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Blockade of the EGF Receptor Induces a Deranged Chemokine Expression in Keratinocytes Leading to Enhanced Skin Inflammation

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During inflammatory skin disorders such as psoriasis, atopic dermatitis, and allergic contact dermatitis, epidermal keratinocytes overexpress large amounts of soluble epidermal growth factor receptor ligands in response to tumor necrosis factor α and interferon γ. These cytokines also promote *de novo* synthesis of numerous chemokines, including CCL2/MCP-1, CCL5/RANTES, CXCL10/IP-10, and CXCL8/IL-8, in turn responsible for the recruitment of different leukocyte populations. This study demonstrates that stimulation of EGFR down-regulates CCL2, CCL5, and CXCL10, while it increases CXCL8 expression in keratinocytes. Conversely, EGFR signaling blockade produces opposite effects, with increased CCL2, CCL5, and CXCL10, and reduced CXCL8 expression. In a mouse model of contact hypersensitivity, a single topical administration of a selective EGFR kinase blocker before antigen challenge results in a markedly enhanced immune response with increased chemokine expression and heavier inflammatory cell infiltrate. Targeting EGFR on epithelial cells may thus have profound impact on inflammatory and immune responses. *(Am J Pathol 2003, 163:303–312)*

Growth factors and their receptors are involved in the response of epithelial cells to injury. Keratinocyte-derived growth factors include several members of the epidermal growth factor (EGF) family such as transforming growth factor α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor, and epiregulin. These factors derive from proteolytic cleavage of integral membrane precursors by metalloproteinases.[1] Although keratinocytes express multiple EGF receptors (c-erbB1–3), c-erbB1 (also called EGFR) is responsible for the bulk of the autocrine activities of EGF family members in keratinocytes.[2,3] Indeed, EGFR activation serves essential functions in skin development, wound healing as well as carcinogenesis, maintaining keratinocytes in an active proliferative state,[4,5] enhancing their motility,[6] and supporting survival and protection from apoptosis.[7] EGFR and its ligand TGF-α are overexpressed in a variety of benign and malignant hyperproliferative skin disorders, including psoriasis,[8–10] and the role of TGF-α in the genesis of epidermal hyperplasia has been confirmed in transgenic mouse models.[11,12] However, no consistent information exists on the contribution of EGFR signaling to skin inflammation.

A common feature of chronic inflammatory skin disorders such as psoriasis, atopic dermatitis, and allergic contact dermatitis, is epidermal hyperplasia and thickening, a phenomenon attributed to leukocyte-derived cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, which are potent inducers of EGF family growth factors and EGFR.[13,14] In the course of T cell-driven skin inflammatory diseases, activated Th1 lymphocytes infiltrating the dermis and the epidermis are the major source of IFN-γ and TNF-α. Among the various leukocyte subsets, Th1 lymphocytes dominate psoriatic and allergic contact dermatitis lesions, but they are present also in chronic atopic dermatitis.[15,16] These cytokines initiate a program of increased keratinocyte expression of inflammatory mediators, including adhesion molecules, cytokines, and chemokines. In particular, prominent keratinocyte expression of CCL2 (monocyte chemoattractant protein 1, MCP-1), CCL5 (RANTES), CXCL8 (IL-8), and CXCL10 (IFN-γ-induced protein of 10 kd, IP-10) is a common finding in T cell-mediated skin diseases, and mediates the recruitment of T cells and other leukocyte populations in the skin.[17–20]

Here we demonstrated that EGFR signaling activation was an early event in keratinocyte response to TNF-α or IFN-γ, and that this mechanism was part of an autocrine loop with regulatory effects on CCL2, CCL5, and CXCL10 expression. In a mouse model of contact hypersensitivity, a T cell-mediated immune response where the contribution of keratinocytes to skin inflammation is well established,[20] administration of a selective EGFR kinase blocker before antigen challenge induced enhanced immune response with increased chemokine expression and heavier inflammatory infiltrate. Thus, EGFR appears to play a relevant role in the control of skin inflammation.

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Materials and Methods

Subjects

Four-mm punch biopsies were taken from lesional skin of adult patients with chronic plaque psoriasis ($n = 5$; three females and two males; age 30 to 48), chronic atopic dermatitis ($n = 5$; three females and two males; age 25 to 42), chronic allergic contact dermatitis ($n = 3$; two females and one male; age 30 to 40), and normal skin of healthy subjects ($n = 4$; two females and two males; age 24 to 39). Epidermal sheets for keratinocyte cultures and full-thickness punch skin explants for organ cultures were obtained from healthy individuals undergoing plastic surgery (mammoplasty or abdominoplasty) ($n = 6$, three females and three males; age 25 to 40). All subjects were not receiving any systemic or topical therapy before sampling. Informed consent was obtained from all subjects and the study design was approved by the local ethical committee.

Cytokines, Reagents, and Antibodies

Recombinant human TGF-α, TNF-α, IFN-γ, and anti-TGF-α neutralizing Ab were purchased from R&D Systems (Abingdon, United Kingdom). PD168393, PD153035, and AG1296 were from Calbiochem (La Jolla, CA). Ilomastat came from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Immunohistochemistry

Four-μm cryostatic sections were fixed with 4% paraformaldehyde for 10 minutes, treated with 0.3% hydrogen peroxide, permeabilized with 0.05% Triton X-100 and then incubated for 1 hour at room temperature with the appropriate dilution of each Ab. For the detection of EGFR in human tissues and of TGF-α, CCL5, CCL2, and CXCL10 in the mouse, we used Abs from Santa Cruz Biotech (Santa Cruz, CA). TGF-α in human tissues was detected with a mAb from Oncogene (Darmstadt, Germany). Abs against human CXCL8 and CXCL10 were from R&D Systems. Anti-mouse CD4, CD8, and CD11b Abs were from BD PharMingen (San Diego, CA). Phospho-ERK1/2 was detected in mouse skin sections with an Ab from Cell Signaling Technologies (Beverly, MA). Secondary biotinylated Abs and staining kits were from Vector Laboratories (Burlingame, CA). Immunoreactivity was revealed using avidin-biotin-peroxidase system and 3-amino-9-ethylcarbazole as chromogen. Sections were counterstained with Mayer's hematoxylin. As negative controls, primary Abs were omitted or replaced with isotype-matched Ig. Infiltrating cells positive for CD4, CD8, or CD11b were counted in high power fields at ×1000 and expressed as cells per area unit ± SD (n [microscopic fields per section] = 6).

Keratinocyte Cultures

Primary cultures of normal human keratinocytes were obtained as described previously.[21] Keratinocytes were routinely grown in serum-free Keratinocyte Growth Medium (Clonetics, San Diego, CA), prepared from the essential nutrient solution Keratinocyte Basal Medium supplemented with 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 2 ml bovine pituitary extract and antibiotics. In the 24 hours preceding the experiments, 80% confluent keratinocyte cultures were switched to EGF-depleted medium.

Organ Cultures of Normal Human Skin

Five mm^3 punches of normal human skin from three healthy donors were placed in Keratinocye Basal Medium with 0.1% normal human serum in a humidified incubator at 37°C, with enough medium to just cover the explants. Cultures were treated or not with IFN-γ (1000 units/ml, 24 hours) following 30 minutes of pre-incubation with PD168393 (2 μmol/L) or its vehicle (0.025% dimethyl sulfoxide (DMSO)). After experiments, the explants were snap-frozen.

TUNEL Staining

TUNEL staining was done on paraformaldehyde-fixed keratinocyte monolayers or paraffin-embedded sections of human and mouse skin using the *In Situ* Cell Death Detection Kit AP (Boehringer Mannheim, Mannheim, Germany), following the manufacturer's instructions. For the evaluation of apoptosis in confluent keratinocyte monolayers, $TUNEL^+$ cells were counted in high power fields at ×1000 and expressed per 10^3 cells ± SD (n [microscopic fields in a chamber slide] = 6). In human whole-skin explants and in mouse skin sections, apoptosis was quantified as $TUNEL^+$ cells/500 basal epidermal cells ± SD (n [microscopic fields per section] = 6). The data were confirmed in three independent experiments.

Flow Cytometry Analysis

Keratinocytes were treated with TNF-α, IFN-γ, or medium alone for 15 minutes at 37°C. Cells were then incubated with anti-TGF-α ectodomain-specific Ab (R&D Systems) or isotype-matched control Ab for 30 minutes on ice, followed by FITC-conjugated rabbit anti-goat Ab (BD PharMingen) for 20 minutes and finally analyzed with FACSCalibur (BD PharMingen).

Enzyme-Linked Immunosorbent Assay (ELISA)

TGF-α was measured in cell-free supernatants using a kit from Oncogene. Chemokines were measured with dedicated kits from BD PharMingen.[17] Data are expressed as nanograms per 10^6 cells ± SD from six independent experiments.

Immunoprecipitation and Western Blot Analysis

Keratinocytes were lysed by adding 1 ml per 10-cm dish of lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L phe-

nylmethylsulphonyl fluoride, 0.15 units/ml aprotinin, 1% leupeptin, 1% pepstatin, 1 mmol/L NaF, and 1 mmol/L Na_3VO_4). EGFR was immunoprecipitated using anti-EGFR Ab from Santa Cruz Biotech, and phospho-EGFR was immunodetected using anti-phospho-tyrosine (PY) Ab from Transduction Laboratories (BD PharMingen). Anti-ERK and phospho-ERK Abs were from Cell Signaling Technologies. Anti-IFN-γR1, phospho-IFN-γR1, TNFR1, and TRAF2 Abs were from Santa Cruz Biotech.

RNase Protection Assay

Total RNA was extracted from cultured keratinocytes using the Trizol reagent (Invitrogen, Carlsbad, CA). The templates of the human chemokines under investigation and of the housekeeping molecule L32, and the kit for RNase protection assay were purchased from BD PharMingen. Total RNA was hybridized overnight with α-^{32}ATP-labeled cDNA templates, and reactions were performed as described.[17] RNase protection assay bands were quantified by using laser densitometry supported by the Quantity One software (Bio-Rad, Hercules, CA). The densitometry value of each chemokine mRNA signal was normalized to the value of the corresponding L32 mRNA signal, and expressed as multifold increase (or decrease) as compared to its respective control, or as relative densitometric units ± SD from four independent experiments.

Contact Hypersensitivity Assay

BALB/c mice (Charles River Italia, Calco, Italy) were sensitized by application of 30 μl of 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich, Milan, Italy) in acetone/olive oil (4/1) on a 2-cm^2 area of the shaved abdomen.[19] Five days later, sensitized and unsensitized animals received 10 μl of 0.15% DNFB on each side of each ear. In selected groups, 10 μl of 4 mmol/L PD168393 dissolved in DMSO/absolute ethanol (1/10 v/v), or 10 μl of the vehicle alone were painted on each side of each ear of sensitized and unsensitized mice 30 minutes before challenge. At least five mice per group were used in each experiment. Ear thickness was measured before challenge and in the following 3 days. Data are expressed as the change (from pre-challenge levels) in ear thickness $\times 10^{-3}$ inches and represent the mean increase ± SEM. In each experimental group, some mice were sacrificed 48 hours after challenge, and the ears were cut and either paraffin-embedded or snap-frozen for hematoxylin & eosin staining or immunohistochemistry, respectively.

Statistics

The Wilkoxon signed-rank test was applied to compare differences in chemokine gene expression and protein release by cultured keratinocytes. In the statistical evaluation of apoptosis in cultured keratinocytes and in mouse experiments, unpaired Student's *t*-test was used to compare the differences between means. Significance was assumed at a *P* value of 0.05 or less.

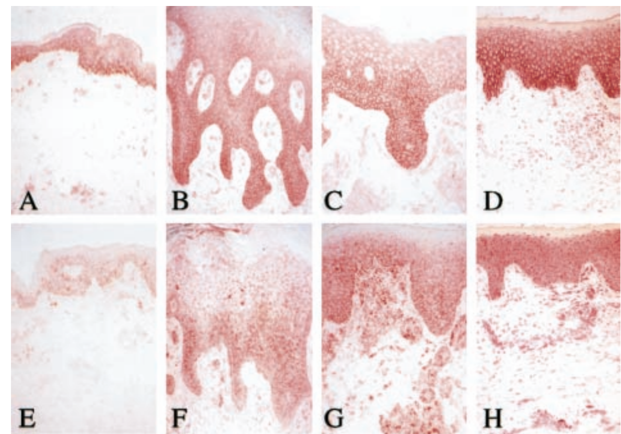


Figure 1. EGFR and TGF-α expression in normal and chronically inflamed skin. Expression of EGFR in the epidermis of a healthy donor (**A**). EGFR expression in lesional skin of chronic plaque psoriasis (**B**), chronic atopic dermatitis (**C**), and chronic allergic contact dermatitis (**D**). Very faint TGF-α immunoreactivity in the epidermis of a healthy donor (**E**). Strong TGF-α expression in the epidermis and dermis of lesional skin of plaque psoriasis (**F**), atopic dermatitis (**G**), and allergic contact dermatitis (**H**). Representative immunohistochemistry results from four healthy donors, five patients with psoriasis, five patients with atopic dermatitis, and three patients with allergic contact dermatitis. Original magnification, ×100.

Results

EGFR and TGF-α Are Overexpressed in Chronic Inflammatory Skin Disorders

EGFR immunoreactivity could be localized throughout the whole epidermis of normal skin from healthy controls as previously reported,[22] although it was more accentuated at the basal cell layer (Figure 1A). In parallel with the increased epidermal thickness, we observed a prominent EGFR expression extended to suprabasal keratinocyte layers, not only in psoriasis as previously documented,[8] but also in lesional skin of atopic dermatitis and allergic contact dermatitis (Figure 1, B–D). Sparse EGFR-positive cells could be detected in the dermis (Figure 1, A–D). In normal skin, TGF-α stained basal keratinocytes quite faintly (Figure 1E). In contrast, accentuated TGF-α immunoreactivity was detected throughout basal and suprabasal keratinocyte layers in psoriasis, atopic dermatitis, and allergic contact dermatitis (Figure 1, F–H). Moreover, the inflammatory infiltrate displayed intense TGF-α staining (Figure 1, F–H), suggesting that it represents a relevant source of this growth factor in the inflamed skin.

EGFR Transactivation by TNF-α and IFN-γ

Both TNF-α and IFN-γ induce TGF-α gene expression in keratinocytes.[13,14] By FACS analysis, we observed that both cytokines also induced a rapid TGF-α shedding from keratinocyte membranes. Compared to untreated cells, TNF-α (100 ng/ml, 15 minutes) induced the loss of 55 ± 9.50% cell-associated fluorescence, whereas a 70 ± 15.25% decrease was caused by IFN-γ (100 units/ml, 15 minutes) ($n = 6$; Figure 2A). Cytokine-induced shedding of TGF-α was efficiently prevented by cell preincubation with the broad-range metalloproteinase inhib-

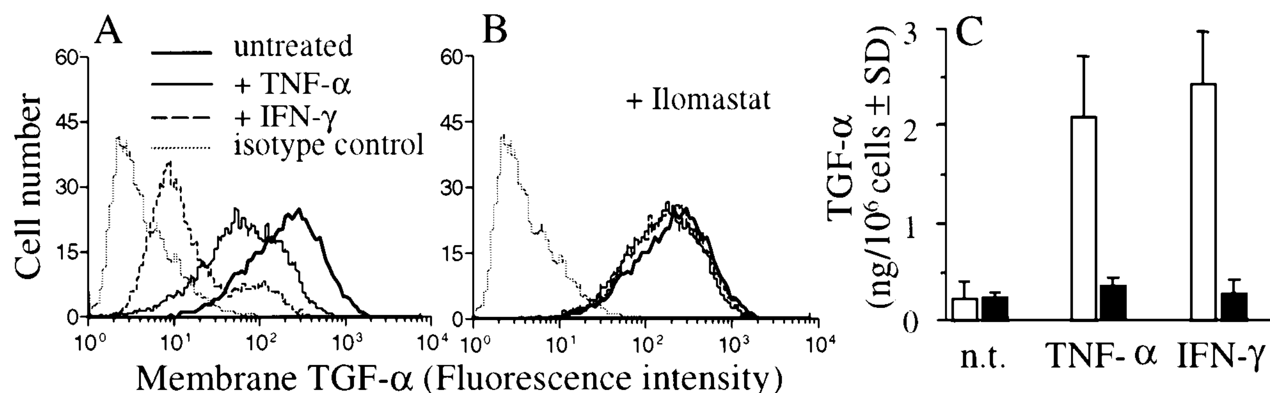


Figure 2. TNF-α and IFN-γ induce metalloproteinase-mediated shedding of membrane TGF-α. Flow cytometric analysis of cell-associated pro-TGF-α following 15 minutes of stimulation with 100 ng/ml TNF-α or 100 units/ml IFN-γ (**A**). TGF-α shedding was prevented by pre-incubation with the broad metalloproteinase inhibitor Ilomastat (**B**). Soluble TGF-α was measured in culture supernatants by ELISA (**C**). Keratinocytes were treated with cytokines or medium alone (no treatment, n.t.) directly (**white columns**), or after 30 minutes pre-incubation with Ilomastat (**black columns**).

itor Ilomastat[23] (25 μmol/L, 30 minutes) (Figure 2B), and was paralleled by over 10-fold increase in soluble TGF-α (Figure 2C). Both cytokines (30 minutes) also increased the levels of EGFR tyrosine phosphorylation, which was efficiently prevented by cell pre-incubation (30 minutes) with PD168393 (2 μmol/L), a selective inhibitor of EGFR/c-erbB2 tyrosine kinase[24] (Figure 3A). EGFR phosphorylation promoted by TNF-α or IFN-γ could be attenuated by pre-incubation with neutralizing anti-TGF-α Ab (10 μg/ml) or Ilomastat, but not by the PDGF receptor-specific tyrphostin AG1296[25] (2 μmol/L) (Figure 3A). Finally, PD168393 totally prevented both TNF-α- and IFN-γ-induced ERK1/2 phosphorylation at the time point of their maximal effect on ERK activation (15 minutes; data not shown) as well as at 30 minutes (Figure 3B), suggesting that EGFR transactivation occurred during keratinocyte response to these cytokines. Treatment with PD168393 did not interfere with IFN-γ-induced phosphorylation of IFN-γR1 or with TRAF2 recruitment by TNFR1 following TNF-α (Figure 3B). Finally, presence of 0.025% DMSO in the medium, used as vehicle for the three pharmacological inhibitors, did not affect signals compared to medium alone (not shown).

TGF-α Modulates Chemokine Expression in Keratinocytes

Keratinocytes are usually cultured in the presence of 10 ng/ml EGF. To test whether EGFR activation was involved in the control of chemokine expression by keratinocytes, they were starved of EGF for 24 hours before treatment with TNF-α (100 ng/ml) or IFN-γ (100 units/ml) alone and together with TGF-α (50 ng/ml). When combined to TNF-α (Figure 4A, left panels), TGF-α invariably induced a prominent down-regulation of CCL2, CCL5, and CXCL10 mRNA at the time points examined. We also registered a twofold increase in the levels of CXCL8 mRNA at 2 hours and 4 hours, but no detectable influence at 8 hours. In contrast, the effects of TGF-α on IFN-γ-driven CCL2, CCL5, and CXCL10 gene expression could be reproducibly observed only after 8 hours co-stimulation (Figure 4A, right panels). At this time point, TGF-α induced a three- to fivefold decrease of IFN-γ-induced expression of CCL2 and CCL5, whereas CXCL10 signal underwent a twofold decrease (from 55.05 ± 7.5 to 23.50 ± 8.25 relative densitometric units, $n = 4$, $P < 0.05$). Finally, in contrast to what was observed with TNF-α, TGF-α induced a persistent up-regulation on IFN-γ-induced CXCL8-specific signal, with an increase from 0.50 ± 0.55 to 2.50 ± 0.50 relative densitometric units ($n = 4$, $P < 0.05$) at 8 hours co-stimulation. In association to either TNF-α (4 hours) or IFN-γ (8 hours), TGF-α displayed a dose-dependent activity at the mRNA (Figure 4B) and at the protein level, as measured by ELISA in the supernatants following 24 hours of stimulation (Figure 5). TGF-α also dose-dependently reduced and augmented basal CCL5 and CXCL8 expression, respectively (Figure 4B and Figure 5) The effects of TGF-α on cytokine-driven chemokine protein release were significant ($P < 0.05$) at concentrations higher than 5 ng/ml.

Chemokine Expression in the Presence of Pharmacologically Impaired EGFR Signaling

We observed that pharmacological impairment of EGFR signaling perturbed keratinocyte response to both cytokines with opposite effects compared to TGF-α (Figure 6). In particular, CCL2, CCL5, and CXCL10 mRNA levels were three to five times higher when stimulation with TNF-α (2 hours) was performed following 30 minutes pre-incubation with TGF-α neutralizing Ab (10 μg/ml) or Ilomastat (25 μmol/L). Inhibition of EGFR kinase activity by PD168393 (2 μmol/L) induced a four- to sevenfold increase in CCL2 and CXCL10 mRNA levels. In contrast, CXCL8 mRNA was significantly reduced by EGFR blockade (Figure 6, left panels). Similar effects could be observed with IFN-γ stimulation (8 hours). Indeed, PD168393 (or Ilomastat) pre-treatment induced three to four times higher CCL2 and CCL5, two times higher CXCL10 (130.05 ± 5.75 *vs*. 54.50 ± 8.55 relative densitometric units; $n = 4$, $P < 0.05$), but down-regulated CXCL8 mRNA levels when compared to IFN-γ alone (Figure 6, right panels). ELISA on 24 hours supernatants showed that PD168393 significantly affected constitutive

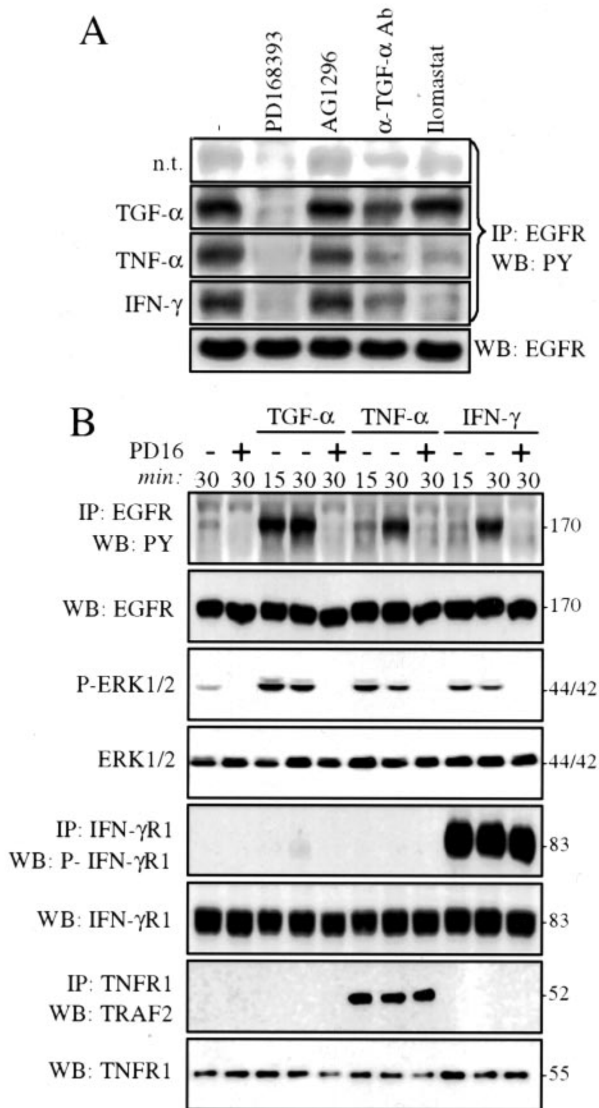


Figure 3. TNF-α and IFN-γ induce EGFR transactivation. EGFR tyrosine phosphorylation (PY) in keratinocytes treated for 30 minutes with medium (n.t.), 50 ng/ml TGF-α, 100 ng/ml TNF-α, or 100 units/ml IFN-γ directly (-), or following 30 minutes pre-incubation with PD168393, AG1296, anti-TGF-α neutralizing Ab, or Ilomastat (**A**). EGFR was immunoprecipitated (IP) using anti-EGFR Ab, and phospho-EGFR was analyzed by Western blot (WB) using anti-phospho-tyrosine (PY) Ab. PD168393 (PD16) abrogated cytokine-induced EGFR and ERK phosphorylation (P-ERK1/2), but did not affect IFN-γR1 phosphorylation (P-IFN-γR1) following stimulation with IFN-γ, or TRAF2 recruitment by TNFR1 in TNF-α-stimulated keratinocytes (**B**). The number on the **right** side of each **panel** represents the weight in kd of the immunodetected molecule(s). Representative results from five independent experiments.

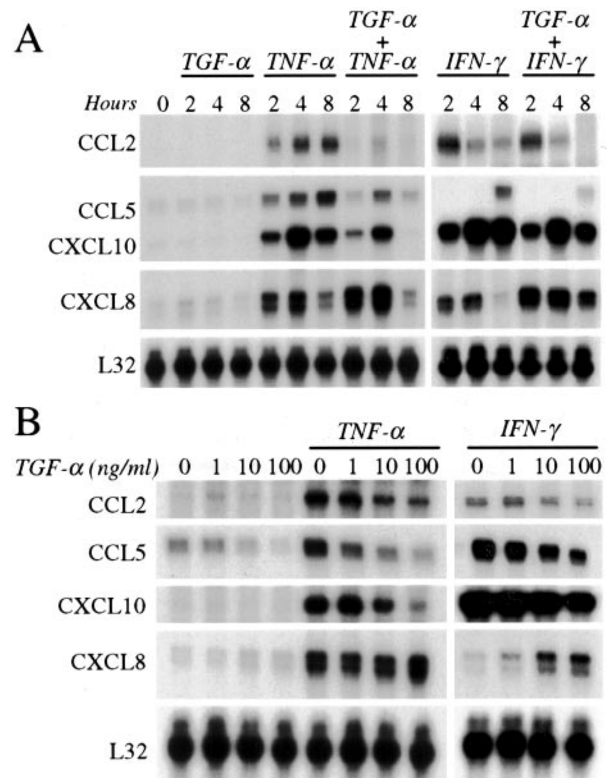


Figure 4. TGF-α down-regulates CCL2, CCL5, and CXCL10, but enhances CXCL8 expression. Time course (**A**) and dose dependence (**B**) of TGF-α effects on chemokine gene expression, as assessed by RNase protection assay. For the time course, TGF-α was used at a concentration of 50 ng/ml. The autoradiographies are representative of four independent experiments.

and cytokine-induced chemokine release, both at 0.2 and 2 μmol/L concentration (Figure 7; $P < 0.05$). Of note, the profile of TNF-α- or IFN-γ-induced chemokine release was similarly perturbed by 0.2 μmol/L PD168393 or 25 μmol/L Ilomastat (data not shown). Moreover, the effects of PD168393 were closely reproduced by a distinct EGFR tyrosine kinase inhibitor, PD153035,[2] both at the mRNA and protein level (data not shown). Neither the unrelated tyrphostin AG1296 (Figure 6 and Figure 7) nor 0.025% DMSO (not shown) could affect chemokine expression as compared to culture medium. Experiments performed on organ cultures of normal human skin confirmed that PD168393 (2 μmol/L) increased CXCL10 and reduced CXCL8 immunoreactivity in the epidermis after a 24-hour stimulation with IFN-γ (1000 units/ml) (Figure 8). Also TGF-α expression was impaired by PD168393, in agreement with previous evidence that TGF-α *de novo* synthesis is principally governed by TGF-α itself through autocrine EGFR stimulation.[26] In the intact epidermis of whole-skin explants, TUNEL$^+$ keratinocytes were not detected either with or without PD168393 treatment, whereas the mean number of TUNEL$^+$ keratinocytes in confluent chamber slides was 4.25 cells ± 3.50 in PD168393-treated cultures, as compared to 4.05 cells ± 2.50 in vehicle-treated controls (per 10^2 cells ± SD; *n* [microscopic fields per chamber slide] = 6), with no significant increase in keratinocyte apoptosis index ($P = 0.5$). Similar results were obtained in three independent experiments.

Mice Treated with an EGFR Inhibitor Show Enhanced Contact Hypersensitivity Response

We next investigated the impact of EGFR-linked signaling impairment in the expression of a prototypic T cell-mediated skin inflammation *in vivo*. Sensitized mice treated with a single application of PD168393 (10 μl of 4 mmol/L solution on each side of each ear) 30 minutes before hapten challenge exhibited a marked increase in contact

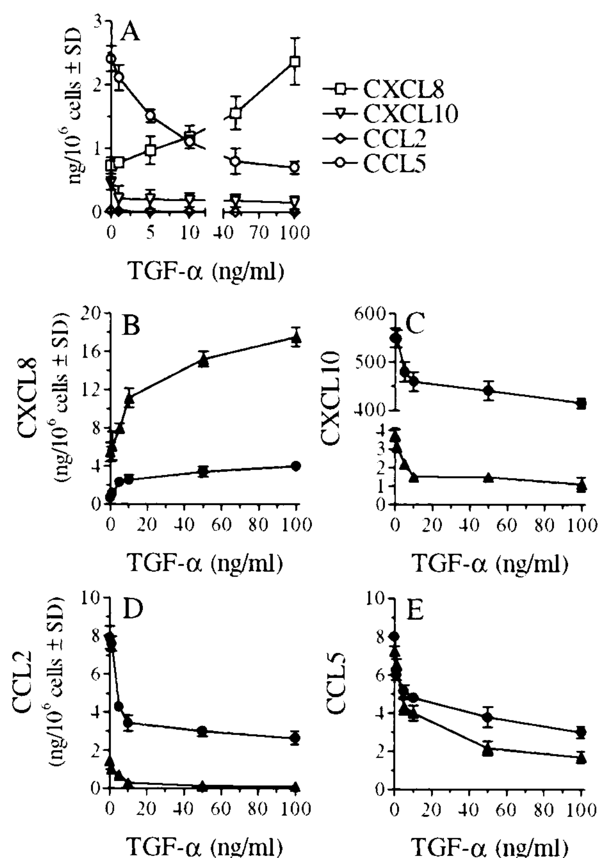


Figure 5. ELISA detection of chemokine levels after a 24-hour stimulation with escalating doses of TGF-α alone (**white symbols**), and together with TNF-α (▲) or IFN-γ (●) (**C**). Values represent ng/10^6 cells ($\pm$ SD) from six independent experiments.

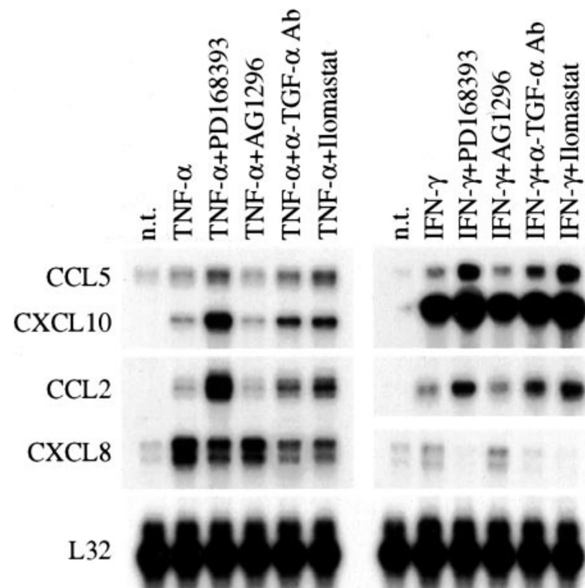


Figure 6. Impairment of EGFR signaling affects cytokine-induced chemokine expression with opposing effects compared to TGF-α, as observed by RNase protection assay. Cell cultures were pre-incubated with culture medium, PD168393, AG1296, neutralizing TGF-α Ab, or Ilomastat for 30 minutes, followed by stimulation with TNF-α for 2 hours (**left panels**) or IFN-γ for 8 hours (**right panels**). The autoradiographies are representative of four independent assays.

hypersensitivity response to DNFB compared to vehicle-treated groups at all time points ($P < 0.02$) (Figure 9A). Also, PD168393 significantly augmented the modest edema evoked by DNFB in unsensitized mice at 24 hours and 48 hours ($P < 0.02$) (Figure 9A), although painting of PD168393 alone did not affect ear swelling or histological features in respect to vehicle (Figure 9, B and C). Increased contact hypersensitivity following PD168393 treatment was associated with stronger edema and heavier inflammatory infiltrate as compared to vehicle-treated ears (Figure 9, D and E), as well as enhanced epidermal immunoreactivity for CXCL10, CCL2, and CCL5, but fainter TGF-α (Figure 10A). In the mouse skin treated with PD168393 we could observe a prominent reduction of epidermis-associated phospho-ERK1/2, which intensely stained keratinocyte nuclei in vehicle-treated sensitized skin (Figure 10A). Finally, CXCL8 was hardly detectable in the epidermis in all experimental conditions, in keeping with previous reports.[18,19] To identify the cell types more numerously recruited in PD168393-treated skin, we counted the number of infiltrating $CD8^+$, $CD4^+$, and $CD11b^+$ cells. We measured a marked increased number of $CD4^+$ T lymphocytes and $CD11b^+$ leukocytes, but not of $CD8^+$ T cells at the site of PD168393 application ($P < 0.02$ *vs*. vehicle-treated skin) (Figure 10B). Finally, no evidence of increased keratinocyte apoptosis could be observed after administration of the inhibitor. In PD168393-treated skin, 11.50 $\pm$ 2.35 $TUNEL^+$ cells were found in the epidermis compared to 10.25 $\pm$ 4.90 $TUNEL^+$ cells in the vehicle-treated skin (per 500 basal cells, n [microscopic fields per section] = 6, P = 0.5). Three independent experiments provided similar values.

Discussion

The EGFR-ligand system plays a fundamental role in the epithelial self-protection and repair to injury, not only in the skin but also in the respiratory and gastrointestinal tract.[27,28] In different animal models, EGFR activation accelerates epithelial cell regeneration while it dampens inflammation following mechanical, chemical, or ischemic tissue damage.[29,30] However, the specific impact of EGFR activation state on immune/inflammatory reactions has received very limited attention. Chronic T cell-mediated skin diseases share the common feature of increased epidermal thickness due to epidermal hyperplasia. We observed that EGFR and TGF-α were overexpressed not only in psoriasis,[8–10] but also in chronic atopic dermatitis and allergic contact dermatitis. Moreover, leukocytes showed strong TGF-α immunoreactivity in chronically inflamed skin. TNF-α and IFN-γ are released by T cells and other inflammatory cells in these disorders, and are potent inducers of EGFR and TGF-α gene expression in keratinocytes.[13,14] Of note, we found that both TNF-α and IFN-γ also promoted a rapid metalloproteinase-mediated TGF-α shedding from keratinocyte membrane, indicating that a "triple-membrane-passing signal mechanism"[31] contributed to early cytokine-induced EGFR transactivation. A variety of proinflammatory

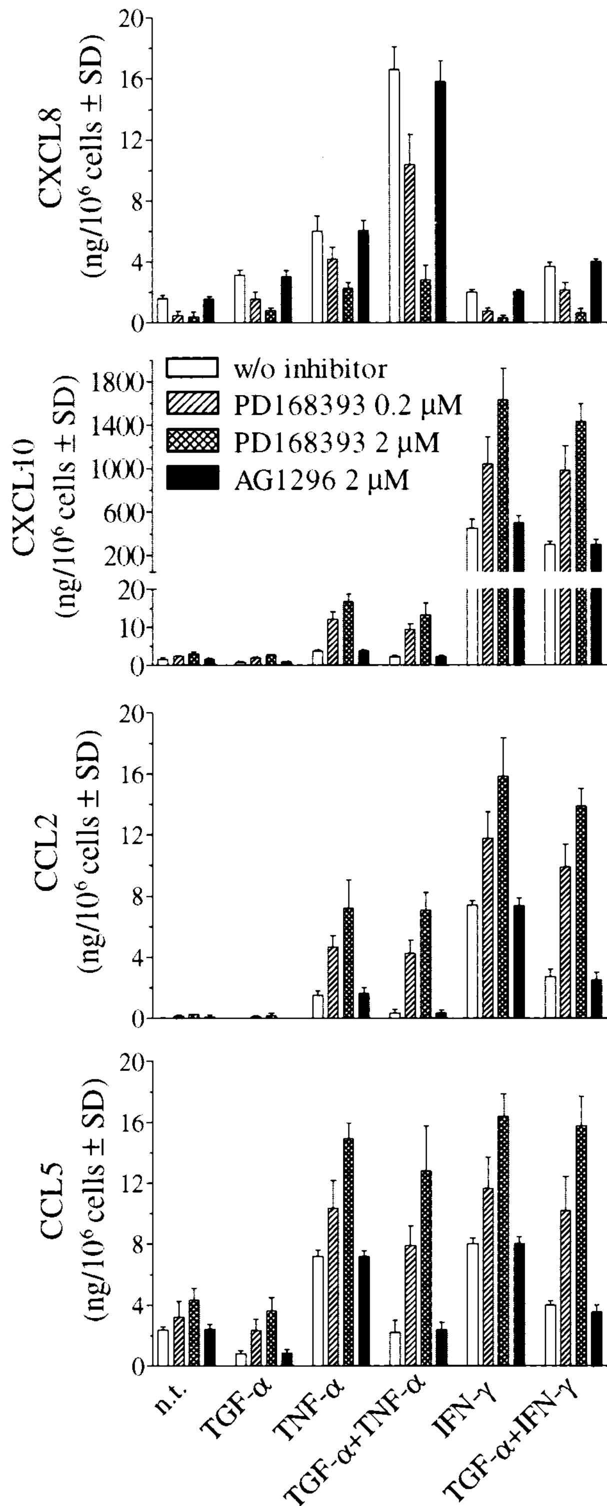


Figure 7. Chemokine release in the supernatants (24 hours) as assessed by ELISA. Values represent ng/10^6 cells (± SD) from six independent experiments. Cell cultures were pre-incubated with PD168393 or AG1296 for 30 minutes, followed by a further 24-hour treatment with TNF-α or IFN-γ, in the presence or not of TGF-α.

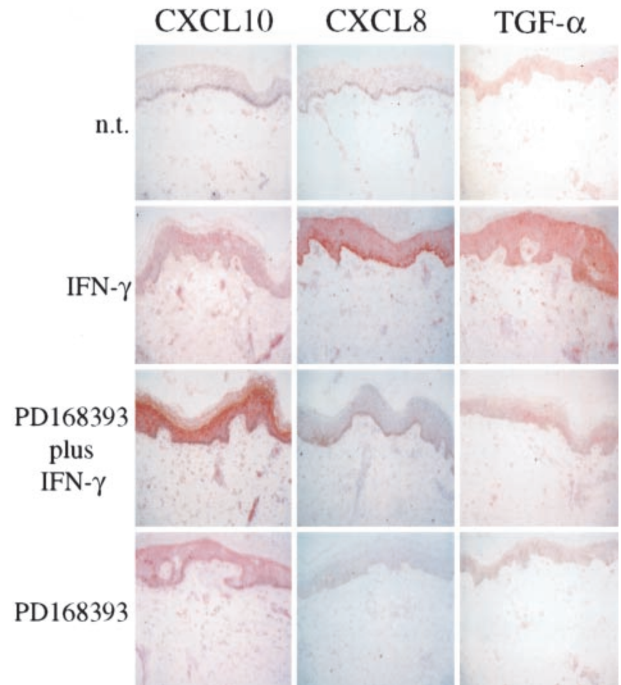
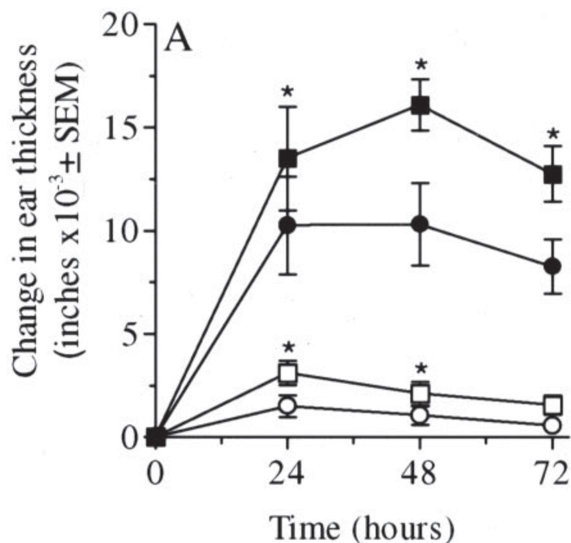


Figure 8. Immunohistochemistry of CXCL10, CXCL8, and TGF-α in cultures of full-thickness human skin explants. Skin explants were pre-incubated with vehicle alone (n.t.) or 2 μmol/L PD168393, and then stimulated or not with 1000 units/ml IFN-γ. Afterward, skin chops were dried by delicate contact with Whatman paper and snap-frozen. Representative results from three independent experiments. Original magnification, ×100.

stimulants are now known to affect epithelial cell functions through this mechanism.[23,31,32] In our system, TGF-α neutralization prevented cytokine-induced EGFR phosphorylation only partially, suggesting that other EGFR ligands could possibly participate in this process. Indeed, we collected some evidence that both TNF-α and IFN-γ triggered the early release of the mature forms of other EGFR ligands, including heparin binding EGF-like growth factor and amphiregulin, although at lower levels than TGF-α (data not shown). However, the observation that the broad-range metalloproteinase inhibitor Ilomastat was not able to prevent EGFR phoshorylation as effectively as the specific inhibitor of its receptor tyrosine kinase PD168393, possibly indicates that both cytokines may act on EGFR activation state also via EGFR ligand-independent, intracellular pathways. The contribution of a ligand-independent, redox-sensitive EGFR transactivation mechanism in the process of NFκB activation in response to TNF-α has been documented in a number of cell types, including the skin carcinoma A431 cells.[33]

In keratinocytes, the early activation of EGFR-driven signaling cascades by both TNF-α and IFN-γ appeared involved in the down-regulation of CCL2, CCL5, and CXCL10, suggesting that this loop is physiologically switched on to modulate the expression of critical proinflammatory mediators, and eventually to dampen skin inflammation. At the mRNA level, EGFR-mediated down-regulation of CCL2, CCL5, and CXCL10 expression varied substantially in its kinetics according to the cytokine used, appearing early (2 hours) with TNF-α, and more delayed (8 hours) with IFN-γ. CXCL10 expression is maximally induced by IFN-γ,[34] which causes the release of very high levels of this chemokine. Of note, TGF-α significantly impaired CXCL10 expression in response to IFN-γ both at the mRNA and protein level, although with less efficiency in comparison to what was observed with



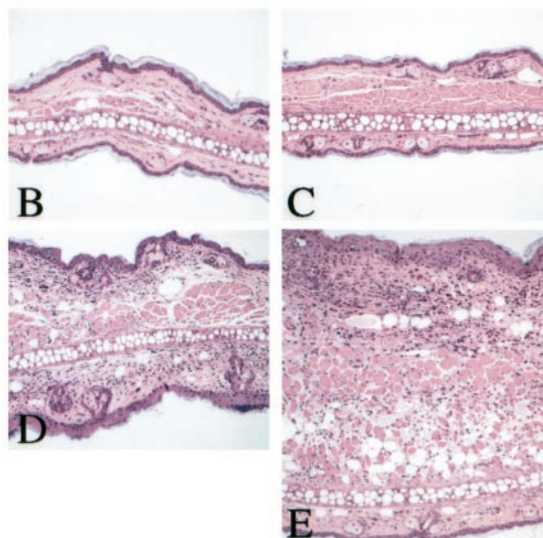
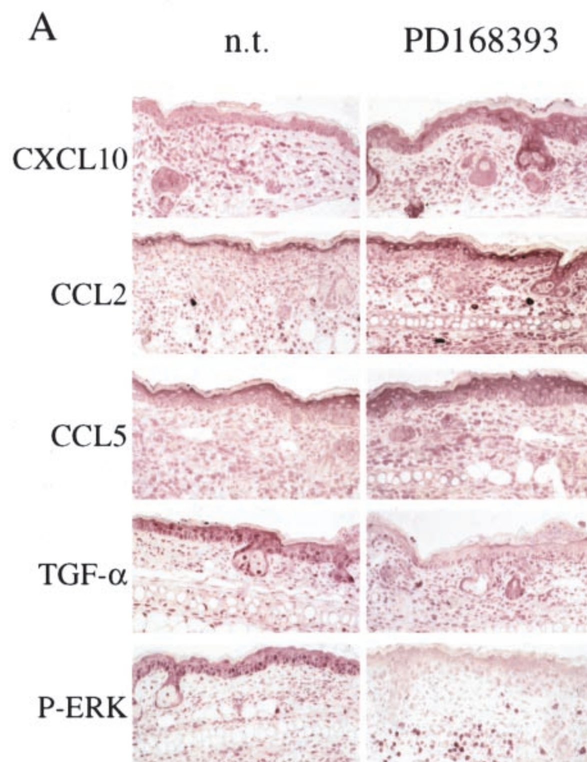


Figure 9. Treatment with PD168393 enhances contact hypersensitivity response. Contact hypersensitivity to DNFB was elicited on the ear skin of immunized BALB/c mice 30 minutes after local painting of 4 mmol/L PD168393 (■) or vehicle alone (10% DMSO in absolute ethanol) (●). DNFB challenge solution was also applied to non-sensitized mice pre-treated with PD168393 (□) or vehicle alone (○) (**A**). Data represent the mean of the mean changes in ear thickness at each data point from five different experiments. *, $P < 0.02$ *vs.* the respective vehicle-treated group. Histological features of ear skin samples collected 48 hours after painting unsensitized mice with vehicle alone (**B**) or PD168393 (**C**), or 48 hours after DNFB challenge of sensitized mice pre-treated with vehicle alone (**D**) or PD168393 (**E**). Ear sections were stained with hematoxylin & eosin. Magnification, ×100.

TNF-α. In contrast, an early EGFR-dependent potentiation of CXCL8 mRNA expression was evident with both cytokines. The increased CXCL8 expression following EGFR activation can be interpreted as a further mechanism of tissue repair, since CXCL8 is a well-recognized autocrine growth factor for epithelial cells.[35] Conversely, impairment of EGFR signaling led to opposite chemokine expression profile, with enhanced CCL2, CCL5, and CXCL10, but reduced CXCL8. Consistent with our *in vitro* findings, skin application of the EGFR blocker PD168393 before antigen challenge led to exaggerated contact hypersensitivity in the mouse, with increased expression of



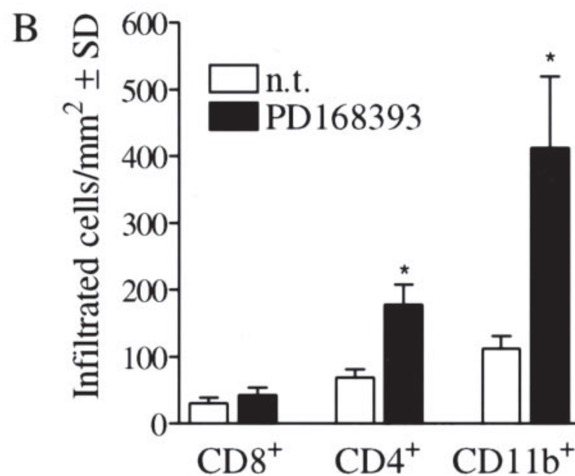


Figure 10. In sensitized mice, a single topical administration of PD168393 30 minutes before challenge enhances keratinocyte-associated expression of CXCL10, CCL2, and CCL5, whereas it reduces TGF-α and phospho-ERK1/2, as assessed by immunohistochemical analyses (48 hours after challenge) (**A**). In control animals (n.t.), the site of DNFB challenge was pre-treated with vehicle alone. Original magnification, ×100. Total number of CD8$^+$, CD4$^+$ and CD11b$^+$ leukocytes at the site of contact hypersensitivity 48 hours after treatment with vehicle alone (**white columns**) or PD168393 (**black columns**) and challenge with DNFB (**B**). Results represent the mean numbers (± SD) per mm^2 (n = 6 microscopic fields per section, and are representative of three independent experiments. *, $P < 0.02$ *vs.* vehicle-treated group (n.t.).

keratinocyte-associated CCL2, CCL5, and CXCL10 and higher numbers of CD4$^+$ T cells and CD11b$^+$ leukocytes. The latter comprise cells of the monocyte/macrophage lineage and activated polymorphonucleates, and are

strongly represented in the lesional skin at the time point of maximal contact hypersensitivity response.[18–20]

EGFR activation encompasses the involvement of a number of signaling cascades implicated in immediate-early gene expression.[36,37] In particular, EGFR control on cell cycling, motility, and rescue from apoptosis is largely mediated by the potent MEK1/2-ERK1/2 activation induced by EGFR ligands.[38–40] Indeed, we have observed that the selective abrogation of ERK1/2 performed by two chemically unrelated inhibitors of MEK1/2 activity closely reproduced the effects of the EGFR blocker PD168393 on chemokine expression in keratinocytes (Mascia F, Dattilo C, Girolomani G, Pastore S, manuscript in preparation). In keratinocyte cultures, PD168393 completely prevented the increase of ERK1/2 phosphorylation due to either TNF-α or IFN-γ stimulation. More importantly, we could observe a strong PD168393-associated reduction of phospho-ERK1/2 immunostaining in the epidermis of contact sensitized mice, 2 days after local painting of this EGFR receptor tyrosine kinase inhibitor. The mechanisms through which abrogation of MEK1/2-ERK1/2 signaling may induce increased CCL2, CCL5, and CXCL10 synthesis are unclear at present. Independent reports have proposed an inhibitory cross-talk between ERK1/2 and p38 MAPK, which could explain an increase in p38 MAPK-dependent gene expression following MEK1/2 blockade.[41,42] Experiments are now underway to evaluate the reciprocal role of the distinct MAPKs in chemokine expression.

EGFR appears to be strongly involved in the control of epithelial cell-triggered inflammation by down-regulating the expression of CCL2, CCL5, and CXCL10, which attract diverse leukocyte subsets. On the other hand, inhibition of EGFR signaling may be exploited to augment the inflammatory response initiated by immunological mechanisms. EGFR blockade by using either anti-EGFR antibodies or EGFR-specific tyrphostins has emerged as a very efficacious strategy against epithelial cell tumors,[43] an activity that has been attributed mainly to neutralization of the proliferative functions mediated by EGFR.[4–7] In addition, prolonged exposure to EGFR-specific tyrphostins has been shown to induce apoptosis in keratinocytes not only in culture systems[7] but also in the skin of patients undergoing anti-EGFR anti-cancer therapy.[43] Our results indicate that inhibition of EGFR signaling might exert anti-cancer activity by favoring the recruitment of inflammatory cells and thus a more pronounced anti-tumor immune response, along with down-regulation of CXCL8, which is an important growth factor for malignant epithelial cells.

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