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Supplemental Information

Gut Microbiota Modulate CD8 T Cell Responses to Influence Colitis-Associated Tumorigenesis

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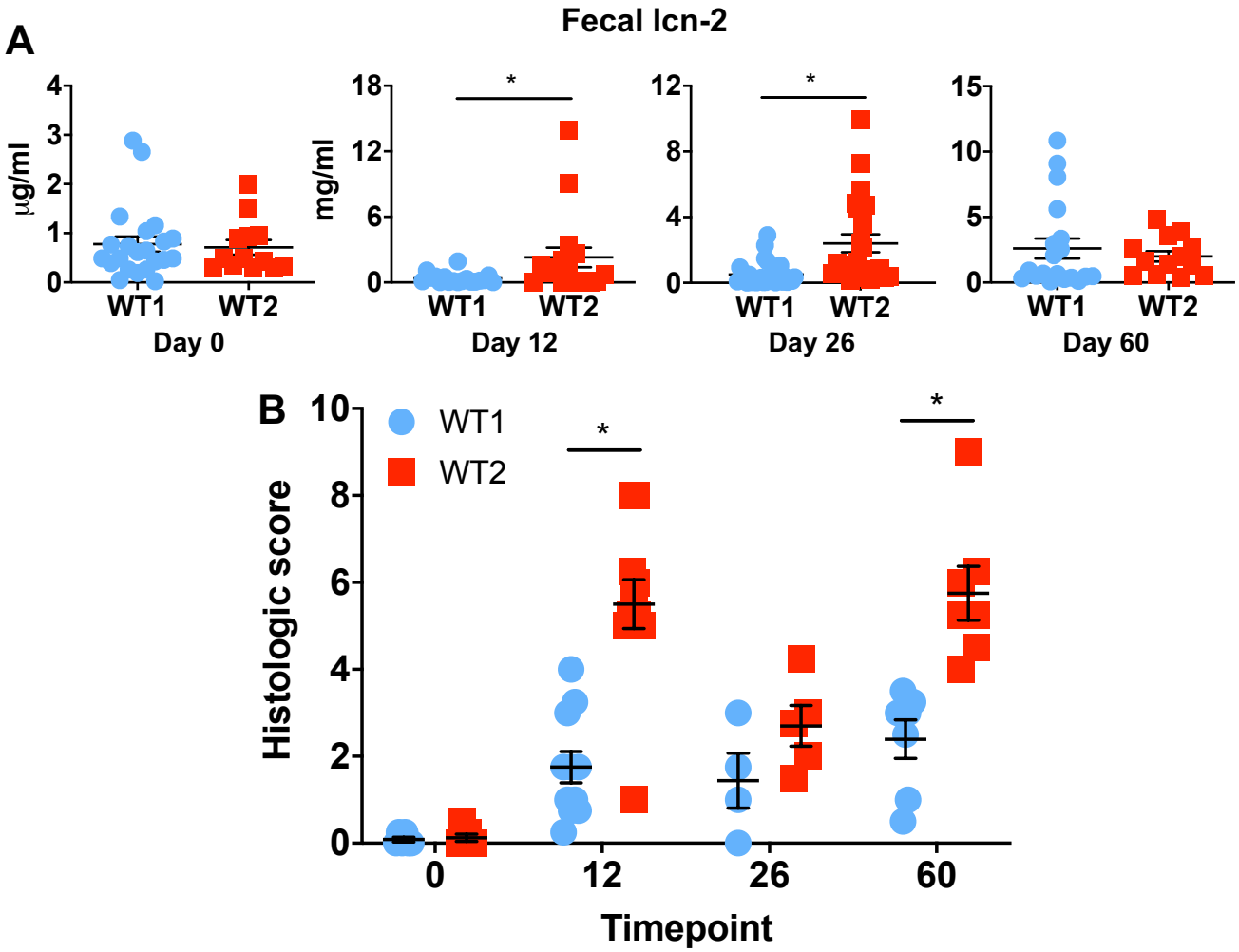

Figure S1. WT2 mice have increased inflammation during AOM/DSS treatment. Related to Figure 1.

(A) Stool lipocalin-2 levels were measured by ELISA on days 0, 12, 26, and 60 of AOM/DSS treatment. D0: WT1 n=22 and WT2 n=13, D12: WT1 n=18 and WT2 n=17, D26: WT1 n=29 and WT2 n=23, D60: WT1 n=19 and WT2 n=13

(B) Severity and extent of inflammatory cell infiltration, epithelial loss and dysplasia of WT1 and WT2 mice on days 0, 12, 26, and 60 of AOM/DSS treatment were assessed by histological scoring. D0: n=6/group, D12: n=11/group, D26: WT1 n=4 and WT2 n=5, D60: n=7/group

Data are mean ± SEM. *p<0.05 by Mann-Whitney

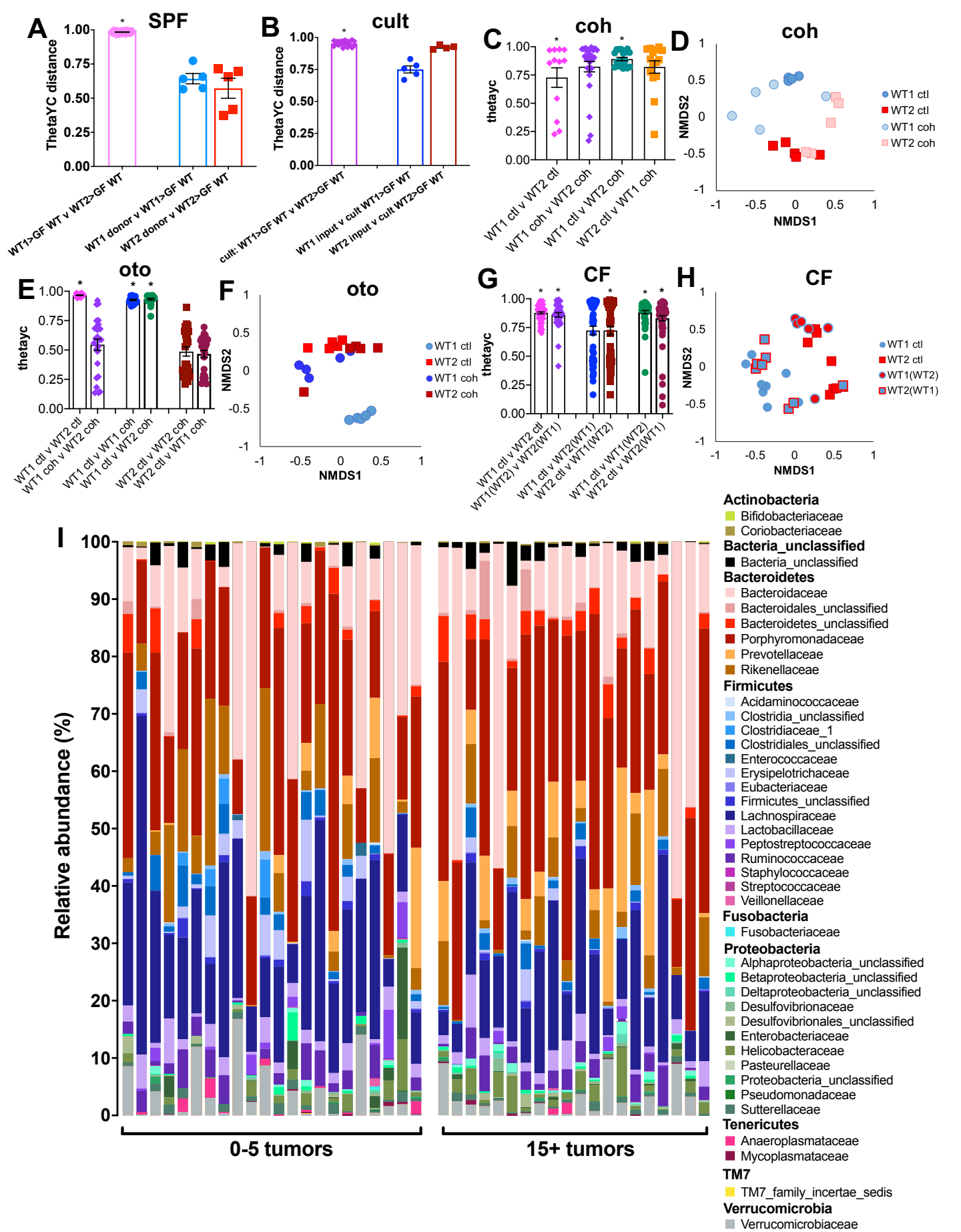
A
SPF
ThetaYC distance
WT1>GF WT v WT2>GF WT
WT1 donor v WT1>GF WT
WT2 donor v WT2>GF WT
B
cult
ThetaYC distance
cult: WT1>GF WT v WT2>GF WT
WT1 input v cult WT1>GF WT
WT2 input v cult WT2>GF WT
C
coh
thetayc
WT1 ctl v WT2 ctl
WT1 coh v WT2 coh
WT1 ctl v WT2 coh
WT2 ctl v WT1 coh
D
coh
NMDS2
NMDS1
WT1 ctl
WT2 ctl
WT1 coh
WT2 coh
E
oto
thetayc
WT1 ctl v WT2 ctl
WT1 coh v WT2 coh
WT1 ctl v WT1 coh
WT1 ctl v WT2 coh
WT2 ctl v WT2 coh
WT2 ctl v WT1 coh
F
oto
NMDS2
NMDS1
WT1 ctl
WT2 ctl
WT1 coh
WT2 coh
G
CF
thetayc
WT1 ctl v WT2 ctl
WT1(WT2) v WT2(WT1)
WT1 ctl v WT2(WT1)
WT2 ctl v WT1(WT2)
WT1 ctl v WT1(WT2)
WT2 ctl v WT2(WT1)
H
CF
NMDS2
NMDS1
WT1 ctl
WT2 ctl
WT1(WT2)
WT2(WT1)
I
Relative abundance (%)
0-5 tumors
15+ tumors
Actinobacteria
Bifidobacteriaceae
Coriobacteriaceae
Bacteria_unclassified
Bacteria_unclassified
Bacteroidetes
Bacteroidaceae
Bacteroidales_unclassified
Bacteroidetes_unclassified
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Firmicutes
Acidaminococcaceae
Clostridia_unclassified
Clostridiaceae_1
Clostridiales_unclassified
Enterococcaceae
Erysipelotrichaceae
Eubacteriaceae
Firmicutes_unclassified
Lachnospiraceae
Lactobacillaceae
Peptostreptococcaceae
Ruminococcaceae
Staphylococcaceae
Streptococcaceae
Veillonellaceae
Fusobacteria
Fusobacteriaceae
Proteobacteria
Alphaproteobacteria_unclassified
Betaproteobacteria_unclassified
Deltaproteobacteria_unclassified
Desulfovibrionaceae
Desulfovibrionales_unclassified
Enterobacteriaceae
Helicobacteraceae
Pasteurellaceae
Proteobacteria_unclassified
Pseudomonadaceae
Sutterellaceae
Tenericutes
Anaeroplasmataceae
Mycoplasmataceae
TM7
TM7_family_incertae_sedis
Verrucomicrobia
Verrucomicrobiaceae

Figure S2

Figure S2. Microbiome colonization after microbiome transfer experiments. Related to Figures 3 and 4.

(A and B) Microbiome composition similarity of GF WT mice colonized with whole SPF stool and cecal contents **(A)** or cultivable bacteria **(B)** of WT1 or WT2 mice was analyzed by ThetaYC distance between groups.

(C and D) WT1 and WT2 mice were cohoused (2:2) and stool was collected after four weeks for microbiome analysis. **(C)** Microbiome composition dissimilarity was analyzed by ThetaYC distance. **(D)** Beta diversity is shown as a non-metric dimensional scaling plot (NMDS).

(E and F) WT1 and WT2 mice were cohoused (1:1) and stool was collected after six weeks for microbiome analysis. Microbiome composition dissimilarity was analyzed by ThetaYC distance **(E)** and shown as an NMDS plot **(F)**.

(G and H) WT1 and WT2 mice were cross-fostered and stool was collected from 6-week-old mice for microbiome analysis. Microbiome composition dissimilarity was analyzed by ThetaYC distance **(G)** and shown as an NMDS plot **(H)**.

(I) Stool was collected on day 0 before AOM injection. Relative abundances of family-level bacteria in naive mice that eventually developed low (0-5) or high (15+) tumors after AOM/DSS treatment are shown. Low tumor n=22, high tumor n=20

Data are representative of or pooled from at least two independent experiments. *$p<0.05$ by AMOVA

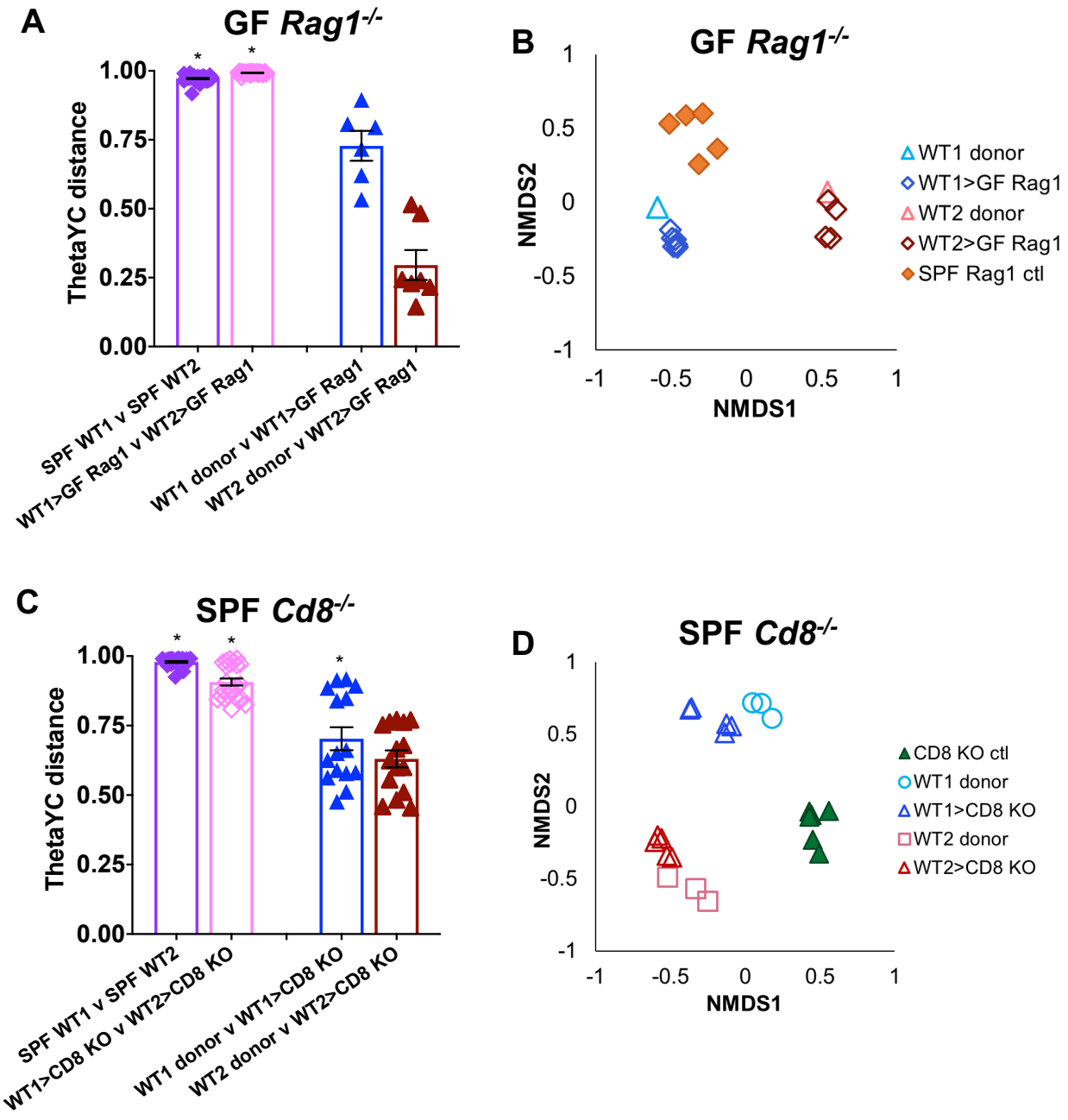

Figure S3. Beta diversity of GF *Rag1*$^{-/-}$ or SPF *Cd8*$^{-/-}$ mice colonized with SPF WT1 or WT2 microbiomes. Related to Figures 5 and 6.

(A and B) 16S rRNA sequencing of fecal microbiota of naïve mice was used to assess microbiome differences between GF *Rag1*$^{-/-}$ mice colonized with SPF WT1 or WT2 stool homogenates. Microbiome composition dissimilarity was analyzed by ThetaYC distance **(A)** and shown as an NMDS plot **(B)**.

(C and D) 16S rRNA sequencing of fecal microbiota of naïve mice was used to assess microbiome differences between SPF *Cd8*$^{-/-}$ mice recolonized with SPF WT1 or WT2 stool homogenates after antibiotic and antifungal treatment. Microbiome composition dissimilarity was analyzed by ThetaYC distance **(C)** and shown as an NMDS plot **(D)**.

n=4-6/group, *p<0.05 by AMOVA

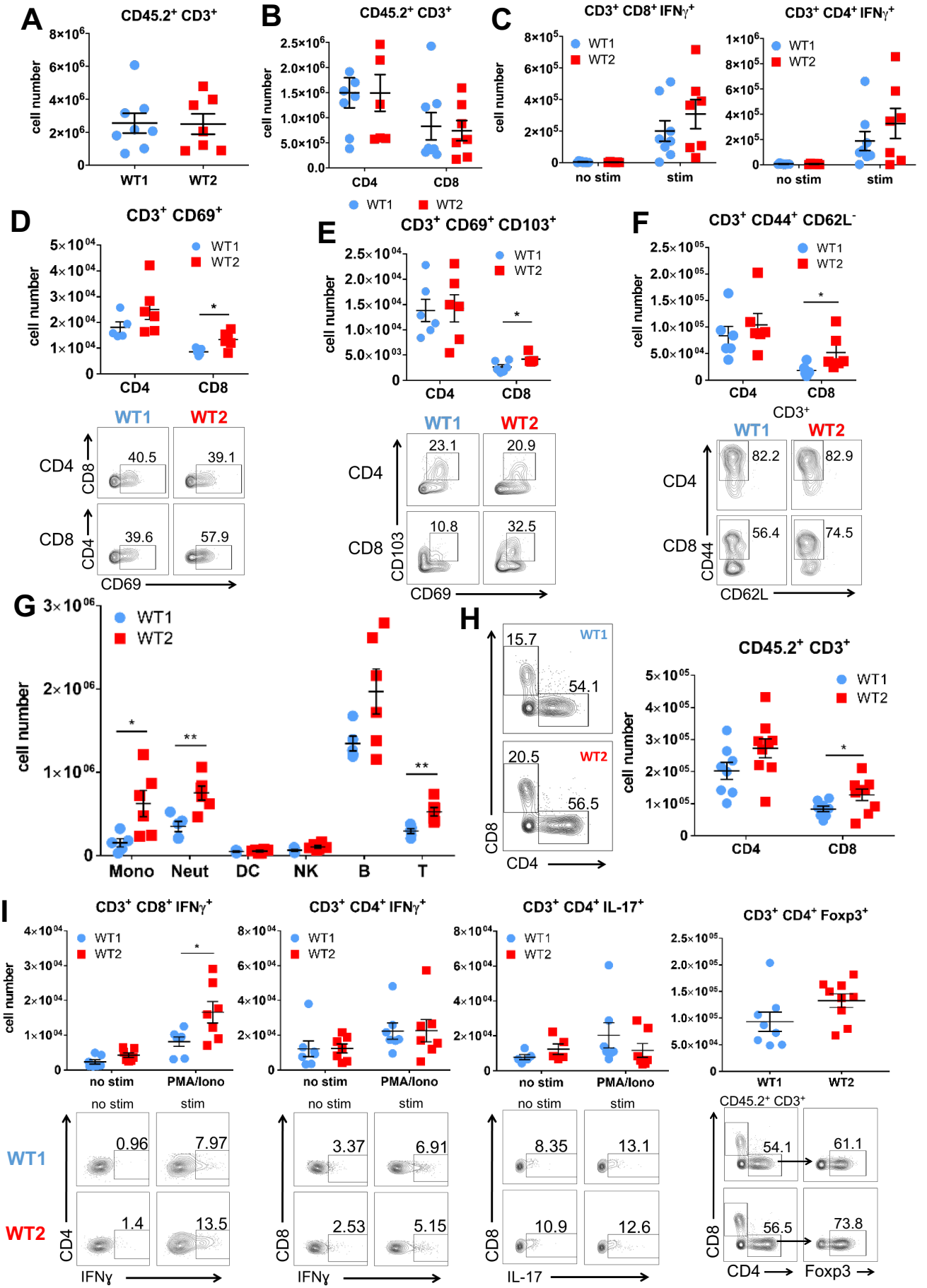
A
CD45.2+ CD3+
cell number
WT1
WT2
B
CD45.2+ CD3+
CD4
CD8
C
CD3+ CD8+ IFNγ+
CD3+ CD4+ IFNγ+
no stim
stim
D
CD3+ CD69+
E
CD3+ CD69+ CD103+
F
CD3+ CD44+ CD62L-
CD3+
40.5
39.1
39.6
57.9
23.1
20.9
10.8
32.5
82.2
82.9
56.4
74.5
CD69
CD103
CD44
CD62L
G
Mono
Neut
DC
NK
B
T
H
15.7
54.1
20.5
56.5
I
PMA/Iono
IFNγ
IL-17
Foxp3
0.96
7.97
1.4
13.5
3.37
6.91
2.53
5.15
8.35
13.1
10.9
12.6
54.1
61.1
56.5
73.8

Figure S4

Figure S4. Naive WT2 mice have increased activated and effector memory colon lamina propria CD8 T cells and no T cell differences in the mesenteric lymph nodes. Colon lamina propria $CD8^+$ $IFN\gamma^+$ T cells are increased in WT2 mice on day 12 of AOM/DSS treatment. Related to Figure 6.

(A-C) Mesenteric lymph node (MLN) cells from untreated WT1 and WT2 mice were *ex vivo*-stimulated. T cell subsets **(A and B)** and IFNɣ activity **(C)** were analyzed by flow cytometry.

(D-F) Naive colon LP T cells were analyzed for activation **(D)**, resident memory T cells **(E)**, and T memory subsets **(F)**. Cells are gated on $CD45.2^+$ $CD3^+$ or $CD3^+$.

(G and H) Mice were injected with AOM and five days later, treated with 2% DSS for five days. Two days after DSS treatment completion, mice were sacrificed for colon LP flow cytometry analysis. Colon LP immune cells **(G)** and T cell subsets **(H)** were analyzed by flow cytometry.

(I) Colon lamina propria immune cells were *ex vivo*-stimulated with PMA and ionomycin and monensin for four hours before flow cytometry analysis. Non-stimulated cells were incubated with monensin for four hours before and flow cytometry analysis.

Data are mean ± SEM and are pooled from at least two independent experiments. n=5-9/group. *$p<0.05$ by Mann-Whitney

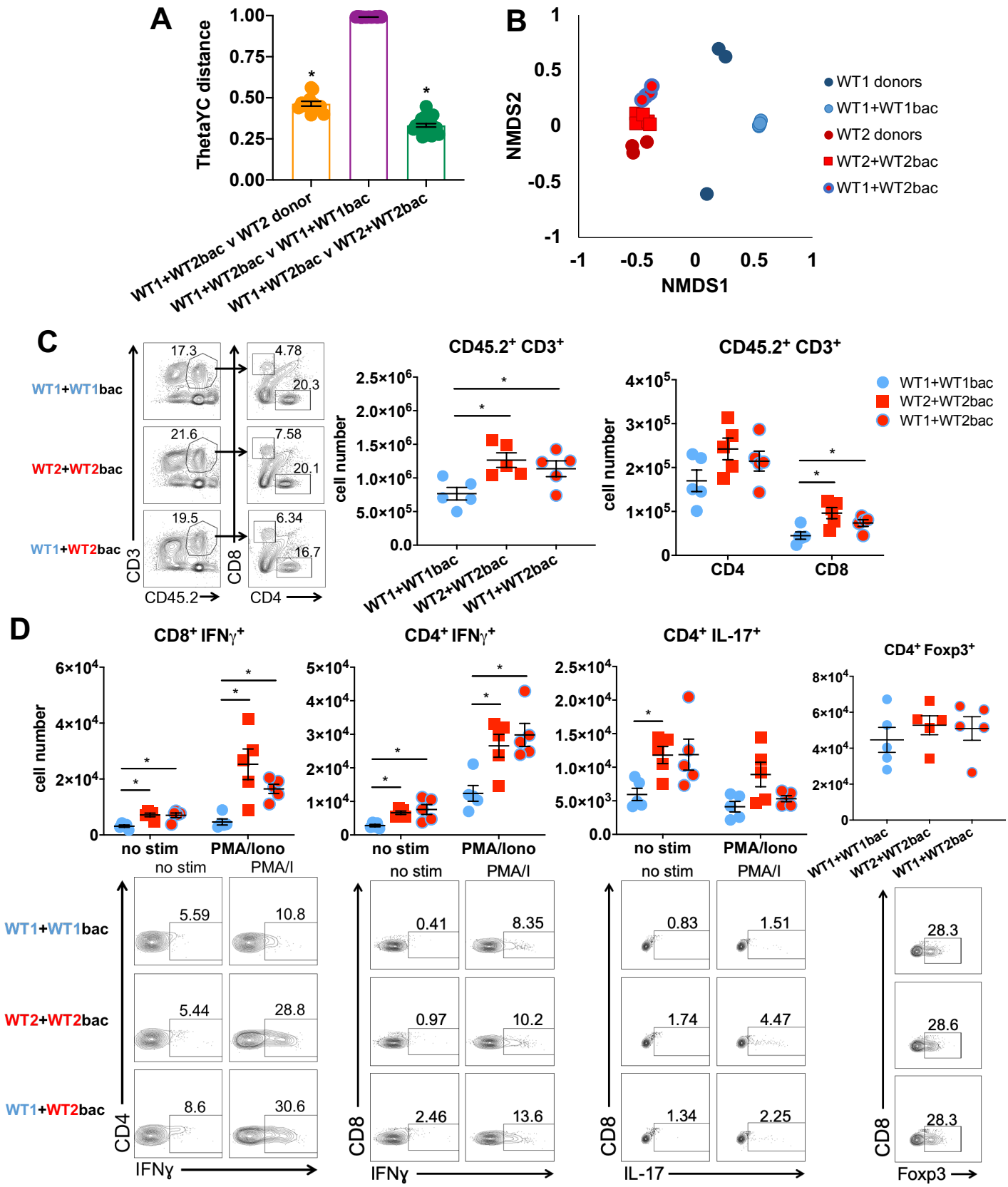
A
ThetaYC distance
1.00
0.75
0.50
0.25
0.00
WT1+WT2bac v WT2 donor
WT1+WT2bac v WT1+WT1bac
WT1+WT2bac v WT2+WT2bac
B
NMDS2
NMDS1
WT1 donors
WT1+WT1bac
WT2 donors
WT2+WT2bac
WT1+WT2bac
C
WT1+WT1bac
WT2+WT2bac
WT1+WT2bac
CD3
CD8
CD45.2
CD4
17.3
4.78
20.3
21.6
7.58
20.1
19.5
6.34
16.7
CD45.2+ CD3+
cell number
WT1+WT1bac
WT2+WT2bac
WT1+WT2bac
CD4
CD8
D
CD8+ IFNγ+
CD4+ IFNγ+
CD4+ IL-17+
CD4+ Foxp3+
cell number
no stim
PMA/Iono
PMA/I
5.59
10.8
5.44
28.8
8.6
30.6
0.41
8.35
0.97
10.2
2.46
13.6
0.83
1.51
1.74
4.47
1.34
2.25
28.3
28.6
28.3
IFNγ
IL-17
Foxp3

Figure S5

Figure S5. SPF WT1 mice colonized with WT2 microbiota develop increased colon lamina propria $CD8^+$ $IFN\gamma^+$ T cells. Related to Figure 6.

SPF WT1 and WT2 mice were treated with antibiotics and antifungal water for one week prior to three consecutive gavages of SPF WT1 or WT2 microbiota. Nine weeks after colonization, the colon LP was analyzed by flow cytometry.

(A and B) Microbiome composition dissimilarity was analyzed by ThetaYC distance **(A)** and shown as an NMDS plot **(B)**. *p<0.05 by AMOVA

(C) CD3 T cell and T cell subsets were analyzed by flow cytometry.

(D) Colon lamina propria immune cells were *ex vivo*-stimulated with PMA and ionomycin and monensin for four hours before flow cytometry analysis. Non-stimulated cells were incubated with monensin for four hours before and flow cytometry analysis.

Data are mean ± SEM and are representative of two independent experiments. n=5/group, *p<0.05 by Mann-Whitney unless otherwise noted

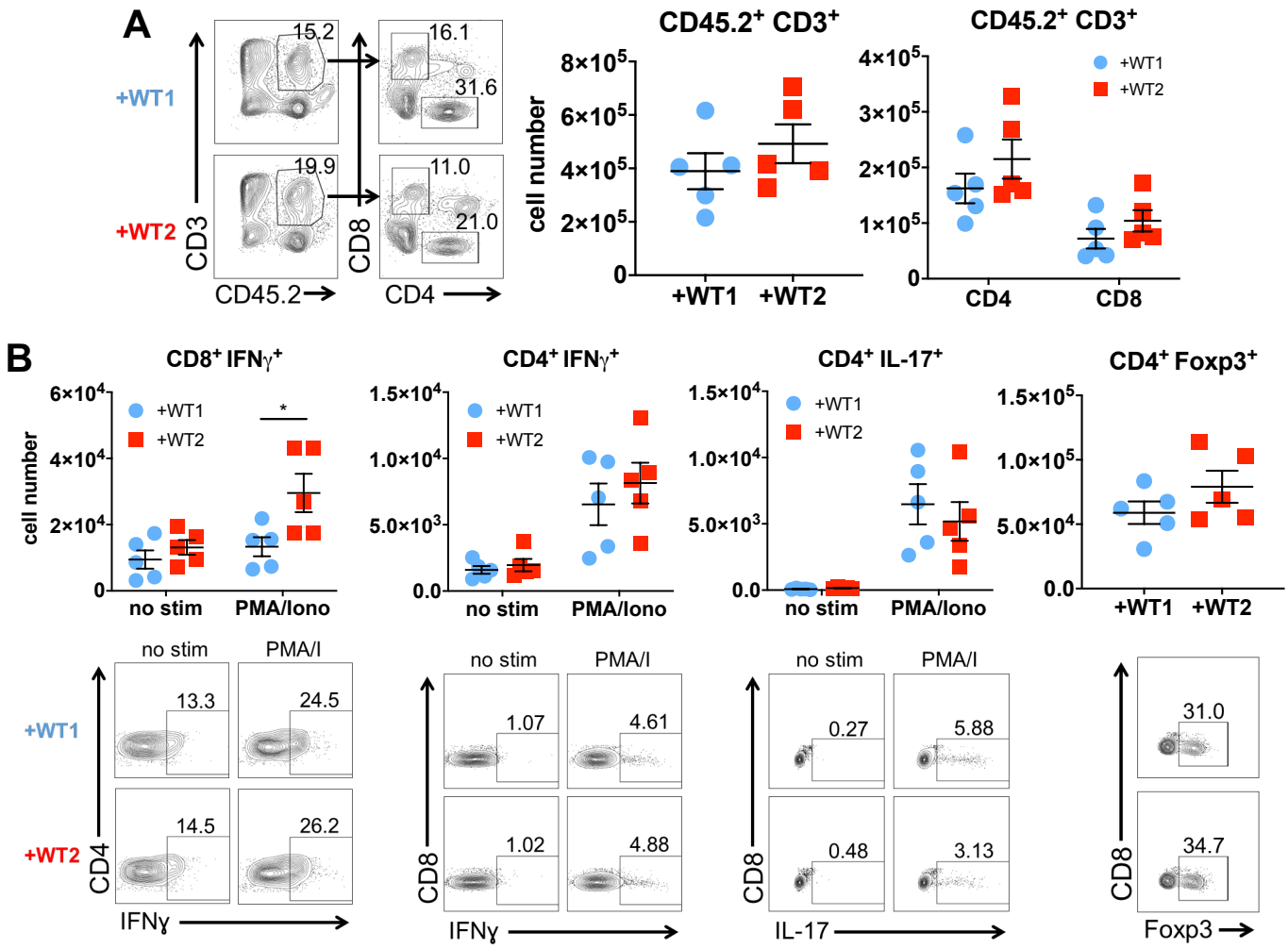

Figure S6. GF WT mice colonized with WT2 microbiota develop increased colon lamina propria CD8⁺ IFNγ⁺ T cells. Related to Figure 6.

GF WT mice were gavaged with SPF WT1 or WT2 microbiota. Eight weeks after colonization, the colon LP was analyzed by flow cytometry.

(A) CD3 T cell and T cell subsets were analyzed by flow cytometry.

(B) Colon lamina propria immune cells were *ex vivo*-stimulated with PMA and ionomycin and monensin for four hours before flow cytometry analysis. Non-stimulated cells were incubated with monensin for four hours before and flow cytometry analysis.

Data are mean ± SEM. n=5/group, *p<0.05 by Mann-Whitney

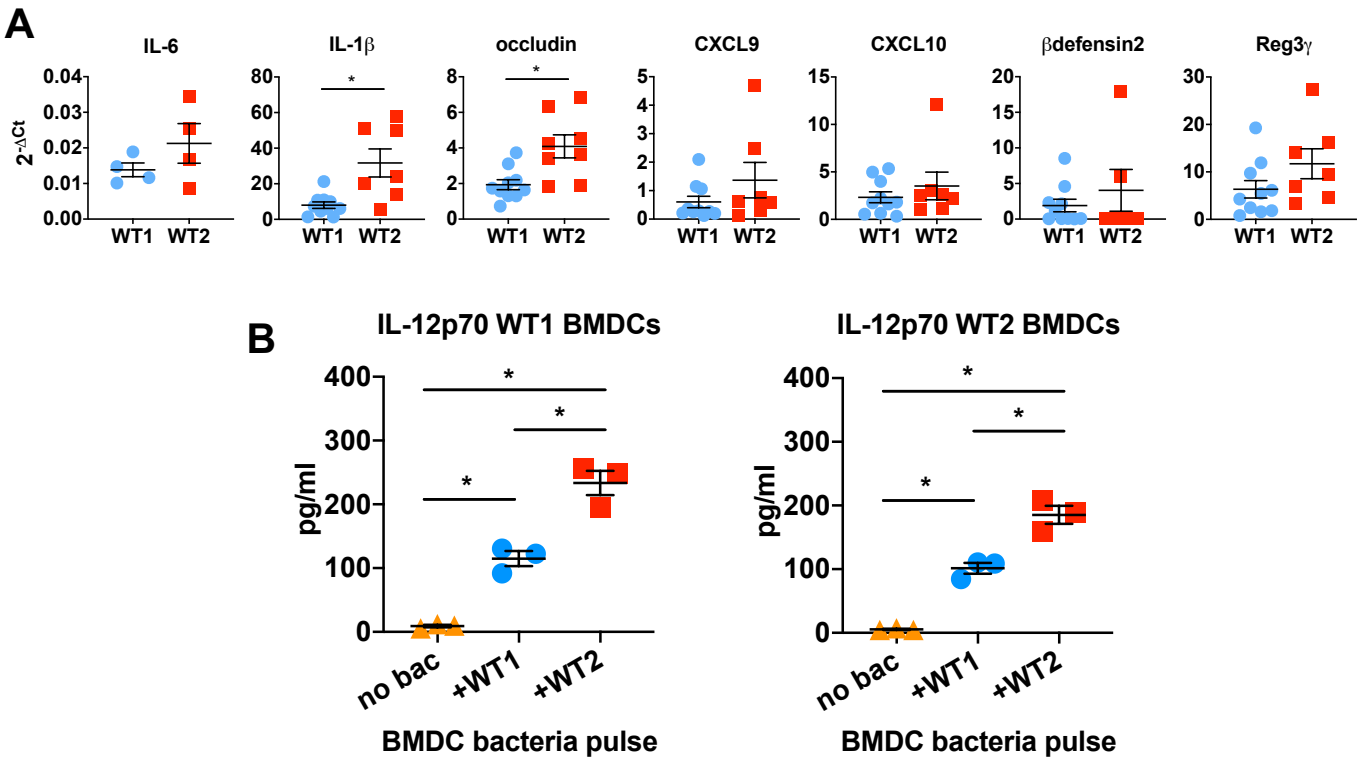

Figure S7. WT2 microbiota results in increased production of IL-12 production by BMDCs. Related to Figure 6.

(A) mRNA expression of several epithelial cell-related genes relative to actin by untreated WT1 and WT2 epithelial cells. IL-6: n=4/group; rest: WT1 n=10, WT2 n=6 (βdef2), 7 (CXCL9, Reg3ɣ, and IL-1β), or 8 (CXCL10 and occludin). *p<0.05 by Mann-Whitney

(B) IL-12p70 levels were measured by ELISA in 24-hour supernatants of bone marrow-derived dendritic cells exposed to heat-killed SPF WT1 or WT2 bacteria cultures. n=3 mice/colony. WT1 data are representative of three independent experiments. WT2 data are representative of two independent experiments. *p<0.05 by Mann-Whitney