Elevated murine HB-EGF confers sensitivity to diphtheria toxin in EGFR-mutant lung adenocarcinoma

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Summary statement:
We report the unexpected finding that systemic diphtheria toxin administration induces rapid regression of murine EGFR-mutant lung adenocarcinomas in the absence of a transgenic allele containing human diphtheria toxin receptor.
Abstract

Conditional ablation of defined cell populations in vivo can be achieved using genetically engineered mice in which the human diphtheria toxin (DT) receptor (DTR) is placed under control of a murine tissue-specific promotor, such that delivery of diphtheria toxin selectively ablates cells expressing the high-affinity human DTR. Cells expressing only the endogenous low-affinity mouse DTR are assumed to be unaffected. Surprisingly, we found that systemic DT administration induced rapid regression of murine EGFR-mutant lung adenocarcinomas in the absence of a transgenic allele containing human DTR. DT enzymatic activity was required for tumor regression, and EGFR-mutant tumor cells were the primary targets of DT toxicity. In FVB mice, EGFR-mutant tumors upregulated expression of HB-EGF, which is the DTR in mice and humans. HB-EGF blockade with CRM197, an enzymatically inactive DT mutant, partially abrogated DT-induced tumor regression. These results suggest that elevated expression of murine HB-EGF (low-affinity DTR) confers sensitivity to DT in EGFR-mutant tumors, demonstrating a biological effect of DT in mice lacking transgenic DTR alleles and highlighting a unique vulnerability of EGFR-mutant lung cancers.

INTRODUCTION

Conditional ablation of defined cell populations in vivo is an essential strategy to probe cell function. In mice, cells can be efficiently ablated using diphtheria toxin (DT), an exotoxin secreted by Corynebacterium diphtheriae (Hadfield, McEvoy et al. 2000). This approach relies on human DT receptor’s (DTR) approximately $10^5$-fold higher affinity for DT compared to mouse DTR (Mekada, Kohno et al. 1982, Pappenheimer, Harper et al. 1982, Saito, Iwawaki et al. 2001). Murine cells expressing high-affinity human DTR under control of a tissue-specific promoter are sensitive to DT, whereas murine cells expressing only the endogenous low-affinity DTR are assumed to be unaffected (Saito, Iwawaki et al. 2001). In sensitive cells, DT inhibits protein synthesis by inactivating elongation factor 2 (EF-2) and inducing apoptotic cell death (Honjo, Nishizuka et al. 1968, Robinson, Henriksen et al. 1974, Morimoto and Bonavida 1992). In addition to the enzymatic domain that mediates toxicity, DT contains receptor-binding and translocation domains that mediate entry into the host cell cytoplasm (Greenfield, Bjorn et al. 1983). In mice and humans, the DT receptor is the membrane-bound precursor to heparin-binding EGF-like growth factor, HB-EGF (Naglich, Metherall et al. 1992).

DTR-based cell ablation systems are widely used to investigate roles of defined cell types in disease (Saito, Iwawaki et al. 2001, Jung, Unutmaz et al. 2002, Kim, Rasmussen et al. 2007, Walzer, Blery et al. 2007, Whiddon and Palmeter 2013, Yamazaki, Sugiyama et al. 2013). However, upon DT administration wildtype (WT) mice lacking transgenic DTR alleles exhibit phenotypes including weight loss (Meyer Zu Horste, Zozulya et al. 2010), proteinuria (Goldwich, Steinkasserer et al. 2012), and cochlear damage (Konishi, Ohgami et al. 2017). Another study detected lung inflammation after DT administration in both C57BL/6 Foxp3<sup>DTR</sup> (Kim, Rasmussen et al. 2007) and WT animals (Chapman and Georas 2013). Although these effects are considered
“off-target” in studies that rely on DTR transgenic animals, they demonstrate that DT has biological effects in WT mice.

We report that DT induces regression of murine EGFR-mutant lung adenocarcinomas in the absence of a transgenic DTR allele. Using the inactive DT mutant, cross-reacting material 197 (CRM197), we confirmed that DT’s enzymatic activity was required for apoptotic tumor cell death. We found that EGFR-mutant tumors upregulate expression of HB-EGF (the DTR) and our data indicate that elevated murine HB-EGF confers sensitivity to DT.

RESULTS

Murine EGFR-mutant lung adenocarcinomas are sensitive to DT administration

We initially investigated the role of Foxp3+ regulatory T cells (Tregs) in EGFR-mutant lung adenocarcinomas. We used tetO-EGFRL858R, CCSP-rtTA mice in which human EGFRL858R is inducibly expressed in type II lung epithelial cells after doxycycline (dox) administration, inducing multifocal lung adenocarcinomas (Politi, Zakowski et al. 2006). We crossed these animals to Foxp3IRESDTR-GFP mice in which DT administration depletes Foxp3+ Tregs (Kim, Rasmussen et al. 2007). Between 6-7 weeks after dox induction, mice received 0.5µg DT or vehicle (PBS) by i.p. injection. Magnetic resonance imaging (MRI) revealed that unlike vehicle controls, DT-treated tumors regressed to approximately 30% of the starting volume after 11 days (Fig. S1A). We confirmed that DT depleted Foxp3+ Tregs in tumor-bearing lungs (Fig. S1B). Consistent with prior studies (Kim, Rasmussen et al. 2007, Joshi, Akama-Garren et al. 2015), Treg depletion increased CD8+ and CD4+ Foxp3− T cell abundance (Fig. S1C). To verify that Treg depletion was indeed the mechanism by which tumors regressed after DT, we treated tumor-bearing Foxp3WT mice with DT. Surprisingly, tumors regressed in mice lacking the DTR allele (Fig. S1A). We also found that a single dose of DT was sufficient to induce rapid yet reversible tumor regression (Fig. S1D).

To understand how DT could induce tumor regression in mice lacking DTR alleles, we treated tumor-bearing FVB tetO-EGFRL858R, CCSP-rtTA mice with 0.5µg DT or CRM197, a DT mutant harboring a single amino acid substitution (G52E) in the catalytic domain. This renders CRM197 inactive, yet it retains an intact receptor-binding domain (Uchida, Pappenheimer et al. 1973). We compared these agents to the tyrosine kinase inhibitor erlotinib or to dox withdrawal, which both induce rapid tumor regression by inhibiting EGFR phosphorylation and turning off oncogenic EGFR expression, respectively (Politi, Zakowski et al. 2006). DT, but not enzymatically inactive CRM197, induced tumor regression in mice lacking transgenic DTR alleles, indicating that DT’s enzymatic activity was required for regression (Fig. 1A). Unlike erlotinib treatment or dox withdrawal, which reduced phosphorylated EGFR (pEGFR) levels in tumor-bearing lungs, DT-treated tumors maintained pEGFR levels comparable to vehicle and CRM197 treated tumors (Fig. 1B). Therefore, DT did not directly inhibit oncogenic EGFR phosphorylation. However, DT, erlotinib, and dox withdrawal reduced the levels of prosurfactant protein C (SPC), which is expressed by type II lung epithelial cells and tumors derived
from them, indicating a reduction in the abundance of epithelial-derived cells (Fig. 1B) (Politi, Zakowski et al. 2006). We further verified that tumor regression quantified from MRI scans was consistent with hematoxylin and eosin (H&E) stained histological sections of DT, CRM197, erlotinib, or vehicle treated tumors. Although vehicle- and CRM197-treated lung tumors were histologically indistinguishable, DT-treated lungs displayed apoptotic cell fragments and reduced papillary tumor cell clusters (Fig. 1C).

Because tumor-bearing mice lacked a transgenic DTR allele, the cellular target for DT’s enzymatic activity was unknown. To test the hypothesis that DT is directly internalized by tumor cells and induces apoptosis, we treated tumor-bearing mice with DT, CRM197, erlotinib, or vehicle for 2 days and quantified apoptotic cells by TUNEL staining (Fig. 1D). We used an EGFR\textsuperscript{L858R}-specific antibody to distinguish between tumor (EGFR\textsuperscript{L858R}+) and non-tumor (EGFR\textsuperscript{L858R}-) cells. Compared to vehicle and CRM197, DT (and to a lesser degree, erlotinib) significantly increased the percentage of apoptotic EGFR\textsuperscript{L858R}+ tumor cells (Fig. 1E). Importantly, DT-treated mice had approximately 3-fold higher rates of apoptosis in EGFR\textsuperscript{L858R}+ tumor cells compared to EGFR\textsuperscript{L858R}- cells, whereas CRM197-treated mice displayed no difference (Fig. 1F). These results demonstrate that EGFR\textsuperscript{L858R}+ tumor cells are the primary targets of DT’s enzymatic activity, which results in apoptotic tumor cell death.

**DT-induced tumor regression alters the inflammatory profile of tumor-bearing lungs**

The inflammatory properties of both DT and CRM197 are well established and form the basis for the use of CRM197 in conjugate vaccines (Schneerson, Barrera et al. 1980, Shinefield 2010). To investigate whether inflammation induced by either agent could impact tumor burden, we examined the frequency and function of inflammatory myeloid cells in the tumor microenvironment after treatment with DT, CRM197, or vehicle. Although vehicle- and CRM197-treated tumor-bearing lungs had decreased neutrophil frequency compared to healthy controls, DT-induced tumor regression restored neutrophil frequency to the levels in healthy lungs (Fig. 2A, B). Vehicle and CRM197 treated tumors had increased alveolar macrophage (AM) abundance compared to healthy controls, yet these AMs produced less TNF\textalpha (Fig. 2C, D). However, DT-induced tumor regression failed to restore AM abundance or cytokine production to the levels found in healthy lungs. Thus DT, but not CRM197, altered the inflammatory profile of tumor-bearing lungs, suggesting these changes were a consequence of tumor regression rather than inflammation induced by the microbial agents.

**Elevated HB-EGF confers DT sensitivity to EGFR-mutant lung tumors**

Next, we investigated the mechanism of DT internalization by EGFR-mutant tumor cells in vivo. In its membrane-bound state HB-EGF is the DT receptor (Naglich, Metherall et al. 1992), yet after cleavage from the cell membrane soluble HB-EGF is a high-affinity EGFR ligand that is upregulated by EGFR-mutant lung tumors (Jones, Akita et al. 1999, Yotsumoto, Fukagawa et al. 2017). Oncogenic EGFR remains sensitive to ligand binding despite sustaining constitutive ligand-independent phosphorylation (Cho, Chen et al. 2013, Red Brewer, Yun et al. 2013). Therefore, we hypothesized that EGFR-mutant lung tumor cells upregulate murine HB-EGF, and that this low-affinity DT receptor could, if expressed at high enough levels, allow for sufficient DT
internalization to induce tumor cell death. Indeed, the entry of one molecule of DT’s catalytic domain was found to be sufficient to kill a cell (Yamaizumi, Mekada et al. 1978). We found that Hbegf as well as two additional EGFR ligands, amphiregulin (Areg) and epiregulin (Ereg), were specifically upregulated in EpCAM+ lung epithelial cells isolated from tumor-bearing lungs compared to healthy controls, and erlotinib-induced tumor regression restored ligand expression to the levels in healthy lungs (Fig. 3A).

Because enzymatically inactive CRM197 retains HB-EGF binding activity, it can compete with active DT for receptor binding and protect sensitive cells from DT toxicity in vitro (Naglich, Metherall et al. 1992). Therefore, we assessed whether increasing doses of CRM197 could compete with DT for HB-EGF binding in vivo. First, we treated tumor-bearing mice with a single dose of 0.125-0.5 µg DT combined with CRM197 (such that the overall bacterial toxin dose was constant across treatment groups) and evaluated tumor burden by MRI on day 3 after treatment (Fig. 3B). This confirmed that DT-induced tumor regression was dose-dependent. We then treated tumor-bearing mice with 0.5 µg DT combined with increasing doses (0, 0.25, or 0.5 µg) of CRM197. We found that CRM197 could partially abrogate DT-mediated tumor regression after 3 days (Fig. 3C). These results suggest that CRM197 competes with DT for HB-EGF binding to protect sensitive tumor cells from DT toxicity.

Although our experiments thus far were performed on FVB mice, many DTR transgenic models are on a C57BL/6 background. Thus, we treated C57BL/6 tetO-EGFR<sup>L858R</sup>; CCSP-rtTA tumor-bearing animals with DT and CRM197. Unexpectedly, we found that these tumors did not regress (Fig. 3D). Despite similar levels of EGFR phosphorylation, tumor-bearing whole lungs on a C57BL/6 background displayed lower expression of Hbegf, Areg, and Ereg compared to those on an FVB background (Fig. 3E, F). Healthy lungs from C57BL/6 mice also had lower Hbegf and Ereg expression compared to those from FVB mice (Fig. 3F), indicating that the strain-specific differences in EGFR ligand expression were not tumor-specific. Healthy lungs from either strain displayed higher Hbegf expression but lower Areg and Ereg expression than tumor-bearing lungs, and in FVB mice erlotinib treatment increased expression of Hbegf and Ereg (Fig. 3F). These results suggest that cells in the lung and tumor other than EpCAM+ epithelial cells (shown in Fig. 3A) can also express these ligands. Collectively, these results suggest that in FVB mice, elevated HB-EGF confers sensitivity to DT in EGFR-mutant tumors.

DISCUSSION

Here we show that in the absence of a transgenic DTR allele, murine EGFR-mutant lung adenocarcinomas are sensitive to DT. We compared the effect of DT vs. the enzymatically inactive mutant CRM197 and found that CRM197 did not induce tumor regression, indicating that the enzymatic activity of DT was required for the effect (Fig. 1A). Because both inactive CRM197 and active DT can induce inflammation, the requirement for DT enzymatic activity also excludes the possibility that inflammation indirectly causes tumor regression. We evaluated whether DT directly kills tumor cells and found that: (1) EGFR<sup>L858R</sup>+ tumor cells are the primary targets for DT’s enzymatic activity, which induces tumor cell apoptosis (Fig. 1D); (2) DT-
sensitive EGFR-mutant tumors on an FVB background have higher HB-EGF levels compared to DT-insensitive EGFR-mutant tumors on an C57BL/6 background (Fig. 3D); and (3) treatment with CRM197 partially abrogates DT-induced tumor regression (Fig. 3C). Collectively, these results suggest that elevated murine HB-EGF confers sensitivity to DT in EGFR-mutant tumors.

In vivo competition assays helped exclude potentially confounding factors. Commercial preparations of DT and CRM197 can be contaminated with endotoxin. However, if endotoxin from DT and/or CRM197 induced tumor regression, increasing doses of either agent should have additive effects, leading to increased regression. DT preparations can also contain nicked DT, in which the catalytic domain has been proteolytically cleaved from the receptor-binding and translocation domains, allowing for indiscriminate entry of the catalytic domain into cells in a receptor-independent fashion. However, receptor-independent entry of nicked DT should not be affected by CRM197. Yet instead of an additive effect (predicted if endotoxin caused regression) or no effect (predicted if nicked DT caused regression), we found that CRM197 delivery partially abrogated DT-induced tumor regression (Fig. 3B, C).

We considered the possibility that the lower expression of EGFR ligands, including HB-EGF, in tumors derived from C67BL/6 mice could be caused by a lower level of EGFR\textsubscript{L858R} transgene expression in these animals, as strain-specific differences in the epigenetic regulation of transgene expression have been reported (Schumacher, Koetsier et al. 2000). However, we found that healthy lung tissue lacking the transgene from C57BL/6 mice also had lower HB-EGF expression compared to healthy lung tissue from FVB mice (Fig. 3F), indicating that the strain-specific difference was not unique to the tumor and not dependent on EGFR\textsubscript{L858R} transgene expression. It is possible that epigenetic regulation of EGFR ligands may vary across different mouse strains: the accessibility of EGFR ligand (\textit{Hbegf, Areg, Ereg}) loci could be reduced in C57BL/6 mice, leading to lower gene expression. Indeed, studies examining DNA methylation patterns across mice of distinct backgrounds have identified strain-specific differences (Zhang, Hoshida et al. 2016).

DT and its derivatives such as CRM197 have been investigated as cancer therapies. In one early example, 24 of 50 cancer patients experienced reductions in tumor volume upon intravenous DT administration, although the mechanism of response was unknown (Buzzi 1982). Subsequent efforts have exploited the toxicity of DT’s catalytic domain. For example, the DT catalytic and translocation domains were fused to human IL-2 to form a fusion toxin, denileukin diftitox (Ontak), which selectively kills cells expressing IL-2 receptors (Williams, Parker et al. 1987, Bacha, Williams et al. 1988). Our results suggest that tumors with elevated HB-EGF may be uniquely susceptible to DT. In humans, where high-affinity binding between DT and human HB-EGF can occur, DT delivered in a targeted fashion to tumors expressing HB-EGF could be used to provoke rapid tumor cell death while reducing the risks of systemic toxicity and may warrant further study.

CRM197 has also been investigated as a cancer therapy due to its ability to block HB-EGF. In contrast to our findings in the murine setting, human lung cancer xenografts expressing HB-EGF were sensitive to CRM197 (Hsieh, Chou et al. 2017, Yotsumoto, Fukagawa et al. 2017). These results are consistent with CRM197’s higher affinity for human versus mouse HB-EGF. Further, in human lung cancers HB-EGF
expression was positively correlated with EGFR expression, regardless of EGFR mutational status (Hsieh, Chou et al. 2017). These results suggest that both EGFR WT and mutant lung tumors can produce EGFR ligands, including HB-EGF, that promote tumor cell survival through autocrine and/or paracrine EGFR signaling. Therefore, lung cancers with elevated HB-EGF may be uniquely susceptible to both CRM197 and DT.

Transgenic DTR systems for targeted cell ablation in vivo have helped define roles for distinct cell populations in a variety of disease settings. However, our results add to a body of literature demonstrating the biological effects of DT on WT mice, which can be strain-specific and must be considered when interpreting results from transgenic DTR models (Meyer Zu Horste, Zozulya et al. 2010, Goldwich, Steinkasserer et al. 2012, Chapman and Georas 2013, Konishi, Ohgami et al. 2017). Importantly, these studies also highlight a unique vulnerability of lung cancers with elevated HB-EGF.

MATERIALS & METHODS

Animal studies and treatments
Animal studies were performed in compliance with Yale University’s Institutional Animal Care and Use Committee (protocol 11354; assurance number D16–00416). Animals were housed in a specific pathogen-free facility and fed chow containing 625ppm doxycycline (Envigo). FVB TetO-EGFR<sup>L858R</sup>; CCSP-rtTA (Politi, Zakowski et al. 2006) and C57BL/6 Foxp3<sup>ires-dTR-GFP</sup> (Kim, Rasmussen et al. 2007) mice have been described. FVB TetO-EGFR<sup>L858R</sup>; CCSP-rtTA mice were used except where noted in Fig. 3, where we used TetO-EGFR<sup>L858R</sup>; CCSP-rtTA mice backcrossed to C57BL/6 for 10 generations (male and female mice aged 9–16 weeks were used). Wild-type (WT) control mice were placed on doxycycline to match tumor-bearing animals. Mice were treated 5 days/week with 25mg/kg erlotinib (MSKCC Organic Synthesis Core) dissolved in 0.5% methylcellulose via intraperitoneal (i.p.) injection. Mice were treated with 0.5µg DT or CRM197 (List Biological) i.p. on day 0 only or on days 0, 4, 7, and 10. For in vivo competition assays, mice were treated on day 0 with 0.125-0.5µg DT and/or CRM197. Lung MRI scans were acquired at the Yale Magnetic Resonance Research Center on a mini-4T scanner (Bruker AVANCE) using respiratory gating on mice anesthetized with a steady flow of oxygen and isoflurane. Volumes of visible lung opacities (tumors) were calculated using BioImage Suite software (Papademetris, Jackowski et al. 2006). Tumor volumes were normalized to the initial volume at the start of treatment.

Immunohistochemistry and Immunofluorescence
Lung tumors were dissected into 4% paraformaldehyde (Electron Microscopy Services), fixed overnight at room temperature, then rehydrated in 70% ethanol. Yale Pathology Tissue Services produced paraffin-embedded and H&E stained sections according to standard methods. After citrate-based antigen retrieval,
immunofluorescence staining was performed with anti-EGFR\textsuperscript{L858R} antibody (Cell Signaling #3197) overnight at 4°C and fluorescent secondary detection for 1hr at room temperature (anti-rabbit AlexaFluor488, ThermoFisher) with TUNEL staining (ApopTag Red \textit{In Situ} Apoptosis Detection Kit, Millipore #S7165). Total DNA was stained with Hoechst-33342 (ThermoFisher).

\textbf{Immunofluorescence quantification}

CellProfiler software (Carpenter, Jones et al. 2006, Kamentsky, Jones et al. 2011) was used to quantify the number and percentage of TUNEL positive tumor (EGFR\textsuperscript{L858R} positive) or non-tumor (EGFR\textsuperscript{L858R} negative) cells. 4-10 images were analyzed from each of 2 mice (Vehicle) or 3 mice (CRM, DT, Erlotinib). CellProfiler was programmed to: (1) separate each image into three channels: Hoechst, TUNEL (rhodamine), and EGFR\textsuperscript{L858R} (AlexaFluor 488); (2) identify primary objects (nuclei) based on Hoechst staining using global minimum cross entropy thresholding; (3) identify secondary objects (cells) based on EGFR\textsuperscript{L858R}-AlexaFluor 488 staining using the global Otsu method with three-class thresholding; (4) identify TUNEL+ nuclei based on rhodamine staining using the global Otsu method with three-class thresholding; (5) retrieve the EGFR\textsuperscript{L858R}-AlexaFluor 488 integrated intensities of each identified cell; (6) classify and filter cells as either EGFR\textsuperscript{L858R} positive or negative based on mean EGFR\textsuperscript{L858R}-AlexaFluor 488 intensity; and (7) correlate TUNEL+ nuclei with EGFR\textsuperscript{L858R}-AlexaFluor 488 positive and negative cells.
**Flow Cytometry**

Lung tumor tissue was minced in RPMI1640 with 2% FBS, 0.5mg/ml collagenase IV (Sigma), and 1 µg/ml DNase (Sigma) and digested at 37°C for 45 min. Samples were filtered through a 70µm cell strainer, incubated with ammonium-chloride-potassium lysing buffer (ThermoFisher) for 3 min, washed with PBS, and resuspended in RPMI1640 with 2% FBS. For intracellular cytokine staining, single cell suspensions were incubated in RPMI1640 with 10% FBS and Brefeldin A (ThermoFisher) at 37°C for 5hr. Single cell suspensions were washed with PBS with 2% FBS, incubated with α-FcγRIII/I antibody (2.4G2) on ice for 15 min, then stained on ice for 30 min. For intracellular staining, samples were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher), then stained on ice for 45 min. The following antibodies were used for surface staining: CD45 BV711 (1:400 dilution; #103147), CD3 APC-Cy7 (1:100; #100222), CD4 PerCP (1:200; #100538), CD8a BV605 (1:200; #100744), CD11b APC-Cy7 (1:200; #101226), CD11c PE-Cy7 (1:200; #117310), Ly6G PerCP (1:200; #127653), Ly6C BV605 (1:200; #128036) (all from BioLegend) and SiglecF BV480 (1:400; BD Biosciences #746668). The following antibodies were used for intracellular staining: Foxp3 AF488 (1:100; #126406) and TNFα PE (1:300; #506306) (both from BioLegend). Dead cells were excluded using Live/Dead Red Fixable Viability Dye (ThermoFisher). Flow cytometry data were acquired on an LSR II (BD) with FACSDiva and analyzed with FlowJo (TreeStar).

**Immunoblotting**

Lung tumors were flash frozen in liquid nitrogen, crushed, and lysed in RIPA buffer containing protease inhibitor. Equal amounts of total protein were separated by gel electrophoresis on Mini-PROTEAN GTX 4-20% gels (Bio-Rad), transferred onto nitrocellulose (Bio-Rad), and probed with the following antibodies: phospho-EGFR tyr1068 (Cell Signaling #3777), EGFR (Cell Signaling #2232), SPC (Abcam #90716), and β-actin (Santa Cruz #47778).

**RNA extraction and real-time quantitative PCR**

Lung tumors were flash frozen in liquid nitrogen and crushed. RNA was extracted using the RNeasy Mini kit (QIAGEN), cDNA was synthesized using the SuperScript III First-Strand Synthesis System (ThermoFisher), and quantitative RT-PCR was performed on a ViiA 7 Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Transcripts were normalized to Gapdh. The following primers were used: Hbegf (forward, 5’-CGCGTTGGTGACCGGTGAGAG-3’; reverse, 5’-CCCGTGGGTAGCAGCTGGTTT-3’); Areg (forward, 5’-GCTGAGGACAATGCAGGTA A-3’; reverse, 5’-GTGACAATGGCAGCTGGTTT-3’); Ereg (forward, 5’-TGCTTTGTCTAGGTTCCCACC-3’; reverse, 5’-GGGAGGAGTACGTATCAGCTCGG-3’); Gapdh (forward, 5’-ATGGTGAAGGTCGGTGTGAA-3’; reverse, 5’-TGGAAATGTGGATGGGCTT-3’) (Kefaloyianni, Muthu et al. 2016).

**RNA sequencing**
RNA sequencing was performed on lung epithelial cells isolated from healthy, tumor-bearing, and erlotinib-treated tumor-bearing mouse lungs as in (Ayeni, Miller et al. 2019). Briefly, EpCAM⁺ CD45⁻ CD11c⁻ lung epithelial cells were sorted on a FACS Aria (BD) at the Yale Flow Cytometry Core. RNA was extracted and purified using the Arcturus PicoPure RNA isolation kit. RNA sequencing was performed on the Illumina HiSeq 2000 platform at the Yale Stem Cell Center Genomics and Bioinformatics Core. Reads were aligned using bowtie2 (Langmead and Salzberg 2012) to the GRCm38 (mm10) reference genome. Normalized reads are shown in Reads Per Kilobase of transcript, per Million mapped reads (RPKM).

**Statistical Analysis**

Analyses were performed using GraphPad Prism software (v7.0a); results are shown as mean ± SEM with p-values from unpaired Student’s t-tests or one-way ANOVA.

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**Competing Interests:**

S.M.K. is Academic Editor at the Journal of Experimental Medicine and has a consultant/advisory board relationship with Celsius Therapeutics and Evolveimmune. K.P. reports receiving commercial research grant (to the Institution) from AstraZeneca and Roche/Genentech; has an ownership interest (including patents) in MSKCC/Molecular MD; and has an unpaid consultant/advisory board relationship with the Lung Cancer Research Foundation. No competing interests are declared by other authors.

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**Contributors:**

References


Figure 1: Diphtheria toxin induces regression of murine EGFR mutant lung adenocarcinomas. (A-C) Tumor-bearing FVB CCSP-rtTA; TetO-EGFR<sub>L858R</sub> mice were treated with vehicle (n=5), CRM197 (n=8), DT (n=5), or erlotinib (n=4), or taken off dox diet (n=4), for 11 days. (A) Tumor growth curves quantified from MR images and normalized to the volume at the start of treatment; p-values comparing vehicle (black) or CRM197 (blue) vs. DT treatment are shown. (B) Immunoblot analysis of phosphorylated EGFR, total EGFR, and SPC expression in lung tumor lysates. (C) Representative hematoxylin and eosin (H&E)-stained lung tumor sections; bottom images focused on remaining tumor cell clusters. (D-F) Tumor-bearing FVB CCSP-rtTA;
TetO-EGFR<sup>L858R</sup> mice were treated with vehicle (n=2), CRM197 (n=3), DT (n=3), or erlotinib (n=3) for 2 days. (D) Immunofluorescence staining for EGFR<sup>L858R</sup> and TUNEL in representative lung tumors. Inset shows 3 TUNEL+ EGFR<sup>L858R</sup> + (tumor) cells; arrows show TUNEL+ EGFR<sup>L858R</sup>- (non-tumor) cells. (E) Percentage of EGFR<sup>L858R</sup>+ (tumor) cells that are TUNEL+ and (F) percentage of EGFR<sup>L858R</sup>+ (tumor) and EGFR<sup>L858R</sup>- (non-tumor) cells that are TUNEL+ quantified using CellProfiler from 4-10 fields of view (FOV) per mouse. Scale bars, 50 µm. Results are shown as mean ± SEM with p-values from unpaired Student’s t-tests (A) or one-way ANOVA (E,F).
Figure 2: DT alters the inflammatory profile of tumor-bearing lungs. Infiltrating immune cells from healthy lungs and from lung tumors derived from FVB CCSP-rtTA; TetO-EGFR<sup>L858R</sup> mice treated with vehicle, CRM197, or DT for 11 days were analyzed by flow cytometry. (A) Representative contour plots demonstrating the gating strategy used to identify distinct myeloid populations and their TNFα production. (B,C) The frequency of (B) CD11b+ Ly6C+ Ly6G+ neutrophils and (C) CD11b- CD11c+ SiglecF+ alveolar macrophages. (D) The frequency of SiglecF+ cells that produce TNFα. Results are shown as mean ± SEM with p-values from one-way ANOVA (B-D).
Figure 3: In FVB mice, HB-EGF blockade with enzymatically inactive CRM197 partially abrogates tumor regression mediated by active DT. (A) mRNA abundance expressed in RPKM of Hbegf, Areg, and Ereg in EpCAM+ epithelial cells isolated from the lungs of healthy, tumor-bearing, and erlotinib-treated tumor-bearing FVB CCSP-rtTA; TetO-EGFR<sup>L858R</sup> mice. (B, C) Normalized tumor volumes quantified from lung MR images of FVB CCSP-rtTA; TetO-EGFR<sup>L858R</sup> mice 3 days after treatment with the listed doses of CRM197 and/or DT. (D) Normalized tumor volumes quantified from lung MR images of FVB and C57BL/6 CCSP-rtTA; TetO-EGFR<sup>L858R</sup> mice 3 days after treatment with DT (FVB n=6, C57BL/6 n=4), or CRM197 (FVB n=3, C57BL/6 n=3). (E)
Immunoblot analysis of phosphorylated EGFR, total EGFR, and SPC expression in lung tumor lysates from vehicle (veh) treated FVB and C57BL/6 CCSP-rtTA; TetO-EGFR<sup>L858R</sup> mice. (F) Relative mRNA abundance of Hbegf, Areg, and Ereg in lung tumors from (E) as well as FVB lung tumors treated with erlotinib (erl; n=3) and healthy lungs (FVB n=5, C57BL/6 (B6) n=5) from WT mice on doxycycline for 7 days. Results are shown as mean ± SEM with p-values from unpaired Student’s t-tests (D) or one-way ANOVA (A-C, F).
Fig. S1. DT treatment in Foxp3^{DTR} tumor-bearing mice induces T_{reg} depletion and transient tumor regression. (A) Normalized tumor growth curves quantified from lung MR images of CCSP-rtTA; TetO-EGFR^{L858R}; Foxp3^{RES-DTR-GFP} mice treated with vehicle (n=7) or DT (n=10) and CCSP-rtTA; TetO-EGFR^{L858R}; Foxp3^{WT} mice treated with DT (n=2) for 12 days. (B, C) Lung tumor infiltrating immune cells were analyzed by flow cytometry after 12 days of DT or vehicle treatment. Quantification of the frequency of (B) Foxp3^{+} Tregs (with representative contour plots) and (C) CD8^{+} and CD4^{+} T cells. (D) Normalized tumor growth curves quantified from lung MR images of CCSP-rtTA; TetO-EGFR^{L858R}; Foxp3^{RES-DTR-GFP} mice treated with a single dose of vehicle (n=2) or DT (n=4) on day 0.