Localized Inflammatory Skin Disease Following Inducible Ablation of I Kappa B Kinase 2 in Murine Epidermis

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Skin inflammation is a complex process that involves interactions between various cell types residing in different skin compartments. Using mice with conditionally targeted I kappa B kinase 2 (IKK2) alleles, we have previously shown that epidermal keratinocytes can play a dominant role in the initiation of an inflammatory reaction. In order to investigate long-term consequences of IKK2 deletion in adult skin, we have generated mice with floxed IKK2 alleles in which expression of a Tamoxifen-inducible Cre recombinase construct is targeted to epidermal keratinocytes (K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice). K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice are born normally and do not show signs of a skin disease until the age of 6 months. Deletion of IKK2 can be observed after Tamoxifen application to the back skin or spontaneously, without Tamoxifen application, in mice older than 6 months. This deletion is accompanied by dramatic, localized skin changes that are characterized by invasion of inflammatory cells, hair follicle disruption, and pseudoepitheliomatous hyperplasia of the epidermis, but not by tumor formation. The hyperplastic epithelium shows increased phosphorylation of signal transducer and activator of transcription 3 and extracellular signal-regulated protein kinase 1/2, typical features of psoriatic epidermis. Our results identify a primary role for IKK2 in the development of skin inflammation and confirm its requirement for the maintenance of skin homeostasis.

Journal of Investigative Dermatology (2006) **126,** 614–620. doi:10.1038/sj.jid.5700092; published online 5 January 2006

INTRODUCTION

Epidermal keratinocytes form the first line of defence to protect the organism against harmful influences of the environment. Recent evidence suggests that epidermal keratinocytes do not only provide mechanical stability and a barrier for liquids but also exert functions in the regulation of immune reactions of the skin. For example, forced activation of a known proinflammatory signaling pathway selectively in epidermal keratinocytes resulted in a psoriasis-like inflammatory skin disease in mice with transgenic expression of a mutant variant of signal transducer and activator of transcription 3 (STAT3) under the control of the keratin 5 promoter (Sano *et al.*, 2005). A primary role of epidermal keratinocytes in the regulation of skin inflammation has also been suggested by our previous results showing that conditional ablation of I kappa B kinase 2 (IKK2) from epidermal keratinocytes leads to the development of a T-cell-independent inflammatory skin disease that requires the presence of the type I receptor for tumor necrosis factor (Pasparakis *et al.*, 2002). IKK2 is a catalytic subunit of the IκB kinase complex that phosphorylates NF-κB-bound IκBs, thus targeting them for ubiquitin-mediated degradation (Karin and Ben-Neriah, 2000; Greten *et al.*, 2004). It has been implicated in the development of malignant epithelial tumors in a mouse model of colitis-associated cancer (Greten *et al.*, 2004). In this model, tissue-specific deletion of IKK2 either in the colon epithelium or in the associated inflammatory infiltrate resulted in impaired NF-κB activation and simultaneously reduced incidence and size of tumors. In contrast, inhibition of NF-κB activation in epidermal keratinocytes was associated with the spontaneous occurrence and promotion of epidermal tumors (Seitz *et al.*, 1998; van Hogerlinden *et al.*, 1999; Dajee *et al.*, 2003). Recently, using transgenic mice that express a nondegradable IκBα mutant, a connection has been proposed between chronic skin inflammation following inhibition of NF-κB activity in the epidermis and the development of squamous cell carcinomas (van Hogerlinden *et al.*, 2004).

Mice with epidermis-specific deletion of IKK2 die by the age of 10 days and therefore do not allow one to investigate the effects of loss of IKK2 in adult murine skin. In order to

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Abbreviations: ERK, extracellular signal-regulated protein kinase; IKK2, I kappa B kinase 2; STAT3, signal transducer and activator of transcription 3

Received 13 June 2005; revised 9 July 2005; accepted 10 September 2005; published online 5 January 2006

delete IKK2 in epidermal keratinocytes in a timely and spatially controlled manner, we generated mice that harbor IKK2 alleles with loxP sites (Pasparakis *et al.*, 2002) and express an inducible Cre recombinase construct under the control of the keratin 14 promoter (Indra *et al.*, 2000).

RESULTS

Spontaneous Cre-mediated deletion and development of skin lesions in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

In order to allow for temporally controlled Cre-mediated IKK2 deletion in epidermal keratinocytes, we crossed mice homozygous for floxed IKK2 alleles (IKK2$^{fl/fl}$ mice) to mice that express the Tamoxifen-dependent Cre-ER^{T2} recombinase under the control of the cytokeratin K14 promoter. The resulting K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice were normal at birth and developed normally without showing any signs of a skin disease until approximately 6 months after birth. At this time, single mice exhibited discreetly thickened, flaky plaques at their neck and around the snout. These lesions enlarged subsequently and developed into hyperkeratotic and focally ulcerated plaques at the neck, sometimes extending to the shoulders, the throat, and the snout (Figure 1a). By the age of 9 months, all K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice had developed lesions that varied in size and severity. One mouse started to develop skin lesions already 3 months after birth. Observation of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice revealed frequent scratching, indicating that the skin lesions were apparently itching. PCR analysis of skin samples from the tail, back, and abdomen showed that Cre-mediated deletion of floxed IKK2 alleles had occurred in the skin of these mice at the time when skin lesions were present (Figure 1c). Sequential analysis of skin samples taken from mice at different age revealed that spontaneous Cre-mediated deletion in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice started not earlier than 12 weeks after birth and occurred at different areas of the body. Extraction of DNA from the epidermis of an inflamed ear of an 8-month-old mouse and subsequent PCR analysis suggested that Cre-mediated deletion of IKK2 within epidermal keratinocytes was complete (Figure 1d).

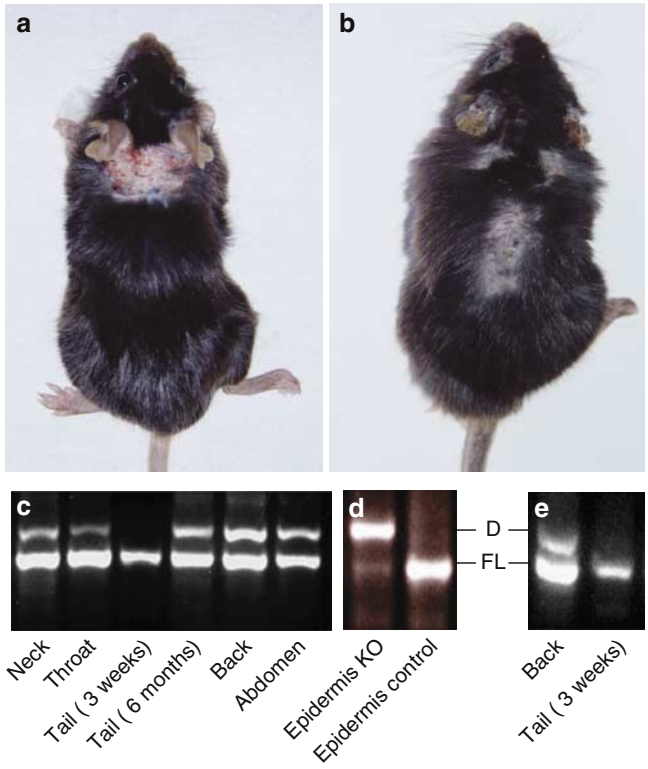

Figure 1. Macroscopic phenotype of mice with spontaneous and Tamoxifen-induced deletion of IKK2 in the epidermis. Phenotype of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice (**a**) with spontaneous epidermis-specific deletion of IKK2 at 6 months and (**b**) 5 weeks after Tamoxifen-induced deletion. (**c**–**e**) DNA isolated from (**c**) various body sites or (**d**) from epidermis of the ear that was separated from the dermis of mice with spontaneous deletion at 6 months (KO) or controls, and (**e**) from a Tamoxifen-treated area 3 weeks after treatment was subjected to PCR analysis. Deletion is detected by the presence of a PCR band of 652 bp; D. The lower band (533 bp; FL) indicates the floxed allele. (**c**, **e**) DNA isolated from tail snips of the corresponding mice 3 weeks after birth served as controls. (**d**) The faint band that can be detected at the expected size of the fragment from the floxed allele is likely to result from invading immune cells.

Tamoxifen-induced deletion of IKK2 in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

In order to induce Cre activity in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice in a timely controlled manner, we treated the back skin of 6-week-old mutant and control mice with Tamoxifen on 5 subsequent days. Three weeks after this treatment, K14-Cre-ER^{T2}IKK2$^{fl/fl}$ animals, but not control mice, started to develop skin lesions on their back skin where they had been treated with Tamoxifen. These lesions progressed in severity until week 5 after Tamoxifen treatment when mice were euthanized for analysis. PCR analysis of skin biopsies revealed that Cre-mediated deletion of IKK2 had occurred in the Tamoxifen-treated area (Figure 1e). The macroscopical aspect of skin lesions induced by Tamoxifen treatment was similar to that of skin lesions following spontaneous deletion. The skin showed a hairless, scaly, indurated plaque (Figure 1b). Mice were euthanized 3 or 5 weeks after Tamoxifen treatment and excised skin lesions were subjected to histological analysis.

Histopathology of skin lesions in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

Microscopical analysis of skin sections of 10 mice after spontaneous deletion of IKK2 and of six mice upon induced deletion following Tamoxifen treatment showed severe hyperplasia of the epidermis (acanthosis) with a thickened cornified layer (hyperkeratosis) that frequently contained nuclei of keratinocytes (parakeratosis), thus indicating an incomplete maturation process of epidermal keratinocytes (Figure 2a–c). The connective tissue of the dermis exhibited increased cellularity and deposition of collagen fibers that replaced the more superficial parts of the subcutaneous fat in severe lesions (Figure 2b, c, e, and h). In all skin lesions, severely distorted hair follicles could be observed. These formed cysts in the upper dermis that were filled with lamellar, eosinophilic material (Figure 2d and e). In several skin samples, both after spontaneous deletion of IKK2 and Tamoxifen treatment structures resembling invasive tumors of

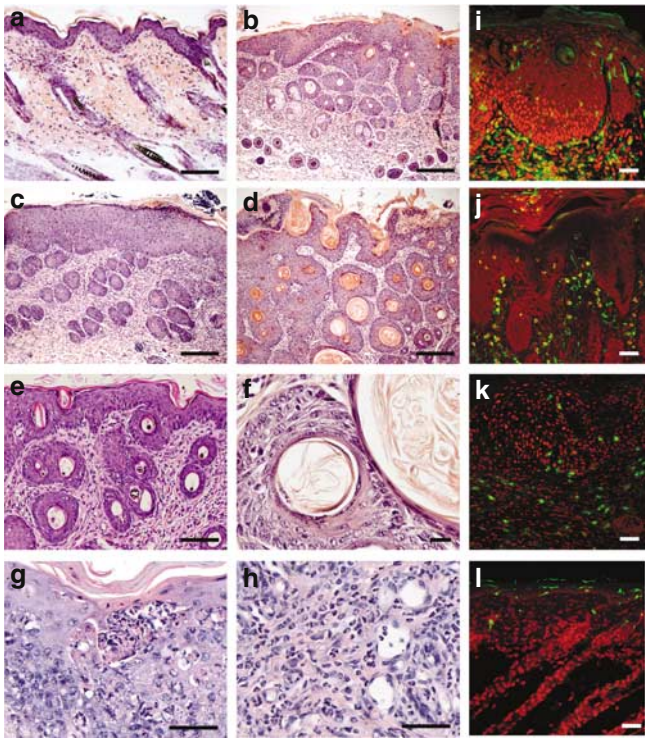

Figure 2. Inflammatory skin disease in mice with localized epidermis-specific deletion of IKK2. Histopathological phenotype of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice. Skin sections from Tamoxifen-induced mice (**a**) 3 weeks; or (**c**, **e**, **i**, **k**) 5 weeks after induction, (**b**, **d**, **f**, **g**, **h**) mice with spontaneous deletion of IKK2, and (**l**) control mice. Skin sections (**a**)–(**h**) were stained with hematoxylin/eosin. Sections (**i**)–(**l**) were immunostained with (**i**) antibodies recognizing macrophages (F4/80), (**j**) granulocytes (Gr-1), and (**k, l**) T cells (CD3). These sections were counterstained with propidium iodide to visualize tissue structure and nuclei. Bars: **a**, 100 μm; **b–e**, 200 μm; **f**, 20 μm; **g, h**, 50 μm; and **i–l**, 40 μm.

the epidermis with the architecture of human basal cell carcinomas (Figure 2c) or highly differentiated squamous cell carcinomas (Figure 2d) were observed. Close inspection of single cells at high power revealed, however, that no pleomorphic or dyskeratotic cells were present in these structures (Figure 2f).

Invasion of inflammatory cells into the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

As we had observed increased cellularity in the dermis of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice, we used antibodies against CD3 (T-lymphocytes), F4/80 (macrophages), and GR-1 (granulocytes) in order to differentiate between different immune cells in the inflammatory infiltrate. Staining of tissue sections of skin from K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice but not from controls showed massive infiltration of macrophages in the upper dermis. Frequently, macrophages were also found within the epidermis (Figure 2i and data not shown). Numerous granulocytes invaded the upper dermis and the epidermis where they formed microabscesses within and below the cornified layer (Figure 2g and i). In several, but not all, samples the infiltrate also contained CD3-positive T-lymphocytes that were localized in the upper parts of the dermis and within the epidermis (Figure 2k and l). T cells were present at variable numbers and were on average less abundant than macrophages or granulocytes. In contrast to invading T-lymphocytes, resident CD3-positive dendritic cells within the epidermis were not found in the inflammatory lesions of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice (Figure 2k and data not shown). We conclude that skin lesions of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice contained a mixed inflammatory infiltrate with variable numbers of T-lymphocytes.

Disturbed proliferation and epidermal differentiation in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

Histopathological findings in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice pointed to a disturbance of the balance between keratinocyte proliferation and differentiation in the skin of these mice. We therefore analyzed the expression of the proliferation marker Ki-67 in the epidermis of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice by means of immunostaining. We counted Ki-67-positive nuclei in 20 high-power fields of skin sections from two individual mice per group. These investigations showed that the number of Ki-67-positive cells was increased 19-fold (spontaneous deletion) and 12-fold (Tamoxifen-induced deletion) in the affected skin lesions of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice as compared to controls. We also carried out immunostainings for keratins 14 and 10 and the late differentiation markers Filaggrin and Loricrin. The skin of mice with spontaneous or Tamoxifen-induced deletion of IKK2 in epidermal keratinocytes showed similar changes with respect to the expression of epidermal keratins, Filaggrin and Loricrin: keratin 14 was present in all layers of the epidermis in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice, but restricted to the basal epidermal layer in control mice (Figure 3 and data not shown). In contrast, expression of keratin 10, Filaggrin, and Loricrin was dramatically downregulated in the interfollicular epidermis of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ skin as compared to control skin (Figure 3 and data not shown). We conclude that epidermal proliferation and differentiation in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice are severely disturbed.

Hair follicle disruption but no tumor formation in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

In order to exclude that the pseudoinvasive epithelial cell formations in the dermis of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice were genuine tumors, we used immunostaining with antibodies against markers of interfollicular and hair follicle differentiation. All epithelial cell islands localized in the dermis stained positive with an antibody against keratin 14, demonstrating their epidermal origin (Figure 4a). In addition, many epithelial islands showed positive staining for keratin 10 and Filaggrin (Figure 4b and c), thus indicating interfollicular epidermal differentiation as it can be found in infundibular cysts. Several epithelial islands in the mid- and deep dermis exhibited positive staining for keratin 6 and CAAT displacement enhancer protein (Figure 4d and g), suggesting hair lineage type keratinocyte differentiation as it occurs in normal hair follicles (Figure 4h). Expression of keratin 6 was also upregulated in the interfollicular epidermis (Figure 4e

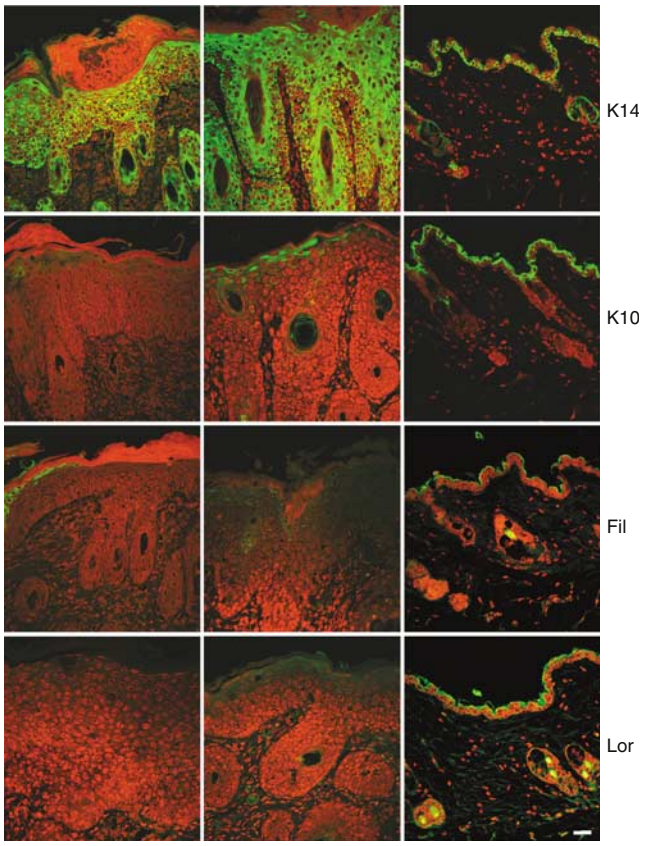

Figure 3. Analysis of epidermal differentiation markers in mice with epidermis-specific deletion of IKK2. Skin sections from K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice spontaneously induced (left panel), tamoxifen-induced (middle panel), and from control mice (right panel) were immunostained for the indicated epidermal differentiation markers (green staining): keratin 14 (K14), keratin 10 (K10), filaggrin (Fil), and loricrin (Lor). Sections were counterstained with propidium iodide to visualize tissue structure and nuclei. Bar = 40 μm.

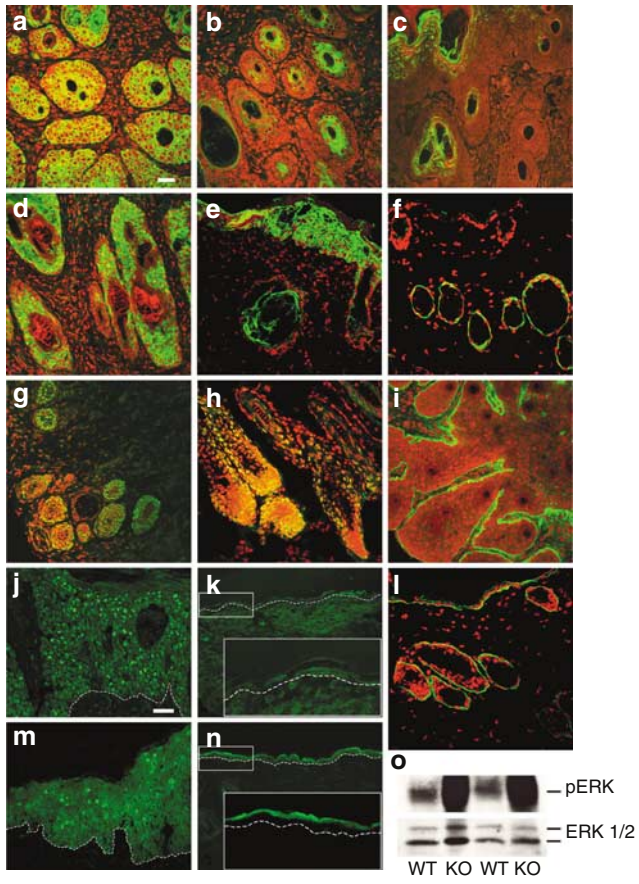

Figure 4. Hair follicle disruption and signal transduction in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice. Confocal images of skin sections from (**c**, **e**, **g**, **i**, **j**) tamoxifen-induced and (**a**, **b**, **d**, **m**) spontaneously induced K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice and (**f**, **h**, **k**, **l**, **n**) controls. The green signal shows immunostaining against (**a**) keratin 14, (**b**) keratin 10, (**c**) filaggrin, (**d–f**) keratin 6, (**g, h**) CCAAT displacement enhancer protein, (**i, l**) laminin 5, (**j, k**) phosphorylated signal transducer and activator of transcription 3 (STAT3), and (**m, n**) phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2). Sections (**a**)–(**i**) and (**l**) were counterstained with propidium iodide to visualize tissue structure and nuclei. Inserts in (**k**) and (**n**) show high-power magnification of the indicated parts of the epidermis. Bars = 40 μm. Western blot analysis of ERK phosphorylation (**o**) of affected skin samples from K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice (KO) and of wild-type skin samples (WT). The upper panel shows phosphorylated ERK (pERK), and the lower panel shows ERK1/2 as loading control (ERK1/2).

and f). In order to prove that the epithelial cell formations did not represent invasive tumors, we stained for the presence of a basement membrane with an antibody against laminin 5 (Figure 4i and l). These staining demonstrated that all cell islands were surrounded by an inconspicuous laminin 5-positive basement membrane, suggesting that they were not parts of an invasive tumor.

Signals of hyperproliferation and inflammation in epidermal keratinocytes of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice

In order to identify signaling events that could be responsible for the phenotype observed in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice, we analyzed the activation status of different signaling pathways *in situ* using phosphorylation-specific antibodies against the mitogen-activated protein kinases extracellular signal-regulated protein kinase (ERK)1/2, protein kinase B (Akt), and signal transducer and activator of transcription 3 (STAT3). Immunostainings with rabbit monoclonal antibodies against different phosphorylated signaling proteins revealed strong nuclear signals for phosphorylated STAT3 and phosphorylated ERK1/2 in the hyperproliferative epidermis of five out of five (STAT3) and three out of five (ERK1/2) K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice with spontaneous or Tamoxifen-induced deletion of IKK2, but not in normal skin of control animals (Figure 4j, k, m, and n). ERK activation was confirmed by Western blot analysis of extracts of affected skin from K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice and controls (Figure 4o). No differences were detected in Akt phosphorylation (data not shown). These results show an activation of STAT3- and ERK1/2-dependent signaling pathways in the skin lesions of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice.

DISCUSSION

Accumulating evidence suggests a role for epidermal keratinocytes in the regulation of skin inflammation (Carroll

et al., 1995; Cook *et al.*, 1997; Pasparakis *et al.*, 2002; Sano *et al.*, 2005). Although it is known that keratinocytes can secrete proinflammatory mediators in bulk, mechanisms that lead to the release of these factors are not well understood. We have shown previously that selective ablation of IKK2 from epidermal keratinocytes of mice results in a widespread inflammatory skin reaction. As these mice die by day 10 after birth, we could not exclude that the inflammatory skin changes observed were due to a modification of mechanisms of epidermal development as they normally occur at this stage. The results of this study demonstrate that adult murine skin responds to epidermis-specific ablation of IKK2 in almost the same manner as skin of newborn mice. This shows that skin inflammation as a consequence of epidermis-specific ablation of IKK2 is not related to epidermal development.

Deletion of IKK2 from all epidermal keratinocytes resulted in a generalized inflammatory skin disease (Pasparakis *et al.*, 2002), leaving open the possibility that skin inflammation following ablation of IKK2 was the consequence of a systemic innate immune response. With the help of a Tamoxifen-dependent Cre recombinase expressed under the control of the keratin 14 promoter, we were able to show that there is spatial correlation between epidermis-specific IKK2 deletion and the inflammatory response. This strongly suggests intrinsic control of the inflammatory reaction by cells within the skin.

In K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice that reached the age of 6 months, we observed spontaneous deletion of IKK2. This is most likely due to a cumulative effect of Cre-mediated deletion caused by incomplete repression of Cre recombinase activity in the K14-Cre-ER^{T2} construct. This view is favored by our observation that Cre-mediated deletion of IKK2 was not detectable in mice at 3 or 5 weeks of age, but was readily seen in the skin of mice above the age of 6 months. Furthermore, Cre-mediated IKK2 deletion was detected in affected skin that showed a hyperplastic inflammatory phenotype as well as unaffected skin without visible phenotype. Possible explanations for this phenomenon are either that the development of the skin phenotype only occurs when a certain threshold level of Cre-mediated deletion is exceeded or that the phenotype requires a combination of epidermis-specific Cre-mediated IKK2 deletion and other factors, the nature of which remains unknown at the moment. In either case, our results demonstrate that epidermis-specific Cre-mediated deletion of IKK2 preceded the inflammatory skin phenotype.

Inhibition of NF-κB signaling in the epidermis by expression of a nondegradable variant of IκBα (IκBα super-repressor) under the control of the keratin 5 promoter (K5-IκB-α) has been reported to cause skin inflammation and epidermal changes that progressed to invasive squamous cell carcinomas 16 weeks after birth in transgenic mice of the FVB/N background (van Hogerlinden *et al.*, 1999, 2004). Although we monitored K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice up to 11 months, we did not find convincing evidence for the presence of squamous cell carcinomas. Using conventional histopathological techniques, we found signs that could at most be classified as mild atypicalities as they can be found, for example, in pseudocarcinomatous hyperplasia, a condition that often occurs in chronic inflammatory skin lesions in humans (Kirkham, 2005). Immunostainings for keratin 6, CAAT displacement enhancer protein, and markers of interfollicular epidermal differentiation revealed that epithelial cell aggregates in the dermis were either epidermal cysts or showed positive staining of hair lineage markers, thus suggesting that they were remnants of destroyed hair follicles. Staining for laminin 5 revealed that epithelial cell islands in the dermis of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice showed an intact basement membrane, suggesting that they are not truly invasive. One possible explanation for this discrepancy is that K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice were in the C57Bl6 background, whereas K5-IκB-α mice were FVB/N, a strain known to be more sensitive to skin tumor development (Hennings *et al.*, 1993; Woodworth *et al.*, 2004). This would be consistent with the fact that K5-IκB-α mice develop skin carcinomas only in the FVB/N background, but not in a pure C57Bl6 background (R Toftgard, personal communication). Another potential explanation that may or may not be related to strain differences is the composition of the inflammatory infiltrate. K5-IκB-α mice show accumulation of inflammatory cells that carry the B-lymphocyte marker B220. We did not observe B220^{+} cells in the skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice.

Recently, forced activation of STAT3 signaling in epidermal keratinocytes has been brought into connection with the development of a psoriasis-like skin phenotype in transgenic mice. In inflamed human skin, STAT3 activation was apparently specific for psoriatic lesions (Sano *et al.*, 2005). The phenotype of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice shows several changes that are reminiscent of human psoriasis such as acanthosis, hyper- and parakeratosis, absence of the granular layer, as well as intraepithelial formation of aggregates of neutrophils. In addition, ERK1/2 were found to be activated in epidermal keratinocytes. We and others have described ERK1/2 activation not only in psoriatic skin but also in healing skin wounds of mice and in other hyperproliferative conditions of human skin, such as Acanthosis nigricans and squamous cell carcinomas (Albanell *et al.*, 2001; Haase *et al.*, 2001; Haase and Hunzelmann, 2002; Chaturvedi *et al.*, 2003). This suggests that ERK activation is not specific for a particular skin disease, but rather a broad functional marker of disturbed proliferation and/or differentiation of keratinocytes. Likewise, although we have observed strong STAT3 activation in hyperproliferative skin of K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice, this was not a specific feature of this skin disease, as phosphorylated STAT3 was also present in hyperproliferative epithelium adjacent to skin wounds of normal mice (data not shown). This may indicate that STAT3 activation is not a specific marker for a certain type of inflammation in murine skin. Alternatively, it is conceivable that skin inflammation following deletion of IKK2 as well as cutaneous wound healing in mice and human psoriasis all share a common signaling pattern with respect to STAT3 and ERK1/2 activity that may be the consequence of similar pathogenic mechanisms. In the future, it will be interesting to explore these possibilities.

MATERIALS AND METHODS

Generation and genotyping of mice

Animal studies were approved by the review board of the local government. Mice with Tamoxifen-inducible, epidermis-specific deletion of IKK2 (K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice) were generated by crossing mice with floxed IKK2 alleles (IKK2$^{FL/FL}$ mice) (Pasparakis *et al.*, 2002) to mice expressing Cre recombinase fused to the human estrogen receptor under the control of a truncated keratin 14 promoter (K14-Cre-ER^{T2} mice) (Indra *et al.*, 2000). All mice used were backcrossed into the C57Bl6 background for at least five generations.

Genotyping was performed by PCR using primers and conditions as described (Pasparakis *et al.*, 2002). Epidermis was mechanically separated from dermis after incubating ears with 3.8% NH_4SCN in 0.1 M $NaKHPO_4$ buffer, pH 6.8, for 1 hour at 4°C.

Tamoxifen treatment

The Cre-ER^{T2} recombinase was activated in K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice at 6 weeks of age by applying 1 mg/mouse per day 4-hydroxy-tamoxifen (Sigma, Schnelldorf, Germany) dissolved in 0.2 ml ethanol to an unshaved area of dorsal skin on 5 subsequent days. Sex- and age-matched K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice treated with ethanol were used as control. Effects of Tamoxifen treatment were monitored for up to 5 weeks.

Histopathology and immunostaining

Sections (4 μm) of paraffin-embedded skin were deparaffinized in xylene and rehydrated using standard procedures. Sections (6 μm) of frozen tissue were fixed in ice-cold acetone. After blocking with 10% normal goat serum in 0.2% fish skin gelatin for 1 hour, the sections were washed in phosphate-buffered saline. Primary antibodies dissolved in fish skin gelatin were applied for 1 hour at room temperature or overnight at 4°C. After washing in phosphate-buffered saline, the sections were incubated with anti-rabbit or anti-rat Alexa 488-coupled IgG and propidium iodide dissolved in fish skin gelatin. For Ki-67 staining, anti-rat Alexa 488 was dissolved in normal mouse serum. Finally, after rinsing in phosphate-buffered saline, the sections were fixed with 4% paraformaldehyde and mounted in Gelvatol. The following primary polyclonal antibodies (BabCo, Richmond, CA) were used: anti-loricrin (1:2,000), anti-filaggrin (1:1,000), anti-keratin14 (1:100), anti-keratin 10 (1:100), AND anti-keratin 6 (1:500). The polyclonal antibodies against CAAT displacement enhancer protein and laminin 5 were kind gifts of Meinrad Busslinger (Vienna, Austria) and Manuel Koch (Cologne, Gemany), respectively. Both antibodies were used on frozen sections at concentrations of 1:100 and 1:500, respectively. Rat monoclonal antibodies were used on frozen sections as follows: anti-CD3 (1:1,000; Chemicon, Temecula, CA), anti-CD4 (1:50; clone GK1.5/4; BD Biosciences Pharmingen, San Jose, CA), anti-Gr-1 (1:20; Ly-6G, clone RB6-8C5, BD Biosciences Pharmingen, San Jose, CA), anti-F4/80 (1:50; Serotec, Oxford, UK), Ki-67 (1:50; clone TEC-3, Dako, Glostrup, Denmark). Phospho-specific monoclonal rabbit anti-STAT3 and anti-ERK1/2 antibodies were obtained from Cell Signaling New England Biolabs (Beverly, MA) and used according to the manufacturer's protocols. Sections were counterstained with propidium iodide to visualize the nuclei. Fluorescent stainings were analyzed using a Leica TCS upright confocal laser-scanning microscope at excitation wavelengths of 488 and 543 nm.

Western blotting

To analyze phosphorylation of ERK, snap-frozen skin samples from affected skin of spontaneously induced K14-Cre-ER^{T2}IKK2$^{fl/fl}$ mice and samples of wild-type murine skin were ground using mortar and pestle and subsequently extracted in modified radioimmunoprecipitation assay buffer containing 5 mM EDTA, 1% Triton X-100, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml soybean trypsin inhibitor, 0.5 mM $NaVO_3$, and 10 mg/ml *p*-nitrophenylphosphate on ice for 1 hour. Lysates were centrifuged at 14,000 $\times$ *g* for 10 minutes and the supernatant was used for Western blot analysis. Equal amounts of protein were separated by SDS-PAGE and blotted onto Hybond-P polyvinylidine difluoride membranes (Amersham, Freiburg, Germany). ERK phosphorylation was detected with antibodies specific for phosphorylated ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:500). Blots were reprobed with antibodies to ERK2 (Santa Cruz Biotechnology; dilution 1:500) to check for equal loading. Protein bands were visualized with horseradish peroxidase-coupled secondary antibodies on Hyperfilm using enhanced chemiluminescence (Amersham Pharmacia Biotech, Amersham, UK).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Meinrad Busslinger and Manuel Koch for providing antibodies. We would like to thank Rune Toftgard for stimulating discussions. This work was supported by Grant TV73 from the Center of Molecular Medicine, University of Cologne, by the Koeln Fortune Program/Faculty of Medicine, University of Cologne and by Deutsche Forschungsgemeinschaft SFB 589.

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