Cutting Edge: NF-κB p65 and c-Rel Control Epidermal Development and Immune Homeostasis in the Skin

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Psoriasis is an inflammatory skin disease in which activated immune cells and the proinflammatory cytokine TNF are well-known mediators of pathogenesis. The transcription factor NF-κB is a key regulator of TNF production and TNF-induced proinflammatory gene expression, and both the psoriatic transcriptome and genetic susceptibility further implicate NF-κB in psoriasis etiopathology. However, the role of NF-κB in psoriasis remains controversial. We analyzed the function of canonical NF-κB in the epidermis using CRE-mediated deletion of p65 and c-Rel in keratinocytes. In contrast to animals lacking p65 or c-Rel alone, mice lacking both subunits developed severe dermatitis after birth. Consistent with its partial histological similarity to human psoriasis, this condition could be prevented by anti-TNF treatment. Moreover, regulatory T cells in lesional skin played an important role in disease remission. Our results demonstrate that canonical NF-κB in keratinocytes is essential for the maintenance of skin immune homeostasis and is protective against spontaneous dermatitis. The Journal of Immunology, 2015, 194: 2472–2476.

Psoriasis is a chronic inflammatory skin disease characterized by epidermal hyperplasia, altered keratinocyte differentiation, and inflammatory infiltrates (1). It remains unclear whether the primary defect fomenting psoriasis lesion development affects keratinocytes or immune cell function. In murine models, forced expression of inflammatory cytokines in keratinocytes produces lesions with characteristics of human psoriasis (2–4). Constitutive activation of several transcription factors that regulate the expression of inflammatory cytokines, such as STAT-3 or NF-κB, can drive cutaneous inflammation (5, 6).

The transcription factor NF-κB is a complex formed by dimerization of its subunits: p65 (RelA), RelB, c-Rel, p50, and p52 (7). The canonical NF-κB pathway culminates in activation of dimers of p65, c-Rel, and p50 subunits. Genome-wide association studies suggest a link between psoriasis and the NF-κB pathway (8) that is supported by mouse models. Ablation of the NF-κB inhibitor IκBα produces cutaneous inflammation and keratinocyte proliferation (6, 9). However, deletion of IKKα, which mediates canonical NF-κB activation, produces a fulminant psoriasis-like disease in mice (10). Expression of an IκBα superrepressor in keratinocytes also results in a similar, although less severe, phenotype (11). These phenotypes are driven by TNF, because IκBα-deficient mice lacking TNFR1 or treated with anti-TNF Abs do not develop the disease (10, 12, 13). Given that IκBα has NF-κB–independent functions in keratinocyte development (14), and IKKα has direct effects on ERK and STAT1 activation (15–17), it is unclear whether the cutaneous inflammation in these mice is fully attributable to defective NF-κB activation. To directly examine the role of NF-κB in skin, we deleted RelA and c-Rel in keratinocytes. Mice lacking both subunits developed dermatitis with some psoriasiform characteristics, which was prevented by TNF blockade. Spontaneous remission was promoted by Foxp3+ regulatory T cells (Tregs). These results highlight a crucial role for canonical NF-κB in the maintenance of skin homeostasis.

Materials and Methods

Animals

Keratin 14 (K14)cre, Relafl/fl, and c-Relfl/fl mice (18–20) were kept in specific pathogen–free conditions in the animal care facility at Columbia University. Experiments were conducted under Institutional Animal Care and Use Committee approval.

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Abbreviations used in this article: DKO, double knockout; K14, keratin 14; LN, lymph node; qRT-PCR, quantitative RT-PCR; Treg, regulatory T cell; WT, wild-type.

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Flow cytometry

Spleen and lymph node (LN) cells were isolated by mechanical desegregation in PBS + 3% FBS. Whole skin was minced, digested for 3 h at 37°C in DMEM (Life Technologies) with 1 mg/ml Collagenase IV and 1 mg/ml DNase I (both from Sigma), and strained. Cell suspensions were stained using the following Abs from eBioscience: TCR-β (H57), CD4 (RM4.5), CD8 (53-6.7), CD19 (Ebio1D3), CD90.2 (53-2.1), CD45 (30F11), CD44 (IM7), NK1.1 (PK136), TCR-γδ (EbioGL3), CD11c (M1/70), IL-33R (ST2), CD25 (PC61, 7D4), and Foxp3 (FJK16s). Foxp3 staining was performed using the eBioscience kit. Cells were acquired on a LSR II (BD Biosciences) and analyzed with FlowJo software.

mRNA expression

For quantitative RT-PCR (qRT-PCR), frozen tissues were dissociated using Lysing Matrix D tubes (MP). Total RNA was isolated using TRIzol reagent and reverse transcribed with Superscript III (Life Technologies). qRT-PCR with SYBR Green (Quanta Biosciences) was performed on a CFX96 or 384 (Bio-Rad); all values are relative to GAPDH. Primers sequences are available upon request.

Histology

Ear or skin specimens were fixed with 4% neutral-buffered formalin for 4 d, transferred to 70% ethanol, and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, stained with H&E or TUNEL, imaged using an Axio M2 (Zeiss) microscope, and processed using AxioVision and ImageJ software. Epidermal thickness was measured on ≥15 random fields/specimen; mean thickness is shown.

Statistical analyses

Statistical significance was calculated using the two-tailed unpaired Student t test.

Results and Discussion

The role of NF-κB in skin biology and pathophysiology remains ambiguous. Although mice lacking p65, cRel, and TNF exhibit defects in epidermal differentiation (21), it is not clear whether this is the result of a keratinocyte-intrinsic requirement for NF-κB. Therefore, we crossed mice carrying either floxed Rela or c-Rel alleles to a transgenic mouse expressing Cre under the control of the K14 promoter. K14creRelafl/fl (K14DRela) and K14crec-Relfl/fl (K14Ac-Rel) pups were born at Mendelian ratios and displayed reduced expression of Rela and c-Rel mRNA, respectively, in the epidermis (Supplemental Fig. 1A). Histological analyses showed normal epidermal thickness and keratinocyte differentiation (data not shown), indicating that deletion of Rela or c-Rel did not affect skin development. Next, we used 2,4-dinitrofluorobenzene to induce contact hypersensitivity. K14DRela and K14Ac-Rel mice exhibited increased ear swelling and TNF and IFN-γ expression compared with littermate controls (Supplemental Fig. 1C–F). Consistent with a recent study using mice lacking RelA in the epidermis (22), these data
suggest that RelA and c-Rel have a nonredundant, keratinocyte-intrinsic, immunoregulatory role in skin.

**Spontaneous dermatitis in mice lacking RelA and c-Rel in keratinocytes**

It was reported previously that RelA has a growth-inhibitory role in keratinocytes (23) and prevents keratinocyte differentiation (24). However, no changes were observed in epidermal differentiation upon keratinocyte-specific deletion of RelA. Therefore, to assess whether they are redundant in epidermal development, we deleted RelA and c-Rel in keratinocytes (double-knockout [DKO] mice), which led to a full ablation of RelA and minimal residual c-Rel in DKO epidermis (Supplemental Fig. 1A, 1B). DKO pups were born at expected Mendelian ratios but exhibited visible skin lesion from day 5, which spread rapidly and covered most of the body by day 12 (Fig. 1A). Early lesions were well-demarcated, scattered, rigid, scaly plaques without edematous or exudative reaction. H&E staining revealed hyperkeratosis and focal parakeratosis, which are features of psoriatic lesions. Epidermal thickening and dermal and epidermal mononuclear infiltrates were observed (Fig. 1B, 1E). DKO mice exhibited apoptotic loci in all layers of the epidermis (Fig. 1F).

No lethality was observed; in >90% of the mice, the skin lesions resolved gradually, and dorsal epidermal thickness returned to normal by day 30 (Fig. 1C–E). Disease reoccurrence was not observed in any animal; however, the abdominal thickening and dermal and epidermal mononuclear infiltrates were decreased in the skin of DKO mice (Fig. 1G, 1H); however, skin barrier function remained intact, as assessed by a Toluidine Blue skin barrier assay (data not shown).

To further explore skin immune homeostasis, we performed flow cytometry on D16 tissues. We observed a 2-fold increase in the proportion of CD45+ leukocytes among total skin cells in DKO mice (Fig. 2A), which correlated with mononuclear cell infiltration (Fig. 1B). TCR-β+ T cells were increased in the skin of DKO mice (Fig. 2B). The CD4+Foxp3+ Treg proportion and number were dramatically enhanced, accounting for up to 60% of the skin-infiltrating CD4+ T cell compartment (Fig. 2C, 2D). We observed a 3–5-fold increase in the numbers of type II innate lymphoid cells, which were implicated in inflammatory responses in the skin (25), in both the LN and skin of DKO mice compared with wild-type (WT) controls (Fig. 2E, 2F). K14 is expressed by medullary thymic epithelial cells (26), which are important for Treg selection and deletion of autoreactive T cells (27). However, no changes were observed in medullary thymic epithelial cells or T cell subsets in the thymus of day-7 DKO mice (Supplemental Fig. 2). Thus, the dermatitis in DKO mice is likely due to a keratinocyte-intrinsic function of NF-κB. Together, these results indicate that canonical NF-κB in keratinocytes is required for their optimal differentiation, as well as maintenance of immune homeostasis in the skin.

**FIGURE 2.** Skin leukocyte infiltration in DKO mice. Spleen, LN (inguinal), and whole skin from day-16 mice were analyzed by flow cytometry. (A) Percentage of CD45+ (leukocytes) among total live cells. (B) Numbers of CD45+ TCR-β+ cells in 10⁶ live cells. (C) Representative dot plot gated on CD45+ TCR-β+CD4+Foxp3+ cells in 10⁶ live cells. (D) Numbers of CD45+ TCR-β+CD4+Foxp3+ cells in 10⁶ live cells. (E) Representative dot plot gated on CD45+ TCR-β+CD25+ST2+ cells in 10⁶ live cells. Data are representative or cumulative of at least three independent experiments. *p < 0.05. n.s., nonsignificant.
Dermatitis in DKO mice can be prevented by TNF blockade

TNF contributes to chronic inflammatory diseases, including psoriasis, and anti-TNF treatments are a first-line treatment for moderate to severe psoriasis (28). Therefore, we asked whether TNF mediated the dermatitis of DKO mice. TNF mRNA was increased in the skin of DKO mice 7 d after birth (Fig. 3A). Injection of an anti-TNF mAb prior to the appearance of the symptoms strongly reduced skin lesions, epidermal thickness, leukocyte infiltration, and apoptosis compared with vehicle treatment (Fig. 3B–D, data not shown). These data definitively establish that TNF can drive psoriasisform dermatitis independent of activation of the canonical NF-κB pathway in keratinocytes.

Tregs play a protective role in the remission of dermatitis

In contrast to K14CreIKK2fl/fl mice (10, 13), DKO mice spontaneously begin to recover 3 wk after birth. This led us to explore the mechanism of “remission.” Because we observed a massive Treg expansion in DKO skin prior to remission, we tested whether Tregs contributed to remission by injecting an anti-CD25 Ab. This protocol achieved a 50% reduction in Foxp3+ T cells 2 d after treatment (Fig. 4A). Treg-depleted DKO mice exhibited worsened pathology, with increased skin immune infiltrate (Fig. 4B–D). These results suggest that Tregs were necessary for disease recovery and highlight the role of Tregs in skin immune homeostasis.

It was proposed that the pathogenesis of psoriasis may follow a two-step model. First, environmental and/or genetic factors drive keratinocyte dysfunction and production of chemokines or inflammatory cytokines; in turn, activation of immune cells, such as dendritic cells and macrophages, may trigger a strong T cell–dependent inflammatory response, leading to increased proliferation of epidermal cells and clinical symptoms. In this article, we show that perturbation of the canonical NF-κB in the epidermis can trigger cell death, immune infiltration, and hyperkeratosis. These data indicate that NF-κB supports skin immune homeostasis and may prevent uncontrolled TNF-dependent leukocyte recruitment and activation.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Increased susceptibility to DNFB-induced hypersensitivity in mice lacking Rela or c-Rel in the epidermis. (A) Epidermis was isolated from 2-day old WT, K14\textsuperscript{Rela\textsuperscript{+}}/\textsuperscript{-}, K14\textsuperscript{c-Rel\textsuperscript{+}}/\textsuperscript{-} and K14\textsuperscript{Rela\textsuperscript{+}}/\textsuperscript{c-Rel\textsuperscript{+}} (DKO) mice, and relative Rela (left) and c-Rel (right) mRNA expression was assessed by RT-qPCR. Values (mean +/-SEM of triplicates) are relative to GAPDH. (B) Expression of RelA and c-Rel in 3-day old epidermis from WT and DKO mice, assessed by Western Blot. GAPDH serves as a loading control. *=unspecific band. (C-F) 6-8 weeks old K14\textsuperscript{cre\textsuperscript{+}}/\textsuperscript{p65\textsuperscript{+}}/\textsuperscript{-} (WT), K14\textsuperscript{Rela\textsuperscript{+}}/\textsuperscript{-} and K14\textsuperscript{c-Rel\textsuperscript{+}}/\textsuperscript{-} were sensitized with DNFB and challenged with vehicle (-) or DNFB (+) 6 days later. Analyses were performed 24 hours after the challenge. (C) Representative H/E staining on ear sections. Bars=100\,\mu m, original magnification= 20X. (D) Cumulative ear thickness; each dot represents an individual mouse, bars represent the mean. (E, F) Tnfa (E) and Ifng (F) mRNA expression (mean +/-SEM in ear skin, assessed by RT-qPCR, relative to GAPDH. Data are representative (A-C, E, F) or cumulative (D) of 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, n.s. non-significant.
Supplementary Figure 2. No early perturbation in thymocyte development in K14<sup>DRel-Rel<sub>A</sub></sup> mice. Thymic cell suspensions from 2-day old WT and DKO mice were analyzed by flow cytometry. (A) Representative CD80 and AIRE expression among gated CD45<sup>-</sup>EpCAM<sup>+</sup>MHC-II<sup>hi</sup> cells. Numbers indicate the % in the gate. (B) Absolute numbers of CD45<sup>-</sup>EpCAM<sup>+</sup>MHC-II<sup>hi</sup>CD80<sup>+</sup>AIRE<sup>+</sup>(AIRE<sup>+</sup>mTEC) cells. (C) AIRE mean fluorescence intensity (MFI) in CD45<sup>-</sup>EpCAM<sup>+</sup>MHC-II<sup>hi</sup>CD80<sup>+</sup> cells. (D) Relative AIRE mRNA expression in the whole thymus, assessed by RT-qPCR. Values are relative to GAPDH. (E) Representative CD4 and CD8 staining in whole thymus cells. Numbers indicate the % in each gate. (F) Absolute numbers per thymus, of DN (CD4<sup>-</sup>CD8<sup>-</sup>), DP (CD4<sup>+</sup>CD8<sup>+</sup>), CD4SP (CD4<sup>+</sup>CD8<sup>-</sup>) and CD8SP (CD4<sup>-</sup>CD8<sup>+</sup>). (G) Absolute numbers of TCR<sup>-β</sup> TCR<sup>-δ</sup> cells. For all histograms, data shown represents the mean +/- S.E.M. of 3 mice/group, from 1 of 2 independent experiments.