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Supplemental Information

The Microbial Metabolite Butyrate Stimulates Bone Formation via T Regulatory Cell-Mediated Regulation of WNT10B Expression

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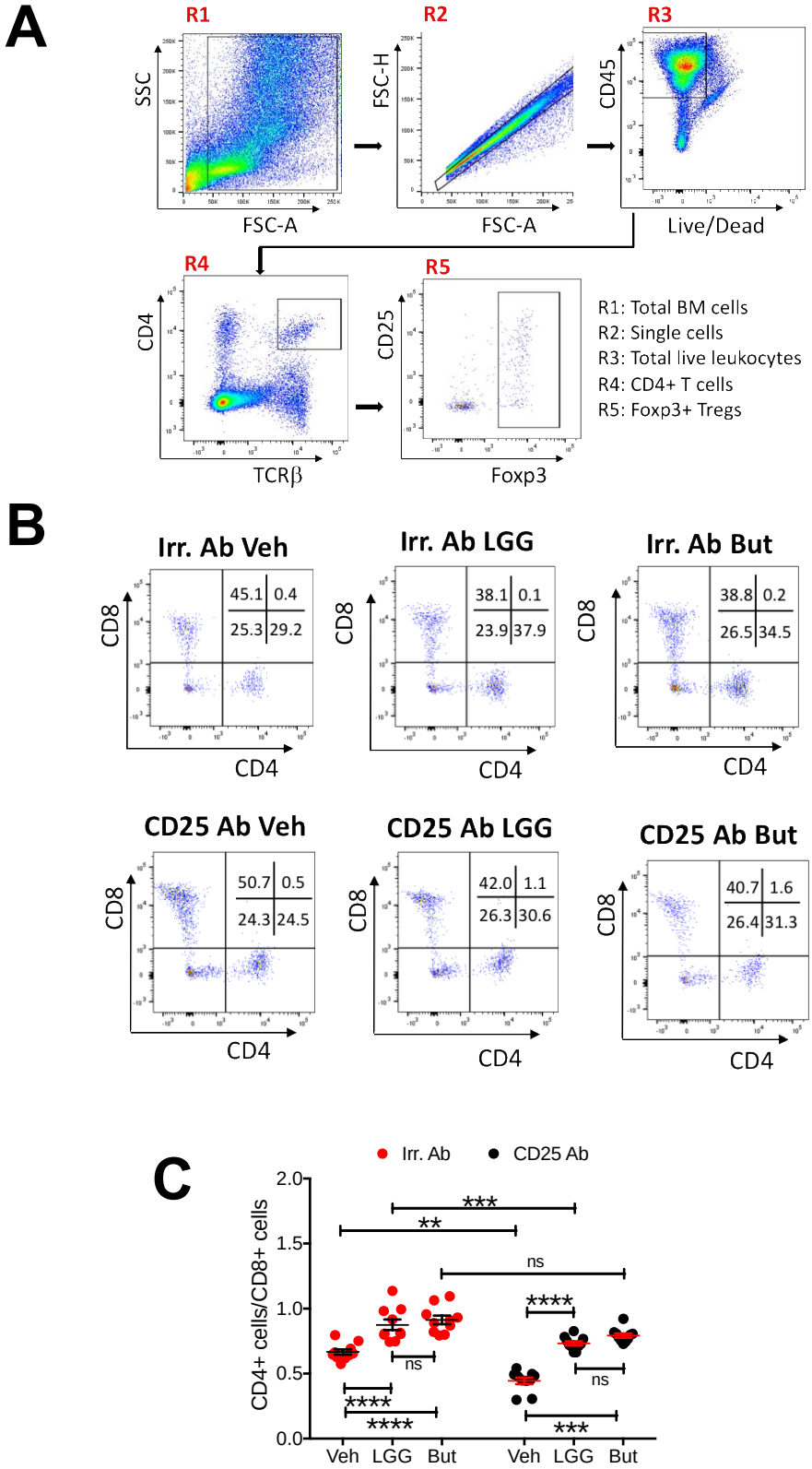

Figure S1 (Related to Figure 1). Gating strategy used to identify bone marrow (BM) Treg cells by flow cytometry and effects of LGG, butyrate (but) or anti-CD25 Ab on the relative frequency of BM CD4+ and CD8+ cells. Following red cells lysis, single cell suspensions were prepared from BM, and stained with antibodies to the indicated antigens and live/dead cell dye. **A.** Gated regions are numbered from R1 to R5. The figure shows one representative gating of flow cytometric plot. **B.** The figure shows one representative flow cytometric plot per group. The numbers in each plot represent the frequency of cells in the corresponding quadrant. **C.** Average CD4+/CD8+ cells ratio. n = 10 samples per group. Data were expressed as Mean ± SEM. Data were normally distributed according to the Shapiro-Wilk normality test. Data and analyzed by two-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$ compared to the indicated group. ns = not significant.

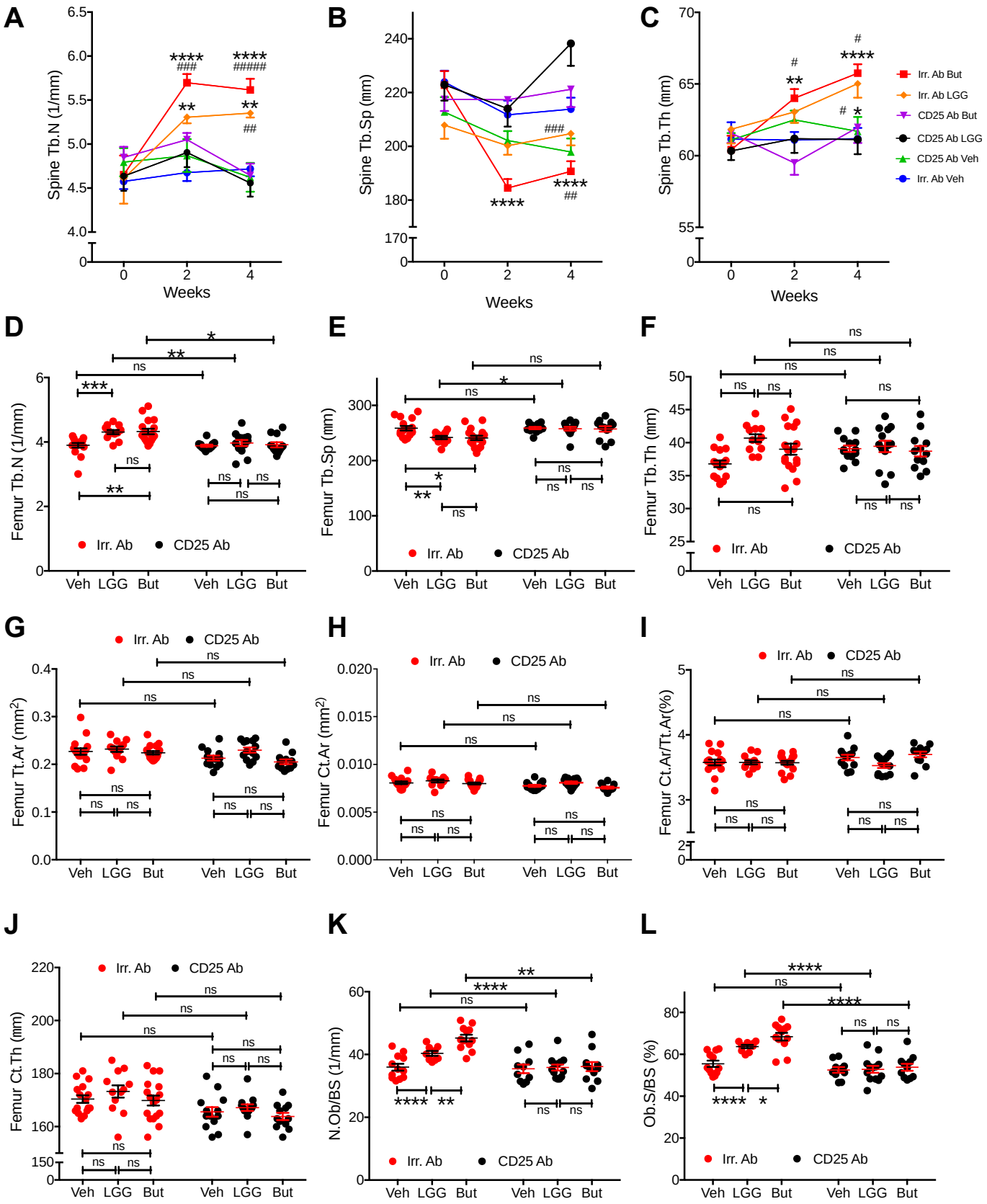
A
B
C
Spine Tb.N (1/mm)
Spine Tb.Sp (mm)
Spine Tb.Th (mm)
Weeks
Irr. Ab But
Irr. Ab LGG
CD25 Ab But
CD25 Ab LGG
CD25 Ab Veh
Irr. Ab Veh
D
E
F
Femur Tb.N (1/mm)
Femur Tb.Sp (mm)
Femur Tb.Th (mm)
Irr. Ab
CD25 Ab
Veh LGG But Veh LGG But
G
H
I
Femur Tt.Ar (mm²)
Femur Ct.Ar (mm²)
Femur Ct.Ar/Tt.Ar(%)
J
K
L
Femur Ct.Th (mm)
N.Ob/BS (1/mm)
Ob.S/BS (%)

Figure S2 (Related to Figure 2). Treatment with anti-CD25 Ab prevents the bone anabolic activity of LGG and butyrate (But). **A-C.** Prospective measurements of vertebral trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) by in vivo μCT scanning. **D-J.** Cross-sectional measurements of femoral Tb.N, Tb.Sp, Tb.Th, total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th) by in vitro μCT scanning. **K, L.** The number of osteoblasts per mm bone surface (N.Ob/BS), and the percentage of bone surface covered by osteoblasts (Ob.S/BS) as measured by bone histomorphometry. n=11-17 mice per group. Data were expressed as Mean $\pm$ SEM. All data were normally distributed according to the Shapiro-Wilk normality test. For Panel A-C data were analyzed by ANOVA for repeated measures. * = $p<0.05$, ** = $p<0.01$, and **** = $p<0.0001$ compared to baseline, # = $p<0.05$, ## = $p<0.01$, ### = $p<0.001$ and ####= $p<0.0001$ compared to Irrelevant (Irr) Ab vehicle. All other data were analyzed by two-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. * = $p<0.05$, ** = $p<0.01$, *** and = $p<0.001$ compared to the indicated group. ns = not significant.

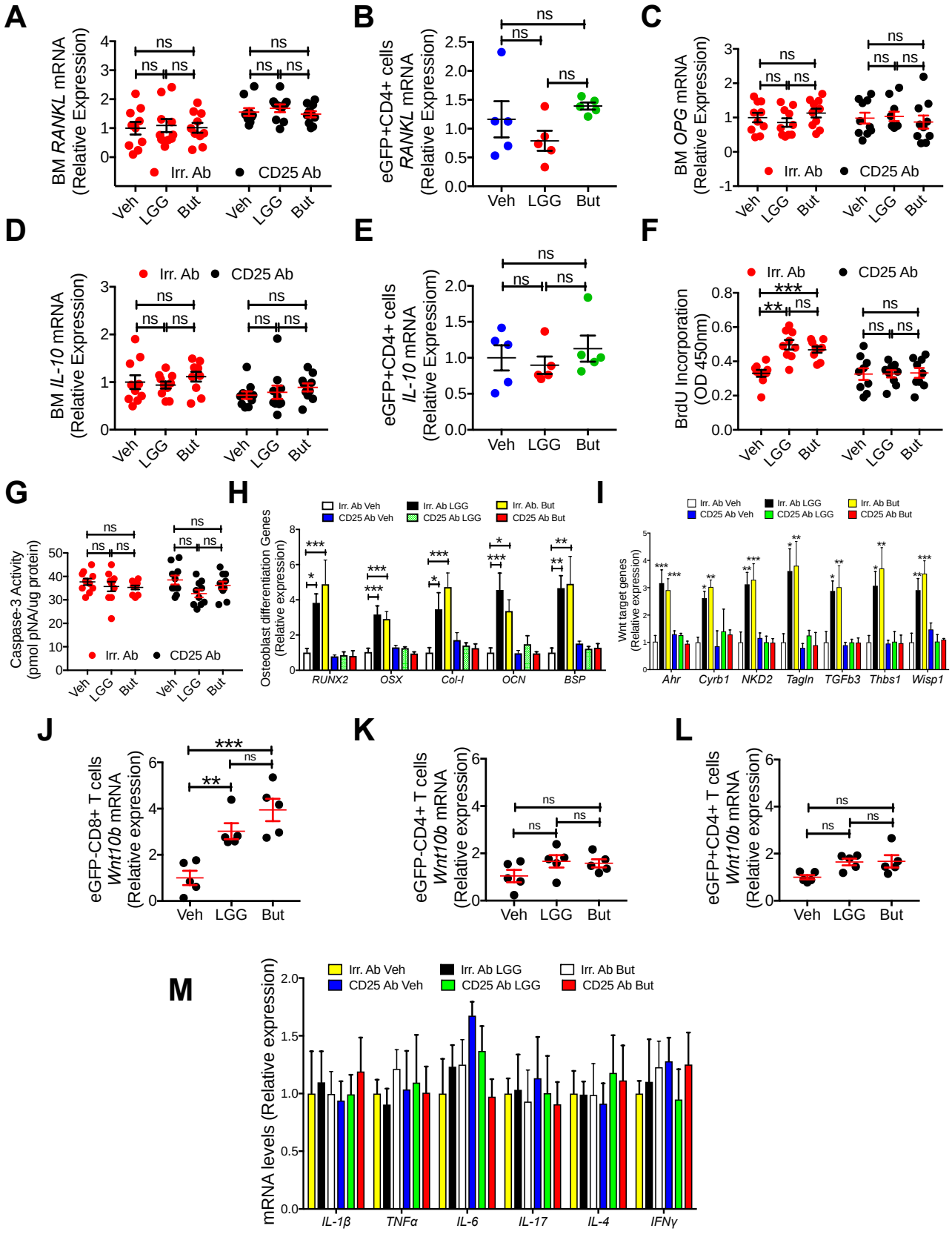
A
BM RANKL mRNA (Relative Expression)
Irr. Ab
CD25 Ab
Veh
LGG
But
B
eGFP+CD4+ cells RANKL mRNA (Relative Expression)
C
BM OPG mRNA (Relative Expression)
D
BM IL-10 mRNA (Relative Expression)
E
eGFP+CD4+ cells IL-10 mRNA (Relative Expressiom)
F
BrdU Incorporation (OD 450nm)
G
Caspase-3 Activity (pmol pNA/ug protein)
H
Osteoblast differentiation Genes (Relative expression)
Irr. Ab Veh
CD25 Ab Veh
Irr. Ab LGG
CD25 Ab LGG
Irr. Ab. But
CD25 Ab But
RUNX2
OSX
Col-I
OCN
BSP
I
Wnt target genes (Relative expression)
Ahr
Cyrb1
NKD2
Tagln
TGFb3
Thbs1
Wisp1
J
eGFP-CD8+ T cells Wnt10b mRNA (Relative expression)
K
eGFP-CD4+ T cells Wnt10b mRNA (Relative expression)
L
eGFP+CD4+ T cells Wnt10b mRNA (Relative expression)
M
mRNA levels (Relative expression)
IL-1β
TNFα
IL-6
IL-17
IL-4
IFNγ

Figure S3 (Related to Figure 2). Effects of LGG and butyrate (But) on BM and Treg cells production of cytokines, stromal cell (SC) proliferation, apoptosis differentiation and Wnt dependent gene expression, and T cell expression of *Wnt10b* mRNA. **A**. BM *RANKL* mRNA expression. **B.** *RANKL* mRNA levels in sorted BM T cells from Foxp3.eGFP reporter mice. **C**. BM *OPG* mRNA expression. **D**. BM *IL-10* mRNA expression. **E.** *IL-10* mRNA levels in sorted BM T cells from Foxp3.eGFP reporter mice. **F**. SC proliferation as measured by BrdU incorporation. **G**. SC apoptosis as measured by Caspase-3 activity. **H**. mRNA levels of factors representative of the differentiation of SCs into osteoblasts, **I.** Transcripts of genes that are specifically increased by Wnt signaling in SCs. The analyzed genes were: aryl-hydrocarbon receptor (*Ahr*), *axin2*, cysteine rich protein 61 (*Cyr61*), naked cuticle 2 homolog (*Nkd2*), transgelin (*tagln*), transforming growth factor β3 (*TGFβ3*), thrombospondin 1 (*Thbs1*), Twist gene homolog 1 (*Twist1*) and Wnt1 inducible signaling pathway protein 1 (*Wisp1*). **J-M**. *Wnt10b* mRNA levels in sorted BM T cells from Foxp3.eGFP reporter mice and cytokine production by unfractionated BM cells. Panel J: eGFP-CD8+ T cells. Panel K: eGFP-CD4+ T cells. Panel L: Treg cells (eGFP+CD4+ cells). Panel M: cytokine production by unfractionated BM cells. For each cytokine, there were no significant differences among groups. n = 5-10 mice per group. Data were expressed as Mean $\pm$ SEM. All data were normally distributed according to the Shapiro-Wilk normality test. All data and analyzed by two-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$ compared to the indicated group. ns = not significant.

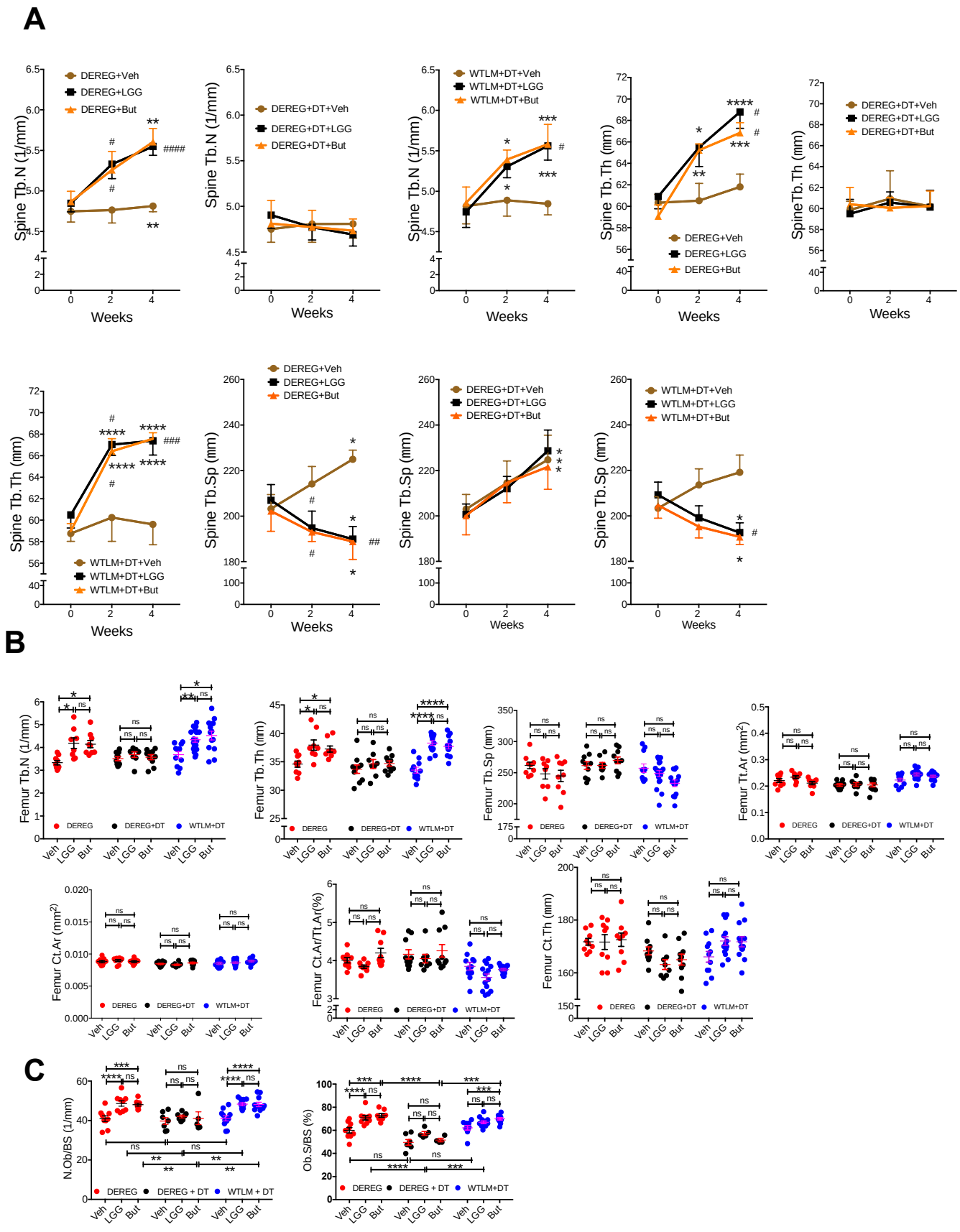
A
Spine Tb.N (1/mm)
DEREG+Veh
DEREG+LGG
DEREG+But
Weeks
Spine Tb.N (1/mm)
DEREG+DT+Veh
DEREG+DT+LGG
DEREG+DT+But
Weeks
Spine Tb.N (1/mm)
WTLM+DT+Veh
WTLM+DT+LGG
WTLM+DT+But
Weeks
Spine Tb.Th (mm)
DEREG+Veh
DEREG+LGG
DEREG+But
Weeks
SpineTb.Th (mm)
DEREG+DT+Veh
DEREG+DT+LGG
DEREG+DT+But
Weeks
Spine Tb.Th (mm)
WTLM+DT+Veh
WTLM+DT+LGG
WTLM+DT+But
Weeks
Spine Tb.Sp (mm)
DEREG+Veh
DEREG+LGG
DEREG+But
Weeks
Spine Tb.Sp (mm)
DEREG+DT+Veh
DEREG+DT+LGG
DEREG+DT+But
Weeks
Spine Tb.Sp (mm)
WTLM+DT+Veh
WTLM+DT+LGG
WTLM+DT+But
Weeks
B
Femur Tb.N (1/mm)
Femur Tb.Th (mm)
Femur Tb.Sp (mm)
Femur Tt.Ar (mm²)
Femur Ct.Ar (mm²)
Femur Ct.Ar/Tt.Ar(%)
Femur Ct.Th (mm)
DEREG
DEREG+DT
WTLM+DT
Veh
LGG
But
C
N.Ob/BS (1/mm)
Ob.S/BS (%)
DEREG
DEREG + DT
WTLM + DT

Figure S4 (Related to Figure 3). Treatment with Diphtheria Toxin (DT) prevents the bone anabolic activity of LGG and butyrate (But) in DEREG mice. **A.** Prospective measurements of vertebral trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) by in vivo μCT scanning. n = 8-12 mice per group. **B.** Cross-sectional measurements of femoral Tb.N, Tb.Sp, Tb.Th, total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th) by in vitro μCT scanning. n = 8-14 mice per group. **C.** The number of osteoblasts per mm bone surface (N.Ob/BS), and the percentage of bone surface covered by osteoblasts (Ob.S/BS) as measured by bone histomorphometry. n=4-11 mice per group. All data were expressed as Mean $\underline{+}$ SEM. All data were normally distributed according to the Shapiro-Wilk normality test. Panel A: Data were analyzed by ANOVA for repeated measures. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$ compared to baseline, # = $p<0.05$, ## = $p<0.01$, ### = $p<0.001$ and #### = $p<0.0001$ compared to Irr. Ab vehicle. Panels B,C. Data were analyzed by two-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. *=$p<0.05$, **=$p<0.01$, and ****= $p<0.0001$ compared to the indicated group. ns = not significant.

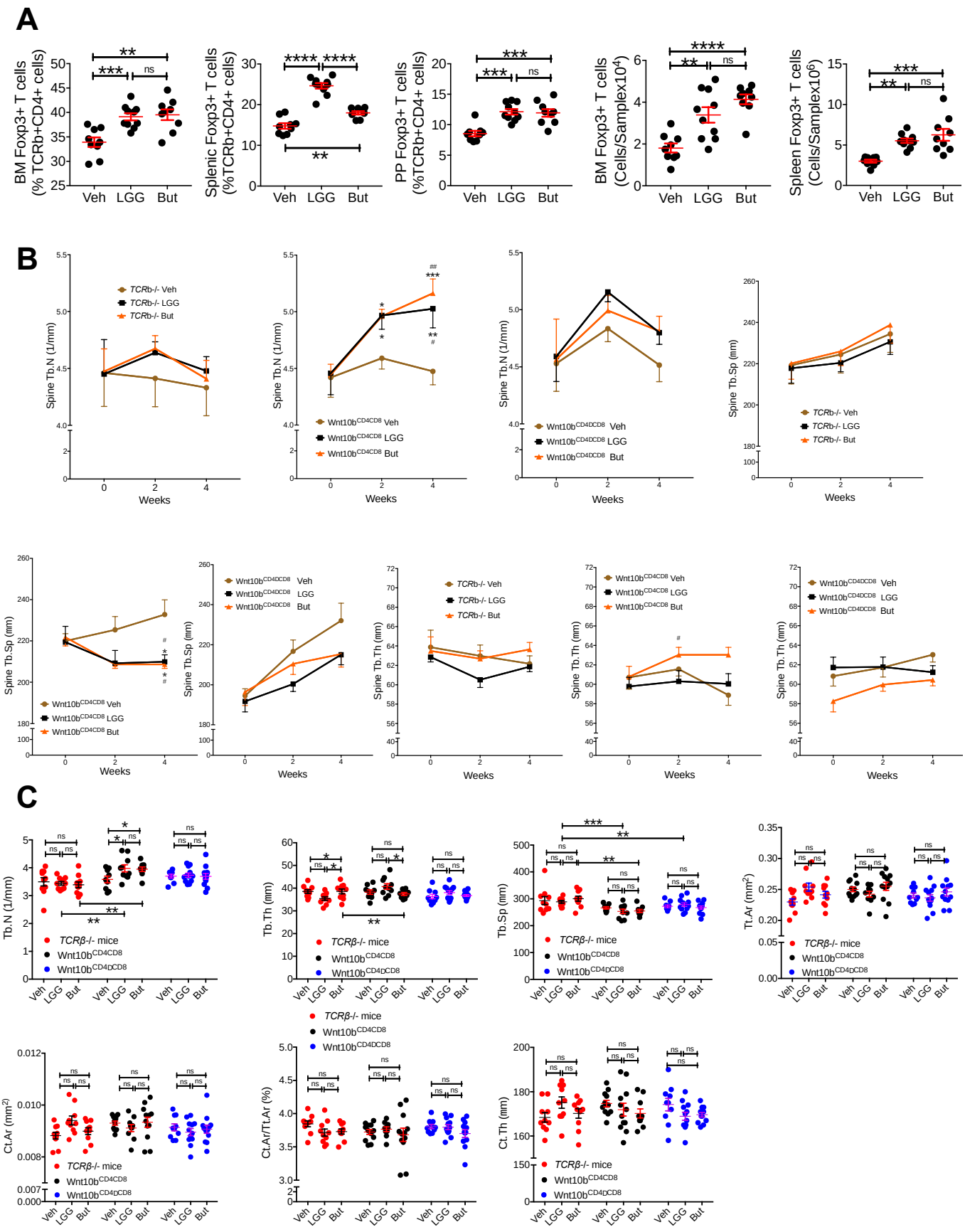
A
BM Foxp3+ T cells (% TCRb+CD4+ cells)
Splenic Foxp3+ T cells (%TCRb+CD4+ cells)
PP Foxp3+ T cells (%TCRb+CD4+ cells)
BM Foxp3+ T cells (Cells/Samplex10^4)
Spleen Foxp3+ T cells (Cells/Samplex10^6)
Veh LGG But
B
Spine Tb.N (1/mm)
Spine Tb.Sp (mm)
Spine Tb.Th (mm)
Weeks
TCRb-/- Veh
TCRb-/- LGG
TCRb-/- But
Wnt10b CD4CD8 Veh
Wnt10b CD4CD8 LGG
Wnt10b CD4CD8 But
Wnt10b CD4DCD8 Veh
Wnt10b CD4DCD8 LGG
Wnt10b CD4DCD8 But
C
Tb.N (1/mm)
Tb.Th (mm)
Tb.Sp (mm)
Tt.Ar (mm2)
Ct.Ar (mm2)
Ct.Ar/Tt.Ar (%)
Ct.Th (mm)
TCRβ-/- mice
Wnt10b CD4CD8
Wnt10b CD4DCD8
Veh LGG But

Figure S5 (Related to Figure 5). Effects of LGG and butyrate (But) on the number of Treg cells, and indices of spinal and vertebral trabecular structure measured by in vivo and in vitro μCT scanning in *TCRβ*-/- mice reconstituted with WT CD4+ and CD8+ T cells or WT CD4+ and Wnt10b-/- CD8+ T cells. **A.** Relative and absolute number of bone marrow (BM), splenic, and Peyer's Patches Treg cells in T cell deficient *TCR*β-/- mice adoptively transferred with WT CD4+ T cells and WT CD8+ T cells. Since the enumeration of the absolute number of PP Treg cells is inaccurate, PP Treg cells are shown as % only. n = 9-12 mice per group. **B.** Prospective measurements of vertebral trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) by in vivo μCT scanning in *TCR*β-/- mice reconstituted with WT CD4+ and CD8+ T cells or WT CD4+ and Wnt10b-/- CD8+ T cells. n = 10-12 mice per group. **C.** Cross-sectional measurements of femoral Tb.N, Tb.Sp, Tb.Th, total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th) by in vitro μCT scanning in *TCR*β-/- mice reconstituted with WT CD4+ and CD8+ T cells or WT CD4+ and Wnt10b-/- CD8+ T cells. n = 9-12 mice per group. Data were expressed as Mean $\pm$ SEM. All data were normally distributed according to the Shapiro-Wilk normality test. Panel A: data were analyzed by one-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. **=p<0.01, ***=p<0.001, and ****=p<0.0001 compared to the indicated group. ns = not significant. Panel B: Data were analyzed by ANOVA for repeated measures and post hoc tests applying the Bonferroni correction for multiple comparisons. * = p<0.05, ** = p<0.01, and *** =p<0.001 compared to baseline, # = p<0.05 and ## = p<0.01 compared to vehicle. Panel C: Data were analyzed by two-way analysis-of-variance and post hoc tests applying the Bonferroni correction for multiple comparisons. * = p<0.05, ** = p<0.01, and *** = p<0.001 compared to the indicated group. ns = not significant

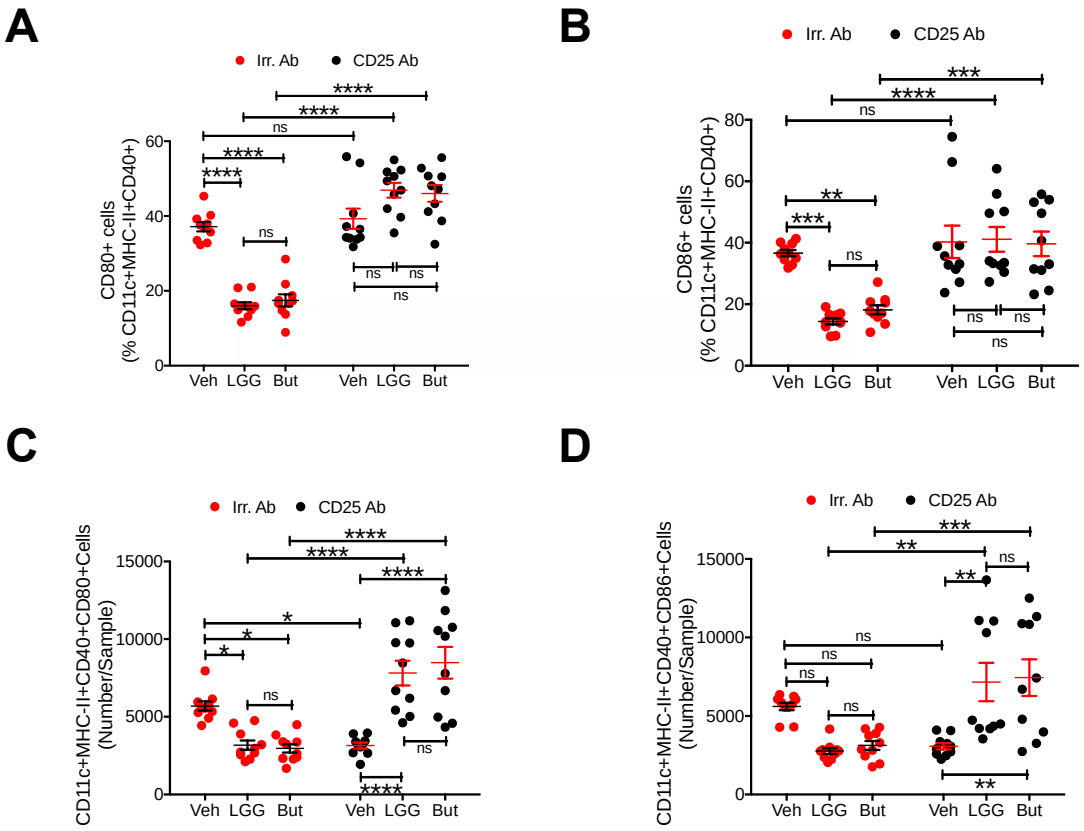

Figure S6 (Related to Figure 7). Effects of LGG and butyrate (but) on the expression of CD80 and CD86 by BM mature dendritic cells (DCs). **A.** Relative frequency of BM CD80+ mature DCs. **B**. Relative frequency of BM CD86+ mature DCs. **C**. Absolute frequency of BM CD80+ mature DCs. **D**. Absolute frequency of BM CD86+ mature DCs. n = 10 mice per group. Data were expressed as Mean ± SEM. All data were normally distributed according to the Shapiro-Wilk normality test. Data were analyzed by two-way ANOVA and post hoc tests applying the Bonferroni correction for multiple comparisons. **= $p<0.01$, ***= $p<0.001$ and ****= $p<0.0001$ compared to vehicle or the indicated group. ns = not significant.

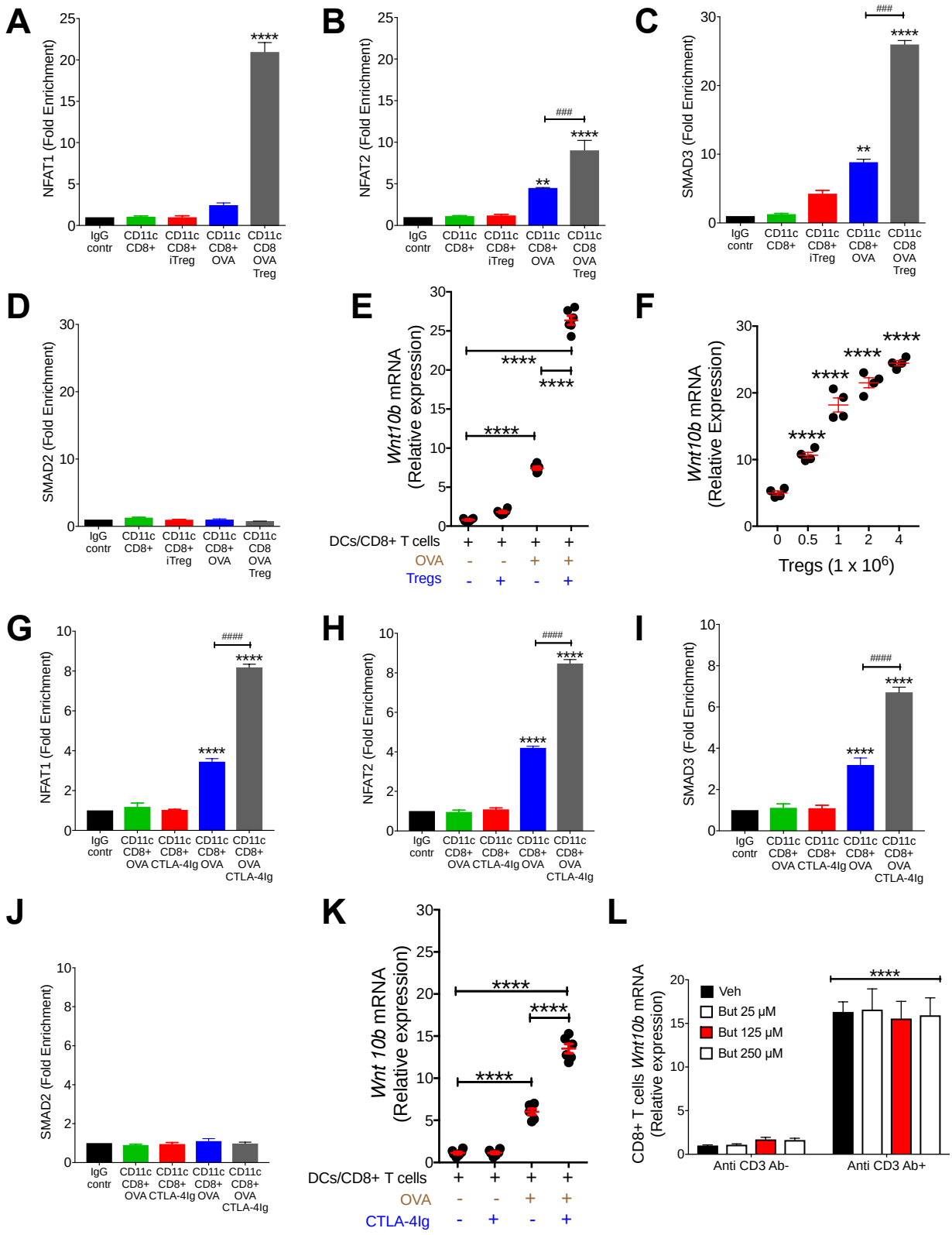
A
NFAT1 (Fold Enrichment)
B
NFAT2 (Fold Enrichment)
C
SMAD3 (Fold Enrichment)
IgG contr
CD11c CD8+
CD11c CD8+ iTreg
CD11c CD8+ OVA
CD11c CD8 OVA Treg
D
SMAD2 (Fold Enrichment)
E
Wnt10b mRNA (Relative expression)
DCs/CD8+ T cells
OVA
Tregs
F
Wnt10b mRNA (Relative Expression)
Tregs (1 x 10^6)
G
NFAT1 (Fold Enrichment)
H
NFAT2 (Fold Enrichment)
I
SMAD3 (Fold Enrichment)
CD11c CD8+ OVA CTLA-4Ig
J
SMAD2 (Fold Enrichment)
K
Wnt 10b mRNA (Relative expression)
CTLA-4Ig
L
CD8+ T cells Wnt10b mRNA (Relative expression)
Veh
But 25 µM
But 125 µM
But 250 µM
Anti CD3 Ab-
Anti CD3 Ab+

Figure S7 (Related to Figure 7). Effects of Treg cells and CTLA-4Ig on NFATs and SMADs binding to the *Wnt10b* promoter and *Wnt10b* mRNA expression. OT-I CD8+ T cells were cocultured with OVA peptide pulsed CD11c+ DCs. **A-D.** Addition of induced Treg cells increases the binding of NFAT1/2 and SMAD3 to the *Wnt10b* promoter, as assessed by ChIP assays. **E**. Addition of induced Treg cells increases the expression of *Wnt10b* by CD8+ T cells. In panels A-E CD8+ T cells were either unstimulated, activated with OVA peptide pulsed DCs and cultured with and without Treg cells. **F**. Treg cells increase the expression of *Wnt10b* by CD8+ T cells in a dose dependent manner. CD8+ T cells were activated with OVA peptide pulsed DCs and cultured with the indicated numbers of Treg cells. **G-J** CTLA-4-Ig increases the binding of NFAT1/2 and SMAD3 to the *Wnt10b* promoter, as assessed by ChIP assays. **K**. CTLA-4-Ig increases the expression of *Wnt10b* by CD8+ T cells. In panels G-K CD8+ T cells were unstimulated, activated with OVA peptide pulsed DCs and cultured with and without CTLA-4-Ig. **L**. Addition of butyrate (But) to cultures of resting and anti-CD3 Ab stimulated CD8+ T cells does not increase *Wnt10b* mRNA expression. Data were expressed as mean + SEM. Panels A-D, G-J and L. n = 3 samples per group. Panels E and K n = 6 samples per group. Panels F n = 4 samples per group. Data were analyzed by Kruskal-Wallis and Dunn's multiple comparisons non-parametric test as they were not normally distributed as assessed by Shapiro-Wilk normality test. **= $p<0.01$, ***= $p<0.001$ and ****= $p<0.0001$ compared to IgG control or the indicated group. ### = $p<0.001$ and #### = $p<0.0001$ compared to the indicated group.

Table S1 (Related to STAR methods). Primer sequences for real-time PCRs, ChIP assays, plasmid construction and mutation of binding sites in murine samples.

Primer Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Mouse *Wnt10b*	GGGACCTCGGGTGACAATAA	CCTCTGTCCTTTTCCAACCG
Mouse *BSP*	GCACTCCAACTGCCCAAGA	TTTTGGAGCCCTGCTTTCTG
Mouse *Col1a1*	CCCTACTCAGCCGTCTGTGC	GGGTTCGGGCTGATGTACC
Mouse *Ocn*	GCCTTCATGTCCAAGCAGGA	GCGCCGGAGTCTGTTCACTA
Mouse *Osx*	GTGTTAGTAACCTGGCCGGG	CATTGGACTTCCCCCTTCTTG
Mouse *Runx2*	CTGTGGTTACCGTCATGGCC	GGAGCTCGGCGGAGTAGTTC
Mouse *TNF*	AACTCCAGGCGGTGCCTAT	TGCCACAAGCAGGAATGAGA
Mouse *IL-4*	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAAGTGAT
Mouse *IL-6*	TAGTCCTTCCTACCCCAATTTC	TTGGTCCTTAGCCACTCCTTCC
Mouse *IL-13*	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
Mouse *IFNγ*	ACAGCAAGGCGAAAAAGGAT	TGGTGGACCACTCGGATGAG
Mouse *IL-17A*	TGACGCCCACCTACAACATC	CATCATGCAGTTCCGTCAGC
Mouse *TGFβ1*	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC
18s ribosomal RNA	ATTCGAACGTCTGCCCTATCA	GTCACCCGTGGTCACCATG
Mouse *IL-10*	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Mouse *RANKL*	CCTGATGAAAGGAGGGAGCA	TGGAATTCAGAATTGCCCGA
Mouse *OPG*		

	CACCTTGAAGGGCCTGATGT	TTTTGGGAAAGTGGGATGTTTT
Mouse *Wnt10b* promoter (ChIP)	GGGAGGGAGTGATCCAGATA	CAGAACCACCCGTGAGTTAG
Mouse *Wnt10b* -2000 bp (Clone)	CGACGCGTAGCAAGACTTCAGAGAGGTTAG	
Mouse *Wnt10b* -1242 bp (Clone)	TTACGCGTACTCAGAAAGAGCACCTCCCG	
Mouse *Wnt10b* -272 bp (Clone)	CGACGCGTCGATGAAAGACCCTGTCATTG	
Mouse *Wnt10b* +216 bp (Clone)		TTTAAGCTTGGGAAACTGTCGGGTTCAG
***NFAT* mut**	taaCGATGAAAGACCCTGTCATTGG	agctTTGCCTCCGAGAGTGGGT
***SMAD* mut**	gcgtCCGTCCTCAGCGTGTCAA	tatgGACCTTAGGGTTACTGACG