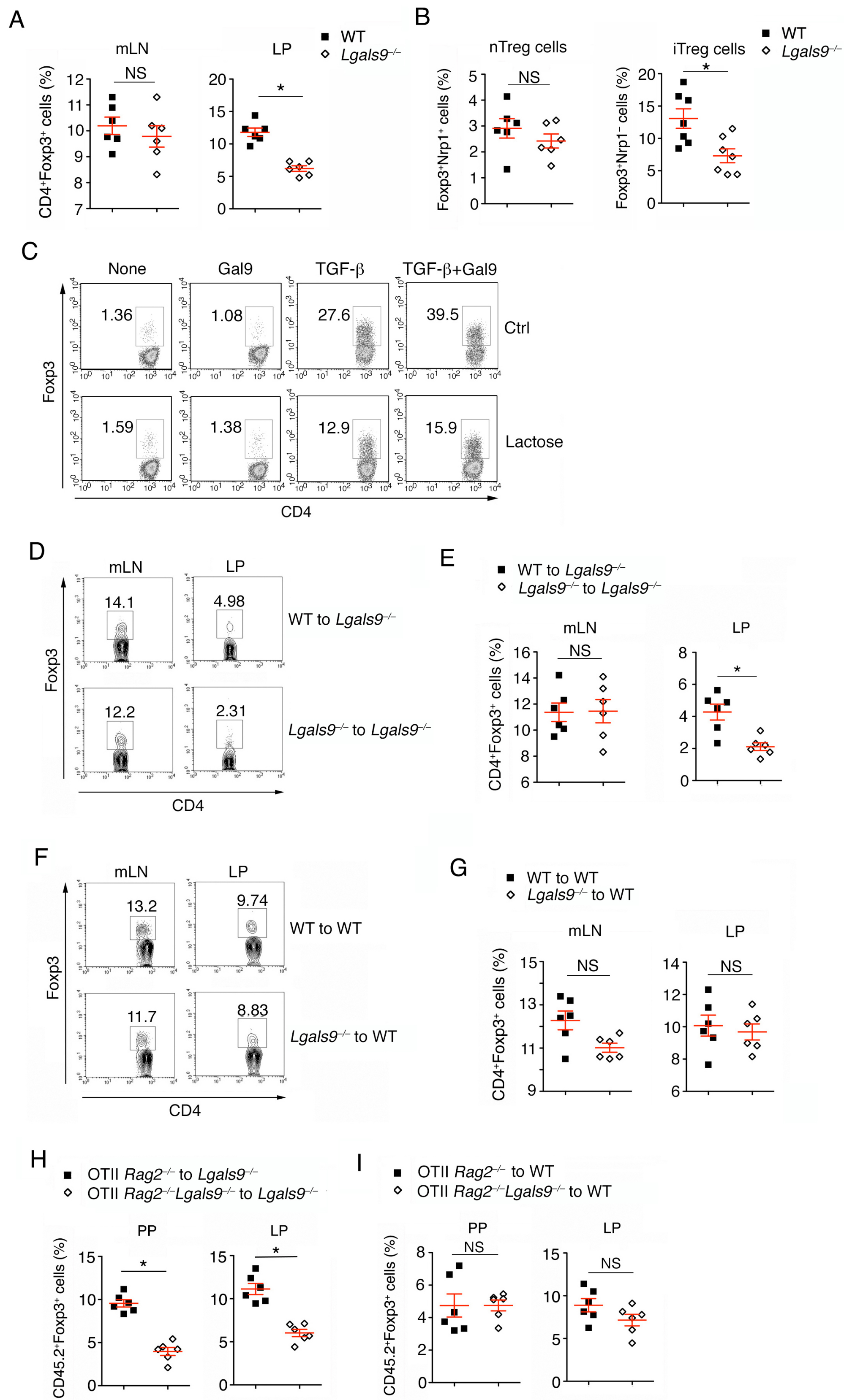
A
mLN
LP
WT
Lgals9−/−
NS
*
CD4+Foxp3+ cells (%)
B
nTreg cells
iTreg cells
Foxp3+Nrp1+ cells (%)
Foxp3+Nrp1− cells (%)
C
None
Gal9
TGF-β
TGF-β+Gal9
1.36
1.08
27.6
39.5
Ctrl
1.59
1.38
12.9
15.9
Lactose
Foxp3
CD4
D
14.1
4.98
WT to Lgals9−/−
12.2
2.31
Lgals9−/− to Lgals9−/−
E
F
13.2
9.74
WT to WT
11.7
8.83
Lgals9−/− to WT
G
H
OTII Rag2−/− to Lgals9−/−
OTII Rag2−/−Lgals9−/− to Lgals9−/−
PP
CD45.2+Foxp3+ cells (%)
I
OTII Rag2−/− to WT
OTII Rag2−/−Lgals9−/− to WT

**Figure S1, related to Figure 1.** (A) Quantification of the frequency of Foxp3$^+$ Treg cells within indicated organs as in **Figure 1A**; (B) Quantification of frequency of Foxp3$^+$Nrp1$^{lo}$ iTreg cells or Foxp3$^+$Nrp1$^{hi}$ nTreg cells as in **Figure 1B**; (C) Activated WT naïve CD4$^+$ T cells were stimulated with combinations of TGF-β and recombinant galectin-9 with or without β-lactose (100mM) for 3 days. The frequency of Foxp3$^+$ cells was determined by flow cytometry; Chimeric mice were generated by transferring WT or *Lgals9*$^{-/-}$ BM into (D-E) *Lgals9*$^{-/-}$ or (F-G) WT host mice. 10 weeks after reconstitution, the percentage of CD4$^+$Foxp3$^+$ Treg cells in mLN and LP was determined by flow cytometry and quantification; (H-I) Quantification of the frequency of CD45.2$^+$CD4$^+$Foxp3$^+$ iTreg cells in PP and LP of recipient mice as in **Figure 1F** and **Figure 1G**. Data are representative of three independent experiments with $n \geq 4$ mice each group. *$P < 0.05$ (Student's *t*-test, error bars, SD).

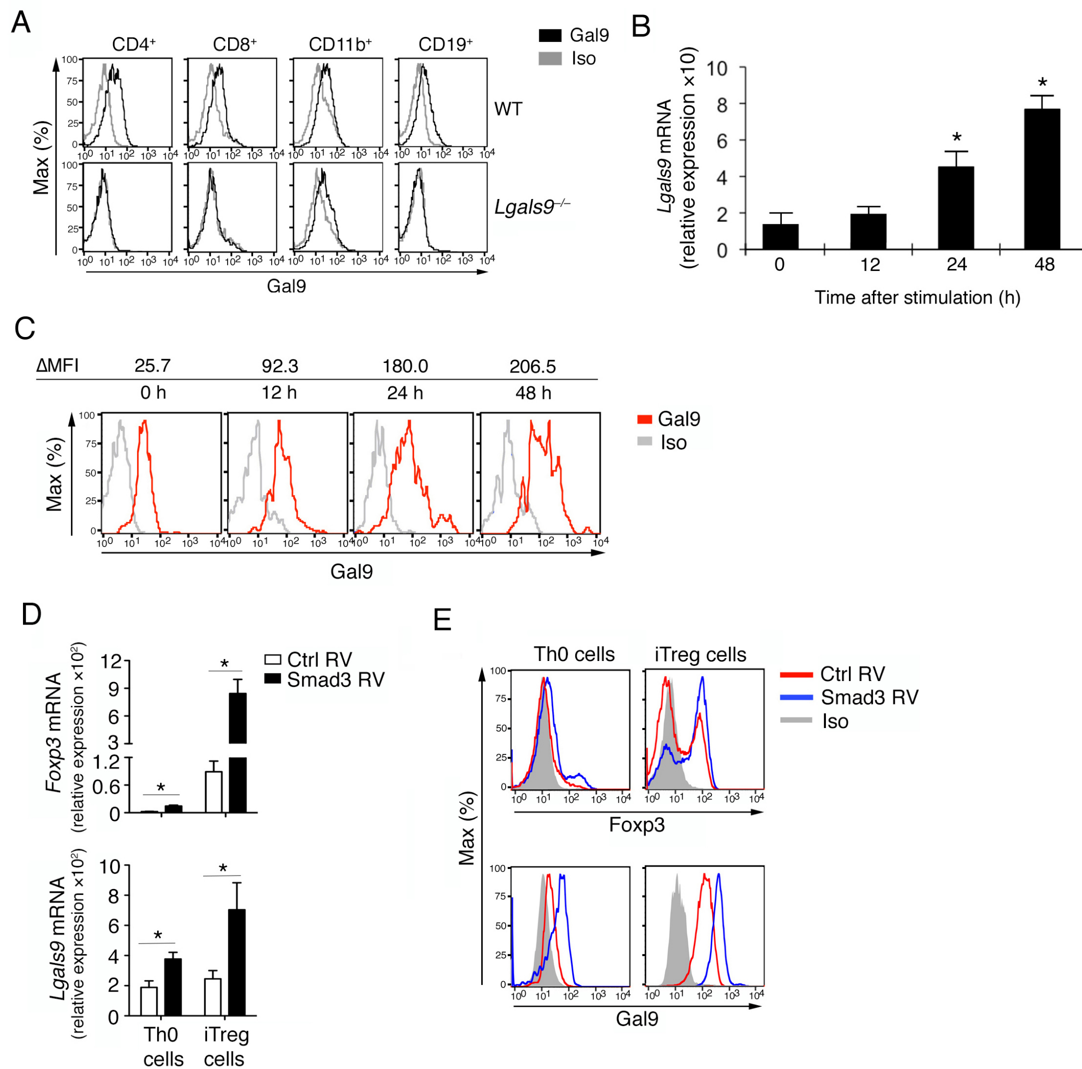


**Figure S2, related to Figure 2.** (A) Protein expression level of galectin-9 was determined in CD4$^+$, CD8$^+$ T cells, CD11b$^+$ macrophages, and CD19$^+$ B cells by flow cytometry; (B) Quantitative real-time PCR analysis of the time course of the expression of *Lgals9* mRNA in iTreg cells; (C) Time course of galectin-9 protein expression in iTreg cells was determined by flow cytometry; (D) mRNA and (E) protein expression levels of Foxp3 and galectin-9 in naive CD4$^+$ T cells transduced with retrovirus expressing control vector (MIG) or Smad3-expressing vector and differentiated into Th0 or iTreg cells; Data

are representative of two independent experiments with n ≥ 5 mice each group. *$P < 0.05$ (Student's *t*-test, error bars, SD).

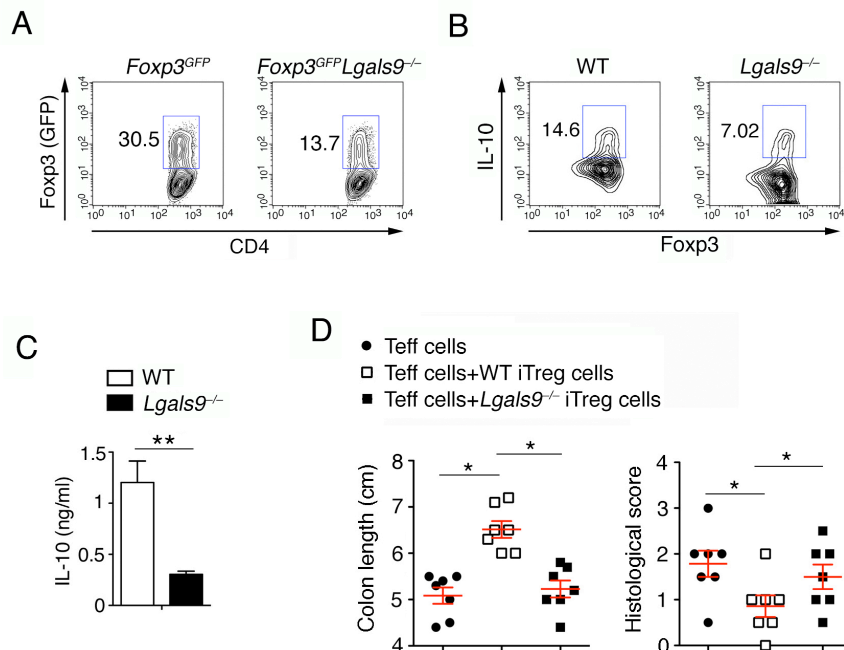


**Figure S3, related to Figure 3.** (A) Naïve *Foxp3*$^{GFP}$ or *Foxp3*$^{GFP}$*Lgals9*$^{-/-}$ T cells were differentiated into iTreg cells with TGF-β. The frequency of GFP (Foxp3$^{+}$) cells was determined by flow cytometry; (B) Intracellular staining of IL-10 production by differentiated WT and *Lgals9*$^{-/-}$ CD4$^{+}$Foxp3$^{+}$ iTreg cells was determined by flow cytometry; (C) WT and *Lgals9*$^{-/-}$ iTreg cells were stimulated for 24 h with PMA and ionomycin, and IL-10 production in the supernatant was determined by ELISA; (D) Left: Colon lengths of *Rag2*$^{-/-}$*Lgals9*$^{-/-}$ mice which had received the indicated cells for transfer as in **Figure 3C**, measured from the colocecal junction to the anal verge; Right: Quantification of pathological changes in the colon of mice as in **Figure 3C**; The data are representative of three independent experiments (A, B, D) or are pooled of three independent experiments (C) with n ≥ 4 mice each group. *$P$ < 0.05, **$P$ < 0.01 (Student's *t*-test, error bars, SD).

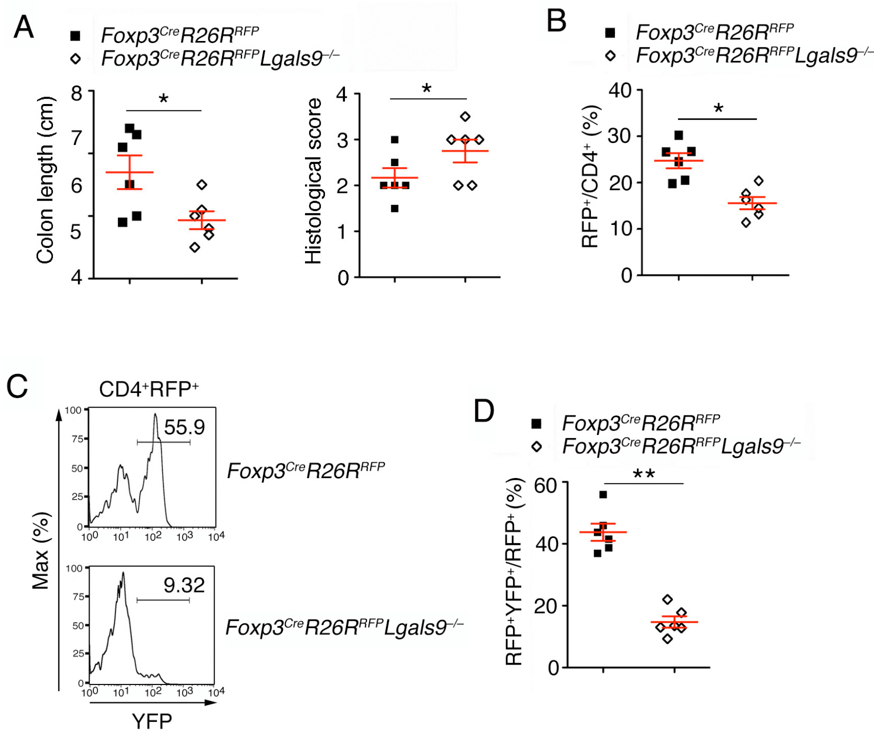


**Figure S4, related to Figure 4.** (A) Left: Colon lengths of $Rag2^{-/-}Lgals9^{-/-}$ mice which had received the indicated cells for transfer as in **Figure 4C**, measured from the colocecal junction to the anal verge; Right: Quantification of pathological changes in the colon of mice as in **Figure 4C** 10 weeks after colitis induction; (B) Quantification of the frequency of $RFP^+$ among $CD4^+$ T cells in the LP of WT and $Lgals9^{-/-}$ fate mapping mice; YFP expression within $CD4^+RFP^+$ T cells isolated from LP of indicated mice as in **Figure 4C** was determined by (C) flow cytometry and (D) quantification. Data are representative of two independent experiments with $n \geq 5$ mice each group. $*P < 0.05$ (Student's *t*-test, error bars, SD).

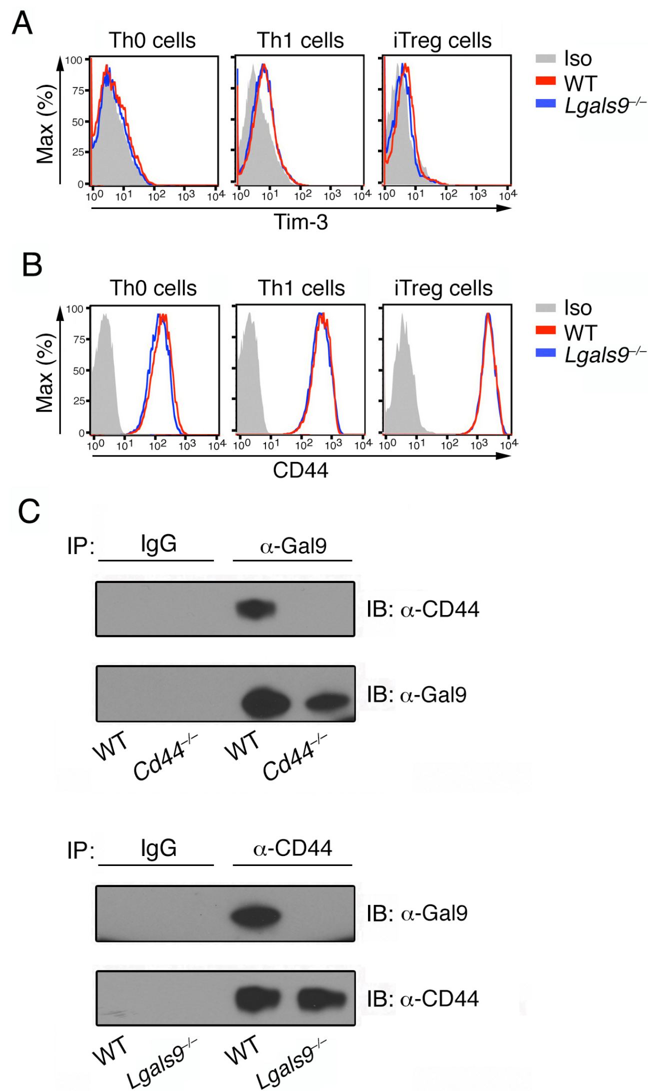


**Figure S5, related to Figure 5.** Flow cytometry of protein expression levels of (A) Tim3 and (B) CD44 on indicated T cell subsets; (C) Immunoprecipitation (with control IgG, anti-galectin-9 or anti-CD44) of lysates of WT and *Cd44*$^{-/-}$ or *Lgals9*$^{-/-}$ iTreg cells, followed by immunoblot analysis with the indicated antibodies. Data are representative of three independent experiments with $n \geq 3$ mice each group.

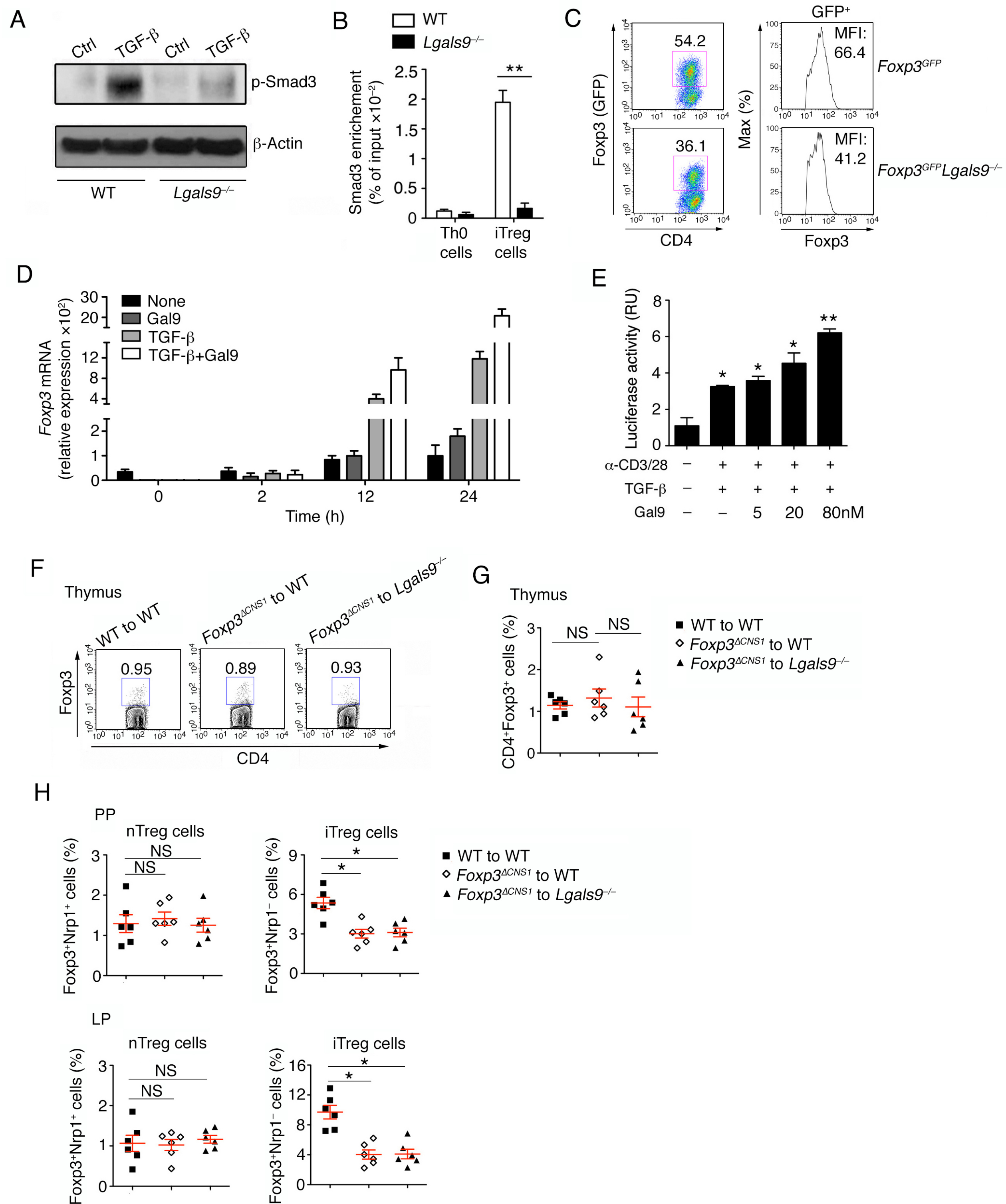


**Figure S6, related to Figure 6.** (A) The level of phosphorylated Smad3 was assessed in WT and *Lgals9*$^{-/-}$ iTreg cells by immunoblot; (B) The binding of Smad3 to the *Foxp3*

CNS1 region in WT and *Lgals9*$^{-/-}$ iTreg cells was determined by ChIP-PCR; (C) Foxp3 expression frequency and mean fluorescence intensity (MFI) from *Foxp3*$^{GFP}$ and *Foxp3*$^{GFP}$*Lgals9*$^{-/-}$ iTreg cells; (D) Activated naïve CD4$^{+}$ T cells were stimulated with TGF-β and/or recombinant galectin-9 for 2, 12 and 24 hours. *Foxp3* mRNA expression was assessed by quantitative real-time PCR analysis; (E) EL4 LAF cells were transfected with a Foxp3 promoter reporter construct containing the CNS1 enhancer and stimulated with anti-CD3 and anit-CD28 antibodies and TGF-β in the presence of increasing concentrations of galectin-9. Luciferase activity was measured 48 hours later; Chimeric mice were generated by transferring WT or *Foxp3*$^{\Delta CNS1}$ BM into WT or *Lgals9*$^{-/-}$ host mice. 10 weeks after reconstitution, the frequency of Foxp3$^{+}$ Treg cells in thymus was determined by (F) flow cytometry and (G) quantification; (H) Quantification of the frequency of Foxp3$^{+}$Nrp1$^{lo}$ iTreg cells or Foxp3$^{+}$Nrp1$^{hi}$ nTreg cells in PP and LP as in **Figure 6E**. Data are representative of three independent experiments (A, C-D, F-H) or are pooled of three independent experiments (B, E) with n ≥ 4 mice each group. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test, error bars, SD).

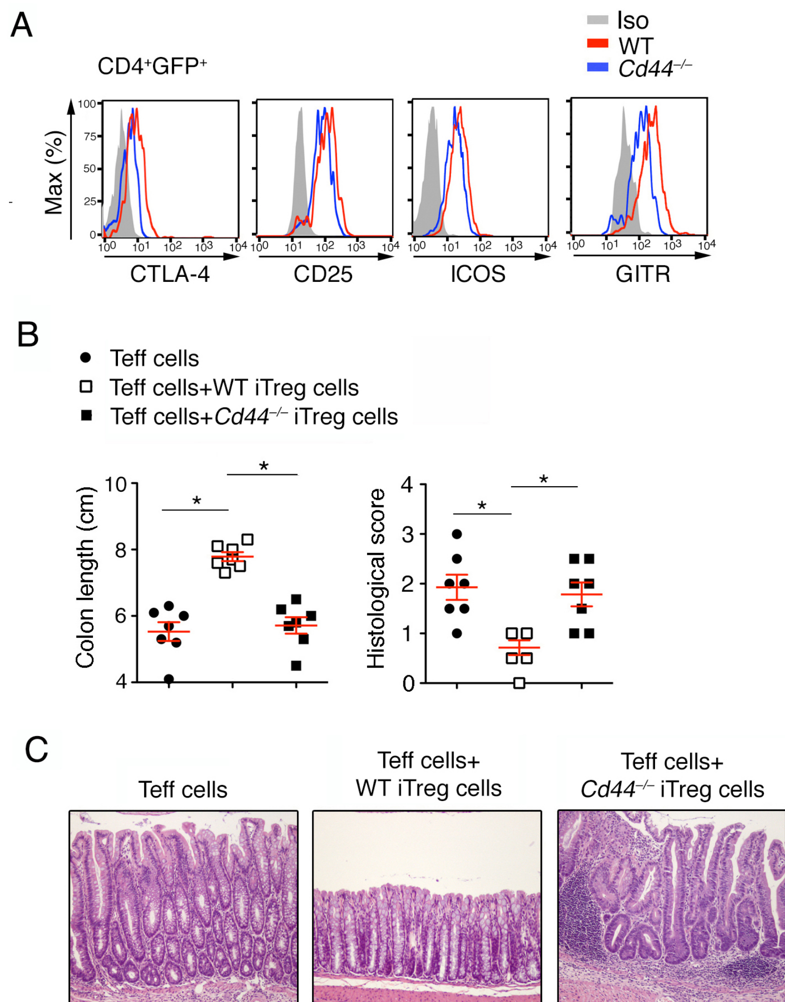


**Figure S7, related to Figure 7.** (A) The expression of indicated co-stimulatory molecules on *Foxp3*$^{GFP}$ or *Foxp3*$^{GFP}$*Cd44*$^{-/-}$ CD4$^{+}$GFP$^{+}$ iTreg cells was determined by flow cytometry; (B) Left: Colon lengths of *Rag2*$^{-/-}$ mice which had received the indicated cells for transfer as in **Figure 7E**, measured from the colocecal junction to the anal verge; Right: Quantification of pathological changes in the colon of mice as in **Figure 7E**; (c) Hematoxylin and eosin staining of colon samples from the different groups as in **Figure 7E** 10 weeks after colitis induction (original magnification, ×20).

The data are representative of three independent experiments with n ≥ 5 mice each group.

*$P < 0.05$, (Student's *t*-test, error bars, SD).