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Supplemental Information

Salt Sensing by Serum/Glucocorticoid-Regulated Kinase 1 Promotes Th17-like Inflammatory Adaptation of Foxp3^{+} Regulatory T Cells

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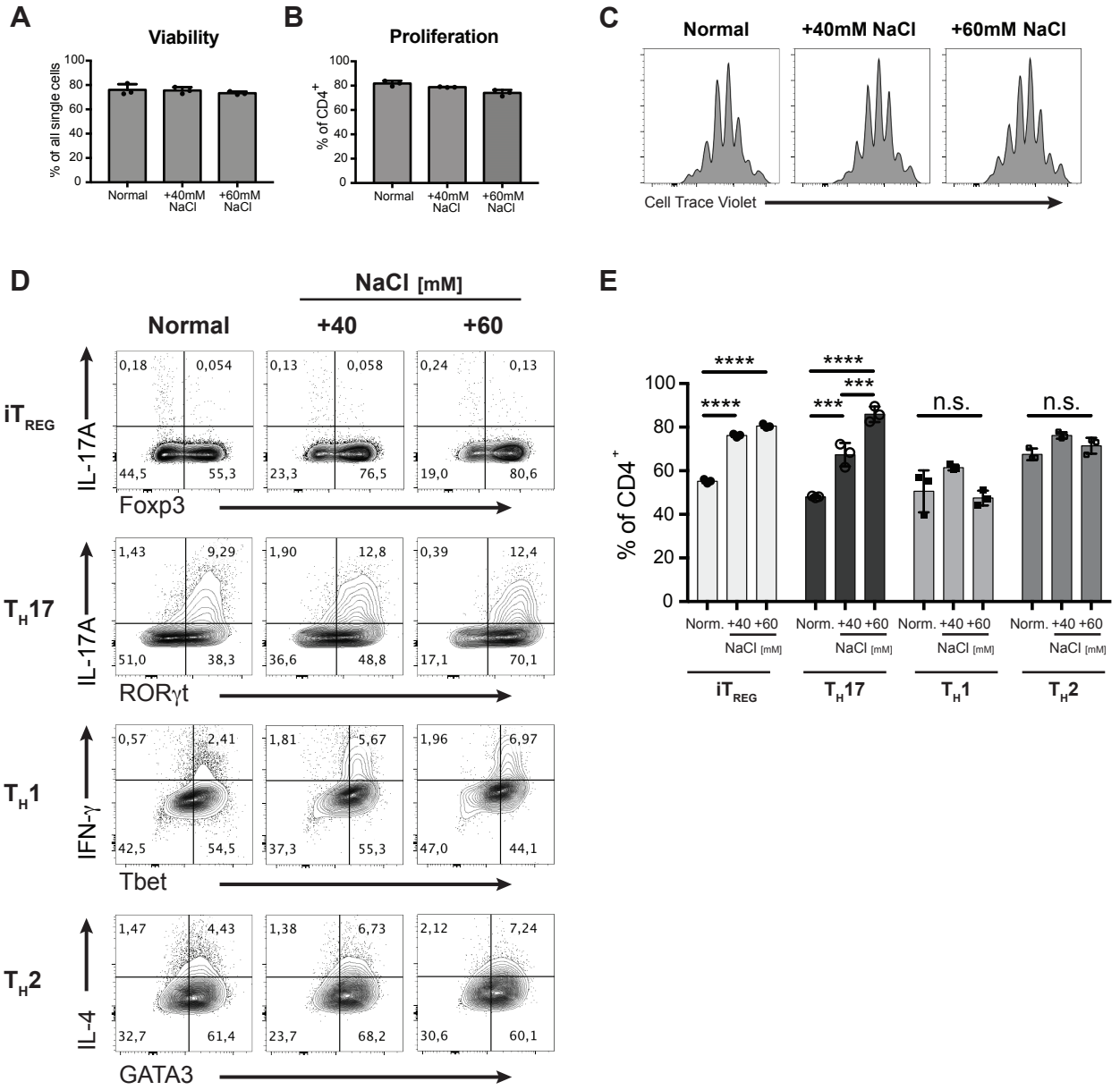
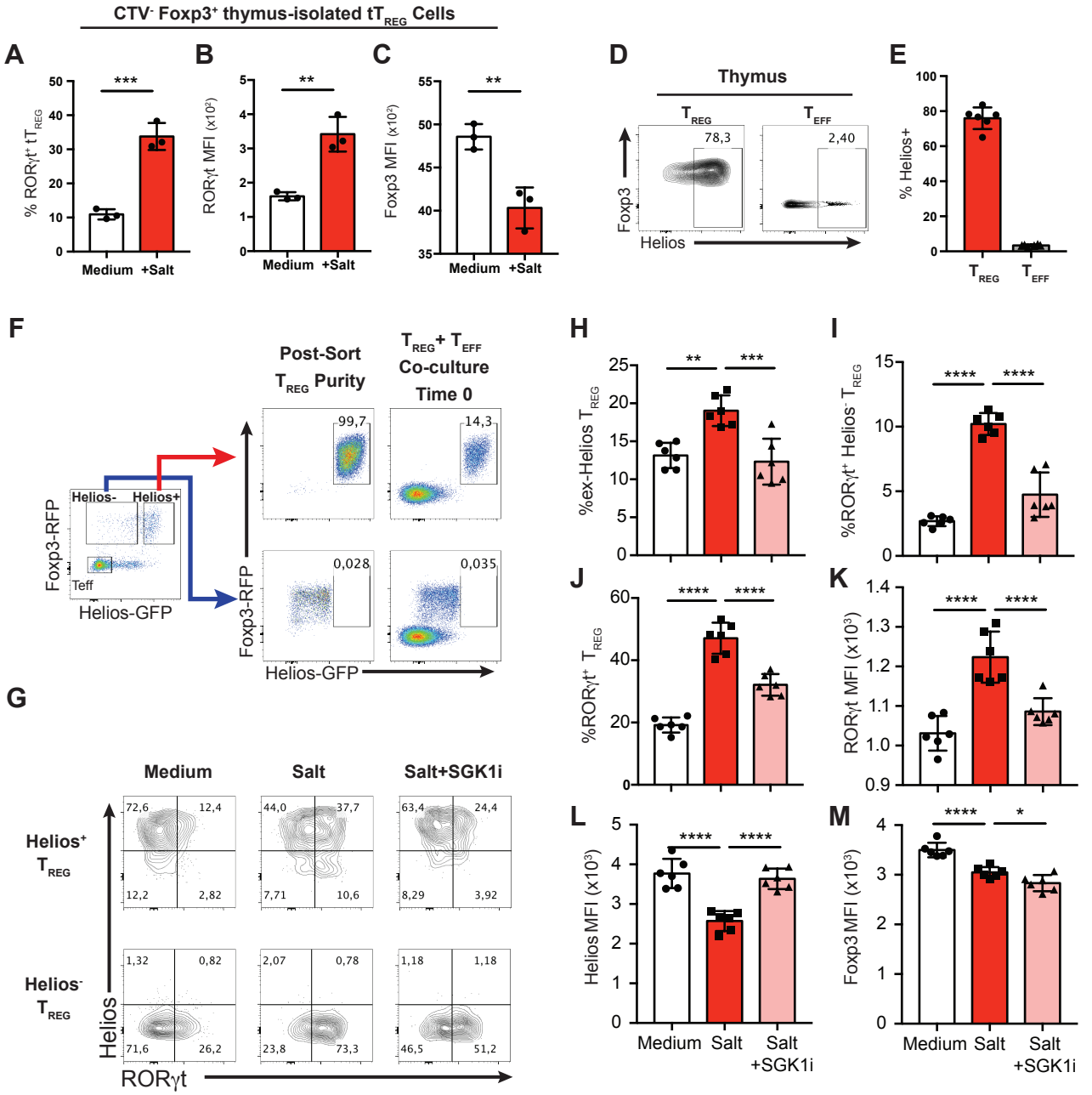


Supplemental Figure 1. Moderate increase in NaCl concentration does not significantly reduce CD4⁺ T cell viability and proliferation, but influence differentiation pathways that involves TGFβ *(Related to Figures 2 and 5)*.

(A) CD4⁺ T cells were labelled with CTV proliferation dye and cultured *in vitro* for 3 days under the indicated concentration of NaCl. Quantification of the frequency of total viable cells at day 3.
(B) Quantification of the frequency of proliferating cells at day 3 assessed by CTV proliferation dye dilution
(C) Representative flow cytometry histograms depicting the proliferative capacity of CD4⁺ T cells cultured under indicated salt concentrations
(D) FACS-purified CD4⁺ T_{EFF} cells were in vitro differentiated with supplementation of polarizing cytokines (IL-12, IL-4, TGFb and IL6, and TGFb) to various lineages (T_H1, T_H2, T_H17, and iT_{REG}, respectively) in the presence of increased concentrations of salt. Representative flow cytometry plots showing the expression of lineage defining master transcription factors and cytokines.
(E) Quantitative analyses of the efficiency of differentiation towards each CD4⁺ T cell lineages at increased concentrations of salt.
Data shown (A-E) are representative of three independent experiments with triplicates of each condition. Error bars represent mean ± standard deviation.



Supplemental Figure 2. NaCl promotes RORγt co-expression in thymic-isolated tT_{REG} cells, as well as Helios$^+$ tT_{REG} cells, in T_H17-Polarizing condition *(Related to Figure 2)*.

(A) Foxp3-GFP$^+$ T_{REG} cells isolated from thymus were co-cultured with CTV-labelled T_{EFF} cells *in vitro* in the same experimental setup described in Figure 2A. Quantitation of the frequency of RORγt$^+$ cells in Foxp3$^+$ CTV$^-$ tT_{REG} cells.

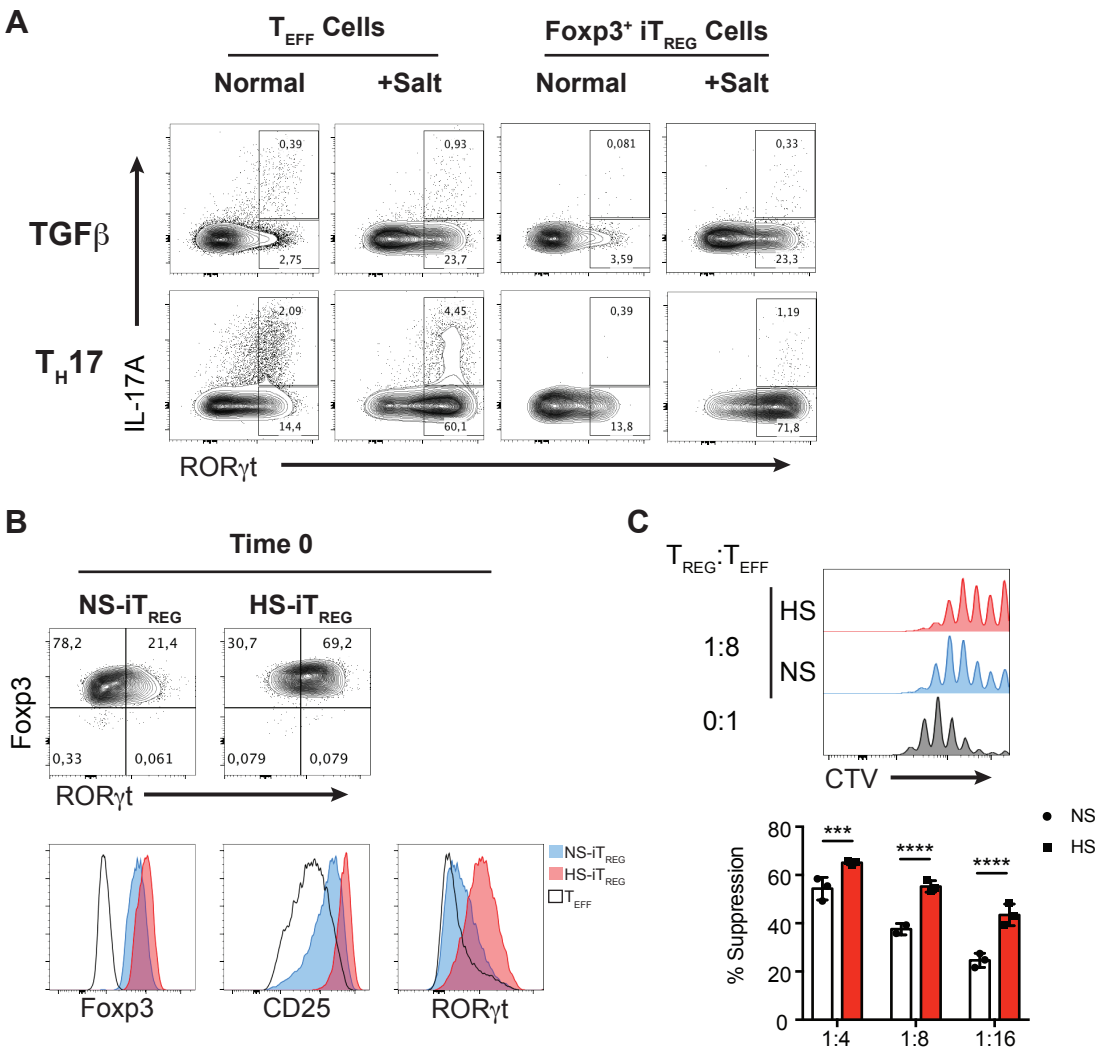
(B and C) Quantitative analyses of RORγt (B) and Foxp3 (C) protein expressions in tT_{REG} cells by the assessment of medium fluorescent intensity (MFI). Data shown are representative of three independent experiments (A-C) with triplicates of each condition.

(D and E) Representative flow cytometry plots and quantification of Helios$^+$ T_{REG} and T_{EFF} cells isolated from thymus of naive mice. Data shown are pooled results from six individual mice.

(F) FACS-purification of Helios$^+$ and Helios$^-$ T_{REG} cell from splenocytes of Helios-GFP/Foxp3-REP dual reporter mice (Left). Purity checks of Helios$^+$ and Helios$^-$ T_{REG} cell post-sort and at time zero of co-culture with T_{EFF} cells (Right). In vitro co-culture was set up the same way described in Figure 2A.

(G-J) Representative flow cytometry plots depicting Helios and RORγt expression in T_{REG} cells that originated from Helios$^+$ or Helios$^-$ subsets after in vitro polarization. Quantification of the frequencies of ex-Helios (H), ex-Helios RORγt$^+$ (I) and RORγt$^+$ (J) cells in T_{REG} cells that are Helios$^+$ origin.

(K-M) Quantitative analyses of RORγt (K), Helios (L) and Foxp3 (M) protein expressions in RORγt$^+$ T_{REG} cells by the assessment of medium fluorescent intensity (MFI). Data shown are representative of two experiments (F-M) with quadruplicate or sexpluticate of each condition. Error bars represent mean ± standard deviation.



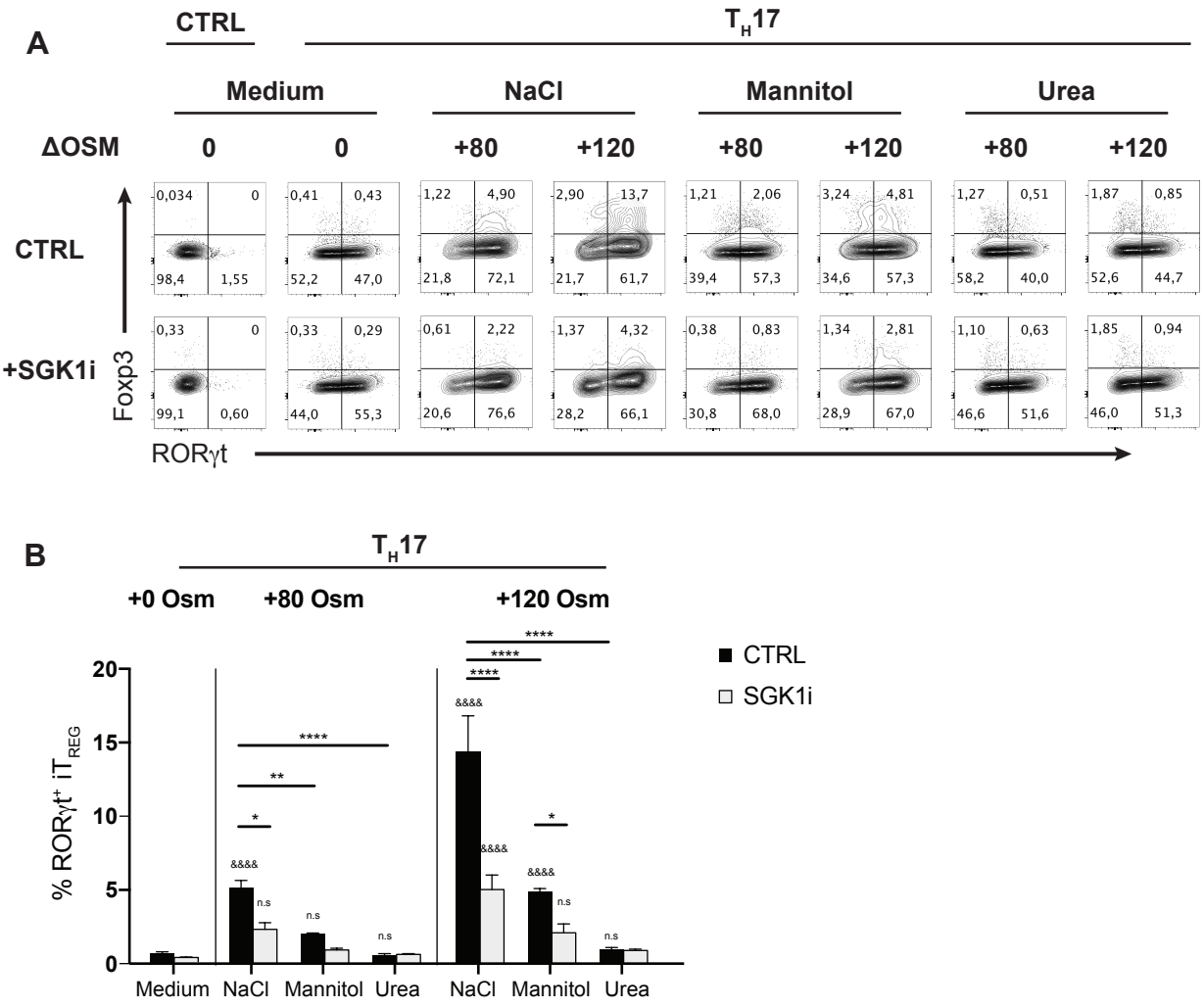
Supplemental Figure 3. RORγt^{+} iT$_{REG}$ cells differentiated under high salt conditions in vitro are refractory to IL-17A production and exhibits enhanced suppressive function *in vitro (Related to Figures 4 and 5).*

(A) Representative flow cytometry plots showing the intracellular staining of IL-17A and RORγt in T$_{EFF}$ cells or induced T$_{REG}$ cells described in experiments from Figure 4 (T$_{H}$17) and Figure 5 (TGFβ).

(B) Phenotyping of iT$_{REG}$ cells generated in control or high salt medium post-FACS-purification for suppression assay. Representative flow cytometry plots depicting the expression of Foxp3, CD25 and RORγt of the iT$_{REG}$ when plated for suppression assay at time zero.

(C) Representative flow cytometry histograms depicting the dilution of Cell Trace Violet proliferation dye of responding T$_{EFF}$ cells in the suppression assay (Top). Quantification of percent suppression at various T$_{REG}$:T$_{EFF}$ ratio (Bottom).

Data shown are representative of three independent experiments (A) or one experiment (B-C) with triplicates of each condition. Error bars represent mean ± standard deviation.

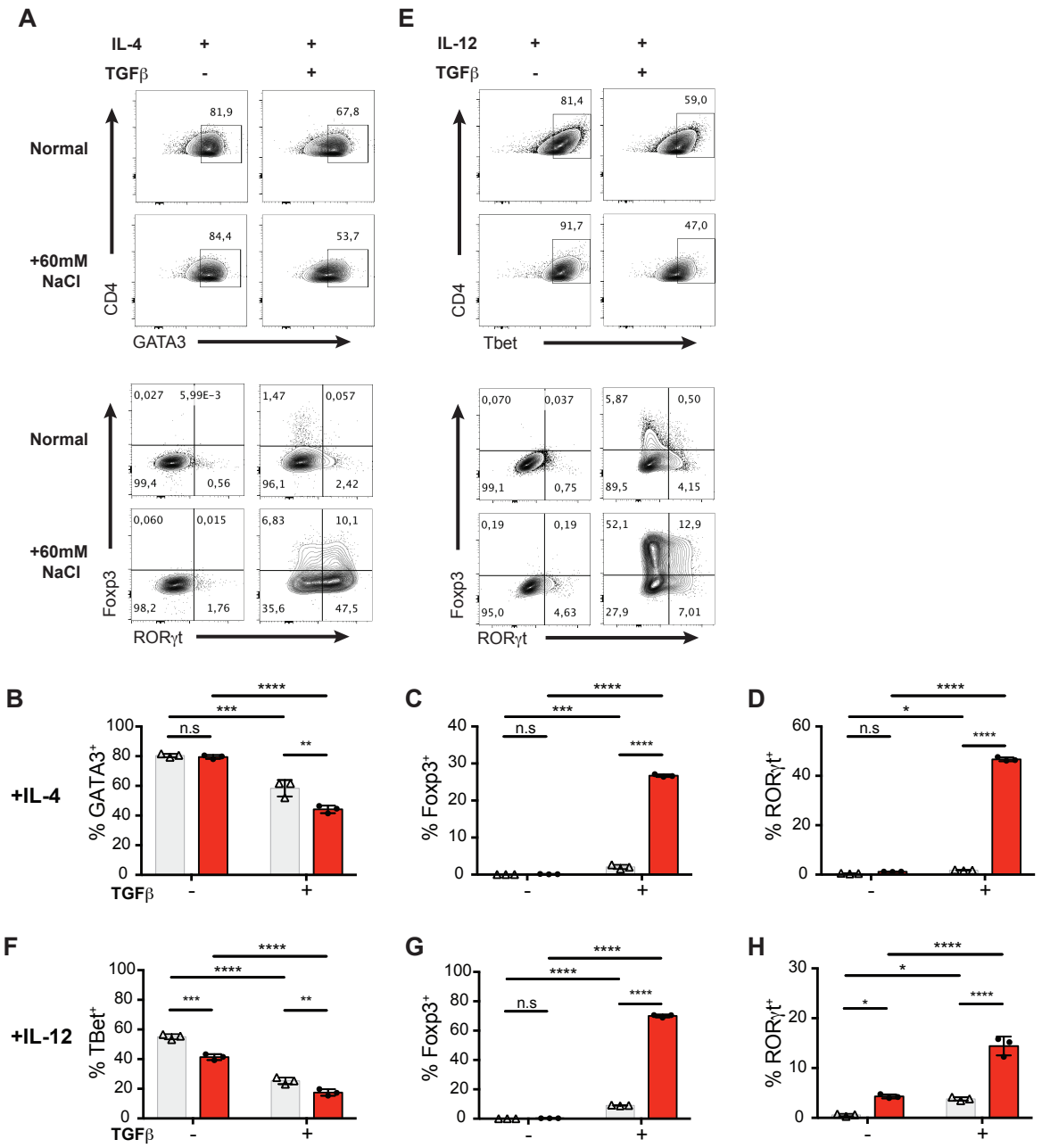


Supplemental Figure 4. The effect on inducing RORγt$^+$ T_{REG} cells are mainly NaCl-dependent, while hypertonic environment exhibits similar effect but to a lesser extent in a SGK1-dependent manner *(Related to Figure 4)*.

(A) Mannitol and urea were used to raise osmolarity to comparable level of various NaCl concentrations used in Figure 4. Representative flow cytometry plots showing the expression of transcription factors Foxp3 and RORγt in CD4$^+$ T cells.

(B) Quantitative analysis of the frequency of RORγt$^+$ iT_{REG} cells.

Data shown are representative of two independent experiments with triplicates of each condition. Error bars represent mean ± standard deviation.



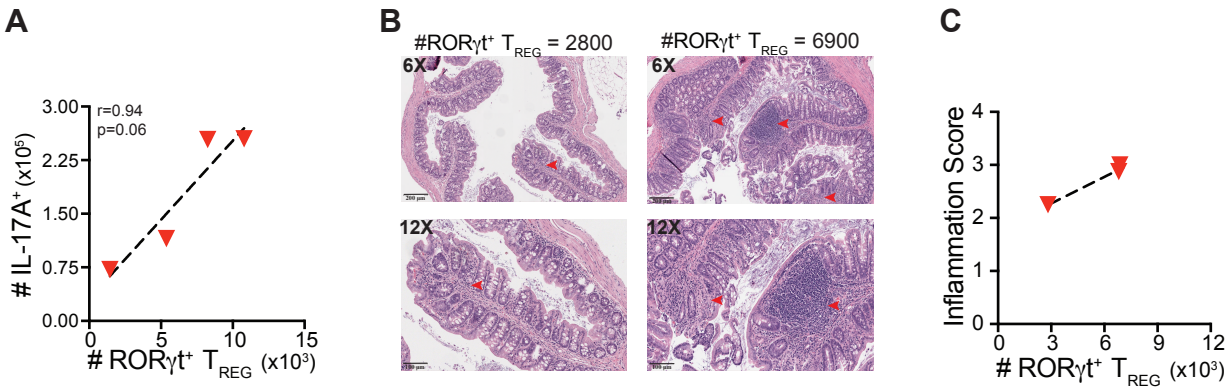
Supplemental Figure 5. High salt skews T cell differentiation and promotes the induction of RORγt$^+$ iT$_{REG}$ cells in strong T$_H$1/T$_H$2 polarizing condition *(Related to Figure 5)*.

(A and E) FACS-sorted T$_{EFF}$ cells were in vitro differentiated with IL-4 for T$_H$2 lineage (A) or IL-12 for T$_H$1 lineage (E), with or without TGFβ, in normal media or media with extra 60mM of NaCl. Representative flow cytometry plots showing the expression of lineage defining transcription factor GATA3 (A) or Tbet (E), or the segregation cells on the expression of Foxp3 and RORγt (lower panels), in CD4$^+$ T cells.

(B-D) Quantitative analyses of the frequency of GATA$^+$ (B), Foxp3$^+$ (C) and RORγt$^+$ (D) cells in CD4$^+$ T cells differentiated in IL-4 or IL-4 and TGFβ in normal media or media with extra 60mM of NaCl.

(F-H) Quantitative analyses of the frequency of Tbet$^+$ (F), Foxp3$^+$ (G) and RORγt$^+$ (H) cells in CD4$^+$ T cells differentiated in IL-12 or IL-12 and TGFβ in normal media or media with extra 60mM of NaCl.

Data shown are representative of three independent experiments with triplicates of each condition. Error bars represent mean ± standard deviation.



Supplemental Figure 6. The colonic pool of RORγt$^+$ T_{REG} cells correlates with IL-17A$^+$ T cells and inflammation scores in a T cell transfer-induced intestinal inflammation model *(Related to Figure 6)*.
(A) The correlation between the absolute number of RORγt$^+$ T_{REG} cells and absolute number of IL-17A$^+$ T cells in colon lamina propria.
(B) Histology of colons from representative mice with low or high number of RORγt$^+$ T_{REG} cells by H&E staining. Red arrows pointing at notable sites of leukocyte infiltration, epithelial hyperplasia or destroyed villous. The top panels are at 6x magnification and bottom panels are at 12x magnification.
(C) The correation between the absolute number of RORγt$^+$ T_{REG} cells and histology scoring of intestinal inflammation. Data shown are representative of three independent experiments (A) or one experiment (B-C). Correlation coefficient (r) is determined by a two-tailed Pearson correlation analysis.