

Epidermal RelA Specifically Restricts Contact Allergen–Induced Inflammation and Apoptosis in Skin

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Strong inhibition of NF- κ B signaling in the epidermis results in spontaneous skin inflammation in mice and men. As there is evidence for linkage between polymorphisms within the NF- κ B signaling pathway and human inflammatory skin phenotypes, we asked whether partial functional inhibition of NF- κ B signaling in epidermal keratinocytes can modulate clinically relevant skin inflammation. We therefore mutated *rela* specifically in the epidermis of mice (RelA^{E-MUT} mice). These mice show no inflammatory phenotype. Induction of contact allergy, but not croton oil–induced irritant dermatitis, resulted in stronger ear swelling and increased epidermal thickness in RelA^{E-MUT} mice. Both contact allergen and croton oil treatment led to increased expression of calgranulins A and B (S100A8/ A9) in RelA^{E-MUT} mice. Epidermal hyperproliferation in RelA^{E-MUT} mice was non-cell autonomous as cultured primary epidermal keratinocytes from RelA^{E-MUT} mice showed reduced proliferation compared with controls. These results demonstrate that epidermal RelA specifically regulates delayed-type hypersensitivity-induced skin inflammation. In addition, we describe here an essential but nonspecific function of RelA in the protection of epidermal keratinocytes from apoptosis. Our study identifies functions of NF- κ B signaling in the epidermis and corroborates a specific role of epidermal keratinocytes in the regulation of skin inflammation.

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INTRODUCTION

Several human skin diseases have been associated with alterations in the activity of the NF- κ B pathway (Smahi *et al.*, 2000; Zonana *et al.*, 2000; Courtois *et al.*, 2003; Janssen *et al.*, 2004; Orstavik *et al.*, 2006; Vinolo *et al.*, 2006). This association is also supported by several studies in mice showing that both activation (Beg *et al.*, 1995a; Klement *et al.*, 1996; Rebholz *et al.*, 2007) and inhibition (Weih *et al.*, 1995; Barton *et al.*, 2000; Pasparakis *et al.*, 2002; Nenci *et al.*, 2006) of NF- κ B signaling can lead to skin inflammation.

Only one known human disease shows spontaneous skin inflammation owing to strong NF- κ B inhibition in epidermal keratinocytes caused by inhibitory mutations in Nf- κ B essential modulator (NEMO): incontinentia pigmenti (Schmidt-Suprian *et al.*, 2000; Smahi *et al.*, 2000). Other disease-associated polymorphisms within the tumor necrosis factor–NF- κ B signaling pathway do not seem to cause spontaneous inflammation. For e.g., epidermis-specific deletion of A20 (TNFAIP3), a regulator of NF- κ B activity, does not cause skin inflammation (Lippens *et al.*, 2011). To investigate how such

polymorphisms could contribute to the expression of an inflammatory skin phenotype, we have asked whether an epidermis-specific mutation that is functionally relevant to signal transduction via NF- κ B but does not cause an overt inflammatory skin phenotype could modify a clinically relevant type of skin immune response.

NF- κ B is a ubiquitous transcription factor that consists of homo- or hetero-dimeric subunits of RelA/p65, RelB, cRel, NF- κ B2/p52, and NF- κ B1/p50 (Verma *et al.*, 1995). Previous reports have suggested that embryonic keratinocytes isolated from mice with complete deficiency of the NF- κ B subunit RelA before E15.5 show cell autonomous hyperproliferation *in vitro* and *in vivo* (Zhang *et al.*, 2004).

The presence of NF- κ B subunits, such as RelA, in the epidermis inhibits epithelial cell death induced by Fas ligand, tumor necrosis factor- α , and microbes (Qin *et al.*, 1999; Seitz *et al.*, 2000; Li *et al.*, 2001). One mechanism by which NF- κ B protects cells from apoptosis is the induction of expression of anti-apoptotic genes such as cellular inhibitors of apoptosis and X-linked inhibitors of apoptosis (XIAPs) (Barkett and Gilmore, 1999; Bowen *et al.*, 2003; Hussain *et al.*, 2011; Meng *et al.*, 2012). One of the nuclear proteins binding to endogenous XIAP and antagonizing its anti-apoptotic functions is XIAP-associated factor 1 (XAF1). Overexpression of XAF1 sensitizes cells to apoptosis (Liston *et al.*, 2001) and reduced expression of XAF1 is frequently seen in tumors and cancer cell lines (Ng *et al.*, 2004; Plenchette *et al.*, 2007; Tu *et al.*, 2009).

Contact hypersensitivity/delayed-type hypersensitivity (DTH) is an immune reaction during which cytotoxic T lymphocytes

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Abbreviations: DTH, delayed-type hypersensitivity; XAF1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis

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attack epidermal keratinocytes in response to an antigen (Watanabe *et al.*, 2002). The role of skin-resident cells in the modulation of this response has not been thoroughly investigated. Nickel has been shown to induce NF- κ B in endothelial cells, facilitating the recruitment of leukocytes to the skin (Goebeler *et al.*, 1995, 2001; Bell *et al.*, 2003; Zhang *et al.*, 2010). In another model of contact allergy, RelB knockout mice showed reduced edema and inflammatory response (Weih *et al.*, 1995).

Cytokines of the S100 family, S100A8 (Calgranulin A) and S100A9 (Calgranulin B), are small calcium-binding proteins expressed by immune cells such as neutrophils and monocytes as well as by keratinocytes. They are associated with epidermal hyperproliferation during wound healing and in psoriasis (Thorey *et al.*, 2001; Eckert *et al.*, 2004). Calgranulins trigger the release of other pro-inflammatory factors from keratinocytes and promote their growth (Nukui *et al.*, 2008).

Using mice with partial functional inhibition of RelA in the epidermis, we describe here that NF- κ B activity in epidermal keratinocytes is specifically required to restrict the severity of a clinically relevant inflammatory skin phenotype.

RESULTS

Generation of mice with epidermis-specific expression of mutant RelA

We generated mice with epidermis-specific expression of a truncated, nonfunctional RelA mutant (RelA^{E-MUT} mice) by crossing mice carrying floxed *rela* alleles (Algul *et al.*, 2007) with transgenic mice expressing Cre recombinase under the

control of K14 promoter (Hafner *et al.*, 2004). Western blot analysis of epidermal lysates from RelA^{E-MUT} mice, but not from control mice, showed expression of a truncated version of RelA (Figure 1a), which has been described previously (Algul *et al.*, 2007). RelA^{E-MUT} mice were indistinguishable from wild-type controls (Figure 1b). Histological and immunohistological analyses revealed normal skin structure and normal expression of epidermal differentiation markers keratin 6 (K6), keratin 14 (K14), keratin 10 (K10), and Loricrin (Figure 1c and d).

Inflammatory response in the absence of epidermal RelA activity

RelA is known to control inflammatory functions in immune cells (Pittet *et al.*, 2011; Powolny-Budnicka *et al.*, 2011). To understand the function of RelA in epidermal keratinocytes during inflammation, we induced a DTH reaction against 1-fluoro-2,4-dinitrobenzene (DNFB) in control and RelA^{E-MUT} mice. Ear thickness was measured at sensitization and challenge as well as at 24, 48, 72, 96, and 120 hours after challenge. Both control and RelA^{E-MUT} mice showed increasing ear thickness with time after challenge until 96 hours. At 120 hours after challenge, ear thickness declined (Figure 2a). In RelA^{E-MUT} mice, however, ear thickness was significantly higher at all time points after challenge as compared with control mice (Figure 2a). Similar results were obtained when we used oxazolone as an antigen (Supplementary Figure S1d online), indicating that the differential response in RelA^{E-MUT} mice is not DNFB specific, but a broader phenomenon in the DTH response.

We asked whether enhanced inflammation in RelA^{E-MUT} mice was specific to the DTH response, and therefore induced

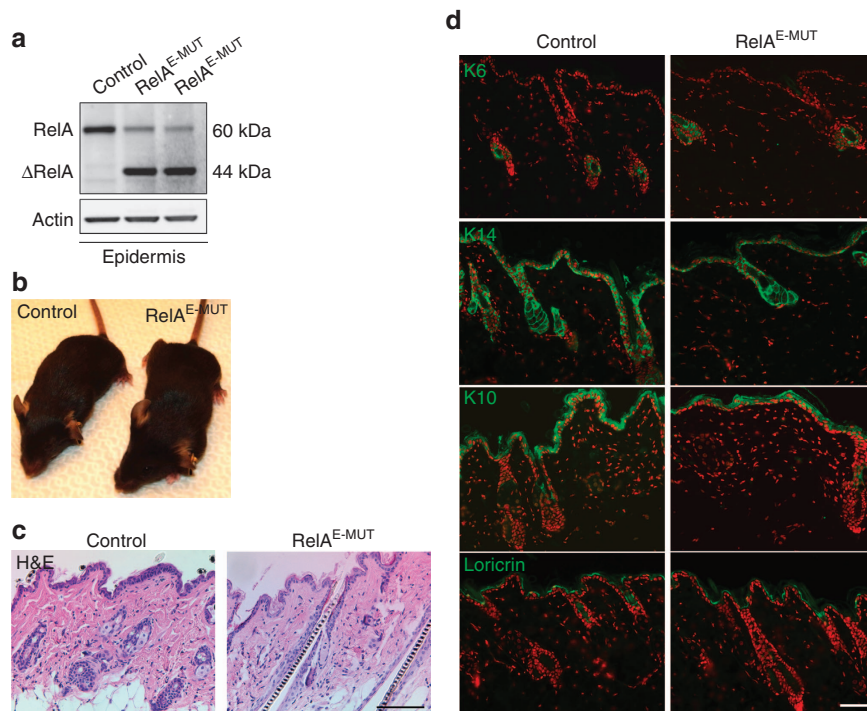


Figure 1. Absence of functionally active RelA from the epidermis does not cause a pathological skin phenotype. (a) Western blot of RelA protein on epidermal lysates from control and RelA^{E-MUT} mice. ΔRelA: truncated, functionally inactive mutant of RelA. (b–d) Macroscopic (b) and histological (c) appearance and keratin 10, keratin 14, and Loricrin staining (d) of skin of control and RelA^{E-MUT} mice at P 60. Scale bars in (b, c) are 100 μm. H&E, hematoxylin and eosin.

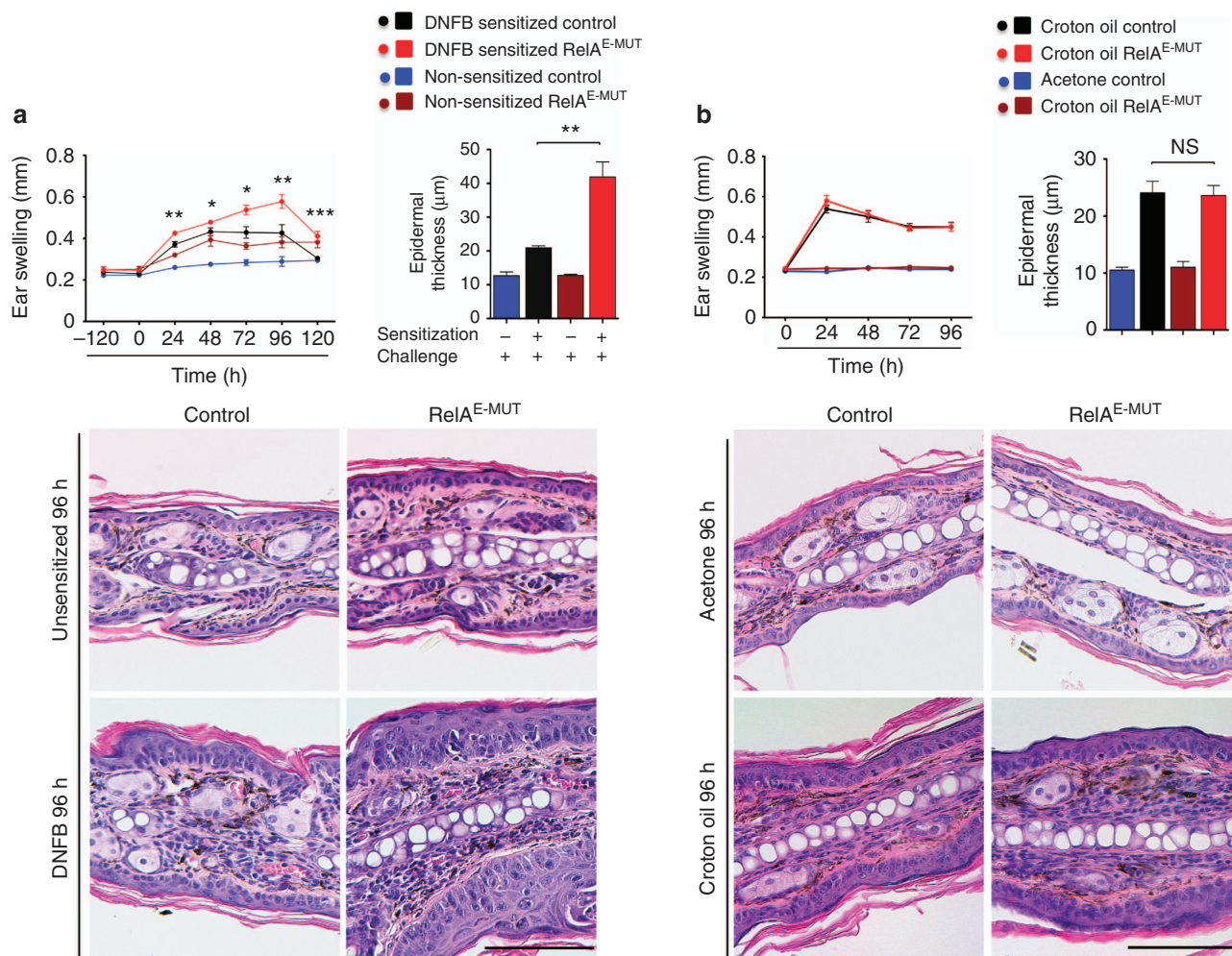


Figure 2. Loss of epidermal RelA activity aggravates inflammation and causes increased epidermal thickness in a delayed-type hypersensitivity response.

Ear thickness measurement after DNFB (0–120 h) or croton oil challenge (0–96 hours) as well as hematoxylin and eosin staining of ear sections and morphometric measurements of epidermal thickness at 96 hours after DNFB challenge (a) or croton oil application (b) in control (DNFB $n = 16$; croton oil $n = 14$) and RelA^{E-MUT} (DNFB $n = 17$; croton oil $n = 16$) mice. Experimental control groups of control ($n = 8$) and RelA^{E-MUT} ($n = 6$) mice were sensitized with acetone: olive oil and challenged with DNFB. Scale bar = 100 μm. NS, not significant.

an irritant contact dermatitis using croton oil. Ear thickness was measured in control and RelA^{E-MUT} mice until 96 hours after treatment. In contrast to the DTH response upon DNFB or oxazolone challenge, ear thickness after croton oil application was comparable in control and RelA^{E-MUT} mice at all time points measured (Figure 2b). These results show that the role of epidermal RelA in the regulation of skin inflammation is variable depending on the type of the inflammatory response.

To exclude an effect of epidermis-specific functional RelA deficiency in the sensitization process to DNFB, we isolated spleen and inguinal lymph node cells from vehicle (acetone:olive oil) and DNFB-sensitized mice. *In vitro* antigen stimulation of these cells after 24 hours showed similar numbers of IFN-γ-producing cells in both control and RelA^{E-MUT} mice (Supplementary Figure S1e online), indicating

that functional RelA deficiency in the epidermis did not influence sensitization to DNFB.

To determine whether hyperplasia of the epidermis (acanthosis) contributes to increased ear thickness in RelA^{E-MUT} mice, epidermal thickness of challenged control and RelA^{E-MUT} mice was measured microscopically. This measurement revealed that the epidermis of RelA^{E-MUT} mice was about two times thicker than that of control mice (Figure 2a). This difference was statistically significant. This increased epidermal thickness in RelA^{E-MUT} skin was due to keratinocyte hyperproliferation, as revealed by a marked increase in BrdU-positive epidermal keratinocytes compared with control mice (Supplementary Figure S1a online). We thus conclude that the observed increase in ear thickness in RelA^{E-MUT} mice is due to both stronger ear swelling and increased epidermal thickness.

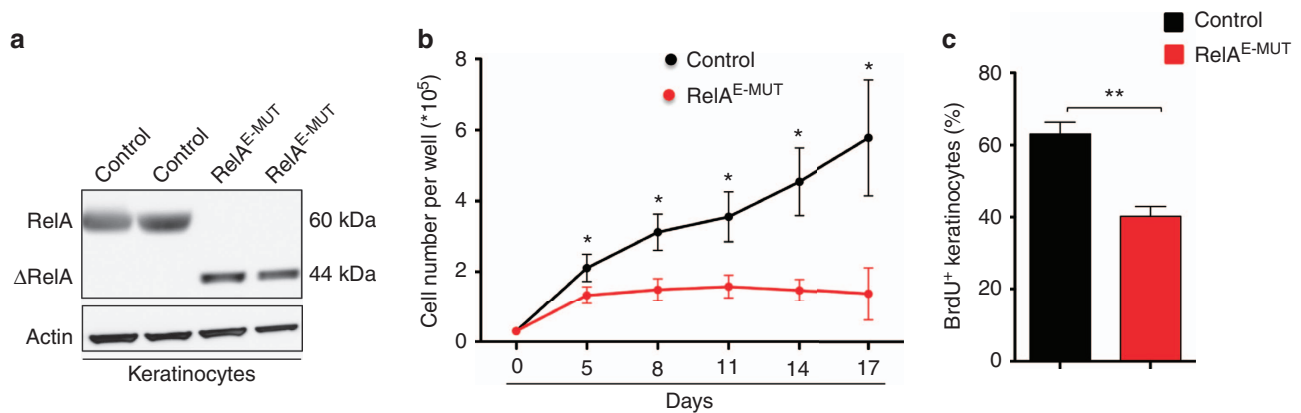


Figure 3. RelA-deficient keratinocytes show decreased proliferation *in vitro*. (a) Western blot analysis of RelA protein on lysates of primary keratinocytes isolated from control and RelA^{E-MUT} mice. (b) Growth curve of control ($n = 7$) and RelA-deficient keratinocytes ($n = 5$) in culture. (c) BrdU incorporation *in vitro* of control ($n = 5$) and RelA-deficient ($n = 4$) keratinocytes. Bars represent mean values $\pm$ SEM. Statistical significance was determined using Student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$).

Reduced *in vitro* keratinocyte proliferation in the absence of RelA activity

Previous studies have suggested a cell autonomous function of NF- κ B transcription factors in restricting keratinocyte proliferation before terminal differentiation (Zhang *et al.*, 2004). To address the question whether increased keratinocyte proliferation in RelA^{E-MUT} mice was cell autonomous, we isolated primary epidermal keratinocytes from control and RelA^{E-MUT} mice and obtained growth curves. Expression of mutant RelA was confirmed by western blot analysis (Figure 3a). Surprisingly, the growth rate of RelA-deficient keratinocytes in culture as well as BrdU incorporation were lower in RelA-deficient keratinocytes than in control keratinocytes (Figure 3b and c). Thus, RelA-deficient keratinocytes show reduced proliferation in culture. We therefore conclude that the increased epidermal thickness in RelA^{E-MUT} mice is not caused by cell autonomous keratinocyte hyperproliferation.

Differential regulation of S100 proteins in RelA^{E-MUT} mice

As the hyperproliferation of RelA^{E-MUT} keratinocytes was not cell autonomous, we asked whether differential immune cell recruitment to the inflamed ear skin of RelA^{E-MUT} mice could account for the observed differences. We stained sections of challenged ears with antibodies against surface markers of inflammatory cells, i.e., myeloid cells (CD11b), macrophages (F4/80), and T lymphocytes (CD3). In these stainings, both control and RelA^{E-MUT} mice showed a comparable pattern and density of immune cell infiltration after 96 hours (Supplementary Figure S1b online).

We therefore asked whether there were functional differences in the production of inflammatory mediators present in the skin during the DTH response. To identify such mediators, we performed microarray analysis on RNA from ear samples at 48 hours after challenge. This analysis revealed that two stress-induced proteins, S100A8 and S100A9, were upregulated in challenged ears of RelA^{E-MUT} mice in comparison with

challenged ears of control mice. This upregulation was confirmed by western blot analysis of protein lysates from DNFB-challenged ears at 48 and 96 hours (Figure 4a) and by immunostaining of skin sections at 96 hours (Figure 4b). In addition to higher numbers of S100A8-positive cells in the dermis, epidermal keratinocytes of RelA^{E-MUT} mice also consistently showed a stronger signal for S100A8 than did keratinocytes of control mice (Figure 4b). Untreated control and RelA^{E-MUT} mice did not show S100A8- and S100A9-positive cells in the skin (data not shown).

To investigate whether the upregulation of S100A8/A9 was DTH specific, we analyzed croton oil-treated ears after 48 hours and found increased expression of both S100A8 and S100A9 in RelA^{E-MUT} mice (Figure 4c). This shows that the absence of RelA function from epidermal keratinocytes results in increased production of S100A8 and S100A9 upon inflammation, regardless of the pro-inflammatory stimulus.

Enhanced apoptosis of RelA^{E-MUT} keratinocytes *in vivo* and *in vitro*

The NF- κ B activity has been implicated in the regulation of apoptosis in keratinocytes and many other cell types (Alcamo *et al.*, 2001; Li *et al.*, 2001). We therefore investigated the consequences of epidermal RelA deficiency for DTH-induced and croton oil-induced keratinocyte apoptosis.

At 96 hours after DNFB challenge or oxazolone challenge or croton oil application, the epidermis of RelA^{E-MUT} mice contained higher numbers of keratinocytes with eosinophilic cytoplasm and hyperchromatic, pycnotic nuclei as compared with those in control mice (Figure 5a; Supplementary Figure S1d online). These cells were morphologically highly similar to sunburn cells observed after UV irradiation of the skin (Bayerl *et al.*, 1995). Staining for cleaved, active caspase-3 confirmed an increased number of apoptotic epidermal keratinocytes in both DNFB-challenged and croton oil-treated RelA^{E-MUT} mice as compared with DNFB-challenged controls (Figure 5b). RelA^{E-MUT} mice without any treatment did not

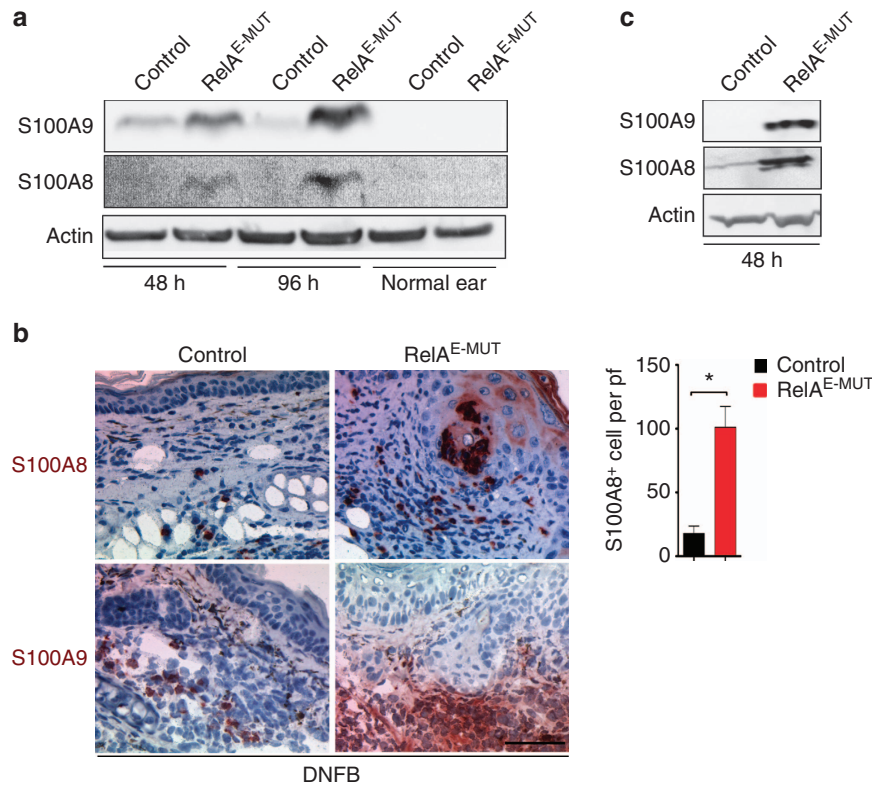


Figure 4. Differential expression of S100A8/9 proteins in RelA^{E-MUT} mice. (a, c) Western blot analysis of RelA protein on ear lysates of control and RelA^{E-MUT} mice 48 and 96 hours after DNFB challenge (a) and 48 hours of croton oil challenge (c). The result is representative for two independent experiments with similar results in a total of six control and six RelA^{E-MUT} mice. (b) Staining (left) and microscopic quantification of S100A8- and S100A9-positive cells per power field (pf) on ear skin sections from control (n = 4) and RelA^{E-MUT} (n = 4) mice 96 hours after DNFB challenge. Nuclei (blue) were visualized by hematoxylin staining. Bars represent mean values $\pm$ SEM. Statistical significance was determined using Student's *t*-test (**P* $\leq$ 0.05). Scale bar in (b) is 50 μ m.

show sunburn cells or caspase-3-positive cells in the epidermis, similar to control mice (Supplementary Figure S1c online). This shows that apoptosis of epidermal keratinocytes is enhanced in the inflamed skin of RelA^{E-MUT} mice, regardless of the nature of the pro-inflammatory stimulus.

Although it is well established that the activity of the NF- κ B signaling pathway protects cells from apoptosis, the mechanisms involved remain unidentified. In our microarray analysis mentioned before, we found that the XAF1 was differentially expressed in the DNFB-challenged ears of RelA^{E-MUT} mice and control mice. This differential expression of XAF1 was confirmed by western blot analysis of protein lysates of ears of RelA^{E-MUT} and control mice 48 and 96 hours after DNFB challenge and also after croton oil treatment (Figure 5c). We conclude that, in the absence of RelA from epidermal keratinocytes, XAF1 is upregulated in inflamed skin. This could be a cause for the observed increase in apoptosis in RelA-deficient epidermal keratinocytes.

DISCUSSION

The NF- κ B signaling pathway is known to be a major regulator of organ inflammation. It is thus not surprising that genome-wide association studies in the chronic inflammatory skin disease psoriasis have revealed linkage with polymorphisms

within this pathway (Nair *et al.*, 2009). These studies cannot explain, however, in which way these polymorphisms could functionally contribute to the pathogenesis of skin inflammation. In this study, we have used genetically modified mice in which exons 7–10 of the *rela* gene were deleted, resulting in the translation of a truncated, nonfunctional mutant of RelA and thereby partial inhibition of NF- κ B signaling (Algul *et al.*, 2007). Unlike other inhibitory mutations within the NF- κ B signaling pathway (Pasparakis *et al.*, 2002; Nenci *et al.*, 2006), this epidermis-specific genetic modification does not result in the spontaneous development of an inflammatory skin phenotype. To analyze whether such partial inhibition of NF- κ B signaling in epidermal keratinocytes can contribute to the pathogenesis of clinically relevant skin inflammation, we have tested the effect of the *rela* mutation on the outcome of DTH and irritative responses of the skin.

To our surprise, RelA^{E-MUT} mice did not show an overt phenotype. Previous work has suggested that, in the epidermis, NF- κ B is responsible for the regulation of the transition of basal, proliferating keratinocytes to suprabasal, differentiating cells. This has been concluded from *in vitro* experiments with cultured epidermal keratinocytes retrovirally transduced with DNA fragments coding for mutant variants of the NF- κ B proteins p50 and p65 (Seitz *et al.*, 1998). In addition,

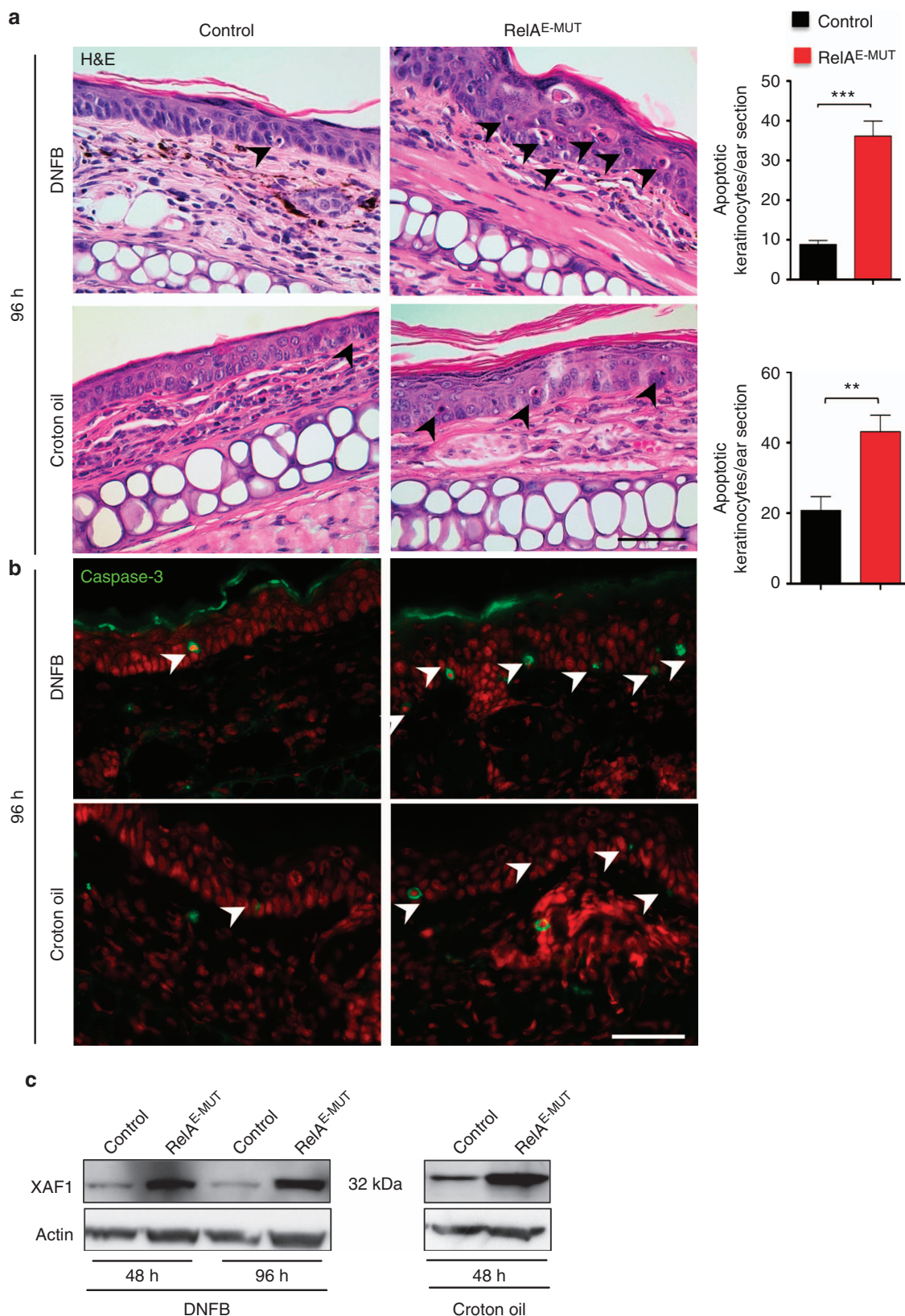


Figure 5. Keratinocyte apoptosis and XIAP-associated factor 1 (XAF1) expression in RelA^{E-MUT} epidermis. Numbers of sunburn cells per ear section (a) and caspase-3 staining (b) of control ($n=9$) and RelA^{E-MUT} ($n=8$) mice 96 hours after DNFB challenge/croton oil treatment. (c) Western blot analysis of XAF1 expression in DNFB (left) or croton oil–treated control ($n=4$) and RelA^{E-MUT} ($n=4$) mice. Bars represent mean values $\pm$ SEM. Statistical significance was determined using Student's t -test (** $P \leq 0.01$, *** $P \leq 0.001$). Scale bars in (a, b) are 50 μ m. H&E, hematoxylin and eosin.

transplanted RelA-deficient embryonic keratinocytes gave rise to a hyperplastic epidermis in severe combined immunodeficient mice (Zhang *et al.*, 2004). In contrast to the results previously achieved with cultured and transplanted keratinocytes, our mice with epidermis-specific abrogation of RelA function (RelA^{E-MUT} mice) did not show a disturbed balance of keratinocyte proliferation and differentiation within the epidermis. The absence of an overt disturbance in epidermal homeostasis is also a feature of mice in which RelA was deleted in an epidermis-specific manner using a different targeting strategy (Manolis Pasparakis, personal communication). In addition, epidermal keratinocytes with inhibited RelA function in culture exhibited a decrease, instead of increase, in cell proliferation and incorporation of BrdU as compared with control keratinocytes. This shows that RelA is not required for the growth arrest of epidermal keratinocytes but could even have a growth-promoting role, at least in culture. In addition, RelA function is dispensable for the formation of a normal epidermis. This obvious difference to the previously published results can be explained in different ways: first, expression of dominant negative NF- κ B mutants using retroviral constructs probably leads to a more broad suppression of NF- κ B activity, whereas the loss of RelA function in RelA^{E-MUT} mice is more specific and would allow for compensation by other NF- κ B subunits, e.g., c-Rel. Second, transplantation of skin inevitably causes a wound-healing reaction of the skin, which creates an extracellular environment entirely different from resting skin. Therefore, our knockout model is more representative for resting skin than a transplantation model. On the other hand, we show that, within an inflammatory environment, the RelA-negative epidermis shows a different behavior than wild-type epidermis, i.e., hyperplasia. Thus, our results are not necessarily inconsistent with those of Zhang *et al.* (2004) as absence or functional impairment of RelA in an altered cytokine milieu shows epidermal hyperplasia in both models. This would also be in agreement with the previous work of Beg *et al.* (1995b), which suggests that RelA controls expression of inducible NF- κ B-regulated genes, but not basal gene expression.

NF- κ B is known to regulate organ inflammation. Conventionally, the onset or enhancement of an inflammatory response has been linked to activation of NF- κ B signaling. Here, we show that it is not activation but functional inhibition of RelA in the epidermis that causes an enhancement of skin inflammation, as evidenced by increased ear swelling and epidermal hyperplasia. How could inhibition of RelA function in epidermal keratinocytes lead to an enhanced DTH response?

One conceivable scenario would be that the RelA-deficient epidermis alters the efficiency of sensitization to the haptens used. Our enzyme-linked immunosorbent spot analysis has, however, revealed that there are no significant differences in the numbers of hapten-specific T cells in the spleens and lymph nodes of RelA^{E-MUT} mice and control mice (see Supplementary Figure S1 online). Thus, increased inflammation in RelA^{E-MUT} mice cannot be explained by differences in the sensitization phase. As functional inhibition of RelA in our mice is epidermis specific, epidermal keratinocytes must be

the target and possibly also the source of one or several factors that modify their proliferative response to the inflammatory environment, which is determined by the presence of hapten-specific T cells. Therefore, the altered proliferative response in the RelA^{E-MUT} epidermis is the consequence of a direct or an indirect communication between hapten-specific T cells and epidermal keratinocytes. As these hapten-specific T cells are not present in croton oil-induced inflammation, there is no difference in the proliferative response of the epidermis.

The fact that functional RelA impairment by itself does not lead to hyperproliferation of the epidermis suggests that epidermal hyperplasia observed in RelA^{E-MUT} mice is due to an indirect mechanism, which involves factors present in the extracellular milieu during the inflammatory response. In our attempt to identify such factors, we focused on differences in the regulation of pro-inflammatory cytokines. Interestingly, upon DNFB challenge, we observed differences in the expression of S100A8 and S100A9 proteins in both keratinocytes as well as in inflammatory cells in the dermis, which are, according to morphological parameters, granulocytes. S100A8 and S100A9 proteins are produced by neutrophils, monocytes, and keratinocytes and are known as pro-inflammatory and chemotactic mediators in the effector phase of organ inflammation (Eckert *et al.*, 2004). They also stimulate keratinocyte growth (Nukui *et al.*, 2008). However, as the expression of S100 proteins was also increased in croton oil-treated ears, which did not show increased swelling and thickening of the epidermis (Figures 2b and 4c), their expression is obviously not sufficient to cause these inflammatory changes.

Nonetheless, our results provide further insights into the regulation of S100A8/ A9 by NF- κ B (Nemeth *et al.*, 2009) and suggest that the presence of epidermal RelA is important to control their production in skin, which could be one of the mechanisms to control the amplitude of an inflammatory response. This finding is in line with previous results showing upregulation of S100 proteins in the skin of mice with epidermis-specific deletion of I κ B kinase 2 (Benoit *et al.*, 2006). As S100A8 and S100A9 have recently been shown to contribute to the pro-inflammatory cross talk between the epidermis and dermis in psoriasis (Lee *et al.*, 2012), the regulatory function of NF- κ B on their expression could be a general mechanism contributing to the maintenance of skin homeostasis (Pasparakis *et al.*, 2002; Stratis *et al.*, 2006a,b).

Our study also reveals a critical role of RelA in protecting keratinocytes from inflammation-induced apoptosis. This is in accordance with described functions of NF- κ B in the protection from cell death (Beg *et al.*, 1995b; Seitz *et al.*, 2000). NF- κ B regulates expression of anti-apoptotic genes such as cellular inhibitors of apoptosis and XIAPs (Barkett and Gilmore, 1999). Expression analysis of apoptosis-related genes revealed an increased expression of XAF1 in challenged ears of RelA^{E-MUT} mice. XAF1 binds XIAP and attenuates its anti-apoptotic function, probably by sequestering XIAP in nuclear inclusions and inhibiting its anti-caspase activity (Liston *et al.*, 2001). This sensitizes cells to undergo apoptosis whenever XAF1 is overexpressed. Such a mechanism could serve as a plausible explanation for the increase in apoptosis observed

in challenged RelA^{E-MUT} mice. It is conceivable that, under inflammatory conditions, XAF1 may sensitize keratinocytes to death receptor ligands known to be active in contact allergy, such as tumor necrosis factor-related apoptosis-inducing ligand or Fas ligand (Trautmann *et al.*, 2000; Schmidt *et al.*, 2010). However, we have shown previously that deletion of Fas from epidermal keratinocytes did not reduce keratinocyte apoptosis in contact allergy and upon UVB irradiation in mice (Hedrych-Ozimina *et al.*, 2011).

Understanding the interplay between extracellular factors and components of the NF- κ B signaling pathway in the regulation of inflammatory and apoptotic responses in the epidermis will be of great relevance to understand and successfully treat potentially life-threatening skin conditions such as drug reactions and graft versus host disease (Gilliam *et al.*, 1996; Lissia *et al.*, 2010).

MATERIALS AND METHODS

Mice

All mice were maintained as per institutional guidelines. RelA-floxed mice (Algul *et al.*, 2007) and K14 promoter-driven Cre mice (Hafner *et al.*, 2004) were maintained on C57Bl/6 background. Genotyping was performed by PCR as described previously (Rebholz *et al.*, 2007). Expression of mutant RelA was tested by western blotting.

Induction of a DTH response by DNFB and oxazolone challenge and contact dermatitis by croton oil application

All experiments were performed on age-matched 8–10-week-old female mice. To induce the DTH response, mice were anesthetized on day 0 and the thickness of the ear was measured using a digital caliper (Mitutoyo, Kawasaki, Japan). Subsequently, 30 μ l of a 0.5% DNFB solution or 100 mg ml⁻¹ oxazolone in acetone/olive oil (4:1) was applied to the shaved abdomen for sensitization. On day 6, mice were anesthetized and ear thickness was measured. Subsequently, mice were challenged with 10 μ l 0.2% DNFB or 10 mg ml⁻¹ oxazolone in acetone/olive oil (4:1) on both sides of the ear.

To induce irritative contact dermatitis, mice were anesthetized and the thickness of the ear was measured. Subsequently, 5 μ l of 1% croton oil in acetone was applied on both sides of the ear.

Ear thickness was determined after 24, 48, 72, 96, or 120 hours of challenge/ croton oil treatment using a digital caliper. Each measurement was performed three times and the mean was calculated.

Keratinocyte isolation and analysis of proliferation and apoptosis

Primary keratinocytes were isolated from newborn pups at P1 and cultured as described previously (Tscharntke *et al.*, 2007).

For growth curves, 30,000 primary keratinocytes were seeded per well of a collagen-coated 24-well plate followed by trypsinization and counting of cell numbers at different time points.

For BrdU incorporation, cells were cultured for 2 days on collagen-coated coverslips and treated with BrdU (1 μ M) for 30 minutes at 37 °C followed by fixation, immunostaining for BrdU, and nuclear staining with 4',6-diamidino-2-phenylindole. Positive cells were counted and expressed as a ratio of BrdU-positive cells to the total cell population.

Immunostaining

K14, K10, Loricrin (Covance, Princeton, NJ, 1:1,000, 1:100, 1:500, respectively), CD11b (eBiosciences, Frankfurt, Germany, 1:500),

F4/80 (AbD Serotech, Oxford, UK, 1:50), CD3 (Chemicon, Temecula, CA, 1:1,000), and Caspase-3 (Cell Signaling Technology, Denver, CO, 1:200) stainings were performed on cryosections as described before (Stratis *et al.*, 2006b; Hedrych-Ozimina *et al.*, 2011). Nuclei were stained with 4',6-diamidino-2-phenylindole. S100A8 staining (Abcam, Cambridge, UK, 1:200) on paraffin sections and S100A9 staining (Abcam, 1:100) on acetone-fixed frozen sections were performed after incubation with an endogenous peroxidase blocker (Dako, Hamburg, Germany) and 10% goat serum, followed by visualization with HRP-AEC (Dako) and hematoxylin and eosin counterstaining. BrdU staining on tissue sections was performed as described previously (Behrendt *et al.*, 2012).

Cleaved caspase-3 was stained on 4% paraformaldehyde-fixed keratinocytes, permeabilized and blocked with 0.2% Triton-X and 10% goat normal serum in 1% BSA/0.2% Triton-X/phosphate-buffered saline using a primary antibody against cleaved caspase-3 (Cell Signaling Technology, 1:200) and Alexa-488-conjugated anti-rabbit IgG (Molecular Probe, Paisley, UK). Nuclei were stained with 4',6-diamidino-2-phenylindole.

Western blot

Epidermis and ear skin samples were homogenized using a mixer mill MM 400 (Retsch GmbH, Haan, Germany) and extracted in RIPA⁺ buffer (50 mM Tris, 5 mM EDTA, 1% Triton-X-100, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg ml⁻¹ soybean trypsin inhibitor, 0.5 mM NaVO₃, and 10 mg ml⁻¹ *p*-nitrophenylphosphate). Cultured keratinocytes were lysed *in situ* using the same buffer. Lysates were centrifuged at 16,000g for 10 minutes at 4 °C and supernatants were used for western blot after standard protein quantification.

In all, 30–50 μ g of protein was resolved on 4–12% NuPAGE SDS-PAGE (Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose or polyvinylidene fluoride membranes using the iBlot transfer system (Invitrogen). After blocking with Roche block (Roche Applied Science, Indianapolis, IN, 1:10) the membrane was probed with polyclonal rabbit anti-p65 (Cell signaling Technology, 1:1,000) or anti-XAF1 (Abcam, 1:500), monoclonal rabbit anti-S100A8 or anti-S100A9 (Abcam, 1:2,000 and 1:1,000, respectively), and monoclonal mouse anti-actin (MP Biomedicals, Aurora, OH, 1:6,000) antibodies. Immunoreactive proteins were visualized by chemiluminescence (PerkinElmer, Waltham, MA; Pierce Thermo Fisher Scientific, Rockford, IL) on an Uvichemi chemiluminescence documentation system (Biometra, Cambridge, UK).

IFN- γ enzyme-linked immunosorbent spot assay

Isolated inguinal lymph node cells and splenocytes were cultured with or without 100 μ g ml⁻¹ 2,4-dinitrobenzenesulfonic acid for 24 hours in RPMI medium supplemented with 10% fetal calf serum. For detection, the Mouse IFN- γ ELISPOT kit (R&D Systems, Minneapolis, MN) was used.

Microarray analysis

Microarray data were generated using the Agilent whole mouse-genome oligo microarrays (one color, Miltenyi Biotec, Cologne, Germany). The method and the raw data are submitted to the NCBI Gene Expression Omnibus with accession number GSE56067.

Statistical analysis

Significance of differences between the samples was calculated using the Student's *t*-test. A *P*-value ≤ 0.05 was considered as statistically significant. **P*-value ≤ 0.05 , ***P*-value ≤ 0.01 , ****P*-value ≤ 0.001 .

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Alcamo E, Mizgerd JP, Horwitz BH *et al.* (2001) Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. *J Immunol* 167:1592–600
- Algul H, Treiber M, Lesina M *et al.* (2007) Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. *J Clin Invest* 117:1490–501
- Barkett M, Gilmore TD (1999) Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 18:6910–24
- Barton D, HogenEsch H, Weih F (2000) Mice lacking the transcription factor RelB develop T cell-dependent skin lesions similar to human atopic dermatitis. *Eur J Immunol* 30:2323–32
- Bayerl C, Taake S, Moll I *et al.* (1995) Characterization of sunburn cells after exposure to ultraviolet light. *Photodermatol Photoimmunol Photomed* 11:149–54
- Beg AA, Sha WC, Bronson RT *et al.* (1995a) Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev* 9:2736–46
- Beg AA, Sha WC, Bronson RT *et al.* (1995b) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376:167–70
- Behrendt K, Klatte J, Pofahl R *et al.* (2012) A function for Rac1 in the terminal differentiation and pigmentation of hair. *J Cell Sci* 125:896–905
- Bell S, Degitz K, Quirling M *et al.* (2003) Involvement of NF-kappaB signalling in skin physiology and disease. *Cell Signal* 15:1–7
- Benoit S, Toksoy A, Ahlmann M *et al.* (2006) Elevated serum levels of calcium-binding S100 proteins A8 and A9 reflect disease activity and abnormal differentiation of keratinocytes in psoriasis. *Br J Dermatol* 155:62–6
- Bowen AR, Hanks AN, Allen SM *et al.* (2003) Apoptosis regulators and responses in human melanocytic and keratinocytic cells. *J Invest Dermatol* 120:48–55
- Courtois G, Smahi A, Reichenbach J *et al.* (2003) A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. *J Clin Invest* 112:1108–15
- Eckert RL, Broome AM, Ruse M *et al.* (2004) S100 proteins in the epidermis. *J Clin Invest Dermatol* 123:23–33
- Gilliam AC, Whitaker-Menezes D, Korngold R *et al.* (1996) Apoptosis is the predominant form of epithelial target cell injury in acute experimental graft-versus-host disease. *J Clin Invest Dermatol* 107:377–83
- Goebeler M, Gillitzer R, Kilian K *et al.* (2001) Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. *Blood* 97:46–55
- Goebeler M, Roth J, Brocker EB *et al.* (1995) Activation of nuclear factor-kappa B and gene expression in human endothelial cells by the common haptens nickel and cobalt. *J Immunol* 155:2459–67
- Hafner M, Wenk J, Nenci A *et al.* (2004) Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. *Genesis* 38:176–81
- Hedrych-Ozimina A, Behrendt K, Hao Z *et al.* (2011) Enhanced contact allergen- and UVB-induced keratinocyte apoptosis in the absence of CD95/Fas/Apo-1. *Cell Death Differ* 18:155–63
- Hussain I, Fathallah I, Accardi R *et al.* (2011) NF-kappaB protects human papillomavirus type 38 E6/E7-immortalized human keratinocytes against tumor necrosis factor alpha and UV-mediated apoptosis. *J Virol* 85:9013–22
- Janssen R, van Wengen A, Hoeve MA *et al.* (2004) The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes. *J Exp Med* 200:559–68
- Klement JF, Rice NR, Car BD *et al.* (1996) IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. *Mol Cell Biol* 16:2341–9
- Lee Y, Jang S, Min JK *et al.* (2012) S100A8 and S100A9 are messengers in the crosstalk between epidermis and dermis modulating a psoriatic milieu in human skin. *Biochem Biophys Res Commun* 423:647–53
- Li M, Shillinglaw W, Henzel WJ *et al.* (2001) The RelA(p65) subunit of NF-kappaB is essential for inhibiting double-stranded RNA-induced cytotoxicity. *J Biol Chem* 276:1185–94
- Lippens S, Lefebvre S, Gilbert B *et al.* (2011) Keratinocyte-specific ablation of the NF-kappaB regulatory protein A20 (TNFAIP3) reveals a role in the control of epidermal homeostasis. *Cell Death Differ* 18:1845–53
- Lissia M, Mulas P, Bulla A *et al.* (2010) Toxic epidermal necrolysis (Lyell's disease). *Burns* 36:152–63
- Liston P, Fong WG, Kelly NL *et al.* (2001) Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. *Nat Cell Biol* 3:128–33
- Meng Z, Lou S, Tan J *et al.* (2012) Nuclear factor-kappa B inhibition can enhance apoptosis of differentiated thyroid cancer cells induced by 131I. *PLoS One* 7:e33597
- Nair RP, Duffin KC, Helms C *et al.* (2009) Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. *Nat Genet* 41:199–204
- Nemeth J, Stein I, Haag D *et al.* (2009) S100A8 and S100A9 are novel nuclear factor kappa B target genes during malignant progression of murine and human liver carcinogenesis. *Hepatology* 50:1251–62
- Nenci A, Huth M, Funte A *et al.* (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* 15:531–42
- Ng KC, Campos EI, Martinka M *et al.* (2004) XAF1 expression is significantly reduced in human melanoma. *J Invest Dermatol* 123:1127–34
- Nukui T, Ehama R, Sakaguchi M *et al.* (2008) S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. *J Cell Biochem* 104:453–64
- Orstavik KH, Kristiansen M, Knudsen GP *et al.* (2006) Novel splicing mutation in the NEMO (IKK-gamma) gene with severe immunodeficiency and heterogeneity of X-chromosome inactivation. *Am J Med Genet A* 140:31–9
- Pasparakis M, Courtois G, Hafner M *et al.* (2002) TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417:861–6
- Pittet LA, Quinton LJ, Yamamoto K *et al.* (2011) Earliest innate immune responses require macrophage RelA during pneumococcal pneumonia. *Am J Respir Cell Mol Biol* 45:573–81
- Plenchette S, Cheung HH, Fong WG *et al.* (2007) The role of XAF1 in cancer. *Curr Opin Invest Drugs* 8:469–76
- Powlony-Budnicka I, Riemann M, Tanzer S *et al.* (2011) RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells. *Immunity* 34:364–74
- Qin JZ, Chaturvedi V, Denning MF *et al.* (1999) Role of NF-kappaB in the apoptotic-resistant phenotype of keratinocytes. *J Biol Chem* 274:37957–64
- Rebholz B, Haase I, Eckelt B *et al.* (2007) Crosstalk between keratinocytes and adaptive immune cells in an IkappaBalpha protein-mediated inflammatory disease of the skin. *Immunity* 27:296–307

- Schmidt M, Hupe M, Endres N *et al.* (2010) The contact allergen nickel sensitizes primary human endothelial cells and keratinocytes to TRAIL-mediated apoptosis. *J Cell Mol Med* 14:1760–76
- Schmidt-Suppran M, Bloch W, Courtois G *et al.* (2000) NEMO/IKK gamma-deficient mice model incontinentia pigmenti. *Mol Cell* 5: 981–92
- Seitz CS, Freiberg RA, Hinata K *et al.* (2000) NF-kappaB determines localization and features of cell death in epidermis. *J Clin Invest* 105:253–60
- Seitz CS, Lin Q, Deng H *et al.* (1998) Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proc Natl Acad Sci USA* 95:2307–12
- Smahi A, Courtois G, Vabres P *et al.* (2000) Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* 405:466–72
- Stratis A, Pasparakis M, Markur D *et al.* (2006a) Localized inflammatory skin disease following inducible ablation of I kappa B kinase 2 in murine epidermis. *J Invest Dermatol* 126:614–20
- Stratis A, Pasparakis M, Rupec RA *et al.* (2006b) Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation. *J Clin Invest* 116:2094–104
- Thorey IS, Roth J, Regenbogen J *et al.* (2001) The Ca2+-binding proteins S100A8 and S100A9 are encoded by novel injury-regulated genes. *J Biol Chem* 276:35818–25
- Trautmann A, Akdis M, Kleemann D *et al.* (2000) T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J Clin Invest* 106:25–35
- Tscharntke M, Pofahl R, Chrostek-Grashoff A *et al.* (2007) Impaired epidermal wound healing in vivo upon inhibition or deletion of Rac1. *J Cell Sci* 120:1480–90
- Tu SP, Liston P, Cui JT *et al.* (2009) Restoration of XAF1 expression induces apoptosis and inhibits tumor growth in gastric cancer. *Int J Cancer* 125:688–97
- Verma IM, Stevenson JK, Schwarz EM *et al.* (1995) Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* 9:2723–35
- Vinolo E, Sebban H, Chaffotte A *et al.* (2006) A point mutation in NEMO associated with anhidrotic ectodermal dysplasia with immunodeficiency pathology results in destabilization of the oligomer and reduces lipopolysaccharide- and tumor necrosis factor-mediated NF-kappa B activation. *J Biol Chem* 281:6334–48
- Watanabe H, Unger M, Tuvel B *et al.* (2002) Contact hypersensitivity: the mechanism of immune responses and T cell balance. *J Interferon Cytokine Res* 22:407–12
- Weih F, Carrasco D, Durham SK *et al.* (1995) Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. *Cell* 80:331–40
- Zhang J, Li L, Lu Q *et al.* (2010) Acute stress enhances contact dermatitis by promoting nuclear factor-kappaB DNA-binding activity and interleukin-18 expression in mice. *J Dermatol* 37:512–21
- Zhang JY, Green CL, Tao S *et al.* (2004) NF-kappaB RelA opposes epidermal proliferation driven by TNFR1 and JNK. *Genes Dev* 18:17–22
- Zonana J, Elder ME, Schneider LC *et al.* (2000) A novel X-linked disorder of immune deficiency and hypohidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). *Am J Hum Genet* 67:1555–62

Supplemental Material

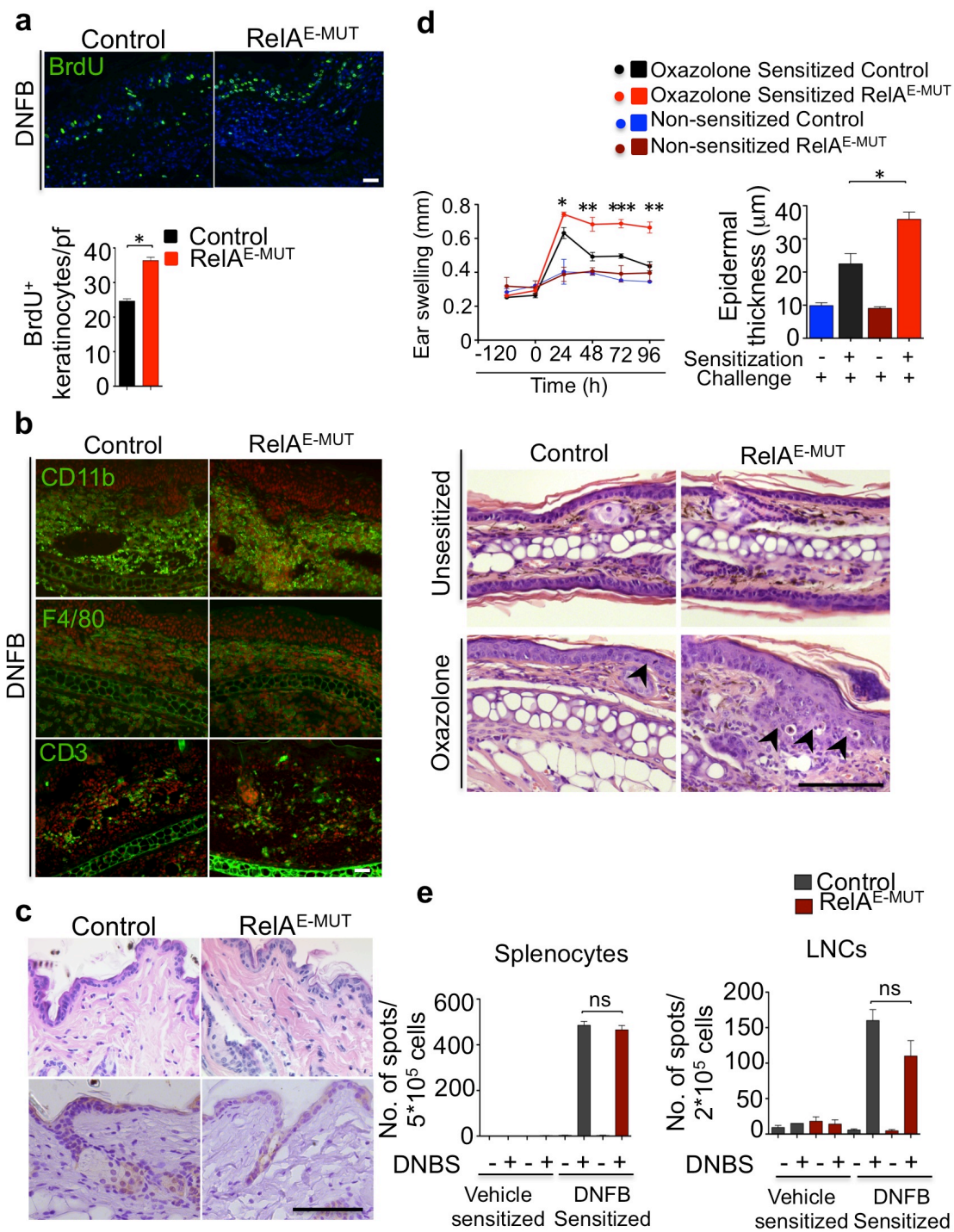


Fig. S1: Keratinocyte proliferation, inflammatory cell invasion and quantification of hapten- specific T cells in DNFB treated mice and ear swelling/ epidermal thickness of RelA^{E-MUT} and control mice in oxazolone-induced DTH

(a, b) Quantification of BrdU positive (green) epidermal cells (a) and immunostaining for CD11b, F4/80 and CD3 (b) on ear sections of control and RelA^{E-MUT} mice 96 after DNFB challenge. Nuclei were visualized with DAPI (a) or propidium iodide (b). Scale bars are 50 μ m. **(c)** H& E (top) and caspase-3 (bottom) staining on skin sections from untreated control and RelA^{E-MUT} mice. Note the absence of spontaneous apoptosis **(d)** Ear swelling response, H&E staining of ear sections and morphometric measurements of epidermal thickness of ears 0- 96 h after oxazolone challenge in control (n =4) and RelA^{E-MUT} (n =4) mice. Non- sensitized controls were sensitized with vehicle only and challenged with oxazolone. Graphs represent mean values $\pm$ SEM. Statistical significance was determined using the Student's *t* test (**P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001). Scale bars are 100 μ m. **(e)** Number of spots representing IFN- γ producing splenocytes per 5×10^5 total cells and IFN- γ producing lymph node cells per 2×10^5 total cells, isolated from vehicle sensitized or DNFB sensitized mice and stimulated *in vitro* with or without DNBS for 24 h. Numbers of spots were counted after assay development in duplicate wells from 4 control and 4 RelA^{E-MUT} mice. Bars represent mean values $\pm$ SEM.