EGFR Signaling Blunts Allergen-Induced IL-6 Production and Th17 Responses in the Skin and Attenuates Development and Relapse of Atopic Dermatitis

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EGFR Signaling Blunts Allergen-Induced IL-6 Production and Th17 Responses in the Skin and Attenuates Development and Relapse of Atopic Dermatitis

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Despite the important role for epidermal growth factor (EGF) in epithelial homeostasis and wound healing, it has not been investigated in atopic dermatitis (AD). We used AD animal models to explore the role of EGF in AD. In an acute AD model, skin transepidermal water loss was significantly attenuated in EGF-treated mice. Blockade of EGFR signaling genetically or pharmacologically confirms a protective role for EGFR signaling in AD. In a chronic/relapsing AD model, EGF treatment of mice with established AD resulted in an attenuation of AD exacerbation (skin epithelial thickness, cutaneous inflammation, and total and allergen specific IgE) following cutaneous allergen rechallenge. EGF treatment did not alter expression of skin barrier junction proteins or antimicrobial peptides in the AD model. However, EGF treatment attenuated allergen-induced expression of IL-17A, CXCL1, and CXCL2 and neutrophil accumulation in AD skin following cutaneous allergen exposure. IL-17A production was decreased in the in vitro restimulated skin-draining lymph node cells from the EGF-treated mice. Similarly, IL-17A was increased in waved-2 mice skin following allergen exposure. Whereas IL-6 and IL-1β expression was attenuated in the skin of EGF-treated mice, EGF treatment also suppressed allergen-induced IL-6 production by keratinocytes. Given the central role of IL-6 in priming Th17 differentiation in the skin, this effect of EGF on keratinocytes may contribute to the protective roles for EGFR in AD pathogenesis. In conclusion, our study provides evidence for a previously unrecognized protective role for EGF in AD and a new role for EGF in modulating IL-17 responses in the skin. The Journal of Immunology, 2014, 192: 859–866.
No skin abnormalities have been detected in mice lacking EGF (34), but the roles of EGF and EGFR have not been evaluated in AD. In the current study, we evaluated the role of EGF and EGFR in AD development and subsequent relapse in experimental AD models. Our findings demonstrate that EGF attenuates AD symptom severity and downregulates IL-17 expression in the skin; this may be due to alteration of the cutaneous immune system caused by EGF- or EGFR-repressed IL-6 induction in keratinocytes.

Methods

Mice

Wild type of C57B1/6, BALB/c (Jackson Laboratory, Bar Harbor, ME) and waved-2 (C57B1/6 background, provided by Tim Le Cras, CCHMC) mice were kept in a specific pathogen–free environment. Procedures were performed in accordance with the ethical guidelines in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal care and Use committee approved by the Veterinary Service Department of the Cincinnati Children’s Hospital Medical Center Research foundation.

Experimental AD model and EGF and erlotinib treatment

Two different experimental models were used to study the development of AD and to study exacerbation. For the acute model described previously (35), mice were anesthetized with Isofluurane (IsFlo; Abbott Laboratories, North Chicago, IL) and then shaved with a hair clipper. Both groups of mice were injected with A. fumigatus conidia without a vehicle (Fig. 1A, B). Mice were euthanized. Paraffin-embedded tissues were cut into 5-μm sections and stained with H&E to assess skin thickness. Epidermal thickness was quantified using morphometric software (Metaphorom, Molecular Devices, Sunnyvale, CA), and an average of 10 random fields (>200) was measured for each sample. T cells and neutrophils were assessed by immunohistochemistry using anti-CD3, anti-CD4, anti-CD8, and Ly6G Abs (BioLegend). Positive cells counted under microscope for 10 to 15 random fields (original magnification ×200), and results were expressed as cells per field.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from homogenized mouse skin using RNeasy Microarray Tissue Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions, and DNase treatment was performed before the reverse transcription reaction with the Ready-To-Go T-Primed First-Stand Kit (Amershams Biosciences, Piscataway, NJ). Quantitative real-time PCR analysis of gene expression was done using LightCycler FastStart DNA master SYBR green I (Roche, San Francisco, CA). cDNA were amplified using the following primers: murine IL-4 (forward, 5′-CTTGAGGCGCTTCAAGGTCGTTTCG-3′, and reverse, 5′-CACTTGTGACATGTTCCTTAGGAC-3′); IFN-γ (forward, 5′-CAGCAACAGGAAGGCAAAAAAGG-3′, and reverse, 5′-TTCCGGTCTCCAGGCTTGAGT-3′); IL-17A (forward, 5′-ACTACCTCAAAGGGTTCACAG-3′, and reverse, 5′-AGAAATCCATGTTGGTCTCA-3′); IL-6 (forward, 5′-TGATGACTTTGACAGAAAAAC-3′, and reverse, 5′-ACCAGGGAAATTTCGACACAG-3′); CCL2 (forward, 5′-CAGGAGGCTCCTGACACAGAG-3′, and reverse, 5′-GCCATCCACACACACACAC-3′); CCL5 (forward, 5′-CCACTCAAGAATTGCTGCG-3′, and reverse, 5′-TCTCCTGTATCTTTGGACAC-3′); IL-22 (forward, 5′-GCAATCAGGTGCTCAGCTG-3′, and reverse, 5′-CGCTCCTGATCTCCTCTCCT-3′); IL-23/p19 (forward, 5′-GACCCCAAGGCTACTAAAGG-3′, and reverse, 5′-GCTCCCTTATGATGCTCA-3′, and TGF-β1 (forward, 5′-GCTCGACACAGGAGGACAAAT-3′, and reverse, 5′-GCTATCCCCTGTTTTCAC-3′). Gene expression was normalized to 36B4 and results were expressed as cells per field.

Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed using Prism 5. One-way ANOVA followed by Bonferroni’s multiple comparison tests was performed on all experiments unless stated otherwise. Significance was set at a p value of 0.05.

Results

EGFR signaling protects from impairment of skin barrier function following cutaneous allergen exposure

To evaluate the effects of EGF on the development of AD, we used the experimental AD model outlined in Fig. 1A. This model, described previously (35), yields key features of atopic dermatitis including erythema, pruritus, excoriation, and epidermal thickening, and is associated with increased expression of Th1, Th2, and Th17 cytokines in skin lesions (Supplemental Fig. 1). To determine the most optimal dose of EGF to use, we gave doses ranging 40–500 μg EGF i.p. to BALB/c mice and then analyzed skin RNA from treated mice for Egr1 expression (known to be induced downstream of EGF signal). Egr1 mRNA expression was maximally increased in the skin of mice treated with 40 μg after 30 min and decreased to baseline after 6 h (Supplemental Fig. 2A); thus, we used this EGF dose for our subsequent studies. Mice were treated with EGF 40 μg i.p. 1 d before the application of the first allergen patch, and then i.p. every other day throughout the rest of the protocol in Fig. 1A. Twenty-four hours after the last allergen patch was removed, TEWL was measured as an indicator of skin barrier function. EGF treatment resulted in attenuation of the AD phenotype that developed as evidenced by attenuated TEWL (Fig. 1B).

To explore the role of EGF in AD further, we used waved-2 mice, which have a spontaneous loss-of-function mutation in EGF and pharmacologic inhibition of EGF kinase activity with erlotinib, a specific EGFR inhibitor (37, 38). Based on our observations, we predicted that genetic (waved-2 mice) or pharmacologic inhibition (erlotinib) of EGFR would result in an enhanced AD phenotype.
Waved-2 mice, repeatedly exposed to cutaneous *A. fumigatus* according to the AD model outlined in Fig. 1A, developed more severe AD compared with strain matched C57BL/6 mice, as evidenced by increased TEWL (Fig. 1C).

BALB/c mice were treated with erlotinib (100 mg/kg) by gavage six times a week during the 3-wk *A. fumigatus* treatment period (Fig. 1A) while control animals were given the same volume of the gavage vehicle (0.5% wt/vol methylcellulose) as described previously (37, 39). Consistent with our previous findings, mice treated with erlotinib had higher TEWL (Fig. 1D) compared with saline-treated mice. These data collectively strongly support a protective role for EGFR in AD.

**EGF treatment attenuates AD exacerbation following cutaneous allergen rechallenge**

AD is a chronic disease characterized by frequent clinical relapses. We next investigated whether EGF treatment could block or attenuate subsequent relapse following AD development. For this purpose, we used a modified AD model (Fig. 2A) in which mice were treated with EGF after AD was established and then rechallenged.

**FIGURE 1.** EGFR signaling protects from skin barrier function impairment following cutaneous allergen exposure. (A) Overview of the acute AD sensitization protocol. Mice were exposed to *A. fumigatus* (ASP) or saline (SAL) patch three times as indicated, and TEWL was assessed 24 h after the last patch was removed. (B) BALB/c mice treated with EGF as indicated. (C) Waved-2 (WD2) mice and C57BL/6 wild type mice. (D) BALB/c mice treated with erlotinib as indicated. Each experiment was performed a minimum of 3 times. *n* = 4–8 mice/group. *p* < 0.05, ***p < 0.001.

**FIGURE 2.** EGF treatment attenuates AD exacerbation following cutaneous allergen rechallenge. (A) Overview of the chronic AD sensitization and rechallenge protocol. Twenty-four hours after the last patch was removed, the following were assessed: (B) TEWL; (C) H&E staining of patched skin; and (D) epidermal thickness of patched skin. Scale bars, 25 μm. Values are expressed as mean ± SEM (*n* = 6–10 mice/group). Each experiment was performed a minimum of three times. *p < 0.05, **p < 0.01, and ***p < 0.001.
with allergen to mimic a relapsing phase. As shown in Fig. 2B, compared with control treatment, the EGF-treated group displayed attenuated TEWL after the allergen re-exposure. Histologic analysis of patched skin revealed that A. fumigatus induced a 2–4-fold increase in epidermal thickness and increased inflammation, whereas the EGF treatment group showed attenuated epidermal thickness (Fig. 2C, 2D). There was significant local infiltration by CD3⁺ cells in skin of the allergen-exposed animals, which was significantly decreased in EGF-treated mice (Fig. 3A, 3B). Infiltrating CD4⁺ and CD8⁺ cells were identified and quantified by immunohistochemistry. As shown in Fig. 3C, both CD4⁺ and CD8⁺ infiltrated the skin following allergen exposure, but EGF treatment blunted infiltration of only the CD4⁺ cells.

Approximately 80% of AD patients have elevated levels of serum IgE and evidence of IgE against allergens, and there is a strong correlation with the disease severity (6). We measured serum total and A. fumigatus–specific IgE levels. As shown in Fig. 3D, the EGF-treated group demonstrated significantly lower levels of total and Asp-specific IgE.

Expression of EGFR and EGFR ligands in the skin of mice following the acute and chronic allergen exposure

Given the important role for the EGF pathway, we evaluated the expression levels of EGFR and EGFR ligands in skin tissue following acute and chronic allergen exposure. The effect of exogenous administration of EGF on the expression of EGFR and EGFR ligands was also evaluated. As shown in Supplemental Fig. 2B–D, TGF-α expression was not significantly changed by either A. fumigates exposure or exogenous EGF treatment. Epiregulin expression was decreased in A. fumigates–exposed mice and exogenous EGF treatment did not alter its expression. In contrast, HB-EGF and amphiregulin were induced following A. fumigates exposure, supporting that EGFR ligands are naturally produced in this AD model. Induction of HB-EGF and amphiregulin, was blunted by exogenous EGF treatment. This may be due to a negative feedback loop. EGF expression level was not affected by A. fumigates exposure or exogenous EGF treatment in the chronic model.

**EGF treatment results in attenuated skin IL-17A levels in allergen rechallenged mice**

To assess the nature of the immune response, we assessed mRNA expression of Th1, Th2, and Th17 in the skin beneath the patched area in this chronic model (Fig. 2A). Although there were no changes in the expression of Th1- and Th2-related cytokines (IL-4 and IFN-γ shown in Fig. 4A and TSLP, CCL17, CCL20 and CCL27, data not shown), IL-17A expression was dramatically decreased in the EGF-treated AD skin (Fig. 4A). Other members of the IL-17 family were also evaluated by quantitative PCR.
As shown in Supplemental Fig. 3, IL-17B and IL-17F, but not IL-17C or IL-17E, were induced following allergen exposure, and induction was unaffected by EGF treatment. Because tight junction abnormalities play an important role during AD development (4, 5), expression of claudin-1, claudin-4, zonulae occludens (ZO)-1, and filaggrin was also assessed in the inflammatory skin. Their expression levels were not affected by EGF treatment (Supplemental Fig. 4); this was confirmed by immunohistochemistry (data not shown). Because EGF is known to stimulate keratinocyte growth and differentiation (40–42), we also measured the keratinocyte proliferation in the skin tissues. Ki67 staining revealed no significant change in the positive keratinocyte percentage during the additional allergen challenge after EGF treatment in this model (data not shown).

We next examined expression of chemokines downstream of IL-17A including CXCL1, CXCL2, CXCL10, and CCL2 (Fig. 4B and data not shown). EGF treatment resulted in attenuated expression of these genes consistent with the observed decrease in IL-17A. Accordingly, neutrophil infiltration was also diminished in EGF-treated mice compared with controls (Fig. 4C, 4D).

To evaluate the nature of the local T cell response further, we assessed cytokine production in the skin-draining lymph nodes. As shown in Fig. 5, IL-17A was decreased in the supernatants taken from allergen-stimulated LN cells from EGF-treated mice compared with saline-treated mice. IL-4 and IFN-γ were unchanged (Fig. 5 and data not shown). These data suggest that less IL-17A is produced by LN cells of EGF-treated mice, and there may be fewer Th17 cells in the draining lymph nodes of EGF-treated mice. These findings are consistent with our previous data showing decreased expression of IL-17A and IL-17A–induced chemokines in the skin from EGF-treated AD mice.
EGFR signaling blunts IL-17A, but not IL-22 response in the AD model

To evaluate the effect of EGFR deficiency on IL-17A induction, we examined IL-17A expression in the patched skin of waved-2 mice. As shown in Fig. 1A, waved-2 mice, repeatedly exposed to cutaneous A. fumigatus developed more severe AD. The patched skin of waved-2 mice also had higher expression of IL-17A (Fig. 6A). Furthermore, assessment of cytokine production by skin-draining lymph nodes also revealed increased levels of IL-17A in the waved-2 mice (Fig. 6B). These data confirm a role for EGFR in the modulation of IL-17 responses. These data collectively support a protective role for EGF and EGFR in the development and exacerbation of AD, and they suggest that EGF attenuates Th17 responses.

In chronic lesions, increased expression of IL-22 has been reported (43). As shown in Fig. 6C, IL-22 expression was increased in chronic AD lesions in exposed mice (assessed 24 h after the fifth allergen patch was removed per the model depicted in Fig. 2A); however, we did not observe any effect of EGF on IL-22 skin mRNA levels at this same time point. Changes in the induction of Th17 responses by EGF may affect IL-22 in lesions that are more chronic.

Th17 cell differentiation environment altered by EGF signaling

Given the crucial role of IL-6, TGF-β, IL-23, and IL-1β in the differentiation and maintenance of Th17 cells (44, 45), we measured mRNA levels of these cytokines in the skin beneath the patched area of mice after the mice were subjected to the AD protocol in Fig. 2A. Although TGF-β1 and IL-23/p19 expression were not altered by EGF treatment, IL-6 and IL-1β expression were attenuated in the skin of EGFR-treated mice (Fig. 7A). IL-6 and IL-1β have been reported to be upregulated after IL-17 stimulation of macrophages and keratinocytes (46, 47). To determine whether EGF modified IL-6 expression by keratinocytes independent of IL-17A, we used a reductionist approach using the HaCat keratinocyte cell line (Fig. 7B) and human primary keratinocytes (Fig. 7C). Keratinocytes exposed to A. fumigatus demonstrated an induction in IL-6 expression, which was nearly abrogated by EGF pretreatment. EGF treatment even decreased IL-6 expression from baseline in unexposed keratinocytes (Fig. 7C). Expression of IL-1β was not altered by EGF treatment (data not shown).

Discussion

We demonstrate a protective role of EGFR signaling in an experimental model of AD. We also provide data demonstrating that EGFR ligands are naturally produced in AD skin and EGFR signaling attenuates IL-17A expression in the skin after cutaneous allergen exposure. Furthermore, our data reveal that EGF negatively regulates IL-6 expression by epidermal keratinocytes, and this might contribute to the observed suppressive effect of EGF on Th17 cell differentiation in the cutaneous immune system.

FIGURE 6. IL-17A levels are further enhanced in waved-2 mice compared with wild type mice following repeated cutaneous challenge. (A) RNA was isolated from patched skin of waved-2 (WD2) and wild type mice after the last patch of the acute AD model shown in Fig. 1A. Expression of IL-17A was determined. (B) Skin-draining LN cells isolated from the same mice were cultured and restimulated with allergen in vitro and IL-17A levels in the medium were assessed by ELISA. (C) Expression of IL-22 was determined by qPCR. Values are expressed as mean ± SEM. Each experiment was performed a minimum of three times. n = 4–6 mice/group. *p < 0.05, ***p < 0.001.

FIGURE 7. EGF treatment alters expression of IL-6 and IL-1β, but not TGF-β1 or IL-23/p19 in patched skin following cutaneous allergen exposure. RNA was isolated from patched skin from the mice from the chronic AD model shown in Fig. 2A and expression of IL-6, IL-1β, TGF-β1, and IL-23/p19 was determined by qPCR (A, n = 6–10 mice/group). RNA was isolated from cultured (B) HaCat cell and (C) primary human keratinocytes treated as indicated, and IL-6 expression was determined by qPCR (n = 3). Values are expressed as mean ± SEM. Each experiment was performed a minimum of three times. *p < 0.05, **p < 0.01, ***p < 0.001.
Prior studies regarding the role of EGFR signaling in the innate immune response have focused mainly on the acute wound healing process, including early recruitment of neutrophils into the wound site, an increase in the expression of antimicrobial proteins, and finally the re-establishment of the physical barrier (23, 24, 48). We assessed each of these possibilities as a potential mechanism for the observed actions of EGFR in our model, but we did not observe any changes following EGF treatment. We also did not observe a change in K667 staining in the skin of AD mice treated with EGF; however, it is possible that that there was an early acute effect of EGFR activation on keratinocyte growth, and we missed it because we are using a chronic model and only examined this at later time points. There was also no change in the expression of the antimicrobial protein, β2-defensin, following EGF treatment (data not shown). Disturbance of the epithelial barrier is a common feature of AD development (4, 5, 7). A defect in the barrier has been argued to favor the penetration or reactivity to microbes and allergens into the dermis. Expression of tight junction proteins including occludin, claudin-1, claudin-4, claudin-23, ZO-1, E-cadherin, and filaggrin (Supplemental Fig. 4 and data not shown) were unaffected by EGFR signaling in the skin in our model.

EGF treatment had a striking and unexpected downregulatory effect on the expression of IL-17A in the skin. These data suggest that EGFR may have a previously unrecognized immunomodulatory role in AD development through regulating IL-17 levels in the inflammatory skin tissue. Interestingly, a common adverse effect of long-term use of EGFR tyrosine kinase inhibitors in patients with cancer is a cutaneous inflammatory rash characterized histologically by a moderate-to-severe inflammatory reaction dominated by neutrophils (49, 50). Studies of these patients have revealed that EGFR activation potently downregulates expression of chemokines, which promotes the migration of neutrophils and T lymphocytes into the skin, including CXCL10, CCL5, and CCL2 from keratinocytes. Similarly, our data revealed that expression of these chemokines is decreased in the skin tissues following EGF treatment. Based on our data, we hypothesize that EGFR inhibitors used in cancer treatment regimens leads to increased IL-17 levels in the skin as we observed in the wavel-2 mice (Fig. 6A, 6B). IL-17 is known to stimulate keratinocyte to secret CXCL10, CCL5, and CCL2 (13, 51) in vivo and in vitro; this is a likely mechanism for the observed skin rash.

The mechanism by which Th17 cells differentiate from naive CD4 T cells has been the subject of much attention, because these cells have been shown to involved in a variety of immune diseases. The differentiation of Th17 cells in vivo and in many in vitro culture conditions requires the presence of IL-6 and several other factors (52–54). IL-6 can induce expression of IL-21 and IL-23 receptor in naive T cells, and these factors act with IL-1β and TGF-β during the Th17 cell priming process (55). Recent data suggest that Th17 cell lineage commitment has differential cytokine requirements depending on the site of priming (56). The priming microenvironment for Th17 cells in the skin and mucosal tissues required IL-6, whereas IL-6 was not essential for Th17 cell priming in the spleen. The investigators found that 20% of the DCs in the skin and other mucosal tissues were CD103+ and made high quantities of TGF-β and retinoic acid, both of which have been shown to suppress Th17 cell differentiation. IL-6 may be required to overcome these suppressive effects (56). In our animal model, IL-6 expression by keratinocytes was induced by A. fumigatus exposure and this induction was abrogated by EGF treatment in vivo and in vitro (Fig. 7). Altered IL-6 expression in the skin microenvironment could affect Th17 cell priming in this model.

In patients with AD, a significant increase in skin IL-6 expression has been observed (57). Herein, we find that EGF treatment results in decreased IL-17A expression in the skin and IL-6 expression is also decreased in the inflammatory skin tissue after EGF treatment. In primary human keratinocytes and HaCat keratinocytes, EGF treatment attenuated IL-6 expression at baseline and after allergen exposure. In vivo, EGF can decrease IL-6 production in the skin environment, resulting in a diminished local Th17 response. IL-6 receptor-blocking mAb (tocilizumab) has been used to treat three patients with severe AD; it was associated with relief in clinical symptoms, although it was associated with bacterial superinfection (58). EGF has been successfully used topically to promote wound healing (20), and our data suggest that topical EGF may be beneficial in preventing AD exacerbation. However, despite solid demonstrations of efficacy of topical EGF in experimental conditions and clinical trials (20), there are obvious unresolved concerns about the potential cancer-enhancing properties of exogously administered EGF. In our studies, EGF is protective in AD, and we have found a novel role for EGF in modulating IL-17 responses. Dissecting and delineating this previously unrecognized EGF pathway will aid in the development of therapies that specifically target this immunomodulatory pathway without affecting cellular proliferation. These findings may have important implications for other inflammatory skin disorders as well.

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