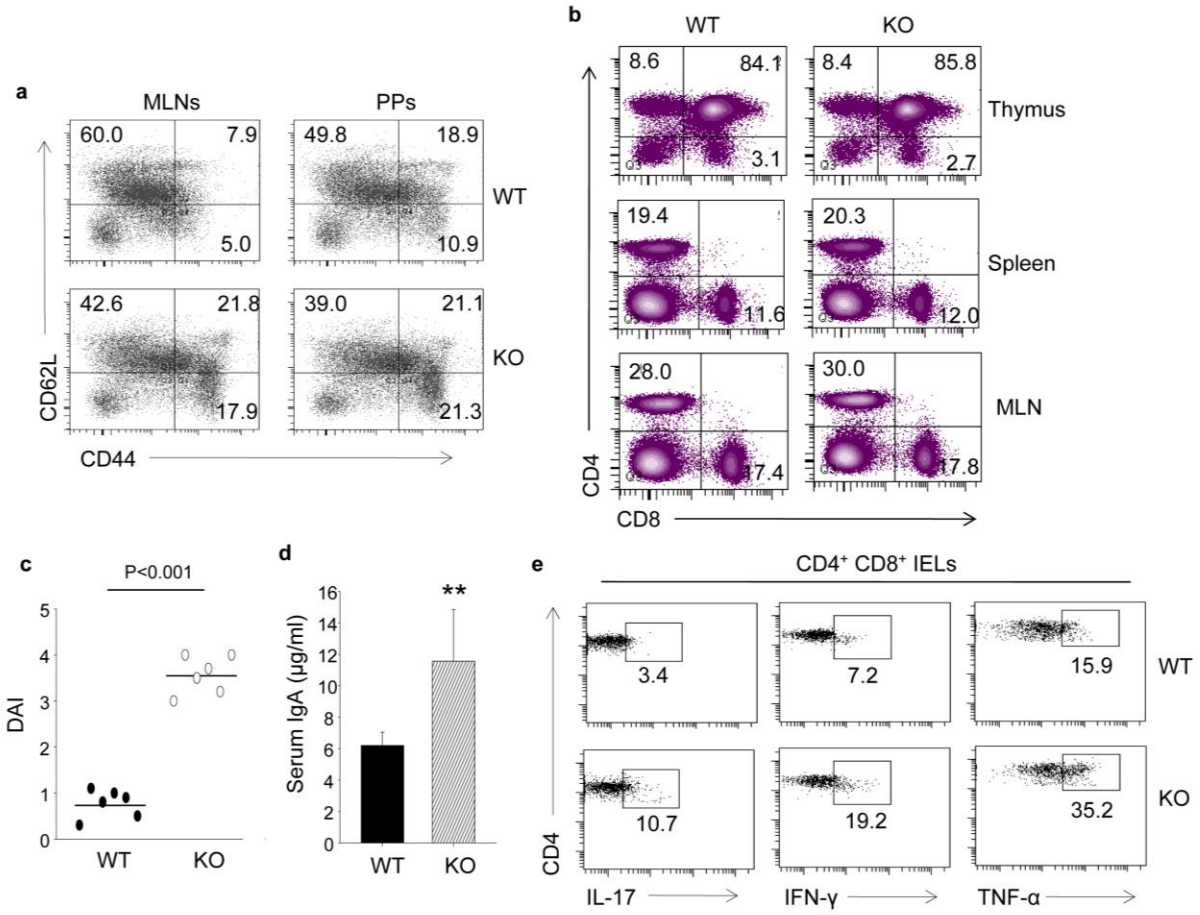
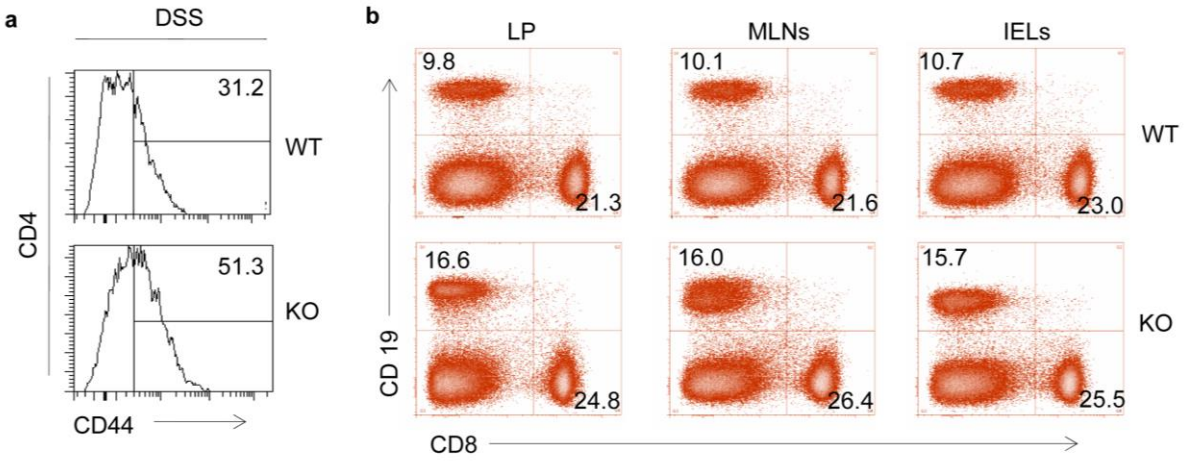
SUPPLEMENTARY DATA



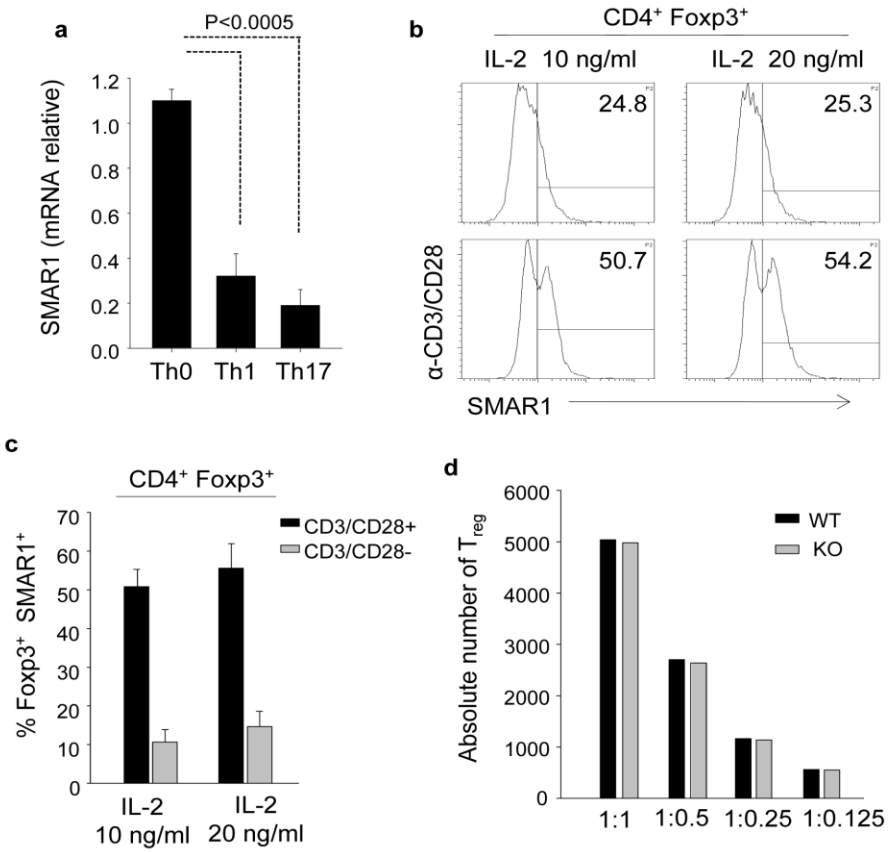
Supplementary Figure 1: Peripheral lymphoid organs of SMAR1$^{-/-}$ mice have an effector memory phenotype. (**a**) Lymphocytes collected from MLNs and Peyer's patches (PPs) of WT and SMAR1$^{-/-}$ mice were stained for CD44 and CD62L and analyzed by FACS. Percentage in the dot plots showed the naive CD62L^{hi}CD44^{lo}, central/memory CD62L^{hi}CD44^{hi}, and effector/memory CD62L^{lo}CD44^{hi} populations, within the gated on CD4. Data is representative for six pairs of mice. (**b**) Representative FACS analysis of absolute number of T cells (CD4^{+} and CD8^{+}) from thymus, spleen and MLN of WT and SMAR1$^{-/-}$ mice. Numbers in quadrant refer to the percentage of each subset. Data shown are representative of 6 mice from 3 independent

experiments. (**c**) Disease activity index (DAI) scores for DSS treated WT and SMAR1$^{-/-}$ mice. Data are shown from three independent experiments (n=6). *P<0.001*. (**d**) Serum IgA levels as determined by ELISA on DSS treated WT and SMAR1$^{-/-}$ mice samples. ***P<0.005*. (**e**) Intracellular cytokine production by colon IEL CD4^{+}CD8^{+} T cells in DSS treated WT and SMAR1$^{-/-}$ mice. Cells were isolated and activated as described in materials and methods, followed by staining for surface CD4 and CD8 and intracellularly for the indicated cytokines, and analyzed by flow cytometry. Frequencies of cytokine secreting cells are indicated within the gated CD4^{+}CD8^{+} T cell population.



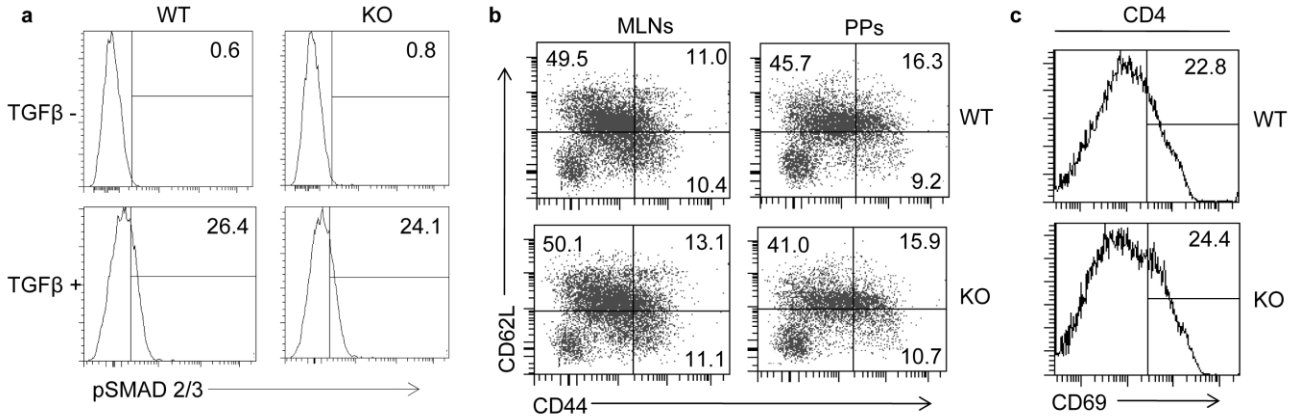
Supplementary Figure 2: SMAR1$^{-/-}$ mice have increased CD8^{+}, CD44^{+} T cells and CD19^{+} B cells in the colon during acute DSS colitis. (**a**) Flow cytometry analysis of surface markers CD44 on IELs in DSS treated SMAR1$^{-/-}$ mice compared to age-matched WT mice. Data is shown as histograms of indicated markers in gated CD4^{+} T cells and represent at least three experiments. (**b**) Frequencies of CD8^{+} T cells and CD19^{+} B cells among the colon LP

lymphocytes, MLNs and IELs in DSS treated WT and SMAR1$^{-/-}$ mice evaluated by flow cytometry. Data is derived from five to six mice per group.

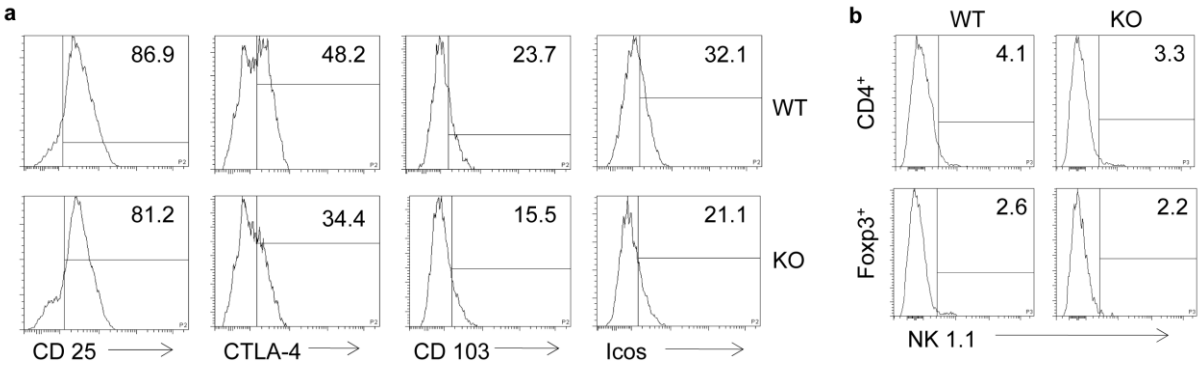


Supplementary Figure 3: SMAR1 is selectively express in T_{reg} cells and SMAR1$^{-/-}$ T_{reg} cells maintained *in vitro*. (**a**) Quantitative real time PCR analysis of SMAR1, presented as mRNA expression relative to β-Actin, in naïve CD4^{+} T cells from WT spleen activated towards Th1 and Th17 phenotype for 5 days (mean ± SD of SMAR1 in three independent experiments). *P<0.0005*. (**b**) IL-2 signaling alone can up-regulate SMAR1 in T_{reg} cells. CD4^{+} T cells were FACS sorted and cultured (*bottom panels*) in presence of anti-CD3 anti-CD28 (2μg/ml) or with various doses of rIL-2 alone for 24 h Top histograms had no anti-CD3 anti-CD28 added. Cells were harvested and stained for CD4, Foxp3 and SMAR1. Histograms are gated on CD4^{+}Foxp3^{+}

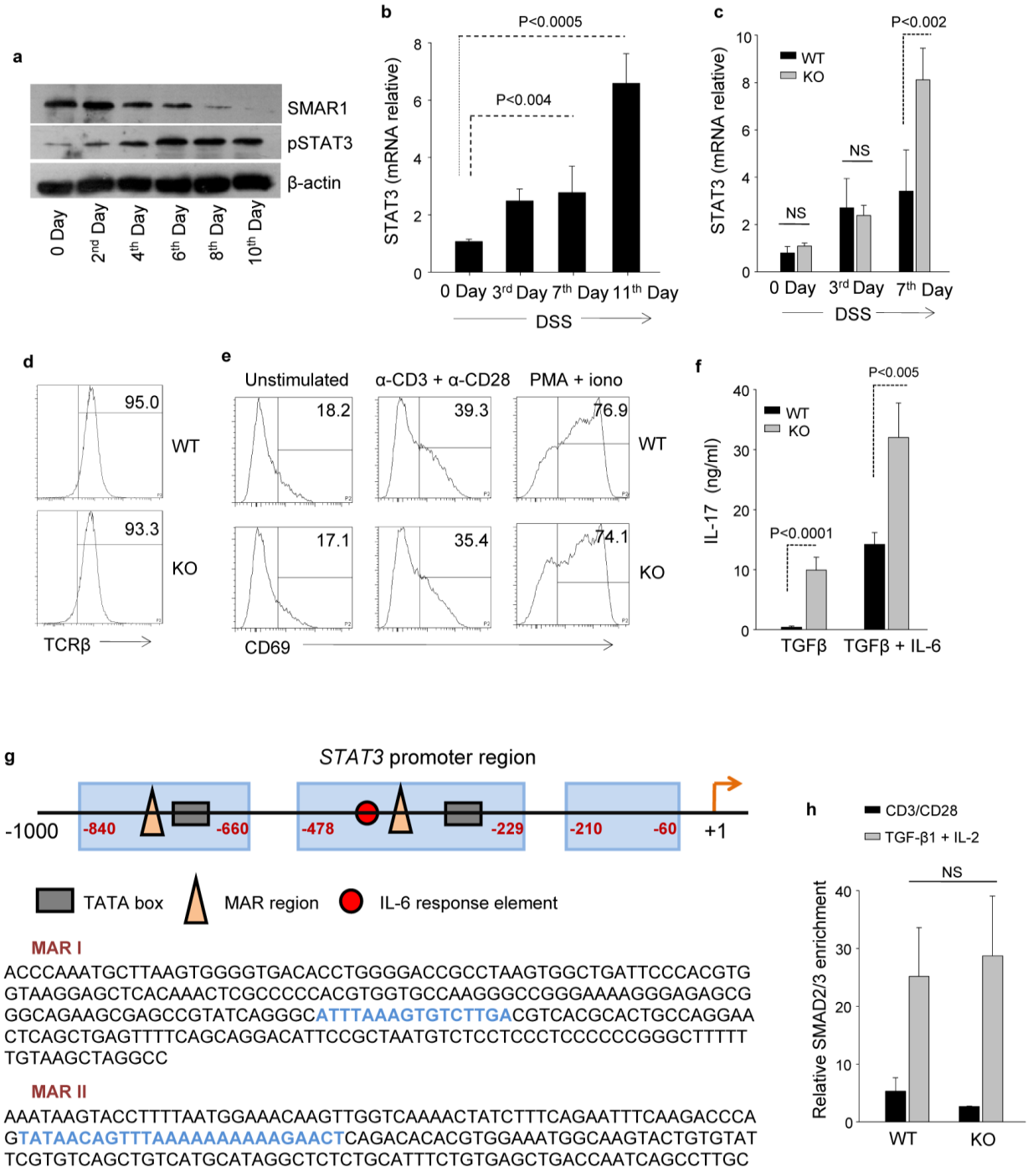
cells. (**c**) Graphical representation of the same. Data shown is representative of 3 independent experiments with similar results. (**d**) *In vitro* maintenance of SMAR1$^{-/-}$ (KO) and WT T_{reg} cells during *in vitro* suppression assay shown in Figure 4e. Cells were stained with CD4 and Foxp3 and analyzed by flow cytometry. Cell statistics were obtained by gating on the CD4^{+}Foxp3^{+} cell populations. Bars represent absolute numbers of T_{reg} cells in each co-culture.



Supplementary Figure 4: Analysis of pSMAD2/3 expression in SMAR1$^{-/-}$ CD4^{+} T cells. (**a**) CD4^{+}CD25^{-} T cells were cultured with anti-CD3 and anti-CD28 in the absence or presence of TGF-β1 (10 ng/ml) for 3 days. Flow cytometry analysis of pSMAD2/3. Data shown is one experiment representative of three. (**b**) Lymphocytes collected from MLNs and Peyer's patches (PPs) of 3 week old WT and SMAR1$^{-/-}$ mice were stained for CD44 and CD62L and analyzed by FACS. Percent in the dot plot showed the naïve CD62L^{hi}CD44^{lo}, central/memory CD62L^{hi}CD44^{hi} and effector/memory CD62L^{lo}CD44^{hi} population within the gated on CD4. Data is representative of five pairs of mice. (**c**) Lymphocytes from 3 week old WT and SMAR1$^{-/-}$ mice were stained for CD4 and CD69 and analyzed by flow cytometry. Histogram showed frequencies of CD69^{+} cells gated on CD4. Data is representative of five pairs of mice.



Supplementary Figure 5: T_{reg} cells of SMAR1$^{-/-}$ mice showed abnormal level of maturation markers and no surface up-regulation of NK 1.1. (**a**) Splenocytes from WT or SMAR1$^{-/-}$ mice were stained for CD4, Foxp3, CD25, CTLA4, CD103 and Icos and analyzed by flow cytometry. Histograms represent the population density for the specified markers, within the gated CD4^{+}Foxp3^{+} T cell population. Data is representative for five pairs of mice. (**b**) Splenocytes from WT and SMAR1$^{-/-}$ mice were stained for CD4, Foxp3 and NK1.1. Numbers in histogram represent the NK1.1 frequencies in CD4^{+} and CD4^{+}/Foxp3^{+} population. Data is representative of five pairs of mice.



Supplementary Figure 6: SMAR1 controls STAT3 expression in Foxp3^{+} T$_{reg}$ cells. (**a**) Total protein lysates, prepared from colon LP lymphocytes of WT mice on days 0, 2nd, 4th, 6th, 8th, and 10th after DSS treatment, and were analyzed for the expression of pSTAT3 and SMAR1 by

western blotting. β-actin was used as loading control. (**b**) RNA from colon LP lymphocytes was isolated from WT mice on day 0, 3^{rd}, 7^{th} and 11^{th} after DSS treatment, and relative STAT3 mRNA expression was measured by quantitative real time PCR. Data represents mean ± SEM of fold induction (n=5-6/genotype). (**c**) Enhanced inflammation and STAT3 activation in DSS treated SMAR1$^{-/-}$ mice. RNA from colon LP lymphocytes was isolated from WT and SMAR1$^{-/-}$ mice on day 0, 3^{rd} and 7^{th} after DSS treatment, and relative STAT3 mRNA expression was measured by quantitative real time PCR. Data represents mean ± SEM of fold induction (n=5-6/genotype). (**d**) Evaluation of TCRβ levels in the CD4^{+} T cells. (**e**) Response of SMAR1 deficient T_{reg} cells to TCR stimulation. Lymphocytes from WT and SMAR1$^{-/-}$ mice were stimulated *in vitro* for 4 h at 37^{o}C, in a complete media with or without cross linked anti-CD3 and anti-CD28 or PMA/ionomycin. Cells were stained for CD4, Foxp3 and CD69 and analyzed by flow cytometry. Histograms showed CD69 on gated CD4^{+}Foxp3^{+} cells, and numbers indicate frequencies of CD69^{+} population. Data are representative of five pairs of mice. (**f**) ELISA of IL-17 in supernatants of naïve CD4^{+}CD25^{-} T cells cultured for 96 h with TCR stimulation along with the addition of TGF-β1 alone or in combination with IL-6. (**g**) Sequence of STAT3 promoter. Blue sequences represent two regions of high MAR potential as determined using the MARFINDER program of Futuresoft software. (**h**) Enrichment of SMAD2/3 at the Foxp3 promoter in WT or SMAR1$^{-/-}$ naïve CD4^{+}CD25^{-} T cells cultured for 96 h with TCR stimulation and treatment along with TGF-β1 and IL-2, assessed with anti SMAD2/3 and presented relative to results obtained with control IgG.

SUPPLEMENTARY METHODS

Antibodies and reagents used in the study. Anti IL-17A (clone TC11-18H10), anti-TNF-α (clone MP6-XT22), anti-IL-4 (clone 11B11), anti-IFN-γ (clone XMG1.2), anti-Foxp3 (clone MF23), anti- CD4 (clone RM4-5), anti-α4β7 (clone DATK32), anti- CCR9 (clone 9B1), anti-CD44 (clone IM7), anti-CD11c (clone HL3), anti-GR-1 (clone RB6-8C5), anti-CD8a (clone 53-6.7), anti-Ki67 (clone 16A8), anti-Helios (clone 22F6), anti-CD-25 (clone PC61), anti-STAT3 (clone 4/P-STAT3), anti-RORγt (clone Q31-378), anti-IL-10 (clone JES7-16E3), anti-CD19 (clone 1D3), anti-CTLA4 (clone UC10-4F10-11), anti-CD304 (Neuropilin-1) (clone 3E12), anti-CD-103 (clone M290), anti-ICOS (clone 7E-17G9), anti-NK1.1 (clone PK136), anti-SMAD2/3 (clone D7G7), anti-GATA-3 (clone L50-823), anti-CD45R/B220 (clone RA3-6B2), anti-CD3e (clone 145-2C11), anti-IgA (clone C10-3), anti-CD3 NA/LE (clone 17A2), anti-CD28 NA/LE (clone 37.51) were obtained from BD biosciences and Biolegend. Anti SMAR1 was purchased from Bethyl laboratories, goat anti rabbit AlexaFluor 647 secondary antibody were obtained from Invitrogen. Recombinant mouse TGF-β1 (240-B), IL-2 (1150-ML), IL-6 (406-ML), anti-IL-6 (AF-406), anti-IL-4 (AF-404), anti-IFN-γ (AF-485), anti-IL-10 (AF-417) were from R&D system.

Adoptive transfer of cells. To determine the ability and suppressive function of T_{reg} cells, WT and SMAR1 deficient $CD4^+CD25^+$ T_{reg} cells were sorted. 2×10^5 sorted T_{reg} cells were cotransferred in to *Rag1*$^{-/-}$ recipient via i.p. injection. The contribution of each donor to the T_{reg} cell population recovered in the host was determined 8 weeks post transfer. To evaluate T_{reg} cell mediated protection of effector T cell elicited IBD *in vivo*, 2×10^5sorted T_{reg} cells were mixed with 4×10^5 $CD4^+CD45RB^{hi}CD25^-$ T cells sorted from WT and SMAR1$^{-/-}$ mice. Cell mixture

was transferred in to *Rag1*$^{-/-}$ mice via i.p. injection. As control 4 × 10^5 $CD4^+CD45RB^{hi}CD25^-$ T cells were also transferred alone in to *Rag1*$^{-/-}$ mice. To monitor IBD development, body weight of the recipient mice was monitored weekly after the transfer. Recipient mice was euthanized 8 weeks after transfer, $CD4^+$ T cells from these mice were harvested and subjected to immunological analysis.

T_{reg} and Th17 differentiation *in vitro*. Naïve $CD4^+CD62L^{hi}CD25^-$ T cells were cultured with plate bound anti-CD3 (2 µg/ml) and soluble anti-CD28 (3 µg/ml), with the addition of TGF-β1 (2 – 20 ng/ml), IL-6 (10 ng/ml), IL-2 (10 ng/ml) and retinoic acid (100 nM) where appropriate. In some experiments, anti- IL-4 (10 ng/ml), anti- IL-6 (10 ng/ml), anti- IFN-γ (10 ng/ml) was included. Culture supernatants were collected after 5th day for cytokine enzyme-linked immunosorbent assay (BD biosciences).

Quantitative Real time PCR. Total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen) and random hexamers in a volume of 20 µl according to the manufacturer's protocol. The primers used for quantitative real time PCR are listed in Table1.

Chromatin immunoprecipitation assay. ChIP assay was performed as per Upstate Biotechnology protocol. In short, cells were cross linked by 1% formaldehyde and lysed in lysis buffer. The lysates were sonicated with a bioruptor sonicator to shear genomic DNA into 200-500 bp fragments. Chromatin prepared from 2 × 10^6 $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ effector T cells were subjected to immunoprecipitation overnight at 4^{o}C with rabbit anti SMAR1, mouse anti SMAD 1/2/3, rabbit anti pSTAT3, mouse anti HDAC1, rabbit anti H3K9 Ac. or with normal rabbit and mouse IgG antibodies. Quantitative real time PCR was performed to determine

the relative abundance of target DNA. Specific primers for analysis of SMAR1 binding to STAT3 or STAT3 binding to Foxp3 and other target loci are listed in Table 2.

iT$_{reg}$ cell induction. CD4^{+}CD62L^{hi}CD25^{-} T cells were sorted and incubated at a density of 2 × 10^5 cells per well in 96 well flat bottom plate for 5 days in a complete media containing cross linked anti-CD3, 20 ng/ml IL-2 and 10 ng/ml TGF-β1. Cells were then stained for CD4, Foxp3 and IL-10 and assessed by flow cytometry.

Cell isolation. Lymphocytes isolated from MLNs, spleen, Peyer's patches and colon were grown in 10% FCS containing RPMI 1640 medium (complete medium). Colon IELs were obtained by incubating cleaned and cut tissue piece in a solution containing 1mM DTT in 1x PBS for 15 min, followed by two 15 min incubations in 10 ml of buffer containing 30 mM EDTA, pH8.0, and 10 mM HEPES in 1x PBS. Remaining tissue containing LP lymphocytes was digested for 90 min in complete medium containing 50 mg/ml collagenase D (Roche), 0.5 mg/ml DNase (Roche). This was followed by a gradient purification in 33% Percoll (Sigma-Aldrich).

***In vitro* Suppression assay.** CD4^{+}CD45RB^{hi} responder T cells and CD4^{+}CD25^{+} T$_{reg}$ cells were sorted. To assess the efficacy of T$_{reg}$ cell mediated immune suppression *in vitro*, 2 × 10^5 sorted responder T cells were labeled with CFSE and mixed with varying amount (as indicated) of T$_{reg}$ suppressor cells. Cell mixture was stimulated with soluble anti-CD3 antibody (2µg/ml), anti-CD28 antibody (1µg/ml) in the presence of 1 × 10^5 irradiated (3000 cGy) T cell depleted Splenocytes as APC. The proliferation of CD4^{+}CD45RB^{hi} responder T cells was assessed by CFSE dilution detected by flow cytometric analysis 72 h post stimulation.

Luciferase reporter assays. Transient transfection assays were performed in HEK 293T cell line using STAT3 promoter luciferase reporter construct. STAT3 promoter sequence was indentified, characterized using the BLAST, Pro-Scan and Clustal W analysis. A construct containing WT STAT3 promoter-pGL3 luciferase, and the mutated promoter-pGL3 luciferase was transfected in to HEK 293T cells. After transfection, cells were either left untreated or treated with IL-6 for 24 h at 37^{o}C. After 24 h, cells were processed using the Luclite "Constant Quanta" assay system (PerkinElmer) as per manufacturer's procedure and luminescence measured on the TopCount in single photon counting (SPC) mode.

Assessment of intestinal inflammation. Samples of proximal, mid and distal colon were prepared and inflammation was graded according to the following scoring system. Each sample was graded semi quantitatively from zero to three according to four criteria: (1) degree of epithelial hyperplasia and goblet cell depletion; (2) leukocyte infiltration in lamina propria; (3) area of tissue affected; and (4) the presence of markers of severe inflammation such as crypt abscesses, submucosal inflammation, and ulcers. Score for each criterion were added to give an overall inflammation score for each sample of 0-12. Scores from proximal, mid and distal colon were averaged to obtain inflammation score for the colon.

Histology. Colons were removed from mice, washed by PBS, fixed overnight in 10% buffered formalin, placed in 70% ethanol and embedded in paraffin. 5-mm sections were stained with H and E. Microscopic examination of the section was performed using Nikon confocal microscope.

Cytokine ELISA. The concentrations of IL-6, IL-17, TNF-α, IL-10 and IFN-γ in supernates of homogenized colon tissue and *in vitro* polarized iTreg cells were measured by ELISA using

commercial BD OptEIA Set kits (BD Biosciences). The assay was conducted according to the manufacturer's instructions.

Immuno-fluorescence staining. Purified naive $CD4^+$ T cells will cultured under neutral (Th0), iT_{reg} and nT_{reg} differentiation conditions. Cells were harvested and fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and incubated with respective antibodies. Counterstained using the anti-mouse and anti rabbit-fluorescently labeled secondary antibodies (Millipore, US). For nuclear staining, cells were treated with 4, 6- Diamidino-2-PhenylindoleDihydrochloride (Fluka). Coverslips were mounted with fluorescent mounting media (Dako) and examined with a Zeiss LSM-510 Meta Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging).

Cytokine and IgA assays. For cytokine analysis, proximal colonic tissue were homogenized in sterile 1× PBS supplemented with 1% triton X-100, protease and phosphatase inhibitor cocktail (sigma) and centrifuged for 15 min at maximum speed at 4^oC. Supernatants were used to measure different proinflammatory and inflammatory cytokine levels using ELISA kits (BD biosciences). IgA protein levels were evaluated on colonic homogenate supernatants or serum samples using an ELISA kit (BD biosciences).

Table1: Primer for real time PCR

Gene	Forward Primer	Reverse Primer
Foxp3	5'-GCATGTTCGCCTACTTCAGAAA-3'	5'-CCACTCGCACAAAGCACTTG-3'
Rorc	5'-CCGCTGAGAGGGCTTCAC-3'	5'-TGCAGGAGTAGGCCACATTACA-3'
IL-10	5′-TGGCCCAGAAATCAAGGAGC-3′	5′-CAGCAGACTCAATACACACT-3′
SMAR1	5′- GCATTGAGGCCAAGCTGCAAGCTC-3'	5′-CGGAGTTCAGGGTGATGAGTGTGAC-3'
STAT3	5'-ACCAACATCCTGGTGTCTCC-3'	5'-CATGTCAAACGTGAGCGACT-3'
β-Actin	5'- TCTACGAGGGCTATGCTCTCC-3'	5'- GGATGCCACAGGATTCCATAC-3'

Table2: Primer for ChIP assay

Promoter	Forward Primer	Reverse Primer
STAT3 (SP1 site)	5'-GTGCGTGTGCGGTACAGC-3'	5'-GGAGAGCAGCTAGGAGAAAGGG-3'
STAT3 (MAR I, IL-6RE)	5'-GTGTCTTGACGTCACGCACTGCCA GGAACT-3'	5'-AGAACTGCAGTGCGTGACGGTCCT TGAGTCG-3'
STAT3 (MAR II)	5'-CATCCCTCTAGCTCCTAAAATAA-3'	5'-TTCTGTGAGCTGACCAATCAG-3'
Foxp3 (SMAD region)	5'-CAGGCTGACCTCAAACTCACAAAG-3'	5'-CATACCCACACTTTTGACCTCTGC-3'
Foxp3 (CNS2 region)	5'-ACAACAGGGCCCAGATGTAGA-3'	5'-GGAGGTTGTTTCTGGGACATAGA-3'