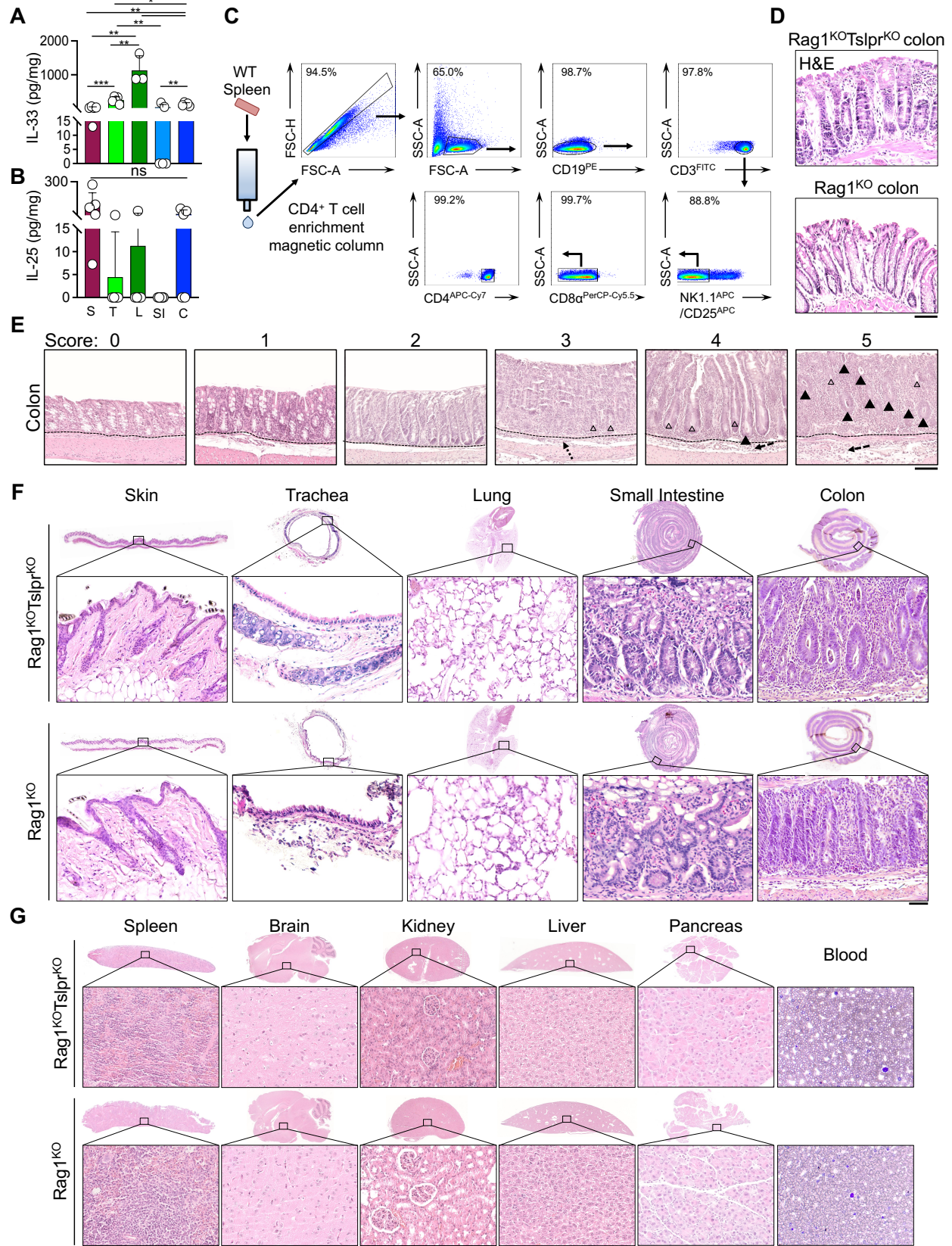


TSLP/Dendritic cell axis promotes CD4⁺ T cell tolerance to the gut microbiome

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SUPPLEMENTAL FIGURES AND TABLES



Supplemental Figure 1. Epithelium-derived alarmins levels in normal barrier organs, CD4⁺ T cells sorting and its impact on vital organs after adoptive transfer.

(A and B) IL-33 (A) and IL-25 (B) protein concentration in WT mouse (n = 5) skin (S), trachea (T), lung (L), small intestine (SI), and colon (C). Bar graphs show mean + sd, Ordinary one-way ANOVA, ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

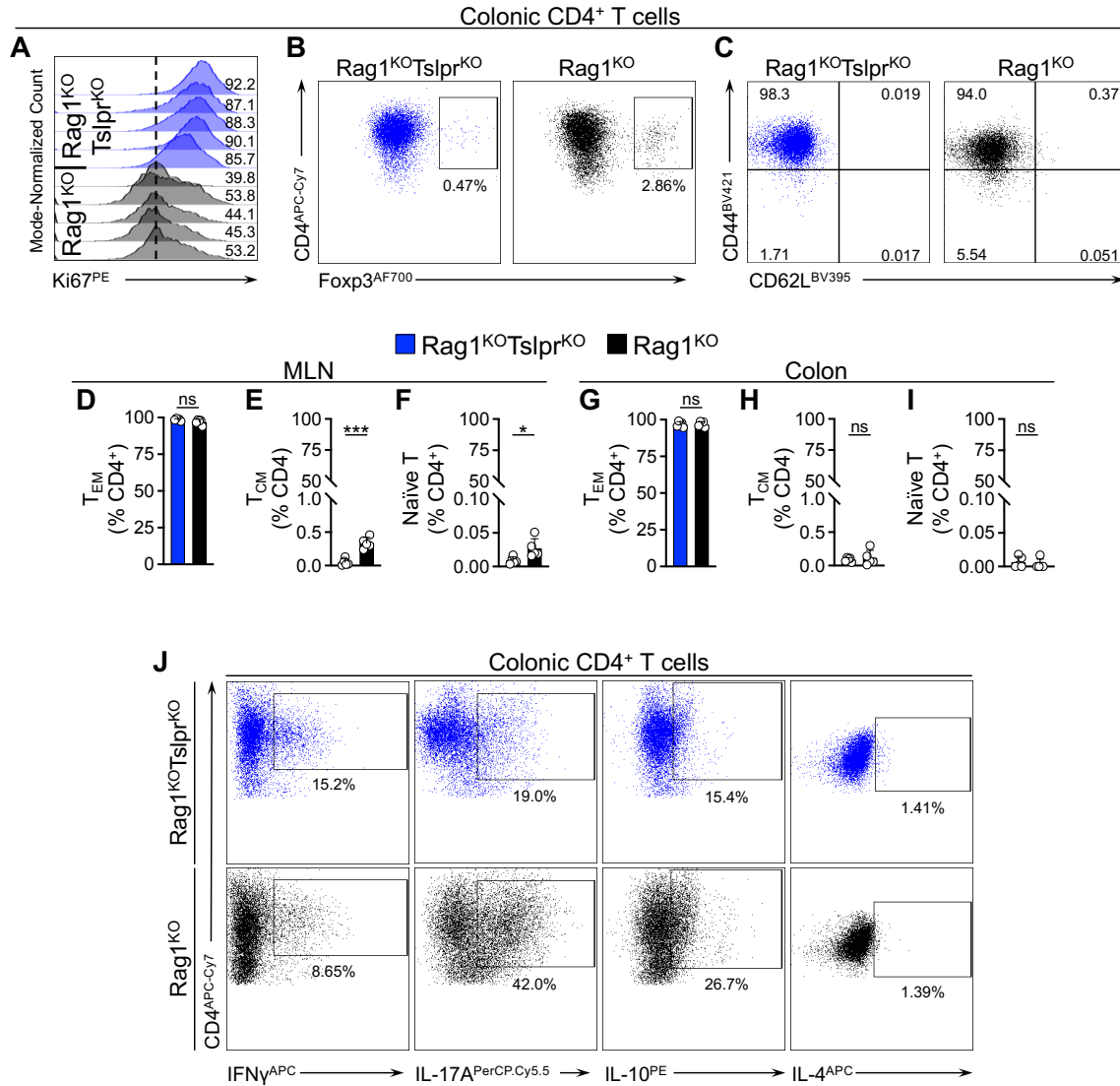
(C) Representative CD4⁺ T cell sorting strategy. WT splenocytes were passed over a CD4⁺ T cell enrichment column before pooling samples and FACS sorting for CD19⁻CD3⁺CD25⁻NK1.1⁻CD8⁻ CD4⁺ T cells using the outlines gating strategy. The percentage of cells within the gate is shown on each flow dot plot.

(D) Representative H&E stains of Rag1^{KO}Tslpr^{KO} cells and Rag1^{KO} colon at the baseline. Scale bars, 50 μ m.

(E) Representative H&E-stained adoptive T cell transfer-induced colitis histology and associated score. This scoring system was used as a rubric to grade specimens in the study, as described previously (1). Note the retention of goblet cells in the mucosal tissue in scores 0-2 with mild increases in crypt height indicating crypt hyperplasia as the scores increase. Overall cellularity increases through the first three scores as well. A score of 3 is notable for the invasion of inflammatory cells beyond the muscularis mucosa (dotted line) and into the submucosal tissue (dotted arrow). A score of 4 includes severe lack of goblet cell presence (open triangles), while a score of 5 is notable for the formation of several crypt abscesses (solid triangles) as well as severe crypt distortion and absorption by inflammatory tissue. Crypt hyperplasia and submucosal infiltration continue throughout scores 3-5 as well. Scale bar, 50 μ m.

(F) Representative H&E stains of barrier organs from Rag1^{KO}Tslpr^{KO} and Rag1^{KO} after adoptive transfer of WT CD4⁺CD25⁻ T cells. Scale bar, 50 μ m.

(G) Representative H&E stains of several vital organs and Giemsa stains of peripheral blood smears from Rag1^{KO}Tslpr^{KO} and Rag1^{KO} after adoptive transfer of WT CD4⁺CD25⁻ T cells. Scale bar, 50 μ m.



Supplemental Figure 2. CD4⁺ T cells status in recipient Rag1^{KO}Tslpr^{KO} and Rag1^{KO} mice.

(A) Representative flow histograms of Ki67 expression in the colonic CD4⁺ T cells from Rag1^{KO}Tslpr^{KO} (blue) and Rag1^{KO} (black) at the end of the study. The percentage of Ki67⁺CD4⁺ T cells is shown on the histograms.

(B) Representative flow cytometric dot plots of Foxp3⁺CD4⁺ T_{reg} in Rag1^{KO}Tslpr^{KO} (blue) and Rag1^{KO} (black) colon.

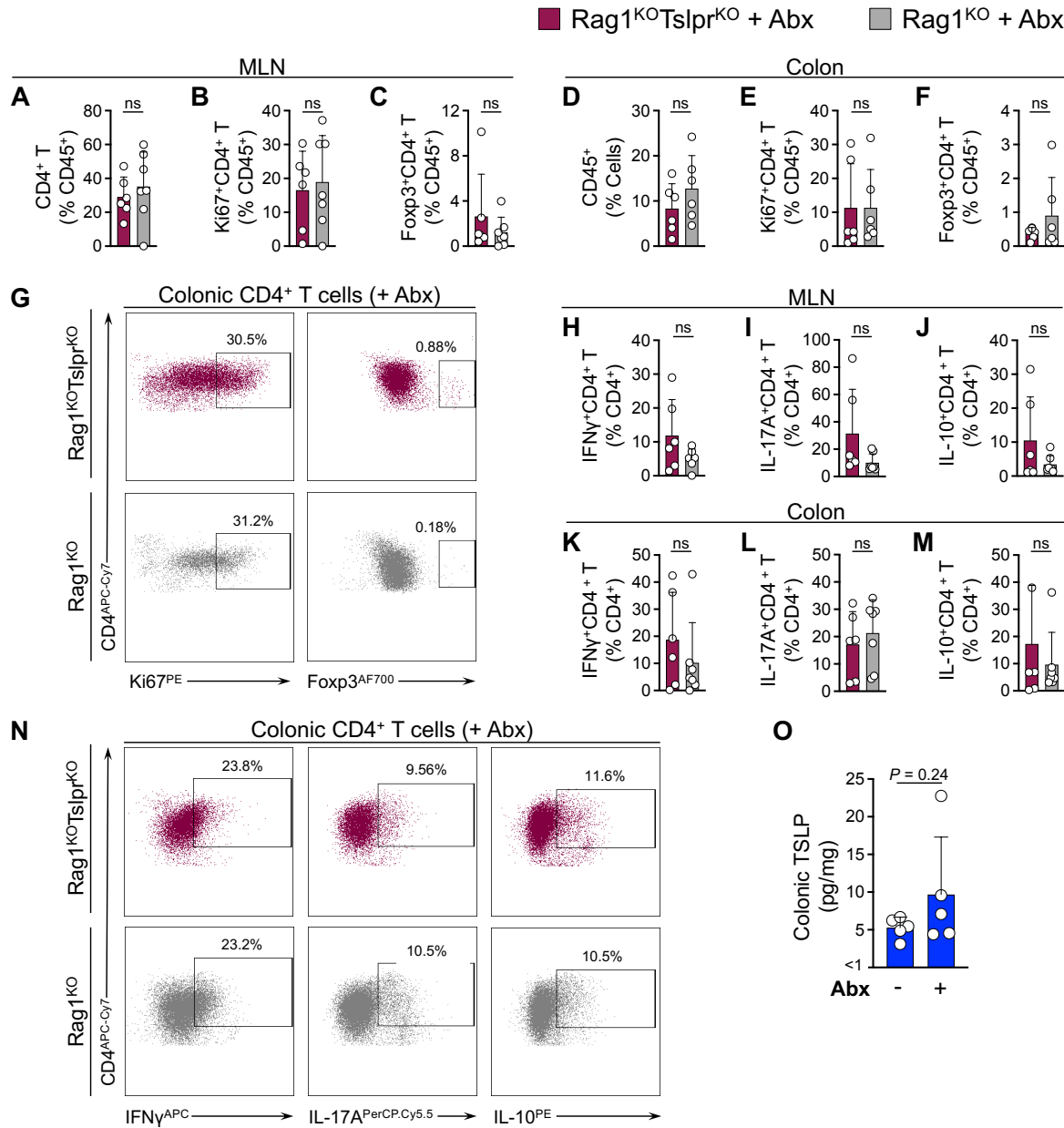
(C) Representative flow cytometric dot plots of CD44 and CD62L expression on CD4⁺ T cells adoptively transferred into Rag1^{KO}Tslpr^{KO} (blue) and Rag1^{KO} (black) colon.

(D-F) Quantification of CD4⁺ T cell phenotypes: T_{EM}, effector memory T cell (CD44⁺CD62L⁻, D), T_{CM}, central memory T cell (CD44⁺CD62L⁺, E) and Naïve T cell (CD44⁻CD62L⁺, F) in Rag1^{KO}Tslpr^{KO} and Rag1^{KO} MLN at the end of the study.

(G-I) Quantification of CD4⁺ T_{EM} (G), T_{CM} (H) and Naïve T cell (I) in Rag1^{KO}Tslpr^{KO} and Rag1^{KO} colon at the end of the study.

(J) Representative flow cytometric dot plots of IFN γ ⁺, IL-17A⁺, IL-10⁺, and IL-4⁺ CD4⁺ T cells in Rag1^{KO}Tslpr^{KO} (blue) and Rag1^{KO} (black) colon.

The percent cells in each gate are shown on the flow plots. Each dot represents one mouse, bar graphs show mean + sd, unpaired *t*-test; ns, not significant, **P* < 0.05, ****P* < 0.001.



Supplemental Figure 3. The microbiome does not alter colonic TSLP expression in adult mice.

(A-C) Flow cytometric quantification of CD4⁺ T (A), Ki67⁺CD4⁺ T (B) and Foxp3⁺CD4⁺ T_{reg} (C) frequency from mesenteric lymph nodes (MLN) of Rag1^{KO}Tslpr^{KO} (n = 5) and Rag1^{KO} (n = 5) mice. Experimental data were verified in a second independent experiment.

(D-F) Flow cytometric quantification of CD4⁺ T (D), Ki67⁺CD4⁺ T (E) and Foxp3⁺CD4⁺ T_{reg} (F) frequency in Rag1^{KO}Tslpr^{KO} (n = 5) and Rag1^{KO} (n = 5) colons. Experimental data were verified in a second independent experiment.

(G) Representative flow cytometric dot plots of Ki67⁺CD4⁺ T and Foxp3⁺CD4⁺ T_{reg} in Rag1^{KO}Tslpr^{KO} and Rag1^{KO} colons. The percent cells in each gate are shown on the flow plots.

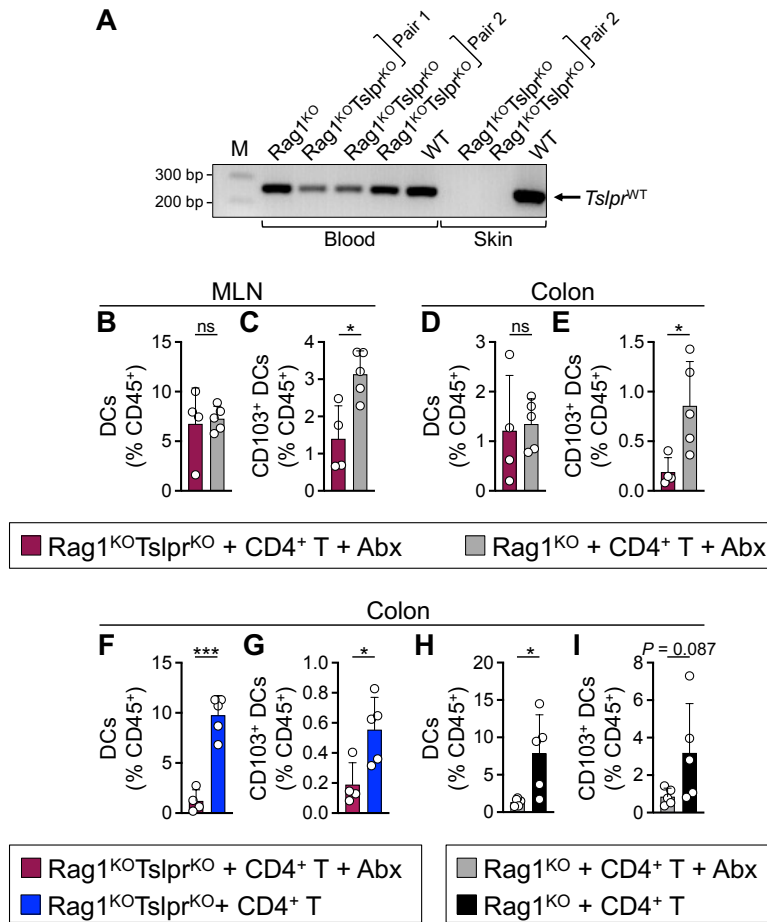
(H-J) Flow cytometric quantification of IFN γ ⁺ (H), IL-17A⁺ (I) and IL-10⁺ (J) CD4⁺ T cell frequency from MLN of Rag1^{KO}Tslpr^{KO} (n = 5) and Rag1^{KO} (n = 5) mice. Experimental data were verified in a second independent experiment.

(K-M) Flow cytometric quantification of IFN γ ⁺ (K), IL-17A⁺ (L) and IL-10⁺ (M) CD4⁺ T cell frequency in Rag1^{KO}Tslpr^{KO} (n = 5) and Rag1^{KO} (n = 5) colons. Experimental data were verified in a second independent experiment.

(N) Representative flow cytometric dot plots of IFN γ ⁺, IL-17A⁺ and IL-10⁺ CD4⁺ T cells in Rag1^{KO}Tslpr^{KO} and Rag1^{KO} colons. The percent cells in each gate are shown on the flow plots.

(O) TSLP expression in the colon of WT adult mice with or without microbiome-depleting antibiotic treatment (Abx).

Single-cell suspensions from MLN and colon were stimulated for 4 h with PMA/ionomycin + Brefeldin A and analyzed for cytokine production. Each dot represents one mouse, bar graphs show mean + sd, unpaired *t*-test, ns: not significant.



Supplemental Figure 4. Confirmation of circulatory chimerism in parabiotic animals and DC status in antibiotic-treated mice.

(A) *Tslpr* wild-type allele (*Tslpr*^{WT}) PCR detection in blood and skin samples from Rag1^{KO}*Tslpr*^{KO} and Rag1^{KO} parabionts following adoptive transfer of WT CD4⁺ T cells into one animal in each parabiotic pair. WT mouse blood and skin are shown as the positive control. Note that Rag1^{KO}*Tslpr*^{KO} + Rag1^{KO}*Tslpr*^{KO} pair (#2) has *Tslpr*^{WT} cells in the blood of both parabionts but not skin. M, marker.

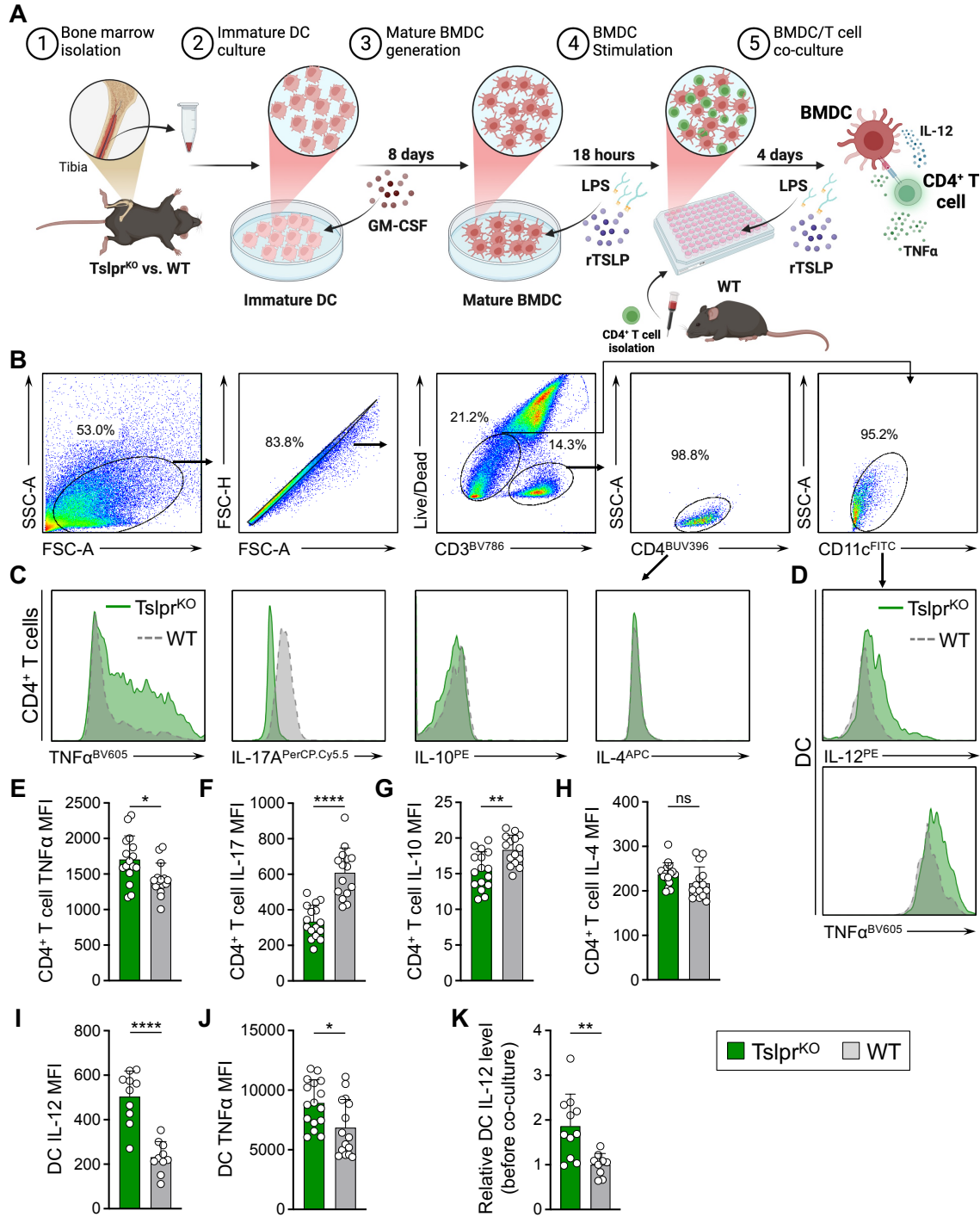
(B and C) Flow cytometric quantification of CD11c⁺ myeloid DC (B) and CD103⁺CD11c⁺ migratory DC (C) frequency in Rag1^{KO}*Tslpr*^{KO} (n = 4) and Rag1^{KO} (n = 5) MLN following antibiotics treatment and adoptive transfer of naïve WT CD4⁺CD25⁻ T cells.

(D and E) Flow cytometric quantification of CD11c⁺ myeloid DC (D) and CD103⁺CD11c⁺ migratory DC (E) frequency in Rag1^{KO}Tslpr^{KO} (n = 4) and Rag1^{KO} (n = 5) colon following antibiotics treatment and adoptive transfer of naïve WT CD4⁺CD25⁻ T cells. Rag1^{KO}Tslpr^{KO} and Rag1^{KO} tissues were harvested at day 15 post-T cell transfer.

(F and G) Flow cytometric quantification of CD11c⁺ myeloid DC (F) and CD103⁺CD11c⁺ migratory DC (G) frequency in Rag1^{KO}Tslpr^{KO} + CD4⁺ T cell and antibiotics (n = 4) versus Rag1^{KO}Tslpr^{KO} + CD4⁺ T cell (n = 5) colon.

(H and I) Flow cytometric quantification of CD11c⁺ myeloid DC (H) and CD103⁺CD11c⁺ migratory DC (I) frequency in Rag1^{KO} + CD4⁺ T cell and antibiotics (n = 5) versus Rag1^{KO} + CD4⁺ T cell (n = 5) colon.

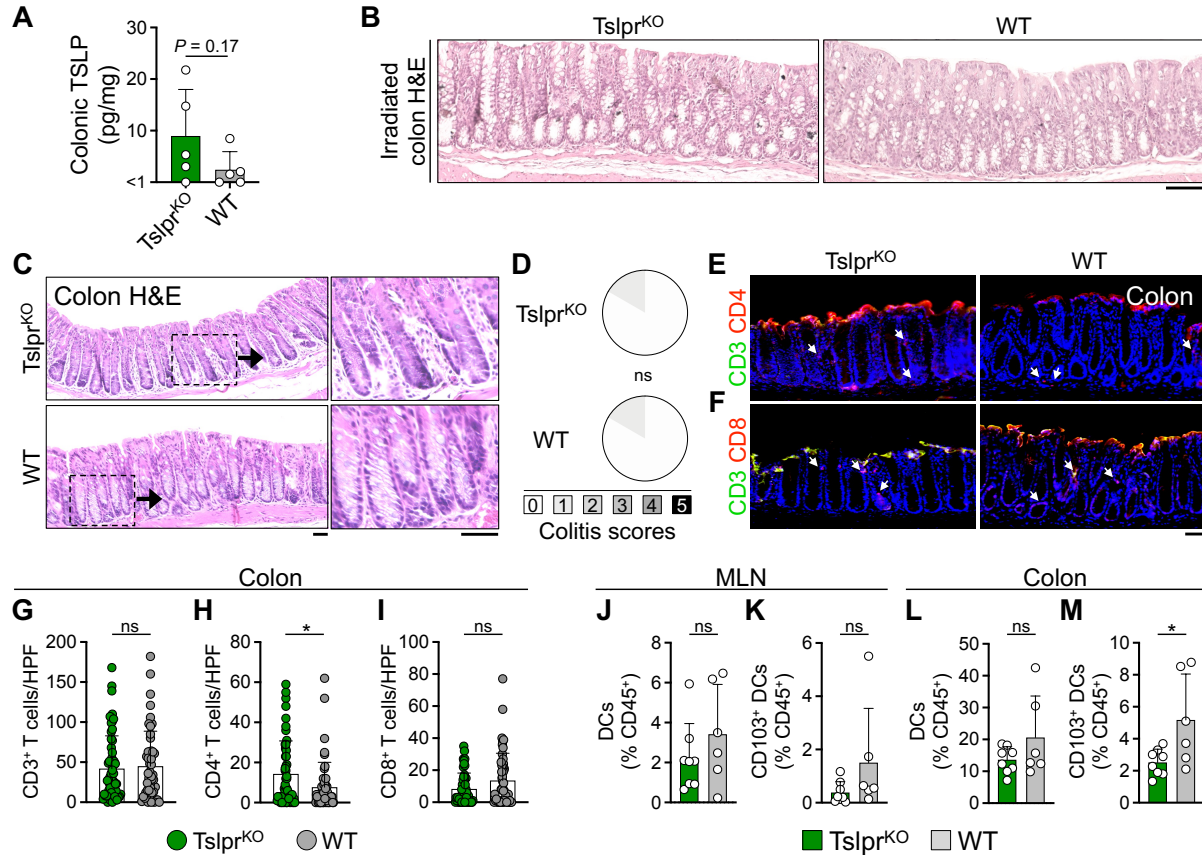
Each dot represents one mouse, bar graphs show mean + sd, unpaired *t*-test, ns: not significant, **P* < 0.05, ****P* = 0.0001.



Supplemental Figure 5. Loss of TSLPR signaling in DCs increases IL-12 expression by DCs and proinflammatory cytokine expression by CD4⁺ T cells in a co-culture assay.

(A) Schematic diagram of BMDC/T cell co-culture assay in which Tslpr^{KO} versus WT BMDC is generated, stimulated and then co-cultured with CD4⁺ T cells.

- (B)** Flow gating strategy to assess BMDCs and CD4⁺ T cells after BMDC/T cell co-culture.
- (C)** Representative flow histogram of TNF α , IL-17A, IL-10 and IL-4 expression in CD4⁺ T cells after co-culture with Tslpr^{KO} versus WT BMDCs.
- (D)** Representative flow histogram of IL-12 and TNF α expression in Tslpr^{KO} versus WT BMDCs after co-culture with WT CD4⁺ T cells.
- (E-H)** Quantification of CD4⁺ T cell TNF α (E), IL-17A (F), IL-10 (G), and IL-4 (H) MFI after co-culture with Tslpr^{KO} versus WT BMDCs.
- (I and J)** Quantification of Tslpr^{KO} versus WT BMDC IL-12 (I) and TNF α (J) MFI after co-culture with WT CD4⁺ T cells.
- (K)** Relative IL-12 levels in the media collected from Tslpr^{KO} (n = 11) versus WT (n = 9) BMDCs after rTSLP plus LPS stimulation for 18 hours before co-culture with CD4⁺ T cells. Data is combined from two independent experiments. Each dot represents media from a BMDC sample, bar graphs show mean + sd, unpaired *t*-test, *: *P* < 0.01, **: *P* < 0.01, ****: *P* < 0.0001, ns: not significant.



Supplemental Figure 6. The inflammatory environment and T cell infiltrates in Tslpr^{KO} and WT colon at the baseline.

(A) TSLP protein levels in Tslpr^{KO} and WT colon at baseline. Each dot represents one mouse.

(B) Representative H&E stains of sublethally irradiated Tslpr^{KO} (n = 5) and WT (n = 5) colon from two independent experiments at day 15 post-irradiation. Note the retention of goblet cells, the relative paucity of immune cells in a thin mucosal interstitium, normal crypt structure and depth. Scale bar, 50 μ m.

(C) Representative H&E stains of colons from Tslpr^{KO} and WT colon at baseline. Scale bars, 50 μ m.

(D) Colitis scoring of Tslpr^{KO} (n = 5) and WT (n = 5) at baseline. Fisher's exact test.

(E-F) Representative CD3/CD4- (E) and CD3/CD8-staining (F) of Tslpr^{KO} and WT colon at baseline. CD3⁺CD4⁺ and CD3⁺CD8⁺ cells are highlighted with white arrows. Scale bar, 50 μ m.

(G-I) CD3⁺ T (G), CD4⁺ T (H) and CD8⁺ T cell (I) quantification in Tslpr^{KO} (n = 5) and WT (n= 5) colon at baseline. CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ mucosal T cells were quantified in 10 randomly selected HPF images per colon at the end of the study. Each dot represents one HPF image.

(J-M) Flow cytometric quantification of DC and CD103⁺ DC frequency in MLN (J and K) and colon (L and M) of Tslpr^{KO} (n = 8) versus WT (n = 6) mice. Each dot represents one mouse.

Bar graphs show mean + sd, unpaired *t*-test, *: *P* < 0.01, ns: not significant.

Supplemental Table 1. Antibodies used in the study.

<u>Flow cytometry Antibodies</u>	<u>Conjugate</u>	<u>Clone</u>	<u>Manufacturer</u>	<u>Cat #</u>	<u>Isotype</u>	<u>RRID</u>
CD3	BV785	17A2	BioLegend	100232	Rat IgG2b, κ	AB_2562554
CD3	FITC	145-2C11	BioLegend	100306	Armenian Hamster IgG	AB_312671
CD4	APC-Cy7	RM4-5	BioLegend	100526	Rat IgG2a, κ	AB_312727
CD8 α	PE-Cy7	53-6.7	BioLegend	100722	Rat IgG2a, κ	AB_312761
CD8 α	FITC	53-6.7	Biolegend	100706	Rat IgG2a, κ	AB_312745
CD8 α	APC	53-6.7	Biolegend	100712	Rat IgG2a, κ	AB_312751
CD8 α	APC-eF780	53-6.7	eBioscience	47-0081-82	Rat / IgG2a, kappa	AB_1272185
CD8 α	PerCP-Cy5.5	53-6.7	BioLegend	100734	Rat IgG2a, κ	AB_2075238
CD19	PE	1D3/CD19	BioLegend	152408	Rat IgG2a, κ	AB_2629817
CD19	FITC	eBio1D3	eBioscience	11-0193-85	Rat / IgG2a, kappa	AB_657666
NK1.1	APC	PK136	BioLegend	108710	Mouse IgG2a, κ	AB_313397
NK1.1	FITC	PK136	BioLegend	108706	Mouse IgG2a, κ	AB_313393
CD45	PerCP-Cy5.5	30-F11	BioLegend	103131	Rat IgG2b, κ	AB_893344
CD45	BV421	30-F11	BioLegend	103133	Rat IgG2b, κ	AB_10899570
CD45	FITC	30-F11	BioLegend	103108	Rat IgG2b, κ	AB_312973
CD11c	APC-Cy7	N418	BioLegend	117324	Armenian Hamster IgG	AB_830649
CD11c	PE	N418	BioLegend	117308	Armenian Hamster IgG	AB_313777
CD103	Alexa Fluor 700	2E7	Invitrogen	56-1031-82	Armenian hamster / IgG	AB_2637111
CD11b	BUV395	M1/70	BD Bioscience	563553	Rat DA, also known as DA/HA IgG2b, κ	AB_2738276
MHCII	PE	M5/114.15.2	BD Bioscience	557000	Rat BN x LEW IgG2b, κ	AB_396546
MHCII	AF647	M5/114.15.2	BioLegend	107618	Rat IgG2b, κ	AB_493525
Foxp3	APC	FJK-16s	eBioscience	17-5773-82	Rat IgG2a, κ	AB_469457
IFN γ	Alexa Fluor 700	XMG1.2	BioLegend	505824	Rat IgG1, κ	AB_2561300
IL-17A	PerCP-Cy5.5	TC11-18H10.1	BioLegend	506919	Rat IgG1, κ	AB_961385
IL-10	PE	JES5-16E3	BioLegend	505007	Rat IgG2b, κ	AB_315361
IL-4	APC	11B11	BioLegend	504106	Rat IgG1, κ	AB_315320
IL-12	PE	C15.6	BioLegend	505204	Rat IgG1, κ	AB_315368
Ki67	Alexa Fluor 700	16A8	BioLegend	652419	Rat IgG2a, κ	AB_2564284
TNF α	BV605	MP6-XT22	BioLegend	506333	Rat IgG1, κ	AB_2562450
<u>Zombie NIR</u>	APC-Cy7	-	BioLegend	423105	-	-
<u>Immunofluorescence Antibodies</u>	<u>Conjugate</u>	<u>Clone</u>	<u>Manufacturer</u>	<u>Cat #</u>	<u>Isotype</u>	<u>RRID</u>
CD3	Purified	CD3-12	Abcam	Ab11089	Rat IgG1	AB_369097

CD4	Purified	EPR19514	Abcam	AB183685	Rabbit IgG	AB_2686917
CD8	Purified	D4W2Z	Cell Signaling Technologies	98941	Rabbit IgG	AB_2756376
TSLP	Purified	73AD11	Merck	GNE01.12F 3.B5.4011	Rat IgG2a	
Cytokeratin	Purified	AE1/AE3	Dako	M3515	IgG1 kappa	
Myeloperoxidase (MPO)	Purified	EPR20257	Abcam	ab208670	Rabbit IgG	
<u>Blocking Antibodies</u>	<u>Conjugate</u>	<u>Clone</u>	<u>Manufacturer</u>	<u>Cat #</u>	<u>Isotype</u>	<u>RRID</u>
PD1	Purified	29F.1A12	BioXCell	BE0273		
CTLA-4	Purified	9D9	BioXCell	BE0164		

Supplemental Table 2. Colon inflammation (colitis) scoring.

Minimal	Mucosa	Minimal hyperplasia		1
Mild	Mucosa, some submucosa	Mild hyperplasia, minimal goblet cell loss +/- erosions		2
Moderate	Mucosa and submucosa	Moderate hyperplasia +/- few crypt abscesses, moderate goblet cell loss +/- erosions		3
Marked	Mucosa and submucosa	Marked hyperplasia +/- several crypt abscesses and or erosions	+/- Irregular crypts or crypt loss +/- ulcerations	4
Marked	Transmural	Marked hyperplasia +/- multiple crypt abscesses	+/- Irregular crypts or crypt loss +/- ulcerations	5

Reference:

1. Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D, Heimesaat MM, et al. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol.* 2014;7(8):4557-76.