Cancer Therapy: Preclinical

Epidermal Growth Factor Receptor Inhibition Augments the Expression of MHC Class I and II Genes

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Abstract

**Purpose:** Diverse immune-related effects occur with the use of epidermal growth factor receptor inhibitors (EGFRI). In addition to the cutaneous inflammation induced by EGFRI, these agents have been associated with the exacerbation of autoimmune skin disease and contact hypersensitivity, antiviral effects, and fatal alveolar damage in the setting of lung transplantation. Because EGFR ligands can modulate MHC class I (MHCI) and II (MHCII) molecule expression, we hypothesized that some of the immune-related effects of EGFRI are due to direct effects on the expression of MHCI and/or MHCII molecules.

**Experimental Design:** Primary human keratinocytes and a malignant keratinocyte cell line (A431) were treated with EGFRI alone or prior to IFN-γ, a potent inducer of MHCI and MHCII molecule expression. CIITA, MHCI, and MHCII RNA expression was measured using quantitative real-time reverse transcriptase PCR, and cell surface MHCI and MHCII protein expression was measured using flow cytometry. Skin biopsies from patients were analyzed for MHCI and MHCII protein expression before and during therapy with an EGFRI using immunohistochemistry.

**Results:** Both EGFR tyrosine kinase inhibitors and ligand-blocking antibodies (cetuximab) augmented the induction of MHCI and MHCII molecules by IFN-γ in primary and malignant human keratinocytes. Unexpectedly, the increase in MHCI protein expression did not require the presence of IFN-γ. Consistent with these in vitro findings, skin biopsies from cancer patients exhibited increased epidermal MHCI protein expression during therapy with an EGFRI as well as increases in MHCI and MHCII molecule RNA.

**Conclusions:** These studies suggest that EGFRI may influence immune/inflammatory responses by directly modulating MHC expression. Clin Cancer Res; 17(13); 4400–13. ©2011 AACR.

Introduction

Aberrant expression or activity of the epidermal growth factor receptor (EGFR) has been linked to a variety of human cancers (1). The importance of the EGFR pathway in human cancer is underscored by the development and clinical use of humanized antibodies and small molecule EGFR tyrosine kinase inhibitors (TKI) that block EGFR activation (2). EGFR inhibitors (EGFRI) were initially developed to block EGFR-dependent prosurvival and mitogenic signals within tumor cells. Regardless of their mechanism, the use of EGFRI in patients is associated with proinflammatory side effects suggesting that EGFR signaling modulates the expression of relevant immunoregulatory genes.

The side effects associated with EGFRI with an apparent immune-related mechanism include the development of skin inflammation (3), exacerbation of autoimmune disease (4), and the development of acute lung injury in the setting of lung transplantation (5). The development of skin inflammation can cause significant morbidity and can be dose limiting thereby interfering with antineoplastic therapy. Despite this, the development of EGFR-induced skin inflammation is associated with prolonged survival and suggests a relationship between EGFR-induced inflammation and antitumor effects (6, 7). Defining how the EGFR pathway impacts immune responses is therefore important because it will foster new approaches to attenuate EGFR side effects and may help identify novel applications for their use.

In this report, we show a novel activity of EGFRI that may help explain the proinflammatory side effects of these medications. We report that both EGFR TKIs and cetuximab potentiate the induction of MHC class I (MHCI) and II (MHCII) molecules in response to IFN-γ. The increase in MHC molecule expression is associated with an increase in...
Translational Relevance

The development of cutaneous inflammation in response to epidermal growth factor receptor inhibitors (EGFRIs) is associated with prolonged survival in patients with advanced cancer. To gain insight into how EGFRIs alter immune responses, we explored the impact of EGFRIs on the expression of MHC class I (MHCI) and II (MHCII) molecules. We found that EGFRIs augment the expression of MHCI and MHCII molecules on normal and malignant human keratinocytes. Consistent with these findings, skin biopsies from patients on EGFRi therapy exhibited an increase in epidermal MHCI protein expression and increases in MHCI and MHCII molecule RNA. These findings may help explain the diverse immune-related effects of EGFRIs. By modulating the expression of MHC molecules, EGFRIs may disturb cutaneous immune homeostasis and alter antitumor immune responses. EGFRIs may be useful in clinical settings where augmented MHC molecule expression is desirable, and conversely, may exacerbate MHC-dependent immune/inflammatory processes.

Reagents and treatment of cells

Human IFN-γ (Peprotech) was resuspended in DMEM (200 μg/mL) and stored at −80°C. For the treatment of cells, each unit of IFN-γ as indicated in the text represents 50 pg/ml of IFN-γ. PD168393 and AG1295 (EMD Biochemicals) were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mmol/L and cells were treated at a final concentration of 1 μmol/L by adding 1 μL of stock PD168393 to 10 mL of media. AG1478 (Cayman Chemical) was dissolved in DMSO to a concentration of 1.58 mmol/L and cells were treated at a final concentration of 1 μmol/L by similarly adding stock AG1478 to 10 mL of media. EGF (Promega) was resuspended in basal keratinocyte media to 100 μg/mL and used at a final concentration of 10 μg/mL. All of the above reagents were stored at −80°C in aliquots until use. Therapeutic antibodies were prepared for human infusion as recommended by the manufacturer and were donated by the Winship Cancer Institute of Emory University as follows: bevacizumab (25 mg/mL; Genetech), cetuximab (2 mg/mL; ImClone), infliximab (10 mg/mL; Centocor Ortho Biotech), and trastuzumab (21 mg/mL; Genetech). Bevacizumab, infliximab, and trastuzumab were diluted to 2 mg/mL in sterile PBS and stored at 4°C, as was cetuximab. All therapeutic antibodies were used at a final concentration of 2 μg/mL for in vitro studies.

RNA isolation, RT reactions, and real-time PCR

RNA isolation and reverse transcription were carried out as previously described (10, 11). Quantitative real-time PCR was carried out using a CFX96 thermal cycler and measuring SYBR green incorporation into double-stranded amplicons. Reactions were carried out in 25 μL volumes containing forward and reverse primers at a final concentration of 100 nmol/L. Primer sequences for CIITA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: CIITA forward 5′-CTGAAGGATGTGGAAGACCTGG-3′, CIITA reverse 5′-GTCCCCGACTTGTCTTAC-TCC-3′; and GAPDH forward 5′-GAAGGTGAAGGTC-TGTAAGAC-3′, GAPDH reverse 5′-GAAGATGGTAGATGG-GATTCC-3′.

Flow cytometry

Following the treatments indicated in the text, cells were trypsinized, washed in fluorescence-activated cell-sorting (FACS) buffer (2 mmol/L EDTA, 1% bovine serum albumin in PBS), pelleted by centrifugation. Cell pellets were then resuspended in 20 to 40 μL of anti-HLA-DR (clone L203, catalogue FAB4869P, R&D Systems) or anti-HLA-A, HLA-B, and HLA-C antibodies conjugated to phycoerythrin (clone G46-2.6, catalogue 557349, Becton Dickinson) or
an isotype control antibody also conjugated to phycoerythrin (catalogue 554680, Becton Dickinson). Cells were incubated on ice for 30 minutes, washed 3 times, and resuspended in 0.5 mL of FACS buffer containing 0.5% paraformaldehyde. Surface HLA-DR or HLA-A, HLA-B, and HLA-C expression was measured using a FACScalibur (BD Biosciences) flow cytometer and MHC expression analyzed on ungated cells using FlowJo software (Tree Star).

Human subjects
The use of human subjects was conducted according to the Declaration of Helsinki principles and was approved by the Emory University Institutional Review Board. Written informed consent was obtained from all patients prior to enrollment in the study. Prior to initiation of therapy with an EGFRI, skin biopsies (4 mm) were conducted on the trunk (chest or back) and medial upper extremity. Three to 4 weeks into therapy with either cetuximab or erlotinib, subjects were biopsied on the trunk (clinically inflamed skin if present and noninflamed skin if not) and noninflamed skin from the upper medial arm.

Immunohistochemistry
Immunohistochemistry (IHC) was carried out at the Winship Cancer Institute Pathology Core Laboratory as previously reported (10). All skin biopsy sections were stained for either MHCI or MHCII at the same time and under the same conditions. The following antibodies were used to examine MHC protein expression: MHCI (Abcam; clone EMR8-5, 1:800) and MHCII (Abcam; clone KU1/05, 1:150). Epidermal MHCI staining intensity between biopsies from the same patient and anatomic site (before vs. after treatment with an EGFRI, skin biopsies (4 mm) were conducted on the trunk (clinically inflamed skin if present and noninflamed skin if not) and noninflamed skin from the upper medial arm.

Quantification of MHCI IHC
Glass slides containing skin biopsies stained with MHCI antibodies as described in the text were scanned at 40× magnification using a Nanozoomer 2.0HT slide scanner (Hamamatsu) at the Emory Pathology Core Laboratory. Scanned images were analyzed using Aperio ImageScope Positive Pixel Count (version 9) software (Aperio). The threshold settings used were intensity weak positive (Iwp = 200), intensity positive (Ip = 155), and intensity strong positive (Isp = 100). A representative region of the epidermis (~1 mm in length) from each slide was selected for analysis using the pen tool. To generate a numerical score for MHCI staining for the selected epidermal regions, the pixel values for the number of weak positive, positive, and strong positive were combined and divided by the total number of pixels analyzed (positive + negative). For statistical analysis, scores before EGFR therapy from each site were averaged and compared with the averages of those from the same site during therapy using a paired Student’s t test (InStat 3, GraphPad Software Inc.).

Statistical analysis
All statistics were conducted using InStat (GraphPad Software Inc.). Either a paired Student’s t test or an ANOVA was conducted as indicated in the text. Tests for Gaussian (normal) distribution were conducted using the Kolmogorov–Smirnov test.

Results
PD168393 augments the induction of CIITA and HLA-DR by IFN-γ
EGFR ligands such as TGF-α are known to be released in response to IFN-γ through a protease-dependent mechanism and activate the EGFR (12, 13). These same ligands can attenuate the induction of MHCI genes by IFN-γ (14). On the basis of this, we hypothesized that the transactivation of the EGFR following IFN-γ exposure might establish a negative feedback loop and thereby limit MHCII molecule expression which is tightly regulated (15). CIITA plays a critical role in controlling the expression of MHCII molecules and is itself tightly regulated (15, 16). Therefore, we first examined the impact of EGFR inhibition on CIITA induction. To this end, we pretreated A431 malignant keratinocytes with the irreversible EGFR PD168393 prior to treatment with IFN-γ. A431 cells have been used extensively to define the mechanisms responsible for the induction of CIITA and MHCI genes (17). PD168393 irreversibly binds to the ATP-binding pocket of the EGFR (18) and was selected because it has been shown to alter immune responses when applied topically to murine skin (13, 19). Following PD168393 pretreatment, A431 cells were incubated with increasing concentrations of IFN-γ and steady-state mRNA levels of CIITA were analyzed using quantitative real-time reverse transcriptase PCR (RT-PCR) at 8, 24, and 48 hours following IFN-γ treatment. These time points were selected because the induction of CIITA by IFN-γ involves the recruitment of transacting factors and chromatin-remodeling enzymes to the promoter of CIITA and peak steady-state CIITA mRNA levels have been shown to occur several hours after IFN-γ treatment (17). The doses of IFN-γ used in these studies ranged from 1 to 20 units/mL. These doses were selected because they approximate serum levels of IFN-γ in humans (20). In addition, we reasoned that at higher doses of IFN-γ the expression of CIITA, and thus its target genes, might be maximal and thus too high to detect any augmentation by EGFR inhibition. As shown in Figure 1A, pretreatment of A431 cells with PD168393 augmented CIITA mRNA levels above those of control cells that were pretreated with vehicle (DMSO) yet treated with the same concentration of IFN-γ. The effect was most noticeable at the 24 and 48 hours time points with statistically significant differences at the 48-hour time point (Fig. 1A). CIITA protein levels were also elevated by pretreatment with PD168393 as shown in Figure 1B.
To determine whether the aforementioned changes in CIITA would impact MHCII cell surface protein expression, A431 cells were treated as above, stained with anti-HLA-DR antibodies, and analyzed by flow cytometry. Pretreatment of A431 cells with PD168393 augmented the IFN-γ–induced cell surface expression of HLA-DR (Fig. 1C and D). Although PD168393 augmented the expression of HLA-DR at all concentrations tested, the effect was most robust at lower concentrations of IFN-γ with statistically significant increases seen at 48 hours using 5 or 1 U/mL of IFN-γ. Indeed, in contrast to lower doses of IFN-γ, at higher doses of IFN-γ (500 U/mL), we saw only a minimal effect of PD168393 on cell surface HLA-DR expression (Supplementary Fig. S1). Thus, in A431 cells, PD168393 potentiates the induction of CIITA and HLA-DR by IFN-γ. Analysis of other cancer cell lines revealed a similar response in SQCCY1 carcinoma cells which are derived from an oral squamous cell carcinoma (SSC; Supplementary Fig. S2; ref. 21). In contrast, pretreatment with PD168393 had no effect on the expression of cell surface HLA-DR protein in other human cancer cell lines tested including those derived from colon carcinomas (WiDR and HCT116), a nodal metastasis of a laryngeal carcinoma (886 LN), and a melanoma (A375; Supplementary Fig. S2).

A431 cells are known to have amplification of the EGFR gene and express levels of the EGFR above that seen in primary and immortalized keratinocytes (22). In addition, there are known signaling differences between keratinocyte cell lines and PHKs (23). Therefore, we repeated the above studies in PHKs using 10 U/mL of IFN-γ. This dose was selected because in our system, it leads to a partial induction of cell surface HLA-DR in PHKs (data not shown) and therefore could be used to detect any increased response in the presence of EGFR inhibition. As observed in A431 cells, pretreatment with PD168393 augmented the induction of CIITA mRNA in PHKs (Fig. 1E) to statistically significant levels above control cells pretreated with vehicle (DMSO). Flow cytometric analysis of cell surface MHCII protein expression showed that PD168393 pretreatment also augmented the cell surface expression of HLA-DR in PHKs (Fig. 1F and G). As a control, we pretreated PHKs with another (although reversible) EGFR inhibitor, AG1478, which also augmented levels of HLA-DR (Fig. 1F and G). In contrast, pretreatment with a platelet-derived growth factor receptor (PDGFR) inhibitor, AG1295, failed to augment levels of HLA-DR (Fig. 1F and G). These findings suggest that EGFR activity following IFN-γ exposure influences the induction of CIITA and MHCII genes in malignant and normal human keratinocytes. The above results support the notion that EGFR activation following IFN-γ serves to attenuate the induction of CIITA. To test this, we treated cells with IFN-γ alone or combined with EGF. We found that in both A431 cells and PHKs, coadministration of EGF with IFN-γ attenuated the induction of CIITA mRNA by IFN-γ (Supplementary Fig. S3). As reported by others, the induction of cell surface HLA-DR protein was also attenuated (Supplementary Fig. S3; ref. 14).

The protease inhibitor marimastat augments the induction of HLA-DR

EGFR ligands, such as TGF-α, can be shed from the cell surface in response to inflammatory cytokines (such as IFN-γ) through a protease-dependent mechanism (13). These same ligands can repress the induction of MHCII molecules by IFN-γ (14). Therefore, we reasoned that inhibition of protease activity would block the release of EGFR ligands following IFN-γ exposure and lead to a more robust induction of MHCII molecules. To test this hypothesis, we pretreated PHKs with the protease inhibitor marimastat prior to IFN-γ treatment. As reported by others, treatment of keratinocytes with IFN-γ resulted in a loss of cell surface TGF-α protein (as measured by flow cytometry) and this effect was prevented by pretreatment with marimastat (Supplementary Fig. S4). Consistent with the aforementioned hypothesis, pretreatment with the protease inhibitor marimastat augmented the induction of HLA-DR by IFN-γ in PHKs (Fig. 1H). The difference between control cells and those pretreated with marimastat was statistically significant and was not due to a direct effect of the protease inhibitor because marimastat alone had no effect on HLA-DR levels.

Cetuximab augments the induction of CIITA and HLA-DR by IFN-γ in human keratinocytes

We next examined how the blockade of EGFR ligand binding influenced the induction of CIITA and HLA-DR by IFN-γ. A431 cells were pretreated with an EGFR-blocking antibody (cetuximab) prior to IFN-γ exposure. As controls, we used other therapeutic humanized antibodies (which like cetuximab are IgG1 antibodies) that target vascular endothelial growth factor (VEGF; bevacizumab), human epidermal growth factor receptor 2 (HER2; trastuzumab), and tumor necrosis factor-α (TNF-α; infliximab). As observed with PD168393, pretreatment with cetuximab, but not the control antibodies, augmented the induction of CIITA mRNA by IFN-γ in A431 cells to statistically significant levels compared with control cells (Fig. 2A). Because the EGFR is known to heterodimerize with other HER family members, and drugs targeting multiple HER family members are in development (2), we examined the effect of combining antibodies that block the EGFR with those targeting the related receptor tyrosine kinase HER2. Although blocking antibodies against HER2 had no effect when used alone, the addition of these antibodies to cetuximab slightly augmented the induction of CIITA mRNA at the 48-hour time point (Fig. 2A). We next examined cell surface MHCII protein expression using flow cytometry and found that pretreatment with cetuximab augmented HLA-DR protein expression on the surface of A431 cells (Fig. 2B and C) to levels that were statistically significant. As we observed with CIITA mRNA, the addition of HER2-blocking antibodies augmented the effect of pretreatment with the EGFR-blocking antibody cetuximab (Fig. 2B and C).

To test whether there is a similar response in primary cells, we repeated these experiments in PHKs. As observed
on A431 cells, cell surface expression of HLA-DR was increased by pretreatment with cetuximab but not with blocking antibodies of other specificity (Fig. 2D and E). Although the combination of EGFR- and HER2-blocking antibodies gave the most robust induction of CIITA mRNA (data not shown), there was no additional increase in cell surface HLA-DR protein levels on PHKs by combining these 2 antibodies at the doses of IFN-γ used in these experiments.

**PD168393 augments the induction of MHCI molecules by IFN-γ**

MHCI genes are not typically expressed in epithelial cells in the absence of inflammatory cytokines such as IFN-γ. In contrast, MHCI genes are constitutively expressed on most nucleated cells (9). In response to IFN-γ, basal MHCI expression is augmented through mechanisms that involve the transcriptional coactivators CIITA and NLRC5 (9, 24). Therefore, we examined the effect of EGFR inhibition on

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**Figure 1.** EGFR TKIs augment the induction of CIITA and HLA-DR. A, A431 cells were pretreated with the EGFR inhibitor PD168393 (1 μmol/L) for 60 minutes prior to IFN-γ and CIITA mRNA quantified. For each dose and time point, values are expressed as fold over control cells that were pretreated with vehicle (DMSO; 0.01%) prior to IFN-γ. Error bars represent the SEM from 3 independent experiments. *, *P < 0.05; 2-tailed paired Student’s t test. B, A431 cells were pretreated with vehicle (DMSO) or PD168393 for 60 minutes prior to IFN-γ treatment (1 U/mL). Whole cell lysates were prepared 48 hours after the addition of IFN-γ and CIITA protein levels visualized by Western blotting. A loading control (GAPDH) is shown. C, a representative flow cytometric experiment is shown. Cells were pretreated as indicated for 60 minutes prior to IFN-γ. After 48 hours, cells were stained with anti-HLA-DR antibodies (clone L203) or isotype control antibodies. Percentages of HLA-DR–positive cells are shown within each panel for treated or untreated (UnTx) cells. D, averaged values from 3 independent flow cytometric experiments are shown. A431 cells were pretreated with DMSO (white) or PD168393 (gray) prior to IFN-γ. The y-axis represents percentage of HLA-DR–positive cells at 48 hours. *, *P < 0.05; 2-tailed paired Student’s t test. E, PHKs were pretreated as above prior to IFN-γ (10 U/mL) and CIITA mRNA measured. Error bars represent the SEM from 3 independent experiments. *, *P < 0.05; 2-tailed paired Student’s t test.
MHCI protein expression. Flow cytometric analysis of MHCI expression (using an antibody that recognizes a monomorphic epitope present on HLA-A, HLA-B, and HLA-C molecules) revealed that pretreatment with PD168393 or anti-EGFR antibodies augmented the expression of MHCI molecules by IFN-γ on A431 cells (Fig. 3A and B). These increases in MHCI protein expression were statistically significant compared with control cells. Even in the absence of IFN-γ, treatment with PD168393 or anti-EGFR antibodies was able to augment the expression of MHCI molecules on A431 cells (Fig. 3A and C). This was not observed with a control TKI (AG1295) or control antibodies (Fig. 3A and C). These results show that EGFR inhibition can directly augment the expression of MHCI proteins in malignant human cells.

To test whether similar biology is observed in primary cells, PHKs were treated similarly and levels of surface MHCI protein assessed using flow cytometry. As we observed with A431 cells, there was a statistically significant increase in MHCI cell surface protein on cells pretreated with an EGFRi prior to IFN-γ treatment (Fig. 3D–F). Again, as with A431 cells, even in the absence of IFN-γ, PD168393, to a lesser extent AG1478, augmented cell surface MHCI protein levels in PHKs (Fig. 3E). Similarly, combining cetuximab and trastuzumab lead to a statistically significant increase in MHCI levels in PHKs (Fig. 3F). Cetuximab alone also augmented MHCI levels albeit to a lesser extent (Fig. 3F).

**EGFRis augment epidermal MHCI protein expression in human skin**

We next examined how EGFRIs impact the expression of MHCI and MHCI proteins in human skin using IHC. Skin biopsies were obtained from patients selected for EGFRi therapy by their treating oncologist. Patients enrolled in the study were biopsied before and 3 to 4 weeks into treatment with cetuximab (2 patients) or the EGFR TKI erlotinib (4 patients). Biopsies were conducted on the trunk (chest or back), common locations for EGFRi-induced skin inflammation, and clinically normal skin on the upper medial arm. The presence of clinical inflammation on the trunk was noted at the time of biopsy. No clinical inflammation was present on the biopsies from the upper medial arm at any time irrespective of inflammation on the trunk. Formalin-fixed, paraffin-embedded (FFPE) tissue sections
were stained with hematoxylin and eosin (H&E) as well as antibodies against MHC-I and MHC-II molecules. A summary of the microscopic and immunohistochemical findings are shown in Table 1. A total of 12 biopsies were available for analysis from 6 patients. Of the 6 patients, 4 developed some degree of cutaneous inflammation on the trunk during EGFRI therapy (3 were described as positive and 1 as minimally positive). Two patients did
not develop any cutaneous inflammation at the time of the biopsy after initiation of EGFRI therapy. The histopathologic changes that we observed were similar to those reported by others (Fig. 4A–D, top, and corresponding figure legends; ref. 7).

Epidermal keratinocytes express MHCI proteins in a graded fashion such that cells of the basal and spinous layers express higher levels of MHCI than keratinocytes within the granular layer (25). To determine whether EGFRI therapy alters MHCI protein expression in vivo, we analyzed the same FFPE sections described above using a monoclonal antibody (EMR8-5) that recognizes an epitope on the heavy chains of HLA-A, HLA-B, and HLA-C molecules (26). Prior to EGFRI therapy, we observed staining of epidermal keratinocytes most noticeably within the basal and lower spinous layers of the epidermis (Fig. 4, middle left, and Supplementary Fig. S5). Most of the cells within the dermis also stained positively with MHCI. Consistent with our in vitro data, we observed increased MHCI protein expression within the epidermis during EGFRI therapy (Fig. 4 and Supplementary Fig. S5). We quantified epidermal MHCI protein expression by combining whole slide scanning with specialized image analysis software as has been reported by others (27). Using this approach, we found that epidermal MHCI protein expression increased in 5 of the 6 patients examined (Table 1 and Supplementary Fig. S6). The increase in MHCI protein expression occurred independent of microscopic or clinical inflammation. We also found corresponding increases in MHCI RNA levels in 2 of the patients for which skin biopsy RNA was available (Supplementary Fig. S7). Thus, in some patients on EGFRI therapy, there is an increase in epidermal MHCI expression independent of clinical inflammation.

To analyze MHCII protein expression, the above skin biopsies were stained using a monoclonal antibody (clone KUL/05) that recognizes MHCII proteins in FFPE tissue specimens (28). Within the epidermis, the primary cell that expresses MHCII molecules constitutively is the Langerhans cell (LC). However, in the setting of inflammation, the above skin biopsies were stained using a monoclonal antibody (clone KUL/05) that recognizes MHCII proteins in FFPE tissue specimens (28). Within the epidermis, the primary cell that expresses MHCII molecules constitutively is the Langerhans cell (LC). However, in the setting of inflammation,
**Figure 3.** EGFR inhibition increases cell surface MHC I protein expression. A, A431 cells were left untreated (UnTx), treated with the indicated compounds (1 μmol/L) alone or 60 minutes prior to the addition of IFN-γ (1 U/mL). Cell surface MHC I molecules (HLA-ABC) were measured using flow cytometry with an antibody that recognizes a monomorphic epitope on MHC I antigens (clone G46-2.6). Averaged mean fluorescence intensity (MFI) values from 5 independent flow cytometric experiments are shown. ***, P < 0.001; repeated measures ANOVA. B, averaged MFI values from 5 independent experiments are shown from A431 cells pretreated with the indicated antibodies (2 μg/mL) prior to IFN-γ (1 U/mL). **, P < 0.01; repeated measures ANOVA. C, averaged MFI values from 4 independent experiments are shown using cells that were treated with the indicated blocking antibodies alone (no IFN-γ). **, P < 0.01; ***, P < 0.001; repeated measures ANOVA. D, representative flow cytometric experiments using PHKs treated with IFN-γ, PD168393 (PD168), or cetuximab alone or those treated with IFN-γ following pretreatment with PD168 or cetuximab. E, averaged MFI values from 5 independent flow cytometric experiments are shown. Pretreatment TKIs included PD168393 (PD168), AG1295 (AG129), and AG1478 (AG147). *, P < 0.05; ***, P < 0.001; repeated measures ANOVA. F, averaged MFI values from 5 independent flow cytometric experiments are shown. Pretreatment antibodies are indicated. *, P < 0.05; ***, P < 0.001; repeated measures ANOVA.
Discussion

Our data support a model (Fig. 5) whereby EGFR activation represses MHCI and MHCII molecule expression. This may occur via its effect on the IFN-γ receptor complex (14), CIITA mRNA, and/or direct effects on the promoters of MHCI and MHCII genes. In addition to direct effects on CIITA mRNA levels, enzymes whose activity is modulated by EGFR signaling may directly alter CIITA protein levels and/or activity via posttranslation modifications (30). By modulating CIITA and the expression of MHCII and MHCII molecules, the EGFR pathway is well poised to modulate how normal and malignant cells interact with T lymphocytes and thus influence immune responses. Furthermore, the molecular events that activate the EGFR during oncogenesis may not only promote tumor cell proliferation and survival but also may facilitate immune escape by repressing the expression of CIITA, MHCI, and/or MHCII molecules.

EGFRI therapy in humans has been shown to alter the production of chemokines that attract T cells to the skin and exacerbate T-cell–driven diseases such as psoriasis (4, 31). Likewise, in mice, EGFRI can alter T-cell responses leading to increased contact hypersensitivity, protection against viral infection, and inhibition of photoimmuno-suppression (13, 19, 31, 32). Our findings may help explain these varied effects because changes in peptide MHC molecule (pMHC) expression levels can influence T-cell biology. Specifically, the impairment of T-cell activation that occurs from prolonged pMHC–TCR interactions does not occur at high pMHC densities (33). In addition, pMHC density can also influence CD8+ memory T-cell differentiation (34). Thus, EGFRI can increase the recruitment of T cells to the skin (and possibly other organs) and alter the expression of molecules that help govern T-cell responses, namely MHCI and MHCII molecules. However, it is important to note that augmented MHC expression in the absence of costimulatory signals may lead to antigen-specific anergy and thereby hamper an effective T-cell response. However, to our knowledge, the development of EGFR-induced antigen-specific anergy has not been reported. Although the level of MHCI and/or MHCII molecule expression can influence T-cell activation, EGFRI may also influence immune homeostasis within the skin through mechanisms that involve altering the pattern of expression of MHCI and/or MHCII molecules as described in the subsequent paragraph.

The cutaneous inflammation seen in response to EGFRI occurs mainly on hair-bearing areas (3, 6). This recapitulates the phenotype of EGFR knockout mice (35). The expression of MHCI and MHCII molecules (and other immune system genes) are downregulated in normal human anagen (growing) hair follicles, and because of this, portions of the hair follicle are considered an anatomic compartment with immune privilege (IP; refs. 36–39). In the setting of autoimmune or inflammatory diseases associated with hair loss, there is felt to be a loss of IP as evidenced by increases in the expression of MHCI and MHCII molecules as well as other immunoregulatory proteins (40). Thus, although there were insufficient terminal hairs in our biopsies to evaluate, if EGFRI alter the expression pattern of MHCI and/or MHCII molecules on follicular keratinocytes they may disrupt the IP at this site.

Table 1. Summary of clinical information and skin biopsy findings from EGFR inhibitor–treated patients

<table>
<thead>
<tr>
<th>Patient number and biopsy site</th>
<th>Medication</th>
<th>Clinical inflammation during EGFRI therapy</th>
<th>Histopathologic changes</th>
<th>Fold change in epidermal MHCI protein expression</th>
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<td>–</td>
<td>+</td>
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<tr>
<td>1, chest</td>
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<td>+</td>
<td>+</td>
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Before therapy

During therapy

A

Epidermis

Dermis

MHC I

MHC II

Patient 1, arm, no cutaneous inflammation

LC

LC

B

Epidermis

Dermis

MHC I

Patient 3, chest, cutaneous inflammation present

MHC II

LC

C

Before therapy

During therapy

Epidermis

Dermis

MHC I

MHC II

Patient 4, chest, no cutaneous inflammation

LC

D

Epidermis

Dermis

MHC I

MHC II

Patient 5, chest, cutaneous inflammation present

LC
Blocking white arrow), and an increase in dermal mononuclear cells (top right; black arrows). There is more intense MHCI staining during therapy (middle, left vs. right). MHCII expression (bottom, brown colored staining) on epidermal LCs (black arrows) is visualized before and during EGFR inhibitor therapy. C, chest biopsies from patient 4 reveal no histopathologic changes (top row, left vs. right). There is an increase in epidermal MHCI staining (middle row, left vs. right). MHCII-positive LCs are present before and during therapy. D, chest biopsies from patient 5 reveal an increase in dermal mononuclear cells (gray arrow) and lymphocytes (black arrows). There is an increase in epidermal MHCI staining (middle, left vs. right). MHCII-positive LCs are present before and during therapy.

Figure 4. Microscopic and immunohistochemical features of patient skin biopsies before and during therapy with an EGFR inhibitor. Biopsy sections were stained with H&E (top row), MHCI antibodies (clone EMR8-5; middle row), and MHCII antibodies (clone KUL/05; bottom row). Scale bars represent 100 μm. A, arm biopsies (no clinical inflammation) from patient 1 show a slight increase in dermal mononuclear cells (top right, black arrows). In addition, an increase in epidermal MHCI protein expression (visualized as brown colored staining) is seen during EGFR therapy versus before therapy (middle, left vs. right; black arrows). MHCI expression (bottom, brown colored staining) on epidermal LCs (black arrows) is visualized before and during EGFR inhibitor therapy. B, chest biopsies from patient 3 reveal an altered stratum corneum (top right; gray arrow), the presence of apoptotic keratinocytes (top right; white arrow), and an increase in dermal mononuclear cells (top right; black arrows). There is more intense MHCI staining during therapy (middle, left vs. right). MHCI staining reveals some MHCII-positive epidermal keratinocytes (Kc; bottom right and insert therein). MHCII-positive LCs are present before and during therapy. C, chest biopsies from patient 4 reveal no histopathologic changes (top row, left vs. right). There is an increase in epidermal MHCI staining (middle row, left vs. right). MHCI-II-positive LCs are present before and during therapy. D, chest biopsies from patient 5 reveal an increase in dermal mononuclear cells (gray arrow) and lymphocytes (black arrows). There is an increase in epidermal MHCI staining (middle, left vs. right). MHCII-positive LCs are present before and during therapy.

Figure 5. Model of EGFR signaling as it relates to MHC expression. In response to cytokines such as IFN-γ, EGFR ligands are released through a protease-dependent mechanism that can be blocked by protease inhibitors such as marimastat. The EGFR ligands cross-activate the EGFR and downstream signaling pathways which have a repressive effect on the expression of CIITA, MHCI, and MHCII genes. Repressive signals emanating from the activation of the IFN-γ receptor complex, the promoter of CIITA, CIITA posttranslational modifications, and/or the promoters of MHCI and MHCII genes. The net result of which is attenuation of CIITA and MHC molecule expression. In response to protease inhibitors, EGFR TKIs, or EGFR ligand-blocking antibodies, the expression of CIITA, MHCI, and/or MHCII genes is derepressed. This leads to an increase in cell surface MHCI protein expression and the potential alteration of immune/inflammatory responses.

and promote inflammation. On nonfollicular epidermis, which lacks IF, our data suggest that an increase in MHCI molecule expression levels is necessary but not sufficient to induce clinical or microscopic inflammation. This is supported by our observation that increases in the expression of MHCI molecules on epidermal keratinocytes occurred without any other evidence of clinical or pathologic inflammation. Thus, it is likely that to influence immune homeostasis, changes in the expression of MHCI and/or MHCII molecules need to occur concomitantly with changes in additional immune elements cellular and otherwise.

The preceding paragraphs illustrate how through an alteration in MHC protein expression, EGFRIs may influence T-cell responses in nonmalignant tissues, and how these drugs may promote inflammation in the skin and other organs. It is also clear that alterations in the expression of CIITA, MHCI, and/or MHCII molecules can influence the immune response against tumors. In both murine models and human studies, the expression of CIITA, MHCI, and/or MHCII molecules can influence antitumor immune responses (41–43). Consistent with this, the expression of MHCI and/or MHCII molecules on tumor cells has been repeatedly shown to impact prognosis (44–46). Thus, our data raise the possibility that in addition to targeting the mitogenic and survival signals emanating from the EGFR, EGFRIs may influence the immune response to tumor cells by altering the expression of MHCI and/or MHCII molecules. We found that EGFRIs augmented the induction of MHCI and MHCII molecules in A431 cells and SQCC/Y1 cells, which were derived from a vulvar carcinoma and buccal carcinoma, respectively. However, we saw no effect of EGFR inhibition on the induction of MHCI and/or MHCII molecules in 2 colon carcinoma cell lines (WiDR and HCT116), 1 melanoma cell line (A375), and a cell line derived from a nodal metastasis of a laryngeal carcinoma (S86LN). This is despite the fact that HCT116, A375, WiDR, and S86LN cells respond to IFN-γ (with regard to the induction of MHCI and/or MHCII molecules) and have been shown to express the EGFR (21, 47–49). Thus, under our experimental conditions, not all tumor cell lines are sensitive to the effects of EGFR inhibition, as it relates to the altered expression of MHCI and/or MHCII molecules.

The direct modulation of MHC expression that we have found complements the work of others and broadens our understanding of how EGFRIs may affect immune/inflammatory responses. Although an increase in MHCI and MHCII molecules may be potentially advantageous in some settings, it may be problematic in others. Indeed, a recent report describes 2 single lung transplant recipients...
who received cetuximab for metastatic cutaneous SSC. Both patients developed rapidly fatal diffuse alveolar damage resembling acute clinical rejection of the transplanted lung (5). Additional work is needed to define how EGFRs modulate MHC expression and how this in turn impacts immune responses in vivo. This information will be helpful to best define how to utilize medications that target the EGFR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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