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

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STATEMENT OF TRANSLATIONAL RELEVANCE

The development of cutaneous inflammation in response to epidermal growth factor receptor (EGFR) 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 major histocompatibility complex (MHC) class I (MHCI) and class 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 anti-tumor 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.
ABSTRACT

Purpose: Diverse immune-related effects occur with the use of epidermal growth factor receptor inhibitors (EGFRIs). In addition to the cutaneous inflammation induced by EGFRIs, these agents have been associated with the exacerbation of autoimmune skin disease and contact hypersensitivity, anti-viral effects, and fatal alveolar damage in the setting of lung transplantation. Since EGFR ligands can modulate MHC class I (MHCI) and II (MHCII) molecule expression, we hypothesized that some of the immune-related effects of EGFRIs 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 EGFRIs 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 RT-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 EGFRis may influence immune/inflammatory responses by directly modulating MHC expression.
INTRODUCTION

Aberrant expression or activity of the 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 (TKIs) that block EGFR activation (2). EGFR inhibitors (EGFRIs) were initially developed to block EGFR-dependent pro-survival and mitogenic signals within tumor cells. Regardless of their mechanism, the use of EGFRIs in patients is associated with pro-inflammatory side effects suggesting that EGFR signaling modulates the expression of relevant immunoregulatory genes.

The side effects associated with EGFRIs 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 anti-neoplastic therapy. Despite this, the development of EGFRI-induced skin inflammation is associated with prolonged survival and suggests a relationship between EGFR-induced inflammation and anti-tumor effects (6, 7). Defining how the EGFR pathway impacts immune responses is therefore important because it will foster new approaches to attenuate EGFRI side effects and may help identify novel applications for their use.

In this report, we demonstrate a novel activity of EGFRIs that may help explain the pro-inflammatory side effects of these medications. We report that
both EGFR TKIs and cetuximab potentiate the induction of major histocompatibility complex (MHC) class I (MHCI) and class II (MHCII) molecules in response to IFN-\(\gamma\). The increase in MHC molecule expression is associated with an increase in the MHCII transactivator (CIITA), a transcriptional regulator of MHCI and MHCII molecules (8, 9). Importantly, even in the absence of inflammatory cytokines, EGFRIs augment the constitutive expression of MHCI molecules. Consistent with our in vitro findings, skin biopsies taken from patients during EGFR1 therapy revealed an increase in epidermal MHCI protein expression and MHCI and MHCII RNA levels when compared to skin biopsies taken prior to EGFR1 therapy. These findings underscore the importance of EGFR signaling in regulating immune system genes and suggest that manipulation of EGFR signaling may offer a new approach to modulate MHC-dependent immune responses.
MATERIALS AND METHODS

Cells

A431 epidermoid keratinocytes were kindly provided by the laboratory of Dr. Jeremy M. Boss (Emory University, Department of Microbiology and Immunology, Atlanta, GA). WiDR cells were purchased from the American Type Culture Collection (Manassas, VA). The melanoma cell line, A375, and the colon carcinoma cell line, Hct116, were kindly provided by Dr. Jack Arbiser (Emory University, Department of Dermatology, Atlanta, GA). A431, A375 and Hct116 cells were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Inc., Logan, UT), penicillin (50 U/ml), streptomycin (50 μg/ml), and L-glutamine (1 mM) (Life Technologies, Grand Island, NY) and maintained at 37 degrees C and 5% CO2 atmosphere. The head and neck carcinoma cell lines (886LN and SQCC/Y1) were kindly provided by Dr. Dong Shin (Winship Cancer Institute, Atlanta GA) and were maintained in DMEM/F12 (1:1) supplemented with L-glutamine (1mM) and 10% FBS at 37 degrees and 5% CO2 atmosphere. Primary human keratinocytes (PHKs) were purchased (LifeLine Technologies, Rockville, MD) and grown in complete defined media as recommended by the supplier and maintained at 37 degrees C and 5% CO2 atmosphere. PHKs were used between passage 3 and passage 6.

Reagents and Treatment of Cells
Human interferon (IFN)-γ (Peprotech, Rocky Hill, NJ) was re-suspended in DMEM (200 μg/ml) and stored at -80 degrees 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, Gibbstown, NJ) were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mM and cells were treated at a final concentration of 1 μM by adding 1 μl of stock PD168393 to 10 ml of media. AG1478 (Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO to a concentration of 1.58 mM and cells were treated at a final concentration of 1 μM by similarly adding stock AG1478 to 10 ml of media. EGF (Promega, Madison, WI) was re-suspended in basal keratinocyte media to 100 μg/ml and used at a final concentration of 10 ng/ml. All of the above reagents were stored at -80 degrees 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 (WCI) of Emory University as follows: bevacizumab (25 mg/ml; Genetech, San Francisco, CA), cetuximab (2 mg/ml; ImClone, New York, NY), infliximab (10 mg/ml; Centocor Ortho Biotech, Horsham, PA) and trastuzumab (21 mg/ml; Genetech, San Francisco, CA). Bevacizumab, infliximab and trastuzumab were diluted to 2 mg/ml in sterile phosphate buffered saline (PBS) and stored at 4 degrees 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 performed as previously described (10, 11). Quantitative real-time PCR was performed using a CFX96 thermal cycler and measuring SYBR green incorporation into double stranded amplicons. Reactions were performed in 25 μl volumes containing forward and reverse primers at a final concentration of 100 nM. Primer sequences for CIITA and GAPDH were: CIITA Forward 5’- CTGAAGGATGTGGAAGACCTGGGAAAGC-3’, CIITA reverse 5’- GTCCCCGATCTTGTTCTCACTC-3’; and GAPDH forward 5’–GAAGGTGAAGGTCGGAGTCA-3’, GAPDH reverse 5’GAAGATGTTAGATGGGATTCC-3’.

Flow Cytometry

Following the treatments indicated in the text, cells were trypsinized, washed in FACS buffer (2mM EDTA, 1% BSA in PBS), pelleted by centrifugation. Cell pellets were then resuspended in 20-40 μl of anti-HLA-DR (clone L203, catalogue # FAB4869P, R & D Systems, Minneapolis, MN) or anti-HLA-ABC antibodies conjugated to phycoerythrin (clone G46-2.6, catalogue # 557349, Becton Dickinson, Franklin Lakes, NJ) or an isotype control antibody also conjugated to phycoerythrin (catalogue # 554680, Becton Dickinson, Franklin Lakes, NJ). Cells were incubated on ice for 30 minutes washed three times and resuspended in 0.5 ml of FACS buffer containing 0.5% paraformaldehyde. Surface HLA-DR or HLA-ABC expression was measured using a FACScalibur (BD Biosciences, Franklin Lakes, NJ) flow cytometer and MHC expression analyzed on ungated cells using Flowjo software (Tree Star, Ashland, OR).
**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 performed on the trunk (chest or back) and medial upper extremity. Three to four weeks into therapy with either cetuximab or erlotinib, subjects were biopsied on the trunk (clinically inflamed skin if present and non-inflamed skin if not) and non-inflamed skin from the upper medial arm.

**Immunohistochemistry**

Immunohistochemistry was performed 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, Cambridge, MA; clone EMR8-5, 1:800) and MHCII (Abcam, Cambridge, MA; clone KUL/05, 1:150). Epidermal MHC Class I staining intensity between biopsies from the same patient and anatomic site (before vs. during EGFRI therapy) were graded as no increase (-) or increased (+) on EGFRI therapy by one of the authors trained in dermatopathology (B.P.P.). Images were captured on a Nikon Eclipse E400 microscope (Nikon, Melville, New York) using SPOT Flex 15.2 64 Mp Shifting Pixel Camera and SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI).
Quantification of MHC Class I Immunohistochemistry

Glass slides containing skin biopsies stained with MHC class I antibodies as described in the main text were scanned at 40X magnification using a Nanozoomer 2.0HT slide scanner (Hamamatsu, Japan) at the Emory Pathology Core Laboratory. Scanned images were analyzed using Aperio ImageScope Positive Pixel Count (version 9) software (Aperio, Vista, CA). 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 MHC class I 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 EGFRI 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, La Jolla, CA).

Statistical Analysis

All statistics were performed using InStat (GraphPad Software Inc., La Jolla, CA). Either a paired Student’s t-test or an analysis of variance (ANOVA) was performed as indicated in the text. Tests for Gaussian (Normal) distribution were performed using the Kolmogorov-Smirnov test.
RESULTS

PD168393 Augments the Induction of CIITA and HLA-DR by IFN-γ

EGFR ligands such as transforming growth factor (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 MHCII genes by IFN-γ (14). Based upon this, we hypothesized that the trans-activation 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 pre-treated A431 malignant keratinocytes with the irreversible EGFRI PD168393 prior to treatment with IFN-γ. A431 cells have been used extensively to define the mechanisms responsible for the induction of CIITA and MHCII 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 pre-treatment, 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 polymerase chain reaction (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 trans-acting 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-\(\gamma\) treatment (17). The doses of IFN-\(\gamma\) used in these studies ranged from 1-20 units/ml. These doses were selected because they approximate serum levels of IFN-\(\gamma\) in humans (20). In addition, we reasoned that at higher doses of IFN-\(\gamma\) 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, pre-treatment of A431 cells with PD168393 augmented CIITA mRNA levels above those of control cells that were pre-treated with vehicle (DMSO) yet treated with the same concentration of IFN-\(\gamma\). The effect was most noticeable at the 24 and 48 hours time points with statistically significant differences at the 48 hour time point (Figure 1A). CIITA protein levels were also elevated by pre-treatment with PD168393 as shown in Figure 1B.

To determine if 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. Pre-treatment of A431 cells with PD168393 augmented the IFN-\(\gamma\)-induced cell surface expression of HLA-DR (Figure 1C and D). While PD168393 augmented the expression of HLA-DR at all concentrations tested, the effect was most robust at lower concentrations of IFN-\(\gamma\) with statistically significant increases seen at 48 hours using 5 or 1 U/ml of IFN-\(\gamma\). Indeed, in contrast to lower doses of IFN-\(\gamma\), at higher doses of IFN-\(\gamma\) (500U/ml) we saw only a minimal effect of PD168393 on cell surface HLA-DR expression (Supplementary Figure S1). Thus, in A431 cells PD168393 potentiates the induction of CIITA and HLA-DR by IFN-\(\gamma\). Analysis of
other cancer cell lines revealed a similar response in SQCCY1 carcinoma cells which are derived from an oral squamous cell carcinoma (Supplementary Figure S2) (21). In contrast, pre-treatment 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 (886LN) and a melanoma (A375) (Supplementary Figure S2).

A431 cells are known to have amplification of the EGFR gene and express levels of the EGFR above that seen in primary keratinocytes and immortalized keratinocytes (22). In addition, there are known signaling differences between keratinocyte cell lines and primary human keratinocytes (PHK) (23). Therefore, we repeated the above studies in primary human keratinocytes (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, pre-treatment with PD168393 augmented the induction of CIITA mRNA in PHKs (Figure 1E) to statistically significant levels above control cells pre-treated with vehicle (DMSO). Flow cytometric analysis of cell surface MHCII protein expression demonstrated that PD168393 pre-treatment also augmented the cell surface expression of HLA-DR in PHKs (Figure 1F and 1G). As a control, we pre-treated PHKs with another (although reversible) EGFR inhibitor, AG1478, which also augmented levels of HLA-DR (Figure 1F and 1G). In contrast, pre-treatment with a platelet-derived growth
factor receptor (PDGFR) inhibitor, AG1295, failed to augment levels of HLA-DR (Figure 1F and 1G). 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, co-administration of EGF with IFN-γ attenuated the induction of CIITA mRNA by IFN-γ (Supplementary Figure S3). As reported by others, the induction of cell surface HLA-DR protein was also attenuated (Supplementary Figure S3) (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 pre-treated 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 pre-treatment with marimastat (Supplementary Figure S4). Consistent with the aforementioned hypothesis, pre-treatment with the protease
inhibitor marimastat augmented the induction of HLA-DR by IFN-γ in PHKs (Figure 1H). The difference between control cells and those pre-treated with marimastat was statistically significant and was not due to a direct effect of the protease inhibitor since 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 pre-treated with an EGFR blocking antibody (cetuximab) prior to IFN-γ exposure. As controls, we used other therapeutic humanized antibodies (which like cetuximab are IgG1κ antibodies) that target vascular endothelial growth factor (VEGF, bevacizumab), human epidermal growth factor receptor 2 (HER2, trastuzumab), and the tumor necrosis factor (TNF)-α receptor (TNFR, infliximab). As observed with PD168393, pre-treatment with cetuximab, but not the control antibodies, augmented the induction of CIITA mRNA by IFN-γ in A431 cells to statistically significant levels compared to control cells (Figure 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 RTK HER2. While 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 (Figure 2A). We next examined cell
surface MHCII protein expression using flow cytometry and found that pre-treatment with cetuximab augmented HLA-DR protein expression on the surface of A431 cells (Figure 2B and 2C) to levels that were statistically significant. As we observed with CIITA mRNA, the addition of HER2 blocking antibodies augmented the effect of pre-treatment with the EGFR-blocking antibody cetuximab (Figure 2B and 2C).

To test if 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 pre-treatment with cetuximab but not with blocking antibodies of other specificity (Figure 2D and 2E). While 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 two antibodies at the doses of IFN-γ used in these experiments.

**PD168393 Augments the Induction of MHC Class I Molecules by IFN-γ**

MHCII 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 co-activators CIITA and NLRC5 (9, 24). Therefore, we examined the effect of EGFR inhibition on MHCI protein expression. Flow cytometric analysis of MHCI expression (using an antibody that recognizes a monomorphic epitope present
on HLA-A, B and C molecules) revealed that pre-treatment with PD168393 or anti-EGFR antibodies augmented the expression of MHCI molecules by IFN-γ on A431 cells (Figure 3A and 3B). These increases in MHCI protein expression were statistically significant compared to 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 (Figure 3A and 3C). This was not observed with a control TKI (AG1295) or control antibodies (Figure 3A and 3C). These results demonstrate that EGFR inhibition can directly augment the expression of MHCI proteins in malignant human cells.

To test if 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 pre-treated with an EGFR inhibitor prior to IFN-γ treatment (Figure 3D, 3E and 3F). Again, as with A431 cells, even in the absence of IFN-γ, PD168393, and to a lesser extent AG1478, augmented cell surface MHCI protein levels in PHKs (Figure 3E). Similarly, combining cetuximab and trastuzumab lead to a statistically significant increase in MHCI levels in PHKs (Figure 3F). Cetuximab alone also augmented MHCI levels albeit to a lesser extent (Figure 3F).

**EGFR Inhibitors Augment Epidermal MHCI Protein Expression in Human Skin**
We next examined how EGFRIs impact the expression of MHCI and MHCII proteins in human skin using immunohistochemistry (IHC). Skin biopsies were obtained from patients selected for EGFRi therapy by their treating oncologist. Patients enrolled in the study were biopsied before and three to four weeks into treatment with cetuximab (2 patients) or the EGFR TKI erlotinib (4 patients). Biopsies were performed 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 MHCI and MHCII 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 six patients, four developed some degree of cutaneous inflammation on the trunk during EGFRI therapy (three were described as positive, one 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 (Figure 4A-4D, top panels and corresponding figure legends) (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 if EGFRI therapy
alters MHCI protein expression \textit{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, B and 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 (Figure 4, middle left panels and Supplementary Figure S5). Most of the cells within the dermis also stained positively with MHCI. Consistent with our \textit{in vitro} data, we observed increased MHCI protein expression within the epidermis during EGFRI therapy (Figure 4 and Supplementary Figure 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 during EGFRI therapy in five out of the six patients examined (Table 1, and Supplementary Figure S6). The increase in MHCI protein expression occurred independent of microscopic or clinical inflammation. We also found corresponding increases in MHCI RNA levels in two of the patients for which skin biopsy RNA was available (Supplementary Figure 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, we stained the above FFPE skin biopsies using a monoclonal antibody (clone KUL/05) that recognizes the beta chains of HLA-DP, DQ and DR molecules that has been reported to work on FFPE sections (28). Within the epidermis, the primary cell that expresses MHCII molecules constitutively is the Langerhans cell (LC). However, in the setting of
inflammation, keratinocytes can express MHCII molecules due to the action of inflammatory cytokines such as IFN-γ (29). Prior to EGFR1 therapy, we saw robust MHCII protein staining of single cells within the epidermis that are consistent with LCs (Figure 4A-D, bottom panels and Supplementary Figure S8). While the majority of the epidermal MHCII protein staining was expressed on cells that most likely represent LCs, we observed some MHCII positivity on epidermal keratinocytes in the setting of clinical inflammation (Figure 4B, bottom right panel and panel insert and Supplementary Figure S8). Consistent with this, we found an increase in HLA-DR mRNA from the two patients for which RNA was available (Supplementary Figure S7).
DISCUSSION

Our data support a model (Figure 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 post-translation modifications (30). By modulating CIITA and the expression of MHCI 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. Further, the molecular events that activate the EGFR during oncogenesis may not only promote tumor cell proliferation and survival but 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, EGFRIs can alter T cell responses leading to increased contact hypersensitivity, protection against viral infection and inhibition of photoimmunosuppression (13, 19, 31, 32). Our findings may help explain these varied effects since 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,
EGFRIs 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 co-stimulatory signals may lead to antigen-specific anergy and thereby hamper an effective T cell response. However, to our knowledge, the development of EGFRI-induced antigen-specific anergy has not been reported. While the level of MHCI and/or MHCII molecule expression can influence T cell activation, EGFRIs 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 below.

The cutaneous inflammation seen in response to EGFRIs 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) (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, while there were insufficient terminal hairs in our biopsies to evaluate, if EGFRIs alter the expression pattern of MHCI and/or MHCII molecules on follicular keratinocytes they may disrupt the IP at this site and promote inflammation. On non-follicular epidermis, which lacks IP, 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 by changing MHC protein expression, EGFRIs may influence T cell responses in non-malignant 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 anti-tumor 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 raises 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 two colon carcinoma cell lines.
(Hct116 and WiDR), one melanoma cell line (A375), and a cell line derived from a nodal metastasis of a laryngeal carcinoma (886LN). This is despite the fact that Hct116, A375, WiDR and 886LN cells respond to IFN-γ (with regards 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. While 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 two single lung transplant recipients who received cetuximab for metastatic cutaneous squamous cell carcinoma. Both patients developed rapidly fatal diffuse alveolar damage resembling acute clinical rejection of the transplanted lung (5). Additional work is needed to define how EGFRIs 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.
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The authors declare no conflicts of interest.
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FIGURE LEGENDS

Figure 1. EGFR Tyrosine Kinase Inhibitors Augment the Induction of CIITA and HLA-DR. (A) A431 cells were pre-treated with the EGFR inhibitor PD168393 (1 μM) 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 pre-treated with vehicle (DMSO, 0.01%) prior to IFN-γ. Error bars represent the SEM from three independent experiments. (*, p<0.05; two-tailed paired Student’s t-test) (B) A431 cells were pre-treated with vehicle (DMSO) or PD168393 for sixty minutes prior to IFN-γ treatment (1U/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 cytometry experiment is shown. Cells were pre-treated 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 three independent flow cytometry experiments are shown. A431 cells were pre-treated with DMSO (white) or PD168393 (gray) prior to IFN-γ. The y-axis represents % of HLA-DR positive cells at 48 hours. (*, p<0.05; two-tailed paired Student’s t-test) (E) Primary human keratinocytes (PHKs) were pre-treated as above prior to IFN-γ (10U/ml) and CIITA mRNA measured. Error bars represent the SEM from three independent experiments. (*, p<0.05; two-tailed paired Student’s t-test) (F) PHKs were pre-treated with DMSO (0.01%), PD168393 (PD168; 1 μM), AG1295.
(1 μM, a PDGFR inhibitor), and AG1478 (1 μM, an EGFR inhibitor) alone or 60 minutes prior to IFN-γ (10 U/ml). HLA-DR expression was analyzed as above by flow cytometry 48 hours later. Isotype control antibody staining is shown. (G) Average values (% HLA-DR+) from three independent flow cytometry experiments. Error bars represent the SEM. (***, \( p < 0.001 \); repeated measures ANOVA) (H) PHKs were pre-treated with 10 μM marimastat (or not pre-treated) for 60 minutes prior to IFN-γ (10U/ml) and HLA-DR levels measured 48 hours later. The error bars represent the SEM from four independent experiments. (**, \( p < 0.01 \); repeated measures ANOVA)

**Figure 2. EGFR Blocking Antibodies Augment the Induction of CIITA and HLA-DR.** (A) A431 cells were treated with IFN-γ alone or pre-treated for 60 minutes with antibodies (2 μg/ml) against VEGF (V, bevacizumab), the EGFR (E, cetuximab), the HER2 (H, trastuzumab), the TNF-α receptor (T, infliximab), or with both cetuximab and trastuzumab (E+H) prior to IFN-γ. Steady state CIITA mRNA levels were quantified as in Figure 1 and are expressed as fold over control cells treated with IFN-γ alone. Error bars represent the SEM from three independent experiments. (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); repeated measures ANOVA) (B) A representative flow cytometry experiment using A431 cells stained with anti-HLA-DR antibodies (clone L203) is shown. Cells were left untreated (UnTx), treated with IFN-γ, or pre-treated with the blocking antibodies indicated prior to IFN-γ (1U/ml) and % HLA-DR positive cells measured 48 hours later. Isotype control staining is shown. (C) Averaged values from three
independent flow cytometry experiments using A431 cells are shown. The y-axis represents % of cells that are positive for HLA-DR. Error bars represent the SEM. (**, p < 0.01; ***, p < 0.001; repeated measures ANOVA) (D) A representative flow cytometry experiment using PHKs is shown. Cells were pre-treated with the antibody indicated prior to IFN-γ (10U/ml) and % HLA-DR positive cells measured 48 hours later. (E) Averaged values from three independent flow cytometry experiments using PHKs are shown. The y-axis represents % of cells that are HLA-DR positive 48 hours following IFN-γ treatment. Error bars represent the SEM. (**, p < 0.01; ***, p < 0.001; repeated measures ANOVA)

Figure 3. EGFR Inhibition Increases Cell Surface MHC Class I Protein Expression. (A) A431 cells were left untreated (UnTx), treated with the indicated compounds (1μM) alone or 60 minutes prior to the addition of IFN-γ (1U/ml). Cell surface MHC class I molecules (HLA-ABC) were measured using flow cytometry with an antibody that recognizes a monomorphic epitope on MHC class I antigens (clone G46-2.6). Averaged mean fluorescence intensity (MFI) values from five independent flow cytometry experiments are shown. (***, p < 0.001; repeated measures ANOVA) (B) Averaged MFI values from five independent experiments are shown from A431 cells pre-treated with the indicated antibodies (2 μg/ml) prior to IFN-γ (1U/ml). (**, p < 0.01; repeated measures ANOVA) (C) Averaged MFI values from four 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 cytometry 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 five independent flow cytometry experiments are shown. Pre-treatment tyrosine kinase inhibitors included PD168393 (PD168), AG1295 (AG129) and AG1478 (AG147). (*, p<0.05; ***, p < 0.001; repeated measures ANOVA) (F) Averaged MFI values from five independent flow cytometry experiments are shown. Pre-treatment antibodies are indicated. (*, p < 0.05; ***, p < 0.001; repeated measures ANOVA)

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 demonstrate a slight increase in dermal mononuclear cells (top right panel, black arrows). In addition, an increase in epidermal MHCI protein expression (visualized as brown colored staining) is seen during EGFR therapy vs. before therapy (middle panels, left vs. right; black arrows). MHCII expression (bottom panels, brown colored staining) on epidermal Langerhans cells (LC;black arrows) is visualized before and during EGFR inhibitor therapy. (B) Chest biopsies from Patient 3 reveal an altered stratum corneum (top right panel; gray arrow), the presence of apoptotic
keratinocytes (top right panel; white arrow) and an increase in dermal mononuclear cells (top right panel; black arrows). There is more intense MHCI staining during therapy (middle panels, left vs. right). MHCII staining reveals some MHCII-positive epidermal keratinocytes (Kc; bottom right panel and insert therein). MHCII positive Langerhans cells (LC) are present before and during therapy. (C) Chest biopsies from Patient 4 reveal no histopathologic changes (top row, left vs. right panel). There is an increase in epidermal MHCI staining (middle row, left vs. right). MHCII positive Langerhans cells 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 panels, left vs. right). MHCII positive Langerhans cells (LC) 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 EGFR may influence the expression of the IFN-γ receptor complex, the promoter of CIITA, CIITA post-translational 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 tyrosine
kinase inhibitors, or EGFR ligand blocking antibodies, the expression of CIITA, MHCI and/or MHCII genes is derepressed. This leads to an increase in cell surface MHC protein expression and the potential alteration of immune/inflammatory responses.
Figure 1

A.

[Bar graph showing CIITA mRNA expression in response to IFN-γ treatment over time. The x-axis represents time (hours) and the y-axis represents CIITA mRNA expression (Fold Over Control). The graph shows increased mRNA expression at various time points for different IFN-γ concentrations, indicated with asterisks for statistically significant differences.]
Figure 1

B. IFN-γ+ IFN-γ+ DMSO PD168

CIITA

GAPDH
Figure 1

C. IFN-γ (U/ml)

Pre-treatment

PD168393

DMSO

SSC

HLA-DR

Isotype control

<table>
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<th>IFN-γ (U/ml)</th>
<th>Pre-treatment</th>
<th>PD168393</th>
<th>DMSO</th>
<th>SSC</th>
<th>HLA-DR</th>
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<td>0.129%</td>
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</table>
**Figure 1**

D.

![Graph showing HLA-DR positive expression with IFN-γ (U/ml) levels.](image-url)

- **x-axis**: IFN-γ (U/ml) with levels 20, 20, 10, 10, 5, 5, 1, 1.
- **y-axis**: HLA-DR Positive with values 90, 80, 70, 60, 50, 40, 30, 20, 10, 0.

Legend:
- **White bars**: DMSO
- **Gray bars**: PD168393

*Significant differences indicated by asterisks.*
Figure 1

E.

![Graph showing the fold change in CIITA mRNA expression over time (hours) with error bars.](graph.png)
Figure 1

F.

UnTx  IFN-γ+DMSO  DMSO  IFN-γ+PD168  PD168

IFN-γ+AG1295  AG1295  IFN-γ+AG1478  AG1478  IFN-γ+PD168

Isotype control

SSC

HLA-DR

0.30%  12.26%  0.29%  62.85%  1.15%

15.91%  0.32%  41.72%  0.61%  0.45%
Figure 1

G.

![Graph showing HLA-DR positive cells for different treatments.](image-url)
Figure 1

H.

![Graph showing HLA-DR positivity](image-url)

- **UnTx IFN-γ**: 0
- **IFN-γ**: 15
- **IFN-γ + marimastat**: 40
- **marimastat**: 0

**Note**: The graph illustrates the effect of IFN-γ and marimastat on HLA-DR positive cells. The comparison of HLA-DR positivity between untreated (UnTx) and treated samples shows a significant increase with IFN-γ and further enhancement with IFN-γ + marimastat, indicated by the **significant difference** (**) in the bar graph.
Figure 2

A.

![Bar graph showing the effect of time and blocking antibody on CIITA mRNA expression.](image)

- **x-axis:** Time (hours) - 8, 24, 48
- **y-axis:** CIITA mRNA (Fold Over Control)
- **Legend:**
  - **Blocking Antibody:** V, E, H, T, E
  - **Time:** H (hours)
  - **Annotations:**
    - *: p < 0.05
    - **: p < 0.01
    - ***: p < 0.001

Research.
Figure 2

B.

UnTx IFN-γ  0.48%  IFN-γ + αVEGF  1.13%  IFN-γ + αEGFR  1.05%

IFN-γ + αHER2  1.81%  IFN-γ + αTNFR  1.16%  IFN-γ + αEGFR/αHER2  12.72%

SSC  HLA-DR  Isotype control
Figure 2

C.

![Graph showing HLA-DR positive expression levels with different treatments.

- Untreated (untx)
- IFN-γ

Pre-treatment: None, αVEGF, αEGFR, αHER2, αTNFR, αEGFR/αHER2

Comparisons indicated:
- **: p < 0.01
- ***: p < 0.001

Graph legend:
- HLA-DR Positive
- Y-axis: 0 to 14
- X-axis: Untx, IFN-γ

Note: The graph shows significant increases in HLA-DR positive expression after IFN-γ treatment compared to untreated samples.
Figure 2

D.

UnTx IFN-γ  IFN-γ+α  VEGF IFN-γ+α  EGFR

0.30%  14.57%  14.03%  29.67%

IFN-γ+αHER2 IFN-γ+αTNFR IFN-γ+αEGFR/αHER2 IFN-γ+αEGFR/αHER2

15.45%  17.04%  34.79%  0.36%

SSC  HLA-DR

Isotype control
Figure 2

E.

![Graph showing HLA-DR positive counts for different treatments and blocking antibodies.](image-url)
Figure 3

A.

Mean Fluorescence Intensity

0 20 40 60 80 100 120 140

UnTx IFN-γ DMSO IFN-γ PD168 IFN-γ AG1295

DMSO PD168 AG1295

*** ***
Figure 3

B.

![Bar chart showing mean fluorescence intensity for different treatments and blocking antibodies.](chart)

- **UnTx:** Un-treated
- **IFN-γ:** Interferon-γ treated
- **Blocking Ab:** Blocking antibodies against VEGF, EGFR, HER2, TNFR, and EGFR/HER2

The chart indicates significant differences (*) and highly significant differences (**) in mean fluorescence intensity across different treatments and blocking antibodies.
Figure 3

C.

![Graph showing mean fluorescence intensity with blocking antibody](image)

- UnTx
- VEGF
- EGFR
- HER2
- EGFR/HER2

** and *** indicate statistical significance.

Blocking antibody
Figure 3

D.

![Graph showing HLA-ABC expression with different treatments.](Image)
Figure 3

E.

Mean Fluorescence Intensity

UnTx IFN-γ DMSO IFN-γ PD168 IFN-γ AG129 IFN-γ AG147

+ + + + + +
DMSO PD168 AG129 AG147

0 100 200 300 400 500 600 700 800 900

*** *
Figure 3

F.

![Bar graph showing mean fluorescence intensity for different conditions and blocking antibodies.](image-url)

- **Blocking Antibody:** VEGF, EGFR, HER2, TNFR, EGFR
- **Conditions:** UnTx IFN-γ, + HER2, + HER2

Legend:
- *: p < 0.05
- ***: p < 0.001
Figure 4A

Before Therapy

During Therapy

Hematoxylin & Eosin

Epidermis

Dermis

MHC Class I

Patient 1, Arm, No cutaneous inflammation

MHC Class II

LC
Figure 4B

Before Therapy

During Therapy

Epidermis

Dermis

Hematoxylin & Eosin

Patient 3, Chest, Cutaneous Inflammation Present

MHC Class I

MHC Class II

Kc

LC

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 4C

Before Therapy

During Therapy

Epidermis

Dermis

Patient 4, Chest, No Cutaneous Inflammation

Hematoxylin & Eosin

MHC Class I

MHC Class II

LC

LC
Figure 4D

Before Therapy

During Therapy

Epidermis

Dermis

Hematoxylin & Eosin

Patient 5, Chest, Cutaneous Inflammation Present

MHC Class I

MHC Class II

LC
IFN-γ receptor complex

Release of EGFR ligands

marimastat

Blocking Antibody

EGFR

Tyrosine Kinase Inhibitor

CIITA

CIITA promoter

Basal Transcription Factors

MHC Class I and II promoters

Altered MHC Expression

Altered Immune / Inflammatory Responses

Figure 5
Table 1. Summary of Clinical Information and Skin Biopsy Findings from EGFR Inhibitor-Treated Patients

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<thead>
<tr>
<th>Patient Number and Biopsy Site</th>
<th>Medication</th>
<th>Clinical Inflammation During EGFR Therapy</th>
<th>Histopathologic Changes</th>
<th>Fold Change in Epidermal MHC I Protein Expression</th>
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Epidermal Growth Factor Receptor Inhibition Augments the Expression of MHC Class I and II Genes

Brian P Pollack, Bishu Sapkota and Todd V Cartee

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