Supplemental Information for:

Synchronization of Mothers and Offspring Promotes Tolerance and Limits Allergy

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Supplementary Materials:

Supplementary Methods:

Figure S1: Kinetics of GAP formation in the small intestine and colon through early life

Figure S2: Naive CD4+ T cells populate the colonic and SI LP and are followed by the presence of a Helios- RORγt+ Treg population

Figure S3: Expression of transcription factors by Foxp3+ CD4+ T cells in multiple tissues

Figure S4: In early life, SI and colon LP-APCs do not have an intrinsic defect in antigen presentation.

Fig S5: The weaning reaction to the gut microbiota is not observed in mice from multiple vivaria.

Figure S6: Transcription factor expression in colon LP T cells.

Figure S7: Altering EGF signals preweaning affects the development of colonic LP T cells and tolerance to a dietary antigen

Figure S8: Asynchronous cross foster results in Th2 skewing of immune responses

Table SI: Antibodies and staining reagents

Supplementary Methods:

Mice Supplemental information: To deplete GCs, Math1$^{f/f}$vil-Cre-ERT2 mice were injected with 5mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) dissolved in sunflower seed oil with 20% ethanol (Sigma-Aldrich) i.p. daily for 4 days. To label Foxp3 cells for YFP expression, Foxp3^{GFPCreERT2}Rosa^{lslYFP} mice were injected with tamoxifen on DOL24. Asynchronously CF mice were obtained by placing DOL1 mice with mothers having delivered a litter 14 days prior (DOL1 ACF), and DOL14 mice with mothers having delivered a litter one day prior (DOL14 ACF). Mice were synchronously CF by placing DOL1 mice with different mothers having delivered one day prior (SCF). At time of cross-fostering the mother's nose, pups, and bedding were swabbed with vanilla to prevent pup rejection. To manipulate GAPs in early life conventionally reared mice were injected with 500µg/kg tyrphostin AG1478 (inhibitor of EGFR phosphorylation) on DOL6 and 8, or administered 10µg EGF by gavage daily from DOL10-21.

Isolation of cellular populations and flow cytometry: Small intestines or colons were harvested, rinsed with PBS, and Peyer's patches or colonic patches were removed. Isolation of splenic and LP cellular populations was performed as previously described (75). Antibodies used for analysis are listed in supplemental Table S1. SI and colonic APCs were identified as 7AAD$^-$, CD45$^+$, CD11c$^+$, MHCII$^+$. Treg subpopulations were identified as 7AAD$^-$, CD45$^+$, CD3$^+$, CD4$^+$, Foxp3$^+$, and either RORγt$^+$ or Helios$^+$. To detect intracellular antigens and cytokines, cells were fixed and permeabilized overnight and stained per the manufacturer's recommendations (eBioscience). Flow cytometry was performed with a FACScan cytometer (BD Biosciences, San Jose, CA) retrofitted with additional lasers, or an Attune NXT four-laser flow cytometer (Invitrogen). Data acquisition and analysis was performed using Attune NXT software and FlowJo software (Tree Star, Ashland, OR). viSNE analysis performed with FlowJo software and Cytobank software.

GAP identification using intravital two-photon (2P) microscopy and fixed tissue sections: Intravital preparation of the intestine was performed as previously described (76). To detect GAPs in mice during early life, tetramethylrodamine labeled dextran 10,000 MW (10mg/mL) or fluorescein labeled dextran 10,000 MW (10 mg/mL) and diamidino-2-phenylindole (DAPI; 10mg/mL) were injected intraluminally 10 minutes prior to imaging and imaging was performed for up to one hour. Tissues were excited using a Ti:sapphire laser tuned to 890nm (Chameleon XR, Coherent). Time-lapse imaging was performed with a custom-built 2P microscope running ImageWarp acquisition software (A&B Software, New London, CT). Following imaging, tissues were placed in 10% formalin buffered phosphate solution (Fisher Scientific) to fix dextrans in place to confirm findings by immunofluorescence microscopy. Alternatively, to identify GAPs using widefield fluorescent microscopy on fixed tissue sections, mice were anesthetized with i.p. ketamine (120 mg/kg) and xylazine (10 mg/kg) and tetramethylrodamine labeled dextran 10,000 MW (10mg/mL) or fluorescein labeled dextran 10,000 MW (10 mg/mL) were injected into the gut lumen. After 30 minutes, tissues were excised, placed in 10% formalin buffered phosphate solution (Fisher Scientific), and embedded in Optimal Cutting Temperature compound (OCT). 7 um sections were cut on a cryostat (Leica) and counterstained with DAPI. Pseudo-colored black and white images from fluorescent microscopy were obtained with an axioskop 2 microscope using Axiovision software (Carl Zeiss, Thornwood, NY).

Assessment of an inflammatory reaction in the terminal ileum: Terminal ileum of day of life 7 and 21 mice were flash frozen. Frozen samples were resuspended in Trizol (Ambion) and homogenized with hand-held homogenizer. RNA was extracted following manufacturer instructions. The extracted RNA was column purified with a RNeasy Mini Kit (Qiagen). 100ng of RNA per sample were combined with SYBR Green mastermix (Bio-Rad), Superscript II reverse transcriptase (Invitrogen) and RNaseOUT (Invitrogen) and used on an ABI 7500 (Applied

Biosystems) for one-step qRT-PCR. Each sample was reverse transcribed and amplified in duplicates for the following genes IFN-γ, TNF-α, and GAPDH with the following conditions 30min at 42°C, 10 min at 95°C, 40 cycles of 15s 95°C, 1min 60°C. Primers were designed to span exon junctions to prevent amplification of residual genomic DNA. Primers are listed in supplemental Table S1 Changes in gene transcription were calculated as ΔΔCT compared to 7 days of life.

Stool protein isolation: Stool specimens were couriered to the laboratory in insulated envelopes containing frozen packs and stored at −80°C until analyzed. Specimens of stool produced on weekends and in the evenings were stored temporarily at −20°C until the next opportunity to transport them on the morning of the next working day. Stool samples were disrupted using a Bullet Blender Tissue Homogenizer (Next Advance) after addition of 500 ul buffer RLT (Qiagen) and 1 mg of 0.1mm zirconium oxide beads. Protein was isolated from lysates using an AllPrep DNA/RNA/protein minikit per the manufacturer's recommendations (Qiagen) and dissolved in PBS after isolation.

Supplemental Data:

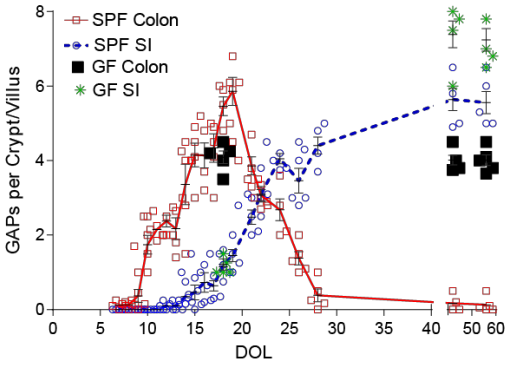

Figure S1*: Kinetics of GAP formation in the small intestine and colon through early life*. Density of GAPs per colonic crypt or SI villus in early life as assessed by 2P imaging in specific pathogen free housed (SPF) or germfree housed (GF) mice. Data are presented as individual data points with the mean +/- SEM, * = $p<0.05$, n= 4 or more mice per timepoint analyzed, offspring from 3 SPF housed litters and 2 germfree housed litters were used in a.

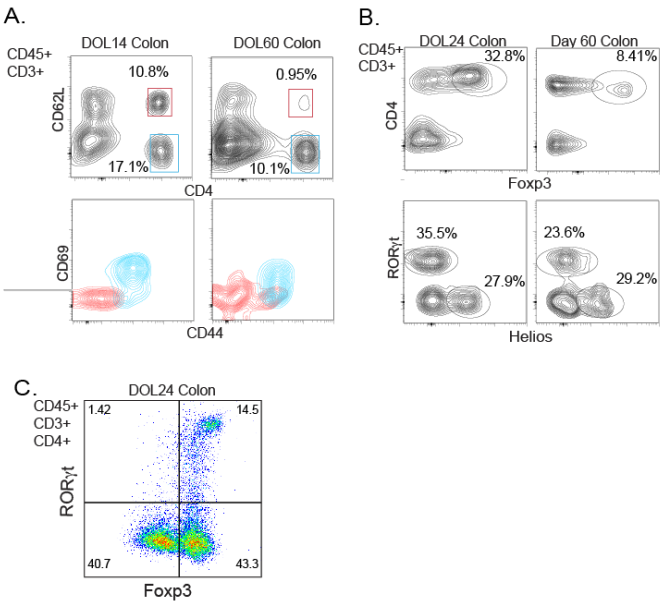

Figure S2: *Naive T cells populate the colonic and SI LP and are followed by the presence of a Helios- RORγt+ CD4+ Treg population*. A) Flow cytometry plots of colonic LP shows CD62L+CD69- CD44- naive CD4 T cells are present at DOL14 and become rare in adulthood. B) DOL24 colon contains a prominent population of CD4+FoxP3+ Tregs that are Helios- and RORγt+, which persists but becomes proportionally smaller in adulthood. C) Flow cytometry plot of colonic LP shows the overwhelming majority of RORγt+ cells are FoxP3+ on DOL24. Flow plots are representative of n= 4 mice per group, offspring from 2 litters per timepoint was used in A-C.

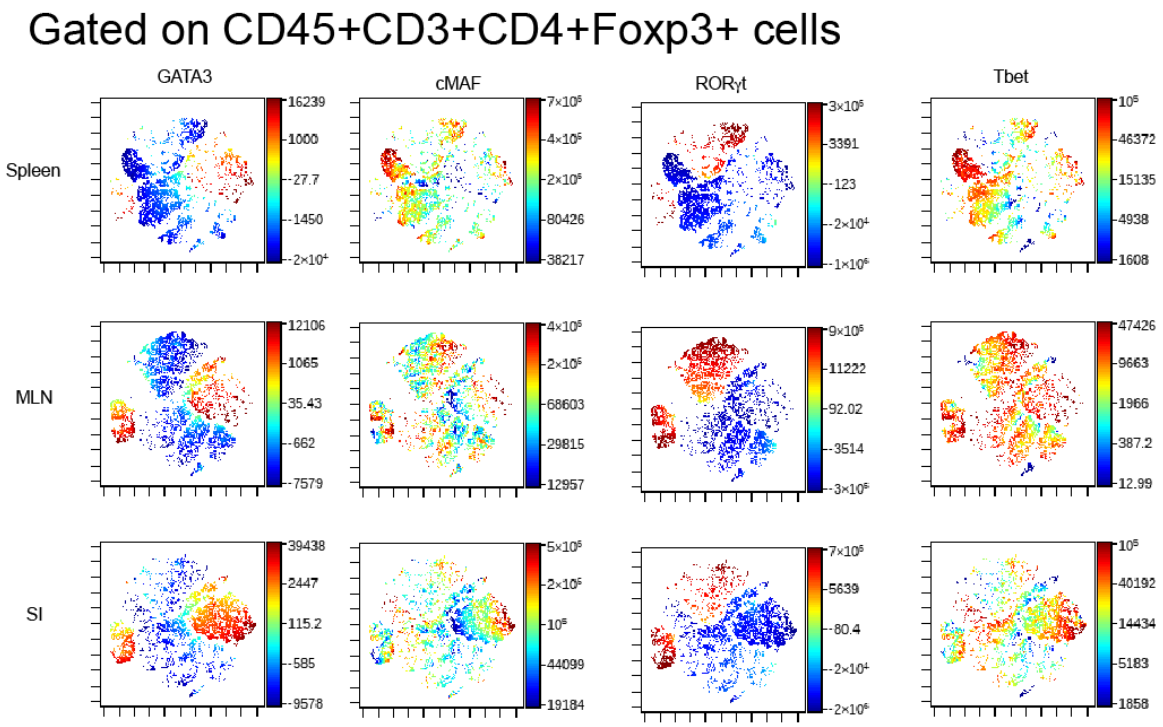

Figure S3: *Transcription factor expression in FoxP3+ CD4+ T cells of the spleen, mesenteric lymph node, and small intestine.* Representative viSNE plots of the expression of GATA3, c-MAF, RORγt, and T-bet by CD45+ CD3+ FoxP3+(GFP+) T cells from the spleen, mesenteric lymph node (MLN) and small intestine (SI) of FoxP3-GFP mice used in Figure 2. n = 4 or more mice from 3 litters.

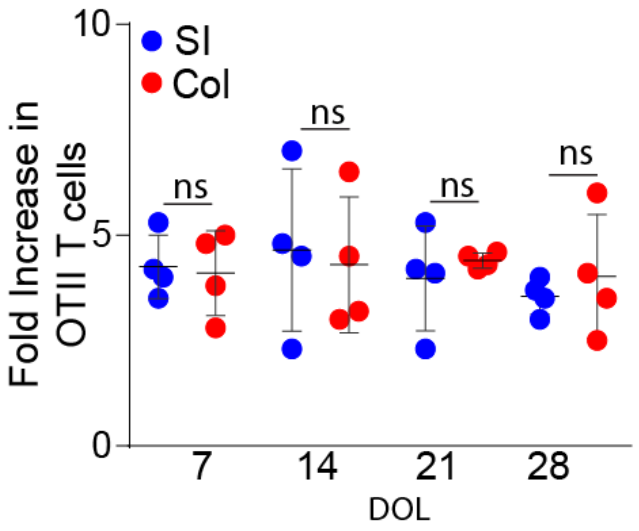

Figure S4: *In early life, SI and colon LP-APCs do not have an intrinsic defect in antigen presentation*. Antigen presentation capacity of SI or colon CD11c+MHCII+ APCs isolated from the LP of DOL7, DOL14, DOL21, or DOL28 mice when exogenous Ova (1 μg/ml) was added directly to the culture as assessed by the increase in Ova specific OTII T-cells after 72 hours of culture. Data are presented as individual data points with the mean +/- SEM, ns = not significant, APCs isolated from 4 mice per group, from two litters, wells ran in duplicate. Significance calculated using student's *t* test.

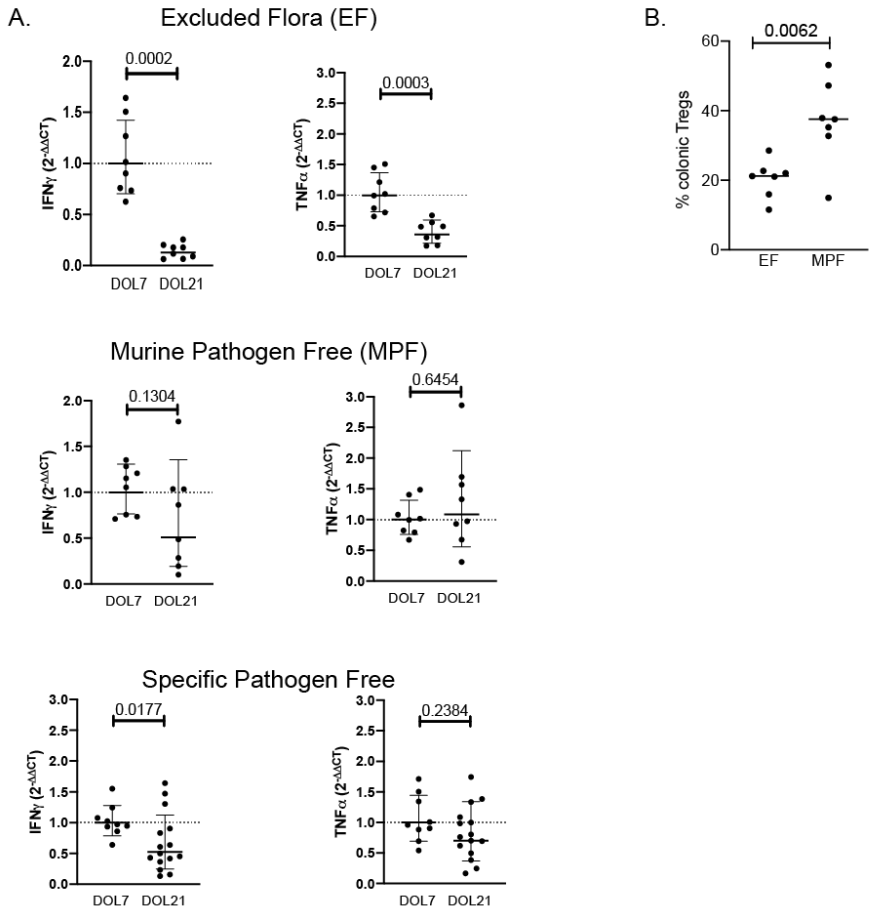

Fig S5: *The weaning reaction to the gut microbiota is not observed in mice from multiple vivaria.* A) Real time PCR was performed on RNA isolated from the terminal ileum of DOL7 and DOL21 C57BL/6 mice with an excluded gut microbial flora (free of overt rodent pathogens and select opportunistic pathogens as well as Segmented Filamentous Bacteria), mice with a murine pathogen free flora (free of overt rodent pathogens), or in house specific pathogen free mice. None of the mice from the different vivaria displayed a significant elevation in TNFα or IFNγ at the time of weaning. B) Murine pathogen free (MPF) mice had a significantly larger population of colonic Tregs when compared with excluded flora (EF) mice with a less rich gut microbial community. * = P<0.05, data presented as the geometric mean +/- SEM in panel A and the mean +/- SEM in panel B. Each dot represents analysis of one mouse, n= at least 7 mice per group, offspring from 2 or more litters. Significance calculated using student's *t* test in A and B.

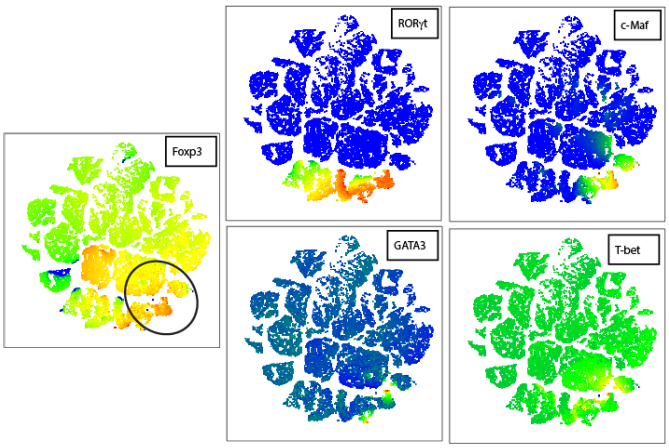

Figure S6: *Transcription factor expression in colon LP T cells.* Representative viSNE plots of the expression of RORγt, c-Maf, Foxp3, GATA3, and T-bet by colon LP (CD45+ CD3+) T cells. Clustering is performed and shown using colon LP T cells from SCF mice, DOL1 ACF mice, DOL14 ACF mice corresponding to viSNE plots in Figure 4d. Ellipse denotes populations that are largely absent in ACF mice.

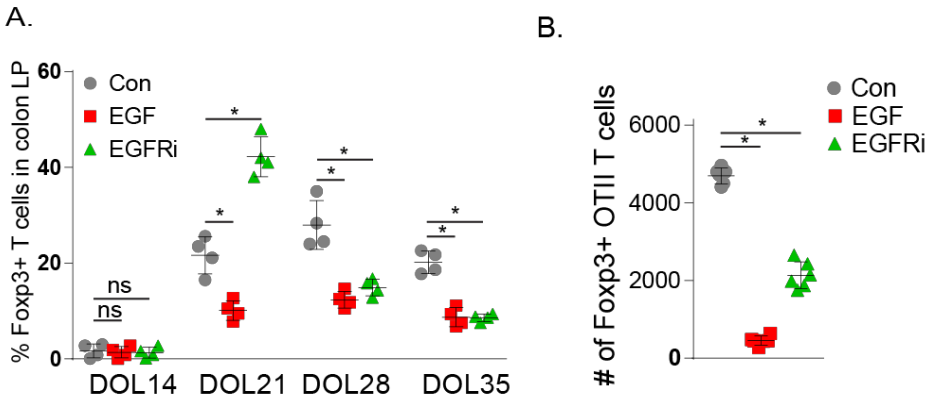

Figure S7: *Altering EGF signals preweaning affects the development of colonic LP T cells and tolerance to a dietary antigen.* A) Percentage of CD45+CD3+CD4+ T cells that are Foxp3+ in the colon lamina propria throughout early life following gavage with EGF (DOL10-21) or i.p. injection with EGFRi (DOL4,6). B) Absolute number of Foxp3+ Ova specific OTII T cells 7 days following transfer of OTIIs into mice on DOL16 treated as in a and given dietary Ova on DOL14-21. Data are presented as the mean +/- SEM, * = $p<0.05$, n= 4 mice or more per group in A and B, offspring from 2 litters per condition and/or timepoint was used in A and B. Significance calculated using one way ANOVA with a Dunnett's post-test in A and B.

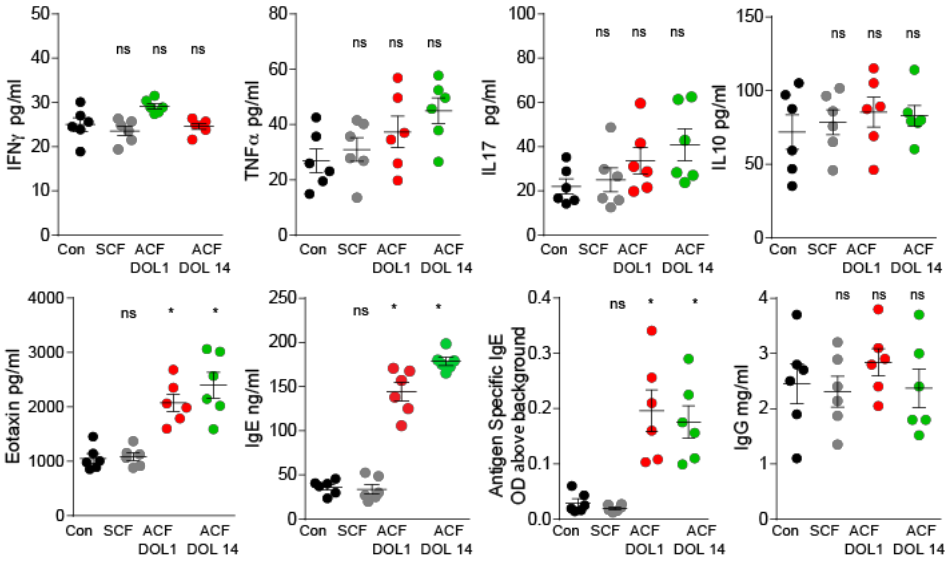

Figure S8: *Asynchronous cross foster results in Th2 skewing of immune responses.* Concentrations of cytokines and antibodies measured by ELISA in the serum of DOL 84 mice. SCF and ACF mice correspond to treatment groups as in Fig 5. Data are presented as the mean +/- SEM, * = $p<0.05$, ns = not significant**,** n= 5 mice or more per group, offspring from 3 litters per condition were analyzed. Significance calculated using one way ANOVA with a Dunnett's post-test.

Table SI: Antibodies, staining reagents, and primers

Reagent	Clone	Manufacturer
Dextran Tetramethylrhodamine		Invitrogen
DAPI (4',6-Diamidino-2-phenylindole, Dihydrochloride)		Invitrogen
7AAD		Invitrogen
Ovalbumin Alexa Fluor 647		Invitrogen
anti-CD45	30-F11	eBioscience
anti-CD45.1	A20	eBioscience
anti-MHCII	NIMR-4	eBioscience
anti-CD11c	N418	eBioscience
anti-Vβ5	MR9-4	eBioscience
anti-Vα2	B20.1	eBioscience
anti-CD62L	MEL-14	BD Pharmingen
anti-CD69	H1.2F3	eBioscience
anti-IL-13	eBio13A	eBioscience
anti-Foxp3	FJK-16s	eBioscience
anti- Helios	22F6	eBioscience
anti-RoRγt	B2D	eBioscience
anti-CD4	GK 1.5	eBioscience
Anti-cMAF	Sym0F1	eBioscience
Anti-GATA3	TWAJ	eBioscience
Anti-T-bet	eBio4B10	eBioscience
INFγ	mINFγ-F	5'-AGGAACTGGCAAAAGGATGGTGAC-3'
	mINFγ-R	5'-TGGGTTGTTGACCTCAAACTTGGC-3'
TNFα	mTNFα-F	5'-AGGGATGAGAAGTTCCCAAATGGC-3'
	mTNFα-R	5'-TGGTGGTTTGCGTGGGCTAC-3'
GAPDH	mGAPDH-F	5'-ACAGAGGACCAGGTGGTCTCCAC-3'
	mGAPDH-R	5'-TCGTTGTCGTACCAGGCAACAAGC-3'