Cell Reports
Report

Protective Roles for Caspase-8 and cFLIP in Adult Homeostasis

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http://dx.doi.org/10.1016/j.celrep.2013.08.045

SUMMARY

Caspase-8 or cellular FLICE-like inhibitor protein (cFLIP) deficiency leads to embryonic lethality in mice due to defects in endothelial tissues. Caspase-8$^{-/-}$ and receptor-interacting protein kinase-3 (RIPK3)$^{-/-}$, but not cFLIP$^{-/-}$ and RIPK3$^{-/-}$, double-knockout animals develop normally, indicating that caspase-8 antagonizes the lethal effects of RIPK3 during development. Here, we show that the acute deletion of caspase-8 in the gut of adult mice induces enterocyte death, disruption of tissue homeostasis, and inflammation, resulting in sepsis and mortality. Likewise, acute deletion of caspase-8 in a focal region of the skin induces local keratinocyte death, tissue disruption, and inflammation. Strikingly, RIPK3 ablation rescues both phenotypes. However, acute loss of cFLIP in the skin produces a similar phenotype that is not rescued by RIPK3 ablation. TNF neutralization protects from either acute loss of caspase-8 or cFLIP. These results demonstrate that caspase-8-mediated suppression of RIPK3-induced death is required not only during development but also for adult homeostasis. Furthermore, RIPK3-dependent inflammation is dispensable for the skin phenotype.

INTRODUCTION

Upon death receptor ligation, caspase-8 is recruited to initiator complexes through the adaptor molecule Fas (TNFRSF6)-associated protein with Death Domain (FADD) and forms active homodimers by induced proximity, propagating the apoptotic signal (Fuentes-Prior and Salvesen, 2004). Cellular FLICE-like inhibitor protein (cFLIP$_L$) (FLIP), a caspase-8 homolog lacking the catalytic cysteine, is recruited to the same complexes, forms heterodimers with caspase-8, and blocks the formation of the proapoptotic caspase-8 homodimers (Krueger et al., 2001). Caspase-8-, FADD-, or FLIP-deficient embryos die around embryonic day 10.5 associated with a failure to remodel the yolk sac vasculature (Varfolomeev et al., 1998; Yeh et al., 1998, 2000), an effect unrelated to the ability of caspase-8 to promote apoptosis. Further, animals with conditional deletion of caspase-8 with endothelium-specific Tie-1 promoter die with the same gross pathology and at the same developmental stage as do caspase-8-deficient embryos (Kang et al., 2004). No embryonic lethality was observed when caspase-8 was deleted elsewhere, including the heart (Dillon et al., 2012), liver (Kang et al., 2004), myeloid cells (Kang et al., 2004), and B or T lymphocytes (Beisner et al., 2005; Salmena et al., 2003). In skin (Kovalenko et al., 2009; Lee et al., 2009), or gut (Günther et al., 2011), deletion of caspase-8 results in postnatal lethality due to loss of barrier function and inflammation.

Receptor-interacting protein kinase-3 (RIPK3) promotes an alternative mode of cell death with characteristics of necrosis, often called "necroptosis" (Zhang et al., 2009). Ablation of RIPK3 fully rescues the development of mice lacking caspase-8, FADD, or both FADD and FLIP (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011). These and other approaches showed that the FADD-induced heterodimer of caspase-8 and FLIP suppresses RIPK3-mediated lethality (Dillon et al., 2012; Oberst et al., 2011). In this paper, we address whether the developmental roles of caspase-8 and FLIP in suppressing the lethal effects of RIPK3 persist into adult life.

RESULTS AND DISCUSSION

In order to achieve acute, systemic deletion of caspase-8 in adult animals, Rosa26.CreER$^+$ (CreER$^+$), caspase-8$^{flox/flox}$ (*casp8*$^{f/f}$) animals were gavaged with tamoxifen. Acute caspase-8 deletion through tamoxifen gavage induced severe weight loss and lethality in Cre$^+$ animals, but not in Cre$^-$ littermates (Figures 1A and 1B). Cre$^+$ animals that survived the treatment recovered weight over time (Figure S1A). Neither the activation of Cre in wild-type (WT) animals (Figure S1B) nor the deletion of an unrelated gene (Figure S1C) induced weight loss. Therefore, the effects were specifically caused by acute caspase-8 ablation.

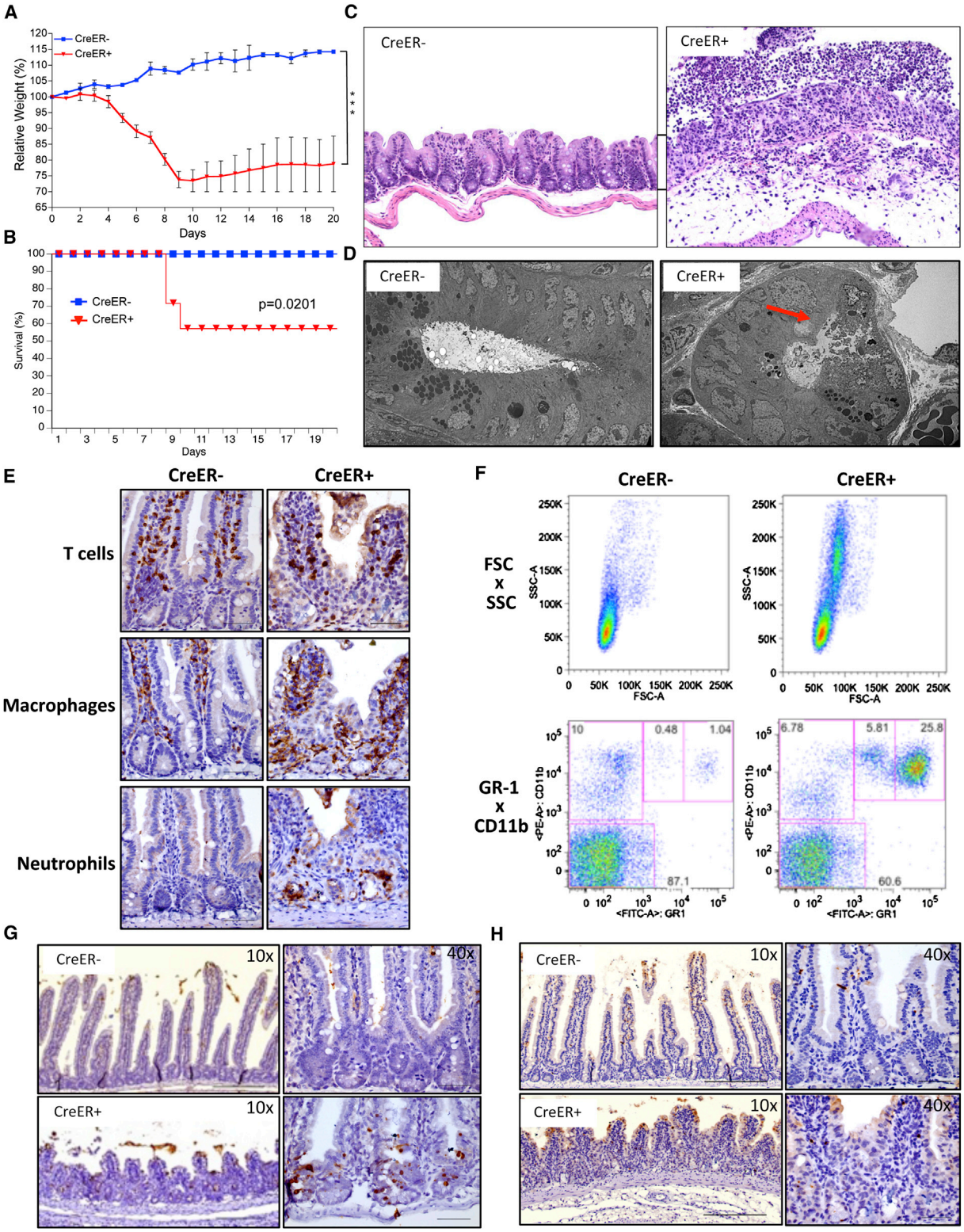

Figure 1. Acute Deletion of Caspase-8 Induces Cell Death, Tissue Damage, Loss of Weight, and Lethality

Rosa26.CreER^{-}, *casp8*$^{f/f}$ (CreER^{-}) and Rosa26.CreER^{+}, *casp8*$^{f/f}$ (CreER^{+}) animals were gavaged with 1 mg tamoxifen per 25 g animal body weight for 6 consecutive days.

(A and B) Animals were observed over 20 days for weight loss (A) and lethality (B). Error bars correspond to SD.

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To assess the efficiency of gene recombination, CreER$^+$ animals were crossed to lox/stop/lox-yellow fluorescent protein (YFP) (LSL-YFP) animals. Upon gavage, these animals showed widespread YFP expression throughout the gut (Figure S1D), lymph nodes, spleen, thymus (Figure S1E), and peripheral blood leukocytes (Figure S1F). Direct assessment of caspase-8 deletion was further performed by PCR in tamoxifen-treated CreER$^+$, *casp8*$^{f/f}$ animals. Gene deletion was detected in all tissues and was nearly complete throughout the gut (Figure S1G), consistent with the YFP expression pattern in the LSL-YFP reporter animal (Figures S1D–S1F).

Histology during the period of acute weight loss showed major disruption of tissue homeostasis and organization, with vacuolation of the villi, enterocyte death, marked inflammation, and infiltration of immune cells (Figures 1C–1E and S1H). The disruption of tissue homeostasis occurred throughout the intestines, including the small intestine (Figure S1H, top row), cecum (Figure 1C), and colon (Figure S1H, bottom row). Infiltration was by macrophages (F4/80$^+$) and neutrophils (Ly6B.2$^+$), with a minor infiltration of T cells (CD3$^+$; Figure 1E). Analysis of the peripheral blood leukocytes showed a marked increase in granulocytes (Figure 1F).

No significant alterations were observed in other organs, including liver, brain, heart, kidney, lungs, and lymphoid organs during the course of these experiments (data not shown). Blood cultures from *casp8*-deleted animals detected gut-resident microorganisms (Figure S2A), and oral-gavaged fluorescein isothiocyanate (FITC)-dextran was detected in the peripheral blood of CreER$^+$, *casp8*$^{f/f}$ animals, indicating abnormal gut permeability (Figure S2B). Therefore, the weight loss and lethality in CreER$^+$, *casp8*$^{f/f}$ animals were most likely related to disruption of the gut epithelial barrier.

Histology of the gut in the pre-weight-loss phase upon *casp8* deletion revealed early enterocyte cell death by hematoxylin and eosin staining (H&E) and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) staining, especially in the crypts and in the base of the villi, before any signs of inflammation (Figures 1G and S1I). Most TUNEL-positive cells did not stain for cleaved caspase-3 (Figure 1H), indicating that apoptosis was not the prevalent mode of cell death. In addition to apoptosis, TUNEL positivity in vivo can result from DNase-II-mediated digestion of engulfed nonapoptotic cell corpses (McIlroy et al., 2000). Indeed, ultrastructure of these dying cells resembled that of necrotic cells (Figure 1D). Although there is no specific marker for necroptosis, the combination of H&E, TUNEL, cleaved caspase-3 staining, and ultrastructure is evidence for nonapoptotic, necrosis-like cell death (Bonnet et al., 2011; Günther et al., 2011; Welz et al., 2011).

The liver, a major site for the conversion of tamoxifen into its active form 4-hydroxytamoxifen (4OHT), was not affected by acute *casp8* deletion (Figure S1G; data not shown). Liver is sensitive to concavalin A (ConA)-induced cell death that can be blocked by the RIPK1 inhibitor, necrostatin-1 (Jouan-Lanhouet et al., 2012). However, ConA-injected, RIPK3-deficient animals displayed the same levels of liver damage as their control littermates (Figures S3A–S3C). Damaged areas in the liver were cleaved caspase-3$^+$, indicating apoptosis (Figures S3C and S3D). This is in agreement with the finding that liver-specific ablation of caspase-8 has no pathological effect (Kang et al., 2004). Moreover, neither tumor necrosis factor (TNF) nor anti-CD95, in combination with the pan-caspase inhibitor zVAD-fmk, induces liver necrosis (Chandler et al., 1998; Künstle et al., 1997), and liver-specific ablation of caspase-8 is protective against anti-CD95-induced liver damage (Kang et al., 2004). Hence, there is no evidence to date that liver cells can undergo RIPK3-dependent necrosis; rather, they seem to be refractory to this type of cell death.

Embryonic lethality in caspase-8$^{-/-}$ mice can be fully rescued by ablation of RIPK3 (Kaiser et al., 2011; Oberst et al., 2011); therefore four genotypes were examined: CreER$^-$, *casp8*$^{f/f}$, *ripk3*$^{+/+}$; CreER$^-$, *casp8*$^{f/f}$, *ripk3*$^{-/-}$; CreER$^+$, *casp8*$^{f/f}$, *ripk3*$^{+/+}$; and CreER$^+$, *casp8*$^{f/f}$, *ripk3*$^{-/-}$. Strikingly, RIPK3 deficiency protected animals from the weight loss induced by acute *casp8* deletion (Figures 2A and S2C). Moreover, no lethality was observed in the CreER$^+$, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ animals (Figure 2B).

A marked reduction in the number of dying enterocytes was observed during the pre-weight-loss phase in CreER$^+$, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ animals as compared to their CreER$^+$, *casp8*$^{f/f}$, *ripk3*^{WT} littermates (Figure 2C), as was confirmed by TUNEL and cleaved caspase-3 staining (Figures 2D, S2D, and S2E) and ultrastructure (Figure 2E). RIPK3-dependent death of enterocytes during the early phases of tamoxifen treatment was associated with more intact gut tissue architecture at later time points (Figure S2F). At the peak of weight loss and disruption of gut homeostasis in CreER$^+$, *casp8*$^{f/f}$, *ripk3*^{WT} animals, CreER$^+$, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ mice showed dramatically lower levels of edema and infiltration of the gut (Figure S2F), including the small intestine (Figure 2F) and the cecum (Figure S2G). No FITC-dextran or bacteria were found in the peripheral blood in the these animals, indicating that there was no gut epithelial barrier breakdown or subsequent infection (Figures S2A and S2B).

Collectively, these findings demonstrate that caspase-8 is required for normal gut homeostasis during adulthood, and upon its acute deletion, RIPK3-mediated effects, including necrosis, can induce rapid and extensive gut damage, leading to epithelial barrier disruption and sepsis.

Skin homeostasis can also be severely affected by tissue-specific loss of caspase-8 during development (Kovalenko et al., 2009; Lee et al., 2009). Therefore, we painted skin with

(C) Day +9 cecum sections from tamoxifen-treated animals were stained with hematoxylin and eosin.
(D) Transmission electron microscopy of proximal small intestine crypts at day +6. Arrow points to necrotic cell.
(E) Proximal small intestine sections at day +9 were immunostained for T cells (anti-CD3), macrophages (anti-F4/80), and neutrophils (anti-LY-6B.2).
(F) Peripheral blood leukocytes at day +9 were stained with anti-CD11b and anti-GR-1 and then analyzed by fluorescence-activated cell sorting. Upper panels show cell size (forward scatter – pulse area [FSC-A]) versus granulosity (side scatter – pulse area [SSC-A]), whereas the lower panels show anti-GR-1 versus anti-CD11b.
(G and H) Proximal small intestine sections from day +6 were stained for TUNEL (G) or cleaved caspase-3 (H). Triple asterisks denote $p < 0.001$.
See also Figures S1 and S2.

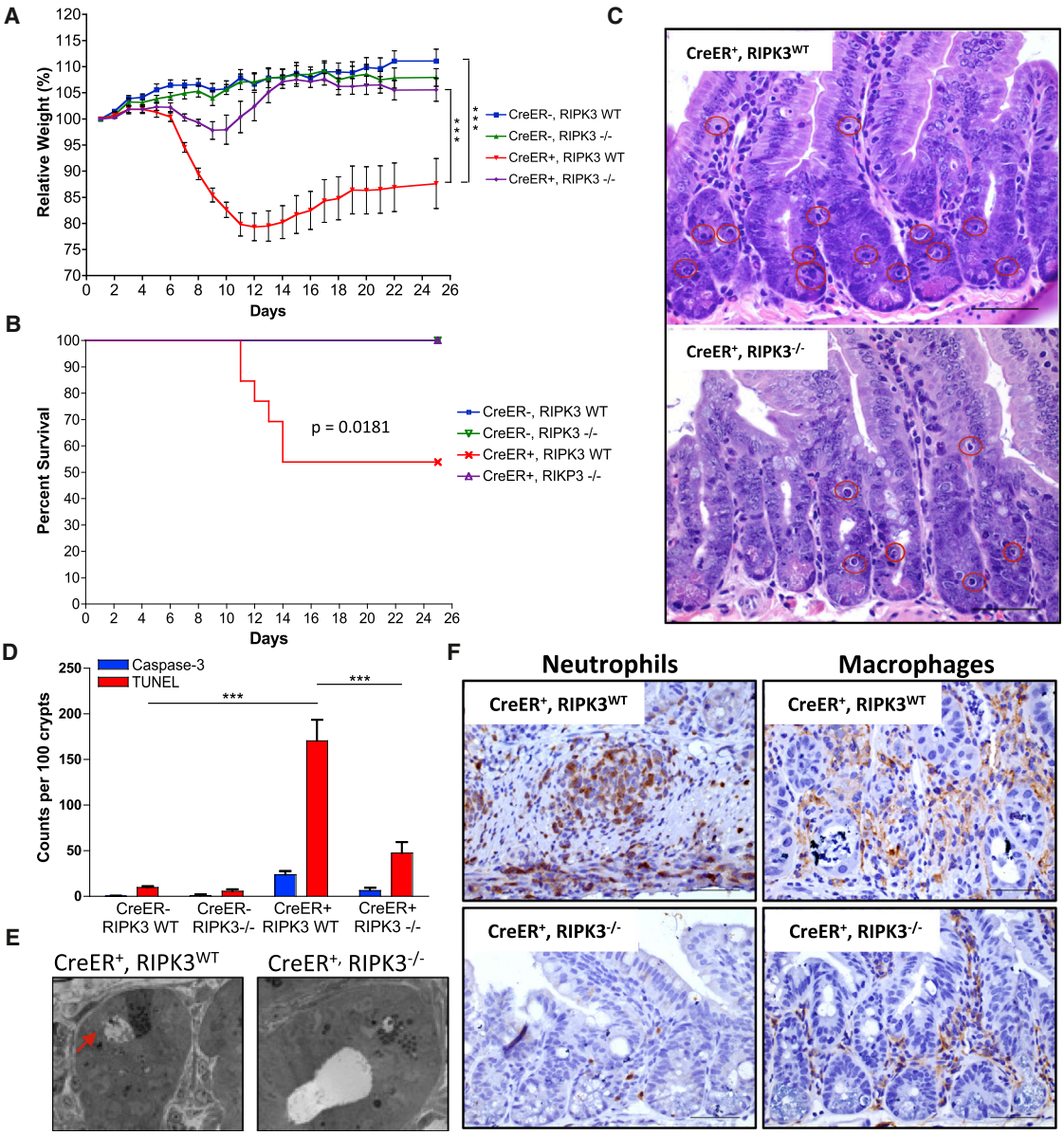


Figure 2. RIPK3 Deficiency Protects from Acute Deletion of Caspase-8

Rosa26.CreER^{-}, *casp8*$^{f/f}$, *ripk3*$^{+/+}$ (CreER^{-}, RIPK3^{WT}); Rosa26.CreER^{-}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{-}, RIPK3$^{-/-}$); Rosa26.CreER^{+}, caspase-8$^{flox/flox}$, RIPK3$^{+/+}$ (CreER^{+}, RIPK3^{WT}); and Rosa26.CreER^{+}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{+}, RIPK3$^{-/-}$) animals were gavaged with 1 mg tamoxifen per 25 g animal body weight for 6 consecutive days.

(A and B) Animals were observed over 25 days for weight loss (A) and lethality (B). Error bars correspond to SD.

(C) Day +6 proximal small intestine sections from tamoxifen-treated CreER^{+}, RIPK3^{WT} and CreER^{+}, RIPK3$^{-/-}$ animals were stained with hematoxylin and eosin. Red circles highlight dead cells.

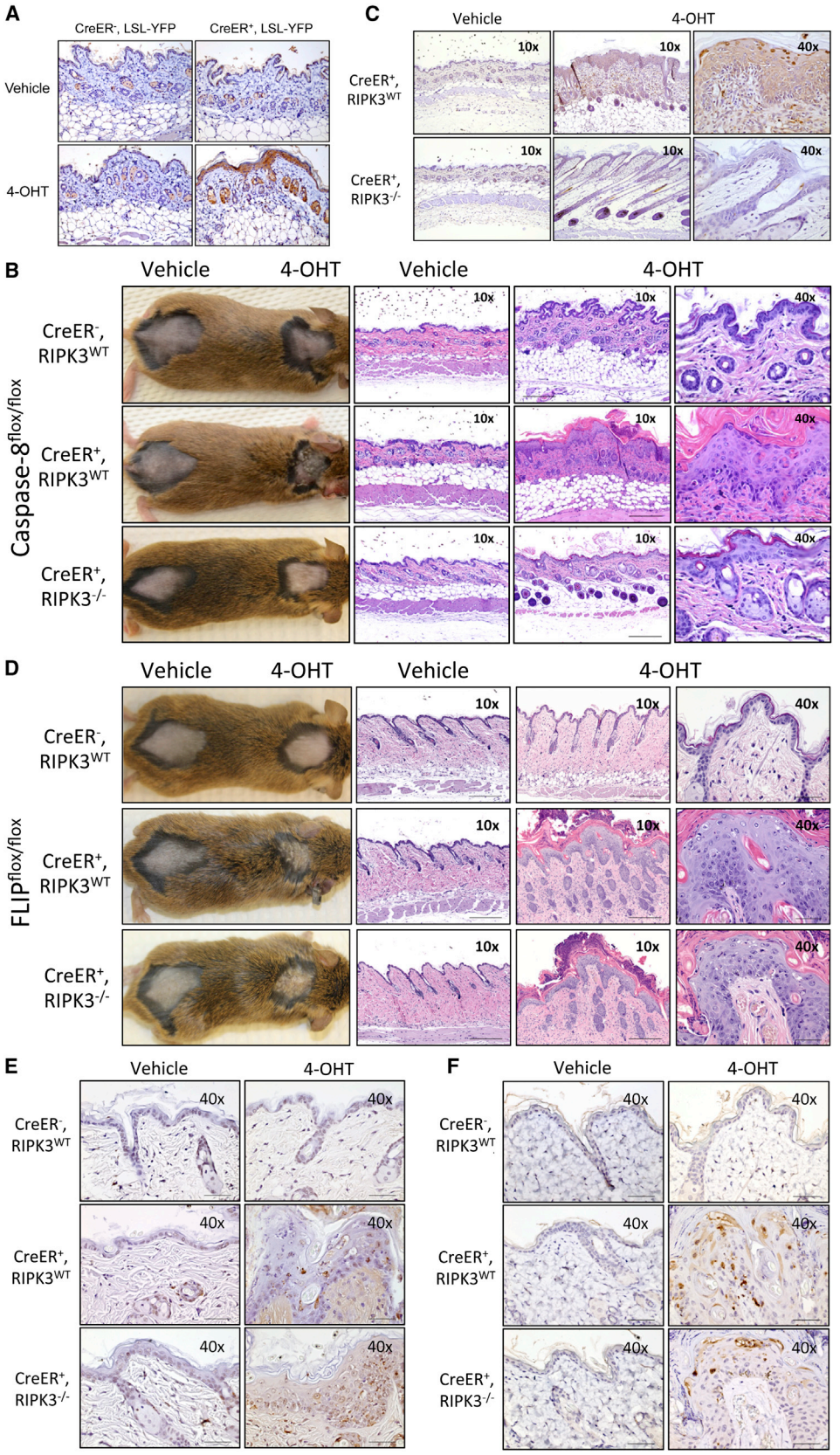
(D) Proximal small intestine sections were stained for TUNEL and cleaved caspase-3, and the number of TUNEL-positive or cleaved caspase-3 positive cells per 100 crypts was determined by manual counts. Error bars correspond to SD.

(E) Transmission electron microscopy of proximal small intestine crypts at day +6. Arrow points to necrotic cell.

(F) Day +9 proximal small intestine sections from tamoxifen-treated CreER^{+}, RIPK3^{WT} and CreER^{+}, RIPK3$^{-/-}$ animals were immunostained for neutrophils (anti-LY-6B.2; left panel) or macrophages (anti-F4/80; right panel). Triple asterisks denote $p < 0.001$.

See also Figures S2 and S3.

4OHT to assess the effects of a localized, acute loss of caspase-8 in adult animals. YFP expression in CreER^{+}, LSL-YFP animals was analyzed 24 hr after the last day of painting showed YFP expression, whereas no YFP^{+} cells were detected in the vehicle-only painted area of the same animal (Figure 3A).



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Local deletion of *casp8* in the skin induced a large ulcerative area, with epidermal hyperplasia and marked superficial hyperkeratosis, forming a thick crust (Figure 3B, left panel), with dyskeratotic foci and central pustule formation, edema, and immune cell infiltration (Figure 3B, right panel). Enhanced keratinocyte death was evidenced by TUNEL staining (Figure 3C). In contrast, 4OHT-painted areas from CreER^{+}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ mice showed minimal epidermal hyperplasia, rare dead keratinocytes, and minimal dermal inflammation (Figures 3B and 3C). Similarly, embryonic skin-specific deletion of FADD induced chronic skin inflammation, which was also blocked by concurrent ablation of RIPK3 (Bonnet et al., 2011).

All previous models of skin-specific Cre-induced deletion of caspase-8 or FADD used either the K5 or the K14 promoters, which are first expressed during embryonic development (Bonnet et al., 2011; Kovalenko et al., 2009; Lee et al., 2009). It is, therefore, possible that the effects seen in young animals were the result of loss during development. This same argument can be made for models of gut-specific deletion of caspase-8, FADD, or FLIP employing the Villin-Cre promoter, which is also expressed during embryogenesis (Günther et al., 2011; Piao et al., 2012; Welz et al., 2011). Our results demonstrate that caspase-8 is required not only during development but also is essential to maintain skin and gut homeostasis in adult animals.

Recently, RIPK3 activation was suggested to be involved not only in necroptosis induction, but also in inflammatory responses (Kang et al., 2013; Vince et al., 2012). *Ripk3*$^{-/-}$ bone marrow-derived macrophages (BMDM), however, presented similar levels of proinflammatory cytokine production compared to RIPK3-sufficient BMDM both after stimulation with lipopolysaccharide (LPS) or infection with *E. coli* (Figure S4A). Likewise, RIPK3 deficiency did not influence LPS-induced cytokine production in vivo (Figure S4B). The detection of RIPK3-dependent necrosis at early time points, when no signs of inflammation were observed (Figure S1I), together with the similar proinflammatory response to LPS between the RIPK3-sufficient and -deficient mice (Figures S4A and S4B) suggests that cell death may be the major driving force behind the damaging outcomes of caspase-8 loss in adult animals.

To directly test this, a model in which *cflar* (encoding FLIP), rather than *casp8*, is acutely deleted in the skin was developed. In the absence of FLIP, caspase-8 fails to block RIPK3-mediated necroptosis (Oberst et al., 2011) and can also sensitize to FADD-caspase-8-dependent apoptosis (Dillon et al., 2012; Irmler et al., 1997; Yeh et al., 2000). 4OHT-painted skin areas from CreER^{+}, *cflar*$^{f/f}$, *ripk3*$^{+/+}$ presented a similar phenotype to that observed in acute *casp8* deletion, with epidermal hyperplasia, superficial hyperkeratosis, and the formation of a thick crust (Figure 3D, left panel), with enhanced edema and infiltration (Figure 3D, right panels) as well as higher levels of cell death, evidenced either by TUNEL (Figure 3E) or cleaved caspase-3 staining (Figure 3F). In contrast to acute *casp8* deletion, apoptosis rather than necrosis was responsible for the cell death. Further, RIPK3 ablation was not able to prevent or lessen the skin disease, as CreER^{+}, *cflar*$^{f/f}$, *ripk3*$^{-/-}$ presented similar levels of skin damage when painted with 4OHT (Figures 3D–3F and S4C).

Therefore, regardless of the mode of cell death, the consequences of acute deletion of either caspase-8 or FLIP appear to produce similar pathologies, yet with different time courses (Figures S4C and S4D), perhaps due to the relative stabilities of the proteins (Fulda et al., 2000). Because, in the latter case, cells can only undergo apoptosis and the phenotype developed independently of RIPK3-mediated pathways, it is reasonable to conclude that RIPK3-induced inflammation is not required in this model. Further, this implies that apoptosis and RIPK3-mediated necrosis must be actively suppressed to maintain skin tissue homeostasis, which is in agreement with the observation that FLIP embryonic lethality is only rescued by ablation of both RIPK3 and FADD (Dillon et al., 2012).

We suggest that either necroptotic or apoptotic cell death is responsible for the inflammatory effects. However, whereas necrosis is known to be proinflammatory, apoptosis is not (Green, 2010). Thus, it is possible that the inflammatory response is due to cell-death-mediated loss of barrier function, rather than an effect of dying cells on the inflammatory response. Indeed, increased production of interleukin (IL)-1β in the skin correlates with the skin damage rather than with RIPK3-induced inflammation (Figures 4A and 4B). Moreover, loss of gut tissue homeostasis by embryonic gut-specific ablation of FADD is attenuated by treatment with antibiotics as well as by germ-free conditions (Günther et al., 2011).

Caspase-8 was suggested to suppress the exacerbated RIPK3-mediated inflammatory response to normal skin cornification via RIG-I and its adaptor protein mitochondrial antiviral-signaling protein (MAVS) (Rajput et al., 2011; Wallach et al., 2010). To assess the contribution of this pathway to the observed skin phenotype, CreER^{+}, *casp8*$^{f/f}$, mavs$^{-/-}$ animals were generated. We found, however, that the pathological effects of acute deletion of *casp8* in the skin were not rescued by ablation of MAVS (Figure S4E), demonstrating that caspase-8 suppression of the RIG-I/MAVS pathway is not responsible for the loss of skin homeostasis.

TNF, via its receptor TNF-R1, is the best-described trigger of necroptosis, both in vitro and in vivo, and it is also a well-known inducer of apoptosis (Weinlich et al., 2011). Upon acute deletion of either *casp8* or *cflar*, TNF is produced at detectable levels in

Figure 3. Acute Deletion of Caspase-8 or FLIP in the Skin by 4-Hydroxytamoxifen Painting Produces Local Inflammation and Tissue Damage

Animals were shaved in two distinct dorsal areas. The shaved area around the neck was painted with 4-hydroxytamoxifen (4OHT), whereas the shaved area near the tail base was painted only with vehicle.

(A) Histological sections from the painted skin areas from Rosa26.CreER^{-}, LSL-YFP (CreER^{-}, LSL-YFP) and Rosa26.CreER^{+}, LSL-YFP (CreER^{+}, LSL-YFP) were taken the day after the last painting and stained with anti-GFP to detect YFP expression.

(B and C) Rosa26.CreER^{-}, *casp8*$^{f/f}$, *ripk3*$^{+/+}$ (CreER^{-}, RIPK3^{WT}); Rosa26.CreER^{+}, *casp8*$^{f/f}$, *ripk3*$^{+/+}$ (CreER^{+}, RIPK3^{WT}); and Rosa26.CreER^{+}, *casp8*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{+}, RIPK3$^{-/-}$) were painted four times, and at day +12, (B) photos and H&E-stained sections or (C) TUNEL-stained sections were produced.

(D–F) Rosa26.CreER^{-}, *cflar*$^{f/f}$, *ripk3*$^{+/+}$ (CreER^{-}, RIPK3^{WT}); Rosa26.CreER^{+}, *cflar*$^{f/f}$, *ripk3*$^{+/+}$ (CreER^{+}, RIPK3^{WT}); and Rosa26.CreER^{+}, *cflar*$^{f/f}$, *ripk3*$^{-/-}$ (CreER^{+}, RIPK3$^{-/-}$) were painted two times, and at day +10, (D) photos and H&E-stained sections, (E) TUNEL-stained sections, or (F) cleaved caspase-3 were produced.

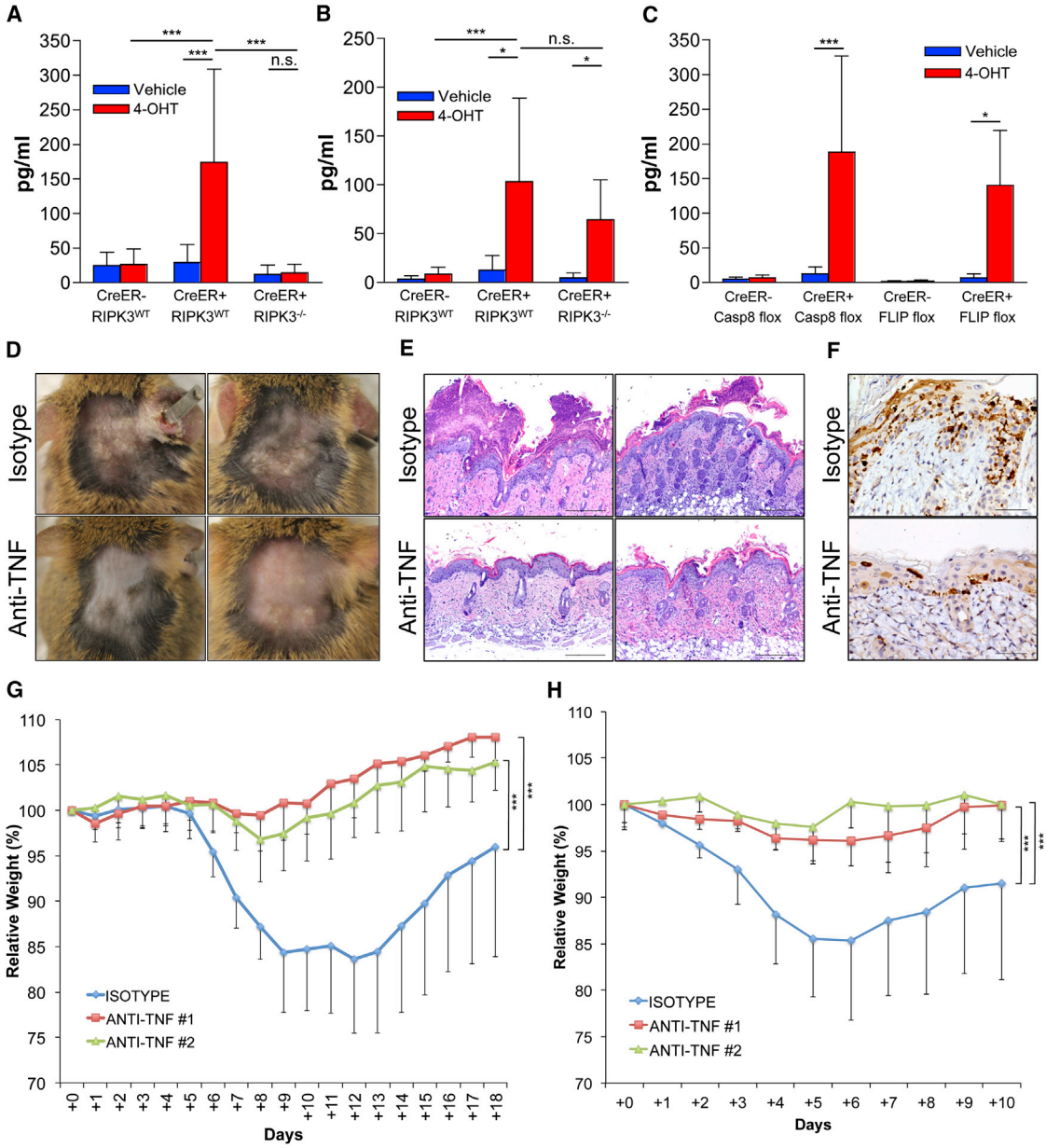

Figure 4. Neutralization of TNF Protects from the Deleterious Effects of Caspase-8 or FLIP Acute Deletion

Animals were shaved in two distinct dorsal areas. The shaved area around the neck was painted with 4-hydroxytamoxifen (4OHT), whereas the shaved area near the tail base was painted only with vehicle.

(A and B) IL-1b levels in the skins of (A) Casp8$^{f/f}$ animals at day +10 or (B) *cflar*$^{f/f}$ animals at day +5. Error bars correspond to SD.

(C) TNF levels in the skins of *casp8*$^{f/f}$ (Casp8 flox) animals at day +10 or *cflar*$^{f/f}$ (FLIP flox) animals at day +5. Error bars correspond to SD.

(D–F) CreER^{+}, *cflar*$^{f/f}$ animals were intraperitoneally (i.p.) injected at days −1, +1, and +3 with 0.5 mg of TNF antibodies (clone XT3.11) or isotype control antibodies (clone HRPN), and examples of (D) photos, (E) sections stained with hematoxylin and eosin, and (F) cleaved caspase-3-staining sections were produced at day +8.

(G) CreER^{+}, *casp8*$^{f/f}$ animals were gavaged with 1 mg tamoxifen per 25 g animal body weight for 6 consecutive days and i.p. injected at days +0, +2, and +4 with 0.5 mg of two different neutralizing TNF antibodies (#1, XT3.11; #2, HB10649) or isotype control (clone HRPN). Weight loss was followed for 18 days. Error bars correspond to SD.

(H) CreER^{+}, *cflar*$^{f/f}$ animals were treated as in (G) but gavaged only twice, at days +0 and +2, and their weights were tracked for 10 days. The asterisk denotes $p < 0.05$. The triple asterisks denote $p < 0.001$. n.s., not statistically significant. Error bars correspond to SD.

See also Figure S4.

the skin at early stages of disease (Figure 4C). Therefore, CreER$^+$, *cflar*$^{f/f}$, animals were injected with neutralizing anti-TNF or control isotype antibodies and painted with 4OHT (Figures 4D and 4E). Whereas all isotype-injected animals fully developed the skin disease, anti-TNF treatment resulted in a marked protection: either they did not show any signs of the disease or presented much milder lesions (Figures 4D–4F).

To further analyze the role of TNF, CreER$^+$, *casp8*$^{f/f}$ or CreER$^+$, *cflar*$^{f/f}$ animals were gavaged with tamoxifen simultaneously with injections of two different neutralizing TNF antibodies or isotype control. Both TNF antibodies protected from the effects of acute deletion of *casp8* (Figure 4G) or *cflar* (Figure 4H). These data indicate that, independent of the cell death type induced, TNF plays a central role in the onset of the disease.

The therapeutic use of pan-caspase inhibitors has been proposed and, to date, no severe toxic effects of these inhibitors have been reported in mice (Callus and Vaux, 2007) or humans (Pockros et al., 2007; Ratziu et al., 2012). Genetic studies that rescued caspase-8 deficiency in vivo (Kaiser et al., 2011; Oberst et al., 2011), as well as in vitro studies on inhibition of RIPK3-dependent necrosis (Oberst et al., 2011), have shown that the protease activity of caspase-8 is required for its protective effects. Therefore, in light of our results, a closer evaluation of how these inhibitors affect caspase-8 activity in vivo, as well as their pharmacokinetics, bioavailability, and tissue distribution, is warranted. This might be particularly important in patients with elevated levels of circulating TNF, as the addition of exogenous TNF to animals lacking intestinal caspase-8 strikingly exacerbated damage to gut tissue (Günther et al., 2011). Furthermore, TNF-induced shock as well as hyperacute shock induced by TNF in combination with caspase inhibitors severely impact the gut and are mainly mediated by RIPK3 (Duprez et al., 2011; Linkermann et al., 2012). Attempts to generate pan-caspase inhibitors that may be more active in vivo may therefore reveal toxicities associated with the effects we describe.

EXPERIMENTAL PROCEDURES

Tamoxifen Treatments

To induce systemic deletion of caspase-8, animals were gavaged for 6 consecutive days with tamoxifen (T5648, Sigma-Aldrich) dissolved in sunflower seed oil (S5007, Sigma-Aldrich) at a concentration of 1 mg tamoxifen per 25 g of animal body weight per day. Weight loss, morbidity, and mortality were assessed every day. Acute deletion of FLIP was induced by two rounds of gavage with a 48 hr interval between them.

To induce localized deletion of caspase-8 or cFLIP in the skin, animals were shaved in two different dorsal areas (one around the neck and one near the tail base) using a hair trimmer. The dorsal area around the neck was painted four times, once every other day, for *casp8*$^{f/f}$ animals and twice, once every other day, for *cflar*$^{f/f}$ animals with 100 μl of 10 mg/ml 4-hydroxytamoxifen (H6278, Sigma-Aldrich) dissolved in ethanol. The dorsal area near the tail base was painted only with vehicle and served as an internal control area.

For further experimental procedures, please see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.045.

ACKNOWLEDGMENTS

The authors thank Patrick Fitzgerald and Laura L. McCormick for providing essential logistical and administrative support and maintenance of the mouse colony, respectively. We thank Sharon Frase and Fara Sudlow from the St. Jude Cellular Image Shared Resource for providing technical support for the electron microscopy analysis. We thank Dr. Peter Murray and Dr. Scott Brown from St. Jude Children's Research Hospital for kindly providing key reagents for this project. We also thank the St. Jude Animal Resource Center and the Veterinary Pathology Core Histology Lab. This work is supported by CA169291, AI44828, and AI47891 from the U.S. National Institutes of Health and by the American Lebanese Syrian Associated Charities.

Received: February 10, 2013
Revised: July 5, 2013
Accepted: August 27, 2013
Published: October 3, 2013

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