Supplementary Materials and Methods

Mice and Cell lines

The female C57BL/6 mice at 6-8 weeks of age were purchased from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). *CD4*^{Cre}and *Foxp3*$^{YFP-Cre}$mice as described previously were purchased from Cyagen Biosciences. *Rag1*$^{-/-}$ mice were purchased from Model Animal Research Center. All strains of mice were housed in a specific pathogen-free facility. The experimental protocols were approved by the Animal Care and Use Committee of Medical School of Zhejiang University (Hangzhou, China). The $CD4^+$Foxp3/$CD25^+CD69^+$ and $CD4^+$Foxp3/$CD25^+CD69^-$ Tregs were sorted using the FACSDiVa system (Becton Dickinson), respectively.

Tregs suppression assay

The suppression of $CD4^+$ T cell proliferation in each group was determined by the CFSE incorporation assay. The murine splenic $CD4^+$ T cells were isolated using a $CD4^+$ T-cell isolation kit II (Miltenyi Biotec, Germany) and were labeled with CFSE (Invitrogen) according to the manufacturer's instruction. CFSE labeled $CD4^+$ T cells (1×10^6/ml) were stimulated with 1 μL of anti-CD3/CD28-coated beads (Invitrogen). Purified $CD69^{fl/fl}$-iTregs, $CD4^{Cre}CD69^{fl/fl}$-iTregs at a ratio of 1: 1, 1: 2, or 1:4 were added, the effector $CD4^+$ T cells served as the control. Three days later, the cells were harvested, and the proliferation of effector $CD4^+$ T cells was analyzed using flow cytometry.

ELISA

IL-10 and TGF-β1 levels in the supernatant of cultured cells and the IL-6, IFN-γ, and IL-17 levels in the colon of mice were measured using specific kits (eBioscience, USA) for ELISA. For analysis of colon explant cultures, the colons of mice were flushed with PBS containing 30% antibiotics, and open along a longitudinal axis. Then, pieces of tissue (~ 3-mm^2) were obtained from the distal colon and incubated for 24 hours in RPMI supplemented with 10% FCS and 20% antibiotics (1 punch biopsy/100 ml medium). Supernatants were collected and kept frozen until assessment. For the detection of TGF-β1**,** 100 μL of supernatants were acidified with 20 μL of 1N HCl at room temperature for 10 min and then neutralized with 20 μL of 1N NaOH to activate latent TGF-β1 into its immunoreactive form.

31 **RNA isolation, cDNA synthesis, and quantitative real-time PCR**

32 Total RNA from the indicated types of cells was extracted using Trizol reagents, and
33 the RNA samples were reverse transcribed into cDNA using a high-capacity cDNA
34 Reverse Transcription Kit (Thermo Fisher, USA). The relative levels of target mRNAs
35 were determined by qRT-PCR. The sequences of primers are listed in Table S1. Data
36 were analyzed using the comparative Ct method using β-actin as the normalization
37 control.

38 **Confocal microscopy**

39 $CD4^+$ cells seeded on chamber slides were cultured overnight. After treatment with
40 MG132 for 2h, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1%
41 Triton X-100/PBS for 10 min. After blocking with 5% bovine serum albumin, cells
42 were incubated with rat anti-HSF1 monoclonal antibody followed by decoration with
43 fluorescein-conjugated anti-rabbit IgG. The images were acquired on confocal
44 microscope.

45 **FACS**

46 To analyze the differentiation of Th1 or Th17 cells, $CD4^+$ T cells were incubated with
47 the cell stimulation cocktail (eBioscience, USA) for 5 h at 37 °C and stained with PE-
48 Cy7-anti-CD4 antibodies. Cells were fixed and stained with PE-anti-IFN-γ (Invitrogen,
49 Clone:XMG1.2; Catalog: 12-7311-82), APC-anti-IL-17 (Invitrogen, Clone: eBio17B7;
50 Catalog: 17-7177-81) antibodies, or isotype controls after permeabilization. The
51 percentages of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells were determined by flow cytometry.

52 **Protein half-life assay**

53 $CD4^+$ T cells were pretreated with or without MG132 (Sigma Aldrich) for 2 hours then
54 incubated with 50 µg/ml CHX (Sigma Aldrich) for 0,6, 12, 18 hours or 25 before
55 Western blot analysis.

56 **Colitis induction**

57 *CD69*$^{fl/fl}$ or *Foxp3*$^{YFP\text{-}Cre}$*CD69*$^{fl/fl}$ Mice were orally given 2% DSS (MW 36,000-
58 50,000, MP Biomedicals,USA) in drinking water for 5 days, followed by regular
59 drinking water without DSS. Body weight was measured every 24 h during the

60 experiment and mice were sacrificed at the indicated day. For T-cell transfer-induced
61 colitis, $CD4^{+}CD45RB^{hi}$ T-cells from C57BL/6 mice were enriched ($CD4^{+}$ T-Cell
62 Isolation Kit; Miltenyi Biotec) and single-cell suspensions were stained with APC-
63 anti-CD4 (GK1.5), and PE-anti-CD45RB (C363.16A), all from eBioscience, followed
64 by cell sorting (FACSAriaII) (purification > 99%). $Rag1^{-/-}$ recipient mice received 5
65 $\times 10^5$ $CD4^{+}CD45RB^{hi}$ T-cells by intravenous (i.v.) injection, 1 $\times 10^6$ Control-
66 iTregs or PSI-iTregs were injected i.v. 21 days later. At the end of the experiment, the
67 large intestines of individual mice were dissected out and fixed in 10% phosphate-
68 buffered formalin.

69 **Statistical analyses**

70 Data are expressed as the mean ± SD. The difference among the groups was were
71 analyzed by one-way ANOVA and Student *t* test where applicable using GraphPad
72 Prism 8. $P < 0.05$ was considered statistically significant.

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74 **Table S1 The sequences of primers**

Real-Time PCR primers		
Gene	**Forward primer（5'-3'）**	**Reverse primer (5'-3')**
IL-10	CCA AGC CTT GGA AA GA	TTT TCA CAG GGG AGA AAT CG
TGF-β1	AACTGCACCCACTTCCCAGTC	CATTAAGGAGTCGGTTAGCAG
HSPA1A	TGGTGCAGTCCGACATGAAG	GCTGAGAGTCGTTGAAGTAGGC
DNAJB1	TTCGACCGCTATGGAGAGGAA	CACCGAAGAACTCAGCAAACA
HSP90AB1	GTCCGCCGTGTGTTCATCAT	GCACTTCTTGACGATGTTCTTGC
Actin	AACAGTCCGCCTAGAAGCAC	CGTTGACATCCGTAAAGACC

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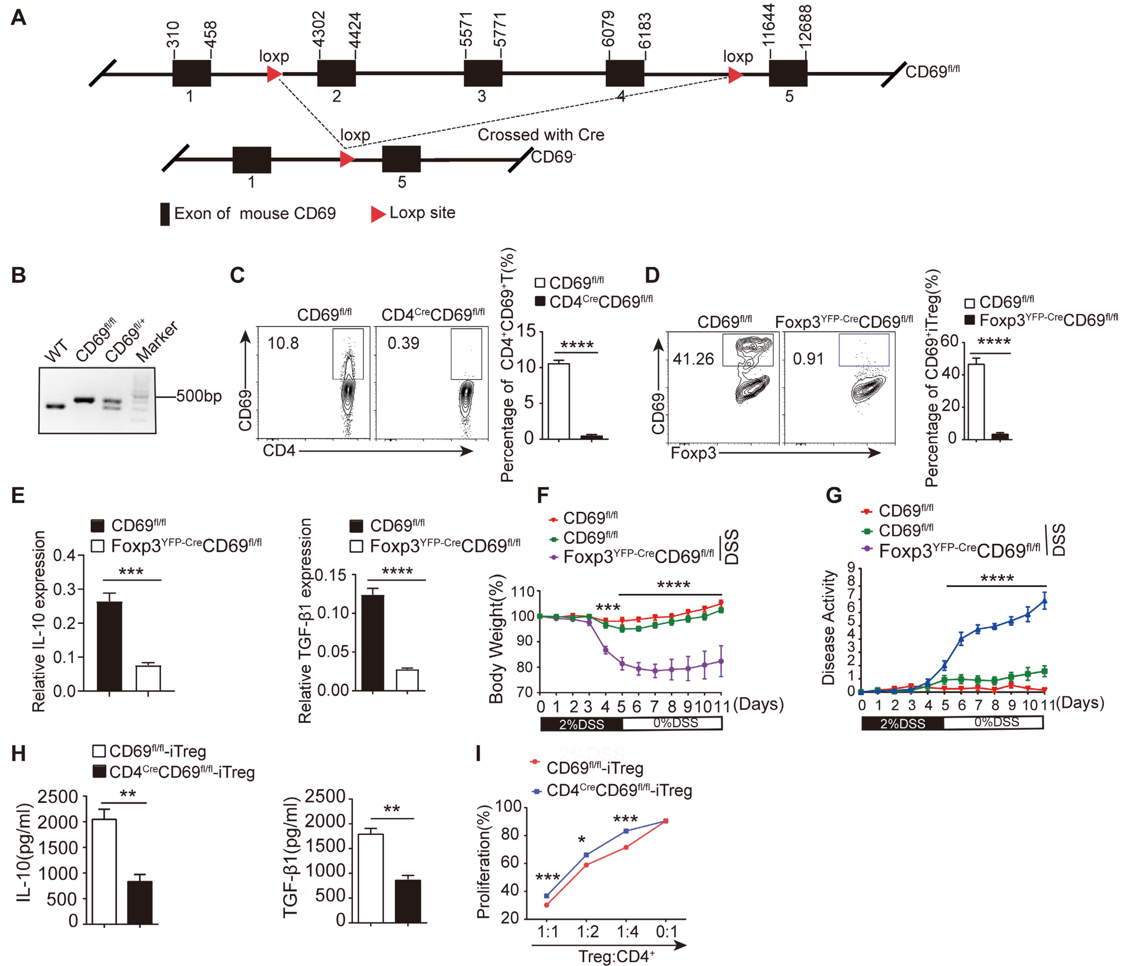

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85 **Figure S1. Conditional targeting of the mouse *CD69* gene in Treg exacerbated**

86 **DSS-induced colitis.** (**A**) Strategy of *CD69* conditional knockout mice. (**B**), Genomic

87 DNA was isolated from tail of indicated mice and then used for PCR analysis of

88 *CD69* deletion and the *LoxP*-flanked allele. (**C** and **D**) Phenotype of $CD69^{fl/fl}$ ×

89 $CD4^{Cre}$ *or* $Foxp3^{YFP\text{-}Cre}$ offspring, MLN from conditional knock-out mice were used

90 for analysis the expression of $CD69^{+}$ Tregs. (**E**) The expression of IL-10 and TGF-β1

91 in Treg from $CD69^{fl/fl}$ or $Foxp3^{YFP\text{-}Cre}CD69^{fl/fl}$ mice were analysis by qRT-PCR. (**F** and

92 **G**) $CD69^{fl/fl}$ and $Foxp3^{YFP\text{-}Cre}CD69^{fl/fl}$ mice were administrated with 2.0% DSS in

93 drinking water for 5 days, and then they were given by normal water in the following

94 days. Average body weight is shown as percentage relative to initial value. Results are

95 means ± SD (n = 7). (**H**) The IL-10 and TGF-β1 in supernatant of induced

96 $Foxp3^{+}CD4^{+}$Tregs from $CD69^{fl/fl}$ and $CD4^{Cre}CD69^{fl/fl}$ mice were analysis by ELISA.

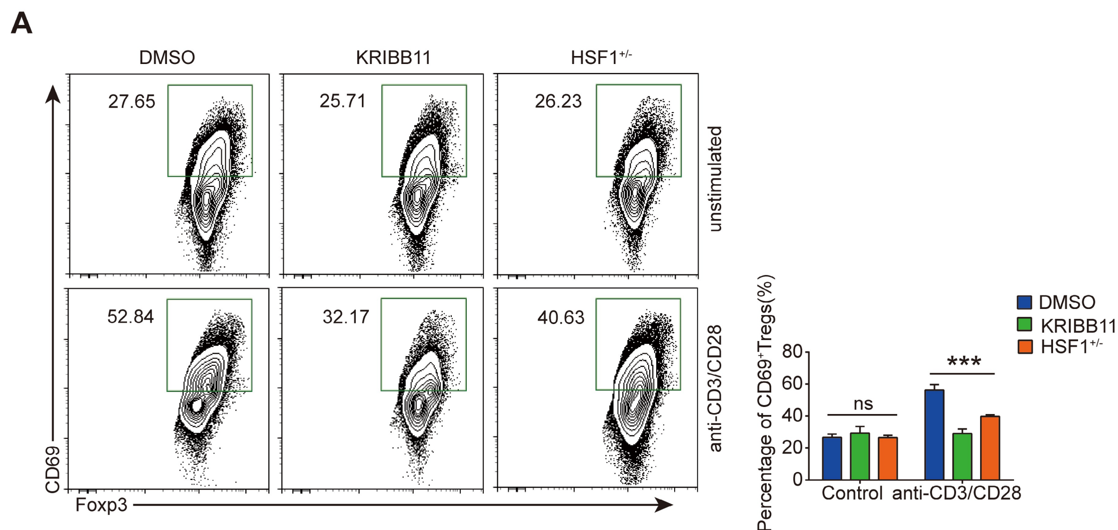
97 (**I**) The proliferation of $CD4^{+}$ T cells was analyzed using flow cytometry. The cells

98 were first gated on living lymphocytes and then on $CFSE^+$ T cells (n = 3). Data are

99 representative of three independent experiments. Comparisons were made using one-

100 way ANOVA or Student's unpaired *t* test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$;

101 $^{****}P < 0.0001$.



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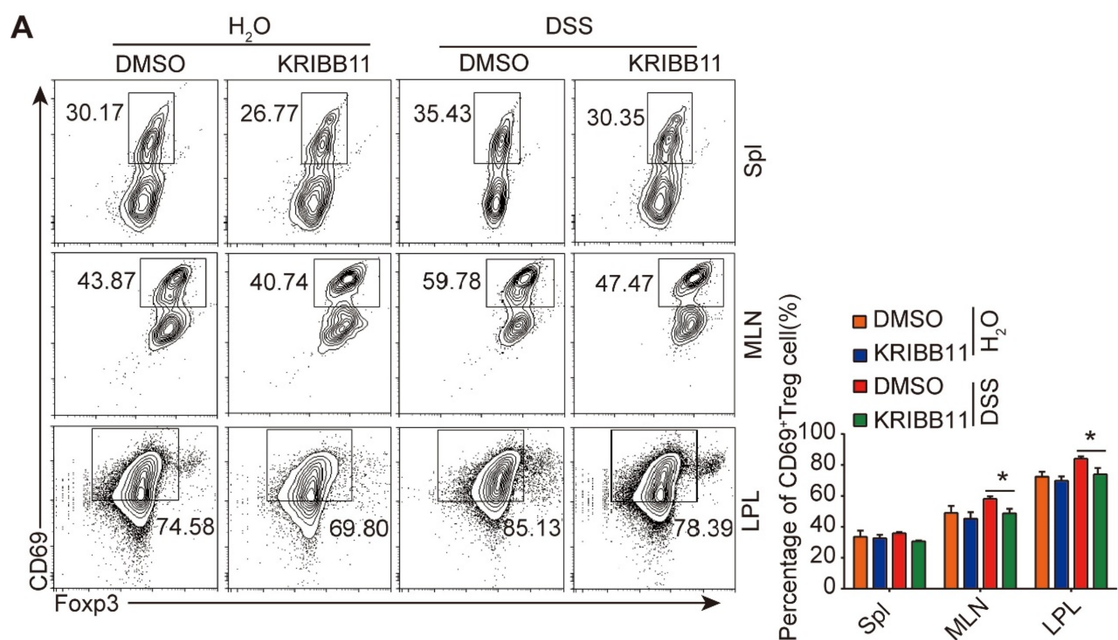
103 **Figure S2. Inhibition of HSF1 reduce CD69 expression of Tregs stimulation with**

104 **anti-CD3/CD28. (A)** 1×10^6/ml Tregs were stimulated with or without anti-

105 CD3/CD28 antibodies and treated with KRIBB11for the indicated time. Relative

106 levels of CD69 expression in different groups of Tregs were determined by FACS.

107 Results are means ± SD. *** $p < 0.001$, as analyzed by Student's unpaired *t* test.



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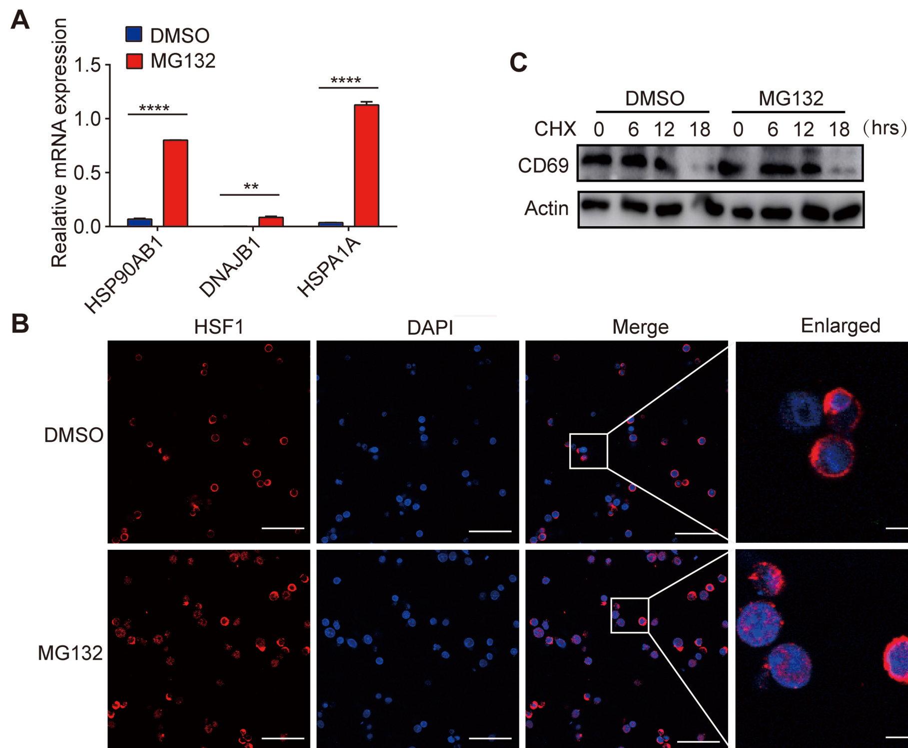
109 **Figure S3. The differentiation of CD69^{+} Treg cells is inhibited in DSS induced**
110 **colitis mice treated with KRIBB11**. (**A**) Spleen, MLN and colonic lamina propria cells
111 were isolated from control or mice injected with HSF1 inhibitor KRIBB11 after
112 administration of DSS for 5 days. The frequency of Tregs and CD69^{+} Tregs was
113 detected by flow cytometry. The graph shows the average percentage of Foxp3^{+}cells
114 among CD4^{+} T cells and CD69^{+} cells among Tregs. Results are means ± SD (n = 5 mice
115 per each group). *$p < 0.05$, as analyzed by Student's unpaired *t* test.



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117 **Figure S4. MG132 stabilized HSF1 protein to activate the transcription of its**
118 **target genes.** (**A**) *HSP90AB1*, *HSP90A1A* and *DNAJB1* normalized to β-actin were
119 analyzed by qRT-PCR; (**B**) CD4^{+} T cells were treated with MG132 for 2 h, HSF1
120 immunoflurescence staining was performed. (**C**) CD4^{+} T cells were treated with CHX
121 alone or pretreatment with MG132 for time periods as indicated and CD69 protein
122 level was measured by western blotting. The date are presented as the means ± SD of
123 three repeated experiments. **$p < 0.01$, ****$p < 0.0001$ as analyzed by Student's
124 unpaired *t* test.
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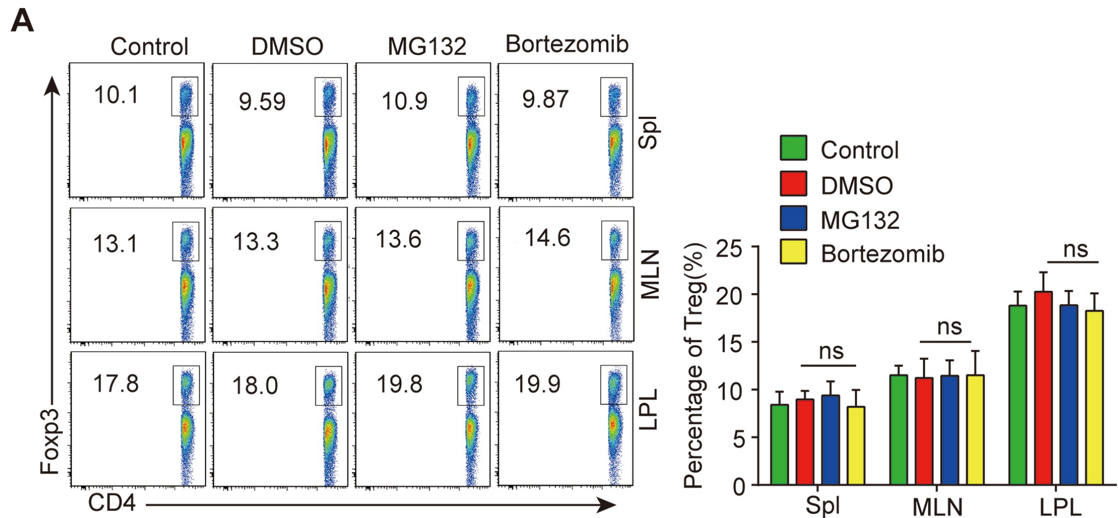

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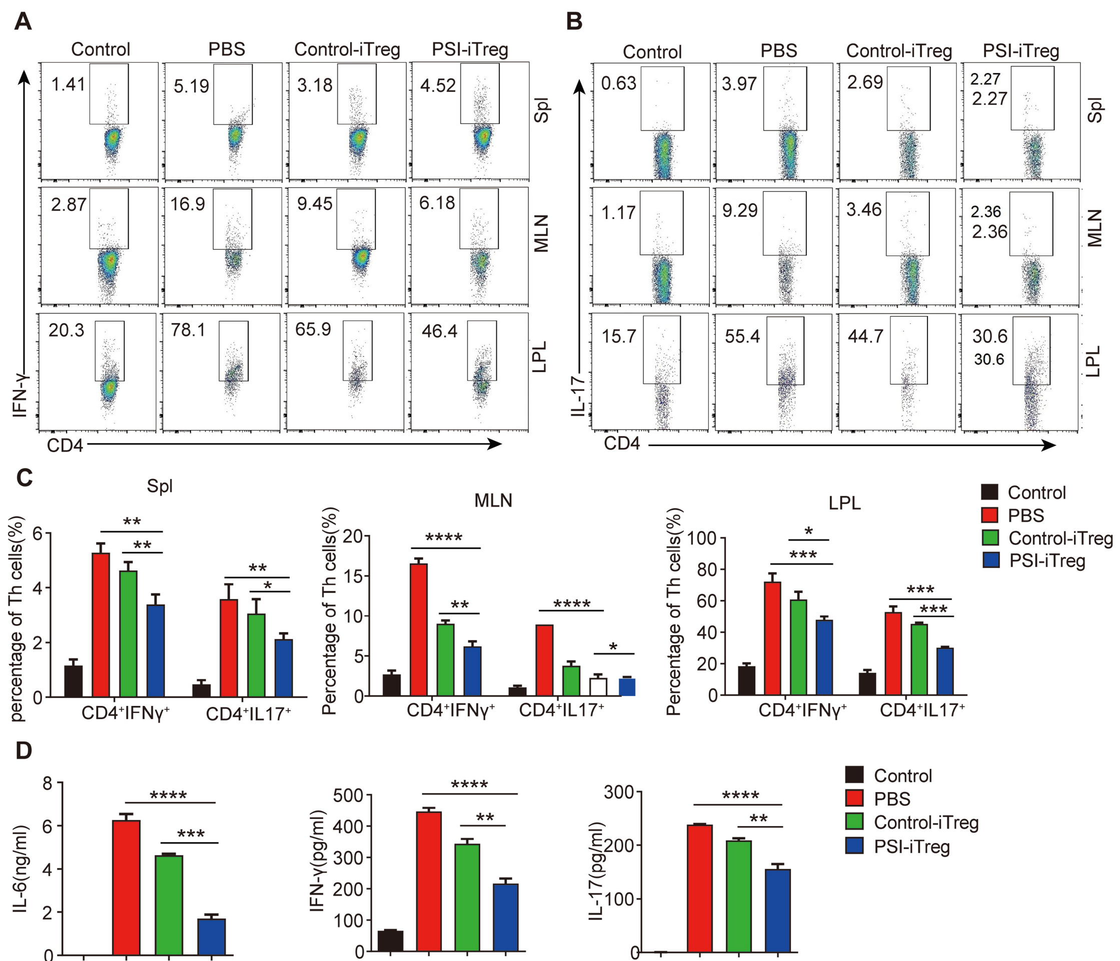
127 **Figure S5. (A)** Density plots showing total $CD4^{+}Foxp3^{+}$ cells from freshly isolated
128 spleen, MLN and colonic LPL in mice treated with MG132 and Bortezomib.
129 Representative images of the data expressed as mean ± SD of three independent
130 experiments. ns, not significant, as analyzed by ANOVA or Student's *t*-test.



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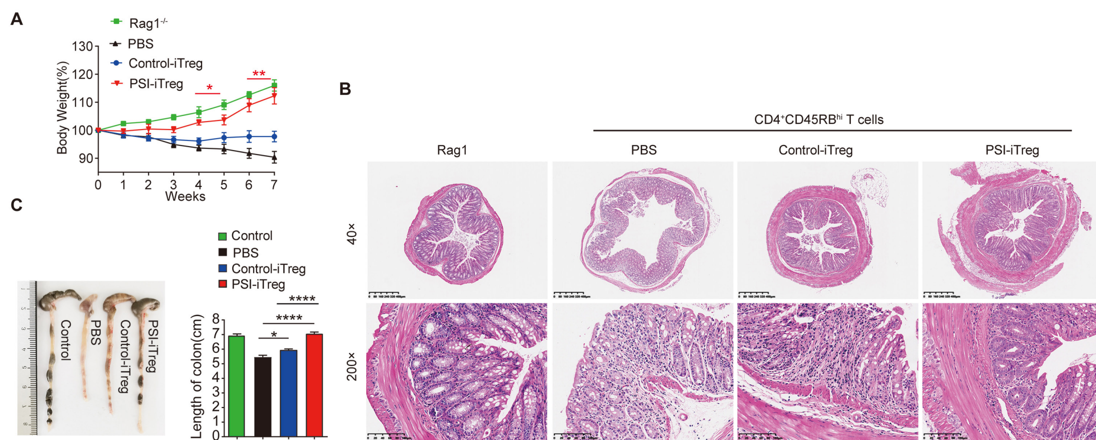
132 **Figure S6. PSI-iTreg prevents mice from exacerbation of DSS-induced colitis.** (**A-**
133 **C**) Each group of T cells was stimulated with the cell stimulation cocktail for 6 h and
134 then stained with anti-mouse CD4 and anti-IFN-γ or anti-IL-17 antibodies, followed
135 by flow cytometry. (**D**) Colon tissue was cultured overnight, and cytokines in the
136 supernatant were measured by ELISA (n = 5). Representative images of the data
137 expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$,
138 $^{***}p < 0.001$, $^{****}p < 0.0001$, as analyzed by One-way ANOVA or Student's
139 unpaired *t* test.



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141 **Figure S7. Adoptive transfer of PSI-iTregs inhibits the disease progression in a**
142 **mouse model of T-cell transfer-induced colitis.** Spleen cells from C57BL/6 mice
143 were enriched for $CD4^{+}$ T-cells and then stained with anti-CD4 and anti-CD45RB
144 monoclonal antibodies. Then $CD4^{+}Foxp3^{-}CD45RB^{hi}$ T-cells were sorted and injected
145 i.v into immunodeficient *Rag1*$^{-/-}$ mice. Groups of mice were injected i.v with Control-
146 iTregs and PSI-iTregs (1×10^{6}/mouse/injection) on days 21 (n = 7). (**A**) The body
147 weights were measured for 7 weeks. Each point represents average weight data
148 pooled from 7 mice ± SD. (**B**) Histological appearance 7 weeks after colitis induction.
149 **(C)** Appearance and statistical analysis of colon length. Representative colonic
150 sections stained with H&E (Magnification: 40 × and 200 ×). Date are representative
151 images or expressed as the mean ± SD of three independent experiments (n = 7 per
152 group). $^{*}P < 0.05$, $^{**}P < 0.01$, analyzed by ANOVA and Student's t-test.