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Supplementary Information for:

ICOS Ligand and IL-10 synergize to promote host-microbiota mutualism.

Ashley E. Landuyt, Barbara J. Klocke, Lennard W. Duck, Keri M. Kemp, Rachel Q. Muir, Melissa S. Jennings, Samuel I. Blum, Hubert M. Tse, Goo Lee, Casey. D. Morrow, Charles O. Elson, and Craig L. Maynard

Corresponding Author: Craig L. Maynard
Email: craigmaynard@uabmc.edu

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Supplemental Methods

Microbiota 16S sequencing and analysis. DNA extraction was performed using bead beating of total fecal material followed by nucleic acid purification using the Zymo Fecal DNA Mini Kit. Amplicon library for bidirectional (2 x 250 bp) sequencing on the Illumina MiSeq platform targeted across 16S rRNA V4 gene hypervariable regions. Multiplex PCR was used with bar codes for 96 samples including, in some runs, negative (no added DNA) controls. PCR products were resolved on agarose gels; DNA was isolated and purified using Qiagen kits and was then quantitated (1). The quality of the raw data was assessed using FASTQC and low-quality data were filtered out using the FASTX toolset. A combination of tools within the QIIME suite were used for clustering reads into operational taxonomic units (OTUs) using the Greengenes 16S rDNA database (2-4) using a 97% identity cutoff. To ensure quality of our results, we performed several filtering steps, removing sequences with low quality scores, sequences that match human databases and low-duplicate sequences. As paired end reads were used, an additional quality control step involved merging of the paired reads and sequences with substantial numbers of differences between the two paired ends were discarded.

Antibiotic treatment. For the duration of the experiment, Mice were treated daily via intragastric delivery with either drinking water (vehicle), colistin (RPI; C70700), metronidazole (Sigma-Aldrich; M1547), vancomycin (Sigma-Aldrich; V2002) or a combination of metronidazole, ampicillin (Sigma-Aldrich; A9518), neomycin (Sigma-Aldrich; N6386), and vancomycin (MANV). Vancomycin was administered at a final concentration of 2.5 mg/ml, all other antibiotics were administered at 5 mg/ml, all in a volume of 200 ml.

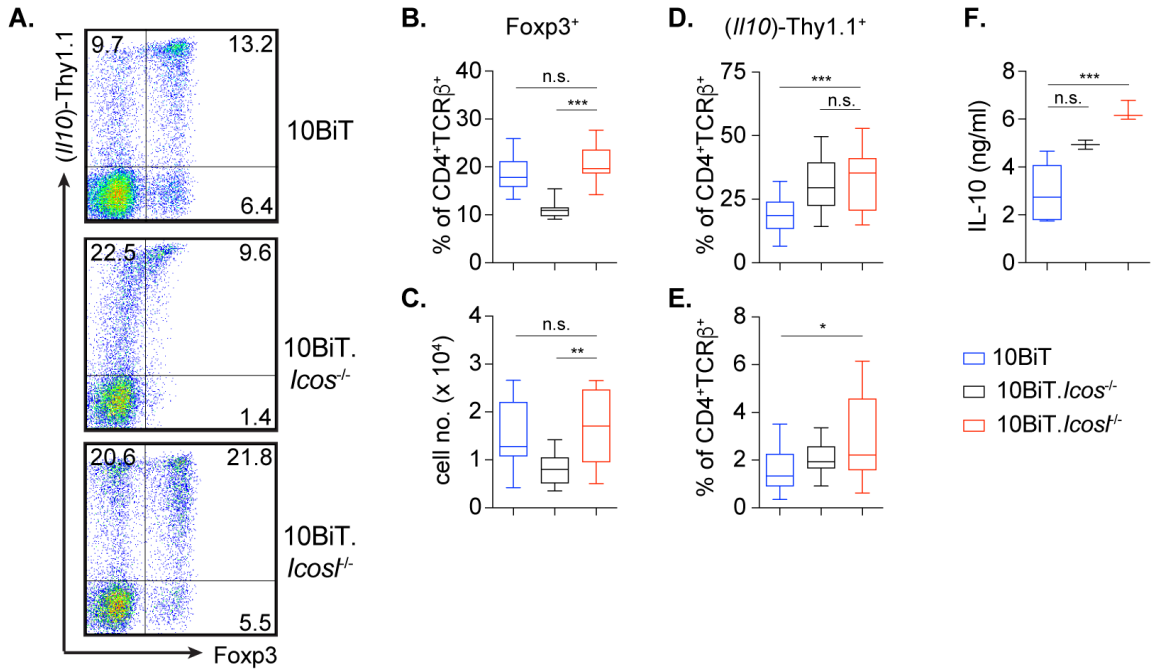
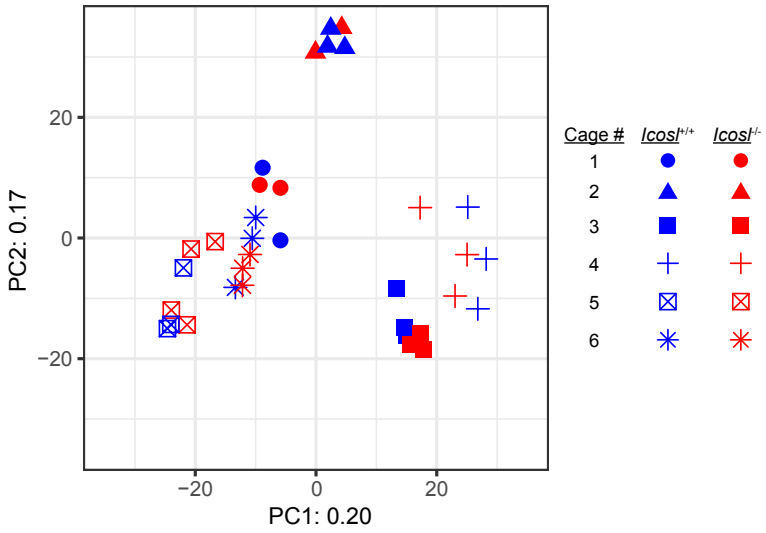


Fig. S1. Differential effects of ICOS- and ICOSL- deficiency on colonic Treg cells.

(**A**) Representative FACS plots showing expression of Foxp3 and Thy1.1 by $CD4^+TCR\beta^+$ cells from the colonic lamina propria of 10BiT, 10BiT.*Icos*$^{-/-}$ and 10BiT.*Icosl*$^{-/-}$ mice. Box and whisker plots illustrate frequencies and numbers respectively of Foxp3+ cells (**B** and **C**) and $Thy1.1^+$ cells (**D** and **E**), analyzed as in (A). 10BiT, n=15; 10BiT.*Icos*$^{-/-}$, n=11, 10BiT.*Icosl*$^{-/-}$, n=13. Data are compiled from four independent experiments. (**F**) IL-10 measured by ELISA of CD4 T cells isolated from colonic lamina propria of 10BiT, 10BiT.*Icos*$^{-/-}$ and 10BiT.*Icosl*$^{-/-}$ mice and stimulated for 24 hours with anti-CD3 and anti-CD28. 10BiT, n=5; 10BiT.*Icos*$^{-/-}$, n=2, 10BiT.*Icosl*$^{-/-}$, n=3. Data are from one of 2 independent experiments. Error bars represent mean ± SD; p values were calculated by ANOVA followed by Tukey's multiple-comparisons test. *p<0.05, **p<0.01, ***p<0.001.

A.



B.

	df	SS	MS	F	R^2	p-value
experiment	2	15302.907	7651.454	10.845	0.319	0.001
experiment:genotype	3	2892.404	964.135	1.367	0.060	0.134
experiment:cage	3	11530.564	3843.521	5.448	0.240	0.001
experiment:cage:genotype	3	2758.205	919.402	1.303	0.057	0.175
Residuals	22	15521.072	705.503		0.323	
Total	33	48005.153				

Fig. S2. Comparison of fecal microbiota composition of *Icosl*$^{+/+}$ and *Icosl*$^{-/-}$ cage mates. **(A)**. Principal coordinates analysis (PCoA) of the gut microbiota of *Icosl*$^{+/+}$ and *Icosl*$^{-/-}$ mice from three separate replicate experiments based on the centered log-ratio Aitchison distance. Three replicate experiments were conducted in which *Icosl*$^{+/+}$ and *Icosl*$^{-/-}$ mice were co-housed starting at weaning (day 21 of life). In the PCoA plots, samples that are closer together are more similar in microbial composition. Genotype is signified by color (blue: *Icosl*$^{+/+}$; red: *Icosl*$^{-/-}$), and samples represented by identical shapes were co-housed. **(B)**. Permutational multivariate analysis (PERMANOVA) was used to assess the association between genotype and gut microbial composition while accounting for the variation due to cage effects and stratifying permutations by experiment replicate. Genotype did not significantly affect the composition of the gut microbiota when accounting for covariates (experiment: genotype, PERMANOVA R^2 = 0.060, p = 0.1; experiment:cage:genotype, PERMANOVA R^2 = 0.057, p = 0.2). Experiment replicate (R^2 = 0.319, p = 0.001 and the interaction between experimental replicate and cage effects

(experiment:cage, PERMANOVA R^2 = 0.240, p = 0.001) accounted for a large proportion of the variation in gut microbial community composition.

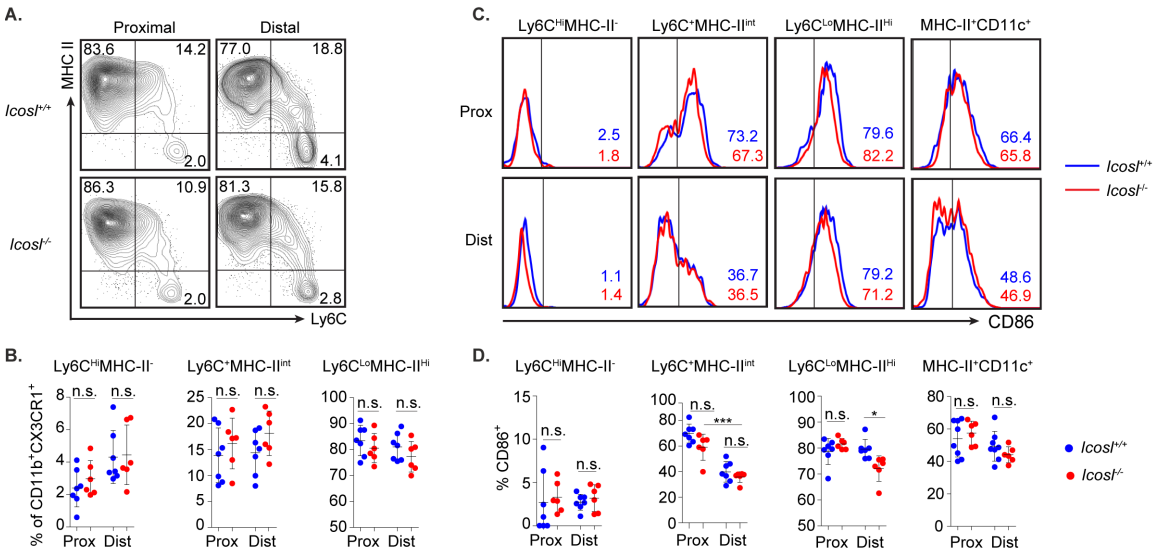


Fig. S3. **ICOSL deficiency does not result in increased or hyper-activated myeloid cells in the colonic lamina propria**. (**A**) Representative flow plots showing colonic monocytes:MHC-II^{int}Ly6C^{+}, newly differentiated inflammatory macrophages: MHC-II^{int}Ly6C^{+}, and mature macrophages: MHC-II^{+}Ly6C^{lo}; among CD11b^{+}CX3CR1^{+} cells in the proximal or distal colon of *Icosl*$^{+/+}$ and *Icosl*$^{-/-}$ mice (**B**) Graphs displaying frequencies of cell subsets as depicted in A. (**C**) CD86 expression by cell subsets defined in A and by dendritic cells (MHC-II^{hi}CD11c^{+}) in the proximal or distal colon of *Icosl*$^{+/+}$ and *Icosl*$^{-/-}$ mice. (**D**) Graphs depicting frequencies of CD86^{+} cells as shown in C.

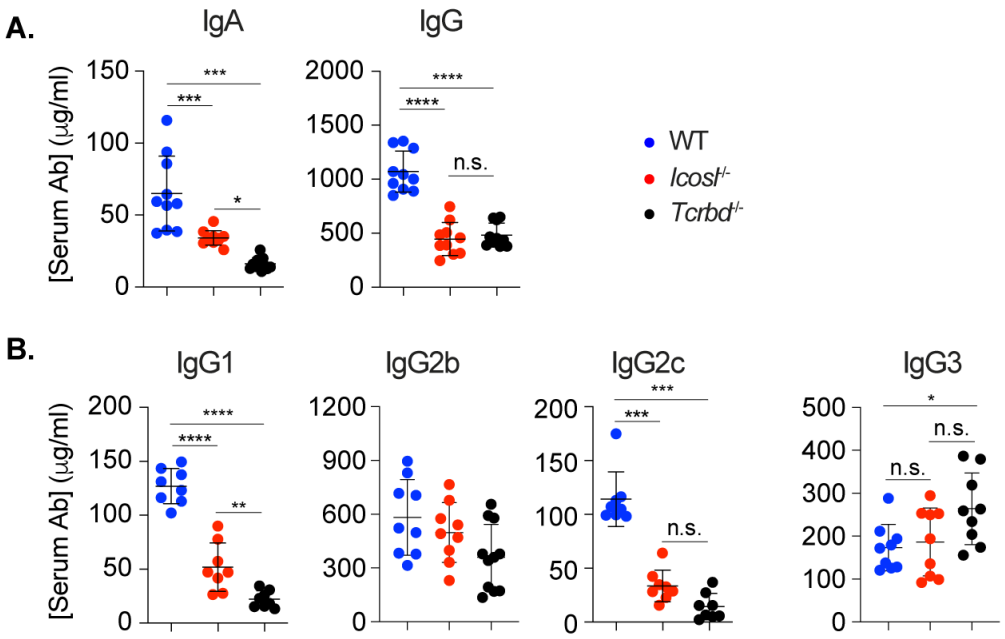


Fig. S4. Comparable effects of TCR- and ICOSL- deficiency on circulating antibody levels at steady state. (**A**) ELISA of IgA and IgG in serum from WT, *Icosl*$^{-/-}$, or *Tcrbd*$^{-/-}$ mice. n=10 mice per group. (**C**) ELISA of IgG1, IgG2b, IgG2c, or IgG3 in serum of WT, *Icosl*$^{-/-}$, or *Tcrbd*$^{-/-}$ mice. WT, n=9; *Icosl*$^{-/-}$, n=10; *Tcrbd*$^{-/-}$, n=9.

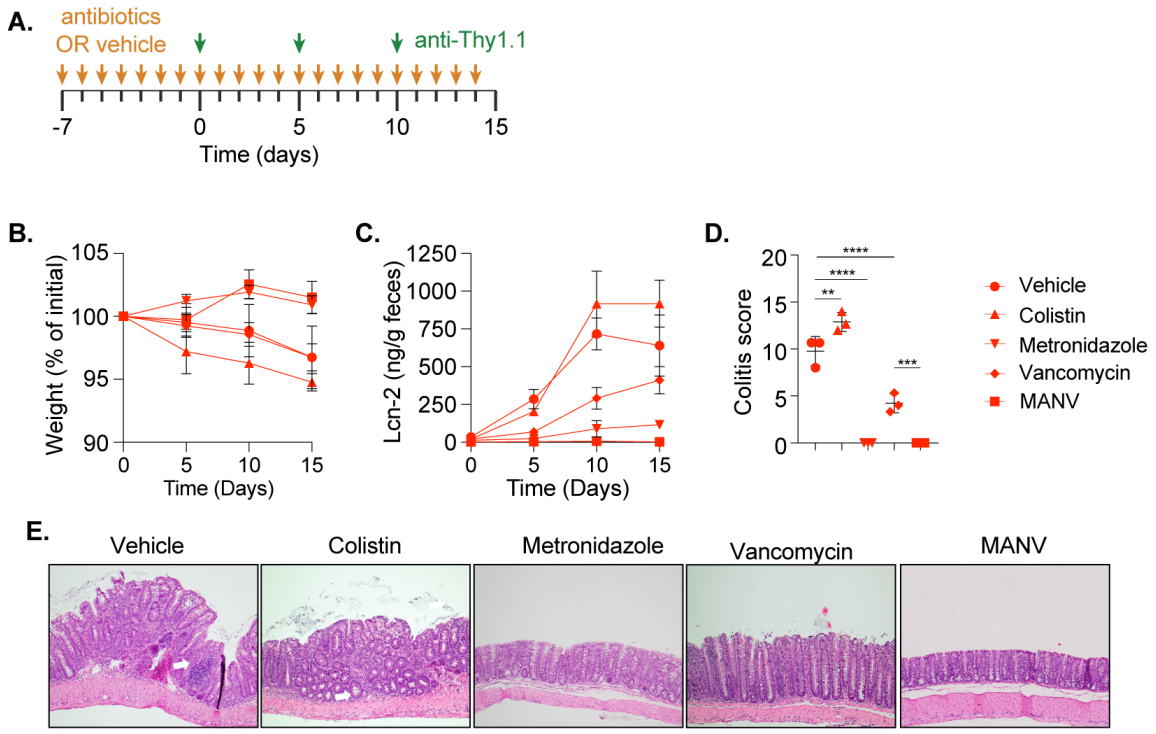


Fig. S5. Colitis induced in ICOSL-deficient mice can be inhibited by pre-treatment with metronidazole. (**A**) Overview of experimental design. 10BiT mice were administered normal drinking water (vehicle) or colistin, metronidazole, vancomycin, or MANV (metronidazole, ampicillin, neomycin, vancomycin) daily from day -7 through day 15. All mice received anti-Thy1.1 on days 0, 5, and 10. (**B**) Relative weight changes, and (**C**) fecal Lcn2 levels, as determined every 5 days. (**D**) Total colitis scores and (**E**) representative histology of H&E-stained colonic tissue sections from one of three independent experiments at day 15, n=3-4 mice/group. Error bars represent mean ± SD; p values were calculated by two-way ANOVA for repeated measures with Bonferroni correction (B and C) or by one-way ANOVA test followed by Tukey's multiple-comparisons test (D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. S6. As expected, *Il10*cKO mice develop adult-onset colitis. (**A**) Weight charts of *Icosl*$^{+/+}$, *Icosl*$^{-/-}$, and *Icosl*$^{+/+}$.*Il10*cKO mice monitored weekly from 8 weeks of age and analyzed grossly for presence or absence of severe colitis at 28 weeks (**B**). (**C**) Representative photomicrographs of H&E-stained colon tissue from the 3 groups represented. n=10 mice/group.

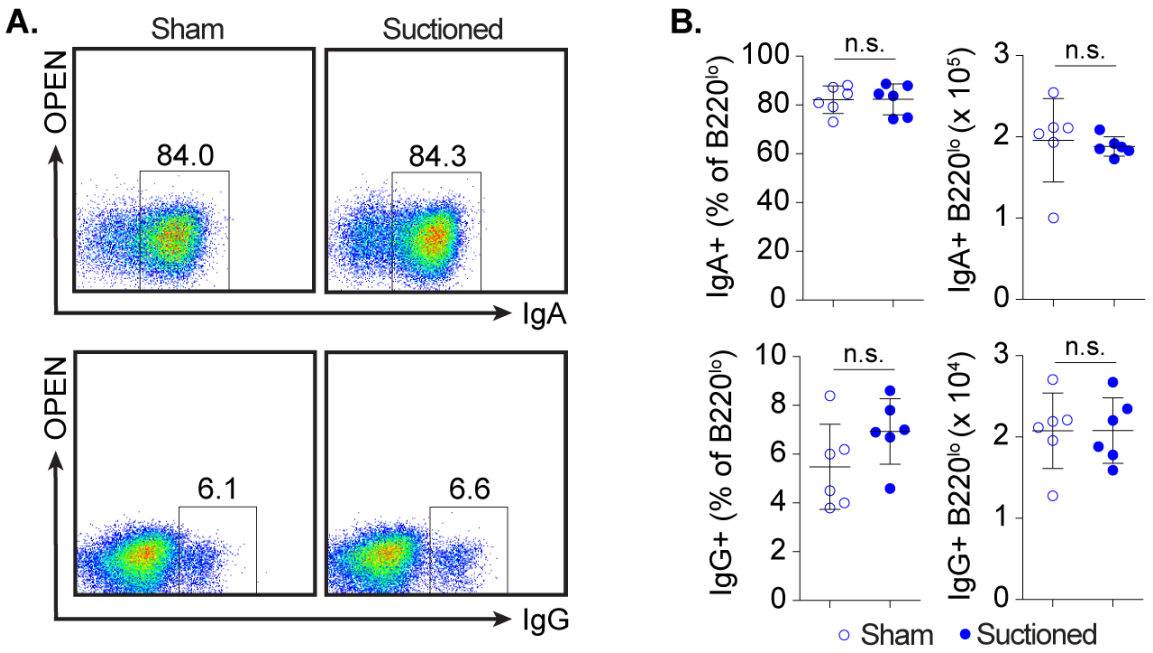


Fig. S7. Mucus suctioning does not disrupt lamina propria plasma cells. Each colon was bisected longitudinally, and one half underwent sham suction without applied vacuum (Sham) while the other half had mucus collected by gentle suction (Suctioned). Remaining tissue was then enzymatically-digested to release total lamina propria cells. **(A)** Representative flow cytometry plots showing IgA and IgG expression by $B220^{lo}$ cells from the lamina propria of the colon. **(B)** Graphs summarizing frequencies and numbers of $B220^{lo}$ cells expressing IgA or IgG of individual mice, collected as depicted in (A). Error bars represent mean ± SD and p values were calculated by unpaired Student's t test; *p<0.05, **p<0.01, ***p<0.001.

SI References

1. Kumar R*, et al.* (2014) Getting started with microbiome analysis: sample acquisition to bioinformatics. *Curr Protoc Hum Genet* 82:18 18 11-29.
2. Wang Q, Garrity GM, Tiedje JM, & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73(16):5261-5267.
3. Cole JR*, et al.* (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* 37(Database issue):D141-145.
4. McDonald D*, et al.* (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 6(3):610-618.