Supplemental materials

Vasoactive intestinal peptide stabilizes intestinal immune homeostasis through maintaining interleukin-10 expression in regulatory B cells

Running title: VIP maintains Breg function

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Reagents

RNAi kits of VIP and TTP, antibodies of IL-10 (3C12C12), TTP (A-8), VIP (H-6), Flag (D-8), c-Maf (6B8), Pol II (E-8), CD3 (CD3-12), CD28 (CD28.1), IgM (A7), FcεRI-FITC (H-5) and mast cell protease 1-PE (CC1) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibody of VIP (ab8556), FcεRI and mMCP-1 was purchased from abcam (Cambridge, MA). ELISA kits of IL-4, IL-13, IFN-γ, TNF, IL-10 and VIP were purchased from BioMart (Beijing, China). AS101 (IL-10 inhibitor) was purchased from Bio-Techne (Shanghai, China). Fluorochrome-labeled antibodies [For mouse: CD71-PerCP-Cy5.5 (C2), CD73-PE (TY/23), CD19-FITC (H1B19), CD25-PE-Cy7 (PC61), TNF-α-BV421 (MP6-XT22). For human: CD71-FITC (M-A712), CD73-BV421 (AD2), CD19-PE-Cy7 (H1B19), CD25-APC (m-A251)] were purchased from BD Biosciences (Franklin Lakes, NJ). TRITC-dextran (40 Kda), oxazolone, MPO kit, IL-10 protein, reagents and materials for immunoprecipitation (IP) and chromatin IP were purchased from Sigma Aldrich (St. Louis., MO). CD40L, IL-3, IL-4 and stem cell factor were purchased from R&D Systems (Minneapolis, MN). Reagents and materials for RT-qPCR and Western blotting were purchased from Invitrogen (Carlsbad, CA). Immune cell isolation kits [B cell (Cat#: 130-091-151; 130-104-443), $CD4^+$ T cell (Cat# 130-104-454; 130-096-533) and $CD25^+$ T cell (130-091-072, 130-092-983)] were purchased from Miltenyi Biotech (San Diego, CA).

Collection of peripheral blood samples from human subjects

Peripheral blood samples were collected from human subjects by an ulnar vein puncture. Peripheral blood mononuclear cells (PBMCs) were isolated from the

samples by gradient density centrifugation. The sera were collected and stored at -80℃ until use.

Isolation of immune cells from PBMCs

Immune cells were isolated from PBMCs or spleen cells by magnetic cell sorting (MACS) or flow cytometry sorting with commercial reagent kits following the manufacturer's instructions. For B cell isolation, PBMC or spleen cells were prepared; the cells were processed with a B cell MACS kit. The purity of isolated B cells was between 95%. For $CD4^+$ $CD25^-$ T cell isolation, PBMCs or spleen cells were prepared; $CD4^+$ T cells were isolated with a $CD4^+$ T cell MACS kit first, then $CD25^+$ T cells were selected out by a MACS kit. The purity of isolated $CD4^+$ $CD25^-$ T cells was between 98%. For Breg isolation, PBMCs or spleen cells were prepared and stained with fluorochrome-labeled antibodies of CD19, CD25, CD71 and CD73; the Bregs ($CD19^+$ $CD25^+$ $CD71^+$ $CD73^-$) were isolated using a BD FACSAria (BD Biosciences, San Jose, CA, USA). The purity of Bregs was between 96%. See reagent section for the information of MACS kits and fluorochrome-labeled antibodies.

Cell culture

Cells were cultured in RPMI1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum. The medium was changed in 2-3 days. The cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Flow cytometry

For the surface staining, cells were stained with fluorochrome-labeled antibodies of interest or isotype IgG for 30 min at 4 °C. For the intracellular staining, cells were treated with fixatives and permeable reagents (BD Bioscience) first, then stained with fluorochrome-labeled antibodies of interest or isotype IgG for 30 min at 4 °C. The cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscience). The data were analyzed with software package Flowjo. In each experiment of flow cytometry, isotype IgG staining was performed. The data of isotype IgG staining were used as the gating reference. To analyze Bregs, $CD19^+$ or/and $CD73^+$ cells were gated first; then further calculated (or gated) the frequency of $CD25^+$ $CD71^+$ cells in $CD19^+$ $CD73^-$ cells. To analyze TNF-α+ mast cells, $mMCP1^+$ cells were gated first, then the TNF-α^+ cells in $mMCP1^+$ cells were gated or calculated.

Real-time quantitative RT-PCR (RT-qPCR)

Cells were collected from relevant experiments and treated with TRIzol reagents to extract RNA. cDNA was synthesized with the RNA and a reverse transcription reagent kit following the manufacturer's instructions. The samples were amplified in a qPCR device with the SYBR Green Master Mix and the presence of relevant primers, including VIP (tgtgtcaagaaatgccaggc and tgaagacggcatcagagtgt) and IL-10 (human: gccaagccttgtctgagatg and aagaaatcgatgacagcgcc; mouse: ataactgcacccacttccca and gggcatcacttctaccaggt). The results are presented as fold change against the housekeeping gene β-actin (human: catggaatcctgtggcatcc and cacacagagtacttgcgctc; mouse: ggaaatcgtgcgtgacatca and gccacaggattccataccca).

Western blotting

Proteins were extracted from cells collected from relevant experiments, fractioned by sodium dodecyl sulphate-poly acrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with primary antibodies of interest overnight at 4 °C, followed by incubating with peroxidase-labeled second antibodies for 2 h at room temperature. Washing with Tris-buffered saline containing 0.1% Tween 20 was performed with the membranes for 3 times after each time of incubation. The immunoblots on the membrane were developed with enhanced chemiluminescence and photographed with an imaging device.

TTP RNA interference (RNAi)

B cells were treated with TTP RNAi reagent kit following the manufacturer's instructions. The effects of RNAi in the cells were assessed by Western blotting 48 h after.

Immunoprecipitation (IP)

Proteins extracted from relevant cells were precleared by incubating with protein G agarose beads for 2 h. The beads were removed by centrifugation. The supernatant was collected and incubated with antibodies of interest or isotype IgG overnight. The immune complexes in the samples were precipitated by incubating with protein G agarose beads for 2 h. Proteins on the beads were eluted with an eluting buffer and analyzed by Western blotting. All the procedures were performed at 4 °C.

Chromatin IP (ChIP)

Cells collected from relevant experiments were fixed with 1% formalin for 15 min to cross link the DAN and surrounding proteins and lysed with a lysis buffer. The lysates were sonicated to shear the DNA into small pieces and then treated with the procedures of IP. The DNA/protein complexes were eluted from the beads with an eluting buffer. DNA was recovered from the samples with a DNA extracting reagent kit following the manufacturer's instructions and analyzed by qPCR in the presence of IL-10 promoter primer (tcaagacaacactactaaggctt and agatggggtggaagaagttga). The results were presented as fold change against the input. All the procedures were performed at 4 °C.

Assessment of VIP binding TTP in HEK293 cells

TTP-expressing plasmids and control plasmids were provided by Sangon Biotech (Shanghai, China). HEK293 cells (ATCC; Manassas, VA) were transfected with TTP-expressing plasmids or control plasmids following the manufacturer's instructions. The cells were cultured in the presence of VIP for 48 h and analyzed by IP.

Mice

The VIP-deficient mice and the littermates, C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Male BALB/c mice (6-8 week old) were purchased from Shanghai Experimental Animal Center (Shanghai, China). Mice were maintained in a specific pathogen-free facility with accessing food and water freely. The animal experimental procedures were approved by the Animal Ethic Committee at Tongji University.

Histology of the colon

Colon segments were fixed with 4% formalin overnight. Paraffin sections were prepared and stained with hematoxylin and eosin. The sections were observed with a light microscope.

Isolation of lamina propria mononuclear cells (LPMCs) in the colon

Colon were excised upon the sacrifice, cut into small pieces and incubated with collagenase (1 mg/ml) at 37 °C with mild agitation for 2 h. Single cells were collected by filtering through a cell strainer (100 µm first, then 40 µm). LPMCs were isolated from the single cell mixture by gradient density centrifugation.

Myeloperoxidase assay (MPO assay)

Following published procedures (Verma N, Verma R, Kumari R, Ranjha R, Paul J. Effect of salicin on gut inflammation and on selected groups of gut microbiota in dextran sodium sulfate induced mouse model of colitis. Inflamm Res. 2014;63:161-9), a small piece (about 0.1 g) of colon tissue was excised and snap-frozen in liquid nitrogen. The tissue was homogenized in 1 ml of HTAB buffer (hexadecyltrimethyl ammonium bromide dissolved in potassium phosphate buffer). The samples were centrifuged at 5,000 rpm for 2 min; the supernatant was collected. 10 µl of supernatant (or HTAB buffer, control) was added to each well in triplicate in 96-well plates. 200 µl of potassium phosphate buffer (pH 6.0) containing 0.5 mM o-dianisidinedihydrochloride (Sigma Aldrich) and 0.05 % hydrogen peroxide was added to each well. Optical density was measured immediately at 450 nm. A further reading was taken between 30 and 60 s. MPO was calculated using the formula:

MPO (U/g tissue) = (Δ0-30 + Δ30-60) ÷ 2 ÷ (T × C × W)

T = Time (s); C = MPO constant (1.13×10^{-2}); W = tissue weight (g).

Assessment of mouse colon epithelial barrier permeability

Four pieces of colon segments were excised and mount on Ussing chambers respectively. 30 min after mounting, TRITC-dextran was added to the epithelial side of the chambers at 10 μg/ml. Samples were taken from the serosal sides 30 min after. TRITC signals in the samples were measured using a fluorometer and used as indicators of intestinal epithelial barrier permeability.

Generation of bone marrow-derived mast cells (BMMC)

Following our established procedures (Feng BS, Zheng PY, Chen X, Liao XQ, Yang PC. Investigation of the role of cholera toxin in assisting the initiation of the antigen-specific Th2 response. Immunol Invest. 2008; 37: 782-97), we collected the bone marrows (BM) from BALB/c mouse femurs. BM cells were cultured in the presence of IL-3 (20 U/ml), IL-4 (50 U/ml), and stem cell factor (200 ng/ml). The culture medium, including the reagents, was changed in every 3 days. About 2-week culture, BM mast cells (BMMCs) were purified from the culture by MACS with the FcεRI and c-kit as the markers of isolation. The purity of isolated BMMCs was greater than 96% as assessed by flow cytometry.

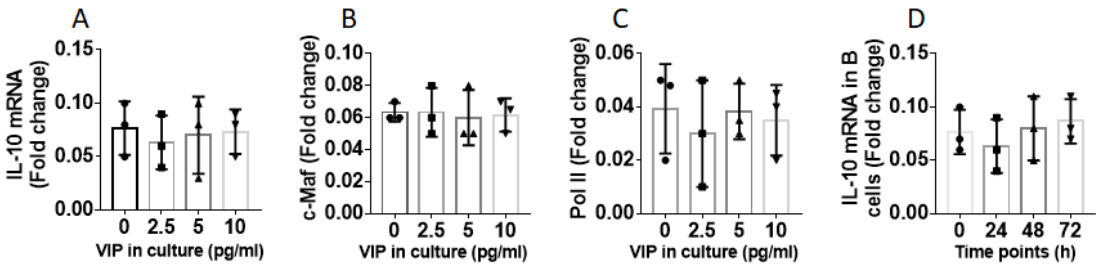

Figure S1. VIP does not alter IL-10 gene transcription. B cells were isolated from 6 naive mouse spleen and exposed to VIP in the culture at indicated concentrations for 3 days. The cells were analyzed by RT-qPCR and ChIP. A, IL-10 mRNA levels in B cells. B, c-Maf (IL-10 transcription factor) levels in IL-10 promoter in B cells. C, Poll II levels in IL-10 promoter in B cells. D, B cells were exposed to VIP in the culture at a concentration of 10 pg/ml. The B cells were collected at indicated time points and analyzed by RT-qPCR. The bars indicate the levels of IL-10 mRNA in B cells at indicated time points. Data of bars are presented as mean ± SEM. Each dot represents data from one independent experiment. The data represent 3 independent experiments.

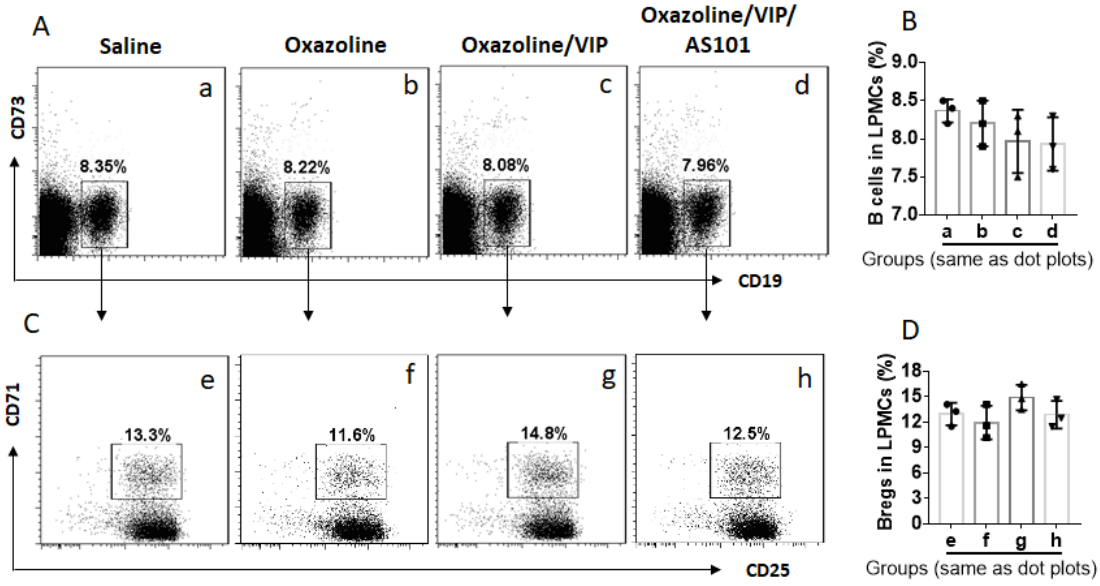

Figure S2. Assessment of Bregs in LPMCs. Mice (6 mice per group) were treated with the procedures denoted above each subpanel of A. LPMCs were prepared and analyzed by flow cytometry. A, the gated dot plots indicate frequency of $CD19^+$ $CD73^-$ B cells in LPMCs. B, bars show summarized data of B cells in LPMCs. C, gated dot plots show frequency of Bregs in LPMCs. D, bars indicate summarized data of Bregs in LPMCs. Data of bars are presented as mean ± SEM. Each dot inside bars present data from an independent experiment. AS101: 10 µg/mouse; ip on day 0, day 3 and day 6, respectively. The data represent 3 independent experiments.

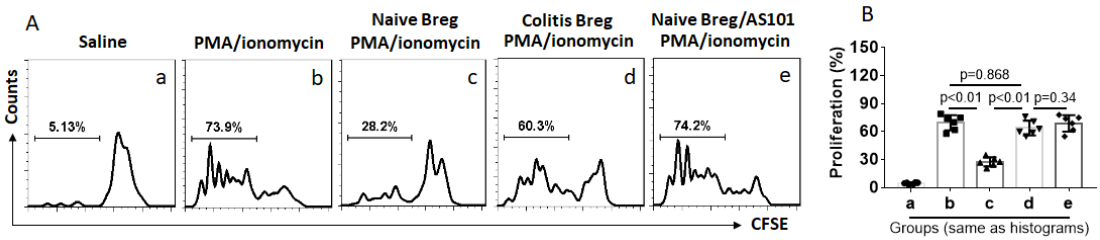

Figure S3. Bregs isolated from the colon of mice with colitis lack immune suppressor function. Bregs ($CD19^+$ $CD25^+$ $CD71^+$ $CD73^-$) were isolated from the colon of naïve mice and mice with colitis. Teffs ($CD4^+$ $CD25^-$ T cells) were isolated from the spleen of naïve mice and labeled with CFSE (Carboxyfluorescein diacetate succinimidyl ester). Bregs and Teffs were cultured together in the presence of non-specific cell activators (PMA: 50 ng/ml and ionomycin: 100 ng/ml) for 3 days. The cells were analyzed by flow cytometry. A, gated histograms indicate proliferating Teffs. B, bars indicate summarized proliferating Teffs. Data of bars are presented as mean ± SEM. Each dot inside bars presents data from an independent experiment. AS101: 10 µg/mouse; ip on day 0, day 3 and day 6, respectively. Statistical methods: t test. Each group consists of 6 mice. Statistical test: ANOVA + Dunnett t test.

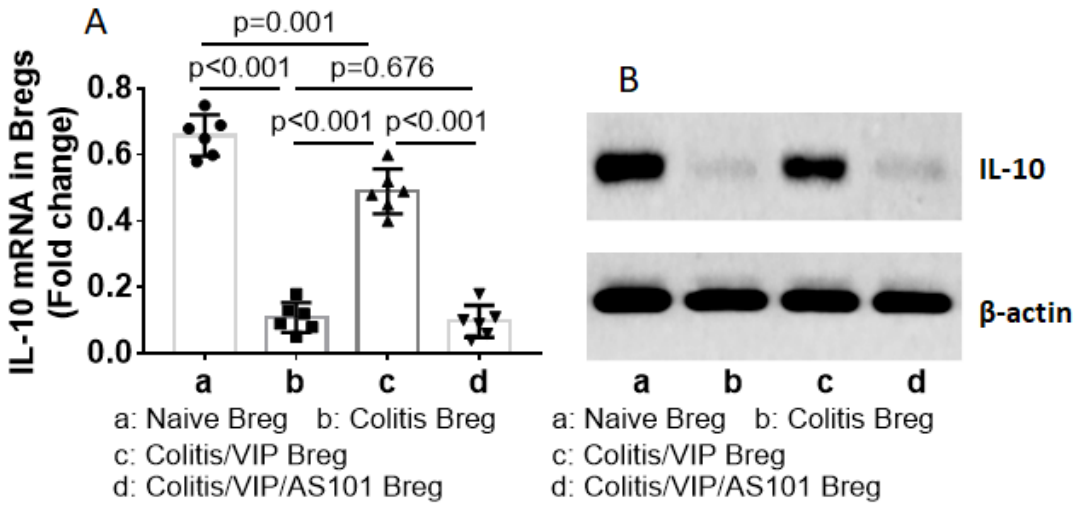

Figure S4. VIP restores IL-10 expression in Bregs. Bregs were isolated from the colon of mice treated with the procedures denoted below x axis of panel A and analyzed by RT-qPCR and Western blotting. A, bars indicate IL-10 mRNA levels in Bregs. B, immunoblots show protein levels of IL-10 in Bregs. Data of bars are presented as mean ± SEM. Each dot inside bars presents data from an independent experiment. AS101: 10 μg/mouse; ip on day 0, day 3 and day 6, respectively. Statistical methods: ANOVA + Dunnett *t* test. Each group consists of 6 mice.