

Cooperation of TRADD- and RIPK1-dependent Cell Death Pathways in Maintaining Intestinal Homeostasis

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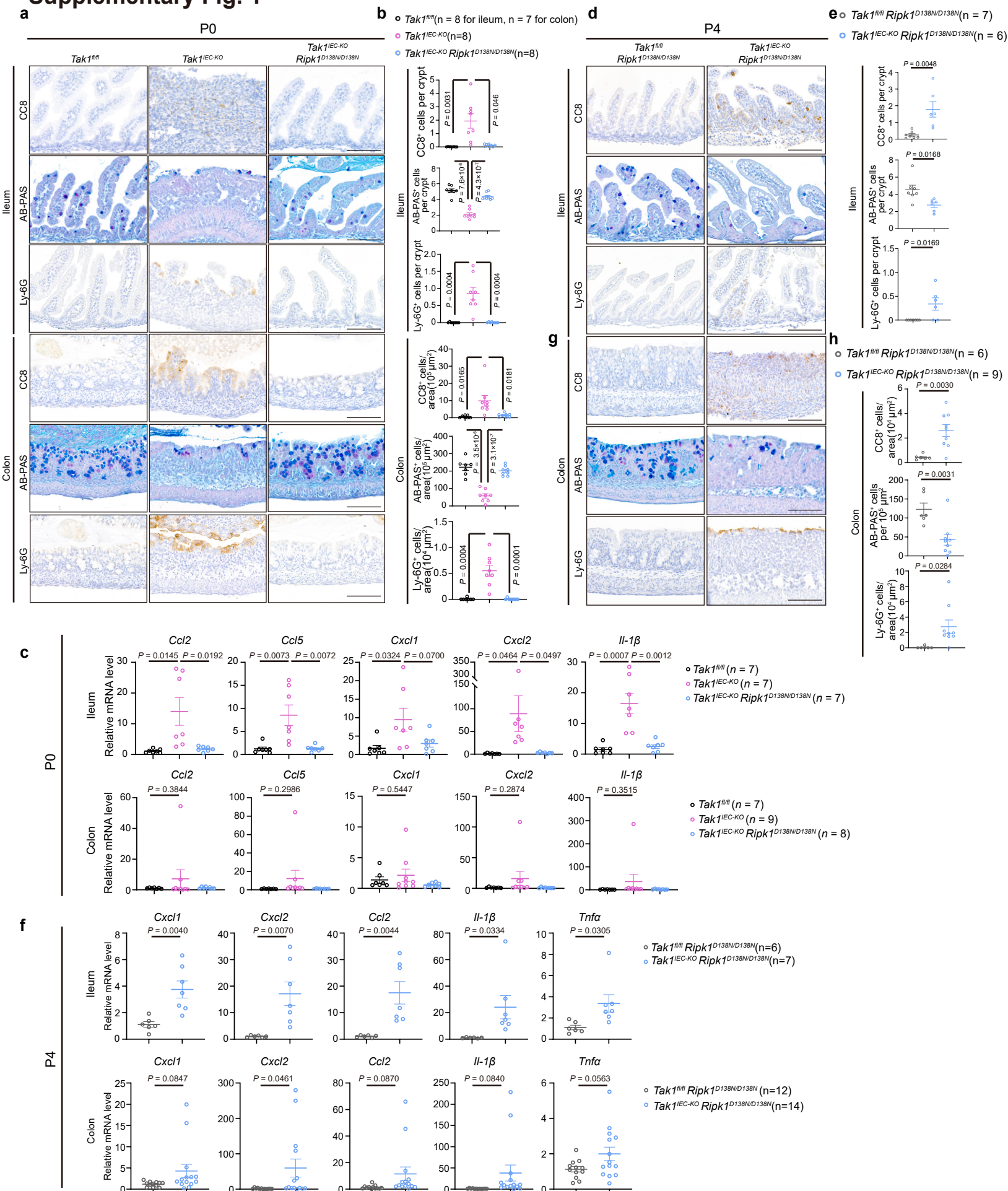
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Supplementary Fig. 1



Supplementary Figure 1. RIPK1 kinase inhibition rescues the caspase-8 activation and inflammation in newborn *Tak1^{IEC-KO}* mice.

(a-b) Representative images of ileum and colon sections from the mice at P0 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (a). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum and colon of the mice with indicated genotypes (b).

(c) The mRNA levels of inflammatory cytokines in ileum and colon of the mice with indicated genotypes at P0 were measured by qRT-PCR.

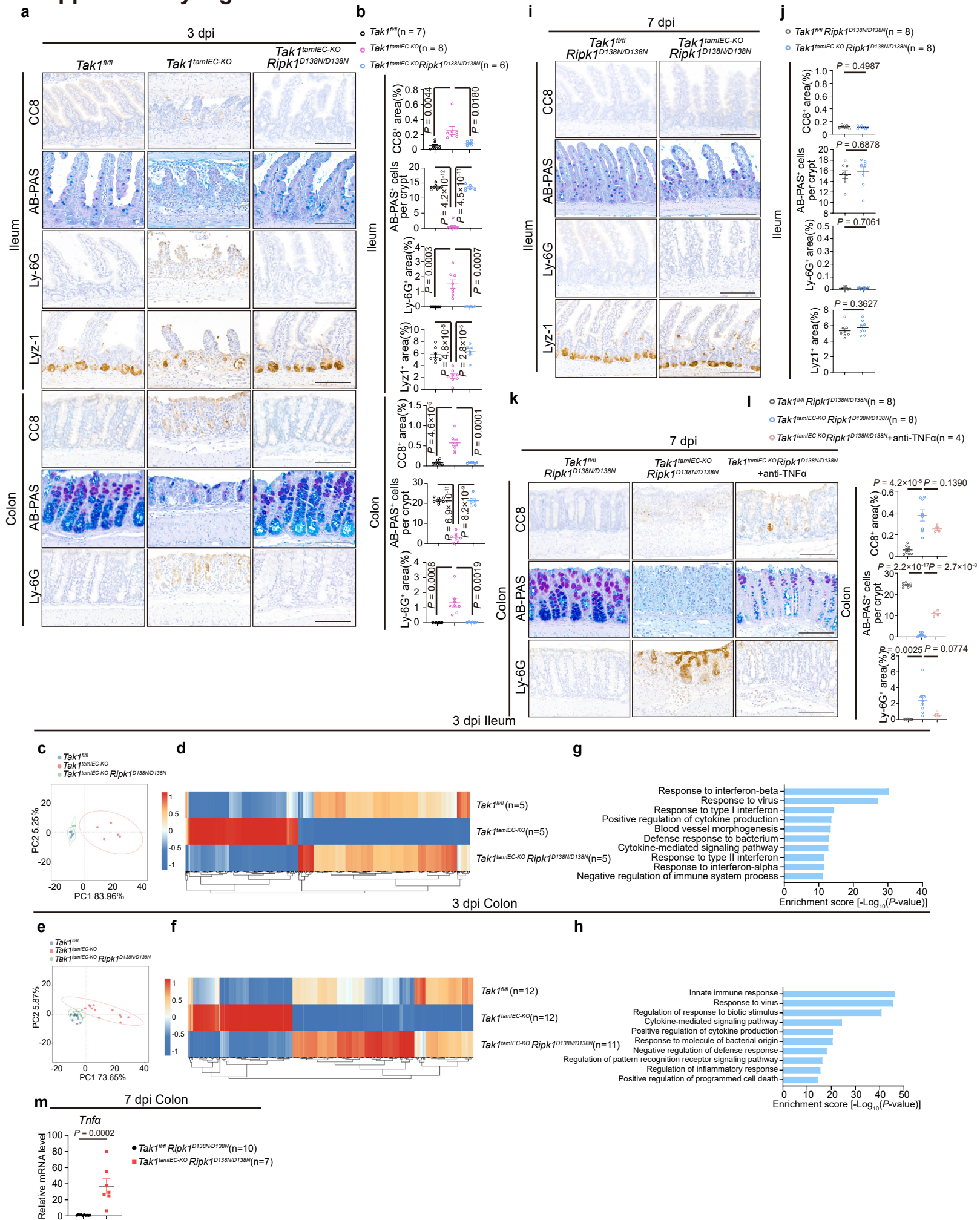
(d-e) Representative images of ileum sections from mice at P4 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (d). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum of the mice with indicated genotypes (e).

(f) The mRNA levels of inflammatory cytokines in ileum and colon of the mice with indicated genotypes at P4 were measured by qRT-PCR.

(g-h) Representative images of colon sections from mice at P4 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (g). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in colon of the mice with indicated genotypes (h).

Scale bar, 100 μ m. Each dot represented one mouse. The results were shown as mean $\pm$ SEM. Unpaired two-tailed t-test was performed.

Supplementary Fig. 2



Supplementary Figure 2. RIPK1 kinase inhibition rescues the caspase-8 activation and inflammation in adult *Tak1^{tamIEC-KO}* mice.

(a-b) Representative images of ileum and colon sections from the mice at 3 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC8, Ly-6G and Lyz-1 (a). Graphs showed the CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum and colon of the mice with indicated genotypes (b). Scale bar, 150 μ m for ileum; 100 μ m for colon;

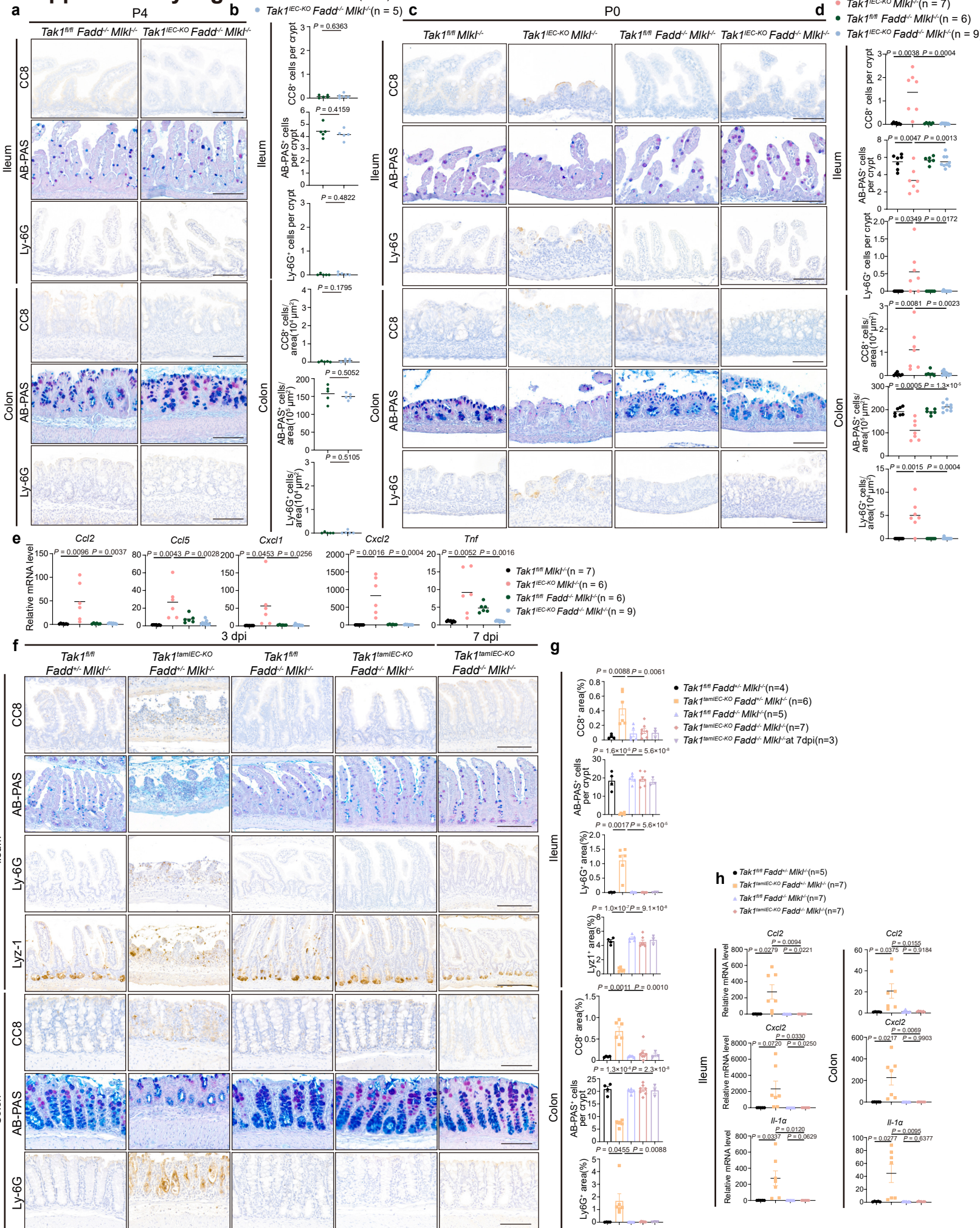
(c-h) PCA on RNA-seq data from ileum (c) and colon(e) of the mice with indicated genotypes at 3 dpi. PCA was based on genes differentially expressed between *Tak1^{fl/fl}* mice and *Tak1^{tamIEC-KO}* mice (cut-off: $\log_2|\text{fold change}| \geq 1$, $p \leq 0.05$). Cluster heat map showing average expression of genes that were significantly changed (cut-off: *Tak1^{tamIEC-KO}* V.S. *Tak1^{fl/fl}* mice $\log_2|\text{fold change}| \geq 1$, $p < 0.05$) in ileum (d) and colon (f) of *Tak1^{tamIEC-KO}* mice at 3 dpi. Z scores are shown. GO analysis of genes upregulated in the ileum (g) and colon(h) of *Tak1^{tamIEC-KO}* mice at 3 dpi in RIPK1 kinase-dependent fashion.

(i-l) Representative images of ileum (i) and colon(k) sections from the mice at 7 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC8, Ly-6G and Lyz-1. Graphs showed the CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum (j) and colon (l) of mice with the indicated genotypes.

(m)The mRNA levels of *Tnfa* in the colon of mice with indicated genotypes at 7 dpi was measured by qRT-PCR. Data was from Fig. 4g.

Scale bar, 150 μ m. Each dot represented one mouse. The results were shown as mean $\pm$ SEM. Unpaired two-tailed t-test was performed.

Supplementary Fig. 3 • *Tak1^{fl/fl} Fadd^{-/-} Mik1^{-/-}* (n = 5)



Supplementary Figure 3. Combined loss of FADD and MLKL prevents the caspase-8 activation and inflammation in newborn and adult TAK1-IEC deficient mice.

(a-b) Representative images of ileum and colon sections from the mice at P4 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (a). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum and colon of the mice with indicated genotypes (b). Scale bar, 100 μ m.

(c-d) Representative images of ileum and colon sections from the mice at P0 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (c). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum and colon of the mice with indicated genotypes (d).

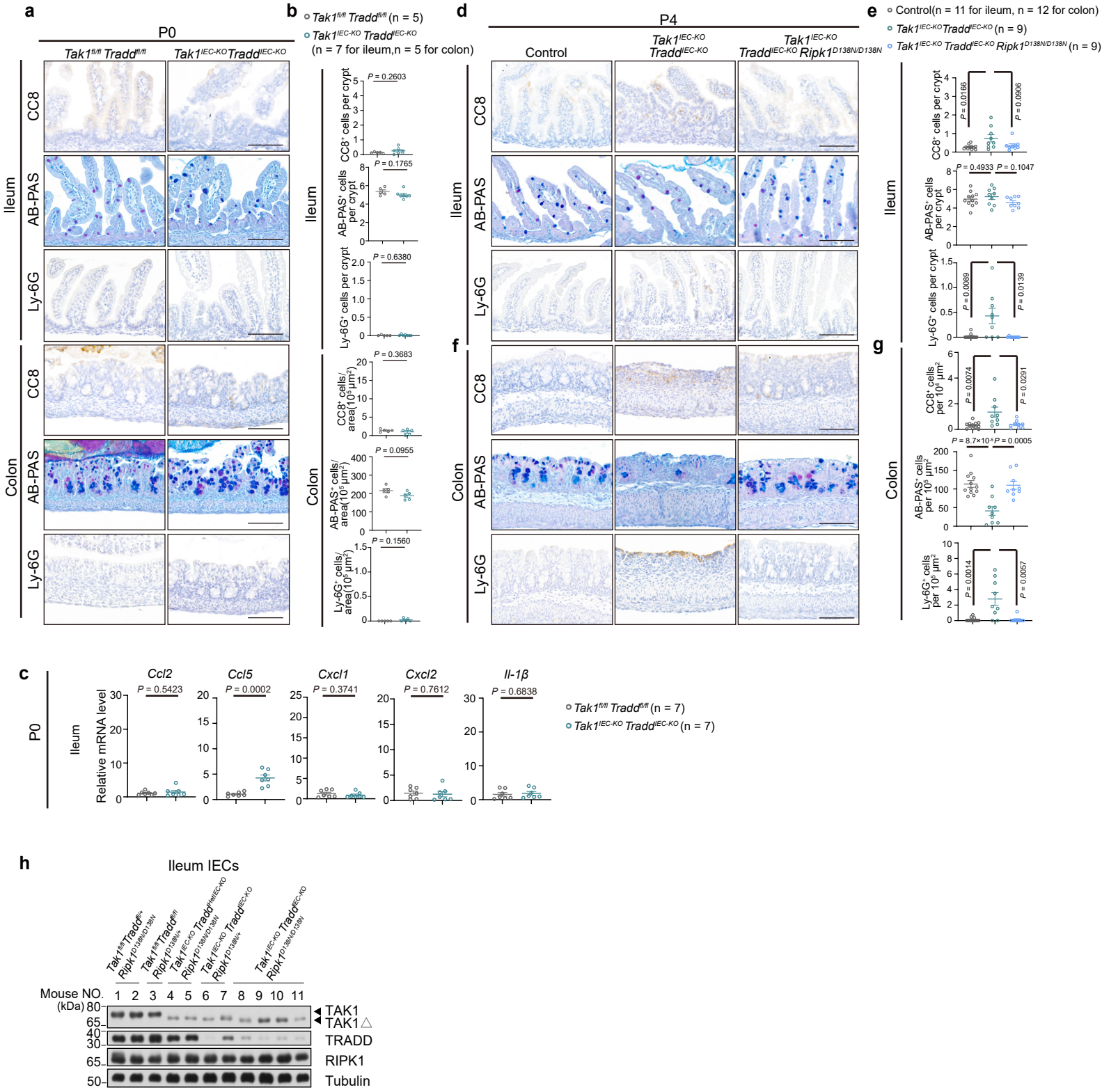
(e) The mRNA levels of inflammatory cytokines in ileum and colon of the mice with indicated genotypes at P0 were measured by qRT-PCR.

(f-g) Representative images of ileum and colon sections from the mice at 3 dpi and 7 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC8, AB-PAS, Ly-6G and Lyz-1 (f). Graphs showed the CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum and colon of the mice with indicated genotypes (g). Scale bar, 150 μ m for ileum; 100 μ m for colon.

(h) The mRNA levels of inflammatory cytokines in ileum and colon of the mice with indicated genotypes at 3 dpi were measured by qRT-PCR.

Each dot represented one mouse. The results were shown as mean $\pm$ SEM. Unpaired two-tailed t-test was performed.

Supplementary Fig. 4



Supplementary Figure 4. Combined inhibition of TRADD and RIPK1 kinase rescues the caspase-8 activation and inflammation in newborn *Tak1^{IEC-KO}* mice.

(a-b) Representative images of ileum and colon sections from the mice at P0 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (a). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum and colon of mice with the indicated genotypes (b).

(c) The mRNA levels of inflammatory cytokines in ileum of the mice with indicated genotypes at P0 were measured by qRT-PCR.

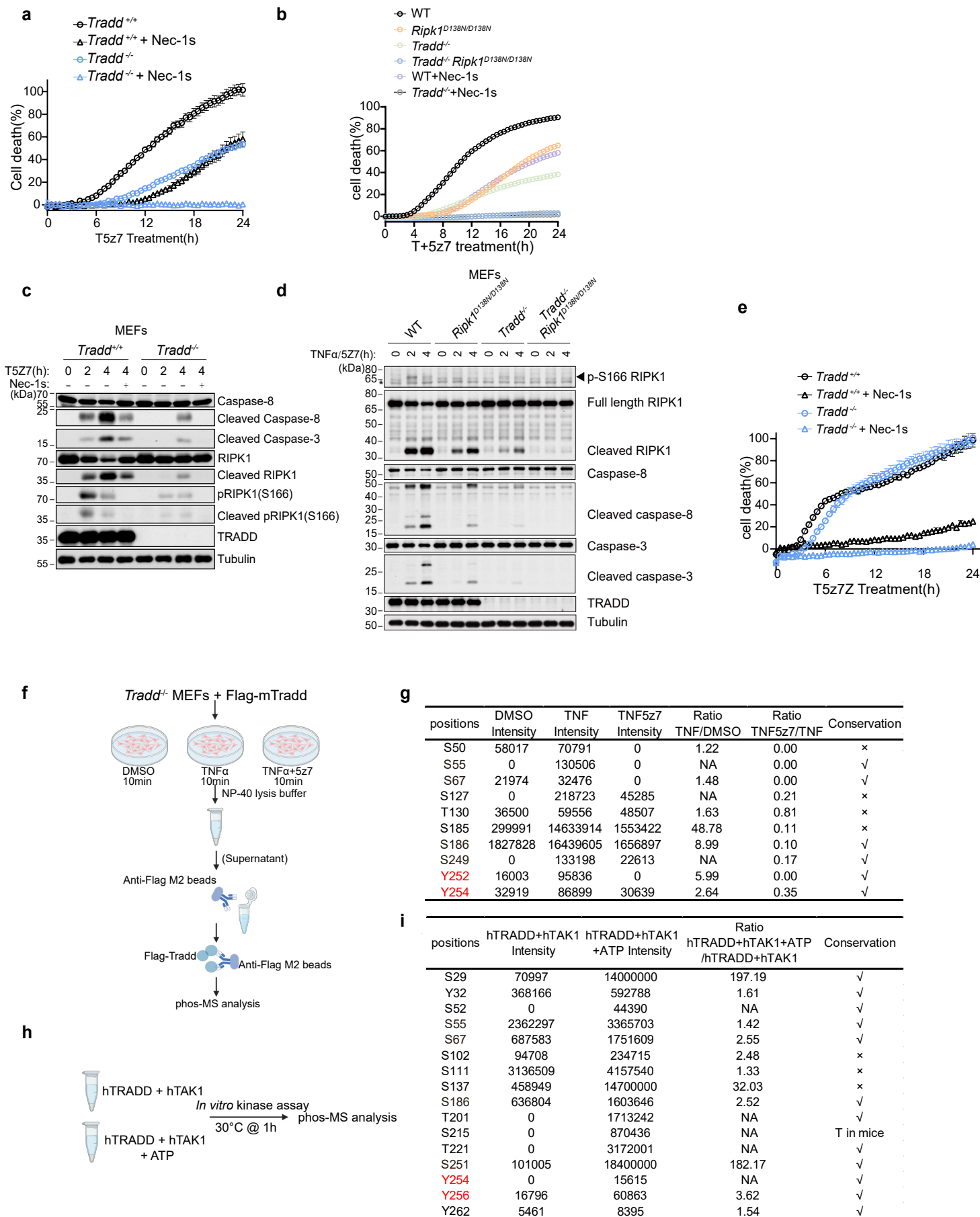
(d-g) Representative images of ileum and colon sections from the mice at P4 with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (d and f). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in ileum and colon of the mice with indicated genotypes (e and g).

(h) Ileum IECs from the mice with indicated genotypes at the age of 7 weeks were isolated and immunoblotting for TAK1, RIPK1 and TRADD.

Scale bar, 100 μ m. Each dot represented one mouse. The results were shown as mean $\pm$ SEM.

Unpaired two-tailed t-test was performed.

Supplementary Fig. 5



Supplementary Figure 5. TAK1 directly modulates the activation of TRADD by phosphorylation.

(a-d) MEFs with indicated genotypes were pretreated with 500 nM (5Z)-7-oxozeaeno with or without 20 μ M Nec-1s for 0.5 hr followed by 20ng/ml mTNF- α treatment. Cell death (a-b) was measured by SytoxGreen and the mean $\pm$ SEM from four (a) or eight (b) biological replicates was shown. The levels of p-RIPK1(S166), RIPK1, Caspase-8, CC8, Caspase-3 and CC3 were determined by immunoblotting (c-d). * denoted unspecific bands.

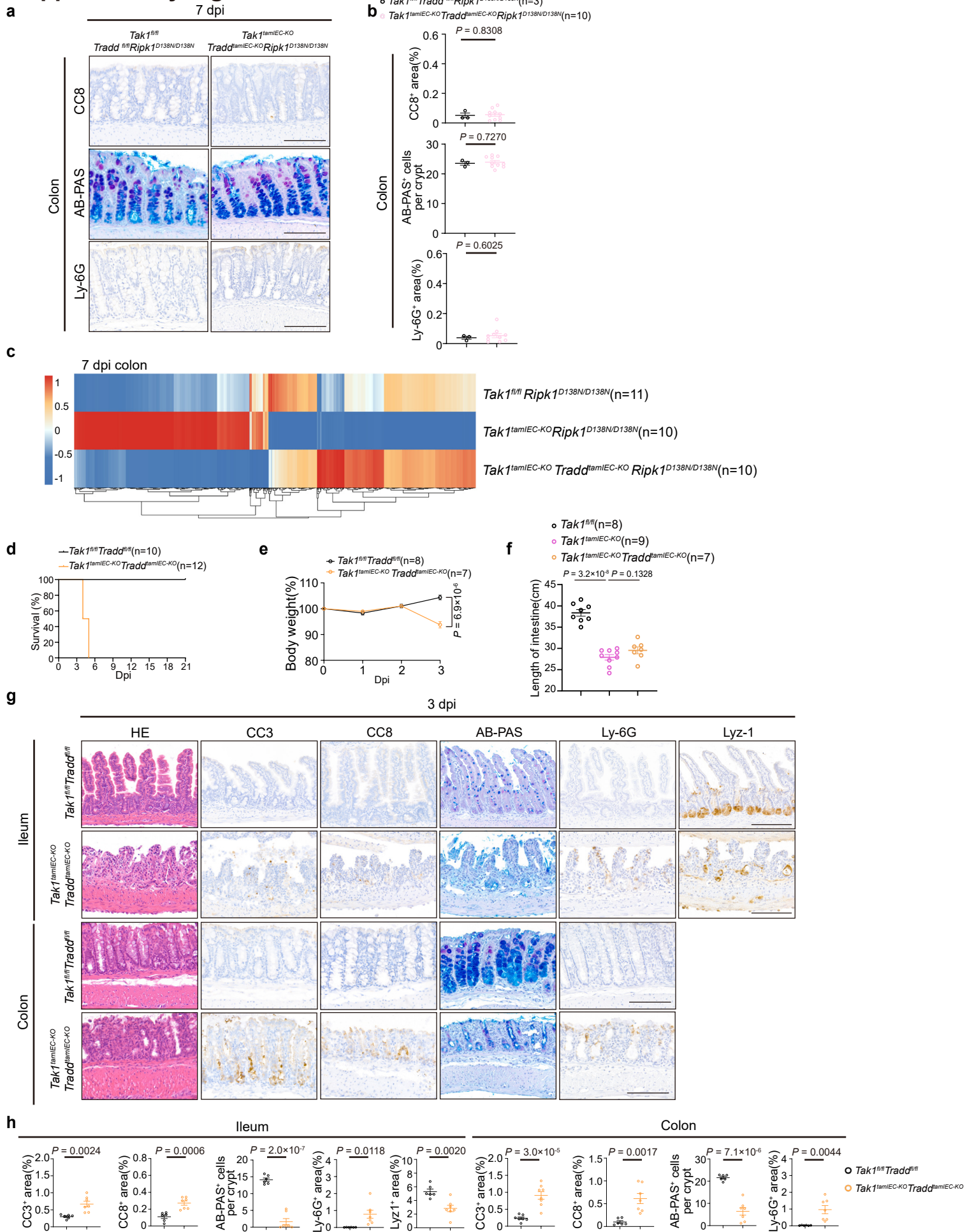
(e) MEFs with indicated genotypes were pretreated with 500 nM (5Z)-7-oxozeaeno, +/- Nec-1s (20 μ M), +/- z-VAD (50 μ M) for 0.5 hr followed by 20ng/ml mTNF- α treatment with or without. Cell death was measured by SytoxGreen and the mean $\pm$ SEM from four biological replicates was shown.

(f-g) The scheme of mass spectrometry sample preparation. Created in BioRender. Yuan, J. (2025) <https://BioRender.com/h59u048> (f). *Tradd*^{-/-} MEFs were retrovirally reconstituted with Flag-mTRADD. Reconstituted cells (20 dishes of 15-cm dish for each group) were stimulated with DMSO, mTNF- α (100ng/ml) or mTNF- α +(5Z)-7-oxozeaeno (500 nM, pretreat for 0.5 hr) for 10min. The cells lysed with NP-40 buffer and the Flag-TRADD in supernatants was then Flag immunoprecipitated. The samples (n=1 for each group) were analyzed by mass spectrometry after phospho- peptide enrichment. The phosphorylation sites of TRADD by mass spectrometry were showed(g).

(h-i) The scheme of mass spectrometry sample preparation. Created in BioRender. Yuan, J. (2025) <https://BioRender.com/k58s496> (h). Recombinant 6*His-TRADD from proteintech (50 μ g) and recombinant TAK1- TAB1 purified from sf9 cells (100 μ g) were incubated in vitro with or without 100 mM ATP at 30°C for 1h. The samples (n=1 for each group) were analyzed by mass spectrometry after phosphor- peptide enrichment. The phosphorylation sites of TRADD by mass spectrometry were showed (i).

(j) A sequence alignment of Death Domain of human TRADD (*Homo sapiens*), and murine TRADD (*Mus musculus*) was shown.

Supplementary Fig. 6



Supplementary Figure 6. Combined inhibition of TRADD and RIPK1 kinase suppresses the caspase-8 activation and inflammation in adult *Tak1^{tamIEC-KO}* mice.

(a-b) Representative images of colon sections from the mice at 7 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (a). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in colon of the mice with indicated genotypes (b).

(c) Cluster heat map showing average expression of genes that were significantly changed (cut-off: *Tak1^{tamIEC-KO} Ripk1^{D138N/D138N}* V.S. *Tak1^{fl/fl} Ripk1^{D138N/D138N}* $\log_2|\text{fold change}| \geq 1$, $p < 0.05$) in the colon of *Tak1^{tamIEC-KO} Ripk1^{D138N/D138N}* mice at 7 dpi. Z scores are shown.

(d) Kaplan–Meier survival curves of the indicated genotypes.

(e) Relative body weight curve of the indicated genotypes mice. Relative body weight of each mouse was normalized to the weight of day0.

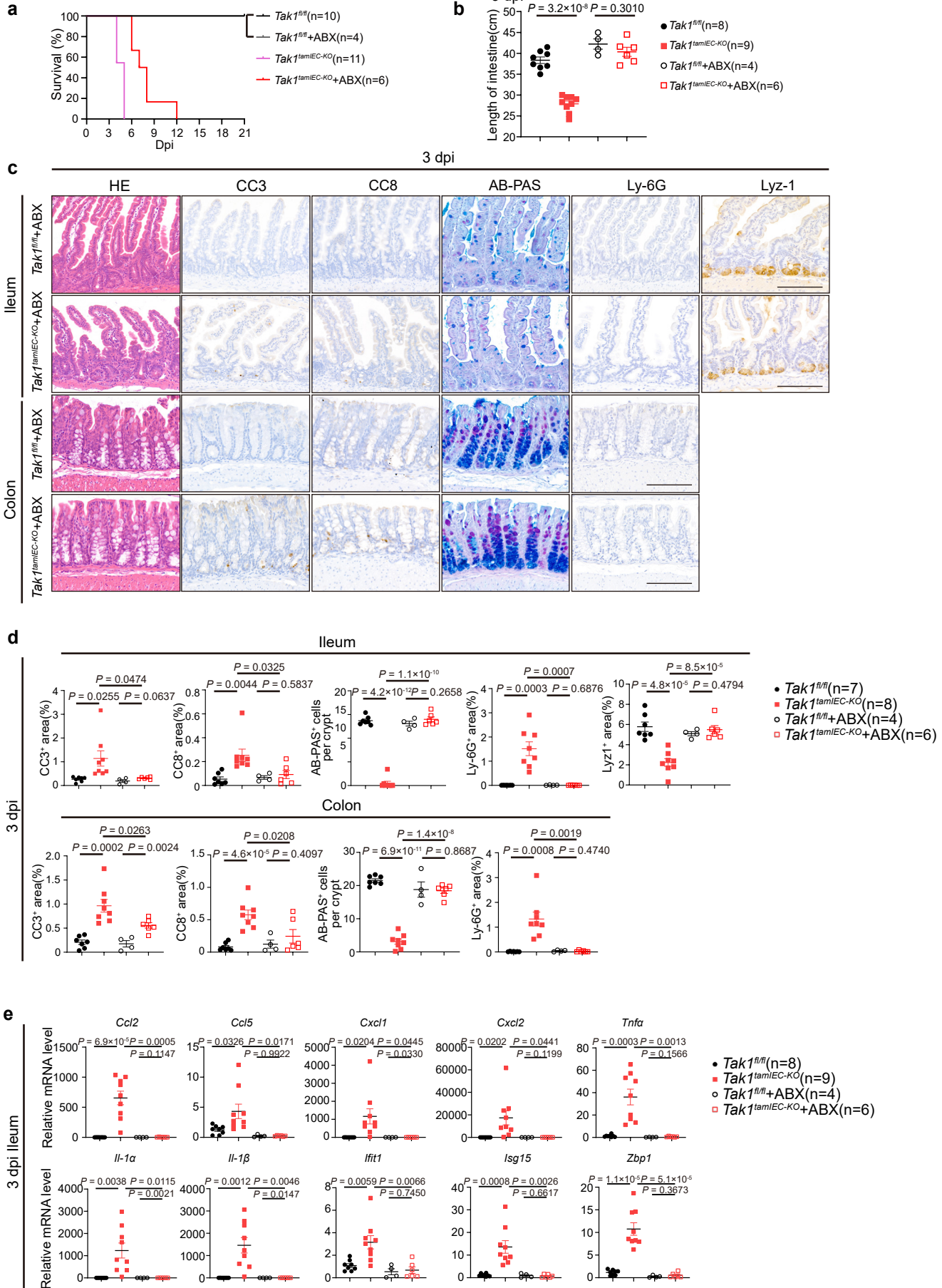
(f) Intestine length of mice at 3 dpi of the indicated genotypes. Intestine length for *Tak1^{fl/fl}* and *Tak1^{tamIEC-KO}* mice at 3 dpi from Fig. 1m were included for comparison.

(g-h) Representative images of ileum and colon sections from the mice at 3 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC3, CC8, Ly-6G and Lyz-1 (g). Graphs showed the CC3⁺, CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum and colon of the mice with indicated genotypes (h).

Scale bar, 150 μm . Each dot represented one mouse. The results were shown as mean $\pm$ SEM.

Unpaired two-tailed t-test was performed.

Supplementary Fig. 7



Supplementary Figure 7. The role of microbiota in driving ileitis and colitis in adult *Tak1^{tamIEC-KO}* mice at 3 dpi.

(a) Kaplan–Meier survival curves of the mice with indicated genotypes. ABX was pretreated for 14-days before and through the tamoxifen injection of the mice with indicated genotypes until sacrificed. Survival data for *Tak1^{fl/fl}* and *Tak1^{tamIEC-KO}* mice from Fig. 1k were included for comparison.

(b) Intestine length of mice at 3 dpi of the mice with indicated genotypes. Intestine length data for *Tak1^{fl/fl}* and *Tak1^{tamIEC-KO}* mice from Fig. 1m were included for comparison.

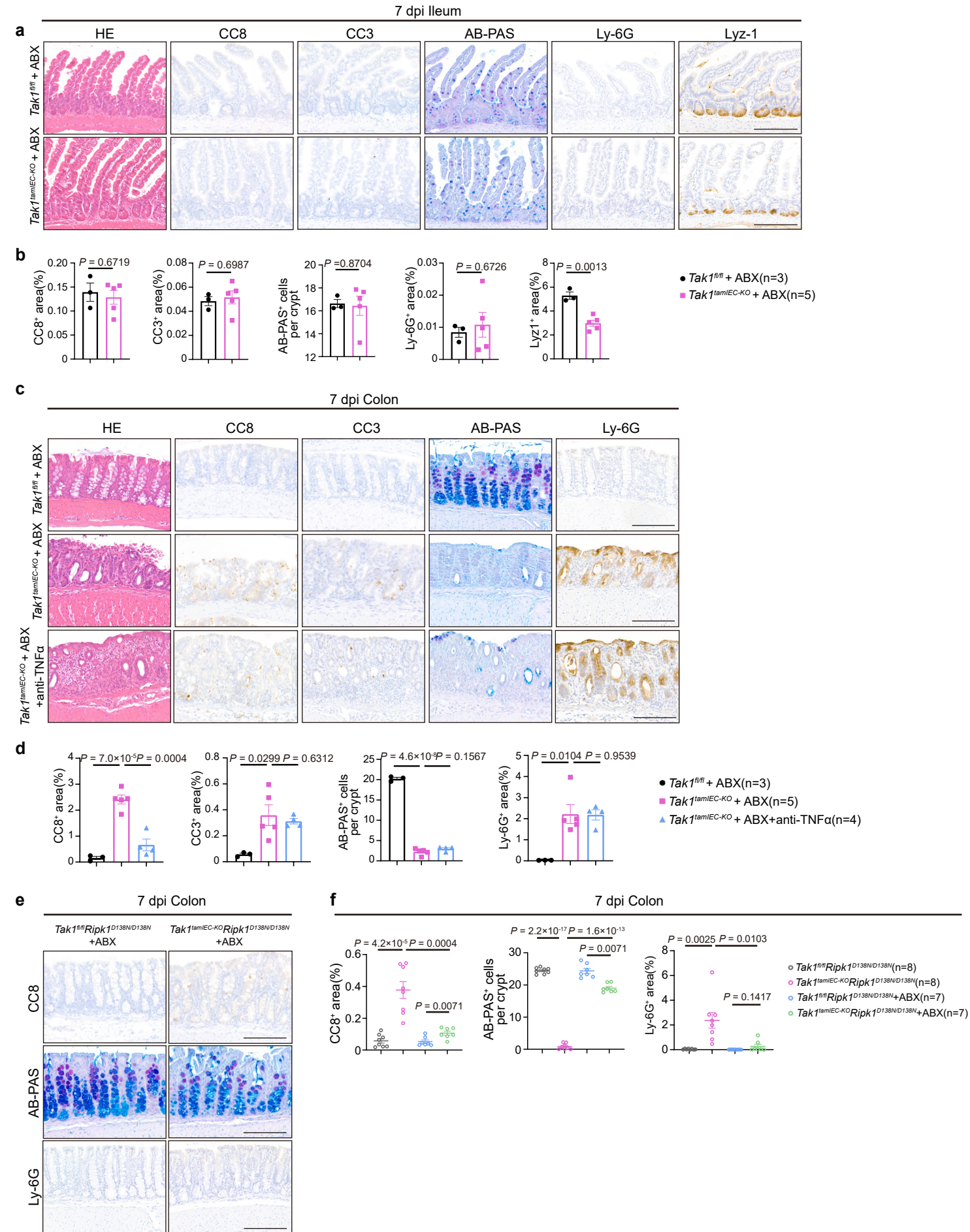
(c-d) Representative images of ileum and colon sections from mice at 3 dpi of the mice with indicated genotypes stained with AB-PAS, or immunostained for CC3, CC8, Ly-6G and Lyz-1 (c). Graphs showed the CC3⁺, CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum and colon of the mice with indicated genotypes (d). Data for *Tak1^{fl/fl}* and *Tak1^{tamIEC-KO}* mice from Fig. 1o and Supplementary Fig. 2b were included for comparison.

(e) The mRNA levels of inflammatory cytokines in ileum of the mice with indicated genotypes at 3 dpi were measured by qRT-PCR.

Scale bar, 150 μ m. Each dot represented one mouse. The results were shown as mean $\pm$ SEM.

Unpaired two-tailed t-test was performed.

Supplementary Fig. 8



Supplementary Figure 8. The role of microbiota in driving ileitis and colitis in adult *Tak1^{tamIEC-KO}* mice at 7 dpi.

(a-d) Representative images of ileum (a) and colon (c) sections from the mice at 7 dpi with indicated genotypes stained with AB-PAS, or immunostained for CC3, CC8, AB-PAS, Ly-6G and Lyz-1 (a and c). Graphs showed the CC3⁺, CC8⁺, AB-PAS⁺, Ly-6G⁺ and Lyz-1⁺ signals in ileum (b) and colon (d) of the mice with indicated genotypes.

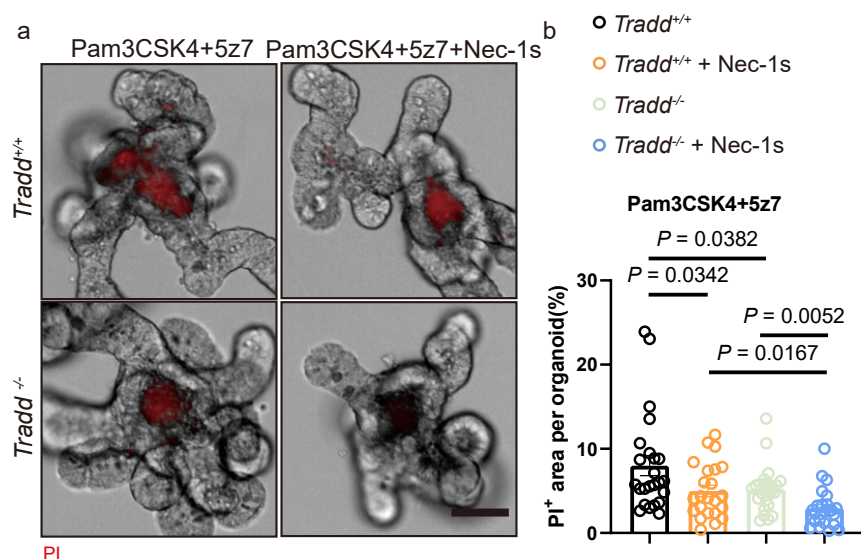
(e-f) Representative images of colon sections from mice at 7 dpi of the mice with indicated genotypes stained with AB-PAS, or immunostained for CC8 and Ly-6G (e). Graphs showed the CC8⁺, AB-PAS⁺ and Ly-6G⁺ signals in colon of the mice with indicated genotypes (f). Signals for *Tak1^{fl/fl} Ripk1^{D138N/D138N}* and *Tak1^{tamIEC-KO} Ripk1^{D138N/D138N}* mice from Fig. 1s and Supplementary Fig. 2l were included for comparison.

Scale bar, 150 μ m. Each dot represented one mouse. The results were shown as mean $\pm$ SEM.

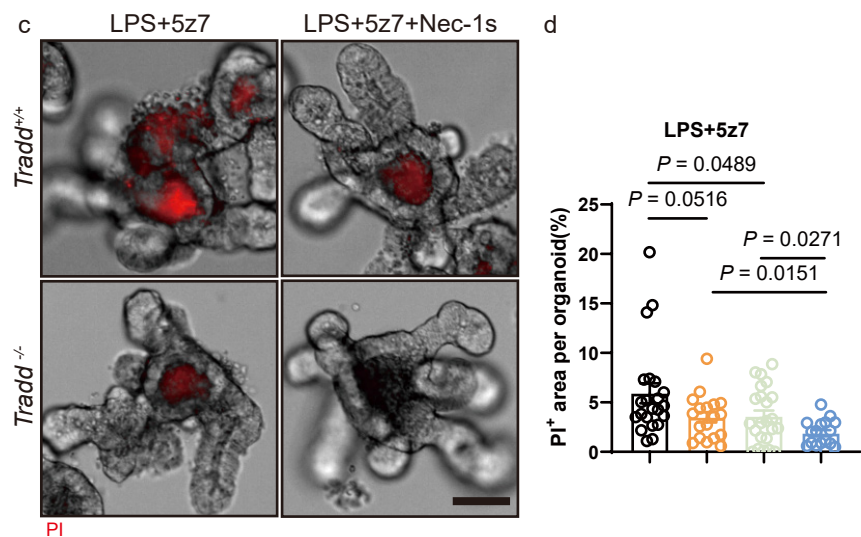
Unpaired two-tailed t-test was performed.

Supplementary Fig. 9

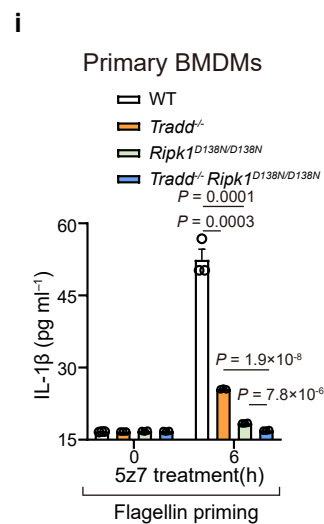
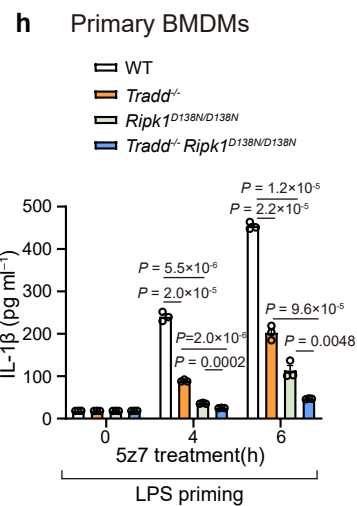
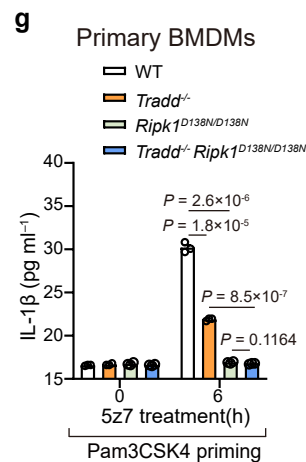
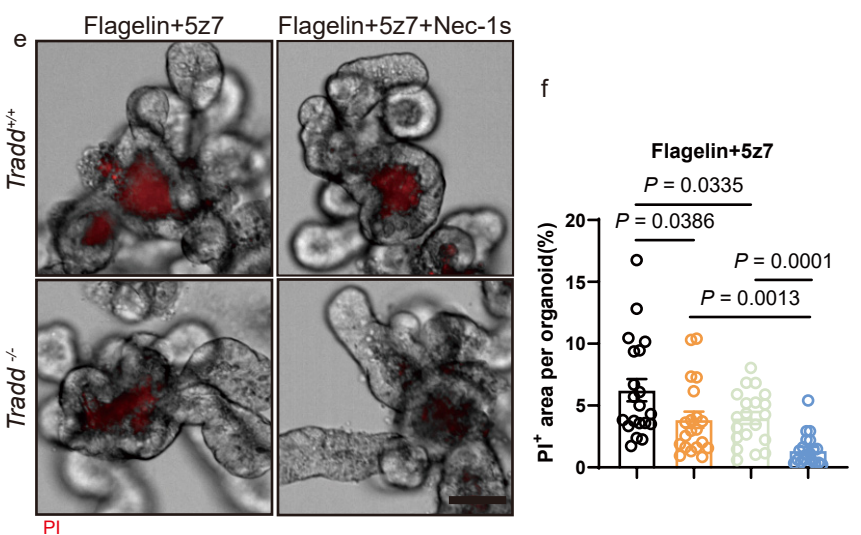
Ileum organoids



Ileum organoids



Ileum organoids



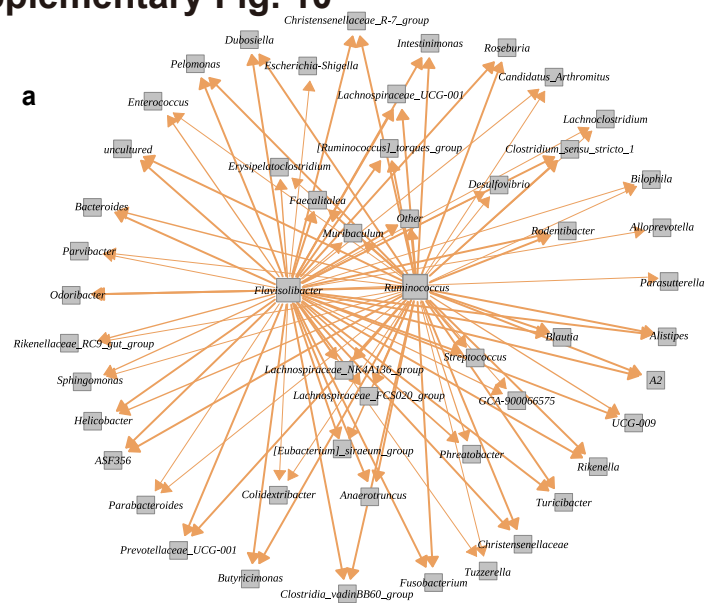
Supplementary Figure 9. Cooperation of TRADD and RIPK1 kinase in TLR signaling pathway.

(a-f) *Tradd*^{+/+} and *Tradd*^{-/-} ileum organoids were treated with 500 nM (5Z)-7-oxozeaeno and Pam3CSK4 (1 µg/ml) (a-b), LPS (1 µg/ml) (c-d) or Flagellin (500ng/ml) (e-f) for 24h with or without 50 µM Nec-1s. Organoid cell death was stained by PI. Each dot represented one organoid. Scale bar, 50 µm. The results were shown as mean ± SEM. Unpaired two-tailed t-test was performed.

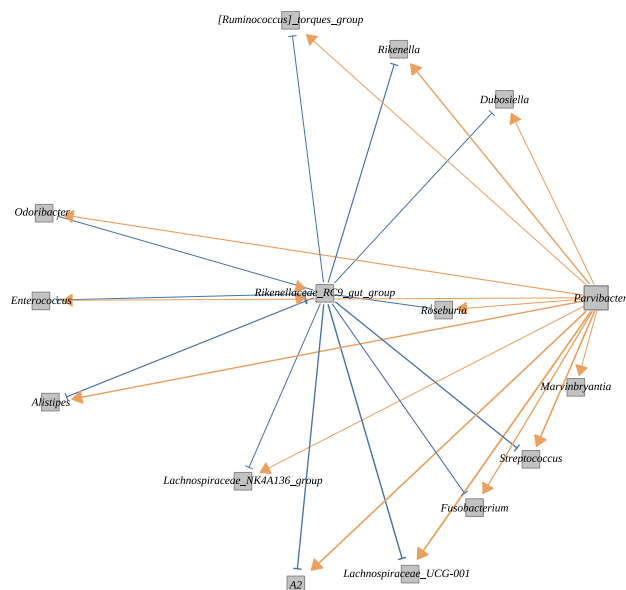
(g-i) Primary BMDMs from the mice with indicated genotypes pretreated Pam3CSK4 (1 µg/ml) (g), LPS (100ng/ml) (h) or Flagellin (100ng/ml) (i) for 4 hrs and followed by 200 nM (5Z)-7-oxozeaeno. The level of IL-1β in supernatant was measured by Elisa. The results were shown as mean ± SEM. Unpaired two-tailed t-test was performed.

Supplementary Fig. 10

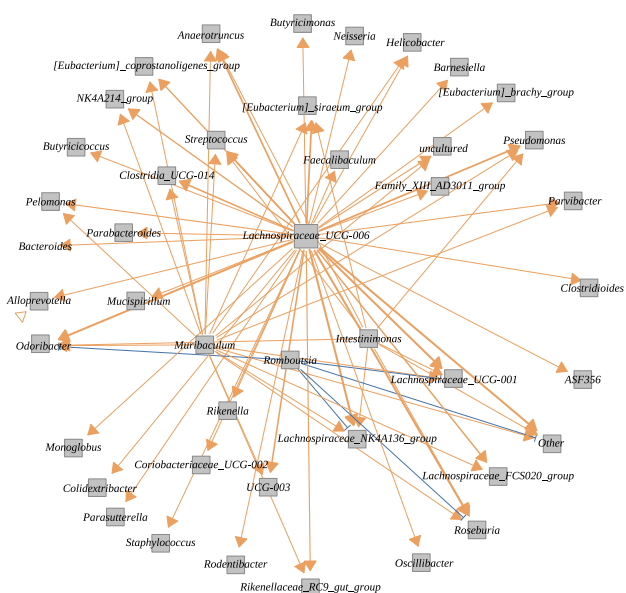
a



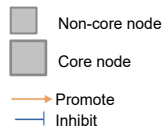
Tak1^{fl/fl}



Tak1^{tamIEC-KO}



Tak1^{tamIEC-KO} Ripk1^{D138N/D138N}



Supplementary Figure 10. Ileum microbiota network using IdopNetwork in adult *Tak1^{tamIEC-}*^{KO} at 3 dpi.

(a) IdopNetwork of microbiota network of ileum from the mice with indicated genotypes at 3 dpi based on 16S rDNA sequencing data.