# Respiratory virus–induced EGFR activation suppresses IRF1-dependent interferon λ and antiviral defense in airway epithelium

Iris F. Ueki,[1,2] Gundula Min-Oo,[3,4] April Kalinowski,[5] Eric Ballon-Landa,[6] Lewis L. Lanier,[3,4] Jay A. Nadel,[1,2] and Jonathan L. Koff[5]

[1]Department of Medicine, [2]Cardiovascular Research Institute, [3]Department of Microbiology and Immunology, and [4]Cancer Research Institute, University of California, San Francisco, San Francisco, CA 94122
[5]Department of Medicine, Yale University, New Haven, CT 06520
[6]University of California, Irvine, Irvine, CA 92697

**Viruses suppress host responses to increase infection, and understanding these mechanisms has provided insights into cellular signaling and led to novel therapies. Many viruses (e.g., Influenza virus, Rhinovirus [RV], Cytomegalovirus, Epstein–Barr virus, and Hepatitis C virus) activate epithelial epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, but the role of EGFR in viral pathogenesis is not clear. Interferon (IFN) signaling is a critical innate antiviral host response and recent experiments have implicated IFN-λ, a type III IFN, as the most significant IFN for mucosal antiviral immune responses. Despite the importance of IFN-λ in epithelial antiviral responses, the role and mechanisms of epithelial IFN-λ signaling have not been fully elucidated. We report that respiratory virus–induced EGFR activation suppresses endogenous airway epithelial antiviral signaling. We found that Influenza virus– and RV-induced EGFR activation suppressed IFN regulatory factor (IRF) 1–induced IFN-λ production and increased viral infection. In addition, inhibition of EGFR during viral infection augmented IRF1 and IFN-λ, which resulted in decreased viral titers in vitro and in vivo. These findings describe a novel mechanism that viruses use to suppress endogenous antiviral defenses, and provide potential targets for future therapies.**

CORRESPONDENCE
Jonathan Koff:
jon.koff@yale.edu

Abbreviations used: AEC, airway epithelial cell; BAL, bronchoalveolar lavage; EGFR, epidermal growth factor receptor; IAV, Influenza A virus; IRF, IFN regulatory factor; MP, metalloproteinase; NHBE, normal human bronchial epithelial; Nox, NADPH oxidase; ROS, reactive oxygen species; RV, Rhinovirus.

Respiratory viral infections, which cause pneumonia and exacerbations of chronic lung diseases, are responsible for significant morbidity and mortality. Despite substantial disease burden, there are limited therapies for treating virus-induced pulmonary disease. Viruses induce inflammation, which impairs host responses. Upon infection of airway epithelial cells (AECs), the primary cell type for respiratory viral infection, viruses induce epithelial production of IL-8 (Choi and Jacoby, 1992; Subauste et al., 1995). Our research, and that of other investigators, has shown that virus-induced AEC IL-8 production requires epidermal growth factor receptor (EGFR) activation (Monick et al., 2005; Koff et al., 2008; Liu et al., 2008). Therefore, we investigated the effect of virus-induced EGFR activation on airway epithelial antiviral responses.

EGFR (ErbB1/HER1), a tyrosine kinase receptor present in epithelial cells, is activated in a ligand-dependent manner (Shao et al., 2003). In AECs, EGFR activation involves an integrated signaling pathway that includes NADPH oxidase (Nox) activation of a metalloproteinase (MP), which cleaves an EGFR pro-ligand that is released to bind to, and to activate EGFR (Shao and Nadel, 2005; Burgel and Nadel, 2008). Recently, viruses have been shown to activate EGFR via this signaling pathway in AECs (Koff et al., 2008; Zhu et al., 2009; Barbier et al., 2012).

IFN signaling is a critical innate antiviral host response. Recent experiments have suggested that IFN-λ, a recently discovered type III IFN, is the most significant IFN in AECs (Khaitov et al., 2009; Mordstein et al., 2010). Studies suggest that IFN-λ is the primary IFN that regulates mucosal responses to viral infection, whereas type I IFNs (e.g., IFN-α and -β) are essential for clearance of systemic infection (Jewell et al.,



The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 10 1929-1936
www.jem.org/cgi/doi/10.1084/jem.20121401

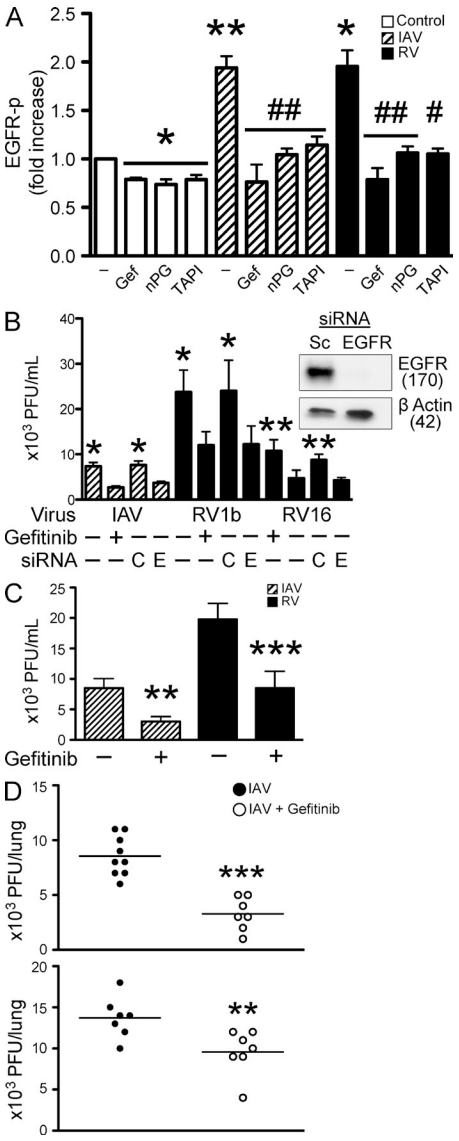


**Figure 1. Role of EGFR in respiratory viral infection.** (A) EGFR-p was measured by ELISA at 10 min in BEAS-2b cell culture lysates. Cells were treated with serum-free medium alone (control), with the selective EGFR tyrosine kinase inhibitor 10 µM Gefitinib, ROS scavenger (nPG, 100 µM), MP inhibitor (TAPI, 10 µM), with IAV and RV1b alone, or with the addition of nPG and TAPI ($n$ = 3–5 independent experiments, mean ± SEM; *, $P < 0.05$ and **, $P < 0.005$ vs. control; #, $P < 0.01$ and ##, $P < 0.005$ vs. each virus alone). (B) BEAS-2b cells were treated with IAV, RV1b, and RV16 alone, with 10 µM Gefitinib alone, or transfected with control (C) or EGFR siRNA (E). After 24 h, cell culture homogenates were collected and virus was quantified by plaque assay ($n$ = 3–4 independent experiments, mean ± SEM; *, $P < 0.05$ and **, $P < 0.01$ vs. virus plus Gefitinib or EGFR siRNA). BEAS-2b cells were transfected with EGFR siRNA and EGFR protein was assessed by Western blot (representative of three independent experiments). Molecular masses are provided in kilodaltons. (C) NHBE cells were treated with IAV and RV16 alone, or with 10 µM Gefitinib for 24 h and viral titers in cell culture homogenates were quantified by plaque assay ($n$ = 4 independent experiments, mean ± SEM; **, $P < 0.005$ and ***, $P < 0.0001$ vs. virus alone). (D) C57BL/6 mice were infected (intranasal) with IAV ($10^{4.5}$ TCID50%) alone, or with 50 mg/kg Gefitinib and viral titers were quantified by plaque assay at 48 h. In a prophylaxis model (top), Gefitinib was given 16 h before viral infection and then continued daily, and in a therapeutic model (bottom), Gefitinib was given 1 h after viral infection and then continued daily ($n$ = 7–9 mice/group repeated twice, mean ± SEM; **, $P < 0.01$ and ***, $P < 0.001$ vs. virus alone).

2010; Mordstein et al., 2010). Despite the importance of IFN-λ in epithelial antiviral responses, the kinetics of airway epithelial IFN-λ production has not been fully elucidated. For example, IFN regulatory factors (IRFs), critical for type I and II IFN signaling (Tamura et al., 2008), have not been analyzed in epithelial IFN-λ production. In addition, the potential for EGFR signaling to suppress IFN-λ has not been explored.

Influenza A virus (IAV) and Rhinovirus (RV) are ssRNA viruses that are significant pathogens that cause viral pneumonia and induce exacerbations of asthma and chronic obstructive pulmonary disease (Johnston, 2005). Recently, both viruses were shown to activate EGFR via Nox and MP-induced release of EGFR ligand (Liu et al., 2008; Zhu et al., 2009; Barbier et al., 2012). Both IAV and RV stimulate epithelial IFN-λ production, and IFN-λ was implicated in effective clearance of these viruses (Contoli et al., 2006; Jewell et al., 2010). Although the role of IRF in epithelial IFN-λ production has not been explored, RV was found to activate IRF1, IRF3, and IRF7 in AECs (Wang et al., 2009b; Zaheer and Proud, 2010).

Here, we examined the interaction between virus-induced EGFR signaling and IFN-λ production in AECs. IAV and RV activated EGFR, and EGFR activation suppressed IRF1-induced IFN-λ production and increased viral infection. In addition, inhibition of EGFR during viral infection augmented IRF1 and IFN-λ production, which resulted in decreased viral titers in vitro and in vivo.

## RESULTS AND DISCUSSION

### Role for EGFR in respiratory viral infection

To confirm a role for respiratory virus-induced EGFR activation in AECs, we measured total EGFR phosphorylation (EGFR-p) by ELISA after viral infection in an airway epithelial (BEAS-2b) cell line. IAV and RV stimulated EGFR-p, and the addition of a reactive oxygen species (ROS) scavenger (nPG) and an MP inhibitor (TAPI-1) decreased IAV- and RV-induced EGFR-p (Fig. 1 A). These results confirmed that virus-induced EGFR activation involves ROS and MP cleavage of an EGFR ligand (Zhu et al., 2009; Barbier et al., 2012). Nox produce ROS and are upstream of EGFR activation (Shao and Nadel, 2005). We found that IAV, RV1b, and RV16 induced Nox in BEAS-2b cells (IAV 91.6 ± 11.5, RV1b 93.6 ± 9.2, and RV16 99.1 ± 10.9 vs. control 58.7 ± 9.4 nM $NADP^+$; $P < 0.001$, $n = 5$), a result which implicates Nox as a shared epithelial signal in response to multiple respiratory viruses.

Epithelial inflammation augments viral infection, and we hypothesized that EGFR plays a role in respiratory viral infection. To assess the effect of EGFR inhibition on viral infection

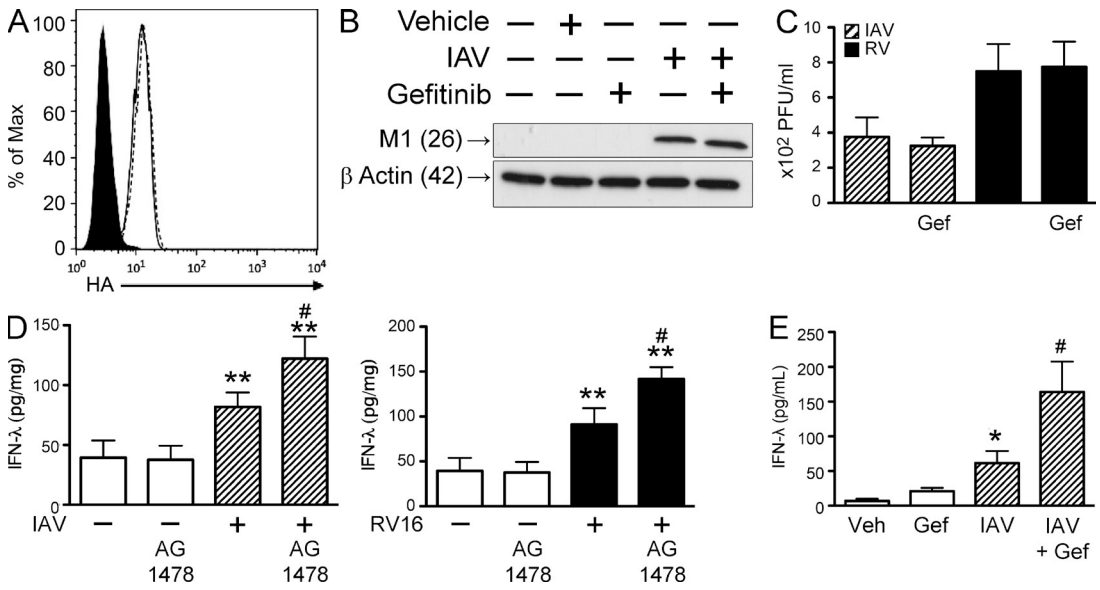


**Figure 2. Effect of EGFR inhibition on viral internalization and IFN-λ production.** (A) NHBE cells were treated with IAV alone, or with 10 µM Gefitinib for 2 h and analyzed by flow cytometry. Cells infected with IAV alone (solid line), or IAV plus Gefitinib (dashed line), were stained with anti-HA Ab or second step Ab alone (solid histogram). Data shown are representative of four independent experiments. Similar findings were obtained with anti-NP and anti-M1 Abs (not depicted). (B) BEAS-2b cells were treated with serum-free medium (first column), DMSO (vehicle; second column), 10 µM Gefitinib (third column), IAV alone (fourth column), or IAV + Gefitinib (fifth column) for 30 min and cell culture homogenates were analyzed by Western blotting for IAV M1 protein (molecular masses are provided in kilodaltons). Data shown are representative of three independent experiments. (C) BEAS-2b cells were treated with IAV and RV alone, or with 10 µM Gefitinib. After 2 h, cell culture homogenates were collected and virus was quantified by plaque assay ($n$ = 4 independent experiments, mean ± SEM). (D) NHBE cell cells were treated with serum-free medium alone (empty bars), 10 µM AG 1478, IAV and RV16 alone, or with AG 1478, and secreted IFN-λ was measured by ELISA at 24 h ($n$ = 3 independent experiments, mean ± SEM; **, $P < 0.005$ vs. control; #, $P < 0.05$ vs. each virus alone). (E) C57BL/6 mice were treated with vehicle (DMSO), 50 mg/kg Gefitinib, or infected (intranasal) with IAV ($10^{4.5}$ TCID50%), or IAV plus Gefitinib. After 24 h, BAL was collected and IFN-λ was measured by ELISA in BAL ($n$ = 6 mice/group representative of three independent experiments, mean ± SEM; *, $P < 0.01$ vs. vehicle alone; #, $P < 0.05$ vs. virus alone).

in AECs, BEAS-2b cells were infected with IAV, RV1b, and RV16 and treated with Gefitinib, a selective EGFR tyrosine kinase inhibitor. Viral titers were quantitated by plaque assay, and we found that EGFR inhibition suppressed viral infection significantly (Fig. 1 B), which has been suggested for IAV (Eierhoff et al., 2010; de Vries et al., 2011; Kumar et al., 2011a,b). These results were confirmed in normal human bronchial epithelial (NHBE) cells with IAV and RV16 (Fig. 1 C). To confirm the specificity of chemical inhibitors, we treated BEAS-2b cells with siRNA, which suppressed EGFR protein significantly (Fig. 1 B, right). Viral infection of BEAS-2b cells treated with EGFR siRNA was reduced, compared with cells stimulated by IAV, RV1b, and RV16 treated with control siRNA (Fig. 1 B). Finally, to test the effect of EGFR inhibition on respiratory viral infection in vivo, C57BL/6 mice were infected with IAV and viral titers were quantified by plaque assay at 48 h. In a prophylaxis model, where Gefitinib was given systemically 16 h before viral infection, and then continued daily, we found significant inhibition of IAV infection (Fig. 1 D, top). In a therapeutic model, Gefitinib was given 1 h after viral infection, and then continued daily (Fig. 1 D, bottom). Again, a significant reduction in IAV infection was observed, although the amount of viral suppression was less than in the prophylactic model. These results implicate an important role for EGFR in viral infection in vitro and in vivo.

### EGFR inhibition increases epithelial antiviral defense

To address the mechanism by which EGFR inhibition decreases respiratory viral infection, we initially investigated the role of EGFR in viral internalization. Although experiments using certain cancer cells have suggested a role for EGFR in IAV internalization (Eierhoff et al., 2010), we were not able to confirm this result in NHBE cells by qPCR measured at 30 min after infection (Ct values: 21.04 ± 0.11 [IAV] vs. 21.09 ± 0.14 [IAV plus Gefitinib]; $n$ = 4 independent experiments) by flow cytometry (Fig. 2 A), and in BEAS-2b cells by Western blot for IAV M1 protein (Fig. 2 B) and plaque assay (Fig. 2 C). Therefore, we evaluated the effect of EGFR signaling on endogenous airway epithelial antiviral responses.

IFNs play a critical role in innate and adaptive antiviral immunity. Recent studies have implicated IFN-λ as the most significant IFN in mucosal responses to viral infection (Khaitov et al., 2009; Mordstein et al., 2010). We confirmed that virus-infected NHBE cells produce significantly more IFN-λ than IFN-α or IFN-β (unpublished data). To investigate the role of EGFR signaling on epithelial IFN-λ, NHBE cells infected with IAV and RV16 were treated with a selective EGFR tyrosine kinase inhibitor, AG 1478. Both viruses induced IFN-λ production, and the addition of AG 1478 augmented IFN-λ production above the amount produced by virus alone (Fig. 2 D). These results were confirmed with RV1b (unpublished data).

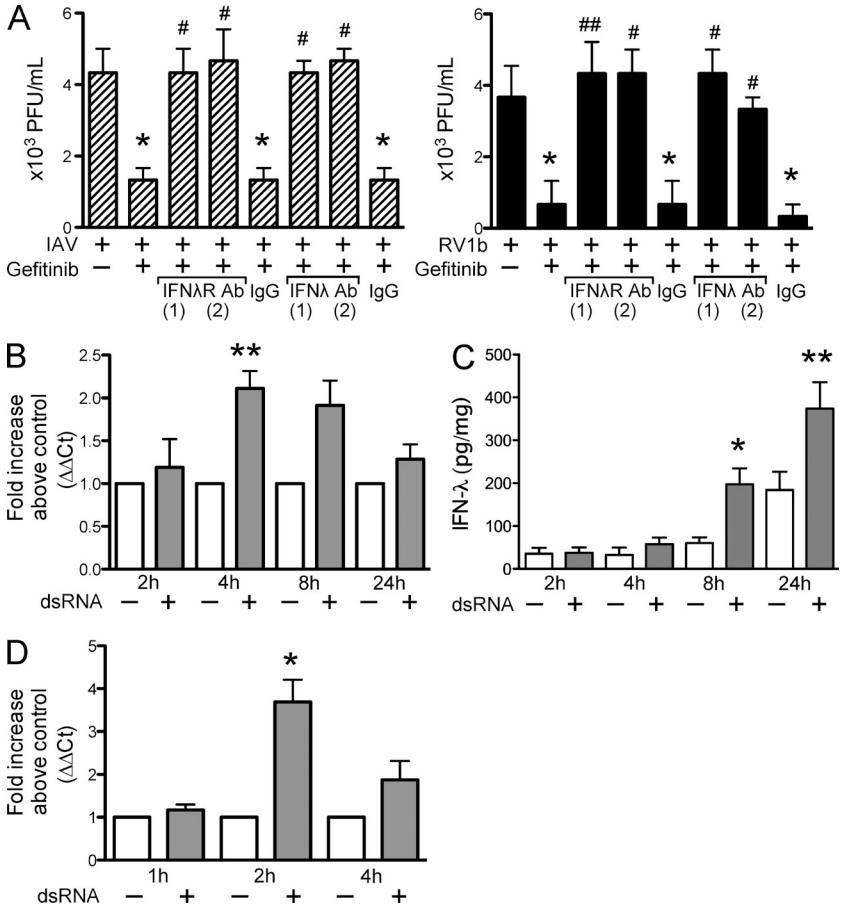


**Figure 3. IFN-λ is required for EGFR inhibitor-induced suppression of viral infection.** (A) BEAS-2b cells were treated with IAV and RV1b alone, with 10 µM Gefitinib, and Gefitinib plus two neutralizing Abs to IFN-λ receptor (columns 3 and 4), two neutralizing Abs to IFN-λ (columns 6 and 7), and isotype-matched Abs (columns 5 and 8) for 24 h and viral titers of cell culture homogenates were assessed by plaque assay ($n = 3$ independent experiments, mean ± SEM; *, $P < 0.05$ vs. virus alone; #, $P < 0.05$ and ##, $P < 0.01$ vs. virus plus Gefitinib). (B) BEAS-2b cells were treated with serum-free medium alone (empty columns), or dsRNA (gray columns), and IFN-λ mRNA was analyzed by quantitative RT-PCR ($n = 3$ independent experiments, mean ± SEM; **, $P < 0.005$ vs. serum-free medium). (C) BEAS-2b cells were treated with serum-free medium alone (empty columns), or dsRNA (gray columns), and secreted IFN-λ was measured by ELISA at 24 h ($n = 3$ independent experiments; *, $P < 0.01$ and **, $P < 0.005$ vs. serum-free medium). (D) BEAS-2b cells were treated with serum-free medium alone (empty columns), or dsRNA (gray columns), and IRF1 mRNA was analyzed by quantitative RT-PCR ($n = 3$ independent experiments; *, $P < 0.05$ vs. serum-free medium).

In addition, we found that Gefitinib-treated mice infected with IAV had increased IFN-λ in bronchoalveolar lavage (BAL) fluid compared with IAV infection alone (Fig. 2 E), which was associated with less IAV infection (Fig. 1 D). EGFR activation is a ligand-dependent process, and we confirmed that blocking ligand-induced EGFR activation increased IAV-, RV1b-, and RV16-induced IFN-λ production in vitro (unpublished data). These results implicated EGFR-p in virus-induced IFN-λ production in vitro and in vivo.

Because EGFR inhibition elevated AEC IFN-λ production, we examined the role of IFN-λ in the antiviral effects of EGFR inhibition. Neutralizing Abs targeting IFN-λ receptor (IFN-λR) and virus-induced IFN-λ were used to inhibit IFN-λ function, either by preventing IFN-λ binding to its receptor or by inactivating secreted IFN-λ. BEAS-2b cells were stimulated with IAV, with the addition of Gefitinib and IFN-λR or IFN-λ Abs, and viral infection was measured by plaque assay. The addition of Abs that suppressed IFN-λ function abrogated the ability of Gefitinib to inhibit IAV and RV1b infection, implicating IFN-λ in this process (Fig. 3 A). These results were confirmed with RV16 (unpublished data). Thus, airway epithelial IFN-λ is required for EGFR inhibition to suppress respiratory viral infection.

Next, we examined the kinetics of AEC IFN-λ production using synthetic dsRNA (poly I:C), an intermediate of ssRNA viral replication that is a common model of ssRNA viral infection. In BEAS-2b cells, dsRNA induced peak IFN-λ mRNA expression at 4 h (Fig. 3 B), which was associated with an increase in protein production at 8 h, which continued to increase at 24 h (Fig. 3 C).

IRFs play a critical role in IFN production. RV stimulates IRF1 in AECs (Zaheer and Proud, 2010) and recently, IRF1 was shown to interact with the IFN-λ promoter (Siegel et al., 2011), implicating IRF1 as a candidate for IFN-λ induction. Consistently, we found increased IRF1 mRNA at 2 h (Fig. 3 D). Furthermore, transfection of BEAS-2b cells with IRF1 siRNA significantly inhibited IFN-λ production in response to IAV, RV1b, and RV16 infection (Fig. 4 A), which implicates IRF1 in IFN-λ production. To examine the role of IRF1 in the antiviral effect of Gefitinib, BEAS-2b cells infected with IAV were treated with Gefitinib and IRF1 siRNA and compared, by plaque assay, with cells treated with Gefitinib and control siRNA. The addition of IRF1 siRNA abrogated EGFR inhibitor-induced IAV viral suppression (Fig. 4 B). These results were confirmed with RV1b and RV16 (unpublished data). In the IAV in vivo model, we found that Gefitinib-treated mice infected with IAV had increased IRF1 in the lung compared with vehicle-treated mice (Fig. 4 C). IRF3 and IRF7 are recognized to be critical for IFN α and β production (Tamura et al., 2008). dsRNA stimulation of BEAS-2b cells increased IRF3 and IRF7 mRNA expression at 12 h (IRF3) and 24 h (IRF7; Wang et al., 2009b), which is later than IFN-λ mRNA

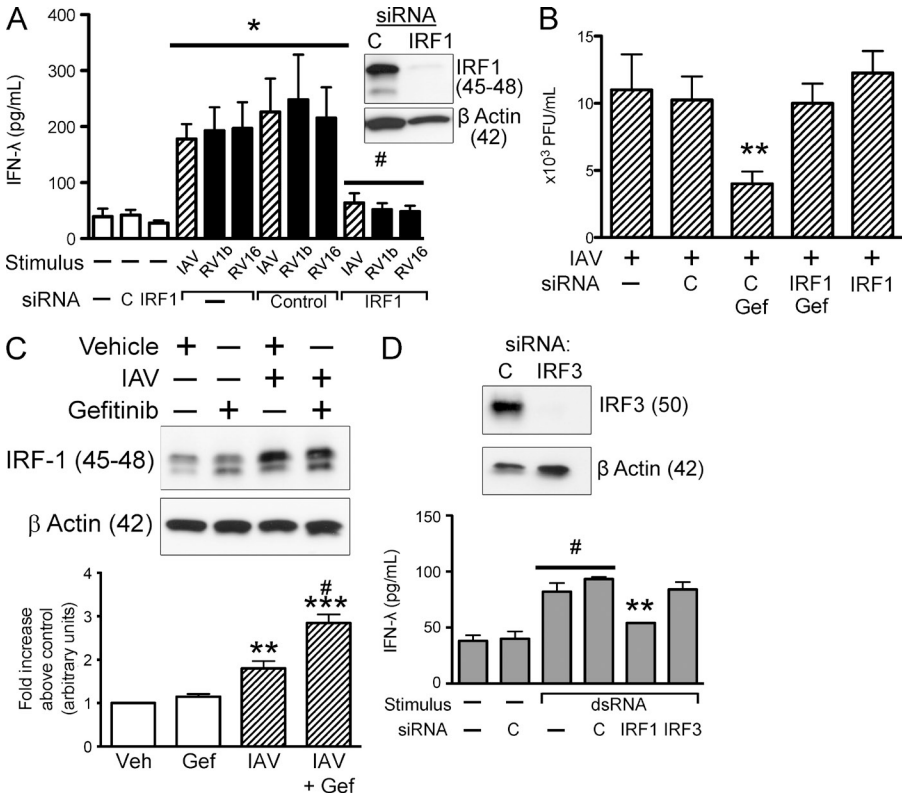


**Figure 4. IRF1-dependent IFN-λ is required for EGFR inhibitor-induced suppression of viral infection.** (A) BEAS-2b cells were treated with serum-free medium alone, or transfected with IRF1 or control (C) siRNA for 24 h and treated with serum-free medium alone (empty column), or IAV (striped column), RV1b (black column), and RV16. 24 h after viral infection secreted IFN-λ was measured by ELISA ($n = 6$ independent experiments, mean ± SEM; *, $P < 0.05$ vs. serum-free medium and C siRNA; #, $P < 0.05$ vs. C siRNA plus virus). BEAS-2b cells were transfected with IRF1 siRNA and IRF1 protein (molecular masses are provided in kilodaltons) was assessed by Western blotting (representative of three independent experiments). (B) BEAS-2b cells were treated with serum-free medium alone, or transfected with IRF1 or control (C) siRNA for 24 h and treated with IAV, or IAV plus 10 µM Gefitinib (Gef). After 24 h, cell culture homogenates were collected and viral titer was quantitated by plaque assay ($n = 4$ independent experiments, mean ± SEM; **, $P < 0.005$ vs. C and IRF1 siRNA plus Gef). (C) C57BL/6 mice were treated with vehicle (DMSO), 50 mg/kg Gefitinib, infected (intranasal) with IAV ($10^{4.5}$ TCID50%), or IAV plus Gefitinib. After 48 h, lungs were collected and IRF1 was measured by Western blotting (representative of five independent experiments; molecular masses are provided in kilodaltons). Densitometry (bottom) was calculated from Western blots ($n = 5$ independent experiments, mean ± SEM; **, $P < 0.01$ and ***, $P < 0.001$ vs. vehicle; #, $P < 0.01$ vs. IAV alone). (D) BEAS-2b cells were treated with serum-free medium alone, or transfected with IRF3 or control (C) siRNA for 24 h and treated with serum-free medium alone, or 25 µg/ml dsRNA. At 24 h, secreted IFN-λ was measured by ELISA ($n = 3$–4 independent experiments, mean ± SEM; #, $P < 0.05$ vs. serum-free medium and C siRNA; **, $P < 0.005$ vs. C siRNA plus dsRNA). BEAS-2b cells were transfected with IRF3 siRNA and IRF3 protein was assessed by Western blotting (representative of three independent experiments). Molecular masses are provided in kilodaltons.

and protein production. In addition, silencing IRF3 in BEAS-2b cells using siRNA showed no inhibition of dsRNA-induced IFN-λ production at 24 h (Fig. 4 D). Together, these results reveal a novel role for IRF1-dependent induction of IFN-λ in AECs, which are required for the antiviral effect of EGFR inhibition on IAV and RV infection.

### EGFR activation decreases epithelial antiviral defenses

Because we found that EGFR inhibition was associated with increased virus-induced IRF1 and IFN-λ, we investigated the effect of EGFR activation on suppression of epithelial antiviral defenses. First, we found that the addition of EGF, an EGFR ligand, decreased IAV-induced IRF1 transcriptional activity in BEAS-2b cells as measured by IRF1 luciferase (Fig. 5 A, left). Next, we showed that EGFR activation suppressed IAV-induced IRF1 protein production in BEAS-2b cells (Fig. 5 A, right). The addition of EGF also suppressed IAV- and RV16-induced AEC production of IFN-λ (Fig. 5 B), and these results were confirmed with RV1b (not depicted). Finally, BEAS-2b cells were stimulated with EGF before viral infection, and virus was quantified by plaque assay after 24 h. We found that EGF increased IAV and RV16 titers significantly (Fig. 5 C), and these results were confirmed with RV1b (not depicted). These results showed that EGFR activation decreases airway epithelial IRF1 and IFN-λ, and increases respiratory viral infection.

In summary, here we have examined the interaction between EGFR signaling and IRF1-induced IFN-λ pathways in the regulation of viral infection. IAV and RV activated EGFR, and EGFR activation suppressed IRF1-induced IFN-λ production, which increased viral infection; inhibition of EGFR augmented IRF1 and IFN-λ, which resulted in decreased viral titers in vitro and in vivo. These findings show that EGFR and IRF1-induced IFN-λ pathways play different roles in respiratory viral infection. Although the signaling intermediates between IRF1 and EGFR remain to be elucidated, future experiments that investigate individual EGFR phosphorylation sites and downstream MAP kinase signaling will be informative. In conclusion, we have uncovered a novel mechanism that viruses use to suppress endogenous epithelial antiviral defenses.

## MATERIALS AND METHODS

**Reagents.** EGFR tyrosine kinase inhibitor AG 1478, EGF, TAPI-1, a matrix MP inhibitor with selectivity for TNF converting enzyme (TACE), neutralizing anti-EGFR (Ab-5) mAb, and an isotype-matched Ab were obtained from EMD Millipore. IFN-λ polyclonal Abs, IFN-λ receptor (IL-28R/IL-10Rβ)

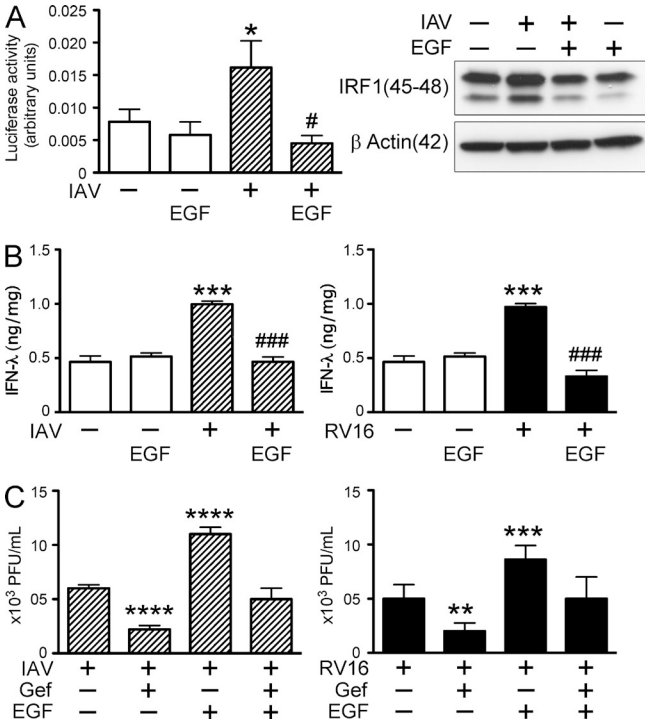


**Figure 5. EGFR activation suppresses IRF1 and IFN-λ.** (A) BEAS-2b cells were transfected with IRF1 luciferase reporter (left), and after 24 h treated with serum-free medium alone (empty columns), 10 ng/ml EGF, IAV (striped columns), and IAV plus EGF for 3 h before luciferase activity was measured ($n = 3$ independent experiments in duplicate; *, $P < 0.01$ vs. serum-free medium; #, $P < 0.01$ vs. IAV alone). IRF1 protein was measured (right) in BEAS-2b cells by Western blot 3 h after treatment with serum-free medium, 10 ng/ml EGF, IAV, and IAV plus EGF (data shown are representative of three independent experiments). Molecular masses are provided in kilodaltons. (B) BEAS-2b cells were treated with serum-free medium alone (empty columns), 10 ng/ml EGF, IAV (striped columns) and RV16 (black columns), or virus plus 10 ng/ml EGF, and secreted IFN-λ was measured by ELISA at 24 h ($n = 6$ independent experiments, mean ± SEM; ***, $P < 0.0005$ vs. control; ###, $P < 0.0005$ vs. each virus alone). (C) BEAS-2b cells were infected with IAV (striped columns) and RV16 (black columns) alone, or with the addition of 10 μM Gefitinib, 10 ng/ml EGF, or both Gefitinib and EGF for 24 h and viral titers in cell culture homogenates were assessed by plaque assay ($n = 5$ independent experiments, mean ± SEM; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0005$ vs. virus alone).

Abs, and isotype-matched Abs were purchased from Santa Cruz Biotechnology, Inc. *N*-propyl gallete (nPG) was purchased from Sigma-Aldrich. Gefitinib was purchased from Tocris Bioscience. The synthetic dsRNA poly I:C was purchased from InvivoGen.

**Viruses.** Purified Influenza A/PR/8/34 (H1N1; Advanced Biotechnologies) was used for in vitro and in vivo experiments. IAV titers were determined by TCID50% and plaque assays using Madin-Darby canine kidney (MDCK; American Type Culture Collection), as previously described (Barbier et al., 2012). RV 16 was a gift from W. Busse (Madison, WI). RV1B was purchased from ATCC. RV16 and 1B were grown in HeLa (Ohio) cells (ATCC) and purified by centrifugation through sucrose gradient, as previously described (Zaheer and Proud, 2010). RV titers were determined using HeLa cells by TCID50% and plaque assay. Cell culture supernatants from MDCK and HeLa cells that were mock infected did not induce IFN-λ in BEAS-2b cells above amounts induced by serum-free medium alone.

**Cell culture.** J. Fahy (San Francisco, CA) provided bronchial epithelial (BEAS-2b) cells. P. Hayden (MatTek Corporation, Ashland, MA) and W. Finkbeiner (San Francisco, CA) provided primary NHBE cells from healthy donors. Cells were seeded at 2–4 × $10^5$ cells/ml and grown in bronchial epithelial growth medium (BEGM; Lonza) supplemented with growth factors, 100 U/ml penicillin, and 100 μg/ml streptomycin. 16 h before viral infection, EGF and hydrocortisone were removed from cell culture medium. After preliminary experiments were completed with different IAV and RV concentrations at 24 h to determine IFN-λ production, subsequent experiments used IAV at a multiplicity of infection (MOI) of 0.5, RV1b at MOI of 1, and RV16 at MOI of 2 in BEAS-2b cells. To maximize viral infection, NHBE cell cultures were infected at 80–90% confluence at MOI = 10, as previously described (Contoli et al., 2006). Chemical inhibitors were added to cell cultures at the time of viral infection, unless stated otherwise. AG 1478 and Gefitinib were used at 10 μM because experiments have shown this concentration to inhibit virus-induced inflammation (Liu et al., 2008; Hewson et al., 2010; Langhammer et al., 2011). We confirmed that AG 1478 and 10 μM Gefitinib inhibited IAV- and RV-induced IL-8 production in AECs, and neither inhibitor induced cell toxicity as measured by LDH production. In addition, higher concentrations of these inhibitors may be active against related HER family members (e.g., erbB2 or erbB4), or other tyrosine kinases (e.g., C-flt). Therefore, we used EGFR siRNA to confirm selectivity for EGFR (Fig. 1 B). For experiments using the EGFR ligand EGF, we used 10 ng/ml because prior investigators have shown that this concentration increased the effect of RV on AECs (Subauste and Proud, 2001).

Cell cultures were incubated at 37°C and cell culture homogenates and supernatants were harvested at the indicated time points. Total EGFR phosphorylation was measured at 10 min by ELISA (R&D Systems) and Nox activity (Cell Technology, Inc.) in cell lysates was measured at 2 h. Virus in cell culture homogenates was measured at 24 h by plaque assay using MDCK cells (for IAV) and HeLa cells (for RV1B and RV16), and by flow cytometry and Western blotting (in BEAS-2b and NHBE cells) at 2 h and 30 min using anti–Influenza A hemagglutinin (HA), anti-nucleoprotein (NP; Santa Cruz Biotechnology, Inc.), and anti-M1 (Abd Serotec) mAbs. IFN-α, -β (R&D Systems), and -λ (eBioscience Inc.) were measured in cell culture supernatants at 24 h by ELISA. BEAS-2b cells cultured in serum-free medium, treated with chemical inhibitors, or siRNA were assessed for cytotoxicity using an LDH assay (Roche) and no significant differences were found.

IFN-λ and IRF1 mRNA expression was assessed by quantitative RT-PCR, as previously described (Wang et al., 2009a; Gencheva et al., 2010). IAV was analyzed by quantitative RT-PCR in BEAS-2b and NHBE cells as previously described (Crowe et al., 2009). Total RNA was extracted using RNeasy kit (QIAGEN). RT-PCR was evaluated with Applied Biosystems Model 7900 sequence detector. The following primers were used: IFN-λ (IL-29; forward), 5′-GGGAACCTGTGTCTGAGAACGT-3′; IFN-λ (IL-29; reverse), 5′-GAGTAGGGCTCAGCGCATAAATA-3′; IRF1 (forward), 5′-CTCTGAAGCTACAACAGATGAGG-3′; IRF1 (reverse), 5′-CTGTAGACTCAGCCCAATATCCC-3′; IAV (forward), 5′-AAGACCAATCCTGTCACCTCTGA-3′; and IAV (reverse), 5′-CAAAGCGTCTACGCTGCAGTCC-3′. The housekeeping gene GAPDH was used as an internal control. Western blotting was used to measure IRF1. In brief, after stimulation of cell cultures, cells were lysed using RIPA buffer (Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitors. Equivalent amounts of protein were loaded onto Mini-PROTEAN TGX 10% gels (Bio-Rad Laboratories). After electrophoresis and blocking with TBST (Bio-Rad Laboratories) containing 5% BSA, blots were then incubated with anti-IRF1 Ab (Cell Signaling Technology) overnight. Membranes were stripped and reprobed with mouse anti–β-actin Ab (Santa Cruz Biotechnology, Inc.). Densitometry was calculated using ImageJ software (National Institutes of Health). Based upon software instructions, a ratio to β-actin was calculated for each condition and compared with the control. siRNA was used to knockdown EGFR, IRF1, and IRF3 in BEAS-2b cells, as previously described (Wang et al., 2009b). Scrambled (control) and EGFR siRNA were purchased from Santa Cruz Biotechnology, Inc. (sense: CUCUGGAGGAAAAGAAAGU; antisense: ACUUUCUUUUCCU CCAGAG). Scrambled

(control) and IRF1 siRNA (duplex UCCCAAGACGUGGAAGGCCAACUUU) were purchased from Invitrogen. Scrambled (control) and pooled sequences of IRF3 siRNA were purchased from Thermo Fisher Scientific (1: CGAGGCCACUGGUGCAUAU, 2: CCAGACACCUCUCCGGACA, 3: GGAGUGAUGA GCUACGUGA, and 4: AGACAUUCUGGAUGAGUUA). siRNA transfection was performed using Lipofectamine (Invitrogen) in subconfluent cells, and 24 h after transfection cell cultures were treated with dsRNA or infected with virus. Unless stated, Gefitinib was added at the same time as viral infection. 24 hours after viral infection, cell culture homogenates were prepared to measure viral titers and cell culture supernatants were collected to measure IFN-λ. siRNA knockdown of EGFR, IRF1, and IRF3 was confirmed by Western blot using anti-EGFR (Santa Cruz Biotechnology, Inc.), -IRF1 (Cell Signaling Technology), and -IRF3 (Santa Cruz Biotechnology, Inc.) Abs. To measure IRF1 transcriptional activity, BEAS-2b cells were transfected using TransIT-2020 reagent (Mirus) with 250 ng IRF1 luciferase reporter and the appropriate negative and positive controls (SABiosciences). After 24 h, cells were stimulated before cell lysates were prepared and IRF1 luciferase activity was assayed by Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

**Mice and in vivo and ex vivo experiments.** C57BL/6 mice were purchased from NCI and maintained in accordance with the University of California, San Francisco and Yale University guidelines of the Institutional Animal Care and Use Committees. All experiments were performed using 6–8-wk-old female mice. For IAV infection, 50 µl IAV ($10^{4.5}$ TCID50%) or sterile PBS were given intranasal after sedation with isoflurane. 50 mg/kg of systemic Gefitinib, at a dose previously used to suppress lung inflammation (Hur et al., 2007), was given 16 h before infection, in a prophylactic model, or 1 h after infection, in a therapeutic model, and continued daily. 24–48 h after viral infection, BAL and lungs were collected. Mice do not produce IFN-λ1 (IL-29); therefore, we measured IFN-λ2/3 (IL-28) by ELISA (eBioscience) in BAL. Lung homogenates were analyzed for IRF1 protein by Western blotting (Cell Signaling Technology) and viral titers were measured by plaque assay in MDCK cells.

**Statistical analysis.** Results are presented as both individual data points and mean ± SE. To determine significance, two-tailed Student's *t* test, ANOVA, and Mann-Whitney test were used as appropriate (Prism version 5; GraphPad Software). P-values of ≤0.05 were considered to be statistically significant.

We would like to thank Drs. Patrick Hayden and Walter Finkbeiner for generously providing primary cells.

J.L. Koff was supported by National Institutes of Health (NIH) grant K08 HL0923288 and L.L. Lanier is an American Cancer Society Professor and is supported by NIH grant AI068129.

The authors declare no competing financial interests.

**Submitted: 28 June 2012**
**Accepted: 31 July 2013**

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