Active vitamin D (1,25-dihydroxyvitamin D$_3$) increases host susceptibility to *Citrobacter rodentium* by suppressing mucosal Th17 responses

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**Abstract**

Active vitamin D (1,25-dihydroxyvitamin D$_3$) increases host susceptibility to *Citrobacter rodentium* by suppressing mucosal Th17 responses. Am J Physiol Gastrointest Liver Physiol 303: G1299–G1311, 2012. First published September 27, 2012; doi:10.1152/ajpgi.00320.2012.—Vitamin D deficiency affects more than 1 billion people worldwide and is associated with an increased risk of developing a number of inflammatory/autoimmune diseases, including inflammatory bowel disease (IBD). At present, the basis for the impact of vitamin D on IBD and mucosal immune responses is unclear; however, IBD is known to reflect exaggerated immune responses to luminal bacteria, and vitamin D has been shown to play a role in regulating bacteria-host interactions. Therefore, to test the effect of active vitamin D on host responses to enteric bacteria, we gave 1,25(OH)$_2$D$_3$ to mice infected with the bacterial pathogen *Citrobacter rodentium*, an extracellular microbe that causes acute colitis characterized by a strong Th1/Th17 immune response. 1,25(OH)$_2$D$_3$ treatment of infected mice led to increased pathogen burdens and exaggerated tissue pathology. In association with their increased susceptibility, 1,25(OH)$_2$D$_3$-treated mice showed substantially reduced numbers of Th17 T cells within their infected colons, whereas only modest differences were noted in Th1 and Treg numbers. In accordance with the impaired Th17 responses, 1,25(OH)$_2$D$_3$-treated mice showed defects in their production of the antimicrobial peptide REG3$. \gamma$. Taken together, these studies show that 1,25(OH)$_2$D$_3$ suppresses Th17 T-cell responses in vivo and impairs mucosal host defense against an enteric bacterial pathogen.

**THE ACTIVE FORM OF VITAMIN D, 1,25-DIHYDROXYVITAMIN D$_3$ [1,25(OH)$_2$D$_3$ or calcitriol], is a hormone that plays a critical role in many different cellular processes, including cell proliferation, apoptosis, and immune modulation (30). In fact, epidemiological studies have implicated vitamin D insufficiency in the pathogenesis of T-cell mediated, inflammation-driven diseases, including the inflammatory bowel diseases (IBD), Crohn’s disease (CD), and ulcerative colitis (27). At present, it is unclear exactly how vitamin D levels impact the development of IBD; however, research has shown that vitamin D deficiency is associated with higher disease activity and longer disease duration in IBD patients (6, 36, 45, 53). Furthermore, CD patients supplemented with vitamin D$_3$ had lower relapse rates compared with placebo-treated patients (35), while a pilot study in CD patients demonstrated a short-term beneficial effect of a 1,25(OH)$_2$D$_3$ analog on disease activity over a 1-yr course (50). Animal models of colitis, including the dextran sodium sulfate (DSS) model, demonstrate that vitamin D deficiency exacerbates disease, while 1,25(OH)$_2$D$_3$ supplementation ameliorates colitis (8, 14, 21, 69), indicating that lack of vitamin D plays a role (albeit poorly defined) in the pathogenesis of IBD. Although the protective mechanisms are unclear, 1,25(OH)$_2$D$_3$ and its analogs have been demonstrated in vitro to inhibit cell proliferation, induce apoptosis, and suppress inflammatory mediators, including TNF-α, IFN-γ, IL-6, and IL-12 in peripheral blood mononuclear cells from patients with IBD (2, 3, 43, 47, 59). Hence, vitamin D therapy may be a novel treatment option for patients with IBD; however, further research is required to understand the underlying mechanisms and potential limitations of treatment with the active compound.

IBD is thought to result from inappropriate immune responses to commensal bacteria found within the gastrointestinal (GI) tract, resulting from a complex interplay among genetic, immunological, and environmental factors. In general, CD is characterized by increased production of Th1/Th17-related cytokines, and evidence suggests that environmental factors, including childhood infections, diet, and sunlight exposure (i.e., vitamin D) may also affect the pathogenesis of CD (40). Although the basis for the beneficial actions of vitamin D during colitis is unclear, studies have suggested that 1,25(OH)$_2$D$_3$ signaling through the vitamin D receptor (VDR) can alter inflammatory responses in the host through a number of mechanisms, including suppressing Toll-like receptor (TLR) expression (55), blocking NF-κB signaling (65), targeting MAPK phosphatase-1 (66), modulating dendritic cell behavior (20), and/or skewing T-cell responses toward a regulatory phenotype (33, 39). In vitro, 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$ can be considered a potent immunosuppressive agent, and we, therefore, wondered whether the immunosuppressive actions of 1,25(OH)$_2$D$_3$ treatment could potentially impair host defenses against enteric
microbes, since Th1/Th17 responses are critical for the clearance of many bacterial infections (5).

Pathogenic strains of Escherichia coli, including enterohemorrhagic E. coli and enteropathogenic E. coli (EPEC) are important causes of infectious diarrhea, with EPEC contributing to as many as 1 million infant deaths per year in developing nations (44). Mucosa-associated E. coli has been observed in greater numbers in patients with IBD compared with healthy controls, and these microbes have been shown to play a role in driving inflammation in the intestine (reviewed in Ref. 51). Since pathogenic strains of E. coli do not colonize mice, researchers rely on the related but mouse-specific attaching and effacing bacterial pathogen Citrobacter rodentium. Following infection, C. rodentium intimately attaches to epithelial cells lining the cecum and colon, resulting in barrier disruption, crypt hyperplasia, loss of goblet cells, mucosal infiltration with immune cells, and a strong Th1/Th17 response (18). The effect of vitamin D levels on host responses to C. rodentium infection have not yet been addressed. To test this, we treated mice undergoing either DSS or C. rodentium-induced colitis with 1,25(OH)2D3 or with vehicle. Interestingly, while 1,25(OH)2D3-treated mice were strongly protected against chemically induced colitis, they were significantly more susceptible to bacteria-induced colitis, and suffered widespread mucosal ulceration and increased pathogen burdens. Through assessment of the host immune response, we found that 1,25(OH)2D3 treatment led to a selective suppression of Th17 T cells and the induction of DSS colitis.

MATERIALS AND METHODS

Mice. Six- to eight-week-old male C57BL/6 mice were obtained from the Centre for Disease Modeling at the University of British Columbia. Mice were maintained in sterilized, filter-topped cages, handled in tissue culture hoods, and fed autoclaved food (PicoLab Rodent Diet 20 no. 5053; Lab Diet, Brentwood, MO) and 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.

1,25(OH)2D3 treatment. Crystalline 1,25(OH)2D3 (SAFC Pharma, Madison, WI) was dissolved in RNA-grade ethanol at 0.025 mg/ml and stored at −20°C. The 1,25(OH)2D3 stock was diluted in sterile PBS immediately prior to injection. Mice were administered either vehicle (PBS with 0.12% ethanol) or 10 ng 1,25(OH)2D3 (0.5 μg/kg) via intraperitoneal injection daily for up to 11 days. The 1,25(OH)2D3 dose was chosen on the basis of previous in vivo studies with mice (32). For additional dose-response studies, mice were administered 5 ng or 15 ng 1,25(OH)2D3 daily.

Induction of DSS colitis. For DSS studies, colitis was induced by adding dextran sodium sulfate (36,000–55,000 kDa, no. 160110; MP Biomedicals, Solon, OH) to sterile drinking water at a concentration of 3% (wt/vol). Animals were treated with DSS for 7 days and then allowed to recover by removing the DSS from their drinking water for an additional 2 days (9 days total). Mice were weighed daily and monitored for signs of distress, including stool consistency and rectal bleeding.

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 ~2.5 × 108 CFU of wild-type or ΔespF streptomycin-resistant C. rodentium (formerly C. freundii biotype 4280, strain DBS100). Mice were weighed daily and monitored for signs of distress.

FITC-dextran intestinal permeability assay. The FITC-dextran assay was performed as previously described (23). Briefly, mice were orally gavaged with 150 μl of 80 mg/ml 4 kDa FITC-dextran (Sigma, St. Louis, MO; FD4) in PBS 4 h prior to death. Mice were anesthetized and blood was collected by cardiac puncture, which was added immediately 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 fluorimeter (Perkin-Elmer Life Sciences, Boston, MA) at excitation wavelengths of 485 nm, emission wavelengths of 530 nm for 0.1 s.

Tissue collection. For DSS studies, mice were killed at day 9 after administration. For C. rodentium studies, mice were killed at day 6 or day 10 postinfection. Mice were anesthetized with halothane and killed by cervical dislocation; tissues were then collected for further analysis. The large intestine was resected and divided into four sections, i.e., cecum, proximal colon, midcolon, and distal colon for further analysis. Tissues were immediately placed in 10% neutral buffered formalin (Fisher) (48 h, 4°C) for histological studies, or placed in RNAlater (Qiagen, Gaithersburg, MD) and stored at −80°C for subsequent RNA extraction. For certain experiments, the colon was opened longitudinally, stool was gently removed, and the tissue was rolled (Swiss roll) from distal to proximal end, fixed with a needle, and placed in 10% neutral buffered formalin (Fisher) (48 h, 4°C) for histological processing.

Serum calcium. Mice were anesthetized with halothane, and blood was collected by cardiac puncture. Blood was allowed to clot naturally at room temperature, and cells were removed by centrifugation. Plasma was collected and stored at −80°C until analysis with the calcium colorimetric assay kit (BioVision Research, Mountain View, CA; cat. no. K380–250). This assay uses the chromogenic complex, which forms between calcium ions and O-cresolphthalein. Samples were quantified at OD 575 nm using a Wallac Victor fluorimeter (Perkin-Elmer Life Sciences, Boston, MA) and compared with the calcium standard provided with the kit.

C. rodentium enumeration. For enumeration of C. rodentium, tissues were prepared as previously described (4). Briefly, whole tissues were collected, weighed, and homogenized in a MixerMill 301 bead miller (Retche). Tissue homogenates were serially diluted in PBS and plated onto Luria-Bertani agar plates containing 100 mg/ml streptomycin (Sigma) and 80°C until analysis with the calcium colorimetric assay kit (BioVision Research, Mountain View, CA; cat. no. K380–250). This assay uses the chromogenic complex, which forms between calcium ions and O-cresolphthalein. Samples were quantified at OD 575 nm using a Wallac Victor fluorimeter (Perkin-Elmer Life Sciences, Boston, MA) and compared with the calcium standard provided with the kit.

Immunofluorescence staining. Immunofluorescence staining of colonic tissues for Ki67 and Tir was performed as previously described (4, 23). 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 10.220.33.6 on July 7, 2017 http://ajpgi.physiology.org/ Downloaded from
mononuclear cell infiltration (0 = none; 1 = mild; 2 = moderate; 3 = severe) and overall tissue damage (0 = none; 1 = up to 50% loss of crypts; 2 = more than 50% loss of crypts; 3 = total disruption of crypts and loss of epithelial cells), as previously described (42). The maximum score that could be obtained with this system is 13 points.

RNA extraction and quantitative RT-PCR. Colon tissues were collected and stored in RNALater (Qiagen) at −80°C, and total RNA was 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 manufacturer’s instructions. Quantitative PCR was carried out using a Bio-Rad 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. The primer sequences and reaction conditions for β-actin, TNF-α, iNOS, IFN-γ, IL-17A, IL-17F, IL-22, IL-23, and RegIIIγ have previously been described (4).


dilute in FACS buffer (fixation buffer) for 10 min and incubated with 2.5 µg/ml brefeldin A (Sigma Aldrich, Oakville, ON, Canada). Samples were then layered on a Percoll gradient (Amersham Biosciences, Uppsala, Sweden), and the LPMC collected from the 40–75% Percoll interface were used in subsequent assays.

Antibody staining and flow cytometry. Surface Ab staining was performed at 4°C in PBS/2% FCS with 0.1% sodium azide (FACS buffer) with fluorescently labeled CD4, CD25, and FoxP3 Ab (BD Pharma and eBiosciences, San Diego, CA). For intracellular cytokine staining, 0.5 × 10^6 cells were resuspended in culture medium (RPMI 1640 containing 10% heat-inactivated FCS and 5 mM EDTA). Tissues were then cut into 1-mm pieces followed by digestion with 0.2 mg/ml type VII collagenase (Sigma Aldrich, Oakville, ON, Canada). Samples were then layered on a Percoll gradient (Amersham Biosciences, Uppsala, Sweden), and the LPMC collected from the 40–75% Percoll interface were used in subsequent assays.

Statistical analysis. Statistical significance was calculated by using either a two-tailed Student’s t-test or the Mann-Whitney U-test unless otherwise indicated, with assistance from GraphPad Prism Software, version 4.00 (GraphPad Software, San Diego, CA, www.graphpad.com). A P value of ≤0.05 was considered significant. The results are expressed as the means ± SE.
mice steadily lost weight as their infection progressed and by day 10 postinfection, they had lost 18% of their initial body weight. Serum calcium levels were higher in 1,25(OH)2D3-treated mice compared with vehicle-treated mice at day 10 postinfection \[10.16 \pm 0.40 \text{ mg/dl vs. } 6.46 \pm 0.27 \text{ mg/dl SE (} P < 0.01; n = 8 \text{ per group)}\]. There was, however, no difference in serum calcium levels between \textit{C. rodentium} infected vs. noninfected mice. There were no observed differences in stool consistency or rectal bleeding between infected mice given vehicle or 1,25(OH)2D3-treated mice; however, the 1,25(OH)2D3-treated mice had shortened colons, compared with vehicle-treated mice \[5.96 \pm 0.18 \text{ cm vs. } 6.67 \pm 0.11 \text{ cm, respectively (} P < 0.01; n = 18 \text{ per group)}\], and 70% of these mice displayed macroscopic mucosal damage and small ulcers in their middle and distal colons at day 10 postinfection, whereas similar pathology was only seen in 20% of vehicle-treated mice \[68.75 \pm 11.96\% \text{ vs. } 18.75 \pm 10.07\%, respectively (} P < 0.01; n = 16 \text{ per group})\] (Fig. 2B).

1,25(OH)2D3-treated mice carry increased \textit{C. rodentium} burdens. To address whether the exaggerated mucosal damage suffered by the 1,25(OH)2D3-treated mice was associated with increased pathogen burdens, colons were Swiss-rolled and immunostained for \textit{C. rodentium}-derived translocated intimin receptor (Tir) (16). As expected, \textit{C. rodentium} were localized to the distal colon in vehicle-treated mice at day 10 postinfection. In contrast, 1,25(OH)2D3-treated mice had strong Tir staining throughout the entire colon, with significant staining found in the midcolon and even reaching the proximal colon.
1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice develop colonic ulcerations and have greater bacterial burdens at day 10 postinfection. A: body weight. Mice were treated with vehicle or 1,25(OH)\textsubscript{2}D\textsubscript{3} intraperitoneally daily throughout infection. Each data point represents the average body weight pooled from 8–16 mice and is expressed as the percentage of the initial body weight with SE. Results are representative of 2–4 independent experiments. 

Two-way ANOVA. B: right: representative digital image of the lower GI tract from mice at day 10 postinfection. Vehicle-treated mice (left) appeared normal, compared with 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice (right), which had shortened, inflamed colons nearly devoid of contents. Left: representative digital image of the lower GI tract, opened longitudinally from proximal (top) to distal (bottom) from 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice at day 10 postinfection. Arrows are pointing to erosion/ulcerated regions in middle and distal colon. C: representative images of formalin-fixed, “Swiss-rolled” colon tissues at day 10 postinfection. Blue denotes DAPI; red denotes Tir. Original magnification = ×50.

(Fig. 2C). To quantify bacteria, tissues were homogenized and plated: at both day 6 and day 10 postinfection, 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice carried significantly higher \textit{C. rodentium} burdens in their colons and ceca, compared with vehicle-treated mice (Fig. 3). \textit{C. rodentium} is a noninvasive pathogen, and bacterial loads are primarily limited to the intestinal lumen and mucosal surface of WT mice. Interestingly, 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice carried more culturable \textit{C. rodentium} from extra-intestinal tissues, including mesenteric lymph node (MLN), spleen, and liver at day 6 postinfection and day 10 postinfection, compared with vehicle-treated mice, indicating greater bacterial translocation to these systemic sites (Fig. 3). These data show that 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment increases pathogen burdens during the course of infection.

1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice have worsened histological damage during \textit{C. rodentium} infection. \textit{C. rodentium} initially colonizes the cecum and then spreads to the distal colon of WT 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 postinfection, 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice had significantly increased submucosal edema (1.42 ± 0.13 vs. vehicle: 0.58 ± 0.80 (P < 0.01; n = 6–7 per group)] and a higher number of mucosal ulcers [0.71 ± 0.28 vs. vehicle: 0 ± 0 (P < 0.05; n = 6–7 per group) throughout the entire colon, as determined by examining Swiss-rolled sections. Tissue cross sections were also examined, and although there was a trend for more damage in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice in the distal colon and cecum between groups at day 10 postinfection, the differences did not reach statistical significance. In contrast, the midcolon of 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice was the site of the greatest damage in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice with significantly elevated scores for edema, goblet cell depletion, hyperplasia, and infiltrating inflammatory cells, compared with vehicle-treated mice (Fig. 4B). Note, that 1,25(OH)\textsubscript{2}D\textsubscript{3} had no effect on histology scores in uninfected mice (data not shown).

1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice show altered epithelial responses during \textit{C. rodentium} infection. To address the mechanisms underlying the mucosal damage that developed in the infected 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice, intestinal epithelial cell proliferation and cell death were determined by Ki67 staining and the TUNEL assay, respectively. As previously shown (23, 62), infected mice displayed more Ki67-positive and TUNEL-positive cells in their colons, compared with uninfected mice at day 10 postinfection (data not shown). However, in the midcolon, infected 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated mice had more TUNEL-positive epithelial cells in the midcolon, compared with vehicle-treated mice at day 10 postinfection (Fig. 4C).
more, 1,25(OH)2D3-treated mice had large numbers of TUNEL-positive cells in both the cecal and colonic lumen at day 10 postinfection, indicating greater epithelial cell sloughing within these mice. These results are in agreement with previous work indicating that 1,25(OH)2D3 can induce apoptosis in human colonic epithelial cells in vitro (reviewed by Ref. 56). However, these changes were only seen during infection, as there were no obvious effects of 1,25(OH)2D3 on cell proliferation or apoptosis between uninfected groups. We did not find any significant difference in Ki67-positive cells in the midcolons between vehicle and 1,25(OH)2D3-treated mice at day 10 postinfection.

To determine whether the observed changes in apoptosis coincided with altered barrier integrity in vivo, mice were orally gavaged with FD4 at day 6 postinfection, and their sera was assessed for levels of translocated FD4. As expected, vehicle-treated mice infected with C. rodentium had higher levels of detectible FD4 in the serum, compared with control mice at day 6 postinfection (Fig. 4D). However, 1,25(OH)2D3-treated mice had significantly higher levels of FD4 in their serum, compared with vehicle-treated mice [4.72 ± 0.89 μg/ml vs. 2.11 ± 0.26 μg/ml respectively (P < 0.05)], indicating that 1,25(OH)2D3 treatment leads to exaggerated barrier disruption during infection with C. rodentium (Fig. 4D).

1,25(OH)2D3-treated mice suffer increased bacterial burdens and ulceration in response to attenuated C. rodentium. To determine whether the observed epithelial pathology that develops in infected mice during 1,25(OH)2D3 treatment reflected exaggerated bacteria-driven pathology, or alternatively, an abnormal host response to C. rodentium, we challenged mice with a C. rodentium mutant lacking the translocated bacterial effector protein EspF (52). Previous studies have shown that the EspF protein plays a critical role in the disruption of tight junctions and the induction of apoptosis in infected colonic epithelial cells (12, 49). Furthermore, this effector has been shown to play a critical role in causing mucosal damage and ulcerations in susceptible mouse strains (24). As expected, vehicle-treated mice challenged with the ΔespF mutant suffered little in the way of weight loss or mucosal damage. In contrast, infected 1,25(OH)2D3-treated mice steadily lost weight, as their infection progressed and by day 10 postinfection, they had lost ~18% of their initial body weight (Fig. 5A).

Furthermore, 1,25(OH)2D3-treated mice developed macroscopic erosions in their middle and distal colons at day 10 postinfection. Histologically, the midcolon of 1,25(OH)2D3-treated mice showed significant edema and inflammatory cell infiltrate, with areas of ulceration (Fig. 5B). In agreement with this increased damage, 1,25(OH)2D3-treated mice had 20-fold higher pathogen burdens in their colons, compared with vehicle-treated mice, indicating greater colonization. 1,25(OH)2D3-treated mice also carried more culturable C. rodentium mutant from extraintestinal tissues at day 10 postinfection, compared with vehicle-treated mice, indicating greater bacterial translocation to these systemic sites (Fig. 5C).
for analysis, since there was comparable histological damage between both groups at this site. The inflammatory mediators TNF-α and iNOS have previously been shown to play an important role in controlling C. rodentium pathogen load (26, 61). Surprisingly, there were no changes in the mRNA levels of these genes between vehicle and 1,25(OH)2D3-treated mice at day 10 postinfection (data not shown). As expected, the expression of Th1- (IFN-γ) and Th17-related cytokines (IL-6, IL-17A) were upregulated in the distal colon of mice challenged with C. rodentium compared with uninfected controls, as previously shown (15, 29, 68). However, at day 10 postinfection, 1,25(OH)2D3 treatment strikingly suppressed the elevated expression of IFN-γ, IL-6, and IL-17A compared with vehicle-treated mice (Fig. 6). There was also a trend for reduced expression of IL-17F in the distal colon of 1,25(OH)2D3-treated mice at day 10 postinfection, compared with vehicle (0.82 ± 0.08- vs. 1.65 ± 0.33-fold expression, respectively); however, this did not reach statistical significance (P = 0.09). We also measured gene expression of other cytokines and growth factors and found no differences in the colonic expression of the anti-inflammatory cytokines IL-10 or TGF-β or other Th17-related cytokines (IL-22, IL-23) between vehicle and 1,25(OH)2D3-treated mice at day 10 postinfection (data not shown).

1,25(OH)2D3-treated mice have fewer CD4+IL-17A+ cells in the colon at day 10 postinfection. On the basis of the reduced expression of IFN-γ and IL-17A, we next asked whether 1,25(OH)2D3 treatment led to alterations in immune cell pop-
1,25(OH)2D3-treated mice were more susceptible to infection with ΔespF C. rodentium mutant. A: body weight. Mice were treated with vehicle or 1,25(OH)2D3 intraperitoneally daily throughout infection. Each data point represents the average body weight pooled from eight mice and is expressed as the percentage of the initial body weight ± SE. Results are representative of two independent experiments. **P < 0.01; ***P < 0.001, two-way ANOVA. B: representative image of cross section of the midcolon at day 10 postinfection. Original magnification = x200. C: ΔespF C. rodentium burden day 10 postinfection; n = 8 per group. Results are representative of 3–5 independent experiments. *P < 0.05; **P < 0.01. Mann-Whitney U-test).

Although there was a trend for 1,25(OH)2D3-treated mice to have more CD4+CD25+FoxP3+ T cells in their colonic lamina propria at day 10 postinfection, compared with vehicle-treated mice, these changes did not reach significance.

1,25(OH)2D3-treated mice have reduced expression of the antimicrobial peptide RegIIIy. Th17-associated cytokines exert strong host-protective roles during C. rodentium infection, although the basis for their protective effects is poorly understood. Considering that a major feature of the 1,25(OH)2D3-treated mice was increased C. rodentium burdens, we decided to assess gene expression for several antimicrobial factors previously linked to host defense against C. rodentium, since these could be the effector molecules through which Th17 cells exert their effects. As previously shown, the expression of RegIIIγ was upregulated in the distal colon of mice challenged with 1,25(OH)2D3-treated mice compared with vehicle-treated mice, although no differences were observed in the expression of RegIIIβ, S100A8, or S100A9 between vehicle and 1,25(OH)2D3-treated mice at day 10 postinfection.
postinfection, indicating a selective effect of 1,25(OH)₂D₃ on RegIIIγ.

**DISCUSSION**

Our work confirms that 1,25(OH)₂D₃ treatment can protect mice against DSS-induced colitis; however, this is the first study to show that providing exogenous and active vitamin D [i.e., 1,25(OH)₂D₃] increases host susceptibility to an enteric bacterial pathogen. It has previously been shown that mice treated with high-dose 1,25(OH)₂D₃ (75 ng, 3 times/wk) developed exaggerated crypt hyperplasia at day 12 postinfection with *C. rodentium*; however, other aspects of disease were not reported (54). We found that 1,25(OH)₂D₃-treated infected mice suffered from increased *C. rodentium* burdens and exag-

**Fig. 6.** 1,25(OH)₂D₃-treated mice have suppressed colonic cytokine mRNA levels during infection with *C. rodentium*. Expression of IFN-γ, IL-17A, and IL-6 in distal colon at baseline and day 10 postinfection as assessed by RT qPCR; *n* = 8 per group. Results are representative of three independent experiments. *P* < 0.05. Mann-Whitney *U*-test).

**Fig. 7.** 1,25(OH)₂D₃-treated mice have fewer CD4⁺IL-17⁺ cells in the colon at day 10 postinfection. A: FACS analysis. Lamina propria (colon + cecum with cecal patch removed) from 2–4 mice were pooled together for each data point. Results are representative of three independent experiments. *P < 0.05, Mann-Whitney *U*-test). B: representative FACS plot of proportion of CD4⁺IL-17⁺ cells in lamina propria at day 10 postinfection (PBS + CR 14.4% vs. Cal + CR 8.82%).
gerated colonic tissue damage, compared with vehicle-treated mice. In agreement with these findings, 1,25(OH)2D3-treated mice showed defects in their expression of colonic IFN-γ, IL-17A, and IL-6 at day 10 postinfection. These cytokines have been shown to play important roles in regulating pathogen burdens, as well as mucosal damage during C. rodentium infection (15, 57, 58). In association with their increased susceptibility, 1,25(OH)2D3-treated infected mice showed substantially reduced numbers of Th17 T cells in their colons during C. rodentium infection, whereas only modest changes were noted in Th1- and Treg T-cell numbers. In agreement with a suppression of Th17 responses, 1,25(OH)2D3-treated mice showed defects in their production of the Th17-associated antimicrobial peptide REG3γ, which has been shown to play a role in mucosal repair and host defense during infection with C. rodentium (68).

As previously shown, mice treated with 1,25(OH)2D3 were protected against DSS-induced colitis (21, 43). Vehicle-treated mice developed severe mucosal damage in the colon post-DSS, characterized by a loss of crypts and infiltration of inflammatory cells, whereas the colons of 1,25(OH)2D3-treated mice appeared relatively undamaged. It is currently unclear how 1,25(OH)2D3 ameliorates DSS-induced colitis. Previous studies have indicated that 1,25(OH)2D3 treatment can suppress several inflammatory mediators in mice challenged with DSS, including TNF-α and IFN-γ (67); however, the underlying mechanism is unknown. We have not investigated the Th17 response in DSS-challenged mice, since the effect of 1,25(OH)2D3 treatment is so dramatic, it is likely that all host inflammatory/immune markers are suppressed. It is also unknown whether Th17 responses play a role during DSS challenge, since T and B cells are dispensable in the acute DSS colitis model (17). However, Alex et al. (1) showed that mice administered 3% DSS for 7 days had increased colonic expression of IL-12 and IL-17, while IL-17A−/− mice were protected against DSS challenge, indicating the IL-17A may play an important role in the pathogenesis of DSS colitis (34).

The newly described Th17 cells are abundant at mucosal surfaces and characterized by the production of IL-17A, a proinflammatory cytokine associated with increased severity of various inflammatory diseases, including Type 1 diabetes, multiple sclerosis, and IBD (reviewed by Ref. 11). As such, strategies to block and/or regulate Th17 responses are currently being investigated, including 1,25(OH)2D3 treatment. Indeed, it was recently shown that 1,25(OH)2D3 can directly suppress IL-17A by dissociation of histone acetylase activity from the IL-17A promoter and recruitment of histone deacetylase and VDR/RXR binding to NFAT sites (37). Furthermore, recent studies have found that 1,25(OH)2D3 can suppress the development of Th17 cells and production of associated cytokines, such as IL-6, IL-17, IL-22, and IL-23, in vitro (9, 10, 33, 37, 60). However, most of these studies have focused on in vitro regulation, whereas in vivo studies are needed to better clarify the potential therapeutic use of modifying vitamin D levels to alter Th17 responses. Our findings indicate that 1,25(OH)2D3 does, indeed, suppress Th17 responses in vivo, and while such suppression may prove protective against autoimmune diseases, it can also impair antimicrobial defenses in the GI tract. In fact, IL-17A has been shown to induce the expression of antimicrobial peptides, stimulate the production of proinflammatory cytokines and induce granulopoietic factors and chemokines to recruit neutrophils in response to infection (reviewed by Ref. 13). As such, Th17 responses are critical for the clearance of extracellular bacterial infections (5).

In agreement with the suppressed colonic Th17 response, we found that 1,25(OH)2D3-treated mice carried significantly higher C. rodentium burdens in their colons and ceca compared with vehicle-treated mice at day 10 postinfection. Furthermore, 1,25(OH)2D3-treated mice suffered C. rodentium colonization throughout their entire colon, as determined by Tir staining on infected epithelial cells, including the middle and proximal regions of the colon, areas not normally colonized by C. rodentium in WT mice. It is currently unknown why 1,25(OH)2D3 treatment altered the typical infection pattern; however, it can be assumed that 1,25(OH)2D3 treatment modified the host response to C. rodentium, rather than having a direct effect on the bacteria, since previous work has shown that 1,25(OH)2D3 does not directly alter bacterial growth or activity, in vitro (63).

Interestingly, we found that 1,25(OH)2D3-treated mice developed worsened histological damage, particularly in the midcolon, an area not typically damaged by C. rodentium in WT mice. In association with the mucosal damage, we found that 1,25(OH)2D3 treatment selectively suppressed IL-6 mRNA levels within the colon. IL-6 is a cytokine shown to play a key role in mucosal protection during challenge with C. rodentium (15). Furthermore, our group has found that mice deficient in TLR2 develop ulceration in the midcolon during C. rodentium challenge, which was attributed to a defect in IL-6 production within the colonic mucosa (24). Normally, C. rodentium colonizes the cecum and then spreads to the distal colon of WT mice, resulting in histological damage to these regions. At first thought, it seems that the exaggerated damage in the midcolon of 1,25(OH)2D3-treated mice was simply caused by higher bacterial burdens in the colon, or perhaps, the immunosuppressive effects of 1,25(OH)2D3 allowed the pathogenic bacteria to colonize areas of the gut it does not normally inhabit. However, we also found similar results when 1,25(OH)2D3-treated mice were challenged with a C. rodentium strain lacking the translocated effector espF (ΔespF), a
strain that does not typically cause mucosal damage in WT mice. These findings indicate that the observed erosions and epithelial damage in 1,25(OH)2D3-treated mice are most likely due to host-driven changes in the epithelial response to C. rodentium challenge, since C. rodentium typically requires EspF to induce mucosal pathology.

The intestinal surface is lined by a single layer of epithelial cells, which function as a barrier to separate the bacteria-rich lumen from underlying host cells. Previous work has shown that vitamin D plays an important role in maintaining barrier integrity in the intestine (21, 38, 41, 67); however, the role of 1,25(OH)2D3 on barrier integrity is less clear. Our results show that 1,25(OH)2D3-treated mice develop worsened intestinal epithelial barrier dysfunction, compared with vehicle-treated mice during infection with C. rodentium, as determined by the FITC/dextran assay. Correspondingly, 1,25(OH)2D3-treated mice suffered from increased bacterial translocation out of the GI tract and to the MLN, spleen, and liver at day 6 and day 10 postinfection, compared with vehicle-treated mice. We also found that 1,25(OH)2D3-treated mice had altered epithelial responses at day 10 postinfection, including increased cell death, as determined by TUNEL staining. Our results are in agreement with previous work demonstrating that 1,25(OH)2D3 can induce apoptosis in human colonic epithelial cells in vitro (reviewed in Ref. 56). However, it is currently unknown whether the defects in barrier integrity are primary or secondary to the immune mediated effects of 1,25(OH)2D3.

Our current understanding of the potential for vitamin D levels to impact on host susceptibility to pathogens is limited. There is an established link between vitamin D deficiency and tuberculosis (22, 64) and viral respiratory infections (25, 48). Moreover, low-vitamin D intake has also been correlated with differences in fecal microbial composition, including elevated levels of Bacteroides in vitamin D-deficient volunteers (46). However, the role of vitamin D during enteric infections is currently unclear. Recently, Edrington et al. (19) found that supplementing cattle with vitamin D3 had no effect of fecal shedding of E. coli 0157:H7; however, the researchers also looked at seasonal variation and found that the proportion of cattle shedding E. coli 0157:H7 was higher in the summer months (16.7% in the summer vs. 6.7% in the winter, P = 0.08), which correlated with higher serum vitamin D levels. Together with our results, these findings raise the question of whether there may be an unexpected benefit to vitamin D deficiency, i.e., potentially, the development of stronger Th17 responses against specific bacterial pathogens. Correspondingly, while vitamin D supplementation has been shown to protect against autoimmune/inflammatory conditions (27), it is currently unknown whether such immune suppression could increase the susceptibility of a host to specific types of infection. It is tempting to speculate that human migration away from the equator and to more northerly climate and its associated risk for vitamin D insufficiency may have yielded both beneficial, as well as detrimental effects on our immune system, leaving individuals at greater risk of autoimmune disease, but more resistant to enteric infections. Future research is necessary to answer these questions and to help clarify the role of vitamin D in maintaining mucosal homeostasis during enteric infections and other inflammatory challenges. Taken together, these studies show that 1,25(OH)2D3 is a potent, yet selective, immunosuppressive agent, and as such, 1,25(OH)2D3 treatment may protect against Th17 T-cell driven damage during experimental, as well as potentially clinical colitis; however, caution should be advised, since Th17 responses are also critical in providing host defense against extracellular bacteria, including those pathogenic strains of E. coli implicated in the pathogenesis of IBD.

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