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Supplementary Materials and Methods:

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. Epithelial cellular populations were released by incubating for 15 min three times in a 37°C rotating incubator in HBSS media (BioWhittaker, Walkersville, Maryland) containing 5 mM EDTA as previously described (*75*). Isolation of splenic, MLN and lamina propria (LP) cellular populations was performed as previously described (*75*). In some experiments, single cell suspensions of MLN were cultured in cell culture media for three days as previously described(*41*). The first wash of HBSS+EDTA was used to isolate goblet cells as below. Antibodies used for analysis are listed in supplemental table S1. SI and colonic LP-MNPs were identified as $7AAD^-$, $CD45^+$, $CD11c^+$, $MHCII^+$ for flow cytometric sorting. Goblet cells were identified as $CD45^-$, $CD24^-$ $CK18^+$, $UEA\text{-}I^+$ for flow cytometric sorting as previously described (*39*). For intracellular antigens and cytokines, cells were fixed and permeabilized overnight and stained per the manufacturers recommendations (eBioscience). Flow cytometry was performed with a FACScan cytometer (BD Biosciences, San Jose, CA) retrofitted with additional lasers. Data acquisition was performed using CellQuest (BD Biosciences) and Rainbow (Cytek,Fremont, CA) or FlowJo software (Tree Star, Ashland, OR). Data analysis was performed on a Macintosh computer running FlowJo software.

Analysis of luminal fluorescent Ova delivery to LP-DCs: 200 µg of Ova conjugated Alexafluor-647 (Molecular Probes) was injected in the SI on colonic lumen and two hours later cell populations were isolated from the SI and colonic LP and analyzed by flow cytometry.

Immunohistochemistry: Immunohistochemistry was performed as previously described(*38*). Antibodies used for immunohistochemistry are listed in supplemental table S1. Pseudo-colored black and white images from fluorescent microscopy were obtained with an axioskop 2 microscope using Axiovision software (Carl Zeiss, Thornwood, NY).

Paracellular leak assay: To detect paracellular leak fluorescein labeled dextran 4,000 MW (50 mg/mL) was gavaged into mice on DOL 18. Four hours later serum was collected and fluorescence was measured on a spectrometer.

Intravital two-photon (2P) microscopy: Mice were anesthetized using nebulized isofluorane in 95% O_2/5% CO_2. Intravital preparation of the intestine was performed as previously described (*38*). To detect GAPs, 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 20 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). Epithelial integrity was assessed by dextran and DAPI staining as previously described (*38*). Following imaging, tissues were placed in 10% formalin buffered phosphate solution (Fisher Scientific) to fix dextrans in place to confirm 2P findings and for further analysis by immunofluorescence microscopy.

Enumeration of GAPs: GAPs were identified using in vivo two-photon imaging and imaging of fixed tissue sections and enumerated as previously described (*38, 39*). The number of GAPs per GCs was quantified by immunofluorescence microscopy of tissues that were fixed following 2P imaging and stained with CK18 (abcam).

Measurement of cytokines, EGF, and immunoglobulins: Cytokines and EGF were measured using the using ELISAs for EGF (R&D systems), phosphorylated MAPK, phosphorylated EGFR,

IL17 (eBioscience), IFNγ and IL-10, (R&D systems) per the manufacturers recommendations. Luminal EGF was measured in stomach, SI, and colonic contents; to isolate contents the stomach was opened and contents was added to 1 ml PBS. For SI and colonic contents, 1 ml of PBS was injected to one open end of the SI or colon using a 24 gauge gavage needle. Contents was then collected in a tube from the other open end of the SI or colon. Phosphorylated MAPK, and phosphorylated EGFR was measured on epithelial cells from the SI or colon isolated by one 15 minute wash with HBSS+EDTA as described above. Cytokines were measured on supernatants from cultured MLNs.

Quantitative real time polymerase chain reaction assay: GCs, identified as $7AAD^{-}$ $CD45^{-}$ $CD24^{-}$ Cytokeratin 18^{+} UEA-I^{+} and IECs identified as $7AAD^{-}$ $CD45^{-}$ $CD24^{-}$ Cytokeratin 18^{-} UEA-I^{-} were flow cytometrically sorted from the epithelial fraction of intestines directly into RLT buffer (Qiagen) for RNA extraction. RNA was extracted from epithelial cellular populations, treated with DNAse, and transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Primers used for RT-PCR are listed in supplemental table S2. The absolute copy number of the target was calculated from standards that were constructed as previously described (*76*).

Treatments to alter GAPs: Acute induction or inhibition of GAPs was performed as previously described (*39*). Mice were given 500µg/kg tyrphostin AG1478 i.p. to inhibit of EGFR activation, 10mg/kg U0126 i.p. to inhibit MAPK/ERK (MEK1/MEK2) activation, 150µg/kg carbamylcholine (acetycholine analogue) i.p., 100mg/kg tropicamide (mAChR4 selective antagonist) i.p., 1 µg recombinant murine EGF (Shenandoah) in 20 µl PBS intracolonic, 10µg LPS intraluminally, or 100µl stomach contents from a DOL10 mice intraluminally, or 100µl heat killed cecal contents

from SPF housed adult mice intraluminally. Reagents from Sigma Aldrich, St. Louis, MO unless otherwise noted.

LP-MNP/T cell co-culture: $1x10^5$ flow cytometrically sorted splenic CD3+CD4+Vβ8.3+ CBir1 T cells or $1x10^5$ flow cytometrically sorted splenic $CD3^+CD4^+V\alpha2^+V\beta5^+$ OTI T cells were cultured with $1x1x10^5$ CD45+ MHCII+ CD11c+ flow cytometrically sorted SI or colonic LP-MNPs isolated from mice two hours following intraluminal administration of 2 mg Ova or PBS. Some wells were cultured with 10 µg Ova or 10µg CBir1 Flagellin as a positive control. After three days, cultured cells were counted and analyzed by flow cytometry to determine absolute number of transgenic cells to calculate fold increase of T cells.

Adoptive T cell Transfer: 10^5 splenic $CD4^+$ T cells from CD45.1 CBir1 TCR transgenic mice, CD45.1+ DP1 TCR transgenic mice, or CD45.1+ OTII TCR transgenic mouse were enriched by magnetic particles (Stemcell Technology, #19752 Vancouver, British Columbia, Canada) and injected i.v. into mice. In some experiments cells were labeled with CFSE prior to injection, and MLNs of recipient mice were analyzed three days posts transfer. Mice were analyzed seven days posts transfer to evaluate the presence of the transferred population in the LP. In some experiments, in figures 5-7, cells were transferred on DOL 16, and analyzed on DOL 30 or after 8 days of DSS treatment on DOL 38.

DSS colitis: Mice were given DSS (36,000-50,000 MW, Reagent grade, MP Biomedicals) in drinking water ad libitum for eight days. $Math1^{fl/fl}$ $ERT2^{ViCre}$ mice and litter mates were given 1.5% DSS due to their increased susceptibility to disease(*41*). Weight was measured daily. On the final day, colons were measured for length to assess shortening, and were fixed in buffered formalin and embedded in paraffin for sectioning. H/E sections were scored for disease as before(*41*). In some experiments, colon LP was isolated on the final day of DSS treatment to analyze endogenous T cell and transferred CBir1 T cell populations.

Bacterial DNA extraction, 16s RNA Quantification, and sequencing: Colonic cecal contents were placed in 750µl lysis buffer (200mM NaCl, 100mM Tris, 20mM ETA, pH 8.0) with 200mg 0.1 mm diameter ziconia silica beads (BioSpec, Bartlesville, OK), and vortexed on bead beater (MP Biomedicals, Fast prep 24) and DNA was isolated using the All prep DNA/RNA extraction kit (cat # 80204, Qiagen, Valencia, CA). Quantification of the copies of 16s was performed using real time PCR and a standard curve, using the primers for total bacteria 5′-GGTGAATACGTTCCCGG-3′ and 5′-TACGGCTACCTTGTTACGACTT-3′(*18*), the CBir1 flagellin epitope 5′-GCTGACACAGGAAATCGATCGT-3′ and 5′-GAGAGTATACATCACCCGTCGCAT-3′ and B. Vulgatus 5′-AAGGGAGCGTAGATGGATG-3′ and 5′-CGAGCCTCAATGTCAGTTGC-3′(*77*). Metagenomic analysis of the bacterial communities using 16s rRNA sequencing was performed and analyzed as previously described(*41*).

Supplementary Figures:

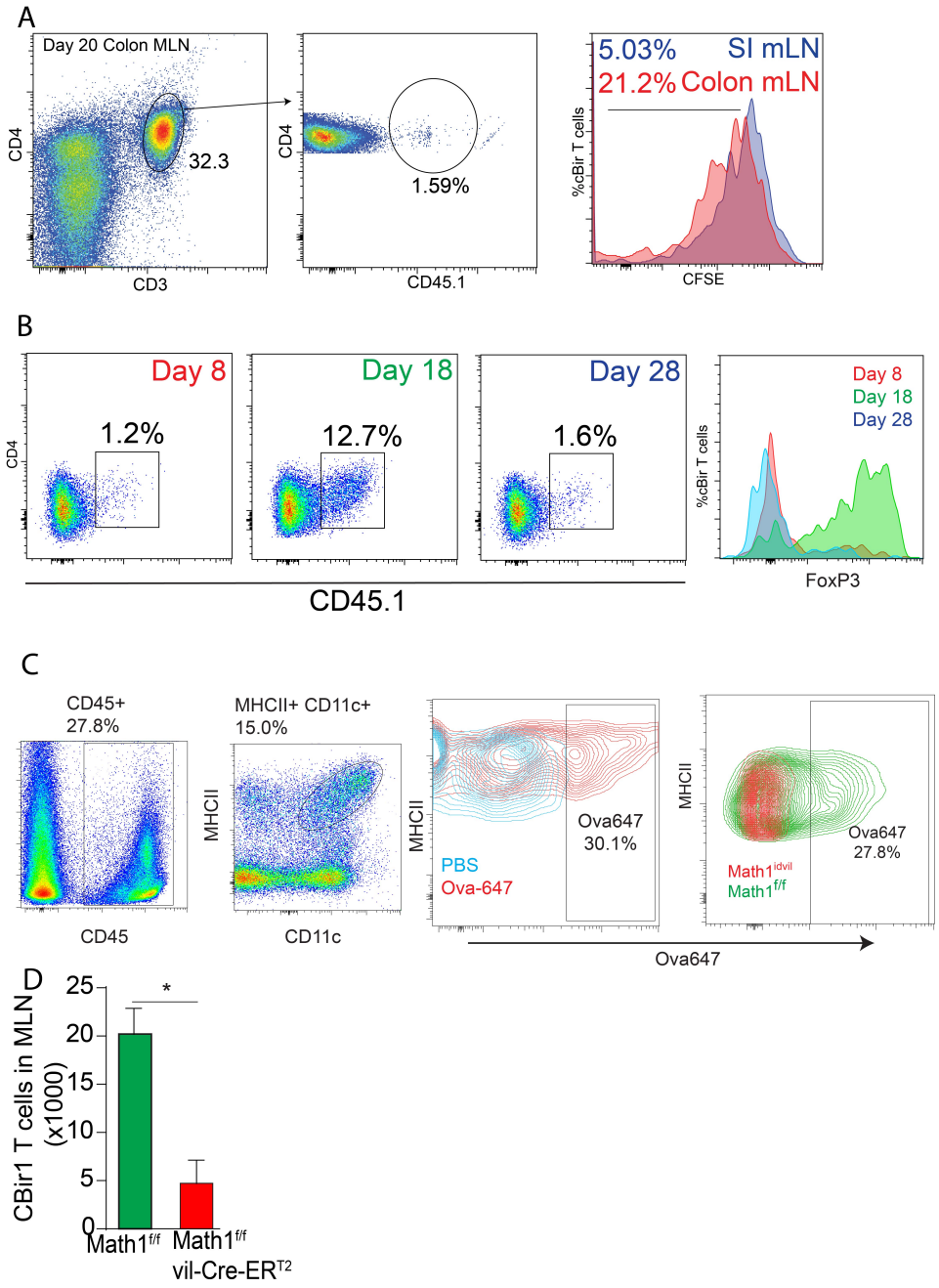

Fig. S1: *Representative flow cytometry plots related to Figure 1.* A) Representative flow plots identifying transferred and proliferating CBir1 T cells in the colon draining MLN in DOL20 mice. B) representative flow cytometry plots identifying Foxp3 expression by transferred CBir1 T cells

in the colon LP on DOL8, 18, and 28. C) Representative flow cytometry plots for identifying LP-MNPs (CD45+MHCII+CD11c+) containing Ova-647 post intraluminal administration. D) Absolute number of CD45.1 CBir T cells in the colon draining MLN three days following transfer. *= $p > 0.05$, n = 4 for panel D.

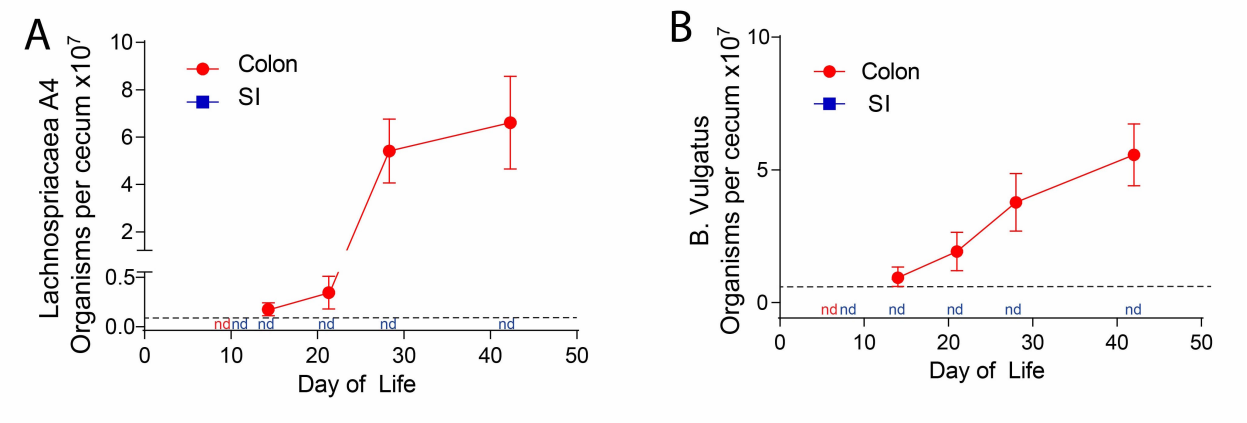

Fig. S2: *CBir1 epitope producing bacteria and B. vulgatus in the small intestinal and colonic contents by DOL.* A) Primers specific for the CBir1 flagellin epitope produced by the *Lachnospiracea* bacteria A4 and *COE1* and B) primers specific for *B. vulgatus* were used to quantitate the number of organisms by performing quantitative real time PCR on DNA isolated from colonic contents. n = 3 for each time point.

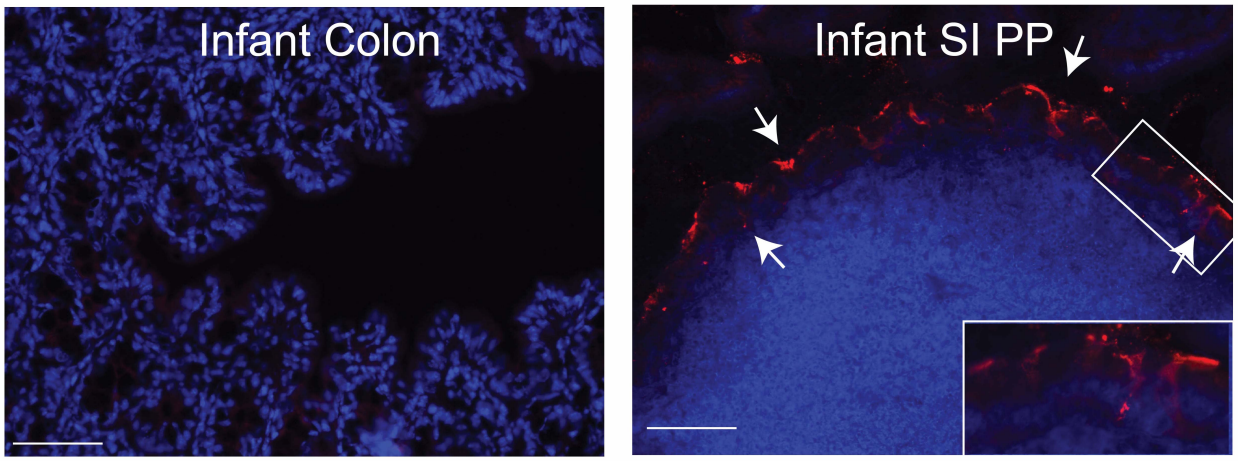

Fig. S3: *M cells are not present on the non-follicle bearing epithelium in the colon in early life.* Immunofluorescent staining for GP2 (red) revealed that M cells are not seen in the colonic epithelium (left panel) but were present in the follicle associated epithelium overlying Peyer's patches (PP) in the SI (right panel) of DOL19 mice. Blue = DAPI nuclear stain, scale bar =50 μm.

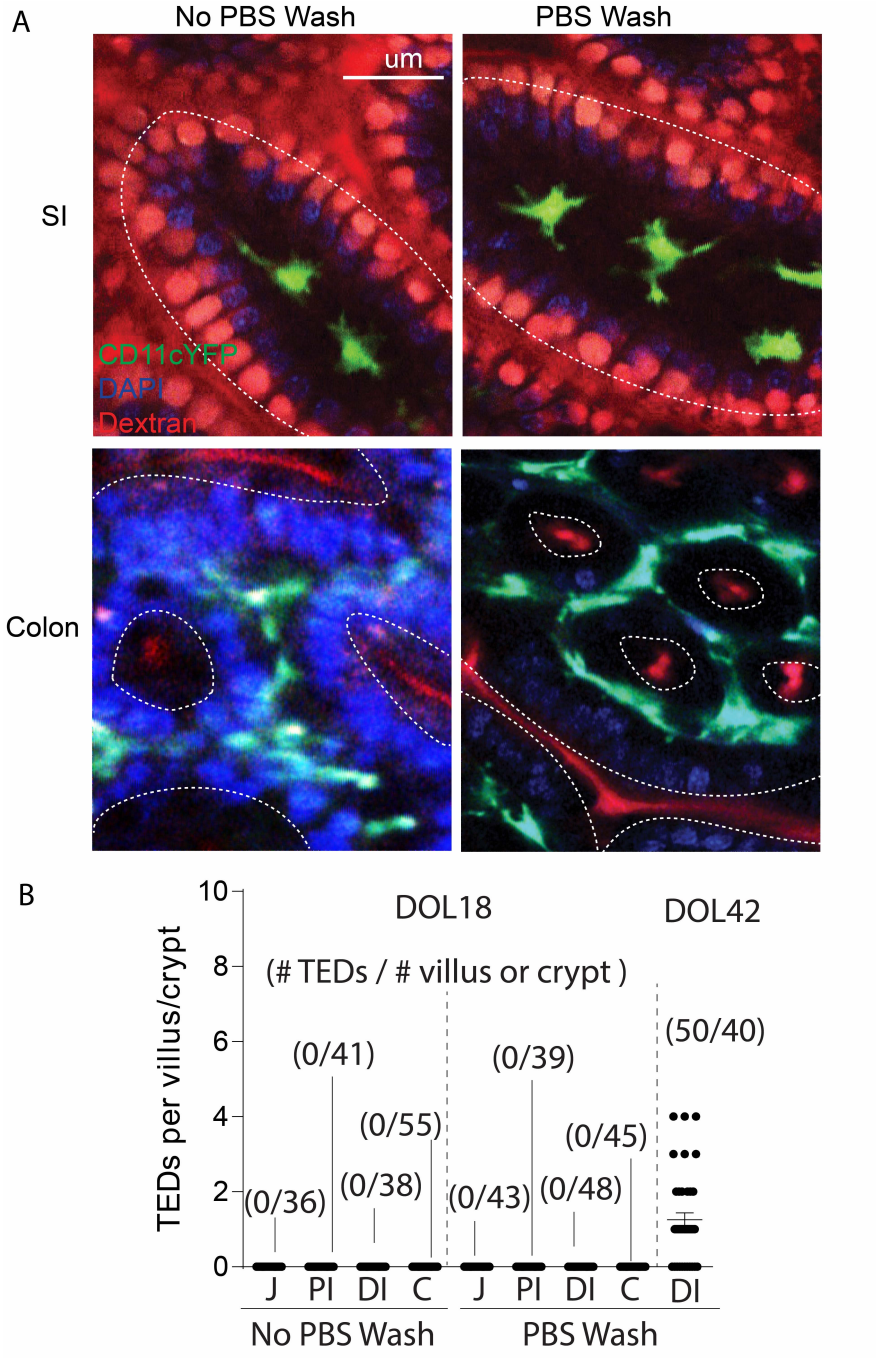

Fig. S4: *The extension of trans-epithelial dendrites by LP-MNPs is rare in the intestine of pre-weaning mice.* A) Two-photon imaging for trans-epithelial dendrites in the intestine imaging of SI or colon of DOL18 CD11c^{YFP} mice 20 minutes after administration of fluorescent dextran (red) to

delineate the lumen and DAPI (blue) to stain epithelial nuclei. Dotted line indicates luminal surface. Vacuolated fetal enterocytes in the SI take up fluorescent dextran top panel. B) Number of trans-epithelial dendrites (TEDs) per small intestinal villus or colonic crypt in the jejunum (J), proximal ileum (PI), distal ileum (DI), or colon (C) measured by two-photon imaging in a DOL18 and DOL42 mice. DOL18 mice were evaluated in the presence and absence of removal of the mucous barrier by washing with PBS. No TEDs were seen in the SI or colon of DOL18 mice, however consistent with prior reports, TEDs were observed in the distal ileum of DOL42 mice following removal of mucus by washing with PBS. Scale bar =50 μm.

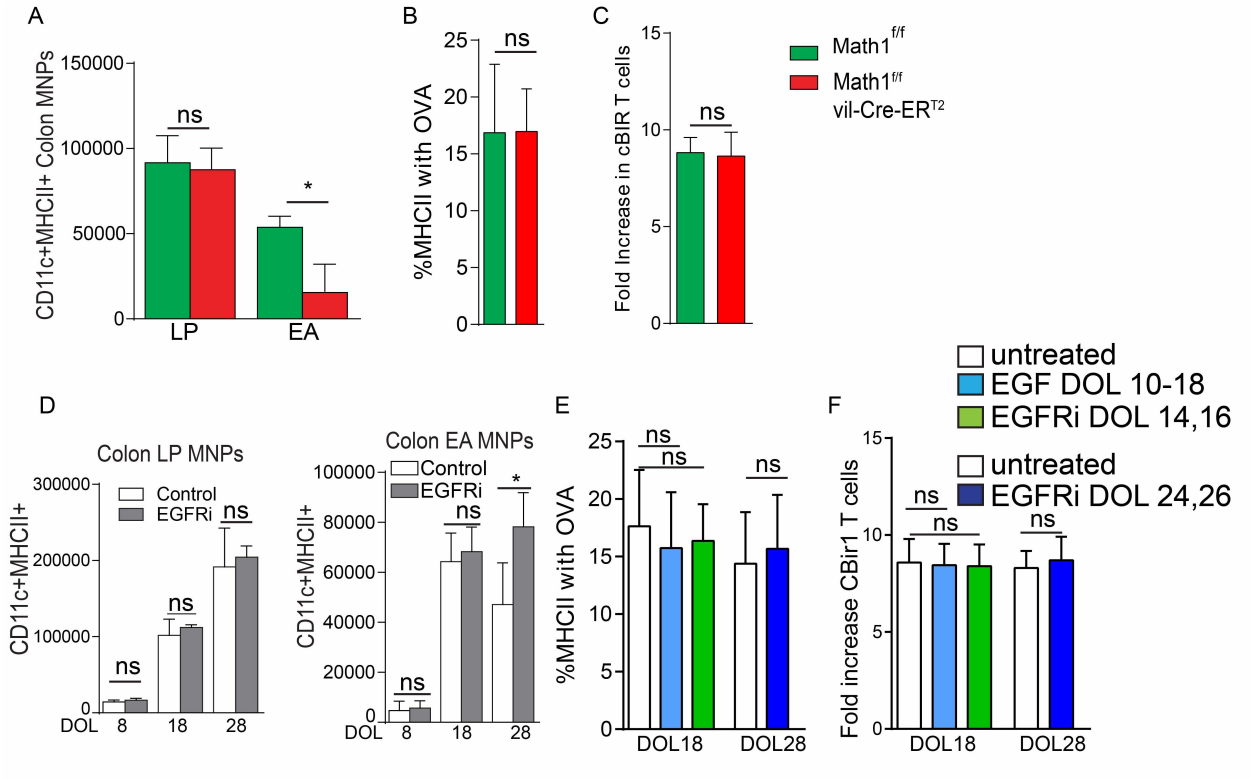

Fig. S5: *GAP manipulations in early life do not affect antigen presenting capacity of colonic lamina propria MNP.* A) Absolute number of CD11c+MHCII+ cells in the colon LP or epithelial fraction (EA) of GC knockout mice or littermate controls. B) Absolute number of CD11c+MHCII+ cells in the colon LP (left) or epithelial fraction (EA) (right) on DOL 8, 18, or 28 with or without EGFRi treatment. C) Percent of CD11c+MHCII+ cells containing fluorescently labled Ova-647 following IV administration on DOL 18 or 28, following GAP manipulation. D) Fold increase in CBir T cells following in vitro culture with LP-MNP cells and 10 ug flagellin. n=4 mice per group panels A and D, n = 3 mice per group panels B, C, E and F.

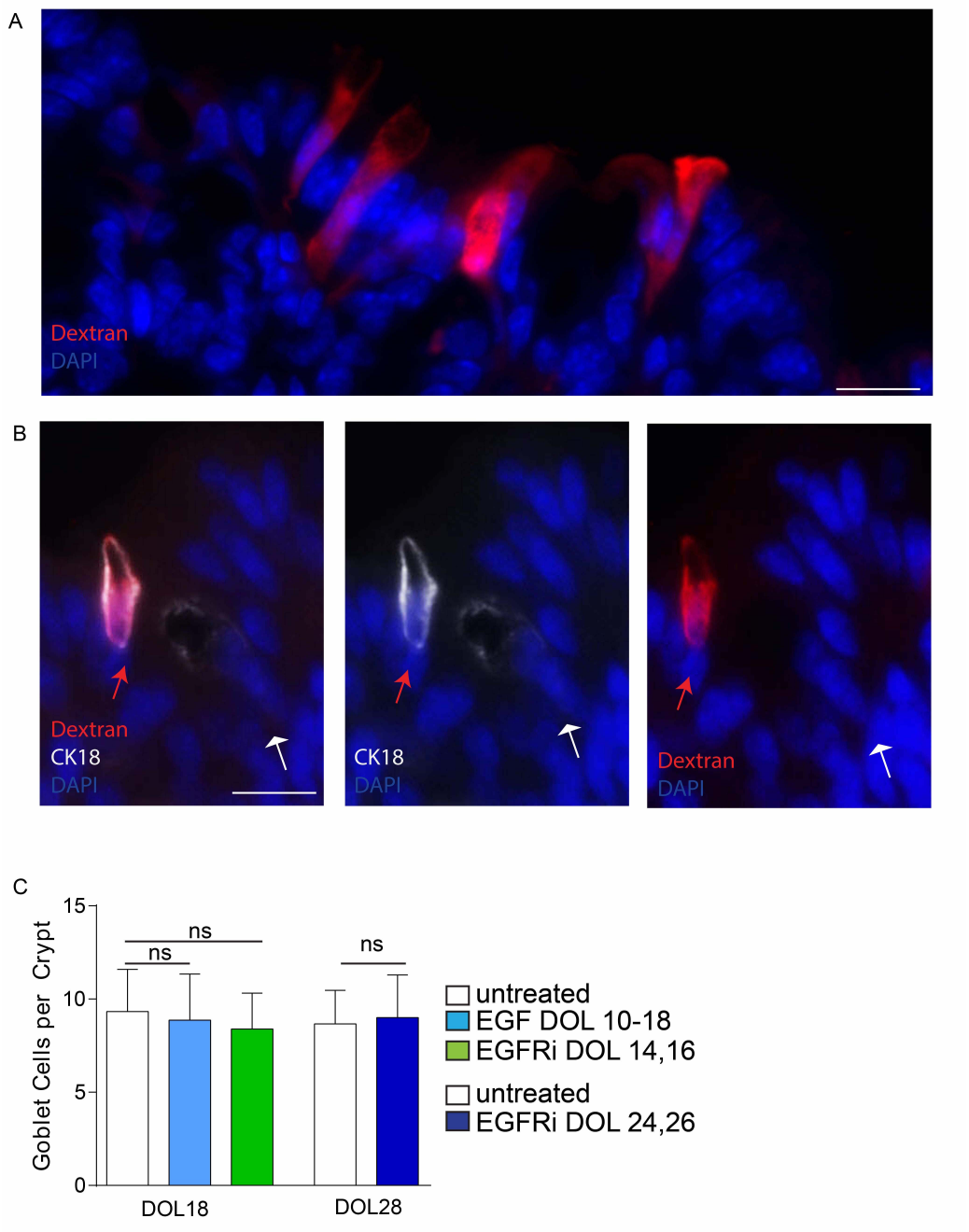

Fig. S6: *Immunofluorescent staining reveals dextran containing epithelial cells express the GC marker cytokeratin 18*. A-B) Intraluminal fluorescent dextran (red) was could be seen within epithelial cells of goblet cell morphology (A) and colocalized with goblet cell marker CK18 (B) in

DOL18 mice. Red arrow denotes CK18+ goblet cell with dextran, or a GAP, white arrow denotes a CK18+ cell not containing dextran. C) Number of goblet cells per colonic crypt is unaffected by GAP manipulations. Colon tissue was isolated on day of life (DOL) 18, or 28, following GAP manipulations and sections were stained for CK-18. n= 15 crypts from 3 mice per group in panel C. Scale bar = 10 μm.

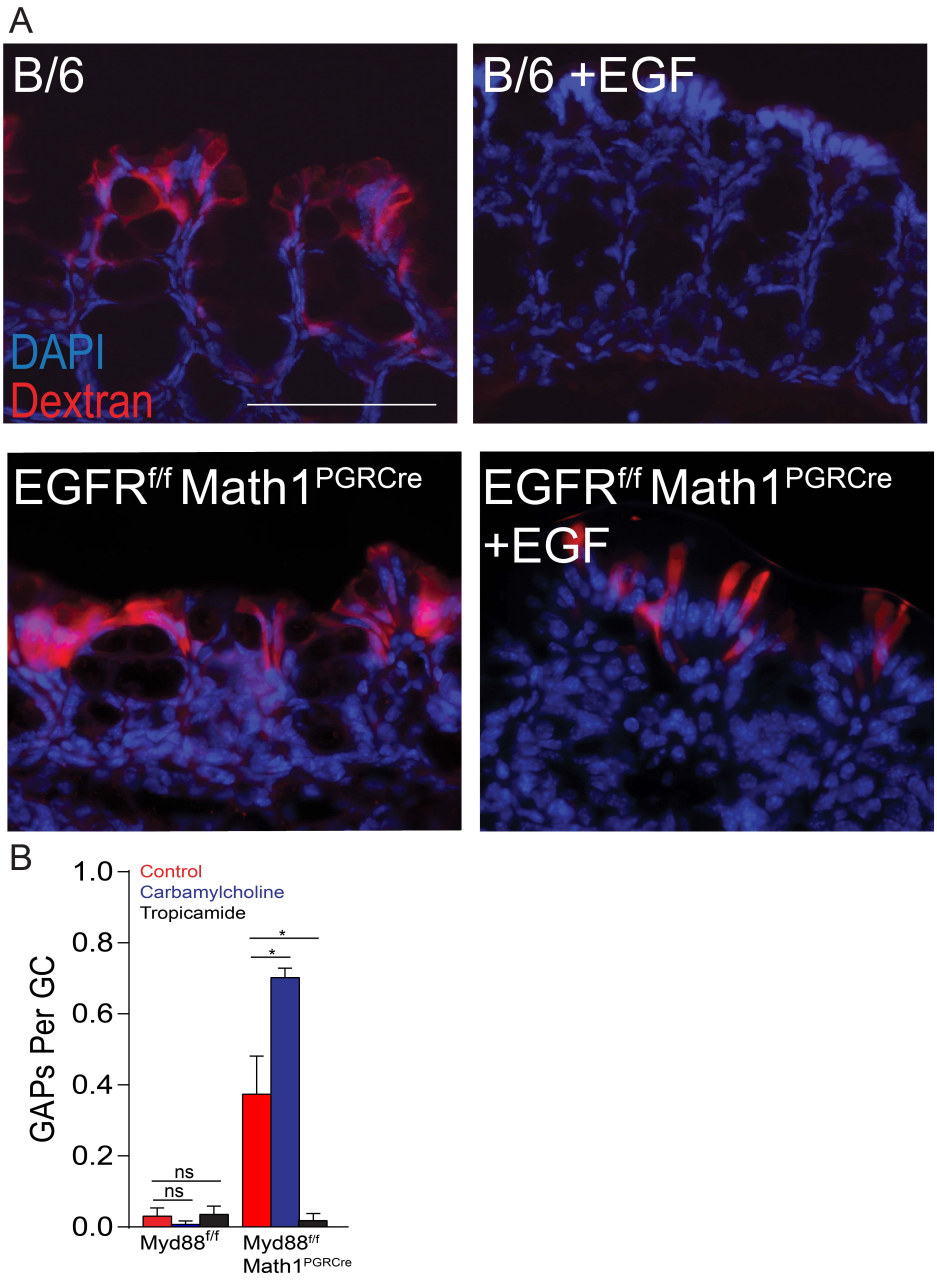

Fig. S7: *EGF inhibits colonic GAPs in the post-neonatal phase of life in a GC intrinsic manner.* Images of colon sections from DOL14 wildtype or EGFR$^{f/f}$Math1^{PGRCre} mice following luminal 10kD fluorescent dextran (red) and luminal vehicle or EGF. Images demonstrate that uptake of dextran by colonic epithelial cells on DOL14 is inhibited by luminal EGF in wildtype mice, but not in mice

lacking EGFR in GCs. Blue = DAPI nuclear stain, scale bar =50 μm. n = 4 mice per group panel B.

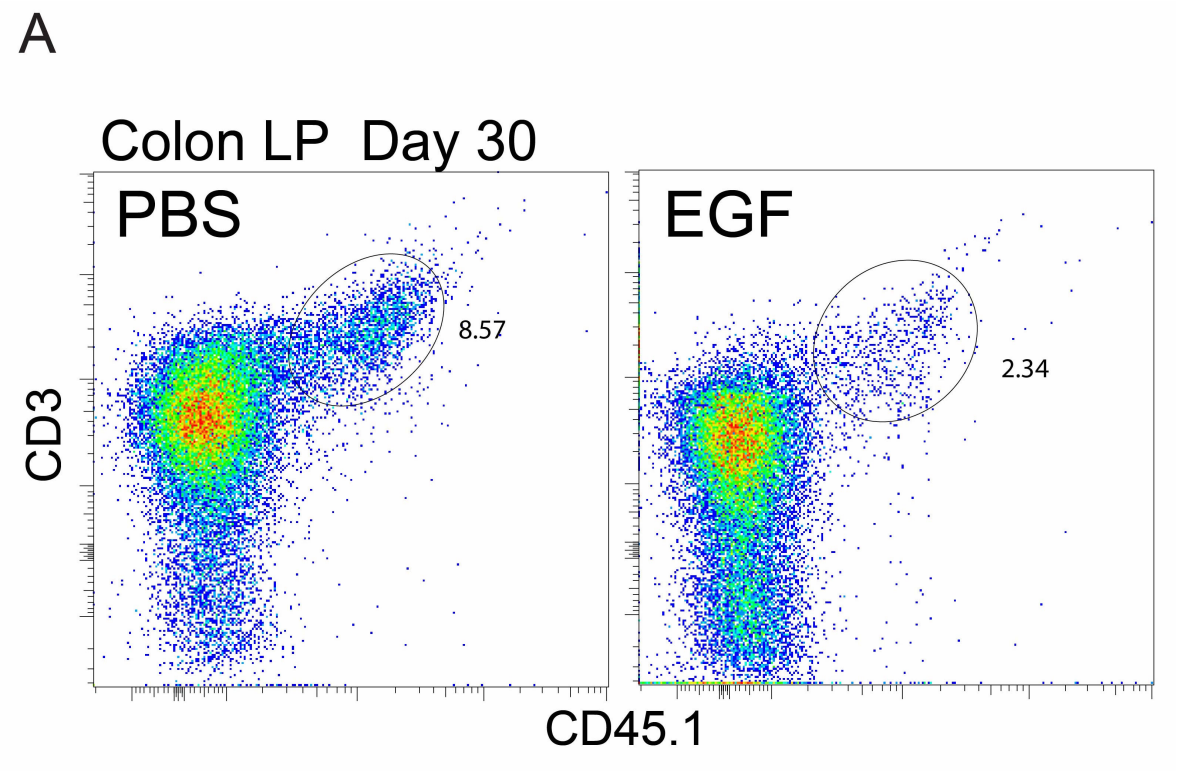

Fig. S8: *Inhibition of GAPs in the post-neonatal phase of life abrogates T cell responses to a gut bacteria.* Representative flow plots A) identifying CD45.1+CD3+CD4+ CBir1 T cells in the colon LP and on DOL30, two weeks after transfer. Mice received intracolonic PBS or EGF, to inhibit colonic GAPS from DOL10-21.

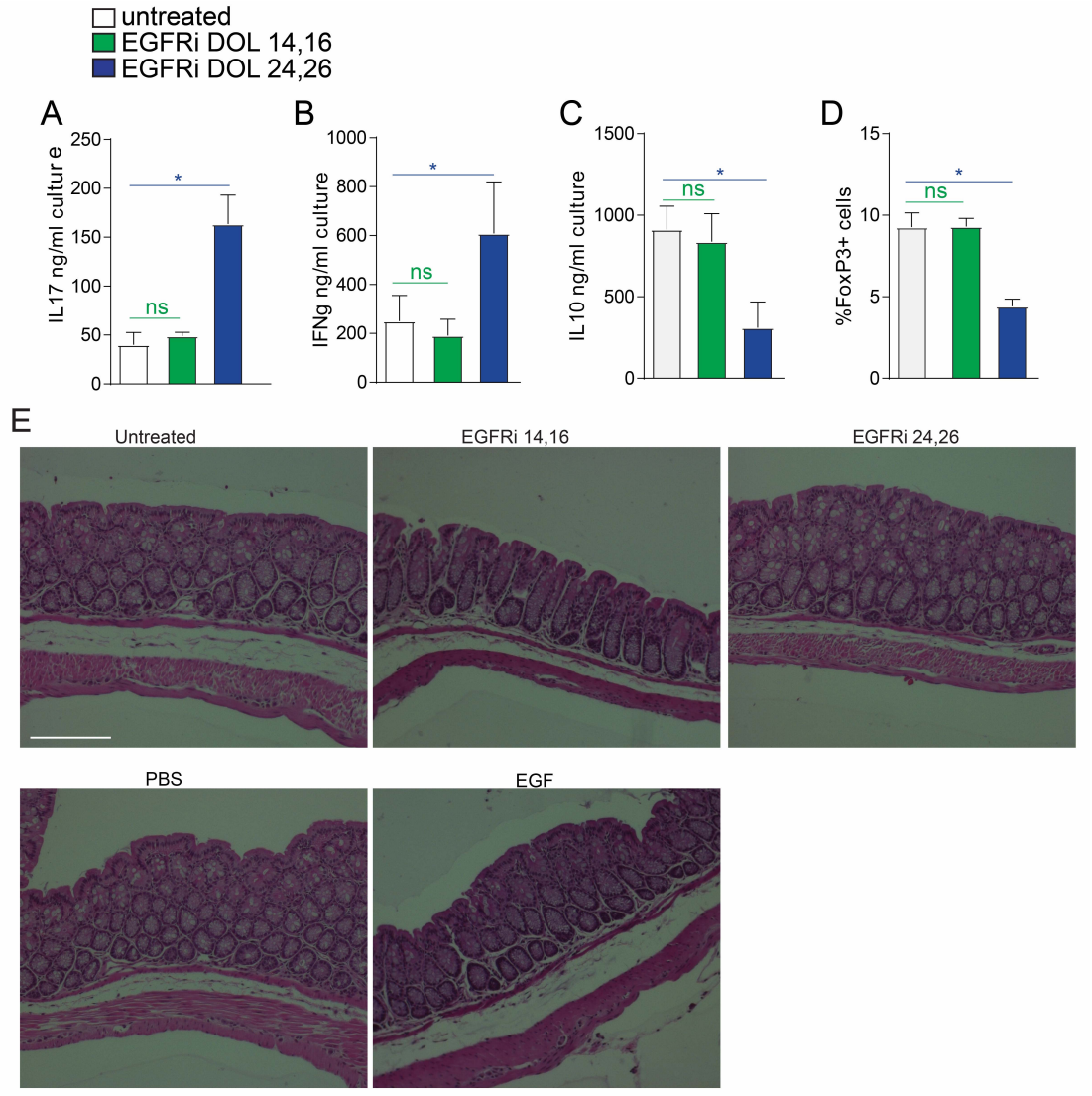

Fig. S9: *EGFR inhibition post-weaning, to inappropriately induce colonic GAPs, but not in the post-neonatal phase, when GAPs are present, results in inflammatory cytokine production in the colon draining MLN, but does not induce overt pathologic changes.* A-C) Cytokine levels in the supernatant after three days of culture of MLNs from DOL35 mice that were untreated or treated with EGFRi on DOL14 and 16, or DOL24 and 26. D) Cellular populations isolated from the colonic MLN of DOL35 mice treated as in A were evaluated for Foxp3+ expression by CD4+ T cells by

flow cytometry. E) H/E sections of colon from DOL35 mice treated as in A or receiving intracolonic PBS or EGF from DOL10-21 show no overt pathology. Scale bar = 100 μm. n = 3 mice per group performed in duplicate panels A-D.

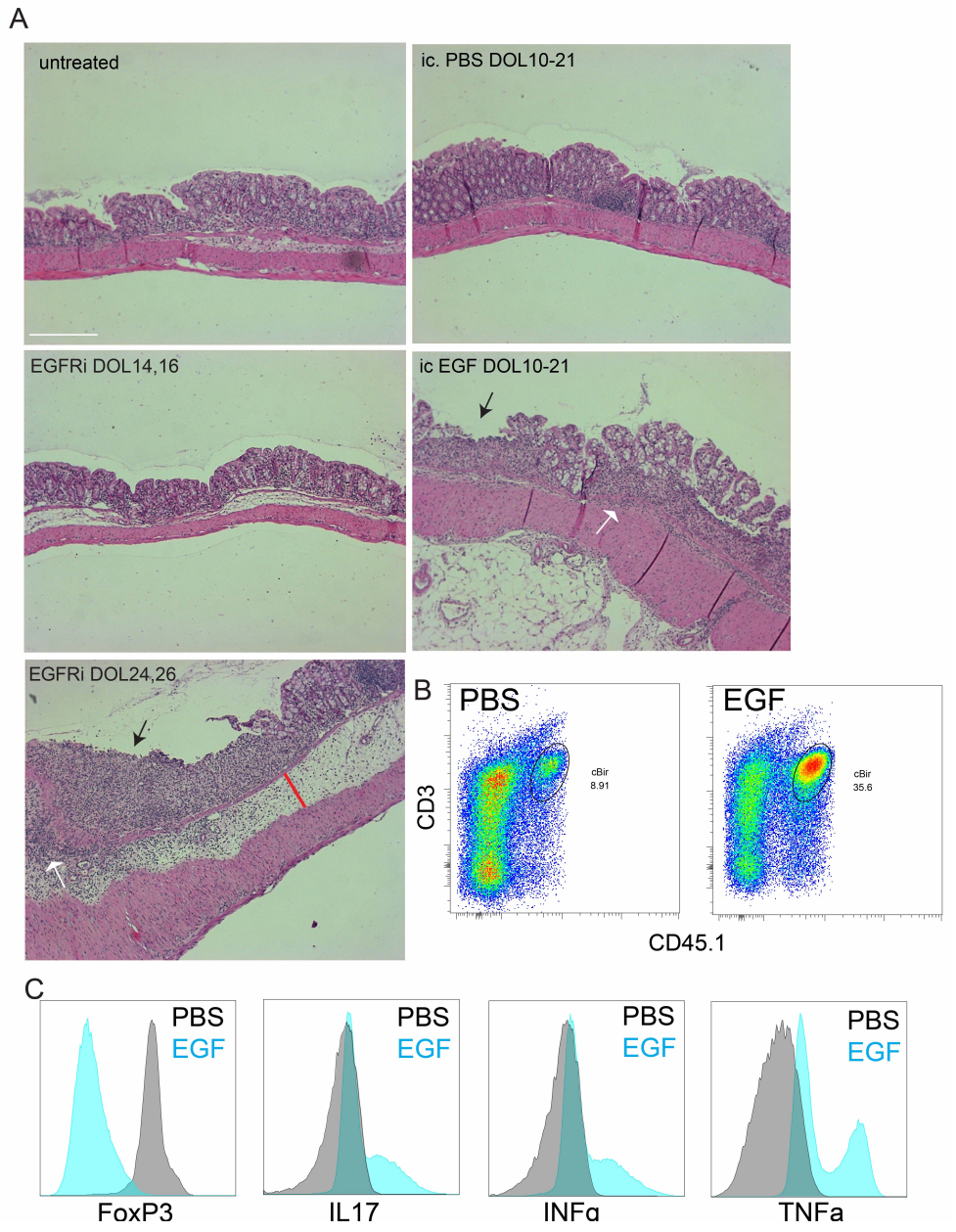

Fig. S10: *Worsened DSS colitis and inflammatory responses to commensal bacterial when encounters with microbial antigens are altered in early life.* A) H/E stained sections of colons after 8 days on 3% DSS in mice receiving intracolonic PBS or EGF from DOL10-21, or mice receiving EGFRi on DOL14 and 16, or DOL24 and 26. Black arrows denotes ulceration, white arrow

denotes infiltrates, red line indicates edema. B and C) Mice treated with intracolonic PBS or EGF on DOL10-21 adoptively transferred with CBir1 T cells on DOL16 and given 3% DSS for 8 days beginning on DOL30 B) Representative flow plots of colonic LP CD45.1+CD3+CD4+ CBir1 T cells and C) expression of Foxp3 or intracellular IL17, IFNγ, or TNFα by colonic LP CBir1 T cells following DSS treatment. Scale bar = 100 μm.