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Targeting NF-κB with a Natural Triterpenoid Alleviates Skin Inflammation in a Mouse Model of Psoriasis[1]

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Psoriasis vulgaris is a common chronic inflammatory skin disease involving cytokines and an activated cellular immune system. At variance to skin from patients with atopic dermatitis or from healthy subjects, human psoriatic skin lesions exhibit strong activation of transcription factor NF-κB that is mainly confined to dermal macrophages, whereas only a few dendritic cells but no CD3$^+$ lymphocytes show activated NF-κB. Since NF-κB signaling is required for the induction and/or function of many cytokines and aberrant cytokine expression has been proposed as an underlying cause of psoriasis, we investigated whether NF-κB targeting would affect the course of the disease in the CD18 hypomorphic (CD18^{hypo}) mouse model of psoriasis. When mice with severe psoriasiform lesions were treated systemically or locally with the IκB kinase inhibitor acetyl-11-keto-β-boswellic acid (AKβBA), NF-κB signaling and the subsequent NF-κB-dependent cytokine production as shown by the TNF-α production of macrophages were profoundly suppressed. Additionally, application of the compound counteracted the intradermal MCP-1, IL-12, and IL-23 expression in previously lesional skin areas, led to resolution of the abundant immune cell infiltrates, and significantly reduced the increased proliferation of the keratinocytes. Overall, the AKβBA treatment was accompanied by a profound improvement of the psoriasis disease activity score in the CD18^{hypo} mice with reconstitution of a nearly normal phenotype within the chosen observation period. Our data demonstrate that NF-κB signaling is pivotal for the pathogenesis in the CD18^{hypo} mouse model of psoriasis. Therefore, targeting NF-κB might provide an effective strategy for the treatment of psoriasis. *The Journal of Immunology,* **2009, 183: 4755–4763.**

Psoriasis vulgaris is a frequently occurring inflammatory skin disease with a prevalence of ~2–3%. The disease is characterized by disfiguring erythematous skin lesions covered with white silvery scales and often leads to discrimination of the patients and a substantial reduction in their quality of life (1).

Previously, psoriasis has been primarily regarded as a T cell-mediated disease (1, 2). However, macrophage-derived proinflammatory mediators such as TNF-α may play a crucial role in human psoriasis (3) and in mouse models of psoriasis (4–6). Accordingly, selective depletion of macrophages by liposome-encapsulated clodronate in mouse models and treatment of the patients with the TNF-α antagonist etanercept resulted in improvement of skin inflammation in murine models and human psoriasis (4, 5, 7). Apart from TNF-α, a considerable number of cytokines, including the NF-κB-induced IL-12, IL-15, IL-20, and IL-23 as well as IFN-α and IFN-β, have been shown to participate in the pathogenesis of psoriasis (8, 9). The pathophysiological significance of TNF-α has been underlined by TNF-α-targeting treatment strategies that have been particularly efficient (8). As TNF-α expression is regulated by activation of the transcription factor NF-κB and TNF-α itself triggers NF-κB activation (10), TNF-α may perpetuate macrophage activation through a positive feedback loop (11, 12). Therefore, enhanced NF-κB activation, for example, in macrophages, might be responsible for the continuation and amplification of the cytokine production, and thus may drive the pathogenic mechanisms of psoriasis.

NF-κB plays a pivotal role in immune and inflammatory responses not only through the regulation of genes encoding proinflammatory cytokines, but also via chemokines and growth factors (10, 13). The active form of NF-κB is a dimer formed by members of the NF-κB/Rel family of proteins, with the p50/p65 dimer being the most abundant. Phosphorylation of the inhibitor IκB by the IκB kinase (IKK)[4] complex is a central step in NF-κB activation, leading to IκB degradation and subsequent nuclear translocation of p50/p65 and other NF-κB subunits (10, 13). The transcriptional activity of the p50/p65 dimer is further enhanced by the IKK-dependent phosphorylation of p65 on Ser^{536} (13, 14).

Despite the substantial progress in understanding the molecular mechanisms underlying psoriasis and the intense search for new drugs, the current treatment modalities are still not satisfactory (15–17). This may at least be partly explained by the failure to deliver the drugs to the proper target cells and tissues. Therefore, the quest continues for compounds that combine therapeutic efficacy with a low profile of adverse events. In this regard, we recently identified acetyl-11-keto-β-boswellic acid (AKβBA) as a NF-κB inhibitor targeting the IKK (18, 19). AKβBA is a natural

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[4] Abbreviations used in this paper: IKK, IκB kinase; AKβBA, acetyl-11-keto-β-boswellic acid; CD18^{hypo}, CD18 hypomorphic mutation; γ-CD, γ-cyclodextrin; K14, keratin 14; PASI, psoriasis activity and severity index; wt, wild type.

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pentacyclic triterpenoid that can be isolated from oleogum resins of various *Boswellia* species commonly known as frankincense. Extracts thereof have been used in traditional medicine and in small clinical trials for the treatment of chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (20).

Studies on the pathogenesis of psoriasis have clearly profited from various animal models (21, 22). In our study, we have used the hypomorphic CD18 ($CD18^{hypo}$) mouse model, which, as previously shown, exhibits increased levels of TNF-α similar to human psoriatic skin (4).

Here, we show that NF-κB is activated in macrophages of human psoriatic plaques and lesional skin of a $CD18^{hypo}$ mouse model of psoriasis. AKβBA inhibited the NF-κB activation along with a profound improvement of the psoriasiform skin disease in $CD18^{hypo}$ mice, revealing a pivotal role for NF-κB signaling in the pathogenesis of psoriasiform skin inflammation. Our data suggest that targeting NF-κB activation might harbor therapeutic potential for the treatment of psoriasis.

Materials and Methods

Mice

Mice with a hypomorphic mutation of the CD18 gene ($CD18^{hypo}$) in the PL/J inbred strain were genotyped by Southern blots (23). $CD18^{+/+}$ littermates ($CD18^{wt}$) resulting from heterozygote crosses served as wild-type controls. For the treatment with AKβBA and the measurement of NF-κB activity and the NF-κB-regulated genes, $CD18^{hypo}$ mice with a strong psoriasiform phenotype were used. All experiments were conducted in compliance with the German Law for Welfare of Laboratory Animals.

Treatment with AKβBA and AKβBA liposomes

AKβBA was isolated from the oleogum resin of *Boswellia carterii* and purified to chemical homogeneity (>99.9% purity) by reversed phase HPLC. The compounds were further identified by mass spectrometry and characterized by one- and two-dimensional nuclear magnetic resonance spectroscopy (24). For the application of AKβBA in the $CD18^{hypo}$ mice we developed a hydrophilic derivative by generating AKβBA-γ-cyclodextrin (γ-CD) complexes, which allow administration of the lipophilic compounds in aqueous solutions in vivo. AKβBA or control vehicle were injected daily i.p. into affected $CD18^{hypo}$ mice (23–28 g) at 30 μmol/kg or 100 μmol/kg for 3 or 5 wk. For pharmacokinetics, AKβBA was analyzed in plasma by serial extractions on diatomaceous earth and graphitized carbon black followed by reversed phase HPLC and photodiode array detection (25).

In a different set of experiments, AKβBA liposomes (10 μM) and PBS liposomes were prepared (26) and were injected intradermally at four sites (50 μl/site) into lesional skin on the back of each $CD18^{hypo}$ mouse. Intradermal injections were done once every week for a period of 21 days. To assess the inhibition of NF-κB activation by AKβBA liposomes or PBS liposomes, we sampled the injected skin areas 24 h after treatment. To evaluate the severity of the psoriasiform phenotype, an adapted psoriasis activity and severity index (PASI) score (27) was used for affected $CD18^{hypo}$ mice before and after treatment with AKβBA or γ-CD or PBS liposome controls.

Immunofluorescence staining

Frozen cryosections of skin from patients or mice with the indicated genotype or treatment were fixed in ice-cold acetone for 10 min before staining. Abs against the following proteins were used: CD163 (Santa Cruz Biotechnology), CD11c (BD Biosciences), CD3 (eBioscience), keratin 14 (K14; Covance), Ki-67 (Abcam), IL-12 (GeneTex), phospo-IκBα Ser^{32} and phospo-p65 Ser^{536} (both from Cell Signaling Technology), IL-23 (Abcam), P-glycoprotein (Calbiochem), TNF-α (BD Biosciences), F4/80 (Caltag Laboratories), CD4 (Serotec), Gr-1 (BD Biosciences), and MCP-1 (Abcam). Immunohistochemistry was performed using a previously described protocol (28). Immunostainings were analyzed with a fluorescence microscope (Zeiss Axioskop 2 plus). Inhibition of Rh123 efflux was analyzed in human keratinocytes 3 h after a 30-min treatment with either solvent, AKβBA (10 μM), or verapamil (50 μM, positive control) by flow cytometry (FACScan; BD Biosciences).

Analysis of NF-κB activation

For the analysis of activated of NF-κB by Western immunoblotting, lysates from liquid nitrogen snap-frozen mouse skin were prepared. Human macrophages were differentiated from monocytes with M-CSF for 6 days (29). Cells (5×10^6 cells/sample), macrophages, HaCaT, or human epidermal keratinocytes obtained from PromoCell, were treated with the indicated concentrations of AKβBA for 30 min, and were subsequently stimulated with TNF-α (1 ng/ml, R&D Systems) for an additional 50 min. Nuclear extracts were prepared and analyzed as described (19). Activation of NF-κB was analyzed in nuclear extracts with the NF-κB TransAM ELISA (Active Motif). Staining of topoisomerase I (Santa Cruz Biotechnology) or actin (Chemicon) served as loading controls. To examine whether AKβBA inhibits NFAT signaling in T cells, human pan-T lymphocytes isolated by negative selection (Miltenyi Biotec) (5×10^6 cells) were either treated with AKβBA or solvent for 30 min and stimulated with immobilized anti-CD3 Ab (5 μg/ml; Orthoclone) for an additional 60 min. Whole-cell lysates were analyzed by Western immunoblotting using Ab against NFAT1 (Abcam).

Statistical analysis

Quantitative results are expressed as means ± SEM. Results were analyzed by the two-tailed heteroscedastic Student's *t* test or, in cases of a non-Gaussian distribution, the Mann-Whitney *U* test. The Newman-Keuls test was used for multigroup comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

Macrophages in human psoriatic lesions exhibit activated NF-κB

Immunofluorescence staining for IκBα phosphorylated on Ser^{32} revealed that almost all epidermal keratinocytes in human psoriatic lesions express phospho-IκBα, whereas in normal skin only suprabasal and basal epidermal keratinocytes show some staining indicating a limited and locally confined activity of NF-κB (Fig. 1, *A* and *B*, *left panels*, and supplemental Fig. 1S*A*).[5] Due to its known staining properties, some staining of the horny layer may be nonspecific (30). Importantly, however, compared with normal controls, where phospho-IκBα was absent in the dermis, skin biopsies taken from psoriatic lesions demonstrated a substantial increase in the levels of phospho-IκBα (Fig. 1, *A* and *B*, *left panels*, and supplemental Fig. 1S*A*). We have previously identified activated macrophages as the major source of TNF-α in the $CD18^{hypo}$ mouse model of psoriasis (4). Because the TNF-α expression is dependent on the activation of NF-κB signaling and TNF-α is a potent activator of NF-κB itself (31–33), we examined whether NF-κB is activated in vivo in macrophages in human psoriasis utilizing double immunofluorescence analysis. Notably, there was a distinguished colocalization of the macrophage-specific marker CD163 (34) and phospho-IκBα in the dermis of psoriatic lesions but not in normal control skin (Fig. 1, *A* and *B*, *center* and *right panels*). The number of CD163 and phospho-IκBα double-positive macrophages was massively increased in the dermis of psoriatic skin compared with control skin (29.4 ± 10.9 vs 1.9 ± 1.6 positive cells/high power field, $p < 0.0001$) (Fig. 1*C*); in relation to the total number of dermal cells, the double-stained macrophages corresponded to 7.5 ± 2.6% vs 1.2 ± 1.1% ($p < 0.001$), respectively. As expected, NF-κB was activated in the cells producing TNF-α (Fig. 1*D*).

In human psoriatic lesions, phospho-IκBα was predominantly found in macrophages and, to a lesser extent, in hair follicles, keratinocytes, and $CD11c^+$ dermal dendritic cells, whereas $CD3^+$ lymphocytes did not exhibit NF-κB activation (Fig. 1, *E* and *F*). In contrast, in biopsy specimens from human atopic dermatitis, NF-κB activation was only weakly detectable in the dermis and

[5] The online version of this article contains supplemental material.

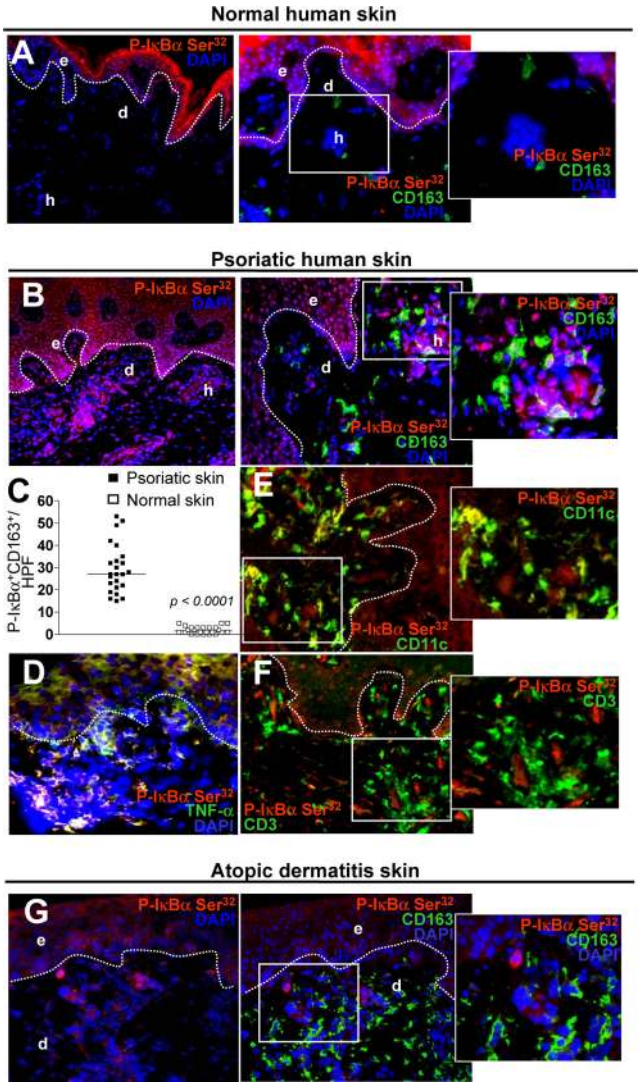

FIGURE 1. NF-κB is activated in macrophages of human psoriatic lesions. Cryosections from skin of normal subjects (*A*) and psoriatic patients (*B*) were double stained for phospho-IκBα (P-IκBα, red) and the human macrophage marker CD163 (green) ($n = 8$ subjects each). *C*, Quantification of the $CD163^{+}$P-IκBα^{+} macrophages in psoriatic and normal skin ($n = 8$ each). The data are presented as percentage of macrophages ($p < 0.0001$, Student's *t* test). *D*, P-IκBα (red) colocalizes with TNF-α (green) in the skin of psoriatic patients, overlay (yellow). *E*, Cryosections from skin of psoriatic patients were double stained for P-IκBα (red) and the marker of dermal dendritic cells CD11c (green), overlay (yellow). *F*, Cryosections from skin of psoriatic patients were double stained for P-IκBα (red) and the lymphocyte marker CD3 (green). *G*, Cryosections from the skin of four atopic dermatitis patients were double stained for phospho-IκBα (P-IκBα, red) and the human macrophage marker CD163 (green). Higher magnification of the boxed areas is shown on the right. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole, when applicable (original magnification × 400). e, epidermis; d, dermis; h, hair follicle. Dotted lines indicate the border between epidermis and dermis.

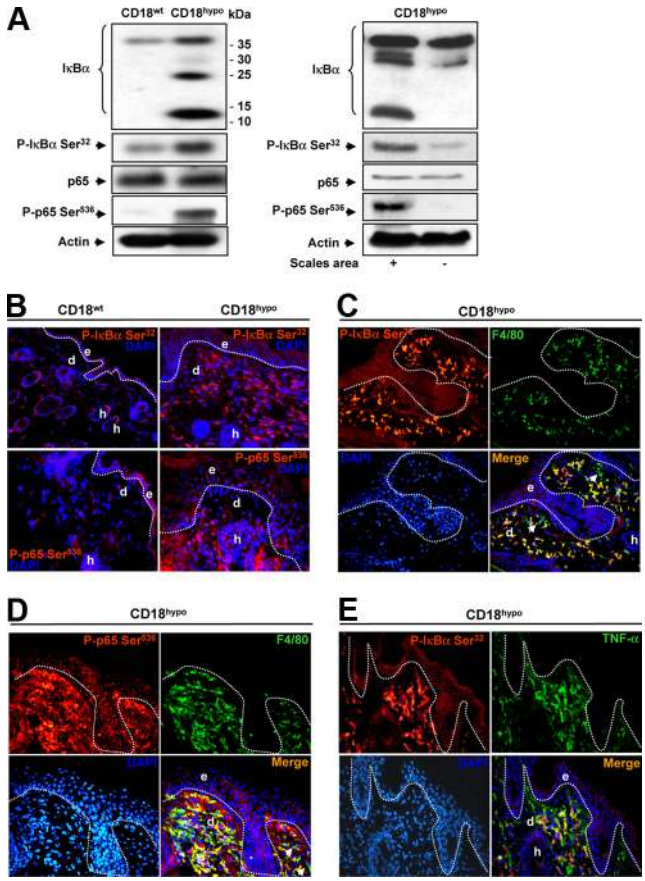

FIGURE 2. NF-κB is activated in macrophages in psoriasiform skin lesions from $CD18^{hypo}$ mice. *A*, Lysates from normal skin of $CD18^{wt}$ or unaffected or affected skin of $CD18^{hypo}$ mice were subjected to Western immunoblotting for IκBα, phospho-IκBα (P-IκBα), and phospho-p65 (P-p65). Actin-loading control. *B*, Skin cryosections from $CD18^{wt}$ and affected $CD18^{hypo}$ mice were subjected to immunofluorescence staining for P-IκBα and P-p65 (both in red). *C* and *D*, P-IκBα and P-p65 colocalize with F4/80 (macrophage marker, green), overlay (yellow). *E*, P-IκBα (red) colocalizes with TNF-α (green), overlay (yellow). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (original magnification ×400). e, epidermis; d, dermis; h, hair follicle. Dotted lines indicate the border between epidermis and dermis. One representative experiment out of seven is shown.

epidermis, and was in any case not associated with dermal macrophages (Fig. 1*G*).

Macrophages in lesional skin of CD18^{hypo} mice exhibit activated NF-κB

Western immunoblotting of skin lysates from $CD18^{hypo}$ mice showed that the degradation and expression of IκBα, the transcription of which is regulated by NF-κB, is greatly enhanced in lesional skin. Accordingly, enhanced phosphorylation of IκBα on Ser^{32} and of p65 on Ser^{536} was observed in scaled skin specimens, whereas those from nonscaling areas of $CD18^{hypo}$ mice exhibited less phospho-IκBα and basically no phospho-p65 (Fig. 2*A* and supplemental Fig. 1S*B*). Consistent with these data, immunohistochemical analyses with Abs against phospho-IκBα and phospho-p65 also detected profound activation of the NF-κB signaling pathway in lesional skin from $CD18^{hypo}$ mice (Fig. 2*B* and supplemental Fig. 1S*B*). In analogy to the NF-κB staining pattern in healthy human skin, phosphorylation of IκBα on Ser^{32} and of p65 on Ser^{536} were observed in some cells of the basal and suprabasal layer of the epidermis in normal skin of $CD18^{wt}$ mice. Thus, at variance to the skin of $CD18^{wt}$ mice, NF-κB signaling in the skin from $CD18^{hypo}$ mice extends beyond the suprabasal cells.

To study whether NF-κB is activated in lesional macrophages in $CD18^{hypo}$ mice, we used double immunofluorescence staining. Consistent with the finding in human psoriatic plaques (Fig. 1, *B*, *center* and *right panels*), a colocalization of the macrophage marker F4/80 with IκBα phosphorylated on Ser^{32} and p65 phosphorylated on Ser^{536} was found in the dermis of lesional skin of

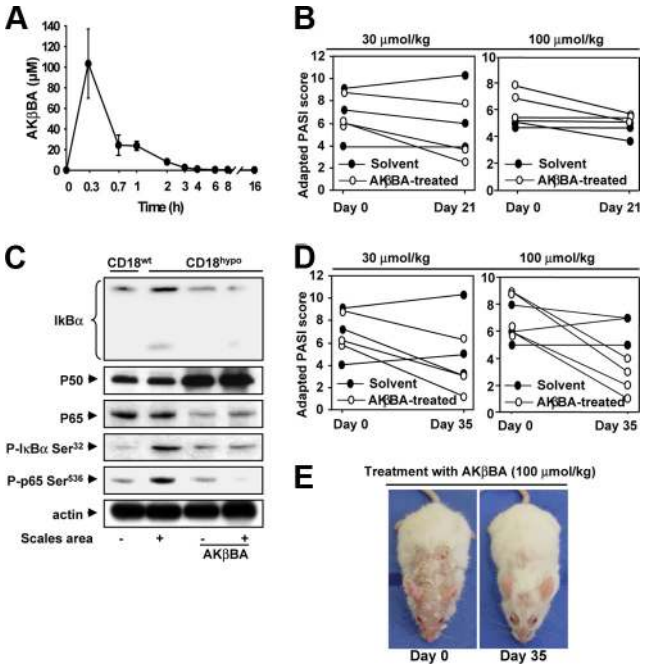

FIGURE 3. Treatment of CD18^{hypo} mice with AKβBA resolves the psoriasiform phenotype. *A*, Plasma kinetics of AKβBA after a single i.p. injection of 100 μmol/kg ($n = 3$). *B*, The severity of the psoriasiform phenotype was assessed by the adapted PASI score in mice injected i.p. once daily with AKβBA or control vehicle at 30 μmol/kg or 100 μmol/kg for 21 days ($n = 3$). *C*, Western immunoblot analyses of skin lysates (100 μmol/kg). Actin-loading control; lysates from three different mice were analyzed individually. *D*, The severity of the psoriasiform phenotype was assessed by the adapted PASI score in mice injected i.p. once daily with AKβBA or control vehicle either at 30 μmol/kg or 100 μmol/kg for 35 days ($n = 4$, $p = 0.0057$). *E*, Representative phenotype of an affected CD18^{hypo} mouse before and after treatment with 100 μmol/kg AKβBA for 35 days.

CD18^{hypo} mice (Fig. 2, *C* and *D*). A small macrophage population in the dermis presented no staining either for phospho-IκBα or for phospho-p65 Ser^{536} (Fig. 2, *C* and *D*, white arrows), suggesting that these cells might be alternatively activated macrophages exhibiting an antiinflammatory phenotype and a reduced expression of proinflammatory cytokines (35). Strikingly, the double immunofluorescence staining using phospho-IκBα and TNF-α Abs revealed that almost all phospho-IκBα-positive cells produced TNF-α in the skin lesions of CD18^{hypo} mice (Fig. 2*E*). Taken together, these data demonstrate that macrophages in lesional skin exhibit NF-κB activation in terms of phosphorylation of IκBα and p65, and that they represent the main source of proinflammatory TNF-α in the CD18^{hypo} mouse model of psoriasis.

Inhibition of NF-κB significantly improves the psoriasiform skin disease in affected CD18^{hypo} mice

We have previously demonstrated that the i.p. administration of AKβBA, an inhibitor of IKK (18, 19), inhibits NF-κB activation in atherosclerotic lesions of apoE$^{-/-}$ mice (36). A single i.p. injection of 100 μmol/kg AKβBA in mice resulted in a rapid but transient increase in the plasma concentrations of AKβBA, which reached maximal plasmatic concentrations of ~100 μM. An initial phase of rapid clearance ($t_{1/2}$ of ~26 min) was followed by a slower, longer lasting elimination phase ($t_{1/2}$ ~68 min). After ~2–3 h, the plasma levels of AKβBA were <10 μM (Fig. 3*A*).

To analyze the effect of the NF-κB inhibition on the psoriasiform skin disease of CD18^{hypo} mice, we treated affected animals with either 30 μmol/kg or 100 μmol/kg AKβBA. In fact, after i.p. administration of AKβBA for 21 days, we observed a slight improvement of the psoriasiform skin disease as assessed by the adapted PASI score (4, 27) (Fig. 3*B*) and a marked decrease in the phosphorylation of IκBα on Ser^{32} and of p65 on Ser^{536} in lysates from affected skin of CD18^{hypo} mice. This effect was paralleled by the accumulation of inhibitory p50 (Fig. 3*C*). p50 lacks a transactivation domain and forms homodimers, which can repress activation of proinflammatory genes. Thus, p50-p50 complexes have been shown to negatively regulate the expression of the proinflammatory cytokine IL-23 (37). On the other hand, p50 homodimers in association with transcriptional activators such as Bcl-3 or p300 can also activate transcription. In macrophages, the p50 homodimers activate the transcription of antiinflammatory IL-10 (37).

After 35 days of treatment, an improvement of the disease was observed in affected CD18^{hypo} mice treated with 30 μmol/kg AKβBA (PASI score of 7.0 $\pm$ 1.7 vs 3.7 $\pm$ 3.1, $p = 0.1987$). When affected CD18^{hypo} mice with a severe inflammatory phenotype were treated for the same time with 100 μmol/kg AKβBA, a remarkable improvement of the psoriasiform skin inflammation was noted (PASI score of 7.5 $\pm$ 1.3 vs 2.5 $\pm$ 1.2, $p = 0.0057$) (Fig. 3, *D* and *E*).

Immunohistochemical analysis of the skin of CD18^{hypo} mice treated for 35 days i.p. with AKβBA (100 μmol/kg) revealed profound suppression of the phosphorylation of IκBα and p65 in the dermal layer indicating inhibition of the NF-κB activation (Fig. 4*A* and supplemental Fig. 1S*C*). Remarkably, the phosporylation of p65 on Ser^{536} in the suprabasal keratinocytes was not inhibited by the AKβBA treatment (Fig. 4*A* and supplemental Fig. 1S*C*). Consistent with the obvious inhibition of NF-κB signaling, the key cytokine TNF-α (38) as well as the monocyte chemoattractant MCP-1 and the proinflammatory cytokines IL-12 and IL-23, which are all dependent on NF-κB-dependent gene induction (32, 33, 39), were significantly reduced in the skin sections from AKβBA-treated mice (Fig. 4*B*). The improvement of these parameters was also accompanied by a significant reduction of the proliferation marker Ki-67 and of K14 as a parameter of the epidermal thickness, clearly indicating normalization of the hyperkeratosis and acanthosis (40) (Fig. 4, *B* and *D*). The AKβBA-dependent improvement of the formerly psoriasiform lesions was further manifested by the virtual resolution of the infiltrates consisting of F4/80$^+$ macrophages, CD4$^+$ lymphocytes, and Gr-1$^+$ leukocytes (Fig. 4, *C* and *D*).

AKβBA inhibits activation of NF-κB in macrophages, but not in keratinocytes

Unexpectedly, we observed that in CD18^{hypo} mice AKβBA effectively inhibited NF-κB signaling in macrophages, but not in the suprabasal keratinocytes. The phosphorylation of p65 associated with the suprabasal keratinocytes was even enhanced (Fig. 4*A*). As NF-κB signaling limits the keratinocyte proliferation and is indispensable for their differentiation (41), we analyzed the effects of AKβBA on the NF-κB activation in macrophages and keratinocytes in vitro. Western immunoblot analyses of nuclear extracts from human macrophages and keratinocytes activated with TNF-α revealed that AKβBA effectively inhibited the phosphorylation of p65, its nuclear accumulation (Fig. 5*A*), as well as the NF-κB activation in macrophages (Fig. 5*B*), but not in keratinocytes (Fig. 5, *A* and *B*). To address the question of specificity of AKβBA on NF-κB signaling, we also investigated its putative interaction with NFAT signaling, specifically because psoriasis has been regarded as a T cell-mediated autoimmune disease (1, 8). However,

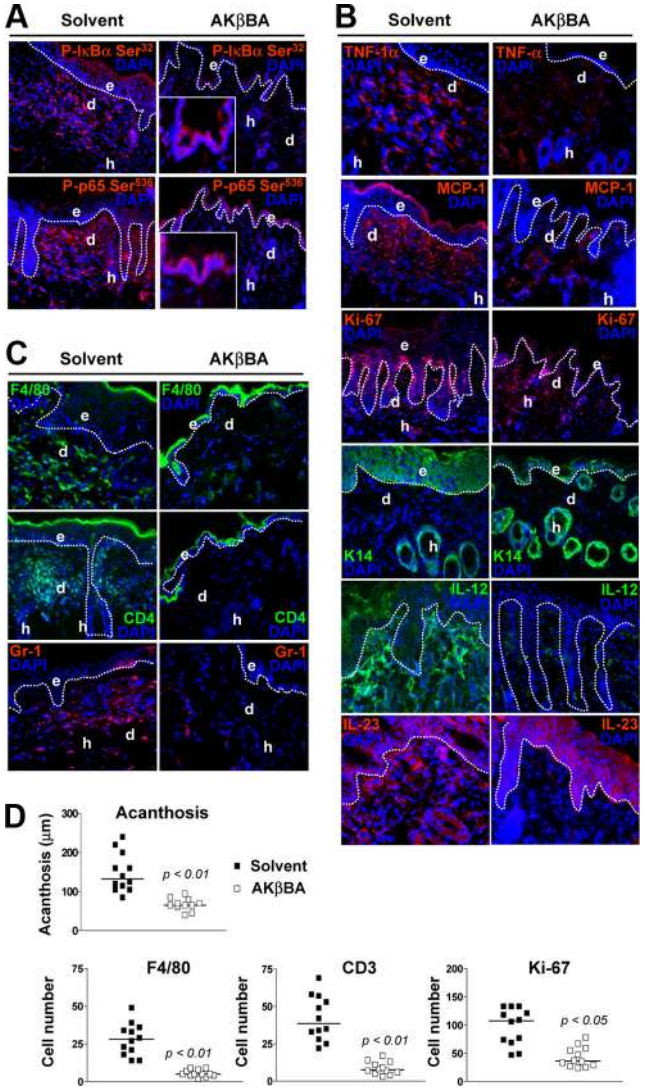

FIGURE 4. Treatment of CD18^{hypo} mice with AKβBA resolves the immunohistochemical parameters of inflammation. *A*, Immunofluorescence staining of phospho-IκBα (P-IκBα) Ser^{32} and phospho-p65 (P-p65) Ser^{536} in cryosections from the skin of CD18^{hypo} mice treated for 35 days i.p. with AKβBA (100 μmol/kg) or control vehicle. The inserts are maginifications showing NF-κB activation in basal keratinocytes. *B*, Immunofluorescence staining of TNF-α (red), MCP-1 (red), Ki-67 (red), K14 (green), IL-12 (green), and IL-23 (red) in cryosections from lesional skin of CD18^{hypo} mice treated as in *A*. *C*, Immunofluorescence staining of macrophages (F4/80, green), lymphocytes (CD4, green), and leukocytes (Gr-1, red) in lesional skin of CD18^{hypo} mice treated as in *A*. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (original magnification ×400). e, epidermis; d, dermis; h, hair follicle. Dotted lines indicate the border between epidermis and dermis ($n = 7$). *D*, Quantification of acanthosis, F4/80 macrophage and CD3$^+$ T cell infiltration, and keratinocyte proliferation (Ki-67$^+$) ($n = 8$, three high power fields from each section were counted, Student's *t* test).

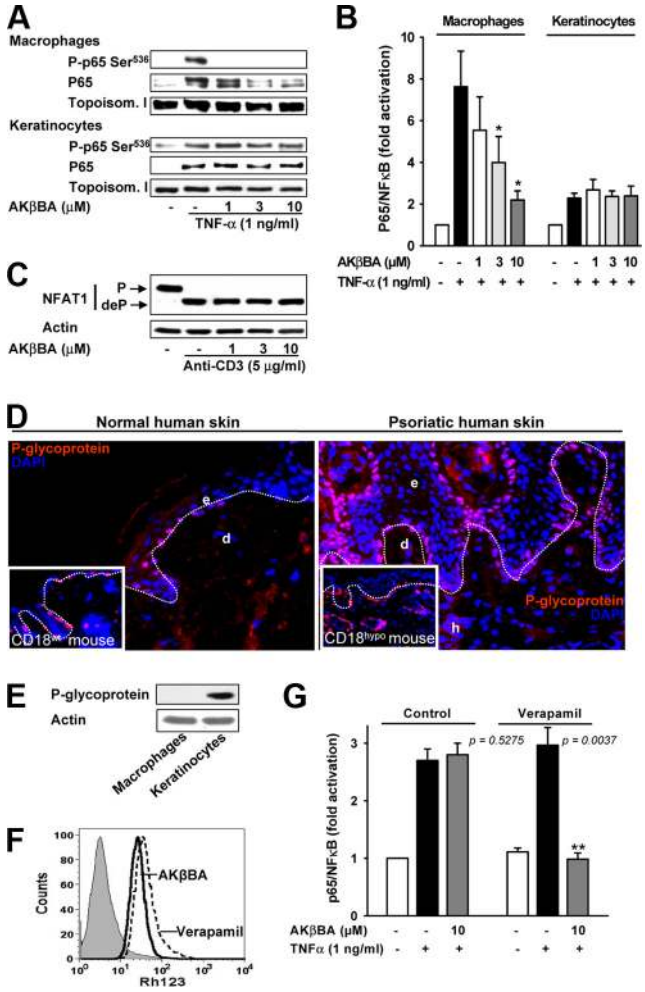

FIGURE 5. AKβBA inhibits NF-κB activation in human macrophages, but not in keratinocytes. *A*, Western immunoblot analyses of p65 phosphorylation and its nuclear accumulation in human macrophages and keratinocytes preincubated with AKβBA (1–10 μM) and activated with TNF-α. Topoisomerase I-loading control of nuclear extracts. *B*, NF-κB activation was measured using the TransAM NF-κB/p65 ELISA in nuclear extracts of cells treated as in *A*. *C*, AKβBA does not inhibit NFAT. Western immunoblot analyses of phosphorylated (P) and dephosphorylated (de-P) NFAT1 in human pan-T lymphocytes preincubated with AKβBA and stimulated with anti-CD3 Ab. Actin-loading control. (*A–C*, $n = 3–5$, HaCaT keratinocytes). *D*, Immunofluorescence staining of P-glycoprotein (red) in cryosections from human psoriatic lesions and normal human skin. Three different areas from each individual/animal were analyzed. e, epidermis; d, dermis; h, hair follicle. Dotted lines indicate the border between epidermis and dermis, $n = 8$. *Insets*: P-glycoprotein staining in wt and CD18^{hypo} mice. *E*, Human macrophages, but not primary human keratinocytes, express P-glycoprotein (170 kDa). Whole cell lysates from (2×10^6 cells) were analyzed by Western immunoblotting. *F*, AKβBA is a substrate for P-glycoprotein. AKβBA (10 μM) similar to verapamil (50 μM) inhibits efflux of Rh123 from primary human keratinocytes ($n = 3$). *G*, Primary human keratinocytes preincubated with the P-glycoprotein inhibitor verapamil (50 μM, 30 min) become sensitive to AKβBA; treated and analyzed as in *B*. Mean ± SEM, $n = 3–4$. Results were normalized for protein contents and expressed as fold activation of p65 as compared with nontreated control. (In *B* and *G*, ∗, $p < 0.05$ and ∗∗, $p < 0.01$, Newman-Keuls test.)

AKβBA did not affect NFAT activation in anti-CD3-stimulated T lymphocytes, indicating specific inhibition of NF-κB (Fig. 5*C*).

Keratinocytes may express various transport-associated enzymes, such as P-glycoprotein (42), which could enhance AKβBA efflux from the cells. Consistently, we observed that suprabasal keratinocytes from both psoriatic lesions and normal skin strongly expressed the multidrug resistance-associated protein, P-glycoprotein (Fig. 5*D*). The expression of P-glycoprotein by keratinocytes, but not by macrophages, was further confirmed by Western immunoblotting (Fig. 5*E*). Indeed, AKβBA was a P-glycoprotein substrate as demonstrated by the inhibition of the Rh123 efflux from human keratinocytes (Fig. 5*F*). Importantly, pretreatment of the keratinocytes with the P-glycoprotein inhibitor verapamil rendered the cells sensitive to AKβBA, leading to efficient inhibition

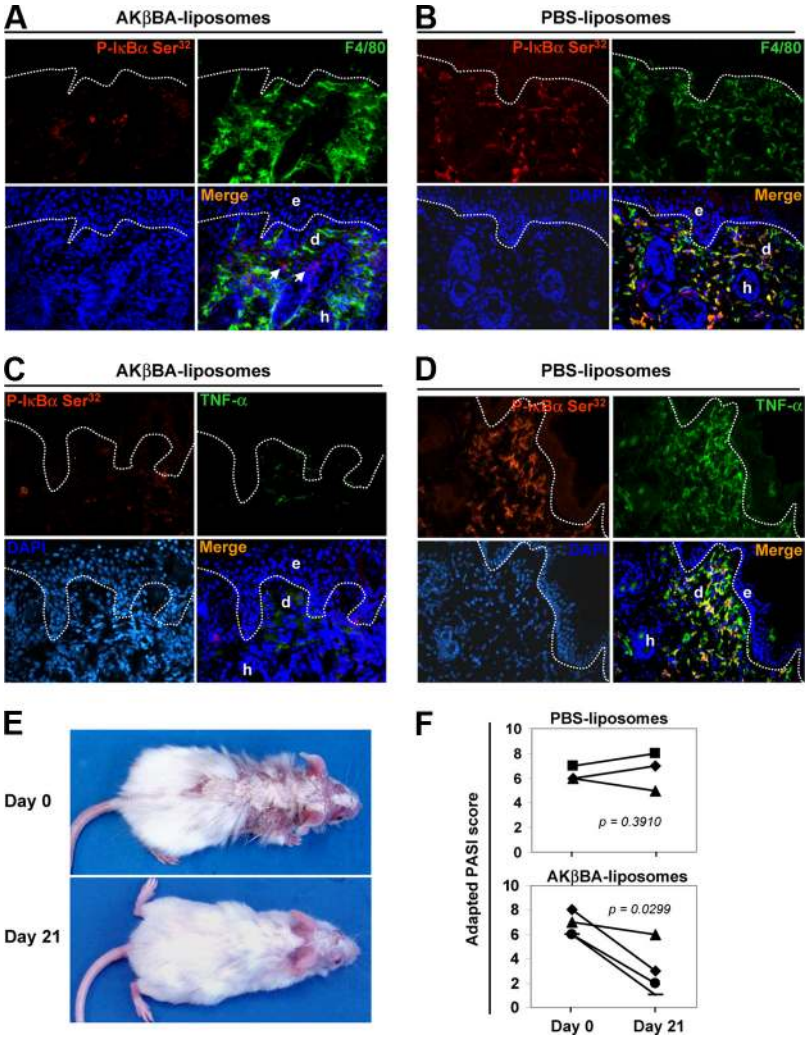

FIGURE 6. Specific targeting of NF-κB signaling in macrophages resolves the psoriasiform skin inflammation of affected CD18^{hypo} mice. AKβBA liposomes or PBS liposomes (10 μM, 4 × 50 μl) were injected intradermally weekly for 3 wk. *A–D*, Down-regulaton of the IκBα phosphorylation and TNF-α production by macrophages 24 h after injection of AKβBA liposomes (original magnification ×400). Immunohistochemical analyses of cryosections from the skin of CD18^{hypo} mice. *E*, Representative phenotype of a CD18^{hypo} mouse with severe psoriasiform dermatitis before and after 21 days of local treatment with AKβBA liposomes. *F*, The severity of the psoriasiform phenotype as assessed by the adapted PASI score was significantly reduced after AKβBA liposome treatment ($n = 4$).

of the NF-κB activity by AKβBA when TNF-α-stimulated cells were treated in the presence of verapamil (Fig. 5*G*). Analysis of AKβBA accumulation by HPLC (25) demonstrated that 1 h after treatment with AKβBA keratinocytes contain only about a fourth of AKβBA found within macrophages (26.9 ± 4.0%, $p < 0.01$, $n = 3$); respective HPLC tracings are shown in supplemental Fig. 2S. Taken together, our data demonstrate that AKβBA inhibits NF-κB activation in macrophages but not in keratinocytes.

Local application of AKβBA liposomes inhibits NF-κB activation in skin macrophages and significantly improves the psoriasiform skin inflammation in CD18^{hypo} mice

To address whether locally injected AKβBA inhibits activation of NF-κB in macrophages in vivo and whether this specific inhibition of NF-κB contributes to the improvement of the psoriasiform skin inflammation in CD18^{hypo} mice, we encapsulated AKβBA in liposomes. Large liposomes (>0.8 μm) are known to be selectively phagocytosed by macrophages (26). Therefore, AKβBA can be selectively targeted to skin macrophages. As expected, 24 h after injection AKβBA liposomes specifically inhibited the phosphorylation of IκBα on Ser^{32} in F4/80^{+} skin macrophages, but they had no inhibitory effect on F4/80^{-} cells, which are nonphagocytotic. Hence, a few single red-stained cells were still present after treatment (Fig. 6*A*, white arrows). In contrast, control PBS liposomes failed to inhibit the phosphorylation of IκBα on Ser^{32} in F4/80^{+} macrophages (Fig. 6*B*). At variance to the treatment with PBS liposomes, AKβBA liposomes almost abolished the phosphorylation of IκBα as well as the TNF-α expression in the skin of affected CD18^{hypo} mice as detected by double immunofluorescence staining of the cryosections (Fig. 6, *C* and *D*).

Most importantly, inhibition of the NF-κB activation in macrophages using the AKβBA liposomes in CD18^{hypo} mice with a very severe inflammatory phenotype led to a remarkable improvement of the psoriasiform skin inflammation after treatment for 3 wk (Fig. 6*E*). Compared with treatment with PBS liposomes, the improvement in terms of severity, extent of erythema, plaque formation, and scaling after treatment with AKβBA liposomes was highly significant as assessed by the adapted PASI score (6.75 ± 0.96 vs 3.00 ± 2.16, $p = 0.0299$) (Fig. 6*F*).

Discussion

Current therapies for the treatment of psoriasis can be divided into two main categories either targeting essential molecular components of the inflammatory cascade such as mediators, adhesion and signaling molecules, or distinct immune cells such as T cells (8, 9, 17). These therapeutic approaches are partly based on complementary pathogenic views, the so-called T cell pathogenesis hypothesis and the aberrant cytokine network theory, both of which do not fully reflect the pathogenic complexity of the disease.

Considering the NF-κB-dependent induction of numerous proinflammatory genes ranging from key cytokines to cytokine

receptors and chemokines (13, 14, 37), intercepting NF-κB signaling could provide a suitable therapeutic target in diseases associated with chronic inflammation such as psoriasis.

Until recently psoriasis has been primarily regarded as a T cell-mediated autoimmune disease (1, 2). Studies in humans and in mouse models of psoriasis revealed also a pivotal role for macrophages and macrophage-produced cytokines in the pathogenesis of psoriasis (3–6). Thus, we found that macrophages within the lesional skin are activated to become the main source of the proinflammatory cytokines such as TNF-α (4). Indeed, we could not detect any macrophages exhibiting activated NF-κB in the skin of $CD18^{wt}$ mice and of healthy control subjects, whereas in lesional skin of affected $CD18^{hypo}$ mice and human psoriasis patients macrophages exhibiting NF-κB activation were abundant. Interestingly, the large quantity of dermal macrophages exhibiting activated NF-κB was characteristic for psoriatic skin, whereas the biopsy specimens from patients with atopic dermatitis revealed no dermal macrophages exhibiting activated NF-κB.

In macrophages, NF-κB promotes proliferation, survival, and release of proinflammatory mediators, including the key cytokine TNF-α (43). In the context of the cytokine network theory, increased TNF-α expression has been implicated in the pathogenesis of autoimmune diseases and psoriasis (44, 45). Hence, anti-TNF-α therapy relieves psoriasis (1, 3, 15, 16), and various TNF inhibitors such as infliximab, adalimumab, and etanercept proved to be remarkably efficient (9, 17). However, Ab-based therapeutics have a number of drawbacks, including costs, reactions against the foreign protein, as well as not well-defined cellular side effects mediated for example via FcγRs such as CD32, which may be causally related to the immune suppression seen with biologics (9). By means of NF-κB inhibition with a small-molecule inhibitor of IKK, AKβBA, we reduced the NF-κB activation and the downstream TNF-α expression in the lesional skin of $CD18^{hypo}$ mice. Most importantly, this led to a profound immunohistochemical and clinical improvement of the psoriasiform skin disease.

Even though we used the TNF-α production occurring predominantly in the macrophages of the skin as a readout for the AKβBA-induced cytokine inhibition, this inhibitory effect of the compound is neither confined to this single cell type nor to this specific cytokine. Thus, taking into consideration that Th1 cells might play a pathogenic role in psoriasis (8), it is of interest that IL-12 required for the differentiation of naive T cells into Th1 cells as well as the Th1 effector cytokines IL-2 and TNF-α require NF-κB activation for their respective gene induction (32, 33, 46–48). This readily implies that the putative therapeutic activity of AKβBA is likely to extend beyond that of the currently available biological TNF-α antagonists. Accordingly, IL-12 expression was also drastically reduced in skin biopsies from mice treated with AKβBA.

Similarly, IL-23, which is a novel cytokine important for autoimmunity, consisting of an IL-23 p19 chain and an IL-23 p40 chain, is regulated by several transcription factors including AP-1 and NF-κB (49). IL-23 is now recognized to play a role in the recruitment of inflammatory cells in Th1-mediated diseases including psoriasis, where it is expressed in monocytes and dendritic cells of lesional human skin (50). Indeed, intradermal administration of IL-23 into murine skin initiates a cascade of events including erythema, mixed dermal infiltrates, and epidermal hyperplasia via induction of the NF-κB-dependent TNF (51). Moreover, a recent genome-wide analysis for psoriasis susceptibility loci revealed an association of psoriasis with IL-12, IL-23, and NF-κB-dependent pathways (52), further stressing the potential value of our approach. Based on the essential role of NF-κB for the induction of IL-23 p19, IL-23 is shown here to be also a target of an AKβBA-based intervention.

While interference of AKβBA with the NF-κB-dependent expression of various pathogenic relevant cytokines might at first glance impose as an attractive target, such an approach could be seriously hampered by interference of the compound with crucial physiological functions of the NF-κB transcription factor. Indeed, NF-κB was found to play an important role in the physiology of keratinocytes, and disruption or modulation of components of the NF-κB signaling affected epidermal proliferation, differentiation, and homeostasis (28, 41, 53–57). Consequently, the terminal differentiation of keratinocytes was perturbed in the absence of proper activation of NF-κB (57) primarily because of the lack of IKKα-dependent expression of a differentiation-inducing factor (57). In suprabasal keratinocytes of the epidermis, IKKα mediates repression of the members of the fibroblast growth factor family, a process independent of the intact kinase activity (56). IKKα has also been implicated in the control of the skin-barrier formation through the regulation of the retinoic acid receptor target genes in keratinocytes (53). An important role in keratinocyte differentiation has also been proposed for IKKβ. Thus, mice with an epidermis-specific deletion of IKKβ were born with histologically normal skin, but epidermal hyperplasia and inflammation with impaired differentiation developed within the first postnatal week (28). Targeted inhibition of NF-κB in the epidermis by overexpression of dominant-negative IκB proteins produced hyperplastic epithelium, whereas overexpression of the active NF-κB subunits p50 and p65 produced growth inhibition and hypoplasia pointing to a growth inhibitory role for active NF-κB (55).

Consistent with the special role of NF-κB in epidermal biology, we observed that suprabasal epidermal keratinocytes exhibit activated NF-κB in normal skin from healthy volunteers. However, it came as a surprise that AKβBA did not inhibit NF-κB signaling in keratinocytes. Nevertheless, the physiological difference in terms of transporter expression between macrophages and keratinocytes led to distinct local pharmacokinetics that allowed sparing the sensitive keratinocytes from NF-κB inhibition. In light of the role of NF-κB in the skin, this might indeed be a very special feature of the compound AKβBA. Thus, systemic application of AKβBA as well as local intracutaneous injection of liposome-encapsulated AKβBA specifically inhibited the activation of NF-κB in macrophages without perturbing the epidermal keratinocytes. Such treatment subsequently resulted in a decreased production of TNF-α in the lesional skin of affected $CD18^{hypo}$ mice.

It has recently been suggested that skin-resident immune cells acting like an autonomous immune pathway might be of predominant importance for the pathogenesis of psoriasis (8, 9). If this concept is correct, localized treatment such as the liposome-encapsulated AKβBA might represent a treatment of choice. Interestingly, this approach proved to be particularly effective in our experimental model. In contrast, cytokine-targeting approaches with neutralizing Abs might in fact enhance the activity of the cytokine in vivo (58). In line with this, induction of psoriasis during anti-TNF-α therapy of rheumatoid arthritis has been reported (59, 60). Apart from other side effects associated with systemic administration of neutralizing anti-cytokine biologics, our present approach would circumvent such problems. Therefore, by the use of liposome-encapsuled AKβBA, it should be possible to target crucial component of the local immune system.

Our results demonstrate that the activation of NF-κB signaling pathway in macrophages is critical in the pathogenesis of the $CD18^{hypo}$ mouse model of psoriasis. Notably, we identified activation of the NF-κB signaling pathway in macrophages in human psoriasis and the $CD18^{hypo}$ mouse model of psoriasis. Inhibition of

the NF-κB activation in macrophages by AKβBA markedly decreased the production of the proinflammatory key cytokine TNF-α and of the chemokine MCP-1, accompanied by resolution of the inflammatory infiltrates and normalization of the hyperkeratosis in $CD18^{hypo}$ mice. Given the necessity for a suprabasal NF-κB activation for epidermal homeostasis, and the anticipated therapeutic effect of the targeted inhibition of the NF-κB in skin macrophages, such an approach might present a promising strategy for the treatment of human psoriasis.

Disclosures

The authors have no financial conflicts of interest.

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