Values are the mean ± SEM of n=7 female mice per group, from 3 independent experiments. Student’s t-test was used to test significance. D) Relative intensity of 1,25D from GF 0, 3, 7, and 14d after CN, d0 (blue) and 3 (pink) were not clearly distinguishable from background noise.
Figure 3-9. Effect of CN on colonic inflammation. A) Colon length from GF 0, 3, 7, and 14d after CN. Values are the mean ± SEM of n=3 females per group. B) Expression of inflammatory cytokine genes, in the colon from GF mice at 0 and 12h, and 1, 2, 3, 7, and 14D after CN. Values are the mean ± SEM of n=3-6 mice per group, 15 males and 6 females, and 1-2 independent experiments. One-way ANOVA with Tukey’s post hoc tests was used to test significance. Bars without a letter in common indicate a significant difference.
Figure 3-10. Endotoxemia reduced 24,25D. A) Vitamin D, B) 25D, and C) 24,25D from +D GF, 6 and 12h after LPS injection. Values are the mean ± SEM of n=4-5 males per group. One-way ANOVA with Tukey’s post hoc tests was used to test significance. Bars without a letter in common indicate a significant difference.
Table 3-1. RT-PCR primer sequences.

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<th>Reverse (5’-3’) Primer</th>
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<td>5’-CCA GTG TCA ATT ATA TCT TCC AC-3’</td>
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<td>5’-GGG TTG ACC TCA AAC TTG GCA-3’</td>
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Chapter 4

Summary and Conclusions
This thesis explores the effect of Abx and the microbiome on host vitamin D metabolism. The microbiome is deeply embedded in our physiology, but regulation of endocrine vitamin D metabolism has not been linked to the microbiome until now. In Chapter 2, the gut microbiota was disrupted with Abx and the effect this had on vitamin D metabolism was assessed. Work by others showed that two Abx, isoniazid and rifampicin, increased expression of vitamin D metabolizing genes in the liver and kidney, but it was not clear if this was due to alterations in the gut microbiota or direct host effects (1). I show that a broad spectrum Abx cocktail increased 25D and 24,25D levels in mice. Increased 25D after Abx was mediated by TLRs, as knockout of TLR signaling eliminated the Abx effect on 25D and vitamin D. Conversely, 24,25D levels were still elevated with Abx treatment in the TLR knockouts. Together the data suggest that the effect of Abx on 25D and 24,25D levels are independent of each other and occur by different mechanisms. 25D increases following Abx are likely due to direct regulation via TLR mediated pathways.

Abx increased FGF23 which regulates 24,25D production via the production of 1,25D (2-4). In the absence of VDR signaling, neither 24,25D nor iFGF23 were affected by Abx, suggesting the changes required 1,25D/VDR (5-7). Transfer of WT bone marrow into VDR KO did not restore the Abx effect on 24,25D, demonstrating that it is unlikely that the Abx effect was mediated by immune production of 1,25D. The data suggest that the 24,25D increase following Abx is due to endocrine regulation of 1,25D levels via changes in FGF23.
Chapter 3 took advantage of GF mice to determine the role of different types of microbial populations in vitamin D metabolism, and the kinetics of altered vitamin D metabolism in response to microbial changes. GF mice had high iFGF23 which was probably suppressing 1,25D (3, 8). Commensals reduced FGF23 levels to those found in conventional mice after 3d, while the increase in 1,25D and 24,25D levels occurred after 7d. Therefore, the early decrease in FGF23 was directly caused by microbial signals while the later changes in 1,25D and 24,25D may be due to re-establishment of homeostasis following decreased FGF23 levels. Increased $\text{tnf-}\alpha$ expression and mild colonic hyperplasia at 2 and 3d demonstrate an immune response in the colon after colonization which might contribute to the reduction in FGF23 (9, 10). TNF-$\alpha$ induced expression of RANKL, which promoted bone resorption (9, 10). Other research showed GF mice had lower expression of TNF-$\alpha$ and fewer T cells compared to conventional mice, suggesting the bone immune compartment is regulated by the microbiome (11). GF mice also had fewer osteoclasts and greater trabecular bone density than conventional mice, and CN increased osteoclasts and decreased trabecular bone density (11). FGF23 is produced in the bone, and changes in bone density altered serum levels of FGF23 (12). Administration of a RANKL inhibitor caused increased bone density and circulating FGF23 (12). FGF23’s role in suppressing Cyp27B1 and 1,25D production is well documented (3, 4, 8). Therefore, increased TNF-$\alpha$ as a result of host-microbial interactions followed by reduced FGF23 following conventionalization might re-establish homeostasis and after 1 wk the 1,25D and 24,25D levels would be normalized.
C. rodentium, a murine enteric pathogen, increased 25D and 24,25D, but the increase was not evident at 2 wks, as it was following conventionalization of GF mice with commensals. 25D and 24,25D increased 4 wks after C. rodentium infection. The peak of inflammation is 10 days after infection, and inflammation is not resolved 2 wks after C. rodentium infection. 25D levels were decreased 2 wks following monocolonization of mice, during which colonic inflammation and infection are ongoing (13). Sterile inflammation that occurs following injection of LPS resulted in a reduction in 24,25D levels at 6 hours. Inflammation has been shown to regulate extra-renal production of 1,25D but not renal production of 1,25D. The rapid (6h) decrease in 24,25D following LPS injection suggests direct renal control of either Cyp24A1 or Cyp27B1 might be occurring. Female mice had higher levels of 24,25D than males. Other work shows bone health and 1,25D levels increase in response to estrogen treatment, and the microbiome drives bone loss in estrogen deficiency by promoting production of osteoclastgeneic cytokines like TNF-α (14-16). My work provides further support for sex hormones, most likely estrogen, as regulators of 1,25D and 24,25D levels. Both males and females should be evaluated in studies of the microbiome and endocrine control of 24,25D production. This chapter demonstrated a new role for the direct regulation of FGF23 by microbial and/or possibly inflammatory signals leading to an increase in 24,25D following conventionalization of GF mice.

Both Chapters 2 and 3 contain the common theme of host-microbial interactions regulating vitamin D metabolism, starting with FGF23. Disruption of homeostatic host-microbial interactions disrupts the production of 25D and 24,25D (Fig. 4-1). Disruption
of homeostasis reduced $tnf-\alpha$ levels after Abx treatment, and increased $tnf-\alpha$ following CN of GF mice. Decreased $tnf-\alpha$ was associated with the inhibition of FGF23 and higher 24,25D probably via the VDR and 1,25D. Normalization of 1,25D levels and FGF23 are required for homeostasis (Fig. 4-1A). When host-microbial interactions are absent or disrupted, as in GF mice or after Abx treatments, regulation of vitamin D metabolism is also disrupted. Increased FGF23 affected endocrine vitamin D metabolism by promoting expression of the $cyp24a1$ gene and inhibiting expression of the $cyp27b1$ gene in GF mice (Fig 4-1B). In GF mice, FGF23 was elevated and 1,25D and 24,25D were low, leading to hypocalcemia. Despite increased FGF23 and expression of the $cyp24a1$ gene, inhibition of Cyp27B1 and 1,25D deficiency meant production of 24,25D by Cyp24A1 was suppressed in GF mice. CN reduced FGF23 and elevated 25,25D levels. In conventional mice after Abx, increased FGF23 correlated with increased levels of 24,25D. Calcium was normal, suggesting that if endocrine 1,25D levels were affected by Abx, changes were not so extreme as to affect mineral homeostasis.

FGF23 is produced primarily in the bone, and acts on the kidney to regulate mineral homeostasis and vitamin D metabolism (3). Immune-derived signals linking the microbiome and bone have already been established (11). Both manuscripts suggest host sensing of microbial changes regulate FGF23, which in turn regulates renal vitamin D metabolism, and this regulation is not antigen specific or limited to specific microbes. Specifically, TNF-\alpha produced by TLR signaling bridges the gap between the microbiome and the 1,25D/FGF23/PTH axis, possibly by regulating FGF23 or PTH directly. In both manuscripts, more microbes correlated with more TNF-\alpha, and less FGF23. Increased FGF23 in response to Abx led to increased 24,25D, whereas CN of GF mice decreased
FGF23 and increased 24,25D. However, GF mice started with low 1,25D and 24,25D levels, and highly elevated FGF23 levels. Abx treated mice started with higher basal 1,25D, and 24,25D levels, and Abx does not completely eliminate the microbiota. Both manuscripts also indicate that 25D levels, that largely reflect vitamin D intakes, were not regulated by the same factors as 1,25D. The increase in 25D following Abx treatment was regulated by TLR signaling (no Abx effect on 25D levels in MyD88 KO mice) and the microbiota directly. However, Abx treatment also had an effect on vitamin D absorption in WT mice but not MyD88 KO mice. It is possible that the microbiota directly regulates vitamin D absorption or one of the 25-hydroxylases, which would be novel.

The findings in this dissertation have implications for many diseases. Overall, the work presented here suggest vitamin D metabolism is regulated by Abx and changes to the microbiome. Dysbiosis is a pathogenic factor in inflammatory bowel disease (IBD), and vitamin D deficiency/insufficiency is also associated with IBD severity (17). Low vitamin D status in IBD is caused in part by poor nutrient absorption in the inflamed gastrointestinal tract; however it would be interesting to know if increased inflammatory signals in IBD are an additional cause of low vitamin D status in IBD. Furthermore, the role of FGF23 in IBD has not been studied. Abx and TNF-α inhibition are common treatments in IBD, and my work shows both treatments could alter FGF23 and vitamin D metabolism. Future work is needed to determine if bone remodeling in inflammatory diseases alters FGF23 production and vitamin D metabolism. Experiments should also be done to determine if host-microbiome interactions alter vitamin D metabolism and FGF23 in IBD models. My results show commensal microbes decreased FGF23 and
increased 1,25D. This could also apply to diseases in which elevated FGF23 and low 1,25D are problematic, such as chronic kidney disease. Intestinal barrier function is compromised in chronic kidney disease, and microbiome-induced inflammation contribute to decreased kidney function (18, 19). Overall host-microbial interactions are novel factors regulating FGF23 and endocrine vitamin D metabolism.
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Figure 4-1. The gut microbiome regulates endocrine vitamin D metabolism. A) During homeostatic host-microbe interactions, the host produces some TNF-α which leads to decreased FGF23, and normal vitamin D metabolism.

B) When host-microbial signals are disrupted, either in GF or as a result of Abx, TNF-α production decreases. This leads to over production of FGF23, which suppresses endocrine vitamin D metabolism.
Appendix

The role of UVR and vitamin D on T cells and inflammatory bowel disease

Adapted from the manuscript entitled:

“The role of UVR and vitamin D on T cells and inflammatory bowel disease.”

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ABSTRACT

Vitamin D deficiency is associated with the development of inflammatory bowel disease (IBD). In experimental IBD the targets of vitamin D that result in protection from IBD include gut epithelial cells, innate immune cells, T cells, and the microbiota. Ultraviolet radiation (UVR) induces production of vitamin D in the skin and suppresses T cell responses in the host. There is limited data demonstrating an effect of UVR on experimental IBD but the mechanisms of UVR suppression in IBD have not been defined. There are several shared effects of vitamin D and UVR on T cells including inhibition of proliferation and suppression of IFN-γ and IL-17 producing T cells. Conversely UVR decreases and vitamin D increases IL-4 production from T cells. Together the data suggest that UVR suppression of T cells and potentially IBD are both vitamin D dependent and independent.
**Inflammatory Bowel Disease (IBD)**

IBD are chronic diseases of the gastrointestinal tract of unknown etiology. In the United States, 1-1.3 million people have IBD and in Europe the incidence of IBD diseases are even higher than in the US.¹ Treating IBD can be a substantial economic burden; in the US the direct cost of living with IBD averages from $5,000-8,000 per year.² Ulcerative colitis and Crohn’s disease make up two distinct forms of IBD. Ulcerative colitis is characterized by inflammation of the lower gastrointestinal tract from the colon to the rectum. Conversely, Crohn’s disease is characterized by inflammation from the esophagus to the rectum, but most commonly occurs in the ileum of the small intestine. In Crohn’s disease inflammation can affect all layers of the intestinal wall, not just mucosal layers.³ For both diseases the immune system is inappropriately activated by the microbes found in the gut. Patients that develop IBD develop persistent inflammation that fails to resolve. Crohn’s disease and ulcerative colitis have shared and distinct risk factors, as well as differences in treatments that point to both shared and unique pathophysiologies. For both Crohn’s disease and ulcerative colitis there are genetic and environmental factors that determine which people develop disease.

IBD is more common among biological relatives, and about 20% of patients (Crohn’s and Colitis Foundation of America, [http://www.ccfa.org/](http://www.ccfa.org/)) have a relative with IBD, demonstrating the role of genetics in IBD. Several IBD susceptibility genes have been identified.⁴ Single nucleotide polymorphisms in the major histocompatibility complex are linked to the development of many different immune-mediated diseases including IBD.⁵ Major histocompatibility genes determine the targets of the immune system since they allow the immune system to identify pathogens but tolerate commensal
microbes. IBD patients have genetic polymorphisms in multiple genes that control the immune response including several cytokines and cytokine receptor genes. Other genetic polymorphisms exist in IBD patients for receptors that sense pathogens or are pattern recognition receptors like nucleotide oligomerization domain 2. Mutations in nucleotide oligomerization domain 2 has been identified as a critical risk factor in Crohn’s disease. Several of the genes linked to IBD susceptibility are important in the regulation of the immune response to microbes.

Identical twin studies have established an important role of the environment in the development of IBD. The concordance rate for ulcerative colitis in identical twins is only 20% and 50% in Crohn’s disease. In addition, the prevalence of IBD world wide has increased and even countries that previously had a low incidence of IBD have seen increases. The incidence of IBD is higher in industrialized countries and in the northern hemisphere, IBD is more prevalent in northern versus southern climates. Environmental factors that may be different in low versus high IBD areas include diet, life-style, pollution, and sunlight (ultraviolet radiation, UVR). One environmental factor that is controlled by changes in diet and life-style are the commensal microbiota that inhabit the gastrointestinal tract. IBD patients have dysbiosis of the microbiota and the diversity of the microbiota is less as compared to healthy controls. Environmental factors that contribute to the development of IBD have been difficult to identify but include the composition of the commensal microbiota, diet, vitamin D and sunlight. Here we will examine the specific role of UVR and vitamin D on IBD.
UVR and IBD

There are only a few studies that have analyzed the effects of UVR on IBD. These studies have been complicated by the fact that UVR exposure of skin results in the production of vitamin D. Vitamin D is produced by UVB radiation of the precursor 7-dehydrocholesterol in the skin to form pre-vitamin D, which is isomerized to vitamin D by heat. Vitamin D from either the diet or UVR is first hydroxylated at the 25-position to produce 25hydroxyvitamin D (25(OH)D) and then by the 1alpha hydroxylase to form active vitamin D or 1,25dihydroxyvitamin D (1,25(OH)2D). There are two sources of vitamin D for production of 1,25(OH)2D, the diet and UVR light.

Incidence of immune-mediated diseases, including IBD, has been correlated with low sun exposure and latitude. The incidence of ulcerative colitis was 40% higher and Crohn’s disease was 80% higher in northern Europe compared to southern Europe. Other studies in France and Scotland showed similar results. In France a study described a clear north-south gradient for the incidence of Crohn’s disease, but not ulcerative colitis. This same group later found a correlation between lower UVR exposure and the increased Crohn’s disease incidence observed in their earlier study. In the US, areas with lower UV exposure, had increased incidence of IBD, increased hospitalizations rates, and increased severity of disease. UV exposure was also associated with the risk of gastrointestinal *Clostridium difficile* infection. In addition, patients with high UV exposure (Arizona), were 16% less likely to die from *C. difficile* infection in the hospital than patients with low UV exposure (Michigan), even when controlling for age, gender, and comorbidities. The above studies did not include
vitamin D measurements but did suggest that changes in vitamin D status might account for the effects of UVR on IBD incidence and severity.

**Vitamin D and IBD**

The vitamin D hypothesis suggests that vitamin D status is one of the environmental factors predisposing for the development of IBD. The effect of UVR on vitamin D status depends on skin color, latitude, skin exposure, season etc. and therefore it has been difficult to determine the UVR contributions to vitamin D status. In addition there are important inter-personal variations in the effect of UVB light on 25(OH)D levels even when controlling for skin color. Studies that use dietary intakes to estimate vitamin D status usually ignore any contributions from UVR. Even if 25(OH)D levels are measured it is difficult to determine how much comes from UVR versus diet and/or supplements. A recent systematic review and meta-analysis was done to determine the association of 25(OH)D levels, UVR exposure, geography and IBD. The conclusions of the study were that both Crohn’s disease and ulcerative colitis patients had lower levels of vitamin D and lower levels of 25(OH)D were associated with higher Crohn’s disease activity indexes (CDAI; there were too few studies to evaluate ulcerative colitis). However, because of a lack of adequate data no correlations could be made between latitude, geography and IBD.

The data linking vitamin D and the propensity to develop IBD have been done without accounting for the possible contributions of UV light to the vitamin D status. The data so far demonstrating an inverse correlation between vitamin D status and IBD severity are stronger for patients with Crohn’s disease than for patients with ulcerative colitis. A prospective study that utilized the Nurses Health study and determined the
relationship between 25(OH)D status and the risk of developing IBD showed a significant inverse risk between 25(OH)D levels and Crohn’s disease but an insignificant inverse association for ulcerative colitis. In two different prospective studies in Crohn’s disease, patients with low 25(OH)D levels (<30ng/ml) required more hospitalizations and surgery compared to patients with higher 25(OH)D levels at entry. One of the two prospective studies included ulcerative colitis patients and showed that low 25(OH)D levels (<30ng/ml) resulted in more morbidity and treatment escalation over the 5 years of the study follow up for ulcerative colitis as well as Crohn’s disease. Crohn’s disease patients in clinical remission had higher 25(OH)D levels than those with mild or moderate disease, and patients who used vitamin D supplements had lower C-reactive protein (P=0.07) and CDAI scores (P<0.05) than those who did not take supplements. The stronger associations of vitamin D on Crohn’s disease versus ulcerative colitis could be because of the differences in the etiology of the diseases as evidenced by the unique as well as shared genetic risk factors for the two diseases. Vitamin D status is strongly inversely associated with IBD and especially Crohn’s disease.

There have only been a few clinical interventions done using vitamin D supplementation of Crohn’s disease patients. In a small open label pilot study, vitamin D supplementation for 6 months (5000 IU/d) improved quality of life scores in Crohn’s subjects with mild to moderate disease, and most of the patients (78%) had CDAI scores below 150 indicating clinical remission after the 6 month intervention. A second small open label study demonstrated a positive effect of vitamin D analog intervention (6 week treatment using 1alpha hydroxy-vitamin D) on CDAI scores. Another small, double-blind randomized controlled study demonstrated an insignificant (P=0.056) decrease in
relapse rate with the 12 month vitamin D intervention (1200 IU/d) as compared to placebo.\textsuperscript{34} Each of the three vitamin D interventions in Crohn’s disease utilized different vitamin D interventions and different study designs.\textsuperscript{32-34} There is currently disagreement by the experts as to the effective dose, frequency of vitamin D delivery and serum 25(OH)D status cut offs for health outcomes; making it more difficult to determine the effects of vitamin D supplementation in clinical studies (reviewed in\textsuperscript{26}). The results from the vitamin D interventions in Crohn’s disease patients, while promising, require additional studies.

**Vitamin D, UVR and experimental IBD**

Mouse models of IBD have been useful for identifying novel therapeutics. There are many experimental models of IBD, and while no model perfectly represents human disease, these models are important in understanding mechanisms of IBD development. A comprehensive review of animal models of experimental IBD has been published by others.\textsuperscript{35} Experimental IBD models fall into three categories: genetic models, transfer models, or chemical-induced injury models. Genetic manipulation to inhibit regulatory immune responses are common in IBD models, such as IL-10 KO.\textsuperscript{35} IL-10 KO animals develop spontaneous colitis as a result of the lack of regulatory T (T reg) cells that produce most of the IL-10 in the gastrointestinal tract.\textsuperscript{35} Transfer of naïve T cells to immunodeficient (no T or B cells) mice is an IBD model that has identified IFN-γ producing Th1 and IL-17 producing Th17 cells as the immunopathologic T cells in experimental IBD.\textsuperscript{35} Co-transfer of naïve T cells with T reg cells that produce IL-10 eliminates disease by suppressing the production of IFN-γ and IL-17 by the naïve T cells in the immunodeficient recipients.\textsuperscript{35} Chemical injury models of IBD have identified the
important contribution of pattern recognition receptors of the innate immune system as important contributors to IBD susceptibility. All of the experimental IBD models are affected by the commensal microbiota in the animal colonies. In dextran sodium sulfate induced colitis the microbes protect from gastrointestinal injury, while in other models (T cell transfer, IL-10 KO) the T cell response is generated against the microbes in the gastrointestinal tract. The experimental IBD models have identified T cells, innate cells and the microbiota as key factors that regulate inflammation and disease in the gastrointestinal tract.

The effects of vitamin D on experimental IBD models have been reviewed. Vitamin D deficient and VDR KO mice develop fulminating forms of several different models of experimental IBD. Treating mice with the active form of vitamin D (1,25(OH)_2D) inhibited IBD in murine models of disease. The targets of vitamin D in experimental IBD include gastrointestinal epithelial cell barrier function and both the innate and adaptive immune system. More recently it has become clear that vitamin D alters the composition of the commensal microbiota probably through regulation of the immune system. The mechanisms by which vitamin D regulates experimental IBD include regulation of gut barrier function, the microbiota and the immune system to maintain gastrointestinal homeostasis.

Two studies have examined the effects of UVR in one model of experimental IBD. The model that was tested is acute dextran sodium sulfate colitis in mice. Colitis in this model is a result of injury in the gastrointestinal tract that is then repaired by the innate immune system, without a contribution of T cells. The experimental design for both studies was similar in that the light therapy was delivered before induction of
Both studies showed a positive effect of the light treatments on experimental IBD symptoms in this model. The dose of light was sufficient in one study to raise serum 25(OH)D levels but in the other was not. The doses of light delivered were 600mJ/cm² (no raise in serum 25(OH)D) on shaved mice versus 14 J/cm² on unshaved mice (raised serum 25(OH)D). The low dose (600mJ/cm²) was associated with an increase in T reg cells. However T reg cells have not been shown to be important in this model of chemically induced colitis. The data suggest that there may be a vitamin D independent effect of UVR on experimental colitis. Future work should focus on the effects of UVR in other experimental models of IBD that involve T cells and the utilization of VDR KO mice to determine the UVR induced versus the vitamin D mediated effects of UVR treatments.

**Vitamin D/UVR regulation of T cells**

While it is clear that innate immune cells, the microbiota, the gut epithelium and T cells are critical regulators of gastrointestinal homeostasis, this commentary will focus on the effects of vitamin D versus UVR on T cells. Dysregulation of T cells leads to IBD and 1,25(OH)₂D and UVR have effects on T cells. Vitamin D is a critical factor in the development and function of T cells. The targets of vitamin D in T cells include inhibition of proliferation of T cells and 1,25(OH)₂D mediated inhibition of T cell produced IL-17 and IFN-γ (Fig. 1). UVR also results in the inhibition of T cell proliferation and a more global inhibition of T cell produced Th1, Th17 and Th2 (IL-4 secreting T cells) cytokine responses in effector T cells (Fig. 1). The immunosuppression following UVR exposure and 1,25(OH)₂D is acute in that antigen sensitization immediately following UVR/1,25(OH)₂D exposure is affected but
subsequent exposures to new antigens are not affected. The induction of FoxP3^+ T reg cells by 1,25(OH)_2D and UVR is also an overlapping function of the two treatments (Fig. 1). 1,25(OH)_2D and UVR increased production of IL-10 by T cells (Fig. 1). Common functions of UVR and 1,25(OH)_2D treatments include suppression of T cell proliferation, inhibition of Th1/Th17 effector cells and induction of T reg cells and IL-10 production (Fig. 1).

Vitamin D is critical in the development of invariant (i) natural killer (NK)T cells and CD8αα T cells. iNKT cells are T cells that respond to lipid antigens and rapidly produce cytokines. iNKT cells have been shown to be important regulators of experimental IBD (dextran sodium sulfate induced colitis). The gastrointestinal tract harbors a unique T cell population that express CD8αα. The CD8αα receptor on these T cells helps to maintain tolerance to the large number of microbial and food antigens found in the gut. VDR KO have fewer iNKT cells and CD8αα compared to WT mice, and the CD8αα T cells from the gut of the VDR KO produced less IL-10 (Fig. 1). Vitamin D status and expression of the VDR are required for the normal development of iNKT cells and CD8αα T cells that regulate experimental IBD (Fig. 1). Reduced iNKT cell or CD8αα T cell functions results in poorly controlled Th1 and Th17 cell responses in the gut (Fig. 1). 1,25(OH)_2D inhibited IL-17 and induced IL-10 production from iNKT cells. There is one study that has looked at the effects of UVR on NKT cells and shown that UVR induces NKT cells that suppress antigen specific responses. The UVR NKT cells were CD1d restricted and showed increased IL-4 production with UVR treatment but may be distinct from the iNKT cells that have been the focus of the vitamin D work. Additional work is needed to determine whether the effects of UVR on NKT
cells is via the production of 1,25(OH)\textsubscript{2}D and induction of iNKT cells (Fig. 1). The targets of vitamin D in T cells, includes regulation of iNKT cell and CD8\textalpha\textalpha T cell development and function (Fig. 1).

The mechanisms by which UVR regulate T cells directly in the absence of induction of vitamin D production have not been well studied. The UVR effects cannot be reproduced \textit{in vitro} which complicates clear determinations of vitamin D versus UVR mediated effects. The effects of UVR on T cells includes inhibition of T cell proliferation and suppression of all antigen specific responses including Th1, Th17 and Th2 (Fig. 1).\textsuperscript{42,48} 1,25(OH)\textsubscript{2}D treatment of T cells induced IL-4 production from Th2 cells.\textsuperscript{41} Mechanisms whereby UVR could regulate the T cell is via prostaglandin-E2 production and the formation of pyrimidine dimers and urocanic acid production.\textsuperscript{49,50} These UVR induced factors have been shown to induce T reg cells and IL-10 production following UVR exposure of skin.\textsuperscript{49,50} UVR was shown to induce functional T regs in VDR KO mice.\textsuperscript{51} Independent of vitamin D, UVR induced T regs and suppressed antigen specific immune responses in vivo.\textsuperscript{51} The factors that mediate regulation of T cells following UVR exposure include both vitamin D independent and dependent effects of UVR (Fig. 1).
Conclusions

UVR and vitamin D are two related environmental factors that have been hypothesized to be etiological factors important in the development of IBD. At present there is strong evidence associating vitamin D status as a risk factor for IBD, especially Crohn’s disease. The evidence for geography, latitude or UVR exposure and IBD does not exist. Animal models have been useful for identifying the targets of vitamin D in experimental IBD, which include T cells, innate immune cells, epithelial cells and the microbiota. A more limited number of studies (two) have studied UVR and experimental IBD without identifying the specific targets in this model. UVR is an effective inhibitor of antigen specific T cell responses. UVR and 1,25(OH)2D have some shared as well as unique effects on T cells. Overall the data support UVR effects on T cells (and potentially IBD) that are both vitamin D dependent and vitamin D independent. IBD patients might be the ideal population for the utilization of UVR therapy. Because of the malabsorption associated with the IBD diseases oral vitamin D supplementation can be ineffective. UVR might increase vitamin D status and have other non-vitamin D mediated benefits in this population.
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Figure A-1. **Vitamin D versus UVR effects on T cells.** The shared effects of both vitamin D and UVR on T cells includes induction of IL-10 producing T regs and suppression of T cell proliferation and Th1 and Th17 cells that produce IL-17 or IFN-γ. UVR suppresses induction of Th2 cells, while 1,25(OH)₂D induces IL-4 production from Th2 cells and iNKT cells. In addition, vitamin D is a critical factor in the development of iNKT cells and CD8αα T cells that help to maintain tolerance in the gastrointestinal tract. It is presently unclear what the effects of UVR are on either iNKT cells or CD8αα T cells.
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