Uncovering Molecular Targets to Overcome Immunosuppression in Non-Small Cell Lung Cancer with Acquired TKI Resistance

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UNCOVERING MOLECULAR TARGETS TO OVERCOME IMMUNOSUPPRESSION IN NON-SMALL CELL LUNG CANCER WITH ACQUIRED TKI RESISTANCE

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A

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DEDICATION

To my sister and best friend Shefali, mom, and dad thank you
for always supporting me
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Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related deaths worldwide. Targeted therapeutic agents, such as epidermal-like growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) or monoclonal antibodies targeting vascular endothelial growth factor (VEGF/R), can effectively inhibit upregulated signaling pathways driving tumorigenesis in NSCLC and many other cancers. Unfortunately, however, resistance to such targeted therapies inevitably arise in most patients and can occur through a variety of resistance mechanisms including genomic alterations and upregulation of bypass pathways. Additionally, patients who have acquired resistance to these targeted agents typically have tumors characterized by an immunosuppressive tumor microenvironment and thus yield minimal clinical benefit to anti-PD-1/PD-L1 immunotherapy. This work seeks to further elucidate the role of molecular targets, like IL-6 and estrogen, in promoting immunosuppression in NSCLC tumors with acquired resistance to an EGFR-TKI or anti-VEGF therapy. Using preclinical in vitro human NSCLC cell lines and in vivo murine NSCLC models in addition to clinical samples from patients with NSCLC tumors, this study has effectively 1) characterized the role of IL-6 in impairing anti-tumor activity of T cells and NK cells in EGFR-mutant NSCLC tumors which have acquired resistance to EGFR-TKIs and 2) determined the impact of estrogen on increasing myeloid infiltration and
secretion of pro-angiogenic factors in NSCLC tumors which are resistance to anti-VEGF therapy. Together, these findings provide a foundation to pursue further studies which determine the clinical utility of therapeutically targeting IL-6 or estrogen signalling in patients with resistant NSCLC disease.
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CHAPTER 1

REVIEW OF THE LITERATURE
Lung cancer is the leading cause of cancer-related deaths worldwide. In the United States, around 230,000 people a year get diagnosed with lung cancer [1]. There are two types of lung cancer - non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer is the less aggressive and more common form making up 80-85% of all lung cancer cases [2]. Non-small cell lung cancer includes the following main three subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

Adenocarcinomas are characterized by neoplastic gland formation and pneumocyte TTF-1 expression and include one-half of all lung cancer [3]. Squamous cell carcinomas are noted by presence of keratin producing cells and typically are detected by p40, p63, CK5, or desmoglein expression [4]. Large cell carcinomas have morphologically undifferentiated tumors diagnosed by exclusion of belonging to any of the other listed classifications. A number of environmental and genetic risk factors such as smoking, exposure to carcinogenic agents, air pollution, and family history of lung cancer can increase a person’s chance of getting lung cancer [5, 6].

Non-small cell lung cancer disease can clinically present in patients through intrathoracic and/or extrathoracic symptoms. Intrathoracic effects include cough, hemoptysis, chest pain, and sometimes pain in the hand or shoulder [7]. Metastasis to the bone occurs in at least 20 percent of all NSCLC cases [8], and brain metastasis can result in headache, vomiting, seizures, or changes in vision [7]. Patients with stage I-III NSCLC are given intent to cure treatment involving surgery, chemotherapy, radiation therapy, immunotherapy, or combined approach [9]. Additionally, the identification of gene mutations or translocations, which can
lead to fusion proteins, (i.e., EGFR, PI3K, KRAS, ALK, etc.) in lung cancer has led to the application of molecularly targeted therapies for these patients [10, 11].
EGFR-MUTANT NON-SMALL CELL LUNG CANCER

MUTATIONAL LANDSCAPE

Approximately 10-15% of NSCLC patients in the United States have activating mutation in the epidermal growth factor receptor (EGFR) gene. In the Asian population, up to 50% of lung cancer patients can harbor an activating EGFR mutation [12]. Mutations in the EGFR gene can occur in exons 18, 19, 20, and 21 with exon 19 deletion, L858R mutation, and combination with a T790M mutation as the most common types of mutations observed clinically. These activating mutations cause constitutive EGFR downstream signaling which ultimately leads to uncontrolled cell proliferation and survival pathway expression. Thus, a majority of patients with EGFR-mutant non-small cell lung cancer (NSCLC) display marked benefit in clinical outcomes when treated with first, second, or third generation EGFR tyrosine kinase inhibitors (TKIs).

EGFR-TKI MECHANISMS OF RESISTANCE

While EGFR-TKIs are effective initially, resistant disease inevitably arises in a majority of patients [13]. Acquired resistance to EGFR-TKIs can be mediated by EGFR dependent and EGFR independent mechanisms [14]. Among patients who are treated with first-generation EGFR-TKIs like erlotinib or gefitinib, nearly ~50% develop resistance through secondary EGFR T790M mutations in an EGFR dependent manner [15]. Third-generation EGFR-TKIs like osimertinib has moved to front-line treatment, overtaking the use of other first and second generation EGFR-TKIs, and effective at initially preventing EGFR T790M – based resistance[16-19].
In instances when secondary EGFR mutations do not arise, EGFR independent mechanisms of resistance can occur through activation of alternate signal pathways through MET amplification [20]. In around ~30% of patients, resistance can occur independent of either EGFR T790M or MET [21]. Previous studies have found that a number of tumors which have developed resistance through EGFR independent mechanisms can be characterized by having undergone histological transformation through epithelial to mesenchymal transition (EMT) [22-25]. These studies have conducted transcriptomic and proteomic analysis which compare the profiles of TKI-naïve EGFR-mutant cells and EGFR-TKI erlotinib resistant (ER) cells which were T790M negative. EGFR-TKI ER cells had undergone EMT as indicated by upregulation of mesenchymal markers including vimentin and ZEB1 and a loss of epithelial markers like E-cadherin [26].

**IMMUNE MICROENVIRONMENT**

EGFR-mutant NSCLC tumors have been previously characterized as having a “cold” or immunosuppressive tumor microenvironment which is highly resistant to immune checkpoint blockade (ICB) therapy. Previous studies have found that EGFR-mutant lung cancer tumors have a lower tumor mutational burden (TMB), evidence of CD8 infiltration and PD-L1 expression compared to EGFR wildtype tumors [27]. These are three factors which are strongly responsible for mediating robust response to ICB, and thus as expected, unfortunately EGFR-mutant tumors have a distinctly poor response rate to ICB. However surprisingly, EGFR-mutant NSCLC patients with high PD-L1 treated with ICB still displayed an overall response rate of 8% compared to 31% in EGFR wildtype patients [28]. These findings indicate that potentially other tumor microenvironment factors likely also contribute to the lack of ICB
response in patients with EGFR-mutant lung cancer. Within the tumor immune microenvironment, cytokines shape the immune milieu and significantly affect the composition and function of immune cells mediating antitumor immunity. EGFR mutations have previously been associated with a microenvironment also marked by impaired dendritic cells, M2 polarized tumor-associated macrophage, and exhausted T cells [29-31].

Additionally, EGFR-mutant NSCLC tumor have been characterized by a strong EMT phenotype. EMT in a tumor has also been associated with a distinct inflammatory immune microenvironment and elevated expression of checkpoint molecules [32]. Increased expression of PD-1/PD-L1 has been noted in mesenchymal tumors recorded in the TCGA and in MD Anderson PROSPECT datasets [32]. Together, there is significant evidence that the EGFR-mutant tumor immune microenvironment is strongly immunosuppressed and therefore minimally responsive to anti-PD-1/anti-PD-L1 immunotherapy.
INTERLEUKIN-6

PRODUCTION OF INTERLEUKIN-6

Interleukin-6 (IL-6) is a 21-28 kDa (184 amino acids) protein encoded by the IL-6 gene, containing 3 transcription start sites and 3 TATA like sequences, found on chromosome 7p21 [33, 34]. IL-6 belongs to the superfamily of cytokines including interleukin-11, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor, cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), and interleukin-27 [35]. These family group members have all been clustered together because they each signal through glycoprotein gp130, a signal transducer subunit. IL-6 was first discovered in 1986, at that time known as BSF-2, and was found to cause B lymphocytes to produce immunoglobulin [36]. Other studies since then have implicated IL-6 in being a critical factor driving several other physiological functions including bone remodeling, acute-phase responses (which occurs in the response to injury, trauma, or infection), inflammation processes, and immune-mediated responses [37-40]. IL-6, a known pleiotropic cytokine, has been noted to be produced by a wide variety of cell types including macrophages, T cells, endothelial cells, fibroblasts, hepatocytes, tumor cells, and more.

REGULATION OF INTERLEUKIN-6 EXPRESSION

The IL-6 gene promoter region is regulated by multiple transcription factors including nuclear factor (NF-κB), Sp-1, interferon regulatory factor (IRF), activator protein-1 (AP-1), and CCAAT enhancer-binding protein (C/EBP), and hormones like estrogen. TNF-α and IL-1 cytokine production has been found to induce of several of these transcription factors part of even further upstream regulation of IL-6 production.
NF-kB, normally found inactive in the cytoplasm when bound to IκBα, translocates to the nucleus to transcribe production of IL-6, amongst other pro-inflammatory cytokines [41]. NF-IL6 also can bind to IL-6’s responsive element and synergistically functions with NF-kB [42]. AP-1 can also bind to the IL-6 promoter and work in conjunction with NF-kB and cAMP to regulate secretion of IL-6 [43]. IRF can induce IL-6 expression following IFN-γ or IFN-β stimulation [44]. IL-6 expression can also be modulated by hypoxic condition in vascular by increasing IL-6 production through the activation of NF-κB [45]. Estrogen has been found to negatively regulate IL-6 expression by disrupting transactivation of NF-κB [46]. Suppressor cytokine signaling 3 (SOCS3) is another major negative regulator of IL-6 dependent signaling which functions in a negative feedback loop [47].

INTERLEUKIN-6 SIGNALING

IL-6 signalling transduction occurs when IL-6 binds to IL-6R made up of IL-6Rα, known as gp80, and IL-6Rβ, known as gp130. IL-6R can be membrane bound or can be found in a soluble secreted form (sIL-6R) [48]. sIL-6R is made when membrane bound IL-6R is cleaved by MMP or ADAM proteins [49]. IL-6 can bind to sIL-6R, and when that complex binds to gp130, it is known as trans-signalling. Binding of IL-6 with IL-6R found on the cell membrane surface is called classical signalling.

Downstream signalling once IL-6 binds to IL-6Rα and IL-6Rβ includes activation of Janus kinase (JAK) a tyrosine kinase which leads to tyrosine phosphorylation and activation of STAT3, which constitutes the IL-6/JAK/STAT3 canonical pathway. This pathway regulates transcription of many genes involved in cell growth differentiation and survival [50]. Ras/MAPK pathway can also be activated by IL-6 through the activation of Grb2 and She
which activate MAPK phosphorylation through activity of c-Myc, c-Jun, and c-Fos [51]. This signaling pathway is involved in the acute phase response and immunoglobulin production. Finally, the PI3K-PKB/Akt pathway is another cell survival pathway downstream of IL-6 signalling whose activation is initiated when PI3K modifies PIP3, which phosphorylates and activates PKB/Akt [52].

**INTERLEUKIN-6 AND CANCER**

In addition to its many other effects, IL-6 has been associated with promoting tumorigenesis. Prolonged activation of STAT3 can occur in the setting of many tumor cells [53]. Specifically, IL-6 signalling appears to induce STAT3 phosphorylation in a biphasic pattern substantiated by both the presence of EGFR and production of new IL-6 [54]. Activation of STAT3 leads to the upregulation of target genes involved in tumor cell survival and proliferation (Bcl-xl, c-MYC, survivin, Mcl-1), angiogenesis (VEGF, HIF-1α), metastasis (MMP2, MMP9, vimentin, Zeb1), and inflammation (CD80, CD86, IL-12, CXCL10) [55]. STAT3 is a pro-oncogenic transcription factor involved in blocking maturation of dendritic cells, preventing T cell activation, and promoting activity of immunosuppressive MDSCs, macrophages, and tumor associated neutrophils [56].

**IL-6 AND EGFR TKI-RESISTANCE**

Acquired resistance to EGFR-TKIs is associated with elevated IL-6. IL-6 was highly upregulated in EGFR-TKI (first-generation) resistant cells that had undergone EMT but not in cells where resistance was mediated by T790M or MET amplification [57, 58]. IL-6 expression can be induced in EGFR-mutant, TKI-naive cells by stress hormones (i.e. norepinephrine) via
β2-adrenergic receptors, thereby promoting EGFR TKI resistance both in vitro and in vivo which can be mitigated by beta blockers or IL-6 targeting [57]. IL-6 has pleiotropic effects on effector cells in the anti-tumor response, promoting CD4+ T cells differentiation into T regulatory cells and inhibiting NK cell cytotoxicity [59-62], suggesting that IL-6 may potentially contribute to an immunosuppression in EGFR-mutant tumors. However, current studies have been limited in understanding IL-6’s effects on the tumor cell and its role in the tumor immune microenvironment.
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**ANGIOGENESIS**

Angiogenesis is the process of new blood vessel development regulated by the shift in balance of pro-angiogenic and anti-angiogenic factors. Angiogenesis is one of the rate-limiting steps that tumors need to overcome to support accelerated tumor growth, spread, and ultimately metastasis [63]. When tumors grow, they can grow hypoxic as it gets harder for oxygen to reach the central core of a tumor. Thus, until angiogenesis begins tumors may have a slower more restricted growth rate. Tumor angiogenesis involves the development of blood vessels which could help sustain tumor growth through the transport of vital nutrients and oxygen. Thus, angiogenesis was proposed as a therapeutic target for cancer in the 1970s, by Dr. Judah Folkman [64] with the hypothesis that targeting angiogenesis would deprive tumors of nutrients for their growth.
VEGF SIGNALING

Vascular endothelial growth factor (VEGF) is a secreted mitogen which is a key mediator of angiogenesis. VEGF is in its active form as a homodimer or heterodimer which binds to a cell membrane-bound tyrosine kinase receptor [65]. Signaling that occurs downstream of VEGF ligand and receptor binding is activation of multiple pathways including PI3K, MAPK, and PKB/AKT pathways [66]. There are multiple VEGF family ligands which regulate the angiogenic process.

VEGF-A can drive major processes involved in cell migration, angiogenesis, and vascular permeability. VEGF-B can increase perivascular cell coverage [67]. VEGF-C can mediate angiogenesis of lymphatic vasculature through unique mechanisms distinct from classical VEGF-A angiogenesis signaling [68]. Meanwhile VEGF-D regulates lymphangiogenesis [69]. These VEGF ligands can bind specific VEGF receptors (VEGFRs). VEGF-A and VEGF-B bind to VEGFR1 on blood vascular endothelial cells. VEGF-A, VEGF-C, and VEGF-D can bind to VEGFR2 is expressed on vascular and lymphatic vessels [70]. VEGF-C and VEGF-D bind to VEGFR3 on vascular and lymphatic endothelial cells [71].

REGULATION OF VEGF IN TUMOR BIOLOGY

Both autocrine and paracrine VEGF signalling has been documented in tumor cells. Tumors can secrete VEGF and express various VEGF receptors. The autocrine nature of VEGF signalling may, in part, explain how aggressive cancers can sustain rapid growth [72]. Paracrine VEGF signalling involves secretion of VEGF by tumor cells to attract endothelial cell growth [73]. Hypoxic tissue has been identified as a major source of pro-angiogenic factors like VEGF-A, and thus VEGF-A can be highly upregulated in growing hypoxic tumors [74,
During hypoxic conditions, hypoxia-inducible factor (HIF-1α) levels increase when HIF-1α is stabilized and not degraded by VHL. Elevated HIF-1α levels lead to VEGF-A transcription [76-78]. VEGF-A can then directly promote tumor invasiveness, motility, and migration [79-81].

VEGF-A can also be upregulated in tumors in hypoxia-independent mechanisms. Abnormal activation of RAS, MAPK, and PI3K pathways can upregulate VEGF production in a hypoxia-independent manner [82]. Similar to the transcriptional regulation of IL-6, multiple transcription factors can control the production of VEGF-A including AP-1, Sp-1, C/EBPB, STAT3, and 17β-estradiol (E2) by binding to the VEGF-A promoter [83-85].

VEGF-A can also directly promote self-renewal of cancer stem cells [86]. Previous studies have associated VEGF-A production with development of cancer stem cells in an angiogenic tumor microenvironment characterized by an epithelial to mesenchymal transition (EMT) phenotype [87]. Additionally, some tumors have upregulated VEGF/VEGFR signaling which sustains further tumor growth through activation of self-renewal and survival pathways involved in chemoresistance [88, 89].

**VEGF EFFECTS ON IMMUNE MICROENVIRONMENT**

VEGF-A is a critical immunomodulatory cytokine which can impact the function of different types of immune cells. VEGF-A can impair the maturation and differentiation process dendritic cells undergo [90, 91]. VEGF-A can even inhibit the apoptotic and antigen-presentation function of already mature dendritic cells through VEGFR-2 signaling [92]. Experiments that evaluated the effects of dendritic cells directly cultured with VEGF showed that VEGF inhibited activation of antigen specific T cells and thus the ability to mediated
cytotoxic killing [93, 94]. VEGF-A can also inhibit activity of hematopoietic progenitor cells (HPCs) which are involved with the activation and regulation of dendritic cell differentiation [95].

VEGF-A has exhibited several immunosuppressive effects on T cells in the tumor microenvironment. VEGFR2 receptor is expressed on T-regulatory cells with high Foxp3 thus allowing VEGF to promote the activity and proliferation of immunosuppressive T-regulatory cells [96-98]. VEGF-A can indirectly regulate induction of T-regulatory cells through impaired activity of dendritic cells and an increase recruitment of MDSCs which both can alter T-regulatory cell function [93, 96]. VEGF-A inhibit CD8+ T effector activity through creating abnormal vasculature which reduces healthy CD8+ T cell trafficking to mediate an effective anti-tumor response [96]. Moreover, defective dendritic cell activity directly impairs the ability for effector T cells to be educated by tumor antigens and activated.

Macrophages are a major source of VEGF. Thus, VEGF signaling has also been implicated in the recruitment and polarization of macrophages, to M2 tumor-associated macrophages, and promoting immunosuppression in the tumor microenvironment [99-101]. Tumor-associated macrophages produce VEGF-A in hypoxic areas of the tumor which results in the recruitment of more macrophages and endothelial cells and the production of MMP9 [102]. VEGF-A functions also as a chemoattract for myeloid derived suppressor cells (MDSCs) and because expression of VEGFR1 and VEGFR2 on their surface supports a positive autocrine feedback loop [103, 104]. Through these various mechanisms (illustrated in Figure 1), VEGF has clear implicated immunosuppressive effects in the tumor microenvironment in addition to its pro-tumorigenic effects via interactions with endothelial cells and tumor cells.
Figure 1. The effect of VEGF on the function and growth of endothelial, tumor, and immune cells in the tumor microenvironment. Signaling with VEGFR-1 on myeloid cells, VEGFR-2 on endothelial cells and tumor cells, and VEGFR-3 on lymphatic endothelial cells is depicted in this figure adapted from an image created with BioRender.com. Reprinted with permission of Clinical Cancer Research.
MECHANISMS OF RESISTANCE TO VEGF TARGETING THERAPIES

Multiple clinical studies have found that anti-VEGF-A blocking therapies yield clinical benefit for patients across several tumor types [105-107]. However, acquired resistance to anti-angiogenic therapy can develop in patients due to a variety of different factors and escape mechanisms [108-110].

First, alternative pro-angiogenic proteins like Ang2, PDGF, and TGF-B1 can be secreted as a bypass mechanism if VEGF-A is blocked also known as redundancy of angiogenic signaling pathways [111]. Additionally, early in the development of VEGF-A therapy resistance is the upregulation of autophagy processes, which result in the survival of hypoxic cells, and other metabolic alterations including increased acidification and induction of glycolysis [112]. Additionally, though initially counter-intuitive, blockade of VEGF-A induces more hypoxic conditions which then mediates resistance to VEGF-A inhibition. In some instances, this process can be induced by upregulation of the HGF/c-met pathway [113]. VEGF-A blockade is administrated in hopes that it targets aberrant tortuous vessel formation. Though, initially productive in reducing angiogenic-driven tumor growth starvation of these same blood vessels of oxygen will cause the tumor to compensate by upregulating angiogenic factor production [114]. In addition to hypoxia, upregulation of HIF-1α in response to prolonged VEGF-A blocking therapies can promote EMT which initiates tumor metastasis and invasiveness [115].

Moreover, several other targets have been found to be overexpressed in a subset of tumors which developed resistance to VEGF targeted therapies. Estrogen has been identified as a mediator of bevacizumab resistance in murine NSCLC xenograft models [116]. Some tumors express high levels of TGF-B or MMPs which mediate enhanced tumorigenesis [117].
Resistance to VEGF therapies can also result through recruitment of pericytes or through a process called vascular mimicry, a mechanism a tumor uses to develop its own endothelial-cell like vascular network using tumors cells [118, 119]. An accumulation of bone marrow-derived cells (BMDCs) in the tumor microenvironment can occur due to rapid restriction to blood vessel development by the anti-angiogenic therapy [120]. In turn, these BMDCs promote angiogenesis and have immunosuppressive properties. In summary, there are a variety of factors involved with response and sometimes these same factors can unfortunately also attribute to the likelihood of developing resistance to VEGF/VEGFR cancer therapies. These preclinical and clinical VEGF studies highlight the need to identify therapeutically targetable vulnerabilities associated NSCLC disease that has developed resistance to anti-VEGF therapy and then effectively evaluate feasible combination treatment options which can be tolerated well and benefit patients.
SUMMARY AND STATEMENT OF THE PROBLEM

Non-small cell lung cancer is the leading cause of cancer-related deaths. 10-15% of all cases harbor activating mutations in EGFR. Despite the use of EGFR-TKI which are effective initially, a majority of patients with EGFR-mutant NSCLC develop resistance and ultimately succumb to their disease. Moreover, while use of anti-PD-1 agents are effective in patients with NSCLC tumors, those with EGFR-mutant NSCLC do not derive clinical benefit. The development of effective therapies is combat TKI-resistant disease and elucidating immunosuppressive mechanisms which drive resistance to immunotherapy are imperative.

NSCLC patients also have several other targeted agents available as suitable treatment options including therapies which target increased angiogenic growth such as vascular endothelial growth factor (VEGF) antibodies and TKIs. Previous analysis of clinical studies found that among NSCLC patients treated with chemotherapy, females derive less benefit from the addition of VEGF monoclonal antibody bevacizumab than males [121]. Thus, it is critical to determine if female sex hormone, estrogen, can in part impact clinical response to anti-angiogenic therapy. NSCLC tumors which have acquired resistance to VEGF targeted therapies also typically have increased infiltration of immunosuppressive cell populations including myeloid cells. However, no studies have yet understood if estrogen directly modulate such an immunosuppressive NSCLC tumor microenvironment.

The goal of my research is 1) to characterize the role of IL-6 on the immune microenvironment, specifically via function of tumor-infiltrating T and NK lymphocytes, of EGFR-mutant tumors and 2) to determine the impact of estrogen on tumor growth and immune infiltration in the bevacizumab-resistant NSCLC disease. The hypothesis to be tested is that 1) secretion of IL-6 by human EGFR-mutant lung tumor cells contributes to the progression of
the disease by promoting immunosuppression of T and NK cell activity and that 2) estrogen potentiates infiltration of immunosuppressive populations which aids growth of NSCLC tumors which are refractory to bevacizumab. To test this hypothesis, I propose the following specific aims:

1. Determine expression of IL-6 in human EGFR-mutant TKI-resistant NSCLC cancer cell lines, patient-derived models, and in vivo models.

2. Elucidate the role of IL-6 in mediating T cell and NK cell activity in the EGFR-mutant microenvironment through in vitro and in vivo assays.

3. Identify if therapeutic blockade of IL-6 affects the sensitivity of EGFR-mutant in vivo models to PD-1 treatment.

4. Understand the regulation of estrogen production and signaling in NSCLC tumor models

5. Determine changes in immune infiltration and angiogenesis in NSCLC models with inhibited estrogen signaling.
CHAPTER 2

INTERLEUKIN-6 EXPRESSION IS ASSOCIATED WITH EGFR-TKI RESISTANCE
*This chapter is largely based on work produced for the following publication in Clinical Cancer Research:


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INTRODUCTION

Elevated IL-6 levels have been associated with the development of several autoimmune diseases and cancers. IL-6 signaling has been linked with chronic inflammation progression of cancer growth through promoting tumor proliferation, invasiveness, and changes in immune cell recruitment. In EGFR-mutant NSCLC patients treated with the first generation EGFR-TKI erlotinib, high levels of IL-6 in their serum was associated with a worse clinical outcomes measured by overall survival [57]. To determine if there was a similar pattern of IL-6 expression in EGFR-mutant NSCLC tumor cells which acquired a resistance to the more now commonly used third-generation EGFR TKI osimertinib, IL-6 secretion was measured in several models of osimertinib-acquired resistance: human EGFR-mutant cancer
cell lines, EGFR-mutant genetically engineered mouse model (GEMM), and patient derived surgical resections.

METHODS

Human Cell lines: EGFR-mutant NSCLC cell lines HCC4006, HCC827, and H1975 were purchased from ATCC and maintained in culture as previously stated [122]. EGFR-TKI resistant cell lines were generated as done previously [26]. YUL-0019 (N771delinsFH) [123] was obtained by Dr. Politi (Yale Medical School). MDA-L-011 (L858R) [26], MDA-L-004K (EGFR exon 20 mutation), MDA-L-0024 (E746_A750del mutation), MDA-L-0046 (EGFR exon 19 del), and MDA-L-0065 (EGFR exon 19 del) were obtained from surgical resected tissue from patients at MD Anderson who progressed on EGFR-TKIs. All patients were provided written informed consent through an Institutional Review Board-approval protocol at MD Anderson Cancer and conducted in accordance with the Declaration of Helsinki and Belmont report for appropriate collection of these surgical specimens. All mentioned cell lines above were cultured in RPMI medium in addition to 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine.

Pharmaceutical reagents: IL-6 neutralizing antibodies were purchased from R&D Systems. Erlotinib and siltuximab were ordered from the University of Texas MD Anderson Cancer Center institutional pharmacy.

Multiplex cytokine array: A mouse Luminex discovery assay was utilized to measure secretion of 25 cytokines from serum collected from the blood of EGFR\textsuperscript{L858R} GEMMs.
Detection of IL-6 in preclinical samples: EGFR-mutant NSCLC cell lines (200,000 cells/well in 6-well plates) were plated in 10% FBS serum RPMI medium and after 24 hours were replaced with serum-free medium. Conditioned medium from these cells was collected. IL-6 ELISA (purchased from R&D Systems) was performed according to manufacturer’s instructions.

Detection of IL-6 in clinical tissue and serum samples: Biospecimens were obtained after patients provided written informed consent under an Institutional Review Board approved protocol and conducted in accordance with the Declaration of Helsinki and Belmont report. The CROSSOVER and NORTHSTAR clinical datasets are comprised of MD Anderson patients who received osimertinib treatment and if applicable local consolidated therapy. We analyzed 12 matched pairs of samples collected prior to progression of disease (after randomization and end of cycle 2 of osimertinib treatment) and at progression of disease. ELISA was run in duplicate technical samples and quantitative measurement of circulating IL-6 serum concentration was calculated. Statistical analysis was used to compare treatment groups using a paired two-tailed Student’s t-test.

RT-PCR: RNA was isolated using TRIzol Reagent (Invitrogen) and RT-PCR was performed in triplicate biological samples.

RNA-sequencing: Total RNA was collected from the EGFR mutant NSCLC GEMM models listed above and extracted and purified using the RNeasy Plus Mini Kit (Qiagen). RNA-seq libraries were prepared and analyzed in triplicate using the Illumina Mouse NovaSeq6000. Gene expression data was also utilized from the human EGFR mutant NSCLC cell line dataset from the GEO repository (GSE 121634) previously reported [26].
**In vivo studies:** CCSP-rtTA EGFR\textsuperscript{L858R} genetically engineered mice were used for in vivo experiments. These mice were generated by Dr. Katherine Politi (Yale School of Medicine) [124]. In the EGFR-independent model experiments, CCSP-rtTA EGFR\textsuperscript{L858R} mice were treated with osimertinib (5 mg/kg, oral once a day). Antibodies were purchased from Bioxcell. Tumor generated in the lung were excised and serum from mice were harvested after three weeks of treatment.

**Immunohistochemistry:** Frozen tissue sections of tumors collected from the CCSP-rtTA EGFR\textsuperscript{L858R} murine model were used to evaluate expression of EMT and lung tissue markers. Specimens were sectioned (8-10 μM thickness) and stained with primary antibodies for E-cadherin, vimentin, SP-C, and TTF-1 at 4°C overnight. Staining was visualized using an AxioCam MRC5 camera and Axio vision software 4.6.

**Timer2.0 analysis:** This online immune deconvolution tool used gene expression from murine RNA-sequencing dataset to estimate immune infiltrates through comparison of multiple deconvolution analyses [125].

**KMplotter analysis:** Online KMplotter database (https://kmplot.com/analysis/) was utilized to analyze clinical outcomes of 1926 NSCLC patients. Overall survival (measured in months) data for these patients was stratified across expression levels of IL-6.

**Statistics:** For all above experiments described, statistical analysis was performed using either a Student’s \textit{t}-test (two-tailed) or one-way ANOVA using GraphPad Prism 9. A \textit{p} value \( \leq 0.05 \) was considered statistically significant (*), \( p \leq 0.01 \) (**), and \( p \leq 0.001 \) (***).
RESULTS

**IL-6 secretion is elevated in human EGFR mutant NSCLC tumor cells with acquired resistance to the EGFR-TKI osimertinib**

Previous studies have shown that EGFR mutant NSCLC tumors cells that have developed an acquired resistance to the first-generation EGFR inhibitor, erlotinib, overexpress IL-6 [14, 15]. The recent FDA approval of osimertinib, a third-generation EGFR inhibitor, has made administration of osimertinib a frontline therapy for patients with EGFR-mutant NSCLC tumors. Thus, it was important to investigate if IL-6 expression in EGFR-mutant NSCLC tumors cells with acquired resistance osimertinib displayed a similar expression pattern. We utilized a panel of osimertinib-resistant tumor cell lines previously shown to have undergone EGFR-TKI resistance [126] through an epithelial to mesenchymal transition (EMT) [126] mechanism. In the other models of acquired EGFR TKI resistance which were mediated by MET amplification, IL-6 was not upregulated [14]. Osimertinib-resistant (OR) variants secreted significantly greater levels of IL-6 compared their respective EGFR mutant parental cell counterparts, HCC4006 and H1975 (Figure 2A-B).
Figure 2. In vitro secretion of IL-6 from human EGFR-mutant NSCLC tumor cells.

Tumor cell secretion of IL-6 is measured by enzyme-linked immunosorbent assay (ELISA) on conditioned medium collected from each cell line (A) HCC4006 and its OR variants and (B) H1975 and its OR variants. Values graphed were performed in triplicate. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.

Next, to determine whether clinical samples also displayed a similar differential expression pattern of IL-6, multiple analyses were performed. Cell lines derived from surgical resected tissue from MD Anderson patients who acquired resistance to an EGFR-TKI were used to measure secreted IL-6. Patient-derived models of acquired EGFR-TKI resistance (MDA-L-004K, MDA-L0011, MDA-L-0024, MDAL-0046, and MDA-L-0065) had higher levels of IL-6 as compared to EGFR mutant TKI naïve cells (HCC827 and YUL-0019) (Figure 3A). To investigate if higher levels of IL-6 were associated with changes in
clinical outcome, analysis of lung cancer data from KMplotter was performed. In this dataset of 1926 patients with lung cancer, higher expression of IL6 was associated with a worse overall survival (HR = 1.48, p<0.0001) than patients with low expression of IL-6 (Figure 3B). Finally, serum of 12 EGFR-mutant NSCLC patients enrolled in the MD Anderson CROSSOVER and NORTHSTAR clinical trial studies was used to investigate changes in circulating IL-6 levels in the blood in patient’s blood collected before and after development of progressive disease after administration of EGFR-TKI. Higher levels of circulating IL-6 levels were measured in patients after progressive disease compared to levels measured in these sample patients prior to progression of disease (p=0.0517). (Figure 3C)
Figure 3. Association of IL-6 and clinical outcomes in patient-derived samples. (A) Measurement of IL-6 secretion by ELISA of patient-derived cells lines from EGFR-mutant NSCLC patients who acquired resistance to EGFR-TKI. Value were performed in triplicate. (B) Kaplan-Meier curves of the overall survival (months) of lung cancer patients with low vs high expression of IL-6. (C) Measurement of circulating IL-6 levels by ELISA of serum from EGFR-mutant NSCLC patients treated with an EGFR TKI before and after development of progressive disease. Values graphed were performed in duplicate. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
EGFR mutant NSCLC tumors with EMT-associated acquired osimertinib resistance
display an immunologically cold phenotype and have higher levels of IL-6

To determine if the tumor microenvironment affects regulation of IL-6 expression in
EGFR-mutant tumor cells, EGFR-mutant genetically engineered mouse models (GEMMs)
were used to conduct in vivo studies. Currently, there are no existing syngeneic murine
models of EGFR-mutant NSCLC tumors which acquired resistance to EGFR-TKIs.
Developing such a model would allow for more nuanced studies which utilize an intact
immune system to better study the immune microenvironment in these tumors.

Previously studies have utilized GEMMs to study KRAS [24, 25] and other
oncogenes of “extinguishing” the driver oncogene-dependent tumor development using an
inducible system. Tumors were induced in CCSP-rtTA EGFR<sup>L858R</sup> mice [21] with
doxycycline (DOX) chow to initiate tumor growth (EGFR dependent tumors; Figure 4A). To
develop oncogene-independent resistance in the EGFR<sup>L858R</sup> GEMM model, DOX chow was
given to the mice. After tumor developed in their lungs visualized by CT imaging, DOX
chow was then removed. This led to an initial shrinking of tumors followed by a slow and
steady growth of tumor volume. The withdrawal of DOX was, as expected, directly
associated to loss of EGFR<sup>L858R</sup> expression (Figure 4B). This process is meant to depict
EGFR-TKI resistance driven independently of EGFR secondary mutations that occurs readily
in the clinic (EGFR independent tumors; Figure 4A).

To evaluate the sensitivity of EGFR independent tumors to osimertinib, mice were
treated with or without osimertinib for 2 weeks. As seen by CT imaging (Figure 4C),
osimertinib treatment led to a marked reduction in the volume of EGFR dependent tumors
(p=0.0002), while EGFR independent tumors increased in size (p = 0.0475; Figure 4D).
**A**

- **EGFR dependent**
  - Induction with dox diet
  - CT image
  - Collect blood & tumor

- **EGFR independent**
  - Induction with dox diet
  - Remove dox
  - CT image
  - Collect blood & tumor

**B**

- RNA expression
- Relative to the Control
- EGFR dependent vs. EGFR independent

**C**

- EGFR dependent
- EGFR independent

- Pre-TKI
- Post-TKI

**D**

- Tumor volume (mm$^3$)
- EGFR dependent
- EGFR independent

- TKI: - (negative), + (positive)

- Statistical significance:
  - *** p < 0.001
  - * p < 0.05
Figure 5. Development of EGFR-TKI resistant EGFR-mutant murine model (A)

Schematic defining methodology used to develop murine models. (B) RNA expression of mutant EGFR levels from tumors of EGFR dependent and EGFR independent mice. (C) CT imaging of lung tumors from EGFR dependent and EGFR independent mice before and after TKI treatment and (D) associated quantitative tumor volume analysis. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
To determine mechanism of resistance that occurs in the EGFR independent tumors, blood and tumors tissues were collected. Gene set enrichment analysis (GSEA) found that EGFR independent tumors displayed an enrichment in EMT-associated gene expression (Figure 5A). The EMT phenotype was further confirmed in the EGFR independent tumors through immunohistochemistry staining of EMT markers, e-cadherin and vimentin. EGFR independent tumors had increased expression of vimentin and a decreased expression of E-cadherin compared to EGFR dependent tumors (Figure 5B).

To understand if these EGFR independent tumors which acquired resistance to osimertinib could be characterized by differential protein secretion serum from the blood of these mice was subjected to a 16 immunomodulatory-related multiplex cytokine assay. IL-6 and IL-9 were the most highly upregulated cytokines in EGFR independent mice compared to EGFR dependent mice (Figure 5C). Increased levels of IL-6 in circulation were confirmed in EGFR independent tumors by ELISA assay (Figure 5D).
A

Gene signature analysis
(EGFR-dependent vs EGFR-independent)
p = 0.030

B

C

Fold change in serum concentration

E-cadherin
Vimentin

D

IL-6 Concentration (pg/mL)

EGFR dependent
EGFR independent
Figure 5. TKI-resistant EGFR independent murine model shows increased mesenchymal marker and IL-6 expression. (A) GSEA analysis finds that EGFR independent tumors have an enrichment of an epithelial-to-mesenchymal gene signature. (B) IHC staining of EGFR dependent and independent tumors for epithelial and mesenchymal marker expression. (C) Multiplex cytokine analysis of serum collected from the blood of EGFR dependent and EGFR independent mice. (D) IL-6 ELISA confirms higher circulating levels of IL-6 in blood collected from EGFR independent mice. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
Prior studies found a positive association between tumors which underwent epithelial to mesenchymal transition (EMT) and a colder immune microenvironment in lung adenocarcinomas [27]. Therefore, the immune infiltration was evaluated in both EGFR dependent and EGFR independent tumors due to their differential expression of EMT marker. Immune cell deconvolution analysis revealed significantly decreased CD8+ T cells (p=0.0194), CD4+ T cells (p <0.0001), T regulatory cells (p = 0.0061; Figure 6A) in EGFR independent tumors. Additionally, total NK cells (p < 0.0001), and decreased activated NK cells (p=0.0122) in EGFR independent tumors when compared to EGFR dependent tumors (Figure 6B).
### A

#### T cell

<table>
<thead>
<tr>
<th>EGFR dependence (DOX)</th>
<th>CD8+ T cells</th>
<th>CD4+ T cells</th>
<th>T regulatory cells</th>
<th>T follicular helper cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>*</td>
<td>****</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>(-)</td>
<td>0.135</td>
<td>0.020</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

### B

#### NK cell

<table>
<thead>
<tr>
<th>NK cells</th>
<th>Resting NK cells</th>
<th>Activated NK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR dependence (DOX)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Relative Ratio</td>
<td>0.40</td>
<td>0.10</td>
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</table>
Figure 6. Tumors from TKI-resistant EGFR-mutant GEMM have reduced immune infiltration. Immune cell deconvolution analysis from RNA-sequencing data collected from EGFR dependent and EGFR independent tumors showed a significant reduction of (A) CD8+ T cells (p = 0.0194), CD4+ T cells (p < 0.0001), (B) total NK cells (p < 0.0001), activated NK cells (p = 0.0122), and T follicular helper cells (p = 0.0061). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
CHAPTER 3

TARGETING IL-6 TO REDUCE IMMUNOSUPPRESSION IN EGFR MUTANT TUMORS
INTRODUCTION

IL-6 is an immunomodulatory cytokine known to have pleiotropic effects in the tumor microenvironment. This includes its ability to promote CD4+ T cell differentiation into different T cell subset including T-regulatory cells and ability to inhibit NK cell cytotoxicity amongst multiple other characterized effects on the immune microenvironment. Therefore, we sought to investigate the role of IL-6 in the immunosuppressive EGFR mutant microenvironment, which is highly refractory to ICB immunotherapy. Because responses to ICB response is heavily dependent on immune effector cells, we sought to investigate the effects of the high levels of IL-6 secreted by EGFR-mutant NSCLC tumors on the function of T and NK cells in the microenvironment.
METHODS

**Flow Cytometry:** The following flow cytometry conjugated antibodies (purchased from Biolegend or Thermofisher) were used at concentrations listed in the manufacturer’s data sheet for immunostaining of mouse tumor cells: Live/Dead Ghost UV450, anti-CD45 BUV805, anti-PD1 BV421, anti-CD8 BV570, anti-NKG2D FITC, anti-CD278 BV785, anti-IFNγ PerCP-Cy5.5, anti-PD-L1 PE-Dazzle 594, anti-NK1.1 Pe-CY5, anti-CD3 Pe-Fire700, anti-CD4 AlexaFluor700, anti-Ki-67 Pacific Blue, anti-FoxP3 Alexa532, anti-Granzyme B PE, anti-IL-17A APC. For intracellular staining for the following antibodies (FoxP3, Granzyme B, IL-17A, and Ki-67), cells were first fixed and then permeabilized using cold 70% ethanol. Immuno-stained cell percentages were assessed by a Cytek Aurora flow cytometer machine and data was recorded to be analyzed by Flow-Jo software.

**RT-PCR and RNA sequencing** were used as described in Chapter 2.

**Functional immune cell assays:** Healthy donors whole blood samples purchased from the Gulf Coast Consortium. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation separation of the whole blood samples. NK cells and T cells were then isolated from human PBMCs by magnetic enrichment. NK cells were expanded ex vivo and activated using K562 feeder cells (1:2 ratio) and 200 U/mL IL-2 and 5 ng/mL IL-15, and 2 mM GlutaMAX purchased from Gibco. Expanded NK cells were cultured in normal media (RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine) for 14 days before being utilized for subsequent co-culture and cytotoxicity assays. Correspondingly T cells were magnetically sorted from PBMCS and
activated with anti-CD3/CD28 Dynabeads (Thermo Fischer Scientific) at a 1:1 ratio in RPMI-1640 complete media (10% FBS, 2 mM GlutaMAX, 100 U/mL penicillin, and 100 μg/mL streptomycin) supplemented with 100 U/mL IL-2 (PreproTech). To assess NK cell or T cell cytotoxicity in vitro, NK cells or T cells were co-cultured with target cells stably transfected with pHIV-Luc-ZsGreen (Addgene plasmid # 39196). Multiple effector to target ratios (10:1, 5:1, 2.5:1, and 1:1) were utilized to generate a cytotoxicity killing curve. Cytotoxic killing was measured by luciferase signal output after 4 hours of target cell and effector cell co-incubation. Luciferase signal was measured and quantified using a FLUOstar OPTIMA multi-mode micro-plate reader (BMG Labtech). Specific lysis was calculated using the following formula: 

\[
\frac{(\text{Target cells only} - \text{Experimental Target})}{(\text{Target cells only} - \text{no target cells})} \times 100\%.
\]

Cytotoxicity assays for T cells utilized the same experimental set up but target cells were transduced with luciferase pHIV-Luc-ZsGreen (Addgene plasmid #39196) and custom OKT3 construct (pPSFG-OKT3-CD86).

**In vivo studies:** CCSP-rtTA EGFR<sup>L858R</sup> genetically engineered mice [124] were crossed with IL-6 knockout mice (IL6<sup>-/-</sup>) obtained from Dr. Seyed Moghaddam (MD Anderson Cancer Center) to generate CCSP-rtTA EGFR<sup>L858R/IL-6-/-</sup> mice. After 6 weeks of doxycycline diet treatment, animals were randomized into treatment groups and treated with a monoclonal antibody against IL-6 (10 mg/kg) or anti-PD-1 (10 mg/kg), intra-peritoneally, twice a week. Antibodies were purchased from Bioxcell. Tumors and serum from mice were harvested after three weeks. IL-6 knockout EGFR-mutant mice were utilized for subsequent survival and immune profiling in vivo studies.

**Timer2.0 analysis** was utilized as described in Chapter 2 Materials.
Statistics: For all studies, statistical analysis was performed using the Student’s $t$-test (two-tailed) or one-way ANOVA. A p value $\leq 0.05$ was considered statistically significant (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) . For RNA-sequencing data, analysis of variance (ANOVA) was used on a gene by gene basis just as described in Chapter 2.

RESULTS

Depletion of IL-6 in murine models of EGFR-mutant NSCLC improves overall survival and increases infiltration of lymphocytes

To evaluate the impact of IL-6 on the EGFR mutant NSCLC tumor immune microenvironment, a syngeneic, immunocompetent mice would be the suitable in vivo model to work with. Doxycycline-inducible EGFR$^{L858R}$ GEMMs [21] were cross with mice with a total body knockout of IL-6 (provided by Seyed Moghaddam at MD Anderson) resulting in EGFR$^{L858R/IL6KO}$ mice. ELISA of IL-6 confirmed the depletion in both the blood and bronchoalveolar lavage fluid (BALF) of EGFR$^{L858R/IL6KO}$ mice as compared to EGFR$^{L858R}$ mice (Figure 7A). Knockout of IL-6 in treatment naïve EGFR$^{L858R}$ mice had a modest yet significant effect on survival (Figure 7B; HR = 1.31, p=0.0212).
Figure 7. Depletion of IL-6 increases the number of infiltrating lymphocytes and overall survival in EGFR mutant NSCLC tumors. (A) IL-6 levels in the serum and BALF of EGFR<sup>L858R</sup> and EGFR<sup>L858R/IL6<sup>KO</sup></sup> tumor-bearing mice. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). (B) Knockout of IL-6 extended the survival of EGFR<sup>L858R</sup> tumor-bearing mice (p = 0.0212) compared to EGFR<sup>L858R</sup> with intact IL-6. Reprinted with permission of Clinical Cancer Research.
To investigate the effects of IL-6 on the tumor immune microenvironment of EGFR mutant tumors, RNA sequencing of EGFR<sup>L858R</sup> and EGFR<sup>L858R/IL6<sup>IKO</sup></sup> tumors were completed to compare gene expression and perform immune cell deconvolution analysis. IL-6 depletion in the EGFR<sup>L858R/IL6<sup>IKO</sup></sup> tumors was associated with an increase in NK cell populations in the tumor (Figure 8A; p=0.0032) in addition to an increase in CD8+ T cell (p=0.0595) and T follicular helper cell populations (p<0.0001). T-regulatory cell populations were however slightly (p=0.1004) decreased in the tumor (Figure 8B).
Figure 8. Immune cell deconvolution analysis from RNA-sequencing data collected from control and IL-6 knockout tumors from EGFR<sup>L858R</sup> mice (A) NK cells (p = 0.0032) and (B) IL-6 knockout mice showed a minor decrease in T-regulatory cells (p = 0.1004), CD4+ T cells (p = 0.0086), and T follicular helper cells (p < 0.0001), and a slight increase of CD8+ T cells (p = 0.0595). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
Next, flow cytometry analysis of EGFR\textsuperscript{L858R}/IL6\textsuperscript{KO} tumors was done to directly identify presence of infiltrating lymphocytes identified which appeared to be modulated in the previous immune cell deconvolution analysis. Total immune infiltration in tumors from EGFR\textsuperscript{L858R}/IL6\textsuperscript{KO} mice was higher compared to immune infiltration in EGFR\textsuperscript{L858R} mice measured by CD45+ expression found within excised lung nodules (p=0.06) (Fig 2E). Specifically, within the total immune cell populations, NK cell (p=0.0471) and T cell (p=0.0408) infiltration were significantly increased in EGFR\textsuperscript{L858R}/IL6\textsuperscript{KO} tumors as compared these lymphocytic populations in EGFR\textsuperscript{L858R} tumors (Figure 9A). Both CD4+ T cells (p=0.0086) and CD8+ T cells (p=0.0595) were increased in EGFR\textsuperscript{L858R}/IL6\textsuperscript{KO} tumors as compared to EGFR\textsuperscript{L858R} tumors while the Foxp3+ T-regulatory cell population was not differentially significantly altered between EGFR\textsuperscript{L858R} and EGFR\textsuperscript{L858R}/IL6\textsuperscript{KO} tumors.

Next, we sought to identify a clinically meaningful and feasible approach to recapitulating a tumor microenvironment depleted of IL-6. Thus, acute blockade of IL-6 signaling through a monoclonal IL-6 antibody was utilized to see if that similarly modulated immune infiltration in the EGFR-mutant microenvironment. Tumors from EGFR\textsuperscript{L858R} mice, were randomized to be treated with a vehicle agent or anti-IL-6 blocking antibodies. IL-6 blockade significantly extended the overall survival of tumor-bearing EGFR\textsuperscript{L858R} mice (HR = 2.301, p=0.0385; Figure 9B). Similar to the knockout of IL-6, there was an increase in infiltration of NK and CD4+ T cells in mice whose tumors were treated with acute IL-6 blocking antibodies as determined by flow cytometry (Figure 9C). Together, these two in vivo experiments illustrate the immunosuppressive effect of IL-6 in the EGFR mutant microenvironment and subsequently depletion of IL-6 can increase lymphocytic infiltration of T cells and NK cells.
Figure 9. Depletion of IL-6 increase infiltration of lymphocytes and is associated with extended survival in mice. (A) Tumor immune cell populations in EGFR\textsuperscript{L858R} tumors with or without anti-IL-6 treatment as determined by flow cytometry. (B) Kaplan-Meier analysis of EGFR mutant GEMM treated acutely with monoclonal blocking antibody to IL-6 (p = 0.0385). (C) Flow cytometry analysis to directly assess infiltrating immune cells in T and NK cell infiltration in EGFR mutant GEMM treated with monoclonal blocking antibody to IL-6. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
High IL-6 levels associated with other disease settings include other cancer types like esophageal cancer have been linked reduced NK activity [28]. Therefore, further studies to document the role of high IL-6 on NK cell function in the EGFR-mutant microenvironment were conducted. First, NK cell proliferation state in EGFR mutant tumors was assessed with or without the presence of IL-6. NK cell proliferation rates were the same in EGFR<sup>L858R</sup> tumors with or without IL-6 knockout (Figure 10A) as determined by flow cytometry analysis. Additionally, treatment of EGFR<sup>L858R</sup> tumor-bearing mice with anti-IL-6 antibodies did not affect NK cell proliferation either(Figure 10B). Activation state of infiltrating NK cells were however increased in mice treated with IL-6 blockade or in the IL-6 knockout mice measured by infiltration of NKG2D+ NK cells (Figure 10C - D).
Figure 10. IL-6 suppresses the activation of NK cells in EGFR mutant NSCLC tumors.

(A) IL-6 knockout and (B) acute blockade of IL-6 increases expression of Ki-67+ proliferating NK cells (p=0.3501, p=0.4698), (C-D) activated NKG2D+ NK cells (p=0.1042, p=0.0418). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
To determine if the high levels of IL-6 in the microenvironment could directly impact immune cell ligand present on tumors a co-culture assay was performed. NK cells isolated from healthy donor peripheral blood mononuclear cells (PBMCs) were expanded ex vivo (See Methods section) and incubated in conditioned media. Conditioned media was collected after 24 hours of culture from human HCC4006 EGFR mutant cell lines and their osimertinib-resistant (OR) variants treated with a vehicle agent or with siltuximab, IL-6 blocking antibody. After 48 hours of co-cultured in the conditioned media, NK cells were analyzed by flow cytometry. IL-6 blockade enhanced expression of the NK activation marker NKG2D in NK cells cultured in conditioned media from HCC4006 OR cells (Figure 11A), but not parental HCC4006 cells. Similarly, IL-6 blocking antibodies increased Granzyme B expression in NK cells incubated in HCC4006 OR4 and OR7 (Figure 11B). NK activation and inhibitory receptors can bind to corresponding ligands expressed on tumor cells. Thus, we investigated the effect of IL-6 on tumor cell expression of NK receptor ligands. Treatment of HCC4006 OR cells with IL-6 blocking antibodies increased expression of MICA, a NK activation ligand which typically binds to NKG2D+ NK cells (Figure 11C).
Figure 11. Blockade of IL-6 in human EGFR-mutant tumor cells increases activation of NK cells during co-culture assay. (A-B) Acute blockade of IL-6 of EGFR mutant NSCLC cell lines co-cultured with human NK cells isolated from healthy donor PBMCs significantly increased expression of NKG2D and Granzyme NK cells. (C) MICA expression on EGFR mutant TKI resistant is increased with IL-6 blockade. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
Next, sensitivity of EGFR-TKI resistant cells that have acquired EGFR-TKI resistance through EMT-mediated mechanisms to NK-mediated killing were assessed. Additionally, further in vitro cytotoxicity assays were performed to determine if the presence of IL-6 affects NK cytotoxic capacity. EGFR mutant TKI resistant cells were more sensitive to NK-mediated cytotoxic killing, measured by specific lysis, compared to EGFR-mutant parental TKI naïve cells. When EGFR-mutant parental TKI naïve cells, including HCC4006 parental cells (low IL-6 expressing), were treated IL-6 neutralizing antibodies they were not as well sensitized NK-mediated killing further proving the association between high IL-6 levels and impaired NK cytotoxic potential (Figure 12). Finally, therapeutic IL-6 blockade may help reduce immunosuppression through increased activation and killing potential of NK cells.
Figure 12. IL-6 blockade modulates NK cell cytotoxicity. IL-6 blockade sensitizes EGFR mutant EGFR-TKI resistant cells to NK cell-mediated cytotoxic killing. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
T cells mediate robust anti-tumor responses and also main driving effector cell mechanistically involved in mediating an anti-PD-1/PD-L1 immunotherapy response. IL-6 has been implicated in modulating activity of T cells in the tumor microenvironment in both immunosuppressive and inflammatory manners [29, 30]. Thus, T cell subpopulation in EGFR<sup>L858R</sup> tumors with and without IL-6 were compared. There was an increase in activated infiltrating CD8+IFNγ+ T cells in tumors from EGFR<sup>L858R/IL6<sup>KO</sup></sup> mice compared to tumors from EGFR<sup>L858R</sup> mice (Figure 13A; p=0.038). EGFR<sup>L858R</sup> mice with tumors treated with IL-6 antibodies also had an increase in the activated CD8+IFNγ+ T cell population compared to mice treated with vehicle agent (Figure 13B; p = 0.060). IL-6 has shown to assist the development of immunosuppressive T cells subpopulation including Th17 T cells [31]. Infiltration of Th17 T cell subpopulation was decreased in tumors from EGFR<sup>L858R/IL6<sup>KO</sup></sup> mice compared to EGFR<sup>L858R</sup> mice (Figure 13C; p = 0.048). Meanwhile Th17 T cells were increased in tumors from EGFR<sup>L858R</sup> mice compared to EGFR<sup>L858R</sup> treated with IL-6 blocking antibodies (Figure 13D; p=0.032).
Figure 13. Knockout of IL-6 modulates activated CD8 and Th17 T cells. (A) IL-6 knockout increased CD8+IFNγ+ T cells (p=0.0375) while (B) IL-6 blockade had slightly more modest effects p=0.0375. (C) Th17 T cell populations were significantly reduced in IL-6 knockout tumors (p=0.048) and those treated with (D) IL-6 blocking antibody (p=0.032). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
To determine if IL-6 secreted from EGFR-mutant tumor cells impacted activity of T cells, T cells isolated from healthy donor PBMCs were cultured in conditioned media from EGFR mutant parental cells HCC4006 cells (low IL-6 expressing), or their OR variants, with or without treatment with IL-6 blocking antibody, siltuximab. T cells incubated with conditioned media from TKI-resistant cells, HCC4006 OR4 and OR7 cells, and treated with IL-6 blocking antibody displayed increased expression of Granzyme B, a marker indicative of T cell activation and cytotoxic potential. (Figure 14).

**Figure 14. Blockade of IL-6 increases Granzyme B expression on T cells.** Human T cells isolated from healthy donor PBMCs co-cultured in conditioned media collected after the acute blockade of IL-6 of EGFR mutant NSCLC cell lines significantly increased expression of Granzyme B expression. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
To directly measure the effect of IL-6 levels in the EGFR-mutant microenvironment on T cell-mediated cytotoxicity, T cells were co-cultured with EGFR mutant tumor cells lines transfected to express membrane-bound anti-CD3 (OKT3), which binds to CD3 on the surface T cells which facilitates T cell killing independent of antigen-specific recognition because T cells isolation was not able to be matched from the same patient that target tumor cells were derived from. When TKI-resistant cells were treated with IL-6 blocking antibody, tumor cells were significantly sensitized to T cell-mediated cytotoxic killing (Figure 15) compared to both TKI-resistant cells not treated with IL-6 blocking antibody and TKI-naïve parental cells. Accordingly, IL-6 blockade did not induce increased T cell-mediated killing against HCC4006 parental cells which express low levels of IL-6.
Figure 15. IL-6 blockade modulates T cell cytotoxicity EGFR mutant NSCLC cell lines osimertinib variants (OR) treated with anti-IL-6, siltuximab, were sensitized to increased T cell-mediated cytotoxicity. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
Because IL-6 levels have been shown to impact T cell activity, an important effector cell mediating ICB response, the next step was to understand if IL-6 levels in part affected sensitivity of EGFR-mutant NSCLC tumors to ICB therapy. To determine if IL-6 blockade could enhance the activity of ICB against EGFR- mutant tumors, EGFR_{L858R} tumor-bearing genetically engineered mice were treated with anti-PD-1, anti-IL-6, or a combination of both. EGFR_{L858R} mice treated with anti-PD-1, did not significantly improve overall survival (HR = 1.53, p= 0.310). On the other hand, single agent anti-IL-6 significantly extended overall survival in EGFR_{L858R} tumor-bearing mice (HR= 2.30, p = 0.039). Concurrent combination treatment with anti-IL-6 and anti-PD-1 agents significantly increased overall survival the most effectively out of all treatment groups (Figure 16A; HR = 3.15, p=0.016). This shows that in part blocking IL-6 levels was able to sensitize tumors from EGFR_{L858R} mice to anti-PD-1 treatment. Of note, this in vivo experiment was performed in TKI-naïve EGFR_{L858R} tumor-bearing mice, and thus it is critical that future investigations evaluate if syngeneic EGFR-mutant models which have develop acquired resistance to EGFR-TKIs display an even more amplified effect if that model would secrete even higher levels of IL-6 compared to those that are TKI-naïve.

Finally, T cell infiltration was evaluated by flow cytometry in the tumors of EGFR_{L858R} mice treated with combination therapy to identify mechanisms which may attribute to the extended overall survival pattern observed. Total CD8+ T cells infiltration (Figure 16B) was not significantly modulated by combination treatment of anti-IL-6 and anti-PD-1. However, tumor infiltration of a subset of activated CD8+ T cells (CD8+ PD-1+) was increased in EGFR_{L858R} tumors treated with the combination of anti-IL-6 and anti-PD-1 compared levels found in single agent-treated tumors (Figure 16C; p=0.047), suggesting that
IL-6 blockade may improve the efficacy of anti-PD-1 treatment via activation of infiltrating CD8 cells exhibited by increased PD-1+ CD8+ T cells.
A

Percent Survival

Days of Treatment

- Vehicle
- anti-IL-6
- anti-PD-1
- anti-IL-6/anti-PD-1

p = 0.0161

B

CD8+

% of total cells

Anti-PD-1
Anti-PD-1/anti-IL6

C

PD-1+ CD8+

% of total cells

Anti-PD-1
Anti-PD-1/anti-IL6
Figure 16. Anti-IL-6 treatment sensitizes EGFR-mutant GEMMs to anti-PD-1 treatment

(A) Anti-IL-6 blockade induces increased survival of EGFR mutant GEMMs treated with anti-PD-1 immunotherapy (p = 0.0161). (B) Infiltration of CD8+ T cells is not significantly modulated between anti-IL-6 and anti-PD-1/anti-IL-6 treatment groups. (C) Anti-IL-6 and anti-PD-1 combination treatment increases infiltration of activated T cells (PD-1+ CD8+) in EGFR mutant GEMMs (p = 0.0468). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Clinical Cancer Research.
CHAPTER 4

ROLE OF ESTROGEN SIGNALLING IN NSCLC TUMORS
INTRODUCTION

While the rate of lung cancer mortality in the United States has declined among male patients, lung cancer related deaths has not decreased in female patients [127]. Female never-smokers are more likely to develop lung cancer compared to males who smoke [128, 129]. Additionally, female smokers experience an increased risk in lung cancer development compared to men who have the same low levels of cigarette exposure [130]. One possible explanation for this disparity is that sex-related hormones may impact lung cancer development. Multiple studies have reported that female patients with lung cancer who had prior menopausal hormone replacement therapy had worse survival outcome compared to female patients who did not undergo menopausal hormone therapy, although the effect of estrogen on lung cancer outcomes remains controversial [131-133]. Thus, we sought to determine the effects of estrogen on NSCLC development and its role in therapeutic resistance.
METHODS

**Cell culture:** The following 3 non-small cell lung cancer (NSCLC) cell lines HCC827, A549, and Calu-6 were purchased from ATCC and maintained in cell culture as previously described [122].

**Cell Proliferation:** Cells were serum-starved for 48 hours in phenol red–free MEM with 5% charcoal-stripped FBS before plating. Cells (3,000 per well) were plated into each well of a 96-well plate and allowed to adhere for 24 hours before the addition of estrogen (E2) \(10^{-9}\) M and fulvestrant \(10^{-8}\) M. Ninety-six hours later, \(3-[4,\ 5\text{-dimethylthiazol-2-yl}]-2,\ 5\text{-diphenyltetrazolium bromide (MTT; thiazolyl blue)}\) was added to each well and incubated at 37°C and 5% CO₂ for 2 hours followed by medium removal and solubilization in 100 μL DMSO. The resulting color change was read at 570 nm and calculated as absorbance above background. Each point condition was performed in quadruplicate.

**Luciferase activity assays:** Cells (\(3\times10^5/well\)) were seeded in 24-well plate with 5% charcoal stripped serum in phenol red free DMEM, transfected with 100 ng of pGL2-VEGF-luciferase, or 100ng of ERE-Luciferase [134] and 2.5ng of pRL-TK (Promega, Madison, WI). Estrogen \(10^{-9}\) M was added 24 hours after transfection. Cells were incubated at 37°C and 5% CO₂ for 24 hours, lysed, and luciferase activity was determined using the dual-luciferase assay kit according to the manufacturer’s instructions (Promega). Relative firefly luciferase activity was normalized to renilla luciferase driven by the thymidine kinase minimal promoter.

**Animals and tumor xenografts:** Female (ovariectomized) athymic nude mice (NCI-nu, 4-8 weeks old) were obtained from the Animal Production Area of the National Cancer Institute (Frederick Cancer Center, Frederick, MD) and housed in facilities approved by the Association
for Assessment and Accreditation of Laboratory Animal Care International. Exogenous E2 (Estradiol 17-β) was supplied with estrogen tubing with approximately 80 pg/ml release [135, 136] was placed under the skin, above the shoulder, and the wound was closed with a wound clip [137]. A549 cells (1x10^6) or HCC827 (3.5x10^6) cells were injected subcutaneously into the dorsal flank. When the tumor reached 250-300 mm³, animals were randomized into treatment groups. Mice were treated with a monoclonal antibody against VEGF, bevacizumab (BV) (10mg/kg), intra-peritoneally, twice a week. Tumors were harvested when volumes reached 1000 mm³ and divided into 3 sections; one was fixed in formalin and embedded in paraffin, another was frozen in OCT compound, and the third was snap frozen in liquid nitrogen. Serum was extracted and stored in -80°C for further analysis.

RESULTS

Presence of estrogen does not induce proliferation of NSCLC tumor cells or stimulate growth of established tumors

To study the effect of estrogen on the growth and initiation of NSCLC tumors, two xenograft models were utilized: A549 and HCC827. These two human NSCLC cell lines were subcutaneously injected into the flank of nude ovariectomized mice, and animals were treated with or without estrogen (Estradiol 17-β) delivered via continuous release. Exogenous E2 (Estradiol 17-β) was supplied with estrogen tubing with approximately 80 pg/ml release [135, 136] was placed under the skin, above the shoulder, and the wound was closed with a wound clip [137]. Mice were randomized and tumor volume was measured in both xenograft models.
Estrogen did not significantly impact on the growth of established tumors starting at 200-400 mm³ (Figure 17A-B).

Tissue sections from these tumors (HCC827 xenograft) were stained for Ki-67 to determine if presence of estrogen influenced proliferation of NSCLC tumor cells. No significant difference in proliferation (indicated by Ki-67 staining) was observed between vehicle and estrogen-treated tumors (Figure 17C).
**Figure 17.** Tumor from A549 (A) and HCC827 (B) NSCLC xenograft models were randomized in treatment groups at 200-400 mm³. Treatment (N = 20 mice per group) included vehicle or estrogen (E2) and experiment was repeated twice. The log-rank (Mantel-Cox) test was used to compare the statistical differences among the groups. NSCLC xenograft tumors were sectioned and stained by immunohistochemistry for Ki-67 to measure tumor proliferation between vehicle and estrogen-treated NSCLC tumors. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
To better understand if estrogen has any direct effects on tumor cell proliferation \textit{in vitro}, Calu-6, A549, and HCC827 NSCLC cell lines were treated with or without estrogen or estrogen in combination with fulvestrant, an ER antagonist, for 4 days. Estrogen did not enhance the \textit{in vitro} proliferation of any cell line tested (\textbf{Figure 18}), and fulvestrant treatment did not inhibit tumor cell proliferation.

\textbf{Figure 18.} Cells were serum starved for 24 hours. $10^{-9}$M of estrogen (E2) and $10^{-8}$ M of estrogen antagonist, fulvestrant (F), was added. An MTT cell viability assay was performed after 96 hours of incubation. Results are presented as a fold change of vehicle-treated proliferation. Results are presented as mean of biological triplicates ± SEM and the experiment was repeated three times. (* \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\)). Reprinted with permission of Journal of Thoracic Oncology.
To determine if estrogen signaling was functional in these NSCLC tumor cell lines, ERα or ERβ were exogenously expressed in A549 and Calu-6 cells whose expression is driven by an estrogen response element. Estrogen stimulation increased luciferase activity in cells ectopically expressing ERα or ERβ (Figure 19). These findings show that while estrogen signaling is functionally intact in NSCLC cells, it does not directly impact tumor cell proliferation or the growth of established tumors.

**Figure 19.** Calu6 and A549 were serum starved and transfected with ERα or ERβ. Luciferase receptor assay was conducted, and results are expressed as fold change of control (B). Results are presented as mean of biological triplicates ± SEM and experiment was repeated three times. (* p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
CHAPTER 5

OVERCOMING ANTI-ANGIOGENIC THERAPY RESISTANCE THROUGH INHIBITION OF ESTROGEN
INTRODUCTION

Anti-angiogenic agents have been extensively studied in patients with NSCLC [138]. The VEGF-targeting antibody, bevacizumab, is approved for the treatment of locally advanced, recurrent, or metastatic non-squamous NSCLC [139, 140]. Unfortunately, not all patients receive sustained clinical benefit from anti-angiogenic agents, and resistance remains a clinical challenge [141, 142]. Sex-related differences have been observed in the therapeutic response of patients with NSCLC to anti-angiogenic agents. The Eastern Cooperative Oncology Group (ECOG) 4599 trial showed that male but not female patients with NSCLC received clinical benefit from bevacizumab treatment when combined with carboplatin plus paclitaxel [121]. Estrogen expression and availability are higher in female patients, which may in part explain this observed effect. Moreover, elevated expression of aromatase, the enzyme which converts testosterone into estrogen, correlates with a worse prognosis in female patients with NSCLC [143].
METHODS

**Immunohistochemistry:** Frozen tissue sections were used to evaluate CD31, desmin, and CD11b expression. Specimens were sectioned (8-10 μM thickness). Frozen sections were stained with anti-Ki-67 antibody (BD Biosciences, 1:500) at 4°C overnight for cell proliferation analysis. For vasculature staining, frozen sections were stained with anti-CD31 antibody (BD Biosciences, 1:500) and anti-desmin antibody (BD Biosciences, 1:400) at 4°C overnight. Immunofluorescence microscopy was performed using a Zeiss Axioplot 2 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Representative images were obtained using an AxioCam MRC5 camera and Axio vision software 4.6. For CD11b staining, frozen sections were stained with anti-CD11b antibody (BD Biosciences, 1:50) at 4°C overnight. Secondary goat anti-rat HRP antibodies (BD Biosciences, 1:200) were diluted and incubated for 1 hour. For VEGF and PDGFB staining, frozen sections were stained with anti-VEGF antibody (BD Biosciences, 1:50) or anti-PDGFB antibody (BD Biosciences, 1:100) at 4°C overnight. Secondary goat anti-rat HRP antibodies (BD Biosciences, 1:200) were diluted and incubated for 1 hour.

**Determination of Microvessel Density (MVD) and Pericyte Coverage:** Tumor MVD was determined as described previously [144]. In brief, tumors were examined microscopically to identify hot spots by low magnification (10X), and the mean MVD was quantified as the total number of CD31+ structures observed in a minimum of five microscopic fields at higher power of vision per tumor (200X). For each group, tumors from 4 mice from each group receiving short and long-term treatment were used. To determine the extent of pericyte coverage on the tumor vasculature, tumor sections were stained for CD31 (red) and desmin (green) as described.
above. Five fields per tumor were randomly identified at original magnification 200X, and blood vessels that were at least 50% covered by green desmin+ cells were considered to be positive for pericyte coverage.

**Determination of CD11b, VEGF, or PDGFBB positive cells:** In brief, we examined tumors microscopically to identify tumor positive area and tumor negative necrotic area. The number of CD11b, VEGF, or PDGFBB positive cells (both cytoplasmic and nuclear staining) were counted in a minimum of eight high power microscopic fields (400X) from periphery to the center/core of the tumor. For each group, tumors from 4 mice were used as indicated in the figure.

**Determination of Serum Levels of G-CSF, PDGFBB, VEGF, and CXCL1:** Serum levels of angiogenic factors were measured in tumor-bearing mice by multiplex bead assay (BioRad, Hercules, CA; Millipore, Billerica, MA) in a 96-well plate according to the manufacturer’s protocol. Serum samples were tested in duplicate, and the mean value used for analysis.

**Human studies:** *ESR1* (encodes ERα), *ESR2* (encodes ERβ), *ITGAM, PECAM, and CXCL1* gene expression levels were determined by microarray analysis (Illumina v3) of surgically resected lung adenocarcinomas from 150 patients (73 female and 77 male) and excluded patients who received any neoadjuvant therapy. This cohort was obtained from the Profiling of Resistance Patterns and Oncogenic Signaling Pathways in Evaluation of Cancers of the Thorax (PROSPECT) study, developed in 2006 at M.D. Anderson Cancer Center [145]. Expression values were log (base 2) transformed. Correlations between genes, separately in male and female LUAD patients, were statistically evaluated using Pearson’s correlation
coefficients and summarized plotted as correlation matrices. All analysis was performed in
the R statistical language and environment (R-project.org; version 3.5.1).

Statistics: Statistical significance was tested using GraphPad Prism 8 software (GraphPad
Software Inc., San Diego, CA). For comparison between groups, Student’s t-test and one-
way ANOVA test were used. A p value < 0.05 on two-tailed testing was considered
significant.

RESULTS

Estrogen promotes resistance to bevacizumab, anti-VEGF, treatment in NSCLC
xenograft models

To investigate whether estrogen is related to acquired resistance NSCLC tumors
develop towards anti-angiogenic therapy, in vivo experiments using A549 and HCC827
xenograft models were performed. A549 and HCC827 cells were injected subcutaneously into
female ovariectomized nude mice treated with or without estrogen (E2). Once tumors reached
a volume of ~250 mm³ mice were randomized to treatments with a vehicle agent or
bevacizumab (10 mg/kg). Presence of estrogen did not directly increase tumor growth rate in
either of the A549 or HCC827 xenograft models compared to mice from both models in the
vehicle-treated group. Tumor growth was significantly reduced by bevacizumab in mice
(A549; Figure 20A and HCC827; Figure 20B) not given estrogen (p = 0.0005;  p = 0.02).
However, tumor growth continued to increase in mice (A549 and HCC827) treated with
estrogen and bevacizumab. A549 tumor-bearing mice had a median survival of over 138 days
when treated with bevacizumab. However, estrogen treated mice given bevacizumab had a
reduced median survival of 25 days (p = 0.006, Figure 20A). HCC827 tumor-bearing mice had a median survival of over 200 days when treated with bevacizumab while mice receiving estrogen and bevacizumab treatment had a median survival of only 56 days (p = 0.0001, Figure 20B). These findings demonstrate that estrogen promotes resistance to anti-angiogenic therapy.
**A549**

- **X-axis:** Days post injection
- **Y-axis:** Percent tumor less than 1000mm³
- **Groups:**
  - Veh
  - Veh+E2
  - BV
  - BV+E2

**HCC827**

- **X-axis:** Days post injection
- **Y-axis:** Percent tumor less than 1000mm³
- **Groups:**
  - Veh
  - Veh + E2
  - BV
  - BV + E2

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Figure 20. Estrogen induces resistance to anti-angiogenic treatment in NSCLC xenografts. Nude ovariectomized mice were treated with bevacizumab (BV) 10mg/kg in the presence or absence of estrogen. Mice were sacrificed when tumor size reaches 1000mm³. Kaplan-Meier plot shows survival distribution of mice treated with BV in (A) A549 (B) HCC827 xenografts. The log-rank (Mantel-Cox) test was used to compare the statistical differences among the groups. N =10 mice per group and experiment was performed twice. Reprinted with permission of Journal of Thoracic Oncology.
Estrogen increases pericyte vessel coverage and pro-angiogenic growth factor secretion in NSCLC xenografts

Because the presence of estrogen is associated with anti-angiogenic therapy resistance in NSCLC xenografts, we sought to determine molecular and cellular factors that may contribute to this effect. To evaluate whether estrogen directly impacted tumor vascularization, microvessel density (MVD), a measure of total vascular endothelial staining, and pericyte coverage, a measure of vascular maturity, were recorded. In HCC827 xenografts, mice treated with vehicle, estrogen, bevacizumab, or the combination of estrogen and bevacizumab had their tumors collected and prepared for immunostaining. Staining for CD31 and desmin and pericyte coverage was calculated as the percentage of CD31+ vessels that co-localize with desmin positivity (Figure 21A). Estrogen treatment was not associated with enhanced MVD (Figure 21B). However, estrogen-treated mice demonstrated a marked 2.3 fold increase in pericyte-covered vessels (p = 0.01, Figure 21C). Increased pericyte coverage has been associated with enhanced tumor endothelial survival and VEGF inhibitor resistance [146-148].
Figure 21. Estrogen increases pericyte vessel coverage and pro-angiogenic growth factor secretion in NSCLC xenografts. Representative of immunofluorescence staining (20X) of CD31 (red), desmin (green) and nuclei (blue) using immunofluorescence microscopy. A minimum of 4-5 microphotograph (20X) for each sample (n=4/group) were collected (A-B). Quantification of microvessel density (C) and pericyte coverage (D) at tumor progression (100mm³) was performed. Vessels were considered pericyte covered if more than 50% of CD31+ vessel co-localized with desmin. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
PDGF-BB is a cytokine critical in regulating pericyte coverage and vascular stability and maturity [149, 150]. Circulating levels of mouse PDGF-BB were greater than 10-fold higher in the blood of estrogen-treated mice compared to vehicle-treated animals (p = 0.03, Figure 22A). Circulating levels of mouse VEGF were 7-fold higher in estrogen-treated mice as compared to the vehicle-treated animals (p=0.08, Figure 22B). These findings suggest that estrogen may modulate secretion of angiogenic factors involved in the accelerated growth of mature tumor vasculature.

**Figure 22.** Increased circulating angiogenic factors, PDGFBB and VEGF were measured during tumor growth in presence of estrogen. Mouse PDGFBB (A) and VEGF (B) serum levels were quantified using the Bio-rad multiplex bead assay. For each sample (n=4/group), blood from tumor bearing mouse was drawn by cardiac puncture. The results were plotted as average of the duplicate. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
Estrogen increases the expression of myeloid recruitment factors CXCL1 and G-CSF and tumor infiltration of CD11b+ myeloid cells

HCC827 and A549 tumor bearing mice were treated with estrogen, bevacizumab, or the combination, and the serum was collected to evaluate circulating factors by multiplex assay in the blood of these mice. In the A549 tumor-bearing animals, estrogen treatment resulted in a 3.5-fold increase in serum levels of mouse G-CSF compared to vehicle-treated animals (p = 0.046, Figure 23A). The combination treatment of bevacizumab and estrogen resulted in an 8-fold increase in serum mouse G-CSF levels, as compared to estrogen alone (p < 0.0001, Figure 23A). A significant increase in serum mouse GCSF was also observed in HCC827 tumor-bearing animals treated with estrogen (p = 0.01, Figure 23B), while G-CSF levels in animals treated with the combination treatment of bevacizumab and estrogen was not significantly modulated compared to animals receiving estrogen alone (p = 0.06, Figure 23B).

Multiplex analysis of circulating growth factors also showed that serum human CXCL1 was significantly increased. In tumor-bearing mice with established A549 or HCC827 xenografts, estrogen treatment induced a significant rise in serum levels of human CXCL1 compared to vehicle-treated animals (p = 0.0019, Figure 23C; p = 0.02, Figure 23D). Circulating levels of human CXCL1 were considerably lower in animals treated with bevacizumab in combination with estrogen as compared to animals receiving estrogen alone (p = 0.03, Figure 23C; p = 0.08, Figure 23D) suggesting that VEGF may directly or indirectly impact levels of this cytokine. Collectively, our results indicate that estrogen induces production of human CXCL1 and mouse G-CSF.
Figure 23. Circulating pro-angiogenic factors, G-CSF from (A) A549 and (B) HCC827 & CXCL1 levels were quantified from (C) A549 and (D) HCC827 mice xenografts in the presence of estrogen. The samples were quantified in duplicate by using the Bio-rad multiplex bead assay. The results were plotted as average of the duplicate. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
CXCL1 and G-CSF regulate mobilization of hematopoietic stem cell progenitors and mature myeloid cells into the circulation [151, 152]. Therefore, we examined the effect of estrogen on the infiltration of myeloid cells into the tumor microenvironment by immunostaining tumors with antibodies directed against CD11b, a pan-myeloid cell marker. In both A549 and HCC827 xenograft models, estrogen induced a significant rise in the number of tumor-infiltrating myeloid cells (p = 0.015, Figure 24A; p = 0.0015, Figure 24B).
**Figure 24.** Quantification of CD11b+ staining of A549 (A) and HCC827 (B) xenografts with and without estrogen treatment. A minimum of eight high power field (400X) was counted for CD11b+ cells from the periphery to the center of the tumor from each sample. The results were plotted as average of eight high power field (400X). (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
Estrogen promotes increased myeloid infiltration and resistance to bevacizumab which can be reversed with estrogen receptor blockade

To therapeutically target estrogen signaling in the NSCLC microenvironment, an estrogen inhibitor was utilized in combination with bevacizumab for in vivo experiments. Consistent with our earlier observations, bevacizumab delayed tumor growth in mice not treated with estrogen (p = 0.05), but in the presence of estrogen, bevacizumab did not reduce tumor growth (p = 0.349, Figure 25). The addition of fulvestrant reversed this effect, significantly enhancing the efficacy of bevacizumab in the presence of estrogen (p = 0.006, Figure 25).
Figure 25. Nude ovariectomized mice injected with HCC827 tumor cells developed tumors that were treated with bevacizumab (BV) 10mg/kg twice in a week, and fulvestrant (F) 200mg/kg, once in a week in with or without the presence of estrogen (E2). Kaplan-Meier plot showing survival distribution of HCC827 xenograft mice treated with BV, F, or E2 (A). The log-rank (Mantel-Cox) test was used to compare the statistical differences among the treatment groups. Reprinted with permission of Journal of Thoracic Oncology.
Images of immunofluorescence stained tumors were obtained to determine if there were any changes in vascular angiogenic growth among different treatment groups (Figure 26).
Figure 26. Representative images (200X) of immunofluorescence staining of CD31 (red), desmin (green) and nuclei (blue) using immunofluorescence microscopy were collected (4-5 microphotograph for each sample with n=4/group). Reprinted with permission of Journal of Thoracic Oncology.
Quantitative analysis of stained tumors revealed that in estrogen-treated mice receiving bevacizumab, fulvestrant significantly decreased MVD (p = 0.002) and pericyte coverage (p = 0.02) compared to estrogen-treated mice receiving bevacizumab alone (Figure 27A-B). Treatment with fulvestrant also reduced infiltration of CD11b+ myeloid cells (p = 0.006) compared to estrogen-treated mice treated with only bevacizumab (Figure 27C) further highlighting the association between presence of estrogen and increased myeloid cell infiltration. Mechanisms outlining the association of increased myeloid compartment infiltration in estrogen treated NSCLC models with resistance to anti-VEGF therapies are summarized in Figure 28.
Figure 27. Quantification of immunostained stained tumor sections to measure (A) microvessel density, (B) pericyte coverage, and (C) CD11b+ myeloid cells. (mean +/- SEM, * p<0.05, ** p<0.01, *** p<0.001). Reprinted with permission of Journal of Thoracic Oncology.
Figure 28. Graphical abstract depicting tumor microenvironmental effects of estrogen blockade in bevacizumab-resistant NSCLC tumors. Summary of the increase angiogenic growth and myeloid cell tumor infiltration during treatment with bevacizumab in a NSCLC model in the presence of estrogen (left) compared to when an estrogen antagonist is administered (right).
CHAPTER 6

DISCUSSION
High levels of IL-6 production has previously been associated with acquired resistance to different types of EGFR-TKIs (first, second, and now generation EGFR-TKIs) in multiple EGFR-mutant NSCLC preclinical studies [57, 58, 153]. High plasma levels of IL-6 were associated with a worse overall survival (OS) than those with lower levels of circulating IL-6 in NSCLC patients treated with the first generation EGFR-TKI, erlotinib [57]. A more recent study found that patients who were refractory to osimertinib, a third-generation EGFR-TKI, also had higher levels of circulating IL-6 in their blood [153].

Identifying suitable therapies for patients with EGFR TKI refractory NSCLC is clear unmet clinical need because other treatment modalities such as immunotherapy have not yield significant clinical benefit to EGFR-mutant NSCLC patients exhibited by an objective response rate (ORR) of 9% [154, 155]. Another retrospective report identified that EGFR-mutant or ALK-altered patients exhibited a 3.6% response rate to treatment with anti-PD-1/PD-L1 inhibitors while EGFR-wildtype and ALK-negative patients displayed a 23.3% response rate [156].

Comprehensive meta-analysis demonstrates that patients with EGFR-mutant NSCLC tumors who are treated with immunotherapy have more poor clinical outcomes compared to those treated with the standard-of-care chemotherapy, docetaxel [157]. Clinical studies have tried to evaluate the concurrent treatment of immunotherapy administrated with EGFR-TKIs. Unfortunately, these trials were prematurely halted due to high incidence of immune-related adverse events for these patients in the EGFR-mutant patient population [158-160]. Thus, it highlights the clear need for studies to identify factors that promote ICB immunotherapy resistance in EGFR mutant NSCLC.
Due to IL-6’s high abundance in EGFR-mutant NSCLC tumors, this study seeks to investigate the role of IL-6 in driving ICB resistance in EGFR mutant NSCLC. This study is the first to identify dysregulated IL-6 signalling across multiple EGFR-mutant NSCLC models which have developed an EGFR independent EMT-associated acquired resistance to the EGFR-TKI, osimertinib. Given the increased use of osimertinib as a first-line treatment modality for patients with EGFR-mutant NSCLC tumors, there will be a growing cohort of patients who may have tumors characterized by this EMT-mediated resistance mechanism. The association between IL-6 and EMT-associated osimertinib resistance will be important to study further. This study dives deeper into elucidating the mechanisms by which these elevated levels of IL-6 in EGFR EMT-associated TKI resistant tumors shape the tumor immune microenvironment.

IL-6 secretion was increased in osimertinib variants of human EGFR-mutant NSCLC tumor cell lines. Additionally, tumor cell lines generated by patient-derived tumor resections also show a similar upregulated secretion of IL-6 compared to TKI-naïve EGFR-mutant tumor cell lines. Moreover, levels of circulating IL-6 in patients after development of progressive disease after osimertinib treatment is higher than measurements from the same patients before progressive disease arose. A novel immune-competent syngeneic model of EGFR-mutant TKI-resistant disease is described as an EGFR independent model characterized by EMT-associated acquired resistance to osimertinib. Mice generated from this EGFR independent model secrete higher levels of IL-6 and exhibit a colder immune microenvironment compared to EGFR dependent models which are TKI sensitive.

Previous studies have outlined the many immunosuppressive effects IL-6 can have across different disease settings including in many cancer types. These studies have noted that
high IL-6 level as associated with a dampened anti-tumor response, specifically in impairing activation and function of T cell and NK cells [161-163]. Thus, this study sought to parse out the specific effects of IL-6 on tumor infiltrating lymphocytes in the EGFR-mutant TKI resistant microenvironment in light on how drastically upregulated expression of IL-6 is in this disease setting. Moreover, IL-6 increased infiltration of immunosuppressive Th17 T cell populations; IL-6 was associated with decreased expression of activation markers on tumor-infiltrating T cell and NK cells in murine EGFR-mutant NSCLC models. Corresponding in vitro assays similarly found that IL-6 impaired cytotoxic potential of T cells and NK cells and their ability to mediate cytotoxic killing.

This study found that therapeutic blockade of IL-6 in TKI-naïve EGFR mutant tumors can in part induce increased immune infiltration and activation of both T and NK cells in the tumor microenvironment. IL-6 blockade further sensitizes TKI-naïve EGFR mutant tumor models, to an improved response to anti-PD-1 therapy treatment. Monoclonal IL-6 antibodies are already FDA approved for other auto-immune indications including Castleman disease and rheumatoid arthritis and thus would be feasible to clinically evaluate in the appropriate cancer setting [164]. Future preclinical studies should perform more in-depth immune function studies using new syngeneic TKI resistant murine tumor cells lines being developed. Based on data from these preclinical experiments across multiple models, future clinical studies should determine if patients with EGFR-TKI refractory NSCLC tumors which acquired resistance independent of secondary EGFR mutations can benefit from combination treatment of immunotherapy and an IL-6 blocking agent. Additionally, EMT-associated immunosuppression is not a unique phenomenon observed solely in EGFR-mutant disease and
thus it would be interesting to evaluate if IL-6 targeting agents can be employed in other TKI-resistant disease settings beyond EGFR (including KRAS, VEGF, etc).

This report also highlights results from a separate investigation focused on the immune tumor microenvironment of NSCLC tumors which developed a resistance to bevacizumab, an anti-VEGF therapy. NSCLC patients treated with the combination of bevacizumab and chemotherapy display clinical benefit as described in the ECOG4599 study [107]. However, among patients in this clinical study, a disparity in response rates aligned based on sex. Female patients had distinctly more poor clinical response compared to their male patient counterparts even after adjustment for other key risk factors like smoking status [121]. It was thus hypothesized that the presence of sex hormones may impact this pattern of response to anti-angiogenic therapy.

Previous clinical research studies have found that expression of ERα and ERβ are associated with prognostic outcomes for patients with NSCLC [165]. Previously, investigators have even implicated estrogen as a mediator of tumor cell proliferation and invasion, specifically in NSCLC models [166-168]. However, it is important to note that a number of these studies utilize estrogen levels at levels that are over 10 times above physiological levels meanwhile this report is constructed to administer estrogen at levels that are closer to physiological levels in this disease setting [135, 136].

In this study, NSCLC xenografts, A549 and HCC827, were established in mice treated with or without estrogen. Estrogen in this setting did not significantly modulate the growth or proliferation rate of established tumors randomized at 200-400 mm³. Additionally, in vitro among 3 NSCLC tumor cell lines which express estrogen ERβ – the predominant form expressed on lung cancer tumor cells, addition of estrogen similarly modulates proliferation
rate. Moreover, therapeutic inhibition of estrogen signaling through treatment with fulvestrant, was not sufficient to reduce proliferation rate. When estrogen receptors, ERα and ERβ were artificially express in NSCLC tumor cell lines, they displayed intact estrogen signaling indicative that estrogen did not directly impact tumor cell proliferation rate. Thus, these data suggest that estrogen promotes growth of NSCLC tumors due to microenvironmental effects.

VEGF has been implicated as a main driver of angiogenic growth in tumors. Thus, targeted therapies which are involved in blocking VEGF/VEGFR signaling have been utilized across multiple tumor types [169-171]. Additionally, pericytes are key structures also involved in the development of angiogenesis. Pericytes cover sections of endothelial cells and control vessel stability [172]. Pericyte recruitment to the tumor microenvironment is primarily regulated by PDGF-BB [173, 174]. This study similarly found that estrogen effectively increased the PDGF-BB levels, and estrogen was associated with increased pericyte coverage of the tumor vasculature.

Levels of other pro-angiogenic cytokines, including G-CSF and CXCL1, were increased in the serum of mice treated with estrogen. Presence of estrogen is also associated with an increased recruitment of myeloid cells to the tumor. Additionally, estrogen promoted resistance to bevacizumab treatment and that blockade of estrogen receptor signaling could mitigate this response. This study illustrates that blockade of estrogen can sensitize NSCLC tumors to anti-VEGF therapy. Combinatorial inhibition of estrogen and VEGF signaling significantly reduced pericyte coverage, secretion of pro-angiogenic factors, myeloid cell tumor infiltration, and ultimately tumor growth. Future studies should be conducted to test if estrogen blocking therapies can help female NSCLC patients overcome resistance to VEGF targeting agents.
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