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Supplemental Information

Dendritic Cell Expression of the Signaling Molecule TRAF6 is Critical for Gut Microbiota-Dependent Immune Tolerance

Daehee Han, Matthew C. Walsh, Pedro J. Cejas, Nicholas N. Dang, Youngmi F. Kim, Jihyun Kim, Laetitia Charrier-Hisamuddin, Lillian Chau, Qin Zhang, Kyle Bittinger, Frederic D. Bushman, Laurence A. Turka, Hao Shen, Boris Reizis, Anthony L. DeFranco, Gary D. Wu, and Yongwon Choi

Supplemental Inventory

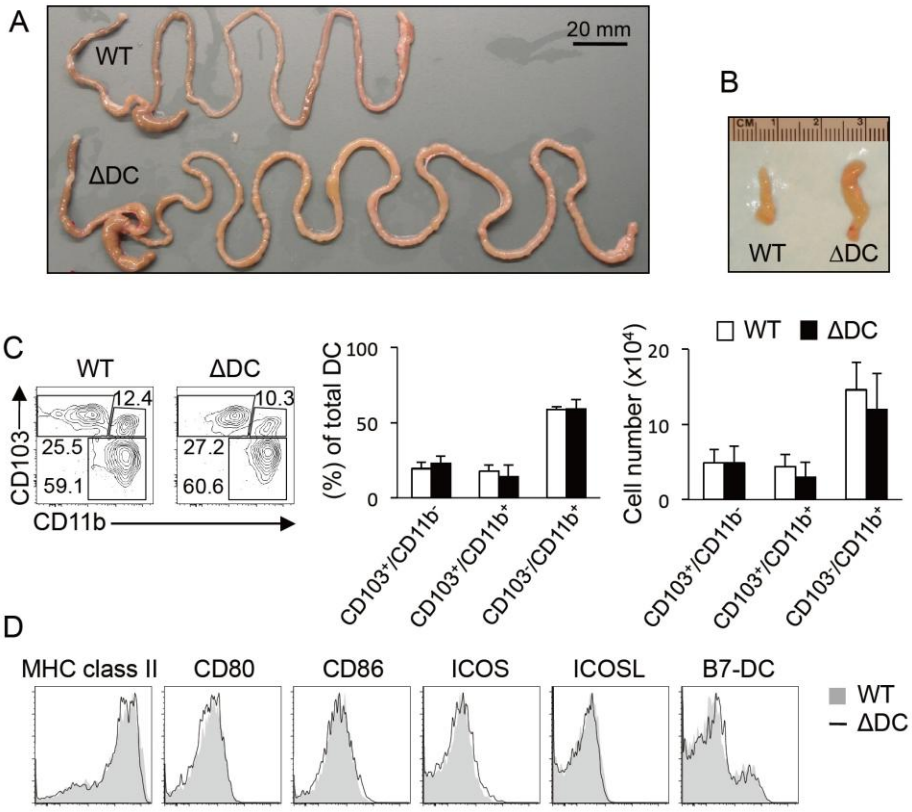

Figure S1. Normal Small Intestine DC Numbers and DC Activation Marker Levels, but Enlarged Small Intestine Morphology in TRAF6ΔDC Mice

(A) Representative 27 week-old control (WT) and TRAF6ΔDC (ΔDC) intestines.

(B) Mesenteric lymph nodes harvested from 30 week-old TRAF6ΔDC (ΔDC) compared to littermate control (WT).

(C) Lamina propria DCs were isolated from small intestine. FACS plots gated on DC markers ($CD11c^+$ and MHC $Class\ II^+$) show subpopulations of DCs (n=5). FACS plots and histogram data (mean ± SD, standard deviation) are representative of at least 3 independent experiments.

(D) Surface marker expression on $CD11c^+$-gated DCs from TRAF6ΔDC gut versus littermate controls. Figure S1, related to Figures 2 and 3.

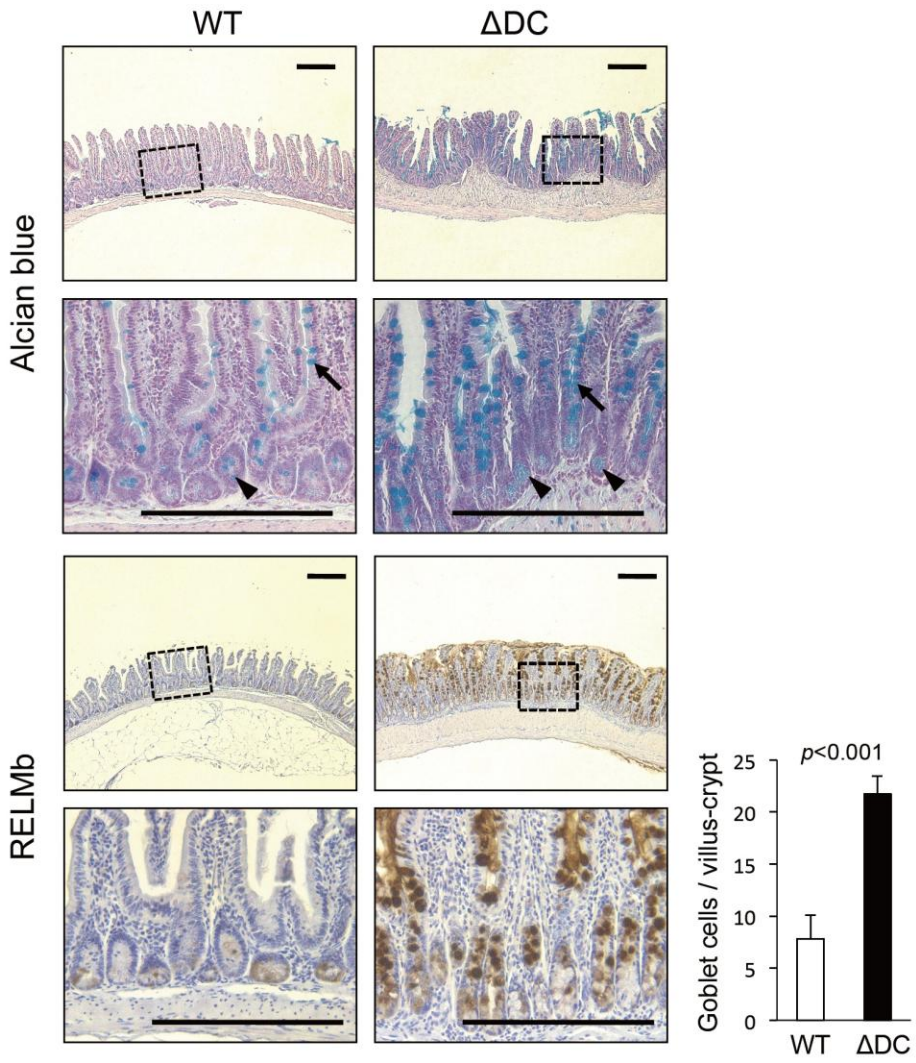

Figure S2. Goblet Cell Hyperplasia in TRAF6ΔDC Small Intestines

Alcian blue and RELM-β staining of the ileum sections of small intestine from TRAF6ΔDC and littermate control (WT, 27 weeks old) mice. The histogram data (mean ± SD) showing goblet cell counts were analyzed with two-tailed, unpaired Student's t-tests. Arrows, goblet cells. Arrowheads, Paneth cells. Scale bars, 300 μm. Figure S2, related to Figure 2.

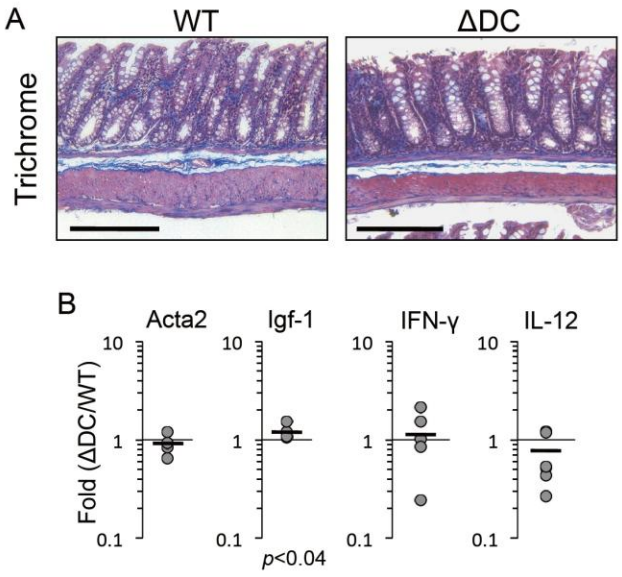

Figure S3. TRAF6ΔDC Colonic Tissue Exhibits Normal Immune Homeostasis

(A) Trichrome staining of colon sections from TRAF6ΔDC and littermate control mice (27 weeks old).

(B) Fold increases in pro-fibrotic (Acta2 and Igf-1), anti-fibrotic (IFN-γ and IL-12) markers and cytokines (IL-13, IL-5 and IL-4) in mRNA isolated from tissue of TRAF6ΔDC (ΔDC) colon were compared to littermate control mice (n≥4; ≥8 week-old littermate groups). Scale bars, 100 μm. Figure S3, related to Figure 2.

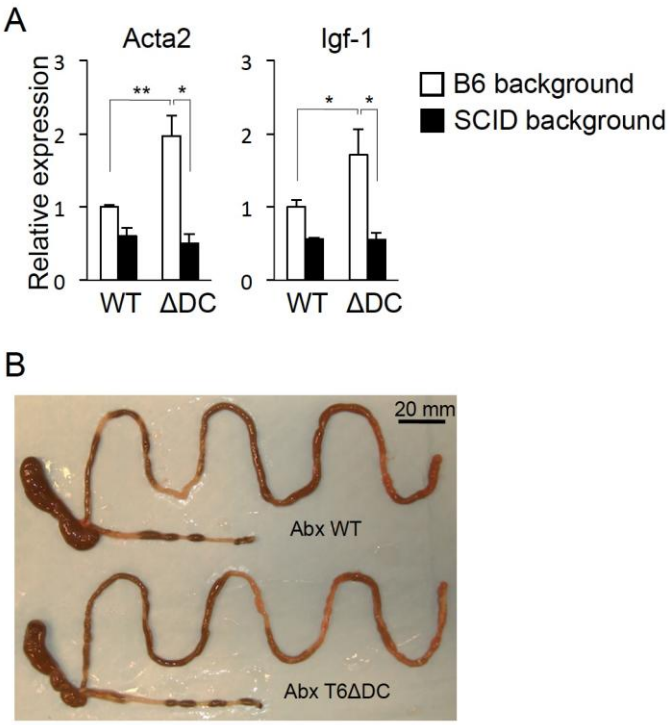

Figure S4. Disease Mitigation in both TRAF6ΔDC.SCID Mice and Antibiotics-treated TRAF6ΔDC Mice

(A) Fibrotic markers (Acta2 and Igf-1) mRNA expression levels in ileum tissues of small intestine from TRAF6ΔDC and littermate control mice were measured on 20 week-old C57BL/6 background or SCID background. The histograms (mean ± SD) are representative of 3 separate experiments. Data were analyzed by one-way ANOVA with Tukey's post-test of multiple comparisons.

(B) Intestines of representative 20 week-old control (WT) and TRAF6ΔDC (T6ΔDC) mice kept on full-spectrum antibiotic (Abx) treatment for the final 6 weeks. Figure S4, related to Figures 2 and 4.

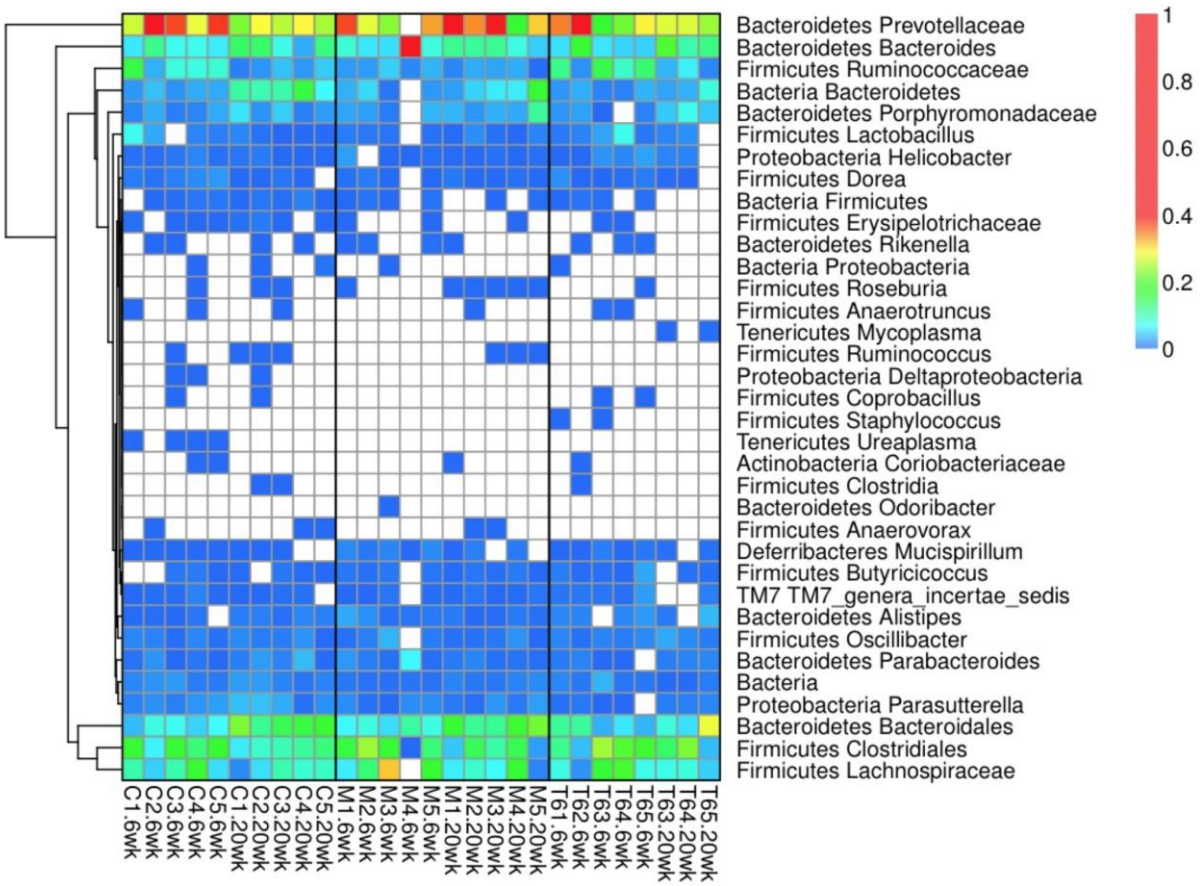

Figure S5. No Major Differences in Taxonomy of Fecal Bacteria Detected in MyD88ΔDC or TRAF6ΔDC Mice

Heatmap analysis shows relative abundance of taxa as a percentage of total 16S rRNA, organized according to genus or most specific assigned taxon. Color scales reflect proportion contributed by each taxon. C#, Wild type control; M#, MyD88ΔDC; T6#, TRAF6ΔDC; 6wk, 6 weeks old; 20wk, 20 weeks old. (e.g., T64.20wk is 20 week-old TRAF6ΔDC mouse, replicate #4). Community analysis using a weighted UniFrac metric (phylogenetic distance) did not show significant differences between controls, TRAF6ΔDC, and MyD88ΔDC when tested at either week 6 or week 20. The MyD88ΔDC samples did show separation when distances were measured by unweighted UniFrac or by the Jaccard metric (non-phylogenetic), indicating that this genotype had a measurable effect on less abundant species (see supplemental sequencing report). Figure S5, related to Figure 5.

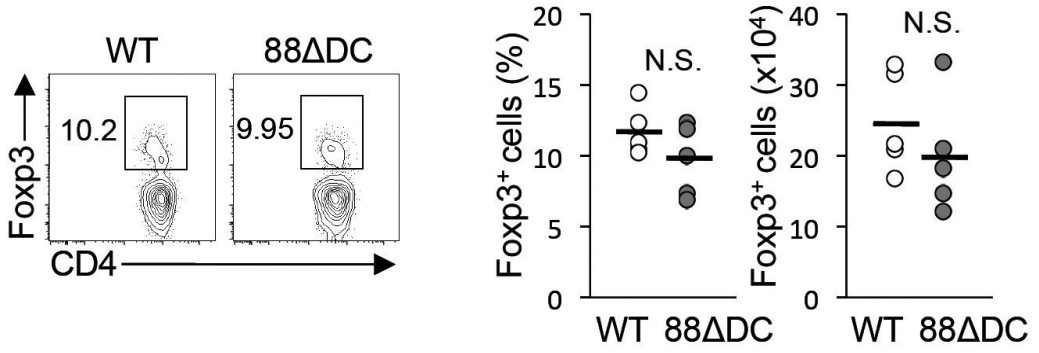

Figure S6. Normal Treg Cell Population in MyD88ΔDC Mice

FACS plots gated on $CD4^+$ T cells show intracellular staining from small intestinal lamina propria from 6-8 week-old MyD88ΔDC mice, and $Foxp3^+$ as percentages of $CD4^+$ populations as well as cell counts. FACS plots are representative of 5 independent experiments. Data were analyzed with two-tailed, paired Student's t-tests. N.S., not significant. Figure S6, related to Figure 6.

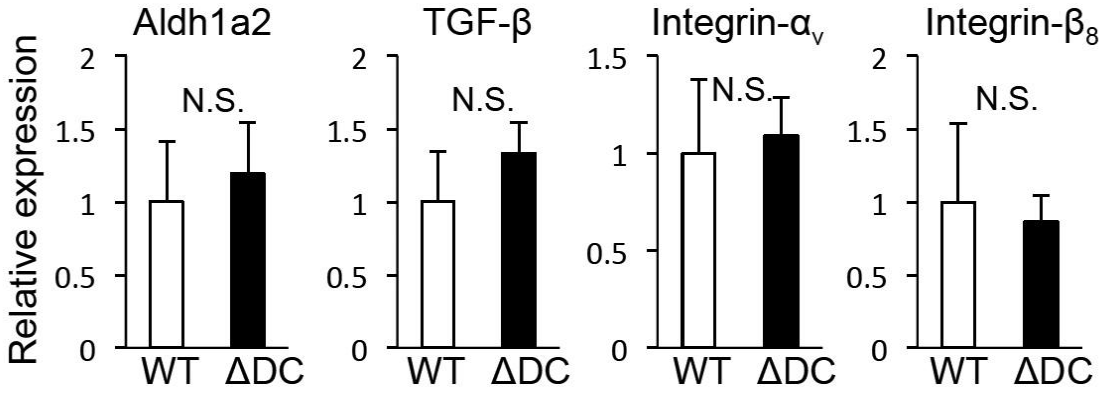

Figure S7. Normal Expression by TRAF6ΔDC Lamina Propria DCs of Treg Cell-Associated Markers

DCs were purified from control (WT) and TRAF6ΔDC (ΔDC) small intestine lamina propria using anti-CD11c MACS, and then Q-PCR analysis of Treg cell-associated genes performed on cDNAs prepared from DCs. The histograms are representative of at least 3 separated experiments and represented as mean ± SD. Student's t-test comparison revealed no statistical difference between control and TRAF6ΔDC for each target gene assayed. Figure S7, related to Figure 6.

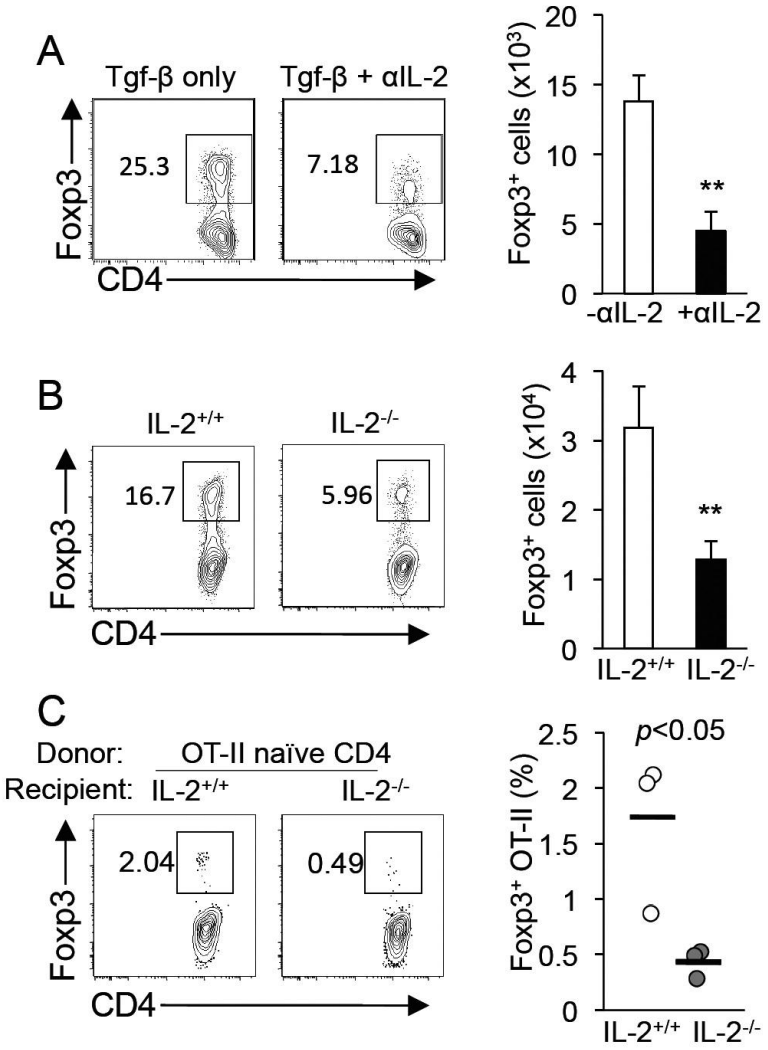

Figure S8. Requirement for DC-expressed IL-2 for Optimal iTreg Cell Induction

(A) Representative FACS plots (left panels) and aggregate counts (right panel) of Foxp3$^+$ cells converted from naïve/effector OT-II CD4$^+$ T cells after 4 days culture with purified lamina propria DCs (5 X 10^4 T cells per 5 X 10^3 DCs), 1 μM OVA 323-339, and 1 ng/ml TGF-β, in the presence or absence of 1 μg/ml anti-IL-2 neutralizing antibody (αIL-2). **, p<0.01.

(B) Representative FACS plots (left panels) and aggregate counts (right panel) of Foxp3$^+$ converted from naïve/effector OT-II CD4$^+$ T cells after 4 days culture with purified lamina propria DCs (5 X 10^4 T cells per 5 X 10^3 DCs) harvested from either control (IL-2$^{+/+}$) or IL-2 knockout (IL-2$^{-/-}$) mice, plus 1 μM OVA 323-339, and 1 ng/ml TGF-β. **, p<0.01.

(C) Representative FACS plots (left panels) and aggregate counts (right panel) of Foxp3$^+$ donor cells recovered from mesenteric lymph nodes of either RAG2^{KO} (IL-2$^{+/+}$) or IL-2/RAG2^{DKO} (IL-2$^{-/-}$) recipient mice 6 days after adoptive transfer of naïve congenic OT-II CD4$^+$ T cells with OVA-feeding on each of the last 5 days. The histograms are represented as mean ± SD. Data were analyzed with two-tailed, paired Student's t-tests. Figure S8, related to Figure 7.

Supplemental Experimental Procedures

Cell Isolation

For small intestinal lamina propria lymphocyte (LPL) isolation, epithelial cells were removed by incubation in stripping RPMI-1640 medium containing 10% FBS, 5 mM EDTA, 1 mM DTT, and 20 mM HEPES at 37 °C for 15 min in an orbital shaker. The remaining pieces were minced and incubated in serum-free RPMI-1640 medium containing 52 U/ml Liverase TM (Roche), 50 μg/ml DNaseI (Sigma), and 20 mM HEPES at 37 °C for 15 min while shaking. Flow cytometry was performed after staining for surface marker antibodies.

Histology and Immunohistochemistry

Intestinal specimens were cut longitudinally and Swiss-rolled inside out. Paraffin-embedded sections were performed after fixation in 10% Formalin solution (Fisher diagnostics). Alcian blue staining or Trichrome staining was performed by the Penn Center for Molecular Studies in Digestive and Liver Diseases. Trichrome staining was used for staining muscle, cytoplasm, and collagen. Immunohistochemistry was performed by using α-RELM-β antibody, followed by DAB staining.

Quantitative PCR

Tissues were homogenized in TRIzol (Invitrogen) after freezing in liquid nitrogen. cDNA was generated using Superscript III (invitrogen) and random hexamer (Qiagen). TaqMan gene probes were used with TaqMan Universal PCR Master Mix (Applied Biosystems) and run on 7300 Realtime PCR System (Applied Biosystems): 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 sec 95 °C, 1 min at 60 °C, and signals were detected during the annealing step (60 °C). Relative mRNA expression levels of all samples were normalized to 18S mRNA.

Fecal Bacteria Taxonomic Analysis

The deep sequencing studies of bacterial composition, including DNA isolation, amplification, sequencing, and sequence analysis, were performed by the Bushman lab (microbiology, UPENN) as previously described (Wu et al.). Briefly, fecal pellets were processed with the PSP Spin Stool DNA Plus (1038110300, STRATEC Molecular, Berlin-Buch, Germany) kit according to manufacturer instructions, and after amplification of 16S rDNA genes, dilutions of broad-specificity V1V2 primers and template, as well as quadruplicate 25 μL PCR reaction assembly, were carried out with liquid-handling robot (epMotion 5075 LH, Eppendorf, Hamburg, Germany). Reactions were performed by AccuPrime Taq DNA Polymerase (12339-016, Invitrogen, Carlsbad, CA), pooled and purified using AMPure magnetic beads (A63881, Agencourt, Brea, CA), and sequencing was performed by a 454 Life Sciences FLX instrument (06372279001, Roche, Branford, CT).

Supplemental References

Wu, G.D., Lewis, J.D., Hoffmann, C., Chen, Y.Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L.*, et al.* Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol *10*, 206.