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The Adaptor Protein FADD Protects Epidermal Keratinocytes from Necroptosis In Vivo and Prevents Skin Inflammation

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SUMMARY

Epidermal keratinocytes provide an essential structural and immunological barrier forming the first line of defense against potentially pathogenic microorganisms. Mechanisms regulating barrier integrity and innate immune responses in the epidermis are important for the maintenance of skin immune homeostasis and the pathogenesis of inflammatory skin diseases. Here, we show that epidermal keratinocyte-restricted deficiency of the adaptor protein FADD (FADD^{E-KO}) induced severe inflammatory skin lesions in mice. The development of skin inflammation in FADD^{E-KO} mice was triggered by RIP kinase 3 (RIP3)-mediated programmed necrosis (termed necroptosis) of FADD-deficient keratinocytes, which was partly dependent on the deubiquitinating enzyme CYLD and tumor necrosis factor (TNF)-TNF receptor 1 signaling. Collectively, our findings provide an in vivo experimental paradigm that regulation of necroptosis in keratinocytes is important for the maintenance of immune homeostasis and the prevention of chronic inflammation in the skin.

INTRODUCTION

The epidermis forms a life-sustaining epithelial barrier preventing water loss, but also provides an important immunological barrier constituting the first line of defense against potentially pathogenic microorganisms (Elias, 2007; Nestle et al., 2009). Epidermal keratinocytes undergo a tightly regulated program of proliferation and differentiation that is essential for the formation of a stratified epidermis and at the same time perform important immune functions that are critical for the regulation of immune responses in the skin. An intense crosstalk between epidermal keratinocytes and nonepithelial cells, including dermal fibroblasts and immune cells, plays a crucial role in the maintenance of physiological skin homeostasis. Keratinocytes exposed to injury and infection transmit signals triggering immune and wound healing responses in the skin, thus providing an early detection system for potential immunological challenges (Nestle et al., 2009). De-regulated keratinocyte responses to injurious, chemical, or pathogen insults could be implicated in the pathogenesis of inflammatory skin diseases.

Members of the tumor necrosis factor (TNF) ligand and receptor superfamily are critical regulators of immune and inflammatory responses. The Fas-associated death domain (FADD) adaptor protein is an essential component of the death inducing signaling complex (DISC), which is formed upon activation of death receptors of the TNF receptor superfamily and induces apoptosis (reviewed in Wilson et al., 2009). FADD is a 28 kDa protein containing a death domain (DD) and a death effector domain (DED). Via its DD, FADD associates either directly with death receptors, as in the case of Fas, or with other intracellular signaling complex components, such as RIP1 and TRADD in the case of TNFR1, to initiate DISC formation. The FADD DED serves as docking site allowing the recruitment and activation of caspase 8 and the induction of apoptosis. FADD-deficient cells are resistant to apoptosis induced by TNFR1, Fas, or TRAIL-R stimulation (Yeh et al., 1998; Zhang et al., 1998). In addition to its well-characterized role in death receptor signaling, FADD has been implicated in different cellular processes including proliferation, differentiation, autophagy, genome surveillance, and innate immunity (reviewed in Tourneur and Chiocchia, 2010).

In addition to apoptosis, death receptors have been shown to induce a particular type of necrotic death in certain cell types, termed programmed necrosis or necroptosis, which is mediated by the kinases RIP1 and RIP3 (Cho et al., 2009; He et al., 2009; Vandenabeele et al., 2010; Zhang et al., 2009). Lack of FADD or caspase-8 or treatment with pan-caspase inhibitors sensitized cells to necroptosis induction after death receptor stimulation, suggesting that apoptosis antagonizes the programmed necrotic death pathway (Cho et al., 2009; Holler et al., 2000; Lüschen et al., 2000; Osborn et al., 2010; Vercammen et al., 1998; Zhang et al., 2009). Mice lacking FADD or caspase-8 die at embryonic day E11.5 as a result of cardiac abnormalities and abdominal hemorrhage (Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998). RIP3 deficiency rescues the

embryonic lethality of caspase-8-deficient mice, and mice lacking both caspase-8 and RIP3 are born normally and reach adulthood without displaying any abnormalities apart from the lymphoproliferative disease characteristic of Fas deficiency (Kaiser et al., 2011; Oberst et al., 2011). In addition, RIP3 deficiency also rescued the T cell defects caused by conditional ablation of caspase-8 in the T cell lineage (Ch'en et al., 2011). Moreover, RIP1 deficiency could also rescue the embryonic lethality and T cell defects associated with FADD deficiency (Zhang et al., 2011). These studies identified a crucial in vivo function of FADD and caspase-8 in preventing RIP kinase-mediated necrosis that is essential for embryonic development and T cell survival. However, the physiological significance of the mechanisms regulating programmed necrosis for tissue homeostasis and disease pathogenesis in vivo remains unclear.

Here, we show that epidermal keratinocyte specific ablation of FADD induces a severe inflammatory skin disease in mice and provide genetic evidence identifying RIP3-dependent necrotic death of FADD-deficient keratinocytes as the essential initiating event triggering the pathogenesis of inflammatory skin lesions. These findings reveal a previously unrecognized important physiological function of FADD in preventing programmed necrosis of epidermal keratinocytes and skin inflammation in vivo.

RESULTS

Mice with Epidermis-Specific FADD Deficiency Develop Skin Lesions

To study the role of FADD in the adult, we generated two different mouse lines with conditional *Fadd* alleles: in the *Fadd*^{FL} line, Cre-mediated deletion of the second exon of the *Fadd* gene results in complete elimination of FADD protein (Mc Guire et al., 2010). In addition, we generated the FADD-IRES-GFP^{FL} mouse line in which a splice acceptor IRES-GFP cassette was introduced after the second loxP site flanking exon 2 (Figure S1 available online). After Cre-mediated excision of the loxP-flanked exon 2, the FADD-IRES-GFP allele produces a hybrid mRNA resulting in the expression of the DED domain of FADD and also of GFP (Figure 1A). Homozygous germline deletion of either the FADD^{FL} or the FADD-IRES-GFP^{FL} alleles resulted in early embryonic lethality (data not shown) similarly to the reported phenotype of FADD-deficient animals (Yeh et al., 1998; Zhang et al., 1998).

To study the role of FADD in the epidermis, we generated mice with keratinocyte-restricted FADD ablation by crossing *Fadd*^{FL} or FADD-IRES-GFP^{FL} mice with a mouse line expressing Cre under the control of the human Keratin 14 (*KRT14*) promoter (Hafner et al., 2004; Pasparakis et al., 2002). Immunoblot and Southern blot analysis showed efficient deletion of the FADD-IRES-GFP^{FL} allele and strongly reduced expression of the full-length FADD protein in the epidermis of these mice (Figures 1A and 1B). In addition, a smaller protein corresponding to the predicted size of the exon 1-encoded fragment including mainly the DED was detected by FADD antibodies in epidermal extracts from these mice, which also showed GFP expression (Figure 1A). *KRT14-cre-Fadd*^{FL} mice showed efficient ablation of FADD protein without expression of the lower molecular weight DED fragment (data not shown). Mice with homozygous epidermal keratinocyte-specific deletion of the FADD-IRES-GFP^{FL} allele

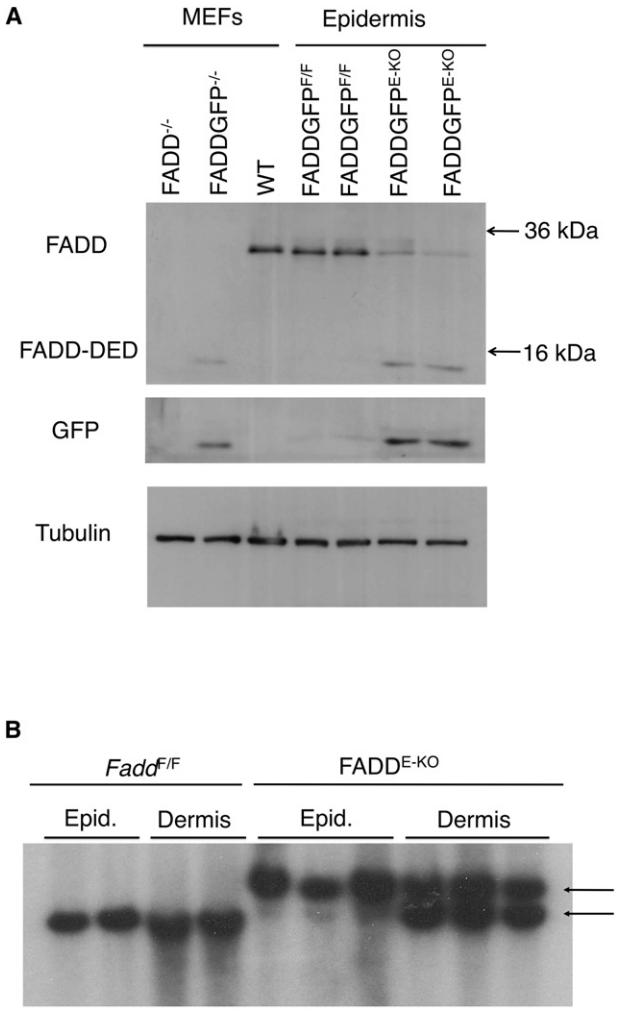

Figure 1. Epidermal Keratinocyte-Specific Knockout of FADD

(A) Immunoblot analysis of FADD, GFP, and tubulin expression in the epidermis of *Fadd*$^{FL/FL}$ and FADD^{E-KO} mice and in MEFs with the indicated genotypes.

(B) DNA isolated from dermis and epidermis of *Fadd*$^{FL/FL}$ and FADD^{E-KO} mice was subjected to Southern blot analysis after digestion with EcoRV. Δ, deleted; F, floxed.

(designated here FADD^{E-KO} for simplicity) were born at the expected Mendelian ratio and were macroscopically indistinguishable from their control littermates until postnatal day 3 (P3). At this stage, these mice started to show signs of skin disease, which progressed rapidly to severe skin lesions characterized by thickened and hard skin showing extensive scaling (Figure S2). All animals with homozygous deletion of FADD in the epidermis died by P8.

To characterize the skin lesions, we performed immunohistological analysis of skin sections from FADD^{E-KO} mice at different time points after birth. Analysis of skin from newborn mice on P0 did not reveal differences between FADD^{E-KO} mice and littermate controls (Figure 2). Immunostaining with antibodies recognizing keratins 14 (K14) and 10 (K10), expressed in basal and suprabasal keratinocytes, respectively, and loricrin, a marker of terminal keratinocyte differentiation, showed normal

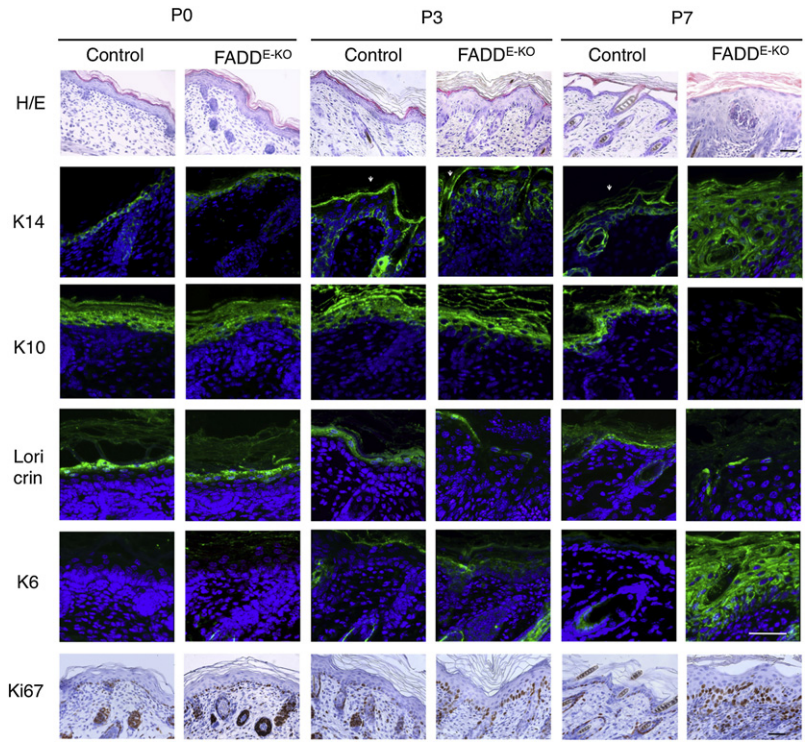

Figure 2. Skin Histology in FADD^{E-KO} Mice

Skin sections from FADD^{E-KO} and littermate control mice at the indicated age were stained with hematoxylin & eosin (H&E) or immunostained for the indicated epidermal differentiation or proliferation markers. Immunofluorescence is shown in green, with nuclei stained with DAPI in blue. Immunohistochemical Ki67 staining shown in dark brown, with nuclei counterstained with hematoxylin. Arrows indicate nonspecific staining of the stratum corneum. Scale bars represent 50 μm. Mice analyzed for each genotype: P0, n = 3; P3, n = 5; P7, n = 10.

epidermal differentiation in FADD^{E-KO} mice. Moreover, keratin 6 (K6) expression was restricted to hair follicles. Histological analysis of skin samples from 3-day-old (P3) mice revealed focal epidermal thickening often associated with the presence of eosinophilic keratinocytes in the upper layers of the epidermis in the skin of FADD^{E-KO} animals (Figure 2). On immunofluorescence analysis, these patches of hyperplastic epidermis in FADD^{E-KO} mice showed increased expression of K14 in suprabasal keratinocytes. Therefore, at P3 the skin of FADD^{E-KO} mice showed patchy areas with epidermal hyperplasia and disturbed keratinocyte differentiation, but also large areas of normal appearing skin without any signs of lesion development. At P7, the skin of FADD^{E-KO} animals displayed marked epidermal hyperplasia, loss of the granular layer, hyperkeratosis, and the presence of epidermal pustules containing inflammatory cells, associated with increased cellularity of the dermis. FADD^{E-KO} mice showed strong expression of K14 and K6 throughout all epidermal layers and nearly complete loss of K10 and loricrin expression at this stage. The epidermal hyperplasia observed in FADD^{E-KO} mice suggested that FADD might be important for the regulation of keratinocyte proliferation. Immunostaining for Ki67 revealed normal keratinocyte proliferation at P0 in FADD^{E-KO} mice compared to littermate controls (Figure 2). Consistent with the presence of focal skin areas showing epidermal hyperplasia, immunostaining for Ki67 revealed increased keratinocyte proliferation in hyperplastic skin patches of FADD^{E-KO} mice at P3. At P7, the entire skin of FADD^{E-KO} mice showed strongly increased Ki67 staining in epidermal keratinocytes not only in the basal but also in suprabasal layers. Epidermis specific deletion of the FADD^{FL} allele caused an identical skin phenotype as with deletion of the FADD-IRES-GFP^{FL} allele (Figure S2), demonstrating that the lesions were caused by the loss of a function performed by full-length FADD in the epidermis and were not related to the expression of the truncated DED domain of FADD.

Inflammation in the Skin of FADD^{E-KO} Mice

The hyperplastic epidermal phenotype seen in the skin of FADD^{E-KO} mice after P3 was associated with increased numbers of inflammatory cells in the dermis but also in epidermal pustules, indicating that lesion development could be driven by an inflammatory reaction. Immunostaining of skin sections with specific antibodies recognizing macrophages (F4/80), granulocytes (Gr-1) and T cells (CD3) did not reveal increased accumulation of immune cells in the skin of FADD^{E-KO} mice at P0. However, at P3 and P7 increased numbers of macrophages, granulocytes, and T cells infiltrated the dermis of FADD^{E-KO} mice (Figure 3A). Taken together, these results showed that epidermal keratinocyte-specific ablation of FADD did not impair epidermal formation, proliferation, and differentiation during development, but triggered the pathogenesis of inflammatory skin lesions starting in the first 2–3 days after birth and culminating in a severe inflammatory hyperplastic epidermal phenotype that resulted in the death of the animals by P8.

To elucidate the potential mechanisms by which FADD deficiency in keratinocytes triggered the development of inflammatory skin lesions, we analyzed cytokine expression in the epidermis of FADD^{E-KO} and littermate control animals. qRT-PCR analysis of cytokine expression did not reveal differences between FADD-deficient and wild-type epidermis at P0 or in primary keratinocytes (Figure 3B). However, similar analysis performed in epidermal mRNA from 3-day-old mice revealed increased expression of the proinflammatory cytokines IL-1β, IL-6 and also of the anti-inflammatory cytokine IL-10 in the epidermis of FADD^{E-KO} mice compared to littermate controls. Moreover, although we did not detect upregulation of TNF mRNA in FADD-deficient epidermis at P3, increased TNF protein amounts were observed by immunostaining in the dermis of FADD^{E-KO} mice at P3 and P7, with a pattern corresponding to infiltrating immune cells (Figure 3A). Collectively, these results showed that FADD deficiency induced increased expression of potentially pathogenic proinflammatory mediators in the epidermis of mice at P3, but not in newborn mice or in cultured primary keratinocytes. Therefore, increased expression of inflammatory mediators in the epidermis of FADD^{E-KO} mice does

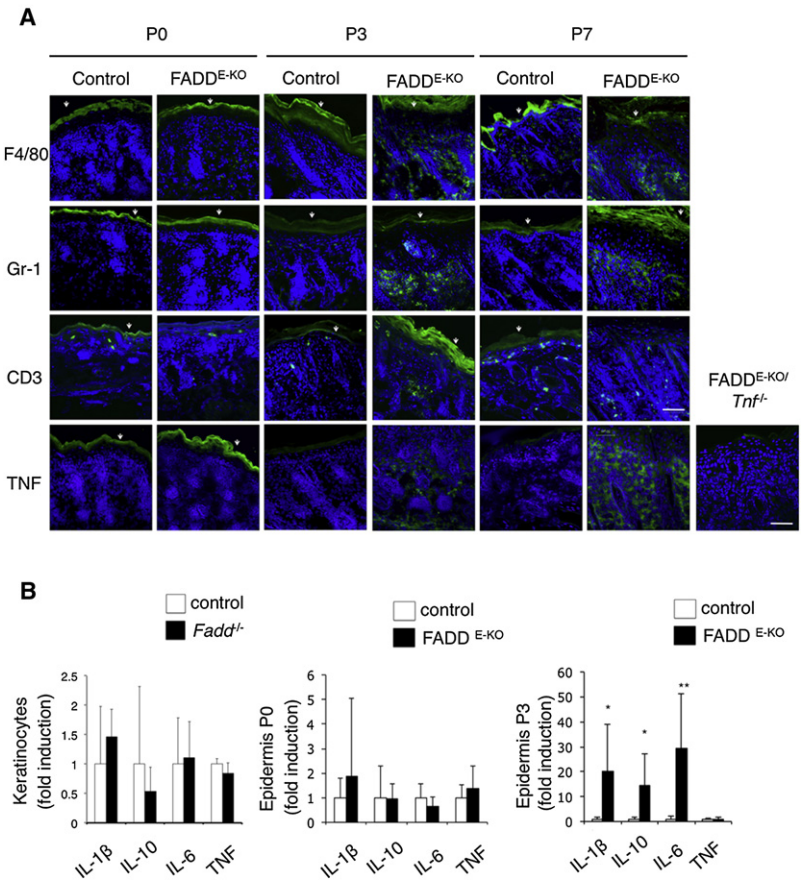

Figure 3. Skin Inflammation in FADD$^{E\text{-}KO}$ Mice

(A) Skin sections from FADD$^{E\text{-}KO}$ and littermate control mice were immunostained (green) with antibodies recognizing macrophages (F4/80), granulocytes (Gr-1), T cells (CD3), or TNF. Nuclei were stained with DAPI (blue). Arrows indicate nonspecific staining of the stratum corneum. Scale bars represent 50 μm. Mice analyzed for each genotype: P0, n = 3; P3, n = 5; P7, n = 10.

(B) qRT-PCR analysis of mRNA expression of the indicated cytokines in the epidermis of mice at P0 and P3 or in primary keratinocytes (P0 and keratinocytes: n = 3 for each genotype, P3: control n = 6, FADD$^{E\text{-}KO}$ n = 8). One representative of two independent experiments is shown. Graphs show mean values ± SD.

not seem to be a primary effect of FADD-deficiency but rather a consequence of additional changes occurring in the epidermis of these mice in the first few days after birth.

Role of Death Receptors in the Development of Skin Lesions in FADD$^{E\text{-}KO}$ Mice

The best-characterized function of FADD is to act as an adaptor recruiting caspase-8 to the DISC downstream of death receptor signaling triggering caspase-8 activation and the induction of apoptosis. We therefore reasoned that FADD deficiency might affect epidermal homeostasis by disturbing death receptor signaling in epidermal keratinocytes. To assess whether FADD triggers skin inflammation by de-regulating Fas signaling, we generated mice lacking both FADD and Fas in epidermal keratinocytes by crossing FADD$^{E\text{-}KO}$ mice with mice carrying loxP-flanked Fas alleles (*Fas*^{FL}) (Hao et al., 2004). FADD$^{E\text{-}KO}$Fas$^{E\text{-}KO}$ mice developed inflammatory skin lesions with identical kinetics and severity compared to FADD$^{E\text{-}KO}$ mice (data not shown), demonstrating that Fas expression in keratinocytes is not required for the pathogenesis of skin lesions triggered by epidermis-specific FADD deficiency.

To assess the potential role of TNF-mediated TNFR1 signaling in the pathogenesis of skin lesions, we crossed FADD$^{E\text{-}KO}$ mice with TNF- (Pasparakis et al., 1996) and TNFR1- (Pfeffer et al., 1993) deficient mice. FADD$^{E\text{-}KO}$ mice lacking TNF showed only minor macroscopic signs of skin inflammation with small patches of scaly skin visible at P7, in contrast to the severely inflamed skin of FADD$^{E\text{-}KO}$ mice at this stage (data not shown). However, FADD$^{E\text{-}KO}$*Tnf*$^{-/-}$ mice developed inflammatory skin lesions during the second and third week of life and needed to be sacrificed by the age of 5 weeks. Histological analysis showed that already at P7 the skin of FADD$^{E\text{-}KO}$*Tnf*$^{-/-}$ mice showed patches of affected skin with signs of mild epidermal hyperplasia, upregulation of K14 expression in suprabasal layers, and expression of K6 in interfollicular epidermis, as well as increased recruitment of myeloid cells in the dermis (Figure 4). TNFR1 deficiency (*Tnfrsf1a*$^{-/-}$) had a stronger effect than TNF deficiency in delaying the development of skin lesions in FADD$^{E\text{-}KO}$ mice. Double FADD$^{E\text{-}KO}$*Tnfrsf1a*$^{-/-}$ mice did not show macroscopic signs of skin disease at P7 (Figure S3) and through the age of 3 weeks; however, after this stage the mice developed inflammatory skin lesions that progressed to severe skin inflammation requiring sacrifice of the animals by the age of 10 weeks. Histological analysis revealed that double FADD$^{E\text{-}KO}$*Tnfrsf1a*$^{-/-}$ mice already at P7 showed the presence of focal areas showing mild signs of skin inflammation, indicated by the upregulation of K14 and K6 and the increased recruitment of macrophages and granulocytes (Figure 4). These lesions progressed to severe inflammatory hyperplastic skin disease in 8-week-old FADD$^{E\text{-}KO}$*Tnfrsf1a*$^{-/-}$ mice characterized by epidermal thickening and increased infiltration of immune cells similar to the skin lesions observed in FADD$^{E\text{-}KO}$ mice at 7–8 days after birth (data not shown). The fact that TNFR1 deficiency had a stronger effect in preventing skin inflammation in FADD$^{E\text{-}KO}$ mice during the first weeks of life compared to TNF deficiency suggests that lymphotoxin-α is also capable of inducing pathogenic TNFR1 signaling in this model. Collectively, these results revealed that TNFR1 signaling plays an important role for the development of skin lesions in FADD$^{E\text{-}KO}$ mice, but other, TNFR1-independent mechanisms also contribute to lesion pathogenesis.

Role of MyD88 Signaling in Skin Lesion Development in FADD$^{E\text{-}KO}$ Mice

To address the potential role of Toll-like receptor (TLR) signaling in the development of inflammatory skin lesions, we crossed FADD$^{E\text{-}KO}$ mice with mice lacking MyD88 (Adachi et al., 1998),

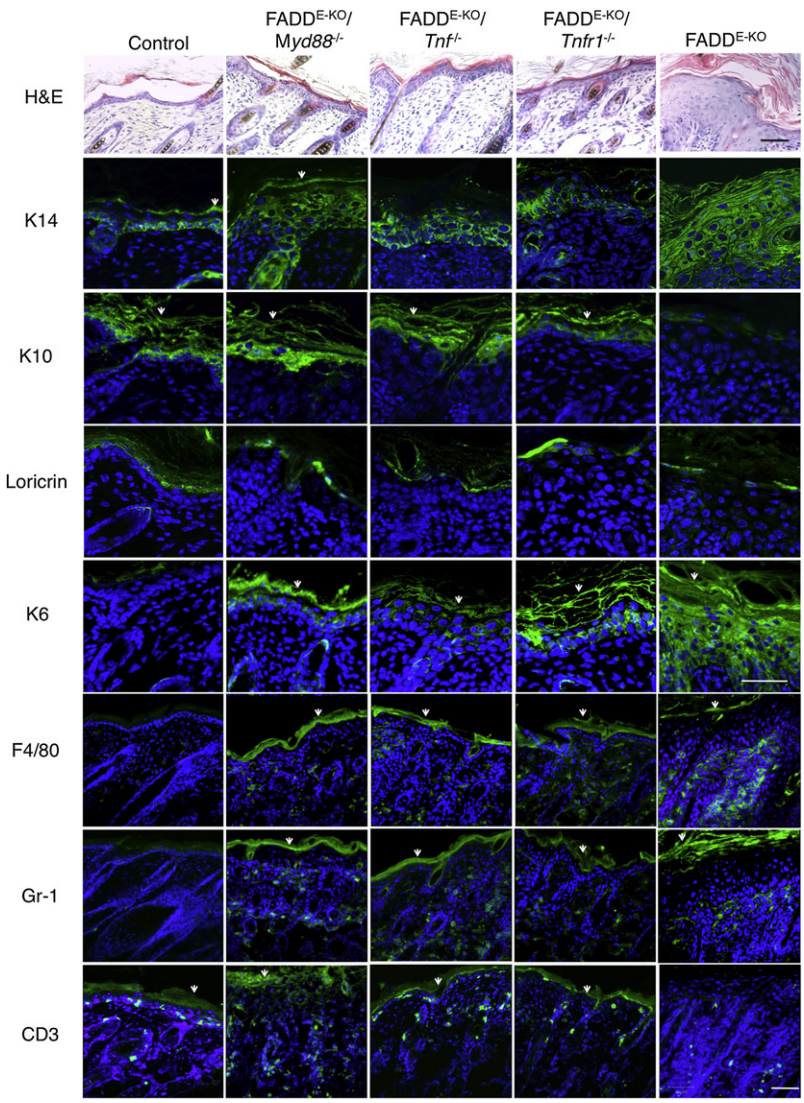

Figure 4. Role of MyD88 and TNF-TNFRI Signaling in Skin Lesion Development in FADD^{E-KO} Mice

Histological and immunofluorescence analysis of skin sections from FADD^{E-KO}*Myd88*$^{-/-}$ mice, FADD^{E-KO}*Tnf*$^{-/-}$ mice, and FADD^{E-KO}/*Tnfrsf1a*$^{-/-}$ mice at P7. Arrows indicate nonspecific staining of the stratum corneum. Scale bars represent 50 μm. Mice analyzed for each genotype: control, n = 10; FADD^{EKO}/*Myd88*$^{-/-}$, n = 4; FADD^{EKO}/*Tnf*$^{-/-}$, n = 6; FADD^{EKO}/*Tnfrsf1a*$^{-/-}$, n = 4; FADD^{EKO}, n = 10.

the main adaptor molecule mediating signaling downstream of most TLRs and of IL-1R (Akira and Takeda, 2004). Double FADD^{E-KO}*Myd88*$^{-/-}$ mice showed a slight delay in the development of skin lesions and looked generally healthy and active at P7 compared to FADD^{E-KO} animals (Figure S3); however, shortly after this stage they developed severe skin lesions and needed to be sacrificed during the third week of life. Histological analysis revealed that already at P7 the skin of FADD^{E-KO}*Myd88*$^{-/-}$ mice showed clear signs of inflammatory lesion development (Figure 4). Given that IL-1R1-deficiency did not have any effect in the development of skin inflammation in FADD^{E-KO} mice (Figure S4), these results suggest that TLR signaling contributes to the pathogenesis of severe inflammation in this model, but in its absence other mechanisms are sufficient for promoting the inflammatory response leading to severe lesion development during the first three weeks of life. Crossing to RAG1-deficient animals did not affect the development of inflammatory skin lesions in FADD^{E-KO} mice (Figure S4), showing that inflammation is driven by an innate immune response that does not require T- or B-lymphocytes.

Caspase-Independent Death of FADD-Deficient Keratinocytes in FADD^{E-KO} Mice

Histological analysis of H/E stained skin sections taken from FADD^{E-KO} mice revealed the presence of small numbers of dying keratinocytes in the epidermis at P1, which increased considerably by P3. These keratinocytes were irregularly shaped and showed an eosinophilic cytoplasm together with hyperchromatic, condensed, and partly fragmented nuclei (Figures 5A–5F). At P3, widened intercellular spaces in the basal compartment of the epidermis were also evident (Figure 5F). Although the epidermis of FADD^{E-KO} mice contained single cells staining positive with antibodies against active caspase-3, a large fraction of the dying keratinocytes did not contain activated caspase-3, indicating that these cells underwent caspase-independent death (Figures 5G–5I). Electron microscopic analysis of skin samples from mice at days 1 and 3 revealed the presence of cells with morphological characteristics of necrosis in FADD^{E-KO} animals, as indicated by swollen, irregular cellular shape, translucent cytoplasm and lack of recognizable cell organelles (Figures 5J–5O). Damaged swollen mitochondria with loss of cristae could also be detected in necrotic keratinocytes. This phenotype affected mostly the suprabasal and basal layers. By contrast, the granular layer appeared unaffected, containing numerous typical keratohyalin granula in flattened keratinocytes with regular cytoplasmic density. Therefore, keratinocytes in the epidermis of FADD^{E-KO} mice undergo caspase-independent necrotic cell death already before signs of inflammation become detectable.

Death receptors such as TNFR1 have been shown to induce caspase-independent necrotic death in certain cell types (Holler et al., 2000; Lüschen et al., 2000; Vercammen et al., 1998). This necrotic death was recently shown to depend on the kinases RIP1 and RIP3 and was termed programmed necrosis or necroptosis (Cho et al., 2009; He et al., 2009; Vandenabeele et al., 2010; Zhang et al., 2009). Pan-caspase inhibitors that efficiently block apoptosis have been shown to sensitize specific cell types to necrotic death (Cho et al., 2009; Vercammen et al., 1998; Zhang et al., 2009), suggesting that apoptosis antagonizes necroptosis. Furthermore, in vitro studies showed that FADD-deficient T cells undergo programmed necrosis in

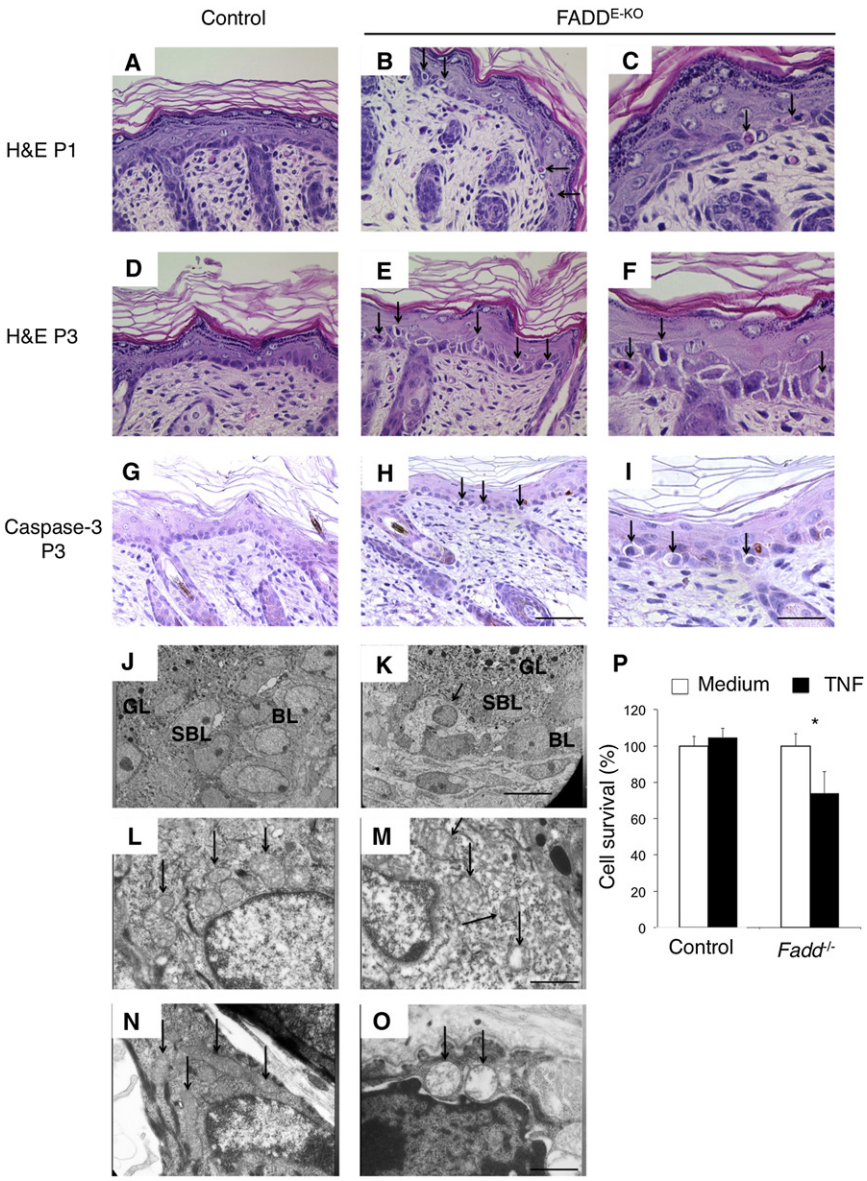

Figure 5. Necrotic Death of Keratinocytes in the Epidermis of FADD^{E-KO} Mice

(A–F) Histological analysis revealed the presence of dying keratinocytes identified from their irregular shape and eosinophilic cytoplasm together with hyperchromatic, condensed, and partly fragmented nuclei in the epidermis of FADD^{E-KO} mice at P1 (three mice analyzed for each genotype) and P3 (n = 8 for each genotype).

(G–I) Skin sections were immunostained for active caspase-3 (dark brown) and counterstained with H&E. Many of the dying keratinocytes in the epidermis of FADD^{E-KO} mice did not stain for active caspase-3 (indicated by arrows), indicating that they undergo caspase-independent death (eight mice analyzed for each genotype).

(J–O) Electron microscopy revealed the presence of necrotic cells in the epidermis of FADD^{E-KO} animals, identified by their swollen, irregular cellular shape, translucent cytoplasm, and lack of recognizable cell organelles (arrow in K). Damaged mitochondria were found in dying keratinocytes in FADD^{E-KO} epidermis at days 1 (L–M) and 3 (N–O) compared to normal mitochondria in control epidermis (arrows). BL, basal layer; SBL, suprabasal layer; GL, granular layer. Mice analyzed for each genotype: P1, n = 3; P3, n = 5.

(P) Primary keratinocytes from control and FADD^{E-KO} mice were treated with 200 ng/ml muTNF. Viability was assessed 24 hr later with Wst1. Graphs show the mean ± SD; *p < 0,05. One representative out of five independent experiments shown. Scale bars represent 50 μm in (A), (B), (D), (E), (G), and (H), 25 μm in (C), (F), and (I), 3.5 μm in (J) and (K), and 0.44 μm in (L)–(O).

response to stimulation by TNF or activation of the T cell receptor (Holler et al., 2000; Osborn et al., 2010). To investigate whether FADD-deficient keratinocytes are also sensitive to TNF-induced killing, we measured TNF-induced death in primary keratinocytes from FADD^{E-KO} and control mice. Indeed, FADD-deficient primary keratinocytes showed increased sensitivity to TNF-induced death compared to control cells (Figure 5P). We therefore hypothesized that FADD deficiency might sensitize epidermal keratinocytes to death by programmed necrosis, which could be a critical early event for the pathogenesis of inflammatory skin lesions in FADD^{E-KO} mice.

Programmed Necrosis of FADD-Deficient Keratinocytes Triggers Skin Lesion Development in FADD^{E-KO} Mice

The deubiquitinating enzyme CYLD was identified in a genome-wide siRNA screen as an important mediator of TNFR1-induced necrosis (Hitomi et al., 2008). Based on in vitro studies, CYLD was proposed to promote necroptosis by removing ubiquitin chains from RIP1 and in this way facilitating the formation of the RIP1 and RIP3 containing "necrosome" complex (Hitomi et al., 2008; Vandenabeele et al., 2010). To assess whether CYLD-dependent necrosis of FADD-deficient keratinocytes was implicated in the pathogenesis of skin lesions, we crossed FADD^{E-KO} mice with mice carrying a conditional *Cyld* allele (*Cyld*$^{\Delta 932FL}$), which upon Cre recombination produces a truncated CYLD protein lacking the last 20 amino acids that are essential for its ubiquitin chain hydrolase activity (Kovalenko et al., 2003). Mice lacking FADD and expressing the catalytically inactive CYLDΔ932 in the epidermis (FADD^{E-KO}CYLD$^{\Delta 932E}$) were smaller than their wild-type control littermates at P7 but did not display macroscopically visible skin lesions at this stage (Figure S5). However, histological analysis revealed mild epidermal hyperplasia accompanied by increased expression of K14 and K6 and increased infiltration of immune cells in the skin of FADD^{E-KO}CYLD$^{\Delta 932E}$ mice at P7 (Figures 6A and 6B). At ~3 weeks of age, FADD^{E-KO}/CYLDΔ932^{E} mice started to show skin lesions that progressed to severe skin inflammation. Notably, these lesions did not develop in control CYLD$^{\Delta 932E}$ mice, showing that they are caused by incomplete rescue of

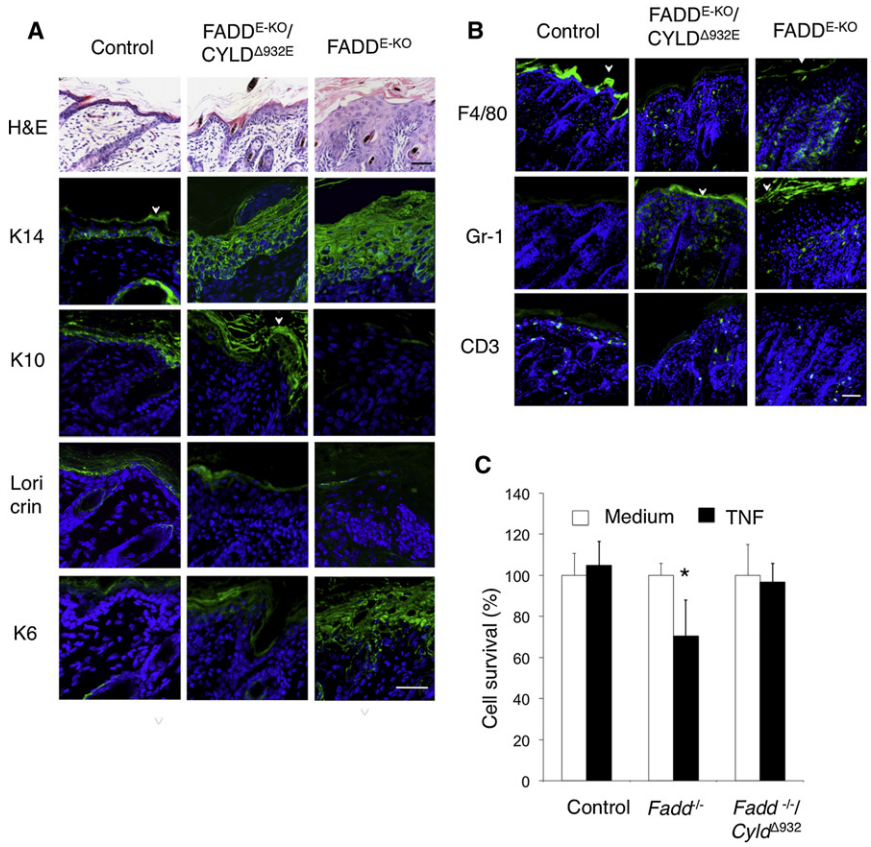

Figure 6. Role of CYLD in the Development of Skin Lesions in FADD^{E-KO} Mice

(A) Histological and immunofluorescence (green) analysis of skin sections from control, FADD^{E-KO} and FADD^{E-KO}CYLD$^{\Delta 932E}$ mice at P7. Nuclei were visualized with DAPI (blue). Arrows indicate nonspecific staining of the stratum corneum. Scale bars represent 50 μm. Mice analyzed for each genotype: Control, n = 10; FADD^{EKO}, n = 10; FADD^{EKO}/CYLD$^{\Delta 932E}$, n = 3.

(B) Skin sections from control, FADD^{E-KO}, and FADD^{E-KO}CYLD$^{\Delta 932E}$ mice at P7 were immunostained (green) with antibodies recognizing macrophages (F4/80), granulocytes (Gr-1), and T cells (CD3). Nuclei were stained with DAPI (blue). Arrows indicate nonspecific staining of the stratum corneum. Scale bars represent 50 μm. Mice analyzed for each genotype: Control, n = 10; FADD^{EKO}, n = 10; FADD^{EKO}/CYLD$^{\Delta 932E}$, n = 3.

(C) Primary keratinocytes from control, FADD^{E-KO}, and FADD^{E-KO}CYLD$^{\Delta 932E}$ mice were treated with 200 ng/ml muTNF. Viability was assessed 24 hr later with Wst1. Graphs show the mean ± SD; *$p < 0.05$. One representative out of three independent experiments shown.

the FADD^{E-KO} phenotype and not a primary effect of CYLD inhibition (Figure S5). Therefore, inhibition of CYLD catalytic activity specifically in epidermal keratinocytes could delay the development of inflammatory skin lesions in FADD^{E-KO} mice, suggesting that CYLD-dependent keratinocyte necroptosis might contribute to the pathogenesis of skin inflammation in these animals. Indeed, primary keratinocytes from FADD^{E-KO} CYLD$^{\Delta 932E}$ mice were resistant to TNF-induced death (Figure 6C), further supporting that CYLD contributes to skin inflammation in the FADD^{E-KO} mice by promoting the death of FADD-deficient keratinocytes.

The kinase RIP3 has been identified as an essential and specific mediator of programmed necrosis (Cho et al., 2009; He et al., 2009; Upton et al., 2010; Vandenabeele et al., 2010; Zhang et al., 2009). Therefore, to unambiguously assess whether programmed necrosis of FADD-deficient keratinocytes triggers skin inflammation in FADD^{E-KO} mice, we crossed them to mice lacking RIP3 (Newton et al., 2004). Strikingly, FADD^{E-KO}*Ripk3*$^{-/-}$ mice appeared totally indistinguishable from their control littermates at P7 but also throughout adulthood (Figure S6), and did not show any signs of skin lesion development at least up to the age of 40 weeks. Histological analysis revealed that FADD^{E-KO}*Ripk3*$^{-/-}$ mice have a normal skin, displaying normal epidermal thickness and proliferation and the expected pattern of keratinocyte differentiation marker expression, with K14 expressed in basal cells, K10 expressed in suprabasal cells, and loricrin expressed in the terminally differentiated upper granular layer, whereas K6 expression was confined to hair follicle keratinocytes (Figure 7A). In addition, RIP3 deficiency prevented infiltration of immune cells and upregulation of cytokine expression in the skin of FADD^{E-KO} *Ripk3*$^{-/-}$ mice (Figures 7A and 7B). Therefore, RIP3 deficiency fully prevented the development of inflammatory skin lesions in FADD^{E-KO} mice, suggesting that RIP3-mediated necrosis of FADD-deficient keratinocytes is critical for the development of skin inflammation in this model. Indeed, immunostaining for activated caspase-3 and EM analysis showed that the epidermis of 3-day-old FADD^{E-KO}*Ripk3*$^{-/-}$ mice did not contain the necrotic keratinocytes detected in FADD^{E-KO} mice at this stage (Figures 7C and 7D), whereas keratinocytes lacking both FADD and RIP3 were protected from TNF-induced death in vitro (Figure 7E). Therefore, RIP3 deficiency fully prevented the development of inflammatory skin lesions in FADD^{E-KO} mice, demonstrating that RIP3-mediated necrosis of FADD-deficient keratinocytes is an essential early event triggering the development of inflammatory skin lesions in FADD^{E-KO} mice. Necrotic keratinocytes could trigger skin lesion development by releasing intracellular components such as the nuclear protein HMGB1 that act as danger-associated molecular patterns (DAMPs) to stimulate innate immune receptors inducing inflammation (Zitvogel et al., 2010). We observed release of HMGB1 in the epidermis of FADD^{E-KO} but not FADD^{E-KO}*Ripk3*$^{-/-}$ mice (Figure S6), suggesting that RIP3-mediated necrosis of FADD-deficient keratinocytes could induce inflammation by triggering the release of DAMPs capable to activate immune responses.

DISCUSSION

Our study revealed that mice with epidermis-specific ablation of FADD showed spontaneous necrosis of keratinocytes in vivo

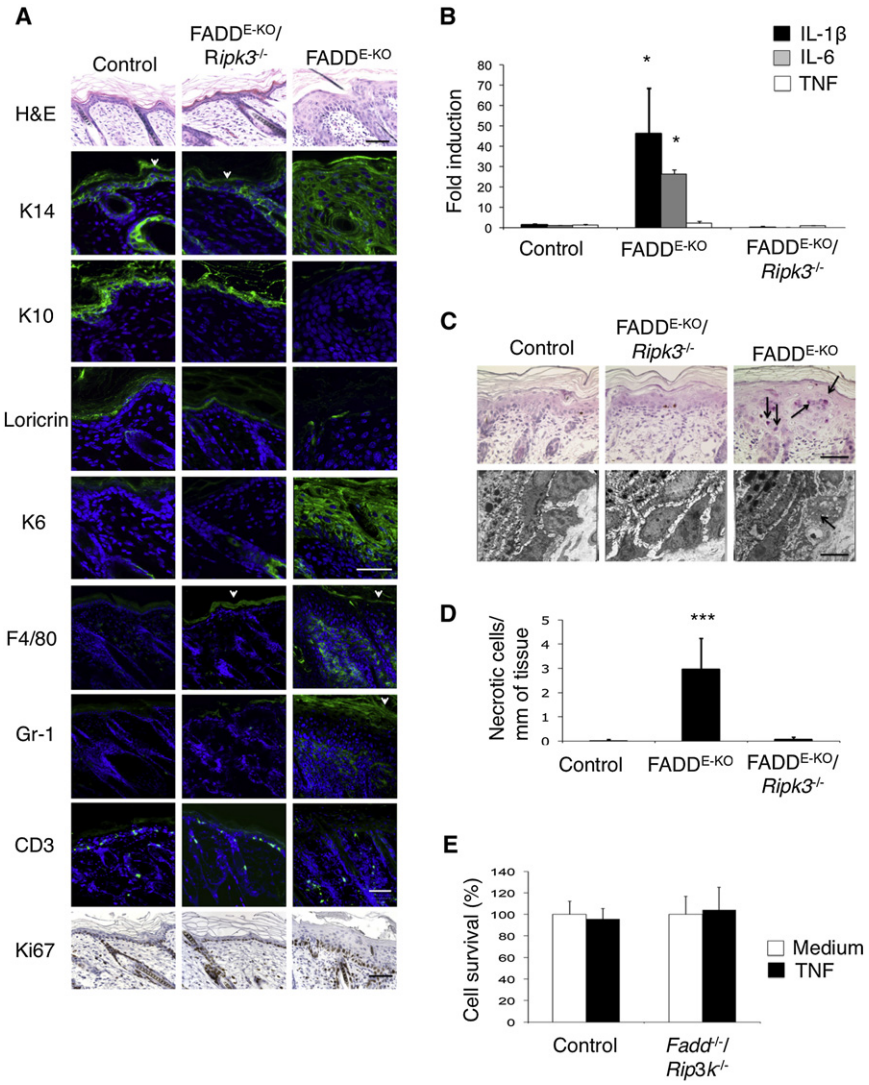

Figure 7. RIP3 Deficiency Prevents Skin Lesion Development in FADD$^{E\text{-}KO}$ Mice

(A) Histological and immunofluorescence analysis of skin sections from control, FADD$^{E\text{-}KO}$ and FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ mice at P7. Arrows indicate non-specific staining of the stratum corneum. Scale bars: 50 μm. The following mice were analyzed: control, n = 10; FADD^{EKO}, n = 10; FADD^{EKO}/*Ripk3*$^{-/-}$, n = 3.

(B) qPCR analysis of IL-1β, IL-6 and TNF mRNA expression in the epidermis of control (n = 5), FADD$^{E\text{-}KO}$ (n = 5) and FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ (n = 4) mice at P3. Graph shows the mean ± SD; *p < 0,05.

(C) Immunostaining for active caspase-3 (brown) and EM pictures of control, FADD$^{E\text{-}KO}$, and FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ mice at P3. Arrows show necrotic keratinocytes in FADD$^{E\text{-}KO}$ epidermis. The scale bar represents 50 μm (upper panels) and 2 μm (lower panels). Mice analyzed: control, n = 5; FADD^{EKO}, n = 5; FADD^{EKO}/*Ripk3*$^{-/-}$, n = 4.

(D) Necrotic cells identified as cells with eosinophilic cytoplasm and hyperchromatic, condensed, and partly fragmented nuclei that did not stain for active caspase 3 were quantified in the epidermis of control (n = 5), FADD$^{E\text{-}KO}$ (n = 5) and FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ (n = 4) mice at P3. Graph shows mean values ± SD; ***p < 0,005.

(E) Primary keratinocytes from control and FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ mice were treated with 200 ng/ml TNF. Viability was assessed 24 hr later with Wst1. Graphs show the mean ± SD; *p < 0.05. One representative out of four independent experiments shown.

and developed severe inflammatory skin lesions within the first few days after birth. RIP3 deficiency fully prevented keratinocyte necrosis and the development of inflammatory skin lesions, demonstrating that RIP3-dependent programmed necrosis of FADD-deficient keratinocytes is the essential initiating event triggering the pathogenesis of severe skin inflammation in FADD$^{E\text{-}KO}$ mice. Therefore, in contrast to its well-established role as a mediator of apoptosis, FADD performs an essential prosurvival function in keratinocytes that is crucial for the maintenance of skin immune homeostasis and the prevention of skin inflammation.

Death receptors are the best-characterized inducers of necroptosis (Vandenabeele et al., 2010). Consistent with the well-established role of TNF as a mediator of necroptosis, FADD$^{E\text{-}KO}$ mice lacking TNF or TNFR1 showed markedly reduced skin lesion formation during the first two weeks of life. However, FADD$^{E\text{-}KO}$*Tnf*$^{-/-}$ and FADD$^{E\text{-}KO}$*Tnfrsf1a*$^{-/-}$ mice developed severe skin lesions later in life, suggesting that although TNF is important during the early stages of the disease, ultimately other TNF-independent mechanisms induce keratinocyte necroptosis and skin inflammation in FADD$^{E\text{-}KO}$ mice. Fas and TRAIL-R have also been shown to mediate necroptosis and could be involved in inducing keratinocyte necrosis and skin inflammation in FADD$^{E\text{-}KO}$ mice. We found that epidermis-specific Fas deficiency did not affect lesion development in FADD$^{E\text{-}KO}$ mice, suggesting that Fas is not implicated in inducing necroptosis of FADD-deficient keratinocytes. However, we cannot exclude that Fas signaling might become important in the absence of TNF, providing an alternative mechanism for the induction of necroptosis in FADD-deficient keratinocytes. Further experiments will be required to address the role of TRAIL-R and the potential redundancy of death receptors in inducing necroptosis of FADD-deficient keratinocytes. Although death receptors are the best-studied inducers of necroptosis, other stimuli, including T cell receptor activation, viral infection, and also activation of pattern recognition receptors, have been implicated in inducing programmed necrosis in a variety of cell types (Vandenabeele et al., 2010). Therefore it is possible that death receptor-independent mechanisms also contribute to the induction of necroptosis in FADD-deficient keratinocytes in vivo.

Two recent studies reported that mice with epidermal keratinocyte restricted caspase-8 ablation developed a severe inflammatory skin phenotype similarly to our FADD$^{E\text{-}KO}$ mice described here (Kovalenko et al., 2009; Lee et al., 2009). Lee et al. (2009) proposed that caspase-8 deficiency triggered skin lesions by

inducing the p38-dependent upregulation of pro-caspase-1 and NALP3 expression in keratinocytes resulting in inflammasome-mediated release of IL-1α, which acted in both epithelial and dermal cells to induce skin inflammation and epidermal hyperplasia. We found that p38α as well as IL-1R1 deficiency did not affect skin lesion development in FADD^{E-KO} mice, suggesting that IL-1 is not important for skin lesion development in our model. In addition, Kovalenko et al. (2009) suggested that caspase-8 deficiency might trigger skin inflammation by inducing an enhanced response to endogenous activators of IRF3 in the epidermis. Although these studies did not address the potential role of necroptosis as a trigger of skin lesion development, considering that inhibition of either FADD or caspase-8 has been shown to sensitize different cell types to necroptosis, our results in FADD^{E-KO} mice strongly suggest that necroptosis of caspase-8-deficient keratinocytes could also contribute to the development of inflammatory skin lesions in mice with epidermis specific caspase-8 deficiency.

The mechanisms by which necroptotic death of a relatively small number of epidermal keratinocytes triggers the development of severe inflammatory skin lesions in FADD^{E-KO} mice remain unclear at present. Whereas apoptosis is considered immunologically silent, necrosis often triggers inflammation due to the release of intracellular components, described as DAMPs, capable for stimulating pattern recognition receptors inducing the production of proinflammatory cytokines by innate immune cells (Zitvogel et al., 2010). For example, HMGB1, HSP70, and nucleic acid fragments activate TLRs, RAGE, NOD-like receptors, and RIG-like helicases, while ATP and uric acid potently induce the inflammasome (Zitvogel et al., 2010). Our finding that MyD88 deficiency delayed but could not prevent skin lesion development in FADD^{E-KO} mice suggests that activation of TLR signaling by DAMPs released by necrotic keratinocytes could be implicated in inducing skin inflammation, but other DAMP sensors might be capable of eliciting a severe inflammatory response in the absence of MyD88-dependent TLR signaling. Indeed, recent studies showed that specific immune cell receptors of the C-lectin family recognize necrotic debris and elicit an inflammatory response (Sancho et al., 2009; Yamasaki et al., 2008). Moreover, RAGE, the main receptor for HMGB1, has been shown to regulate skin inflammation (Gebhardt et al., 2008). RAG1 deficiency did not affect skin lesion development in FADD^{E-KO} mice, showing that inflammation is induced by a B and T cell-independent innate immune response. DAMPs released by necrotic keratinocytes could activate the expression of proinflammatory mediators in resident myeloid cells of the skin but also in neighboring keratinocytes, as indicated by the increased expression of cytokines by FADD-deficient epidermis at P3. Systematic genetic analysis of the role of the different pattern recognition receptor families in the pathogenesis of inflammatory skin lesions in FADD^{E-KO} mice will be required in order to fully understand the mechanisms by which keratinocyte necroptosis induces skin inflammation.

An important question remaining to be answered is why do FADD-deficient keratinocytes undergo necroptosis under physiological in vivo conditions and in the absence of any experimental challenge. FADD deficiency does not seem to merely change the fate of cells destined to die by apoptosis because we did not find evidence that an equivalent number of epidermal keratinocytes undergoes apoptosis in wild-type epidermis, at least as detected by immunostaining for active caspase-3 and by TUNEL staining. Necroptosis has been shown to be important for host-defense against certain viruses (Challa and Chan, 2010). Necroptosis provides an alternative way to kill cells infected with poxviruses encoding potent inhibitors of apoptosis such as CrmA (Chan et al., 2003; Cho et al., 2009). Moreover, mouse cytomegalovirus (MCMV) encodes the M45 inhibitor that potently prevents RIP3-dependent necrosis (Upton et al., 2010). M45 mutant MCMV could establish productive infection in RIP3-deficient but not in wild-type mice, demonstrating that necroptosis of virus-infected cells is an essential mechanism for the control of MCMV infection (Upton et al., 2010). Therefore, sensitization to necroptosis seems to be a mechanism allowing the detection and elimination of virus-infected cells. The release of DAMPs from necroptotic virus-infected cells could also further enhance antiviral responses resulting in effective clearance of the virus. It is tempting to speculate that constant surveillance of epithelial tissues such as the skin for the presence of cells sensitive to necroptosis could be an evolutionarily conserved mechanism for the early detection of viral infection and the potent elicitation of inflammatory responses aiming to clear the infection. In this context, we envisage that FADD deficiency might be detected by this surveillance mechanism as a potential infection resulting in the elicitation of a strong inflammatory response culminating in severe lesion formation. It is noteworthy that similar conditions exist in human skin, the most striking example being rashes such as erythema multiforme, a severe adverse reaction induced by drugs and/or viral infections (Torres et al., 2009). In these patients, skin inflammation is associated with a variable degree of keratinocyte death in the epidermis. Although it is thought that these keratinocytes undergo apoptosis, a systematic analysis of cell death mechanisms in erythema multiforme has not been performed. Our studies suggest that the potential role of keratinocyte necroptosis in conditions such as erythema multiforme deserves further investigation.

In conclusion, our results identify sensitization of keratinocytes to RIP3-mediated necrosis as a potent mechanism triggering skin inflammation and suggest that genetic or exogenous factors sensitizing keratinocytes to programmed necrosis could be implicated in the pathogenesis of inflammatory skin diseases. In light of our findings and considering the efficacy of anti-TNF therapies for the treatment of psoriasis (Mössner et al., 2008), it is tempting to speculate that TNFR1-induced programmed necrosis of epithelial cells might be an important mechanism contributing to the pathogenesis of chronic inflammatory skin diseases. While it remains to be seen whether similar mechanisms function in other epithelial tissues, our findings provide an in vivo experimental paradigm that regulation of necroptosis is important for the maintenance of tissue immune homeostasis and the prevention of chronic inflammation.

EXPERIMENTAL PROCEDURES

Mice

The following mouse lines were used: *Fadd*^{FL} (Mc Guire et al., 2010), K14-Cre (Pasparakis et al., 2002), *Rag1*$^{-/-}$ (Mombaerts et al., 1992), *Fas*^{FL} (Hao et al., 2004), *Tnf*$^{-/-}$ (Pasparakis et al., 1996), *Tnfrsf1a*$^{-/-}$ (Pfeffer et al., 1993),

Myd88$^{-/-}$ (Adachi et al., 1998), *Il1r1*$^{-/-}$ (Glaccum et al., 1997), *Mapk14*^{Fl} (Heinrichsdorff et al., 2008) and *Ripk3*$^{-/-}$ (Newton et al., 2004). The generation of the CyldΔ932^{FL} mice will be described elsewhere. All animal procedures were conducted in accordance with European, national, and institutional guidelines and protocols and were approved by local government authorities.

Immunostainings

Cryo- or paraffin sections were stained according to standard procedures and images were taken with a Leica DM550B and a Zeiss LSM 510 Meta microscope. The following antibodies were used: K14, K10, loricrin, and K6 from Covance (Princeton, NJ); K14 from Neomarkers (Fremont, CA); Ki67 and CD3 from DAKO (Glostrup, Denmark); F4/80 (clone A3-1); Gr-1 (clone RB6-8c5); anti-TNF from Becton Dickinson; anti-active caspase-3 from R&D Systems; and anti-HMGB1 from Abcam. Secondary antibodies were coupled to Alexa 488 or 594 (Molecular Probes). Sections were counterstained with DAPI (Sigma) for nuclei visualization. Immunostainings for Ki67 and active caspase-3 were performed with the DakoCytomation System (Dako) and counterstained with hematoxylin or H&E.

Immunoblotting

Protein extracts were prepared as described previously (Pasparakis et al., 2002), subjected to SDS-PAGE and transferred to Immobilon P membranes (Millipore). Membranes were blocked with 5% milk and probed with antibodies against FADD (M-19, Santa Cruz, CA), GFP (Abcam) or tubulin (Sigma, Munich, Germany).

RNA Isolation

RNA was prepared with Trizol (Invitrogen, Karlsruhe, Germany) with additional DNase digest and purification on RNEasy columns (QIAGEN. Hilen, Germany).

Quantitative RT-PCR Analysis

RNA (1 μg) was reverse transcribed with SuperScript II (Invitrogen). The cDNA produced was diluted 1/10 and 2 μl were used for qRT-PCR using Taqman probes (Applied Biosystems). Normalization was done with GAPDH.

Preparation of Skin Samples for Electron Microscopy

Freshly prepared back skin from mice was fixed in 2% paraformaldehyde, 2% glutaraldehyde, and 0.1M cacodylate buffer at pH 7.35 and postfixed with ruthenium tetroxide.

Keratinocyte Culture and Cell Death Assay

Primary epidermal keratinocytes were isolated from the skin of mice between days 0 and 3 and cultured in minimal calcium medium (0.05 mM $CaCl_2$) supplemented as previously described (Nenci et al., 2006). For cell death assays, keratinocytes were seeded (10.000 cells/well) in collagen-coated 96-well plates (Biocoat, Becton Dickinson). Cells were then treated with 200 ng/ml TNF for 24 hr and cell viability was assessed with Wst1 (Cell Proliferation Reagent, Roche). Absorbance was measured at 450 nm with a 96-well plate reader (Paradigm Detection Platform, Beckman Coulter) after 1 hr of incubation with Wst1 at 34°C.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.immuni.2011.08.014.

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REFERENCES

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity *9*, 143–150.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. *4*, 499–511.

Ch'en, I.L., Tsau, J.S., Molkentin, J.D., Komatsu, M., and Hedrick, S.M. (2011). Mechanisms of necroptosis in T cells. J. Exp. Med. *208*, 633–641.

Challa, S., and Chan, F.K. (2010). Going up in flames: Necrotic cell injury and inflammatory diseases. Cell. Mol. Life Sci. *67*, 3241–3253.

Chan, F.K., Shisler, J., Bixby, J.G., Felices, M., Zheng, L., Appel, M., Orenstein, J., Moss, B., and Lenardo, M.J. (2003). A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J. Biol. Chem. *278*, 51613–51621.

Cho, Y.S., Challa, S., Moquin, D., Genga, R., Ray, T.D., Guildford, M., and Chan, F.K. (2009). Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell *137*, 1112–1123.

Elias, P.M. (2007). The skin barrier as an innate immune element. Semin. Immunopathol. *29*, 3–14.

Gebhardt, C., Riehl, A., Durchdewald, M., Németh, J., Fürstenberger, G., Müller-Decker, K., Enk, A., Arnold, B., Bierhaus, A., Nawroth, P.P., et al. (2008). RAGE signaling sustains inflammation and promotes tumor development. J. Exp. Med. *205*, 275–285.

Glaccum, M.B., Stocking, K.L., Charrier, K., Smith, J.L., Willis, C.R., Maliszewski, C., Livingston, D.J., Peschon, J.J., and Morrissey, P.J. (1997). Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J. Immunol. *159*, 3364–3371.

Hafner, M., Wenk, J., Nenci, A., Pasparakis, M., Scharffetter-Kochanek, K., Smyth, N., Peters, T., Kess, D., Holtkötter, O., Shephard, P., et al. (2004). Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. Genesis *38*, 176–181.

Hao, Z., Hampel, B., Yagita, H., and Rajewsky, K. (2004). T cell-specific ablation of Fas leads to Fas ligand-mediated lymphocyte depletion and inflammatory pulmonary fibrosis. J. Exp. Med. *199*, 1355–1365.

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009). Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell *137*, 1100–1111.

Heinrichsdorff, J., Luedde, T., Perdiguero, E., Nebreda, A.R., and Pasparakis, M. (2008). p38 alpha MAPK inhibits JNK activation and collaborates with IkappaB kinase 2 to prevent endotoxin-induced liver failure. EMBO Rep. *9*, 1048–1054.

Hitomi, J., Christofferson, D.E., Ng, A., Yao, J., Degterev, A., Xavier, R.J., and Yuan, J. (2008). Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell *135*, 1311–1323.

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.L., Schneider, P., Seed, B., and Tschopp, J. (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol. *1*, 489–495.

Kaiser, W.J., Upton, J.W., Long, A.B., Livingston-Rosanoff, D., Daley-Bauer, L.P., Hakem, R., Caspary, T., and Mocarski, E.S. (2011). RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature *471*, 368–372.

Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israël, A., Wallach, D., and Courtois, G. (2003). The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature *424*, 801–805.

Kovalenko, A., Kim, J.C., Kang, T.B., Rajput, A., Bogdanov, K., Dittrich-Breiholz, O., Kracht, M., Brenner, O., and Wallach, D. (2009). Caspase-8

deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J. Exp. Med. *206*, 2161–2177.

Lee, P., Lee, D.J., Chan, C., Chen, S.W., Ch'en, I., and Jamora, C. (2009). Dynamic expression of epidermal caspase 8 simulates a wound healing response. Nature *458*, 519–523.

Lüschen, S., Ussat, S., Scherer, G., Kabelitz, D., and Adam-Klages, S. (2000). Sensitization to death receptor cytotoxicity by inhibition of fas-associated death domain protein (FADD)/caspase signaling. Requirement of cell cycle progression. J. Biol. Chem. *275*, 24670–24678.

Mc Guire, C., Volckaert, T., Wolke, U., Sze, M., de Rycke, R., Waisman, A., Prinz, M., Beyaert, R., Pasparakis, M., and van Loo, G. (2010). Oligodendrocyte-specific FADD deletion protects mice from autoimmune-mediated demyelination. J. Immunol. *185*, 7646–7653.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Mössner, R., Schön, M.P., and Reich, K. (2008). Tumor necrosis factor antagonists in the therapy of psoriasis. Clin. Dermatol. *26*, 486–502.

Nenci, A., Huth, M., Funteh, A., Schmidt-Supprian, M., Bloch, W., Metzger, D., Chambon, P., Rajewsky, K., Krieg, T., Haase, I., and Pasparakis, M. (2006). Skin lesion development in a mouse model of incontinentia pigmenti is triggered by NEMO deficiency in epidermal keratinocytes and requires TNF signaling. Hum. Mol. Genet. *15*, 531–542.

Nestle, F.O., Di Meglio, P., Qin, J.Z., and Nickoloff, B.J. (2009). Skin immune sentinels in health and disease. Nat. Rev. Immunol. *9*, 679–691.

Newton, K., Sun, X., and Dixit, V.M. (2004). Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol. Cell. Biol. *24*, 1464–1469.

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363–367.

Osborn, S.L., Diehl, G., Han, S.J., Xue, L., Kurd, N., Hsieh, K., Cado, D., Robey, E.A., and Winoto, A. (2010). Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. Proc. Natl. Acad. Sci. USA *107*, 13034–13039.

Pasparakis, M., Alexopoulou, L., Episkopou, V., and Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: A critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med. *184*, 1397–1411.

Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., et al. (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. Nature *417*, 861–866.

Pfeffer, K., Matsuyama, T., Kündig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Krönke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Sancho, D., Joffre, O.P., Keller, A.M., Rogers, N.C., Martínez, D., Hernanz-Falcón, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature *458*, 899–903.

Torres, M.J., Mayorga, C., and Blanca, M. (2009). Nonimmediate allergic reactions induced by drugs: Pathogenesis and diagnostic tests. J. Investig. Allergol. Clin. Immunol. *19*, 80–90.

Tourneur, L., and Chiocchia, G. (2010). FADD: A regulator of life and death. Trends Immunol. *31*, 260–269.

Upton, J.W., Kaiser, W.J., and Mocarski, E.S. (2010). Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe *7*, 302–313.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: An ordered cellular explosion. Nat. Rev. Mol. Cell Biol. *11*, 700–714.

Varfolomeev, E.E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J.S., Mett, I.L., Rebrikov, D., Brodianski, V.M., Kemper, O.C., Kollet, O., et al. (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity *9*, 267–276.

Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., Grooten, J., Fiers, W., and Vandenabeele, P. (1998). Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J. Exp. Med. *187*, 1477–1485.

Wilson, N.S., Dixit, V., and Ashkenazi, A. (2009). Death receptor signal transducers: Nodes of coordination in immune signaling networks. Nat. Immunol. *10*, 348–355.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat. Immunol. *9*, 1179–1188.

Yeh, W.C., Pompa, J.L., McCurrach, M.E., Shu, H.B., Elia, A.J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., et al. (1998). FADD: Essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science *279*, 1954–1958.

Zhang, J., Cado, D., Chen, A., Kabra, N.H., and Winoto, A. (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature *392*, 296–300.

Zhang, D.W., Shao, J., Lin, J., Zhang, N., Lu, B.J., Lin, S.C., Dong, M.Q., and Han, J. (2009). RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science *325*, 332–336.

Zhang, H., Zhou, X., McQuade, T., Li, J., Chan, F.K., and Zhang, J. (2011). Functional complementation between FADD and RIP1 in embryos and lymphocytes. Nature *471*, 373–376.

Zitvogel, L., Kepp, O., and Kroemer, G. (2010). Decoding cell death signals in inflammation and immunity. Cell *140*, 798–804.

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Supplemental Information

The Adaptor Protein FADD Protects Epidermal Keratinocytes from Necroptosis In Vivo and Prevents Skin Inflammation

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Inventory of Supplemental Information:

Figure S1: Generation of FADD-IRES-GFP^{FL} mice (related to Figure 1). Targeting strategy used to generate the FADD-IRES-GFP^{FL} mice.

Figure S2: FADD$^{E\text{-}KO}$ and FADD-GFP$^{E\text{-}KO}$ mice develop severe inflammatory skin lesions with identical severity and disease course (related to Figure 2). Macroscopic pictures and histological analysis of FADD$^{E\text{-}KO}$ and FADD-GFP$^{E\text{-}KO}$ mice at P7.

Figure S3: Development of skin inflammation in FADD$^{E\text{-}KO}$ mice is delayed in the absence of TNFR1 or MyD88 (related to Figure 4). Macroscopic phenotype of FADD$^{E\text{-}KO}$/*Tnfrs1a* $^{-/-}$ and FADD$^{E\text{-}KO}$/*Myd88* $^{-/-}$ mice at P7.

Figure S4: Inflammatory skin lesions in FADD$^{E\text{-}KO}$ mice develop independently of IL-1 and p38α signalling and in the absence of B and T lymphocytes (related to Figure 4). This figure shows that the development of skin lesions in FADD$^{E\text{-}KO}$ mice is not inhibited by genetic ablation of the IL-1R and RAG1 and by epidermis specific ablation of p38α.

Figure S5: Impairment of CYLD catalytic activity in keratinocytes does not cause spontaneous skin abnormalities but partially protects FADD$^{E\text{-}KO}$ mice from the development of inflammatory skin lesions (related to Figure 6). This figure shows that FADD$^{E\text{-}KO}$/CYLD$^{\Delta 932E}$ mice at P7 do not show macroscopic signs of skin inflammation and that epidermis specific inhibition of CYLD in CYLD$^{\Delta 932E}$ does not trigger spontaneous skin lesions.

Figure S6: RIP3 deficiency prevents the development of inflammatory skin lesions in FADD$^{E\text{-}KO}$ mice (related to Figure 7). This figure shows that RIP3 deficiency prevents skin inflammation also in adult FADD$^{E\text{-}KO}$ mice and that HMGB1 release is detected in the epidermis of FADD$^{E\text{-}KO}$ but not control or FADD$^{E\text{-}KO}$*Ripk3*$^{-/-}$ mice.

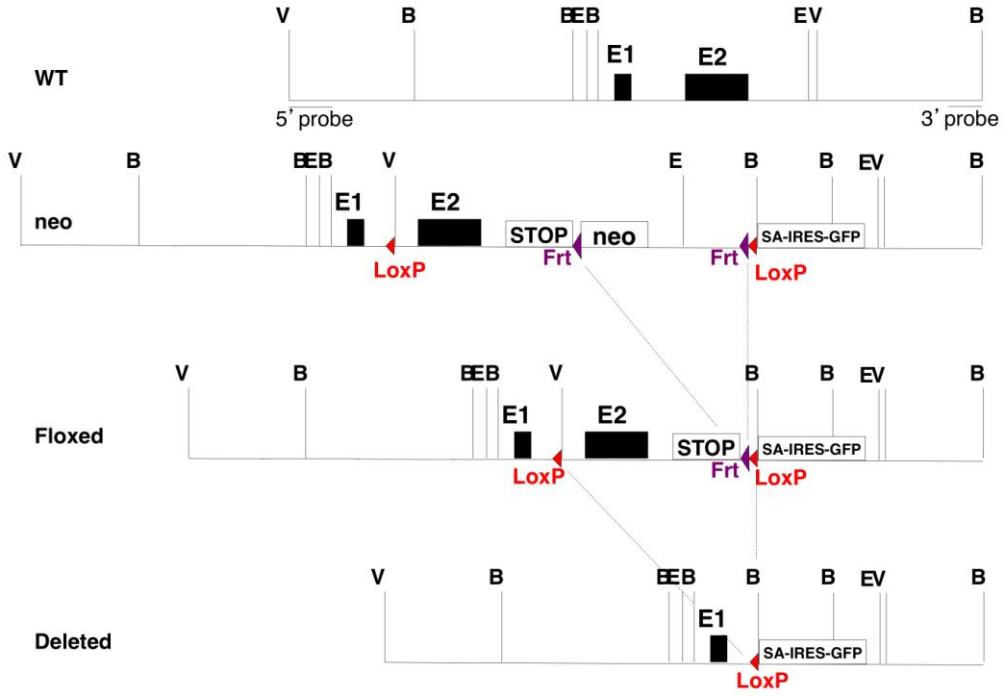

Figure S1: Generation of FADD-IRES-GFP^{FL} mice. Diagram showing the wild-type *Fadd* genomic locus (WT), the neomycin resistance-containing (neo), the loxP-flanked allele after Flpe-mediated neo excision (Floxed) and the Deleted allele after Cre-mediated deletion. Filled boxes indicate exons. A loxP site was inserted in the intron and a STOP cassette was introduced after the 3' end of the *Fadd* gene, followed by an FRT-site-flanked neomycin resistance cassette. A second loxP site was introduced after the FRT-flanked neo cassette, followed by a splice acceptor (SA)-IRES-GFP cassette. Restriction enzyme sites and the location of the probes used for Southern Blot analysis are depicted. B, *BamHI*, E, *EcoRI*, V, *EcoRV*.

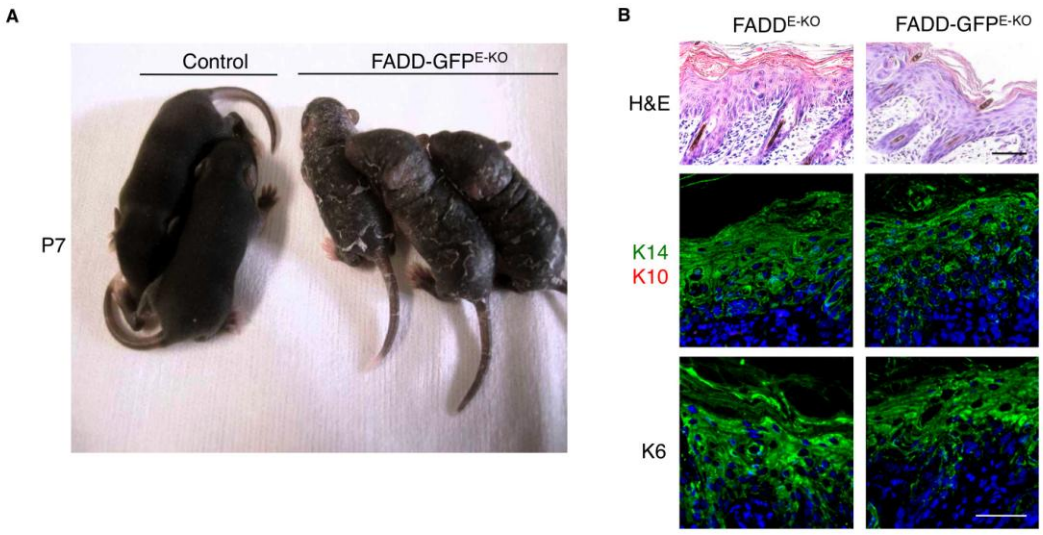

Figure S2: FADD^{E-KO} and FADD-GFP^{E-KO} mice develop severe inflammatory skin lesions with identical severity and disease course. (A) Representative pictures of FADD-GFP^{E-KO} mice at P7 (B) Histological and immunofluorescence analysis of skin sections from FADD^{E-KO} and FADD-GFP^{E-KO} mice at P7. Nuclei were stained with DAPI (blue). Scale bar: 50 μm.

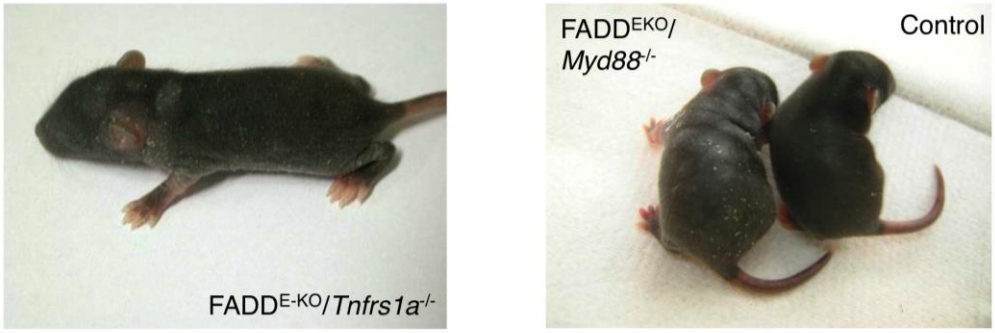

Figure S3: Development of skin inflammation in FADD^{E-KO} mice is delayed in the absence of TNFR1 or MyD88.

Representative pictures of FADD^{E-KO}*Tnfrs1a*$^{-/-}$ and FADD^{E-KO}*Myd88*$^{-/-}$ mice at P7, showing that the double deficient mice do not show macroscopic signs of severe skin inflammation at this stage.

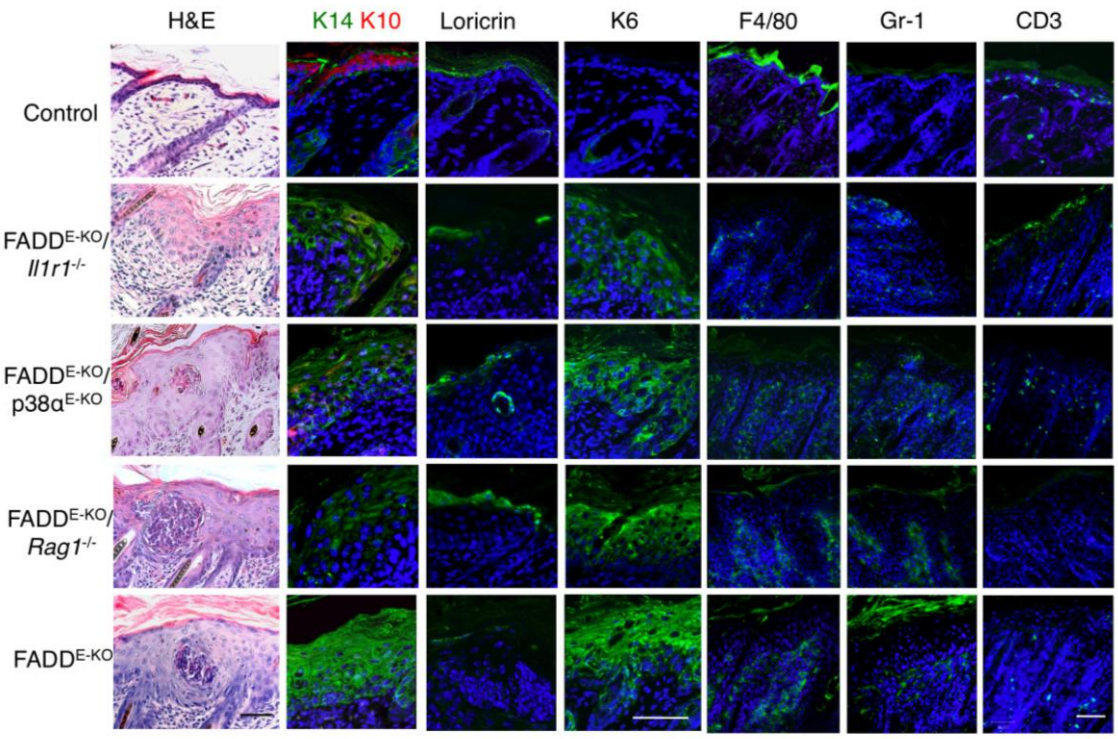

Figure S4: Inflammatory skin lesions in FADD^{E-KO} mice develop independently of IL-1 and p38α signalling and in the absence of B and T lymphocytes. Histological and immunofluorescence analysis of skin sections from control, FADD$^{E-KO,}$ FADD^{E-KO}/*Il1r*1$^{-/-}$, FADD^{E-KO}/p38α^{E-KO} and FADD^{E-KO}/*Rag1*$^{-/-}$ mice at P7. Scale bar: 50 μm.

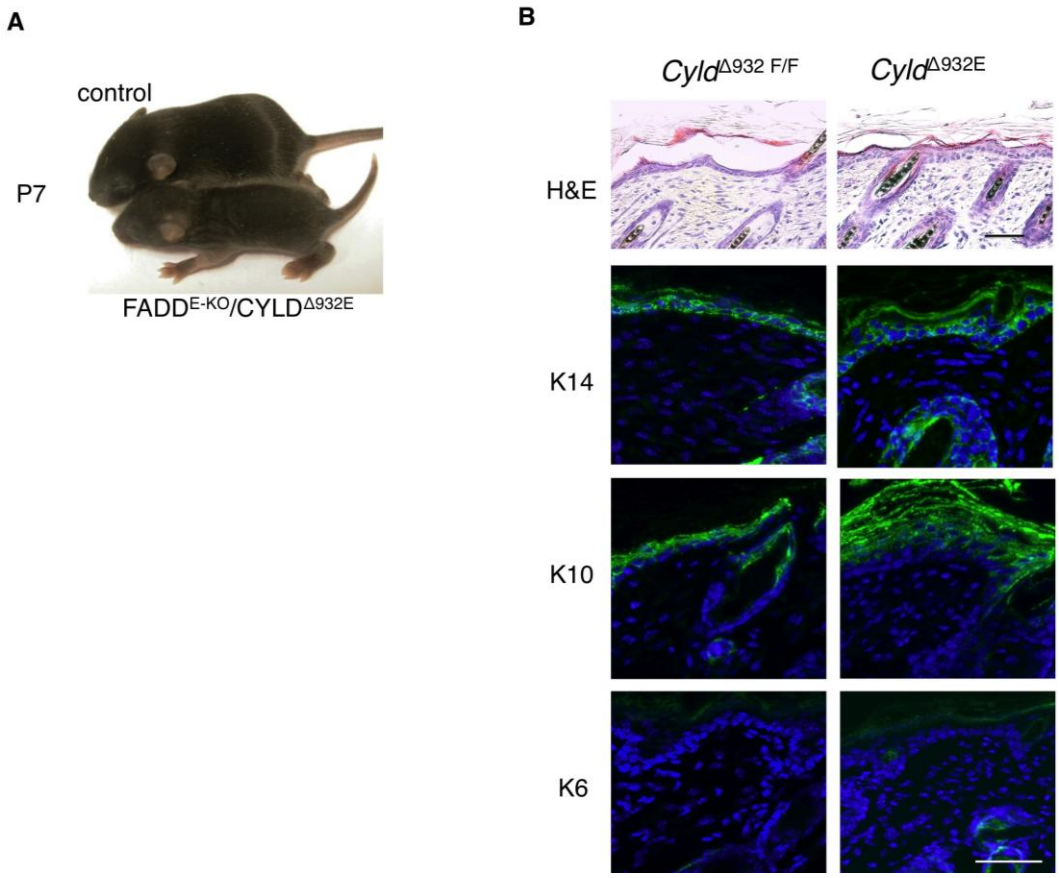

Figure S5: Impairment of CYLD catalytic activity in keratinocytes does not cause spontaneous skin abnormalities but partially protects FADD$^{E\text{-}KO}$ mice from the development of inflammatory skin lesions (a) Representative pictures of control and FADD$^{E\text{-}KO}$CYLD$^{\Delta 932E}$ mice at P7. (b) Histological and immunofluorescence analysis of skin sections from *Cyld*$^{\Delta 932FL/FL}$ and CYLD$^{\Delta 932E}$ mice at P7, demonstrating that epidermal keratinocyte specific inhibition of CYLD catalytic activity does not induce skin lesions. Scale bar: 50 μm.

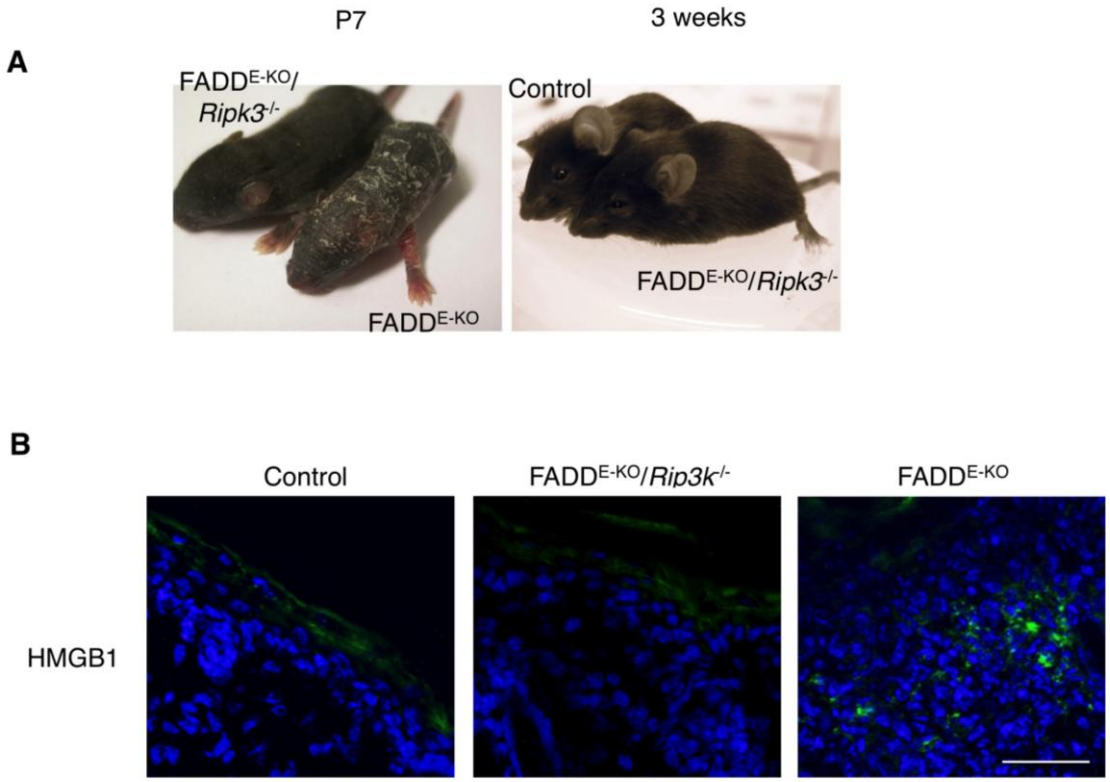

Figure S6: RIP3 deficiency prevents the development of inflammatory skin lesions in FADD$^{E\text{-}KO}$ mice. (A) Representative pictures of FADD$^{E\text{-}KO}$/*Ripk3*$^{-/-}$ mice. (B) Immunostaining of non-permeabilized skin cryosections with anti-HMGB1 antibody revealed areas showing the presence of HMGB1 in the extracellular space in the epidermis of FADD$^{E\text{-}KO}$ mice but not in control or FADD^{EKO}/*Ripk3*$^{-/-}$ mice. Scale bar: 50 μm.