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cFLIP Regulates Skin Homeostasis and Protects against TNF-Induced Keratinocyte Apoptosis

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SUMMARY

FADD, caspase-8, and cFLIP regulate the outcome of cell death signaling. Mice that constitutively lack these molecules die at an early embryonic age, whereas tissue-specific constitutive deletion of FADD or caspase-8 results in inflammatory skin disease caused by increased necroptosis. The function of cFLIP in the skin in vivo is unknown. In contrast to tissue-specific caspase-8 knockout, we show that mice constitutively lacking cFLIP in the epidermis die around embryonic days 10 and 11. When cFLIP expression was abrogated in adult skin of cFLIP$^{fl/fl}$-K14CreER^{tam} mice, severe inflammation of the skin with concomitant caspase activation and apoptotic, but not necroptotic, cell death developed. Apoptosis was dependent of autocrine tumor necrosis factor production triggered by loss of cFLIP. In addition, epidermal cFLIP protein was lost in patients with severe drug reactions associated with epidermal apoptosis. Our data demonstrate the importance of cFLIP for the integrity of the epidermis and for silencing of spontaneous skin inflammation.

INTRODUCTION

Death receptor (DR) activation initiates the formation of a death-inducing signaling complex (DISC) that contains the adaptor protein FADD, cellular FLICE-inhibitory protein (cFLIP), and the initiator cysteinyl-aspartate specific protease (caspase)-8, a close homolog of cFLIP with proteolytic activity (Dickens et al., 2012). Studies in mice deficient for caspase-8, cFLIP, or FADD demonstrated the essential role of these molecules in embryonic development (Varfolomeev et al., 1998; Yeh et al., 1998, 2000). Recent data suggest that within intracellular signaling platforms, beyond activation of apoptosis, the core components FADD and caspase-8, and the proteolytic activity of caspase-8, play an equally important role in inactivation of necroptosis, although the exact molecular mechanism remains to be determined. The impact of cFLIP for these divergent signals remains to be resolved in vivo.

In previous studies, loss of central elements of the apoptotic machinery in the skin, such as caspase-8 (Kovalenko et al., 2009) or FADD (Bonnet et al., 2011), unexpectedly resulted in severe inflammatory skin disease. In this context, the role of cFLIP as a direct binding partner of caspase-8 is not yet fully understood. A current hypothesis is that activity of the caspase-8/cFLIP$_L$ heterodimer within different signaling platforms is able to inactivate pronecroptotic signaling (Geserick et al., 2009; Oberst et al., 2011). Furthermore, intracellular localization of caspase-8's activity within or outside of the signaling platforms, as well as its proteasomal degradation-dependent half-life in the cytoplasm (Gonzalvez et al., 2012), is of major importance for the differential regulation of apoptosis versus necroptosis. These aspects of caspase-8 activity are critically regulated by cFLIP and its different isoforms (Feoktistova et al., 2011; Geserick et al., 2009). Thus, both apoptosis and necroptosis signaling pathways are closely intertwined in a number of caspase-8 and cFLIP isoform-containing intracellular death platforms that are involved in the outcome of cellular stress signaling in a number of organs, including the skin.

We previously noted a strong expression of cFLIP in the basal layer of human epidermis (Armbruster et al., 2009), suggesting an important role of cFLIP in the epidermis. We thus investigated the role of cFLIP in vivo by generating and analyzing epidermis-specific, cFLIP-deficient animals. Of note, whereas prenatal constitutive deletion of cFLIP in the skin resulted in predominant embryonic lethality, postnatal acute deletion of cFLIP in keratin 14 (K14)-positive keratinocytes induced severe dysregulation

of epidermal homeostasis. We found that an early critical event leading to cell death in cFLIP-deficient keratinocytes is TNF-dependent apoptosis. In addition, studies of cFLIP in different skin diseases demonstrated a reduction of cFLIP protein expression in conditions associated with epidermal cell death (dyskeratosis). Our results thus suggest that epidermal loss of cFLIP may be a prerequisite for the life-threatening cell death seen in patients with toxic epidermal necrolysis (TEN)/Stevens-Johnson syndrome (SJS) or erythema exudativum multiforme (EEM). Altogether, our findings highlight the importance of cFLIP in skin homeostasis and apoptosis protection, whereas under those conditions, necroptosis seems to be of lesser relevance.

RESULTS

Constitutive Epidermal Deficiency of cFLIP Results in Embryonic Lethality

We generated skin-specific cFLIP knockout mice by using Cre/loxP-mediated recombination for conditional gene targeting. In order to specifically target cFLIP in the basal layer of epidermis, we used mice strains that express Cre recombinase (Cre) under the control of human K14 promoter (Hafner et al., 2004). However, a high rate of abortion (60%) in embryos from embryonic day 11 (E11) to E14 was detected (data not shown), and no progeny was born when cFLIP was deleted by K14-dependent Cre expression (Table S1). Western blot analysis of epidermal extracts from the few cFLIP$^{fl/fl}$-K14-Cre animals that were born did not reveal epidermal loss of cFLIP, indicating that these animals may have silenced K14-dependent Cre expression or counter selected against cFLIP-deleted keratinocytes during earlier phases of embryogenesis (data not shown). The embryonic lethality of epidermal cFLIP$^{fl/fl}$-K14Cre mice precluded a vigorous analysis of the role of cFLIP in the skin, but indicated the relevance of cFLIP for epidermal homeostasis during embryonic development.

Inducible Postnatal Deletion of cFLIP Leads to Dramatic Dermal Inflammation and Epidermal Cell Death

We next generated K14CreER^{tam} transgene mouse strains that allowed temporally and spatially controlled expression of cFLIP in keratinocytes. The transgene expresses a fusion protein between Cre and a tamoxifen- or 4-hydroxytamoxifen (4-HT)-responsive hormone-binding domain of the estrogen receptor (Vasioukhin et al., 1999). All animals with the cFLIP$^{fl/fl}$-K14CreER^{tam} genotype were born at the expected Mendelian ratio, and showed normal health and fertility, and no indication of cFLIP deletion in the absence of 4-HT as determined by genotyping PCR (an example of an initial set of genotyped progeny is shown in Table S2). To analyze the effect of inducible cFLIP deletion in restricted skin areas of adult animals, cFLIP$^{fl/fl}$-K14CreER^{tam} and cFLIP$^{+/+}$-K14CreER^{tam} control mice were depilated and subsequently treated with tamoxifen (2.5 mg/mice) for 5 consecutive days. Shortly after initiation of tamoxifen treatment, cFLIP$^{fl/fl}$-K14CreER^{tam} mice, but not control mice, developed macroscopic skin abnormalities starting with skin redness. At later time points, the animals showed yellowish crusts in treated skin regions (Figures 1A and S1). These changes quickly progressed to severe alterations and macroscopically detectable inflammation and thickening of the skin at later time points (Figure 1A). This skin disease was independent of bacterial overgrowth in skin with a perturbed skin barrier, as indicated by the same kinetics and severity of disease in animals treated with daily topical disinfection (Figure S1B). Successful deletion of the floxed cFLIP alleles in the epidermis was confirmed by multiplex PCR (Figures 1B and 1C). Of interest, 12 weeks after the treatment, most of the depilated hair had regrown in ethanol-treated areas, whereas local scarring alopecia was still evident in tamoxifen-treated skin of the same animals, indicating that loss of epidermal cFLIP also results in irreversible loss of hair follicles and scarring (Figure S1C).

Immunohistological analysis of skin sections from cFLIP$^{fl/fl}$-K14CreER^{tam} and control mice was performed. Despite the detected loss of cFLIP protein expression predominantly in the basal layer 48 hr after initiation of tamoxifen treatment (T48h), we were unable to detect any overt histological differences between cFLIP$^{fl/fl}$-K14CreER^{tam} and the respective control mice at this time point (Figure 1D). Moreover, at that stage, epidermal differentiation appeared normal, as demonstrated by the expression of K14 and K10 in basal and suprabasal keratinocytes, respectively. Staining with the terminal differentiation marker loricrin or the proliferation marker Ki67 excluded differentiation or proliferation defects of the epidermis at these early time points (Figures 1D, S2A, and S2D). However, at T72h, cFLIP$^{fl/fl}$-K14CreER^{tam} mice fully lacked cFLIP expression in the epidermis (Figures 1D, 1E, and S3). Furthermore, we detected marked acanthosis of the epidermis accompanied by a dramatically increased dermal infiltration of neutrophilic granulocytes and lymphocytes concomitantly with a regular occurrence of eosinophilic keratinocytes with pyknotic (commonly known as dyskeratotic) nuclei (Figures 1D and 1E; Schwarz et al., 1995). Although high K14 expression was found, reduced expression of K10 and loricrin in the epidermis of cFLIP$^{fl/fl}$-K14CreER^{tam} mice was accompanied by increased keratinocyte proliferation at these time points (Figures S2A and S2D). At the T72h stage, the skin of tamoxifen-treated cFLIP$^{fl/fl}$-K14CreER^{tam} mice displayed both patchy areas of epidermal hyperplasia and dysregulated keratinocyte differentiation (Figure 1D). At T96h, the epidermis of cFLIP$^{fl/fl}$-K14CreER^{tam} animals was fully disintegrated in large regions, along with extensive infiltration of inflammatory cells in the dermis, abundant pustules containing neutrophils, and the frequent appearance of subepidermal blistering (Figure 1F). Taken together, these results indicate that epidermis-specific inducible deletion of FLIP resulted in complete loss of K14- and K10-positive cells at late time points, consistent with complete loss of epidermis due to extensive cell death. Thus, based on acute postnatal ablation of cFLIP, our data demonstrate that cFLIP is critical for epidermal homeostasis.

The early skin erythema after tamoxifen application was suggestive of increased inflammation of knockout, but not diluent-treated, mice (Figure S1). Our analysis of the inflammatory response after keratinocyte-specific loss of cFLIP in the epidermis detected early development of a T cell- and granulocyte-dominated dermal inflammatory infiltrate accompanied by dysregulated proliferation and keratinocyte differentiation (Figure S2; Supplemental Results; Supplemental Discussion).

These findings suggest that damaged or dying keratinocytes may release danger signals in order to activate the innate immune system and/or the adaptive immune system.

cFLIP-Deficient Keratinocytes Undergo Apoptosis, but Not Necroptosis, In Vivo

It has been demonstrated that epidermal loss of caspase-8 or FADD results in uncontrolled necroptosis (Bonnet et al., 2011; Kovalenko et al., 2009). In contrast, loss of cFLIP was shown to result in increased apoptotic cell death in vivo (Zhang and He, 2005) and in human primary keratinocytes in vitro (Kavuri et al., 2011). Therefore, we next examined which type of cell death took place in cFLIP-deficient epidermis. Hematoxylin and eosin (H&E) staining of skin sections showed hypereosinophilic suprabasal cells with pyknotic nuclei, indicative of increased keratinocyte cell death in the epidermis of cFLIP$^{fl/fl}$-K14CreER^{tam} mice. Dyskeratosis was rarely detectable at T48h after acute ablation of cFLIP, but substantially increased at T72h (Figures 2A and 2E). We next examined skin sections with an antibody to a cleavage-specific epitope of caspase-3. Importantly, we were unable to detect dyskeratotic (dying) cells without concomitant caspase-3 positivity at early and later time points after initiation of tamoxifen treatment (Figures 2A and 2B). Cleaved caspase-3-positive cells were first detected 48 hr after the initial tamoxifen application, with a further increase up to 72 hr. Quantification of caspase-3-positive cells as well as double-staining experiments using cleaved caspase-3 Abs and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 2D) showed that cells with active caspase-3 were far more numerous predominantly at earlier time points than dyskeratotic keratinocytes or TUNEL-positive keratinocytes. In line with our notion of highly increased levels of apoptotic cell death, electron microscopy of the epidermis from tamoxifen-treated cFLIP$^{fl/fl}$-K14CreER^{tam} mice at T48h and T72h revealed the characteristic features of early and late apoptosis. Control mice showed a regular stratification of the epidermis and normal cellular and nuclear morphology. In contrast, after 48 hr, the keratinocytes of tamoxifen-treated cFLIP$^{fl/fl}$-K14CreER^{tam} mice showed numerous cytoplasmic vacuoles containing organelles indicative of autophagocytosis (Figure 2C, white arrowheads). Different stages of apoptosis could be observed in cFLIP$^{fl/fl}$-K14CreER^{tam} mice, i.e., early chromatin condensation (black arrowhead), cell shrinkage, and advanced chromatin condensation (white arrowheads) followed by nuclear pyknosis (asterisks). After 72 hr, infiltrating granulocytes (G) emerged in the epidermis in addition to the ongoing autophagy and apoptosis (Figure 2C). Taken together, our data demonstrate that loss of cFLIP in the epidermis results in a biphasic response. After an initial inflammatory response, the subsequent dramatic increase of cell death results in loss of the full thickness of the epidermis, mostly due to apoptotic cell death. Of note, the presence of one wild-type (WT) allele of cFLIP was sufficient to protect the epidermis from spontaneous apoptosis or caspase activation (Figures 2E and 2F). These experiments suggest that cFLIP protein expression from one allele of cFLIP is sufficient to protect epidermal keratinocytes from cell death and a macroscopic phenotype upon acute ablation of the cFLIP locus.

cFLIP-Deficient Keratinocytes Are Sensitive to Autocrine TNF-Induced Apoptosis In Vitro

To further mechanistically investigate why keratinocyte cell death occurs upon acute ablation of cFLIP, we studied cultured primary murine keratinocytes (PKs) of cFLIP$^{fl/fl}$-K14CreER^{tam} mice. Since keratinocytes can undergo either apoptosis or necroptosis (Feoktistova et al., 2011), we further investigated the sensitivity to CD95L-induced cell death in PKs isolated from cFLIP$^{fl/fl}$-K14CreER^{tam} animals upon cFLIP deletion (4-HT) or diluent-treated cells (control). We demonstrated increased sensitivity to CD95L-induced cell death (Figures 3C and 3D). Interestingly, cell death was completely blocked by pretreatment with zVAD, whereas necrostatin-1 was ineffective in preventing cell death, indicative of an intrinsic block of RIP1-dependent death signaling pathways in PKs despite a lack of cFLIP expression (Figure 3C). However, we observed in these experiments that the deletion of the cFLIP locus was incomplete in cFLIP$^{fl/fl}$-K14CreER^{tam} PK, possibly due to a loss of K14 promoter activity in vitro (Figure 3A). Therefore, we applied a different experimental approach in which the floxed cFLIP allele was excised in vitro using lentiviral (LV)-mediated Cre expression in murine PKs. In cFLIP$^{fl/fl}$ PKs, LV transduction led to complete deletion of the cFLIP allele (Figures 3A and 3B). Loss of both cFLIP alleles resulted in loss of cFLIP protein (Figure S3). Complete cFLIP deletion in PKs resulted in increased spontaneous cell death (50%–70%) at day 4 posttransduction in PKs isolated from cFLIP$^{fl/fl}$-K14CreER^{tam} mice, but not in those isolated from heterozygous or WT animals (Figure 3E). These data suggest that Cre-mediated DNA damage can be excluded as an inducer of cell death in our experimental system. Moreover, PKs derived from cFLIP$^{fl/fl}$ and transduced by Cre-LV were exquisitely sensitive to cell death induced by CD95L or TNF (Figure 3F), in line with data generated using other cell types of cFLIP knockout or conditional knockout animals (Yeh et al., 2000; Zhang and He, 2005). To further assess the role of DR signaling in PKs lacking cFLIP, we analyzed the cellular viability of PKs isolated from homozygous cFLIP$^{fl/fl}$-K14CreER^{tam} and heterozygous cFLIP$^{fl/+}$-K14CreER^{tam} animals in the presence and absence of different soluble DR fusion proteins. Intriguingly, cFLIP-deficient PKs were protected from spontaneous cell death by TNF-R2-Fc, whereas addition of TRAIL-R2-Fc or CD95-Fc was unable to block cell death (Figure 3G). We reasoned that loss of cFLIP in our in vitro experimental model based on cFLIP-deficient murine PKs may promote autocrine TNF production, which ultimately translates into TNF-induced spontaneous cell death. TNF mRNA induction and protein secretion of cFLIP$^{fl/fl}$-K14CreER^{tam} PKs treated with Cre virus confirmed that TNF induction occurs as a response to the loss of cFLIP rather than to nonspecific effects of viral transduction or DNA damage generated by expression of Cre (Figures 3H and 3I). Taken together, our data suggest that loss of cFLIP results in increased sensitivity to death ligand-induced cell death and that induction of autocrine TNF production driven by loss of cFLIP is sufficient to kill cFLIP-deficient PKs. In this context, we also studied the contribution of other signaling pathways, such as interleukin-1β (IL-1β) induction by active caspase-8 (Vince et al., 2012) or lipopolysaccharide (LPS)-induced cell death, as a cause of the epidermal inflammation (Bannerman et al., 2004). However, neither IL-1β

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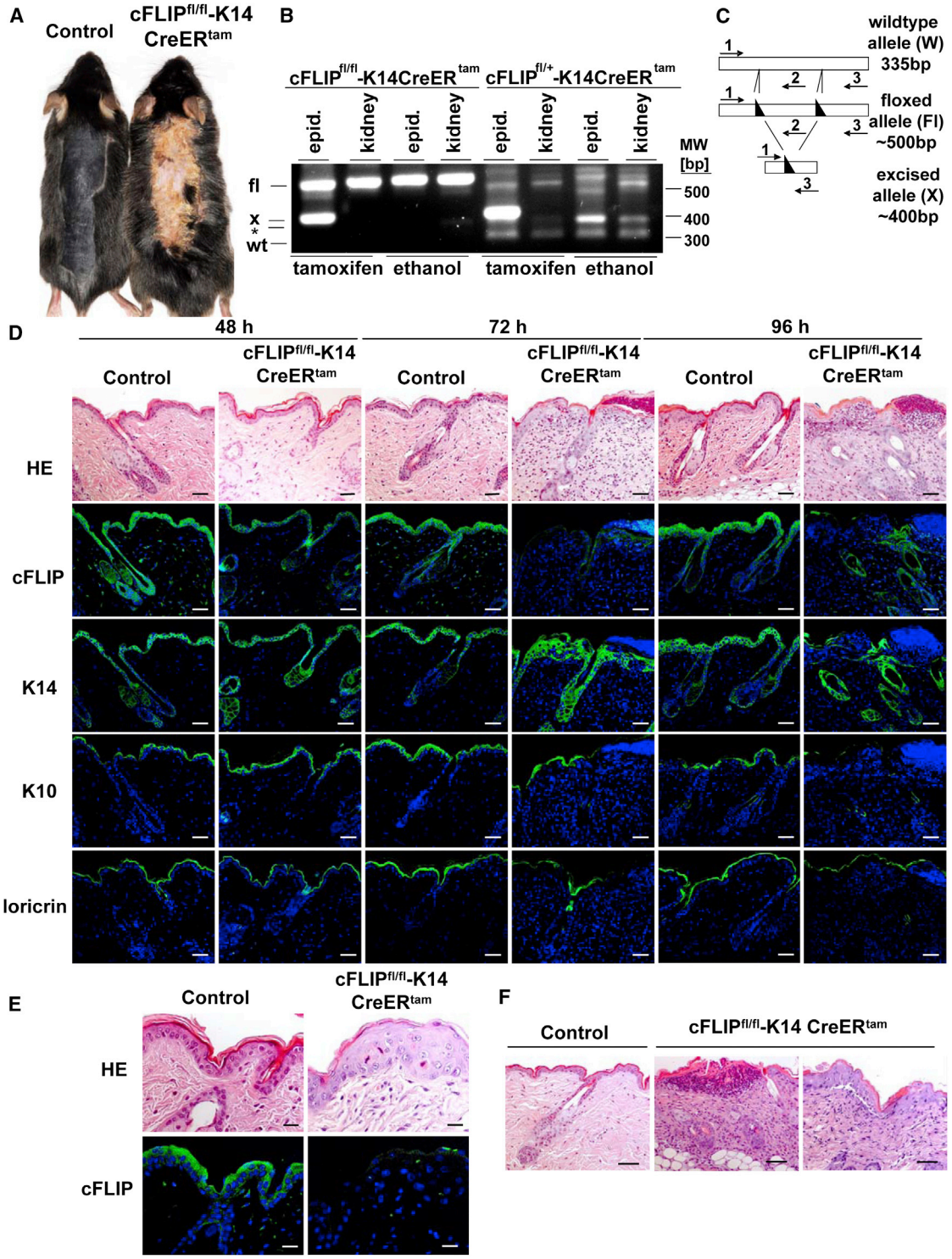

Figure 1. Deletion of cFLIP in Epidermal Keratinocytes Results in Early Hyperproliferation and Later Destruction of the Epidermis

(A) Macroscopic aspect of control cFLIP$^{+/+}$-K14CreER^{tam} mice and cFLIP$^{fl/fl}$-K14CreER^{tam} mice 10 days after the first tamoxifen application. A representative mouse and the respective control from a total of ten mice are shown.

(B) Detection of tamoxifen-induced excision of the cFLIP allele (x) in the epidermis. DNA was isolated from different organs of cFLIP$^{fl/fl}$-K14CreER^{tam} and cFLIP$^{fl/+}$-K14CreER^{tam} mice and analyzed by multiplex PCR. fl, floxed allele; WT, wild-type allele; x, excised allele.

(C) Schematic representation of the design of the multiplex PCR for detection of the deleted cFLIP allele.

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nor LPS-induced cell death was modulated by loss of cFLIP (data not shown). These results indicate that these inflammatory pathways are of lesser relevance in our model system. Taken together, the in vitro experiments argue that a TNF-mediated autocrine loop in the skin is the cause of TNF-mediated apoptosis of cFLIP-deficient PKs in vivo.

Inhibition of Autocrine TNF Signaling Partially Protects cFLIP-Deficient Epidermis against Early Apoptotic Cell Death

In order to investigate whether induction of TNF is the cause of cell death induced by acute cFLIP ablation, we next performed in vivo experiments using a soluble receptor fusion protein (recombinant TNF-R2-Fc) to inhibit TNF-dependent signaling. To that end, we treated cFLIP$^{fl/fl}$-K14CreER^{tam} animals with tamoxifen following repetitive injection of TNF-R2-Fc. Mice pretreated with TNF-R2-Fc developed weaker, but not completely abolished, macroscopic skin abnormalities at T96h and T120h as compared with control animals pretreated with immunoglobulin G (IgG) only (Figure 4A). However, this macroscopic difference was much less pronounced at later time points, indicating that additional signaling pathways become operative for cell death independently of TNF signaling. Histological analysis of skin sections from TNF-R2-Fc pretreated mice at T72h and T96h also showed a (nonsignificant) tendency to decreased hyperkeratosis, acanthosis, pustule formation, epidermal disintegration, etc., as compared with control IgG-pretreated animals (Figures 4B and 4E). Staining of active caspase-3, however, demonstrated a significant decrease in the number of apoptotic keratinocytes in the TNF-R2-Fc-treated animals at T72h (Figures 4C and 4D). Thus, it seems likely that epidermal cell death in cFLIP$^{fl/fl}$-K14CreER^{tam} mice is mediated by paracrine or autocrine TNF. In this model, TNF may be produced by either infiltrating cells or in an autocrine manner by keratinocytes, as suggested by our in vitro and in vivo studies. Acute loss of cFLIP in the epidermis therefore initiates early TNF-dependent responses, which suggests that cFLIP is a critical inhibitor of TNF-mediated cell death in the skin.

Epidermal cFLIP Expression Is Lost Concomitantly with Increased Caspase-8 Activation in Skin Disease Associated with Epidermal Cell Death

Given the dramatic cell death phenotype of cFLIP-depleted mouse skin that was evident within 48–72 hr after cFLIP locus deletion, we reasoned that cFLIP may represent a major roadblock to epidermal cell death in human skin disease. We thus next investigated disease entities that have long been known to be associated with increased cell death in the skin, namely, TEN/SJS (Harr and French, 2012), EEM, and cutaneous lupus erythematodes (CLE). We detected a strong cFLIP expression in the basal layer of healthy skin (Figure 5A, healthy skin). In contrast, detectable caspase-8 cleavage, as determined by staining of a cleavage-generated neoepitope of this caspase, was absent in healthy skin. Interestingly, epidermal staining of cFLIP was substantially decreased in lesional skin of patients with TEN/SJS and EEM, whereas lesional skin of CLE patients did not show a loss of cFLIP (Figure 5A, right), consistent with a granzyme B-mediated form of cell death in this disease, as proposed by Wenzel and Tüting (2008). Concomitantly, increased cleaved (active) caspase-8 was detected in these skin regions, as also indicated by double stainings of cFLIP and caspase-8 (Figure 5A, right). Our data indicate that epidermal loss of cFLIP in keratinocytes may be an important prerequisite for the increased caspase-8-mediated epidermal cell death seen in patients with TEN/SJS.

DISCUSSION

Tight control of the programmed cell death machinery is of critical importance to ensure undisturbed skin development and the maintenance of skin homeostasis. Whereas early studies that examined the role of DRs focused on the elicitation of cell death (Strasser et al., 2000), more recent studies highlighted the fact that DR signaling pathways and their apical signaling molecules have additional important functions beyond the mere initiation of cell death responses. This includes activation of signals such as NF-κB and gene induction of cytokines and chemokines (Leverkus et al., 2003; Cullen et al., 2013). Moreover, it was shown that caspase-8 regulates wound-healing responses (Lee et al., 2009) and downregulates metastasis in cancer cells (Stupack et al., 2006). We previously suggested that the control of inflammatory responses of the skin to allergens or other extrinsic "danger signals" may be profoundly influenced by the qualitative outcome of DR triggering in the skin, because DR-induced inflammatory signaling (e.g., activation of the transcription factor NF-κB) may impact skin diseases (Kerstan et al., 2009; Leverkus and Trautmann, 2006). When one considers the numerous functions of caspase-8, it is evident that the procaspase-8-like, protease-deficient homolog cFLIP is of great interest as a central regulator of caspase-8 function. Here, we investigated the role of cFLIP in the skin in vivo. Constitutive ablation of the cFLIP gene using K14-Cre mice revealed that loss of cFLIP is lethal during embryonic life, which is indicative of its importance during skin development. Because constitutive ablation may result in a multitude

(D) Skin sections at the indicated time points from control cFLIP$^{+/+}$-K14CreER^{tam} and cFLIP$^{fl/fl}$-K14CreER^{tam} mice stained with H&E, cFLIP, K14, K10, and loricrin. Scale bars represent 50 μm.

(E) Skin sections from tamoxifen-treated cFLIP$^{fl/fl}$-K14CreER^{tam} and control cFLIP$^{+/+}$-K14CreER^{tam} mice at T72H were stained with H&E and Abs to cFLIP, respectively. cFLIP Abs (H-202) stained the epidermis of control animals, but did not label the epidermis of animals with epidermal deletion of cFLIP. This staining pattern was controlled for by genetic typing (data not shown) and was seen at antibody concentrations up to 5 μg/ml. However, cFLIP Abs (H-202) were not suitable for the detection of murine cFLIP by western blotting (Figure S3). Scale bars represent 100 μm. Data presented in (D) and (E) are representative slides from a total of 23 mice.

(F) Skin sections from control cFLIP$^{+/+}$-K14CreER^{tam} mice and cFLIP$^{fl/fl}$-K14CreER^{tam} 96 hr after initiation of tamoxifen treatment, stained with H&E. Different histological features, such as pustule formation, epidermal disintegration, and appearance of subepidermal blisters (arrow), are shown. Data are representative of a total of 20 analyzed mice and their respective controls.

See also Figures S1 and S2.

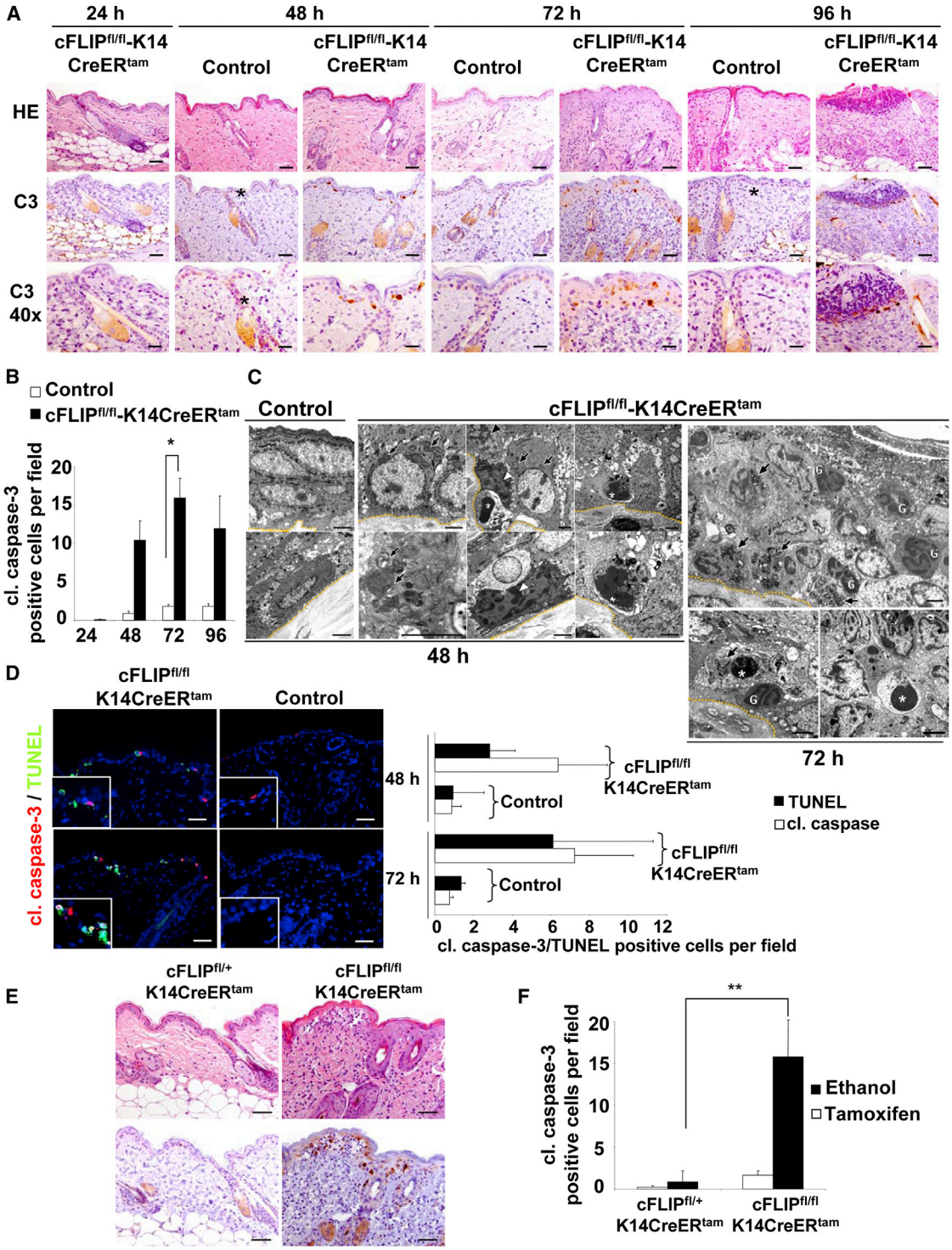

Figure 2. cFLIP Protects against Epithelial Apoptotic Cell Death In Vivo

(A) Skin sections at the indicated time points from control cFLIP$^{+/+}$-K14CreER^{tam} and cFLIP$^{fl/fl}$-K14CreER^{tam} mice were stained with H&E or Abs to cleaved caspase-3, respectively. Asterisks indicate nonspecific staining. Scale bars represent 50 μm. The data are representative of a total of 26 mice.

(B) Quantification of caspase-3-positive cells per microscopic field at the indicated time points from cFLIP$^{fl/fl}$-K14CreER^{tam} mice compared with controls. A total of 12 mice were analyzed.

(C) The skin of control and cFLIP$^{fl/fl}$-K14CreER^{tam} mice was investigated by transmission electron microscopy after 48 and 72 hr. Basement membranes are highlighted by a dotted yellow line. Scale bars correspond to 2 μm. The data shown are representative of ten analyzed animals.

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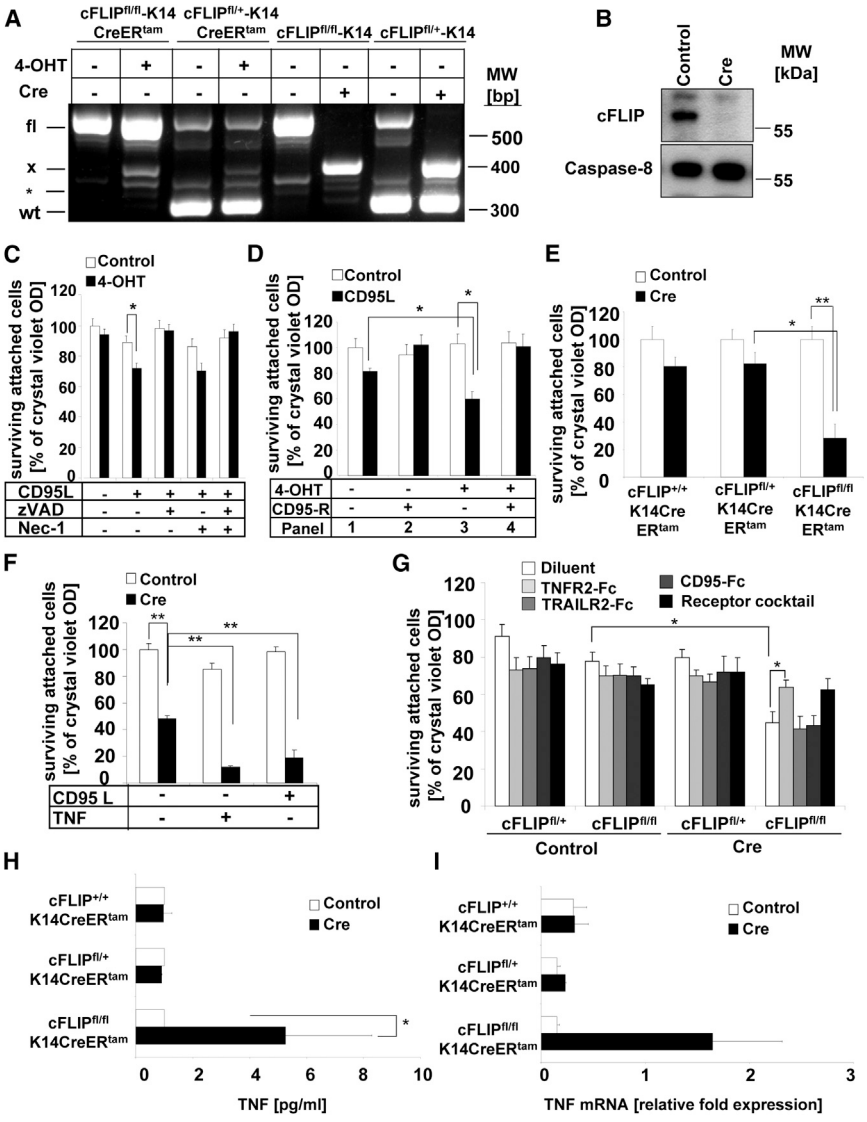

Figure 3. cFLIP Deletion In Vitro Results in Increased Sensitivity to Death-Ligand-Induced Cell Death of PKs and Is Caused by TNF-Induced Apoptosis

(A) Comparison of cFLIP allele deletion in PKs isolated from cFLIP$^{fl/fl}$-K14CreER^{tam} mice. The comparison of cells treated with either 4-HT or LV-Cre transduction is shown. Isolated DNA was analyzed by multiplex PCR. Asterisks indicate nonspecific bands. The data shown are representative of five independent experiments. fl, floxed; WT, wild-type; x, excised allele.

(B) Western blot for cFLIP and caspase-8 of control-LV and Cre-LV transduced PKs isolated from cFLIP$^{fl/fl}$-K14CreER^{tam} mice. The western blot shown is representative of two independent experiments.

(C) Cellular viability of PKs isolated from cFLIP$^{fl/fl}$-K14CreER^{tam} mice treated as indicated. Error bars represent the SEM of eight independent experiments.

(D) cFLIP$^{fl/fl}$-K14CreER^{tam} mice were treated as indicated and assayed by crystal violet staining. Error bars represent the SEM of two independent experiments.

(E) Control-LV and Cre-LV transduced PKs isolated from cFLIP$^{+/+}$-K14CreER^{tam}, cFLIP$^{fl/+}$-K14CreER^{tam}, and cFLIP$^{fl/fl}$-K14CreER^{tam} were treated as indicated. Error bars represent the SEM of three independent experiments.

(F) PKs of cFLIP$^{fl/fl}$-K14CreER^{tam} mice were infected with control-LV or Cre-LV and treated as indicated. Error bars represent the SEM of three independent experiments.

(G) cFLIP$^{fl/fl}$-K14CreER^{tam} and cFLIP$^{fl/+}$-K14CreER^{tam} mice were Cre-LV transduced and treated as indicated. Error bars represent the SEM of three independent experiments.

(H) PKs isolated from cFLIP$^{+/+}$-K14CreER^{tam}, cFLIP$^{fl/+}$-K14CreER^{tam}, and cFLIP$^{fl/fl}$-K14CreER^{tam} mice were transduced with control-LV and Cre-LV. At T72h, cell-free culture supernatants were analyzed for TNF production by ELISA. Error bars represent the SEM of two independent experiments. In (C)–(H), *$p < 0.05$ and **$p < 0.01$.

(I) mRNA expression of TNF in control and Cre-LV transduced PKs isolated from cFLIP$^{+/+}$-K14CreER^{tam}, cFLIP$^{fl/+}$-K14CreER^{tam}, and cFLIP$^{fl/fl}$-K14CreER^{tam} mice. Error bars represent the SEM of two independent experiments. All experiments with PKs were performed with pooled cells from four to six animals per pool. See also Figure S3.

of secondary effects in the developing skin or in the early postnatal period, we decided to focus on the acute deletion of cFLIP from adult skin. We found that within days, loss of cFLIP disturbed skin homeostasis, and this was associated with a dual response. At early time points, an inflammatory response with marked acanthosis accompanied by infiltration of T cells and neutrophils was followed by a marked increase in dyskeratotic keratinocytes in the epidermis. As early as 4 days after initiation of cFLIP deletion, we detected subepidermal blisters, pustules, and ultimately complete loss of the epidermis. These data demonstrate that cFLIP is indispensable for skin homeostasis and protects against cell death. The macroscopic skin

(D) Skin sections at the indicated time points from control cFLIP$^{+/+}$-K14CreER^{tam} and cFLIP$^{fl/fl}$-K14CreER^{tam} mice with double staining of TUNEL/cleaved caspase-3. Scale bars represent 100 μm. Quantification of cleaved caspase-3- or TUNEL-positive cells per microscopic field of the double-stained skin sections. Inset shows higher magnification. A total of nine mice were analyzed.

(E) Skin sections of cFLIP$^{fl/+}$-K14CreER^{tam} and cFLIP$^{fl/fl}$-K14CreER^{tam} mice 72 hr after initiation of tamoxifen treatment, stained with H&E and Abs to cleaved caspase-3. Scale bars represent 100 μm. The data shown are representative of ten analyzed animals.

(F) Quantification of caspase-3-positive cells per microscopic field of skin sections from cFLIP$^{fl/+}$-K14CreER^{tam} and cFLIP$^{fl/fl}$-K14CreER^{tam} mice at T72h. A total of 12 mice were analyzed. *$p < 0.05$, **$p < 0.01$.

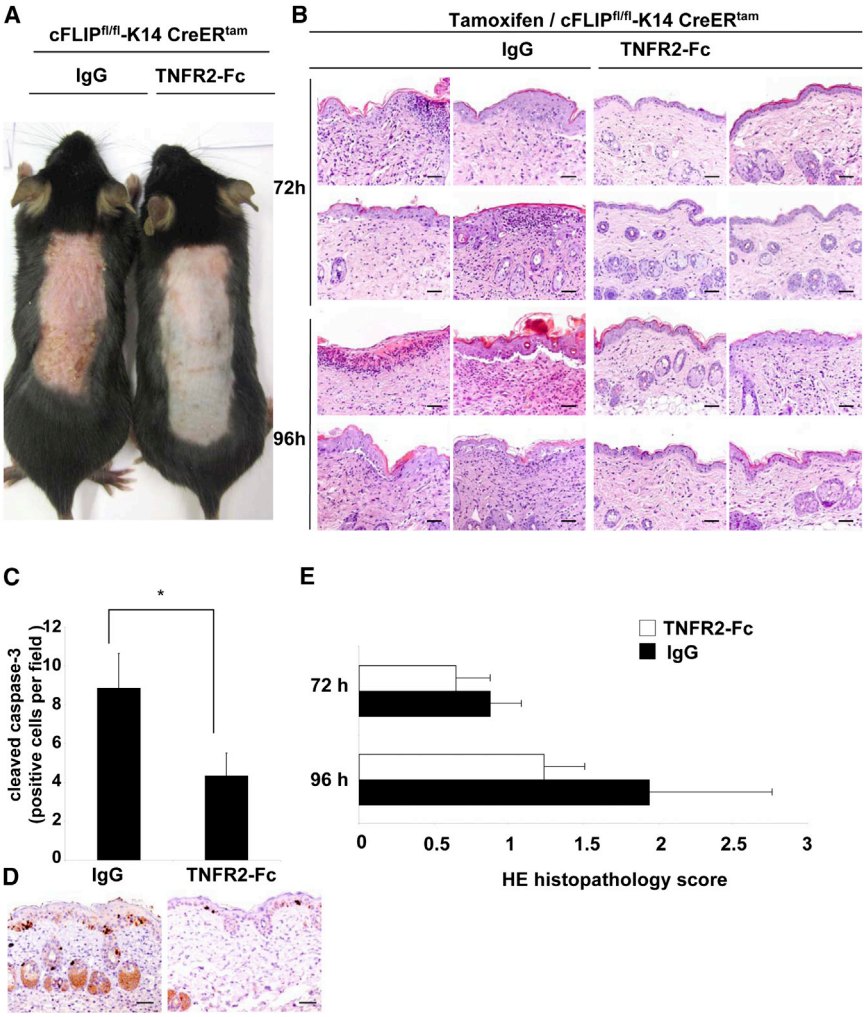

Figure 4. cFLIP-Deficient Epidermis Is Protected from Early Caspase-3 Activation and Cell Death by TNF-R2-Fc

(A) Macroscopic appearance of FLIP$^{fl/fl}$-K14CreER^{tam} mice 5 days after the first tamoxifen application after TNF-R2-Fc (TNFR2-Fc) or IgG pretreatment, respectively. A total of six mice were analyzed for this time point. A representative picture is shown.

(B) Skin sections stained with H&E at T72h and T96h. Scale bars represent 100 μm. A total of 20 animals were analyzed and four different examples are shown for each time point/condition.

(C) Quantification of caspase-3-positive cells per microscopic field of skin sections from cFLIP$^{fl/fl}$-K14CreER^{tam} mice at T72h pretreated with TNF-R2-Fc or IgG, respectively. Skin sections of a total of ten animals treated with either IgG or TNF-R2-Fc were analyzed.

(D) Representative skin sections from cFLIP$^{fl/fl}$-K14CreER^{tam} mice at T72h pretreated with TNF-R2-Fc or IgG stained with active caspase-3 antibody.

(E) Semiquantitative analysis of H&E pathological scoring as outlined in Experimental Procedures. A total of 20 animals (ten treated with TNF-R2-Fc [TNFR2-Fc] and ten treated with IgG) were analyzed.

phenotype was accompanied by the rapid appearance of caspase-3 activity in the epidermis, which was detectable prior to the profound histological changes of cFLIP-deficient skin. Moreover, electron microscopy further supported the apoptotic phenotype of cFLIP-deficient keratinocyte death. Taken together, our morphological studies demonstrate that acute loss of cFLIP results in fulminant apoptosis, but not necroptosis, of the epidermis within days. Our data contrast with interesting reports that necroptosis in the skin was evident in either constitutive caspase-8- or FADD-deficient skin that contained numerous dyskeratotic keratinocytes a few days after birth (Bonnet et al., 2011; Kovalenko et al., 2009) but lacked active caspase-3 (Bonnet et al., 2011). This implies that the predominant function of caspase-8 is antinecroptotic rather than antiapoptotic, at least upon chronic deletion of FADD or caspase-8. In contrast to these findings, acute loss of cFLIP in the skin reveals that a major function of cFLIP in vivo is to protect against epidermal apoptosis. The disparate effects of cFLIP and caspase-8 were also recently highlighted in a developmental mouse model. Whereas acute or chronic loss of caspase-8 was rescued by concomitant loss of RIP3, a major effector of necroptosis, the constitutive or acute removal of cFLIP was not rescued by the accompanying loss of RIP3 (Dillon et al., 2012; Weinlich et al., 2013). It is therefore tempting to speculate that apoptosis, rather than necroptosis, is the predominant mode of cell death during development when cFLIP is absent. Our data from a postnatal model of acute deletion of cFLIP in the skin further support the notion that cFLIP primarily serves an antiapoptotic function, in contrast to the strikingly different function of caspase-8 in the epidermis.

It remains to be dissected how the striking macroscopic and microscopic phenotypes of cFLIP-deficient skin are ruled at the molecular level. We investigated this important question using several in vitro model systems. Primary cFLIP-deficient PKs showed an increased sensitivity to death ligands, exemplified by CD95L or TNF. Moreover, enforced deletion of cFLIP in most PKs by LV-Cre infection resulted in more than 50% spontaneous cell death that was largely blocked by TNF inhibition, but not by either CD95L or TRAIL blockade. These in vitro experiments argue that a TNF-mediated autocrine loop in the skin is required to initiate TNF-mediated apoptosis of cFLIP-deficient PKs in vivo. Inhibition of TNF in vivo by TNF-R2-Fc protected against early inflammation, caspase-3 activation, and cell death, and reduced the early extent of skin disease. This argues for an important role of TNF in the early phase of skin disease and suggests that a major function of cFLIP is to protect keratinocytes against TNF-induced apoptosis in vivo. Our data are in line with results from mice lacking caspase-8 or FADD in the skin. In these

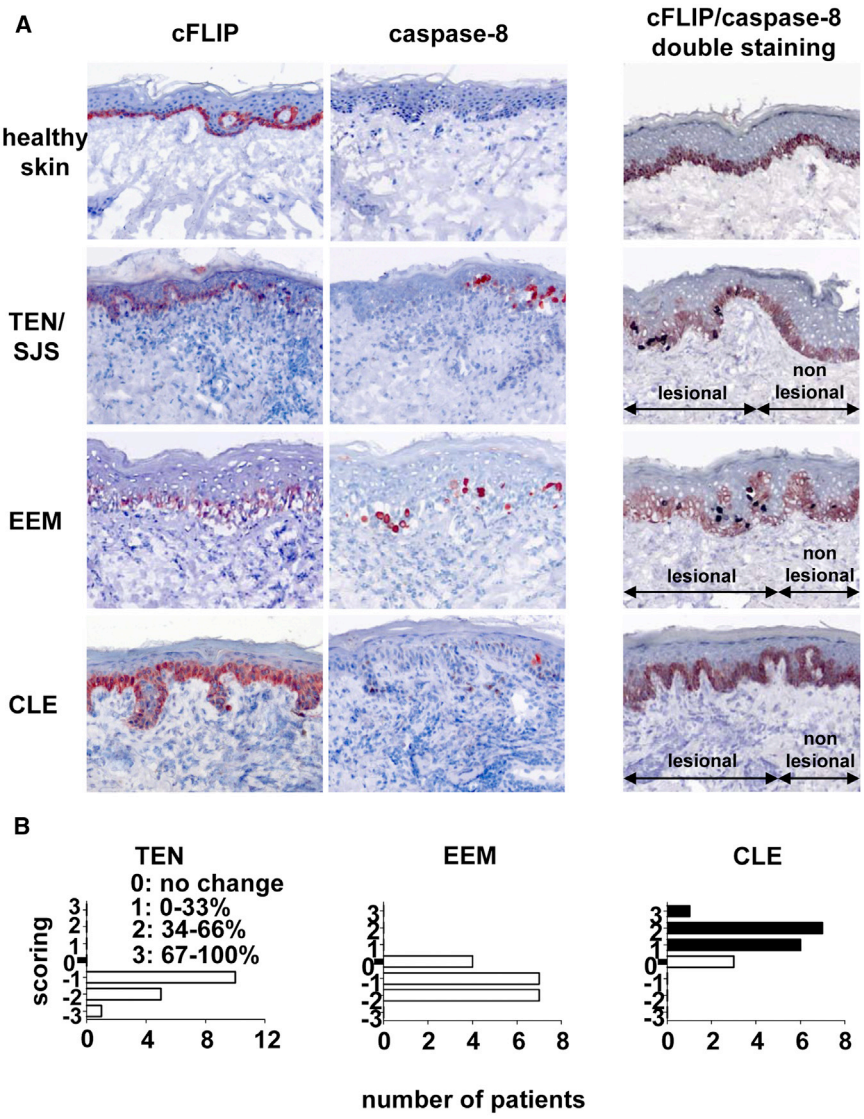

Figure 5. Epidermal cFLIP Expression Is Lost Concomitantly with Increased Caspase-8 Activation in Skin Disease Associated with Excessive Epidermal Cell Death

(A) Serial skin sections (left) or representative skin sections (cFLIP/caspase-8 double staining) of patients with TEN/SJS, EEM, or CLE were stained with Abs to cFLIP and cleaved caspase-8, and compared with the expression in healthy skin.

(B) Semiquantitative analysis of cFLIP expression in a series of patients. Relative expression was evaluated by three independent investigators as described in Experimental Procedures. Black bars identify histopathology scoring > 0, and white bars depict scoring < 0.

animals, TNF or TNF-R1 deletion delayed the macroscopic phenotype, but did not prevent it at later time points (Bonnet et al., 2011; Kovalenko et al., 2009). Therefore, it is most likely that alternative cFLIP-inhibited pathways contribute to the macroscopic phenotype following acute ablation of cFLIP in keratinocytes. Our data suggest that early induction of TNF in the skin and subsequent TNF-induced apoptosis represent a major mechanism of dramatic epidermal cell death in our mouse model.

In combination, the kinetics of skin morphology and the pattern of molecular changes in PKs suggest the following mechanism of cell death in our mouse model: initial apoptotic cell death caused by loss of cFLIP triggers the development of an inflammatory skin disease that precedes the disintegration of the epidermis. In this regard, the earliest detectable event after initiation of tamoxifen treatment was the appearance of inflamed skin around 3 days after initiation of cFLIP locus deletion (Figure S2A). The remaining cFLIP protein may then be able to protect against TNF death signaling for this period of time. However, TNF may already have been induced in the epidermis at earlier time points, as suggested by our in vitro studies. The early expression of TNF may explain the inflammatory response in cells that do not die in our model, because TNF complex II is still inhibited by the remaining cellular levels of cFLIP protein (Micheau and Tschopp, 2003).

How can the endogenous caspase-8 inhibitor cFLIP block apoptosis and not allow for necroptosis in vivo? Our current findings and previous biochemical work using different cFLIP isoforms (Feoktistova et al., 2011; Geserick et al., 2009), together with elegant evidence from mouse genetics (Oberst et al., 2011), suggest the following scenario: cFLIP is critically needed to guide and keep the enzymatic activity of the cFLIP/caspase-8 heterodimer within different death signaling platforms. In these signaling platforms, caspase-8 activity associated with the uncleaved caspase-8/cFLIP heterodimer is needed to inhibit necroptosis, most likely by cleavage of RIP1 (Feoktistova et al., 2011; O'Donnell et al., 2011; Rajput et al., 2011). RIP1 cleavage thus interferes with the assembly or stability of the necrosome, ripoptosome, TNF complex II, or RIG-I complex, thereby blocking necroptosis. If this important function of caspase-8 within these signaling complexes is not maintained by its heterodimer with $cFLIP_L$, caspase-8 is further autoprocessed to heterotetramers and released from these death platforms. In the absence of cFLIP, apoptosis without overt necroptosis is found, and a major function of cFLIP is to keep the caspase-8 activity localized within the death platforms. Regardless of the exact stimulus of death-platform assembly after cFLIP deletion in the epidermis (beyond TNF complex II, as suggested by our inhibition studies), the lack of cFLIP protein promotes rapid and fulminant apoptosis. The resulting high caspase activity outside of the assembled death platforms then inhibits further necroptosis

signaling, likely by RIP1 and RIP3 cleavage (Feng et al., 2007; Martinon et al., 2000). The fact that autocrine TNF is associated with the early phenotype and is spontaneously induced after cFLIP loss in cultured PKs suggests a critical function of cFLIP in the blockade of TNF complex II, as previously suggested (Micheau and Tschopp, 2003). In the future, it will be interesting to study the role of mitogen-activated protein kinase (MAPK) and the NF-κB signaling pathways in TNF transcription and mRNA stability upon cFLIP deletion in keratinocytes (Leverkus et al., 1998). Our data highlight that tissue distribution of the core proteins of the death signaling complexes (including cFLIP, caspase-8, FADD, RIP1, and other known or unknown proteins) of both apoptosis and necroptosis pathways determine the outcome of cellular stress signals, including TNF. For example, tissue-specific constitutive knockout of cFLIP in the liver produced divergent results, with either no spontaneous disease (Schattenberg et al., 2011) or perinatal death (Piao et al., 2012), whereas liver-specific, cFLIP-deficient animals rapidly succumbed to fulminant hepatitis upon poly I:C challenge in a TNF-, TRAIL-, and CD95L-dependent manner generated most likely by Kupffer cells. In contrast, deletion of cFLIP in intestinal epithelial cells resulted in perinatal lethality (Piao et al., 2012), whereas cFLIPL deficiency in T lymphocytes led to altered regulation of apoptosis, autophagy, and necroptosis (He and He, 2013). This suggests that epithelial cells may be particularly susceptible to loss of cFLIP, as shown in our study in keratinocytes. It is possible that epithelial cells are particularly vulnerable to loss of cFLIP due to their constant encounters with noxious stimuli that activate pattern-recognition receptors (PRRs), DRs, or the RIG-I signaling complex.

Previous reports indicated that the fulminant cell death phenotype seen in patients with TEN is induced by the death ligand CD95L (Viard et al., 1998) or granulysin produced by subsets of natural killer (NK) or CD8 T cells (Chung et al., 2008). Our data now add to this body of evidence by indicating that epidermal loss of cFLIP in keratinocytes may be an important prerequisite for the dramatic cell death seen in patients with TEN/SJS. This suggests that additional signals induced by the quality and/or quantity of infiltrating immune cells mediate cFLIP protein loss and subsequent fulminant epidermal cell death in these diseases. Our data highlight that cFLIP is necessary to protect against excessive apoptosis in the epidermis and is a potential target of therapeutic efforts (i.e., by transcriptional induction or posttranslational upregulation of cFLIP) in these potentially life-threatening diseases. Although this conclusion is based on only indirect and correlative evidence obtained from patients with TEN/SJS as compared with patients with CLE, the dramatic phenotype of cFLIP-deficient mouse skin resembling TEN after acute loss of cFLIP renders it an attractive possibility. Taken together, our findings provide insights into the regulation of the cell death machinery of the epidermis and warrant future studies to dissect the impact of loss of cFLIP in a number of inflammatory and neoplastic diseases of the skin.

EXPERIMENTAL PROCEDURES

Mice

Transgenic mice expressing Cre under the control of the K14 promoter, mice with conditional K14-Cre expression, and mice carrying the *loxP* FLIP allele were previously described (Hafner et al., 2004; Vasioukhin et al., 1999; Zhang and He, 2005). To generate knockout mice with epidermal-specific cFLIP deletion, we bred floxed cFLIP to either K14-Cre or K14CreER^{tam} mice. Cre expression was induced by the application of tamoxifen (Sigma). The back skin of the mice was treated daily with a 100 μl ethanol solution of tamoxifen at a concentration of 25 mg/ml for 5 consecutive days. Control animals expressing Cre but lacking loxP-flanked alleles were included in all experiments.

Histology and Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were used for all histopathological and immunofluorescence studies of murine skin. We used the following antibodies: K14, K6, K10, and Loricrin (Covance); cleaved caspase-3 (Asp175; Cell Signaling); cleaved caspase-8 (Asp391) clone 18C8; Cell Signaling); and cFLIP (H202; Santa Cruz). Secondary biotinylated antibody and Cy2-Streptavidin were purchased from Vector Laboratories and Jackson ImmunoResearch, respectively. The TUNEL assay was obtained from Millipore. Image processing was applied identically to all samples and controls.

Quantification of Immunohistochemical or Immunofluorescent Labeling of Histological Slides

Skin sections were semiquantitatively evaluated by three independent investigators blinded to the treatment of the respective mouse in at least 30–40 fields of each sample. Pathological changes of the epidermis (H&E staining) were scored as 0 (healthy skin), 1 (single dead cells, hyperkeratosis, acanthosis), 2 (small regions of confluent cell death, small ulcers, subepidermal blistering), 3 (wide ulcers, wide regions of confluent cell death per microscopic field), or 4 (fully disintegrated epidermis).

Electron Microscopy

The skin was fixed in Karnovsky's glutaraldehyde (2% paraformaldehyde, 2.5% glutaraldehyde, 0.2 M cacodylate buffer pH 7.4) and embedded in araldite (Serva). Ultrathin sections were stained with lead citrate and uranyl acetate. Photographs were taken on a transmission electron microscope (Zeiss EM 900; Carl Zeiss).

Primary Keratinocyte Isolation and Cell Death Assays

PKs were isolated from the skin of newborn cFLIP$^{fl/fl}$ K14CreER^{tam} mice and cultured for 5 days in CnT-07 medium (CELLnTEC). Cre expression was induced with 100 μM 4-HT. The prestimulation and stimulation conditions were as follows: zVAD (10 μM), Necrostatin-1 (Nec-1; 50 μM), TNF-R2-Fc (10 μg/ml), TRAIL-R2-Fc (10 μg/ml), CD95-Fc (10 μg/ml), TNF (10 ng/ml), and agonistic CD95L-Fc (625 U/ml).

LV Expression of Cre in PKs and Immunoblotting

The pFU Cre SV40 puro W construct was kindly provided by John Silke (WEHI, Melbourne, Australia). Cre-LV was generated in human embryonic kidney (HEK) cells. Total protein was probed with cFLIP (clone Dave-2; Apotech) and caspase-8 (clone 1G12; Axxora).

Statistical Analysis

The SEM was determined for three to five independent experiments performed with PKs pooled from three to five newborn animals for each experiment. Student's t test was used for statistical data analysis. Significance is stated whenever the p value was < 0.05.

RNA Isolation and Real-Time qPCR

RNA isolation from skin or cultured PKs, respectively, was performed with the RNeasy Kit (QIAGEN). cDNA was synthesized by SuperScript II Reverse Transcriptase (Invitrogen) using a mixture of random nonamers and oligo dT primers. Each experiment was normalized to β-actin expression.

Patient Specimens

Cryosections of patients were obtained either from healthy donors or from patients with EEM, TEN, or CLE as approved by the ethics committee of the University of Würzburg Medical School. Slides were examined on an Olympus BX41 microscope (Olympus) at constant illumination settings. The staining intensity was semiquantitatively evaluated by three independent observers at

200× magnification. Epidermal expression of cFLIP was scored as 0 (no difference), −1 (1%–33% reduction), −2 (34%–66% reduction), or −3 (67%–100% reduction), and vice versa for increased expression as compared with healthy skin or normal skin appearing at the margins of the biopsy. Double staining was performed by sequential incubation and development of cFLIP and caspase-8 using the polymerized peroxidase enzyme staining system (ImmPRESS; Vector Laboratories). 3-Amino-9-ethylcarbazole (AEC) and Vector SG were used as substrates, followed by counterstaining with hematoxylin. Sections incubated with the appropriate isotype monoclonal antibody or polyclonal rabbit IgG were used as negative controls.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, Supplemental Discussion, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.035.

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