



Vitamin D Regulates the Microbiota to Control the Numbers of ROR γ t/FoxP3+ Regulatory T Cells in the Colon

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The active form of vitamin D (1,25(OH)₂D) suppresses experimental models of inflammatory bowel disease in part by regulating the microbiota. In this study, the role of vitamin D in the regulation of microbe induced ROR γ t/FoxP3+ T regulatory (reg) cells in the colon was determined. Vitamin D sufficient (D+) mice had significantly higher frequencies of FoxP3+ and ROR γ t/FoxP3+ T reg cells in the colon compared to vitamin D deficient (D-) mice. The higher frequency of ROR γ t/FoxP3+ T reg cells in D+ colon correlated with higher numbers of bacteria from the *Clostridium* XIVa and *Bacteroides* in D+ compared to D- cecum. D- mice with fewer ROR γ t/FoxP3+ T reg cells were significantly more susceptible to colitis than D+ mice. Transfer of the cecal bacteria from D+ or D- mice to germfree recipients phenocopied the higher numbers of ROR γ t/FoxP3+ cells and reduced susceptibility to colitis in D+ vs. D- recipient mice. 1,25(OH)₂D treatment of the D- mice beginning at 3 weeks of age did not completely recover ROR γ t/FoxP3+ T reg cells or the *Bacteriodes*, *Bacteriodes thetaiotaomicron*, and *Clostridium* XIVa numbers to D+ values. Early vitamin D status shapes the microbiota to optimize the population of colonic ROR γ t/FoxP3+ T reg cells important for resistance to colitis.

Keywords: vitamin D, T regulatory (T reg) cells, inflammatory bowel diseases, microbiota, gnotobiotic mice

INTRODUCTION

The intestinal microbiota is critical for maintenance of gut homeostasis and experimental colitis fails to develop in germfree (GF) mice. The composition of the microbiota regulates diseases of the gastrointestinal (GI) tract; including, celiac and inflammatory bowel diseases (IBD) (1–3). IBD is associated with decreased diversity and dysbiosis of the microbiota and the decreased abundance of *Firmicutes* and *Bacteroides* and increased *Gammaproteobacteria* (4). Th17/Th1 cells that target the normal microbiota contribute to colitis (5). Conversely, FoxP3-expressing CD4+ regulatory T (T reg) cells play a crucial role in suppressing IBD (6, 7). The microbiota determines the types of T cells that are found in the GI tract (8, 9). The presence of segmented filamentous bacteria in a mouse colony is sufficient for the development of robust Th17 cells (8, 9).

In addition, *Bacteroides* and *Clostridium* species have been found to suppress experimental colitis via the induction of T reg cells (10, 11). Colitis is a result of a T cell response to the microbiota and dysbiosis of the microbiota.

Vitamin D is an important regulator of GI homeostasis. Vitamin D and or vitamin D receptor (VDR) deficiency increases the susceptibility of mice to several different models of experimental colitis (5, 12–14). The active form of vitamin D (1,25(OH)₂D, 1,25D) suppresses experimental colitis (5, 12–14). The mechanisms by which vitamin D regulates experimental colitis includes a role for vitamin D in shaping the microbiota (15). Th17 cells are direct targets of 1,25D (16, 17). *In vitro*, 1,25D inhibited Th17 cells and induced FoxP3+ T reg cells (17, 18). 1,25D treatment of mice induced the expansion of FoxP3+ T reg cells *in vivo* (19). However, expression of the VDR is not required for normal development of peripheral FoxP3+ T reg cells (14). Vitamin D inhibits Th17 cells and induces peripheral T reg cells to maintain GI homeostasis.

Within the colon are a unique population of T reg cells that also express the transcription factor retinoic acid receptor-related orphan receptor (ROR) γ t and are ROR γ t/FoxP3+ T reg cells (20). The colon also contains FoxP3+ single positive T reg cells and GATA3/FoxP3+ T reg cells which are derived from the thymus (21). The majority of the T reg cells found in the colon are ROR γ t/FoxP3+ T reg cells and this population of cells are low/absent in GF mice (22, 23). Microbial transplants into GF mice can recover the ROR γ t/FoxP3+ T reg cells (22, 23). Mice without ROR γ t/FoxP3+ T reg cells developed severe inducible experimental colitis (22, 23). ROR γ t/FoxP3+ T reg cells from the colon were better suppressors of colitis than splenic CD25+ T reg cells in the T cell transfer colitis model (23). The ROR γ t/FoxP3+ T reg cells in the colon are important for the maintenance of GI homeostasis and to prevent the development of colitis.

The effect of vitamin D on microbiota-dependent ROR γ t/FoxP3+ T reg cells were determined in the colons of vitamin D sufficient (D+) and vitamin D deficient (D-) mice. D- mice had significantly fewer total FoxP3+ T reg cells in the colon. The reduction in FoxP3+ T reg cells was due to reduced numbers of ROR γ t/FoxP3+ T reg cells, which were 80% of the FoxP3+ T reg cells in the colon. Microbial transplants from D+ and D- mice into GF mice resulted in lower ROR γ t/FoxP3+ T reg cells in the D- vs. the D+ recipients. D- microbiota had fewer numbers of *Bacteroides* and *Clostridium* XIVA species that were correlated significantly with the ROR γ t/FoxP3+ T reg frequencies. 1,25D treatments of D- mice were ineffective for inducing ROR γ t/FoxP3+ T reg cells or increasing *Bacteroides* or *Clostridium* XIVA numbers to the D+ values. D- mice and GF recipients of D- microbiota were more susceptible to dextran sodium sulfate (DSS) induced colitis than their D+ counterparts. Vitamin D status is an important factor that shapes the microbiota and impacts the expansion and development of protective colonic ROR γ t/FoxP3+ T reg cells.

METHODS

Mice

C57BL/6 mice were originally from Jackson Labs (Bar Harbor, MN) and maintained at the Pennsylvania State University

(University Park, PA). All mice used were housed within the same rooms in the animal facility. Mice were fed purified diets made in the lab as described that either contained vitamin D (D+) or did not (D-) (24). GF C57BL/6 mice were bred and maintained at the Pennsylvania State University gnotobiotic animal research facility. For some experiments, 1,25D was started in the diets of 3–5 week old mice (25 ng/d until 5 weeks of age) and mice that were >5 weeks old were fed 50 ng/d 1,25D exactly as described (24). For microbial transplantation experiments (two independent experiments), the 4 week old GF mice were gavaged with 100 μ l/10 μ g cecal contents from D+ or D- mice, and used for experiments after 2 weeks (25). All of the experimental procedures were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

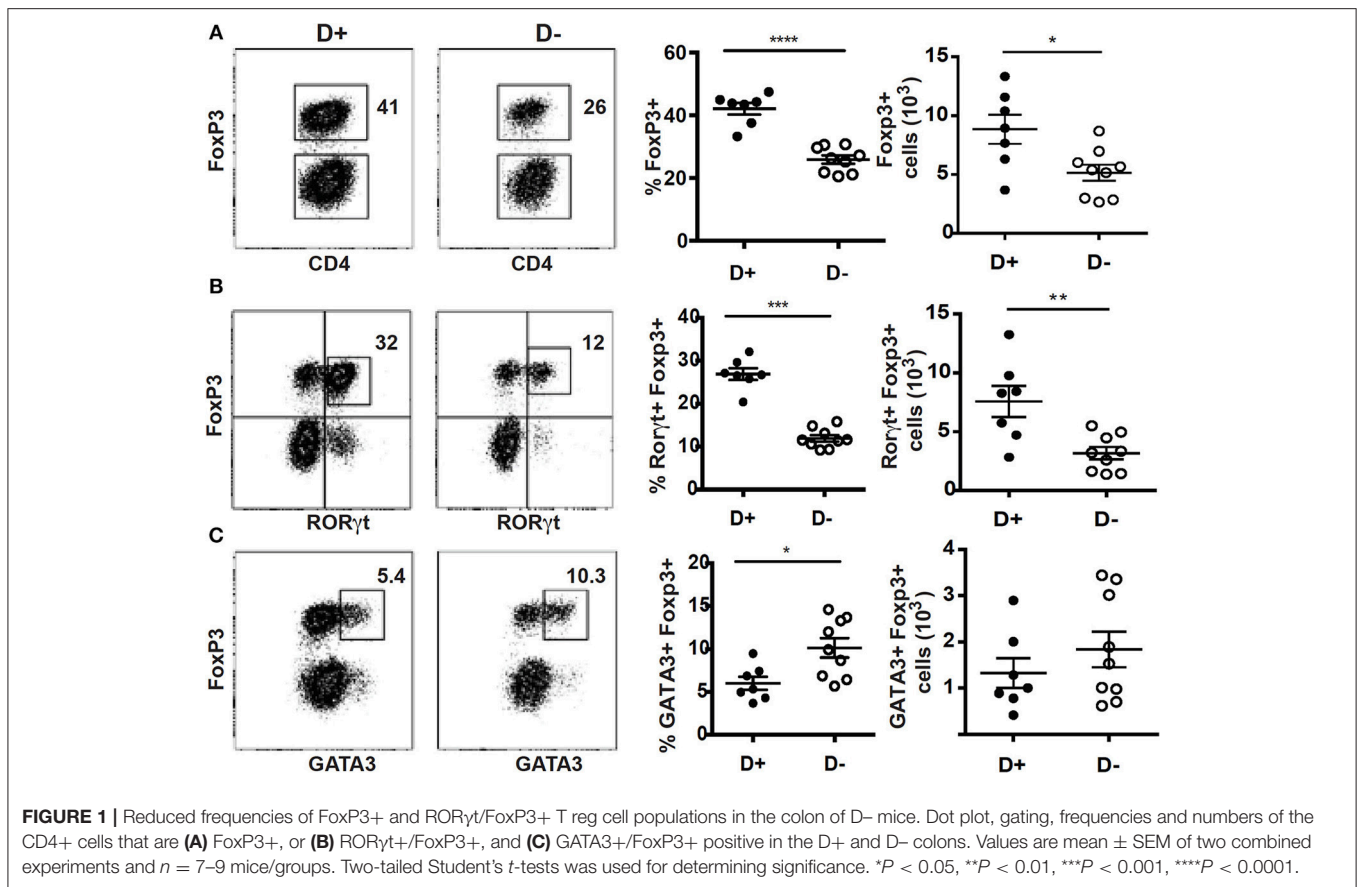
Cell Isolation and Flow Cytometry

Colon lamina propria (LP) cells were isolated as described previously and stained for flow cytometry (26). Briefly, the colon tissues were washed and cut into 1–1.5 cm sections incubated in Hanks' Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) with 5 mM EDTA at 37°C, and then digested in RPMI-1640 containing 1 mg/ml collagenase type 1 (Worthington, Lakewood, NJ) and 10% FBS at 37°C for 1 h in a shaking incubator. The cells were collected from the interface of 40/80% Percoll gradients (Sigma-Aldrich). The fluorescent dye conjugated-antibodies listed below were used for flow cytometry: CD3 (clone 145-2C11), CD4 (clone GK1.5) from Biolegend (San Diego, CA), CD45.2 (clone 104) from BD Biosciences (San Jose, CA), FoxP3 (clone FJK-16s), ROR γ t (clone B2D) from eBioscience (San Diego, CA). The cells were fixed and permeabilized using kits for intracellular staining (eBioscience) and the manufacturer's instructions. All data were collected on a BD Fortessa LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Gut Microbiota Analysis

The bacterial DNA in the cecal contents was extracted using the E.Z.N.A stool DNA isolation kit (OMEGA Bio-tek, Norcross, GA) according to the manufacturer's instructions. A 100 μ l aliquot of the isolated bacterial DNA was created with a bacterial DNA concentration of 10 ng/ μ l. PCR was completed as previously described (27). Bacteria were then analyzed by qPCR using primers targeted at 16S ribosomal RNA gene (28, 29). Primers for all of the species in the *Clostridium* clusters XIVA, IV, and XVIII were used and listed in **Supplementary Table 1** (28, 29). qPCR assays were carried out using SYBR Green qPCR Master Mix on a StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA) (30). The results were normalized to 16S ribosomal (universal) DNA sequences and expressed as the relative difference compared to D+ control group using the $\Delta\Delta C_T$ method.

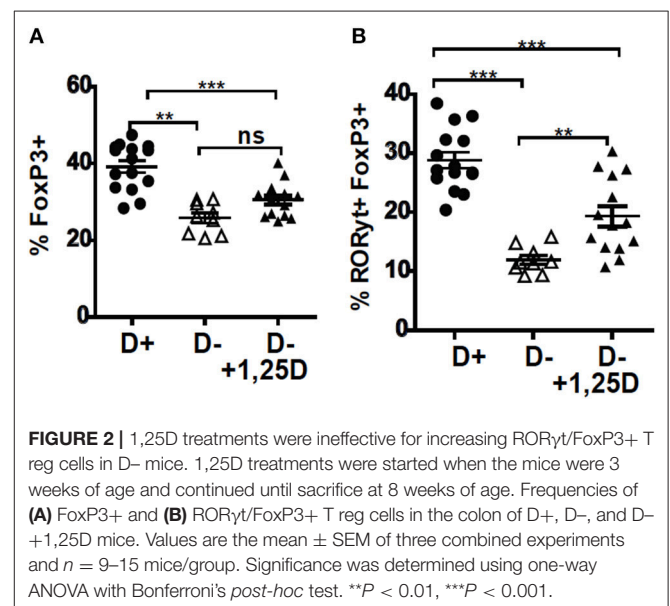
16S rRNA sequencing was performed using the Illumina MiSeq platform by the Pennsylvania State University Genomics Core Facility. Data analysis was done using mothur and aligned with the SILVA databases as described previously (27, 30–32). The raw data can be accessed on the National



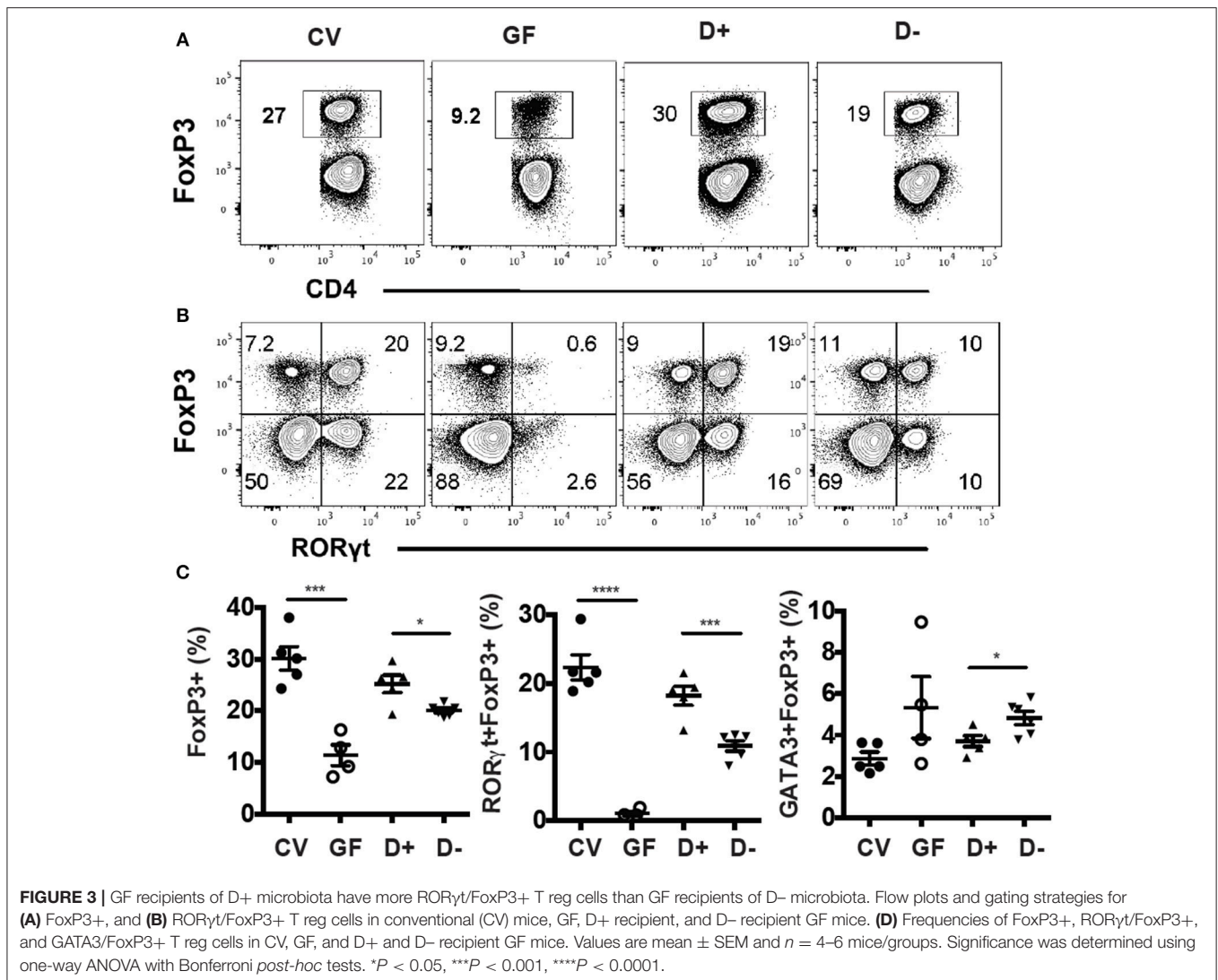
Center for Biotechnology Information (NCBI) with the accession number PRJNA506977.

DSS Colitis

Mice were administered DSS (MP Biomedicals, Solon OH) for 5 days, after which mice were given regular drinking water. Animals were weighed daily, and monitored for rectal bleeding, diarrhea, and signs of morbidity. Because of differences in initial starting weights (Figure 7) the females received 3.5% DSS and the males received 4% DSS. Based on their identical initial starting weights the male and female GF recipients of the D+ or D- microbial transplants received 3.5% DSS (Figure 8). Diarrhea was scored based on a scoring system as described (33). Briefly: 0- firm normal stool, 2- semi-formed stool, and 4- liquid stool that adhere to the anal region (33). The entire colon from cecum to anus was removed and the length was measured and reported as colon length. Distal colon was fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin (Pennsylvania State University Animal Diagnostic Laboratory). Histological analysis for severity of inflammation, injury, and crypt damage was performed blinded by two independent investigators as described previously (13, 15, 34). Inflammation was scored from 0-3 (0 = none, 1 = slight, 2 = moderate, 3 = severe). Injury was scored from 0-3 (0 = none, 1 = mucosal, 2 = mucosal and submucosal, 3 = transmural). Crypt damage was scored for 0-4 (0 = none, 1 = basal 1/3 damaged,



2 = basal 2/3 damaged, 3 = only surface epithelium intact, 4 = entire crypt and epithelium lost). Each score was added to obtain a total histology score where the maximum score was 10.



Statistics

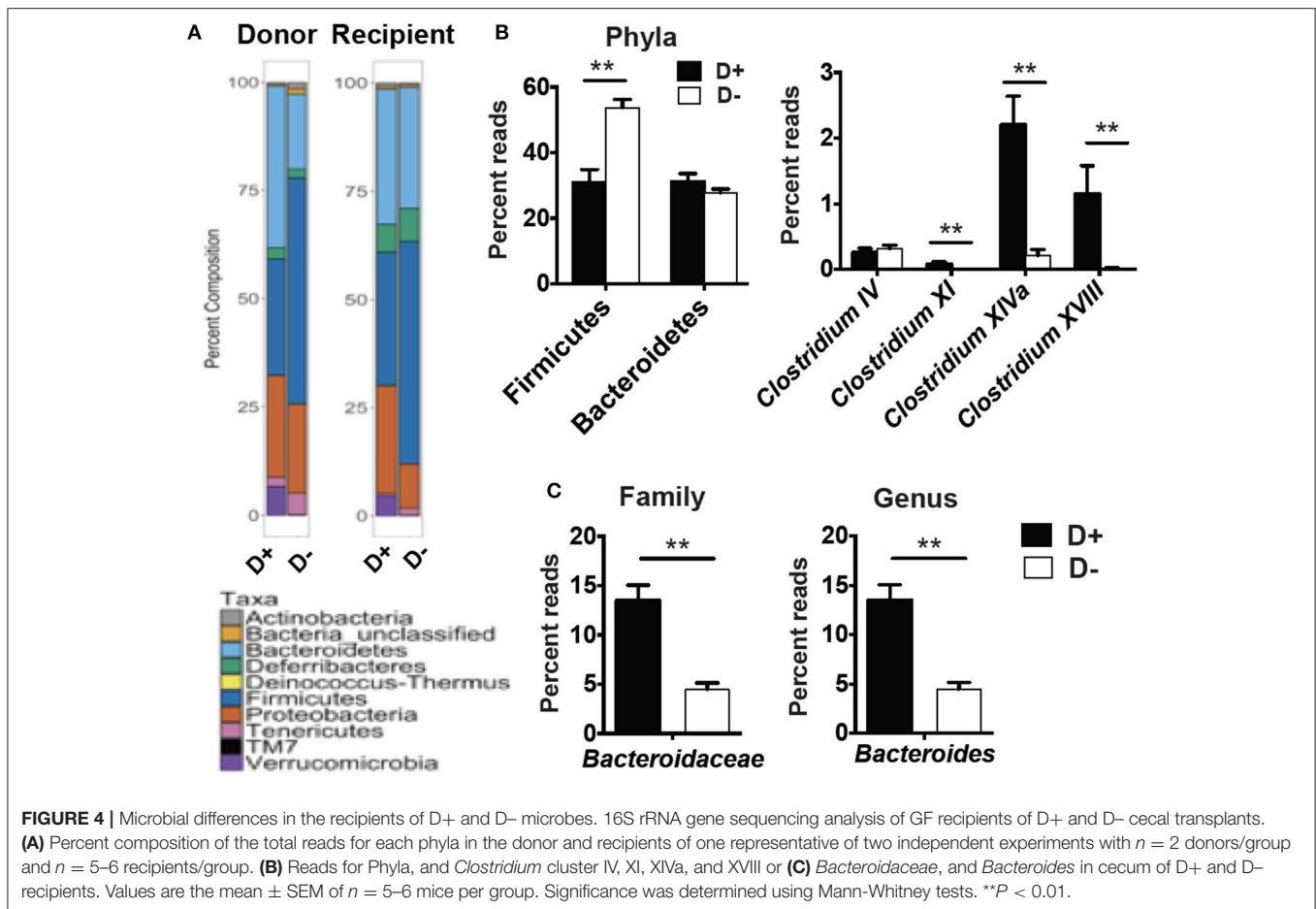
Statistical analyses were performed with PRISM software by (GraphPad, La Jolla, CA). Two-tailed Student's *t*-test, Mann-Whitney tests or one-way ANOVA with Kruskal-Wallis or Bonferroni *post hoc* tests were used for data analyses. * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ were used to indicate significance in the figures.

RESULTS

Reduced FoxP3+ and ROR γ t/FoxP3+ Colonic Regulatory T Cells in D- Mice

Mice with defects in vitamin D metabolism or without the VDR develop dysbiosis of the microbiota, which was associated with alterations in mucosal immune function (15, 35). Here we determined the effect of changes in vitamin D on the microbially dependent colonic T reg cells. There were no significant differences in the total cells isolated from D+ and D- colon (Supplementary Figure 1A). The frequencies

of $\alpha\beta$ T cells, CD4+ and CD8+ cells were not different in D+ and D- colon LP (Supplementary Figure 1B). The D- mice had significantly fewer ILC3 cells, and reduced IL-22 compared to D+ mice [Supplementary Figure 1B; (36)]. In addition, the Th17 cells from the D- colon were unable to expand after gastrointestinal infection (36). The frequency and numbers of T reg cells in the D+ colon were significantly higher than in D- mice (Figure 1A). Most of the T reg cells in the D+ colon were ROR γ t/FoxP3+ (80% of the FoxP3+ cells, Figure 1B), the remainder of the T reg cells either co-expressed GATA3 or singly express FoxP3 (10% each, Figure 1B). D+ mice had significantly more ROR γ t/FoxP3+ T reg cells than D- mice (Figure 1B). The frequency of GATA3/FoxP3+ T reg cells was lower in D+ than D- colon (Figure 1C). However, there were no differences in the total cell numbers of GATA3/FoxP3+ T cells in the D+ and D- colons (Figure 1C). The colon of D- mice had fewer T reg cells and in particular fewer of the ROR γ t/FoxP3+ T reg cells in the colon than D+ mice.



1,25D Treatment Fails to Completely Recover Colonic ROR γ t/FoxP3+ T regs in D- Mice

The D- mice were treated with 1,25D to determine whether 1,25D treatment could recover the T reg frequencies in the colon. Beginning the 1,25D treatments at either 5 weeks of age or 3 weeks of age significantly increased the frequencies of ROR γ t/FoxP3+ T reg over those in the D- mice (Supplementary Figure 2 and Figures 2A,B). However, 1,25D treatment of D- mice failed to completely recover the FoxP3+ T reg or ROR γ t/FoxP3+ T reg frequencies to D+ levels (Figures 2A,B). D+ mice had significantly more FoxP3+ T reg and ROR γ t/FoxP3+ T reg than the 1,25D treated D- mice (Figure 2). 1,25D treatment of D- mice did completely recover ILC3 frequencies to those in D+ mice (36), suggesting that the failure of 1,25D to completely recover the T reg cell population was selective.

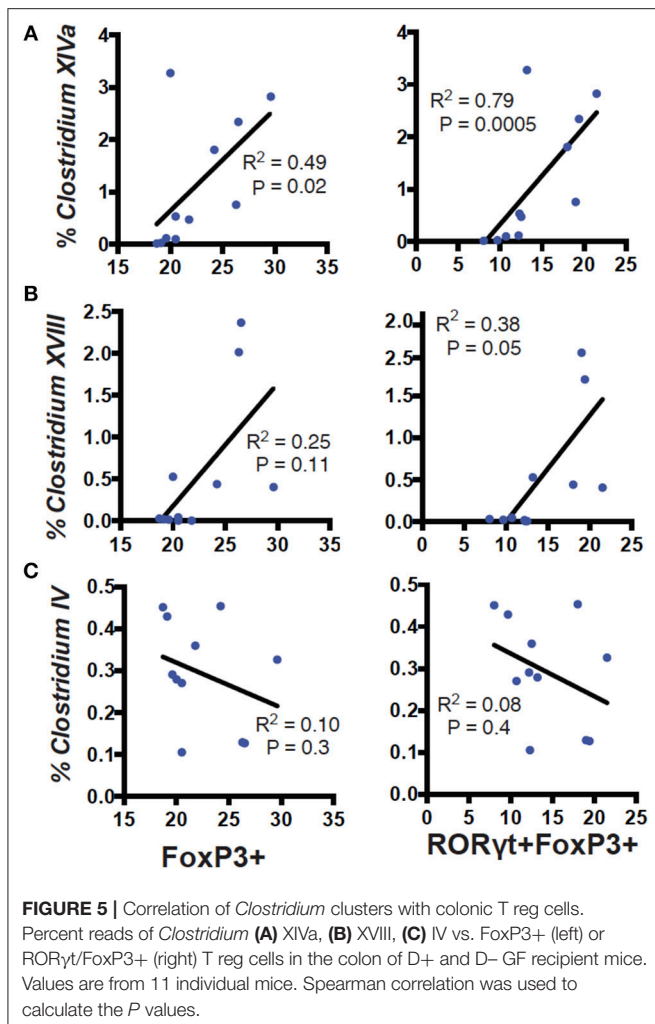
Reconstitution of GF Mice With D- Microbiota Resulted in Fewer Colonic ROR γ t/FoxP3+ T reg Cells

Conventional (CV) mice had significantly more colonic FoxP3+ T reg cells compared to GF mice (Figure 3A). ROR γ t/FoxP3+

T reg cells were almost completely absent in GF mice (Figure 3B). Colonization of GF mice with D+ cecal microbiota resulted in colonic FoxP3+ T reg cell and ROR γ t/FoxP3+ T reg cell frequencies that were similar to those in CV colons (Figures 3A,B). Mice that received D+ microbiota had significantly higher FoxP3+ T reg and ROR γ t/FoxP3+ T reg cells than mice that received D- microbiota (Figure 3C). The GATA3/FoxP3+ T reg cell frequencies were the same in GF and CV mice (Figure 3C). Recipients of D+ microbiota had fewer GATA3/FoxP3+ T reg cells than recipients of D- microbiota (Figure 3C). Microbial transplants from D+ and D- mice into GF mice phenocopied the original ROR γ t/FoxP3+ T reg frequencies in the D+ and D- donor mice.

Microbial Dysbiosis in D- Mice

ROR γ t/FoxP3+ T reg cells develop and expand following microbial signals (23). The cecal microbial composition of the D+ and D- GF recipients were sequenced (Figure 4A). Reconstitution of GF mice with D+ microbiota resulted in the recipients having microbiota that grouped with the D+ donors as seen with generalized unifracs (Supplementary Figure 3A). Conversely, the GF mice that were reconstituted with D- microbes resulted in microbe populations that were different from the original D- donor populations



(Supplementary Figure 3). The microbes in D+ and D- recipients were significantly different from one another (Supplementary Figure 3). *Firmicutes* phyla members were lower in D+ than D- recipient mice (Figure 4B). Within the *Firmicutes* phyla, D+ recipient mice had more bacteria from the *Clostridium* clusters XI, XIVA, and XVIII as compared to D- recipient mice, while the *Clostridium* cluster IV numbers were similar between groups (Figure 4B). D+ recipient mice had a larger population of the family *Bacteroidaceae* and the genus *Bacteroides* (Figure 4C). D+ recipient mice had a smaller *Firmicutes* population but a larger population of the genus *Clostridium* and *Bacteroides* than D- recipient mice. There was a significant, linear relationship between the numbers of *Clostridium* XIVA, and XVIII and the frequency of colonic RORγt/FoxP3+ T reg cells (Figures 5A,B). Conversely, the *Clostridium* cluster IV was not significantly related to RORγt/FoxP3+ T reg cells frequencies in the colon (Figure 5C). Reduced frequencies of RORγt/FoxP3+ T reg cells in D- mice were associated with reductions in *Clostridium* XIVA and XVIII numbers.

Additional analyses of the cecal microbial composition was done in the cecum of D+, D-, and D- + 1,25D treated

mice by PCR (Figure 6). D- mice had fewer *Bacteroidetes*, *B.thetaiotaomicron*, and *Clostridium* XIVA numbers than D+ mice (Figures 4, 6). The reduced numbers of *Clostridium* XVIII numbers in the D- microbiota found in the sequencing data was not found with PCR of expanded numbers of D+ and D- mice (Figures 4, 6). The numbers of *B. fragilis*, *Clostridium* IV, and *Clostridium* XVIII were similar in D+, D-, and D- + 1,25D (Figure 6). The 1,25D treatments were ineffective for raising *Bacteroidetes*, *B.thetaiotaomicron*, and *Clostridium* XIVA numbers to D+ levels (Figure 6). Conversely, 1,25D treatments significantly decreased the *Firmicutes* population in the cecum to D+ levels (Supplementary Figure 3B). 1,25D treatment of D- mice was ineffective for restoring *Bacteroidetes*, *B.thetaiotaomicron*, and *Clostridium* XIVA numbers to D+ values.

The metabolites found in the cecum of mice describe the functioning of microbial communities of mice (31). OPLS-DA plots showed that the cecal metabolites were distinct between donor D+ and D- mice (Supplementary Figure 4A), recipient D+ and D- mice (Supplementary Figure 4B) and 1,25D treated vs. D+ mice (Supplementary Figure 4C). There were no consistent signatures that could explain the higher RORγt/FoxP3+ T reg cells in D+ donor and recipient mice as compared to D- donor and recipient mice or D- + 1,25D mice (Supplementary Figure 4). In particular, the short-chain fatty acids (SCFA) in D+ donor cecal contents had higher propionate than the D- donors (Supplementary Figure 4A) and the D+ recipients had lower butyrate than the D- recipients (Supplementary Figure 4B). There were no differences in SCFAs in the cecal contents from D+ and D- + 1,25D mice (Supplementary Figure 4C). SCFAs may not be the cause of changes in RORγt/FoxP3+ T reg populations between D+, D-, and D- + 1,25D treated mice.

Increased Susceptibility of D- Mice and GF Recipients of D- Microbiota to DSS Colitis

FoxP3+ T reg cells have been shown to protect mice from DSS induced colitis (10). DSS induced weight loss in D+ and D- mice; however, D- mice lost significantly more weight than D+ mice following DSS administration (Figure 7A). The colons of D+ and D- mice shortened following 10 days of DSS (Supplementary Figure 5 and Figure 7B). D- mice had significantly shorter colons than D+ mice at day 10 post-DSS (Supplementary Figure 5 and Figure 7B). Histopathology scores from untreated D+ and D- mice were low and DSS induced inflammation, crypt damage, and injury in the colon of D- mice (Supplementary Figure 5 and Figure 7C). Inflammation, crypt damage and injury were all higher in D- mice than in D+ mice at day 10 post-DSS (Supplementary Figure 5). The total histology scores of D- mice treated with DSS was significantly higher than untreated D+ and D- mice and DSS treated D+ mice (Figure 7C). D- mice were more susceptible to DSS-induced colitis compared to D+ mice.

GF recipients of D+ and D- microbiota were challenged with DSS. The GF recipients of D+ microbiota largely maintained their starting weight following DSS treatment (Figure 8A). In contrast the GF recipients of D- microbiota lost significantly

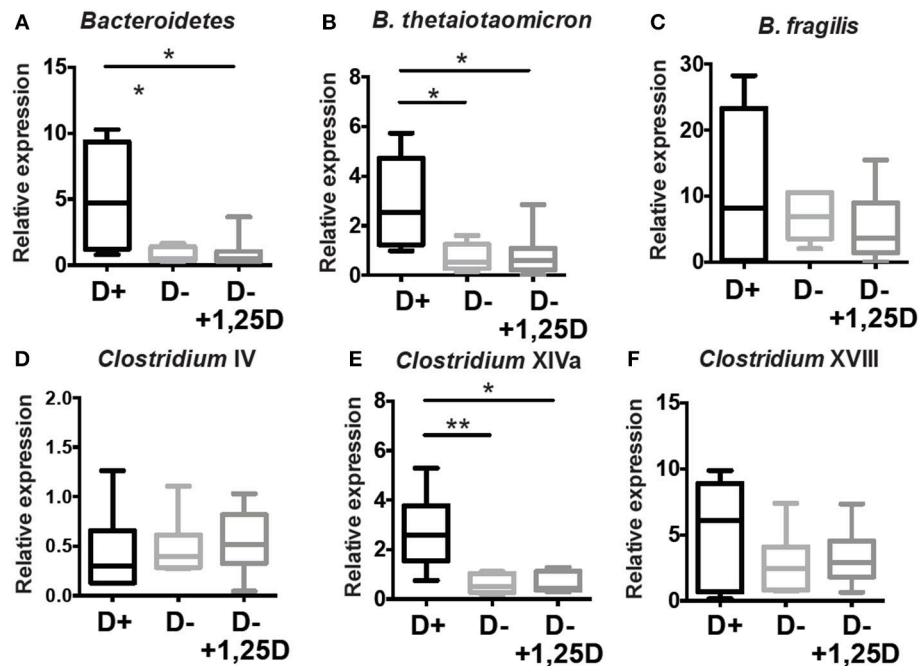


FIGURE 6 | 1,25D treatment of D- mice is ineffective for raising *Bacteroidetes*, *B. thetaiotaomicron*, and *Clostridium XIVa* to D+ levels. qPCR analysis of the proportion of (A) *Bacteroidetes*, (B) *B. thetaiotaomicron*, (C) *B. fragilis*, (D) *Clostridium IV*, (E) *Clostridium XIVa*, and (F) *Clostridium XVIII* in the cecum of D+, D- and D- +1,25D mice. Values are the mean \pm SEM two combined experiments and $n = 6-14$ mice/group. Significance was determined using one-way ANOVA with Kruskal-Wallis post-hoc tests, * $P < 0.05$, ** $P < 0.01$.

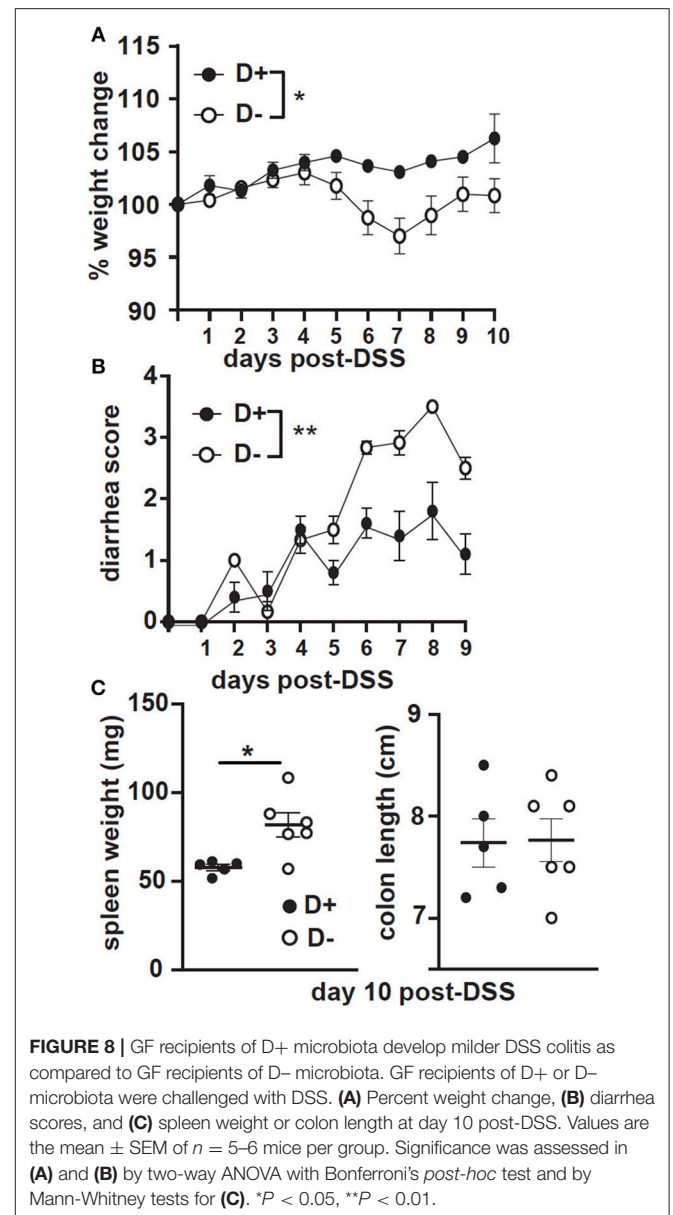
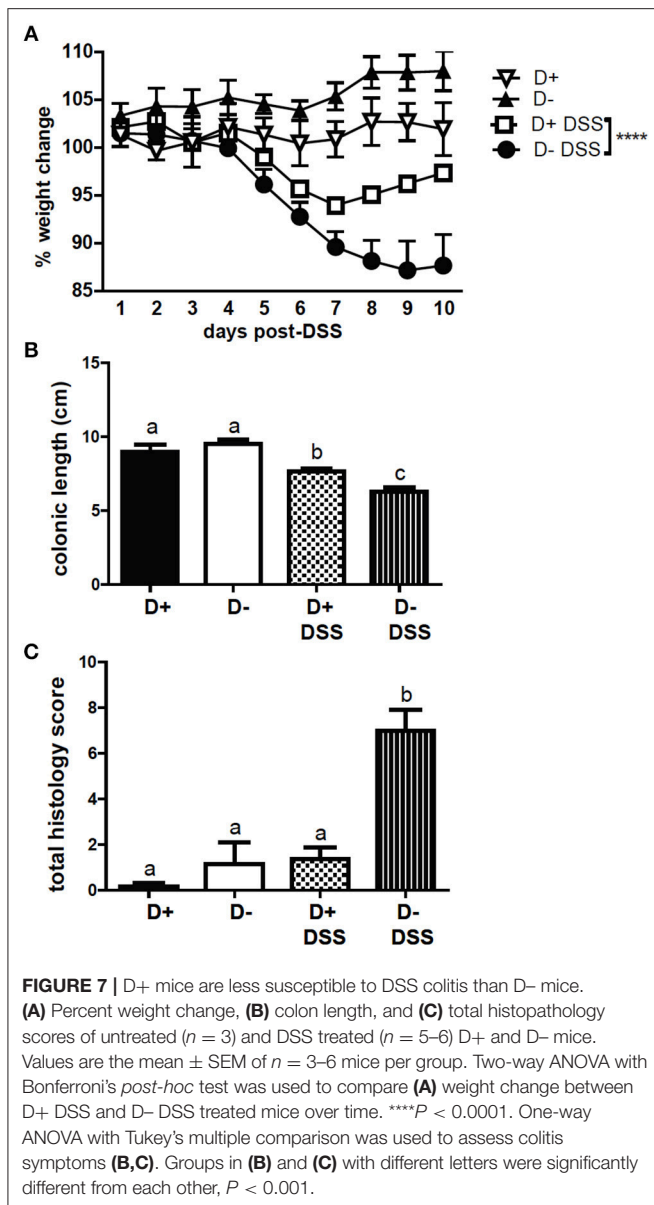
more weight than the GF recipients of the D+ microbiota (Figure 8A). The GF recipients of the D+ microbiota developed mild diarrhea while the GF recipients of the D- microbiota developed significantly more severe diarrhea following DSS induced colitis (Figure 8B). The colon lengths were not different, but the spleen weight was significantly heavier at day 10 post-DSS in the D- vs. D+ recipients (Figure 8C). The GF recipients of D- microbiota developed more severe DSS colitis than the GF recipients of D+ microbiota.

DISCUSSION

D- mice had fewer colonic ROR γ t/FoxP3+ T reg cells that resulted in reduced total FoxP3+ T reg cells compared to D+ mice. The cause of the reduced ROR γ t/FoxP3+ T reg cells was the differences in the microbiota of D+ and D- mice. Microbial transplants into GF recipients using D+ and D- cecal contents recapitulated the colonic ROR γ t/FoxP3+ T reg cell deficiency of the D- mice. D- mice had fewer *Clostridium* species from the XIVa cluster that have been shown to expand colonic ROR γ t/FoxP3+ T reg cells (10, 37). 1,25D treatments of D- mice failed to completely recover the colonic ROR γ t/FoxP3+ T reg cells even when started at weaning (3 weeks of age). The 1,25D treatments also failed to recover some of the microbes to D+ levels. D- + 1,25D treated mice had D- levels of *Bacteroides*, *B. thetaiotaomicron*, and *Clostridium* cluster XIVa species. Colonization of GF mice with only *B. thetaiotaomicron* was enough to restore conventional numbers of ROR γ t/FoxP3+ T reg cells (23). Here 1,25D treatments partially restored colonic

ROR γ t+ T reg cell frequencies but not to D+ levels. Recent data showed that D- mice took significantly longer than D+ mice to clear *Citrobacter rodentium* infection (36). Others have shown that depletion of ROR γ t+ T reg cells inhibited the clearance of *C. rodentium* (38). 1,25D treatments of D- mice resulted in faster clearance of *C. rodentium* infection than in D- mice, but slower clearance than D+ mice (36). Our data here suggests that the partial protection of 1,25D treated D- mice from *C. rodentium* infection could be due to the partial recovery of ROR γ t+ T regs [Figure 2; (36)]. The early effects of vitamin D are required to shape the microbiota and establish the colonic population of ROR γ t/FoxP3+ T reg cells.

T reg cells in the colon are low just after birth and increase to adult levels at 4 weeks of age (10). The temporal increase in T reg cells in the intestine with weaning and the induction of ROR γ t+ T reg cells in the colon with microbial colonization of GF mice demonstrates the critical role of microbes in the development of colonic T reg cells (10). The data show that intervention with 1,25D at weaning in D- mice failed to induce the microbial changes needed to increase the colonic ROR γ t+ T reg cells to D+ levels. Microbial exposure following birth is critical for immune development and alterations in the gut microbiota during development can have negative effects for allergy and other immune mediated diseases later in life (39). Early life colonization (before 5 weeks of age) with diverse microbes is important for the regulation of IgE; demonstrating a critical window of microbial regulation of immune cells during development (40). Like GF mice, VDR KO and D- mice have hyper IgE (41). The D- mice and GF recipients of D- microbiota



that lacked ROR γ t+ T reg cells, *B.thetaiotaomicron* and the *Clostridium* cluster XIVa, developed more severe colitis following DSS than D+ mice or GF recipients of D+ microbiota. Others have shown that mice with reduced or absent ROR γ t+ T reg cells develop more severe colitis (22, 23). Vitamin D and the microbiota are both environmental factors interacting to affect immune development and the risk for immune mediated disease.

A number of individual bacterial strains have been shown to induce colonic T reg cells *in vivo*. *Clostridium ramosum* from the *Clostridium* XVIII cluster and a collection of 42 *Clostridium* species (cluster IV and XIVa) induced T reg cells in the colon of GF mice (22, 23). *B.thetaiotaomicron* has also been shown to induce colonic ROR γ t+ T reg cells in mice (22, 23). Induction of colonic T reg cells depends on toll-like receptor (TLR) signaling since colonization of GF mice without the TLR adapter proteins

failed to induce T reg cells in the colon (42). In addition, the induction of T reg cells by *B.fragilis* required TLR2 (43). Butyrate production by microbial fermentation of fiber increased FoxP3+ T reg cells and ROR γ t+ T reg cells in the colon (22, 44-46). Conversely, Sefik and colleagues found no role for SCFA in the induction of ROR γ t+ T reg cells (23). We also did not find any significant correlation between cecal SCFA levels and ROR γ t+ T reg cells. Other potential mechanisms underlying the induction of ROR γ t+ T reg cells by the microbiota include regulation of toll like receptor signaling, induction of transforming growth factor- β , matrix metalloproteinases, and indoleamine 2,3-dioxygenase. The reduced numbers of ROR γ t+ T reg cells in D- and D- +1,25D treated mice corresponded to the lower levels of *Clostridium* species from cluster XIVa and *B.thetaiotaomicron*.

Bacteria do not express the VDR and therefore direct regulation of the microbiota by vitamin D seems unlikely. 1,25D treatments *in vivo* have been shown to induce peripheral T reg cells (19). The direct effects of vitamin D in the colon include regulation of immunity, barrier function, and/or the production of anti-microbial peptides (5). Vitamin D regulates innate immune responses via TLR and pattern recognition receptors (42). ILC3 cells were completely recovered in the 1,25D treated D⁻ colon (36). ILC3 production of IL-22 depends on vitamin D in the colon and IL-22 induces antibacterial peptide production by epithelial cells (36). NOD2 a pattern recognition protein linked to the development of IBD is a vitamin D target (47). TNF- α , IL-17, and IFN- γ were all reduced in D⁺ as compared to D⁻ or VDR KO mice (5, 48). Vitamin D regulates gut inflammation and the vitamin D alterations in mucosal immunity underlies the vitamin D effects on the microbial community structure.

Vitamin D status regulated the composition of the microbiota in the gut and the induction of colonic ROR γ t⁺ T reg cells. The implications of the work include that early adequate vitamin D status could determine the composition of the microbiota and early vitamin D deficiency may preclude optimal ROR γ t⁺ T reg cell expansion. Vitamin D interventions might improve vitamin D status but be ineffective for colonic ROR γ t⁺ T reg cells. Early nutrition is critical for the establishment of the community of microbes and the development of tolerance in the GI tract. Vitamin D is a micronutrient critical for optimal numbers of microbial induced ROR γ t⁺ T reg cells.

DATA AVAILABILITY

The datasets generated for this study can be found in the National Center for Biotechnology Information (NCBI) the accession number PRJNA506977.

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ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Pennsylvania State University.

AUTHOR CONTRIBUTIONS

MC, Y-DL, SB, and AP conceptualized and designed the experimental studies. JA, Y-DL, SB, YT, and RN performed the experiments and acquired and analyzed the data. MC drafted the manuscript with the help of JA, Y-DL, YT, RN, and SB. AP critically revised the manuscript. All authors approved the publication of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01772/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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