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Dysbiosis caused by vitamin D receptor deficiency confers colonization resistance to *Citrobacter rodentium* through modulation of innate lymphoid cells

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Abstract

Vitamin D receptor (VDR) knockout (KO) mice had fewer *Citrobacter rodentium* in the feces than wild-type (WT) mice and the kinetics of clearance was faster in VDR KO than WT mice. VDR KO mice had more IL-22 producing innate lymphoid cells (ILC), and more anti-bacterial peptides than WT mice. The increased ILC in the VDR KO mice was a cell autonomous effect of VDR deficiency on ILC frequencies. BM transplantation from VDR KO mice into WT resulted in higher ILC and colonization resistance of the WT mice. Disruption of the gut microbiota using antibiotics in VDR KO mice reversed colonization resistance to *C. rodentium* infection. Confirming the role of the microbiota in the colonization resistance of VDR KO mice, transfer of the VDR KO microbiota to WT GF mice, resulted in colonization resistance. Once colonization resistance is overcome, VDR KO mice had increased susceptibility to *C. rodentium*. VDR expression is a regulator of ILC frequencies, IL-22, dysbiosis and *C. rodentium* susceptibility.

Keywords

vitamin D receptor; microbiota; innate lymphoid cells

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INTRODUCTION

All cells of the immune system that have been analyzed express the vitamin D receptor (VDR) including dendritic cells (DCs), macrophages, T cells and B cells[1]. The VDR is a nuclear receptor that is part of the steroid hormone superfamily of receptors that regulate gene transcription. The active form of vitamin D and the high affinity VDR ligand is 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$)[2, 3]. The effects of 1,25$(OH)_2D_3$ include direct effects on IFN-γ and IL-17 production by T cells and indirect effects of 1,25$(OH)_2D_3$ on DC and macrophage that reduce the Th1 and Th17 response *in vivo*[4]. The effects of vitamin D include inhibition of the generation of Th1 and Th17 responses, induction of regulatory T cells and amelioration of experimental immune-mediated disease.

Because of the suppressive effects of 1,25$(OH)_2D_3$ on Th1 and Th17 responses, it seemed possible that vitamin D might compromise the anti-infectious response. Paradoxically, when therapeutic doses of 1,25$(OH)_2D_3$ (for immune-mediated disease) were tested, there was no effect of the 1,25$(OH)_2D_3$ treatment on the ability of the host to fight a *Herpes simplex* or *Candida albicans* infection[5]. In addition, vitamin D supplementation of humans during tuberculosis treatment was shown to be beneficial in some studies and not to have an effect in others[6–9]. *In vitro* and *in vivo* vitamin D induced the production of anti-bacterial peptides that might be beneficial in protecting the host from infection[10, 11]. VDR-KO mice infected with *Schistosoma mansoni* showed no effect of VDR expression on worm burdens, weight and fibrosis compared to wild-type (WT) mice[12]. VDR-KO mice had decreased *Leishmania major* parasite burdens compared to WT controls[13]. VDR-KO mice exhibited a delayed clearance of *Listeria monocytogenes* following infection but the VDR-KO mice were able to clear the infection[14]. How vitamin D could inhibit Th1 and Th17 responses in one context (immune-mediated disease) but not another (infectious disease) has not been established.

Citrobacter rodentium is a gram-negative murine pathogen that naturally colonizes and infects mice and forms attaching and effacing lesions[15]. In WT mice, *C. rodentium* colonizes the cecum and colon transiently and is cleared over three weeks [16]. Th17-mediated immune responses are required for clearing the infection[17]. T cell, B cell and T and B cell (Rag) KO mice fail to clear a *C. rodentium* infection[16]. In the gut, innate lymphoid cells (ILC) that produce IL-22 and IL-17 (ILC3) are critical for early protection against *C. rodentium* infection[17], ILC3 cells are the major source of IL-22 during the first 6 days following infection[18]. The innate immune system induces the development of protective acquired immunity required for clearance of *C. rodentium*.

Here we determined the role of the VDR on host resistance to *C. rodentium* infection. Surprisingly, VDR-KO mice were resistant to colonization with *C. rodentium*. The colonization resistance of the VDR-KO mice was associated with increased IL-22, increased ILCs, higher expression of RegIIIγ, Ang-4 and dysbiosis of the bacterial microbiota. Antibiotic (ABX) disruption of the gut microbiota reversed colonization resistance to *C. rodentium* in VDR-KO mice. Cecal transplantation of the microbiota from VDR-KO mice to WT germfree (GF) mice conferred colonization resistance compared to recipients of WT microbiota. In addition, VDR-KO bone marrow (BM) transplantation transferred colonization resistance to WT mice, which was associated with increased IL-22 and

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increased ILCs. Double VDR/Rag (D)KO mice had increased ILCs and IL-22 production in the gut. However, colonization resistance was not observed in the DKO mice. Both the DKO mice and the ABX treated VDR-KO mice developed more severe infections, including increased mortality, than their respective controls (Rag KO and ABX WT) to *C. rodentium* infection. Our data demonstrate that increased IL-22-producing ILCs contribute to dysbiosis and *C. rodentium* colonization resistance of VDR-KO mice. Taken together, our data suggest that the VDR regulates the gut microbiota, colonization and susceptibility to *C. rodentium*.

RESULTS

Reduced bacterial shedding and inflammation in VDR-KO mice

In WT mice, *C. rodentium* was detectable in the feces at d1 and increased through d7 when it peaked and then declined until d21 (Figure 1A). At d1 post-infection, VDR-KO mice had 3 logs fewer bacteria in the feces than WT mice (Figure 1A). The bacterial shedding in the feces was lower in VDR-KO mice compared to WT and the VDR-KO mice cleared the infection by d18 (Figure 1A). VDR-KO mice were less susceptible to *C. rodentium* than WT mice (Figure 1A). VDR-KO mice were resistant and WT mice were susceptible to *C. rodentium* infection, regardless of ancestry (breeders), sex or housing of the mice.

The recruitment of inflammatory cells into the colonic lamina propria (LP) was measured in VDR KO and WT mice. In uninfected (d0) WT and VDR-KO mice, the frequencies of either inflammatory monocytes ($CD11b^+Gr\text{-}1^{high}F4/80^+$, Figure 1B) or neutrophils ($CD11b^+Gr\text{-}1^{high}F4/80^-$, Figure 1C) were low (0.1%). Frequencies of inflammatory monocytes and neutrophils increased at d10 and fell significantly at d21 in WT mice (Figure 1B, C). Conversely, the frequencies of inflammatory monocytes remained low in VDR-KO mice following infection (Figure 1B). The neutrophils in VDR-KO mice were significantly lower than WT mice at d10 post-infection (Figure 1C). Infection increased the frequency of $CD3^+$T cells in the WT colonic LP at d10 and d21 post-infection (Figure 1D). The increase in CD3 frequencies occurred after d21 of infection in VDR-KO mice (Figure 1D). VDR-KO mice had fewer $CD3^+$T cells compared to WT mice at d10 post-infection (Figure 1D). The total numbers of LP lymphocytes isolated from WT and VDR-KO mice were not different. The expression of *Ifng*, *Il17a* and *Il6* were low in uninfected colon from WT mice and increased significantly at d10 post-infection in WT mice (Figure 1E–1G). There was no increase in *Ifng*, *Il17a* and *Il6* expression with infection of VDR-KO mice (Figure 1E–1G). In addition, VDR-KO mice had significantly lower expression of *Ifng*, *Il17a* and *Il6* than WT mice at d10 (Figure 1E–1G). Lower colonization of VDR-KO mice with *C. rodentium* was associated with lower numbers of immune cell infiltrates and reduced cytokine expression.

Disruption of the microbiota in VDR KO mice reverses colonization resistance

The intestinal commensal bacteria compete with *C. rodentium* for colonization[19]. Previously we had shown that VDR-KO and WT mice had different commensal bacteria in the feces and that the difference in the microbiota occurred between VDR-KO and WT littermates suggesting an effect of VDR expression on the microbiota[20]. Here we used quantitative real-

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time PCR to determine the frequencies of bacterial phyla in the feces from mice used for these experiments (Figure 2A). Confirming the published data, VDR-KO mice had decreased quantities of bacteria from the Firmicutes phylum, and increased quantities of bacteria from the Bacteroidetes and the Proteobacteria phyla in the feces compared to WT mice (Figure 2A)[20]. In addition, several bacterial genus members were also different in VDR-KO mice compared to WT, including Eubacterium, Bacteroides and Salmonella (Supplementary Figure S1A–S1C). Similar phyla differences were also shown in the colon and small intestine (SI) from VDR-KO and WT mice (Supplementary Figure S1D–SII).

To determine the role of commensal bacteria in the colonization resistance of VDR-KO mice to *C. rodentium* infection, ABX was used to disrupt the bacteria. The one-dose ABX treatment significantly reduced the total amount of bacterial 16S rDNA in the feces of the ABX-treated WT and ABX-treated VDR-KO mice compared to the values before the ABX treatment (Figure 2B). In addition, ABX-treated WT and VDR-KO mice had decreased bacterial diversity with ABX treatment (fewer bands, Supplementary Figure S2). ABX treatment eliminated the bacterial differences in feces, colon and SI (Supplementary Figure S1). There was no effect of ABX on the WT clearance or susceptibility to *C. rodentium* infection, and none of the ABX-treated WT mice died following infection (Figure 2C, 2D). ABX VDR-KO mice had significantly higher bacterial shedding than VDR-KO mice starting at d2 post-infection and throughout the infection (Figure 2C). In addition, the ABX VDR KO mice took longer to clear the infection than VDR-KO, ABX WT or WT mice (Figure 2C). ABX VDR KO mice were extremely susceptible to *C. rodentium* infection and at peak infection 35% of them died following infection (Figure 2D). ABX treatment reduced total bacterial numbers, changed the bacterial composition and removed *C. rodentium* colonization resistance in VDR KO mice.

Changes in the expression of antimicrobial peptides and mucin in VDR KO mice

To determine the cause of the altered microbiota and *C. rodentium* colonization resistance, the expression of mRNA for several antimicrobial peptides and mucin were measured in WT and VDR KO mice. The expression of mRNA for RegIIIγ, angiogenin (Ang) 4, mucins (Muc) 1–4, RegIIIβ, cathelicidin-related antimicrobial peptide (CRAMP) and mouse β-defensin (mBD) 3 were not different at d0 in WT and VDR-KO mice (Figure 3A–3C and Supplementary Figure S3). By d10, the amount of RegIIIγ, Ang-4, RegIIIβ, mBD-3 were significantly increased in infected WT and VDR-KO mice, while the amount of Muc2, CRAMP, Muc1 were only increased in infected VDR-KO mice but not infected WT mice (Figure 3 and Supplementary Figure S3). The amount of Muc3 and Muc4 were not affected by infection or genotype (Figure 3A–3C and Supplementary Figure S3). At d10, VDR-KO mice had higher expression levels of RegIIIγ, Ang-4 and Muc 2 than WT mice at d10 post-infection (Figure 3A–3C). ABX treatment eliminated the d10 differences in the expression for RegIIIγ, Ang-4 and Muc2 between VDR-KO and WT mice (Figure 3A–3F). VDR-KO mice had increased expression of anti-microbial peptides compared to WT mice following *C. rodentium* infection and ABX treatment eliminated those changes.

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Increased IL-22^{+} ILCs in VDR-KO mice

IL-22 has been shown to induce epithelial cell production of antimicrobial peptides. RORγt^{+} ILCs (predominately ILC3 cells) have been identified to be the main source of IL-22 production during the early stage of *C. rodentium* infection[21]. The frequencies of ILCs (CD3^{-}RORγt^{+}) were measured in VDR-KO and WT mice. There were higher frequencies of ILCs in the SI of VDR-KO mice than WT mice, but the amount of ILCs were not different in the colons of these mice (Figure 4A). In addition, VDR-KO mice had more ILC3 (IL-22 producing) in the SI than WT mice (Figure 4B). Furthermore, VDR-KO mice also had more CD3^{-}RORγt^{+}NKp46^{+} (ILC1 or NK22) cells in the SI LP than WT mice (Supplementary Figure S4A). The frequencies of other ILC (LTi$_{4,}$ CD3^{-}RORγt^{+}NKp46^{-}CD4^{+} and LTi$_{0}$, CD3^{-}RORγt^{+}NKp46^{-}CD4^{-}) cells were increased in both SI and colonic LP in VDR-KO mice (Supplementary Figure S4). The total cell numbers isolated from the SI or colonic LP were not different between WT and VDR-KO mice, so the changes in frequencies reflect the changes in absolute numbers.

A 1:1 ratio of WT and VDR-KO BM was injected into lethally irradiated WT recipients. 8 wks later the WT recipients had 1/2 of the blood and colonic lymphocytes of WT origin and the other 1/2 of VDR-KO origin (Figure 4C). The frequencies of ILCs in the colonic LP showed preferential reconstitution with the VDR-KO ILC compared to WT resulting in higher ILC of VDR-KO origin in the chimeric WT mice (Figure 4D). VDR deficiency results in more IL-22 producing ILCs in the GI tract and BM reconstitution of WT mice with VDR-KO BM demonstrates a cell autonomous effect of the VDR on ILC frequencies in the gut.

Colonization resistance is induced in WT mice by VDR-KO BM

WT BM was transplanted into WT (WT-WT) and VDR-KO (WT-VDR KO) recipients and VDR-KO BM was transplanted into VDR-KO (VDR KO-VDR KO), and WT (VDR KO-WT) recipients. Following confirmation of reconstitution the recipient mice were infected with *C. rodentium*. WT recipient mice receiving WT BM cells had 5 logs of *C. rodentium* at d2 post-infection and the numbers of *C. rodentium* shed increased to 8 logs by d7 (Figure 4E). The VDR KO-VDR KO mice had only 4 logs of *C. rodentium* at d2 post-infection and the numbers remained the same at d4 and 7 post-infection (Figure 4E). Like the VDR KO-VDR KO mice the VDR KO-WT mice had reduced numbers of *C. rodentium* in the feces at d4 and d7 compared to the WT-WT mice (Figure 4E). Conversely, the WT-VDR KO mice had higher numbers of *C. rodentium* shed in the feces and the shedding resembled the WT-WT mice more than the VDR KO-VDR KO (Figure 4E). The difference in the *C. rodentium* between recipients of VDR-KO BM and WT BM was less (Figure 4E) than that between VDR-KO and WT mice (Figure 1). This may be due to the incomplete reconstitution of the colonic LP cells in the gut (70–75%) of the BM recipients and the older age of the BM recipients (16–20wk versus 8–12wk) at the time of infection. However, partial colonization susceptibility and resistance to *C. rodentium* could be transferred by BM transplantation.

The frequencies of donor-derived ILCs from the mice in Figure 4E were measured at d10 post-infection. Recipients of WT BM (both WT and VDR-KO) had fewer ILCs, fewer IL-22+ ILCs and less total IL-22+ cells in the LP of the SI compared to recipients of VDR-

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KO BM (both WT and VDR-KO, Figure 4F–4H). The frequencies of ILCs, IL-22+ ILCs and total IL-22+ cells were significantly higher in the SI of VDR-KO BM recipients and this corresponded with colonization resistance to *C. rodentium* (Figure 4). Surprisingly the colonization resistance was not associated with measurable changes in the bacterial phyla in the feces of the BM reconstituted mice (Figure 4I) and this may be another reason that the colonization resistance of the VDR-KO BM recipients was less than that in the VDR-KO mice. Partial colonization resistance and susceptibility to *C. rodentium* was transferred via the BM. In addition, the increased numbers of IL-22 producing ILCs in the VDR-KO mice was a cell-autonomous effect of VDR deficiency.

Colonization resistance is transferred to GF WT mice by cecal transplants from VDR KO mice

In order to determine whether the changes in the VDR-KO microbiota could mediate colonization resistance, WT GF mice received cecal transplants from WT and VDR-KO mice for 48h and then were infected with *C. rodentium*. WT recipients of WT cecal contents had significantly higher fecal shedding of *C. rodentium* compared to WT recipients of VDR-KO cecal contents (Figure 5A). The reduced *C. rodentium* fecal shedding in the WT recipients of VDR-KO microbiota was not due to a change in ILC numbers since there was no difference in the frequencies of ILC or IL-22^{+} ILC compared to WT mice that received WT microbiota (Figure 5B, 5C). The WT mice with the VDR-KO microbiota did have higher total IL-22^{+} cells in the SI than the WT recipients of WT microbiota (Figure 5D). VDR-KO microbiota transferred *C. rodentium* colonization resistance to GF WT mice.

VDR/Rag KO mice are more susceptible to *C. rodentium* than Rag KO mice

RORγt^{+} ILCs are also present in Rag KO mice. The uninfected Rag KO mice had 7.5% ILCs in the SI LP which is higher than those in WT mice (5%). ILC1, LTi$_4$ and LTi$_0$ cells were significantly higher in the SI (no differences in the colon) of DKO as compared to Rag KO mice (Suppl. Figure 4D–F). Despite the differences in ILC numbers between uninfected DKO and Rag KO mice, there were no differences in the Firmicutes or Bacteroidetes phyla members in the feces from DKO and Rag KO mice (Supplemental Figure S5A). There were increased numbers of the Proteobacteria phylum in the feces from DKO mice compared to Rag KO mice (Supplemental Figure S5A). DKO and Rag KO mice were infected with *C. rodentium* to determine whether DKO mice would be colonization resistant. Bacterial shedding in Rag KO mice increased and peaked at d7 post-infection and the Rag KO mice failed to clear the infection (Supplemental Figure S5B). Rag KO mice remained colonized with large numbers of *C. rodentium* that decreased survival beginning at d21 post-infection (Supplemental Figure S5B,S5C). Early post-infection DKO mice had significantly higher bacterial shedding than Rag KO mice and the DKO mice died quickly following systemic spread of the infection and weight loss (Supplemental Figure S5). DKO mice were not colonization resistant and instead were significantly more susceptible to *C. rodentium* infection than Rag KO mice.

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DISCUSSION

VDR-KO mice were found to be significantly more resistant to *C. rodentium* infection than WT mice. The intestinal microbiota is in competition with *C. rodentium*. A previous study demonstrated that fecal transplants from resistant mice into susceptible mice conferred partial resistance to *C. rodentium* infection, and this protection was associated with increased IL-22 and anti-microbial peptides[22]. Consistent with this study, we demonstrate that transplant of the microbiota from VDR-KO mice into WT GF mice conferred protection from *C. rodentium* infection compared to WT microbiota. In addition, overproduction of IL-22 predominantly by ILCs in the VDR-KO gut induced antimicrobial peptides that altered the microbiota in the gut. The data further demonstrate that the VDR-KO microbiota induced more IL-22 than WT microbiota in GF WT recipients. However, the number of ILCs was not changed with VDR-KO cecal transplant and the colonization resistance was less than that seen in the VDR-KO controls. Since the microbiota don't respond to changes in vitamin D (they don't express a vitamin D receptor), it seems likely that the change in IL-22 and anti-microbial peptides precedes the change in microbiota. However, our results fall short of showing a direct link between higher IL-22 producing ILC and the shifts in the microbiota that result in colonization resistance. The VDR-KO mice overproduced IL-22 and ILCs that likely caused a shift in the commensal bacteria such that the VDR-KO host is resistant to infection with *C. rodentium*.

BM transplantation studies demonstrated that the higher number of ILCs in the VDR-KO mouse was due to a cell-autonomous effect of the VDR deficiency on ILC numbers. ILCs are critical for the control of homeostasis in the gut, but during intestinal inflammation, ILC function is dysregulated[23]. In particular, ILC3 cells that make IL-22 can be both protective and pathogenic[23]. Other nutrients and the microbiota influence ILC development and function[23, 24]. Our results suggest that the VDR regulates ILC3 cell numbers and possibly function. It might be possible that simple vitamin D interventions could normalize ILC function help control dysbiosis to maintain homeostasis.

Interestingly, vitamin D deficiency did not result in colonization resistance to *C. rodentium* (data not shown)[25]. There are several circumstances where deficiencies of the vitamin D ligand result in different outcomes than VDR deficiency[26, 27]. VDR-KO BM transferred partial colonization resistance to WT mice. Conversely, WT BM transplantation into VDR-KO mice resulted in *C. rodentium* colonization. The VDR is important for maintaining tight junction protein expression and barrier function in gut epithelial cells[28]. Furthermore, the VDR negatively regulates NF-κB expression in gut epithelial cells[29]. Our study now demonstrates an epithelial-independent effect of the VDR in controlling the microbiota and IL-22 production in the gut.

VDR-KO mice were extremely susceptible to *C. rodentium* infection after ABX treatment. The ABX treatment disrupted the microbiota and eliminated colonization resistance. A previous study demonstrated that GF mice infected with *C. rodentium* were unable to clear the infection, suggesting the critical role of commensal bacteria in eradicating *C. rodentium* in the gut[19]. However, none of the monoassociated GF mice died of systemic infection despite high *C. rodentium* numbers in the gut[19]. Consistent with this, ABX-treated WT mice

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did not succumb to systemic infection. In contrast, our data showed that ABX VDR-KO mice were unable to control the *C. rodentium* and 35% of them died. In addition, large numbers of *C. rodentium* were detected in systemic organs of ABX VDR-KO mice, including the spleen, kidney and liver (data not shown), suggesting that the VDR must be important to control infection and prevent systemic spread. T cells and B cells are important for containing *C. rodentium* in the gut. Mice deficient in T cells or B cells are unable to clear *C. rodentium* infection and develop lethal systemic infections[16]. The increased lethality of the ABX VDR-KO occurs within a time frame that suggests that the failure to induce a protective acquired T and/or B cell response might be preventing the VDR-KO mice from containing the infection to the gut. The data suggest that acquired immunity in the VDR-KO host is impaired such that once colonized the VDR-KO mouse is unable or too slow in mounting a protective T/B cell response required to clear *C. rodentium*.

The role of the VDR and vitamin D in the control of gastrointestinal infections is complicated. *Salmonella* infection was more severe in VDR-KO mice due in part to a change in NFkB activation in colonic epithelial cells[29]. It should be noted that in order for *Salmonella* to infect mice antibiotic pretreatment is required in both WT and VDR-KO mice[29]. Similarly here following antibiotic pretreatment VDR-KO mice were more susceptible to GI infection than WT mice. Recently Assa et. al demonstrated that feeding mice vitamin D deficient diet for 5 wk was associated with increased barrier dysfunction, dysbiosis of the microbiota and more intestinal inflammation following *C. rodentium* infection[25]. Paradoxically, $1{,}25(OH)_2D_3$ injections increased *C. rodentium* numbers in the colon and spleen of mice[30]. The $1{,}25(OH)_2D_3$-mediated increase in *C. rodentium* numbers was attributed to the suppression of the mucosal Th17 response[30]. Our data demonstrate that the VDR is a negative regulator of ILC, and infection-induced IL-22. ILC are important for the generation of both T and B cell responses in the gut[23, 31]. Interestingly diseases like IBD result in the reduced expression of the VDR in the gut [32]. Our data predicts that with disease development the expression of the VDR will go down creating local areas of VDR deficiency, which might induce alterations in ILC and/or dysbiosis.

Our data highlights a role for the VDR in regulation of the composition of the microbial flora in the gut. Dysbiosis in the VDR-KO mouse was associated with increased ILCs and IL-22 producing cells in the gut that induced the production of anti-bacterial peptides. The increase in ILC numbers in the VDR-KO gut is a cell autonomous effect of the VDR. Understanding the role of vitamin D and the VDR in regulating ILC function and/or development would be critical for management of gastrointestinal homeostasis. In addition, expression of the VDR is a critical regulator of both the innate and acquired immune response since ABX VDR-KO and VDR/Rag DKO mice had high numbers of *C. rodentium* in the gut that led to the premature lethality of the mice following infection. VDR expression and regulation of VDR expression is critical for protection from infection and the maintenance of gastrointestinal homeostasis.

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METHODS

Mice

Age and sex matched WT, VDR-KO, Rag KO and DKO mice on the C57BL/6 background were bred (breeders were VDR+/−) and housed in the same room at the Pennsylvania State University (University Park, PA). Experimental procedures were approved by the Office of Research Protection, Institutional Animal Care and Use Committee at the Pennsylvania State University.

Citrobacter rodentium infection

The *C. rodentium* strain ICC169 was a kind gift of Gad Frankel (London School of Medicine and Dentistry, London UK). *C. rodentium* was cultured in Luria-Bertani (LB) broth containing 20 μg/ml nalidixic acid (EMD Chemicals). Mice were infected by oral gavage with 200 μl of *C. rodentium* suspension, which contained 5×10^9 CFU. Mice were housed 1 per cage for the infection to prevent mouse to mouse transmission of *C. rodentium*. The *C. rodentium* numbers in the feces were determined by plating on LB agar plates with 50 μg/ml nalidixic acid. Some mice were treated with the ABX vancomycin (20mg/ml) the day before infection.

BM transplantation

BM cells from WT (CD45.1) and VDR KO (CD45.2) donor mice were transplanted either alone or in a 1:1 ratio into lethally irradiated WT (CD45.1/CD45.2) or VDR KO (CD45.2) recipient mice as previously described[33]. 7–8 wks following BM transplantation mice were evaluated for reconstitution in the blood (98–99%). The reconstitution of the gut was much less (70–75% of the total colon LP) but consistent with what has been described previously for reconstitution of the gut[34].

Cecal transplant

Fresh cecal contents from 1 donor WT or 1 donor VDR KO mouse was collected and placed in PBS (0.1 g cecal content/1 ml PBS) on ice. The cecal contents from WT or VDR KO mice were homogenized and 200 μl of the homogenates were orally inoculated to WT GF mice. 48 h after cecal transplant, the recipient mice were orally infected with *C. rodentium* (5×10^9 CFU per mouse).

Cell isolation and flow cytometry

Isolation of intestinal LP lymphocytes were done as described previously [35]. Pieces of the SI or colon were incubated twice in HBSS containing 5 mM EDTA, 0.15 μg/ml DTT and 5% FBS for 20 min at 37°C under 250 rpm rotation with stirring bar. The supernatant was discarded and the tissue was further incubated in RPMI-1640 containing 1 mg/ml collagenase type 1 (Worthington, Lakewood, NJ) and 10% FBS for 1.5 h at 37°C under 250 rpm rotation to obtain LP cells. The LP cells were collected from the interface of 40/80% Percoll gradients (Sigma-Aldrich). Cells were stained with FITC CD11b, FITC CD4, PE Gr-1, APC NKp46, PECy5 F4/80, PECy7 CD3 or isotype controls (eBiosciences, San Diego, CA). Sample staining histograms are shown in Supplementary Figure S6.

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Cells were stimulated with PMA (0.1μg/ml, Sigma-Aldrich), ionomycin (0.5μg/ml, Sigma-Aldrich) for 5 h and for the final 3 h, Brefeldin A (10μg/ml, Sigma-Aldrich) was added to the culture medium. For the measurement of IL-22, cells were stimulated with mouse recombinant IL-23 (0.04μg/ml, R&D systems) for 5 h and for the final 3 h, Brefeldin A (10μg/ml, Sigma-Aldrich) was added to the culture medium. After surface staining, cells were fixed with 4% paraformadehyde (Sigma-Aldrich), permeabilized with 0.1% saponin (Sigma-Aldrich), and stained with FITC IFNγ, PE IL-17A, APC IL-22 or the FITC/PE/APC labeled isotype controls (eBiosciences). For RORγt staining was done using the transcription factor staining buffer kit and the manufacturer's instructions (eBioscience). Cells were analyzed on BD Fortessa LSRII (BD Biosciences) and the data was analyzed with FlowJo 7.6.5 software (TreeStar, Ashland, OR).

PCR

Fecal DNA was isolated using QIAamp DNA stool minikit (Qiagen, Valencia, CA). Fecal DNA was amplified with universal 16S rDNA primers or specific primers for different bacterial phyla or genus using SYBR green mix (BioRad, Hercules, CA). Relative 16S rDNA quantities were calculated using Ct method and were normalized to the amount of universal bacteria. The fecal DNA was amplified with universal 16S rDNA primers and DGGE was done exactly as described [36].

Total RNA was isolated (Qiagen). cDNA was synthesized using the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA) with SYBR green mix (BioRad) by MyiQ Single-Color Real-Time PCR machine (BioRad). Expression levels of these molecules were normalized by GAPDH and calculated with the Ct method. The primer sequences are listed in Supplementary Table 1.

Statistics

Statistical analyses were performed using GraphPad software (PRISM software, La Jolla, CA). Two-tailed Student's t tests were used to test differences between genotype (WT vs. KO). Two-way ANOVA with Bonferroni post-hoc tests were used to test the effects of experimental groups, time and their interactions. Log-rank tests were used to test the survival rates. Some of the data was transformed (square root transformation) to eliminate unequal variances, followed by a repeated-measures (mix model) two-way ANOVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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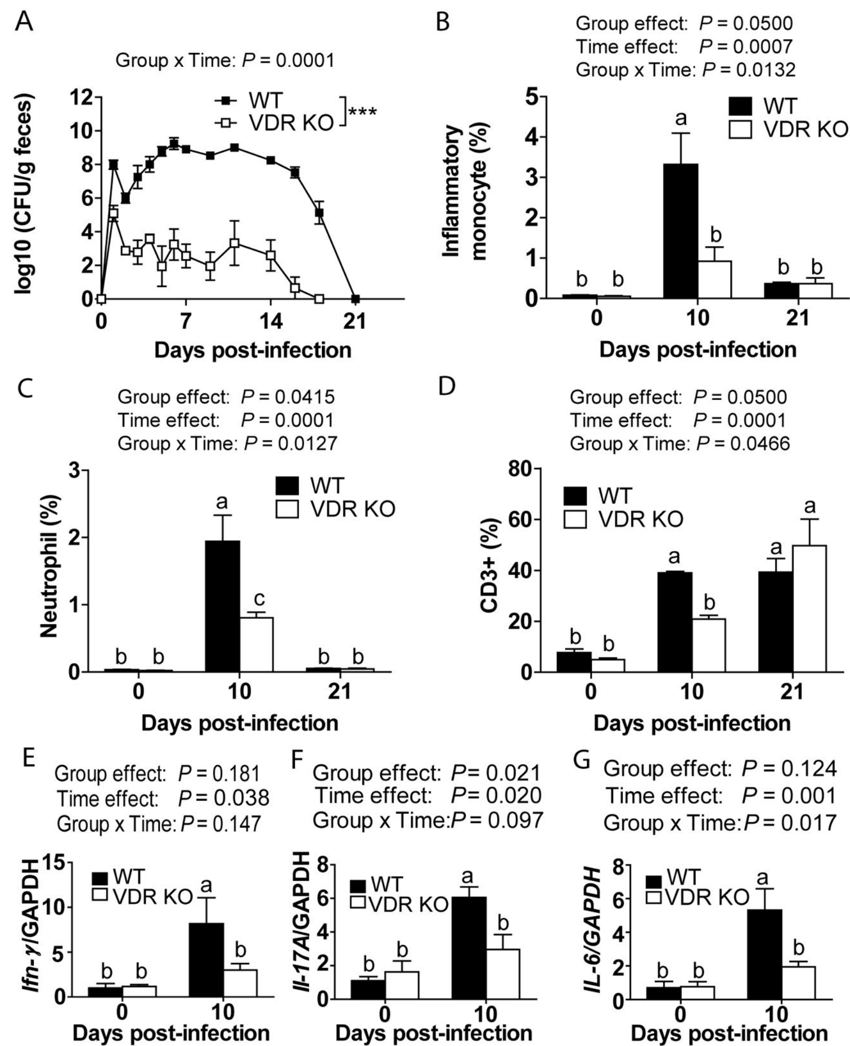

Figure 1. The kinetics of *C. rodentium* infection in WT and VDR KO mice

(A) *C. rodentium* numbers in the feces. The frequencies of (B) inflammatory monocytes, (C) neutrophils and (D) T cells in the colonic LP. mRNA expression for (E) *Ifn-γ*, (F) *Il-17A* and (G) *Il-6* in the colon. Data is from n=6–12 mice per group and the values represent the mean of three independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A–G), *** $P<0.001$. Groups without a common letter differ at the indicated time point (B–G), $P<0.05$.

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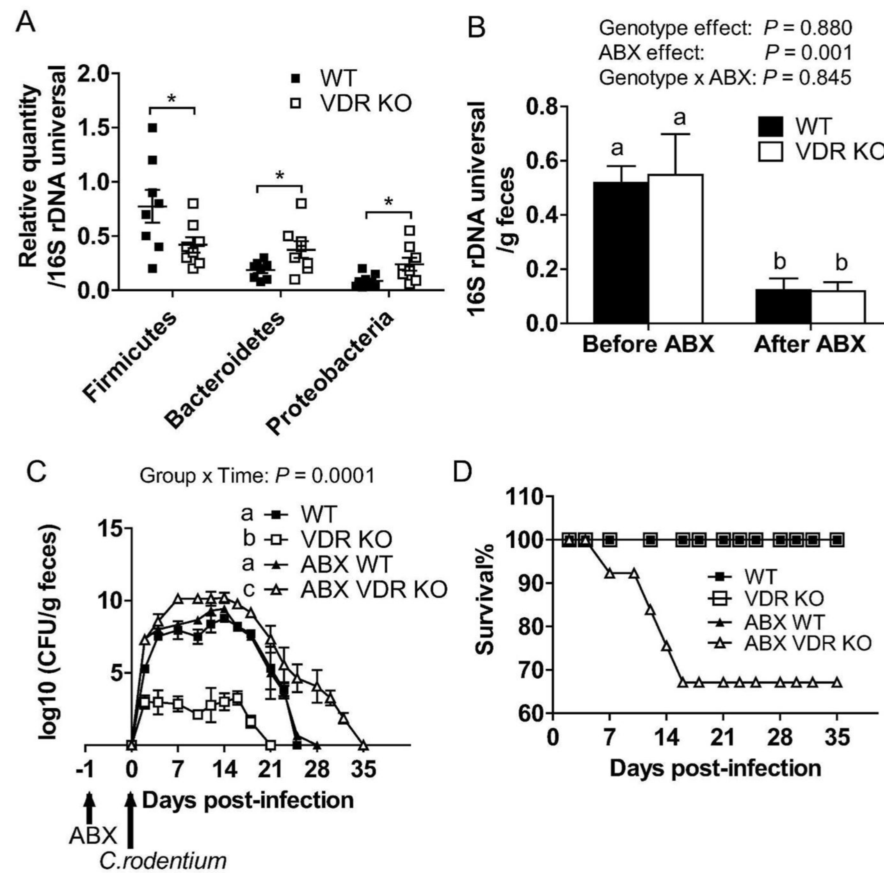

Figure 2. The effect of gut microbiota on infection with *C. rodentium*

(A) The relative quantity of bacteria from the Firmicutes phylum, the Bacteroidetes phylum and the Proteobacteria phylum in the feces of WT and VDR KO mice. (B) The relative total amount of universal 16S rDNA in the feces of WT and VDR KO mice before and after ABX treatment. The values were normalized to the fecal weight. (C) Shedding of *C. rodentium* in the feces of WT, VDR KO, ABX WT and ABX VDR KO mice. (D) The survival rate of the WT, VDR KO, ABX WT and ABX VDR KO mice following infection with *C. rodentium*. Data shown is mean ± SEM using n=4–10 mice/group. Two-tailed student's t tests (A), two-way ANOVA with Bonferroni post-hoc tests (B, C), and log-rank test (D), *P <0.05. Values (B and C) without a common letter are significantly different.

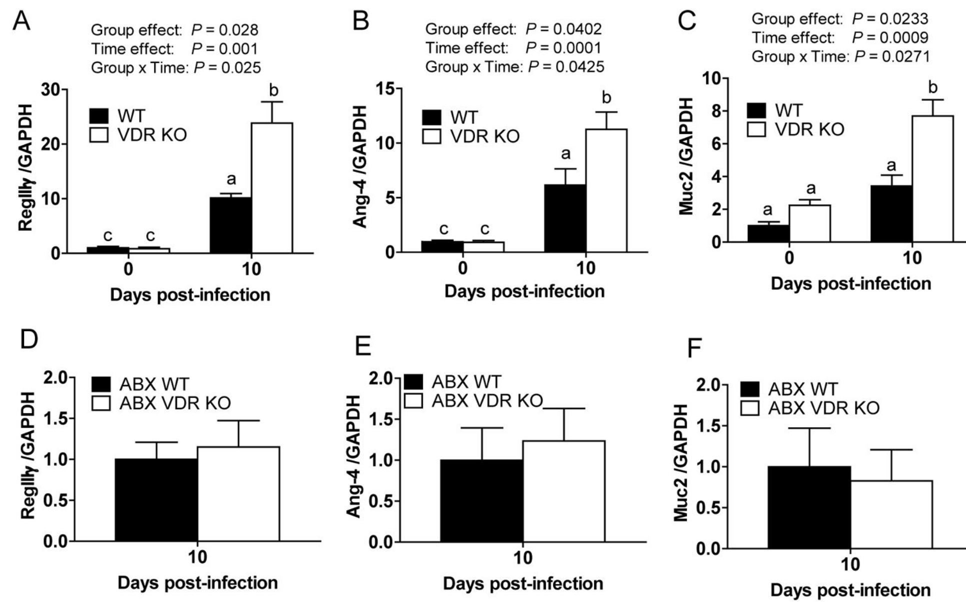

Figure 3. Expression of antimicrobial peptides and mucins in the colon of WT and VDR KO mice

mRNA expression for (A,D) RegIIIγ, (B,E) Ang-4 and (C,F) Muc2 in the colons of WT and VDR KO (A–C) and ABX treated WT and VDR KO (D–F) mice. Data is from n=6–8 mice per group and the values represent the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A–C), two-tailed Student's t tests (D–F). Values (A–C) without a common letter are significantly different.

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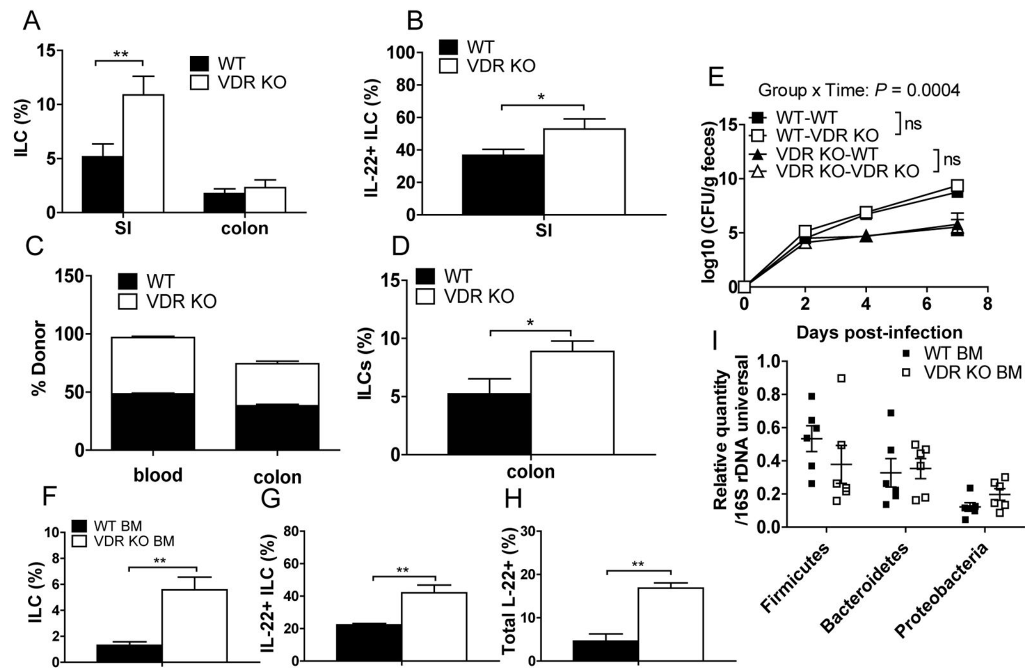

Figure 4. Cell-autonomous VDR control of ILC number and colonization resistance

The frequencies of (A) ILCs (CD3-RORγt+) and (B) IL-22 producing ILCs in the SI and colon LP of uninfected WT and VDR KO mice. BM cells from WT, VDR KO, or a 1:1 ratio of WT and VDR KO mice were transplanted into lethally irradiated WT or VDR KO recipient mice. The frequency of VDR KO (CD45.2) and WT (CD45.1) cells in the (C) blood and colon LP and the donor derived-ILC (D) in the colon of recipient mice. WT donor BM was transplanted into WT (WT-WT) or VDR KO recipient (WT-VDR KO) mice. VDR KO BM was transplanted into WT (VDR KO-WT) or VDR KO recipient (VDR KO-VDR KO). 7–8 weeks post-BM transplantation recipient mice were infected with *C. rodentium* and the (E) CFU in the feces were determined. The frequencies of (F) ILCs (CD3-RORγt+), (G) IL-22+ ILCs and (H) total IL-22+ cells in the SI LP of the recipients of WT BM or VDR KO BM at d10 post-*C. rodentium* infection. (I) The relative quantities of bacteria in the feces of mice 7 wks post-BM transplantation and without *C. rodentium* infection. Values are the mean ± SEM of n=3–8 recipient mice/group. Two-tailed Student's t tests (A–D, F–I), Two-way ANOVA (E), * $P<0.05$, ** $P<0.01$.

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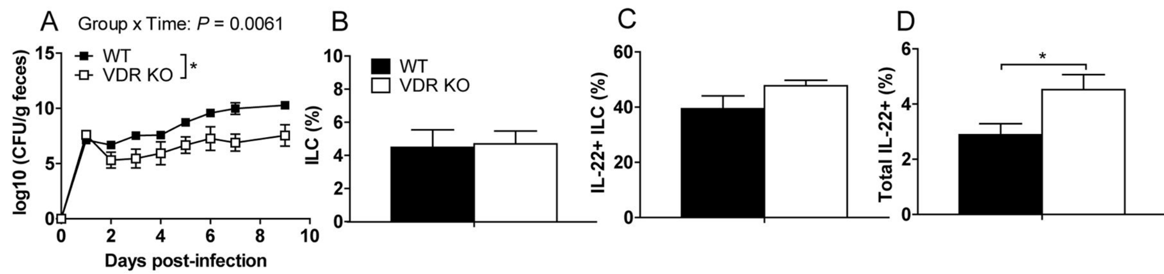

Figure 5. Cecal transplants into WT GF mice transfers colonization susceptibility to *C. rodentium*

Cecal contents from WT or VDR KO mice were orally inoculated into WT GF mice. 48 hours after cecal transplantation the WT recipient mice were infected with *C. rodentium*. (A) *C. rodentium* CFU in the feces of WT GF mice receiving WT or VDR KO cecal contents. The frequencies of (B) ILCs (CD3-RORγt+), (C) IL-22+ ILCs and (D) total IL-22+ cells in the SI LP of mice in (A) at d10 post-*C. rodentium* infection. Values are the mean ± SEM of n=3–8 mice/group. Two-tailed Student's t tests (B–D), one-way ANOVA (A) with Bonferroni post-hoc tests, * $P<0.05$.