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Genetic ablation of epidermal EGFR reveals the dynamic origin of adverse effects of anti-EGFR therapy

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Abstract

Cancer patients treated with anti-EGFR drugs often develop a dose-limiting pruritic rash of unknown etiology. The aims of our study were to define causal associations from a clinical study of cutaneous and systemic changes in patients treated with gefitinib and use these to develop and characterize a mouse model that recapitulates the human skin rash syndrome caused by anti-EGFR therapy. We examined the patients' plasma before and after treatment with gefitinib and documented changes in chemokines and leukocyte counts that were associated with the extent of rash or the presence of pruritus. We established a parallel mouse model by ablating EGFR in the epidermis. These mice developed skin lesions similar to the human rash. Prior to lesion development, we detected increased mRNA expression of chemokines in the skin associated with early infiltration of macrophages and mast cells and later infiltration of eosinophils, T cells and neutrophils. As the skin phenotype evolved, changes in blood counts and circulating chemokines reproduced those seen in the gefitinib-treated patients. Crossing the mutant mice with mice deficient for TNF α receptors, MyD88, NOS2, CCR2, T or B cells failed to reverse the skin phenotype. However, local depletion of macrophages provided partial resolution, suggesting that this model can identify targets that may be effective in preventing the troublesome and dose limiting skin response to anti-EGFR drugs. These results highlight the importance of EGFR signaling in maintaining skin immune-homeostasis and identify a macrophage contribution to a serious adverse consequence of cancer chemotherapy.

One Sentence Summary:

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Data and materials availability: MBP antibody was obtained with an MTA from Mayo Clinic, Arizona and can be requested from Dr. James Lee; mice can be requested from David Threadgill at North Carolina State University (EGFR flx) and Jose Jorcano at Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas in Madrid (K5Cre) or from us with their approval.

Epidermal EGFR ablation causes a localized cutaneous rash and a systemic inflammatory syndrome

Introduction

Epidermal Growth Factor Receptor (EGFR) activation through over-expression or mutation contributes to tumor development and progression in multiple tissues. Both small molecules and monoclonal antibodies designed to block EGFR activation have proven effective in cancers dependent on EGFR activity (1). However, all EGFR inhibitors produce skin and skin adnexa specific toxicity in patients (2). Starting as early as the first two weeks after therapy initiation, many patients develop the typical papulo-pustular follicular rash and frequently suffer from pruritus, skin xerosis, scaling of the epidermis and non-scarring alopecia. Depending on severity, the skin response can negatively affect the quality of life necessitating discontinuation of therapy or dose reduction that may interfere with treatment efficacy. An interesting feature of the rash is that its presence and severity often coincide with a positive tumor response suggesting a shared mechanism of action of the drug within the skin and the tumor compartments (3–5). For this reason, there is considerable interest in characterizing the pathogenesis of the rash beside the obvious necessity to minimize the negative effects on patients' quality of life.

Altered epithelial differentiation is characteristic of the skin of patients undergoing anti EGFR treatment and this has been interpreted as the main trigger of the inflammatory process (6). It is worth noting that in biopsies from lesional skin, the inflammatory response and the aberrant epithelial differentiation often coincide (7). Thus, it is not possible to identify the initial causative event solely from the examination of human lesional skin. Transgenic models with reduced or absent EGFR expression also display alterations in the differentiation of the follicular and interfollicular keratinocytes, but the relationship between these alterations and the inflammatory process is unknown (8–10).

In normal mice, acute pharmacological inhibition of EGFR or one of its downstream effector kinases ERK1/2, is associated with exacerbation of skin inflammatory responses and enhanced expression of chemokines in keratinocytes (11, 12). Interfering with EGFR signaling through blockade of growth factor shedding, preventing ligand-receptor interaction through antibody administration or blocking tyrosine kinase activity with small inhibitors all enhanced inflammation outcome in human keratinocytes *in vitro* and *in vivo* in patients (1, 11, 13). These data indicate that the blockade of EGFR is linked to an inflammation-prone behavior of keratinocytes. Clinical evidence suggests that what may start as a local cutaneous response has systemic repercussions, because biomarker analyses have documented changes in circulating cytokines and chemokines in treated patients (14–17). However, a temporal analysis documenting the dynamics of these local cutaneous and systemic changes in patients and a model system that can link the systemic effects of the anti-EGFR treatment to the skin response is lacking. Such an analysis is important to determine the primacy of the response, because clinical samples reveal only the fully formed characteristics of the skin lesions. Using data from a clinical trial of gefitinib treatment for ovarian cancer, we have been able to monitor the pattern of systemic changes in circulating

chemokines, cytokines and blood cells after initiation of treatment and relate this to onset of pruritus and rash. We constructed a mouse model of epidermal targeted ablation of EGFR and observed local inflammation, pruritus, altered keratinocyte differentiation, alopecia and systemic inflammation. We used this model to document the temporal changes involved in the developing phenotype and applied genetic and pharmacological methods for prevention.

Results

Inflammatory mediators and blood cell count changes in plasma of gefitinib-treated patients are associated with rash and pruritus

We examined changes in inflammatory mediators in plasma collected from ten patients participating in a gefitinib trial for epithelial ovarian cancer (NCT00049556) (18) to investigate a relationship between rash severity and pruritus. Because of the small number of patients, we looked for trends and significant differences. Although we saw increases in most circulating factors after treatment, the patients whose pretreatment levels of these inflammatory factors were the lowest tended to have greater rash and pruritus after treatment (Fig. 1A, B). Lower IL-18 concentrations before and after the first month of treatment with gefitinib were associated with higher grade rash (each $p=0.038$; Fig. 1A); lower CCL11 showed a similar trend at both times, but did not reach statistical significance (each $p=0.067$). Lower CCL11 concentrations both before and after one month of treatment with gefitinib, and lower IL-18 before treatment also showed a trend to associate with pruritus, which was not significant (each $p=0.095$; Fig. 1B). Lower concentrations of IL-1ra and CCL22 after the treatment were associated with higher grade rash ($p=0.0095$ and $p=0.019$ respectively; Fig. 1A), but only lower concentrations of IL-1ra showed a trend to associate with pruritus ($p=0.056$; Fig. 1B). In contrast, higher concentrations of CCL5 before the treatment were associated with trends toward pruritus ($p=0.064$; Fig. 1B). We analyzed the available blood cell counts for eight of the ten patients, looking for trends indicating association with the rash severity or presence of pruritus (Fig. 1C, D). The magnitude of increase in the percentage of granulocytes and decrease in the percentage of lymphocytes in the blood cell counts were associated with rash severity ($p=0.029$) and pruritus ($p=0.036$; Fig. 1C, D). Larger differential increases in the absolute number of platelets were also associated with pruritus ($p=0.036$; Fig. 1D) but no association of platelets was found with the rash. These data may indicate that the changes in circulating granulocytes, lymphocytes and platelets of individual patients can be related to the anti-EGFR treatment but do not discriminate between the effect of the drug on the tumor and a possible role of EGFR blockade in other target organs.

Epidermal ablation of EGFR in mice recapitulates the skin phenotype of anti-EGFR treated patients

To gain insight into the role of EGFR inhibition in the skin, we developed an epidermally-targeted mouse model of EGFR ablation by crossing Keratin5/promoter-driven Cre-Recombinase transgenics (19) with mice containing loxP sites flanking exon 3 of the EGFR gene (20). Double transgenics or epidermal EGFR deleted mice ($K5Cre^{WT/+}$ EGFR $flx^{+/+}$, indicated as KO) appeared phenotypically and histologically normal at birth and up to 5–6 days of age (Fig. 2A-C). After the first week of age, the skin of EGFR ablated mice

progressively developed the hallmarks of the skin lesions of patients treated with anti EGFR agents (Fig. 2D-L): infiltration of leukocytes and pruritus, scaly skin, neutrophilic pustules (red arrows), keratin plugs (yellow arrows), altered sebaceous glands (green arrows), pigment masses (black arrows) and hair follicle destruction. Thus, the local consequences of the genetic ablation of EGFR in the epidermis mimicked the adverse effects observed with anti-EGFR drugs in patients (6, 7).

The epidermal ablation of EGFR has systemic consequences

We measured the plasma levels of cytokines and chemokines in WT and KO littermates at two, three and twelve weeks of life to verify that our model could also reproduce the systemic imbalance of inflammatory mediators observed in patients after one month of gefitinib treatment. In mice with EGFR-ablated epidermis, we observed increases in plasma concentration of CCL11, CCL22, IL1ra as in human patients, as well as CCL2, CCL17, IL6 and IL17 (Fig. 3A, table S4). In contrast, plasma concentrations of the cytokine IL18 were not significantly different between WT and KO mice. Interestingly, blood cell counts reflected the pattern of gefitinib-treated patients: increased circulating neutrophils and platelets and decreased lymphocytes in KO mice versus normal littermates (Fig. 3B). With aging, systemic symptoms of myeloproliferative disease became evident with extramedullary hematopoiesis and enlarged spleen and lymph nodes (Fig. 3C).

Immune cell infiltration precedes changes in expression of epidermal differentiation markers

Skin barrier alterations are often associated with the establishment of local and systemic inflammatory conditions (21). Changes in epidermal differentiation markers and cornified envelope proteins are detected in the skin of patients with anti-EGFR rash, but there is not a common consensus if they are primary or secondary events (7). Immuno-blot analysis of total protein from skin of EGFR- ablated mice compared to WT littermates (Fig. S1A) showed an early increase in the levels of CD45 antigen (total leukocytes) and keratin 6 (7 days of age) that anticipated the up-regulation of keratin 1, keratin 10 and loricrin (2 weeks of age). Filaggrin total protein levels remained unchanged. In the absence of epidermal EGFR, the skin barrier formed normally, as shown by the dye permeation assay in newborn mice (Fig. S1B). Moreover, follicular and interfollicular keratinocytes maintained a normal expression of differentiation markers at day 7 when higher numbers of leukocytes (CD45 positive cells) were already invading the tissue as shown by immunohistochemistry (Fig. S2). At the same time, keratin 6 expression was slightly altered in the hair follicles of EGFR-ablated mice. At 21 days of age, keratin 6, keratin 1, loricrin and filaggrin were aberrantly distributed and overexpressed throughout the skin of KO mice (Fig. S2). Thus, changes in leukocyte population recruited in the EGFR-ablated skin anticipated changes in differentiation markers expression.

We further characterized which subsets of leukocytes contributed to the increase of the total CD45 population, starting when the KO mice had no apparent histological skin phenotype (3 days), to the later stages of life (20 weeks). Immunohistochemical analysis of skin tissue (Fig. 4A, B, table S4) indicated early infiltration of macrophages (detected with F4/80 antibody), mast cells (Toluidine Blue staining), followed by dermal T cells (CD3) and

eosinophils (Major Basic Protein, MBP) in KO skin. B cells (CD45R) were not differentially recruited or retained in the skin of the two genotypes. Infiltration of neutrophils occurred later and detectable myeloperoxidase levels were observed after 2 weeks (Fig. 4C, D). Neutrophils accumulated in numerous pustules were often associated with keratin plug formation and hair follicle destruction (Fig. 4D). Cultures of skin scrapings for bacteria and fungi were performed on multiple young and old WT and KO mice from two background strains (Table S1). Several bacterial species were detected in scrapings from the skin of young and old mice of both genotypes of both backgrounds without evidence of a pattern or predominant pathogen associated with skin phenotype. No fungi were isolated.

Local overexpression of cytokines and chemokines is an early event in the EGFR-ablated skin

We examined early changes in chemokines and their receptors to provide insights into the character of the skin infiltrates. Real time PCR of skin mRNA showed up-regulation of a subset of inflammatory mediators. In particular, Ccl5, Ccl11, Ccl2, Ccl22, Il1 β , Tnfa and Nos2 were expressed at higher level in EGFR-ablated skin within the first week of life (Fig. 5, table S4). Ccl5 was one of the earlier mediators to be upregulated in KO skin. The mRNA for Ccl5 receptors Ccr5, Ccr3 and Ccr1 (Fig. 5, table S4), were also elevated in KO skin starting at 3 days (Ccr5) and further increased at 1 week (Ccr3, Ccr5) and 2 weeks (Ccr1, Ccr3, Ccr5). Chemokine receptors Ccr1 and Ccr5 are expressed on macrophages, and we observed those in higher numbers in EGFR-ablated skin at 3 days (Fig. 4A, table S4). Interestingly, supernatants from keratinocytes isolated from EGFR-null mice (22) showed enhanced basal and cytokine (cocktail of IL1 β , IL6, IL17 and TNF α)-induced CCL5 protein expression when compared with supernatants from cells isolated from WT littermates (Fig. S3A, table S4). We tested these supernatants as chemotactic stimuli on the macrophage cell line RAW264.7. Supernatants from EGFR-null keratinocytes, both untreated and after stimulation with a cocktail of cytokines, showed a stronger chemotactic activity toward macrophages (Fig. S3B, C). In the presence of CCL5 neutralizing antibodies or a combination of CCL5 receptor inhibitors (DAPTA and UCB35625), the chemotactic activity induced by EGFR-null supernatants was significantly reduced ($p < 0.0001$, Fig. S3D). Besides Ccl5, another ligand promoting the infiltration of Ccr3 positive cells (mast cells and eosinophils) is Ccl11, which is expressed at high levels in 3-day-old KO skin (Fig. 5, table S4). Isolated keratinocytes do not release detectable levels of CCL11 in supernatants in vitro (Fig. S3A, table S4). We performed CCL11 immunohistochemistry combined with Toluidine Blue staining and observed high expression of this chemokine in dermal mast cells (Fig. S3E). While levels of both ccl2 and its cognate receptor ccr2 were highly expressed in KO skin starting from the first week of life, abundant levels of the chemokine ccl22 were not associated with enhanced expression of the corresponding receptor ccr4 (Fig. 5, table S4). Many of the inflammatory mediators described as elevated in the skin in the early phase were persistently elevated in the circulation of older KO mice (Fig. 3A and Fig. S4, table S4).

Macrophage recruitment and hair phenotype in KO mice is not altered by crosses with mice deficient for CCR2, MyD88, TNFR1/2, B and T cells and NOS2

In the attempt to neutralize or ameliorate the skin inflammatory phenotype as a potential therapeutic strategy, we crossed the epidermal targeted EGFR KO mice with several strains of mice defective in major pathways regulating immune signaling: TNFR1/R2^{-/-} (23), MyD-88^{-/-} (24), NOS2^{-/-} (25), CCR2^{-/-} (26) and Rag1^{-/-}. None of these regulatory mutants prevented or reversed the induction or maintenance of the skin phenotype in the EGFR KO mice (Fig. 6). The persistent infiltration of F4/80 macrophages recruited in the skin of the multiple mutants in absence of EGFR (Fig. 6) prompted us to determine the contribution of this cell type to the skin phenotype.

Macrophage depletion improves KO skin phenotype

Reducing macrophages by subcutaneous injection of liposomes containing clodronate (a bisphosphonate) (27) caused changes in the architecture of KO skin with more parallel growing hair follicles, less dermal cellularity and fewer keratin plugs (Fig 7A and Fig. S5A, B). Those effects are visible as a gradient radiating from the skin immediately adjacent to the injection site (red arrow Fig. S5B) to more distal areas. Interestingly, the reduction of F4/80 positive cells (Fig. 7B) was associated with a decrease of Keratin 1 levels in skin of clodronate treated KO mice. Clodronate also influenced WT skin by stimulating an anagen-like phenotype (elongated hair follicles, expanded dermal compartment), suggesting a role for macrophages in maintaining telogen (Fig. 7B).

Macrophage depletion alters the expression of inflammatory genes and reduces epithelial differentiation markers in KO mouse skin

RNA from clodronate-treated KO skin was compared to RNA from KO skin treated with PBS liposomes. Changes in RNA expression of inflammatory and differentiation markers were expressed as percent of change (clodronate/PBS) to globally visualize factors that may contribute to the amelioration of the phenotype (Fig. 8A, B) and factors that may prevent further normalization (Fig. 8C). The levels of chemokine receptors *ccr1*, *ccr3* and *ccr5*, chemokines like *ccl11* and cytokines like *Il17*, highly expressed in 3-week-old KO skin, were normalized or reduced in clodronate-treated animals (Fig. 8A). A similar trend was followed by epithelial differentiation markers (Fig. 8B). Clodronate-induced macrophage apoptosis also stimulated some unwanted adverse effects like enhanced presence of neutrophils (*Cxcr2* and its ligands *Cxcl1* and *Cxcl2*), higher expression of *Il6*, *Icam-1*, *Nos2* and super-induction of *Gcsf* expression (Fig. 8C) (28). Interestingly, clodronate treatment also reduced infiltrating mast cells (Fig. S5C). Although there were some quantitative differences, the responses to clodronate/liposomes were qualitatively similar in mice of both FVB/N and C57Bl/6 backgrounds.

Discussion

Although the clinical data reported here are limited by the small number of patients and thus, the lack of statistical power for some of the endpoints, the study has the advantages of monotherapy with gefitinib and both baseline and defined after-treatment measurements to correlate with the more dynamic mouse studies. Similarly, the advantage of the mouse

model is its ability to use epidermal targeting to distinguish primary skin events from events caused by systemic anti-EGFR therapy. The disadvantage of the mouse model is that it lacks the setting of neoplastic disease characteristic of chemotherapy. Mouse studies have shown that cutaneous EGFR activity is important for the proper development and cycling of the hair follicles (8–10) and for maintaining cutaneous immune-homeostasis (11, 12, 29). Thus, the adverse skin phenotype characteristic of cancer patients on EGFR inhibitory drugs is consistent with prior mouse studies. However, data illuminating the underlying pathways involved in either species are sparse. The human studies presented here suggest that individual variation in the baseline concentration of chemokines or cytokines, due to disease, therapy or individual genetic variation could potentially influence the severity of adverse skin reactions. Confirmation of this finding in a larger group of patients could be helpful in predicting those patients who are most likely to experience cutaneous reactions. However, the similarity in skin phenotype, hematological changes and common chemokine and cytokine alterations in these patients and KO mice suggest that the mouse model will be useful to further dissect the mechanism that causes the skin adverse effect of anti-EGFR drugs. Our genetic approaches to inhibit the skin phenotype with complementary knockout of a pro-inflammatory pathway, demonstrated the complexity of the inflammatory response that follows EGFR blockade. The neutralization of a single pathway was not sufficient to ameliorate the phenotype of KO mice. It is likely that a subset of inflammatory mediators act in concert during lesion formation and that infiltrating cells respond to multiple stimuli at the same time. The antagonists of both TNF α and IL1 pathways have been suggested to alleviate the skin lesions induced by an anti-EGFR antibody in mice (30). Crossing our KO mice with mice lacking MyD-88 or TNFR1/2 indicated that these pathways are not essential for the development and exacerbation of the lesions.

From the mouse data, it appears that macrophages and mast cells are early responders and actively contribute to the lesion formation. Because these cells are part of the infiltrate documented in the skin of treated patients (6, 31) they, or their attractants, could be clinical targets for prevention or resolution of the rash. Both bone osteoclasts and macrophages are targeted by bisphosphonates, a class of drugs approved for clinical use to prevent bone fractures in cancer patients (32). Future studies in patients treated with EGFR inhibitors could assess a possible improvement of the skin lesions upon bisphosphonate administration. Patients' lesions are currently managed with anti-inflammatory and immune-modulatory agents. Tetracyclines, administered to patients to prevent superinfection of the lesions, might function in a dual mode by modulating macrophage differentiation as shown in other systems (33). Another aspect illuminated by our model is the early induction of the inflammatory response followed by the development of the aberrant epithelial differentiation program. In patients, protein alterations involved in cornified envelope formation and the development of skin xerosis are not an immediate consequence of EGFR blockade but develop later in the areas of the papulo-pustular rash (7, 34). Thus, EGFR blockade impairs the proper differentiation of epithelial cells and skin appendages both in a cell autonomous way and via the modulation of the immune cell recruitment and activity. Interfering with this mechanism improves the adverse effects caused by EGFR ablation showing the crosstalk of the two mechanisms.

Recent observations highlight how the EGFR driven control of inflammatory homeostasis is not restricted to the skin compartment but also to colon epithelium. Diarrhea is another common adverse reaction to anti-EGFR therapy. EGFR expression is necessary to prevent excessive local inflammation in experimental colitis (35, 36). Moreover, a syndrome of inflammatory skin and bowel disease in children (13) has been linked to a loss of function mutation in ADAM 17, an EGFR ligand sheddase. Thus, the EGFR pathway may control inflammatory homeostasis in multiple lining epithelia requiring intense effort to determine how to counteract dysregulation of the pathway in non-target tissues with expanding use of EGFR inhibitors in the clinical setting.

Materials and Methods

Study Design

Patient sample study—Full details of the phase II gefitinib monotherapy trial (NCT00049556) in ovarian cancer patients have been reported previously (18). The protocol mandated monthly blood collection and punch biopsy of tumor tissue before the trial and at one month. Human samples were all blinded for assays reported here. For this analysis, patients had to have remaining matched aliquots of pre-treatment and one month plasma. These aliquots were provided with unique patient identifiers (UPI) after IRB approval for their use; cytokine results were unblinded and matched to clinical findings (toxicity and clinical laboratory results) by the clinical team, and the re-blinded dataset provided to the statistician. Samples had been isolated and frozen at -80°C within 4 hours of receipt from the patient and the aliquots provided had never been thawed. Patients are presented according to rash NCI Common Toxicity Criteria v2 with, grade 1 signifying limited acneiform or maculopapular rash and limited or no symptoms, and grade 2 representing more extensive rash and/or plaques, symptoms and/or requiring medical intervention. Table S2 provides limited demographic characteristics of the patients. All clinical investigation has been conducted according to Declaration of Helsinki principles. Human studies have been approved by the institutional review board of the Clinical Center for Cancer Research. Written informed consent was received from participants prior to inclusion in the study.

Mouse study—Mouse studies were performed under a protocol approved by the National Cancer Institute and the NIH Animal Care and Use Committee. The epidermal deletion of EGFR was achieved by crossing Keratin5/promoter-driven Cre-Recombinase (19) male transgenics with female mice in which loxP sites were inserted flanking exon 3 of the EGFR gene (20). Double transgenic $\text{K5Cre}^{\text{WT}/+}/\text{EGFR flx}^{+/+}$, indicated in the text and figures as KO, were compared with $\text{K5Cre}^{\text{WT}/\text{WT}}/\text{EGFR flx}^{+/+}$ or $\text{K5Cre}^{\text{WT}/+}/\text{EGFR flx}^{+/-}$ littermates indicated in the text and figures as WT. Mice were observed on a C57Bl/6 and FVB/N background. In the text all the data are from C57Bl/6 mice unless otherwise stated. No blinding was performed, due to the visibly different phenotype of EGFR-deficient animals and controls. For rescue experiments, we backcrossed our mice on a $\text{TNFR1/R2}^{-/-}$ (23), $\text{MyD-88}^{-/-}$ (24), $\text{NOS2}^{-/-}$ (25), $\text{CCR2}^{-/-}$ (26) or $\text{Rag1}^{-/-}$ (Jackson Lab) background. All of these strains were on C57Bl/6 background except the NOS2 mice which are FVB/N. Mice were genotyped using small distal tail samples. *Taq* polymerase mixes were used according to the manufacturer instructions, with Platinum PCR Supermix (Invitrogen) used for

EGFRlox3, NOS2, MyD88, and TNFR primers sets and *Taq* Master Mix (Qiagen) used for Cre, CCR2, and RAG1 sets. GAPDH was used as a housekeeping gene with Cre genotyping to ensure adequate genomic amplification occurred. PCR products were run using standard electrophoresis methods. The list of primers used is in supplementary material. Design of individual experiments is reported below.

Clodronate treatment

Clodronate liposomes and control PBS liposomes were purchased from Encapsula NanoSciences. 50 μ L of clodronate or PBS liposomes were injected subcutaneously in the upper and lower dorsum of mice every other day from age 6 days to 18 d. At age 21 d, dorsal skin sections spanning from the neck to the tail were taken for histological analysis as well as for skin RNA preparation.

Mouse peripheral blood analysis

The mice were anesthetized with Nembutal (Abbott). Blood for differential white blood cell counts was collected by intracardiac puncture using heparinized syringes to prevent coagulation. Total blood cell counts were performed at the Department of Laboratory Medicine at the NIH with an automated hematology analyzer (CELL-DYN® 3700, Abbott).

Myeloperoxidase assay (MPO)

Back whole skin samples were used for MPO assays as previously described (37). In brief, 6 mm punch biopsies taken from the back skin of mice at different ages were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), sonicated, and freeze-thawed 3 times, after which sonication was repeated. The suspension was centrifuged at 40,000 *g* for 15 minutes, and 10 μ L of supernatant was added to 290 μ L of potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL *o*-dianisidine dihydrochloride (SigmaAldrich) and 0.0005% hydrogen peroxide. Changes in OD were monitored at 460 nm at 25°C, over a 4-minute period.

Dye Permeation Assay

For toluidine blue staining, newborn mice were sacrificed and immediately dehydrated by sequential incubation in 25, 50, 75, and 100% methanol. After rehydration in PBS, they were incubated for 10 min in 0.01% toluidine blue and destained with PBS.

Multiplex biomarker analysis

Concentrations of cytokines and chemokines in plasma of patients or mice or cell supernatants were measured using Aushon protein arrays (Aushon BioSystems). Samples were incubated on array plates pre-spotted with capture antibodies specific for each protein biomarker. Plates were decanted and washed before cocktail of biotinylated detection antibodies was added to each well. After incubating with detection antibodies, plates were washed and incubated with streptavidin-horseradish peroxidase conjugate. Plates were again washed before adding a chemiluminescent substrate. The plates were immediately imaged using the Aushon CCD imaging system, and data were analyzed using Aushon software. Concentrations were interpolated from a standard curve.

Cell cultures

Mouse keratinocytes were isolated from WT and EGFR null mice (22) and cultured as extensively described in (38). 24 hours before stimulation, cells were starved with medium containing 0.2% serum and then treated with a cocktail of cytokines (IL-1 β , IL-6, IL-17 and TNF- α , all 5 ng/ml) for an additional 24 hours. RAW264.7 cells were obtained from ATCC and maintained in 8% serum DMEM. Subconfluent cultures were starved with 0.2% serum 24 hours before being harvested for the chemotactic assay.

Migration assay

Twenty-six microliters of keratinocyte medium were placed in the bottom of the well of a 48-well microchemotaxis plate (Neuroprobe). A framed filter (5 μ m) was then placed over the microplate and 50 μ L of the prepared cells (10,000) were placed on the top of the filter. For experiments involving inhibitors, cells were pre-treated for 30 minutes with the indicated inhibitor before being placed in the chamber wells. The plate was then incubated at 37°C in humidified air with 5% CO₂ for 45 min. After incubation, a clean smooth edge wiper was used to quickly wipe the non-migrated cells from the top of the filter. The filter was then fixed and stained according to Protocol Heme 3 Stain Set (Biochemical) and placed between 2 glass slides. Image J software was used to calculate densitometry on a scanned image of the filter. CCL5 neutralizing antibody and normal goat IgG (R&D) were used at 200 ng/ml, UCB35625 (or J-113863), an inhibitor of CCR1/3, was used at 5 μ m and DAPTA, an inhibitor of CCR5, at 50 nm (Tocris).

Immunohistochemical analysis

Tissues were fixed in 4% Neutral Buffered Formalin and embedded in paraffin. Antigen retrieval was performed using Target Retrieval Solution (Citrate pH 6, DAKO) for CD3, CD45R/B220, K1, K6, K10, filaggrin, loricrin and MPO, proteinase K (DAKO) for F4/80 and pepsin for MBP. Antigen retrieval was not performed for CD45. Slides were treated with 3% hydrogen peroxide for 15 min and blocked for a minimum of 30 minutes with Serum-Free Protein Block (DAKO). Sections were incubated for 1 hour at room temperature (CD3, CD45R/B220, F4/80, MBP, filaggrin, K1, K10, loricrin, K6) or overnight at 4°C (CD45, MPO) with primary antibodies. Slides were treated for 1 hour at room temperature with the corresponding biotinylated secondary antibody. Primary and secondary antibodies were diluted in Antibody Diluent with background reducing components (DAKO). Slides were then incubated for 20 minutes at room temperature with the Vector R.T.U. ABC system. PBS washes were performed between each step. ImmPACT DAB Vector was used for staining development. Counterstaining was performed with Hematoxylin QS Vector. All slides were dehydrated and mounted using VectaMount Vector. Primary antibodies and dilutions used: CD3 (AbD Serotec MCA1477, 1:50), CD45 (R&D Systems AG114, 1:200), F4/80 (eBioscience 14-4801-82, 1:100) K1 (Covance PRB-165P, 1:1000), K6 (Covance PRB-169P, 1:500), K10 (Covance MMS-159S 1:1000), filaggrin (Covance PRB-417P, 1:500), loricrin (Covance PRB-145P, 1:1000), MBP antibody (James Lee, Mayo-Rochester 1:100). For immunohistochemical analysis, skin sections of 3 mice for each time point were analyzed. Positive cells were counted in 10 independent fields per slide/mouse and averaged.

Toluidine blue

Slides were stained for mast cells by immersing the sections in Toluidine Blue Working Solution (50 mg Toluidine Blue O, 5 mL 70% ethanol, 45 mL 1% sodium chloride, pH 2.0–2.5) for 3 minutes. Slides were then washed with dH₂O.

Skin preparation and protein lysates

Mouse skin was isolated, snap-frozen in liquid nitrogen, and cold processed at 2,000 rpm in a Mikro-Dismembrator S (Sartorius). Protein lysates were obtained from powderized skin using cold RIPA (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) lysis buffer supplemented with 1 mM sodium orthovanadate, cocktail of anti-proteases (Roche complete, 1836145) and 1 mM sodium fluoride. Lysates were vortexed and incubated on ice for 10 minutes and then separated into two fractions. One fraction was centrifuged at 14,000 rpm for 15 minutes with supernatants quantified using the Bradford method. The second fraction was supplemented with Beta-mercaptoethanol (1.5 M) and SDS (5%) to optimize keratin and cornified envelope protein extraction.

Immunoblot analysis

Protein samples were analyzed according to standard immunoblotting protocols with 10% Tris/HCl gels and nitrocellulose membranes from BioRad. 15 µg protein was loaded to detect the differentiation markers, and 70 µg protein was loaded to detect CD45. All primary antibodies were diluted in 1% BSA in TBST and incubated overnight at 4°C. Primary antibodies were the same as those used for immunohistochemistry. Chemiluminescent substrates were purchased from Pierce Biotechnology.

Quantitative RT-PCR

Total RNA was isolated from powderized skin by using TRIzol Reagent (Invitrogen) and cleaned of genomic DNA contamination by RNeasy Mini Kit (Qiagen). For cDNA synthesis, 1 µg total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was diluted 1:100 and Real Time PCR was performed using BioRad iQ SYBR Green Supermix on myiQ and iQ5 PCR systems (BioRad). Relative expression was calculated using the C_T method with GAPDH as the housekeeping gene. Melt curves were analyzed from 55 to 95°C at 0.5°C increments to confirm specificity of amplification products. The list of primers used is in supplementary material.

Statistical analysis

For mRNA, cell counts, migration assays, MPO assays, mouse plasma assays and supernatants assay the data were tested for differences using a two-tailed two-sample t test. For these analyses, statistical significance was identified when $p \leq 0.05$ and single p values correspondent to * in the figures are reported in Table S3. For patient plasma analyses, the difference between groups of patients classified by their rash grade or presence or absence of pruritus was determined by a Wilcoxon rank-sum test. Given the small number of patients and the large number of exploratory tests performed on the parameters, $p < 0.01$ was considered statistically significant, while $0.01 < p < 0.10$ reflected trends in the differences. For this group, individual p values are discussed in the Results paragraph and in Fig. 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1.

Inflammatory mediators and blood cell count changes in gefitinib-treated patients. Patients with ovarian cancer were treated with gefitinib (500 mg daily for 28 days) and matched plasma samples were studied before treatment (T=0) and after 1 cycle (T=1 month). Patients were characterized as having rash grade 1 (limited acneiform or maculopapular rash, limited or no symptoms; blue line) or grade 2 (more extensive rash and/or plaques, symptoms and/or requiring medical intervention; red line), and complaining of pruritus (red) or not (blue). Cytokine and chemokine concentrations were measured by ELISA-based multiplex assay.

Associations of cytokine and chemokine concentrations (pg/ml) with rash (A) and pruritus (B) were analyzed. Blood cell count data were available for 8 of the 10 patients and were analyzed by calculating the percentage of granulocytes and lymphocytes present in the blood after one month of treatment minus the percentage before the treatment (Difference %) and correlating these differences with the extent of rash (C) and presence of pruritus (D). Changes in platelet count (absolute numbers k/ μ l) were reported as the number of platelets after treatment minus the number before treatment (D). P-values described in the text were calculated with a Wilcoxon rank sum test. Given the small number of patients and the large number of exploratory tests performed, $p < 0.01$ was considered statistically significant, while $0.01 < p < 0.10$ indicated trends.

Fig. 2. Consequences of epidermal ablation of EGFR in mice. Hematoxylin and eosin staining in skin sections and corresponding macroscopic phenotype of mice at different ages are shown at 3 days (A, B, C), 7 days (D, E, F), 21 days (G, H, I) and 4 months (J, K, L). Neutrophilic pustules (red arrows), keratin plugs (yellow arrows), sebaceous glands (green arrows) and pigment masses (black arrows) are highlighted in KO mouse skin sections and photographs (H, K, L). Objective 10X, scale bar 100 μ m. Images were captured by staining skin sections from $n > 3$ independent litters.

Fig. 3. Systemic consequences of EGFR ablation in the epidermis. (A) Multiplex assay of plasma concentration of chemokines and cytokines expressed in pg/ml for 2, 3 and 12-week-old WT (white bars) and KO (black bars) mice. Graphs show mean pg/ml $\pm$ SD (n=3 individual readings, each from a pool of plasma from 6–7 mice/genotype/time point, * p 0.05 by t test for KO compared to WT). Individual p values are listed in table S3. (B) Neutrophil (p=0.0001), platelet (p=0.0007) and lymphocyte (p=0.0008) mean counts $\pm$ SD in 3-week-old mice (blue WT and red KO, n=4 mice, by t test). (C) Spleen and lymph nodes from 2-month old WT and KO littermates are shown.

Fig. 4. Time course analysis of inflammatory infiltrate in EGFR-ablated mouse skin. (A, B) Immunohistochemical analysis of skin infiltrating cells was performed: F4/80 (macrophages), Toluidine blue (mast cells), CD3 (T cells in the dermis and gamma delta T cells in the epidermis), CD45R (B cells) and MBP (major basic protein/ eosinophils). The cells were counted in ten 200X fields at the indicated ages (A) for each mouse and expressed as the mean cell counts $\pm$ SD ($n=3$ mice, * $p < 0.05$ by t test, individual p values listed in table S3), WT white bars, KO black bars. (B) Representative 100X fields at day 7 in WT and KO mouse skin (scale bar 100 μ m). (C) Myeloperoxidase (MPO) assay of skin biopsies isolated from the back of WT (blue) and KO (red) mice. The enzyme activity is monitored as changes in OD (optical density) over time. MPO activity is not detectable earlier than 2 weeks in both WT and KO mice. $p=0.011$ at 2 weeks, $p=0.039$ at 3 weeks. $n=4$ mice/time point/genotype, p calculated by t test. (D) MPO staining in sections from back skin and bone

marrow of 3 month old WT and KO mice. Red arrows are pointing to microabscesses. Scale bars are 50 μ m.

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Fig.5. Time course of cytokine and chemokine changes in EGFR-ablated mouse skin. Sybr green based Real Time PCR analysis of cytokines, chemokines and chemokine receptors expressed over time after birth in mouse skin. Values are expressed as fold induction over WT values at 24 h, normalized to GAPDH. Mean expression $\pm$ SD, n=4 mice/genotype/time point, * p 0.05 by t test of KO (black) versus corresponding WT (white), individual p values listed in table S3.

Fig. 6.

Macrophage recruitment and hair phenotype in EGFR KO mice crossed with mice deficient for CCR2, B and T cells, MyD88, TNFR1/2, and NOS2. Representative F4/80 (left panels) and hematoxylin and eosin staining (right panels) in skin sections of TgK5Cre^(WT/+)/Egfr^(f/f)/CCR2^{-/-}, TgK5Cre^(WT/+)/Egfr^(f/f)/Rag1^{-/-}, TgK5Cre^(WT/+)/Egfr^(f/f)/MyD88^{-/-}, TgK5Cre^(WT/+)/Egfr^(f/f)/TNFR1/2^{-/-}, all on a C57Bl/6 background, TgK5Cre^(WT/+)/Egfr^(f/f)/NOS2^{-/-} on a FVB/N background. All mice were 21 days old. Objective 10X, scale

bar 100 μ m. Images were captured by staining skin sections from n = 3 independent litters per genotype.

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Fig.7. Macrophage depletion and changes in KO skin phenotype and differentiation marker expression. (A) Hematoxylin and eosin staining in skin sections from WT and KO littermates treated with PBS liposomes or clodronate liposomes. Objective 20X, scale bar 100 μ m. (B) Immunohistochemistry for F4/80 macrophages and Keratin 1 in mice treated as in (A). Objective 20X, scale bar 100 μ m. Images were captured by staining skin sections from n=3 independent litters treated with clodronate injections.

Fig. 8. Differential effect of macrophage depletion on mRNA in EGFR KO mouse skin. (A, B, C) Sybr green based Real Time PCR analysis of chemokines, chemokine receptors, inflammatory mediators and epithelial differentiation markers. Values are expressed as percent change in clodronate treated KO compared to PBS-treated KO. (A, B) Values below zero on the y axis represent a decrease in KO mRNA levels after treatment with clodronate, with -100% corresponding to WT levels. (C) Changes with positive values (above zero on the y axis) represent a further increase in the mRNA expression in KO skin upon clodronate

administration. No effect of clodronate is represented in values around zero. Experiments were performed on littermates from two litters of C57Bl/6 (black circles) and one litter of FVB/N mice (empty circles).

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