Dietary Vitamin D3 deficiency alters intestinal mucosal defense and increases susceptibility to *Citrobacter rodentium* induced colitis

Natasha R. Ryz¹, Arion Lochner¹, Kirandeep Bhullar¹, Caixia Ma¹, Tina Huang¹, Ganive Bhinder¹, Else Bosman¹, Xiujuan Wu¹, Sheila M. Innis², Kevan Jacobson³#, and Bruce A. Vallance¹#

Department of Pediatrics, ¹ Division of Gastroenterology, ² Division of Neonatology, the Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada, V5Z 4H4

#co-corresponding author

Address correspondence to:
Bruce A. Vallance, PhD
Room K4-188, 4480 Oak Street
Ambulatory Care Building, BC Children's Hospital
Vancouver, British Columbia, Canada, V6H 3V4
Phone: 604-875-2345 ext. 5112
Fax: 604-875-3244
Email: bvallance@cw.bc.ca

Kevan Jacobson, MD
Room K4-181, 4480 Oak Street
Ambulatory Care Building, BC Children's Hospital
Vancouver, British Columbia, Canada, V6H 3V4
Phone: 604-875-2332 ext 1
Fax: 604-875-3244
Email: kjacobson@cw.bc.ca
Vitamin D deficiency and *C. rodentium* induced colitis

**ABSTRACT**

Vitamin D deficiency affects more than 1 billion people worldwide. Although thought to increase risk of bacterial infections, the importance of vitamin D on host defense against intestinal bacterial pathogens is currently unclear since injection of the active form of vitamin D, 1,25(OH)2D3 increased susceptibility to the enteric bacterial pathogen *Citrobacter rodentium* by suppressing key immune/inflammatory factors. To further characterize the role of vitamin D during bacteria-induced colitis, we fed weanling mice either vitamin D3 deficient or vitamin D3 sufficient diets for 5 weeks and then challenged with *C. rodentium*. Vitamin D3 deficient mice lost significantly more body weight, carried higher *C. rodentium* burdens, and developed worsened histological damage. Vitamin D3 deficient mice also suffered greater bacterial translocation to extra-intestinal tissues, including mesenteric lymph nodes, spleen and liver. Intestinal tissues of infected vitamin D3 deficient mice displayed increased inflammatory cell infiltrates as well as significantly higher gene transcript levels of inflammatory mediators TNF-α, IL-1β, IL-6, TGF-β, IL-17A and IL-17F as well as the antimicrobial peptide REG3γ. Notably, these exaggerated inflammatory responses accelerated the loss of commensal microbes and were associated with an impaired ability to detoxify bacterial lipopolysaccharide. Overall, these studies show that dietary-induced vitamin D deficiency exacerbates intestinal inflammatory responses to infection, also impairing host defense.

Key Words: Vitamin D, *Citrobacter rodentium*, Inflammation, Colitis, Segmented FilAMENTous Bacteria
Vitamin D deficiency and *C. rodentium* induced colitis

**INTRODUCTION**

It is being increasingly recognized that vitamin D plays an important role in host defense against pathogenic microbes. Vitamin D deficiency affects more than 1 billion people worldwide and is associated with an increased risk of respiratory infections, including *Mycobacterium tuberculosis* (18, 53) and *Pseudomonas aeruginosa* (8, 48). Recently, vitamin D status was also shown to influence the risk and severity of *Clostridium difficile* infection, which is the most common cause of hospital-acquired infectious diarrhea (2, 51). Furthermore, vitamin D deficiency is associated with an increased risk of systemic bacterial infection leading to sepsis (31). Animal models of vitamin D deficiency, including the vitamin D receptor (VDR)-/- mice also have increased susceptibility to infection and carry higher bacterial burdens during challenge with *Salmonella typhimurium* (56). Surprisingly, despite its broad linkage to host defense against predominantly mucosal bacterial pathogens, the mechanisms and pathways through which vitamin D affects a host’s susceptibility to these infections is poorly understood.

Vitamin D refers to a group of fat-soluble prohormones, including vitamin D3 or cholecalciferol, which is produced endogenously in the skin during exposure to UVB light and is also naturally present in certain foods such as fatty fish (23). Studies of people in Northern latitudes, such as Canada suggest that endogenous synthesis of vitamin D is minimal from October to March due to limited UVB exposure (22). Furthermore, recent efforts to limit sun exposure and increased usage of sunscreen have dramatically reduced the levels of vitamin D3 produced in the skin of people worldwide (22). Dietary and supplemental forms of vitamin D3 are thus becoming more important sources of this vitamin. Most dietary vitamin D3 is absorbed via passive diffusion in the small intestine, together with dietary fats and bile salts in chylomicrons. Classically, vitamin D3 enters the systemic circulation bound to the D-binding protein and is transported to the liver and kidney, where it is metabolized by various enzymes, including CYP27B1 to yield 1,25(OH)2D3 - the active hormonal form of vitamin D, also known as calcitriol (23). 1,25(OH)2D3 signals through the VDR, a nuclear receptor located in most cells in the body. Studies in mice have shown the VDR is highly expressed throughout the small intestine and colon, with the highest expression found in
the cecum and proximal colon (29, 56). Ligand binding activates the VDR to heterodimerize with the retinoid X receptor (RXR), and the VDR-RXR complex binds to specific DNA sequences, known as vitamin D-responsive elements (VDRE) to induce transcription (6). 1,25(OH)2D3 directly or indirectly regulates many different genes through the VDR and is involved in diverse cellular functions including calcium metabolism, cell proliferation and immune modulation (6), all of which could impact intestinal host defense.

Another important group of mucosal bacterial pathogens comprise the attaching and effacing (A/E) family of pathogenic Escherichia coli including enterohemorrhagic E. coli (EHEC) and enteropathogenic E. coli (EPEC). Both microbes are important causes of infectious diarrhea, with EPEC causing as many as 1 million infant deaths per year in developing nations (37). Since these clinically important pathogens do not colonize mice, these infections have been modeled using the related mouse-specific A/E bacterial pathogen Citrobacter rodentium. Following infection, C. rodentium intimately attaches to epithelial cells lining the cecum and colon, resulting in barrier disruption, crypt hyperplasia and goblet cell depletion as well as immune and inflammatory cell infiltration of the intestinal mucosa (14). More specifically, Toll-like receptor (TLR) based recognition of C. rodentium products, such as peptidoglycan and lipopolysaccharide (LPS) drives the inflammatory response during infection (19, 32). Ultimately the host immune/inflammatory response not only helps control these infections, but it also causes significant pathophysiology changes. Similarly while the induction of antimicrobial genes at the mucosal surface helps to eventually clear C. rodentium from the host, these responses also remove competing commensal microbes and reduce intestinal colonization resistance (45).

At present, the impact of vitamin D on host defense against C. rodentium is poorly understood. Previous research from our group has shown that treatment with the active form of vitamin D, 1,25(OH)2D3 can increase the susceptibility of mice to C. rodentium by suppressing key inflammatory factors required for bacterial clearance (41). This is in keeping with several studies showing that 1,25(OH)2D3 can inhibit inflammatory
Vitamin D deficiency and *C. rodentium* induced colitis

responses in the host through a number of mechanisms, including suppressing TLR expression (42), targeting MAPK phosphatase-1 (58), blocking NF-κB signalling (57), modulating dendritic cell and macrophage behaviour (15) and skewing T-cell responses toward a regulatory phenotype (26, 33). Moreover 1,25(OH)2D3 acts directly on T-cells to inhibit proliferation and production of inflammatory cytokines, including IL-2, IFN-γ, TNF-α and IL-17 (7). As such, 1,25(OH)2D3 can be considered a potent immunosuppressive agent, and may inadvertently impair host defenses against enteric microbes.

Correspondingly, studies suggest that loss of vitamin D3 signaling promotes exaggerated intestinal inflammatory responses. Mice fed a vitamin D3 deficient diet and mice deficient in the VDR gene have both been shown to exhibit a higher baseline colonic inflammatory tone, as well as elevated serum levels of IL-6 (3, 56). Published studies that have examined the impact of vitamin D3 deficiency during *C. rodentium* infection have shown an exaggerated colitic phenotype (3, 9). Chen et al. (9) showed that VDR-/- mice are resistant to colonization with *C. rodentium*, but their resistance appeared to depend on a dysbiotic microbiota rather than a direct effect of VDR deficiency. Assa et al. (3) did not directly examine pathogen burdens but focused on the impact of a vitamin D3 deficient diet on epithelial barrier function. To complement these studies, we sought to test whether a vitamin D deficient diet would be beneficial or detrimental to intestinal host defense, as well as host specific inflammatory/anti-microbial factors that during *C. rodentium* infection are regulated by vitamin D.

We fed C57Bl/6 mice either a vitamin D3 deficient or sufficient diet for 5 weeks and challenged them with *C. rodentium*. Interestingly, despite having a higher baseline inflammatory tone in their intestines, vitamin D3 deficient mice carried significantly higher pathogen burdens in the ceca and in the mesenteric lymph nodes (MLN), spleen and liver at day 10 post-infection (pi), indicating greater susceptibility to pathogen translocation. In accordance with their heightened immune responses, intestinal tissues of infected vitamin D3 deficient mice showed significantly higher gene transcript levels of the inflammatory mediators TNF-α, IL-1β, IL-6, TGF-β, IL-17A and IL-17F as well as
the antimicrobial peptide REG3γ. Vitamin D3 deficient mice also carried significantly more segmented filamentous (SFB) bacteria in their stool, compared to vitamin D3 sufficient mice. Moreover, they showed defects in the ability to detoxify bacterial LPS and carried higher serum levels of CD14. Overall, these findings show that dietary-induced vitamin D deficiency alters host mucosal defense and increases susceptibility to an enteric bacterial pathogen.
MATERIALS AND METHODS

Mice and Experimental Diets
Weanling (3-week-old) female C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada). Mice were fed either a vitamin D3 deficient diet (0 IU) or a vitamin D3 sufficient diet (1000 IU) for 6 weeks, as previously described (35). All diets were procured from Research Diets, Inc. (New Brunswick, NJ). Mice were maintained in sterilized, filter-topped cages, handled in tissue culture hoods and given free-access to water under specific pathogen-free conditions in the animal facility at the Child and Family Research Institute. Sentinel animals were routinely tested for common pathogens. The protocols used were approved by the University of British Columbia’s Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

Bacterial Strains and Infection of Mice
Mice were infected by oral gavage with 0.1 ml of an overnight culture of luria broth (LB) containing approximately $2.5 \times 10^8$ CFU of streptomycin resistant C. rodentium (formerly C. freundii biotype 4280, strain DBS100). Mice were weighed daily and monitored for signs of illness or distress.

FITC-Dextran Intestinal Permeability Assay
The FITC-Dextran assay was performed as previously described (4). Briefly, mice were orally gavaged with 150 μl of 80 mg/ml 4 kDa FITC-dextran (Sigma; FD4) in PBS 4 h prior to sacrifice. In subsequent studies, mice were given 100 μl of 80 mg/ml 4 kDa FITC-dextran intra-rectally 2 h prior to sacrifice. Mice were anaesthetized and blood was collected by cardiac puncture. Blood was then immediately added to a final concentration of 3% acid-citrate dextrose (20 mM citric acid, 100 nM sodium citrate, 5 mM dextrose) (Harald Schulze, Shivdasani Laboratory, DFCI). Plasma was collected and fluorescence was quantified using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) at excitation 485 nm, emission 530 nm for 0.1 s.

Tissue and Serum Collection
Mice were anesthetized with halothane and blood was collected by cardiac puncture. Blood was allowed to clot naturally at room temperature, the cells were removed by centrifugation, and then serum was collected and stored at -80°C until analysis. Anesthetized mice were euthanized by cervical dislocation and cecal and colonic tissues were collected and immediately placed in 10% neutral buffered formalin (Fisher) (48 hrs, 4°C) for histological studies, or placed in RNAlater (Qiagen) and stored at -80 °C for subsequent RNA extraction.

**Serum Calcium Analysis**

Serum samples were analyzed with the calcium colorimetric assay kit (BioVision Research, Mountain View, CA; Cat #K380-250). This assay utilizes the chromogenic complex, which forms between calcium ions and 0-cresolphthalein. Samples were quantified at OD 575 nm using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) and compared to the calcium standard provided with the kit.

**Serum 25(OH)D3 Analysis**

Serum samples were processed and 25(OH)D3 was assessed using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) as previously described (12).

**Citrobacter rodentium Enumeration**

For enumeration of *C. rodentium*, tissues were prepared as previously described (4). Briefly, tissues were collected, weighed and homogenized in a MixerMill 301 bead miller (Retche). Sample homogenates were serially diluted in PBS and plated onto LB agar plates containing 100 mg/ml strep, incubated overnight at 37°C, and *C. rodentium* colonies were enumerated the following day, normalizing them to the tissue or stool weight (per gram). For fecal bacterial burden analysis, stool pellets were collected from live mice at different time points pi and processed as described above. For cecal and colonic samples, tissues were opened longitudinally, luminal contents were collected and then tissues were gently cleaned 3x in PBS rinse prior to collection.
Assessment of Total Microbes Via DAPI Staining

Enumerating total microbes was performed as previously described (45). Briefly, 2 fecal pellets were collected from each animal. After homogenization, samples were placed in 10% Neutral Buffered Formalin diluted to a final concentration of 3%. Samples were further diluted 1:10 in PBS, vortexed briefly, and stored at 4°C. Next, 2–5 ml of the 1:10 diluted sample was further diluted in 1 ml PBS and filtered onto Anodisc 25 filters (Whatman International Ltd) with a pore size of 0.2 mM and 2.5 cm diameter. The samples were thoroughly dried and then mounted on glass slides with ProLong Gold Antifade reagent containing DAPI (Molecular Probes) and sections were viewed on a Zeiss AxioImager microscope and images taken using an AxioCam HRm camera operating through AxioVision software. The mean number of DAPI positive microbes was counted in 3 to 6 randomly chosen fields per disc (1000x). The total number of commensal microbes was calculated based on the mean numbers of all the counted fields and the dilution factor. The total number of commensal microbes was presented as the percentage of uninfected controls.

Commensal Microbe Analysis

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

**Histopathological Scoring**

To assess tissue pathology, paraffin-embedded cecal and colonic tissue sections (5 μm) were stained with haematoxylin and eosin, and were examined by two blinded observers. Tissue sections were assessed for submucosal edema (0 = no change; 1 = mild; 2 = moderate; 3 = profound), epithelial hyperplasia (scored based on percentage above the height of the control where 0 = no change; 1 = 1–50%; 2 = 51–100%; 3 = > 100%), epithelial integrity (0 = no change; 1 = < 10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; 4 = epithelial ulceration with severe crypt destruction) and neutrophil and mononuclear cell infiltration (0 = none; 1 = mild; 2 = moderate; 3 = severe), as previously described (4). The maximum score possible was 15 points.

**Immunofluorescence Staining**

Immunofluorescence staining of cecal tissues for Ki67, F4/80 and Ly6G was performed as previously described (4, 19, 49). Briefly, paraffin-embedded sections were deparaffinized and then rehydrated, followed by antigen retrieval using 0.1 M citric acid monohydrate (Sigma) with 0.05% Tween 20 (pH 6.0) and steam for 45 min. Slides were blocked in PBS with 2% normal goat serum, 1% BSA, 0.1% Triton X-100 and 0.05% Tween 20. Primary antibodies used were rabbit antisera generated against Ki67 (1:200; Abcam), F4/80 (1:8K; Serotec) or Ly6G (Abcam). This was followed by secondary Alexa568-conjugated goat anti-rabbit or anti-rat IgG antibodies (Molecular Probes) and Prolong® Gold antifade reagent containing 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen). Sections were viewed at 350 and 594 nm on a Zeiss AxioImager microscope. Images were obtained using a Zeiss AxioImager microscope equipped with an AxioCam HRm camera operating through AxioVision software (Version 4.4).
RNA Extraction and Quantitative RT-PCR

Cecal tissues were collected and stored in RNAlater (Qiagen) at -80°C with total RNA extracted using the Qiagen RNeasy kit, as previously described (4). Total RNA was quantified using a NanoDrop Spectrophotometer (ND1000). RNA was reverse-transcribed using a Qiagen Omniscript RT kit (Qiagen), according to the manufacturer’s instructions. Quantitative PCR was carried out using a BioRad MiniOpticon or Opticon2, as previously described (4). Melting point analysis confirmed the specificity for each of the PCR reactions. Quantitation was performed using GeneEx Macro OM 3.0 software.

Primer sequences and annealing temperatures were as follows: IL-10 (Fwd: 5’-GTT GCC AAG CCT TAT CGG AA-3’; Rev: 5’-CCA GGG AAT TCA AAT GCT CCT-3’; Annealing 55°C), TGF-β (Fwd: 5’-GAC TCT CCA CCT GCA AGA CCA T’; Rev: 5’-GGG ACT GGC GAG CCT TAG TT; Annealing 59°C); beta-defensin 1 (Fwd: 5’-TCC TCT CTG CAC TCT GGA CC’; Rev: 5’ - ATC GCT CGT CCT TTA TGT CC; Annealing 72°C); beta-defensin 3 (Fwd: 5’- CTC CAC CTG CAG CTT TTA GC’; Rev: 5’ - GCT AGG GAG CAC TTG TTT GC; Annealing 72°C). The primer sequences and reaction conditions for β-actin, TNF-α, IL-6, IL-1β, IL-17A, IL-17F, mcramp and Reg3γ have previously been described (4).

Serum CD14 Measurement

Serum samples were collected and prepared as described above. CD14 was measured using the Quantikine ELISA Mouse CD14 Immunoassay Kit (R&D Systems Inc, Minneapolis, MN; Cat # MC140), and the assays were performed according to directions provided by manufacturer. This assay employs the quantitative sandwich enzyme immunoassay technique. Samples were quantified at OD 450 nm using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) and compared to the standard provided with the kit.

Lipopolysaccharide-Dephosphorylation Assay

LPS-dephosphorylating activity was measured by the malachite green assay, which measures free phosphate release, as previously described (17). In brief, intestinal tissues were homogenized in 500 mL of homogenization buffer and then centrifuged at 11,000
rpm for 3 min to remove insoluble material. To determine the protein concentrations, a Bradford assay (Bio-Rad) was performed on the tissue samples according to the manufacturers instructions. A standard curve was created using a stock solution of 1 mg/mL BSA (Sigma) in triplicates with multiple concentrations. 40 mL of a 5 mg/mL solution of \textit{Escherichia coli} 055:B5 LPS (Sigma L2880) was then added to 15 mL lysate and left for 2 h at room temperature. 40 mL of a solution composed of 0.01% malachite green (Sigma), 16% sulfuric acid (Fisher), 1.5% ammonium molybdate (Sigma) and 0.18% Tween-20 (Sigma) was incubated with the lysate for 10 min. LPS-dephosphorylating activity was determined from colorimetric measurements taken at an absorbance of 620 nm.

\textbf{Statistical Analysis}
Statistical significance was determined using either a two-tailed Student’s t-test or the Mann-Whitney test unless otherwise indicated, with assistance from GraphPad Prism Software Version 4.00 (GraphPad Software, San Diego California USA, www.graphpad.com). A \( P \) value of \( \leq 0.05 \) was considered significant. The results are expressed as the mean value ± the standard error of the mean (SEM).
Vitamin D deficiency and *C. rodentium* induced colitis

**RESULTS**

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

To determine the role of vitamin D3 during enteric infection, we first fed weanling mice either a vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks. There was no difference in food intake or body weight between the two groups during the 5-week feeding trial (data not shown). Dietary vitamin D3 is converted in the liver into 25OHD3, which is the major circulating form of vitamin D in the body and used to assess vitamin D status. After the 5-weeks, vitamin D3 deficient mice had significantly lower levels of serum 25OHD3, compared to vitamin D3 sufficient mice (Figure 1A), in agreement with previous studies (3, 35). Vitamin D also plays an important role in regulating calcium metabolism in the body, and supplemental dietary calcium has previously been shown to protect against *C. rodentium* infection (40, 50), but we found no difference in serum calcium between the diet groups (Figure 1B), similar to findings by Lagishetty et al. (35), whose feeding protocol (including diet manufacturer) we replicated.

On challenge with *C. rodentium*, vitamin D3 deficient mice lost 5-8% of their body weight by day 2 pi, significantly greater than vitamin D3 sufficient mice, with this greater weight loss maintained until the mice were euthanized at day 10 pi (Figure 1C). Vitamin D3 deficient mice had thicker colons and shrunken ceca, which were often devoid of stool contents when compared to infected vitamin D3 sufficient mice (Figure 1D). To determine pathogen burdens, tissues were homogenized and plated to quantify *C. rodentium*. While no significant differences were found regarding pathogen burdens in the colon between the groups at day 10 pi, vitamin D3 deficient mice were found to carry 5-10 fold higher *C. rodentium* burdens in their ceca (cecal tissue + contents) than the vitamin D3 sufficient mice (Figure 2A). Interestingly, vitamin D3 deficient mice also carried significantly more culturable *C. rodentium* from extra-intestinal tissues, including MLN, spleen and liver compared to vitamin D3 sufficient mice at day 10 pi, indicating greater bacterial translocation to these systemic sites with vitamin D3 deficiency (Figure 2B).
To determine if the exaggerated pathogen translocation in vitamin D deficiency reflected increased disruption of intestinal barrier integrity, mice were administered FD4 through oral or intra-rectal routes as measures of proximal or distal intestine, respectively, and their serum was collected and assessed for translocated FD4. We found no significant differences in translocated FD4 levels between vitamin D3 deficient and sufficient mice at day 6 pi (data not shown) or day 10 pi (Figure 2C), indicating the exaggerated bacterial translocation may not reflect an overt epithelial barrier defect. Overall these findings indicate that vitamin D3 deficient mice are more susceptible to infection with *C. rodentium*.

**Vitamin D3 deficient mice carry heavier *Citrobacter rodentium* burdens in their cecal contents at day 10 post infection**

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

During infection, *C. rodentium* initially colonizes the cecum and then spreads to the distal colon of wildtype mice, resulting in characteristic histological damage to these regions, including goblet cell depletion, crypt hyperplasia, loss of epithelial integrity and inflammatory cell infiltration. At day 10 pi, the ceca of vitamin D3 deficient mice displayed worsened histological damage, with significantly more submucosal edema and crypt hyperplasia compared to vitamin D3 sufficient mice, whose ceca had modest damage, as typical for a wildtype C56Bl/6 mouse at day 10 pi (Figure 4). We also examined Swiss-rolled sections of the colon for histological damage. Although we found a trend for worsened *C. rodentium*-induced damage in the distal colon of vitamin D3 deficient mice at day 10 pi, the differences between dietary groups did not reach statistical significance (data not shown). Furthermore, there was no difference in histological scores between dietary groups under uninfected conditions in the colon or cecum (data not shown). Since we found higher pathogen burdens and greater tissue damage in the ceca of vitamin D3 deficient mice, we focused our additional analysis on cecal tissues. To further characterize the mucosal pathology and responses to infection, epithelial cell proliferation in the cecum was determined by Ki67 staining. At day 10 pi, vitamin D3 deficiency mice displayed significantly more Ki67+ve intestinal epithelial cells when compared to vitamin D3 sufficient infected mice (Figure 4C and Figure 4D). These results are in agreement with previous work showing that active vitamin D (1,25(OH)2D3) can inhibit cell proliferation in human colonic epithelial cells *in vitro* (43). However, these changes were only seen during infection, as there were no overt differences in intestinal epithelial cell proliferation between uninfected groups.

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

To determine if the increased susceptibility of vitamin D3 deficient mice to *C. rodentium* infection could reflect an altered host immune response, we assessed cecal tissues for inflammatory cells and mediators. While no differences in macrophage/neutrophil numbers were noted in the cecum of vitamin D3 deficient or sufficient groups under
uninfected conditions, at day 10 pi, vitamin D3 deficient mice showed more infiltrating macrophages and neutrophils in their cecal tissues, particularly in the submucosal regions, compared to vitamin D3 sufficient mice (data not shown).

We next assessed cecal tissues for the transcription of genes encoding cytokines that influence susceptibility to *C. rodentium* infection. Interestingly, vitamin D3 deficient mice showed higher transcript levels for the acute inflammatory cytokines TNF-α, IL-1β and IL-6 under both uninfected and day 10 pi conditions (Figure 5), indicating a higher inflammatory tone. As expected, infection also induced an increase in transcript levels for Th17-related cytokines (IL-17A, IL-17F) in the ceca, similar to findings previously described in the infected colon (10, 60). However the infection induced increase in IL-17A and IL-17F transcripts was significantly greater in vitamin D3 deficient mice as compared to vitamin D3 sufficient mice (Figure 6A). Interestingly, the vitamin D3 deficient mice also showed higher transcript levels of the anti-inflammatory cytokines IL-10 and TGF-β (Figure 6B), suggesting an attempt to counteract the increased inflammatory tone.

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

Considering the higher luminal and systemic pathogen burdens carried by infected vitamin D3 deficient mice, we next examined whether their increased susceptibility could be attributed to differences in their commensal intestinal microbiota. It has recently been shown that vitamin D3 deficient mice have an altered fecal microbiome composition (3), while mice with altered vitamin D signalling; including VDR-/- mice and Cyp27b1-/- mice have been described as suffering intestinal microbial dysbiosis (39). Assessing the microbiota within the stool (using Sybr Green stain) of our two dietary groups under uninfected and infected conditions, we found no difference in the number of commensal bacteria/gram between the dietary groups under uninfected conditions (Figure 7A). Interestingly, vitamin D3 deficient mice carried significantly more segmented filamentous bacteria (SFB) in their stool at day 0, compared to vitamin D3 sufficient mice (Figure 7B), as determined by qPCR. In contrast, we found no significant
Vitamin D deficiency and *C. rodentium* induced colitis

... differences in Bacteroidetes or γ-Proteobacteria in the stool between dietary groups during uninfected conditions (Figure 7B), and although there was a trend (p = 0.0850) for vitamin D3 deficient mice to carry fewer Firmicutes, it did not reach statistical significance.

Previous studies have shown that *C. rodentium* infection is associated with a host driven depletion of commensal microbes that potentially aids pathogen colonization by reducing colonization resistance (45). We assessed commensal microbe numbers over the course of infection and found a modest but significant acceleration in commensal loss in the vitamin D3 deficient mice by day 6 pi, in keeping with the increased inflammatory response seen in these mice (Figure 7C). The makeup of the cecal microbiota at day 10 pi was also assessed, (Figure 7D) with vitamin D3 deficient mice found to carry significantly higher levels of Actinobacteria and Bifidobacteria, compared to vitamin D3 sufficient mice (Figure 7E). There was also a trend for higher levels of γ-proteobacteria and SFB in the cecal contents of vitamin D3 deficient mice at day 10 pi (Figure 7E), but no significant differences in the major bacteria phyla- Firmicutes or Bacteroidetes were found between dietary groups at day 10 pi (Figure 7D, Figure 7E).

**Vitamin D3 deficient mice upregulate expression of the antimicrobial peptide REG3γ during infection with *Citrobacter rodentium***

To understand a potential basis for the altered commensal microbes seen in the vitamin D3 deficient group, as well as explore why higher numbers of *C. rodentium* would be only found within their cecal lumen as opposed to being tissue adherent, we assessed gene transcripts for several antimicrobial factors including REG3γ, which has been shown to play a role in mucosal repair and host defense during infection with *C. rodentium* (60). Moreover in our previous studies, supplementation with the active form of vitamin D, 1,25(OH)2D3 suppressed transcription of REG3γ, thereby impacting on the susceptibility of mice to *C. rodentium* infection (41). Notably, REG3γ transcription was dramatically elevated in vitamin D3 deficient mice (Figure 8), supporting the belief that it is regulated by vitamin D3 and suggesting it may be protecting the cecal mucosa from exaggerated *C. rodentium* colonization. In contrast, no difference between diet groups...
Vitamin D deficiency and *C. rodentium* induced colitis

was detected for transcript levels of other antimicrobial genes that encode beta-defensin 1, beta-defensin 3 or mcramp at day 10 pi (data not shown).

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

Lastly, we explored why the intestinal inflammatory response was exaggerated in the vitamin D3 deficient mice. We previously showed that the inflammatory response during *C. rodentium* infection is largely LPS dependent, as it is significantly reduced in TLR4 deficient mice (32). We therefore examined whether vitamin D3 deficient mice might have altered responses to bacterial LPS. Many of the immune activating abilities of LPS can be attributed to the lipid A unit, which contains two phosphate groups coupled to glucosamines. Removal of one of the phosphate groups generates a monophosphoryl lipid A that is a 100-fold less toxic than the unmodified lipid A (44). Using the malachite green assay to measure LPS dephosphorylation, we found LPS dephosphorylation was significantly increased in vitamin D3 sufficient mice over that seen in uninfected mice both in the duodenum and ileum (Figure 9A), likely as a means to limit inflammatory responses against *C. rodentium*. In contrast, this increase in LPS activity did not occur in vitamin D3 deficient mice during challenge with *C. rodentium* (Figure 9A), indicating that vitamin D3 deficient mice may possess reduced LPS dephosphorylation activity during infection.

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

In keeping with the assessment of responses against bacterial LPS, we examined serum levels of cluster of differentiation 14 (CD14), a pattern recognition receptor responsible for the detection of several bacterial products including LPS (55). CD14 is found in two forms, membrane bound CD14 (mCD14) on the surface of monocytes, macrophages and neutrophils (55), and soluble CD14 (sCD14), which is secreted into bodily fluids including tears, blood and breast milk (52). In combination with TLR4, detection of LPS by CD14 results in a pro-inflammatory immune response, therefore the more CD14, the greater the inflammation. While we noted no difference in serum CD14 levels between
uninfected groups, at day 10 pi however, vitamin D3 deficient mice carried significantly higher levels of serum CD14, compared to vitamin D3 sufficient mice (Figure 9B), confirming that in the absence of vitamin D3, responses against bacterial LPS are exaggerated.
Vitamin D deficiency and *C. rodentium* induced colitis

**DISCUSSION**

Vitamin D has been shown to modulate a wide variety of immune responses; however its potential to impact host defense against enteric pathogens within the gastrointestinal (GI) tract is poorly understood. The most significant impact of vitamin D3 in our study was its effect on the host inflammatory response within the intestine. Even under uninfected conditions, vitamin D3 deficient mice showed a higher intestinal inflammatory tone, with elevated cecal expression of TNF-α, IL-1β, IL-6 and IL17A gene transcripts. Similarly, Assa et al. (3) has shown that vitamin D3 deficient mice had elevated expression of IL-17A and IL-17F in the distal colon compared to vitamin D3 sufficient mice. Moreover infection with *C. rodentium* was associated with an appropriate, but exaggerated response with a further rise in cecal transcript levels of TNF-α, IL-1β, IL-6, IL17A and IL17F with levels that were significantly elevated above vitamin D3 sufficient infected mice. It has previously been shown that TNF-α, IL-1β and IL-6 play an important role in controlling *C. rodentium* burdens in the gut and preventing tissue injury during infection (1, 10, 21). However, excess TNF-α and IL-1β are also known to promote tissue injury during infection with *C. rodentium* (1, 21). Indeed, along with the heightened inflammatory response, vitamin D3 deficient mice also suffered worsened cecal histological damage at day 10 pi, compared to vitamin D3 sufficient mice. Taken together, our results demonstrate that vitamin D3 deficiency results in dysregulated inflammatory responses in the ceca during bacteria challenge.

The basis for this exaggerated inflammatory response was uncertain, however the elevated levels of TNF-α, IL-1β and IL-6, even under baseline conditions suggested the involvement of innate signalling. Bacterial LPS is a major component of the cell wall of Gram-negative bacteria and is recognized by the host innate receptor TLR4, in combination with CD14, MD-2 and the LPS binding protein, found on the surface of monocytes, macrophages and intestinal epithelial cells. Activation of TLR4 and its co-factors by bacterial LPS induce a signaling cascade that leads to the production of inflammatory mediators and localized recruitment of inflammatory cells. Considering that the active form of vitamin D, 1,25(OH)2D3 has previously been shown to inhibit LPS-induced inflammatory responses in the host through a number of mechanisms,
including suppressing TLR4 expression, blocking NF-κB signaling and targeting MAPK phosphatase-1 (42, 58), we decided to examine whether vitamin D3 deficient mice show exaggerated responses to LPS.

In the current study, we found that vitamin D3 deficient mice showed a defect in their ability to dephosphorylate bacterial LPS, compared to vitamin D3 sufficient mice, at day 10 pi, as determined by the malachite green assay. LPS dephosphorylation can occur by intestinal alkaline phosphatase (IAP)- a small intestinal brush border enzyme that plays a critical role in host defense and in maintaining intestinal homeostasis. Indeed, IAP has been shown to reduce intestinal inflammation and limit bacterial translocation into systemic sites (36). Interestingly, although IAP is secreted mainly in the duodenum, it has been shown to retain its activity throughout the small intestine and colon (20) and increased IAP activity has been observed in the colon during infection with *C. rodentium* (17). A previous study showed that vitamin D deficient rats exhibited lower total alkaline phosphatase activity in their duodenum, compared to vitamin D sufficient rats (13). Furthermore, 1,25(OH)2D3 has been shown to stimulate the activity of IAP in the duodenum of chicks (38). Overall these findings indicate that vitamin D may play a role in detoxification of LPS and future studies should investigate IAP.

Along with impaired LPS dephosphorylation, we also noted an increase in serum levels of CD14, a pattern recognition receptor responsible for the detection of several bacterial products including LPS (55). Soluble CD14 (sCD14) is secreted into bodily fluids and in combination with TLR4, detection of LPS by CD14 can result in a pro-inflammatory immune response (52). However, soluble CD14 may also decrease immune responses to LPS by binding LPS and keeping it from mCD14-expressing cells and providing clearance of LPS through the liver (52). Interestingly, the active form of vitamin D, 1,25(OH)2D3 has been shown to increase the expression of CD14 in human monocytes, while suppressing the expression of TLR4 (11, 42). Furthermore, a vitamin D derivative 1α,25-dihydroxy-22-oxavitamin D3 (Oxa-D3) has been shown to increase the release of soluble CD14 from intestinal HT-29 cells through ERK1/2 activation, *in vitro* (25). While we noted no difference in serum CD14 levels between uninfected groups, at day 10 pi
Vitamin D deficiency and *C. rodentium* induced colitis

vitamin D3 deficient mice carried significantly higher levels of serum CD14, compared to vitamin D3 sufficient mice, confirming that in the absence of vitamin D3, responses against bacterial LPS are exaggerated.

Despite their heightened baseline inflammatory tone, vitamin D3 deficient mice proved more susceptible to *C. rodentium* infection, carrying higher bacterial burdens both in the cecal lumen and at systemic sites. Interestingly, although several previous studies have found that vitamin D can help maintain the intestinal epithelial barrier (3, 16, 34, 59), we did not find any difference in barrier integrity (FITC/dextran assay) between dietary groups at either day 6 pi or day 10 pi, suggesting the translocation to systemic sites was not due to an overtly weaker gut barrier. Although an overt alteration in epithelial barrier integrity was not observed in the vitamin D3 deficient mice, bacteria can enter the systemic circulation through several routes including through intestinal-epithelial microfold cells (M-cells), which are permeable to bacteria as well as macromolecules (24). More likely the increased systemic spread of *C. rodentium* reflects the impaired ability of vitamin D3 deficient mice to dephosphorylate LPS, permitting greater *C. rodentium* translocation out of the gut. With respect to their increased luminal burdens, previous studies showed that VDR-/- mice carried higher pathogen burdens in their ceca during infection with *Salmonella typhimurium* (56), although the basis for this was not defined. A potential explanation reflects the concept that pathogens such as *C. rodentium* actually benefit from intestinal inflammation, since it helps deplete competing commensal bacteria, creating a niche within the intestine where *C. rodentium* can colonize and proliferate. Furthermore, studies have shown that enteric bacterial pathogens can utilize nutrients and metabolites released within the inflamed intestine that are not used by commensal species (30, 54). We recently demonstrated that mice deficient in SIGIRR, a negative regulator of innate signaling not only developed greater inflammatory/antimicrobial responses during *C. rodentium* infection, but they also proved more susceptible to infection, carrying much higher *C. rodentium* burdens than wildtype mice (45). Notably, in the current study, we found no difference in the total number of commensal bacteria/gram between the dietary groups under uninfected conditions or at day 2 pi. However, by day 6 pi, vitamin D3 deficient mice showed a
significant drop in commensal microbe populations, in keeping with the increased inflammatory response seen in these mice. Moreover we found an exaggerated induction of the gene encoding the antimicrobial factor REG3γ in the vitamin D3 deficient mice. We have previously shown that 1,25(OH)2D3 treatment in vivo can increase susceptibility to *C. rodentium* infection by suppressing Th17-mediated immune responses, as well as REG3γ transcription, leading to increased tissue adherent pathogen burdens (41). Thus our studies show REG3γ is clearly dependent on vitamin D status and its upregulated expression in vitamin D3 deficient mice might explain the accelerated loss of commensal microbes in these mice, as well as selective increase in *C. rodentium* burdens in the cecal lumen, rather than at the mucosal surface where REG3γ is expressed.

While the increased inflammatory tone seen in the uninfected intestines of vitamin D3 deficient mice did not affect total commensal numbers, vitamin D3 deficient mice were found to carry more segmented filamentous bacteria (SFB) in their gut luminal contents under both uninfected and infected conditions, compared to vitamin D3 sufficient mice. SFB can induce the differentiation of Th17 cells in the small intestine and can also potentially protect hosts against extracellular bacterial infections, or promote inflammatory diseases (28). It is notable that by simply altering dietary vitamin D levels, we were able to alter SFB populations in the gut and change Th17 response. As for other commensals, we found no significant differences in Bacteroidetes or γ-Proteobacteria in the stool between dietary groups during uninfected conditions, and although there was a trend for vitamin D3 deficient mice to carry fewer Firmicutes, it did not reach statistical significance. At day 10 pi, vitamin D3 deficient mice had significantly higher levels of Actinobacteria as well as γ-Proteobacteria in the stool, in agreement with findings from Assa et al. (3). Interestingly, infected vitamin D3 deficient mice also carried significantly higher levels of Bifidobacteria, compared to vitamin D3 sufficient mice, however we found no differences in the major bacteria phyla- Firmicutes or Bacteroides between dietary groups at day 10 pi, similar to findings by Assa et al. (3).

There is an established link between vitamin D deficiency and respiratory infections (18) and systemic infections (31). However our understanding of the potential for vitamin D
Vitamin D deficiency and *C. rodentium* induced colitis

levels to impact host susceptibility to GI infections is limited. Our current study demonstrates that a short 5-week dietary induced vitamin D3 deficiency increases bacterial overgrowth during infection with the enteric pathogen *C. rodentium* by altering host factors required for bacterial detoxification, indicating that vitamin D plays a protective role during enteric infection. However, these results should be interpreted with caution, since we have previously shown that treating *C. rodentium* infected mice with active vitamin D, 1,25(OH)2D3 led to increased pathogen burdens and exaggerated tissue pathology. In association with their increased susceptibility, 1,25(OH)2D3-treated mice showed less expression of IL-6 and IL-17A, and substantially reduced numbers of Th17 T cells within their infected colons. Th17 responses play a protective role during *C. rodentium* infection (27, 46, 47). Therefore, too much active vitamin D can suppress Th17-mediated inflammatory responses, which can impair host defense against *C. rodentium*. To summarize- during 1,25(OH)2D3 supplementation- there is suppression of Th17 mediated immune responses that are normally important for clearing *C. rodentium* infection. In contrast- during vitamin D deficiency, we showed there is a higher baseline intestinal inflammatory and antimicrobial tone, which leads to a faster loss of commensal microbes, reducing commensal microbial competition with *C. rodentium*, and allowing the pathogen to overgrow and cause increased intestinal damage. Overall these results suggest that to promote gut health, there is likely an optimal range of vitamin D and that too little or too much of this vitamin may promote gut inflammation and/or increase susceptibility to enteric infections.
ACKNOWLEDGEMENTS

We thank the ACF staff at the CFRI for their help with animal care. We also thank Roger Dyer for help with HPLC analysis of 25(OH)D3. This work was supported by two Grants in Aid awarded by Crohn’s and Colitis Canada (CCC) to BAV and KJ, and a discovery grant to BAV from the National Science and Engineering Research Council (NSERC). NRR and KB were both supported by Vanier Canada Graduate Scholarship Doctoral Research Awards, and by Four-Year Doctoral Fellowships from the University of British Columbia. B.A.V. is the Children with Intestinal and Liver Disorders (CHILD) Foundation Research Chair in Pediatric Gastroenterology. KJ is a Senior Clinician Scientist supported by the CHILD Foundation and the Child and Family Research Institute (CFRI) Clinician Scientists Award Program, University of British Columbia.
REFERENCES


Vitamin D deficiency and *C. rodentium* induced colitis

19. Gibson DL, Ma C, Rosenberger CM, Bergstrom KS, Valdez Y, Huang JT, Khan MA, and 
    Vallance BA. Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during Citrobacter 

20. Goldberg RF, Austen WG, Jr., Zhang X, Munene G, Mostafa G, Biswas S, McCormack M, 
    alkaline phosphatase is a gut mucosal defense factor maintained by enteral nutrition. *Proc Natl Acad Sci U 

    Simmons CP, and MacDonald TT. Critical role for tumor necrosis factor alpha in controlling the number 
    of lumenal pathogenic bacteria and immunopathology in infectious colitis. *Infect Immun* 69: 6651-6659, 

    S, Krieger J, Richards JB, Goldzman D, and CaMos Research G. 25-Hydroxyvitamin D in Canadian 


25. Hidaka M, Wakabayashi I, Takeda Y, and Fukuzawa K. Vitamin D(3) derivatives increase 
    soluble CD14 release through ERK1/2 activation and decrease IL-8 production in intestinal epithelial cells. 

    1alpha,25-Dihydroxyvitamin D3 and all-trans retinoic acid synergistically inhibit the differentiation and 

    Y, Akitsu A, Kotaki H, Sudo K, Nakae S, Sasakawa C, and Iwakura Y. Differential roles of 
    interleukin-17A and -17F in host defense against mucop epithelial bacterial infection and allergic responses. 

28. Ivanov, II and Littman DR. Segmented filamentous bacteria take the stage. *Mucosal Immunol* 3: 

    and Cross HS. Colon-specific regulation of vitamin D hydroxylases--a possible approach for tumor 

30. Kamada N, Seo SU, Chen GY, and Nunez G. Role of the gut microbiota in immunity and 

31. Kempker JA, Tangpricha V, Ziegler TR, and Martin GS. Vitamin D in sepsis: from basic 

32. Khan MA, Ma C, Knodler LA, Valdez Y, Rosenberger CM, Deng W, Finlay BB, and 
    Vallance BA. Toll-like receptor 4 contributes to colitis development but not to host defense during 

33. Khoo AL, Joosten I, Michels M, Woestenenk R, Preijers F, He XH, Netea MG, van der Ven 
    AJ, and Koenen HJ. 1,25-Dihydroxyvitamin D3 inhibits proliferation but not the suppressive function of 

    of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol 
    Gastrointest Liver Physiol* 294: G208-216, 2008.

    JS, and Hewison M. Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to 

36. Lalles JP. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* 72: 82- 

37. Lebeis SL, Sherman MA, and Kalman D. Protective and destructive innate immune responses 

38. Moreno J, Cortes CS, Asteggiiano CA, Pereira R, Tolosa N, Canas FM, and Blanco A. 
    Changes of intestinal alkaline phosphatase produced by cholecalciferol or 1,25-dihydroxyvitamin D3 in 
Vitamin D deficiency and *C. rodentium* induced colitis


Vitamin D deficiency and C. rodentium induced colitis

FIGURE LEGENDS

Figure 1: Vitamin D3 deficient mice lose more body weight during infection with Citrobacter rodentium and have thickened colon and shrunken ceca at day 10 post infection

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with C. rodentium for 10 days. A) Serum 25(OH)D3. Results are representative of 3 independent experiments, n = 5-6 per group, * p < 0.05, Mann-Whitney test. B) Serum Calcium. Results are representative of 3 independent experiments, n = 6-8 per group. C) Body weight. Each data point represents the average body weight pooled from 8 mice and is expressed as the percentage of the initial body weight with SEM. Results are representative of 3 independent experiments, n = 8 per group, Student’s t-test was conducted at each time point, * p < 0.05, Mann-Whitney test. D) Macroscopic images of lower gastrointestinal tract (cecum + colon) taken at day 10 pi are representative of group phenotype. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = C. rodentium; D10 = Day 10; pi = post infection

Figure 2: Vitamin D3 deficient mice are more susceptible to Citrobacter rodentium infection and carry higher bacteria burdens in cecum and extra-intestinal tissues at day 10 post infection

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

Figure 3: Vitamin D3 deficient mice carry higher *Citrobacter rodentium* burdens in the cecal contents at day 10 post infection and show delayed clearance of pathogen

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

Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection

Figure 4: Vitamin D3 deficient mice suffer worsened histological damage with increased cell proliferation in the ceca at day 10 post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Representative image of cross section of the cecum at day 10 pi. Original magnification = 50X for top panels, 200X for lower panels, Scale bar = 200 μm. B) Cecum was assessed for histological damage by scoring system for *C. rodentium* described in Methods. Results are representative of 3 independent experiments, n = 8 per group, * p < 0.05, Mann-Whitney test. C) Representative images of formalin fixed cross section of cecum at day 10 pi. Immunofluorescence stained: Blue = DAPI; Red = Ki67. Original magnification = 50X, Scale bar = 200 μm. D) Cecum was assessed for number
of Ki67+ve cells in the lumen (percent of +ve Ki67 per +ve DAPI per area measured). Results are representative of 3 independent experiments, n = 4 per group (uninfected) and n = 11 per group at Day 10 pi, ** p < 0.01, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = C. rodentium; D10 = Day 10; pi = post infection

**Figure 5:** Vitamin D3 deficient mice have higher cecal expression of tumor necrosis factor-α, interleukin-1β and interleukin-6

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

**Figure 6:** Vitamin D3 deficient mice have higher cecal expression of interleukin-17A, interleukin-17F and transforming growth factor-β

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

**Figure 7:** Vitamin D deficient mice have more segmented filamentous bacteria in stool at baseline conditions and have a more dramatic drop in commensal bacteria at day 6 post infection with *Citrobacter rodentium*
Vitamin D deficiency and *C. rodentium* induced colitis

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

**Figure 8:** Vitamin D3 deficient mice have higher cecal expression of Reg3γ at day 10 post infection with *Citrobacter rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. Expression of Reg3γ in cecum during uninfected conditions and day 10 pi as assessed by RT qPCR. Results are representative of 2 independent experiments, n =4-7 per group, * p<0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection

**Figure 9:** Vitamin D3 deficient mice have impaired detoxification of bacterial lipopolysaccharide and higher serum levels of CD14 at day 10 post infection with *C. rodentium*

Weanling (3-week-old) female C57Bl/6 mice were fed vitamin D3 deficient (0 IU) or vitamin D3 sufficient (1000 IU) diets for 5 weeks and then orally infected with *C. rodentium*. A) Relative LPS dephosphorylating activity in duodenum and ileum at Day
1019 Vitamin D deficiency and *C. rodentium* induced colitis

1020 10 pi as determined by the malachite green assay, which measures free phosphate release. Results are representative of 2 independent experiments, n = 6 per group, ** p<0.0043, Mann-Whitney test. B) Serum CD14 during uninfected conditions and at day 10 pi. Results are representative of 3 independent experiments, n = 7-8 per group, * p < 0.05, Mann-Whitney test. Abbreviations: VD3 = Vitamin D3; Def = Deficient; Suff = Sufficient; CR = *C. rodentium*; D10 = Day 10; pi = post infection; LPS = lipopolysaccharide
A

IL-17A in Cecum

IL-17F in Cecum

Uninfected

D10 pi

Relative Expression IL-17A

Relative Expression IL-17F

VD3 Def

VD3 Suff

VD3 Def + CR

VD3 Suff + CR

B

IL-10 in Cecum

TGFβ in Cecum

Uninfected

D10 pi

Relative Expression IL-10

Relative Expression TGFβ

VD3 Def

VD3 Suff

VD3 Def + CR

VD3 Suff + CR

* p = 0.0823