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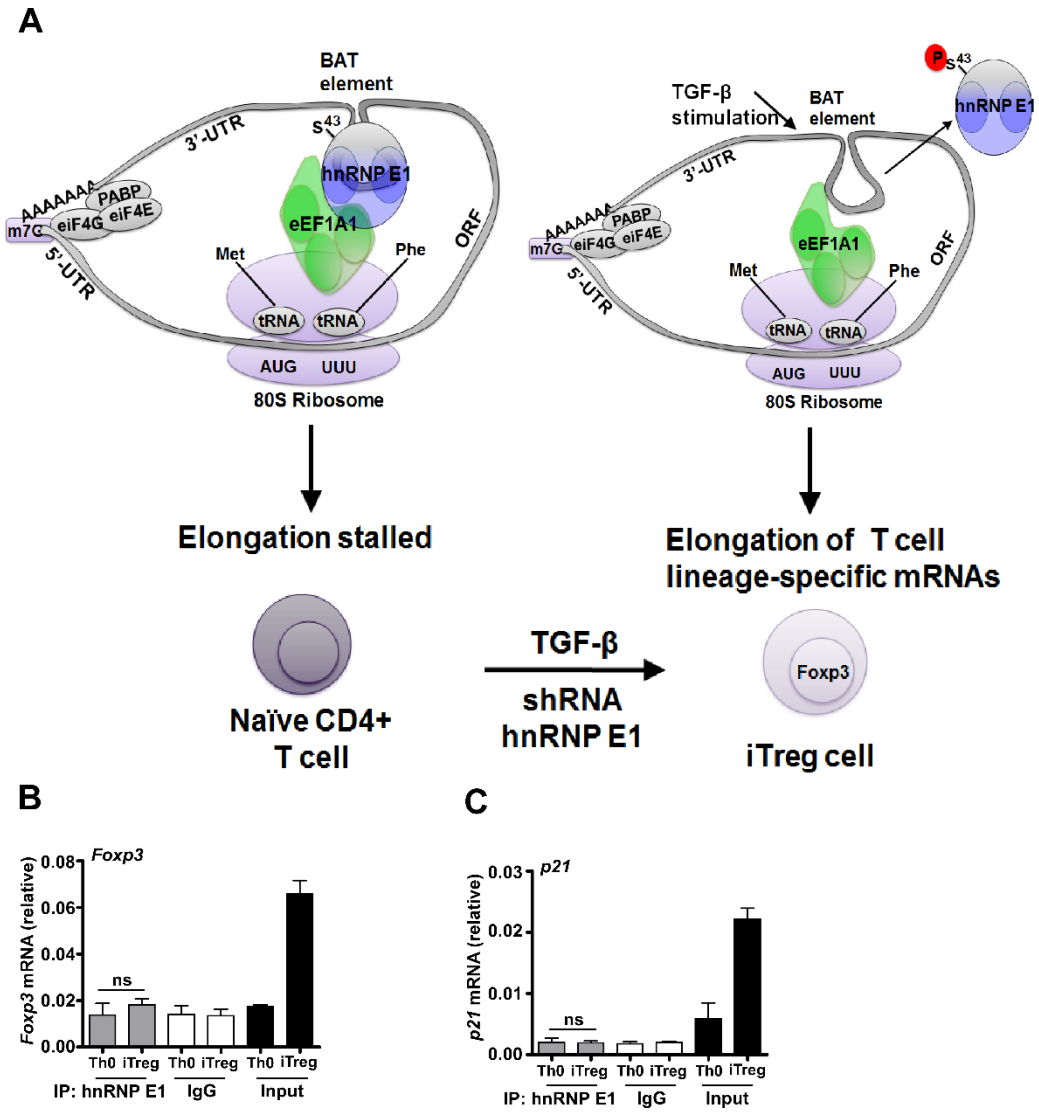
Supplemental data

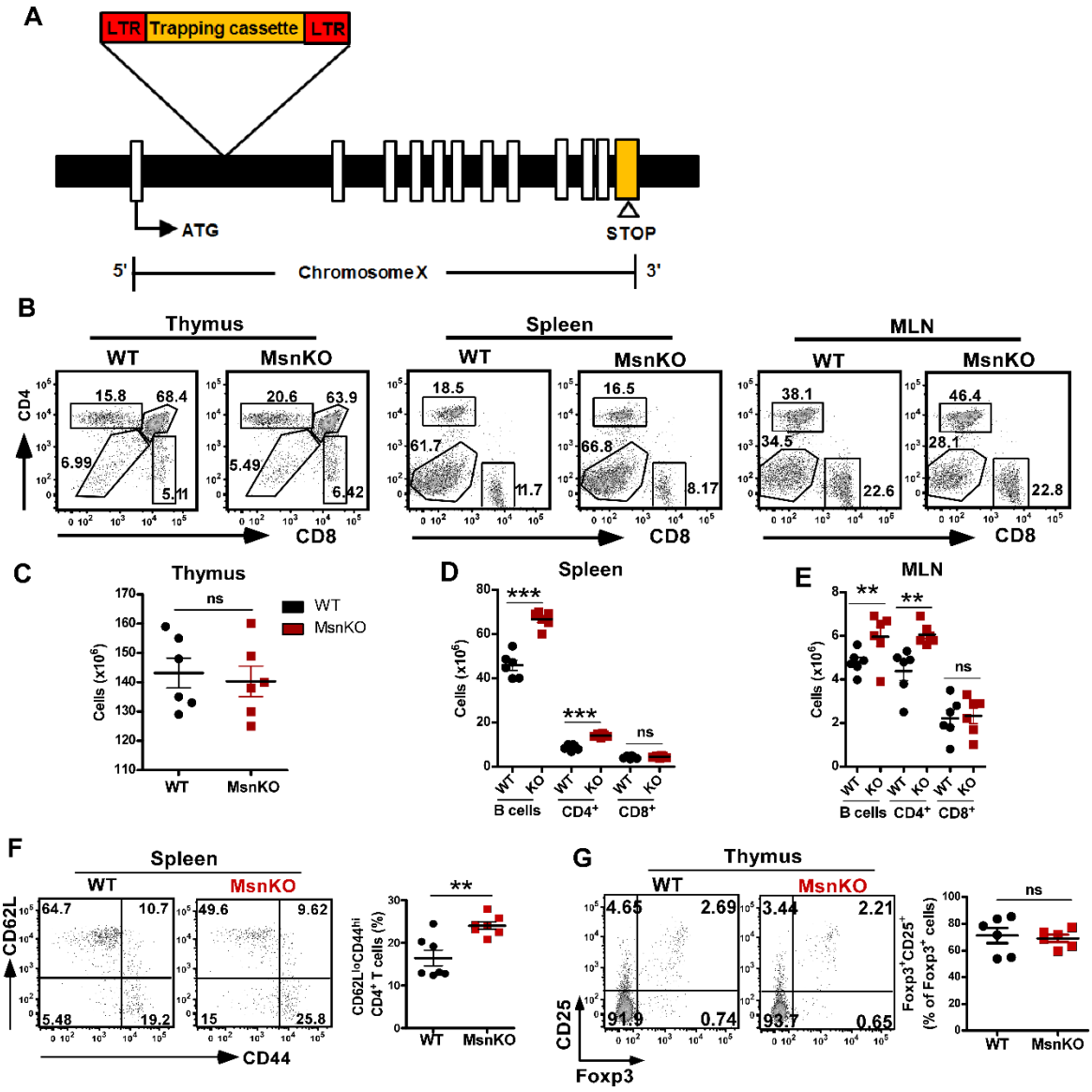
Moesin controls TGF-β signaling and iTregs and attenuation improves adoptive T cell therapy

Ephraim A. Ansa-Addo,[1] Yongliang Zhang,[1] Yi Yang,[1] George S. Hussey,[2] Breege V. Howley,[2] Mohammad Salem,[1] Brian Resenberg,[1] Shaoli Sun,[3] Don C. Rockey,[4] Serhan Karvar,[4] Philip H. Howe,[2] Bei Liu,[1] and Zihai Li[1]

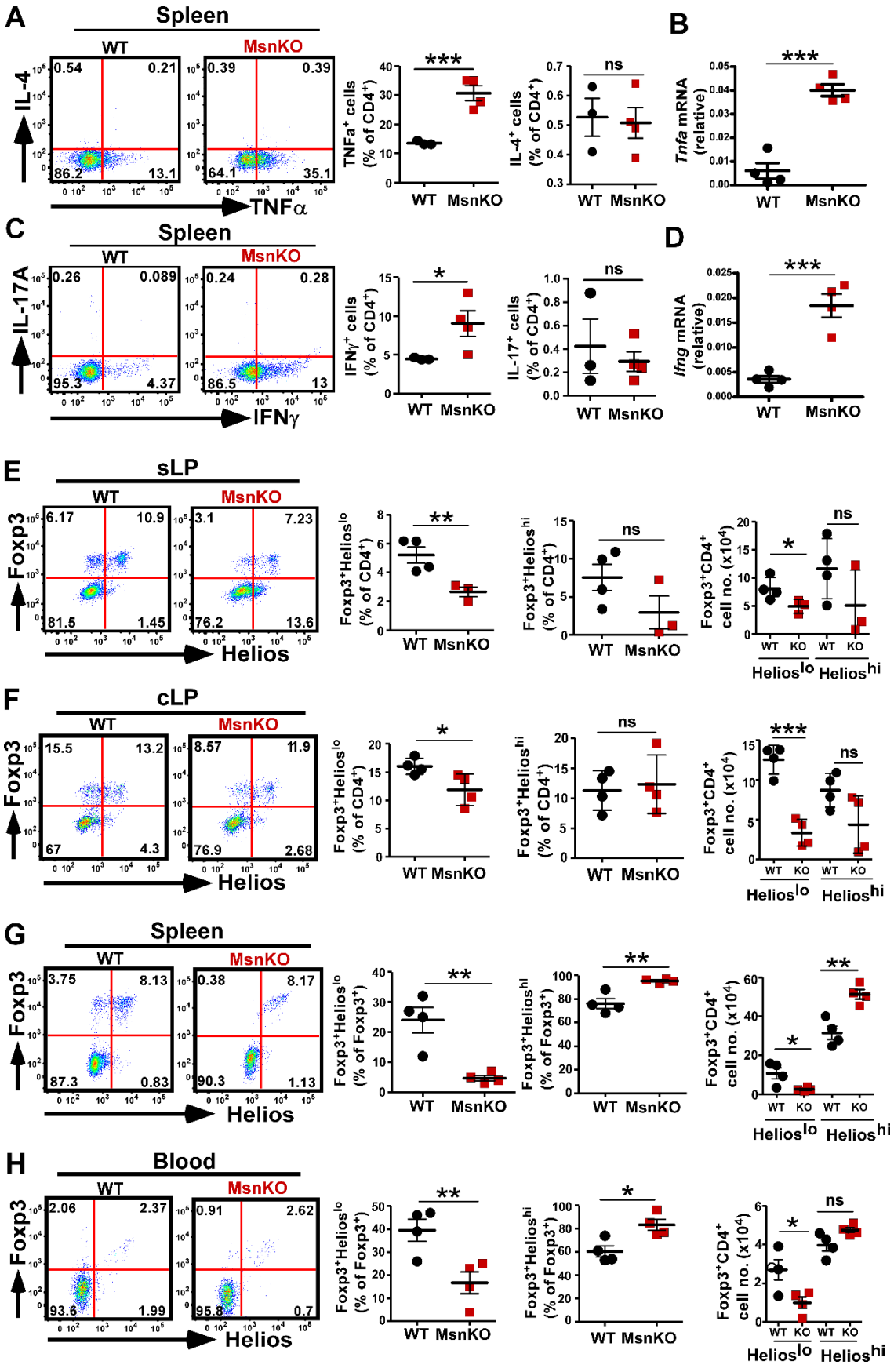
7 Figures



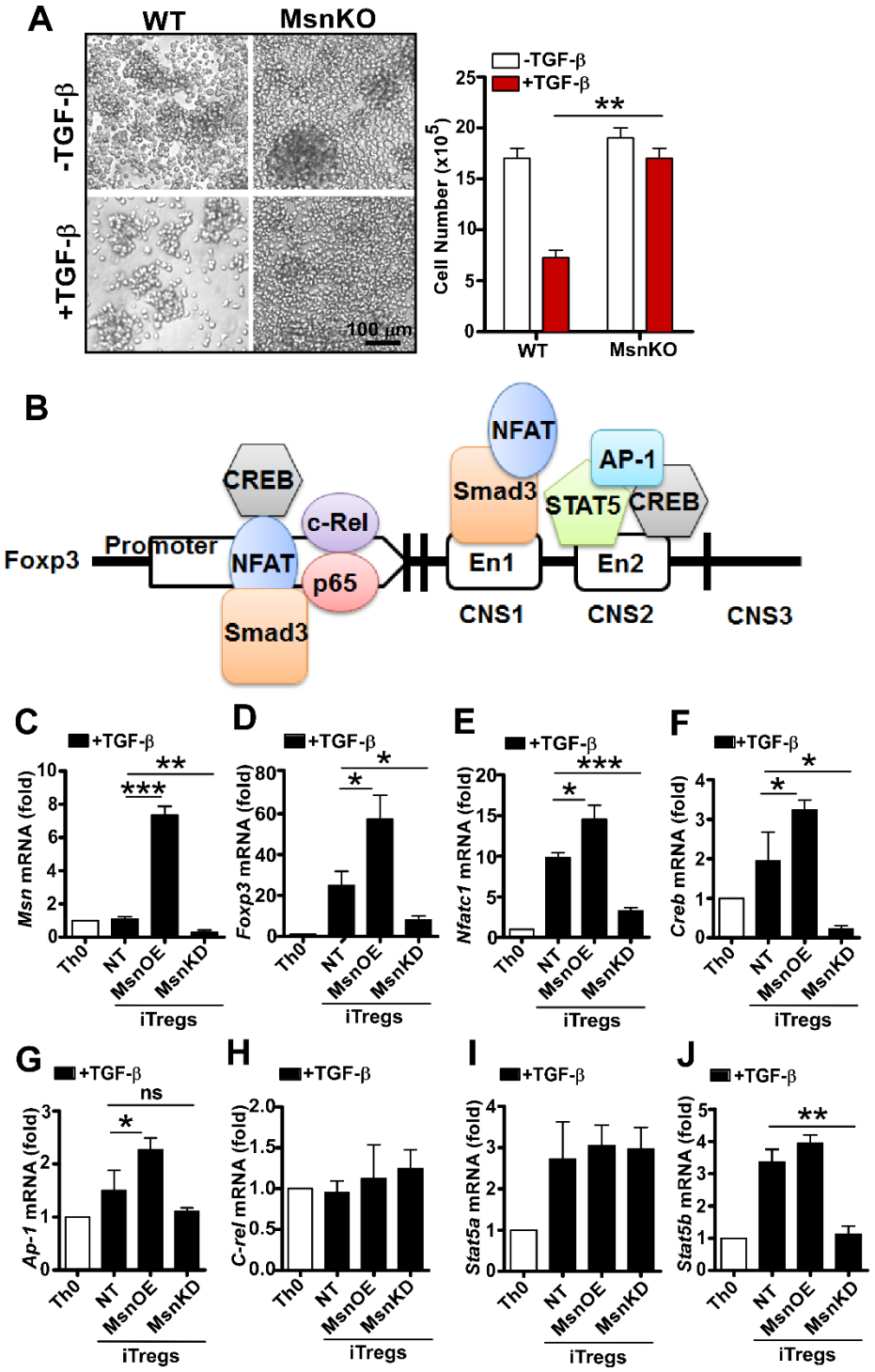
Supplemental Figure 1. Schematic models depicting the TGF-β activated translation regulatory mechanism. (**A**) hnRNP E1 binds to a 3' UTR of TGF-β activated translation element in target transcripts and silences gene translation in a similar fashion in both epithelial and T cells. TGF-β activation leads to phosphorylation of E1 at Ser43, causing its release from the target gene and subsequent translation of the mRNA. (**B** and **C**) hnRNP E1 does not bind to *Foxp3* and *p21* mRNAs. RNA Immunoprecipitation was performed using rabbit anti-hnRNP E1 antibody or isotype IgG control in Th0 cells and Tregs, followed qRT-PCR analysis of *Foxp3* and *p21* transcripts. Th0 cells are defined as naïve $CD4^+$ T cells activated in presence of anti-CD3 and anti-CD28 antibodies, but without TGF-β. Data represent the mean ± SD of at least three independent experiments performed in triplicate. ns, not significant by Student's *t*-test (**B** and **C**).



Supplemental Figure 2. Generation of moesin knockout mice and phenotypic analysis of T cells in lymphoid organs. (**A**) Strategy for generating moesin knockout (MsnKO) mice by insertion of the gene trapping cassette to the first intron of the *Msn* gene. (**B-E**) Representative flow cytometry analysis of individual organs and cell numbers for $CD4^+$, $CD8^+$ and B cells isolated from the thymus (**C**), spleen (**D**) and mesenteric lymph node (MLN) (**E**) of WT and MsnKO littermate mice. (**F**) Flow cytometry analysis and frequency of $CD62L^{lo}CD44^{hi}$ activated $CD4^+$ T cells from the spleen of WT and MsnKO littermate mice at steady state. (**G**) Flow cytometry analysis and quantification of thymic Treg cells in the thymus. Data are reported as the mean ± SEM; ns, not significant. $^{**}P$ <0.01, $^{***}P$<0.001 by Student's *t*-test. n = 6 mice per group (**C-E** and **G**); n = 7 mice per group (**F**).

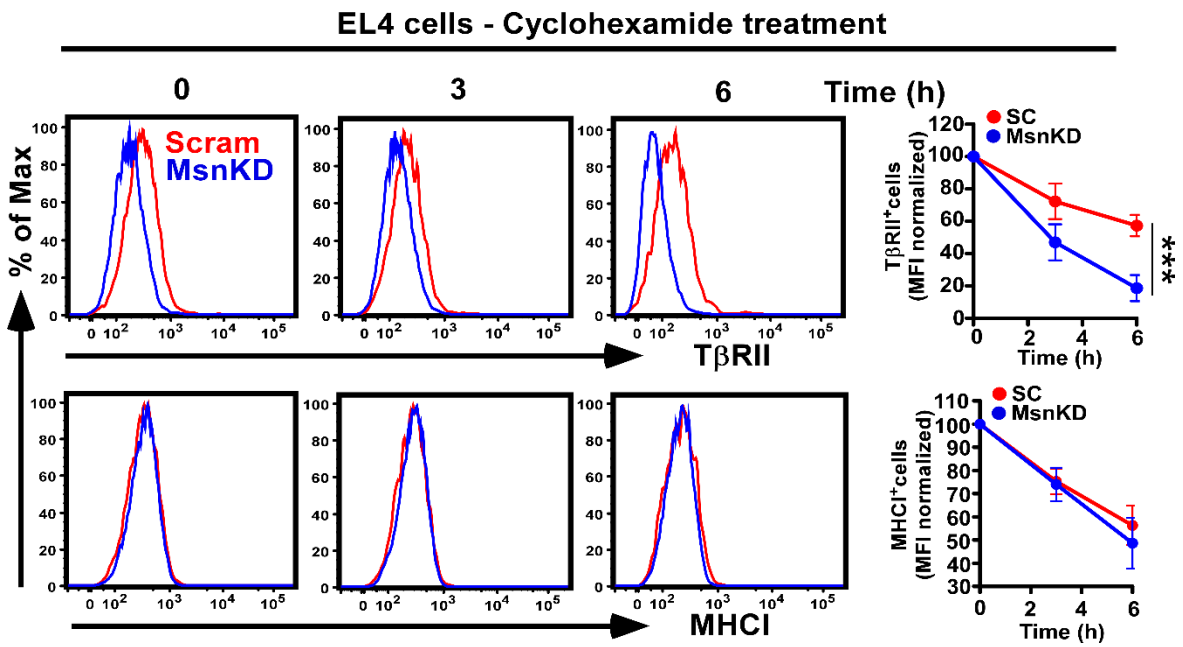

A
Spleen
WT
MsnKO
IL-4
TNFα
0.54
0.21
86.2
13.1
0.39
0.39
64.1
35.1
TNFa+ cells (% of CD4+)
***
IL-4+ cells (% of CD4+)
ns
B
Tnfa mRNA (relative)
***
C
Spleen
IL-17A
IFNγ
0.26
0.089
95.3
4.37
0.24
0.28
86.5
13
IFNγ+ cells (% of CD4+)
*
IL-17+ cells (% of CD4+)
ns
D
Ifng mRNA (relative)
***
E
sLP
Foxp3
Helios
6.17
10.9
81.5
1.45
3.1
7.23
76.2
13.6
Foxp3+Helioslo (% of CD4+)
**
Foxp3+Helioshi (% of CD4+)
ns
Foxp3+CD4+ cell no. (x10^4)
*
ns
Helioslo
Helioshi
F
cLP
15.5
13.2
67
4.3
8.57
11.9
76.9
2.68
Foxp3+Helioslo (% of CD4+)
*
Foxp3+Helioshi (% of CD4+)
ns
Foxp3+CD4+ cell no. (x10^4)
***
ns
G
Spleen
3.75
8.13
87.3
0.83
0.38
8.17
90.3
1.13
Foxp3+Helioslo (% of Foxp3+)
**
Foxp3+Helioshi (% of Foxp3+)
**
Foxp3+CD4+ cell no. (x10^4)
*
**
H
Blood
2.06
2.37
93.6
1.99
0.91
2.62
95.8
0.7
Foxp3+Helioslo (% of Foxp3+)
**
Foxp3+Helioshi (% of Foxp3+)
*
Foxp3+CD4+ cell no. (x10^4)
*
ns
WT
MsnKO
KO

Supplemental Figure 3. Moesin knockout mice have fewer peripherally-derived Treg (pTreg) cells and evidence of polyclonal T cell activation. (**A** and **B**) Flow cytometry of inflammatory cytokines, TNF-α and IL-4 (**A**) and mRNA expression of *Tnf* (**B**), as well as flow cytometry of IFN-γ and IL-17A (**C**) and mRNA expression of *Ifng* (**D**) produced by $CD4^+$ T cells from spleens of WT and MsnKO mice after PMA-Ionomycin stimulation for 4 h. Data are reported as the mean ± SEM; **P* <0.05 and ****P* <0.001 by Student's *t*-test. WT n = 3, MsnKO n = 4. (**E** and **F**) Flow cytometry analysis and absolute number of pTregs ($Foxp3^+Helio^{lo}$) in the small intestine lamina propria (sLP) (**E**) and colon lamina propria (cLP) (**F**) of 10-12 weeks old mice. Data are reported as the mean ± SEM; **P* <0.05, ***P*<0.01 and ****P* <0.001 by Student's *t*-test. n = 4 per group, MsnKO sLP n = 3. (**G** and **H**) Flow cytometry analysis and absolute number of pTregs ($Foxp3^+Helios^{lo}$) in the spleen (**G**) and peripheral blood (**H**) of 10-12 weeks old mice. Data are reported as the mean ± SEM; ns, not significant; **P* <0.05 and ***P* <0.01 by Student's *t*-test. n = 4 per group.

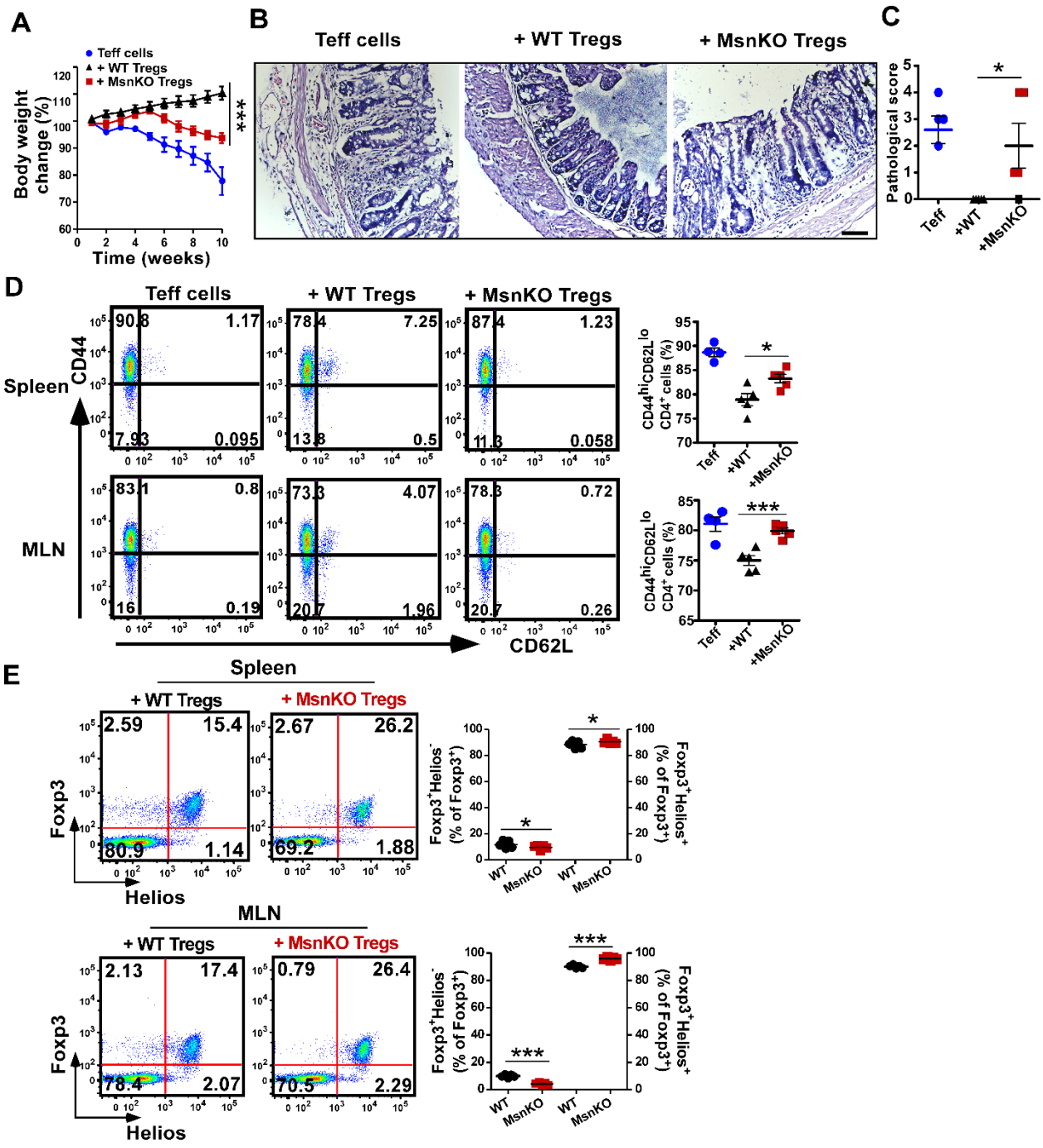


Supplemental Figure 4. Moesin knockout cells show increased proliferation and changes in Foxp3 locus-binding transcription factors. (**A**) Significant cell proliferation observed in moesin KO (MsnKO) primary $CD4^{+}CD25^{-}$ T cells cultured *in vitro* in response to anti-CD3/anti-CD28 antibodies and IL-2, and

with or without TGF-β1. (**B**) Multiple transcription factors associate at the Foxp3 locus during TGF-β-mediated iTreg cell induction. (**C-J**) mRNA expression of known Foxp3 locus binding transcription factors in primary $CD4^+CD25^-$ T cells transduced with either lentiviral shRNA to silence moesin (MsnKD), with moesin expression plasmids (MsnOE) or with control vectors (NT, non-targeting). Cells were then differentiated with TGF-β1 into iTregs or activated without TGF-β (Th0 cells) for 3 days. Th0 cells are defined as purified primary $CD4^+CD25^-$T cells activated in the presence of anti-CD3 and anti-CD28 antibodies, but without TGF-β1. Data are reported as the mean ± SD of at least three independent experiments performed in triplicate; ns, not significant. **P* <0.05, ***P* <0.01, ****P* <0.001 by Student's *t*-test.

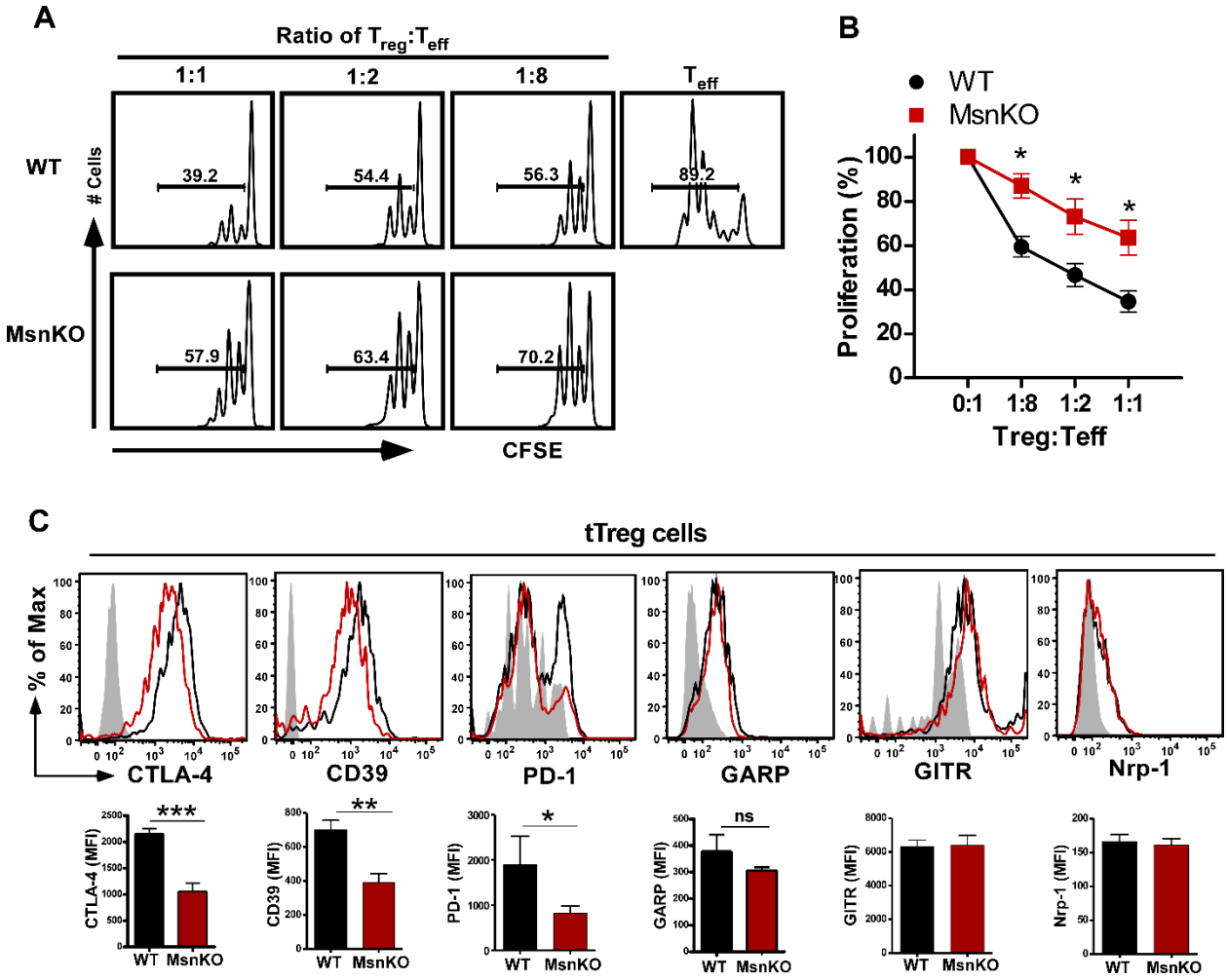


Supplemental Figure 5. Moesin stabilizes TβRII expression on the cell surface. Flow cytometry analysis of surface TβRII and MHCI levels following treatment of EL4 cells with cyclohexamide (100 µg/ml) at the indicated times. Data represents the mean ± SD of at least three independent experiments in triplicate. ****P* <0.001 by two-way analysis of variance (ANOVA).



Supplemental Figure 6. Loss of moesin compromises the suppressive functions of Treg cells. (**A**) Change in body weight of B6 *Rag2*$^{-/-}$ host mice 0-10 weeks after transfer of sorted CD4^{+}CD45RB^{hi}CD25^{-} T cells alone for the induction of colitis (Teff cells) or together with Treg cells from WT or MsnKO donor mice. (**B** and **C**) Representative disease severity (of two experiments) and blinded pathological score in the host mice in A, evaluated by hematoxylin-and-eosin staining of colon sections at end-point. (**D**) Flow

cytometry analysis of $CD44^{hi}CD62L^{lo}$ activated $CD4^{+}$ T cells from the spleen and mesenteric lymph node (MLN) of recipient mice. (**E**) Flow cytometry analysis of $Foxp3^{+}Helio^{lo}$ and $Foxp3^{+}Helio^{hi}$ Treg cells in the Spleen and MLN of mice subjected to T cell transfer-induced colitis. Data are reported as the mean ± SEM of two independent experiments. **P* <0.05 and ****P* <0.001 by Student's *t*-test (**A**, **D** and **E**) or by the Wilcoxon signed-rank *non*-parametric test (**C**). Scale bar = 50 µm (**B**). n = 4 Teff; n = 5 mice per group WT and MsnKO.



Supplemental Figure 7. (**A** and **B**) Moesin-deficient $CD4^+CD25^+$ Treg cells are less suppressive. *In vitro* suppression of the proliferation of CFSE-labelled responder $CD4^+$ T cells (Teff) by $CD4^+CD25^+$ Treg cells isolated from the spleen of WT and MsnKO mice (**A**) and quantification (**B**), after co-culture at various ratios and stimulation with gamma ray-irradiated splenocytes. Proliferation was assessed after 3 days by the dilution of the CFSE dye and by flow cytometry. (**C**) Expression overlay of indicated markers in $CD4^+Foxp3^+Helios^{hi}$ Tregs from WT (black line) vs MsnKO (red line). Data are reported as the mean ± SD of three independent experiments in triplicate (**B**) or the mean ± SEM of three experiments (**C**). ns, not significant. **P* <0.05, ***P* <0.01 and ****P* <0.001 by Student's *t*-test (**B** and **C**).