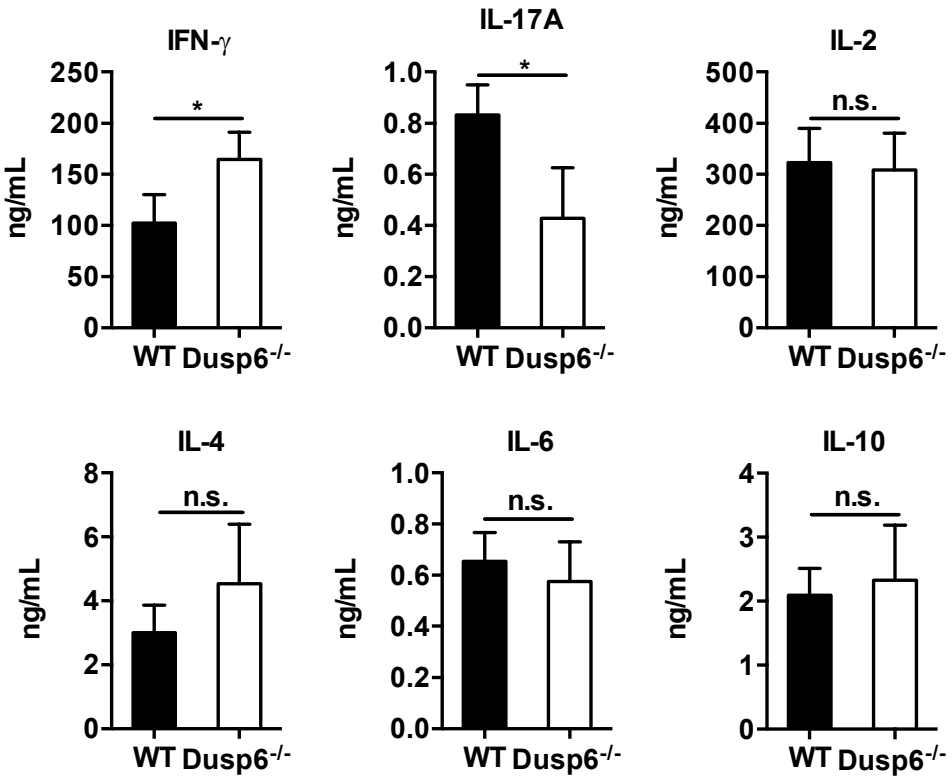
SUPPLEMENTARY MATERIAL FOR

Dual Specificity Phosphatase 6 (DUSP6) regulates CD4+ T cell functions and restrains the spontaneous colitis in IL-10 deficient mice

Samuel Bertin[2*], Beatriz Lozano-Ruiz[1*], Victoria Bachiller[1], Irma García-Martínez[1], Scott Herdman[2], Pedro Zapater[1], Rubén Francés[1], José Such[1], Jongdae Lee[2], Eyal Raz[2] and José M González-Navajas[1,2]

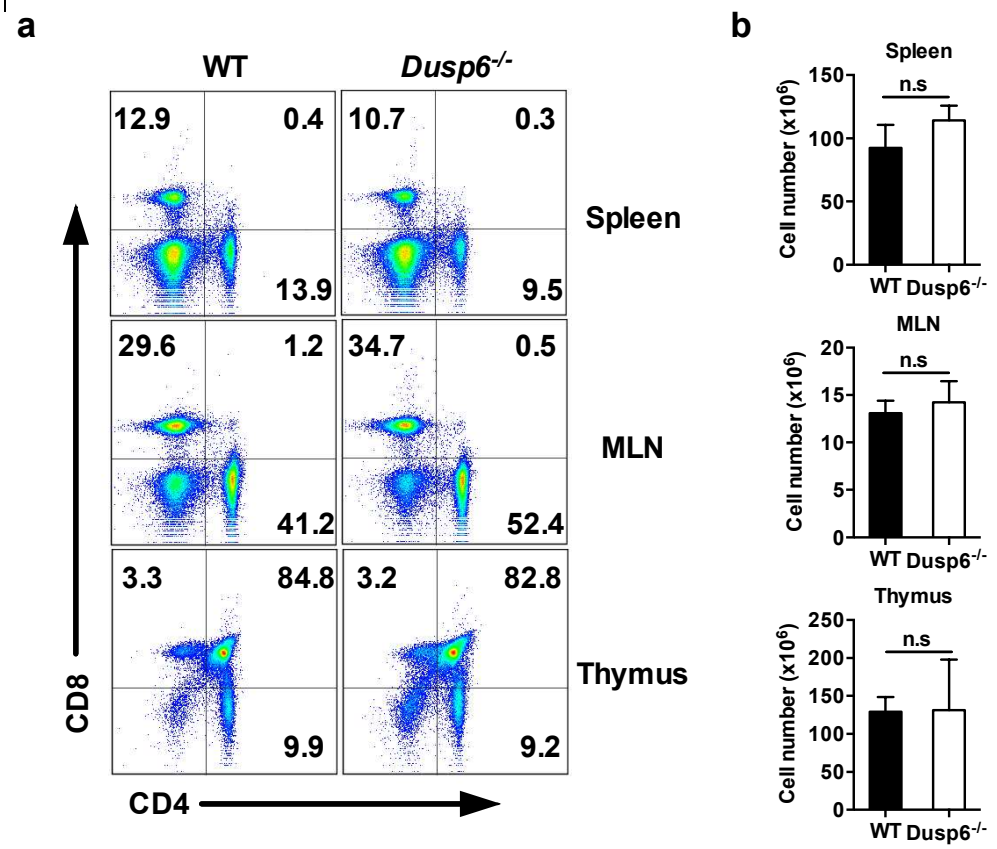
* These authors contributed equally to this work

1. Networked Biomedical Research Center for Hepatic and Digestive Disease (CIBERehd). Institute of Health Carlos III, Madrid. Spain.
2. Division of Rheumatology, Allergy and Immunology. University of California San Diego. La Jolla, CA. USA.



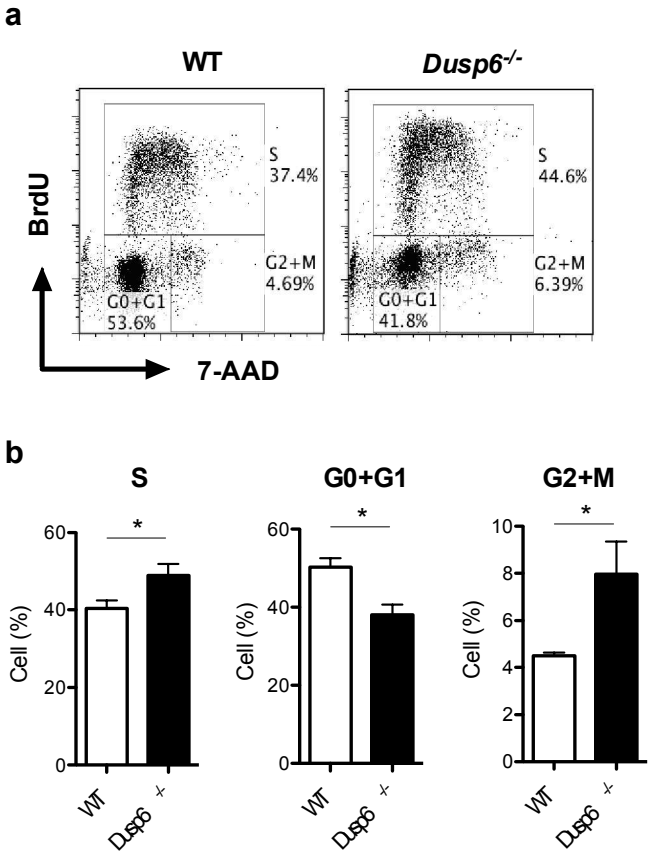
Supplementary Figure S1: Cytokine production in splenic CD4$^+$ T cells from WT and *Dusp6*$^{-/-}$ mice

Cytokine levels in culture supernatants of splenic CD4$^+$ T cells, isolated from WT and *Dusp6*$^{-/-}$ mice and stimulated with anti-CD3/28 antibodies for 24 hours. Data represent pooled results from three independent experiments with at least 3 mice per group. Error bars represent mean ± standard deviation. n.s: not significant, *P<0.05 (two-tailed Mann-Whitney U test).



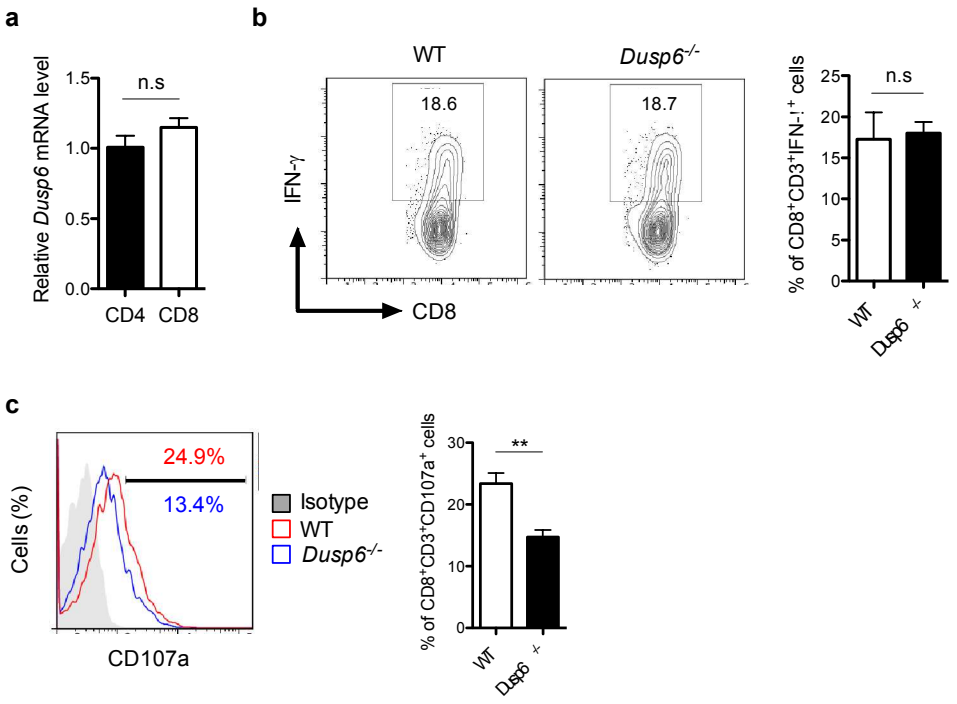
Supplementary Figure S2: T cell development in the thymus is unaltered by DUSP6

(a) Flow cytometry analysis of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ populations in the thymus, spleen, and mesenteric lymph nodes (MLN) of 5 weeks-old WT and *Dusp6*$^{-/-}$ mice. Data are representative of two independent experiments with 3 mice per group. **(b)** Microscopic counting of total $CD4^+$ cell numbers in the organs of 5 weeks-old WT and *Dusp6*$^{-/-}$ mice. Data represents pooled results from two independent experiments. Error bars represent ± standard deviation. n.s: not significant (two-tailed Mann-Whitney *U* test)



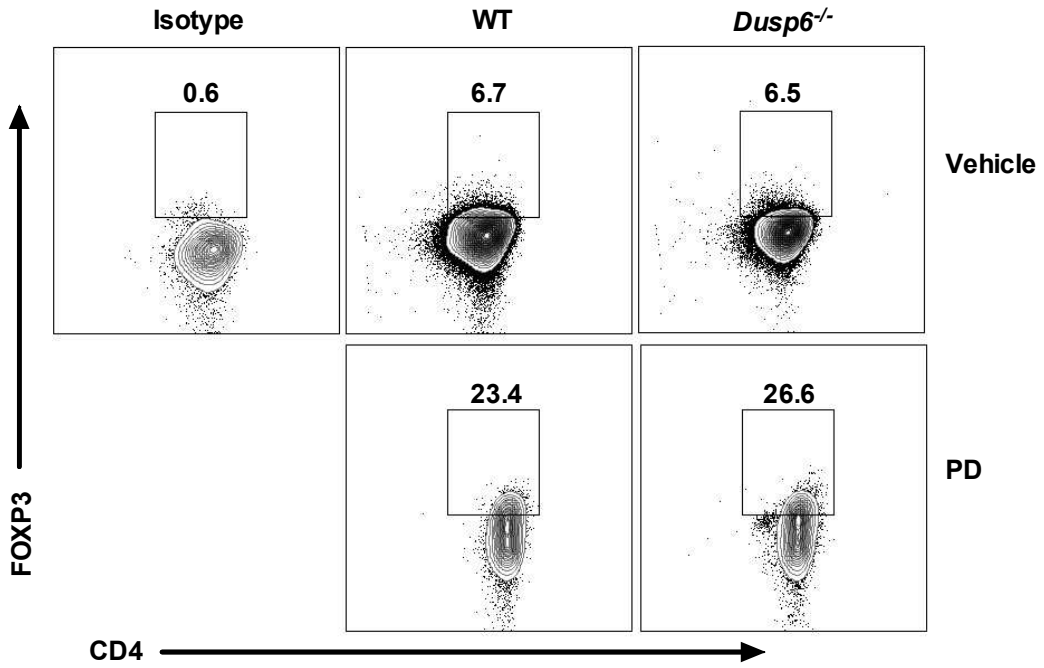
Supplementary Figure S3: *Dusp6*$^{-/-}$ CD4^{+} T cells display increased proliferation capacity

Naïve CD4^{+} T cells were isolated and stimulated with 1 μg/mL of goat anti-hamster antibody (plate-bound) in complete RPMI medium with soluble anti-CD3 antibody (1 μg/mL) and anti-CD28 antibody (2 μg/mL) for 72 hours. Cells were pulsed with BrdU 1 hour before collection and stained with FITC-conjugated anti-BrdU Ab and 7-AAD following the manufacturer's instructions (BrdU Flow kit #559619;BD Biosciences). **(a)** BrdU incorporation and DNA content on a per-cell basis were analyzed by flow cytometry. Cell cycle phases are clearly distinguished in plots showing 7-AAD *vs.* BrdU-FITC and presented as dot plots. **(b)** Statistical analysis of the differences in the percentage of cells in each cell cycle between WT and *Dusp6*$^{-/-}$ naïve CD4^{+} T cells. Data represent results from two independent experiments. *$P < 0.05$ (two-tailed Mann-Whitney *U* test).



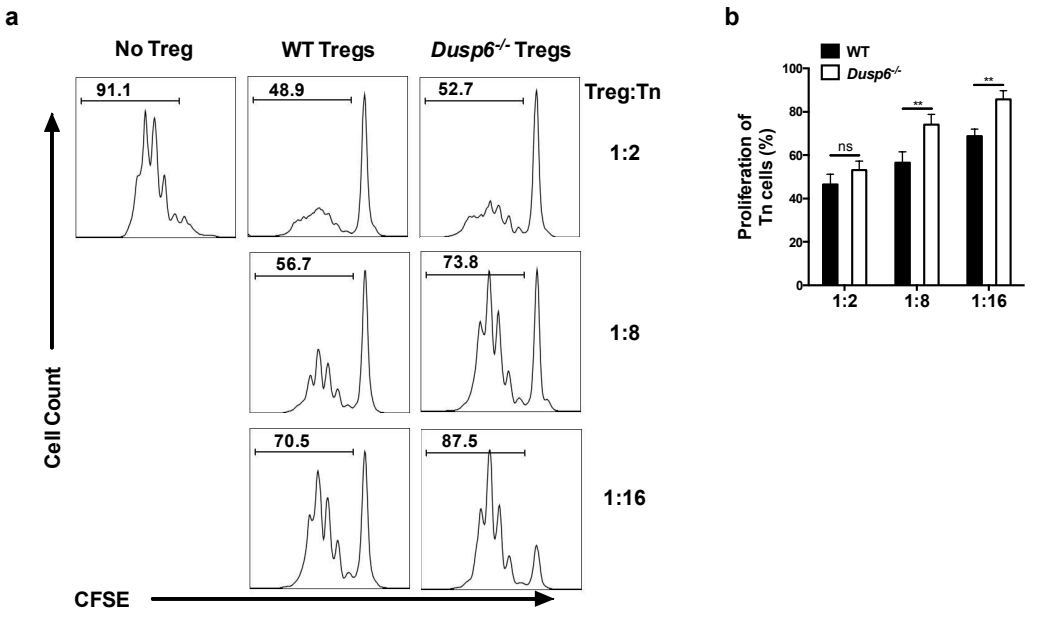
Supplementary Figure S4: *Dusp6*$^{-/-}$ CD8^{+} T cells have decreased CD107a (LAMP-1) mobilization but normal IFN-γ production

(a) Assessment of *Dusp6* mRNA expression levels by qPCR in CD4^{+} and CD8^{+} T cells isolated from the spleen of WT mice. **(b)** Splenocytes were isolated from WT and *Dusp6*$^{-/-}$ mice and cultured for 48h in medium supplemented with concanamycin A (ConA; 2 μg/mL) and IL-2 (20 ng/mL) in round-bottom 96-well plates. The stimulated lymphoblasts were then re-stimulated with 1 μg/mL of goat anti-hamster antibody (plate-bound) in complete RPMI medium with soluble anti-CD3 antibody (1 μg/mL) and anti-CD28 antibody (2 μg/mL). After 2 hours, Golgi Plug was added to the culture and the cells were harvested after another 4 hours to analyze IFN-γ production by intracellular cytokine staining on gated CD3^{+}CD8^{+} T cells. **(c)** CD8^{+} cells were stimulated as in (b) for 6 hours and then harvested and stained with APC-conjugated anti-CD8, PE-Cy7-conjugated anti-CD3, and PE-conjugated anti-CD107a antibodies and the expression of CD107a was analyzed on gated CD3^{+}CD8^{+} T cells. Data represents mean ± SEM (n=4 mice per group). n.s: not significant, $^{**}P<0.01$ (two-tailed Mann-Whitney *U* test).



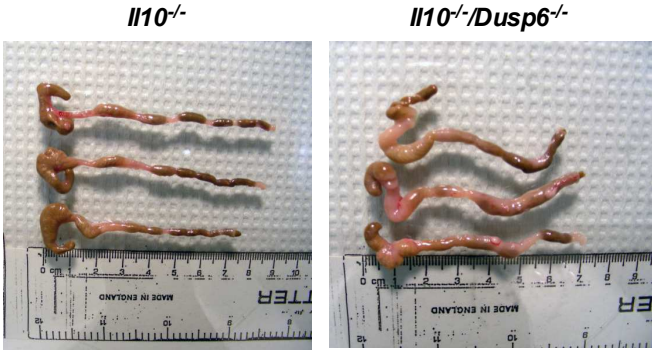
Supplementary Figure S5: ERK inhibition promotes regulatory T cell differentiation *in vitro*

Representative contour plots of FOXP3 expression in FACS-sorted naïve $CD4^+$ T cells from WT and *Dusp6*$^{-/-}$ mice cultured in regulatory T cell (Treg) differentiation conditions for 5 days, treated with the MEK1/2 inhibitor PD0325901 (PD) or left untreated (vehicle solution). Numbers within the graphs denote the percentage of $FOXP3^+$ cells compared with cells stained with isotype antibody. Data are representative of two independent experiments with 3 mice per group.



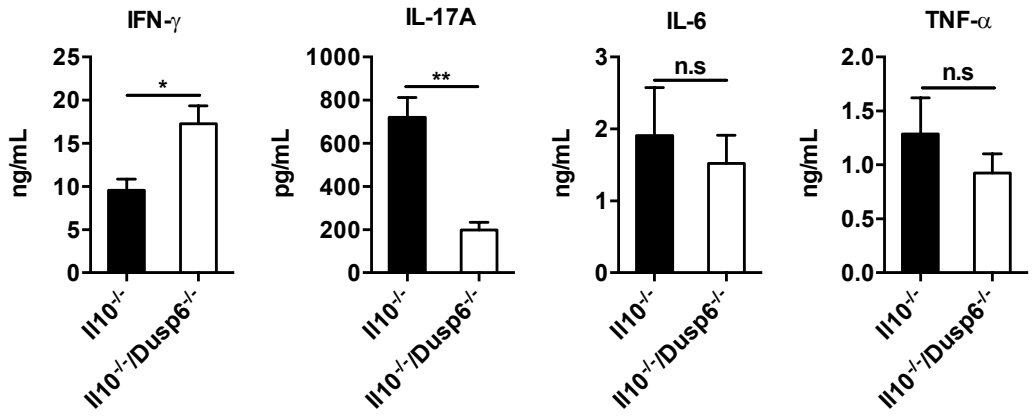
Supplementary Figure S6: Suppressive ability of WT and *Dusp6*$^{-/-}$ Tregs

(a) Flow cytometry analysis of CFSE-labeled naïve T cells from WT mice stimulated with anti-CD3/28 antibodies for 72 hours and co-cultured with regulatory T cells (Tregs; $CD4^{+}CD45RB^{low}CD25^{+}$) isolated from spleens of WT or *Dusp6*$^{-/-}$ mice. Naïve and regulatory cells were co-cultured at a ratio of 1:2, 1:8, and 1:16 (Treg:Tnaive). (n=2 mice per group). **(b)** Percentage of proliferating naïve T cells from WT mice co-cultured with either WT or *Dusp6*$^{-/-}$ Tregs at a ratio of 1:2, 1:8, and 1:16. Data are representative of 2 independent experiments. Error bars represent standard deviation. ns: not significant, $^{**}P<0.01$ (two-tailed Mann-Whitney *U* test).



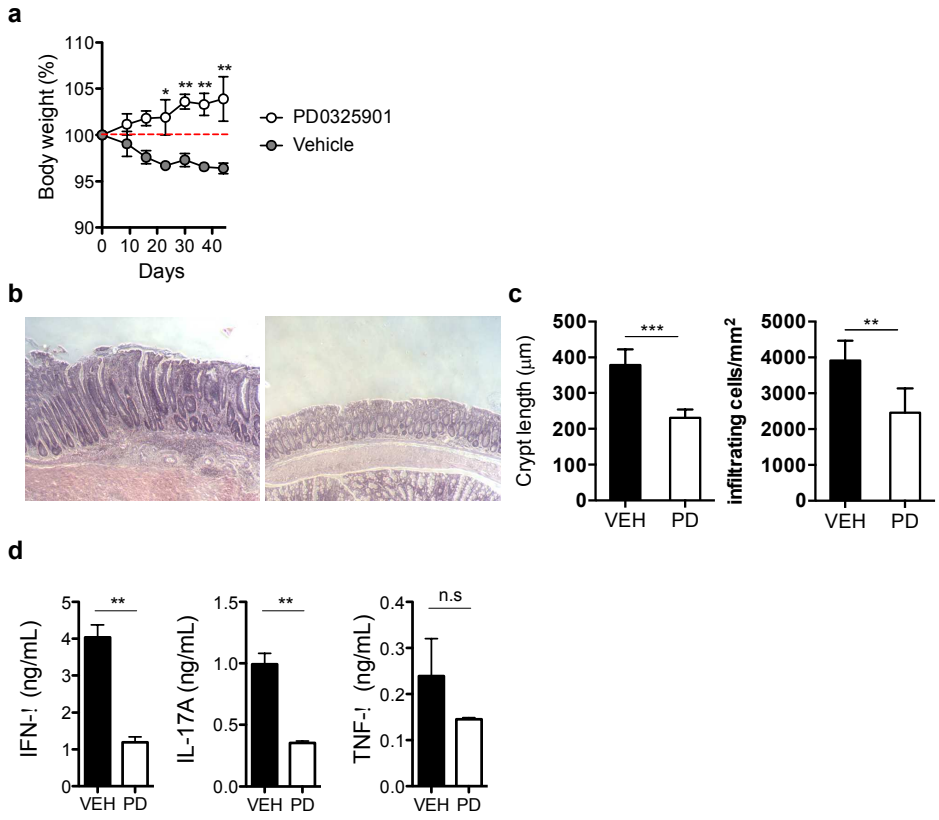
Supplementary Figure S7: Overt signs of inflammation in *Il10*$^{-/-}$*/Dusp6*$^{-/-}$ mice at 10 weeks of age

Representative pictures of colons obtained from *Il10*$^{-/-}$ and *Il10*$^{-/-}$*/Dusp6*$^{-/-}$ mice. Macroscopic signs of intestinal inflammation such as thickening of the intestinal wall and diarrhea were evident in the colon of *Il10*$^{-/-}$*/Dusp6*$^{-/-}$ mice at 10 weeks of age.



Supplementary Figure S8: Cytokine production in splenic CD4+ T cells from *Il10*$^{-/-}$ and *Il10*$^{-/-}$*/Dusp6*$^{-/-}$ mice

Cytokine levels in culture supernatants of splenic CD4^{+} T cells isolated from *Il10*$^{-/-}$ and *Il10*$^{-/-}$*/Dusp6*$^{-/-}$ mice and stimulated with anti-CD3/28 antibodies for 24 hours. Data represent pooled results from two independent experiments with at least 6 mice per group. Error bars represent standard deviation. n.s: not significant (two-tailed Mann-Whitney *U* test).



Supplementary Figure S9: ERK inhibition ameliorates preexisting colitis in *Il10*$^{-/-}$/*Dusp6*$^{-/-}$ mice

Six months old *Il10*$^{-/-}$/*Dusp6*$^{-/-}$ mice with signs of overt colitis were treated with the ERK1/2 inhibitor PD0325901 (PD) at a dose of 25 mg/Kg, or its vehicle (VEH), three times a week by gavage for a period of 6 weeks. **(a)** Percentage of initial body weight of *Il10*$^{-/-}$/*Dusp6*$^{-/-}$ mice treated with either PD or vehicle. **(b)** Representative micrographs of colon sections stained with H&E (magnification x100). **(c)** Quantitative measurement of crypt length and cellular infiltration in PD-treated or vehicle-treated mice. **(d)** Cytokine levels in culture supernatants of MLN-derived CD4^{+} T cells isolated from both groups of mice and stimulated with anti-CD3/28 antibodies for 24 hours. Error bars represent standard deviation (n=3-4 mice per group). n.s: not significant, **P*<0.05, ***P*<0.01,

***P<0.001 (**[a]** two-way ANOVA with post hoc Bonferroni test; **[b-d]** two-tailed Mann-Whitney *U* test).