Supplementary material

Supplementary Methods

Intestinal epithelial cell culture and preparation of conditioned-medium (CM). Young adult mouse colonic (YAMC) epithelial and mouse small intestinal epithelial (MSIE) cell lines were generated from transgenic mice harboring thermolabile simian virus 40 (SV40) large tumor (T) antigen (TAg) from a SV40 strain, tsA58. The functional expression of SV40 TAg is induced by an IFNγ-inducible H-2Kb promoter at the permissive temperature (33°C). Expression of this gene is required for cell proliferation. Cells die when cells are cultured in medium without IFN-γ at the non-permissive temperature (37°C) for three passages.

YAMC and MSIE cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 5 U/ml of murine IFNγ, 100 U/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid at 33°C with 5% CO_2.

To prepare CM, YAMC and MSIE cells were cultured in RPMI 1640 medium containing 1% FBS and 100 U/ml penicillin and streptomycin for 18 hours at 37°C. Then cells were treated with p40 (10 μg/ml) for 6 to 24 hours for preparation of p40-CM. CM from cells without treatment was used as control. Cell numbers were counted for normalizing the ratio of the volume of medium to the cell number as $1x10^5$ cells/ml medium.

ELISA analysis. The level of TGFβ in CM from YAMC and MSIE cells was measured using a mouse TGFB1 ELISA kit (ThermoFisher Scientific), according to the manufacturer's instruction. Purified TGFβ was used to generate the standard concentration curve. The TGFβ concentration was calculated as of TGFβ ng/ml medium ($1x10^5$ cells/ml medium).

Isolation, culture, and treatment of $CD4^+$ T cells. $CD4^+$ T cells were isolated from the spleen of C57BL/6 mice using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec Inc.), according to the manufacturer's instructions. $CD4^+$ T cells (10^5/well) were cultured in 96-well dish in 100 μL of RPMI 1640 medium containing Dynabeads® Mouse T-Activator CD3/CD28 (at 1:10 ratio to T

cells), 10% FBS, 5 μM 2-mercaptoethanol (2-ME), and 100 U/ml penicillin and streptomycin. $CD4^+$ T cells were treated with p40 (10 ng/ml) in T cell culture medium and CM from YAMC and MSIE cells in the presence and absence of 40 and a monoclonal anti-TGFβ neutralizing antibody (ThermoFisher Scientific). The concentrations of CD3/CD28, FBS, and 2-ME in CM were adjusted to the same levels as those in T cell culture medium. T cells were cultured for 3 days before analyzing differentiation of $CD4^+$ T cells using flow cytometry.

Preparation of albumin-containing hydrogels and mice treatment. Albumin-containing hydrogels were developed using the method for preparation of p40-containing hydrogels. Bovine serum albumin (Sigma-Aldrich) was dissolved in pectin in water (2.0% w/v). Zein solution (1.0% w/v) was prepared in 75% ethanol solution containing 0.5% (w/v) of $CaCl_2$. Then, the albumin-containing pectin solution was dropped into the zein solution through a 27G needle connected to a syringe. After hydrogels (4 μl/drop) became solid in the zein solution and formed round sheets, hydrogels were washed with water, air-dried, and stored at 4°C. Two concentrations of albumin-containing hydrogels, 0.5 and 1.0 μg of albumin/hydrogel, were prepared.

Two female mice from the same litter and one male mouse were housed in the same cage until one to three days before delivery. Pups in one litter were treated with albumin at the same dosage as p40 treatment, which was 0.5 μg/day (postnatal days 2-6), 1 μg/day (postnatal days 7-13), and 1.5 μg/day (postnatal days 14-21). Pups from the other litter were treated with hydrogels only (without albumin or p40) or p40-containing hydrogels at 0.5 μg/day (postnatal days 2-6), 1 μg/day (postnatal days 7-13), and 1.5 μg/day (postnatal days 14-21).

Isolation of lymphocytes from mouse Peyer's patches and lamina propria of the small intestine and the colon. Peyer's patches were removed from the small intestine. Lymphocytes from Peyer's patches were isolated by gently crushing the patches and forcing cells through 70 μm cell strainer. Single cells through the filter were suspended in RPMI.

To isolate lymphocytes from lamina propria, small intestinal and colonic tissues were flushed with ice-cold calcium- and magnesium-free HBSS containing 2% FBS, cut into small pieces, and incubated in HBSS supplemented with 1 mM DTT for 40 minutes with shaking at 37 °C, followed by HBSS containing 1.3 mM EDTA for 1 hour at 37°C. Tissues were further incubated in 1 U/mL collagenase D in RPMI medium for 1.5 hours at 37 °C. After flowing through 70 μm cell strainer, lymphocytes were isolated on a 44/67% Percoll density gradient.

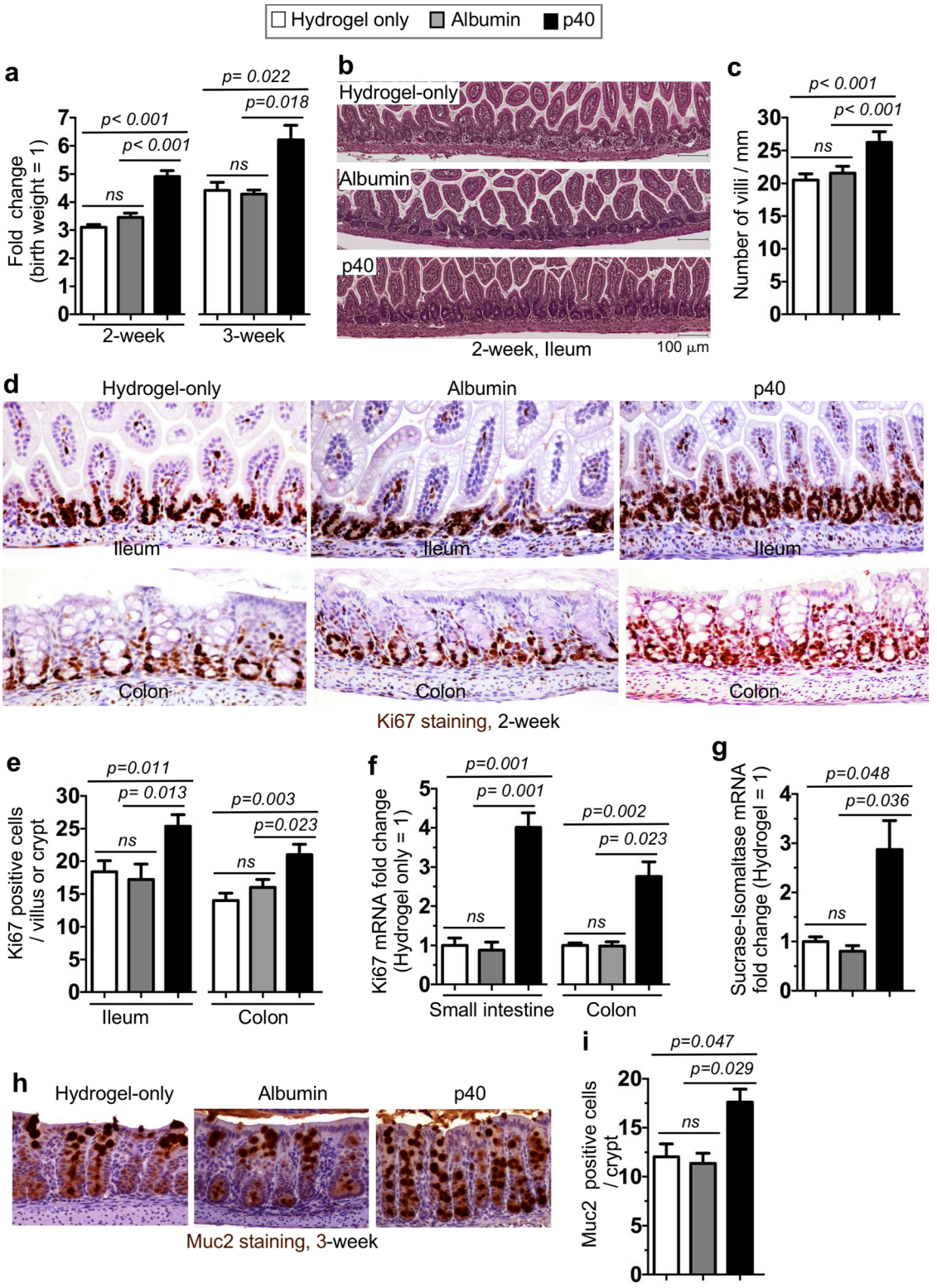
Myeloperoxidase assay. Colonic mucosal lysates were suspended in tissue suspension buffer containing 50 mM potassium phosphate (pH 6.0) and 5 mg/ml hexadecyl-trimethylammonium bromide (Sigma-Aldrich). Myeloperoxidase (MPO) level was detected using the reaction buffer containing 17% o-dianisidine (Sigma-Aldrich), 5 mM potassium-phosphate, and 0.0005% H_2O_2. Pure MPO (Calbiochem/EMD Biosciences, Darmstadt, Germany) was used to generate a standard concentration curve. Tissue suspension buffer was used as negative control. The results were presented as U MPO/g colonic mucosal soluble proteins.

Real-time PCR assay. Total RNA was isolated from small intestinal and colonic mucosal tissues using an RNA isolation kit (Qiagen) and was treated with RNase-free DNase. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using Taqman Gene Expression Master Mix and primers, Ki67 (Mm01278617), MUC2 (Mm01276696), Sis (Mm01210305), CLDN3 (Mm00515499), April (Mm03809849-s1), TNF (Mm00443259), IFN-γ (Mm99999071), IL-6 (Mm00446190), KC (Mm00433859), and TGFβ (Mm01178820). The relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize levels of the mRNAs of targeted genes. All cDNA samples were analyzed in triplicate.

Immunhistochemistry. Paraffin-embedded tissue sections were deparaffinized. After unmasking antigens, tissue sections were blocked using 5% goat serum in PBS. For Ki67 and MUC2 staining, tissue sections were incubated with a rabbit anti-ki67 monoclonal antibody

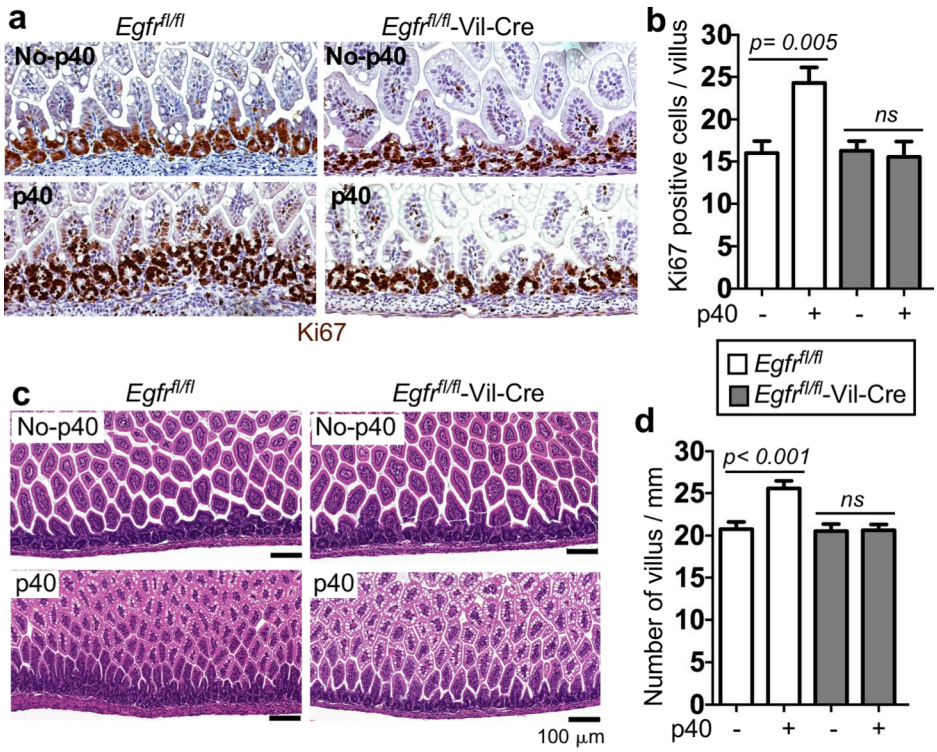
(Biocare Medical, LLC) and a rabbit anti-MUC2 antibody (Santa Cruz Biotechnology, INC) overnight at 4°C and followed by MACH 2 Universal HRP Polymer Detection reagents (Biocare Medical, LLC) for 1 h at room temperature. The sections were developed using the ImmPACT™ DAB substrate (Vector laboratories, Inc.), counterstained with hematoxylin, and observed using light microscopy. The number of Ki67 and MUC2 positive cells were determined by counting the absolute number of positive stained cells in at least 300 villi or crypts for each mouse.

For sucrose-isomaltase, ZO-1, IgA and Lgr5 immunostaining, sections were incubated with mouse anti-sucrose-isomaltase (Santa Cruz Biotechnology, INC), rabbit anti-ZO-1 (Invitrogen Corporation, Carlsbad, CA), rat anti-IgA (Cell Signaling Technology), and mouse Lgr5 (OriGene Technologies, Inc.) antibodies respectively, overnight at 4°C, followed by FITC- or Cy3-conjugated anti-rabbit, anti-mouse (Cell Signaling Technology) and anti-rat (Life Technologies) secondary antibodies for 1 h at room temperature. Slides were mounted using Mounting Medium with DAPI and observed using fluorescence microscopy. FITC, Cy3, and DAPI images were taken from the same field. The number of IgA positive cells was determined by counting the absolute number of positive stained cells in at least 300 villi in the ileum.

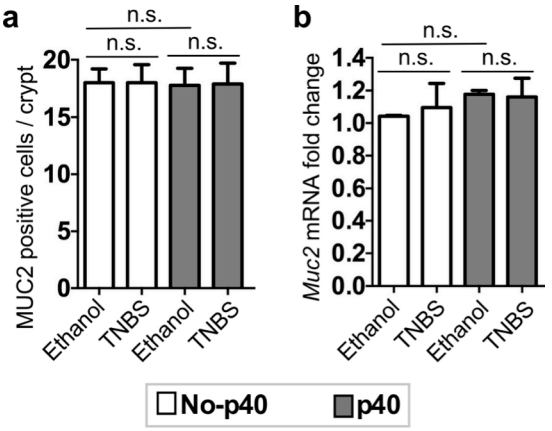


Supplementary Figure 1. Neonatal supplementation of albumin does not have effects on intestinal functional maturation in mice. Pups were treated with p40- and albumin containing hydrogels at 0.5, 1, and 1.5 μg/day at postnatal days 2-6, 7-13, and 14-21, respectively. Pups were also treated with hydrogels without p40 or albumin (hydrogel only).

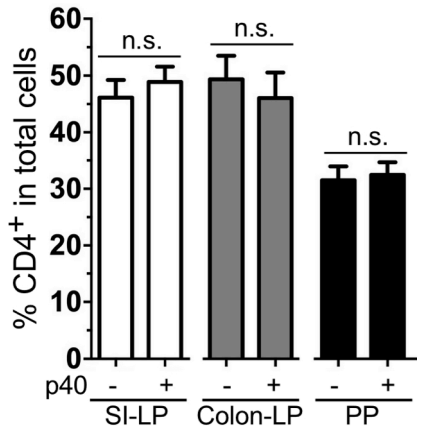
Bodyweight was recorded. (a) The fold change of bodyweight was calculated by comparing the bodyweight at the indicated postnatal day to the bodyweight at birth of the same pup. (b-c) Ileal tissues from 2-week old mice were prepared for H&E staining and the number of villi per mm is shown. (d-e) Ileal and colonic tissues from 2-week old mice were immunostained using an anti-Ki67 antibody and a horseradish peroxidase-conjugated secondary antibody. Slides were developed using DAB and counterstained with hematoxylin. The numbers of positively stained cells are shown. (f-g) Real-time PCR analysis was performed to detect Ki67 gene expression in the small intestine and the colon and *Sucrase-isomaltase* in the small intestine of 2-week old mice. The average of mRNA expression levels in the hydrogel only group was set as 100%, and the mRNA expression level of each mouse was compared to the average. (h-i). The colonic tissue sections were immunostained using an anti-Muc2 antibody and a horseradish peroxidase-conjugated secondary antibody (brown staining) and were developed using DAB. Sections were counterstained with hematoxylin. The numbers of MUC2 positively stained cells are shown. In a-c, hydrogel only group: n=7, albumin group: 9, p40 group: n=7. In d-i: n = 5-6 in each group.



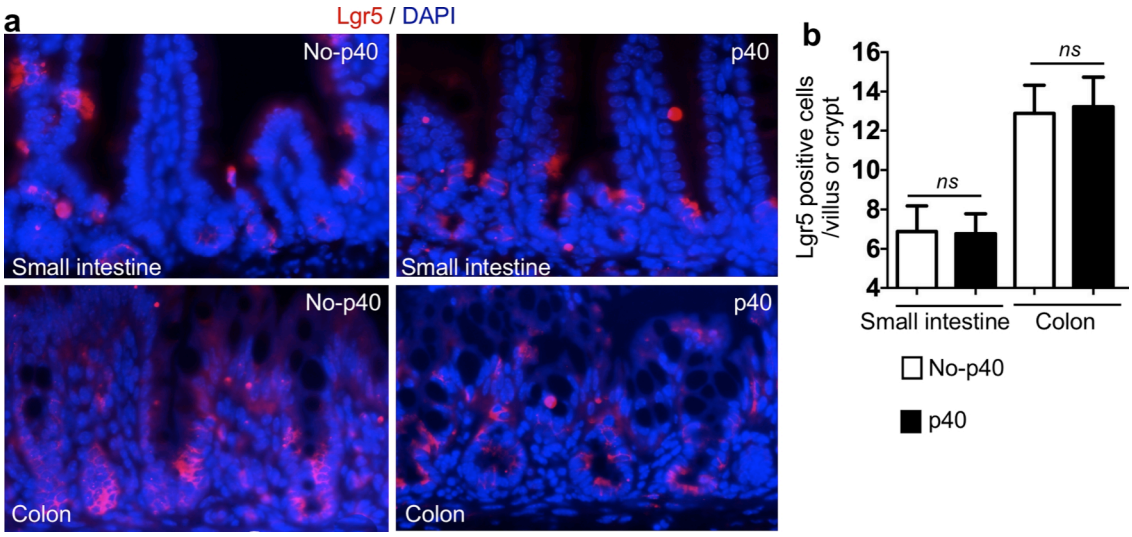
Supplementary Figure 2. EGFR in intestinal epithelial cells mediated p40 stimulated proliferation in 2-week old mice. Mice were treated with p40 as described in Fig. 2. (a-b) Ileal tissues from 2-week old mice were immunostained using an anti-Ki67 antibody and a horseradish peroxidase-conjugated secondary antibody. Slides were developed using DAB, counterstained with hematoxylin. The numbers of positively stained cells are shown. (c-d) Ileal tissues were prepared for H&E staining and the number of villi per mm is shown. n= 5-9 in each group.



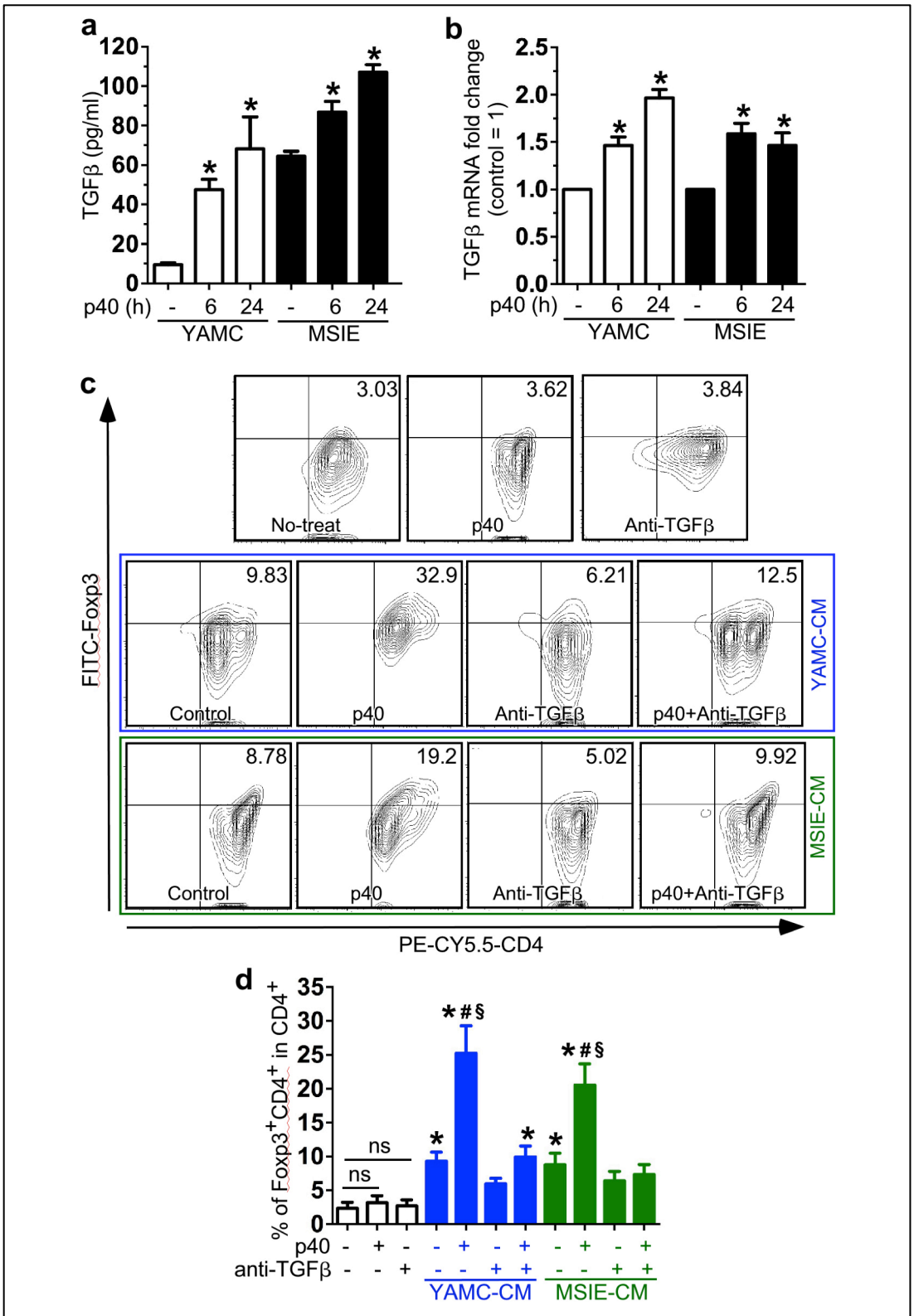
Supplementary Figure 3. Neonatal p40 treatment does not affect MUC2 production in adult mice. Mice were treated with p40 from postnatal day 2 to day 21, as described in Figure 2. Colitis was induced in 7-week old mice by TNBS, as described in Figure 7. (a) The colonic tissue sections were immunostained with MUC2 and counterstained with hematoxylin. The numbers of MUC2 positively stained cells/crypt are shown. (b) mRNA was isolated from the colonic tissues for real-time PCR analysis of *Muc2* mRNA expression levels. The average of mRNA expression levels in ethanol-treated mice of no-p40 group was set as 100%, and the mRNA expression level of each mouse was compared to this average. n=5-7 in each group.



Supplementary Figure 4. Neonatal p40 treatment does not affect the total number of $CD4^+$ T cells in the small intestine and the colon and in Peyer's patches in adult mice. Mice were treated with p40 from postnatal day 2 to day 21, as described in Figure 2. Lymphocytes from lamina propria (LP) of the small intestine (SI) and the colon and Peyer's patches (PP) were isolated from 7-week old wt mice. CD4 expressing cells were assessed using flow cytometry analysis. The percentages of $CD4^+$ cells in total LP and PP are shown. N=7-10 in each group.

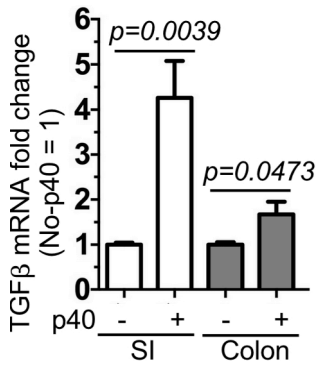


Supplementary Figure 5. p40 does not affect stem cell proliferation in the intestine in 2-week old mice. Mice were treated with p40 as described in Figure 2. (a) Ileal and colonic tissues from 2-week old mice were immunostained using an Lgr5 antibody and a Cy3-conjugated secondary antibody (red staining). Nuclei were stained with DAPI (blue staining). (b) The numbers of Lgr5 positively stained cells/crypt are shown. n= 5 in each group.



Supplementary Figure 6. p40 stimulates TGFβ production in intestinal epithelial cells for up-regulating differentiation of regulatory T cells (Trgs).

(a-b) YAMC and MSIE cells were treated with p40 at 10 ng/ml for 6 to 24 hours for preparing conditioned media (CM) ($1x10^5$ cells/ml medium). (a) The TGFβ levels in CM were measured using ELISA (a). RNA was isolated from cells for real-time PCR analysis of the TGFβ mRNA level (b). The TGFβ mRNA expression level in the control group was set as 1, and mRNA expression levels in treated groups were compared to the control group of the same cell line. (c-d) $CD4^+$ T cells in 96-well-dish (10^5 cells/well) were cultured for 3 days with indicated treatments, including direct p40 (10 ng/ml) treatment and CM from untreated (control) and 6-hour p40-treated YAMC and MSIE cells with and without co-treatment of an anti-TGFβ neutralizing antibody (1 μg/ml). Induction of Tregs was examined by flow cytometry analysis to detect CD4 and Foxp3 expressing cells. In a and b, * $p<0.05$ compared to the control group of the same cell line. Results are quantified from three independent experiments. In d, * $p<0.05$ compared to the no-treat T cell group, $^{\#}p<0.05$ compared to T cells with control CM treatment of the same cell line. $^{\S}p<0.05$ compared to T cells with p40-CM and anti-TGFβ antibody treatment of the same cell line. Results are quantified from two to three independent experiments.



Supplementary Figure 7. Neonatal p40 treatment increases TGFβ gene expression in the small intestinal and colonic mucosa in adult mice. Mice were treated with p40 from postnatal day 2 to day 21, as described in Figure 2. RNA was isolated from the small intestinal (SI) and the colonic mucosa of 7-week old mice. Real-time PCR analysis of gene expression levels of TGFβ was performed. The average of mRNA expression levels in the no-p40 group were set as 100%, and the mRNA expression level of each mouse was compared to the average. N=10 in no-p40 group, N=7 in p40 group.