The E3 ligase Hrd1 stabilizes regulatory T-cells by antagonizing inflammatory cytokine-induced ER stress response

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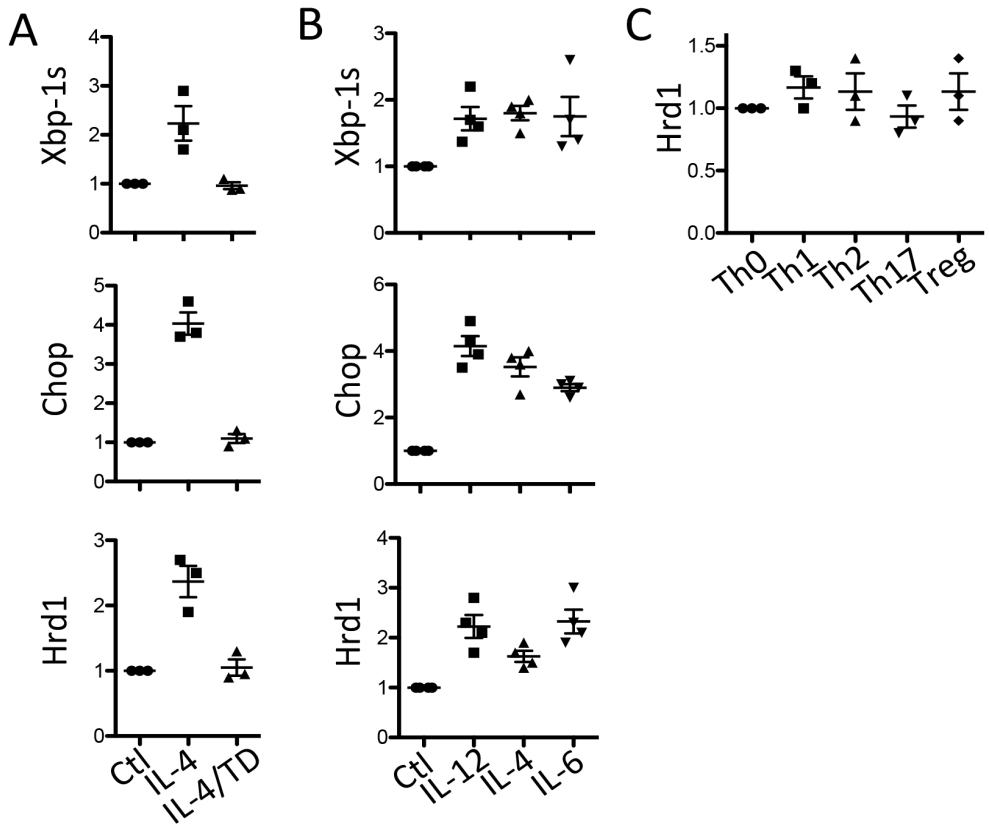
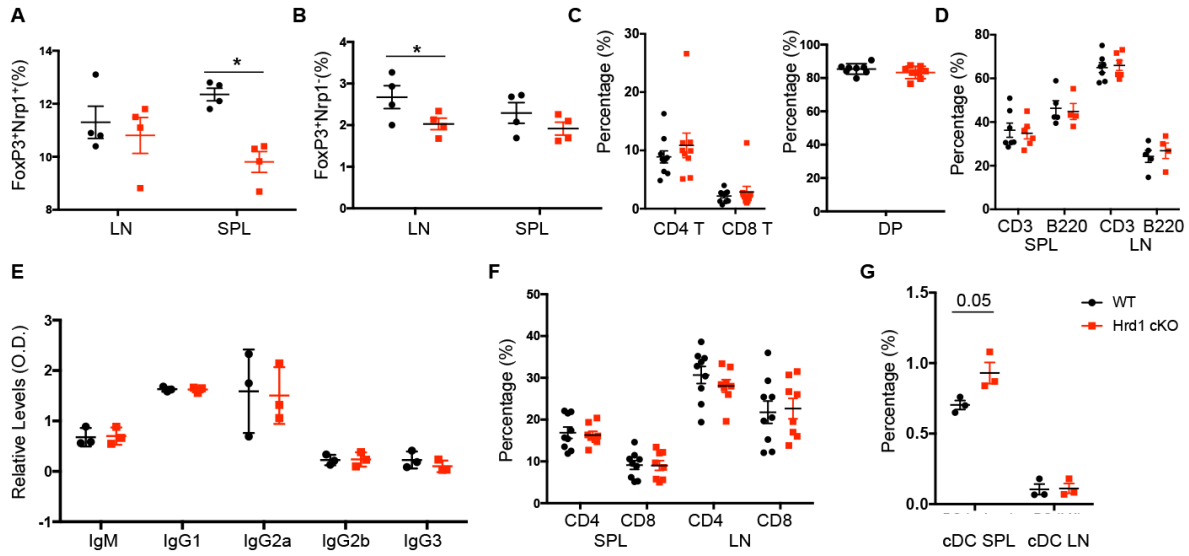
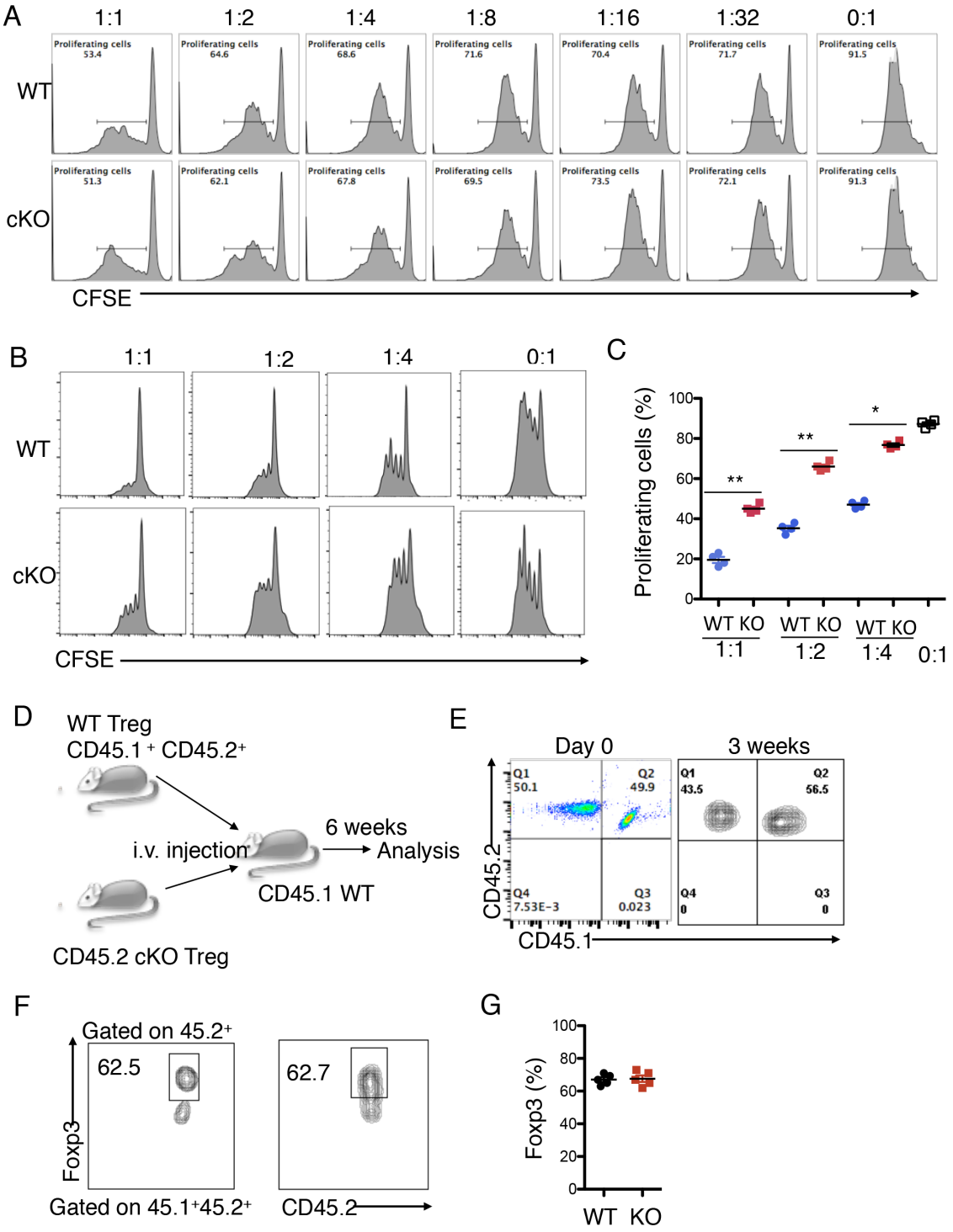
Supplementary Figures 1-9 and legends:



Supplementary Figure 1. Analysis of ER stress responsive gene expression in Tregs. (A) Tregs were cultivated with IL-4 or further with the ER stress inhibitor TD for two days, the expression of ER stress responsive genes including Xbp-1s, Chop and Hrd1 was analyzed by real-time RT-PCR. **(B)** Tregs were cultivated with or without each indicated cytokines for two days, the expression of ER stress responsive genes including Xbp-1s, Chop and Hrd1 was analyzed by real-time RT-PCR. **(C)** The expression levels of Hrd1 in polarized Th0, Th1, Th2, Th17 and Tregs was analyzed by real-time RT-PCR. Data are shown as mean ± SD. Student t test was used for the statistical analysis: *$p<0.05$; **$p<0.01$ and *** $p<0.001$.

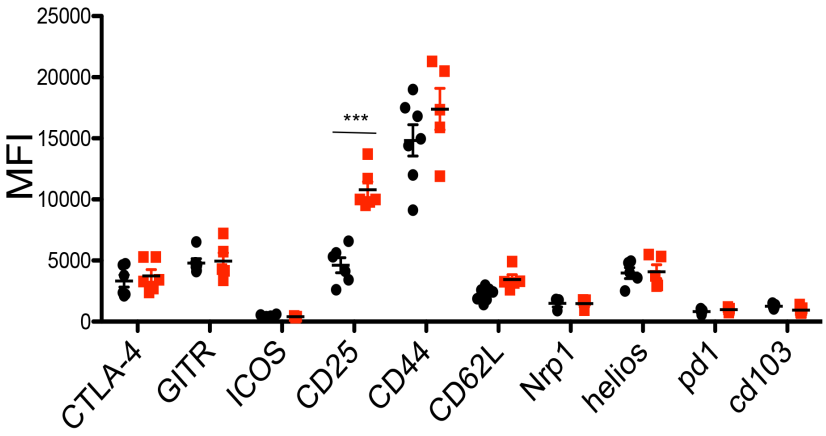


Supplementary Figure 2. Analysis of immune cells in Hrd1$^{fl/fl}$-FoxP3^{cre} mice. (**A, B**) Flow cytometry analysis of the expression of Nrp1 in CD4^{+} cells from the pLN and SPL in WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice. (**C**) Frequencies of CD4^{+}, CD8^{+}, and CD4^{+}CD8^{+} double-positive cells in the thymus from WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice (n=7-9 per group). (**D**) Frequencies of CD3^{+} and B220^{+} cells in the SPL and pLN from WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice. (n=4-6 per group). (**E**) The levels of antibodies in the sera of WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice were analyzed by enzyme-linked immunosorbent assay (ELISA). Error bars represent data from 3 pairs of mice. (**F, G**) Single-cell suspensions of SPL and pLN from WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice were stained with CD4, CD8, CD11c, and MHCII antibodies and analyzed by flow cytometry (n=3-9 per group). Data are shown as mean ± SD.

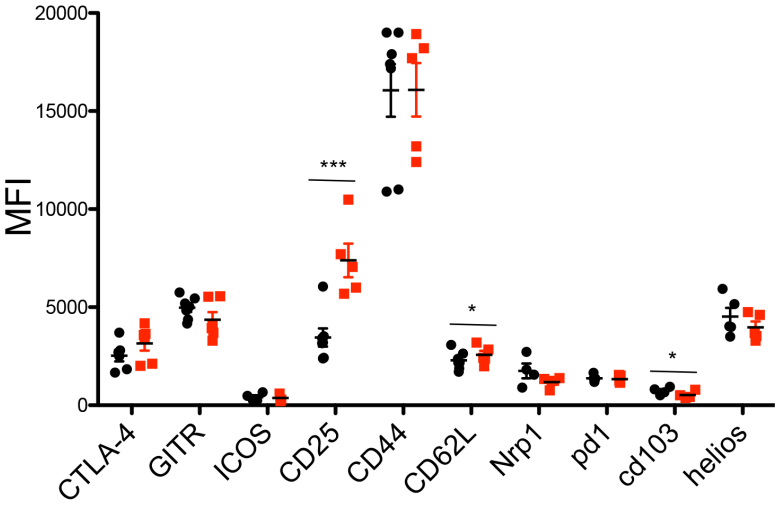


Supplementary Figure 3: In vitro Treg suppressive activity analysis. **(A)** Tregs sorted from WT and Hrd1 cKO mice were cocultivated with CFSE prestained WT naïve CD4 T cells at each indicated ratio in the presence of anti-CD3, anti-CD28 and IL-2. Three days after $CD4^+$ T cell proliferation was analyzed by flow cytometry.**(B & C)** In vito Treg suppressive assay were performed in the presence of IL-4. Representaive immages (B) and data from three independent experiments (C) are shown. Student t test was used for the statistical analysis: *p<0.05 and **p<0.01. **(D-G)** $CD45.1^+CD45.2^+$ WT Treg and

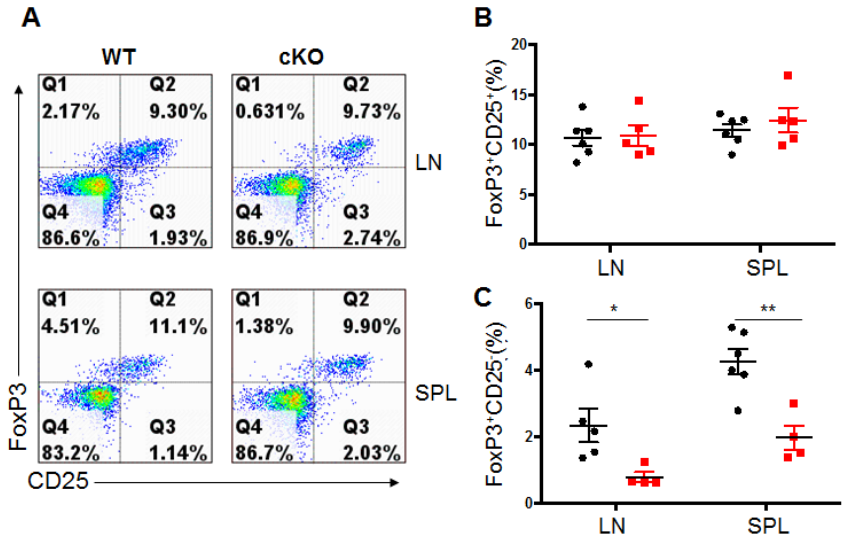
CD45.2^{+} Hrd1-null Tregs were co-transferred into lethally irridiated CD45.1+ WT mice for six weeks (D). CD45.2^{+} Tregs were gated and the expression levels of FoxP3 was analyzed (E-G). The average FoxP3^{+} CD45.2^{+}CD45.1^{+} WT and CD45.2^{+}CD45.1^{-} Hrd1-null Tregs were analyzed. Representative immages (F) and data from 5 mice (G) are shown. Data are shown as mean ± SD.



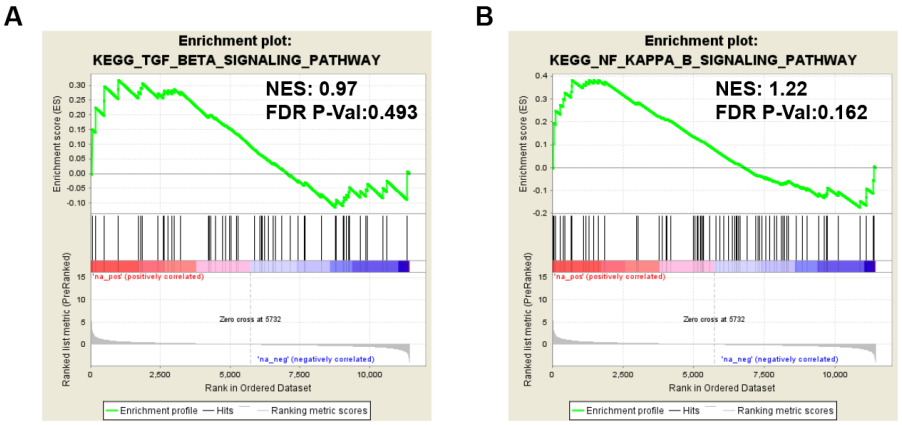
Supplementary Figure 4. Expression profiles of Treg-specific molecules in the pLN. Mean fluorescence intensity of CD25, CD44, CD62L, CD103, ICOS, GITR, Helios, CTLA4, Nrp1, and PD-1 in Treg from the pLN of WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice (n=5-7 per group). Data are shown as mean ± SD.



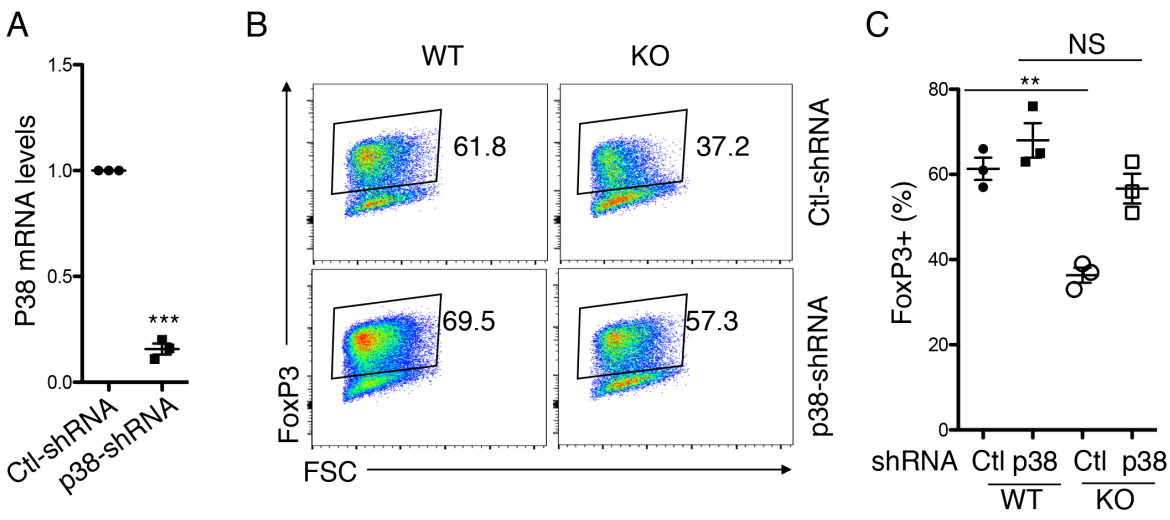
Supplementary Figure 5. The expression profiles of Treg specific molecules in the SPL. Mean fluorescence intensity of CD25, CD44, CD62L, CD103, ICOS, GITR, Helios, CTLA4, Nrp1, and PD-1 in Treg from the SPL of WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice (n=5-7 per group). Data are shown as mean ± SD.



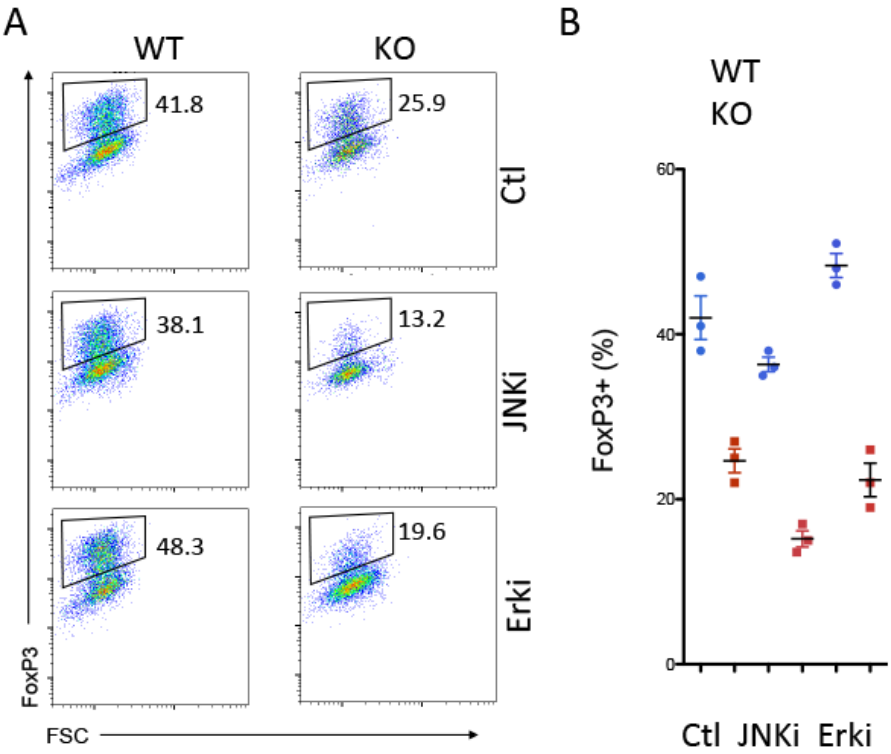
Supplementary Figure 6. Reduced FoxP3^{+}CD25^{-} population in Hrd1$^{fl/fl}$-FoxP3^{cre} mice. CD4^{+} T cells were gated, the levels of CD25 and FoxP3 were analyzed. **(A)** Representative images and (**B-D**) data of FoxP3^{+}CD25^{+} (B) and FoxP3^{+}CD25^{-} expression in Treg from the SPL and pLN in WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice (n=4-6 per group). Student t test was used for the statistical analysis: *p<0.05 and **p<0.01.



Supplementary Figure 7. GSEA of iTreg and nTreg in WT and Hrd1$^{fl/fl}$-FoxP3^{cre} mice. (**A-B**) GSEA of the TGFβ signaling pathway and NF-κB in WT and Hrd1$^{fl/fl}$-FoxP3^{cre} TGFβ-converted iTreg (n=3 per group).



Supplementary Figure 8. P38 suppression by sh-RNA on Hrd1-null Treg polarization. $CD4^+$ T cells were infected with lenti virus that carrying control or p38-specific shRNA. **(A)** the mRNA levels of p38 in GFP^+ cells were analyzed by real-time RT-PCR. **(B & C)** Cells were cultivated in Treg polarization condition with 1 ng/ml TGF-β during and after infection. $FoxP3^+$ cells were analyzed. Data are shown as mean ± SD. Student t test was used for the statistical analysis: *p<0.05 ; **p<0.01 and *** p<0.001.



Supplementary Figure 9. JNK and Erk suppression on Hrd1-null Treg polarization. WT and Hrd1-null Tregs were cultivated with JNK-specific inhibitor SP600125(20 μM) or with Erk inhibitor II (FR180204, 2 μM). $FoxP3^+$ cells were determined by intracellular staining. Representative images (A) and data from four independent experiment are shown.