MyD88-Dependent Signaling Decreases the Antitumor Efficacy of Epidermal Growth Factor Receptor Inhibition in Head and Neck Cancer Cells

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Abstract

EGFR is upregulated in the majority of head and neck squamous cell carcinomas (HNSCC). However, many patients with HNSCC respond poorly to the EGFR inhibitors (EGFRIs) cetuximab and erlotinib, despite tumor expression of EGFR. Gene expression analysis of erlotinib-treated HNSCC cells revealed an upregulation of genes involved in MyD88-dependent signaling compared with their respective vehicle-treated cell lines. We therefore investigated whether MyD88-dependent signaling may reduce the antitumor efficacy of EGFRIs in HNSCC. Erlotinib significantly upregulated IL6 secretion in HNSCC cell lines, which our laboratory previously reported to result in reduced drug efficacy. Suppression of MyD88 expression blocked erlotinib-induced IL6 secretion in vitro and increased the antitumor activity of erlotinib in vivo. There was little evidence of Toll-like receptor or IL18 receptor involvement in erlotinib-induced IL6 secretion. However, suppression of IL1R signaling significantly reduced erlotinib-induced IL6 production. A time-dependent increase of IL1α but not IL1β was observed in response to erlotinib treatment, and IL1α blockade significantly increased the antitumor activity of erlotinib and cetuximab in vivo. A pan-caspase inhibitor reduced erlotinib-induced IL1α secretion, suggesting that IL1α was released because of cell death. Human HNSCC tumors showed higher IL1α mRNA levels compared with matched normal tissue, and IL1α was found to be negatively correlated with survival in patients with HNSCC. Overall, the IL1α/IL1R/ MYD88/IL6 pathway may be responsible for the reduced antitumor efficacy of erlotinib and other EGFRIs, and blockade of IL1 signaling may improve the efficacy of EGFRIs in the treatment of HNSCC.

Introduction

EGFR is a receptor tyrosine kinase that activates numerous pro-survival pathways including Akt and STAT3 signaling pathways (1). Given that EGFR signaling is upregulated in many cancers especially head and neck squamous cell carcinoma (HNSCC), several drugs that target EGFR have been developed and approved for cancer therapy such as monoclonal antibodies that block the extracellular ligand binding domain (e.g., cetuximab, panitumumab) and small molecule tyrosine kinase inhibitors (TKI) that prevent activation of the cytoplasmic tyrosine kinase domain (e.g., gefitinib, erlotinib; ref. 1). To date, only cetuximab is FDA-approved for use in HNSCC; however, it should be noted that response rates to cetuximab as a single agent are quite low (13%).

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Notes: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cells and culture conditions
Cal-27 and FaDu human HNSCC cells were obtained from the ATCC. SQ20B HNSCC cells (20) were a gift from Dr. Anjali Gupta (Department of Radiation Oncology, The University of Iowa, Iowa City, IA). All HNSCC cell lines are EGFR-positive and are sensitive to EGFR inhibitors. All cell lines were authenticated by the ATCC for viability (before freezing and after thawing), growth, morphology, and isoenzymology. Cells were stored according to the supplier’s instructions and used over a course of no more than 3 months after resuscitation of frozen aliquots. Cultures were maintained in 5% CO2 and air humidified in a 37°C incubator.

In vitro drug treatment
Erlotinib (Tarceva), anakinra (Kineret), and N-acetyl cysteine (NAC; Acetadote) were obtained from the inpatient pharmacy at the University of Iowa Hospitals and Clinics. Drugs were added to cells at final concentrations of 5 µmol/L erlotinib, 10 or 50 ng/mL anakinra, and 20 mmol/L NAC. Human IL1, IL18, and DMOs were used as controls and were obtained from Sigma-Aldrich. PEGylated catalase (CAT; Sigma-Aldrich) was used at a concentration of 100 U/mL. Human IL1α, IL1β, and IL18Rα neutralizing antibodies were obtained from R&D Systems and were used at a concentration of 0.5 µg/mL. Recombinant human IL1α was obtained from Life Technologies and administered at a concentration of 5 ng/mL. Recombinant human IL1β was obtained from ATCC. SQ20B HNSCC cells (20) were a gift from Dr. Anjali Gupta (Department of Radiation Oncology, The University of Iowa, Iowa City, IA). All HNSCC cell lines are EGFR-positive and are sensitive to EGFR inhibitors. All cell lines were authenticated by the ATCC for viability (before freezing and after thawing), growth, morphology, and isoenzymology. Cells were stored according to the supplier’s instructions and used over a course of no more than 3 months after resuscitation of frozen aliquots. Cultures were maintained in 5% CO2 and air humidified in a 37°C incubator.

Microarray analyses
Gene expression analysis of HNSCC cells treated with DMSO or erlotinib (5 µmol/L; 48 hours) has been described previously (GeneBank accession no. GSE45891; ref. 10). Downstream pathway, network, process, and disease analyses of the resultant gene expression data for all cell lines (n = 3 experiments per cell line) was carried out using MetacoreTM (GeneGo) using a threshold of +1.3 and a P value of 0.05. Enrichment analysis of the resultant gene expression profiles of SQ20B and Cal-27 HNSCC cells exposed to erlotinib versus DMSO was performed by mapping gene IDs from the resultant dataset onto gene IDs in built-in functional ontologies, which include cellular/molecular process networks, disease biomarker networks, canonical pathway maps, and metabolic networks.

Real-time quantitative PCR
Total RNA was extracted from cells after indicated time points using RNaseasy Plus mini kit (Qiagen). Conversion of RNA into cDNA was accomplished with the iScript cDNA Synthesis Kit (Bio-Rad) and a thermocycler with the following conditions: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Subsequent RT-PCR analysis was performed in a 96-well optical plate with each well containing 6 µL of cDNA, 7.5 µL of Sybr Green Universal SuperMix (Bio-Rad), and 1.5 µL of oligonucleotide primers (sense and antisense; 4 µmol/L) for a total reaction volume of 15 µL. Oligonucleotide primers for human genes were obtained from Integrated DNA Technologies (IDT) and are as listed in Supplementary Table SI. RT-PCR was performed on ABI PRISM Sequence Detection System (model 7000, Applied Biosystems) with the following protocol: 95°C for 15 seconds (denaturing) and 60°C for 60 seconds (annealing), repeated for 40 cycles. Threshold cycle (Ct) values for analyzed genes (in duplicate) were normalized as compared to GAPDH (cell lines) or 18S (human samples) Ct values. Relative abundance was calculated as 2^(-ΔΔCt), with ΔCt being the Ct value of the analyzed gene minus the Ct value of the reference gene (GAPDH or 18S).

Western blot analysis
Cell lysates were standardized for protein content, resolved on 4% to -12% SDS-PAGE, and blotted onto nitrocellulose membranes. Membranes were probed with rabbit anti-MyD88 (1:500, Cell Signaling), anti-IL1R1 (1:500, Santa Cruz), and anti-β-actin (1:5000, Thermo Scientific). Antibody binding was detected by using an ECL Chemiluminescence kit (Amersham).

Adenoviral vectors
Construction and characterization of adenoviral vectors encoding wild-type and dominant-negative NADPH oxidase-4 (NOX4) have each been described previously (10, 21). An empty vector lacking the NOX4 construct was used as a control. All vectors were obtained from the University of Iowa Gene Vector Core. HNSCC cells in serum-free media were infected with 100 multiplicity of infection (MOI) of the above-described adenoviral vectors for 24 hours. Biochemical analyses were performed 72 to 96 hours after transfection.

siRNA/shRNA transfection
MyD88, TLR2, TLR5, and control siRNA (Santa Cruz) were transfected into HNSCC cells at a concentration of 40 to 80 nmol/L with equal volume Lipofectamine RNAiMAX (Invitrogen). Cells were incubated in Opti-MEM for 4 hours before addition of siRNA and 16 hours after addition of siRNA. For shRNA transfection, SQ20B cells were transfected with 1 µg/mL of pSilRNA-h5SKGFpzeo, pSilRNA-shMyD88, or pSilRNA-shhIL1R (Inviogen) in the presence of Opti-MEM and Lipofectamine RNAiMAX. Cells were allowed to recover 48 to 72 hours in antibiotic-free DMEM with 10% FBS before 48-hour erlotinib treatment. Knockdown was confirmed by RT-PCR and/or Western blotting.

Clonogenic survival assay
Clonogenic survival was determined as previously described (22). Individual assays were performed with multiple dilutions with at least 4 cloning dishes per data point, repeated in at least three separate experiments.

Tumor cell implantation
Male and female athymic nu/nu mice (4–5 weeks old) were purchased from Harlan Laboratories. Mice were housed in a
pathogen-free barrier room in the Animal Care Facility at the University of Iowa and handled using aseptic procedures. All procedures were approved by the IACUC committee of the University of Iowa and conformed to the guidelines established by the NIH. Mice were allowed at least 3 days to acclimatize before beginning experimentation, and food and water were made freely available. Tumor cells were inoculated into nude mice by subcutaneous injection of 0.1-mL aliquots of saline containing 2 × 10^6 SQ20B cells into the right flank using 26-gauge needles.

**In vivo drug administration**

Mice started drug treatment 1 week after tumor inoculation. For the MyD88 knockdown experiments, female mice were randomized into two treatment groups and orally administered either water or 12.5 mg/kg erlotinib daily. For the IL1α neutralization experiments, male and female mice were randomized into four treatment groups as follows. Control group: Mice were administered water orally daily and 1 mg/kg IgG intraperitoneally once per week. Neutralizing IL1α antibody (nIL1αab) group: A human IL1α neutralizing antibody (XBiotech) was administered intra- peritoneally at 100 μg/mouse once per week. Erlotinib group: Erlotinib was administered orally 12.5 mg/kg daily. Erlotinib + nIL1αab group: Erlotinib was administered orally 12.5 mg/kg daily in addition to nIL1αab administered intraperitoneally at 100 μg/mouse once per week. For experiments involving cetuximab, cetuximab was administered intraperitoneally 0.2 mg per mouse twice per week and control mice were given IgG twice per week. All treatments were given for the duration of 3 weeks. Mice were evaluated daily and tumor measurements taken 3 times per week using Vernier calipers. Tumor volumes were calculated using the formula: tumor volume = (length × width^2)/2 where the length was the longest dimension and width was the dimension perpendicular to length. Mice were euthanized via CO2 gas asphyxiation or lethal overdose of sodium pentobarbital (100 mg/kg) when tumor diameter exceeded 1.5 cm in any dimension.

**Bioinformatics**

The Cancer Genome Browser (University of California-Santa Cruz; https://genome-cancer.ucsc.edu) was used to download the level 3 dataset HNSCC dataset (TCGA_HNSC_exp_HiSeqV2_PANCAN) from The Cancer Genome Atlas (TCGA). RNAseq data was normalized across all TCGA cohorts and reported as log2 values. Corresponding level 3 clinical data were available for most of the 467 samples. Selected tumors (n = 41) also had RNAseq data for matched normal tissue. Matched tumor and normal samples were analyzed. Linear fold change was calculated to emphasize difference between groups. Kaplan–Meier survival curves were generated by comparing survival of the highest quartile of expressing tumors (for indicated gene) against the lowest quartile. In some cases, Kaplan–Meier curves were generated using an aggregate of several genes. The genes aggregated are as follows: TLR (TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10), IL18R (IL18Rαa, IL18Rαb), and IL1R (IL1R1, IL1Rα). Tumors were ranked according to expression of each gene, and ranks were averaged to determine highest and lowest quartile of tumors expressing the given receptor family.

**Statistical analysis**

Statistical analysis was done using GraphPad Prism version 5 for Windows (GraphPad Software). Differences between 3 or more means were determined by one-way ANOVA with Tukey post tests. Linear mixed-effects regression models were used to estimate and compare the group-specific change in tumor growth curves. Differences in survival curves were determined by Mantel–Cox test. All statistical analysis was performed at the P < 0.05 level of significance.

**Results**

**Erlotinib induces processes involved in inflammation**

Of the top 10 upregulated cellular process networks identified by erlotinib treatment, 6 processes were related to immune response or inflammation for both cell lines (Fig. 1A and B). The top 10 significant diseases that were identified from erlotinib treatment were predominantly systemic inflammatory disorders in both cell lines such as rheumatic diseases/disorders (rheumatic arthritis, rheumatic fever, rheumatic heart disease; Fig. 1C and D). Similarly, the majority of the top 10 upregulated canonical pathways were immune response/inflammation related in both cell lines, which included IL6 and IL1 signaling in SQ20B cells (Fig. 2A) and TLR and IL1 signaling in Cal-27 cells (Fig. 2B).

The top network identified for SQ20B and Cal-27 was the NF-kB, MyD88, IκB, IRAK1/2, NF-xB2 (p100) network (Fig. 2C) and TRAF6, TAK1 (MAP3K7), NF-kB, IκB, IKK-γ network (Fig. 2D), respectively. The genes and processes in these networks were both related to MyD88-dependent TLR signaling and NF-kB activity (Supplementary Tables S2 and S3). Altogether, the gene expression analyses suggested that erlotinib activates inflammatory processes and pathways that may be mediated by MyD88.

**Loss of MyD88 increases tumor sensitivity to erlotinib**

We have previously shown that erlotinib induces the secretion of IL6 and other proinflammatory cytokines via NF-kB activation in HNSCC cells (10), which supports the gene expression results (Figs. 1 and 2). Transient knockdown of MyD88 significantly suppressed baseline and erlotinib-induced IL6 production in both SQ20B (Fig. 3A) and Cal-27 cells (Fig. 3B). MyD88 stable knockout clones (shMyD88#2, shMyD88#9) also demonstrated significantly reduced IL6 in the absence and presence of erlotinib compared with control (Fig. 3C) supporting the role of MyD88-dependent signaling in erlotinib-induced IL6 production. Both MyD88-knockout clones showed reduced tumor growth when treated with erlotinib compared with erlotinib-treated control xenografts (Fig. 3D–G). Notably, xenografts bearing the shMyD88 #9 clone showed reduced tumor growth in both treated and untreated groups (Fig. 3D and G). Altogether these results suggest that MyD88-dependent signaling is involved in erlotinib-induced IL6 secretion and suppresses the antitumor activity of erlotinib.

**TLR5 signaling may be involved in erlotinib-induced IL6 secretion**

A general trend of increased TLR, IL1R, and IL18R RNA expression was found in HNSCC human tumors [obtained from the Tissue Procurement Core (TPC) in the Department of Pathology] compared with matched normal tissue (Fig. 4A and B). Notably, both tumors showed large increases in expression of TLR2 compared with normal matched tissue (Fig. 4A and B). IL6 secretion was significantly increased after treatment with agonists to TLR1/2, TLR2/6, and TLR3 in all 3 cell lines (Fig. 4C), although...
TLR5 appeared to be active in only SQ20B cells (Fig. 4C). Erlotinib increased TLR8 expression in SQ20B cells and TLR10 in Cal-27 cells, although the absolute levels of these TLRs were very low and most likely not of biologic significance (Fig. 4D). As the TLR1/2 and TLR2/6 dimers both depend on TLR2, the activity of these dimers were suppressed using siRNA targeted to TLR2 (Fig. 4E and F). Knockdown of TLR2 expression did not decrease erlotinib-induced IL6 (Fig. 4E). However, knockdown of TLR5 expression partially but significantly suppressed erlotinib-induced IL6 secretion in SQ20B cells (Fig. 4G and H), which was not observed in Cal-27 cells (data not shown). TLR3, which is not a MyD88-dependent receptor also, was not involved in erlotinib-induced IL6 in both cell lines (Supplementary Fig. S1). Altogether, these results suggest that of the TLRs, only TLR5 signaling may contribute to IL6 secretion induced by erlotinib in select HNSCC cell lines.

IL1 signaling is critical for erlotinib-induced IL6 expression in HNSCC cells

To investigate the contribution of other MyD88-dependent signaling pathways, the IL18R and IL1R pathways were studied. Neutralization of IL18R in SQ20B (Fig. 4I) and Cal-27 (Fig. 4J) failed to suppress erlotinib-induced IL6. However, anakinra, a recombinant IL1R antagonist (IL1RA/IL1RN) significantly reduced baseline and erlotinib-induced IL6 in both SQ20B (Fig. 5A) and Cal-27 (Fig. 5B). In addition, transient (Supplementary Fig. S2) and stable knockdown of IL1R suppressed erlotinib-induced IL6 (Fig. 5C), suggesting that IL1R signaling may be involved in erlotinib-induced IL6. Sequenced HNSCC tumors and matched normal tissue (n = 40) were analyzed from TCGA for mRNA levels of ligands of the IL1 pathway. IL1α and IL1β were found to be increased in tumors by 4.8- and 2.5-fold, respectively, compared with normal samples while IL1RA/IL1RN was decreased by 2.5-fold (Fig. 5D). IL1α was also upregulated in both HNSCC tumors analyzed in Fig. 4A and B while IL1β was only upregulated in one of these tumors (Supplementary Fig. S3). IL1α but not IL1β was detectable after erlotinib treatment and increased across all time points measured in both cell lines (Fig. 5E). Exogenous IL1α increased IL6 secretion in the presence and absence of erlotinib (Fig. 5F) and blockade of IL1α abut not of IL1β activity significantly reduced IL6 secretion in the absence and presence of erlotinib (Fig. 5G), suggesting that IL1α release may be responsible for erlotinib-induced IL6 production.
Erlotinib-induced cell death triggers IL1α release

IL1α unlike IL1β is not secreted but is typically released by cell death. To confirm this, we showed that Z-VAD-fmk (ZVAD), a pan-caspase inhibitor, significantly reduced baseline and erlotinib-induced levels of IL1α (Fig. 6A) and blocked erlotinib-induced cell death (Supplementary Fig. S4), suggesting that IL1α is likely released because of erlotinib-induced cell death. These results were not observed with the caspase-1 inhibitor, Ac-Y-VAD-cho (YVAD, Fig. 6A). Our laboratory has previously shown that erlotinib induces mixed cell death via H2O2-mediated oxidative stress due to NOX4 activity (23). To confirm that oxidative stress is involved in IL1α release, we showed that the antioxidants NAC and CAT significantly suppressed erlotinib-induced IL1α in addition to IL6 in both SQ20B (Fig. 6B) and Cal-27 cells (Fig. 6C). We have previously shown that these antioxidants significantly protect these HNSCC cell lines from erlotinib-induced cytotoxicity (23). Moreover, overexpression of dominant-negative NOX4 (N4dn) decreased erlotinib-induced IL1α, IL6 production (Fig. 6D and E) and cytotoxicity (Fig. 6F) in both SQ20B (Fig. 6D and F) and Cal-27 (Fig. 6E and F). The opposite results were observed with wild-type NOX4 (N4wt; Fig. 6D–F). The ability of N4wt (and not N4dn) to significantly induce oxidative stress in these cell lines has been demonstrated in our previous publications (10, 21). Altogether, these results suggest that erlotinib-induced oxidative stress (via NOX4) results in cell death, leading to IL1α release resulting in activation of IL1R signaling in unaffected/surviving cells leading to IL6 expression and secretion.

IL1α is negatively correlated with survival in HNSCC

Sequenced HNSCC tumors (TCGA, n = 467) with high expression of MyD88, TLRs, IL1R, IL1α, IL1β, and IL1RA were plotted for survival against low expressing tumors (Fig. 7A–H). MyD88, TLRs, IL1R, IL1α, IL1β, and IL1RA were not significantly correlated with survival (Fig. 7A–C, G, and H).
High IL1R expressing tumors showed a trend ($P = 0.06$) toward a negative correlation with survival (Fig. 7D) while IL1α mRNA expression was negatively correlated ($P = 0.04$) with survival (Fig. 7E). Selected tumors from patients who received targeted molecular therapy (TMT, $n = 40$) showed an increased negative correlation with survival ($P = 0.02$, Fig. 7F), suggesting that IL1α expression may be an important prognostic marker in HNSCC.

Finally, we showed that SQ20B cells treated with an IL1α neutralizing antibody (XBiotech; ref. 24) in combination with erlotinib displayed a significant reduction in survival compared with the other treatment groups in vitro (Fig. 7I) and in vivo (Fig. 7J). Similar results were observed with cetuximab in vivo (Fig. 7K), suggesting that blockade of the IL1 pathway may increase the sensitivity of erlotinib and other EGFRIs. Altogether, our results and previous findings suggest that erlotinib (and
perhaps other EGFRIs) induce cell death via H$_2$O$_2$-mediated oxidative stress due to NOX4 activity leading to IL1-a release and activation of the IL1R/MyD88/NF-$\kappa$B signaling axis on surviving tumor cells resulting in IL6 secretion (Fig. 7L). Our results also propose that another unidentified DAMP may be released that activates the TLR5/MyD88/NF-$\kappa$B signaling axis, resulting in IL6 secretion. This IL6 signaling is believed to reduce the antitumor activity of EGFRIs and promote tumor progression (Fig. 7L).

Discussion

Our laboratory has previously shown that EGFRIs increased IL6 secretion and that IL6 levels played a critical role in the antitumor effect of erlotinib in vitro and in vivo (10), which has been supported and studied in depth by other groups (15–18). The studies presented here now indicate that MyD88-dependent IL1R signaling is most likely responsible for the IL6 production induced by EGFRIs. Therefore, targeting IL1 signaling may be a novel strategy to increase the antitumor activity of EGFRIs and promote tumor progression (Fig. 7L).

Figure 4.
Role of TLR signaling in erlotinib (ERL)-induced IL6 in HNSCC cells. A and B, RNA isolated from two HNSCC tumors [#9 (A) and #13 (B); gray bars] and matched normal tissue (black bars) was analyzed for TLR1–10, IL1R, and IL18R gene expression by RT-PCR. C, SQ20B, Cal-27, and FaDu cells were treated with TLR agonists as described in Materials and Methods. Secreted IL6 was measured by ELISA. D, SQ20B and Cal-27 were treated with DMSO or 5 μmol/L erlotinib for 48 hours. Cells were analyzed by RT-PCR for the expression of TLR genes. Values were normalized to 18S mRNA levels and reported as fold change over DMSO (set at 1, dotted line). E–H, SQ20B or Cal-27 cells were transfected with scrambled siRNA control (siCON), siRNA targeted against TLR2 (sTLR2; E and F), or siRNA targeted against TLR5 (sTLR5; G and H), treated with DMSO or 5 μmol/L erlotinib, and then analyzed for IL6. Knockdown of respective TLRs was confirmed by RT-PCR. I–H, SQ20B or Cal-27 cells were treated with IgG or an IL18R neutralizing antibody (nIL18Rab, 0.5 μg/mL) for 2 hours before DMSO or erlotinib (5 μmol/L) before IL6 analysis. n = 3; errors bars, SEM. * P < 0.05 versus control; ** P < 0.05 versus erlotinib.

of the ligands in the IL1 family, IL1-a is the most well-studied and its production is dependent on inflammasome-mediated caspase-1 activity (28). In the present studies, we believe that IL1-a and not IL1-b is involved in the activation of the IL1R/MyD88/IL6 pathway by erlotinib, as we were unable to observations support one other study showing that the EGFRI PD153035 upregulated genes related to inflammation and innate immunity (25). Interestingly, the inflammatory profile displayed by erlotinib treatment was remarkably similar to that of rheumatic diseases and other systemic inflammatory disorders (Fig. 1C and D). In fact, inhibition of the IL1 pathway is a well-documented strategy for the treatment of rheumatoid arthritis (RA), as IL1R ligands (IL1-a and IL1-b) are particularly abundant in the synovial lining of the joint (26). Anakinra is a humanized recombinant IL1R antagonist (IL1RA) that is FDA-approved for use in the treatment of RA. IL1RA is an IL1R ligand that inhibits the IL1 pathway through competition with the other IL1R ligands (27). In support of this, we have shown that anakinra effectively blocked erlotinib-induced IL6 in HNSCC cell lines (Fig. 5A and B).
detect any secreted IL1β and suppression of IL1β using a neutralizing IL1β antibody or a caspase-1 inhibitor did not affect erlotinib-induced IL6 (Figs. 4E and G and 6A). On the other hand, we were able to detect IL1α (Fig. 5E) and suppression of IL1α significantly blocked erlotinib-induced IL6 (Fig. 5G), suggesting that IL1α was the ligand responsible for activating the IL1 pathway.

Unlike IL1β, IL1α is not secreted from the cell but is released during cell death and acts as a DAMP (29). It is likely that the cell death induced by erlotinib treatment resulted in IL1α release, as the use of ZVAD blocked erlotinib-induced cell death (Supplementary Fig. S4) and IL1α release (Fig. 6A). Furthermore, our laboratory has previously shown that erlotinib induces cell death via H2O2-mediated oxidative stress due to NOX4 activity (23). We have now extended these findings to show that IL1α release in addition to downstream IL6 secretion is mediated by erlotinib-induced cell death due to NOX4-induced oxidative stress (Fig. 6B–F).

Our gene expression analyses also implicated TLR/MyD88 signaling (especially TLR2) as a possible mediator of erlotinib-induced IL6 (Fig. 2); however, we found no evidence of TLR2 involvement despite TLR2 being present and active on HNSCC tumors and cell lines (Fig. 4A–C). Surprisingly, we found that TLR2 knockdown increased IL6 secretion (Fig. 4E). An explanation for these results is unclear, although one prior report has shown that activation of TLR2 resulted in decreased NF-kB activity via increased miR-329 leading to decreased IL6 expression in human trophoblast cells (30). Perhaps in our HNSCC cell model, inhibition of TLR2 expression decreased levels of miR-329 resulting in increased NF-kB and IL6 secretion, which would be consistent with the previous findings in trophoblast cells (30).

Interestingly, TLR5 was active in only SQ20B cells (Fig. 4C) and IL6 secretion, which would be consistent with the previous findings in trophoblast cells (30).

Given that IL1α appears to be the ligand that triggers the IL1R/MyD88/IL6 cascade that we believe is responsible for poor response to EGFRIs, then in theory, neutralization of IL1α should increase the antitumor efficacy of EGFRIs in the same manner as blockade of IL6 as previously shown by our laboratory (10, 15–18). Indeed we observed that IL1α neutralization significantly increased the antitumor efficacy of erlotinib (Fig. 7I) in addition to cetuximab (Fig. 7K) in SQ20B cells. These exciting results suggest that IL1α plays an important role in response to EGFRIs. Moreover, we want to highlight that the observed effects of erlotinib in our studies are believed to be directly due to cell death mediated by EGFR inhibition and not due to off-target effects of the drugs as (i) we are using clinical achievable doses (31) and (ii) we have already confirmed the ability of EGFR knockdown (using siRNA targeted to EGFR) to induce oxidative stress, cell death, and cytokine secretion (10, 23).

To further stress the importance of IL1α in the management of HNSCC, we found that HNSCC tumors expressed high levels of IL1α compared with matched normal tissue (Fig. 5D) and high IL1α-expressing tumors have worse prognosis than low IL1α-expressing tumors (Fig. 7E). Furthermore, when
we selected for tumors from patients receiving TMT, we found an increased separation and significance between the survival curves (Fig. 7F), suggesting that IL1α expression may not only predict overall survival in HNSCC but also predict response to TMT. Unfortunately, the clinical information associated with the tumors from patients that received TMT did not reveal what treatment regimen was administered; therefore, we cannot make firm conclusions from this analysis. However, as the only TMT currently used in HNSCC is EGFR-targeting drugs and the only approved EGFRI for HNSCC to date is cetuximab, it is more likely than not that the TMT involved cetuximab in our analysis.

Suppression of MyD88 effectively blocked erlotinib-induced IL6 production and suppressed tumor growth in the presence of erlotinib (Fig. 3), which is likely due to the ability of MyD88 knockdown to block all potential proinflammatory signaling from MyD88-dependent receptors. It is unclear why control-treated shMyD88 #9 tumors displayed such a pronounced inhibition of tumor growth (Fig. 3E) compared with control-treated shMyD88 #2 tumors (Fig. 3D). Previous reports have shown that MyD88 signaling may induce EGFRI ligands such as amphiregulin (AREG) and epiregulin (EREG), resulting in the activation of EGFRI (32). Perhaps knockdown of MyD88 expression in the shMyD88 #9 clone led to the inhibition of EGFRI via downregulation of AREG/EREG in addition to suppression of IL6, which may explain our observations. Nevertheless, these results suggest that MyD88 inhibition may also be a promising strategy to increase the effect of erlotinib.

It should be noted that global inhibition of MyD88, IL1α, or any factor in the IL1R/MyD88/IL6 signaling axis in vivo may have unexpected results. Our model takes into account only the activity of MyD88 or IL1α within cancer cells. Inhibition of these inflammatory components in innate immune cells may change the inflammatory microenvironment especially in an immunocompetent mouse model, conceivably altering recruitment of immune cells and unpredictably altering growth of the tumor. This remains to be studied.

On the basis of these findings and our prior studies (10, 21, 23), we propose a model in which EGFR inhibition causes cell death and release of IL1α, which we believe binds its receptor IL1R on surviving cells, activates MyD88, and induces IL6 secretion via NF-κB (Fig. 7L). IL6 signaling pathways typically lead to phosphorylation of STAT3, which is well known to compensating for the loss of EGFR signaling due to cross-talk (33). As such, we believe that the poor response and possibly acquired resistance to erlotinib in the clinical setting may be due to IL1R/MyD88/IL6 signaling triggered by release of IL1α from dying cells, which is different from other proposed mechanisms of poor response/acquired resistance (acquired mutations, alternative signaling pathways; refs. 6–9). To our knowledge, the studies presented here are the first to connect IL1α and MyD88-dependent signaling with response to EGFR-targeted therapy and this novel mechanism may offer insight.
into why other methods of overcoming EGFRi resistance have failed and proposes new clinical targets that may enhance the efficacy of EGFRis in HNSCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A.T. Koch, A.L. Simons
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Figure 7.
IL1α expression affects response to EGFR inhibitors in HNSCC. A dataset (n = 88) of HNSCC tumors from TCGA was analyzed for MyD88 (A), TLRs (B), IL18R (C), IL1α (E), IL1β (G), and IL1RN (H) expression. A dataset (n = 48) of HNSCC tumors from patients who received TMT was also analyzed for IL1α expression (F). The highest quartile of expressing tumors was plotted against the lowest quartile in Kaplan–Meier survival curves. SQ20B cells were treated with IL1α (anti-IL1α) or IL1β (anti-IL1β) neutralizing antibodies for 2 hours before treatment with DMSO (black bars) or erlotinib (ERL; 5 μmol/L, gray bars) for 48 hours and then analyzed for clonogenic survival, n = 3 (I). J and K, athymic (nu/nu) mice bearing SQ20B xenograft tumors were treated as described in Materials and Methods. Data points represent the average tumor volume values for 10 to 11 mice (J and K). L, schematic representing the proposed role of IL1 signaling in the reduced effect of erlotinib in HNSCC. Error bars, SEM. *, P < 0.05 versus control; **, P < 0.05 versus erlotinib.
References


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