Targeting interleukin-6 trans-signaling in head and neck squamous cell carcinoma

Rachel A. Dahl

University of Iowa

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This is to certify that the Master’s thesis of Rachel A. Dahl has been approved by the Examining Committee for the thesis requirement for the Master of Science degree in Pathology at the May 2018 graduation.

Thesis Committee:

_____________________________ Andrean Simons-Burnett, Thesis Supervisor

_____________________________ Douglas Laux

_____________________________ Frederick Quelle

_____________________________ Weizhou Zhang
Dedication

To my family
Acknowledgments

Many thanks to Ann Simons-Burnett, for giving me a home in her lab and helping me learn how to make my presentations more professional; to Tom Waldschmidt, for always being supportive and diligently ensuring my progression through the program; to Carla Hartl, for ordering all our lab supplies without complaint; to Dr. Sushmita Sinha and Isaac Jensen for taking the time to teach me the basics of flow cytometry; to Amanda Kalen for her help with zapping mice; to Yinwen Cheng and Sam Rodman, for being great labmates, and Sam again for being my advanced mouse handler; and last but not least, to Madelyn Espinosa-Cotton – all I can say is that my inner kayak is sinking and I am stuffing the hole with gummyworms.
Abstract

**Title:** Inhibition of interleukin-6 trans-signaling by sgp130Fc is anti-tumorigenic in head and neck squamous cell carcinoma.

**Background:** Head and neck squamous cell carcinoma (HNSCC) is a highly inflammatory cancer type, and interleukin-6 (IL-6) is associated with this phenotype. Elevated expression of IL-6 is linked to tumor progression, recurrence, metastasis, and resistance to therapy in HNSCC. However, targeting IL-6 or IL-6 receptor (IL-6R) has demonstrated little to no clinical efficacy.

IL-6 signals through a classical signaling pathway via membrane IL-6R or a trans-signaling pathway via soluble IL-6R (sIL-6R). Recent evidence suggests that classical signaling induces acute, transient inflammation, eventually resulting in homeostasis; whereas trans-signaling may induce chronic, pro-tumorigenic inflammation. Therefore we propose that IL-6 trans-signaling is associated with the pro-inflammatory phenotype observed in HNSCC. We wanted to determine whether inhibition of IL-6 trans-signaling by sgp130Fc would better demonstrate anti-tumor efficacy and increase HNSCC tumor response to radiation, chemotherapy, and targeted therapy (cetuximab) compared to global IL-6 pathway inhibition.

**Method/Results:** Baseline levels of IL-6, IL-6R, sIL-6R, and sgp130 proteins in HNSCC cells were determined using ELISA and flow cytometry. Cisplatin, radiation, and cetuximab treatments each induced HNSCC cell secretion of IL-6 and sIL-6R in vitro, yet adding sgp130Fc to those treatments did not further reduce clonogenic survival. Sgp130Fc treatment significantly suppressed SQ20B tumor growth in nude mice, whereas global IL-6 pathway inhibition by IL-6R antagonist tocilizumab did not; however, cetuximab reduced the efficacy of sgp130Fc in this animal model. Sgp130Fc also sensitized SQ20B xenograft tumors to radiation and chemotherapy in nude mice and suppressed SCCVII tumor growth in male but not female C3H/HeJ mice.

**Conclusion:** Inhibition of IL-6 trans-signaling by sgp130Fc displayed significant anti-tumor effects as a single therapy and sensitized resistant HNSCC tumors to radiation and chemotherapy in vivo; however, sgp130Fc did not reduce survival of HNSCC cells in vitro. These results suggest that the efficacy of sgp130Fc relies on targeting another part of the microenvironment instead of tumor cells directly. Sgp130Fc has promise both as a single therapy and potentially as combined therapy with radiation and chemotherapy in HNSCC.
Public Abstract

The overall rate of survival for head and neck cancer squamous cell carcinoma (HNSCC) patients has increased only modestly in the past twenty years, and so there is a strong need to continue to improve treatments for HNSCC, particularly for incurable resistant and recurrent/metastatic cancers. Interleukin-6 (IL-6) cell signaling is an important part of the inflammatory process associated with progression of HNSCC. A promising new drug, sgp130Fc, can blockade pro-inflammatory (pro-tumorigenic) IL-6 trans-signaling, without interfering with beneficial IL-6 classical signaling. This study showed that sgp130Fc can inhibit tumor growth in HNSCC mouse models and may have clinical potential to improve survival of HNSCC patients, especially those with incurable HNSCC.
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List of Abbreviations

ACA Affordable Care Act

ADAM 10/ADAM 17 a disintegrin and metalloproteinase domain-containing protein

ADCC antibody-dependent cellular cytotoxicity

AJCC American Joint Committee on Cancer

CAR-T chimeric antigen receptor therapy

CT-1 cardiotrophin-1

CNTF ciliary neurotrophic factor

DEN diethylnitrosamine

DMEM Dulbecco’s Modified Eagle Medium

FBS fetal bovine serum

FDA Food and Drug Administration

HNC head and neck cancer

HNSCC head and neck squamous cell carcinoma

HPV human papilloma virus

hr hour

IFNβ interferon beta

IL-6 interleukin-6

IL-6R interleukin-6 receptor

IL-11 interleukin-11

IL-27 interleukin-27

IL-31 interleukin-31
IMRT intensity-modulated radiation therapy

IL-1ra interleukin-1 receptor antagonist

i.p. intraperitoneal injection

IRF7 interferon regulatory factor 7

IRF9 interferon regulatory factor 9

JAK Janus kinase

LIF leukemia inhibitory factor

MCD multicentric Castelman’s disease

min minute

MM multiple myeloma

m/r metastatic/recurrent

NK natural killer cell

NT not treated

OSCC oral squamous cell carcinoma

OSM oncostatin-M

PBMC peripheral blood mononuclear cell

PD-1 programmed cell death receptor 1

PD-L1 programmed cell death ligand 1

RT radiation therapy

R/M recurrent/metastatic

SCID severely combined immunodeficient

sec second
sIL-6R soluble interleukin-6 receptor

gp130 soluble gp130

gp130Fc soluble gp130-human IgG Fc chimera

SOC standard of care

TCGA The Cancer Genome Atlas

TNFα tumor necrosis factor alpha

WB Western blot
Chapter 1: Introduction

Section I: Head and neck squamous cell carcinoma (HNSCC)

Etiology and statistics of HNC/HNSCC

Head and neck cancer (HNC) originates from tissues in the oral cavity, nasal cavity, pharynx, larynx, or salivary glands, at least 90% of which is squamous cell carcinoma (HNSCC) [1]. More than 65,000 new incidences of HNC were estimated to occur in the United States in 2017 (Table 1) [2, 3].

Table 1. Overall HNC incidence and survival in United States [3].

<table>
<thead>
<tr>
<th>Cancer origin</th>
<th>5yr O/S (%)</th>
<th>Estimated new cases in 2017</th>
<th>Estimated new deaths in 2017</th>
<th>Estimated # living in U.S. 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>18.8</td>
<td>16,940</td>
<td>15,690</td>
<td>45,547</td>
</tr>
<tr>
<td>Larynx</td>
<td>60.7</td>
<td>13,360</td>
<td>3,660</td>
<td>99,914</td>
</tr>
<tr>
<td>Oral cavity/pharynx</td>
<td>64.5</td>
<td>49,670</td>
<td>9,700</td>
<td>346,902</td>
</tr>
</tbody>
</table>

Up to 90% of HNC cases might be preventable and are caused by long-term exposure to cancer initiators such as alcohol and tobacco use, human papilloma virus (HPV) infection, and in the case of lip cancer, UV exposure [4]. A greater variability in risk for HNC is due to alcohol use than due to smoking [5]. A case-cohort study of 395 HNC and 4,288 control participants showed that alcohol use and cigarette smoking were not only independently and positively associated with HNC risk, but together augmentative [6].

Men are about three times as likely as women to be diagnosed with HNC (Table 2), which may be due to higher use of alcohol and tobacco by men than women [6]. Some studies also support that estrogen may be a protective factor against HNC development [7-9]. Age is also a risk factor, as HNC is more common in patients above the age of 45. However in both the United States and Europe, the rate of incidence of oropharyngeal cancer in younger (20-44 years) white populations has increased recently, which may correspond to an increase in the rate of HPV infection in that population, associated with having oral sex and multiple sexual partners [10, 11].
Risk of mortality in HNC typically depends on how early the patient is diagnosed and on the stage and location of the tumor at diagnosis. For example, based on 2007-2013 SEER data, the 5-year overall survival rates of patients with cancers of the oral cavity/pharynx or larynx were more than three times as high as that of patients with cancers of the esophagus [3]. In a 2009 meta-analysis of 15 case-control studies, smoking had a higher association with laryngeal cavity cancer, whereas alcohol use had a higher association with pharyngeal and oral cavity cancer [5]. Therefore, different risk factors associated with HNC are also associated with different tumor sites and so indirectly with rates of survival.

Table 2. HNC incidence and deaths by sex in United States in 2017 [2].

<table>
<thead>
<tr>
<th>Cancer origin</th>
<th>Estimated new cases</th>
<th>Estimated new deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Esophagus</td>
<td>13,360</td>
<td>3,580</td>
</tr>
<tr>
<td>Larynx</td>
<td>10,570</td>
<td>2,790</td>
</tr>
<tr>
<td>Mouth</td>
<td>7,800</td>
<td>5,410</td>
</tr>
<tr>
<td>Other oral cavity</td>
<td>2,260</td>
<td>800</td>
</tr>
<tr>
<td>Pharynx</td>
<td>13,780</td>
<td>3,220</td>
</tr>
<tr>
<td>Tongue</td>
<td>11,880</td>
<td>4,520</td>
</tr>
</tbody>
</table>

African-Americans have lower incidence of oropharyngeal cancer, yet have a higher mortality rate from it than do whites in the United States [3]. Evidence shows that this disparity is not only due to less access to health care, but may also be due to discriminatory practices; for example, African-American HNC patients are less likely to be offered radiation therapy (RT) as treatment [12]. Though access to medical care has improved overall for African-Americans in the United States since the enactment of the Affordable Care Act (ACA) in 2010, it remains to be seen whether the ACA will result in improvements in survival of African-American HNC patients [13, 14]. Moreover, the results of ACA reforms that only came into effect in 2014 might not be visible yet in current epidemiological data.

HNC 5-year overall survival rates have increased from 54.7% in 1992–1996 to 65.9% in 2002–2006 in the United States, which is believed in part to be due to reductions in smoking and alcohol use [15]. However, an alternative reason for this increase in survival is the recent
increase in HPV-related HNC, which is generally associated with improved prognoses compared to HPV-negative HNC. HPV-positive HNC tumors are highly responsive to RT; additionally, HPV-positive tumors are more commonly seen in younger patients, who typically respond better to cancer treatments than do older patients [15, 16].

Late stage diagnosis of HNC and recurrent/metastatic (R/M) HNC are associated with much lower survival rates. For example, for oropharyngeal cancer patients in the United States, the 5-year overall survival rate is 64%; however the 5-year survival rate rises to 83% for patients with early stage cancers (though only 79% for African-American patients), compared to 38% for patients with R/M cancer [2].

**Staging in HNSCC**

HNSCC is generally described as local, locoregionally advanced, or recurrent/metastatic depending on the invasiveness, aggressiveness, and recurrence of the primary tumor and the presence of distant metastases. In the United States, staging for HNC is described using TNM (primary tumor size/lymph node involvement/presence of metastases) guidelines from the American Joint Committee on Cancer (AJCC). Staging differs slightly depending on the primary tumor site, which may include: oral cavity; oropharynx; larynx; hypopharynx; nasal cavity and paranasal sinuses; salivary glands; or neck, excluding nasopharynx and thyroid [17].

**Treatments for HNSCC**

RT may be definitive care for HNSCC on its own, used concomitantly with chemotherapy, or given as an adjuvant to surgery [17]. Developments of 3-D conformal RT, intensity-modulated RT (IMRT), and now 4-D RT have significantly improved the ability to target cancerous cells with less off-target damage compared to conventional RT [18-20]. Yet radiation is not always curative, and even when effective, is still associated with severe side effects. One of the most common side effects, oral mucositis, causes pain and dysphagia, which can reduce quality of life and be severe enough to require cessation of treatment [21]. Additionally, RT poses a risk for initiating new disease.

Chemotherapy (such as cisplatin, fluorouracil, methotrexate, carboplatin, paclitaxel, and docetaxel) can be given as an adjuvant to surgery, concomitantly with RT, or as palliative
therapy [17]. Chemotherapy also is associated with a range of side effects, including fatigue, anemia, loss of appetite, nausea and vomiting, that can significantly reduce quality of life.

Surgery to resect the primary tumor may be curative in some cases and is frequently performed with adjuvant therapies such as RT and/or chemotherapy. However, some tumors cannot be resected due to their location or their extent of local invasion, and surgery can lead to painful recovery, cause disfigurement, or cause dysfunction of essential organs. Some tumors recur months after resection, which (as said previously) is associated with poorer survival.

Cetuximab (Erbitux) is the first targeted therapy approved by the U.S. Food and Drug Administration (FDA) for HNSCC. Cetuximab is a recombinant, human/mouse chimeric monoclonal antibody that binds with high affinity to the extracellular portion of epidermal growth factor receptor (EGFR) and inhibits EGFR-mediated signaling associated with tumor proliferation and survival [22]. Additionally, cetuximab can induce antibody-dependent cell-mediated cytotoxicity (ADCC) against target tumor cells, by means of natural killer (NK) activation through its IgG1 Fc domain [23-25]. Between 90-100% of HNSCC tumors exhibit some expression of EGFR, and higher levels of EGFR expression correlate negatively with survival [26-28]. In a randomized, multinational, phase III clinical trial involving 424 patients with locoregionally advanced HNSCC, patients receiving a combination of cetuximab and RT treatment had significantly improved median survival of 49.0 months compared to patients only receiving RT (29.3 months) [29]. This study was the basis for FDA approval of cetuximab for HNSCC in 2006, which was indicated for locoregionally advanced HNSCC in combination with radiation therapy and for recurrent or metastatic HNSCC progressing after platinum-based therapy [30, 31]. That label was amended in 2011 to further indicate cetuximab as first-line treatment for recurrent locoregional disease or metastatic HNSCC in combination with platinum-based therapy with 5-FU [31, 32].

However, cetuximab treatment is only efficacious in a small percentage of HNC patients, with a 13% overall response rate and median time of 70 days to progression when given as a single therapy [33]. Tumor resistance may be innate, such as through the presence of polymorphism EGFRK521 that decreases cetuximab’s affinity for EGFR [29]. Resistance also may be acquired in response to EGFR inhibition, such as through tumor mutations and activation of alternative proliferative and survival signaling pathways [34]. Additionally, cetuximab treatment may cause serious side effects. The majority of patients develop a rash that may be painful or
other dermatological disorders which can significantly reduce quality of life and also be treatment-limiting [35, 36]. A very small number of HNC patients (2-3%) have undergone cardiopulmonary arrest or sudden death upon cetuximab infusion [31, 32]. Therefore, there is a strong clinical need to improve cetuximab’s efficacy, as well as to search for additional HNSCC biomarkers aside from EGFR that will lead to novel and improved targeted therapies.

In 2016, two immunotherapies, pembrolizumab (Keytruda) and nivolumab (Opdivo) received accelerated FDA approval for recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) with disease progression on or after platinum-containing chemotherapy [37-39]. These monoclonal antibodies can bind extracellularly to the immune cell checkpoints programmed cell death 1 (PD-1) to create a blockade that prevents tumor cells from performing immune escape by suppressing or inactivating immune cells through this checkpoint, thus increasing the overall anti-tumor immune response [37]. However, the major risk in immunotherapy is an overactive, unregulated immune response, which in turn can cause a wide spectrum of side effects, ranging from fatigue, nausea, rash, diarrhea, pneumonia, colitis, hepatitis, to respiratory failure [38, 39]. Globally, there are currently more than five dozen active or actively recruiting clinical trials that are investigating the effects of immunotherapy with other treatments in different subsets of HNSCC patients.

The basis of approval for pembrolizumab were the results of a Phase II study of 171 R/M platinum- and cetuximab-refractory HNSCC patients, in which pembrolizumab treatment yielded an overall response rate of 16% with a median duration of response of 8 months [40]. In a Phase III trial, nivolumab increased overall survival of R/M HNSCC patients to 7.5 months compared to 5.1 months in the group receiving only standard therapy [41]. Additionally, nivolumab was shown to stabilize disease in R/M HNSCC patients longer than did single therapies of methotrexate, docetaxel, or cetuximab [42]. Although there is excitement that these two immunotherapies displayed clinically significant improvements for R/M and refractory HNSCC, these statistics still show relatively limited success.

Therefore, there remains a strong need to further improve the clinical efficacy of treatments that already are approved for HNSCC and to develop novel therapies that will increase the rate of progression-free survival of HNSCC patients, particularly for resistant and R/M cancers.
Section II: Inflammation and interleukin-6 signaling in cancer

Inflammation and cancer

In a healthy body, inducing inflammation is one of the best defense mechanisms for the host’s tissues in order to survive an attack by a pathogen. Increased leakiness of blood vessels and other mechanisms offer fast access of immune cells to the site under attack. The leakiness results in swelling at the site from fluids, which helps to trap the pathogens in the area. Inflammation is used successfully by the body for such discrete, acute episodes. However, chronic inflammation is now known to be involved in the development and progression of many different diseases, including cancer.

In a landmark 1986 article and in a revised 2015 version of that article, Dvorak referred to tumors infamously as “wounds that do not heal”, referring to the ability of tumors to “disguise themselves as wounds and call upon the host to ‘heal’ them” by co-opting their microenvironment [10, 11]. He meant that tumors can cause their surrounding stroma to adapt to better promote tumor proliferation and survival. In 2000, Hanahan and Weinberg first described six of the common hallmarks of cancer: the abilities of tumor cells to provide their own growth signals for proliferation; evade apoptosis; promote and sustain angiogenesis; invade other tissues; ignore anti-growth signals; and display unlimited replication [43]. In 2011, Hanahan and Weinberg published an updated version of “hallmarks of cancer” to also include four newly discovered cancer hallmarks or enabling characteristics, one of which was “tumor-promoted inflammation” [44]. From the research described in these reviews and from related studies, it is now known that persistent or chronic inflammation can initiate cancer as well as be an important factor in tumor progression, in which tumor cells may either release or harness components of a pro-inflammatory environment to better promote tumor proliferation, survival, and metastasis [45]. Tumor cells may do so by taking advantage of growth factors and other cytokines in the microenvironment to internally upregulate signaling pathways associated with growth and survival; or they may do so externally, by promoting inflammation through secretion of interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), or other inflammatory-associated cytokines. For example, chronic inflammation plays a role in the development and progression of HPV-associated cancers [46], breast cancer [47], and colitis-associated colorectal cancer [48]. In the absence of prior chronic inflammation, tumor elicited inflammation has been
implicated in the progression of colorectal cancer, and more specifically, through IL-6 signaling [49].

**Role of IL-6 signaling in inflammation**

IL-6 signaling is key player in inflammation, most known for its role in inducing expression of acute phase response genes as an early mechanism against infection. However, there are additional known members of the IL-6 cytokine family, such as interleukin-11 (IL-11), interleukin-27 (IL-27), interleukin-31 (IL-31), oncostatin-M (OSM), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), cardiotrophin-like cytokine factor-1 (CLCF1), and ciliary neurotrophic factor (CNTF) [50-52]. Though their roles in cellular differentiation, proliferation, and survival may overlap, members of this cytokine family may each have specialized and localized functions as well [53, 54].

IL-6 family cytokines are also referred to as gp130-related cytokines because they all initiate signaling through unique cell-surface receptors that interact with one or two gp130 membrane proteins (also known as IL-6 signal transducer or IL-6ST) to form a complex. The cytoplasmic portion of gp130 has a stable association with Janus kinase (JAK) family proteins. Upon receptor complex formation, the associated JAK can phosphorylate tyrosine residues on gp130 to create docking sites for proteins such as SHP2 (at gp130 Tyr757/Tyr759) and STAT3 (within gp130 YXXQ motif), where these proteins also can be phosphorylated by JAK proteins [52]. When SHP2 is recruited to gp130, it may activate MAPK and PI3K/Akt signaling pathways, while sterically inhibiting STAT3 recruitment. However, when STAT3 is recruited instead and is phosphorylated on Tyr705, it can dimerize, which is required for its entry into the nucleus where the STAT3 dimer acts as a transcription factor [52]. STAT3 can either upregulate or downregulate different genes, which again are most known to be associated with acute phase signaling and immune cell differentiation, recruitment, growth and survival. However, STAT3’s actions can be oncogenic in tumor cells.

STAT3α and STAT3β isoforms, created by alternate splicing, have been described to have independent characteristics, including duration of activation, retention in the nucleus, and gene targets [55]. The STAT3β isoform may act as a dominant-negative protein, thus inhibiting transcription, and a proportional shift from α-isoform to β-isoform expression may lead to apoptosis or cell-cycle arrest in some cancers [56, 57].
IL-6 is the most well-known of the gp130-related cytokines. It was formerly named as B cell stimulating factor 2 for its ability to promote B cell maturation and activation, and also named as a hepatocyte-stimulating factor for its ability to induce the acute phase response in the liver [58]. Yet IL-6 is highly pleiotropic and therefore is associated with a wide spectrum of other phenotypes, such as: T cell activation, proliferation, and survival; changes in regulatory T cell differentiation to a pro-inflammatory Th17 type; and immune cell recruitment and trafficking [59].

IL-6 can initiate signaling in two different ways: through membrane IL-6 receptor (IL-6R or IL-6Rα), which is termed classical signaling (Fig. 1a), or through soluble IL-6R (sIL-6R), which is called trans-signaling (Fig. 1b). Membrane IL-6R is expressed on only a few cell types (e.g. hepatocytes, megakaryocytes, and some leukocytes), but the ubiquity of signal transducer gp130 allows almost any cell to be able to perform IL-6 trans-signaling using sIL-6R. sIL-6R is formed either from alternative mRNA splicing or by starting as a membrane protein and then shed from the plasma membrane by proteases ADAM10 or ADAM17 to become soluble [60].

Figure 1. Initiation of IL-6 signaling, displaying a) classical signaling through membrane IL-6R, or b) trans-signaling through sIL-6R.
There also exists a soluble form of gp130 (sgp130) created by alternative splicing or alternative polyadenylation of gp130 mRNA that acts as an endogenous inhibitor of IL-6 trans-signaling due to its selective affinity for the IL-6/sIL-6R complex [61, 62]. When sgp130 binds to the IL-6/sIL-6R complex, it sterically prevents interactions with membrane gp130, thus inhibiting downstream signaling (Fig. 2a-b). There are several different known endogenous isoforms of sgp130 that may each have unique, localized functions [62, 63]. Although sgp130 is considered to be a selective inhibitor of IL-6 trans-signaling and not classical signaling, when sIL-6R exceeds IL-6 by one molar or more, it may “trap” IL-6 protein within IL-6/sIL-6R/sgp130 complexes, thereby sequestering IL-6 and indirectly inhibiting classical IL-6 signaling [64].

![Figure 2](image)

**Figure 2. Inhibition of IL-6 trans-signaling by sgp130.** a) Initiation of IL-6 trans-signaling. b) Inhibition of IL-6 trans-signaling by sgp130.

Though IL-6 signaling is more known for its pro-inflammatory functions, it also has been shown to display to a limited extent certain anti-inflammatory effects. IL-6 treatment induced production of anti-inflammatory IL-10-producing type I Treg cells from naïve CD4 T cells in C57BL/6 mice [65]. Infusions of recombinant IL-6 caused a rapid increase of interleukin-1 receptor antagonist (IL-1ra) plasma levels in cancer patients, although this effect was transient [66]. Macrophages stimulated with IL-6 *in vitro* produced high amounts of IL-1ra [66].
Recent studies suggest that IL-6 classical signaling and trans-signaling may be responsible for different effects, dependent on cell type and localization. For example, IL-6 classical signaling, not trans-signaling, was shown to induce IL-1ra secretion in BALB/C and C57Bl/6 mice [67]. IL-6 trans-signaling has been found to upregulate intercellular adhesion molecule 1 (ICAM-1), induce angiogenesis, and induce endothelial cell activation [59, 68]. Furthermore, recombinant sgp130 suppressed tumor growth and reduced liver collagen formation in a hepatocellular carcinoma mouse model, suggesting that IL-6 trans-signaling is involved in tumorigenesis [69]. IL-6 trans-signaling induced by sIL-6R released by lamina propria macrophages was found to be critical for endothelial cell turnover in the colon of Balb/C mice in a study of inflammatory bowel disease and colon cancer [70]. However, there are still many gaps in our knowledge about the differences in function of IL-6 classical signaling and trans-signaling.

Section III: IL-6 role in HNSCC

IL-6 signaling is implicated in disease progression in multiple myeloma (MM) and in breast, colorectal, and pancreatic cancers, as well as in HNSCC [71-74]. Elevated IL-6 signaling is associated with tumor resistance to chemotherapy, radiation, and targeted therapy in HNSCC [75-79]. High levels of tumor IL-6 protein were significantly associated with high lymph node metastasis and poor tumor differentiation in a study of 337 oral squamous cell carcinoma (OSCC) patients [80]. Interestingly, in that study, IL-6 signaling was a prognostic factor in male but not female HNSCC patients. STAT3 has been shown to display oncogenic activity in tumors, such as through mutations causing constitutively active JAK/STAT3 signaling [81]. Ligand-independent, constitutive gp130 signaling leading to downstream STAT3 activation in collaboration with oncogene MYC induced an MM phenotype in a murine model [82]. Activated STAT3 protein suppressed pro-apoptotic type I interferon-β (IFNβ) signaling by downregulating expression of STAT1, STAT2, and interferon regulatory factors 7 and 9 (IRF 7 and IRF 9) in diffuse large B cell lymphoma models, in addition to upregulating NF-κB expression [83]. In certain types of breast cancer, tumor IL-6-mediated JAK/STAT3 signaling was shown to upregulate NFκB activation, which feeds forward to induce more IL-6 synthesis [84]. Therefore, IL-6 signaling externally (in stroma) and internally (in tumor cells) can each affect the overall microenvironment to promote tumor growth, proliferation, metastasis, and survival [54].

Furthermore, newer evidence more specifically implicates IL-6 trans-signaling, not
classical signaling, in cancer. IL-6 trans-signaling promoted development of KRAS-driven lung
cancer in a gp130 knock in mouse model [85]. In that study, it was also observed that both lungs
and sera from 24 lung adenocarcinoma patients displayed elevated levels of IL-6 and sIL-6R
proteins compared to that of control patients [85]. In a hepatocellular carcinoma DEN mouse
model, IL-6 trans-signaling suppressed p53 activity (preventing apoptosis) and increased β-
catenin activation in hepatocytes, and additionally induced angiogenesis, thereby promoting
tumor proliferation [86].

**Preliminary results: TCGA data of IL-6 signaling in HNSCC tumors**

Data from the Cancer Genome Atlas (TCGA) of HNSCC tumor mRNA expression was
analyzed to investigate whether expression levels of IL-6 signaling genes were significantly
associated with changes in patient survival. Patient subsets were divided into groups of highest
and lowest quartiles of tumor mRNA expression in order to generate Kaplan-Meier survival
curves. Surprisingly, higher expression of tumor IL-6 mRNA was not significantly associated with
change in survival of HNSCC patients from all tumor stages and treatments (Fig. 3a); nor was
expression of gp130 (survival curve not shown, p=0.6831) or ADAM10 (survival curve not shown,
p=0.2773) in this larger patient group. However, higher tumor expression of IL-6 (Fig. 3b), gp130
(Fig. 3c) and ADAM10 (Fig. 3d) were each significantly associated with reduced survival in HNSCC
patients who survived at least two years from diagnosis, suggesting that IL-6 signaling has a
higher association with HNSCC tumor progression than initiation. High tumor expression of IL-6
mRNA also was associated with reduced survival of HNSCC patients who had undergone
radiation (Fig. 3e) or who had a Stage III or IV tumor (Fig. 3f); however, tumor IL-6 expression
was not associated with differences in survival in HNSCC patients who had not undergone
radiation (survival curve not shown, n=139, p=0.5946) or who had a Stage I or II tumor (survival
curve not shown, n=116, p=0.4970). These results also suggest that tumor IL-6 signaling is more
highly associated with tumor progression than initiation in HNSCC.

TCGA data does not differentiate between membrane IL-6R or sIL-6R mRNAs generated
by splicing, so differences in levels of these tumor mRNAs could not be compared. No
differences in survival were seen in any of these patient subset groups when comparing survival
to total IL-6R mRNA expression.
Figure 3. Kaplan-Meier plots associating HNSCC tumor mRNA expression to patient survival. Survival curves were generated from HNSCC tumor mRNA data from The Cancer Genome Atlas, sorted into highest and lowest quartiles of expression of a) IL-6 in HNSCC patients from all stages and treatment categories; b) IL-6, c) gp130, d) ADAM10 in HNSCC patients who survived two years after diagnosis; e) IL-6 in HNSCC patients who underwent radiation; and f) IL-6 in HNSCC patients with stage III or IV cancers.
Preliminary results: Comparison of IL-6 trans-signaling protein levels in healthy versus HNC patient plasma

Through an ongoing study with Dr. Douglas Laux in the Holden Comprehensive Cancer Center at University of Iowa Hospitals and Clinics and through collaboration with Dr. Stephanie Gilbertson-White in the Department of Nursing at University of Iowa, we were able to obtain plasma from either two or three time points from HNSCC patients (n=12; 1F/11M; mean age 58.00 ± 1.87 yrs) and from healthy patients (n=12; 4F/8M; mean age 63.17 ± 2.40 yrs) for analysis. Plasma was assayed using QuantiKine ELISA to determine the concentrations of the IL-6 trans-signaling proteins IL-6, sIL-6R, and sgp130. Each patient’s serum sample values (from two or three time points) were first averaged together into one mean value per patient. Then the mean values from each patient in each treatment group were averaged and analyzed to

Figure 4. ELISA QuantiKine results displaying mean concentrations of a) IL-6, b) sIL-6R, and c) sgp130 proteins in healthy vs. HNSCC patient plasma. *p<0.05 HNSCC patient plasma displayed significantly higher mean plasma IL-6 concentration (9.23 ± 1.69 pg/ml) compared to healthy patient plasma (1.82 ± 0.30 pg/ml).
determine group means and standard deviation. Not surprisingly, mean plasma levels of IL-6 were significantly higher in HNSCC patients (9.23 ± 1.69 pg/ml) compared to that of healthy patients (1.82 ± 0.30 pg/ml) (Fig. 4a). However, mean plasma levels of sIL-6R (HNSCC 24,947.01 ± 2,354.68 pg/ml; healthy 25,726.25 ± 2,117.53 pg/ml) and sgp130 (HNSCC 216.90 ± 16.43 pg/ml; healthy 207.11 ± 14.62 pg/ml) were not significantly different between patient groups (Figs. 4b-c). Additionally, no significant differences in IL-6, sIL-6R, or sgp130 concentrations were seen in HNSCC plasma from blood that was drawn prior to the start of cetuximab treatment versus blood drawn from after cetuximab treatment (data not shown). Plasma IL-6 ranges from about 1-5 pg/ml in healthy individuals [87]. Mean plasma sIL-6R measured in 24 healthy individuals in one study ranged from 41683 ± 11497 pg/ml [88].

Section IV: Targeting IL-6 signaling in cancer

Targeting IL-6 signaling in cancer with IL-6/IL-6R inhibitors and neutralizers

With the better understanding in the last ten years that inflammation is a key component of tumor progression, the novel idea of testing anti-inflammatory medications as potential anti-cancer therapeutics became popular. However, targeting IL-6 or IL-6R has yielded little clinical efficacy in cancer clinical trials so far, in efforts to either improve the efficacy of already existing cancer treatments or to identify anti-inflammatories that display anti-tumor efficacy on their own.

Siltuximab and tocilizumab are two of the most common inhibitors of IL-6 signaling that have been tested in animal models and clinical trials as potential cancer therapies. They are both humanized monoclonal antibodies, that bind to IL-6 and IL-6R respectively, and were first developed as potential therapies for highly inflammatory diseases such as rheumatoid arthritis.

Siltuximab was approved by the FDA in 2014 and directed for patients with multicentric Castleman’s disease (MCD) who are HIV negative and human herpesvirus 9 negative [89]. MCD is a lymphoproliferative disorder that can be fatal and is also associated with elevated IL-6 signaling. Siltuximab was highly successful as a treatment in a trial of 171 MCD patients, where it both mitigated symptoms as well as increased the 5 yr survival rate to 96% compared to the 55-77% 5 yr survival rate of MCD patients given traditional treatments [90]. Siltuximab treatment stabilized disease in more than 50% of metastatic renal cell carcinoma patient participants in a phase II clinical trial [91]. However, siltuximab did not significantly improve
progression-free or overall survival in a Phase II trial of 281 patients with relapsed/refractory MM who were also being treated with bortezomib (clinical trial ID NCT00401843) [92] nor did it improve survival in a Phase II study of newly diagnosed MM patients also receiving standard care (clinical trial ID NCT00911859) [93]. Siltuximab displayed no clinical efficacy against advanced, solid tumors in a Phase I/II study of 84 patients (35 colorectal, 29 ovarian, 9 pancreatic, and 11 other cancer types that included HNC patients whose tumors were resistant to EGFR inhibition) (clinical trial ID NCT00841191) [94, 95].

Tocilizumab was first approved by the FDA in 2013 as treatment for adult rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, and in patients 2 years of age and older, for active polyarticular juvenile idiopathic arthritis [96]. Tocilizumab (at approximately 4 mg/kg dose every 48 hrs) suppressed tumor growth, reduced tumor STAT3 phosphorylation, and reduced tumor expression of VEGF in an OSCC xenograft severely combined immunodeficient (SCID) mouse model; however, these mice were initially treated with tocilizumab 24 hrs after inoculation with tumor cells instead of after actual tumor development, which may have influenced the results [97]. Our lab previously has shown that tocilizumab increased the efficacy of the small molecule EGFR inhibitor erlotinib in HNSCC xenograft nude mouse models, though it displayed no tumor suppression alone [98], and that it sensitized erlotinib-resistant HNSCC cells to erlotinib treatment [75]. Yet tocilizumab failed as a combined therapy with Peg-Intron (interferon α) in a phase II clinical trial for recurrent ovarian cancer [99]. Tocilizumab is currently being tested in trials for trastuzumab-resistant breast cancer and as combined treatment with gemcitabine in pancreatic cancer, with no results as of yet.

Both siltuximab and tocilizumab have been used off-label to treat cytokine release syndrome after chimeric antigen receptor therapy (CAR-T), for which tocilizumab is now FDA approved as of March 2018 [100, 101]. It is possible that although both drugs have disappointed in cancer clinical trials, they may still prove to be beneficial therapies against the side effects of cancer treatments.

Ruxolitinib, a JAK1 and JAK2 inhibitor, has been tested as another potential cancer therapeutic in several dozen clinical trials. However, ruxolitinib treatment had a zero response rate in a trial of 21 patients with inflammatory or triple negative STAT3+ overexpression breast cancers (clinical trial ID NCT01562873) and displayed no significant improvement in progression-free or overall survival of patients with advanced or metastatic adenocarcinoma of the pancreas.
who also received capecitabine (clinical trial ID NCT01423604) [102]. Several other trials that tested ruxolitinib as potential treatment for pancreatic, breast, or colorectal cancers were terminated early due to progression in most patients or patient deaths; additionally, cessation of ruxolitinib is associated with severe side effects [102, 103]. Tofacitinib, a pan-JAK inhibitor, was approved by the FDA in 2012 for adult patients with moderately or severe active rheumatoid arthritis who did not respond to or who could not tolerate methotrexate, with an updated approval for adults with active psoriatic arthritis in 2017 [104]. Tofacitinib has yielded some success in animal cancer models, but has not yet been tested in cancer clinical trials, perhaps because of the lack of success of ruxolitinib.

**Development of sgp130Fc**

The sgp130Fc chimera is a newer compound that was first developed by Stefan Rose-John of the Conaris Research Institute and is now continuing development through Conaris-Ferring Pharmaceuticals with the compound name FE999301. FE999301 (which hereafter in this paper will be referred to as sgp130Fc) completed Phase I in November 2016 and now is in a Phase II study for treatment for active ulcerative colitis. This study is anticipated to conclude in summer of 2019 [105].

The structure of sgp130Fc is made of recombinant sgp130 protein conjugated to the Fc domain of human IgG1 (Fig. 5). Adding an Fc domain is a popular addition to recombinant protein pharmaceuticals, as it offers improved solubility and overall stability in plasma [106]. Additionally, the Fc domain stabilizes the dimeric structure of sgp130, which increases the specificity of this compound for the IL-6/sIL-6R complex and improves its efficacy as an inhibitor; it is believed to be 10-100 times more effective than its endogenous counterpart, sgp130 [107]. Furthermore, the IgG1 Fc domain might enable the molecule to interact with Fc-receptors (like CD16/FcyIII) on immune cells such as NK cells and macrophages to activate them. Sgp130Fc currently is available in both mouse and human forms from R&D Systems (Minneapolis, MN). The sgp130 domain is of either mouse or human origin, and the Fc domain is from human IgG1. This offers the opportunity to be able to test the effects of the drug in both immunodeficient and immunocompetent mouse models. Using immunoprecipitation, it was shown that human IL-6 will bind to mouse sIL-6R with low affinity, but mouse IL-6 has no affinity for human sIL-6R; whereas human gp130 and mouse gp130 will each bind to either mouse or human IL-6/sIL-6R complexes [107]. This indicates that the use of human sgp130Fc in an immunodeficient mouse
model would not selectively target just IL-6 trans-signaling induced by tumor cells, but also target to some extent trans-signaling that was induced by mouse cells.

Figure 5. Structure of sgp130Fc.

The selectivity of sgp130Fc for IL-6/sIl-6R was determined in a cell proliferation model in which BAF/3 cells, which are normally gp130-deficient, were transfected with gp130 or gp130 and IL-6R and tested for proliferation after treatments with IL-6 or hyper IL-6 (fused IL-6/sIL-6R). In this experiment, sgp130Fc treatment was shown to inhibit IL-6 trans-signaling but not classical signaling at a range of doses from 1-1000 ng/ml; additionally, sgp130Fc was shown to partially antagonize LIF and OSM signaling at higher doses, but had no effect on CNTF signaling[107]. Currently, sgp130Fc is being tested as potential treatment in a variety of mouse models of different disorders, including autism, inflammatory bowel disease, seizures, and emphysema [108-111].

Sgp130Fc also has been proven to display anti-tumor properties. In a pancreatic ductal adenocarcinoma mouse model, sgp130Fc was more potent than tocilizumab as a single therapy in suppressing tumor growth and reducing angiogenesis; however, both tocilizumab and sgp130Fc treatments prevented tumor recurrence equally when used as an adjuvant to surgery in the same mouse model [112]. Sgp130Fc suppressed tumor growth in a diethylnitrosamine (DEN) hepatocellular carcinoma C57BL/6 mouse model [69, 86, 112]. Additionally, sgp130Fc prevented development of lung adenocarcinoma in a gp130 knock in mouse model [85].

The ability of sgp130Fc to selectively inhibit IL-6 pro-inflammatory trans-signaling, without significantly inhibiting IL-6 classical signaling (like siltuximab or tocilizumab), nor broadly
inhibiting JAK activation (like ruxolitinib or tofacitinib), may allow sgp130Fc to succeed as an effective treatment for HNSCC where other treatments have failed.

Section V: Purpose of study

Although upregulated IL-6 signaling is clearly implicated in the progression of inflammatory cancer types such as HNSCC, IL-6 and IL-6R have been targeted with little success in HNSCC animal models and clinical trials.

IL-6 is highly pleiotropic, and therefore IL-6 signaling is associated with myriad phenotypes. This may explain why IL-6 signaling has been so difficult to target in cancer. Recent evidence supports that IL-6 classical signaling and trans-signaling largely promote transient and chronic inflammatory states respectively. Though evidence is yet limited to support this, we hypothesize that classical signaling and trans-signaling are also anti-tumorigenic and pro-tumorigenic respectively, in which classical signaling supports the immune response against tumor cells, whereas trans-signaling creates a microenvironment supportive to tumor cells. The inhibitor sgp130Fc has a selective affinity for the IL-6/sIL-6R complex, thus enabling it to completely antagonize IL-6 trans-signaling without impeding IL-6 classical signaling while only partially antagonizing other IL-6 family cytokine signaling. Therefore we believe that sgp130Fc might target only the chronic inflammatory effects of IL-6 signaling in HNSCC that induce tumor growth, survival, and metastasis.

There are limited treatment options for recurrent and metastatic HNSCC and for HNSCC tumors that are resistant to radiation, chemotherapy, and targeted therapy. Upregulated IL-6 signaling is implicated in HNSCC progression, tumor resistance, and metastasis. Therefore we also hypothesize that sgp130Fc might be a good adjuvant to enhance other HNSCC treatments, particularly in the case of resistant and R/M tumors.

Section VI: Hypothesis and specific aims

Hypothesis: Inhibition of IL-6 trans-signaling by sgp130Fc is anti-tumorigenic in HNSCC.

Aim 1: Investigate the effect of sgp130Fc on HNSCC tumor cell growth in vitro and in vivo.
**Aim 2a**: Investigate the combination of standard of care treatments (radiation and chemotherapy) and sgp130Fc on HNSCC tumor cell growth *in vitro* and *in vivo*.

**Aim 2b**: Investigate the combination of targeted therapy (cetuximab) and sgp130Fc on HNSCC tumor cell growth *in vitro* and *in vivo*. 
Chapter 2: Materials and methods

Treatments and reagents Tocilizumab (Actemra), cetuximab (Erbitux), cisplatin (CISplatin), and tofacitinib (Xeljanz) were obtained from the inpatient pharmacy at the University of Iowa Hospitals and Clinics (Iowa City, IA). Human immunoglobulin G (IgG) was purchased from Sigma-Aldrich (St. Louis, MO). Human and mouse recombinant sgp130Fc chimera proteins were purchased from R&D Systems (Minneapolis, MN). Drugs were diluted in Dulbecco’s PBS for both in vitro and in vivo experiments. A standard dose of 5 μg/ml of human IgG, sgp130Fc, or tocilizumab, was chosen for the majority of cell culture assays to correspond to in vivo experiments.

ELISA ELISA kits were obtained from R&D Systems. Human and mouse IL-6, sgp130, and IL-6Rα Duo-Set kits were used for in vitro experiments. Human IL-6, soluble gp130, and IL-6Rα Quantikine kits were used for cytokine quantification of patient plasma samples. Levels of IL-6, sIL-6R, and sgp130 in fetal bovine serum (FBS) were determined using ELISA and were either not detected or barely detectable. Plasma from healthy patients from two different time points was obtained through collaboration with Dr. Stephanie Gilbertson-White (IRB ID# 201506780) in the Department of Nursing at the University of Iowa. Plasma from HNC patients from three different time points was obtained through an ongoing study with Dr. Douglas Laux (IRB ID# 201302782) in the Holden Comprehensive Cancer Center at the University of Iowa Hospitals and Clinics. Plasma was obtained following the guidelines of the University of Iowa Institutional Review Board in accordance with the Department of Health and Human Services regulations 45CFR46.

Western blot Goat anti-rabbit IgG horseradish peroxidase (HRP) secondary antibody and antibodies against human β-Actin, STAT3, and phosphoSTAT3Y705 were purchased from Cell Signaling Technologies, Inc. (Danvers, MA). Precision Protein™ StrepTactin-HRP Conjugate was purchased from BioRad (Hercules, CA). Proteins were run on Thermo Fisher (Waltham, MA) Scientific NUPage™ 4-12% Bis-Tris Gels. Blots were imaged with Thermo Fisher SuperSignal West Femto™ Maximum Sensitivity Substrate.

Cell culture Cell lines were verified by ATCC (Manassas, VA) and tested for mycoplasma by Iowa Institute of Genetics Genomics Division at the University of Iowa. Cal27 and FaDu cell lines were purchased from ATCC. The mouse squamous cell carcinoma SCCVII cell line was a gift from the Weiner lab (Department of Internal Medicine, The University of Iowa); the cell line was initiated
in a Harvard lab from a spontaneous abdominal tumor in a C3H mouse [113]. The SQ20B cell line
was a gift from Dr. Anjali Gupta (Department of Radiation Oncology, The University of Iowa). Pt6
is a HNSCC cell line that was initiated in this lab from a patient-derived tumor sample obtained
from the University of Iowa Tissue Procurement Core (University of Iowa Hospitals and Clinics,
Holden Comprehensive Cancer Center). Cell lines were grown in complete DMEM (10% FBS,
50μg/ml Gentamicin, sodium pyruvate) and incubated at 37°C in 5% CO₂. In vitro assays were
performed in triplicate and independently unless otherwise stated.

**Flow cytometry** Cancer cell lines or mouse cell samples were incubated at room temperature
with 2.5 μg Human BD Fc Block (BD Biosciences, San Jose, CA) per 10⁶ cells in FACS buffer (PBS
1% BSA 0.05% NaN₃) for 10 min at room temperature and washed with FACS buffer twice before
staining at 4°C for 30 min protected from light and then fixed with 2% paraformaldehyde in PBS
(See Table 3 for a list of reagents, manufacturers, and concentrations). Data was acquired with a
BD Facs Canto II flow cytometer using BD FACSDiva software and analyzed using BD FlowJo v10.

**Immune cell subset assay** Mouse spleens and tumors were harvested, washed in RPMI, forced
through 70 μm filters by syringe, washed in RPMI, incubated with ACK lysing buffer (155 mM
NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.3), at room temperature for 2 min, washed and then
suspended in RPMI on ice for counting prior to staining. In the first panel, cell samples were
gated first for CD45⁺. NK, T, and B cells were gated by NKp46, CD3, and CD19 expression
respectively, and then CD3⁺ populations were further differentiated by CD4 and CD8 expression.
In the second panel, cell samples were gated for CD45⁺, and then NKp46⁺, CD3⁺, and CD19⁺
populations were excluded. CD11b, CD11c, Ly6C, and Ly6G expression were used to identify
dendritic cell, neutrophil, and macrophage/monocyte populations.

**Baseline IL-6R cell surface expression assay** Ligand removal was performed by a 30 sec low pH
acetate buffer wash (0.05 M Acetate pH 4.0, 0.085 M NaCl, 0.005 M KCl, 2% FBS) on ice followed
by neutralization with equal volume 0.1 M Tris (pH 8.0, 2% FBS) and two washes with FACS
buffer prior to staining for flow cytometry [114]. Cell surface expression of IL-6R was compared
to isotype controls, with or without ligand removal.

**NK cell activation assay** Peripheral blood mononuclear cells (PBMCs) were isolated from healthy
donor blood obtained from the DeGowin Blood Center (University of Iowa Hospitals and Clinics)
using Ficoll-Paque density gradient centrifugation and then frozen at -80°C until use. 0.25 x 10^6 HNSCC cells in complete DMEM were plated into wells of 96-well plate and given several hours in which to adhere. Medium was aspirated from wells and then treatments of 2.5 μg/ml human IgG, cetuximab, human sgp130Fc, and/or tocilizumab were added to corresponding wells within 50 μl/well RPMI medium. Non-treated wells received only 50 μl of medium. 0.25 x 10^6 PBMCs were then added to each well within 200 μl RPMI medium (for a total of 250 μl per well) and incubated between 20-24 hrs at 37°C prior to staining for flow cytometry. After gating for PBMCs, CD3+ and CD19+ populations were excluded and CD56+ populations were selected to identify NK cells. Activation was associated with an increase in CD54 and decrease in CD16 expression.

Table 3. Flow cytometry reagents, manufacturer, and concentrations.

<table>
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<tr>
<th>Product/Catalogue #</th>
<th>Manufacturer</th>
<th>Dilution</th>
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<tbody>
<tr>
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<td>Anti-human CD56-APC 362504</td>
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<tr>
<td>Anti-human CD126(IL-6R)-APC 352806</td>
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<tr>
<td>Mouse IgG1 κ isotype APC 400122</td>
<td>BioLegend</td>
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<tr>
<td>Anti-mouse CD3-PE 100205</td>
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<tr>
<td>Anti-mouse CD3-PE/Cy7</td>
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<tr>
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<tr>
<td>Anti-mouse NKp46-PE 137603</td>
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**qRT-PCR** was performed using Thermo Fisher ViiA7 Real-Time PCR System and software. Primers for membrane IL-6R were: F: CATTGCCATTGTTCTGAGGTTC; R: GTGCCACCCAGCCAGCTATC [112].

**In vivo experiments** 4-6 week old athymic Foxn1\(^{nu/nu}\) male and female mice were purchased from Envigo (United States). 2 x 10\(^6\) SQ20B cells were injected subcutaneously on right flank to form xenograft tumors.

4-6 week old C3H/HeJ male and female mice were purchased from Jackson Labs (Bar Harbor, ME). 1 x 10\(^6\) SCCVII cells were injected subcutaneously on right flank to form allograft tumors.

Mice were housed with 12 h light/dark cycles with free access to standard diet and water and were treated according to protocols established by the University of Iowa Office of Animal Resources Institutional Animal Care and Use Committee (IACUC).

Treatments began once tumors were palpable and measurable, which was approximately 3-4 days after inoculation. Treatment was given by intraperitoneal injection (i.p.) twice weekly. Mice were sacrificed when tumors reached a diameter of 15 mm or when experiment was ended. Mice received doses of 0.25 or 0.5 mg/kg of human IgG, human or mouse sgp130Fc, or tocilizumab depending on the experiment. For *in vivo* experiments, “SOC” (standard of care) treatments were defined as a combination of cisplatin (2 mg/kg, two or three times a week) and radiation (2 Gy from x-ray source, two times a week) treatments. PBS was used in equal volume as the control for cisplatin.

**Irradiation** of cells and mice by an x-ray source, Pantak Orthovoltage unit, Bipolar Series 2, HF320, Pantak Inc. (East Haven, CT) was performed by the Free Radical and Radiation Biology Research Core (University of Iowa). Mice were sedated with 60 µl i.p. injection containing 1.05 mg ketamine hydrochloride and 0.15 mg xylazine hydrochloride (obtained from the inpatient pharmacy at the University of Iowa Hospitals and Clinics) prior to irradiation of mice from SOC treatment groups. Mice were lead shielded and hind leg irradiated with 2 Gy (dose rate, 1.38 Gy/min).

**Bioplex** Bio-Plex Pro™ Mouse Cytokine 23-Plex Assay (detecting TNFα, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFNγ, RANTES, MCP-1, MIP-1a, MIP-1b) was purchased from Bio-Rad to determine mouse plasma cytokine concentrations.
**RNAseq** Total RNA was isolated using Qiagen RNeasy Plus Mini Kit with DNase DNA-removal. RNAseq was performed by the Iowa Institute of Genetics Genomics Division (University of Iowa) and analyzed by Dr. Diana Kolbe (Bioinformatics Division, University of Iowa Institute of Human Genetics).

**Statistical analysis** was performed (GraphPad Prism 7.03 software) using one-way or two-way ANOVA followed by Tukey’s test. Error bars represent standard deviation (SD). Comparisons of Kaplan-Meier survival curves were determined by log-rank (Mantel-Cox) test. Statistical significance in all experiments was set at $p \leq 0.05$. 
Chapter 3: Results

Section I: Aim 1: Investigate the effect of sgp130Fc treatment on HNSCC tumor cell growth in vitro and in vivo.

First we wanted to determine whether HNSCC cells performed IL-6 trans-signaling and IL-6 classical signaling at baseline. The secretion of IL-6 signaling proteins IL-6, sIL-6R, and sgp130, from human HNSCC cell lines (Cal27, FaDu, Pt6, SCC-25, and SQ20B) and from the SCC-VII mouse squamous cell carcinoma cell line were determined using ELISA (Fig. 6a-c). We also used flow cytometry to determine expression of membrane IL-6R in Cal27, FaDu, and SQ20B cells (Fig. 7a-e). Each cell line displayed unique, characteristic protein profiles.

Figure 6. HNSCC cell baseline expression of IL-6 trans-signaling proteins. HNSCC cells (Cal27, FaDu, Pt6, SCC-25, SQ20B) and mouse squamous carcinoma cells (SCCVII) were plated and medium was removed at different time points after plating. ELISA was used to determine levels of cell secretion of a) IL-6, b) sIL-6R, and c) sgp130 proteins into the medium at 24, 48, or 72 hr.
Figure 7. HNSCC cell baseline expression of membrane IL-6R protein. HNSCC cells were grown to confluence and then stained for flow cytometry to determine cell surface IL-6R expression compared to isotype control. Ligand removal was performed (see Methods) to show expression of only membrane, not soluble, IL-6R (Untx = ligand removal was not performed; Post-tx = ligand removal was done). HNSCC cell lines a) Cal27 without ligand removal, b) Cal27 after ligand removal, c) FaDu without ligand removal, d) FaDu after ligand removal, and e) SQ20B after ligand removal, were analyzed.

Cal27, Pt6, and SCC-25 cell lines secreted the highest amounts of IL-6, whereas SCC-25 and SQ20B displayed the highest secretion of sIL-6R and sgp130. Though sIL-6R was detectable in the medium of SCCVII cells, IL-6 and sgp130 were not.

In order to remove sIL-6R from the cell surface to be able to detect expression of membrane IL-6R by flow cytometry, HNSCC cells were grown to confluence, collected, and then half of the samples were subjected briefly to an acid wash to remove surface ligands, followed by neutralization, washing, and staining. Interestingly, Cal27 cells displayed negligible differences in fluorescence for IL-6R compared to isotype control prior to ligand removal (Fig. 7a), but did display differences after ligand removal treatment (Fig. 7b). In contrast to Cal27 cells, FaDu cells exhibited no change in profile following ligand removal (Fig. 7c-d), indicating that FaDu cells express either no or very minimal membrane IL-6R protein (Fig. 7d). Additionally,
both Cal27 and SQ20B cells displayed bimodal membrane IL-6R-expressing populations in this assay, although it is not clear why.

![Figure 8. Effects of sgp130Fc inhibition on HNSCC cells.](image)

Cells were treated with 5 µg/ml of human IgG, sgp130Fc, or tocilizumab for 24 hr time course. 50 µM pan-JAK inhibitor tofacitinib treatment was used as a control. Cell samples were removed at 2, 6, and 24 hr time points and prepared for WB or clonogenic survival assays. WB displaying pSTAT3 and total STAT3 protein from a) SQ20B and b) FaDu cell lysates. A similar trend was seen with Cal27 (WB data not shown). Normalized surviving fraction of c) Cal27, d) SQ20B, or d) FaDu cells by treatment groups. *p<0.05
Figure 9. RNAseq results from SQ20B cells treated with IgG, sgp130Fc, or tocilizumab in vitro. Cells were treated for 48 hrs with 5 μg/ml human IgG, human sgp130Fc, or tocilizumab prior to RNA isolation. Samples were prepared in triplicate. Sample code: E= IgG; F= tocilizumab; G= sgp130Fc. a) Heat map of mRNA expression by sample. b) Genes that were upregulated (up arrow) or downregulated (down arrow) by sgp130Fc treatment.

The human HNSCC cell lines that we tested by ELISA and flow cytometry all had displayed secretion of IL-6 trans-signaling pathway proteins, and Cal27 and SQ20B displayed membrane IL-6R; therefore we wanted to determine whether selective IL-6 trans-signaling inhibitor sgp130Fc or global IL-6R inhibitor tocilizumab treatments would affect JAK/STAT3 signaling in vitro. A dose of 5 μg/ml of sgp130Fc was used in this assay (and in most other in vitro assays for this thesis project) to correspond to in vivo doses, which were chosen based on other studies using sgp130Fc [69, 112]. Cells were treated for a 24 hr time course with 5 μg/ml human IgG (control), sgp130Fc, or tocilizumab, and then cells were either pelleted in preparation for Western blot (WB) or else plated for clonogenic assays. Tofacitinib, a pan-JAK inhibitor, was used as a positive control.

Tocilizumab but not sgp130Fc treatment inhibited STAT3 phosphorylation at 6 and 24 hr time points in SQ20B and FaDu cells (Fig. 8a-b). Sgp130Fc and tocilizumab treatments actually
increased clonogenic survival of SQ20B cells compared to IgG treatment by approximately 1/3- to 1/2-fold (Fig. 8d); however, no significant changes in survival were seen between treatment groups in Cal27 (Fig. 8c) or FaDu (Fig. 8e) cells.

To investigate how sgp130Fc or tocilizumab treatments would affect HNSCC cell gene expression, total RNA were isolated from SQ20B cells that had been treated for 48 hrs with 5µg/ml human IgG, sgp130Fc, or tocilizumab. Assay was performed in triplicate. After RNA purification, RNAseq was performed and analyzed (Fig. 9a). Possible contamination in one of the IgG-treated control samples prevented obtaining quantitative results because it reduced the IgG sample size to n=2; however, qualitative results showed that tocilizumab treatment had broader and more dramatic effects on HNSCC tumor cell gene expression than did sgp130Fc. After excluding the contaminated IgG sample, sgp130Fc treatment did show a trend of modestly affecting SQ20B gene expression (Fig. 9b) compared to the remaining IgG samples, but again due to not having the proper sample size of the IgG-treated control cells, we could not perform a quantitative statistical analysis of these trends.

Though sgp130Fc did not reduce clonogenic survival in vitro, we still wanted to test its effects in vivo because we anticipated that it might inhibit IL-6 trans-signaling in the tumor microenvironment and therefore inhibit tumor growth indirectly. In order to determine whether sgp130Fc treatment would suppress HNSCC tumor growth in vivo, athymic Foxn1\(^{nu/nu}\) male and female mice were injected subcutaneously on the right flank with SQ20B cells to produce xenograft tumors. After tumor development, mice were treated twice a week by i.p. with 0.25 or 0.5 mg/kg doses of human IgG, human sgp130Fc, or tocilizumab. Mice treated with 0.25 mg/kg sgp130Fc displayed significantly reduced mean tumor growth from day 14 of treatment compared to mice treated with corresponding doses of IgG or tocilizumab (Fig. 10a). Interestingly, no significant differences in tumor growth were seen between the 0.5 mg/kg treatment groups (data not shown). After sacrifice, tumors and blood were harvested for histological analysis and plasma cytokine quantification. A Bioplex with a panel that recognizes 22 different inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFNγ, RANTES, MCP-1, MIP-1a, and MIP-1b) was performed on the plasma to identify whether sgp130Fc or tocilizumab treatments had resulted in changes in plasma inflammatory cytokine levels compared to the control IgG group. Eotaxin was the only cytokine with significantly different plasma concentrations between treatment groups (Fig. 10b; p=0.0059 Eotaxin level is significantly different in IgG group
Figure 10. Effects of sgp130Fc treatment in SQ20B xenograft athymic nu/nu mouse model. Mice were treated with 0.25 mg/kg human IgG, human sgp130Fc, or tocilizumab by i.p. twice a week. a) Mean tumor volume of treatment groups: IgG n=8 (3F, 5M); sgp130Fc n=8 (3F, 5M); tocilizumab n=6 (2F, 4M); *p<0.05, sgp130Fc group mean tumor volume was significantly lower than that of IgG or tocilizumab groups on Day 13. b) Bioplex results of plasma cytokine concentrations. Eotaxin was the only cytokine that displayed significantly different plasma levels between treatment groups according to ANOVA (*p=0.0059 IgG is significantly different than tocilizumab; p <0.0001 sgp130Fc is significantly different than tocilizumab). No significant differences were observed between treatment groups using c) Ki67 or d) H&E staining.
Figure 11. Effects of sgp130Fc treatment in immunocompetent C3H/HeJ SCCVII allograft tumor model.

Mice were treated with 0.25 or 0.5 mg/kg of human IgG or mouse sgp130Fc by i.p. twice a week. a) Mean tumor volume of male mice by treatment group: 0.25 mg/kg IgG n=3; 0.5 mg/kg IgG n=5; 0.25 mg/kg sgp130Fc n=5; 0.5 mg/kg sgp130Fc n=4. *p<0.0026 sgp130Fc (0.5 mg/kg) group had significantly lower tumor growth than IgG (0.5 mg/kg) group on Day 8. **Both 0.25 mg/kg (p=0.0017) and 0.5 mg/kg (p=0.0110) sgp130Fc treatment groups had significantly lower tumor growth than their respective IgG control groups on Day 9. b) Mean tumor volume of female mice by treatment group: 0.25 mg/kg IgG n=5; 0.5 mg/kg IgG n=5; 0.25 mg/kg sgp130Fc n=5; 0.5 mg/kg sgp130Fc n=4.

compared to tocilizumab group; p<0.0001 Eotaxin level is significantly different in sgp130Fc group compared to tocilizumab group). No significant differences in histology or tumor proliferation were observed from H&E or Ki-67 staining of the tumors (Figs. 10c-d).

We wanted to find out whether sgp130Fc would retain its anti-tumor effects in an immunocompetent mouse model. C3H/HeJ mice were inoculated by subcutaneous injections on the right flank with SCCVII cells to form allograft tumors. After tumor development, mice were
treated twice a week by i.p. with 0.25 or 0.5 mg/kg of human IgG or mouse sgp130Fc. Human IgG was used as a control for this experiment because mouse sgp130Fc contains a human IgG1 Fc domain. Both doses of sgp130Fc treatments resulted in suppressed mean tumor growth in male mice compared to IgG controls (Fig. 11a), however no significant differences in tumor growth were seen between treatment groups of female mice (Fig. 11b).

In order to elucidate the mechanism of the anti-tumor efficacy of sgp130Fc, we wanted to find out whether sgp130Fc affected immune cell recruitment. Therefore, we repeated the in vivo experiment in which we used the immunocompetent C3H/HeJ SCCVII allograft tumor model (this time using only male mice) and treated the mice with 0.25 mg/kg human IgG or mouse sgp130Fc. We then used flow cytometry in order to identify whether sgp130Fc treatment had altered proportions of immune cell subsets in mouse tumors and spleens compared to IgG treatment. In this experiment, the mean tumor growth of the sgp130Fc-treated mice trended behind that of the IgG-treated mice, but there was no statistically significant difference in mean tumor growth between treatment groups at the point that at least one mouse from either group had to be sacrificed. Spleens and tumors were harvested and pushed through filters by syringe to prepare the cells for staining for flow cytometry (see Methods for gating protocol).

![Figure 12. Immune cell subsets in spleens and SCCVII allograft tumors of IgG- or sgp130Fc-treated C3H/HeJ mice. Percentage of immune cell populations in a) spleens and b) tumors as determined by flow cytometry (see Methods for gating protocol).*p<0.05 There were significant differences in neutrophil and dendritic cell percentages in sgp130Fc-treated mice compared to IgG-treated mice.

We observed subtle but significant differences in immune cell populations between treatment groups, with slight increases in mean percentages of neutrophils in spleens (Fig. 12a) and of dendritic cells in tumors (Fig. 12b) in the sgp130Fc-treated mice compared to IgG-treated mice. No significant differences were seen in natural killer (NK) cell, B cell, CD4 T cell, or CD8 T
cell populations between treatment groups (data not shown). We also ran a panel to determine proportions of T cell subsets (i.e. Th1, Th17, Treg, etc.); however, the intracellular staining protocol failed, and we were unable to get results from that panel. This is a potential area of research to explore in the future to better identify how sgp130Fc treatment may target the immune system.
Section II: Aim 2a: Investigate the combination of standard of care treatments (radiation and chemotherapy) and sgp130Fc on HNSCC tumor cell growth in vitro and in vivo.

There is no universal standard of care (SOC) protocol for HNSCC in the United States, but frequently combined radiation and chemotherapy is considered SOC for locoregionally advanced cancers. IL-6 signaling has been found to be associated with resistance to both radiation and chemotherapy in HNSCC, and so we hypothesized that this resistance is due more specifically to IL-6 trans-signaling. HNSCC cells were treated with dose-response levels of cisplatin and radiation to determine whether those treatments affected levels of HNSCC cell secretion of IL-6 trans-signaling proteins.

Figure 13. HNSCC cell secretion of IL-6 trans-signaling proteins in response to SOC treatment compared to clonogenic survival. Cells were treated with a dose response of cisplatin for 24 hr prior to a dose response of radiation from an x-ray source, and then media was removed for ELISA assays before plating cells for clonogenic survival assays. ELISA results of IL-6 and sIL-6R secretion compared to clonogenic survival of a-b) SQ20B and c-d) Cal27 cells. Secretion of endogenous sgp130 shared a similar trend to that of IL-6 and sIL-6R (data not shown).
Using ELISA, we found that SQ20B (Fig. 13a-b), Cal27 (Fig. 13c-d), and FaDu cells (data not shown) upregulate secretion of IL-6, sIL-6R, and sgp130 proteins in response to radiation and chemotherapy in a synergistic and dose-dependent manner, though each cell line again displayed their own characteristic profile (the sgp130 results are not shown here but had displayed a similar dose-dependent curve). These results suggested that HNSCC cells use IL-6 trans-signaling as a survival response. To determine whether sgp130Fc would inhibit this upregulation and thereby improve the anti-tumor efficacy of SOC treatments, HNSCC cells were treated with 5 µg/ml human IgG, sgp130Fc, or tocilizumab and either 1 µM of cisplatin or the same volume of PBS for 24 hrs prior to radiation by x-ray source. Just after radiation, cells were plated for clonogenic survival assays; colonies were counted two weeks later. Combining sgp130Fc or tocilizumab with SOC treatments did not significantly reduce clonogenic survival of SQ20B cells (Fig. 14a) or FaDu cells (Fig. 14b) compared to SOC treatment alone. In SQ20B cells that did not receive radiation, sgp130Fc and tocilizumab treatments each individually significantly increased cell survival by about half-fold (Fig. 14a). In FaDu cells that received 2 Gy of radiation, sgp130Fc treatment slightly increased cell survival. The Cal27 cell line was also tested using this assay, but the Cal27 assay was only performed twice, so statistics on the Cal27 clonogenic results were not performed (data not shown).

SQ20B cells are known to be resistant to both radiation and cisplatin [115-117]. To find out whether sgp130Fc treatment would increase the sensitivity of SQ20B tumors to SOC treatment in vivo, athymic Foxn1^{nu/nu} male and female mice were given SQ20B xenografts by subcutaneous injection using the same method as described previously. After tumor development, mice from SOC treatment groups were treated twice a week. In order to treat the mice, the mice were removed from their housing facility and brought to the radiation facility by cart. All mice from all treatment groups were first sedated by i.p. with ketamine/xylazine. Then the mice in the SOC treatment groups were placed into lead coffins with their tumor-bearing flank pulled out of the coffin and received 2 Gy x-ray radiation, after which all mice received i.p. injections of 0.25 mg/kg IgG or sgp130Fc and 2 mg/kg cisplatin or equivalent volume of PBS. The combination sgp130Fc and SOC treatment group but not the SOC control (IgG + SOC) group displayed significantly reduced mean tumor growth compared to the IgG control group on treatment day 14 (Fig. 15a). One mouse from the IgG group had to be sacrificed on Day 14 due to tumor size so we were unable to continue the graph to be able to compare between
Figure 14. Normalized surviving fractions of HNSCC cells treated with IgG, sgp130Fc, or tocilizumab and SOC. Cells were treated with 5 μg/ml of human IgG, human sgp130Fc, or tocilizumab with or without SOC (1 μM cisplatin and dose response of radiation). a) Normalized surviving fractions of SQ20B cells. b) Normalized surviving fractions of FaDu cells. Cal27 cells displayed similar trends to FaDu cells (data not shown).

treatment groups after that day. Both male and female mice in the combined sgp130Fc and SOC group shared similar trends of reduced tumor growth (Fig. 15b-c). It is not clear why sgp130Fc
treatment as a single therapy did not result in decreased tumor growth in male mice in this experiment, as had been seen in our previous SQ20B xenograft nude mouse experiments;

![Tumor growth graph](image)

**Figure 15. Effects of combined sgp130Fc and SOC treatments in SQ20B xenograft athymic nu/nu mouse model.** Mice from all treatment groups were sedated by ketamine/xylazine before SOC treatment groups were irradiated at 2 Gy dose by x-ray source and then all mice were consecutively treated by i.p. with 0.25 mg/kg human IgG or sgp130Fc and 2 mg/kg cisplatin or same volume PBS twice a week (IgG n=7; sgp130Fc n=7; IgG + SOC n=6; sgp130Fc + SOC n=7). a) Mean tumor volume of all mice by treatment group.*p=0.0045 Sgp130Fc + SOC treatment resulted in significantly reduced mean tumor volume compared to IgG group on Day 12. Mean tumor volume of IgG + SOC group was not significantly different than IgG group on Day 12. b) Mean tumor growth in males by treatment group (IgG n=3, sgp130Fc n=4, IgG + SOC n=4, sgp130Fc + SOC n=3). c) Mean tumor growth in females by treatment group (IgG n=4, sgp130Fc n=3, IgG + SOC n=2, sgp130Fc + SOC n=4).
however, as the mice were treated differently in this experiment than in those previous experiments (they were sedated by ketamine/xylazine and had to travel from their housing facility to the radiation facility in another building twice a week), it is possible that these differences affected their cytokine levels, either from stress or from the effects of the ketamine.

This experiment was repeated using the same SQ20B xenograft nude mouse model but with an increased cisplatin dose (6 mg/kg/wk, given as 2 mg/kg three times a week) to determine whether it would result in a significant decrease in tumor growth in the control SOC treatment group and even more improved tumor reduction in the combined sgp130Fc and SOC treatment group. However, no significant differences in tumor growth or survival were seen in this experiment (data not shown), which may have been due to the change in dose or dosing schedule of cisplatin.

This experiment was repeated again, this time using the immunocompetent SCC-VII allograft C3H/HeJ model in only male mice. Treatments (2 Gy x-ray radiation, consecutively followed by i.p. injection of 0.25 mg/kg human IgG or mouse sgp130Fc and 2 mg/kg cisplatin or equivalent volume PBS) were given twice a week. No significant difference in tumor growth was found between treatment groups (data not shown).
Section III: Aim 2b: Investigate the combination of targeted therapy (cetuximab) and sgp130Fc on HNSCC tumor cell growth in vitro and in vivo.

As said in the introduction, the EGFR inhibitor cetuximab is not a very effective targeted therapy for HNSCC overall due to innate and acquired tumor resistance. Therefore there is a need to improve cetuximab’s efficacy in HNSCC. This lab had previously shown that cetuximab treatment induces HNSCC cell secretion of IL-6 and other cytokines associated with inflammation; moreover, this lab found that the global IL-6R inhibitor tocilizumab improved the efficacy of small molecule EGFR inhibitor erlotinib against erlotinib-resistant SQ20B cells [98]. It is believed that when EGFR-mediated signaling is inhibited, HNSCC tumors respond by activating alternative proliferation and survival pathways, including IL-6 JAK/STAT pathways. Genes targeted by IL-6-mediated JAK/STAT3 signaling overlap with EGFR signaling targets, including cyclin D1 and E2F1 [84], suggesting one reason why IL-6 JAK/STAT3 signaling might be “turned on” by tumor cells whose EGFR signaling has been “turned off” by cetuximab treatment.

![Figure 16](image.png)

Figure 16. Mean tumor volume of SQ20B xenograft nude mice treated with human IgG, cetuximab, tocilizumab, or combined cetuximab and tocilizumab. Mice were treated by i.p. with: 9 mg/kg IgG 1x/week + 1 mg/kg IgG 2x/week (n=8); 8 mg/kg cetuximab 1x/week (n=5); 1 mg/kg tocilizumab 3x/week (n=8); or 8 mg/kg cetuximab 1x/week + 1 mg/kg tocilizumab 2x/week (n=8). *p=0.0209 Mean tumor volume of cetuximab and tocilizumab treatment group was significantly higher than mean tumor volume of cetuximab treatment group on Day 11.

Prior to our work with sgp130Fc, we had wanted to investigate whether tocilizumab would improve cetuximab’s efficacy as it had for erlotinib. SQ20B xenograft nude mice were
treated with 8 mg/kg IgG or cetuximab once a week and with 2 mg/kg IgG or tocilizumab three times a week. Surprisingly, tocilizumab rescued cetuximab’s anti-tumor effects in SQ20B xenograft nude mouse models (Fig. 16). We hypothesized that the reason was because tocilizumab was not only inhibiting pro-tumorigenic IL-6 signaling (trans-signaling), but was also inhibiting anti-tumorigenic IL-6 signaling (classical signaling), that might be required by cetuximab, but not erlotinib.

**Figure 17. SQ20B IL-6 trans-signaling response to cetuximab treatment.** a) SQ20B cells were treated for 48 hrs with a dose response of cetuximab (50 – 200 μg/ml). Medium was removed for ELISA. b-c) SQ20B cells were treated for 48 hrs with 200 μg/ml human IgG or cetuximab and 50 μM tocilizumab or equivalent amount of human IgG. Medium was removed for ELISA. d) SQ20B cells were treated for 48 hrs with 200 μg/ml or a dose response of cetuximab (50 – 200 μg/ml) prior to RNA isolation for qRT-PCR. *p<0.05

In order to identify whether HNSCC cells upregulate IL-6 trans-signaling in response to cetuximab treatment, SQ20B cells were treated with a dose-response of cetuximab for 48 hrs. ELISA confirmed that cetuximab treatment induced SQ20B cell secretion of IL-6 (Fig. 17a), though not in a dose-dependent manner. Cetuximab treatment appeared to result in a trend of higher SQ20B cell secretion of sIL-6R (Fig. 17b) and sgp130 (Fig. 17c), and interestingly, tocilizumab treatment appeared to reduce cetuximab-mediated sgp130 secretion in SQ20B cells.
(Fig. 17c); however this assay was only performed twice and therefore these results were not significant. Cetuximab treatment reduced HNSCC cell expression of membrane IL-6R mRNA, as seen by qRT-PCR (Fig. 17d). Together, these results indicate that SQ20B cells respond to cetuximab treatment with downregulation of IL-6 classical signaling and upregulation of IL-6 trans-signaling.

![Tumor growth graph](image)

**Figure 18.** Mean SQ20B xenograft tumor growth in nude mice treated with combined therapies of cetuximab and sgp130Fc or cetuximab and tocilizumab. (Ctx = cetuximab; Tocil = tocilizumab). Mice were treated by i.p. twice a week with: 4 mg/kg IgG (n=5); 1 mg/kg Ctx (n=4); 1 mg/kg Ctx + 3 mg/kg IgG (n=6); 2 mg/kg tocilizumab (n=4); 1 mg/kg Ctx + 2 mg/kg tocilizumab (n=6); 0.5 mg/kg sgp130Fc (n=5); or 1 mg/kg Ctx + 0.5 mg/kg sgp130Fc. Dose of cetuximab was increased to 2 mg/kg for all mice receiving cetuximab after one week of treatment because cetuximab treatment group was not showing significant differences in mean tumor size compared to other treatment groups. One mouse in the cetuximab group had higher tumor growth than other mice in the group which affected mean growth, but due to the small size of the group, was not an outlier. *p<0.05 Mean tumor growths of sgp130Fc, IgG + Ctx, and tocilizumab + Ctx treatment groups were significant different than IgG; sgp130Fc mean tumor growth is significantly different than IgG or IgG + Ctx.

Therefore, we hypothesized that sgp130Fc, which selectively inhibits IL-6 trans-signaling but not classical signaling, would have an improved effect compared to tocilizumab when combined with cetuximab. However, when we repeated this experiment using the SQ20B xenograft nude mouse model with additional sgp130Fc and combined cetuximab/sgp130Fc treatment groups, we found that sgp130Fc and cetuximab resulted in increased tumor growth compared to cetuximab or sgp130Fc alone, although sgp130Fc proved efficacious on its own (Fig. 18). There was an error in this experiment: the dose of cetuximab in the experiment in Fig.
16 had been too high, so a lower dose of cetuximab was used in this experiment (Fig. 18), but it did not significantly suppress tumor growth. Additionally, the graph appears as though the combined cetuximab and tocilizumab treatment group had lower mean tumor growth than the cetuximab treatment group, yet this was not significant. The results suggested that either cetuximab requires IL-6 trans-signaling for its anti-tumor efficacy or else there is another interaction in which sgp130Fc and cetuximab interfere with each other’s actions.

One possibility that we hypothesized is that since sgp130Fc and tocilizumab both contain IgG1 Fc regions, there might have been antibody-antibody interference between either of them and the Fc region of cetuximab in the two experiments shown in Fig. 16 and Fig. 18. It has been seen in mouse models that one of the anti-tumor mechanisms of cetuximab is the ability to induce ADCC against cancer cells [25], so we thought that this antibody-antibody interference might explain why neither sgp130Fc nor tocilizumab had increased cetuximab’s efficacy in vivo. To test this, a flow cytometry in vitro assay was performed to identify changes in NK cell CD54 and CD16 expression associated with activation in response to treatments [118, 119]. In this assay we additionally wanted to determine whether individual sgp130Fc or tocilizumab treatments could induce NK cell activation at the same doses that were used in our HNSCC mouse models. Unfortunately, that dose was saturating in this assay, and so a lower dose had to be used.

Peripheral blood mononuclear cells (PBMCs) were treated in the presence of SQ20B or Cal27 cells with 2.5 μg/ml IgG, cetuximab, sgp130Fc, or tocilizumab, alone or in combination, for 20-24 hrs prior to staining for flow cytometry. Gating was performed to identify NK cell populations (see Methods section for protocol). Activation of NK cells was associated with an increase in CD54 expression and a corresponding decrease in fluorescence associated with CD16, as CD16 would no longer be accessible to the fluorophore antibody reagent if it was bound to an Fc conjugate. PBMCs treated with cetuximab in the presence of cancer cells were used as positive controls, as cetuximab was already shown in other studies to cause NK cell activation. As another control, PBMCs also were treated without the presence of cancer cells; this resulted in no significant differences in NK cell activation in any of the treatment groups, indicating that cetuximab requires target cells in order to cause NK cell activation (data not shown).
Figure 19. Effects on NK cell activation by sgp130Fc, cetuximab, and/or tocilizumab treatments. Cancer cells were plated and allowed to adhere. Medium was removed and treatments were added to plate followed by addition of PBMCs. (Ctx=cetuximab). Cells were treated for 20-24 hrs prior to staining for flow cytometry. a-h) Results from one assay displaying cell populations by treatment group (a=NT, b=IgG, c=Cetuximab, d=sgp130Fc, e=tocilizumab, f=Ctx + IgG, g=Ctx + sgp130Fc, h=Ctx + tocilizumab). i) Cumulative results from all assays. Assays were only performed twice for each cell type so ANOVA was not performed.
This NK cell activation assay was performed only twice, so though standard deviation is shown on the graph, ANOVA was not performed; however the trends in the results suggest that neither sgp130Fc nor tocilizumab treatments individually induce NK cell activation at this dose, nor does there appear to be any difference in NK cell activation in any of the combination treatment groups (IgG and cetuximab, sgp130Fc and cetuximab, or tocilizumab and cetuximab) compared to cetuximab treatment alone, which displayed a trend of approximately double the percentage of activation of other individual treatments (Fig 19i). Overall, these results suggest that neither sgp130Fc nor tocilizumab impair NK cell activation at these doses, although additional experiments (perhaps using an in vivo system) are necessary to confirm this result.
Chapter 4: Discussion

Five different human HNSCC cell lines were shown to all secrete IL-6 trans-signaling proteins IL-6, sIL-6R, and sgp130 by using ELISA (Fig. 6). Each cell line displayed unique and characteristic profiles of expression. It is of note that this lab repeatedly has seen that when HNSCC cell sIL-6R secretion rises, sgp130 secretion correspondingly rises as well, which suggests that sgp130 naturally regulates sIL-6R. Also notable (with the exception of SCC-25 cells in which all protein secretion levels were high) there appeared to be a “see-saw” effect of HNSCC cell secretion of IL-6 trans-signaling proteins at baseline; when IL-6 secretion levels went up, sIL-6R and sgp130 secretions went down, and vice versa. As mentioned in the introduction, sIL-6R may buffer IL-6 in the plasma by sequestering IL-6 in IL-6/sIL-6R/sgp130 complexes [64]; therefore it is possible that our ELISA results might represent this buffering and might not indicate the exact levels of IL-6 secretion from HNSCC cells (i.e. because the IL-6 was trapped in the complex and therefore not detectable by the ELISA detection antibody). This is something that could be tested in the future by using immunoprecipitation to determine the amount of IL-6 trapped in these complexes; however, at the time when these ELISAs were performed, we did not know about this potential capacity for buffering. Cal27 and SQ20B cell lines also displayed expression of membrane IL-6R as detected by flow cytometry following ligand removal (Fig. 7). Interestingly, each of these two cell lines displayed bimodal populations of higher and lower expression of IL-6R. It is not clear what these two populations represent, which might be an interesting area of inquiry. Though literature suggests that FaDu cells express membrane IL-6R, this experiment indicated that FaDu cells no longer express IL-6R on the plasma membrane after ligand removal (Fig. 7). This experiment should be repeated again to confirm these results.

Although the Cal27, FaDu, and SQ20B cell lines all secreted IL-6 trans-signaling proteins, the IL-6 trans-signaling inhibitor sgp130Fc did not reduce phosphorylation of STAT3 or reduce clonogenic survival of these cell lines in vitro; in fact, sgp130Fc treatment significantly increased clonogenic survival of SQ20B cells (about 1/3 to 1/2-fold), as did global IL-6R inhibitor tocilizumab (Fig. 8). However, sgp130Fc treatment was efficacious as a single therapy at a lower dose (0.25 mg/kg) in an immunodeficient (SQ20B xenograft athymic nude) mouse model compared to the same dose of tocilizumab (Fig. 10). Interestingly, a higher dose of sgp130 (0.5 mg/kg) did not result in tumor suppression compared to the same dose of IgG or tocilizumab in this experiment. However, something similar was seen in an experiment in a different lab, in
which a lower dose of sgp130Fc (0.5 mg/kg/wk) resulted in higher tumor suppression than did a higher dose of sgp130Fc (2.5 mg/kg/wk) in a pancreatic cancer mouse model [112]. This may indicate that modulating rather than completely blocking IL-6 trans-signaling may result in higher anti-tumor efficacy, perhaps because completely blocking IL-6 trans-signaling induces activation of alternative pathways; this is an area for investigation. In order to pursue the mechanism of the anti-tumor efficacy of sgp130Fc, we investigated whether there were differences in tumor histology or in plasma inflammatory cytokine levels in these mice. No differences in H&E or Ki-67 staining of tumors and only a negligible difference in concentration of one cytokine (eotaxin) were seen between treatment groups (Fig. 10). These results did not help to elucidate the mechanism of sgp130Fc. It was noted in the introduction that in one immunoprecipitation experiment, human sgp130Fc was shown to be able to bind to mouse IL-6/sIL-6R complexes [107]; therefore, the results from this in vivo experiment may not be all from direct interactions of the human sgp130Fc drug against the human HNSCC SQ20B cells in the xenograft, but also against other parts of the tumor microenvironment.

Sgp130Fc treatment displayed tumor suppression in an immunocompetent (SCCVII allograft C3H/HeJ) in vivo model in male but not female mice (Fig. 11). Interestingly in this experiment, the higher dose of IgG showed a (non-significant) trend of tumor suppression in the female mice. We have seen similar results of human-IgG-mediated tumor suppression in female mice before in this lab, not in this thesis project but in other projects, and this would be worth investigating to be able to understand how the use of human IgG can affect the results of our in vivo experiments. It would be interesting to determine whether the female mice simply have stronger immunogenic reactions to i.p. injections of human proteins than do male mice.

The sexual differences that we saw in these results may suggest that IL-6 trans-signaling is linked to a protective mechanism against HNSCC progression in females that can only be seen in an immunocompetent model, for sgp130Fc treatment appeared to (non-significantly) promote tumor growth in the females in this experiment. The fact that sgp130Fc displayed anti-tumorigenic properties in immunocompetent male but not female mice suggests other avenues of research: to investigate whether IL-6 trans-signaling is on an axis with estrogen signaling and whether IL-6 trans-signaling or this axis actually offer protective effects against HNSCC development and progression, just in females. Interestingly, tumor levels of IL-6 were prognostic in male but not female OSCC patients in a study of 263 male and 74 female patients [80].
Women have lower risk of incidence and mortality from HNSCC, colorectal cancer and MM, which are all cancers that are associated with highly inflammatory phenotypes. IL-6 signaling is known to be linked to estrogen signaling; however, it has not yet been investigated whether IL-6 trans-signaling is implicated in the IL-6/estrogen axis. HNC patients (both male and female) have been shown to have abnormal estrogen metabolism compared to healthy controls [9]. Estrogen can inhibit NFκB signaling by interfering with NFκB dimer formation, inhibiting NFκB DNA binding, increasing IkappaB expression and decreasing IKappa B phosphorylation; additionally estrogen receptors can be present in monocytes and macrophages and PBMCs, and be part of activation and modulation of inflammatory cytokine signaling [120]. Estrogen receptors can interfere with protein complexes binding to C/EBP-NF-kB site, reducing inflammation [121]. Loss of anti-inflammatory effects due to G protein-coupled estrogen receptor deficiency can accelerate liver tumorigenesis [122]. Interestingly, some of the proposed treatments that have been approved for cancer treatments or are currently being tested for anti-tumor efficacy in clinical trials (including tamoxifen, raloxifene, and bazedoxifene) are selective estrogen receptor modulators (SERMs) that have also shown to inhibit gp130-JAK/STAT3 signaling. Future experiments might look more closely at these mechanisms to identify whether IL-6 trans-signaling is involved.

Our results from ELISA and clonogenic assays indicated that radiation and cisplatin treatments could induce HNSCC cell secretion of IL-6 trans-signaling proteins in a dose-dependent manner (Fig. 13). However, human sgp130Fc did not enhance the efficacy of SOC treatments against Cal27, FaDu, or SQ20B by reducing clonogenic survival in vitro at the dose that we tested (Fig. 14); in fact, sgp130Fc increased clonogenic survival of SQ20B cells treated at 0 Gy and of FaDu cells treated at 2 Gy. Sgp130Fc treatment did sensitize SQ20B xenograft tumors (which are known to be radiation- and cisplatin-resistant) to SOC treatments in a nude mouse model in both male and female (Fig. 15), but in this experiment, sgp130Fc displayed no anti-tumor efficacy on its own. As mentioned in the results section, the mice in this experiment (Fig. 15) were not treated exactly the same way as those in the experiment in Fig. 10, because in this experiment the mice had to be sedated with ketamine/xylazine and also had to be transported from their housing facility to the radiation facility twice a week. We hypothesize that these differences may have increased the stress levels of the mice. Additionally, ketamine can influence plasma inflammatory cytokine levels, including IL-6; ketamine may potentially have dual effects, in which in some contexts, ketamine can increase IL-6 levels, and in other
contexts, decrease IL-6 levels [123, 124]. Ketamine can also affect the activity of immune cells [124, 125]. Therefore it is possible that a higher dose of sgp130Fc might have been needed in this experiment for anti-tumor efficacy. We attempted to repeat this experiment in both the SQ20B xenograft nude mouse model (though using a different dose and dosing schedule of cisplatin) and in the immunocompetent SCCVII C3H/HeJ model, and we were unable to yield the same results as in Fig. 15. These results may indicate that sgp130Fc requires a very specific dose or dosing schedule. These experiments should be repeated to determine whether the results in Fig. 15 are real.

The success of in vivo results and lack of success in vitro in this thesis project suggest that the anti-tumor efficacy of sgp130Fc may lie more in its ability to target other cells in the microenvironment than just tumor cells, such as immune and endothelial cells. A similar contrast (high in vivo efficacy but poor in vitro efficacy) was also seen in a pancreatic cancer study, in which sgp130Fc treatment did not affect cancer cell proliferation, but did suppress tumor growth in an orthotopic pancreatic cancer mouse model [112]. IL-6 trans-signaling has been shown to upregulate CD31 and ICAM-1 expression in mouse cancer models, and may be necessary for maturation, trafficking, and activation of certain types of immune cells, so sgp130Fc very likely targets the immune system whether or not it targets tumor cells directly [112]. Unfortunately, we were unable to determine the specific mechanism of sgp130Fc’s anti-tumor efficacy in our experiments. An immune cell subset assay revealed that sgp130Fc treatment slightly but significantly increased the percentages of neutrophils and dendritic cells in spleens and tumors respectively in an immunocompetent SCC-VII allograft C3H/HeJ mouse model (Fig. 13); however, in this experiment, there were no significant differences in mean tumor growth between sgp130Fc and control IgG treatment groups at the point at which we had to sacrifice a mouse from each treatment group (though the sgp130Fc group trended behind the IgG group), and so this experiment should be repeated to confirm these results. This would be a significant area of research to investigate.

To further investigate the mechanism of sgp130Fc and its direct action on HNSCC cell gene expression compared to global IL-6 inhibition by tocilizumab, an RNAseq experiment on IgG-, sgp130Fc- or tocilizumab-treated SQ20B cells was performed (Fig. 9). Due to one contaminated IgG sample, these results could not be analyzed for statistics; however, qualitative results showed clearly that tocilizumab had much broader and greater effects on SQ20B cell
gene expression than did the same dose of sgp130Fc. It is possible that the lack of efficacy of tocilizumab that we saw in vivo was due to the tocilizumab treatment turning on or off pathways that sgp130Fc did not affect. This experiment should be repeated in order to yield statistically significant results and to allow investigation of the HNSCC tumor genes that sgp130Fc treatment does alter, particularly in comparison with those genes affected by tocilizumab treatment. Additionally, repeating this experiment using different HNSCC cell lines such as Cal27 or FaDu might further elucidate whether sgp130 directly affects HNSCC cells.

We determined that cetuximab treatment induced HNSCC cell secretion of IL-6 and reduced expression of membrane IL-6R mRNA. Additionally, cetuximab appears to induce HNSCC cell secretion of sIL-6R and sgp130, though this assay was only performed twice and therefore was not statistically significant. However, tocilizumab rescued the efficacy of cetuximab (Fig. 16) and cetuximab rescued the efficacy of sgp130Fc (Fig. 18) in our SQ20B xenograft nude mouse model. To determine whether the loss of efficacy from these combination treatments were due to inhibition of ADCC, a flow cytometry assay using PBMCs treated in the presence of HNSCC cells was performed to determine levels of NK activation in response to treatment in vitro. This assay was performed twice so we did not have statistically significant results, but cetuximab treatment alone appeared to increase NK cell activation by about double compared to single treatments of IgG, sgp130Fc, or tocilizumab; however, combination treatments of IgG and cetuximab, sgp130Fc and cetuximab, or tocilizumab and cetuximab did not reduce the level of cetuximab-mediated NK cell activation in this in vitro model. Therefore, these results did not explain why combined treatments of sgp130Fc and cetuximab or tocilizumab and cetuximab lose their efficacy in vivo. This assay could be repeated or refined by investigating different markers of NK cell activation, though an in vivo experiment might be necessary in order to confirm these results.

A drawback to this overall project was the inability to identify where the action of sgp130Fc occurs and whether it directly affects tumor cells, other cells in the tumor microenvironment, or both. Furthermore, this project did not confirm whether sgp130Fc truly acted as a selective inhibitor of IL-6 trans-signaling in these experiments: i.e. whether sgp130Fc also inhibited other gp130-cytokine family signaling pathways that are pro-tumorigenic, and whether sgp130Fc indirectly inhibits IL-6 classical signaling by sequestering or buffering IL-6. Another drawback was the lack of time to perform in vivo experiments using different cell lines.
Overall, the results support that sgp130Fc has anti-tumor efficacy as a single therapy in HNSCC and shows promise as an adjuvant to chemotherapy and radiation to enhance HNSCC treatment.
Chapter 5: Future directions and conclusion

An important area in the future is to investigate the mechanism or mechanisms behind the anti-tumor efficacy of sgp130Fc, to identify the locations that sgp130Fc targets and determine whether it targets tumors directly, indirectly through other areas of the microenvironment, or both. Additionally, the interesting sex differences in response to sgp130Fc treatment that we saw in our in vivo models should be explored, to investigate how closely IL-6 trans-signaling is linked to estrogen signaling and whether IL-6 trans-signaling is part of an axis of protection against HNSCC tumor progression in females. Our results may suggest that females have a different IL-6 buffering system than males. In future experiments, it might be beneficial to compare levels of plasma IL-6 trans-signaling proteins to staging of the mouse menstrual cycle and to choose sgp130Fc doses according to these levels.

It was not clear why Cal27 and SQ20B displayed bimodal membrane IL-6R expressing populations, and it would be of interest to compare and investigate those populations. Other directions to be explored include: investigating the functions of ADAM10 and ADAM17 in the production of sIL-6R and whether this mechanism is linked with tumor cell survival; identifying why tocilizumab worked well with erlotinib but did not work well with cetuximab and neither did sgp130Fc; and investigating the effects of sgp130Fc on IL-6-mediated MAPK and PI3K/Akt pathways. Additionally, investigating the different isoforms of endogenous sgp130 to determine how they function locally might yield results that could be used to manipulate the structure of sgp130Fc in order to increase its selectivity of targets.

A final drawback to sgp130Fc is that it is yet very expensive, which hopefully will change once the drug is approved and becomes more widely available after it is marketed. However, sgp130Fc has high potential as a single therapeutic agent in males and as a combination therapy with radiation and chemotherapy in both males and females for HNSCC. One of the exciting parts of sgp130Fc’s potential is the possibility of being an effective agent in resistant and R/M HNSCC, which are associated with upregulated IL-6 signaling. Though we saw no differences in levels of sIL-6R or sgp130 in metastatic HNC and healthy patient plasma samples, perhaps IL-6 trans-signaling may act in a localized fashion that may not be obvious compared to overall plasma levels. This further suggests the importance of investigating and trying to identify the specific mechanism of sgp130Fc: discovering which cells it targets and where it targets them.


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