Immunity
Article

Tumor Necrosis Factor Receptor Signaling in Keratinocytes Triggers Interleukin-24-Dependent Psoriasis-like Skin Inflammation in Mice

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SUMMARY

Psoriasis is a common chronic inflammatory skin disease with a prevalence of about 2% in the Caucasian population. Tumor necrosis factor (TNF) plays an essential role in the pathogenesis of psoriasis, but its mechanism of action remains poorly understood. Here we report that the development of psoriasis-like skin inflammation in mice with epidermis-specific inhibition of the transcription factor NF-κB was triggered by TNF receptor 1 (TNFR1)-dependent upregulation of interleukin-24 (IL-24) and activation of signal transducer and activator of transcription 3 (STAT3) signaling in keratinocytes. IL-24 was strongly expressed in human psoriatic epidermis, and pharmacological inhibition of NF-κB increased IL-24 expression in TNF-stimulated human primary keratinocytes, suggesting that this mechanism is relevant for human psoriasis. Therefore, our results expand current views on psoriasis pathogenesis by revealing a new keratinocyte-intrinsic mechanism that links TNFR1, NF-κB, ERK, IL-24, IL-22R1, and STAT3 signaling to disease initiation.

INTRODUCTION

The skin forms an essential structural and immunological barrier protecting the organism from water loss and from mechanical, chemical, and microbial challenges. A tightly regulated cross-talk between epithelial, mesenchymal, and immune cells is essential for both the maintenance of a healthy skin homeostasis and its reestablishment after injury. Disruption of skin homeostasis results in chronic inflammatory skin diseases such as psoriasis. Psoriatic skin inflammation evolves through interactions between epidermal keratinocytes and cells of the immune system (Nestle et al., 2009). Although the immune mechanisms contributing to cutaneous inflammation in psoriatic skin have been extensively characterized, the factors responsible for triggering the initiation of skin inflammation are not known. Although it is generally believed that T lymphocytes and dendritic cells contribute to its pathogenesis in an essential manner (Lowes et al., 2005; Nickoloff and Wrone-Smith, 1999), the contribution of epidermal keratinocytes to the onset of psoriasis remains poorly understood.

The efficacy of tumor necrosis factor (TNF)-neutralizing drugs in the treatment of psoriasis demonstrates an essential role for TNF in its pathogenesis (Lowes et al., 2007). TNF is a highly potent proinflammatory cytokine (Apostolaki et al., 2010). The ubiquitously expressed TNF receptor 1 (TNFR1) mediates the majority of TNF responses by activating intracellular signaling cascades inducing inflammation but also cell death (Walczak, 2011). However, the molecular mechanisms responsible for its pathogenic role and the cellular targets of TNF in psoriasis remain unknown. More recently, cytokines of the interleukin-10 (IL-10) family, including IL-19, IL-20, IL-22, and IL-24, have been implicated in the pathogenesis of psoriasis (Kunz et al., 2006; Leng et al., 2011; Rømer et al., 2003; Weiss et al., 2004; Wolk et al., 2009a). Increased expression of IL-19, IL-20, IL-22, and IL-24 was detected in psoriatic compared to normal skin

(Kunz et al., 2006; Rømer et al., 2003; Wang et al., 2012; Weiss et al., 2004; Wolk et al., 2009b). In the skin, IL-22 is produced mainly by T cells, whereas IL-19, IL-20, and IL-24 are produced by keratinocytes, myeloid cells, and T cells (Conti et al., 2003; Kunz et al., 2006; Zheng et al., 2007). The receptors for IL-19, IL-20, IL-22, and IL-24 are expressed at high amounts in keratinocytes and signal primarily by activating signal transducer and activator of transcription 3 (STAT3) (Dumoutier et al., 2001; Kunz et al., 2006; Parrish-Novak et al., 2002). STAT3 is overexpressed and activated in lesional psoriatic skin (Sano et al., 2005; Wolk et al., 2009a), and transgenic expression of constitutively active STAT3 in epidermal keratinocytes induced psoriasis-like skin inflammation in mice (Sano et al., 2005), suggesting an important role for epidermal STAT3 signaling in psoriasis.

We showed previously that nuclear factor kappa B (NF-κB) inhibition in keratinocytes by deletion of IκB kinase 2 (*K14-Cre Ikk2*$^{fl/fl}$) (Pasparakis et al., 2002) or by transgenic IκBα superrepressor expression (*K5-IκBαSR*) (van Hogerlinden et al., 1999) triggers inflammatory skin lesions in mice. Histologically detectable signs of inflammation appear between postnatal days P3 to P4 in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice and rapidly progress to a severe inflammatory skin condition, resulting in the death of all animals between P8 and P10. Skin inflammation in K5-IκBαSR mice starts around 3–4 weeks after birth and progresses more slowly than in the *K14-Cre Ikk2*$^{fl/fl}$ mice. The skin lesions induced by epidermis-specific NF-κB inhibition in these mouse models closely resemble human psoriasis, both clinically and histologically. They consist of widespread scaly plaques, which upon histological analysis show acanthosis, hyperkeratosis, dilation of dermal blood vessels, and infiltration by a mixed inflammatory infiltrate with neutrophils forming aggregates in the upper epidermis (Pasparakis et al., 2002; van Hogerlinden et al., 1999). Genetic ablation of TNFR1 in the whole body prevents the development of inflammatory skin lesions in both the *K14-Cre Ikk2*$^{fl/fl}$ and the *K5-IκBαSR* mice (Lind et al., 2004; Pasparakis et al., 2002), demonstrating that TNF regulates the pathogenesis of skin inflammation in these models similarly to psoriasis in humans. Skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice occurs independently of αβ T cells but requires the presence of macrophages in the dermis (Stratis et al., 2006).

Here we investigated how keratinocyte-intrinsic inhibition of NF-κB causes psoriasis-like skin inflammation. Our study uncovers a mechanism that links NF-κB, TNF, IL-24, and STAT3 signaling in epidermal keratinocytes to the initiation of psoriasis-like skin inflammation in mice. We also provide evidence that a similar mechanism could be implicated in the early stages of the development of skin inflammation in human psoriasis.

RESULTS

TNFR1 in Keratinocytes Triggers Skin Inflammation in Mice with Epidermis-Specific NF-κB Inhibition

To address whether TNFR1 signaling in epidermal keratinocytes is needed for the development of skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice, the mice were crossed with animals carrying "floxed" TNFR1 alleles to generate mice lacking both IKK2 and TNFR1 specifically in keratinocytes (*K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{fl/fl}$). Flow cytometry analysis of TNFR1 expression confirmed efficient deletion of TNFR1 in epidermal keratinocytes in *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{fl/fl}$ mice (Figure 1A). In contrast to *K14-Cre IKK2*$^{fl/fl}$, which developed severe skin inflammation shortly after birth and died before P9 (Pasparakis et al., 2002), *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{fl/fl}$ mice appeared macroscopically normal and reached adulthood without showing inflammatory skin lesions, similar to *K14-Cre Ikk2*$^{fl/fl}$ mice with ubiquitous deletion of TNFR1 (*K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{-/-}$) (data not shown). Histological analysis revealed normal epidermal thickness and differentiation and absence of inflammatory infiltrates from the skin of *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{fl/fl}$ mice (Figures 1B and 1C). These results demonstrate that TNFR1 signaling in keratinocytes is essential for the pathogenesis of the inflammatory skin disease in this model.

To address whether TNFR1 expression in keratinocytes was sufficient to induce skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice, we employed the TNFR1^{cneo} mouse model, in which the expression of TNFR1 is disrupted by insertion of a loxP-flanked *neo* cassette in intron 5 of the *Tnfr1* gene (Victoratos et al., 2006). By crossing *K14-Cre Ikk2*$^{fl/fl}$ with *Tnfr1*^{cneo} mice we generated *K14-Cre Ikk2*$^{fl/fl}$ animals expressing TNFR1 exclusively in epidermal keratinocytes (*K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{cneo/cneo}$ mice) (see Figure S1A available online). These animals macroscopically displayed milder skin lesions at P7 compared to *K14-Cre Ikk2*$^{fl/fl}$ mice, which progressively developed to severe skin inflammation by the age of 4 weeks (data not shown). Histological analysis revealed epidermal hyperplasia and inflammation in the skin of *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{cneo/cneo}$ mice at P7 (Figure 1B; Figure S1B), demonstrating that TNFR1 expression in keratinocytes was sufficient to trigger skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice.

To address the role of epidermal TNFR1 signaling in the *K5-IκBαSR* model, we generated transgenic mice expressing mouse TNFR1 under the control of the keratin 5 promoter and crossed them to *K5-IκBαSR Tnfr1*$^{-/-}$ mice. The resulting *K5-IκBαSR Tnfr1*$^{-/-}$ *K5-Tnfr1* mice were deficient for endogenous TNFR1 but expressed IκBαSR and TNFR1 as transgenes in epidermal keratinocytes. Whereas no skin inflammation or epidermal hyperplasia could be detected in either *K5-IκBαSR Tnfr1*$^{-/-}$ or *Tnfr1*$^{-/-}$ *K5-Tnfr1* mice, the *K5-IκBαSR Tnfr1*$^{-/-}$ *K5-Tnfr1* mice developed epidermal hyperplasia and inflammatory changes in the skin within the first 3–4 days after birth, with a rapid progression resulting in the death of most of these animals within 10 days (Figure S1C). Expression of transgenic TNFR1 was increased 3–4 fold in epidermal sheets of 4-day-old *Tnfr1*$^{-/-}$ *K5-Tnfr1* mice compared to expression of endogenous TNFR1 in wild-type (WT) mice (data not shown), which might contribute to the accelerated kinetics of the disease in *K5-IκBαSR Tnfr1*$^{-/-}$ *K5-Tnfr1* mice compared to the *K5-IκBαSR* animals. Collectively, these results demonstrate that keratinocyte-specific TNFR1 signaling is both necessary and sufficient for the pathogenesis of skin inflammation in mice with epidermis-specific NF-κB inhibition.

IL-19 and IL-24 Expression in the Epidermis Precedes Skin Inflammation in *K14-Cre Ikk2*$^{fl/fl}$ Mice

Our results showing that TNFR1 acts in epidermal keratinocytes to induce skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice suggest that IKK2-deficient keratinocytes might produce signals triggering an inflammatory response when stimulated by TNF. We previously showed that myeloid cells are recruited to the skin of *K14-Cre Ikk2*$^{fl/fl}$ mice as early as 3–4 days after birth and have an

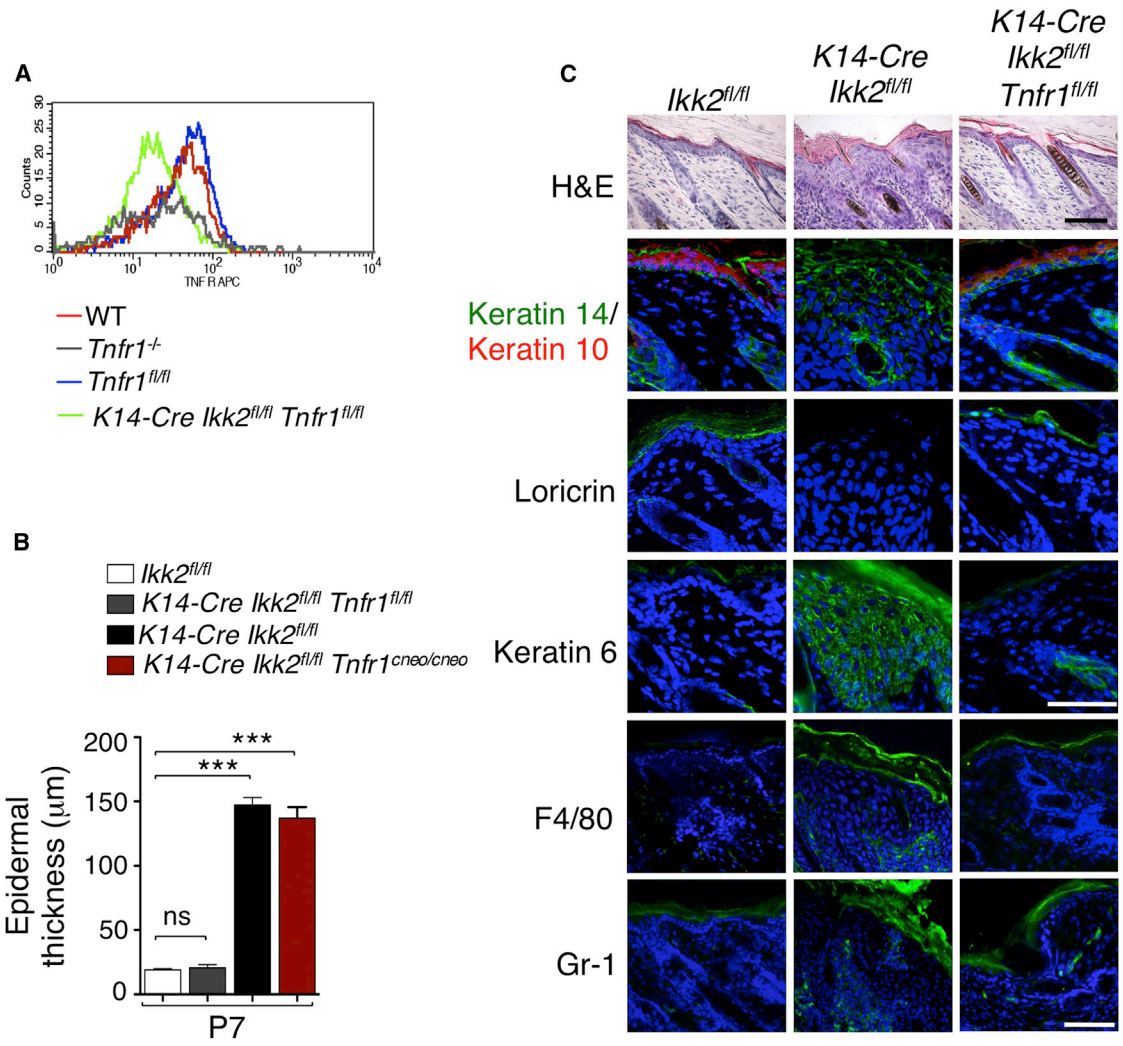

Figure 1. TNFR1 Expression in Keratinocytes Is Necessary and Sufficient to Induce Skin Inflammation in *K14-Cre Ikk2*$^{fl/fl}$ Mice

(A) FACS analysis of TNFR1 expression in epidermal keratinocytes from mice with the indicated genotypes.

(B) Bar graph showing microscopic measurement of the epidermal thickness on skin sections obtained from mice of the indicated genotypes (n = 3). Results are expressed as mean values ± SEM. ***p ≤ 0.001.

(C) Histological (H&E) and immunofluorescence analysis of epidermal differentiation markers (Keratin 14, 10, 6, and loricrin) and immune cells (F4/80 and Gr-1) in skin sections from mice with the indicated genotypes at P7. Nuclei were stained with DAPI (blue). Scale bar represents 50 μm. See also Figure S1.

important role in the pathogenesis of skin inflammation (Stratis et al., 2006). FACS analysis of the immune cell infiltrate in the skin of *K14-Cre Ikk2*$^{fl/fl}$ mice at P8 revealed that macrophages (CD11b$^+$F4/80$^+$) and dendritic cells (CD11c$^+$) constituted the main immune cell populations, whereas CD3$^+$ T lymphocytes and neutrophils (F4/80$^-$Gr-1$^+$) were less abundant (Figure 2A; data not shown). We therefore reasoned that IKK2-deficient epidermal keratinocytes might trigger skin inflammation by producing factors attracting myeloid cells to the skin and analyzed the expression of several cytokines and chemokines in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice during the first 4 days after birth. At P2, P3, and P4, we detected elevated transcript levels of CCL3, CCL4, and CCL5, as well as of IL-6 and GM-CSF in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice compared to their WT littermates (Figures 2B and 2C). Because these proinflammatory mediators were not upregulated at P1, we asked whether their production was preceded and perhaps induced by an initiating factor. In an unbiased approach to detect this factor by using microarray analysis, we identified upregulation of IL-19 and IL-24 messenger RNA (mRNA) as early as P1 (data not shown), which was confirmed with qRT-PCR (Figure 3A). Because the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice does not contain increased numbers of myeloid cells at P1 and no expression of IL-19 and IL-24 could be detected in dermal RNA preparations (Figure S2), epidermal keratinocytes are the major source of the elevated epidermal IL-19 and IL-24 mRNA. IL-19 and IL-24 were not upregulated in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice at P0, suggesting that their expression is induced during the first 24 hr after birth. The increase in mRNA levels correlated with increased production of IL-24 protein, as revealed by immunoblot analysis of whole-skin protein lysates and by immunostaining of skin sections from *K14-Cre Ikk2*$^{fl/fl}$ and control mice (Figure 3B). IL-20

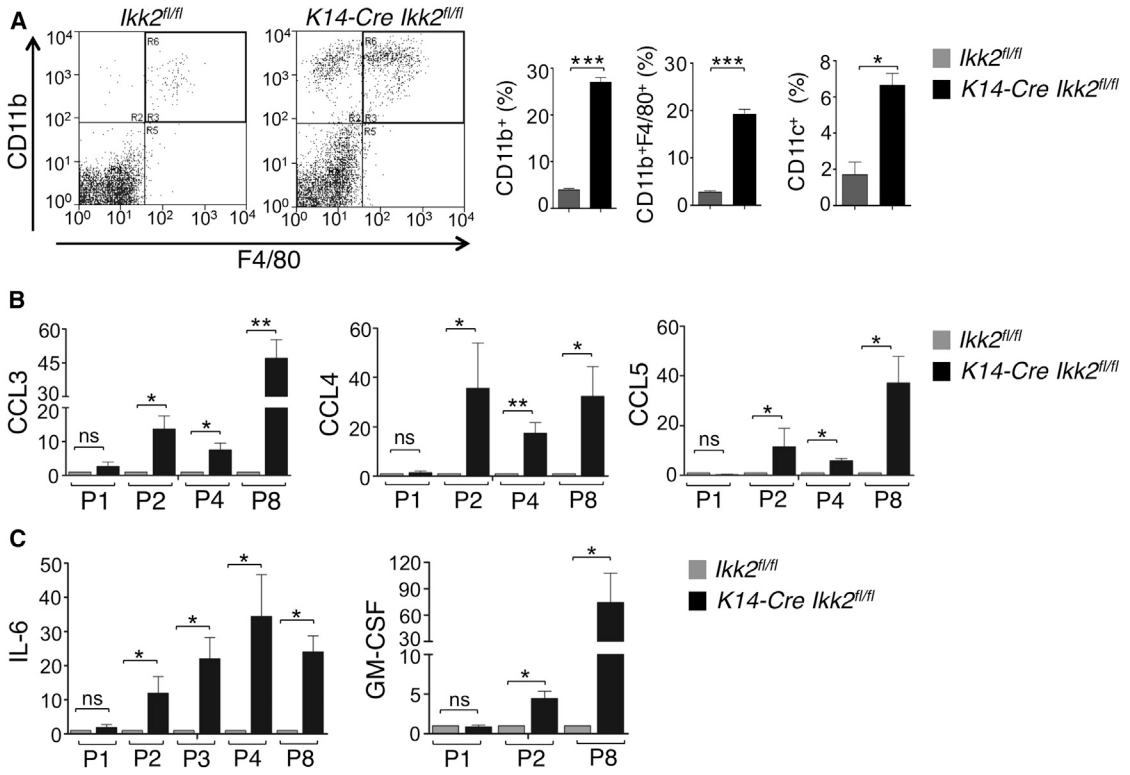

Figure 2. Upregulation of Chemokines and Proinflammatory Cytokines in the Skin of *K14-Cre Ikk2*$^{fl/fl}$ Mice

(A) FACS analysis of cells isolated from whole skin of *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ mice at P8. Bar graphs show the percentage of positive cells in relation to the whole live-cell population (n = 3). Results are expressed as mean values ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(B and C) qRT-PCR analysis of chemokine (B) and cytokine (C) expression in the epidermis (P1–P4) and in whole skin (P8) of 3–8 *Ikk2*$^{fl/fl}$ and 3–8 *K14-Cre Ikk2*$^{fl/fl}$ mice. Bars show fold regulation of chemokine and cytokine mRNA in *K14-Cre Ikk2*$^{fl/fl}$ over *Ikk2*$^{fl/fl}$ mice normalized to HPRT mRNA. All qRT-PCR results are expressed as mean values ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

and IL-22, two other members of the IL-10 cytokine family, were upregulated in the skin at P4 and P8, but not at P2 (Figure 3C).

IL-19 and IL-24 bind to specific receptors, which signal via STAT3 (Dumoutier et al., 2001; Parrish-Novak et al., 2002). Consistent with the elevated expression of IL-19 and IL-24, we detected increased nuclear staining for phosphorylated STAT3 in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$, but not of control mice at P1, which was further increased at P3 (Figure 3D). Thus, early upregulation of IL-19 and IL-24 correlated with STAT3 activation in epidermal keratinocytes of *K14-Cre Ikk2*$^{fl/fl}$ mice already at P1, a stage when no histological signs of skin inflammation can be detected in these animals. Considering that activation of STAT3 in keratinocytes induces psoriasis-like skin inflammation (Sano et al., 2005), IL-19- and IL-24-mediated activation of STAT3 in the epidermis could provide the pathogenic signal that triggers skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice.

Aberrant TNFR1 Signaling Induces IL-19 and IL-24 Expression in IKK2-Deficient Keratinocytes

Because TNFR1 signaling in keratinocytes is essential for triggering skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice, we asked whether the upregulation of IL-19 and IL-24 in the epidermis was induced by TNFR1. Indeed, IL-19 and IL-24 were not upregulated in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{-/-}$ mice at P3 (Figure 4A), demonstrating that their expression was dependent on TNFR1 signaling. Consistent with these results, IL-24 upregulation in the skin of *K5-IκBαSR* mice was also induced by TNFR1 signaling in epidermal keratinocytes (Figure 4B). Although TNFR1 was required for the early upregulation of IL-24 in the NF-κB-deficient epidermis, we could not detect increased TNF mRNA in the skin of these animals during the first postnatal days (Figure 4C; data not shown). These results indicate that IL-24 upregulation in IKK2-deficient epidermis is not induced by increased TNF expression but rather by an aberrant response of keratinocytes to TNF concentrations present in normal skin.

To investigate how TNFR1 signaling induces IL-24 expression in IKK2-deficient keratinocytes, we analyzed their response to TNF stimulation. As shown in Figure 4D, IKK2-deficient keratinocytes showed increased expression of IL-24 and GM-CSF but decreased expression of the NF-κB-dependent IL-6 gene in response to TNF stimulation compared to control cells. This response was specific for TNF because IL-1β stimulation did not induce increased IL-24 and GM-CSF expression in IKK2-deficient keratinocytes (Figure 4E). These in vitro findings confirm that upregulation of IL-24 and GM-CSF in epidermal keratinocytes of *K14-Cre Ikk2*$^{fl/fl}$ mice is the result of an aberrant response to TNF. Because ERK activation was previously shown to regulate IL-24 expression (Yang et al., 2011), we tested the involvement of ERK signaling in the upregulation of IL-24 and GM-CSF expression in IKK2-deficient keratinocytes. Inhibition

of MEK and ERK signaling by the specific MEK-1 and MEK-2 inhibitor UO126 suppressed the TNF-dependent expression of IL-24 and GM-CSF in both control and IKK2-deficient keratinocytes (Figure 5A). In addition, IKK2-deficient keratinocytes showed increased ERK phosphorylation after TNF stimulation compared to control keratinocytes (Figure 5B), suggesting that an overactivation of ERK signaling contributes to the increased IL-24 expression in these cells. NF-κB is proposed to limit TNF-dependent MAPK activation by inhibiting the accumulation of reactive oxygen species (ROS) (Sakon et al., 2003). Indeed, IKK2-deficient keratinocytes had increased concentrations of ROS compared to control cells (Figure 5C), and treatment with the ROS scavenger N-acetylcysteine (NAC) strongly inhibited both TNF-induced ERK phosphorylation and IL-24 expression in IKK2-deficient and control keratinocytes (Figures 5B and 5D). Thus, TNF-induced IL-24 expression requires ROS-mediated activation of MEK and ERK signaling.

Pathogenic Role of IL-24 in the Pathogenesis of Psoriasis-like Skin Inflammation in *K14-Cre Ikk2*$^{fl/fl}$ Mice

The early TNF-dependent upregulation of IL-19 and IL-24 in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ mice identifies them as probable proinflammatory triggers. IL-19 and IL-24 signal via distinct heterodimeric receptors. The IL-19 receptor, which is also used by IL-20 and IL-24, consists of IL-20R1 and IL-20R2 chains (Dumoutier et al., 2001). In addition, IL-24 mainly signals via the IL-22R1 and IL-20R2 heterodimer. The IL-22R1 chain forms an additional heterodimeric receptor with IL-10R2 that is essential for signaling by IL-22 (Sabat, 2010). To assess IL-24 and IL-19 function in the pathogenesis of the observed skin inflammation, we crossed the *K14-Cre Ikk2*$^{fl/fl}$ mice with mice deficient for IL-20R1 (*Il20ra*$^{-/-}$) or IL-22R1 (*Il22ra1*$^{-/-}$). IL-20R1 deficiency resulted in a delayed onset and decelerated development of the skin lesions in *K14-Cre Ikk2*$^{fl/fl}$ mice. Double-deficient *Il20ra*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice showed macroscopically visible patches of inflamed skin at P8 (data not shown); however, the skin inflammation was milder as indicated by a mildly reduced epidermal thickness compared to *K14-Cre Ikk2*$^{fl/fl}$ mice (Figure 6). Moreover, unlike *K14-Cre Ikk2*$^{fl/fl}$ animals, these mice did not die by P8, but the skin lesions progressed to severe skin inflammation requiring sacrificing the animals by P25.

IL-22R1 deficiency had a stronger protective effect in *K14-Cre Ikk2*$^{fl/fl}$ mice, with double-deficient *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice showing only very few flaky patches on the skin by P8, which covered about 3%–10% of the total visible body surface area (data not shown). Immunohistological analysis confirmed that most of the skin area in 8-day-old *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice did not show signs of inflammation, with normal K14, K10, and K6 expression and an absence of immune cell infiltration (Figure 6). The affected areas (3%–10%) of the skin of *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice showed epidermal hyperplasia, deregulation of keratin expression, and infiltration of immune cells, a similar but much milder phenotype compared to the skin lesions of *K14-Cre Ikk2*$^{fl/fl}$ mice (data not shown). Consistent with the improved clinical signs of skin inflammation, quantification of the epidermal thickness showed strongly reduced epidermal hyperplasia in *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ compared to *K14-Cre Ikk2*$^{fl/fl}$ and *Il20ra*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice at P8 (Figure 6). Furthermore, all double-deficient *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice survived to adulthood, with 11 out of 14 mice showing only very mild signs of skin inflammation up to the age of 7 weeks (Figure S3A; data not shown). After 14 weeks, all of the *Il22ra1*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice developed cutaneous inflammation to a variable degree, which affected their abdomen, the flanks, and the throat. Therefore, whereas ablation of IL-20R1 had a mild protective effect, IL-22R1 deficiency strongly inhibited, although it did not completely prevent, the development of inflammatory skin lesions in the *K14-Cre Ikk2*$^{fl/fl}$ mice.

To address the potential role of IL-22, which also signals via IL-22R1 (Sabat, 2010), we crossed the *K14-Cre Ikk2*$^{fl/fl}$ mice to IL-22-deficient mice (Kreymborg et al., 2007). *Il22*$^{-/-}$ *K14-Cre Ikk2*$^{fl/fl}$ mice developed severe inflammatory skin lesions and died before P9 similarly to *K14-Cre Ikk2*$^{fl/fl}$ mice, demonstrating that IL-22 does not play an important pathogenic role in this model (Figure S3B).

The TNF-IL-24 Axis in Human Psoriasis

Overactivation of ERK and STAT3 signaling have been reported in psoriatic epidermis (Haase et al., 2001; Sano et al., 2005; Takahashi et al., 2002; Wolk et al., 2009a), suggesting that the mechanism triggering skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice could be relevant for human psoriasis. As shown previously and similar to *K14-Cre Ikk2*$^{fl/fl}$ epidermis (see Figures 2 and 3), we found that several cytokines and chemokines were upregulated in psoriatic epidermis, including IL-24, IL-19, IL-6, IL-1β, GM-CSF, and CCL4 (Figures 7A and 7B). In addition, consistent with our findings in *K14-Cre Ikk2*$^{fl/fl}$ skin, TNF transcripts were upregulated in whole skin, but not in the epidermis of human psoriasis lesions (Figures 7A and 7B). To investigate whether the upregulation of IL-24 in human psoriatic epidermis could also be induced by an aberrant signaling response of human keratinocytes to TNF, we examined TNF-induced IL-24 expression in primary human keratinocytes treated with a highly specific IKK2 inhibitor, BI605906 (Clark et al., 2011). Similar to IKK2-deficient murine keratinocytes, primary human keratinocytes showed increased expression of IL-24 mRNA and protein upon stimulation with TNF when pretreated with BI605906 (Figure 7C). Therefore, inhibition of IKK2-mediated NF-κB activation led to increased IL-24 expression in response to TNF stimulation in both mouse and human primary keratinocytes.

To examine whether IL-24 expression in psoriatic epidermis is driven by TNF, we measured IL-24 mRNA in the skin of psoriasis patients before and after treatment with anti-TNF antibodies. Anti-TNF therapy almost completely suppressed IL-24 expression in psoriatic skin, suggesting that IL-24 production is mainly driven by TNF in psoriasis (Figure 7D). To test whether IL-24 is capable to induce the expression of proinflammatory genes in human epidermis, we used a three dimensional (3D) human skin culture system (reconstituted human epidermis, RHE). As shown in Figure 7E, IL-24 stimulation induced the expression of many of the inflammatory mediators detected in psoriasis, including psoriasin, LCN2, IL-20, CXCL1, CXCL8, and CCL20. An increase in epidermal thickness was also observed in IL-24 stimulated compared to unstimulated RHE (Figure S4). Taken together, our results suggest that TNF-driven IL-24 expression in keratinocytes contributes to the induction of inflammation in psoriasis, similar to our findings in the *K14-Cre Ikk2*$^{fl/fl}$ mouse model.

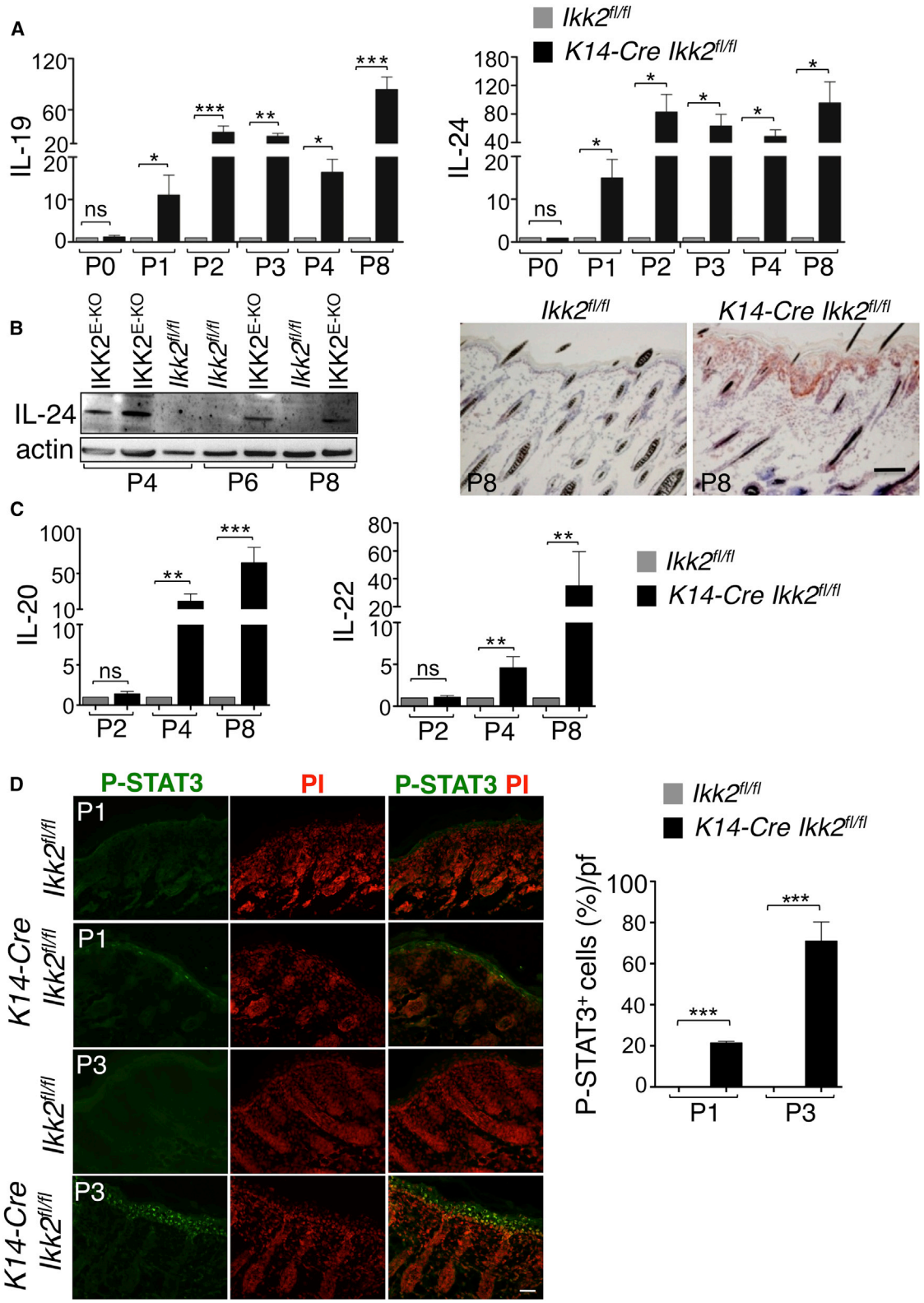

Figure 3. Upregulation of IL-19 and IL-24 and Activation of STAT3 in the Epidermis of *K14-Cre Ikk2*$^{fl/fl}$ Mice

(A) qRT-PCR analysis of IL-19 and IL-24 expression in whole skin (P8) or epidermal extracts (P1–P4) of *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ mice.

(B) Immunoblot and immunohistochemical analysis of IL-24 expression in the skin of *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ (IKK2^{E-KO}) mice with specific antibodies. Actin served as loading control. Red signal in immunostaining shows IL-24. Scale bar represents 50 μm.

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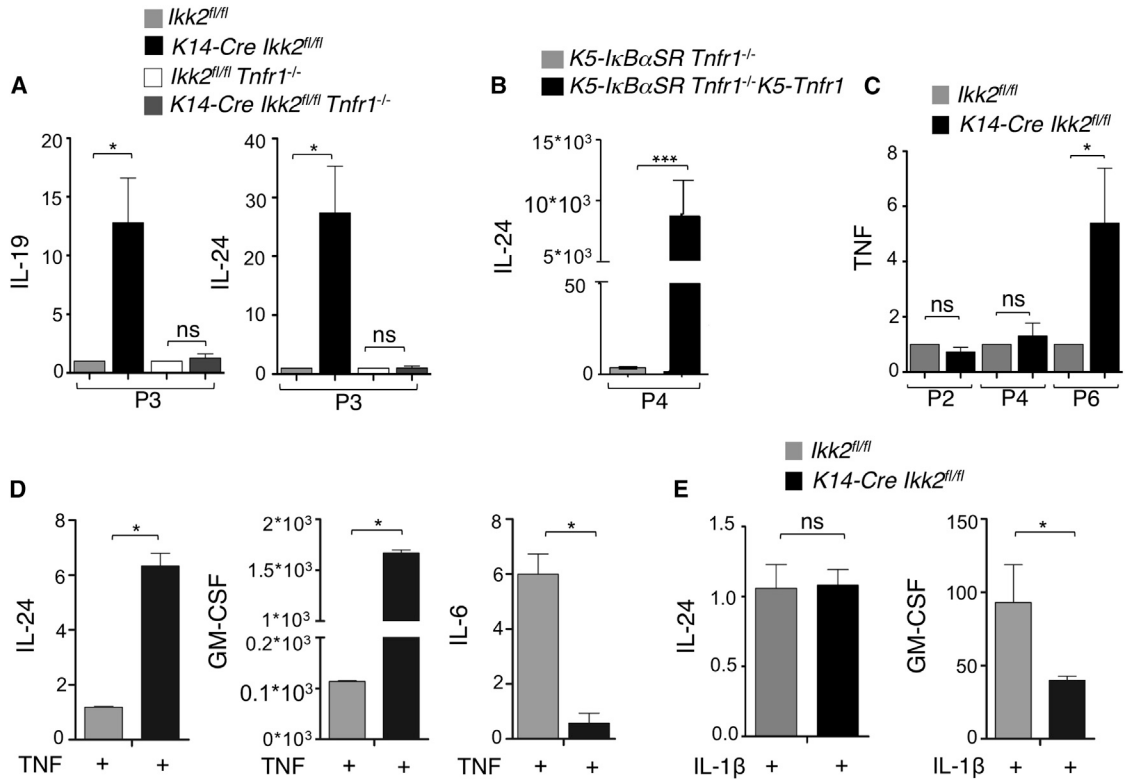

Figure 4. TNF-Dependent Regulation of IL-19, IL-24, and GM-CSF Expression in IKK2-Deficient Epidermal Keratinocytes

(A–C) qRT-PCR analysis of expression of IL-19 and IL-24 in the epidermis of *K14-Cre Ikk2*$^{fl/fl}$ and *Ikk2*$^{fl/fl}$ mice each at P3 (A), IL-24 expression in the skin of *Tnfr1*$^{-/-}$ *K5-Tnfr1* and *K5-IκBαSR Tnfr1*$^{-/-}$ *K5-Tnfr1* mice at P4 (B), and TNF expression in the whole skin of *K14-Cre Ikk2*$^{fl/fl}$ and *Ikk2*$^{fl/fl}$ mice at P2, P4, and P6 (C). Bars show fold regulation of TNF, IL-19, and IL-24 in *K14-Cre Ikk2*$^{fl/fl}$, *K14-Cre Ikk2*$^{fl/fl}$ *Tnfr1*$^{-/-}$, and *K5-IκBαSR Tnfr1*$^{-/-}$ *K5-Tnfr1* mice over the respective control mice normalized to HPRT mRNA.

(D and E) qRT-PCR analysis of cytokine expression in primary murine keratinocytes isolated from *Ikk2*$^{fl/fl}$ (gray bars) and *K14-Cre Ikk2*$^{fl/fl}$ (black bars) mice 6 hr after stimulation with 100 ng/ml rmTNF (D) or 20 ng/ml IL-1β (E). Bars show fold regulation in IKK2-deficient keratinocytes over the respective unstimulated control keratinocytes normalized to HPRT mRNA. All qRT-PCR results are expressed as mean values ± SEM. *$p \leq 0.05$, ***$p \leq 0.001$.

DISCUSSION

Our results presented here unravel a keratinocyte-intrinsic mechanism triggering psoriasis-like skin inflammation in mice with epidermis-specific NF-κB inhibition that is relevant for the understanding of the pathogenesis of psoriatic skin inflammation in humans. We show that TNFR1 signaling in IKK2-deficient epidermal keratinocytes is both necessary and sufficient for the development of skin inflammation, identifying skin epithelial cells as the major cellular target of the pathogenic TNF signals in this model. In addition, we identify TNFR1-induced, ROS-, and ERK-dependent expression of IL-24 in keratinocytes as a key early event contributing to skin inflammation. Our genetic experiments showing that IL-22R1 deficiency strongly ameliorated skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice provide functional evidence for the key role of IL-24 as an early upstream cytokine inducing skin inflammation in this model. This is further supported by our findings that IL-20 and IL-22, which also signal via IL-22R1, were not upregulated at early stages of phenotype development. Indeed, IL-22 deficiency did not affect skin lesion development in these mice. However, although its expression pattern argues against a critical role in the initiation of skin inflammation in *K14-Cre Ikk2*$^{fl/fl}$ mice, in the absence of functional evidence we cannot exclude that IL-20 also contributes to lesion development. Our findings are in line with recent results showing that transgenic expression of IL-24 in mouse epidermis was sufficient to induce skin inflammation (He and Liang, 2010). In addition, the very mild protective effect of IL-20R1-deficiency suggests that IL-19, which signals exclusively via this receptor, is unlikely to play an important role in this type of skin inflammation, consistent with the findings that transgenic mice overexpressing IL-19 in the epidermis did not develop skin lesions (Parrish-Novak et al., 2002).

What is the relevance of our mouse model findings for the pathogenesis of human psoriasis? As shown recently (Wang et al., 2012), we also found that IL-24, together with IL-19, was

(C) qRT-PCR analysis of IL-20 and IL-22 expression in whole skin (P2, P4, and P8) of *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ mice.

(D) Representative images (left) and quantification (right) of immunofluorescence staining for phosphorylated STAT-3 (green) on skin sections of *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ mice at P1 and P3. Nuclei were stained with PI (red). Scale bar represents 50 μm. All qRT-PCR results are expressed as mean values ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. See also Figure S2.

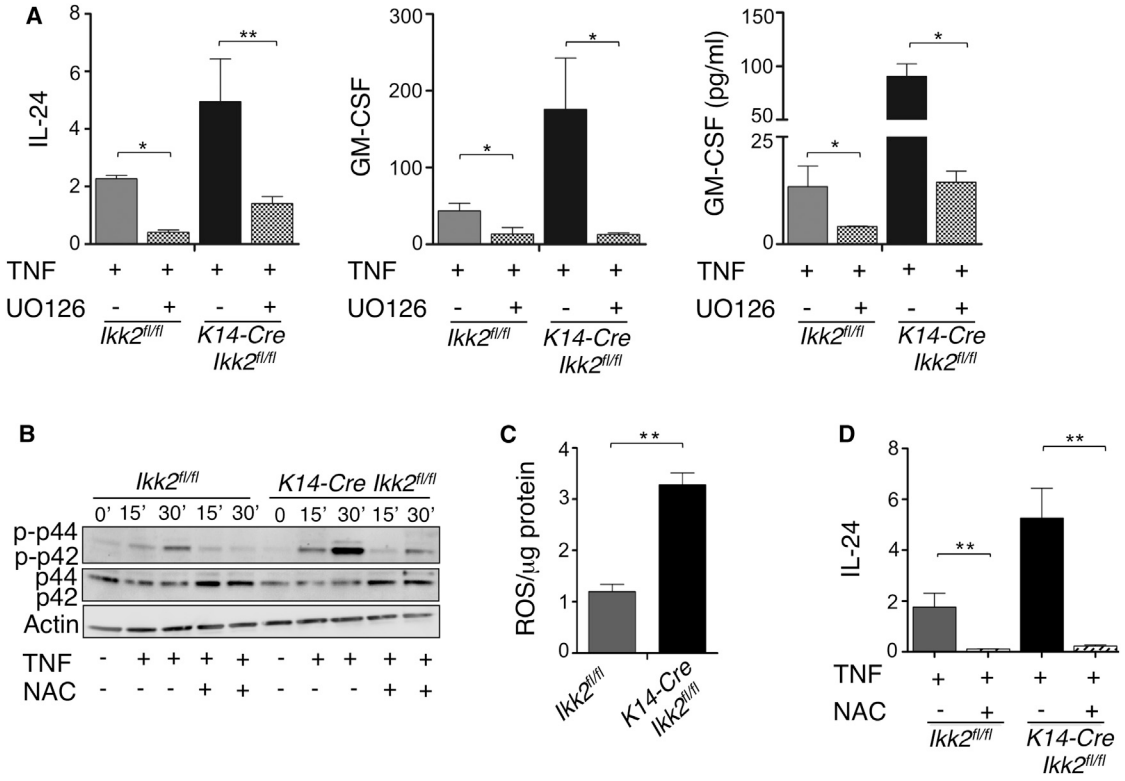

Figure 5. TNF-Induced Expression of IL-24 and GM-CSF in Keratinocytes Depends on ROS and MEK-ERK Signaling

qRT-PCR analysis of IL-24 and GM-CSF expression (A, D), as well as ELISA of GM-CSF protein (A), measurement of ROS production (C), and immunoblot analysis of p44 and p42 phosphorylation after TNF stimulation with or without NAC (B) in primary epidermal keratinocytes isolated from *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ mice. qRT-PCR analysis was performed with RNA obtained 6 hr after stimulation and ELISA on supernatants harvested 8 hr after stimulation of cells with 100 ng/ml rmTNF in the presence or absence of the MEK inhibitor UO126 (1 µM) or NAC (10 mM) as indicated. Bar graphs show fold regulation of cytokine expression in TNF-stimulated *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ keratinocytes in the presence or absence of UO126 or NAC over vehicle-stimulated keratinocytes from the same isolate, respectively. Constitutive ROS production in primary keratinocytes isolated from *Ikk2*$^{fl/fl}$ and *K14-Cre Ikk2*$^{fl/fl}$ keratinocytes were measured with the fluorescent dye CM-H2DCFDA. All data are expressed as mean values ± SEM of two different isolates of primary keratinocytes. *p ≤ 0.05, **p ≤ 0.01.

upregulated in psoriatic epidermis, similarly to the *K14-Cre Ikk2*$^{fl/fl}$ and *K5-IκBαSR* mice. In addition, our results showing that pharmacological inhibition of IKK2-mediated NF-κB activation strongly increased TNF-induced IL-24 expression in primary human keratinocytes suggest that the TNF-dependent mechanism triggering skin inflammation in our mouse models is relevant for humans. Consistent with a recent study showing that anti-TNF treatment acutely suppressed the expression of IL-19, IL-20, and IL-24 in psoriatic epidermis (Wang et al., 2012), we also detected downregulation of IL-24 expression in the skin of anti-TNF-treated psoriasis patients, suggesting that the epidermal keratinocyte is an important and direct cellular target of pathogenic TNF signaling also in human psoriasis. Our results in a human epidermis model showing that IL-24 strongly induces the expression of proinflammatory mediators known to be expressed in psoriatic skin also provide experimental evidence that IL-24 might act as an important upstream mediator in the pathogenesis of psoriatic skin inflammation, similarly to skin inflammation in our mouse models. There is still an ongoing debate concerning primary pathogenic functions of epidermal keratinocytes versus T lymphocytes in psoriasis, and there is evidence for both cell types playing important roles in disease development (Nickoloff et al., 2000). Previously, a possible contribution of epidermal keratinocytes to the pathogenesis of psoriasis has been discussed primarily in the context of the dramatic changes in proliferation and differentiation that these cells undergo during disease development. Our results show that, in addition to their well-characterized role in epidermal hyperplasia, keratinocytes exhibit a potent capacity to initiate skin inflammation and could therefore be a crucial cellular component triggering the onset of the inflammatory response in psoriasis. More recent concepts of psoriasis pathogenesis allocate functions of different cell types to the changing phases of the disease process: it is thought that disease initiation is driven by innate immune responses, whereas T cell-dependent adaptive immune mechanisms take control in the maintenance phase (Nestle et al., 2009; Sabat et al., 2007). We showed previously that the disease in *K14-Cre Ikk2*$^{fl/fl}$ mice develops independently of αβ T cells (Pasparakis et al., 2002), suggesting that this model is more relevant for the innate immunity-driven initial phase of the disease. This concept is also compatible with our results that IL-24, but not IL-22, is critical for disease initiation. The idea of functional redundancies between these two cytokines in the development of psoriatic skin inflammation is tempting and is compatible with the two-phase model of the pathogenesis of psoriatic inflammation (Nestle et al., 2009; Sabat et al., 2007). As IL-24

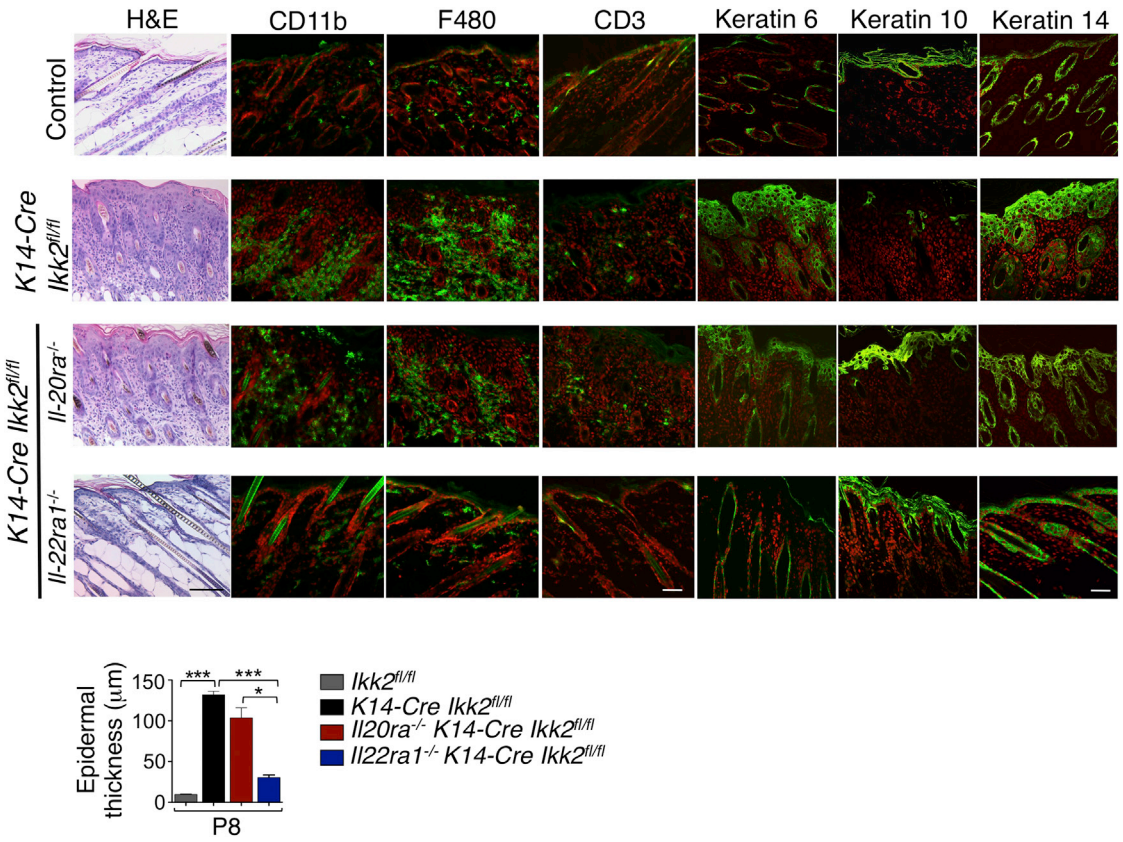

Figure 6. IL-22RI-Deficiency Inhibits the Development of Skin Inflammation in *K14-Cre Ikk2*$^{fl/fl}$ Mice

Histological analysis of skin from mice with the indicated genotypes at P8. Skin sections were stained with H&E or immunostained with antibodies against CD11b, F4/80, CD3, Keratin 6, Keratin 14, and Keratin 10 (green). Nuclei were stained with PI (red). Scale bar represents 100 μm (H&E), 50 μm (immune marker stainings), and 40 μm (keratin stainings). Bar graph in the lower panel shows microscopic measurement of the epidermal thickness on skin sections obtained from mice of the indicated genotypes (n = 3–4). Results are expressed as mean values ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. See also Figure S3.

is mainly produced by epithelial and innate immune cells and IL-22 by T- lymphocytes (Conti et al., 2003; Kunz et al., 2006; Ouyang et al., 2011; Zheng et al., 2007), it is conceivable that IL-24 is particularly relevant for the initiation phase, whereas IL-22 could be mainly involved in the chronic phase of the disease. We therefore consider our mouse models as valuable for understanding the mechanisms governing the initiation phase of psoriasis-like skin inflammation.

Anti-TNF therapy is currently one of the most effective treatments for psoriasis; however, the TNF-dependent mechanisms causing psoriatic inflammation are unknown. Our results suggest that epidermal keratinocytes are critical targets of pathogenic TNF signaling in psoriasis. Unexpectedly, it is not the upregulation of TNF expression but rather the deregulation of TNFR1 signaling that provides the pathogenic signal. In addition to NF-κB inhibition, other alterations disturbing TNF-TNFR1 signaling in epidermal keratinocytes also induced skin inflammation. For example, epidermis-specific FADD deficiency sensitizes keratinocytes to TNF-induced RIP kinase 3 (RIPK3)-mediated necrosis and spontaneously triggers severe skin inflammation in mice (Bonnet et al., 2011). Given that a TNF-TNFR1-dependent surveillance mechanism is present in normal, resting skin, any intracellular change leading to altered TNFR1 signaling responses in epidermal keratinocytes could trigger the initiation of skin inflammation. This TNF-dependent surveillance mechanism could have an important physiological function to ensure the rapid induction of skin inflammation that is essential for host defense, wound healing, and tissue regeneration. The development of psoriatic lesions after wounding of the skin is a typical clinical characteristic of the disease known as Koebner phenomenon and could be explained by such a mechanism. Many polymorphisms that have been associated with psoriasis in genome-wide association studies lie within genes encoding proteins of the TNF-NF-κB signaling pathway or associated regulatory proteins (Jordan et al., 2012; Nair et al., 2009). Although the functional consequences of these genetic polymorphisms are largely unknown, it is conceivable that some of these alleles could contribute to skin inflammation by altering TNFR signaling in epidermal keratinocytes.

Considering that chronic skin inflammation clinically identified as psoriasis most likely represents a range of diseases with distinct etiologies, it is likely that different mechanisms affecting the response of epidermal keratinocytes to TNF and most likely other immune surveillance mechanisms could trigger the disease. Indeed, previous studies showed that disturbance of different signaling pathways in keratinocytes can induce skin

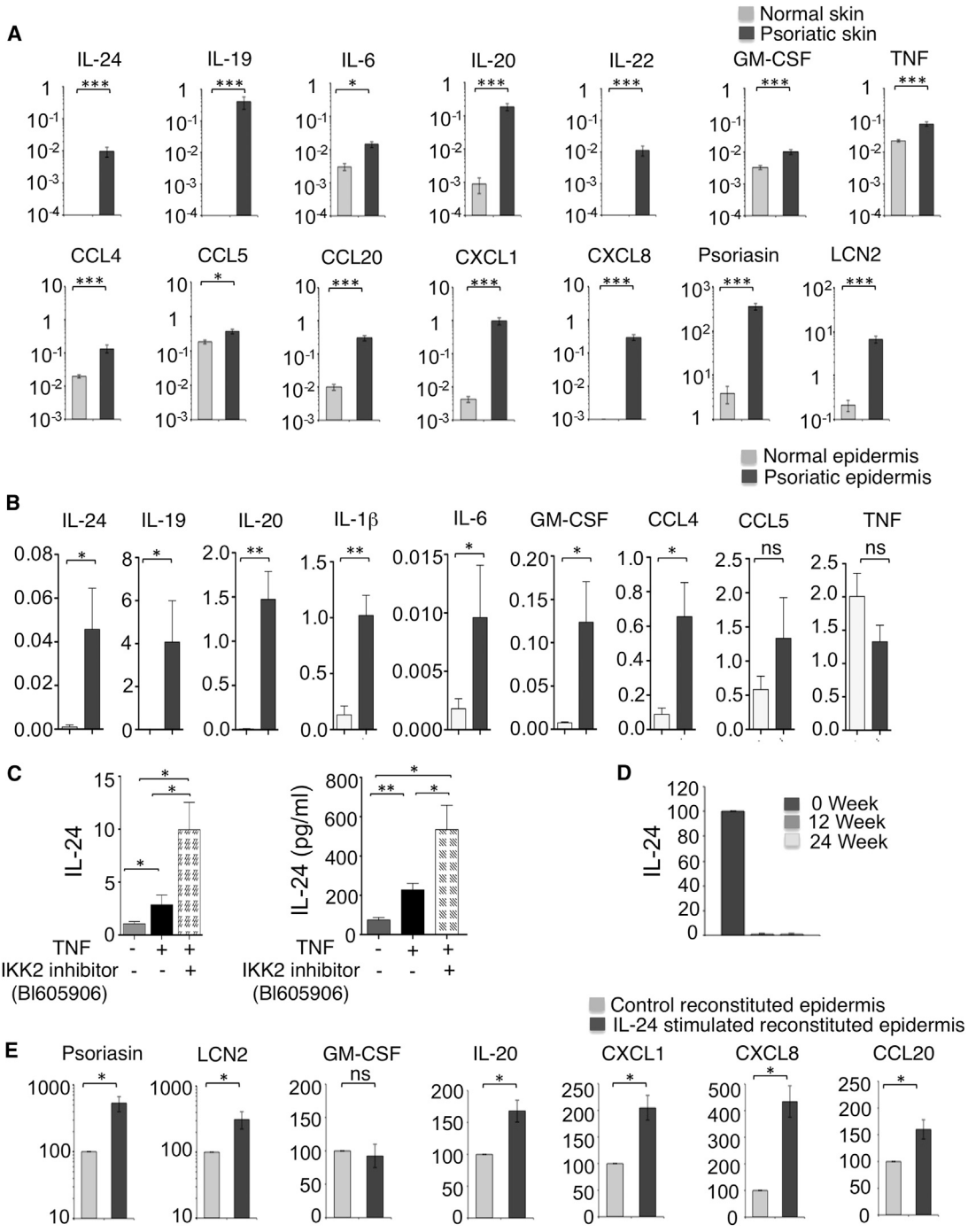

Figure 7. TNF-Induced IL-24 Expression by Epidermal Keratinocytes Is Relevant for Human Psoriasis

(A and B) qRT-PCR analysis of the expression of the indicated cytokines and chemokines in whole skin (A, n = 8–11) and epidermis (B, n = 3–5) from normal or psoriatic skin samples. Graphs show arbitrary gene expression relative to HPRT.

(C) Left shows human primary keratinocytes (n = 3) that were stimulated for 6 hr with 100 ng/ml TNF in the presence or absence of the IKK2 inhibitor BI605906 (5 μM) and IL-24 mRNA levels that were determined by qRT-PCR. Graphs show fold regulation compared to unstimulated cells normalized to HPRT mRNA. Right shows human primary keratinocytes (n = 4) that were stimulated for 48 hr with 10 ng/ml TNF in the presence or absence of the IKK2 inhibitor BI605906 (5 μM). IL-24 concentrations in culture supernatants were determined by ELISA. Data are shown as mean values ± SEM.

(D) qRT-PCR analysis of IL-24 expression in skin before (0) and 12 and 24 weeks after anti-TNF treatment. Bars show percentage of IL-24 expression in anti-TNF treated skin after 12 weeks and 24 weeks over 0 weeks.

(E) qRT-PCR analysis of the expression of the indicated cytokines and chemokines in reconstituted human epidermis (n = 6) 72 hr after stimulation with IL-24 (20 ng/ml). Bars show percentage of mRNA expression in IL-24 stimulated over unstimulated 3D epidermis normalized to HPRT mRNA. Results are expressed as mean values ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. See also Figure S4.

inflammation, including activation of NF-κB (Rebholz et al., 2007), MEK1/ERK (Hobbs et al., 2004; Hobbs and Watt, 2003), or STAT3 signaling (Sano et al., 2005) and also inhibition of Jun-AP1 signaling (Zenz et al., 2005). Because all of these pathways are involved in the regulation of keratinocyte reactions to stress, infection, or injury, it is tempting to hypothesize that deregulation of normal keratinocyte responses to environmental challenges could be a major pathogenic factor triggering the initiation of psoriatic inflammation. By identifying a keratinocyte-intrinsic mechanism that links TNF, NF-κB, ERK, and STAT3 signaling with the initiation of psoriasis-like skin inflammation, our results provide an important step toward the better understanding of the pathogenesis of psoriasis.

EXPERIMENTAL PROCEDURES

Mice

The following mouse lines were used: *Ikk2*^{FL} (Pasparakis et al., 2002), *K14-Cre* (Hafner et al., 2004; Pasparakis et al., 2002), *Tnfr1*$^{-/-}$ (Pfeffer et al., 1993), *Tnfr1*^{cneo} (Victoratos et al., 2006), *K5-IκBαSR* (van Hogerlinden et al., 1999), *Il22*$^{-/-}$ (Kreymborg et al., 2007), and *Tnfr1*^{FL} (Van Hauwermeiren et al., 2013). IL-20R1 and IL-22R1 knockout mice will be described elsewhere. All animal procedures were conducted in accordance with European, national, and institutional guidelines, and protocols and were approved by local governmental authorities.

Immunostainings

Keratin staining on paraffin sections and CD11b, F4/80, Gr-1, CD3, and STAT-3 staining on cryosections were performed as described (Stratis et al., 2006). IL-24 staining was performed on cryosections with anti-IL-24 antibodies (Santa Cruz Biotechnology). Details of the procedures are provided under Supplemental Experimental Procedures.

Isolation, Culture, and Stimulation of Keratinocytes

Primary epidermal keratinocytes were isolated from newborn mice and cultured as described before (Tscharntke et al., 2007). Primary human epidermal keratinocytes were obtained from Cascade Biologics and Promocell and cultured in KGM Gold Medium (Lonza). Cells were starved overnight in 1% FCS containing medium before stimulation with TNF or IL-1β. Inhibitors UO126 (EMD, Calbiochem), N-Acetyl-L-Cysteine (Sigma Aldrich), or BI605906 (Clark et al., 2011) were added 1 hr prior to cell stimulation.

Reconstituted Human Epidermis Culture and Stimulation

Underdeveloped EpiDerm-201 reconstituted human epidermis (RHE) tissues composed of stratified human keratinocytes derived from neonatal foreskins were obtained from MatTek and were cultured as described previously (Wolk et al., 2011). Briefly, they were cultured in inserts at the air-liquid interface with hydrocortisone-free Epi-201-DM differentiation medium (MatTek). For stimulation, culture medium was supplemented or not (control) with 20 ng/ml IL-24 (R&D Systems). After 72 hr, biopsies were taken from the tissues and either snap-frozen for histology (determination of thickness of the living cell layers) or lysed for mRNA analysis.

Separation of Epidermis, RNA Isolation, and cDNA Synthesis

Human tissue samples and cells were used after approval by the Ethics Committee of the Medical Faculty of Cologne University and the clinical institutional review board of the University Medicine Charité, Berlin. Punch skin biopsies were obtained from adult control participants (30 to 67 years old) and patients with plaque psoriasis (26 to 63 years old, ca. 65% moderate to severe disease). Mouse and human epidermis were peeled off after incubating skin samples in 0.5 M ammonium thiocyanate (NH_4SCN) in phosphate buffer, pH 6.8 (0.1 M NH_2HPO_4, 0.1 M KH_2PO_4) for 30 min on ice. Total RNA was extracted from homogenized tissue or cultured keratinocytes with TRIzol reagent (Invitrogen) and purified with an RNeasy mini kit (QIAGEN, Hilden) or Invisorb® RNA kit II (Invitek/Stratec molecular GmbH) as per manufacturer's protocol. Single-stranded cDNA was synthesized from 1 μg of total RNA with the cDNA synthesis kit (Applied Biosystems, Thermo Fisher/Fermentas or Life Technologies/GIBCO) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed on cDNA with Power SYBR Green PCR mix (Applied Biosystems) or Maxima Probe/ROX qPCR Master Mix (Thermo Fisher Scientific/Fermentas) and systems with double-labeled fluorescent probes as described previously (Wolk et al., 2004) with a 7300 or StepOnePlus Real Time PCR system (Applied Biosystems). Details of the analysis and primer sequences (Tables S1 and S2) are provided under Supplemental Experimental Procedures.

Immunoblot

Tissue and cell lysates were resolved on SDS PAGE and probed with primary antibodies, and immunoreactive proteins were visualized with HRP-coupled secondary antibodies and chemiluminescence reagents (PerkinElmer; Pierce Thermo Fisher Scientific) on an Uvichemi chemiluminescence documentation system (Biometra). Details of the method are provided under Supplemental Experimental Procedures.

Statistical Analysis

Statistical significance between in vivo-derived samples and in vitro-generated samples with different genotypes was determined with the unpaired Student's t test or the Mann-Whitney U-test (two-tailed) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Paired in vitro generated samples were tested for significant differences between treatment groups with the Wilcoxon matched-pairs signed- rank test or paired student t test (two-tailed) (*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.10.009.

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Supplemental Information

Tumor Necrosis Factor Receptor Signaling in Keratinocytes Triggers Interleukin-24-Dependent Psoriasis-like Skin Inflammation in Mice

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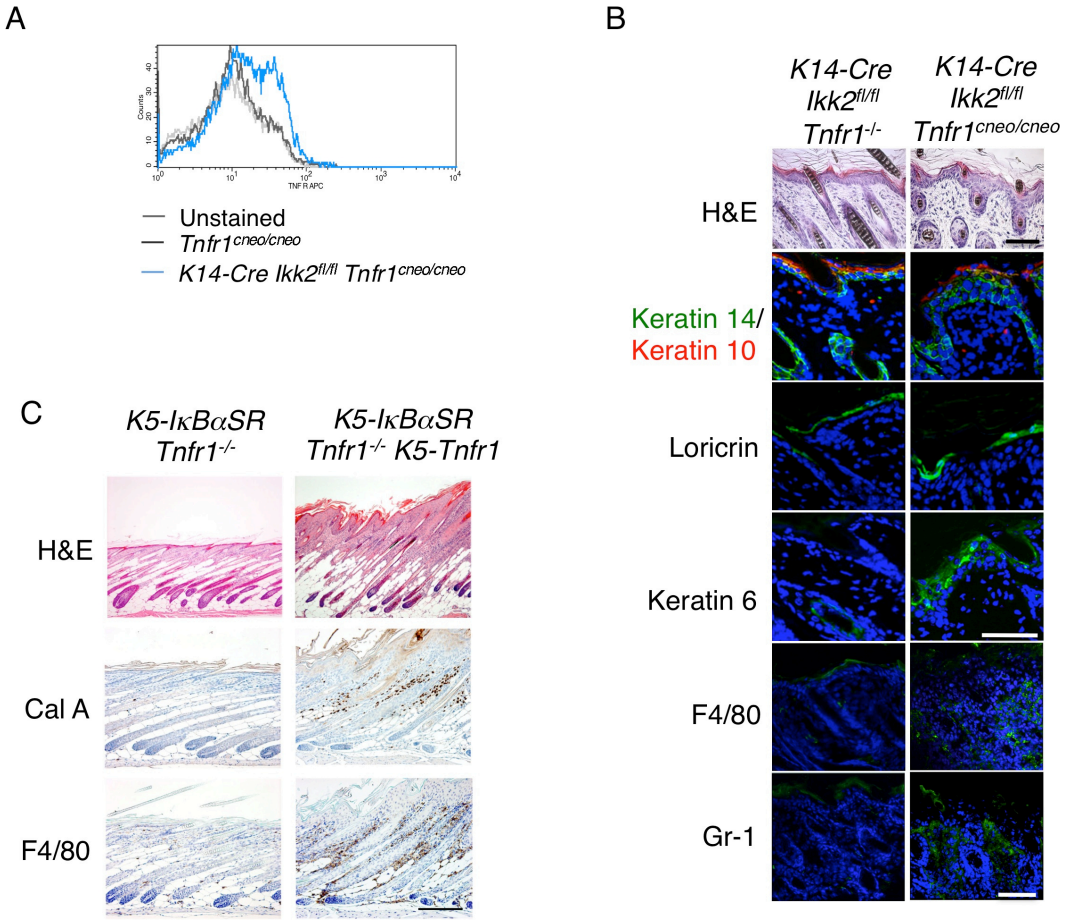
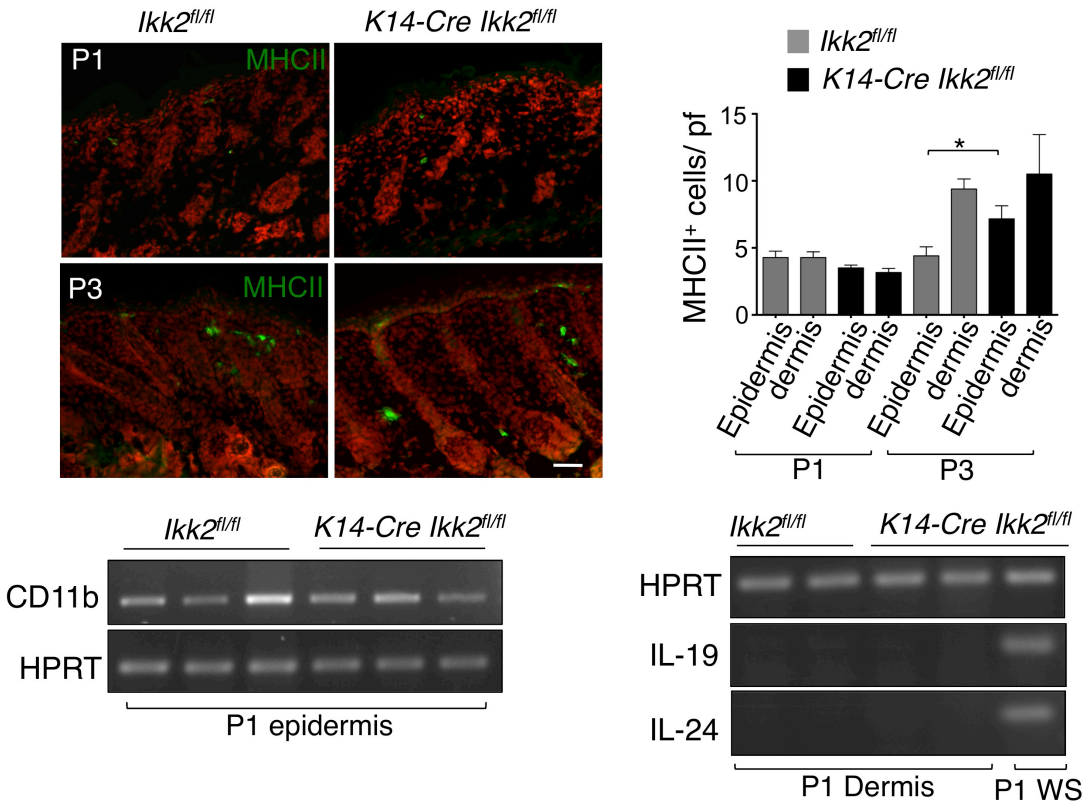
Supplemental Inventory

Supplementary Figures



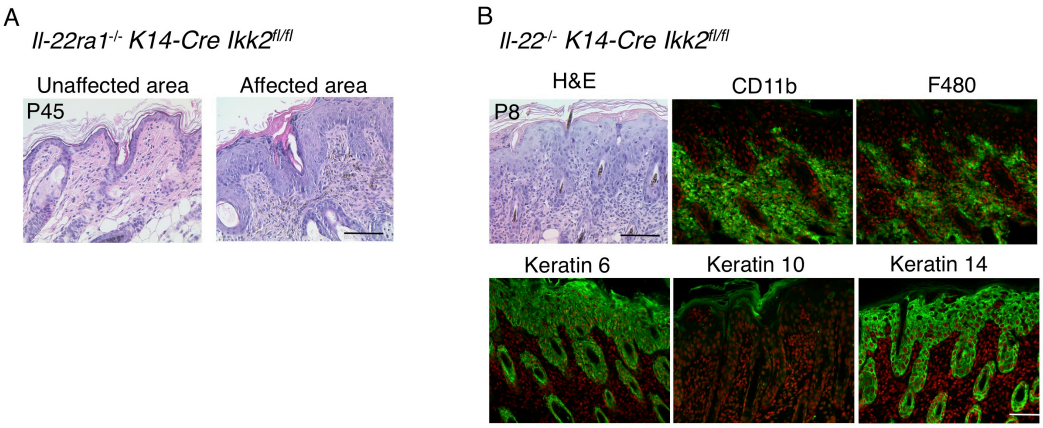
Supplementary Figure 1, related to Figure 1: Keratinocyte-specific expression of TNFR1 is sufficient to induce skin inflammation in *K14-Cre Ikk2$^{fl/fl}$* and *K5-IκBαSR Tnfr1$^{-/-}$* mice.

(A) FACS analysis of TNFR1 expression in epidermal keratinocytes from mice with the indicated genotypes. **(B)** Histological (H&E) and immunofluorescence analysis of epidermal differentiation markers (Keratin 14, 10, 6 and loricrin) and immune cells (F4/80 and Gr-1) in skin sections from mice with the indicated genotypes at P7. Nuclei were stained with DAPI (blue). **(C)** Histological analysis and immunostaining (F4/80 and Calgranulin A (CalA) in skin sections from *K5-IκBαSR Tnfr1$^{-/-}$* and *K5-IκBαSR Tnfr1$^{-/-}$ K5-Tnfr1* mice at P8. Scale bar is 100 μm.



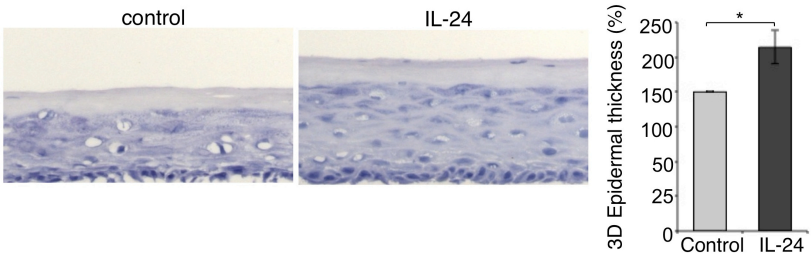
Supplementary Figure 2, related to Figure 3: Unaltered expression of myeloid cell markers in the epidermis and absence of IL-24 expression in the dermis of *K14-Cre Ikk2$^{fl/fl}$* mice.

Immunofluorescence staining (*left*) and quantification (*right*) of MHCII positive cells (green) in the epidermis and dermis of *Ikk2$^{fl/fl}$* and *K14-Cre Ikk2$^{fl/fl}$* mice at P1 and P3 (upper panel), and semi quantitative RT-PCR analysis of CD11b expression in the epidermis and IL-24 in the dermis of *Ikk2$^{fl/fl}$* and *K14-Cre Ikk2$^{fl/fl}$* mice at P1 (lower panel). Nuclei were stained with PI (red) ; pf (power field). Scale bar is 50 μm. Results are expressed as mean values ± SEM. *$P \leq 0.05$.



Supplementary Figure 3, related to Figure 6: *K14-Cre Ikk2$^{fl/fl}$* mice bred on a IL-22RI-deficient genetic background show mild signs of skin inflammation in adulthood, while IL-22-deficiency does not prevent skin inflammation in K14-Cre *Ikk2$^{fl/fl}$* mice.

(A) H&E staining of skin sections from a representative double deficient *Il-22r1$^{-/-}$ K14-Cre IKK2$^{fl/fl}$* mouse at P45. Scale bar is 100 μm. **(B)** H&E and immunofluorescence staining of immune cells (CD11b and F4/80) and keratins 6, 14 and 10 in skin sections of double deficient *Il-22$^{-/-}$ K14-Cre Ikk2$^{fl/fl}$* mice at P8. Nuclei were stained with PI (red). Scale bar is 100 μm (H&E) and 50 μm (immunofluorescence stainings).



Supplementary Figure 4, related to Figure 7: IL-24 treatment of reconstituted human epidermis induces acanthosis.
Mayer's hemalum staining and quantification of the thickness of the living layer in RHE tissue upon IL-24 treatment (20 ng/ml) for 72 h. Representative pictures from five experiments are given. Bar graph shows mean values ± SEM from five experiments. *$P \leq 0.05$.

Table S1: Primer sequences for SYBR Green qRT-PCR and semi quantitative RT-PCR

mHPRT	FP: ATCATTATGCCGAGGATTTGGAA RP: TGAAGGACTCTGGCTTTGTCT
mIL-1β	FP: CGACAAAATACCTGTGGCCT RP: TCTTCTTTGGGTATTGCTTGG
mIL-6	FP: ATGGATGCTACCAAACTGGAT RP: TGAAGGACTCTGGCTTTGTCT
mIL-19	FP: GCCAACTCTTTCCTCTGCGT RP: GGT GGCTTCCTG ACTGCAGT
mCCL3	FP: TGCCCTTGCTGTTCTTCTCT RP: GTGGAATCTTCCGGCTGTAG
mCCL4	FP: GCTCTGTGCAAACCTAACCC RP: GAGAAACAGCAGGAAGTGGG
mCCL5	FP: CCCTCACCATCATCCTCACT RP: AGAGGTAGGCAAAGCAGCAG
mGM-CSF	FP. GATATTCGAGCAGGGTCTAG RP: ATGAAATCCGCATAGGTGGT
mTNF	FP: TGCCTATGTCTCAGCCTCTTC RP: GAGGCCATTTGGGAACTTCT
hHPRT	FP: TGACCTTGATTTATTTTGCATACC RP: CGAGCAAGACGTTCAGTCCT
hCCL4	FP: CTTCCTCGCAACTTTGTGGT RP: CAGCACAGACTTGCTTGCTT
hCCL5	FP: ATCCTCATTGCTACTGCCCTC RP: GCCACTGGTGTAGAAATACTCC
hGM-CSF	FP: AGAAATGTTTGACCTCCAGGA RP: TTGCACAGGAAGTTTCCG
mCD11b	FP: GCCGGTGAAATATGCTGTCT RP: GCGGTCCCATATGACAGTCT

Table S2: Primer sequences for Taqman qRT-PCR

hIL-19	5'-CATGCACCATATAGAAGAGAGTTTCC-3' 5'-TGGACAGGATAGTGACATTTGGG-3' 5'-AGAAATCAAAAgAGCCATCCAAGCTAAGGACAC-3'
hIL-20	5'-GGAGGACTGAGTCTTTGCAAGAC-3' 5'-CCGGAGAGTATAATGGTCAGGG-3' 5'-CAAAGCCTGCGAATCGATGCTGC-3'
hIL-22	5'-ACAACACAGACGTTCGTCTCATTG-3' 5'-GAACAGCACTTCTTCAAGGGTGA-3' 5'-TTCCACGGAGTCAGTATGAGTGAGCGCT-3'
hIL-24	5'-TTTCAACAGAGGCTGCAAAGC-3' 5'-GCACAACCATCTGCATTTGAGA-3' 5'-ACTTTAGCCAGACCCTTCTGCCCTCCTTT-3'
hHPRT	5'-AGTCTGGCTTATATCCAACACTTCG-3' 5'-GACTTTGCTTTCCTTGGTCAGG-3' 5'-TTTCACCAGCAAGCTTGCGACCTTGA-3'

Supplemental Extended Experimental Procedures

Generation of K5-Cre IκBαSR *Tnfr1*$^{-/-}$ K5-Cre TNFR1 mice

Mouse cDNA encoding TNFR1 was a kind gift from Professor Kiyoshi Mik, (Department of Cell and Developmental Biology, The University of North Carolina at Chapel Hill, USA), and was subcloned by blunt-end ligation into the *SnaB*I restriction site of the p59BK5 expression vector (Ramirez *et al*., 1994), a kind gift from Professor José Jorcano (CIEMAT, Madrid, Spain). *Tnfr1*$^{-/-}$ mice (Pfeffer et al., 1993) were kindly provided by Professor Tak Mak (Advanced Medical Discovery Institute, Toronto, Canada), and were backcrossed 10 generations into FVB/N. The transgene was excised from the expression plasmid with *Sal*I and *Not*I and microinjected into the pronuclei of fertilized *Tnfr1*$^{-/-}$/FVB/N oocytes. Transgene integration was confirmed by PCR analysis of genomic DNA extracted from tail biopsies. Two independent founder lines were established. Both lines showed a similar phenotype. At least 5 mice in every group were analyzed. Mice were kept according to Swedish national requirements and ethical permission was obtained for all experiments.

Isolation of skin cells and Flow Cytometry

Immune cells from whole skin of mice at P8 were isolated by a combination of enzymatic digestion and mechanical dissociation using liberase blendzyme 3 (Roche Applied Science) and BD^{TM} Medimachine (BD Biosciences, San Jose, USA), respectively. The isolated cells were fluorescently labeled with primary antibodies; anti-CD11b-APC (MACS, Miltenyi Biotech, 1:50), anti-F4/80-Alexa488 (Caltag, Invitrogen, 1:100), anti-CD11c-FITC (BD Pharmingen, 1:200) in FACS buffer (PBS, 2 mM EDTA and 0.5% BSA) for 30 minutes at 4°C. Primary epidermal keratinocytes, cultured in minimal calcium medium (0.05 mM) supplemented as previously described (Nenci et al., 2006) and dermal fibroblasts (grown in DMEM 10% FCS) were stained with anti-TNFR1- APC (Biolegend, San Diego, CA) in PBS-BSA for 1

hour at 37°C (34°C for keratinocytes), then fixed in 4% PFA for 30 minutes at RT. For each sample a minimum of 20,000 cells were counted by flow cytometry. Non-viable cells were gated using 7AAD (BD Pharmingen).

Histopathological Analysis and Immunostainings

Tissue samples were excised and either fixed in 4% paraformaldehyde overnight, then embedded in paraffin or embedded freshly in OCT compound (Sakura, Netherlands). H&E- and Keratin- stainings were performed on sections of paraffin embedded skin which were de- paraffinized/ re- hydrated according to standard procedures and boiled in 1X target retrieval buffer, pH 6.0 (Dako) when applicable. Biopsies taken from reconstituted human epidermis cultures were embedded in tissue-freezing medium (Leica Microsystems). Sections were fixed in -20°C acetone and stained with Mayer's hemalaun (Dr. K. Hollborn & Söhne GmbH & Co.). The thickness of living cell layers was measured by means of AxioVision Release 4.6.3 image software (Zeiss).

For IL-24 staining, acetone fixed frozen tissue sections were incubated with polyclonal anti-IL-24 antibody after blocking with 10% goat serum. Endogenous peroxidase was blocked using peroxidase block (Dako). EnVision^{+} system- HRP labeled Polymer anti-rabbit IgG and AEC substrate (Dako) were used for visualization.

The following primary antibodies were used: IL-24 (Santa Cruz Biotechnology, 1:200), P-STAT3 (Cell Signaling, 1:200), K14 (Neomarkers and Covance, 1:1000), K10, Loricrin and K6 (Covance, 1:100, 1:600), F4/80 (AbD Serotech, clone A3-1, 1:50), Gr-1 (eBiosciences, clone RB6-8C5, 1:20). Secondary antibodies were coupled to Alexa 488 or 594 (Molecular probes, 1:500). Sections were counterstained with DAPI or PI for nuclei visualization.

ROS Measurement in keratinocytes

Constitutive ROS production from primary keratinocytes was measured using the fluorescent dye CM-H2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate; Invitrogen). After starvation for 4 h, the medium was removed, cells were washed with PBS and incubated with 10 μM CM-H2DCFDA in PBS for 10 minutes at 32°C. Subsequently, the dye was removed and the cells were incubated in culture medium for 10 minutes at 32°C. After washing, the cells were lysed in buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $Cacl_2$, 300 mM sucrose/saccharose, 1% Triton X-100). The fluorescence of the lysates was measured in a Victor Multilabel reader (Perkin Elmer, USA) with an excitation of 490 nm and emission of 530 nm and normalized by total protein content of the same lysate as determined with the BCA Protein Assay (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

Quantitative real time PCR

HPRT (Hypoxanthine-guanine phosphoribosyltransferase) as a housekeeping gene and efficiency matched primers of the target genes were used for the PCR. The comparative method ($2^{-\Delta\Delta Ct}$) of relative quantification was used to determine the expression levels of target genes normalized to HPRT. Data were expressed as fold change over control mice or control keratinocytes. SYBR Green qRT-PCR primers for murine genes, IL-24, IL-22, IL-20, human genes IL-19, IL-24, IL-6, IL-1β, TNF were purchased from Qiagen. Taqman qRT-PCR primers and probes were purchased from Applied Biosystems. Primers for murine genes, HPRT, IL-19, IL-1β, CCL3, CCL4, CCL5, TNF, GM-CSF and human genes HPRT, GM-CSF, CCL4 and CCL5 were designed using standard software and synthesized, murine IL-6 primer sequence was taken from the RT Primer database. Primer sequences are provided in Tables S1 and S2.

Western Blot and ELISA

Separated epidermis and whole skin samples were homogenized for 3 minutes using a mixer mill MM 400 (Retsch, Germany) in $RIPA^+$ buffer (5 mM EDTA, 1% Triton 100, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 20 mM leupeptin, 1 mM PMSF, 0.5 mg/ml soybean trypsin inhibitor, 0.5 mM $NaVO_3$ and 10 mg/ml p-nitrophenylphosphate). Keratinocytes were lysed *in situ* in $RIPA^+$ buffer and sonicated for 30 s. Lysates were centrifuged at 16,000 g for 10 minutes at 4°C. The supernatants were collected and used for protein and western blot analysis after protein quantification as described above.

Protein (30 - 50 μg) was separated on 4 - 12% NuPAGE SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and blotted onto nitrocellulose membranes using the iBlot gel transfer stacks and gel transfer system (Invitrogen, Carlsbad, CA, USA). After blocking with Roche block (Roche Applied Science, 1:10) or with 5% milk powder solution the membrane was probed with polyclonal antibodies against the following proteins: pERK, (Cell signaling, 1:1000), ERK and IL-24 (Santa Cruz Biotechnology, 1:1000 and 1:200 respectively), and a mouse monoclonal anti- actin antibody (MP Biomedicals, Aurora, OH, USA, 1:6000).

GM-CSF ELISA on culture supernatants of primary murine keratinocytes and IL-24 ELISA on culture supernatant of primary human keratinocytes were performed using ELISA kits from R&D systems according to the instructions of the manufacturer.

Supplementary references

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