Immunity, Volume *49*

Supplemental Information

The Adaptor Protein CARD9 Protects against Colon Cancer by Restricting Mycobiota-Mediated Expansion of Myeloid-Derived Suppressor Cells

Tingting Wang, Chaogang Fan, Anran Yao, Xingwei Xu, Guoxing Zheng, Yun You, Changying Jiang, Xueqiang Zhao, Yayi Hou, Mien-Chie Hung, and Xin Lin

Supplemental Information

The adaptor CARD9 protects against colitis-associated colon cancer by restricting mycobiota-mediated expansion of myeloid-derived suppressor cells

Tingting Wang, Chaogang Fan, Anran Yao, Xingwei Xu, Guoxing Zheng, Yun You, Changying Jiang, Xueqiang Zhao, Yayi Hou, Mien-Chie Hung, and Xin Lin

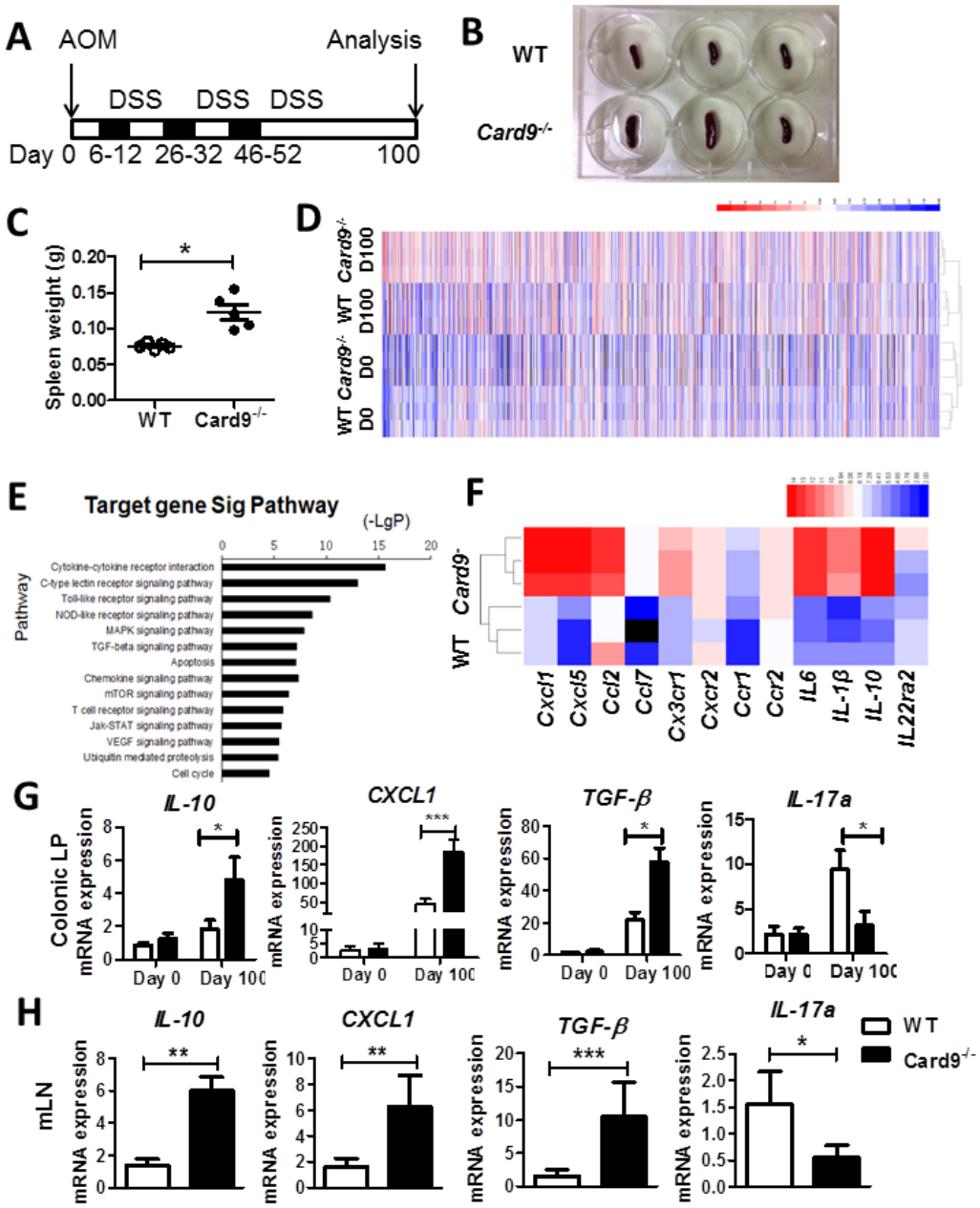

Figure S1. *Card9*$^{-/-}$ mice have increased tumor-burden upon AOM-DSS treatment than WT mice. Related to Figure 1.

(A) WT mice and *Card9*$^{-/-}$ mice (n=5 for each group) were separated at least 4 weeks before use. Mice were injected intraperitoneally with AOM (10 mg/kg) on day 1. After 5 days, 2% DSS was added to the drinking water for 7 consecutive days. Three cycles of DSS treatment were used. Mice were euthanized on day 100. (B-C) Spleens from mice were photographed and weighted. (D) Colon tissues were separated from AOM-DSS treated or untreated WT and *Card9*$^{-/-}$ mice. Gene expressions were determined by mRNA array. (E) Pathway analysis based on differentially expressed genes in mRNA array. (F) Cluster analysis of cytokine and chemokine in colon tissues based on mRNA array. (G) Gene expression in colonic LP cells was determined by qPCR. (H) Gene expression in mLNs was determined by qPCR. Data with error bars are presented as mean $\pm$ SD. Each panel is a representative experiment of at least 3 independent biological replicates. * P <0.05, ** P < 0.01, *** P < 0.001 as determined by unpaired Student's t test.

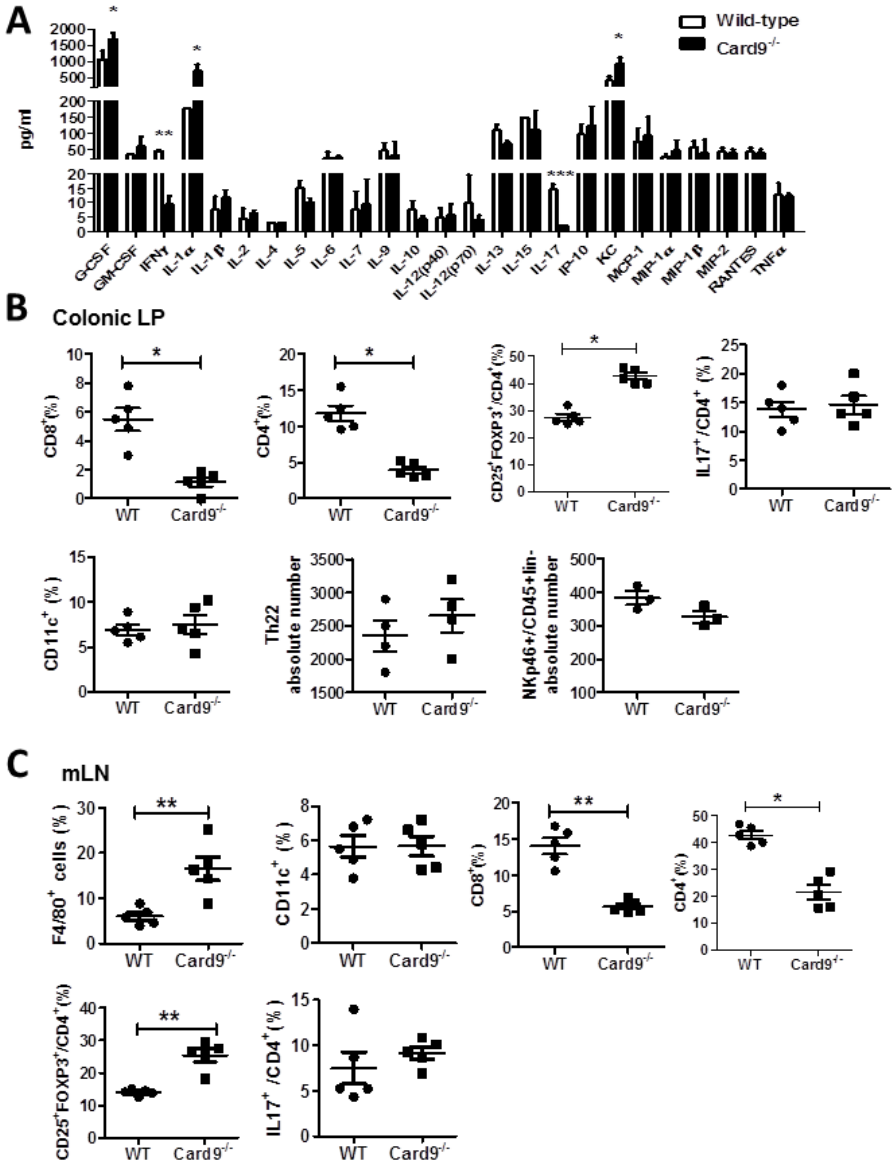

Figure S2. Impaired immune responses in *Card9*$^{-/-}$ mice during the development of CAC. Related to Figure 1.

(A) Mice were treated as described in Figure 1A (n=5 per group). Multiple cytokine production in the serum of tumor-bearing WT and *Card9*$^{-/-}$ mice was determined by a suspension array. (B) Colon tissues were collected on Day 100 and colonic LP cells were isolated. The proportion of immune cells in LP cells was determined by flow cytometry. (C) mLNs were isolated from each mouse. The proportion of immune cells in mLN was determined by flow cytometry. Data with error bars are presented as mean ± SD. Each panel is a representative experiment of at least 3 independent

biological replicates. * P <0.05, ** P < 0.01 as determined by unpaired Student's t test.

A

	WT (n=20)	Card9$^{-/-}$(n=22)
Mouse has no tumors (n)	12	14
Mouse has only one tumor (n)	6	5
Mouse has two tumors (n)	2	3
Percentage of mouse with tumors	40.0%	36.4%

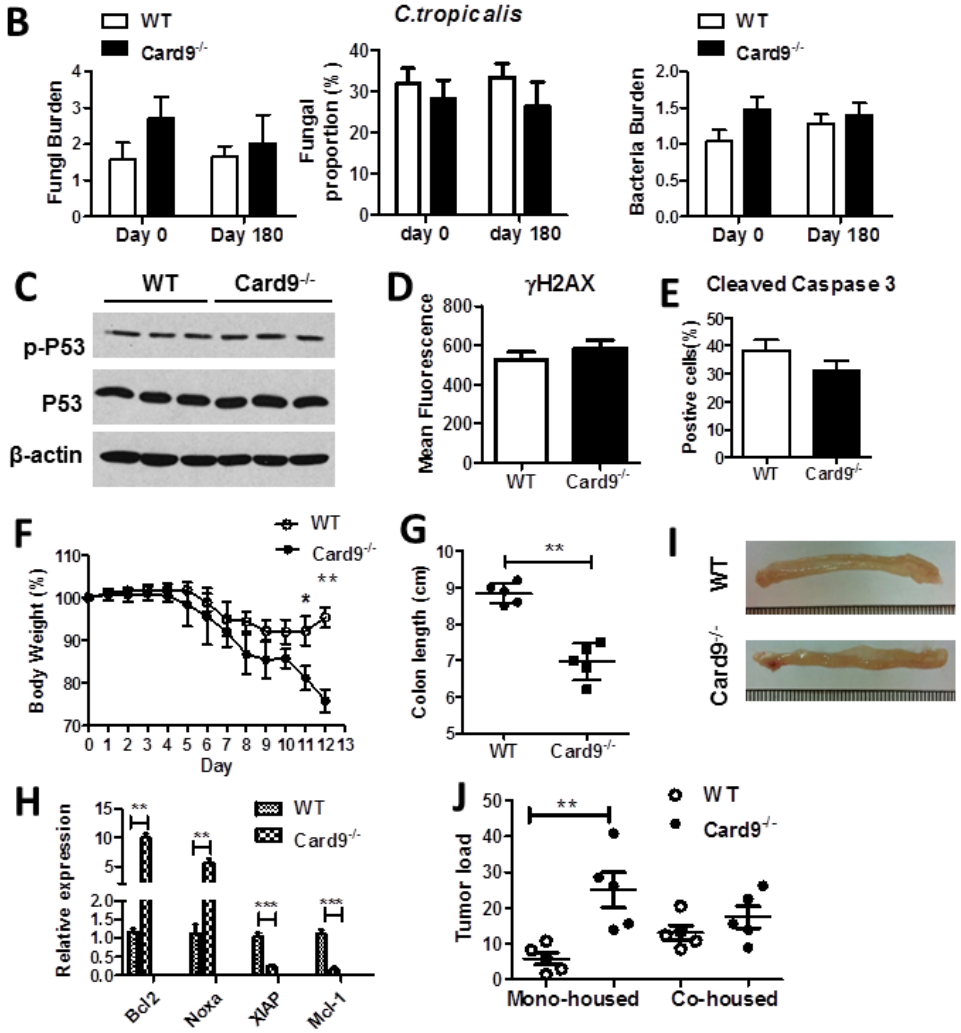

Figure S3. Low induction rate of tumorigenesis upon treatment with either AOM or DSS in both WT and *Card9*$^{-/-}$ mice. Related to Figure 1.

(A-E) WT (n=20) and *Card9*$^{-/-}$ mice (n=22) were given AOM (10 mg/kg) on day 1 and were kept till 6 months without DSS administration. Mice were euthanized on day 180. (A) Tumor numbers were detected in each mouse. (B) Total fungal burden in the

feces of AOM-treated or untreated mice was quantified using 18S rDNA qPCR. Proportions of *C. tropicalis* in the feces were quantified using qPCR with specific primers. Total bacteria burden was quantified using 16S rDNA qPCR. (C-D) Tumors were separated from AOM-treated WT and *Card9*$^{-/-}$ mice. Expression of p-P53 and P53 was detected by using Western Blot (one mouse per lane). Amounts of γ H2AX were detected by using flow cytometry. (E) Tumors from WT and *Card9*$^{-/-}$ mice were stained for Cleaved-caspase 3 and positive cells were quantified. (F-H) For the acute colitis model, mono-housed WT and *Card9*$^{-/-}$ mice (n=5, each group) were given 2.5% DSS in the drinking water for 7 days and then given regular drinking water until day 12. (F) Body weight was recorded during the DSS treatment. (G) Colon lengths were measured in each mouse. (H) Colon tissues were isolated. Expressions of *Bcl2*, *Noxa*, *Xiap* and *Mcl-1* were detected by using qPCR. (I) Mono-housed WT and *Card9*$^{-/-}$ mice (n=5, each group) were given three cycles of 2% DSS and were kept till 6 months without AOM administration. Representative images of colons were shown. (J) *Card9*$^{-/-}$ and WT mice were co-housed for at least 4 weeks before and throughout the CAC experiments. Tumor loads in colons were measured. Experiments in A-E were repeated three times with totally 20 mice. Data presented in F-J were one of three independent biological replicates. Data with error bars are presented as mean ± SD. * P <0.05, ** P < 0.01, *** P < 0.001 as determined by unpaired Student's t test.

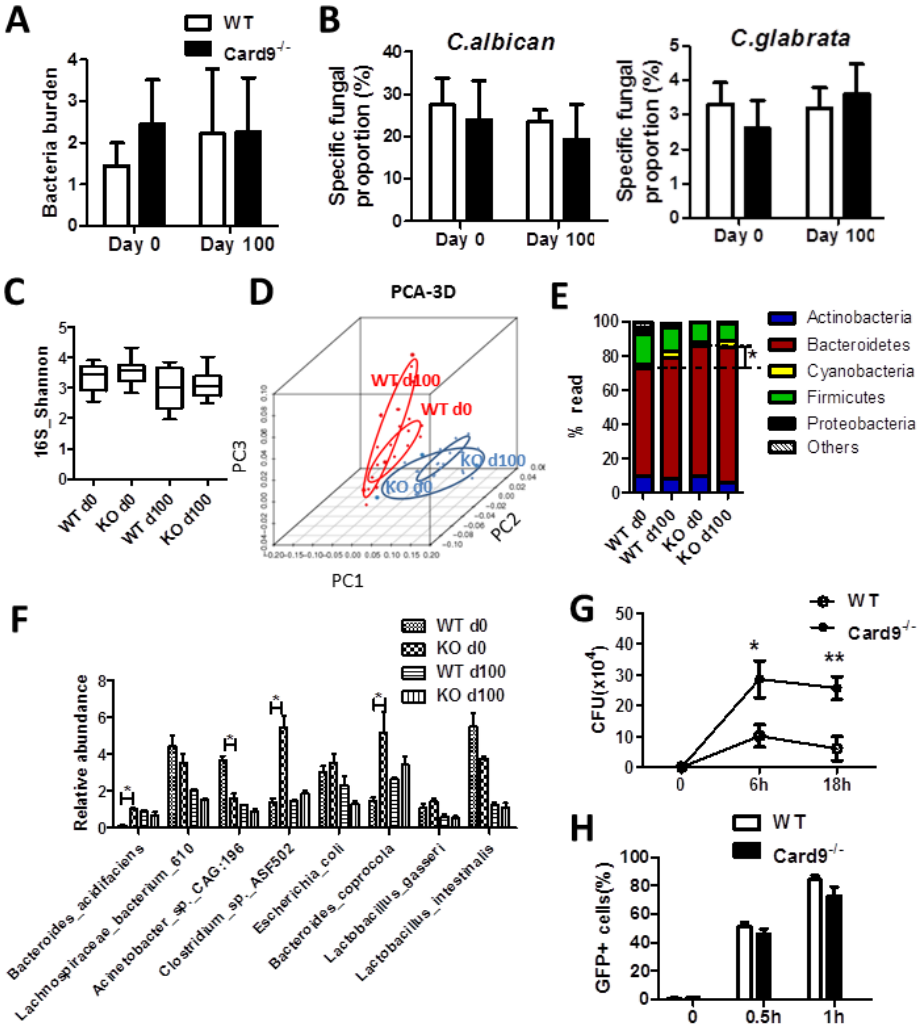

Figure S4. Bacteria burden shows no difference between tumor-bearing WT and *Card9*$^{-/-}$ mice. Related to Figure 2.

Mice were treated as stated in Figure 1. Feces was collected on tumor-bearing mice on Day 100. (A) Total bacteria load in feces of WT and *Card9*$^{-/-}$ mice was quantified using 16S rDNA qPCR. (B) Proportions of *C. albican* and *C. glabrata* in feces of WT and *Card9*$^{-/-}$ mice were quantified using qPCR. (C) Bacterial diversity was analyzed by alpha-diversity analysis showing Shannon index. (D) Three-dimensional (3D) principal component analysis (PCA) based on 16S rDNA gene sequence abundance in the feces. X axis: principal components 1 (PC1), Y axis: principal components 2 (PC2), Z axis: principal components 3 (PC3). (E) Bacteria-taxon-based analysis at the phylum level in the feces. (F) Bacteria-taxon -based analysis at the species level in the feces. (G) Bone marrow cells were acquired from WT and *Card9*$^{-/-}$ mice and were induced to BMDMs by adding supernatant from L929 cells. GFP-*C. tropicalis* (5×10^6)

was added onto 1×10^6 BMDMs and incubated for 1 hour. Culture wells were washed, and fresh media containing fluconazole (300 μg/ml) were added. At 6-hour and 18-hour, *C. tropicalis* CFUs inside BMDMs were calculated by plating onto YPD agar. (H) GFP-*C. tropicalis* (5×10^6) was added onto 1×10^6 BMDMs for indicated times. GFP^+ BMDMs were calculated by flow cytometry. d, days. Data with error bars are presented as mean ± SD. Each panel is a representative experiment of at least 3 independent biological replicates. * P <0.05, ** P < 0.01 as determined by unpaired Student's t test.

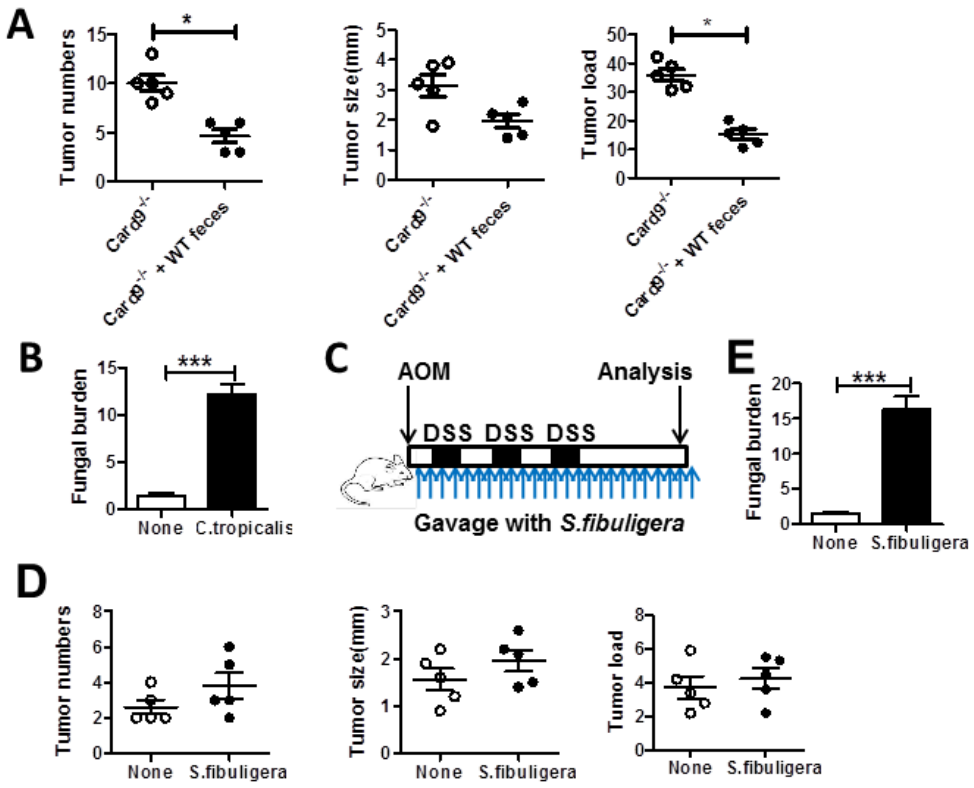

Figure S5. Transferring WT feces to *Card9*$^{-/-}$ mice partly inhibits CAC development. Related to Figure 3.

(A) *Card9*$^{-/-}$ mice were orally gavaged with feces (400μl each time, twice a week) from WT mice during administrated with AOM-DSS (n=5, each group). Mice were euthanized on day 100. Tumor number, tumor size and tumor load in colons were measured. (B) GF mice were treated as described in Figure 3H. Total fungal burden in feces was detected by using 18S rDNA qPCR. (C-D) GF mice were orally gavaged with *S.fibuligera* (1×10^7, twice a week) during administrated with AOM-DSS (n=5, each group). Mice were euthanized on day 100. Tumor number, tumor size and tumor load in colons were measured. (E) Total fungal burden in feces was detected by using 18S rDNA qPCR. Data with error bars are presented as mean ± SD. Each panel is a representative experiment of at least 3 independent biological replicates. * P <0.05, *** P < 0.001 as determined by unpaired Student's t test.

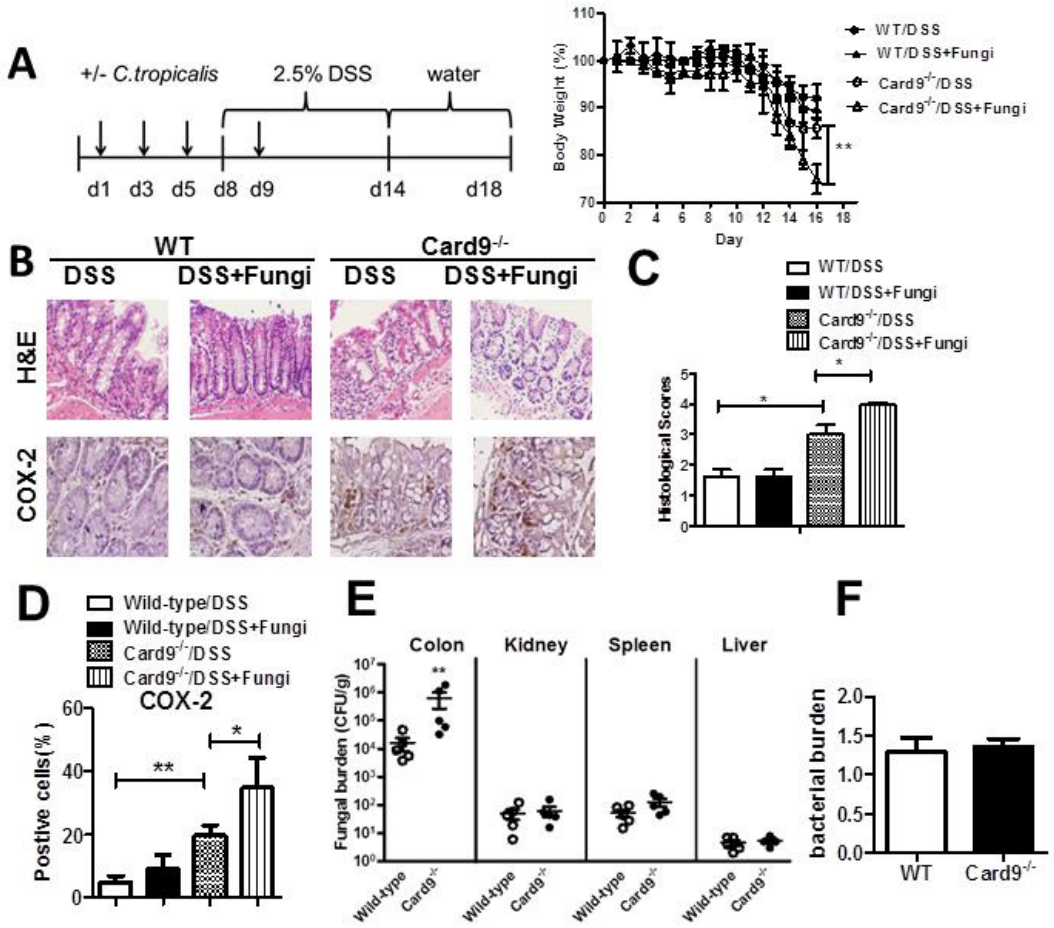

Figure S6. Intragastrical administration of *C. tropicalis* aggravates colitis in *Card9*$^{-/-}$ mice. Related to Figure 3.

(A) Mono-housed WT and *Card9*$^{-/-}$ mice (n = 5, each group) were intragastrical administration of *C. tropicalis* for 7 days, and were then administrated to 2.5% DSS for 7 days. Mice were euthanized on day 18. Body weight was measured for each mouse. (B) Histological analysis of colitis was shown by hematoxylin and eosin (HE) staining. (C) Histological colitis scores were evaluated based on inflammation cell infiltration and damage of epithelial cells. (D) Colon tissues were stained for COX-2. COX-2-positive cells were quantified. (E) Fungal burden was detected in different organs using CFU-counting assay. (F) Total bacteria burden in the feces of *C.tropicalis* -DSS treated WT and *Card9*$^{-/-}$ mice was quantified using 16S rDNA qPCR. Data with error bars are presented as mean ± SD. Each panel is a

representative experiment of at least 3 independent biological replicates. * P <0.05, ** P < 0.01 as determined by unpaired Student's t test.

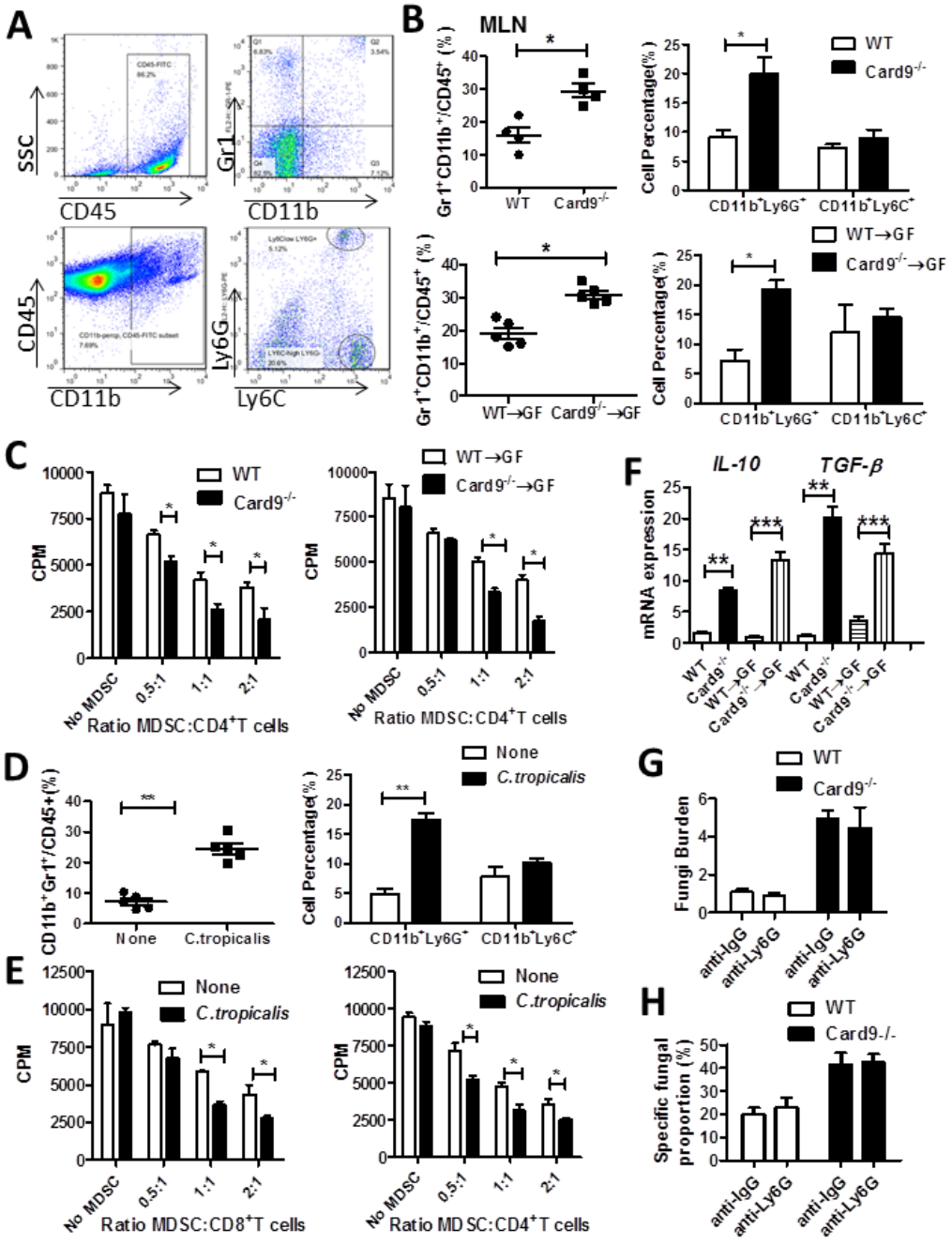

Figure S7. MDSCs are expanded and activated in tumor-bearing *Card9*$^{-/-}$ mice. Related to Figure 5.

(A) FACS gating strategy of total MDSCs, G-MDSC and M-MDSCs was provided. (B) Mice were treated as described in Figure 1 and Figure 3A. Proportions of MDSCs

($Gr1^+CD11b^+$), M-MDSCs ($CD11b^+Ly6C^+$) and G-MDSCs ($CD11b^+Ly6G^+$) in mLN of AOM-DSS treated mice were determined by flow cytometry. (C) Primary MDSCs were isolated from mLN in ex-GF tumor mice and were co-cultured with $CD8^+$ T and $CD4^+$ T cells. The suppressive function of MDSCs was determined by [^{3}H] thymidine in-corporation. (D) Mice were treated as described in Figure 3H. Proportions of MDSCs ($Gr1^+CD11b^+$), M-MDSCs ($CD11b^+Ly6C^+$) and G-MDSCs ($CD11b^+Ly6G^+$) in colon LP cells were determined by flow cytometry. (E) Mice were treated as described in Figure 3H. Primary MDSCs were isolated from mLN in exGF tumor mice and were co-cultured with $CD8^+$ T and $CD4^+$ T cells. The suppressive function of MDSCs was determined by [^{3}H] thymidine in-corporation. (F) Mice were treated as described in Figure 1 and Figure 3A. mRNA expressions of *IL-10* and *TGF-β* in LP cells were determined by qPCR. (G-H) Mice were treated as described in Figure 4E. Total fungal burden and the proportion of *C.tropicalis* were determined by qPCR. Data with error bars are presented as mean ± SD. Each panel is a representative experiment of at least 3 independent biological replicates. * P <0.05, ** P < 0.01, *** P < 0.001 as determined by unpaired Student's t test.

Table S1. Analysis of fungal alpha diversity in feces from tumor-bearing WT and Card9$^{-/-}$ mice. Related to Figure 2.

Index	WT	Card9$^{-/-}$	P value	FDR
Chao1	302.2 ± 22.6	326.5 ± 31.3	0.40	0.90
Shannon	2.97 ±0.35	3.09 ± 0.25	0.78	0.92
Simpson	0.09±0.005	0.08±0.004	0.93	0.93
Coverage	0.99±0.05	0.98±0.06	0.52	0.90

Data were presented as Mean ± SD. Difference was compared using unpaired Student's t test.

Table S2. Histologic criteria of tumors. Related to Figure 1.

Histologic criteria of tumors		**Score**
Number of lesions	0-2	1
	3-5	2
	6-8	3
	9-	4
Crypts	normal	0
	goblet cell depletion	1
	branching	2
	Complex budding	3
Epithelium	normal	0
	Hyperplasia/aberrant crypt foci	1
	low-grade dysplasia	2
	High-grade dysplasia	3
Submucosal invasion	absent	0
	present	1

Table S3. Primers for PCR.
Related to EXPERIMENTAL PROCEDURES

Gene name	Primers
18S rDNA	FP: 5'- ATTGGAGGGCAAGTCTGGTG-3' RP：5'- CCGATCCCTAGTCGGCATAG-3'
ITS-2	FP: 5′-GTGARTCATCGAATCTTT-3′ RP：5′-GATATGCTTAAGTTCAGCGGGT-3′
C. albicans	FP: 5'-CTGTTTGAGCGTCGTTTC-3' RP：5'- ATGCTTAAGTTCAGCGGGTAG-3'
C. tropicalis	FP: 5'-TTTGGTGGCGGGAGCAATCCT-3' RP：5'- CGATGCGAGAACCAAGAGATCCGT-3'
C. glabrata	FP: 5'-CTGCGCTTAACTGCGCGGTT-3' RP：5'-TGCGAGAACCAAGAGATCCGTTGC-3'
16S rDNA	FP: 5'-AGAGTTTGATCMTGGCTCAG-3' ; RP：5'-CTGCTGCCTYCCGTA-3'
Mouse S100A9	FP：5'- AAAGGCTGTGGGAAGTAATTAAGAG-3' RP：5'- GCCATTGAGTAAGCCATTCCC-3'
Mouse Arg-1	FP：5'- GAAGGAGCCACTGAGGTCTG-3' RP：5'-CACGGCACCTCCTAAATTGT-3'
Mouse IL-6	FP: 5'- GAGGATACCACTCCCAACAGACC-3' RP：5'- AAGTGCATCATCGTTGTTCATACA-3'
IL-10	FP: 5'- GCTCTTACTGACTGGCATGAG-3' RP：5'- CGCAGCTCTAGGAGCATGTG-3'
Mouse TGF-β	FP：5'- TGACGTCACTGGAGTTGTACGG-3' RP：5'- GGTTCATGTCATGGATGGTGC-3'
IL-17a	FP：5'- TTTAACTCCCTTGGCGCAAAA-3' RP：5'- CTTTCCCTCCGCATTGACAC-3'
CXCL1	FP：5'- CTGGGATTCACCTCAAGAACATC-3' RP：5'- CAGGGTCAAGGCAAGCCTC-3'
iNOS	FP: 5'- GGGCTGTCACGGAGATCA-3' RP: 5'- CCATGATGGTCACATTCTGC-3'
TNF-α	FP：5'- GCCTCCCTCTCATCAGTTCT-3' RP：5'- CACTTGGTGGTTTGCTACGA-3'
β-actin	FP：5'- ATGACCCAGATCATGTTTGA-3' RP：5'- TACGACCAGAGGCATACAG-3'
Human CARD9	FP: 5'-CCCTCACGCATCACACCTTAC-3' RP: 5'-GCACACCCACTTTCCGTTTG-3'
Human GAPDH	FP: 5'-GGAGCGAGATCCCTCCAAAAT-3' RP: 5'-GGCTGTTGTCATACTTCTCATGG-3'

FP：Forward primers

RP：Reverse primers