Protein Kinase Cα-Mediated Chemotaxis of Neutrophils Requires NF-κB Activity but Is Independent of TNFα Signaling in Mouse Skin In Vivo[1]

Christophe Cataisson,* Andrea J. Pearson,* Sara Torgerson,* Sergei A. Nedospasov,† and Stuart H. Yuspa[2]*

Protein kinase C (PKC) isoforms are major regulators of cutaneous homeostasis and mediate inflammation in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We have previously reported that transgenic mice overexpressing PKCα in the skin exhibit severe intraepidermal neutrophilic inflammation and keratinocyte apoptosis when treated topically with TPA. Activation of PKCα increases the production of TNFα and the transcription of chemotactic factors (MIP-2, KC, S100A8/A9), vascular endothelial growth factor, and GM-CSF in K5-PKCα keratinocytes. In response to PKCα activation, NF-κB translocates to the nucleus and this is associated with IκB phosphorylation and degradation. Preventing IκB degradation reduces both the expression of inflammation-associated genes and chemoattractant release. To determine whether TNFα mediated NF-κB translocation and subsequent expression of proinflammatory factors, K5-PKCα mice were treated systemically with a dimeric soluble form of p75 TNFR (etanercept) or crossed with mice deficient for both TNFR isoforms, and keratinocytes were cultured in the presence of TNFα-neutralizing Abs. The in vivo treatment and TNFR deficiency did not prevent inflammation, and the in vitro treatment did not prevent NF-κB nuclear translocation after TPA. Together these results implicate PKCα as a regulator of a subset of cutaneous cytokines and chemokines responsible for intraepidermal inflammation independent of TNFα. PKCα inhibition may have therapeutic benefit in some human inflammatory skin disorders. *The Journal of Immunology,* **2005, 174: 1686–1692.**

Many of the common skin diseases have an inflammatory basis. Such conditions includes psoriasis, allergic contact dermatitis, and atopic dermatitis (1). Although rarely life-threatening, psoriasis and atopic dermatitis have significant morbidity including profound effects on the quality of life. Overall, inflammatory skin diseases have a significant socioeconomic impact (2).

Skin represents the body's largest immune organ; the role of skin's dendritic cells, infiltrating T cells, and granulocytes on immune surveillance is well established (3, 4). Interestingly, keratinocytes are also major contributors to local and systemic cytokines (reviewed in Ref 5) but have received less attention in the field of classical immunology.

We have previously demonstrated that transgenic mice that overexpress protein kinase Cα (PKCα)[3] in basal keratinocytes exhibit an acute inflammatory response when mice were painted with the PKC activator 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (6). The cutaneous inflammation was characterized by intraepidermal neutrophilic microabcesses that became confluent and trapped beneath the statum corneum; this particular inflammation has not been described for transgenic mice with other PKC isoforms targeted to the epidermis (7–9). We have also reported that the intraepidermal inflammatory response in skin-targeted PKCα mice is independent of the AP-1 transcription factor pathway, although that pathway is a known target of PKC activation (6).

NF-κB transcriptional response plays an important part in cutaneous inflammation and is a crucial element of innate immunity (3). NF-κB is composed of hetero- or homodimers of five related members in mammals: Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100) (10). In resting cells, most NF-κB dimers are bound to IκBs and retained in the cytoplasm. The IκBs are also members of a gene family of which the most common are IκBα, IκBβ, and IκBϵ. In response to stimuli, such as TNFα, IL-1, or LPS, the IκB kinase (IKK) complex (IKKα, IKKβ, IKKγ/NF-κB essential modulator) is activated and phosphorylates IκB, leading to its polyubiquitination and degradation. The NF-κB dimer is now free to enter the nucleus and regulate transcription (11). Involvement of the NF-κB transcription factors in skin physiology has been defined using gain or loss of functions studies in mice. Transgenic models have demonstrated a critical role for NF-κB on the control of keratinocyte proliferation and viability (12, 13). Heterozygous IKKγ-deficient mice exhibit a transient dermatosis characterized by granulocytic infiltration and increased keratinocyte apoptosis (14, 15). Skin-targeted deletion of IKKβ leads to a severe inflammatory skin disease mediated by TNFα (16). Sustained NF-κB activity leads to severe dermatitis in mouse models (17, 18) while inhibiting cutaneous NF-κB activity is associated with the development of squamous cell carcinomas (12, 19). Furthermore, the phenotype of K5-IκBα mice is also characterized by an intense neutrophilic cutaneous

*Laboratory of Cellular Carcinogenesis and Tumor Promotion, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892; and †Basic Research Program, SAIC-Frederick and Laboratory of Molecular Immunoregulation, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

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[2] Address correspondence and reprint requests to Dr. Stuart Yuspa, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Center for Cancer Research, National Cancer Institute, 37 Convent Drive, MSC-4255, Building 37, Room 4068, Bethesda, MD 20892-4255. E-mail address: yuspas@mail.nih.gov

[3] Abbreviations used in this paper: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IKK, IκB kinase; WT, wild type; VEGF, vascular endothelial growth factor; SLPI, secretory leukocyte proteinase inhibitor.

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inflammation (20). Overall, theses studies suggest a critical role of NF-κB signaling in the maintenance of skin homeostasis.

We have previously reported that PKCα activation up-regulates the transcription of IL-1 and TNFα. TNFα is among the best-characterized inducers of NF-κB activity. Two structurally related but functionally distinct receptors (TNFR1/p55 kDa and TNFR2/p75 kDa) mediate the biological activities of TNF. The majority of inflammatory responses classically attributed to TNFα occur through TNFR1. In contrast, TNFα-induced thymocyte proliferation and apoptosis of activated T cells are mediated by TNFR2 (21–23). Mouse keratinocytes express both TNFR1 and TNFR2 (24). The current study was designed to test whether TNFα or NF-κB signaling contributes to the intraepidermal inflammation.

Materials and Methods

Mice

The construction and characterization of K5-PKCα mice were previously described (6). These mice express a full-length murine PKCα cDNA under the control of the bovine keratin 5 promoter (6) that targets the transgene to the basal layer of the epidermis and the outer root sheath of the hair follicle. For the studies reported here, an FVB/N strain expressing a 10-fold excess of PKCα over the endogenous level in the target sites was used (6).

K5-PKCα mice were crossed to homozygous mice deficient in TNFR1 and TNFR2 ($TNFR1^{-/-}TNFR2^{-/-}$). F_1 mice K5-PKCα $TNFR1^{-/+}TNFR2^{-/+}$ were backcrossed to $TNFR1^{-/-}TNFR2^{-/-}$ to generate K5-PKCα mice deficient for either TNFR1, TNFR2, or doubly deficient. At weaning, tail samples were collected and screened by separate PCR for each of the alleles. K5-PKCα allele was identified by PCR using primers TGCATATAAATTCTGG CTGGCG and GCATGAACATGGTTAGCAGAGGG that span a 166-nt sequence of the β-globin intron (6). TNFR alleles were detected using the following primers: allele detected: *R1 wt*; forward primer, AGAAATGTCC CAGGTGGAGATCTC; reverse primer, GGCTGCAGTCCAAGCACTGG; allele detected: *R1 KO*; forward primer, TGCTGATGGGGATCCATC; reverse primer, CCGGTGGATGTGGAATGTGTG; allele detected: *R2 wt*; forward primer, CCTCTCATGCTGTCCCGGAAT; reverse primer, AGCTC CAGGCACAAGGGCGGG; and allele detected: *R2 KO*; CGGTTCTTTTT GTCAAGAC; reverse primer, ATCCTCGCCGTCGGGCAT.

To neutralize TNFα pharmacologically in K5-PKCα mice, a first dose of etanercept (100 μg) was administrated i.p. and 12 h later a second identical treatment was performed before TPA painting (2 μg). Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75-kDa TNFR linked to the Fc portion of human IgG1 and is produced in genetically engineered Chinese hamster ovary cells (25). Etanercept was previously shown to neutralize mouse TNFα by the i.p. route (26). Etanercept was reconstituted at a concentration of 25 mg/ml in sterile water as suggested by the manufacturer (Immunex) and was further diluted in PBS to a final concentration of 500 μg/ml.

Reagents

For in vivo application, TPA (LC Laboratories) was dissolved in acetone and the indicated concentrations were applied topically in 200 μl. TNFα was purchased from Calbiochem, anti-mouse TNFα Ab from R&D Systems, LPS (*Escherichia coli* serotype 0111:B4) from Sigma-Aldrich, and etanercept was purchased through the Division of Veterinary Resources (National Institutes of Health, Bethesda, MD). The IκBαSR adenovirus was a generous gift from D. C. Guttridge from the University of North Carolina (Chapel Hill, NC).

Myeloperoxidase assay

Back whole skin samples were used for myeloperoxidase assay following the method of Bradley et al. (27). In brief, samples were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, sonicated, and freeze-thawed three times, after which sonication was repeated. The suspension was centrifuged at 40,000 × *g* for 15 min, and 10 μl of supernatant was added to 290 μl of potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide. Changes in OD were monitored at 460 nm at 25°C over a 4-min period. Isolated mouse neutrophils obtained from thioglycolate-induced peritonitis were processed in the same manner and used to generate a calibration curve for neutrophils, which were enumerated by light microscopy.

Cell culture

Primary mouse keratinocytes and hair follicle buds were isolated from newborn transgenic and wild-type (WT) littermate epidermis as described elsewhere (28). Primary keratinocytes were seeded at a density of 5×10^6 cells per 60-mm dish (or equivalent concentrations) in Ca^{2+}- and Mg^{2+}-free MEM (Invitrogen Life Technologies) supplemented with 8% Chelex (Bio-Rad Laboratories)-treated FBS (Gemini Bioproducts) and 0.2 mM Ca^{2+}. After 24 h, cultures were switched to the same medium with 0.05 mM Ca^{2+} to select for basal cells. TPA was reconstituted in DMSO, and primary keratinocytes were treated with 5 ng/ml TPA for various times as indicated in individual experiments. This dose of TPA was shown previously to selectively activate PKCα in K5-PKCα keratinocytes (6).

Transfection and luciferase reporter assay

The NF-κB-luciferase plasmid DNA was a gift from Dr. Z.-G. Liu (National Cancer Institute, National Institutes of Health). Primary keratinocytes were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen Life Technologies). Transient transfection was conducted for 48 h before treatment with TPA. After treatment, cells were rinsed twice in PBS and harvested in reporter lysis buffer (BD Clontech). Luciferase activity was measured by using the luciferase reporter assay kit (BD Biosciences Clontech) according to the manufacturer's protocol. Results were normalized to the total protein content.

Immunofluorescence

Primary keratinocytes were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed with PBS five times, and permeabilized in prechilled MeOH for 10 min. After blocking with 100 mM glycine for 30 min, the cells were permeabilized with 0.2% Triton X-100 for 10 min. After washing the cells three times in PBS, they were blocked again for 30 min in 0.5% BSA and then incubated overnight with mouse mAb to p65 (Santa Cruz Biotechnology) diluted 1/100 in BSA or rabbit polyclonal Ab to PKCα (Sigma-Aldrich) diluted 1/10,000 in BSA. After being washed with PBS, cells were incubated for 1 h with goat anti-mouse or goat anti-rabbit Ab conjugated with FITC diluted 1/200 in BSA. Nuclei were stained using 4′,6′-diamidino-2-phenylindole (Roche Diagnostics) diluted 1/5000 in PBS for 3 min. After additional washes, coverslips were mounted (Vector Laboratories) and slides were stored at 4°C in the dark until examined by confocal microscopy.

RT-PCR analysis

RNA was isolated from cultured keratinocytes with TRIzol per the manufacturer's protocol (Invitrogen Life Technologies). For cDNA synthesis, 2 μg of total RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen Life Technologies). PCR amplifications were performed in a volume of 20 μl using Platinum PCR SuperMix (Invitrogen Life Technologies). The primers used for this analysis are as follows: gene: *actin*; forward primer, CAGATCATGTTTGAACCTTC; reverse primer, ACTTCATGATG GAATTGAATG; gene: *GM-CSF*: forward primer, GCCATCAAAGAAGC CCTGAA; reverse primer, GCGGGTCTGCACACATGTTA; gene, *KC*: forward primer, GCTTGTTCAGTTTAAAGATGGTAGGC; reverse primer, CGTGTTGACCATACAATATGAAAGACG; gene, *IL-1α*: forward primer, CAAACTGATGAAGCTCGTCA; reverse primer, TCTCCTTGAGCGCT CACGAA; gene, *IL-6*: forward primer, TTGCCTTCTTGGGACTGATG; reverse primer, CTGAAGGACTCTGGCTTTGT; gene, *MIP-2*: forward primer, CTGCCGCTCCTCAGTGCTGCACTG; reverse primer, GCCTTGC CTTTGTTCAGTATCTTTTGG; gene, *MIP-3α*: forward primer, AGCCAG GCAGAAGCAAGCAATAC; reverse primer, CTGTGTCCAATTCCATC CCAAAAA; gene, *S100A8*: GGAAATCACCATGCCCTCTA; reverse primer, GCTGTCTTTGTGAGATGCCA; gene, *S100A9*: forward primer, TCATCGACCCTTCCATCAA; reverse primer, GATCAACTTTGCCAT CAGCA; gene, secretory leukocyte proteinase inhibitor (*SLPI*): forward primer, GATGCTATCAAAATCGGAGC; reverse primer, CACAGCACTT GTATTTGCCG; gene, *TNFα*: forward primer, ATGAGCACAGAAAG CATGATCCG; reverse primer, GCAATGACTCCAAAGTAGACCTGCCC; and gene, vascular endothelial growth factor (*VEGF*): CCCTCCGAAAC CATGAACTT; reverse primer, GGCTTTGGTGAGGTTTGATCC.

To avoid saturation or the plateau effect of amplification, PCR was limited to a total of 20 cycles for actin and MIP3-α; 25 cycles for IL-1α, S100A9, SLP1, and TNF; 28 cycles for MIP-2, KC, and IL-6; 30 cycles for S100A8 and VEGF; and 32 cycles for GM-CSF. Each reaction was performed from at least three independent experiments.

Chemotaxis assays

Chemotaxis assays were performed using 48-well chemotaxis chambers (NeuroProbe) as described previously (29). Twenty-six to 28 μl of conditioned medium collected 6 h after TPA treatment of cultured keratinocytes

was placed in the wells of the lower compartment of the chamber and 50 μl of peritoneal lavage containing 1.5×10^5 neutrophils was placed in the wells of the upper compartment. A polyvinylpyrolidone-free polycarbonate membrane (3-μm pore size; NeuroProbe) separates the upper and lower compartments. The pore size was chosen to allow the migration of neutrophils but not monocytes. After incubation at 37°C for 1 h, the filters were removed, washed on the upper side, fixed, and stained with Diff-Quik. Cells migrating across the filters were counted under light microscopy after coding the samples. The results were expressed as chemotaxis index, which represents the fold increase in the number of migrated cells in six high-powered fields in response to TPA-stimulated supernatants over the spontaneous cell migration in response to control medium.

Adenovirus transduction

The IκBαSR or PKCα adenoviruses were introduced into primary keratinocytes using an adenoviral construct driven by a CMV promoter (30) and empty adenovirus was used as control. The cells were infected for 30 min in serum-free medium with a multiplicity of infection of 5 viral particles/cell and 2.5 μg/ml Polybrene (Sigma-Aldrich) to enhance uptake. Serum-containing medium was added to the cells for the next 48 h after the infection.

Immunoblotting

Cultured cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 20 μM leupeptin, 200 μM $NaVO_3$, and 10 mM NaF. Proteins were quantified by the Bradford method (Bio-Rad) and separated by 7.5% SDS-PAGE followed by immunoblotting. Phospho-IκBα (Ser^{32}) and IκBα Abs were used at 1/1000 (Cell Signaling) and β-actin Ab was used at 1/1000 (Chemicon). An ECL Super-Signal (Pierce) detection system was used.

TNFα ELISA

TNFα levels in plasma and culture supernatants were measured by ELISA according to the manufacturer's protocol (BD Biosciences).

Statistical methods

Data were analyzed by PRISM software and significance values were assigned through Student's *t* test. A value of $p < 0.05$ was considered to be significant.

Results

PKCα activates NF-κB signaling in keratinocytes

The dimeric NF-κB complex is present in an inactive state bound to inhibitory IκB protein in the cytosol. Phosphorylation of IκB leads to its degradation and allows NF-κB subunits to translocate to the nucleus. To test whether PKCα activation would cause NF-κB translocation to the nucleus, primary keratinocytes from K5-PKCα mice and their WT littermates were treated with 5 ng/ml TPA, and p65 cellular localization was monitored by confocal microscopy. Within 30 min, p65 was detected in the nucleus of virtually all transgenic cells while it remained mostly cytoplasmic in WT cells (Fig. 1). To determine whether NF-κB translocation triggered by TPA correlated with IκB hyperphosphorylation, K5-PKCα keratinocyte lysates were separated by SDS-PAGE and immunoblotted for phospho-IκBα and total IκBα using specific Abs. PKCα activation increases phosphorylation within 20 min and this activity results in a decrease of total IκBα, presumably associated with enhanced protein degradation as previously reported (31) (Fig. 2). The functional relevance of these observations was evaluated in WT and K5-PKCα primary keratinocytes that were transfected with a NF-κB reporter construct and stimulated with TPA. As shown in Fig. 3, TPA treatment substantially increased NF-κB transcriptional activity in transgenic cells while only a marginal induction was detected in their WT counterpart. These results suggest that selective PKCα activation leads to NF-κB nuclear translocation and transcriptional response. The limited response detected in WT keratinocytes along with the transient induction of NF-κB-responsive genes reported in TPA-treated WT keratinocytes previously (6), probably reflects the activation of endogenous PKCα in these cells.

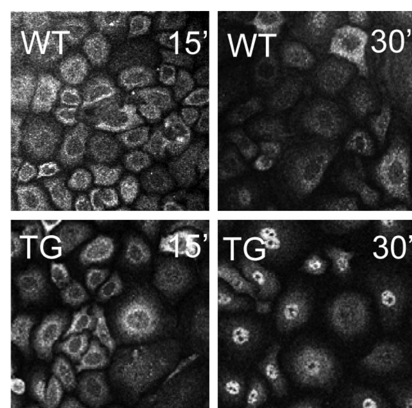

FIGURE 1. NF-κB subunit p65 translocates to the nucleus of K5-PKCα primary keratinocytes in response to TPA. Newborn mouse primary keratinocytes from WT or K5-PKCα littermates (transgenic (TG)) were treated with TPA for either 15 or 30 min and fixed with paraformaldehyde, immunostained with p65-specific Abs, and analyzed by confocal microscopy. TG, Transgenic.

Blocking NF-κB activation reduces inflammatory gene expression and chemoattractant release in primary keratinocytes

To test a functional role for NF-κB downstream of PKCα, primary keratinocytes were transduced with an adenoviral IκBα mutant construct (referred to as IκBα superrepressor, or IκBαSR). IκBαSR harbors mutations on Ser^{32} and Ser^{36} that renders the mutant protein resistant to phosphorylation and subsequent proteasome degradation (30). The inhibitory activity of IκBαSR was demonstrated by its ability to inhibit TPA-induced NF-κB reporter activity in K5-PKCα primary keratinocytes (Fig. 4*A*). In addition to the up-regulation of TNFα, MIP-2, cyclooxygenase 2, and MIP-3α that we have previously reported (6), we now report that cytokine-induced neutrophils chemoattractant (KC), S100A8 (calgranulin A, MRP-8), S100A9 (calgranulin B, MRP-9), IL-1α, IL-6, GM-CSF, secretory leukocyte proteinase inhibitor (SLPI), ICAM-1, and VEGF transcripts are up-regulated in response to PKCα activation in the first 3 h after TPA treatment (Fig. 4*B*, cf *lanes 1* and *2*). PKCα activation also up-regulates the expression of G-CSF transcripts 30 min after TPA treatment (data not shown). Consistent with the abrogation of the NF-κB reporter activity, the PKCα mediated increased expression of MIP-2, MIP-3α, TNF-α, GM-CSF, and IL-6 transcripts was completely blocked by IκBαSR (Fig. 4*B*). NF-κB transcriptional activity contributes only partially to the PKCα-mediated regulation of the KC, IL-1α, S100A8, and VEGF genes. We have previously demonstrated that culture supernatant from TPA-treated K5-PKCα keratinocytes contains chemotactic activity, confirming that functional chemokines are released from keratinocytes after activation of PKCα (6). To determine whether NF-κB-induced chemokines mediate the chemotactic activity of these supernatants, TPA-treated IκBαSR-transduced K5-PKCα primary keratinocyte supernatants were prepared. Figure 4*C* indicates that blockade of NF-κB transcriptional activity reduced the chemotactic activity by 40% compared with supernatants from K5-PKCα keratinocytes transduced with a control adenovirus. This result suggested that the NF-κB signaling pathway is a major but not exclusive contributor to the production of chemotactic molecules by keratinocytes, which leads to the migration of neutrophils.

NF-κB nuclear translocation is independent of TNF signaling

To test whether PKCα activation would cause TNFα release in culture supernatants, keratinocytes isolated from K5-PKCα newborn pups and their WT littermates were plated in duplicate in

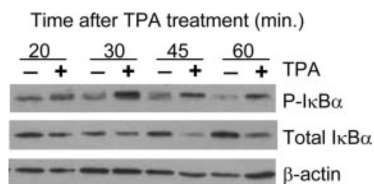

FIGURE 2. PKCα activation results in IκBα phosphorylation and decreased total IκBα levels. K5-PKCα primary keratinocytes were treated for the indicated periods of time and cell lysates were analyzed by Western blots.

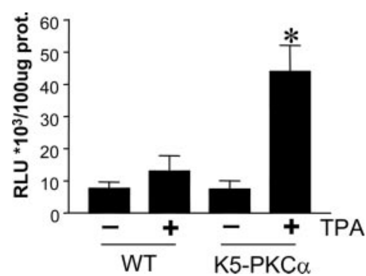

FIGURE 3. PKCα activation increases NF-κB reporter activity in K5-PKCα keratinocytes. WT and K5-PKCα primary keratinocytes were transiently transfected with a NF-κB reporter activity plasmid construct and after 48 h were treated with DMSO (−) or TPA (+) for 6 h. Cells were harvested and assayed for luciferase activity. Bars represent mean ± SEM of quadruplicate determinations. Values are expressed as relative light units (RLU) per 100 μg total protein as determined according to the method of Bradford et al. Results are representative of three independent experiments. *, $p < 0.05$ compared with respective DMSO control.

six-well plates. The cells were treated with either the vehicle DMSO or 5 ng/ml TPA for 3 h. The TNFα released was normalized to the number of cells at the collection time. The results indicate that keratinocytes from K5-PKCα mice released TNFα into the medium in response to PKCα activation (Table I). To determine whether the observed activation of NF-κB was a direct effect of PKCα activation and not due to the secondary induction of TNFα, we examined NF-κB translocation in the presence of neutralizing anti-TNFα Abs in cultured keratinocytes. The specificity of the neutralizing anti-TNFα Abs was confirmed by using IgG isotype Abs. Pretreatment of K5-PKCα primary keratinocytes with such anti-TNFα Abs abrogates the translocation of NF-κB by rTNFα (Fig. 5*A*) but has no effect on PKCα-mediated NF-κB translocation (Fig. 5*B*). TNFα associated with the cell surface is biologically active (32) and could be active even in the presence of neutralizing Ab. To confirm the independence of NF-κB activation from TNFα induction, primary keratinocytes from newborn mice homozygous with targeted disruption of the two known TNFα cell surface receptors, TNFR1 (p55) and TNFR2 (p75) were transduced with a PKCα adenoviral construct and treated with TPA. Nuclear translocation of NF-κB is not abrogated in the double knockout cells, confirming that PKCα causes NF-κB activation independently of TNFR expression (Fig. 5*C*). The PKCα expression level in adenovirus-transduced TNFR-deficient keratinocytes was similar to that of primary K5-PKCα cells (data not shown).

Neutrophil infiltration in the epidermis is TNFα independent

To determine whether TNFα activity is required for leukocyte recruitment to the epidermis, we analyzed neutrophil migration into the skin of mice that were pretreated systemically with a dimeric soluble form of p75 human TNFα receptor (etanercept). This molecule has been proven to be efficient at blocking TNFα activity in various murine models of inflammation (25, 26). As shown in Fig. 6*A*, etanercept given i.p. at 200 μg was ineffective at blocking the formation of microabscesses in response to PKCα activation. In addition, neither a higher dose (400 μg) nor a s.c. injection blocked neutrophil infiltration. To confirm the dispensable function of TNFα on PKCα-mediated skin inflammation, K5-PKCα mice were crossed with mice homozygous for targeted disruption of both TNFR1 and TNFR2 ($TNFR1^{-/-}$ $TNFR2^{-/-}$). None of the three K5-PKCα TNFR-deficient strains displayed any gross phenotypic or reproductive anomalies as reported for individual strains (6, 33). It has also been reported that genetic deletion of TNFR1 or TNFR2 individually does not affect expression of the remaining TNFα receptor subtype in the skin (34). As shown in Fig. 6*B*, the migration of neutrophils to the epidermis and the inflammatory response were indistinguishable in mice lacking TNFR1, TNFR2, or both isoforms from that of K5-PKCα expressing both TNFR. The neutrophil content was further evaluated by measurement of the myeloperoxidase activity in skin, confirming that migration of neutrophils in the skin of K5-PKCα was independent of TNFα (Fig. 6*C*).

Discussion

In the cutaneous lesions of TPA-painted K5-PKCα mice, inflammatory changes are associated with neutrophil infiltration and intraepidermal microabscess formation. In the present study, we have demonstrated that PKCα activation promotes NF-κB transcriptional activity in keratinocytes. Blocking NF-κB activity in primary keratinocytes partially abolishes the chemotaxis of neutrophils. Although TNFα is a potent inducer of inflammatory mediators through NF-κB, our results show that PKCα mediates infiltration of neutrophils in the epidermis independently of TNFα signaling. These data suggest that K5-PKCα mice are a valuable inducible (through TPA painting) model to study the critical mediators of neutrophilic inflammatory lesions.

We had previously shown that activation of PKCα in the FVB/N strain produces keratinocyte growth arrest and apoptosis that is dependent on intact AP-1 transcriptional activation but AP-1 activity is not required for the inflammatory infiltrate (6). Together it would appear that PKCα controls two distinct responses in keratinocytes, one related to growth and viability that is AP-1 dependent and the other related to chemotaxis and inflammation that involves NF-κB. Our results suggest that PKCα is an important regulator of NF-κB activation in the skin. Interestingly, NF-κB activation in the suprabasal layer of the epidermis causes growth arrest, a condition necessary for the proper differentiation of keratinocytes (12) and a response that is also regulated by PKCs (35). For most known stimuli, the degradation of IκB is an essential step for releasing NF-κB and its subsequent activation (11). Our results provide evidence that PKCα activation leads to phosphorylation of amino-terminal serine residues of IκBα, presumably through IKK activation. Although the signaling cascade emanating from PKCα causing IKK activation in K5-PKCα keratinocytes deserves further attention, it has been demonstrated that PKCα can activate IKKβ through PKCθ in T lymphocytes (35, 36). Peripheral T cells of $PKC\theta^{-/-}$ mice cannot be activated in response to Ag due to a striking deficiency in NF-κB activation (37, 38). However PKCθ is not a direct IKK activator and the signaling components that connect PKCθ and IKKβ have not been fully defined.

NF-κB is one of the pivotal regulators of proinflammatory gene expression in K5-PKCα primary keratinocytes. As a consequence, NF-κB blockade causes a significant reduction of the keratinocyte-derived chemotactic activity. Recent studies using transgenic models have demonstrated a critical role for NF-κB for the control of keratinocyte proliferation and viability (12, 13). However, blockade of NF-κB function in the skin of K5-IκBα mice leads to neutrophils-mediated inflammation of the skin (39), a condition also

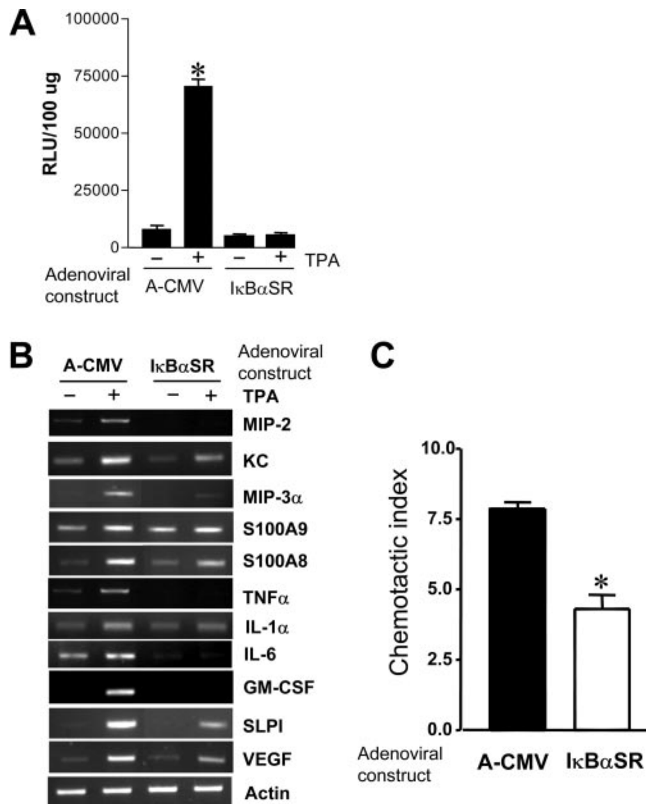

FIGURE 4. Blocking NF-κB transcriptional activity reduces inflammatory gene expression and chemoattractant release in response to PKCα activation. *A,* K5-PKCα primary keratinocytes were transiently transfected with the NF-κB reporter activity plasmid construct and 24 h later cells were transduced with an adenovirus encoding a degradation-resistant IκBα (IκBαSR) or a control adenovirus (A-CMV). After an additional 24 h, cells were treated with DMSO (−) or TPA (+) for 6 h. Values are expressed as relative light units (RLU) per 100 μg total protein as determined by Bradford. Results are representative of three independent experiments. *, $p < 0.001$ compared with respective DMSO control. *B,* Agarose gel stained with ethidium bromide showing RT-PCR product for MIP-2 (281 bp), MIP-3α (260 bp), S100A9 (227 bp), S100A8 (189 bp), TNFα (700 bp), GM-CSF (113 bp), SLPI (273 bp), VEGF (352 bp), and actin (472 bp). RNA was extracted from A-CMV (control) or degradation-resistant IκBα (IκBαSR) adenovirus-transduced primary keratinocytes treated with DMSO (−) or TPA (+) for 3 h. *C,* Chemotactic activity of culture supernatant collected from K5-PKCα keratinocytes transduced with A-CMV (control) or degradation-resistant IκBα (IκBαSR) adenovirus after a 6-h TPA treatment. Fold increase (chemotaxis index) of neutrophil migration in response to TPA was compared with supernatant collected after DMSO treatment. *, $p < 0.05$ compared with A-CMV control. Bars represent the mean ± SEM of two triplicate determinations.

seen in IKKγ-deficient mice (14, 15). Characteristic neutrophil accumulation under the stratum corneum can be observed in highly inflamed areas of human psoriatic lesions where neutrophils are chemotactically attracted by synergistic activity of IL-8 and growth-related oncogene α chemokines produced by keratinocytes (40). In the mouse, the local recruitment of polymorphonuclear leukocytes can be mediated by cytokine-induced neutrophil chemoattractant (KC) (41), the homologue of the human GRO, and MIP-2, considered as the functional analogue of human IL-8. We have previously excluded MIP-1α as a possible mediator of PKCα-induced intraepidermal inflammation since CCR1 deficiency did not prevent neutrophilic infiltration in K5-PKCα mouse epidermis. Our results demonstrate that both KC and MIP-2 are PKCα regulated through NF-κB activation. Interestingly, although blocking NF-κB activation completely abrogates the induction of MIP-2 by PKCα, KC mRNA induction was not completely prevented. This might reflect an alternative regulatory mechanism for that gene and could contribute to the residual chemotactic activity upon NF-κB blockade in this model. Constitutive expression of KC in the epidermis of transgenic mice causes infiltration of neutrophils in the skin, but the inflammatory reaction was very moderate (41). Inducible expression of KC in transgenic grafts causes a greater inflammatory response by circumventing CXCR2 (KC's

Table I. *TNFα levels released from cultured keratinocytes*

Time (h)	Keratinocytes	Picograms per Milliliter medium TNFα
3	WT-DMSO	33.3 ± 0.49
	WT-TPA	41.3 ± 2.2
	K5-PKCα-DMSO	35.4 ± 0.2
	K5-PKCα-TPA	158.8 ± 2.9*
6	WT-DMSO	37.1 ± 3.8
	WT-TPA	47.0 ± 1.3
	K5-PKCα-DMSO	37.1 ± 2.9
	K5-PKCα-TPA	226.4 ± 8.4*

*, $p < 0.002$ compared to respective DMSO treatment.

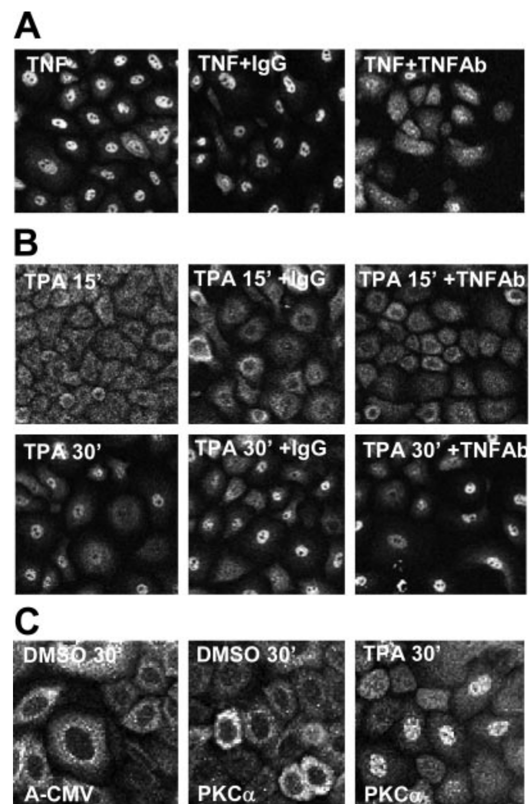

FIGURE 5. NF-κB nuclear translocation is independent of TNFα signaling. *A,* K5-PKCα primary keratinocytes were treated with TNFα alone for 15 min (TNF) or pretreated with control IgG for 1 h followed by a 15-min treatment with TNFα (TNF + IgG) or pretreated with TNFα-neutralizing Ab for 1 h followed by a 15-min treatment with TNFα (TNF + TNFAb). B, K5-PKCα primary keratinocytes were treated with TPA alone for 15 or 30 min (TPA) or pretreated with control IgG for 1 h followed by a 15- or 30-min treatment with TPA (TPA + IgG) or pretreated with TNFα-neutralizing Ab for 1 h followed by a 15- or 30-min treatment with TPA (TPA + TNFAb). *C,* TNFR1R2 double knockout keratinocytes transduced with A-CMV (control) or PKCα adenovirus were treated with DMSO or TPA for 30 min. Cells were fixed with paraformaldehyde, immunostained with p65-specific Abs, and analyzed by confocal microscopy.

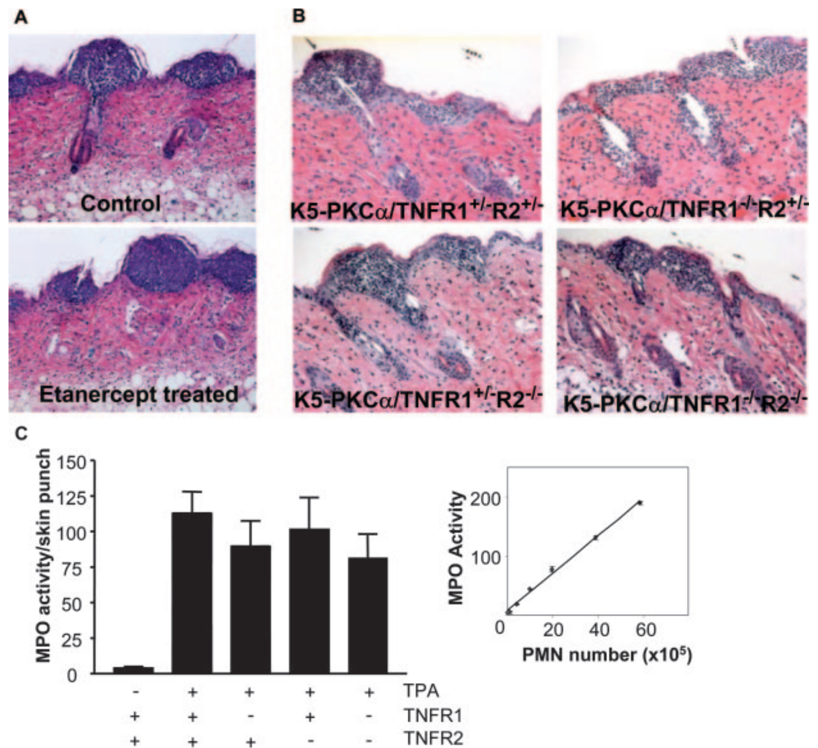

FIGURE 6. Neutrophil infiltration in the epidermis is TNFα independent. *A,* A single dose of TPA (2 μg) in acetone was applied to the shaved backs of K5-PKCα mice that had been injected 12 h earlier with PBS (control) or the TNF inhibitor (etanercept treated, 200 μg i.p.). Skin was collected 12 h later and stained with H&E. *B,* K5-PKCα mice expressing both TNFRs (K5-PKCα/TNFR1$^{+/-}$R2$^{+/-}$), K5-PKCα mice deficient for TNFR1 (K5-PKCα/TNFR1$^{-/-}$R2$^{+/-}$), K5-PKCα mice deficient for TNFR2 (K5-PKCα/TNFR1$^{+/-}$R2$^{-/-}$), and K5-PKCα mice deficient for both TNFRs (K5-PKCα/TNFR1$^{-/-}$R2$^{-/-}$) were TPA painted, skin was collected 24 h later, and sections were stained with H&E. Original magnification, ×200). *C,* Neutrophil infiltration in the epidermis of the mouse used in *B* was quantified using leukocyte myeloperoxidase activity (MPO). Bars represent the mean ± SEM of four independent animals and results are representative of at least five independent experiments. *Right panel,* A calibration curve for myeloperoxidase activity vs neutrophil number (see *Materials and Methods*). The differences among the treated groups are not significantly different. PMN, Polymorphonuclear leukocytes.

receptor) desensitization caused by high-level constitutive expression of KC that prevents a more marked inflammatory response (42). In that regard, the K5-PKCα mice may prove to be an inducible model well suited for the analysis of the contribution of CXCR2 ligands role on neutrophil migration in the skin. In addition, it appears that PKCα up-regulates the transcription of the *S100A8* and *S100A9* genes. S100A8 and S100A9 are calcium binding and regulated proteins for which an increasing body of information suggests a prominent role during inflammation (43). The presence of the S100A8/A9 complex in sera of patients with various inflammatory conditions, including cystic fibrosis and rheumatoid arthritis, suggests an extracellular role. S100A8 and S100A9 are up-regulated in psoriatic lesional skin (44), and dimers are constitutively secreted by human Hacat keratinocytes (45) and released through a PKC-dependent mechanism in human monocytes (46). Interestingly, recent reports suggest chemotactic properties for S100A8 and S100A9 on the local recruitment of neutrophils in response to LPS in a mouse air pouch model (47, 48). Our results indicate that S100A8 and S100A9 transcription is up-regulated independently of NF-κB downstream of PKCα; this could in turn also contribute to the residual chemotactic activity observed upon NF-κB blockade. Overall, our results confirm the potential for keratinocyte-derived chemotactic factors to promote neutrophil infiltration in the epidermis. Interestingly, the up-regulation of both G-CSF/GM-CSF and IL-6 gene expression suggests that PKCα activation in keratinocytes could also increase the central production of neutrophils, a response that would contribute to systemic neutrophilia.

Our observation that K5-PKCα mice lacking both TNFR1 and TNFR2 still develop neutrophilic microabscesses in the epidermis strongly indicates that in this model neutrophil emigration is TNFα independent even though TNFα is up-regulated and released. Transgenic mice overexpressing high levels of TNFα under the K14 promoter develop only a moderate dermal inflammatory response (49). In contrast, recruitment of inflammatory cells during UVB exposure, chemical irritation, s.c. TNFα injection, and wound healing requires TNFR1 expression (50–52). TNFR1 signaling pathways also contribute to cutaneous permeability barrier repair (53). Mice having a defect in NF-κB signaling through skin-targeted deletion of *ikkβ* or keratin 5-driven expression of the IκBα repressor mutant develop a strong epidermal inflammation that can be prevented by TNFR1 deficiency (16, 39). The K5-IκBα repressor mice display a mixed inflammatory infiltrate dominated by neutrophils and concomitant up-regulation of TNFα (20). Collectively, these reports support a critical role for TNFα/TNFR1 on the initiation of inflammation and recruitment of inflammatory cells in the skin. However, neutrophil emigration in the lung in response to LPS does not require TNFR expression (33). Similarly, careful analysis of RelA$^{-/-}$tnfr1$^{-/-}$ mice revealed a defect in neutrophil emigration in the lung not due to TNFR1-defective signaling but rather defective induction of KC and MIP-2 because of RelA deficiency (54). Therefore, clearly a precedent exists where the recruitment of neutrophils at the inflammation site is TNF independent, and TPA-mediated inflammation through PKCα appears to be one of them.

Clinical trials with several different TNF antagonists have established that this cytokine has an important pathogenic contribution to cutaneous inflammation (55). However, our results suggest that in the skin there are situations where TNF-independent pathways exist for the chemotaxis of neutrophils. These situations would challenge the clinical efficacy of TNF antagonists. PKCα-specific inhibitors could have potential to provide therapeutic benefit in skin inflammatory disorders presenting neutrophilic granulocyte accumulation in which TNF antagonists are ineffective.

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