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Supplemental information

Environmental enteric dysfunction induces regulatory T cells that inhibit local CD4+ T cell responses and impair oral vaccine efficacy

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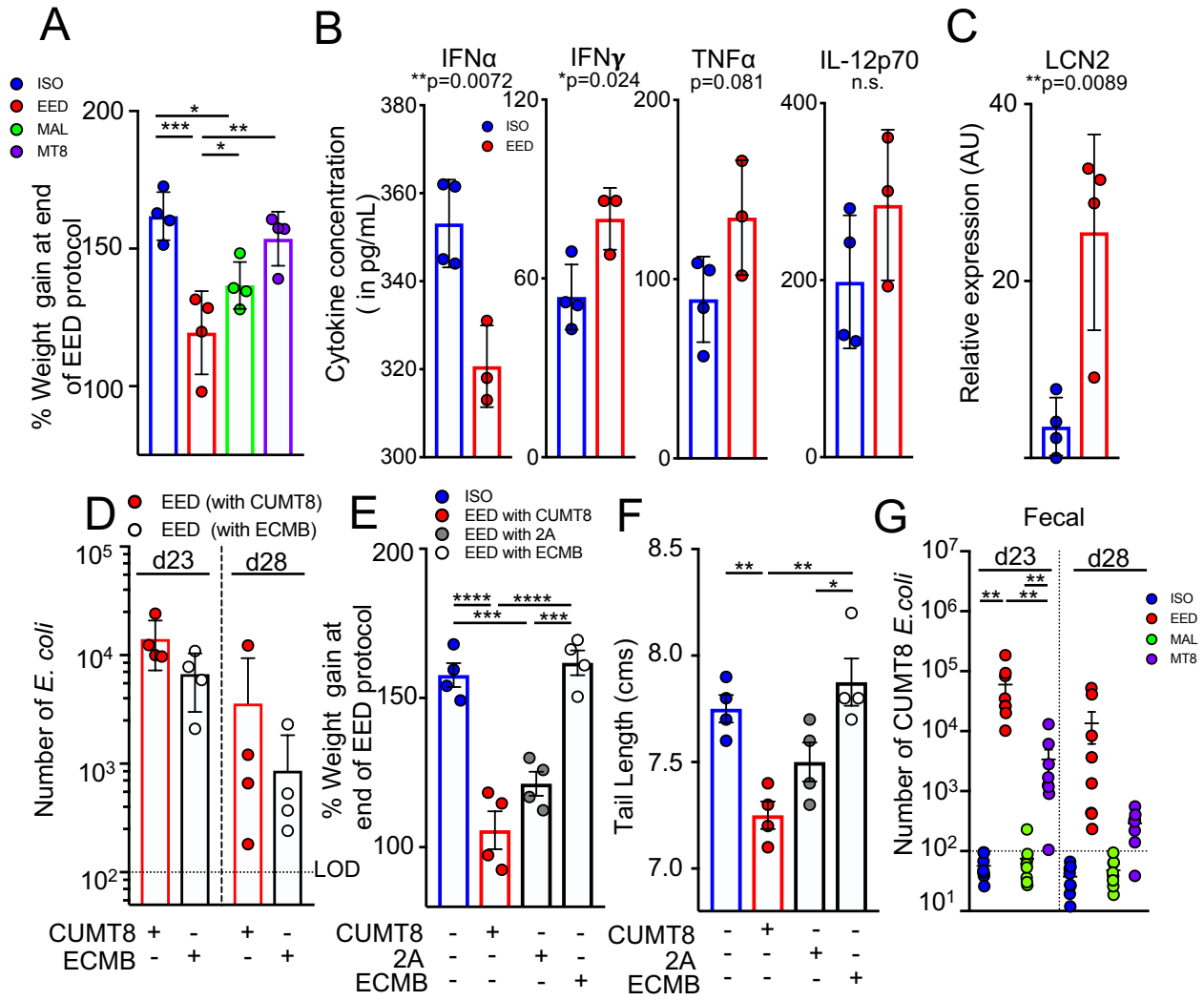
Supplemental Figure 1



Supplemental Figure 1 Development of murine EED-like disease requires colonization with adherent invasive *E.coli* (AIEC) and is associated with the induction of intestinal inflammation. (Related to Fig.1)

Mice were placed on the EED or control protocols as indicated and sacrificed on d28 .

A) Percent weight gain at the end of of the EED protocol (day 28). Data are representative of 3 independent experiments (n=4).

B) Luminex bead-based quantification of various cytokines from homogenized ileal tissue. Data shown are representative of 2 independent experiments (n=3-4/experiment).

C) Relative expression of *Lcn2* from ileal tissues of ISO and EED mice as measured by qPCR. Data are representative of 2 independent experiments (n=4/experiment).

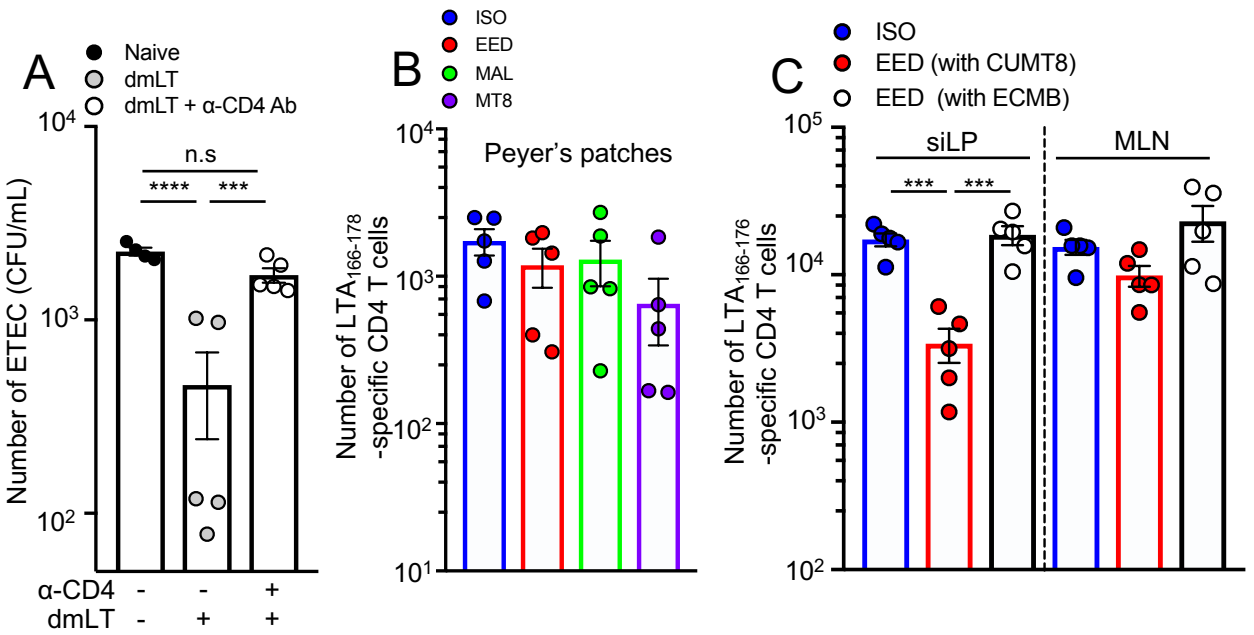
D) Level of CUMT8 or ECMB *E. coli* in fecal samples from day 23 and day 28 of the EED protocol. Genomic DNA was isolated from fresh stool samples and the abundance CUMT8 or ECMB was measured by qPCR and compared against a standard curve. Dotted lines represent limit of detection. Data shown is representative of 2 experiments (n=3-4/experiment).

E) Percent weight gain and **F)** tail lengths at the end of the EED protocol comparing EED induction with CUMT8(mouse derived AIEC) vs E coli 2A (AIEC isolated from patient with Crohn's disease) and ECMB (non-adherent/non-motile *E coli*). Data are representative of 2 independent experiments (n=4/experiment)

G) Level of CUMT8 *E. coli* in fecal samples from day 23 and day 28 of the EED protocol. Genomic DNA was isolated from fresh stool samples and the abundance CUMT8 was measured by qPCR and compared against a standard curve. Dotted lines represent limit of detection. Data are pooled from 2 independent experiments(n=3-4/experiment).

(C,E-G). Data points represent a single mouse. Graphs show the mean ±SEM. Statistics for A and E-G were calculated by one-way ANOVA. Statistics for B-D were calculated by Students t-test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$)

Supplemental Figure 2



Supplemental Figure 2 Measurement of the number and function of oral vaccine-specific CD4+ T cells in mice with EED-like Disease (Related to Figure 2)

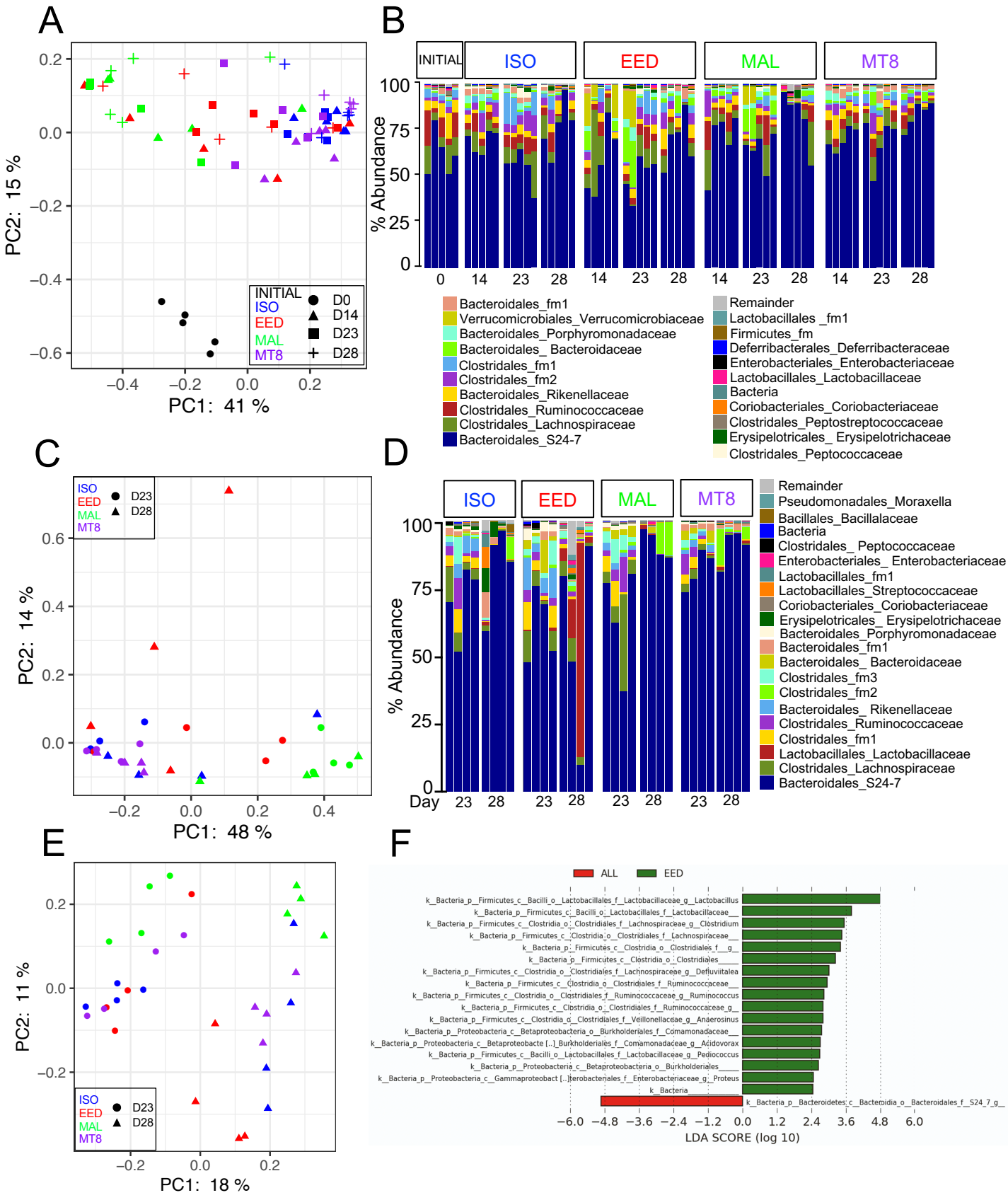
A) WT B6 mice were immunized with 20µg of dmLT and boosted 7 days later. During the boost (from day 8 to 14), some mice were depleted of $CD4^+$ T cells (GK1.5 clone; anti-CD4) and all mice were then challenged with 1 x 10^4 CFU of LT-expressing Kan^{Res} ETEC (jf786). After 24 h, bacteria were recovered from the small intestine in PBS and plated onto LB/Kanamycin agar. Pooled from 2 experiments

B) Mice in the EED protocol were immunized as in Fig. 2A. Shown are the number of $LT_{166\text{-}176}$:I-A^b specific $CD4^+$ T cells isolated from Peyer's Patches. Data shown is pooled from 2 experiments where 2-3 groups of PPs were analyzed/experiment.

C) Comparison of the numbers of $LT_{166\text{-}176}$:I-A^b specific $CD4^+$ T cells after prime/boost oral vaccination in the siLP and MLN of ISO, EED and a third group of mice fed the low protein/fat chow but colonized with a non-adherent/non-motile *E. coli* ECMB. Data shown is representative experiment of 2 independent experiments.

Graphs show the mean ±SEM. Statistics calculated by one-way ANOVA (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

Supplemental Figure 3



Supplemental Figure 3 Murine EED is associated with shifts in the small intestinal microbiome (Related to Figure 3)

Genomic DNA was isolated from fecal and ileal samples of singly housed mice in the EED or control protocols and the structure of the microbiome analyzed via sequencing and enumeration of 16S rRNA genes using QIIME2.

A) Principal Coordinate Analysis (PCoA) of fecal samples collected on experimental day 0,14, 23 and 28 based on bacterial community similarity. Clustering of samples by Bray-Curtis method.

B) Stacked bar chart showing the mean relative abundances of the most abundant OTUs in fecal samples (n=4-5) at the family level on experimental day 14, 23 and 28. Indicated are the 20 most abundant taxa at the family level. When QIIME2 was unable to identify family-level taxa specificity, the lowest taxon level was identified with “fm1” and “fm2” denoting different families within this taxon.

C) PCoA of ileal samples on experimental day 23 and 28 based on bacterial community similarity. Clustering of samples by Bray-Curtis method.

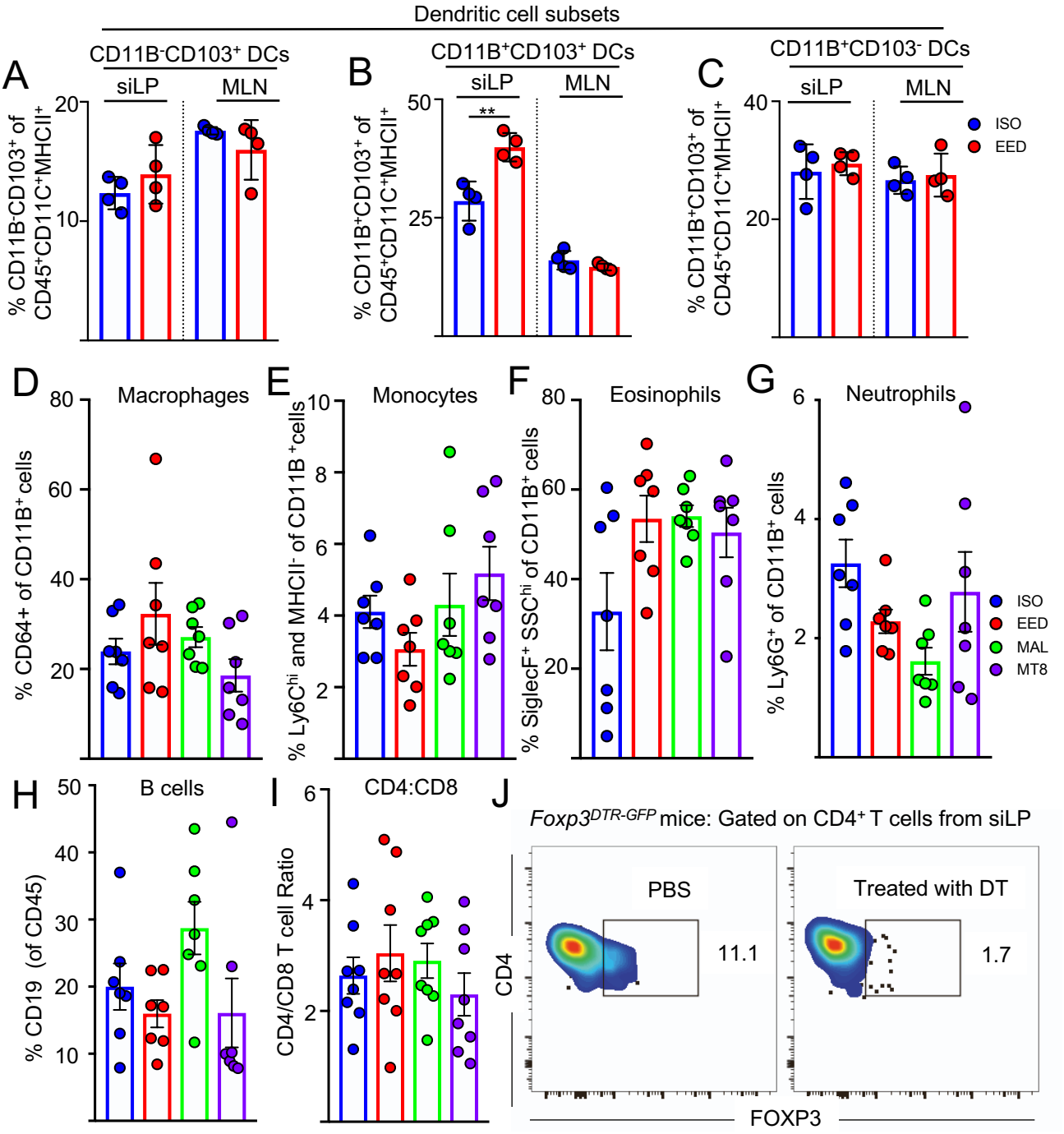
D) Stacked bar chart showing the mean relative abundances of the most abundant OTUs in fecal samples (n=3-5) at the family level on experimental day 14, 23 and 28. Indicated are the 20 most abundant taxa at the family level. When QIIME2 was unable to identify family-level taxa specificity, the order was identified with “fm1” and “fm2” denoting different families within this order.

E) PCoA of ileal samples on experimental day 23 and 28 based on bacterial community similarity. Clustering of samples by Jaccard method.

F) LEfSe plots showing the genera differing significantly (LDA score (log10)>2.4) in relative abundance between the ileum of EED mice (day 28) and all other groups(ISO, MAL and MT8)

Data in this figure are representative of 2 independent experiments (n=3-5)

Supplemental Figure 4



Supplemental Figure 4 Analysis of intestinal immune cell populations in murine EED (Related to Fig. 4)

A-I) ISO, EED, MAL or MT8 protocols were induced in mice as shown in Fig.1A.

A-C) Percents of dendritic cell (DC) subsets (Gated on Live $CD45^+$ $Ly6G^-$ $SiglecF^-$ $Ly6C^-$ $CD90^-$ $CD19^-$ $CD64^-$ $CD11c^+$ $MHCII^+$). Data shown is representative of 2 independent experiments (n=4/experiment)

A) Percents of $CD11B^-CD103^+$ DCs in the siLP and MLN.

B) Percents of $CD11B^+CD103^+$ DCs in the siLP and MLN.

C) Percents of $CD11B^+CD103^-$ DCs in the siLP and MLN.

(**D-I**) Immune cell phenotyping of siLP resident immune cells. Data shown is pooled from 2 separate experiments (n=3-4/experiment)

D) Relative frequency of macrophages (Gated Live $CD45^+$ $Ly6G^-$ $SiglecF^-$ $Ly6C^-$ $CD90^-$ $CD19^-$ $CD64^+$ $CD11b^+$)

E) Relative frequency of monocytes (Gated Live $CD45^+$ $Ly6G^-$ $SiglecF^-$ $CD90^-$ $CD19^-$ $CD64^-$ $CD11c^-$ $MHCII^-$ $Ly6C^+$ $CD11b^+$)

F) Relative frequency of eosinophils (Gated Live $CD45^+$ $Ly6G^{-/dim}$ $SiglecF^+$ $CD90^-$ $CD19^-$ $CD11c^-$ $MHCII^-$ $Ly6C^-$ $CD11b^+SSC^{hi}$)

G) Relative frequency of neutrophils (Gated Live $CD45^+$ $Ly6G^+$ $SiglecF^-$ $CD90^-$ $CD19^-$ $CD64^-$ $CD11c^-$ $MHCII^-$ $Ly6C^+$ $CD11b^+$)

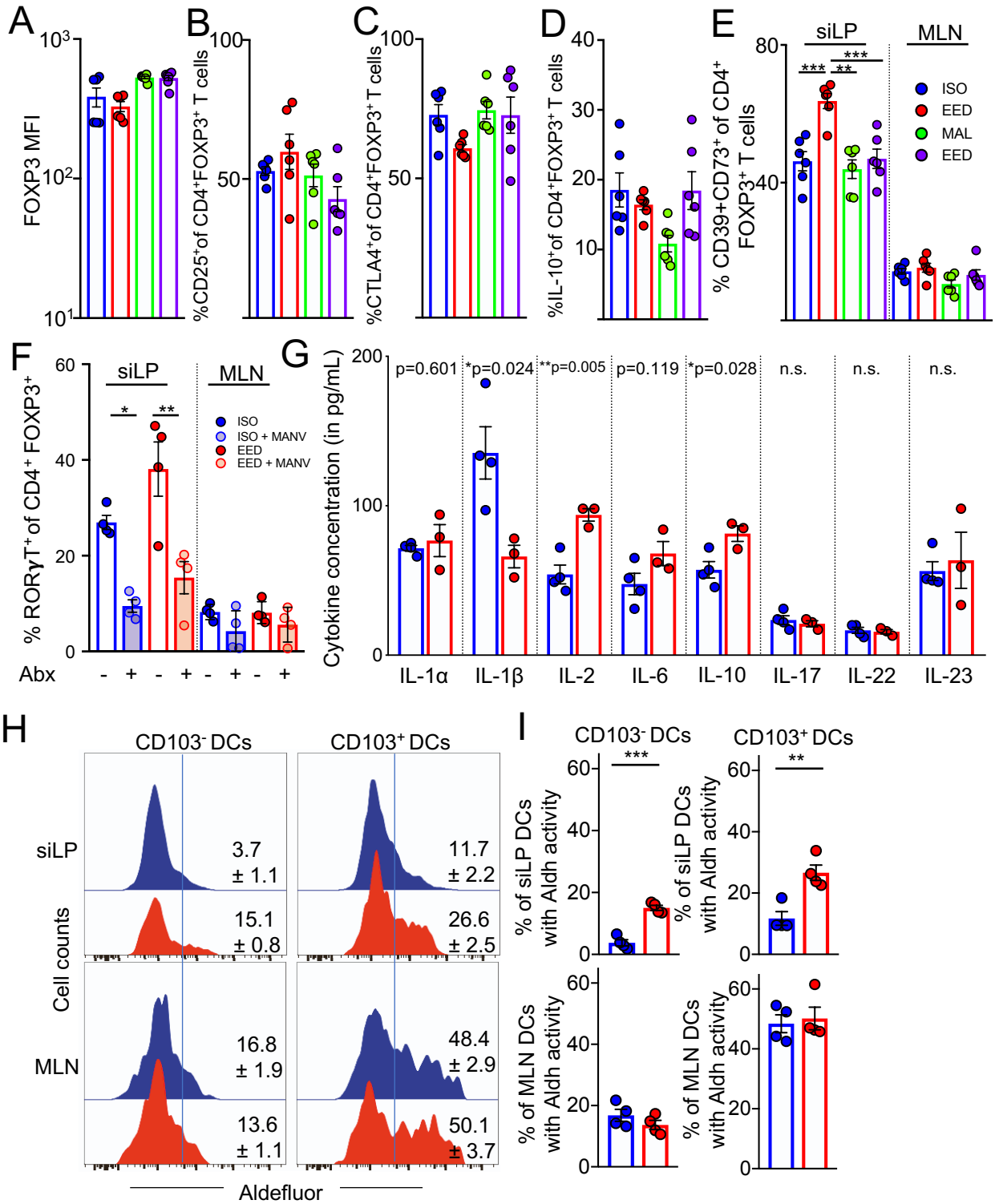
H) Relative frequency of B cells (Gated Live $CD45^+$ $Ly6G^-$ $SiglecF^-$ $CD90^-$ $CD19^+$)

I) Ratio of $CD4^+$ to $CD8\beta^+$ T cells (T cells gated Live $CD45^+$ $CD90^+$ $TCRb^+$ $CD4^{+/-}$ $CD8\beta^{+/-}$

J) Representative flow cytometric plot showing depletion of $CD4^+$ $FOXP3^+$ cells from the siLP of *Foxp3*$^{DTR\text{-}GFP}$ mice following diptheria toxin administration as described in Fig. 4C**.** Data is representative of 2 separate experiments.

All data points represent a single mouse. Graphs show the mean ±SEM. Statistics for A-C were calculated by a Student's t-test. Statistics for D-I were calculated by one-way ANOVA (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$)

Supplemental Figure 5



Supplemental Figure 5 Mice with murine EED have intestinal Treg cells with increased expression of CD39 and CD73 and intestinal dendritic cells with increased aldehyde dehydrogenase activity. (Related to figure 5)

A-E) Mice were placed on the ISO, EED, MAL or MT8 protocols and sacrificed on d28 as in Fig.1

A-D) Expression of transcription factors, surface proteins and cytokines of intestinal Treg cells in mice with EED. The expression of **A**) FOXP3, **B**) CD25, **C**) CTLA4, or **D**) IL-10 was assessed by flow cytometry. IL-10 was assessed by intracellular flow cytometry on cells stimulated with PMA/ionomycin and Brefeldin A Treg cells were gated as: Live, $CD90^+$, $TCRb^+$, $CD8\beta^-$, $CD4^+$ $FOXP3^+$. Data are pooled from 2 experiments (n=3/experiment).

E) Percent of $FOXP3^+$ $CD4^+$ Treg cells expressing CD39 and CD73 in the siLP and MLN of EED and control mice. Data are pooled from 2 experiments (n=3/experiment).

F) Percent $ROR\gamma T^+$ cells of $FOXP3^+$ $CD4^+$ Treg cells in the siLP and MLN of mice in the ISO or EED protocols, treated with antibiotics as in Fig.3A. Antibiotic treated mice are represented as shaded bars. Data are representative of 2 independent experiments (n=3-4/experiment).

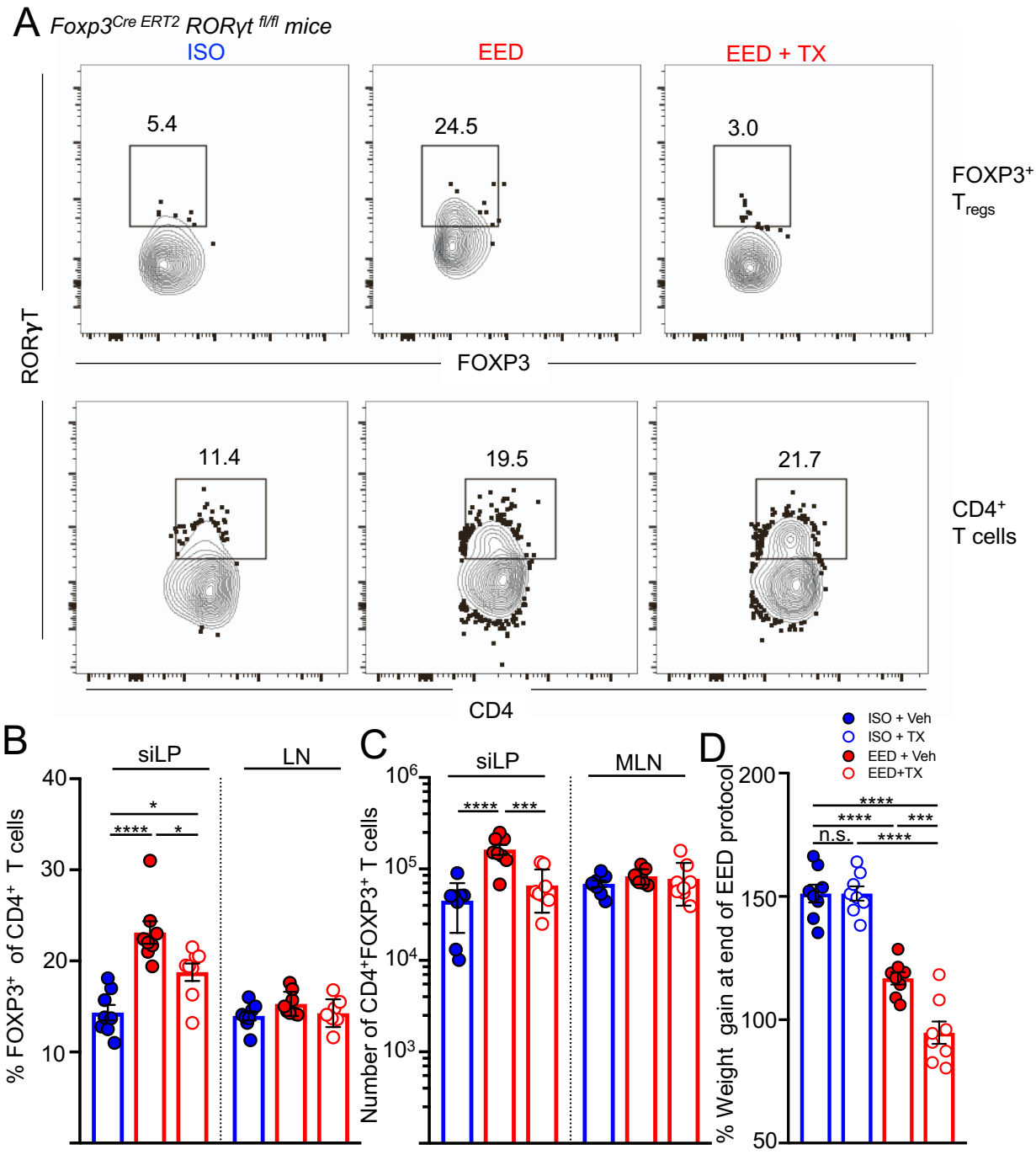
G) Luminex bead-based quantification of cytokines from homogenized ileal tissue of mice from the ISO or EED protocols at day 28. Data are representative of 2 independent experiments (n=3-4/experiment).

H-I) Determination of Aldh activity in DCs using the ALDEFLUOR Kit. Data shown are representative of 2 independent experiments (n=3-4/experiment). Samples were isolated from ISO or EED mice at day 28 of the protocol. Numbers on flow plots indicate mean percents of DCs that show ALDH activity ±SEM.

H) Representative histograms showing percents of DCs that were positive for the $FITC^+$ Aldefluor conversion product (an indicator of Aldh activity) after 30 min of substrate addition.(Gated on Live $CD45^+$ $Ly6G^-$ $SiglecF^-$ $Ly6C^-$ $CD90^-$ $CD19^-$ $CD64^-$ $CD11c^+$ $MHCII^+$)

I) Percents of siLP (top 2 panels) and MLN (bottom 2 panels) DCs showing Aldh activity from (H). Data points represent a single mouse. Graphs show the mean ±SEM. Statistics for H and I were calculated by a Student's t-test. Statistics for A-F were calculated by one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$).

Supplemental Figure 6



Supplemental Figure 6 Targeted deletion of Rorc from Treg cells in EED mice reduces the total number of Treg cells and increases EED-associated stunting. (Related to Fig. 6)

A) Representative flow cytometric contour plots showing RORγT expression in FOXP3^{+} CD4^{+} Treg cells, and CD4^{+} Tconv cells following tamoxifen treatment of FOXP3$^{Cre\ ERT2}$ Rorc$^{fl/fl}$ mice . Data are representative of 2 independent experiments

B) Percent FOXP3^{+} CD4^{+} Treg cells from the siLP and MLN after tamoxifen treatment of FOXP3$^{Cre\ ERT2}$ Rorc$^{fl/fl}$ mice at day 28 of the EED protocol. Data shown are pooled from 3 independent experiments (n=2-3).

C) Number of FOXP3^{+} CD4^{+} Treg cells from (B).

D) Percent weight gain at the end of the EED protocol (day 28) from FOXP3$^{Cre\ ERT2}$Rorc$^{fl/fl}$ mice with and without tamoxifen treatment. Data shown are pooled from 2 experiments (n=4/experiment).

B-D).Data points represent a single mouse. Graphs show the mean ±SEM. Statistics calculated by one-way ANOVA (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).