Supplemental Information

Extended Experimental Procedures

Mice

caspase-8^{flox} (Salmena et al., 2003), Rosa26.CreER (Badea et al., 2003), Rosa26.LSL-YFP (Srinivas et al., 2001), *mavs*$^{-/-}$ (Sun et al., 2006) and *bax*^{flox}bak$^{-/-}$ (Takeuchi et al., 2005) mice have been previously described. *cflar*^{flox} animals were kindly provided by Richard Pope (Huang et al., 2010). RIPK3-deficient animals were obtained from V. Dixit (Newton et al., 2004). Mice were bred to generate animals with the following genotypes in combination with the presence or absence of Rosa26.CreER: *casp8*$^{f/f}$, *cflar*$^{f/f}$, *bax*$^{flox/flox}$*bak*$^{-/-}$ or Rosa26.LSL-YFP. For some experiments, these animals were crossed to *ripk3*$^{-/-}$ or *mavs*$^{-/-}$ animals.

All experiments were performed using age and gender matched littermates. Genotypes were confirmed by tail snip PCR as described previously. To determine the efficiency of caspase-8 deletion in tamoxifen-treated animals, PCR was performed on DNA extracted from different tissues to detect the floxed and deleted alleles of caspase-8, as described previously (Salmena et al., 2003). The St. Jude Institutional Animal Care and Use Committee approved all procedures and animal maintenance in accordance with the Guide for the Care and Use of Animals.

Concavalin A (ConA)-induced hepatitis

RIPK3$^{+/-}$ and RIPK3$^{-/-}$ animals were injected intravenously with 12mg/kg Concavalin A (cat# C5275, Sigma) in a final volume of 200ul. Blood was harvested 6h-48h post-injection from the retrorbital sinus from animals anesthetized with 2.5%

isoflurane in oxygen. Serum liver enzymes levels were assayed using a Trilogy Multi-Purpose Analyzer System from Drew Scientific. Livers were taken 12h post-injection and submitted for histological analysis at the St. Jude Veterinary Pathology Core facility.

Flow cytometry

For immune cell staining of peripheral blood leukocytes, blood was harvested from the retrorbital sinus from animals anesthetized with 2%–2.5% isoflurane in oxygen. Red blood cells were lysed in hypotonic buffer, and samples were stained with the appropriate antibodies, including anti-CD11b and anti-GR-1 antibodies (eBiosciences). Data were acquired using a FACSCalibur or LSRII using FlowJo Collectors or FACSDiva software, respectively. Data analysis was performed using FlowJo (Tree Star).

Immunohistochemistry

Tissues from treated animals were fixed in 10% formalin and embedded in paraffin. Four to six μm serial sections were cut, deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E). For immunostaining of T cells, macrophages and neutrophils, slides were stained with anti-CD3 (cat# sc-1127, Santa Cruz Biotechnology), anti-F4/80 (cat#MS48000, Caltag) and anti-LY-6B.2 (cat#RM6500, Caltag), respectively. YFP detection was performed by anti-GFP staining (cat#A11122, Invitrogen). For caspase-3 activity, sections were submitted to heat-induced epitope retrieval (cat# S2367, DAKO) and then stained with anti-cleaved caspase-3 (cat#CP229C, BioCare Medical). Histological analysis and scoring were performed blindly by a veterinary pathologist.

TUNEL

Slides of 4-6 μm sections cut from formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated. TUNEL assays were performed using the Dead End kit (cat#PRG7130, Promega) with the following modifications: TBST buffer (cat#TA-999-TT, ThermoShandon) was used for all washes, proteinase K (cat#S3020, DAKO) was substituted for the kit proteinase K, and endogenous peroxidases were blocked for 5 min with 3% aqueous hydrogen peroxide. Slides were counterstained lightly with hematoxylin.

Electron Microscopy

For TEM analysis, tissues were fixed overnight with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, washed with 0.1M cacodylate buffer in 5% sucrose, post-fixed in 2% OsO_4 with 0.3% potassium ferrocyanide, enbloc stained with uranyl acetate, embedded, sectioned, and mounted for imaging. The grids were viewed on a JEOL 1200 EXII Transmission Electron Microscope (JEOL, Peabody, MA) at 80 kV and images were captured using an 11 megapixel AMT digital camera (Advanced Microscopy Techniques, Danvers, MA).

Anti-TNF treatment

Some of the animals that were either skin painted with 4-OHT or gavaged with tamoxifen (as described in the Experimental Procedures Section), received three intraperitoneal injections of 0.5mg of neutralizing anti-TNF antibodies (clone XT3.11 from BioXCell or clone HB-10649 from ATCC) or 0.5mg of isotype control antibodies clone HRPN, BioXCell). For the Rosa26.CreER^{+}, FLIP$^{flox/flox}$ animals, anti-TNF antibodies were injected at days -1, +1 and +3 and for the Rosa26.CreER^{+}, Caspase-8$^{flox/flox}$, injections were performed at days +0, +2 and +4.

In vivo permeability assay using FITC-Dextran

Animals were gavaged for six consecutive days with tamoxifen (cat#T5648, Sigma) dissolved in sunflower seed oil (cat#S5007, Sigma) at a concentration of 1mg tamoxifen per 25g of animal body weight per day. In the following day, animals were fasted for 8h and then gavaged with 0.5mg of FITC-Dextran (cat#FD-4, Sigma) per gram of animal body weight using a solution of 50mg/mL of FITC-Dextran dissolved in PBS. Three hours later, serum was collected from heart blood and FITC-Dextran concentration was assessed using a fluorescent spectrophotometer at 490nm excitation and 520nm emission.

Cytokine Detection

RIPK3 wild-type or knockout animals were injected with 20ug of LPS per gram of animal body weight and serum was collected after 12h. Alternatively, bone marrow derived macrophages from these animals were stimulated *in vitro* with 10ng/mL LPS for 4h or infected with 25MOI of *E. coli* for 12h and supernatants from these cultures were collected. The concentration of cytokines (IL-6, TNF, KC and MCP-1) was detected using the Milliplex Multiplex immunoassays (Millipore).

For IL-1b and TNF quantitation in the skin, samples were snap frozen with liquid nitrogen and protein lysates were generated in RIPA lysis buffer supplemented with complete protease inhibitor cocktail (Cat#1697498001, Roche) and PhosSTOP (cat# 04906837001, Roche) using a tissue homogenizer. Debris was pelleted and the supernatants were assessed via ELISA according to manufacturers' instructions (IL-1b cat #88-7013, TNF cat#88-7324, eBiosciences). Cytokine levels were standardized according to grams of tissue.

Statistical analysis

Data was analyzed using the software Prism version 4.0 for Mac (GraphPad Software Inc, 2005). One-way ANOVA or two-way ANOVA followed by Bonferroni post-test was used to analyzed line graphs or bar graphs, respectively. One asterisk (*) represents $p<0.05$, (**) is $p<0.01$ and (***) is $p<0.001$. For survival curve graphs, curves were compared two by two using logrank test and the p value is shown for each comparison.

Graphical Abstract Legend

Schematic model of the different outcomes for caspase-8 and FLIP acute deletion. In response to various inducing signals (i.e. TNF), a complex containing RIPK1, RIPK3, FADD, FLIP, and caspase-8 inhibits both the formation of caspase-8 homodimer and the activation of RIPK3, preventing both apoptosis and necrosis, respectively. In the absence of caspase-8, no homodimer can form, and therefore apoptosis cannot proceed. However, the lack of caspase-8-FLIP heterodimer caused by caspase-8 deficiency allows RIPK3 to become activated and leads to necrosis and tissue damage. Ablation of RIPK3 in this context prevents cell death and loss of tissue homeostasis. FLIP deficiency also prevents the RIPK3-inhibiting caspase-8-FLIP heterodimer from forming, which leads to necrosis. However, inhibition of the caspase-8 homodimer is also lost when FLIP is absent, so apoptosis can also occur. Loss of RIPK3 in the context of FLIP deficiency only prevents necrosis from occurring. Under these conditions, apoptosis can drive tissue damage that occurs even in the absence of RIPK3-mediated inflammation.

Supplemental Figure Legends

Figure S1. Weight loss of tamoxifen-gavaged animals and efficiency of Cre-induced recombination. Related to Figure 1. **(A-C)** Animals were gavaged six consecutive days with 1mg tamoxifen per 25g body weight. Weight curves were determined over twenty days for **(A)** $CreER^-$, *casp8*$^{f/f}$ ($CreER^-$) and $CreER^+$, *casp8*$^{f/f}$ ($CreER^+$) animals, with the latter being split in two distinct groups according to their response to treatment (moribund and survivors); **(B)** $CreER^-$ and $CreER^+$, both with wild type alleles for caspase-8; and **(C)** $CreER^-$, *bax*$^{flox/flox}$, *bak*$^{-/-}$, and $CreER^+$, *bax*$^{flox/flox}$, *bak*$^{-/-}$ animals. **(D-F)** $CreER^-$, LSL-YFP and $CreER^+$, LSL-YFP animals were treated as above and the expression of YFP was detected with anti-GFP antibodies by immunohistochemistry (IHC) in **(D)** the gut and in **(E)** the spleen (SP), lymph nodes (LN) and thymus (TO) or **(F)** by flow cytometry for peripheral blood leukocytes. **(G)** Caspase-8 deletion was assessed in tamoxifen-treated *casp8*$^{f/f}$ animals in the indicated tissues by PCR. Upper panel represent animals that do not express Cre recombinase ($CreER^-$) and the lower panel represents animals that express it ($CreER^+$). Prox. SI is proximal small intestine, Dist. SI is distal small intestine and LN is lymph node. **(H-I)** $CreER^-$, c*asp8*$^{f/f}$ ($CreER^-$) and $CreER^+$, *casp8*$^{f/f}$ ($CreER^+$) animals were gavaged six consecutive days with 1mg tamoxifen per 25g body weight. Sections from the small intestine and colon were taken during **(H)** the weight loss phase, which corresponds to days 8-9 after the start of the treatment, and **(I)** the pre-weight loss phase, which corresponds to the last day of tamoxifen gavage, and stained with hematoxylin and eosin. (**) $p<0.01$. (***) $p<0.001$.

Figure S2. RIPK3 deficiency protects from the effects of acute caspase-8 deletion. Related to Figures 1 and 2. CreER^{-}, *casp8*$^{f/f}$, *ripk3*^{WT} (CreER^{-}, RIPK3^{WT}), CreER^{-}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{-}, RIPK3$^{-/-}$), CreER^{+}, *casp8*$^{f/f}$, *ripk3*^{WT} (CreER^{+}, RIPK3^{WT}) and CreER^{+}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{+}, RIPK3$^{-/-}$) animals were gavaged with 1mg tamoxifen per 25g animal body weight for 6 consecutive days. **(A)** Peripheral blood was collected at day +9 and cultures were performed in order to isolate and identify bacteria present in the blood. **(B)** Animals were orally gavaged with FITC-Dextran at day +6 and its presence in the blood was assessed by fluorescence after 3h post-gavage and **(C)** weights were followed for 25 days. CreER^{+}, RIPK3^{WT} animals were split in two distinct groups according to their response to treatment (moribund and survivors). **(D-E)** Sections from the cecum were taken at day 6 of tamoxifen-treated CreER^{+}, RIPK3^{WT} and CreER^{+}, RIPK3$^{-/-}$ animals and stained for **(D)** TUNEL or **(E)** cleaved caspase-3. **(F-G)** Sections from the cecum were taken at day 9 of tamoxifen-treated CreER^{+}, RIPK3^{WT} and CreER^{+}, RIPK3$^{-/-}$ animals and stained with **(F)** hematoxylin and eosin or **(G)** for the presence of macrophages (anti-F4/80), neutrophils (anti-LY-6B.2) and T cells (anti-CD3). (*) $p<0.05$. (**) $p<0.01$. (***) $p<0.001$.

Figure S3. Concavalin A (ConA)-induced liver damage is not dependent on RIPK3. Related to Figure 2. RIPK3$^{+/-}$ and RIPK3$^{-/-}$ animals were injected with 12mg/kg ConA intravenously. **(A-B)** Animals were bled retro-orbitally at the indicated time points and serum was analyzed for **(A)** ALT and **(B)** AST levels. Light bars are RIPK3$^{+/-}$ and dark bars are RIPK3$^{-/-}$ **(C-D)** Livers were taken 12h post-ConA injection and sections were

stained with **(C)** H&E or **(D)** cleaved caspase-3. (*) $p<0.05$. (n.s.) not statistically significant.

Figure S4. RIPK3 and MAVS-mediated inflammation is not required for the deleterious effects of Caspase-8 or FLIP acute deletion. Related to Figure 4. **(A)** LPS or *E.coli*-induced cytokine production in bone marrow derived macrophages from $RIPK3^{WT}$ or $RIPK3^{-/-}$ animals. **(B)** *In vivo* production of pro-inflammatory cytokines in response to LPS injection in $RIPK3^{WT}$ or $RIPK3^{-/-}$ animals. **(C)** Time course of the onset of the skin disease in animals painted with 4OHT. $CreER^{+}$, $cflar^{f/f}$, $ripk3^{WT}$ (FLIP flox, $RIPK3^{WT}$) and $CreER^{+}$, $cflar^{f/f}$, $ripk3^{-/-}$ (FLIP flox, $RIPK3^{-/-}$) animals were painted two times, at days +0 and +2. $CreER^{+}$, $casp8^{f/f}$, $ripk3^{WT}$ (Casp8 flox, $RIPK3^{WT}$) and $CreER^{+}$, $casp8^{f/f}$, $ripk3^{-/-}$ (Casp8 flox, $RIPK3^{-/-}$) animals were painted four times, at day +0, +2, +4 and +6. **(D)** Photos from 4OHT-painted skins from $CreER^{+}$, $cflar^{f/f}$ (FLIP) and $CreER^{+}$, $casp8^{f/f}$ (Caspase-8) animals. **(E)** Photos from 4OHT-painted skin from $CreER^{-}$, $casp8^{f/f}$, $mavs^{+/-}$ ($CreER^{-}$, $MAVS^{+/-}$), $CreER^{-}$, $casp8^{f/f}$, *mavs* ($CreER^{-}$, $MAVS^{-/-}$), $CreER^{+}$, $casp8^{f/f}$, $mavs^{+/-}$ ($CreER^{+}$, $MAVS^{+/-}$) and $CreER^{+}$, $casp8^{f/f}$, $mavs^{-/-}$ ($CreER^{+}$, $MAVS^{-/-}$) animals were taken at day +12. (*) $p<0.05$. (**) $p<0.01$. (***) $p<0.001$. (n.s.) not statistically significant.

Figure S1

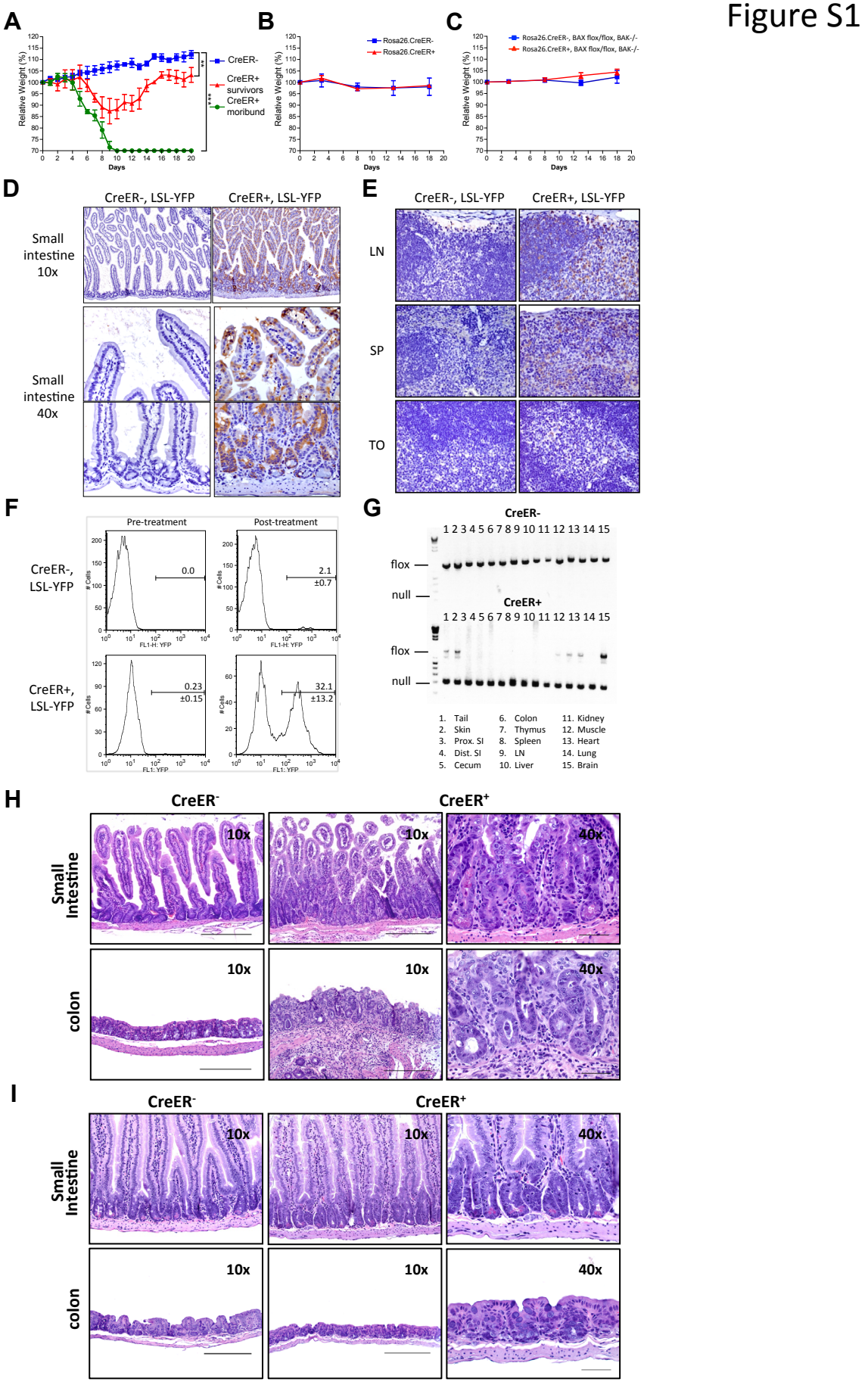
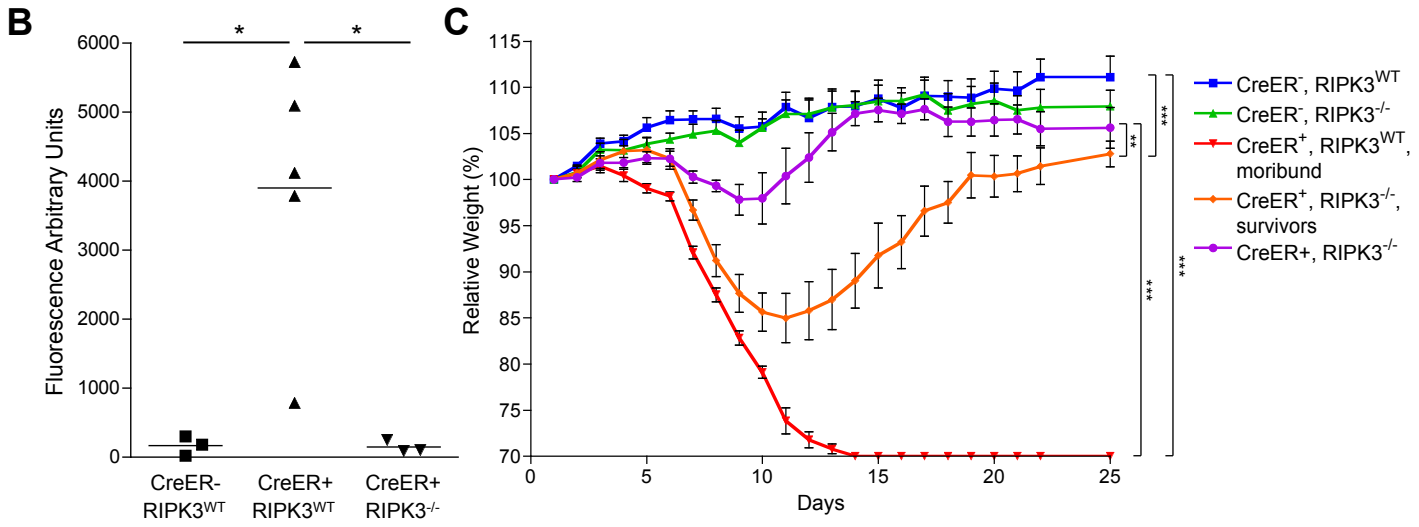

A
B
C
Relative Weight (%)
Days
CreER-
CreER+ survivors
CreER+ moribund
Rosa26.CreER-
Rosa26.CreER+
Rosa26.CreER-, BAX flox/flox, BAK-/-
Rosa26.CreER+, BAX flox/flox, BAK-/-
D
CreER-, LSL-YFP
CreER+, LSL-YFP
Small intestine 10x
Small intestine 40x
E
LN
SP
TO
F
Pre-treatment
Post-treatment
CreER-, LSL-YFP
CreER+, LSL-YFP
0.0
2.1 ±0.7
0.23 ±0.15
32.1 ±13.2
Cells
FL1-H: YFP
G
CreER-
CreER+
flox
null
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1. Tail
2. Skin
3. Prox. SI
4. Dist. SI
5. Cecum
6. Colon
7. Thymus
8. Spleen
9. LN
10. Liver
11. Kidney
12. Muscle
13. Heart
14. Lung
15. Brain
H
CreER⁻
CreER⁺
Small Intestine
colon
10x
40x
I
CreER⁻
CreER⁺
Small Intestine
colon
10x
40x

Figure S2

A

CreER^{-}, RIPK3^{WT}	CreER^{+}, RIPK3^{WT}	CreER^{+}, RIPK3$^{-/-}$
none	*Enterococcus faecalis* *Bacillus spp* *Lactobacillus spp* *Enterococcus spp*	none



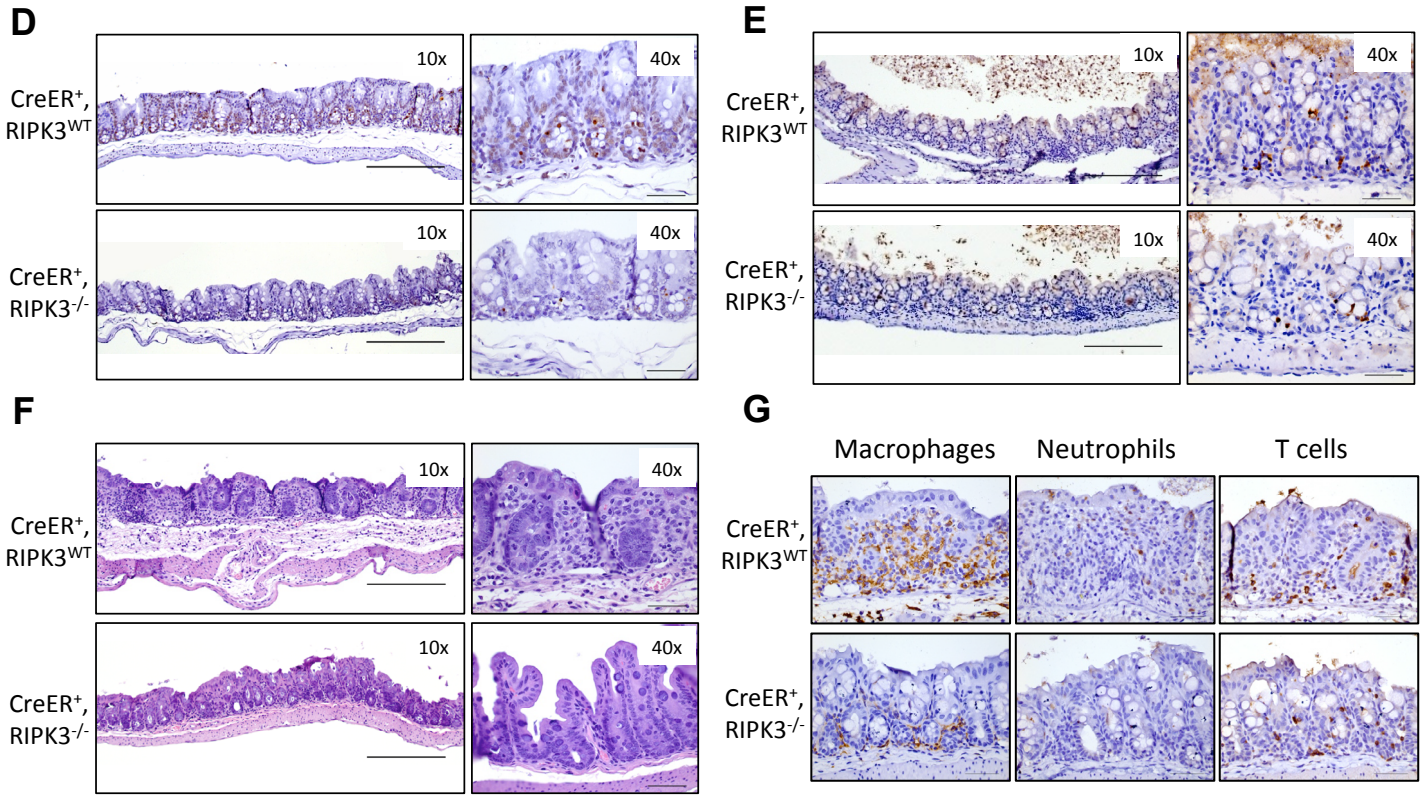
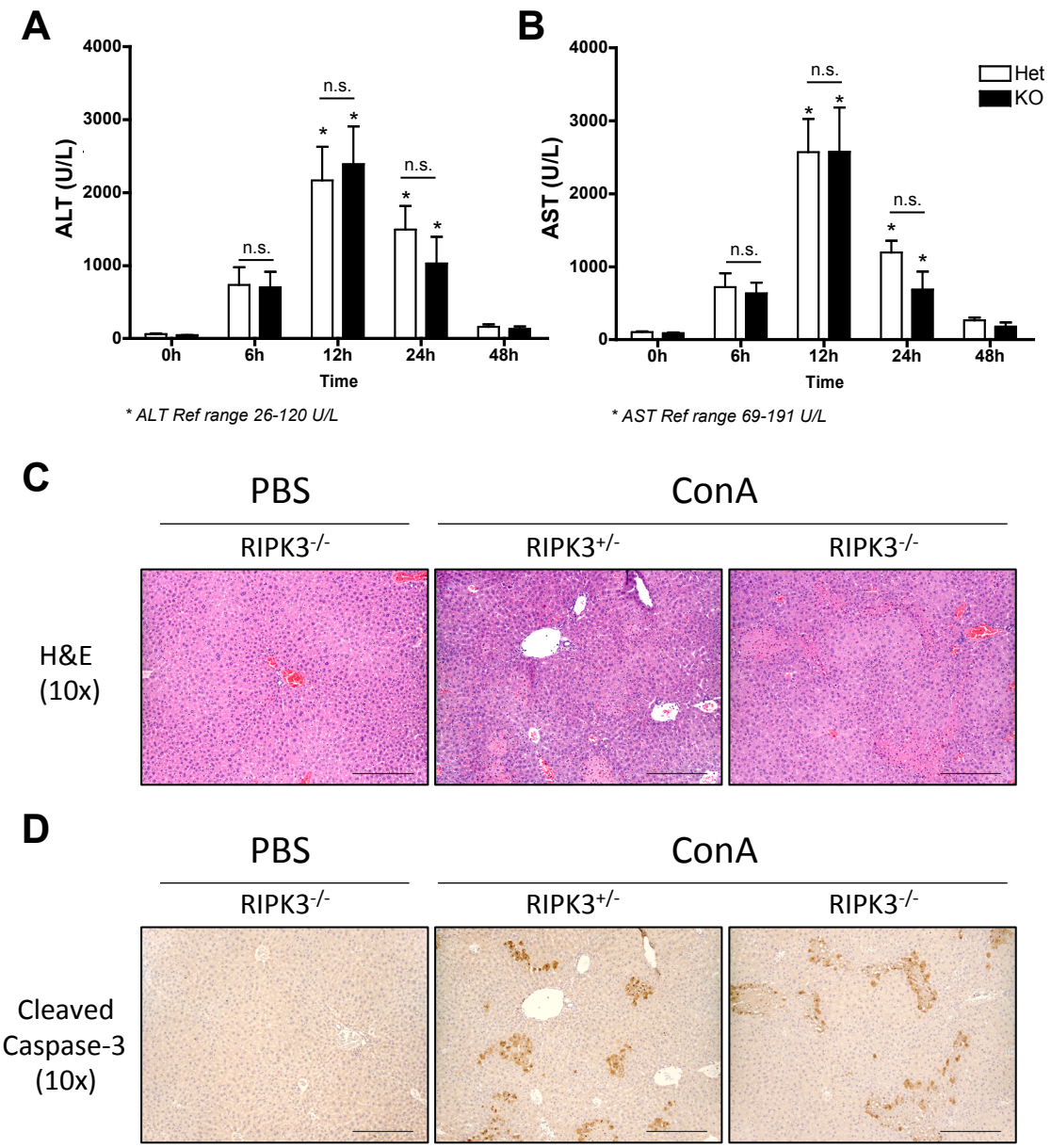


Figure S3


A
ALT (U/L)
4000
3000
2000
1000
0
n.s.
n.s.
n.s.
0h
6h
12h
24h
48h
Time
* ALT Ref range 26-120 U/L
B
AST (U/L)
Het
KO
n.s.
n.s.
n.s.
Time
* AST Ref range 69-191 U/L
C
PBS
ConA
RIPK3-/-
RIPK3+/-
RIPK3-/-
H&E
(10x)
D
PBS
ConA
RIPK3-/-
RIPK3+/-
RIPK3-/-
Cleaved
Caspase-3
(10x)

Supplemental Figure 4

Figure S4