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I, McKenzie Crist, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Pathobiology & Molecular Medicine.

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Understanding metformin mediated natural killer cell activation in head and neck squamous cell carcinoma

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Understanding metformin-mediated natural killer cell activation in head and neck squamous cell carcinoma

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by

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Abstract

Head and neck cancer (HNC) is the 6th most common cancer worldwide, the majority of which are head and neck squamous cell carcinomas (HNSCC). Recurrent or metastatic HNSCC are often treated with immune checkpoint inhibitors (ICIs), which have dramatically increased survival, but unfortunately, only 20% respond to treatment necessitating improved therapies. The diabetes drug, metformin, was shown previously to have both anti-cancer and immune stimulating (primarily T cells) activity. In a phase I clinical trial (NCT02325401), metformin was administered to HNSCC patients prior to standard of care treatment. Metformin specifically increased NK cell numbers as well as activation more robustly than T cells in HNSCC patients. Therefore, we studied the direct effects of metformin on NK cells. Pre- and post-metformin-treated tumor tissue from a clinical trial (NCT02083692), was analyzed by immunofluorescence. Post-metformin tissue exhibited an increase in infiltrating NK cells. *Ex vivo* metformin treatment of HNSCC NK cells resulted in increased perforin production correlating with higher cytotoxicity of tumor cells. To determine pathways in which metformin may be regulating in order to promote cytotoxicity in NK cells, we utilized bulk RNA-sequencing (RNA-seq) on *ex vivo* pre- and post-metformin treated HNSCC patient NK cells. The chemokine, CXCL1, expression was significantly reduced with metformin treatment. Exogenous CXCL1 prevented metformin-mediated NK cell perforin release, but this could be reversed by an inhibitor of the CXCL1 receptor, CXCR2. We next investigated the mechanism in which metformin mediated CXCL1 inhibition may be preventing perforin release and subsequent cytotoxicity. Given metformin is known to inhibit mTOR and pSTAT3, we investigated the roles of these pathways in perforin release. Perforin was decreased by pSTAT1 inhibition and increased by mTOR inhibition suggesting that both pathways may be important. In order to understand if the CXCR2/CXCL1 pathway regulated the STAT pathways, NK cells were treated *ex vivo* with metformin, CXCL1, and a CXCR2 inhibitor and phosphorylation of STATs was determined by western blot analyses. Metformin resulted in increased pSTAT1 and decreased pSTAT3 as expected. However,

CXCL1 reversed the effect of metformin mediated pSTAT3 inhibition which was then rescued by a CXCR2 inhibitor. We gathered supporting evidence that metformin directly increases NK cell infiltration, and perforin production by inhibiting CXCL1 likely through reduction of pSTAT3 and subsequent inhibition of CXCL1. Importantly, CXCR2 inhibition could reverse CXCL1 mediated inhibition of perforin release. In the future, CXCR2 inhibition in NK cells could be further explored to determine if knockdown of CXCR2 in NK cells could result in increased NK cell function and anti-tumor activity. Finally, we have identified a novel pathway by which metformin increases NK cell cytotoxicity, which could be utilized in the treatment of HNSCC in future studies.

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Chapter I: Introduction

Section 1.1 - Head and Neck Cancer

In 2023 an estimated 609,000 people died from cancer in the U.S. and 1.1 million were newly diagnosed, costing the United States roughly $150 billion.[1] The immense loss of life, reduction in quality of life for survivors, and financial cost have led to cancer as one of the most studied diseases in the world. Despite all the resources allocated to cancer research and drug development, outcomes have only improved by about 1.5% per year.[2]

Worldwide, approximately 900,000 people are diagnosed with head and neck cancer (HNC) each year, and nearly half will die from the disease.[3,4,5] The annual number of cases of HNC are expected to rise 30% by 2030.[6] HNC includes cancers of the oral cavity, salivary gland, pharynx, and larynx and is currently more prevalent in men than women (3:1 diagnosis, respectively) (Figure 1).[4–10] This thesis will focus primarily head and neck squamous cell carcinoma (HNSCC), which makes up about 90% of head and neck cancers. There are currently no reliable screening strategies for HNSCC, but risk factors include smoking or tobacco use, excessive alcohol consumption, and human papilloma virus (HPV) infection.[4–13] Infection can occur from open mouth kissing or oral sexual contact.[14] HPV can integrate into the genome of a cell and result in genomic instability, increasing the chances that tumor enhancing and suppressing genes can be altered[14–20] HPV-related HNSCC can appear in younger patients, with an average diagnosis age of 50 years compared to average age of 66 in HPV- cancers.[14–16] HPV+ cancers also tend to have better prognoses, with a 53% reduction in mortality as compared to HPV- in similar sites and stages.[7]

Despite intensive multimodal care, up to 50% of patients will relapse after entering remission and those who survive report lower quality of life, including inability to effectively swallow and speak, increased anxiety, and depression.[3,10,13,21–23]

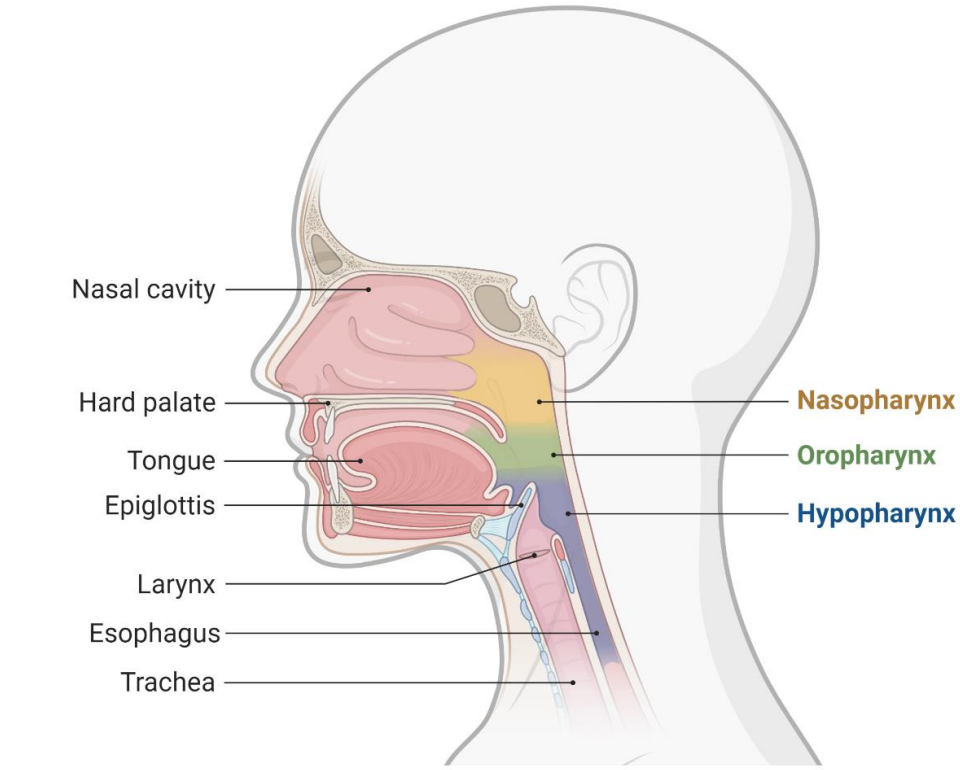

Figure 1.1: Head and neck cancer sites. Figure created with Biorender.

Section 1.1.1 Diagnosis of Head and Neck Cancer

At diagnosis, the site of primary tumor presentation and relevant risk factors are first taken into consideration.[3,24,25] Oral cavity tumors, often originating from the mobile tongue and hard palate, usually present as non-healing ulcers or lesions, and are often caught early.[3,24,25] These tumors can also be identified early with disclosed risk factors of tobacco use, alcohol consumption, or poor oral hygiene.[3,24] Oropharyngeal tumors originate from the base of the tongue and tonsils, with the first presentation including difficulty swallowing or eating.[3,24,25] Laryngeal cancers will present as difficulty speaking or hoarseness, with progression to difficulty breathing if not caught early.[3] Biopsies are taken in the office and stained by hematoxylin-eosin stain, which can then be analyzed for invasive front and tumor budding qualities that are signs of tumor tissue versus normal tissue.[24,25]

Section 1.1.2 Head and Neck Cancer Standard of Care Therapies

Standard of care treatment is dependent location of tumor, stage, and whether the tumor has metastasized.[3] The first line of treatment includes surgery, radiation, and/or chemotherapy.[26–30] Oncologists take into consideration how to preserve the best quality of life for the patients. Patients with small, primary tumors can often achieve remission with resection or radiation, depending on the size and accessibility of the tumor.[3,18] For patients with more advanced tumors, combination therapy of radiation and chemotherapy reduces risk of recurrence.[3,26–30] Utilizing all three therapies (surgery followed by radiation and chemotherapy) is avoided as often as possible as it can increase the toxicity and subsequent morbidity.[3,26] Cancers that have metastasized into multiple nodes or are recurrent may need additional systemic treatment. Immunotherapy has also demonstrated benefit in recurrent/metastatic HNSCC patients. [31,32]

Section 1.2 - Immunotherapy in Head and Neck Squamous Cell Carcinoma

The introduction of immunotherapy over the past 3 decades has increased options for patients and allowed for novel drug combinations.[2] Immunotherapy activates a cancer patient's immune system, allowing immune cells to better target cancer cells.[33,34] Antibody based immunotherapies target markers on immune cells or the tumor cell surface that interact with immune cells to alter signaling and increase cancer cell targeting.[33–35] Immunotherapies approved for HNSCC include immune checkpoint inhibitors (ICIs), which block immune cell regulations that hinder cancer response.[33] This thesis focuses on the two approved immunotherapies in HNSCC: PD-1 inhibitors pembrolizumab and nivolumab.

Section 1.2.1 PD-1 and PD-L1 Inhibitors

The most common immunotherapies in solid tumors are PD-1 and PD-L1 inhibitors.[33–35] PD-1 is a surface marker on T cells and NK cells that can deactivate cytotoxic signaling when it encounters its ligand, PD-L1.[36] Normally PD-L1 acts as a checkpoint, keeping immune cells from attacking self-cells.[36] However, tumor cells can take advantage of PD-L1 and often upregulate it to evade the immune system.[33–37] PD-1 and PD-L1 inhibitors can block the activation of PD-1, which allows immune cells to exert toxicity against PD-L1 expressing cells.[36] PD-1 inhibitors pembrolizumab and nivolumab are approved for use in recurrent and metastatic HNSCC.[37] Tumors expressing 1% or more PD-L1 on the tumor surface are eligible for pembrolizumab, and about 50-60% of patients qualify.[37] However, only about 18% of patients see a true response of 20% reduction in tumor volume.[33] There is gap between those who are expected to respond to immunotherapies and those who fail to respond. To fill this gap, investigators have mainly targeted T cells.[38–40] T cells target cancer cells after antigen exposure and have direct cytotoxicity to tumor cells. This makes T cells an ideal target to activate for potential increased response.

Section 1.2.2 Current Combination Therapies

To increase response rates to pembrolizumab, several combination studies have been launched to increase T cell activation. Below is a tabular review of combinations currently or formerly in clinical trials within HNSCC.

Table 1.1: Non-exhaustive list of combinational therapies with pembrolizumab in head and neck squamous cell carcinoma

Drug Name	Clinical Trial Identifier Number	Drug type	Results
Duvelisib[41]	NCT04193293	PI3K inhibitor	Suspended
Epacadostat[42]	NCT02752074	2,3-dioxygenase enzyme	Recruiting Phase III
Abemaciclib[43]	NCT03938337	CDK4/CDK6 inhibitor	Terminated for increased incidence of pulmonary toxicity
Evorpaept[44]	NCT04675294	CD47 inhibitor	Recruiting Phase II
Alisertib[45]	NCT04555837	Aurora A Kinase inhibitor	Still enrolling
Enolituzumab[46]	NCT02475213	anti-B7-H3 antibody	No more beneficial than pembrolizumab and chemotherapy
SD-101[47]	NCT02521870	Toll-like receptor 9 stimulator	Increased immune trafficking
PDS0101[48]	NCT04260126	HPV antigen mix	41% response rate
NC410[49]	NCT05572684	Leukocyte-associated immunoglobulin like receptor-2 antibody	Still enrolling
INBRX-106[50]	NCT04198766	OX40 agonist antibody	40% response rate (low n, still enrolling)
Itacitinib[51]	NCT02646748	PI3K inhibitor	25% response
CDX-1140[52]	NCT03329950	CD40 antibody	21% response
INCB001158[53]	NCT02903914	Arginase inhibitor	28% response rate
Tabelecleucel[54]	NCT03769467	Allogenic Epstein Bar associated T cells	Failed safety
Ilixandencel[55]	NCT03735290	Allogenic DCs with PAMP recognition	Failed safety
GSK3359609[56]	NCT04428333	ICOS antibody	Failed safety
PLZ3397[57]	NCT02452424	CSF-1R inhibitor	Terminated for insufficient clinical efficacy

LY3435151[58]	NCT04099277	TIGIT antibody	Halted development
Carvotolimod[59]	NCT03684785	TLR9 agonist	Terminated
Clopidogrel[60]	NCT03245489	Anti-platelet therapy	Recruiting
Ipatasertib[61]	NCT05172258	AKT Inhibitor	Recruiting
Xeloda[62]	NCT02842125	Chemotherapy	Recruiting
GR-MX-02[63]	NCT04987996	Galectin-3 inhibitor	Suspended, 2/6 response
ADU-S100[64]	NCT03937141	STING activator	No anti-tumor activity
BNT113[65]	NCT04534205	CAR-T Therapy	Recruiting
BEMPEG[66]	NCT04969861	IL-2 stimulator	No identified benefits

Additionally, there are over 100 clinical trials registered on Clinicaltrials.gov that combine pembrolizumab with existing therapies and chemoradiation. Those completed have resulted in little to no improvement in response rates. Many of these studies target T cell receptors and cytotoxic granule stimulation, such as Bempegaldesleukin (BEMPEG), or target other upregulated pathways of cancer cells such as epidermal growth factor (EGFR).[67] Only recently have other cell types, such as natural killer (NK) cells become a topic of potential activation and therapy in combination with pembrolizumab in some solid tumors.[68,69]

Section 1.3 - Natural Killer Cells

Section 1.3.1 Natural Killer Cell Functions

NK cells were first discovered in the 1960s, having formerly been mistaken for a subset of T cells.[70] They are found in blood, bone marrow, tonsils, the spleen, and lymph nodes. [71,72] Within mice they are known to mature within bone marrow and secondary lymphoid tissues.[71,72] It is unknown exactly where human NK cells mature.[71] They are a type of lymphocyte, a white blood cell that resides in blood and lymph nodes. Lymphocytes begin as pluripotent stem cells and can become innate lymphoid cells, B-cells, T cells, or NK cells.[70,71] Activation of the CD132 receptor results in commitment to either T cells or NK cells.[71,73] Outside signals, such as IL-15 and IL-17, can induce these cells to become NKP cells that express early NK receptors such as

NKp46 and NKG2D.[70,71,73] Additional receptors and functions arise with continued maturation. There are two major subsets of NK cells: CD56bright, CD16- negative cells, known as cytokine producing NK cells, and CD56dim, CD16+ NK cells, known as cytotoxic NK cells.[70–73] These subsets can further be divided, such as memory-like NK cells, but this dissertation will focus only on the two main subtypes. Cytokine producing NK cells are vital to whole immune cell function. When responding to tumors and pathogens, these cells produce IFNγ, TNFα, and GM-CSF, which activate T cells.[70–74] They also secrete chemotactic cytokines called chemokines to recruit other lymphocytes to the infected tissue. Cytotoxic NK cells are part of the body's first line of defense against pathogens and cancer.

Adaptive immune cells recognize self-cells (and therefore do not attack normal cells in the body) by major histocompatibility complex (MHC) I and II .[70–75] When the surface protein is a mismatch to known cells, the cytotoxic cells engages and exerts toxicity.[76]Cancer cells can downregulate MHC to evade T cells, but NK cells have the unique ability to exert cytotoxicity in the absence of MHC. They mainly achieve this through activation of NKG2D.[70–75] The ligand for NKG2D is often expressed on infected cells, allowing the NK cell to lyse the infected cell and release the pathogen to then be recognized by DCs, macrophages, and T cells for further elimination in the body.[71,74] NK cells are also able to bypass many signals that cancer cells upregulate to evade the immune system. Attacking these cancer cells can then help the rest of the immune system recognize the offending cells as cancer. Activated NK cells also release recruiting signals that can bring additional immune cells to the site of the tumor. Therefore, ensuring NK cells are functional in the tumor environment can further power the immune system to fight cancer.

Section 1.3.2 Natural Killer Cell Receptors

NK cells have several receptors which change with maturity. These receptors are vital in signaling to an NK cell when to respond to a cell as a threat and give the cell many opportunities to overcome confounding signals. These signals can also tell researchers how active a cell is, if it has become exhausted, or if a certain signal has been taken advantage of by a pathogen or cancer.[70,71,81–86,72–75,77–80]

Receptors on the NK cell surface play a role in various downstream actions by the cell. Receptors of particular interest in this thesis are TIM-3, PD-1, and NKG2D.[77] NK cells expressing both TIM-3 and PD-1 are considered functionally exhausted and have lower cytotoxicity but can be recovered.[71,77] NKG2D is considered a vital receptor for cytolytic granule release and can be used as a general marker for the overall activation of a patient's NK cells.[71]

Section 1.3.3 Natural Killer Cells in Head and Neck Squamous Cell Carcinoma

NK cell counts in HNSCC patients are known to be lower than non-cancer counterparts, and lower NK cell counts portend a poor prognosis for many solid tumors.[87–90] NK cells are vital to the overall cancer response. Outside of their direct cytotoxic abilities, cytokine production can aid in activating and recruiting other immune cells. In the tumor microenvironment, NK cells are initiated for T cell trafficking, and a high NK:T cell ratio has also been identified as a positive prognosis factor.[74,91] In PD-1 non-responders, NK cells are lower in number and attenuated.[92] It is not clear exactly what causes NK cell attenuation or how to determine if an NK cell is irreversibly dysfunctional.[93,94] However, the tumor microenvironment (TME) secretes many cytokines and activates exhausting signals known to downregulate NK cell activity.[95,96] In HNSCC, pro-tumorigenic cytokines such as IL-6, CXCL1-5, IL-10, and TGF-β are increased both in the TME and in plasma.[94–97] Many of these cytokines increase the expression of pSTAT3 in NK cells.[97] Although pSTAT3 can be activating and aid in proliferation of NK cells, chronic

upregulation of pSTAT3 causes dysregulation, slower growth, decreased IFNγ, and inhibition of anti-cancer STATs such as pSTAT1 and pSTAT5.[97–100] pSTAT3 can reliably be knocked out of NK cells without impacting maturation, therefore therapies targeting the pathway or reducing cytokines that activate the pathway are becoming increasingly explored.[101] Additionally, the presence of other cells in the TME can inhibit NK cell functionality.[94,100] The hypoxic environment of tumors is known to reduce NK cell proliferation and cytotoxicity, but the mechanism by which hypoxia changes NK cell function is not well known.[102] T-regs, T cells that regulate immune cell surveillance, are known to be increased in HNSCC and can reduce both T cell and NK cell activity.[94,100,103] Additionally, 'crowding' cells that are not cytotoxic such as macrophages and neutrophils can keep NK cells from accessing the tumor cells, preventing direct killing.[94,103]

Section 1.3.4: Role of STATs in Natural Killer Cell Function

Throughout maturation and activation, signal transducer and activator of transcription (STATs) play a vital role in NK cell function. Many receptors on NK cells phosphorylate Janus Kinase (JAK) when activated, which in-turn phosphorylates various STATs. There are 4 JAK proteins: JAK1, JAK2, JAK3, and TYK2. All can activate various STATs. The table below illustrates JAK/STAT/Receptor pairings.[101,104]

Table 1.2: STAT receptors and downstream effects

STAT	Receptors	Downstream effects in NK cells
STAT1	IL-2r, IL-5r, IL-9r, IL-21r, Insulin-r, IFN receptors	Maturation, cytotoxicity, IFN release
STAT2	IL-27r, IFN receptors	Viral load control
STAT3	IL-6r, IL-2r, IL-9r, IL-10r, IL-15r, IL-21r, EGFR, VEGFR, IFN receptors	IL-6 and IL-10 release, IFN inhibition, cytotoxicity inhibition
STAT4	IL-12r, IL-27r	IFN release, maturation, cytotoxicity
STAT5	IL-2r ,IL-3r, IL-9r, IL-15r, IL-21r, GM-CSFR, IFN receptors	Proliferation, survival, maturation, cytotoxicity
STAT6	IL-4r, IL-5r IL-13r	cytotoxicity inhibition

STAT1 is vital to NK cell cytolytic activity.[101] STAT1 knockout NK cells have reduced maturation and are under-responsive.[101] However, there is mounting evidence that STAT1 after maturation controls IFNγ and perforin release and can potentially reverse NK cell exhaustion.[94,101] STAT3 seems to be the main driver of NK cell tolerance and preventing autoimmunity.[101,105] STAT3 overexpression has been implicated in poor prognosis in solid tumors and can prevent NK cells from tumor killing.[101] STAT5 phosphorylation early in NK cell development pushes cells towards maturation and supports cytotoxicity.[101] STAT5 deficiencies in leukemia result in poor prognosis, and NK cells with STAT5 knocked down do not develop into cytolytic cells.[101] STATs 2,4, and 6 are less defined in NK cell biology. Current therapies have largely targeted STAT3, but there may be promise in activating STAT1 in mature, cytolytic NK cells.[94,101,104]

Section 1.3.5: Why Focus on Natural Killer cells?

NK cells are understudied immune cells in the context of cancer. Therapies focusing only on T cells are limited, and patients in clinical trials have not benefited from sole T cell therapies. It is important to understand the entire immune system in the context of cancer and elucidating how to activate NK cells in the cancer environment will add another piece to the puzzle of cancer therapeutics.

Section 1.4 – Metformin

Metformin is a biguanide that was first discovered in the 1940s.[105] Metformin has been FDA approved to treat type II diabetes since 1995.[105] It has also been prescribed off label for polycystic ovary disease, weight loss, and diabetes prevention.[106] However, there has been recent interest in metformin for its anti-inflammatory properties.[107–111] Metformin is a known activator of AMPK, which in turn inhibits several inflammatory pathways such as NFkB and mTOR.[106–112] It is relatively safe and easily combined with many medications.[106] This thesis will discuss current information and studies of metformin within the context of solid tumors.

Section 1.4.1 Metformin in Cancer

In a study following patients with diabetes on metformin, a decreased rate of colon cancer incidence was observed in those consistently taking metformin.[113] This influenced additional studies into metformin's potential anti-cancer properties. Metformin inhibits the insulin receptor and directly activates AMPK.[106,110] Both of these pathways downregulate mTOR and NFkB. mTOR is a main driver of tumor growth, and NFkB drives inflammation and secretion of many inflammatory cytokines.[110] Theoretically, metformin can directly impact the growth and immune inhibiting/exhausting of tumors by impacting these pathways.

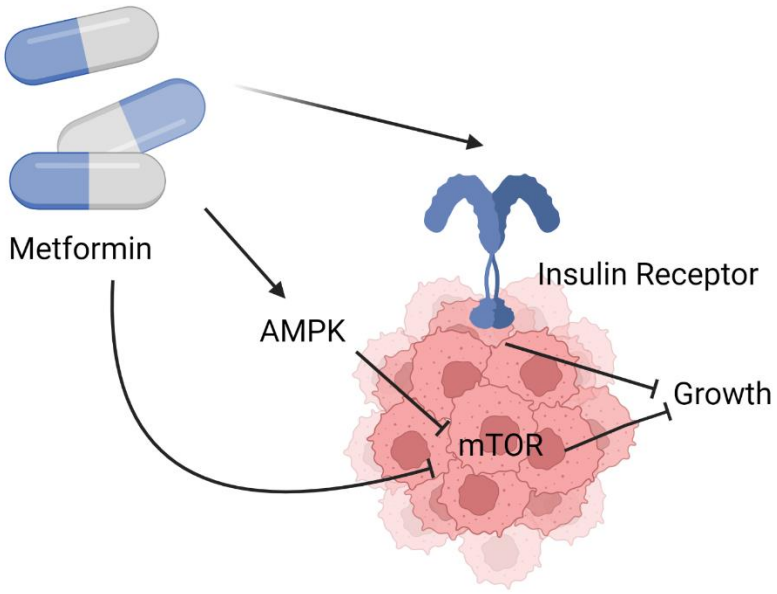

Figure 1.2: Metformin regulation of tumor cells. Figure created with Biorender.

In *ex vivo* studies, metformin has been identified as delaying cancer transformation of stem cells by inhibiting IL-1, IL-6, VEGF, and NFkB.[113] In radioresistant cancers, metformin reduced colony formation and increased apoptosis through STAT3 and TGFβ inhibition.[114] At the time of this thesis development, there are approximately 138 clinical trials utilizing metformin as cancer treatment either in combination with existing standard of care or alone. In widely explored cancers such as colorectal, ovarian, and breast, metformin has been shown to have protective effects but has had mixed results in patients with prevalent cancer. Although there seems to be some tumor growth inhibition with metformin, how metformin also impacts the immune system must be considered.

Section 1.4.2 Metformin in Immuno-Oncology

The effects of metformin on the immune system are less elucidated than those on tumor cells. Meta analysis shows there are many studies exploring macrophages and T cells in the context of metformin treatment, with little on innate cells.[115]

T cells have two major subtypes: CD4+ and CD8+.[116] CD4+ cells respond to MHC class II and produce cytokines, while the subset regulatory T cells can dampen the immune system.[116] CD8+ T cells respond to MHC class I and are directly cytotoxic to diseased cells such as cancer.[116,117] These mature into effector T cells that may eventually become memory T cells, which have long-term immunological effects against remembered pathogens.[116] Several studies have investigated the effects of metformin on both CD8+ and CD4+ T cells in many disease models. In lung cancer patients, metformin increased microRNA (miRNA) that induced memory phenotypes but did not change overall T cell counts.[118] This mechanism was AMPK dependent and overcame mTOR inhibition that would normally discourage memory cell differentiation.[118] In inflammatory models of lupus, T cell proliferation was decreased by metformin and increased regulatory T cell (Treg) differentiation.[119] In CD4+ T cells, metformin has been identified as increasing autophagy and mitochondrial function while reducing the type I interferon (IFN) response.[120,121] The type I IFN response can be a driving factor for anti-tumor response, but prolonged exposure to type I IFN cytokines, such as IL-10 and IL-6, can exhaust or lyse T cells.[122]

Macrophages are essential innate cells that can clear pathogens and debris, while also secreting a number of cytokines that modulate the immune system.[123] There are two major subsets of macrophages: M1 and M2.[123] M1 macrophages are inflammatory and secrete cytokines, such as IL-6 and IL-1β, while M2 macrophages are more healing and anti-inflammatory that secrete IL-10 and TGF-β.[123,124] Metformin has been identified as polarizing macrophages to M2, which reduces inflammatory responses and decreases IL-1β

secretion.[119,124] Metformin also reduces reactive oxidation species (ROS) and collection of foam cells, which are dysfunctional macrophages clogged with cholesterol.[115,125]

B-cells produce antibodies for T cell recognition and aid in their cytotoxicity. Metformin has been identified as reducing autoantibodies, differentiating B cells into plasma cells, creating germinal centers, and reducing TNFα production.[119,126] Plasma cells created from germinal centers have high-affinity antibody production against pathogens and tumor cells and have been positively correlated with a better cancer prognosis.[127,128]

Dendritic cells present antigens on their surface to help other cells of the immune system identify a pathogen. Studies have indicated metformin can decrease MHC complex and presentation of T cell stimulating molecules CD54, CD80, and CD86 , as well as IL-23 secretion.[119,129] IL-23 is vital to pSTAT3 balance in the tumor and immune system environment, but whether it is tumorigenic or anti-tumorigenic has yet to be determined.[130]

NK cells have not been as extensively studied in response to metformin. One study utilized the immortalized NK cell line NK92, through which metformin was observed to increase NK cell activity through STAT5 activation and mTOR inhibition, independent of AMPK.[131] Most studies of NK cells in response to metformin have focused on how changes to the tumor increase NK cell functions. In leukemia, metformin increased cytotoxicity by increasing intercellular adhesion molecule-1 (ICAM-1), a molecule that aids in recognition for cytotoxicity in both T cells and NK cells.[132] In another, tumor cells had reduced PD-L1 expression when exposed to metformin.[133]

Metformin has varying effects on the immune system. Most of its action seems to be anti-inflammatory, which can be beneficial to the state of cancer when many cytotoxic cells become exhausted from the constant flood of signals from both the tumor and other immune

cells. Studying how metformin affects both the entire immune system and individual cells could give valuable information on how to balance immune signaling in cancer.

Section 1.5.3: Metformin and Discovery of Novel Therapeutics

Metformin has varying systemic effects on cancer. While it has been beneficial in directly inhibiting cancer growth in vitro or preventing the onset of cancer, responses in advanced and/or metastatic cancer have been disappointing. Metformin is a non-specific drug with many targets and may have both positive and negative effects. By isolating the pathways that have a positive effect in HNSCC, we may be able to identify a more direct therapeutic as a single or combination therapy.

Section 1.5 – How This Thesis Contributes to Head and Neck Squamous Cell Carcinoma Outcomes

We hypothesize that NK cell activation by metformin will increase NK cell numbers and functionality measures such as cytotoxicity. NK cell therapies are understudied in cancer, especially in HNSCC. NK cell function is important for HNSCC outcomes, but how to target NK cells to increase function and number remains unclear. This thesis aims to determine if a recently appreciated anti-cancer drug, metformin, may affect NK cells in a positive way, and how to isolate those pathways to develop new therapeutics that target NK cells. By improving NK cell function, overall immune cell function can be improved in patients. In addition, increased immune function could lead to increased tumor responses and cancer remissions.

Chapter II: Metformin Activation of Natural Killer Cells

Section 2.1 – Background

Over 70% of new HNSCC cancers present as locally advanced disease (LAHNSCC), requiring multi-modality treatment.[134] For patients with locally advanced disease ineligible for resection, concurrent cisplatin-based chemoradiotherapy (CRT) is still recognized as the standard of care treatment.[135] Despite improved outcomes with CRT, there is still a high level of disease recurrence, especially in the HPV-negative, smoking population, necessitating improved treatment regimens.[135,136] Alternative drug development studies have begun to explore repurposing medications originally intended to treat other non-cancer diseases. Metformin has increasing evidence of anti-cancer properties and is relatively safe alone and in combination with other drugs.[137] It has been identified as improving prognosis in several cancers, such as colorectal and ovarian cancer, but has not been largely explored in HNSCC. In accordance with the phase I open-label single site dose escalation study combining metformin and CRT in LAHNSCC, we investigated the effects of metformin on peripheral immune cells from patients on a clinical trial and further investigated these responses *ex vivo*.[138] We also briefly investigated the effects of metformin on tumor cell lines to determine if effects were direct or indirect.

Section 2.2 - Materials and Methods

2.2.1 Human Samples

Peripheral blood and serum were obtained from patients from the phase 1 clinical trial of metformin in combination with CRT in patients with LAHNSCC ((stage III/IV); NCT02325401) at the University of Cincinnati. Key eligibility criteria for patient inclusion in the trial were confirmation by tissue biopsy of newly diagnosed LAHNSCC that was unresectable with no prior chemotherapy or radiation treatment. Key exclusion criteria included metastatic disease, known history of diabetes requiring insulin, or nasopharyngeal carcinoma as the primary tumor site. In addition, it was confirmed that patients had not received metformin prior to study entry. Blood samples were collected before and after 14-days of treatment with metformin and prior to chemotherapy and radiation. Primary tumor specimens were obtained from subjects enrolled in another previous investigator-initiated HNSCC surgical trial carried out in accordance with the recommendations of the Thomas Jefferson University IRB (NCT02083692). Criteria for NCT02083692 can be found in Amin et al.[139] Samples used in this study were from patients with no prior history of chemoradiation prior to surgical resection. Patients received metformin for 9-24 days prior to surgery. Additional *ex vivo* studies were performed on age matched peripheral blood obtained from IRB-approved studies UCCI-UMB-14-01 (IRB #2014-4755) and general specimen collection protocol (IRB #2017-2137) to investigate differences in molecular and immune cell markers compared to clinical outcomes in HNSCC patients and normal healthy controls. The studies were approved by the Institutional Review Board at the University of Cincinnati and were conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was received from all participating patients prior to enrollment.

2.2.2 Cell Lines

Human-derived HNSCC cell lines UMSCC-47 (kindly gifted by Randall Kimple, University of Wisconsin),Cal27 (kindly gifted by Nira Ben-Jonathan, University of Cincinnati), and HN5 were grown and maintained in 1x DMEM high glucose (Corning), 8mM L-glutamine (Corning), 10% FBS (Omega Scientific), 1% pen/strep (Corning) and 1x nonessential amino acids (Corning). All cell lines were cultured at 37°C with 5% CO_2. Mycoplasma presence was checked every 3 months (MycoProbe, R&D Systems, Minneapolis, MN) and reported no cases of contamination within these cell lines as of February 2022. UMSCC47, HN5 and Cal27 cell lines were STR Profiled confirmed by Cincinnati Children's Hospital Medical Center Cytogenics Labs in May 2020.

2.2.3 Flow Cytometry for Peripheral Immune Cell Characterization

Flow cytometry was performed on thawed peripheral blood mononuclear cells (PBMC). Cells were quickly thawed, washed in flow buffer (FB) (1x PBS + 2% FBS), and fixed for 1hr at room temperature (RT) in 1.6% paraformaldehyde (PFA, Electron Microscope Sciences, Hatfield, PA). Cells were then washed and stained with the appropriate antibodies for 1hr at RT in the dark, washed in FB, and analyzed by flow cytometry. For intracellular staining, cells were quick-thawed, washed in FB, and fixed with 1.6% PFA for 1hr at RT, followed by washing with FB. Cells were then permeabilized with buffer (Thermo Fisher Scientific) and stained with intracellular antibodies for 1hr at RT in the dark. Cells were washed and analyzed by flow cytometry. Flow cytometry was performed at Shriner's Hospital for Children (Cincinnati, OH), USA, using a 5-laser BD LSRII equipped with a UV laser (355nm). Data were analyzed with FlowJo V10. Populations are defined as follows: $CD56^{bright}CD16^{-}CD3^{-}$ were considered cytokine

producing NK cells, $CD56^{dim}CD16^{+}CD3^{-}$ were considered cytotoxic NK cells, $CD56^{+}CD3^{+}$ were considered NKT cells, $CD56^{-}CD3^{+}CD8^{+}$ were considered cytotoxic T cells, and $CD56^{-}CD3^{+}CD4^{+}$ were considered helper T cells.

2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits for IFN-γ, TGF-β, CXCL1, IL-4, IL-6, IL-8, and IL-10 were obtained from R&D Systems; the ELISA kit for perforin was obtained from ABCAM. ELISA was performed according to the manufacturer's protocol. Protocols included overnight coating of capture antibody diluted by lot number recommendation, 1hr block with 400uL 2% BSA in PBS, 2hr sample incubation at room temperature of 100uL sample (no dilution of sample was performed in these experiments), 2hr capture antibody incubation with 100uL capture antibody diluted by lot number recommendation, 20min Streptavidin incubation with 100uL of 1:40 dilution of stock, and 15min incubation with 100uL included TMB ELISA reagent. 50uL stop solution of in house 2N sodium sulfide was added to stop reaction before reading at 500nm wavelength on plate reader. All plates included standard curve prepared per lot number recommendation with range of 35-2000pg.

2.2.5 Single-Cell Multiplex Cytokine Profiling

PBMCs were thawed and stimulated with 100U/mL of IL-2 for 16hrs. PBMCs were washed in PBS+ 2% FBS and resuspended in 20uL solution per 10^7 cells. 20uL CD56 microbeads (Miltenyi Biotec #130-050-400) were added for 20min. Cells were run through Miltenyi SL columns, and labeled CD56 cells were flushed using plunger. Isolated CD56+ cells were washed and resuspended in 100uL RPMI. 10uL CD56 stain included in IsoPlexis' Isocode

Single Cell Polyfunctional Strength kit was added incubated with cells at 36ºC for 20min. Cells were washed and resuspended in RPMI containing 12mM metformin or sterile water. Approximately 30,000 cells were loaded onto IsoPlexis Single Cell Secretome IsoCode chips (IsoPlexis, Haven, CN) and analyzed with the IsoLight system.

2.2.6 Tissue Staining

Tissue slides were incubated for 1hr at 60º C. Slides were deparaffinized and incubated in Antigen retrieval solution (Biogenex HK086-9K) for 20min at 90ºC. Slides were then washed, blocked in 10% goat serum and 1% BSA in TBS overnight, incubated in primary in the block agent overnight, washed in TBS and incubated overnight with secondary (Invitrogen 2149786). Slides were washed, incubated overnight with conjugated antibodies, washed, and stained with 1:1000 diluted DAPI in block agent for 15min. Slides were washed and mounted with Fluoromount-G (Invitrogen 00-4958-02) and imaged on Zeiss Axio Observer Z1 inverted microscope connected to a Zeiss LSM710 confocal with available laser lines of 405, 458, 488, 514, 561 and 633nm. Images were analyzed using ImageJ (NIH). Number of NK cells were determined by using the measure particles function on NKp46 only images. The number of infiltrating NK cells was determined as particles within pan-cytokeratin (PCK) stained areas.

2.2.7 Natural Killer Cytotoxicity Assay (NKCA)

NK cells were isolated from PBMCs using the EasySep Human NK Cell Isolation Kit (Stem Cell Technologies). Cells were washed in PBS + 2% FBS and treated with drug indicated in legend for 24hrs in culture medium. One day before co-culture, cells were collected, washed, and stained with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE; StemCell

Technologies). Untreated or treated UMSCC47, Cal-27, or matched primary HNSCC tumor cell lines cells were plated at 10,000 cell/ml and assumed to double overnight. NK cells were resuspended in RPMI 1640 (Corning) with 5% human serum at a density of 200,000 cells/ml. Cells were co-cultured for a target:effector ratio of 1:5 for 4hrs, collected, and washed in flow buffer. Cells were then stained with 7AAD Viability Staining Solution (Biolegend). Cells were immediately run on a 4-laser BD Fortessa Instrument (University of Cincinnati Cancer Cell Biology Department, Cincinnati, Ohio). Target cells were considered any cell that was CSFE. Any 7AAD+ cells were considered dead cells. NK killed cells were calculated by the following equation: (%CSFE+7AAD+ experimental co-culture) – (%CSFE+7AAD+ control no co-culture [baseline death]).

2.2.8 RNA-Sequencing (RNA-seq)

HNSCC NK cells were isolated from PBMCs using the EasySep Human NK Cell Isolation Kit (Stem Cell Technologies). NK cells were resuspended at 10^6 cell/mL density and incubated for 24hrs with 12mM metformin or sterile water vehicle. After 24hrs, all cells were washed, suspended in 1mL CyroStor, and frozen in -80ºC fridge. Samples were shipped to Genewiz/Azenta for standard RNA-seq profiling. At least 10^6 cells per sample were shipped. Poly(A) selection was performed by Azenta. RNA was run on Illumina NovaSeq with 2x150bp configuration by Azenta. Data were trimmed, mapped, and returned to investigators. Sequence reads were aligned to the current reference mouse genome (GRCh38) using the STAR aligner and the reads aligned to each known gene were counted based on the latest GENCODE definitions of gene features (44,45).

2.2.9 Western Blot Analysis

NK92, Cal27, UMSCC47 or HN5 cells were cultured with drug as described. Cells were collected in RIPA (0.05% sodium deoxycholate, 150nM NaCl, 50nM Tris HCL, 0.1% SDS, 1% NP-40). Protein content was analyzed by Pierce BCA kit (Thermo 23225). 50ug of protein was diluted in 1x loading buffer (SDS, bromophenol blue, 47% glycerol, Tris 0.5M pH 6.8, 0.2mM DTT-G) and heated to 90ºC for 3 minutes. Prepared lysate was loaded into gradient gels (Biorad 4561093) and run at 85V for 1.5 hour in Biorad casing with a Biorad powerpack in 1x running buffer (Tris, Glycine, SDS). Gels were transferred on nitrocellulose and run at 100V on bench for 1 hour in 1x transfer buffer (Tris, Glycine). Membranes were blocked in 5% BSA in TBS for 1hr, washed, and incubated in primary diluted in 2% BSA in TBS overnight. Membranes were washed and incubated in Licor secondary (Licor 926-32211 and 925-68070) in 5% BSA in TBS for 1hr. Membranes were washed and imaged on a Licor Odyssey Clx. Images were analyzed in Image Studio Lite V 5.2.

2.2.10 qPCR and PCR

Treated or infected cells were collected in Qiagen RNAeasy (Cat# 74004) buffer and spun at 10xg for 5min in QiaShred tubes (Cat# 79656). RNA collection was continued per Qiagen protocol. RNA was eluted into RNA-free water and analyzed on Nanodrop, cDNA production was continued per Qiagen cDNA kit protocols (Cat# 205211). cDNA was checked for purity and concentration on Nanodrop. Thermo-fisher qPCR kit protocol (Cat#4472903) was followed for the following primers:

IL-6 Forward: AGACAGCCACTCACCTCTTCAG
IL-6 Reverse: TTCTGCCAGTGCCTCTTTGCTG
IL-8 Forward: CCTGATTTCTGCAGCTCTGTG

IL-8 Reverse: CCAGACAGAGCTCTCTTCCAT

IL-4 Forward: CCGTAACAGACATCTTTGCTGCC

IL-4: Reverse: GAGTGTCCTTCTCATGGTGGCT

IL-10 Forward: TCTCCGAGATGCCTTCAGCAGA

IL-10 Reverse: TCAGACAAGGCTTGGCAACCCA

Actin Forward: CACCATTGGCAATGAGCGGTTC

Actin Reverse: AGGTCTTTGCGGATGTCCACGT

2.2.11 Lentivirus Infection of Tumor Cells

pSTAT3 flag lentivirus was kindly gifted by the Zhao lab at Case Western Reserve University. This lentivirus adds a tag to STAT3 so that it cannot be phosphorylated in the nucleus. 10uL of lentivirus stock with 10ng polybrene were added to Cal27, HN5, or UMSCC47 cells in optiMEM for 20min. optiMEM was removed and replaced with culture media for 72hrs. Media was replaced with fresh culture medium containing 10ng/mL kanamycin. Surviving cells were expanded and confirmed for reduction of phosphorylated STAT3 by western blot.

2.2.12 Statistics

An unpaired 2-tailed *t* test with Welch's correction was used for analysis between flow cytometry in controls versus HNSCC patients. A 2-tailed Students *t* test was used to compare differences between HNSCC patients before and after treatment in flow cytometry and cytokine experiments. Differences in immune cell populations between three groups were compared by one-way ANOVA with specific post-hoc contrasts. NKCA data were compared using a paired *t* test. Statistical analysis was performed in GraphPad Prism (V10). Differences between groups were considered statistically significant when $P < 0.05$. Paired-sample differential gene expression analysis of RNA-seq data was performed by fitting a two-factor generalized linear

model based on negative-binomial distribution of read counts as implemented in the *edgeR* Bioconductor package. In the two-factor model, the factor of interest was the metformin treatment and the blocking factor corresponded to five patients from whom the HNSCC NK cells were isolated.[140,141] The p-values for the metformin treatment effect were adjusted based on false discovery rate (FDR).[142,143]

Section 2.3 – Results and Discussion

Section 2.3.1: Metformin Increases Peripheral Blood Natural Killer Cells in HNSCC Patients.

Low NK cell counts in peripheral blood is a poor prognostic factor in patients with both solid and non-solid tumors, while the presence of highly activated NK cells can improve prognosis.[87,88] Circulating levels of NK cells in patients with HNSCC are significantly lower compared to their healthy counterparts.[89,144] To confirm this in the population of patients seen in the clinic from which our translational samples were sourced, evaluation of peripheral populations of NK cells in patients with HNSCC compared to healthy control patients were evaluated via flow cytometry. **Figure 2.1** is a representative scatterplot of the cytokine-producing $CD56^{bright}CD16^{-}$ and cytotoxic $CD56^{dim}CD16^{+}$ NK cell subpopulations in a healthy control sample and HNSCC patient. Due to the known decline of NK cells with age, healthy controls were age-matched by selecting controls in the age range 50-100 years old. Flow cytometry was performed on PBMCs isolated from patient peripheral blood. The gating strategy was as follows: lymphocytes à live cells à CD3- à CD56/CD16.

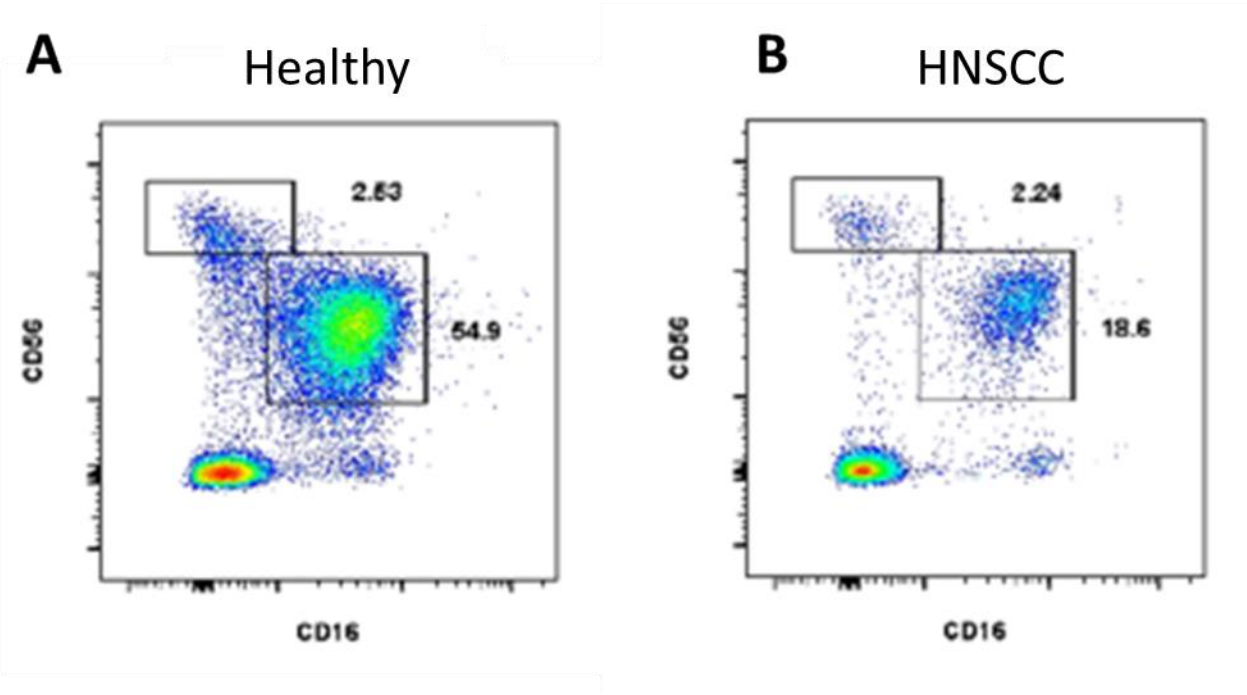

Figure 2.1: Head and neck squamous cell carcinoma (HNSCC) patients have lower natural killer (NK) cell counts in the peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated from non-diseased (healthy) donors (A) or HNSCC patients (B) and evaluated by flow cytometry for NK cell populations (CD56+, CD16-/+ cells). Data collected was performed by former lab member Benjamin Yaniv.

After confirming our HNSCC population has lower NK cell counts than healthy controls, we determined whether there was a difference in NK cell subpopulations. There were fewer NK cell populations in HNSCC patients (n = 8) compared to healthy controls (n = 5) for $CD56^{bright}CD16^{-}$ ($P < 0.002$), and $CD56^{dim}CD16^{+}$ ($P < 0.05$) **(Figure 2.2)**. The gating strategy was as follows: lymphocytes à live cells à CD3- à CD56/CD16.

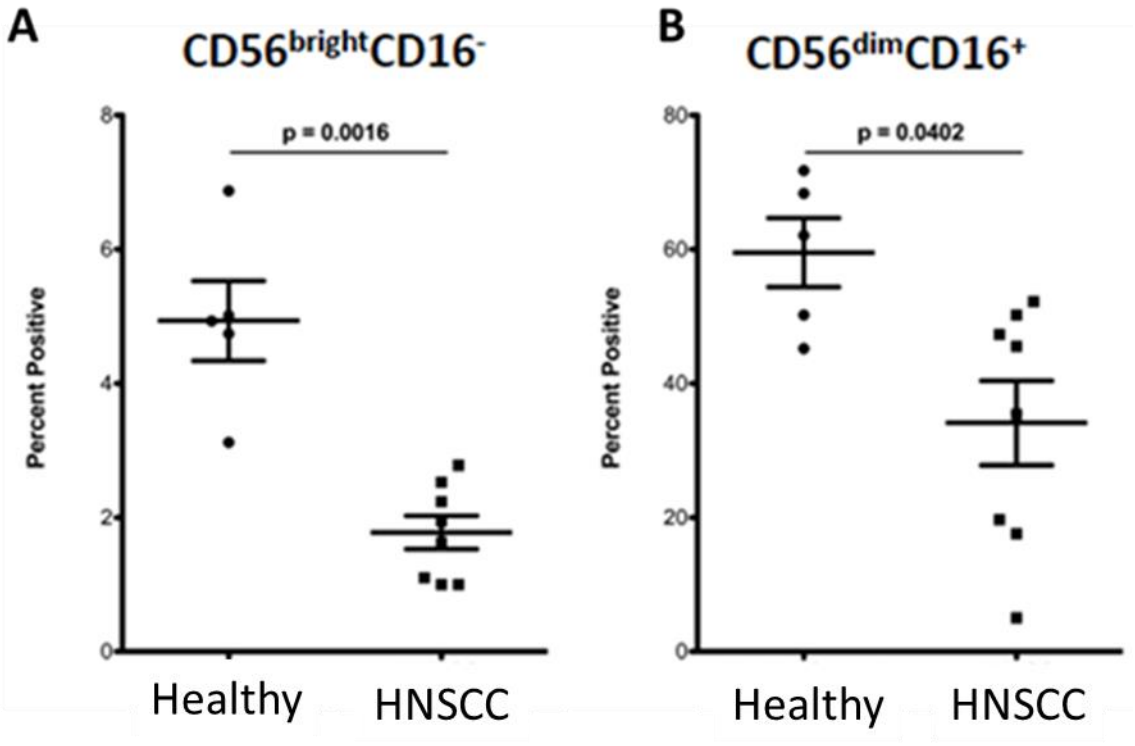

Figure 2.2: Head and neck squamous cell carcinoma (HNSCC) patients have lower proportions of all natural killer (NK) cell types. NK cell populations were evaluated by flow cytometry in both HNSCC (n = 8) and control samples (n = 5) Data was analyzed by unpaired Student's t-test. Data was collected by former lab member Benjamin Yaniv.

Impaired NK cell activity is associated with tumor progression.[88] A measure of activity, production of IFN-γ, was measured from NK cells negatively selected from cryopreserved PBMCs and primed overnight with low dose IL-2. Isolated NK cells were then activated with 1ng IL-12 and 10ng IL-18 for 24hrs to induce IFN-γ release and measured by ELISA. Overall, baseline IFN-γ production was higher in healthy controls (*n* = 4) compared to HNSCC patients (*n* = 4; p = 0.005). Upon activation, HNSCC patient-derived NK cells increase IFN-γ production significantly but not to healthy donor levels. Altogether, these data support previous observations of decreases in circulating NK cell populations and decreased functional capacity in patients with HNSCC. (**Figure 2.3**).

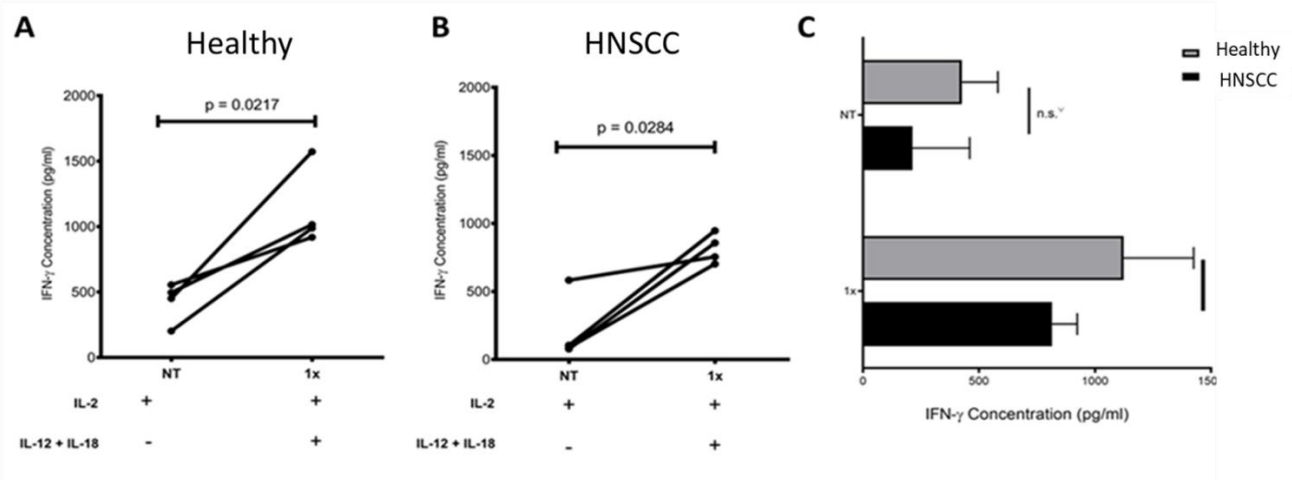

Figure 2.3: Head and neck squamous cell carcinoma (HNSCC) patient-derived natural killer (NK) cells have lower IFN-γ activation. NK cells that were primed with either IL-2 or IL-2, IL-12 and IL-18 were analyzed by ELISA for IFN-γ secretion. (A) Comparison of activated NK cells from healthy donors (n = 5). (B) Comparison of activated NK cells from HNSCC patients (n = 5). (C) Comparison of NK cells from healthy donors to those from HNSCC patients. Data were analyzed by paired Student's t-test for (A, B) and unpaired Student's t-test for (C). Data was collected by former lab member Benjamin Yaniv.

With support that HNSCC patient-derived NK cells are lower in counts and activity, we utilized blood samples from a clinical trial ("Dose-finding study of metformin with chemoradiation in locally advanced head and neck squamous cell carcinoma"; NCT02325401) to investigate metformin as a possible activator of HNSCC immune cells. Late stage/advanced HNSCC patients were identified and had blood drawn pre-treatment. Patients were then administered metformin for 2 weeks and had their blood drawn post-metformin (**Figure 2.4)**.

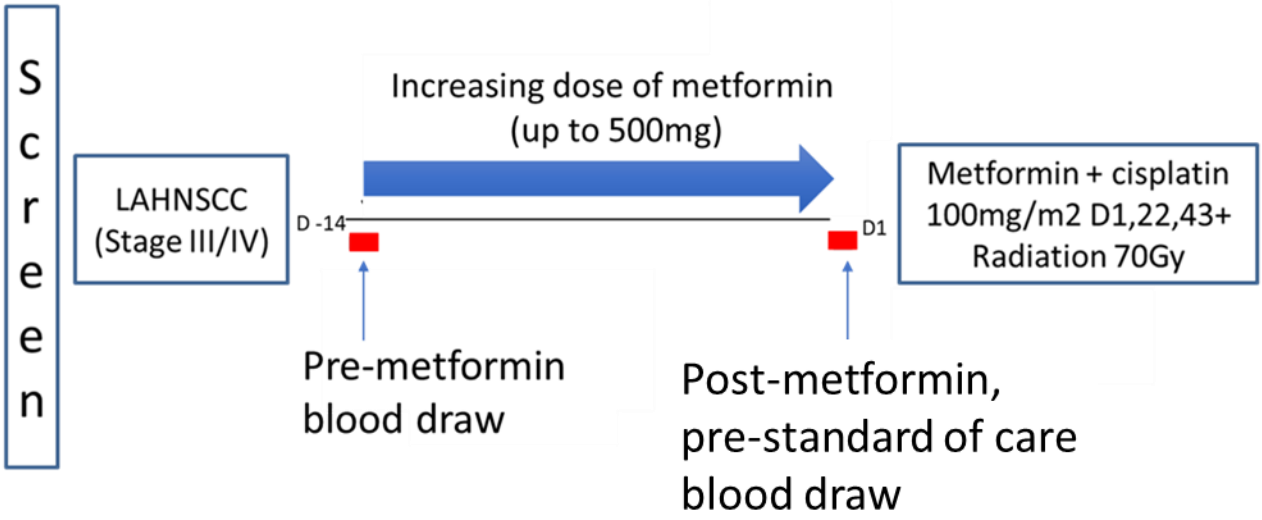

Figure 2.4: Schema of blood draws from clinical trial "Dose-finding study of metformin with chemoradiation in locally advanced head and neck squamous cell carcinoma" (NCT02325401).

To determine if metformin changed overall immune cell counts, whole patient blood was measured for white blood cells (WBC; **Figure 2.5A**) and absolute leukocyte count (ALC; **Figure 2.5B**). Some patients had a slight increase (largest increase: 1.4-fold), but there was no significant difference in ALC or WBC before and after metformin treatment overall.

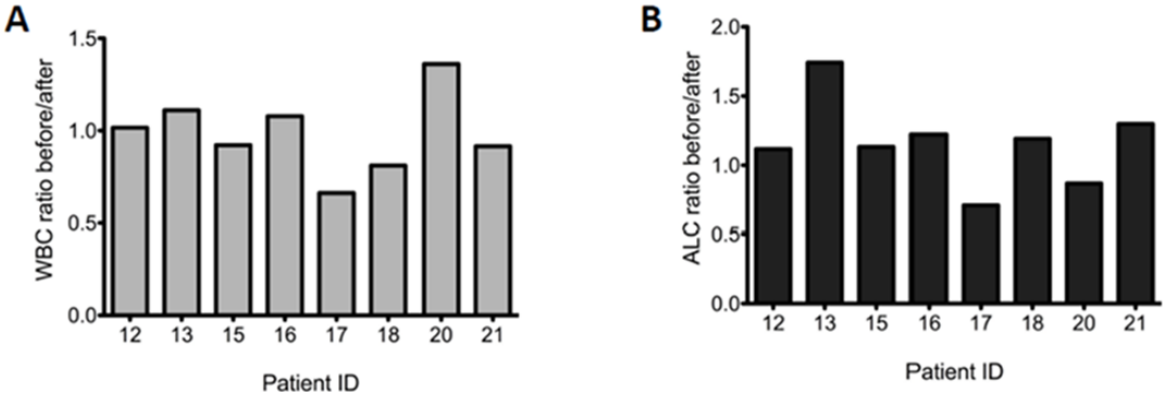

Figure 2.5: Metformin does not change overall white blood cell numbers. (A) white blood cell count ratio of patients pre/post metformin treatment (n = 8). (B) Absolute lymphocyte count pre/post metformin. Data was collected by former lab member Benjamin Yaniv and clinical fellow Vidhya Karivedu.

We next utilized flow cytometry on patient PBMCs to investigate changes in T cell populations. There was no significant change in the overall CD3 population (**Figure 2.6A**) or the CD8+/CD4+ ratio (**Figure 2.6B**). Therefore, there was no detectible change in numbers of cytotoxic T cells after metformin treatment.

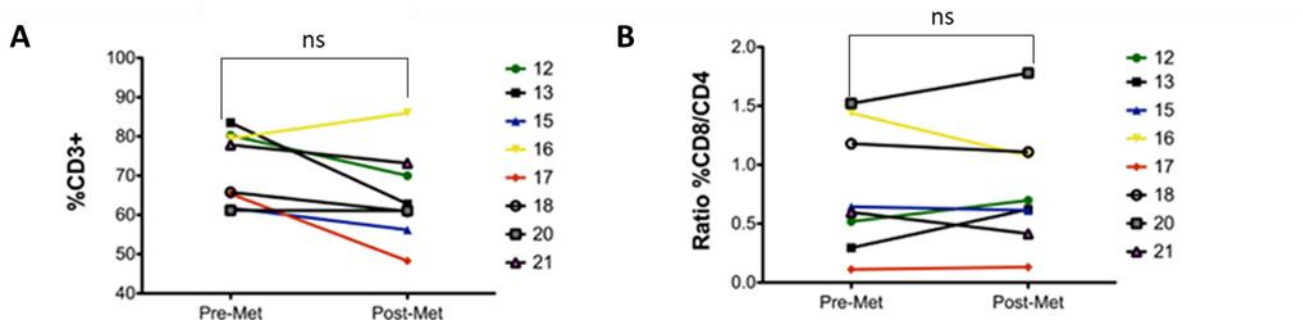

Figure 2.6: Metformin does not change overall numbers of T cells. (A) Overall T cell counts pre/post metformin treatment (n = 8). (B) CD8 (Cytotoxic) and CD4 (cytokine producing) T cell ratios pre/post. shift in T cell populations. Data in (B,C) analyzed by paired Student's t-test. Data was collected by former lab member Benjamin Yaniv.

We further divided T cells into naïve, central memory, effector memory, and effector T cells. Naïve T cells are available to prime against new antigens, effector T cells are active but not committed to a pathogen, central memory T cells proliferate rapidly and express honing chemokines in response to a committed pathogen, and effector memory T cells rapidly release granzyme and perforin but proliferate slowly.[145,146] Utilizing flow cytometry, CD8+ T cells were gated into CCR7+/- and CD45RO+/- (**Figure 2.7A**) and quantified in **Figure 2.7B**. T effector memory cells increased upon metformin treatment with a p value of 0.02 and there was no significant change in other cell types.

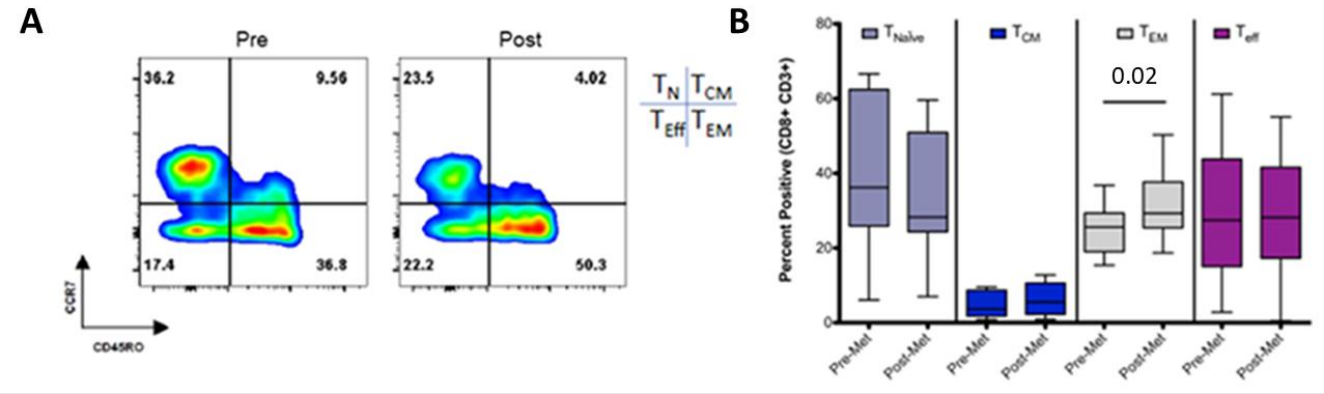

Figure 2.7: Metformin shifts memory T cell phenotypes (A) Flow cytometry graph depicting shift in T cell populations. (B) Percentage of T cell types naïve (CCR7+CC45RO-), central memory (CCR7+CC45RO+), effector (CCR7-, CD45RO-), and effector memory (CCR7-,CD45RO+). Data in (B) analyzed by paired students t-test. N=8 from metformin treated HNSCC patients. Data collected by former lab member Benjamin Yaniv.

After observing minor changes in T cell populations, we evaluated whether patient NK cell phenotypes shifted after treatment with metformin. Metformin treatment resulted in a relative but not significant increase of cytokine-producing $CD56^{bright}CD16^{-}$ (**Figure 2.8A**) and cytotoxic $CD56^{dim}CD16^{+}$ (**Figure 2.8B**) NK cells. Additionally, most patients (7 of 8) experienced an increase in the ratio (**Figure 2.8C**) of percent positive $CD56^{dim}CD16^{+}$:$CD56^{bright}CD16^{-}$ cells. The gating strategy was as follows: lymphocytes à live cells à CD3- à CD56/CD16

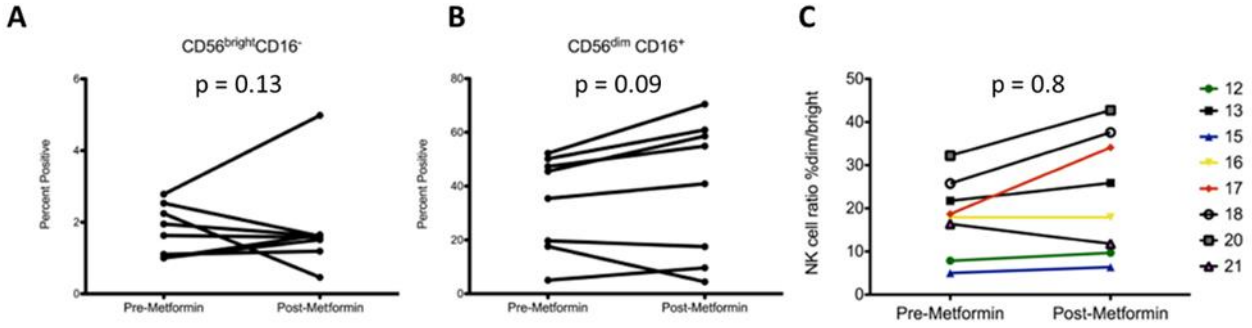

Figure 2.8: Metformin increases natural killer (NK) cell populations. (A) Cytokine producing NK cell populations pre- and post-metformin treatment in locally advanced head and neck squamous cell carcinoma (LAHNSCC; n = 8). (B) Cytotoxicity NK cells populations pre- and

post-metformin in LAHNSCC. (C) Ratio of cytotoxic cells to cytokine producing cells. (A, B, C) analyzed by paired students t-test. Data was collected by former lab member Ben Yaniv.

To determine if these increased cells were active, we analyzed expression of the activation marker NKG2D (killer cell lectin like receptor K1 [KLRK1], CD314) on NK cells and other cytotoxic lymphocytes.[147] At baseline, a higher number of $CD56^{+}CD3^{-}$ NK cells of healthy controls are $NKG2D^{+}$ compared to patients with HNSCC (**Figure 2.9A**). After metformin treatment, NKGD2 activity is partially restored in HNSCC patients to 35.89%. Mean fluorescence intensity (MFI) demonstrates increased surface expression of NKG2D on both $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells in HNSCC patients after metformin treatment (**Figure 2.9B**).

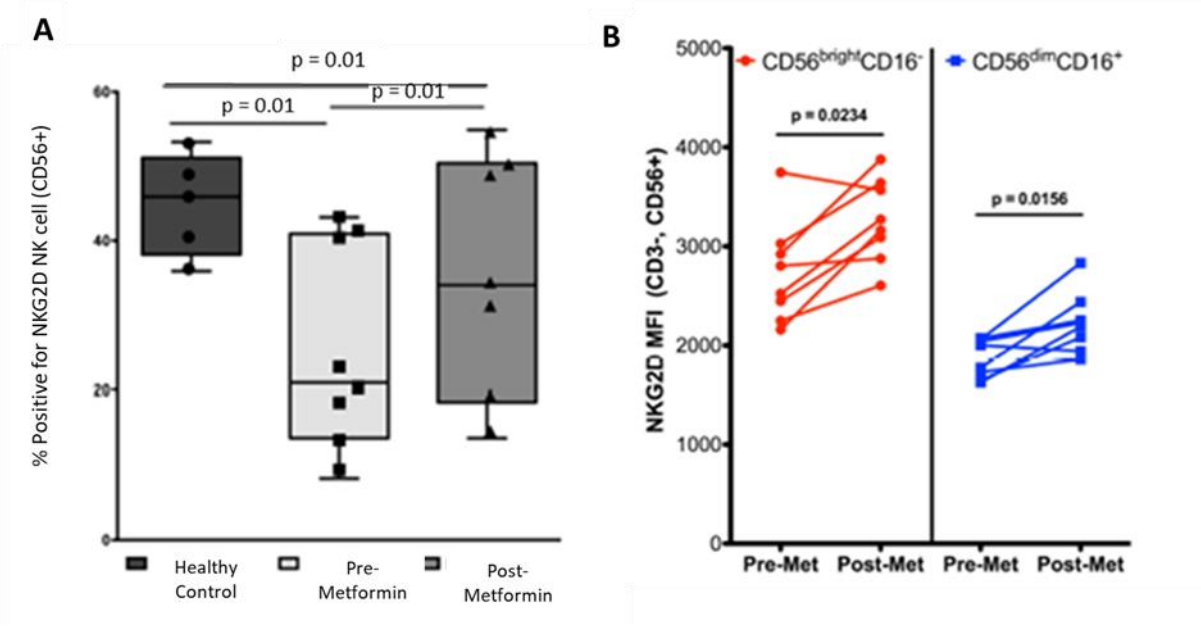

Figure 2.9: Metformin increases activation marker NKG2D. (A) NKG2D expression in healthy donors, as well as in head and neck squamous cell carcinoma (HNSCC) patients pre- compared to post-metformin treatment. (B) NKG2D expression in cytokine producing vs cytotoxic NK cells pre- and post-metformin. (A) Analyzed by two-way ANOVA and (B) analyzed by paired student's t-test. n = 8. Data collected by lab member Ben Yaniv.

We next examined if the increase in NKG2D expression on NK cells in the peripheral blood translated to an increase in NK cell tumor infiltration. In an HNSCC surgical window of opportunity trial conducted by Curry et al., patient pre-treatment (before) biopsy tissue was collected at enrollment.[139,148] Patients received 500mg of metformin daily for 3 days, and then 1000mg daily up until day of surgery for at least 9 days. Surgical resection (pre-metformin treatment) tissue was also collected. Tumor slides were stained with the tumor marker pan-cytokeratin (PCK), T cell marker CD3, and NK cell activation marker NKp46 and analyzed by immunofluorescence (**Figure 2.10**). 5 images (bottom left and right, top left and right, and center) were taken at 10x on confocal per slide.

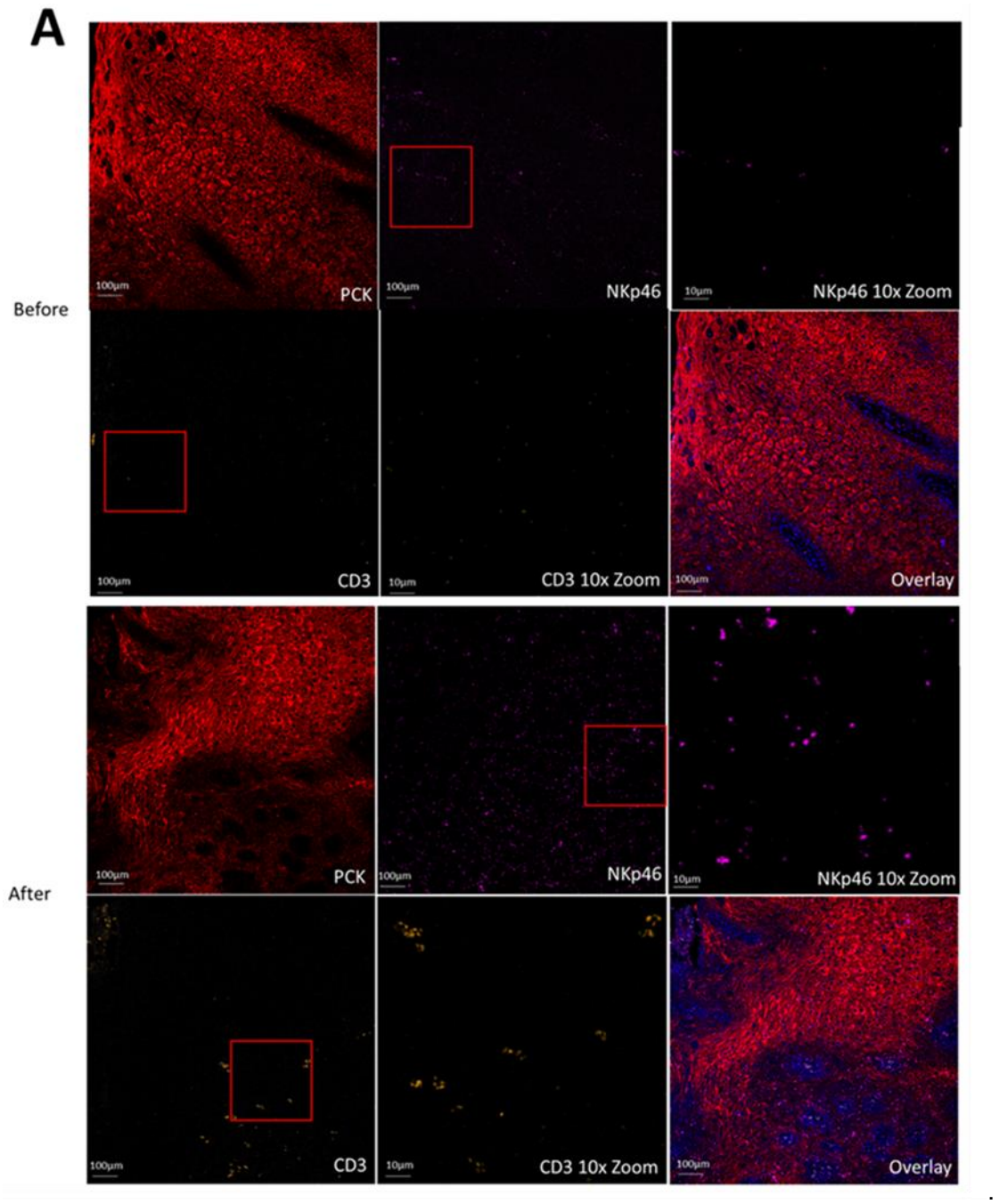

Figure 2.10: Metformin increases expression of NKp46 and CD3 protein expression in head and neck squamous cell carcinoma (HNSCC) tissue. (A) immunofluorescence of pan-cytokeratin (Red), NKp46 (pink), and CD3 (Orange) in biopsy (pre-metformin) and resection (post-metformin) tissue in HNSCC patients.

Consistent with earlier results, CD3 tumor infiltration was increased in response to metformin treatment.[139,148] Total NKp46 expressing NK cells were increased on the tissue slides and NKp46 expressing cells infiltrated into PCK expressing tumor were also increased. It should be noted that these would be tumor infiltrating NK cells (NKILs), which were not examined in the clinical trial source from figures 2.1-2.9.

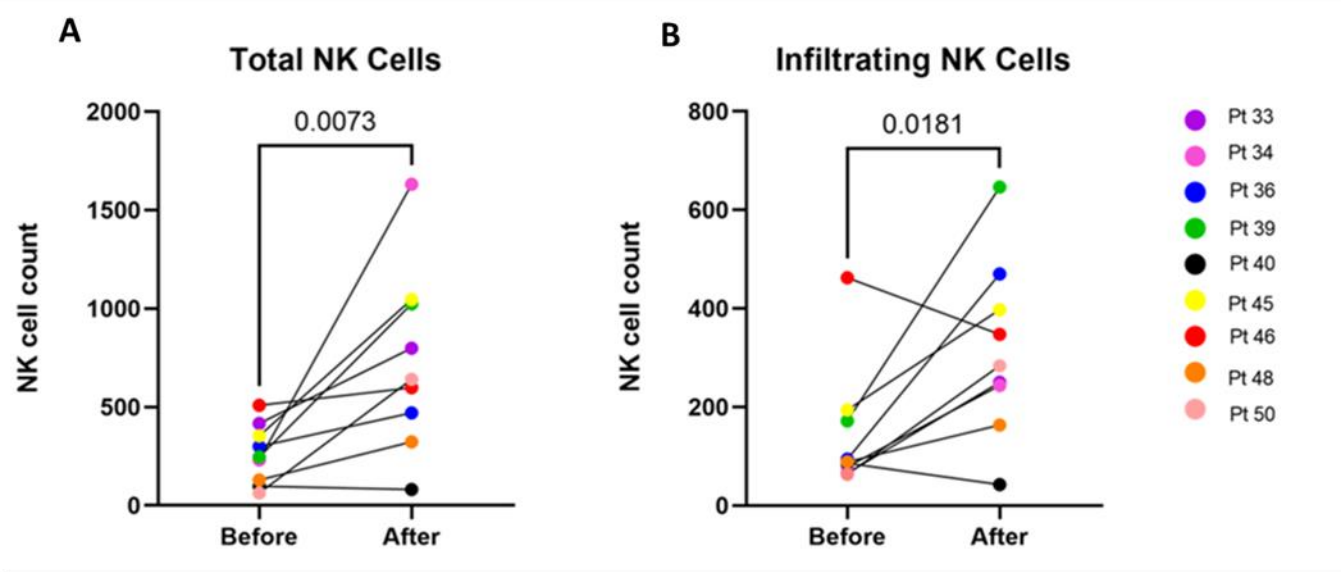

Figure 2.11: Metformin increases natural killer (NK) cell infiltration in head and neck squamous cell carcinoma (HNSCC). (A) Quantification of immunofluorescence in Figure 2.10 of all NKp46 positive points in tumor tissue (n = 9). Each point was an average of 5 images taken at 10x per tissue slide. (B) Quantification of immunofluorescence in Figure 2.10 of all NKp46 positive points within PCK positive tissue. Individual punctuated marks representing immune cells were counted using Image J. A border was drawn around PCK positive tissue and punctuated marks within the border were counted as infiltrating cells. Patient totals are averages of all 5 images. (A) and (B) were analyzed by paired Student's t-test.

Section 2.3.2: Indirect Activation of Peripheral Blood Natural Killer Cells

To determine if metformin treatment of tumor cells and NK cells enhanced NK cell cytotoxicity *ex vivo*, we treated the Cal27 HNSCC cell line with 12mM metformin for 24 hours. Cells were then co-cultured with untreated HNSCC patient or healthy donor NK cells and analyzed by flow for tumor cell cytotoxicity. Although baseline cytotoxicity was higher, there was no change in healthy donor NK cell mediated tumor cytotoxicity (**Figure 2.12A**) but HNSCC NK cell cytotoxicity did significantly increase upon HNSCC metformin treatment (**Figure 2.12B**).

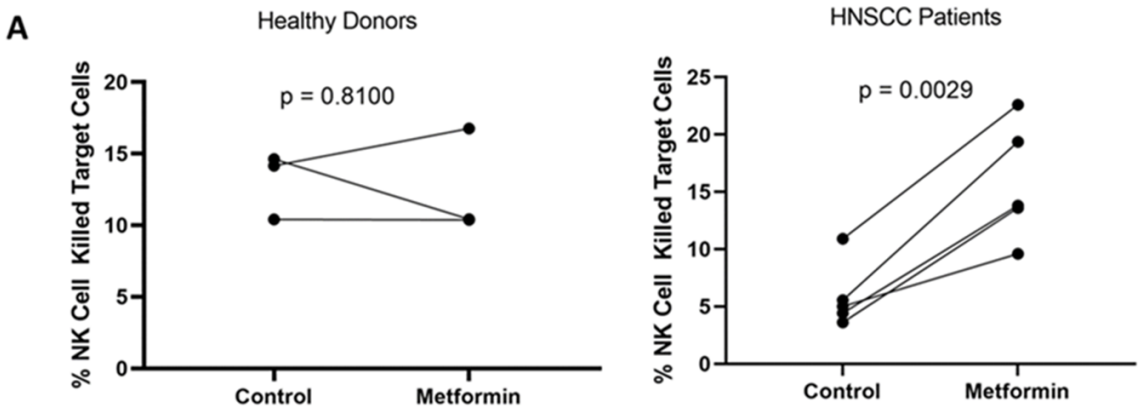

Figure 2.12: Metformin treated tumor cells are more susceptible to natural killer (NK) cell killing. (A) Healthy donor NK cell killing against vehicle or metformin treated Cal27 cells. (B) HNSCC NK cell killing against vehicle or metformin treated Cal27 cells. Baseline death of cells treated with vehicle or metformin and not exposed to NK cells was subjected from total death counts to consider any potential toxicities of metformin to tumor cells. (A, B) analyzed by paired student's t-test. n = 3 for healthy donors, n = 5 for head and neck squamous cell carcinoma (HNSCC).

When compared directly, HNSCC metformin treated tumor and NK cell co-culture resulted in increased NK cell cytotoxicity to level of healthy untreated donors co-cultured with untreated tumor cells (**Figure 2.13**).

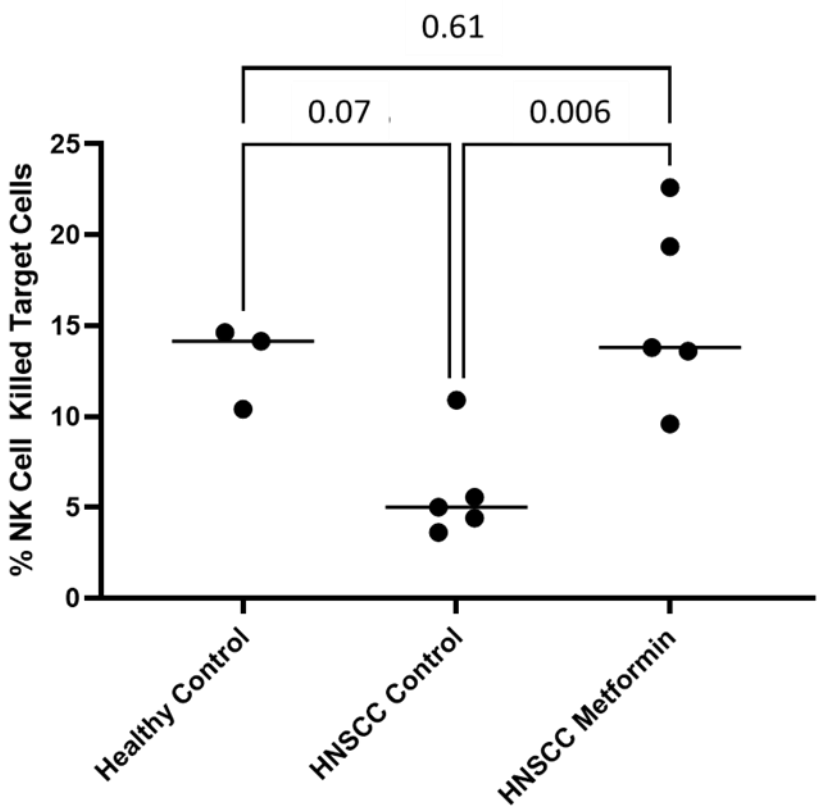

Figure 2.13: Metformin treated tumor cells are as susceptible to head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cell killing as healthy donor NK cells. Comparison of healthy donor NK cells co-cultured with vehicle treated Cal27 cells with HNSCC NK cells co-cultured with Cal27 cells treated with vehicle or metformin. Baseline death of cells treated with vehicle or metformin and not exposed to NK cells was subjected from total death counts to consider any potential toxicities of metformin to tumor cells. Data analyzed by two-way ANOVA. . n = 3 for healthy donors, n = 5 for HNSCC.

Treatment with metformin could modulate tumor cell lines to either be more susceptible to NK cell killing or changes cytokine secretion to cause NK cell activation. To determine how metformin may be changing tumor cell cytokine secretion, we treated tumor cells lines Cal27, HN5, and UMSCC47 with metformin or vehicle for 24 hours and collected RNA. RNA was converted to cDNA and analyzed by qPCR for changes in cytokine expression (**Fig 2.14**). Fold-change was an average of 3 technical replicates. Metformin treatment of HN5 cells resulted in a reduction of IL-6, IL-8, and IL-10 expression, all of which are STAT3 activated cytokines

generally not beneficial for mature NK cells. IL-4 was not detectable in HN5 cells. IL-6 was reduced and IL-4 was increased in metformin treated Cal27 cells. IL-4 generally leads to NK cell proliferation. Similarly, metformin resulted in an increase in IL-4 and decrease in IL-6 and IL-10 in UMSCC47 cells. Metformin modulated tumor cell cytokine secretions, but not at the same levels for every cell type. The direct tumor response to metformin may be patient specific. However, it is not clear whether these changes and subsequent pathway activation would change NK cell behavior in the four hours the cells are exposed to one another in co-culture.

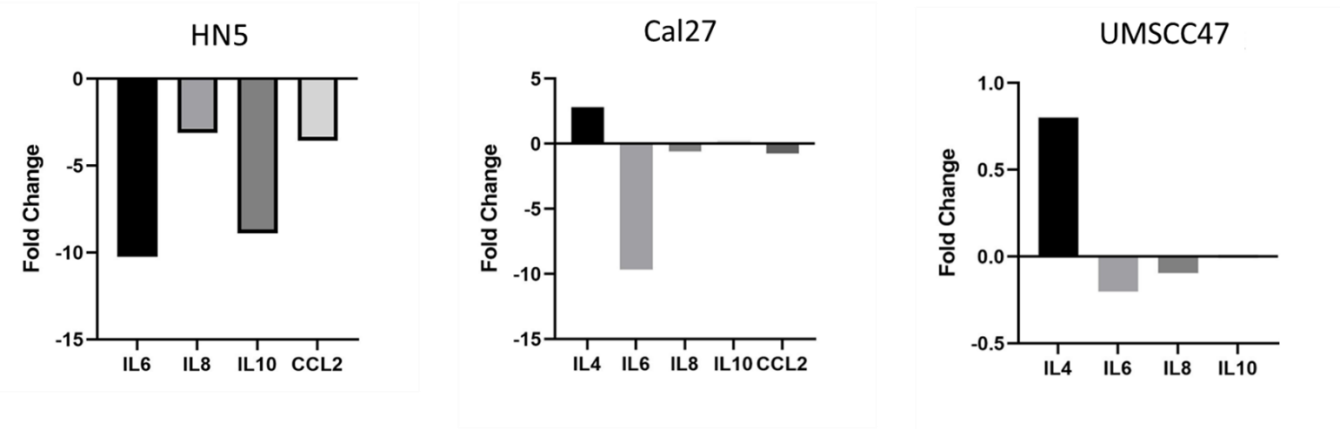

Figure 2.14: Metformin reduces pSTAT3 related cytokine expression in head and neck squamous cell carcinoma (HNSCC) cell lines. HNSCC cell lines HN5, Cal27, and UMSCC47 were treated with vehicle or 12mM metformin for 24hrs and RNA was collected for cDNA conversion and finally qPCR. Each point is fold change of 3 replicates. Data collected by lab members Reece Swoverlandand Makenzie Fourman.

In addition, RNA expression does not always correlate with protein expression. Using ELISA and supernatant collected from the plates of the qPCR samples, there was no significant change in cytokine production of those evaluated (data not shown). The discrepancy between RNA and protein may be due to some post translational modifications that occur intracellularly or degradation of cytokines post-collection. Cancer cells are known to have unstable mRNA, which may lead to changes in protein that are not consistent with changes to RNA detected by qPCR or PCR.[149]

Modulation of RNA had the highest fold change in pSTAT3 related cytokines IL-6 and IL-10. We investigated if the mechanism of action for metformin on tumor cell lines could be pSTAT3 dependent. As discussed, tumor cells express high levels of pSTAT3 which in turn activates the secretion of inhibitory signals to the immune system. We confirmed that tumor cell line Cal27 and UMSCC47 had significant decreases in pSTAT3 in response to metformin treatment (**Figure 2.15**) when probed by western blot.

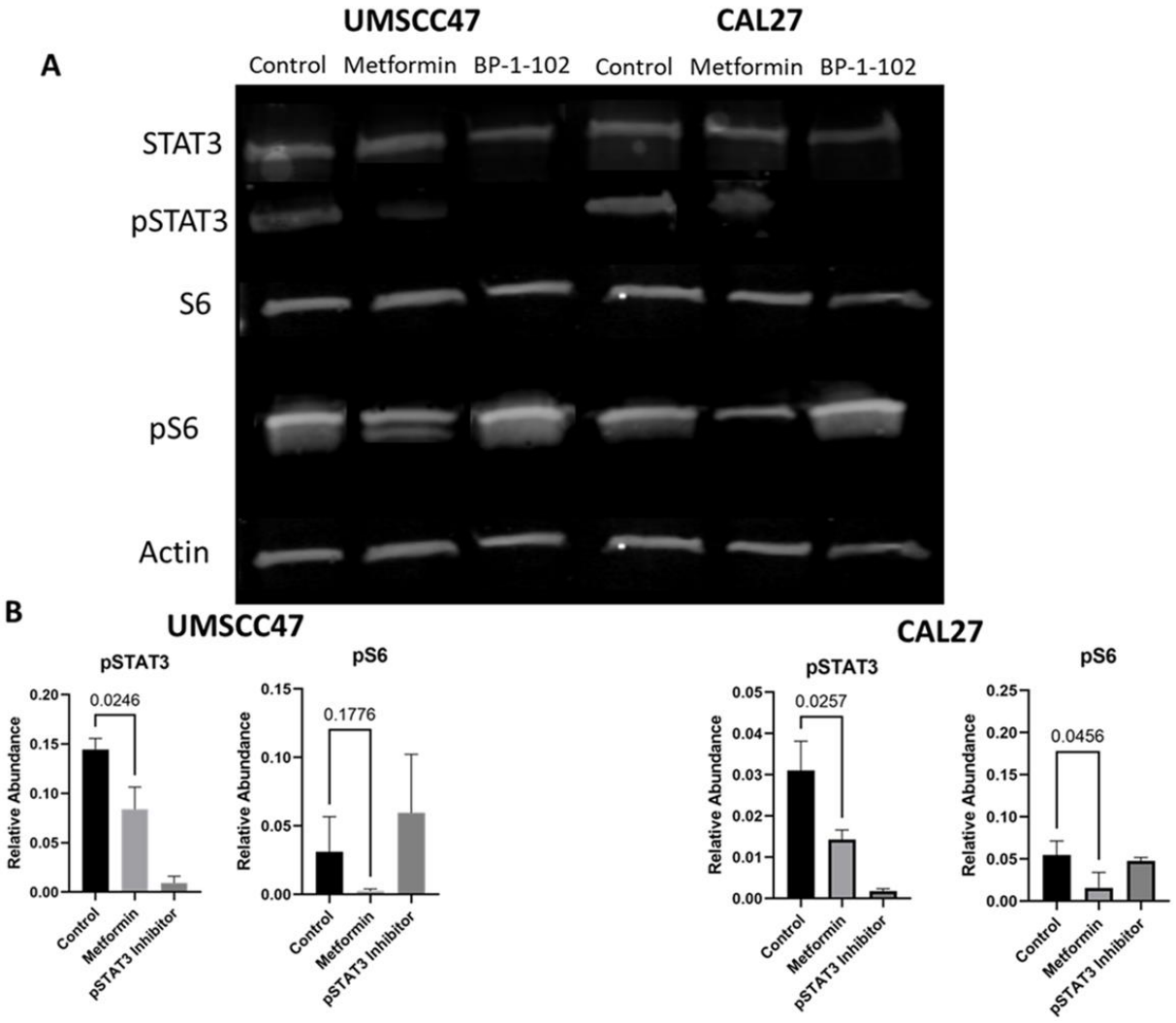

Figure 2.15: Metformin reduces pSTAT3 in tumor cells. (A) Western blot analysis of UMSCC47 and Cal27 head and neck squamous cell carcinoma (HNSCC) tumor cell lines. BP-1-102 is a pSTAT3 inhibitor used as a control. (B) Quantification of A. (B) Analyzed by one-way repeated measures ANOVA. Blot was repeated 3 times for quantification

pS6K was used as a control to confirm metformin activity, as metformin is known to reduce pS6K over 24 hours. We utilized BP-1-102, a specific pSTAT3 inhibitor, to ensure we properly probed for pSTAT3 on our western. Quantification of 3 repeats (2 blots not shown) indicated that pSTAT3 was significantly reduced with metformin in both cell lines, although not to levels of a pSTAT3 inhibitor. pS6 was unaffected by the inhibitor.

After observing the reduction in pSTAT3 after metformin treatment, we explored if pSTAT3 was necessary for metformin's mechanism of action on tumor cells. We utilized a flagged lentivirus that prevents STAT3 from moving into the nucleus and therefore cannot be phosphorylated. This keeps levels of total STAT3 the same as wildtype lines and decreases pSTAT3 alone.

One ng lentivirus supplied by the Zhang lab (Case Western Reserve University) was added to the HN5, Cal27, and UMSCC47 cell lines and incubated for 4 hours. Cells were washed and incubated 72hrs before all cell lines were kept in kanamycin selection media and probed for loss of STAT3 phosphorylation by western blot analysis (**Figure 2.16**). See appendix Figure 1 for lentiviral map.

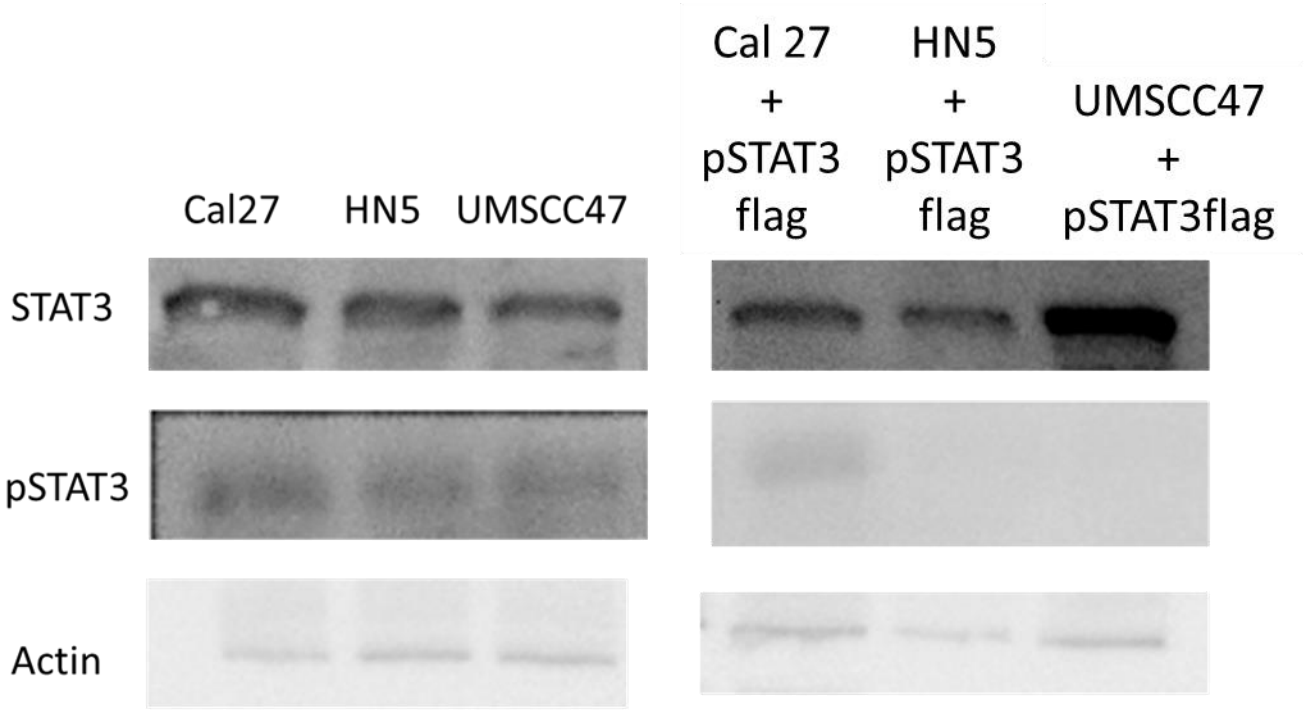

Figure 2.16: Lentivirus infection reduces phosphorylation of STAT3: Cal27, HN5 and UMSCC47 cells controls or lentiviral infected cells were collected for lysates and analyzed by western blot analysis.

Although this data is interesting, we decided not to proceed with continued analysis of the mechanism of metformin effects in HNSCC tumor cells and subsequent effects on NK cells for this thesis. Several papers in the past reported pSTAT3 inhibition as a vital mechanism of action of metformin in solid tumors.[109,110,150–152] pSTAT3 knockdown cell lines remain in the lab and could be further explored later. For these reasons, we decided to turn our focus to the direct effects of metformin on NK cells and its mechanism.

Section 2.3.3: Direct Activation of NK Cells

To determine how metformin alters cytokine and cytotoxic molecule secretion of individual NK cells, we isolated NK cells from PBMCs collected from newly diagnosed HNSCC patients prior to any anti-cancer treatment and subjected them to 12mM metformin or vehicle. The cells were analyzed on an IsoLight machine for 16hrs to evaluate single cell multiplex cytokine secretion (**Figure 2.17**). Metformin treatment significantly increased the cytotoxic molecule, perforin (p=0.00009).

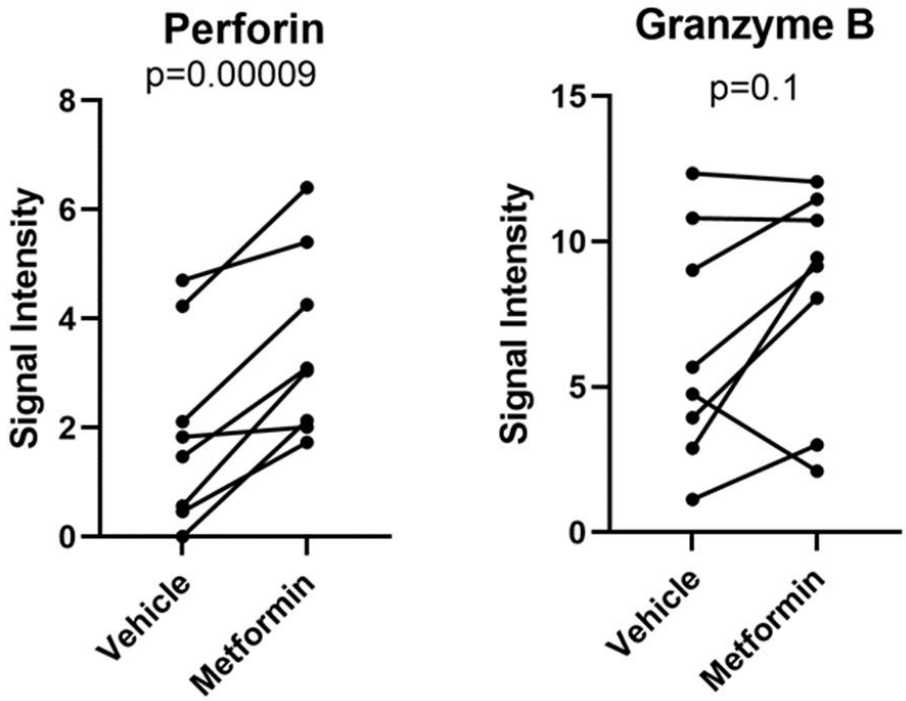

Figure 2.17: Metformin increases perforin expression in NK cells. Signal intensity of cytokines produced by natural killer (NK) cells treated with vehicle (12uL sterile water) or 12mM metformin for 16hrs. Analyzed by paired student's t-test. n = 8.

When analyzing cytokinesIL-9 IL-8, IL-10, and IL-6 trended towards a decrease in response to metformin (**Figure 2.18**). Interleukins 6,8,9 and 10 increase STAT3 phosphorylation, and are considered tumorigenic in the tumor microenvironment.[97]

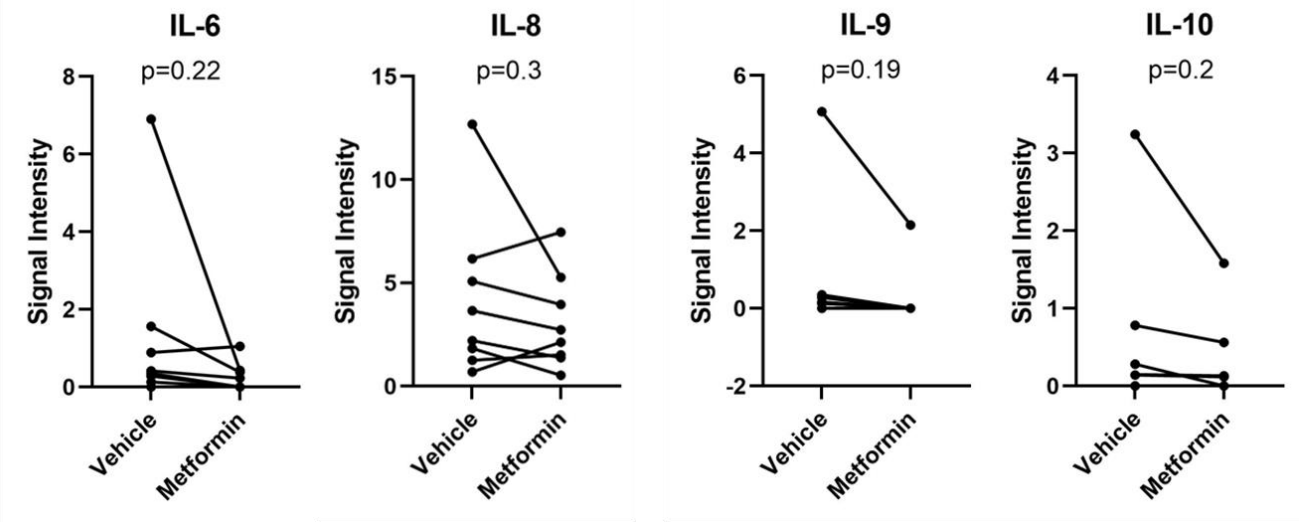

Figure 2.18: Metformin reduces pSTAT3 responding cytokines. Signal intensity of cytokines produced by natural killer (NK) cells treated with vehicle (12uL sterile water) or 12mM metformin for 16hrs. Analyzed by paired student's t-test. n = 8.

With support of increased perforin secretion, we next investigated if this was associated with enhanced NK cell cytotoxic function. We utilized a flow cytometry based NKCA to determine HNSCC tumor cell killing by HNSCC patient-derived NK cells. Metformin treatment resulted in a significant increase of HNSCC NK cell cytotoxicity in Cal27 tumor cells lines **(Figure 2.19**). Cal27 cells are HPV-, oral tumor cells.

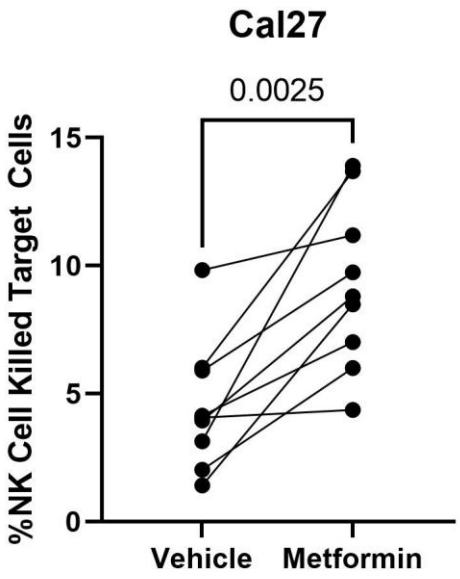

Figure 2.19: Metformin increases cytotoxicity of peripheral natural killer (NK) cells against Cal27 cells. Calculated cytotoxicity of peripheral NK cells against Cal27 cells at a 1:5 ratio. Analyzed by paired student's t-tests. n = 9.

To support that isn't a one cell type phenomenon, we next co-cultured the peripheral NK cells with HPV+, oral cell line UMSCC47 (**Figure 2.20)**. There was once again a significant increase in toxicity.

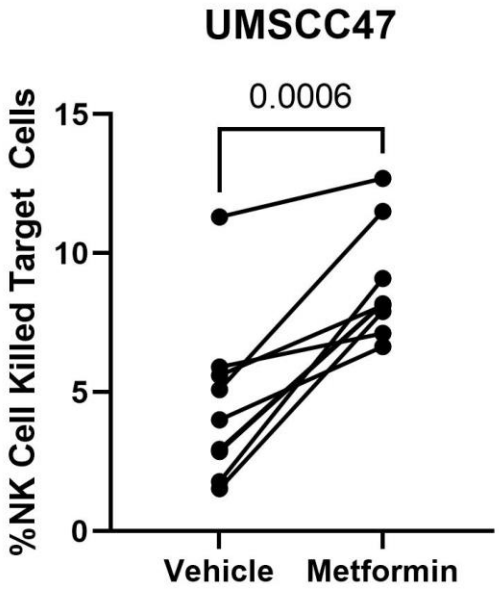

Figure 2.20: Metformin increases cytotoxicity of peripheral natural killer (NK) cells against UMSCC47 cells. Calculated cytotoxicity of peripheral NK cells against UMSCC47 cells at a 1:5 ratio. Analyzed by paired student's t-tests. n =12.

We also observed the effects of metformin on normal donor NK cells. Metformin does not significantly affect healthy NK cell cytotoxicity (n = 8), although metformin does rescue HNSCC NK cell mediated cytotoxicity to near normal donor levels (**Figure 2.21**).

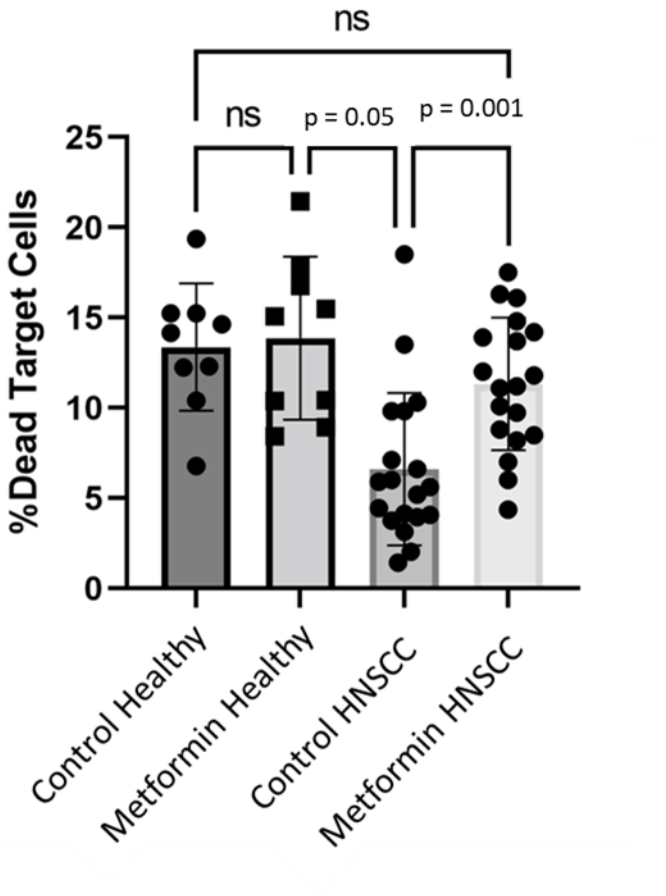

Figure 2.21: Metformin does not change healthy donor natural killer cell activity. Cytotoxicity of healthy donors peripheral natural killer (NK) cells treated with vehicle or metformin compared against head and neck squamous cell carcinoma (HNSCC) samples. Analyzed by two-way ANOVA. n = 9 for healthy donor and n = 20 for HNSCC.

We also collected evidence that normal NK cells treated with metformin did not have a significant change in cytokine secretion on a single cell basis, despite probing for similar cytokines that were reduced in HNSCC patients with metformin treatment (data not shown). Due

to this lack of change, we focused only on the mechanism by which metformin changed HNSCC cells.

To determine by what mechanism metformin exerts its mechanism of action on NK cells, we treated pre-treatment HNSCC NK cells with vehicle or metformin for 24 hours and analyzed by bulk RNA-seq. (See methods for collection methods and equipment; **Figure 2.22**). Expression of two genes were significantly downregulated (See appendix for non-adjusted significant genes): *Thioredoxin interacting protein (TXNIP)* and *C-X-C motif chemokine ligand 1 (CXCL1)*. TXNIP regulates glucose and is known to be decreased with metformin due to its insulin interaction. Therefore, we chose to further investigate how CXCL1 plays a role in metformin mediated activation.

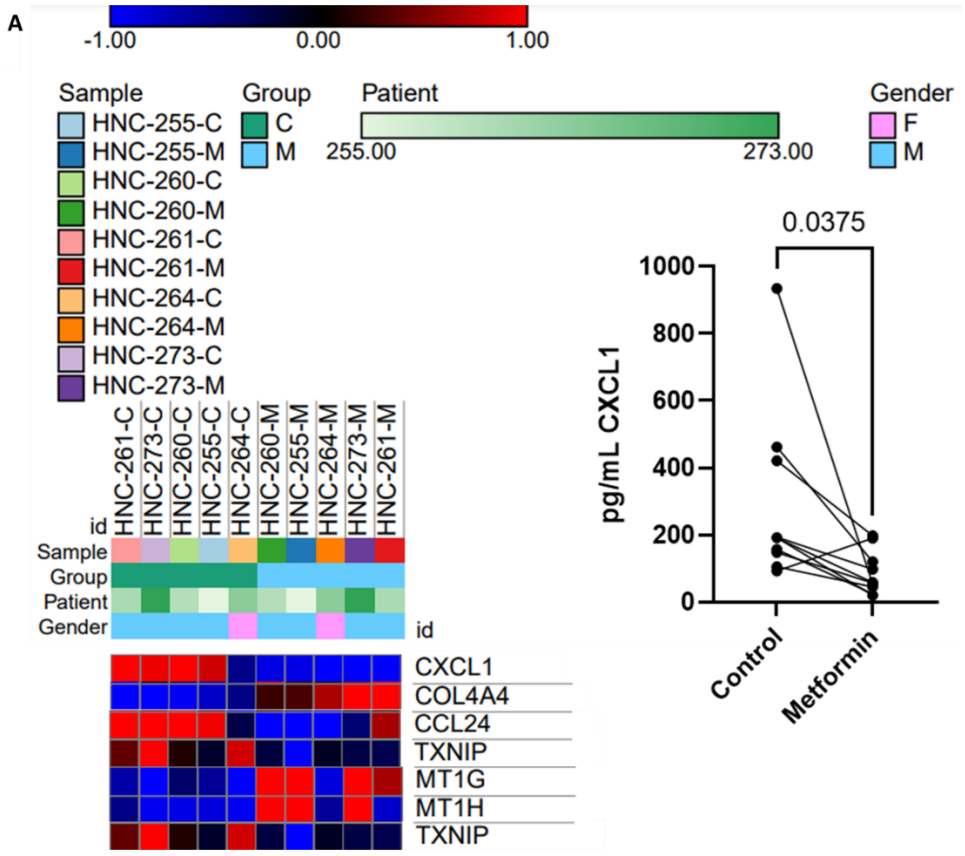

Figure 2.22: Metformin suppresses CXCL1 RNA expression of head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells: NK cells were treated with vehicle or 12mM

metformin for 24hrs and whole frozen cells were sent for RNA-seq. Blue indicates lower expression and red indicates higher expression. See methods for statistical analysis. n =5.

We next treated HNSCC patient-derived NK cells with metformin for 24 hours and collected the supernatant for ELISA analysis to confirm if the observed differential mRNA expression translated to differential protein expression. Metformin decreased CXCL1 protein expression in NK cells (**Figure 2.23**). How this occurs is further explored in Chapter 3.

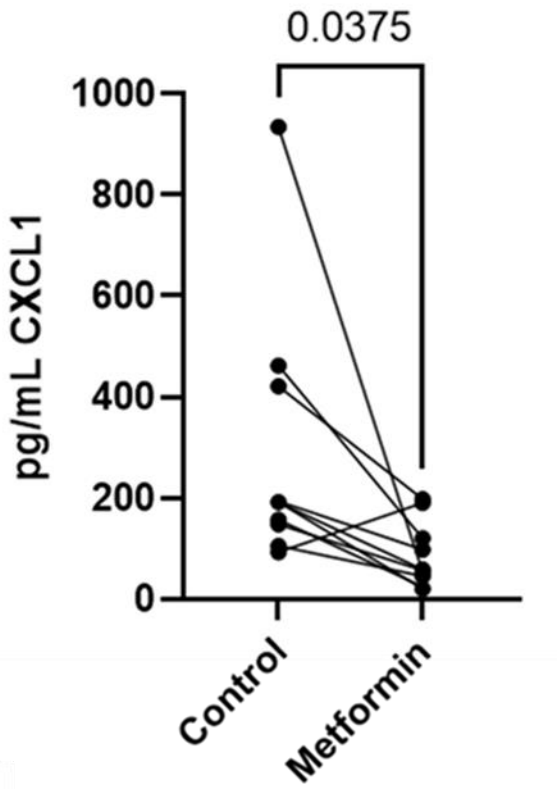

Figure 2.23: Metformin suppresses CXCL1 protein expression of head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells: NK cells were treated with vehicle or metformin for 24hrs and supernatant was collected for ELISA analysis. Analyzed by paired students t-test. n = 10.

Section 2.4 – Summary

There is a gap between patients that are predicted to respond to immunotherapy and those who do. There has been recent interest in further boosting the immune system in addition to blocking the PD-1 checkpoint. Much of this interest has been focused on T cells, which can directly kill cancer cells. Metformin has recently garnered attention as a potential safe, immune system boosting drug. Utilizing patient samples from the phase 1 clinical trial NCT02325401, we found that T cells remained relatively unaltered in HNSCC patients treated with metformin, but NK cells, which are also capable of directly killing tumor cells, were more abundant and more active upon metformin treatment. We also observed increased NK cell infiltration of the tumor post-metformin treatment in tissues from another phase I clinical trial NCT0208369 in which patients were treated with metformin pre-surgery.

We first investigated if metformin was directly impacting the tumor which could change the immune cell landscape and found that tumor cells treated with metformin were more susceptible to NK cell killing. When RNA was collected from metformin treated samples, there was a reduction in pSTAT3 related cytokine expression, but this change did not correlate with enhanced cytokine protein expression in the supernatant. Western blot analysis showed pSTAT3 is downregulated in tumor cells treated with metformin and we created tumor cell line with decreased pSTAT3. However, multiple studies have shown this link between metformin and solid tumors, so we focused on the effects of metformin on NK cells directly.

NK cell *ex vivo* newly diagnosed HNSCC patient-derived NK cells treated with metformin for twenty-four hours had increased perforin and decreased inflammatory cytokines. They were also more cytotoxic against HPV- and HPV+ cell lines. This same effect was not observed in age matched healthy donor NK cells, possibly because healthy NK cells have lower activation of pSTAT3, a JAK/STAT known to be upregulated in the TME and downregulated by metformin. To investigate how metformin was exerting this activation on HNSCC NK cells, we analyzed

control and treated patient NK cells by RNA-seq and surprisingly saw a significant decrease in CXCL1, which was repeated on a protein level using an ELISA. CXCL1 suppression may play a role in the activation of NK cells in the presence of metformin. How that may play into existing known activation and deactivation signals in NK cells will be discussed in Chapter 3.

Chapter III: Mechanism of CXCL1 and Metformin in Natural Killer Cells

Section 3.1 – Background

As mentioned in Chapter 2, we discovered a unique mechanism of action for metformin in HNSCC NK cells: CXCL1 inhibition. CXCL1 is a neutrophil recruiting chemokine expressed mainly by macrophages and mast cells and circulated at low levels during normal conditions.[150–152] These neutrophils are usually recruited in response to microbial infections.[150] Under-expression of CXCL1 and lack of recruitment causes sepsis, while overexpression and crowding of neutrophils leads to tissue damage.[150] CXCL1 can bind to two receptors: CXCR1 and CXCR2. Both are highly expressed on neutrophils and NK cells.[153] All chemokines can also bind to the Duffy antigen/Chemokine Receptor (DARC), which can nullify the chemokine.[154] Interestingly, CXCR1 is not expressed in mice and normally leads to bursting after activation in neutrophils in humans.[155] CXCR2, on the other hand, promotes proliferation of neutrophils and when mutated constitutively activates CXCL1 secretion.[156] CXCR1 mutations do not change CXCL1 secretion.[156]

Expression of CXCL1 and CXCR2 has been formerly investigated in cancer. Overexpression of CXCL1 can encourage cancer neoplasms, migration, angiogenesis, and metastasis.[156–158] In breast cancer, silencing CXCL1 expression by tumor associated macrophages reduced incidences of metastasis.[158] In melanocytes, knocking in CXCL1 increased cancer growth.[156] In HNSCC, high CXCL2 was correlated with a positive prognosis while CXCL1 and CXCL8 (both ligands of CXCR2) were associated with poor outcomes.[159]

CXCR2 is known to activate JAK/STAT and mTOR. These activations further downstream cause transcription of more CXCL1, which can cause an autocrine loop.[155]

JAK/STATs and mTOR can increase tumor cell growth, which may explain why CXCL1 may be beneficial to the tumor itself.[160] What is unknown is how CXCL1 may be changing NK cell activation. Metformin is known to inhibit similar pathways to those that CXCR2 activates (**Figure 3.1**)

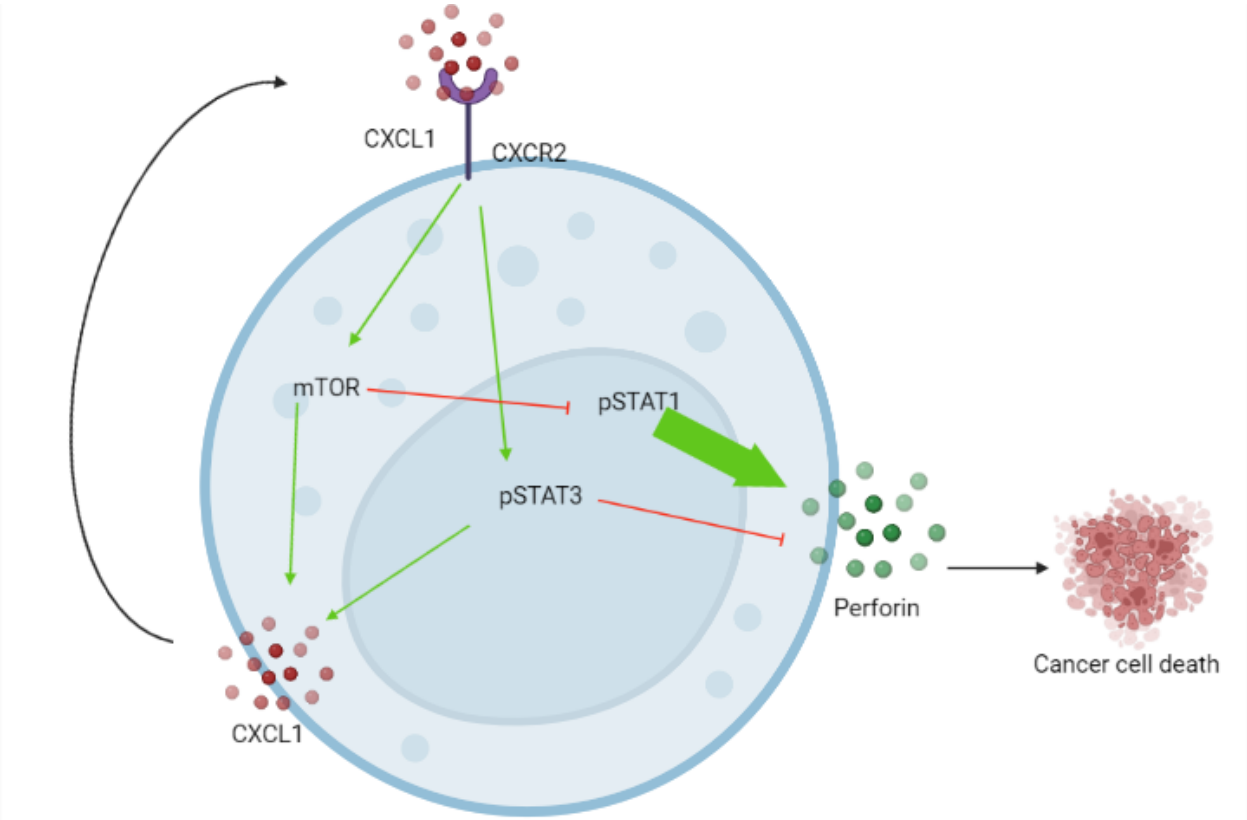

Figure 3.1: Pathways activated by CXCR2 binding of CXCL1. Figure created in Biorender

In this chapter, we explore how CXCL1 is modulated in HNSCC by metformin and what mechanistic role it plays in NK cell activation. This information can further lead to honing therapeutics for combination with current therapies to potentially improve patient outcomes.

Section 3.2 – Materials and Methods

3.2.1 Human Samples

Peripheral blood and serum were obtained from patients from the phase 2 clinical trial “Phase II investigation of adjuvant combined cisplatin and radiation with pembrolizumab in resected head and neck squamous cell carcinoma” (NCT02641093) at the University of Cincinnati, University of Louisville, University of Michigan, Medical Center of South Carolina, and MD Anderson Cancer Center. Key eligibility criteria for patient inclusion on trial were confirmation by tissue biopsy of locally advanced HNSCC that was resectable. Key exclusion criteria included metastatic disease, or nasopharyngeal carcinoma as the primary tumor site. Blood samples were collected before and after 1-3 weeks of treatment with pembrolizumab prior to surgery. Peripheral blood and serum were obtained from patients from the clinical trial “Combining pembrolizumab and metformin in metastatic head and neck cancer patients” (NCT04414540) at the University of Cincinnati. Key eligibility criteria for patient inclusion on trial were confirmation by tissue biopsy of recurrent and/or metastatic HNSCC. Key exclusion criteria included known history of diabetes requiring insulin, or nasopharyngeal carcinoma as the primary tumor site. Samples were collected before treatment, 1 week after metformin treatment, and 2 weeks after addition of pembrolizumab. Additional *ex vivo* studies were performed on age matched peripheral blood obtained from IRB approved studies UCCI-UMB-14-01 (IRB #2014-4755) and general specimen collection protocol (IRB #2017-2137) to investigate differences in molecular and immune cell markers compared to clinical outcomes in HNSCC patients and normal healthy controls. The studies were approved by the Institutional Review Board at the University of Cincinnati and were conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was received from all participating patients prior to enrollment.

3.2.2 Cell Lines

Human-derived HNSCC cell lines CAL27 and HN5 were grown and maintained in 1x DMEM high glucose (Corning), 8mM L-glutamine (Corning), 10% FBS (Omega Scientific), 1% Pen/Strep (Corning) and 1x essential amino acids (Corning). All cell lines were cultured at 37°C with 5% CO_2. Mycoplasma presence was checked every 3 months (MycoProbe, R&D Systems, Minneapolis, MN) and no cases of contamination within these cell lines were reported as of February 2022. Cell lines were STR Profiled confirmed by Cincinnati Children's Hospital Medical Center Cytogenics Labs in May 2020.

3.2.3 Western Blot

NK92, Cal27, UMSCC47 and HN5 cells were cultured with drug as described. Cells were collected in RIPA (0.05% sodium deoxycholate, 150nM NaCl, 50nM Tris HCL, 0.1% SDS, 1% NP-40) and protein content was analyzed by Pierce BCA kit (Thermo 23225). 50ug of protein was diluted in 1x loading buffer (SDS, bromophenol blue, 47% glycerol, Tris 0.5M pH 6.8, 0.2mM DTT-G) and heated to 90ºC for 3 minutes. Prepared lysate was loaded into gradient gels (Biorad 4561093) and run at 85V for 1.5 hour in Biorad casing with a Biorad powerpack in 1x running buffer (Tris, Glycine, SDS). Gels were transferred on nitrocellulose and run at 100V on bench for 1 hour in 1x transfer buffer (Tris, Glycine). Membranes were blocked in 5% BSA in TBS for 1hr, washed, and incubated in primary diluted in 2% BSA in TBS overnight. Membranes were washed and incubated in Licor secondary (Licor 926-32211 and 925-68070) in 5% BSA in TBS for 1hr. Membranes were washed and imaged on a Licor Odyssey Clx. Images were analyzed in Image Studio Lite V 5.2.

3.2.4 Drugs

Metformin (13118) and BP-1-102 (28368) were obtained from Caymen Chemicals. Dosomorphin (S7306), Everolimus (S1120), MHY1485 (S7811), and Flubarabine (S1491) were obtained from Selleck Chemicals. Navarixin (HY-10198) was obtained from Medchem Express.

3.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

For NK cell perforin assays, NK cells were treated with drug for 24hrs, washed, and co-cultured with Cal27 cells for 4 hours. Supernatant was then collected, snap frozen in liquid nitrogen and stored at -80 until thawed for single use. For patient samples, undiluted plasma was added directly to ELISA. ELISA kit for perforin was obtained from ABCAM. ELISA was performed according to the manufacturer's protocol. Protocols included overnight coating of capture antibody diluted by lot number recommendation, 1hr block with 400uL 2% BSA in PBS, 2hr sample incubation at room temperature of 100uL sample (no dilution of sample was performed in these experiments), 2hr capture antibody incubation with 100uL capture antibody diluted by lot number recommendation, 20min Streptavidin incubation with 100uL of 1:40 dilution of stock, and 15min incubation with 100uL included TMB ELISA reagent. 50uL stop solution of in house 2N sodium sulfide was added to stop reaction before reading at 500nm wavelength on plate reader. All plates included standard curve prepared per lot number recommendation with range of 35-2000pg.

3.2.6 Statistics

An unpaired 2-tailed *t* test with Welch's correction was used for analysis between flow cytometry in controls versus HNSCC patients. A 2-tailed Student's *t* test was used to compare differences

between HNSCC patients before and after treatment in flow cytometry and cytokine experiments. Differences in immune cell populations between three groups were compared by one-way ANOVA with specific post-hoc contrasts. NKCA data were compared using a paired *t* test. Statistical analysis was performed in GraphPad Prism (V10). Differences between groups were considered statistically significant when $P < 0.05$.

Section 3.3 – Results and Discussion

Given our focus on CXCL1, we next investigated by what mechanism metformin decreases CXCL1 expression, and how that may be beneficial to NK cell activity. We first determined if HNSCC NK cells produced higher levels of CXCL1 compared to healthy patients and the effect of metformin treatment on NK cells *ex vivo*. We found that HNSCC NK cells secreted significantly more CXCL1 than normal NK cells and metformin significantly reduced CXCL1 supernatant levels in HNSCC patient-derived NK cell culture (**Figure 3.2**)

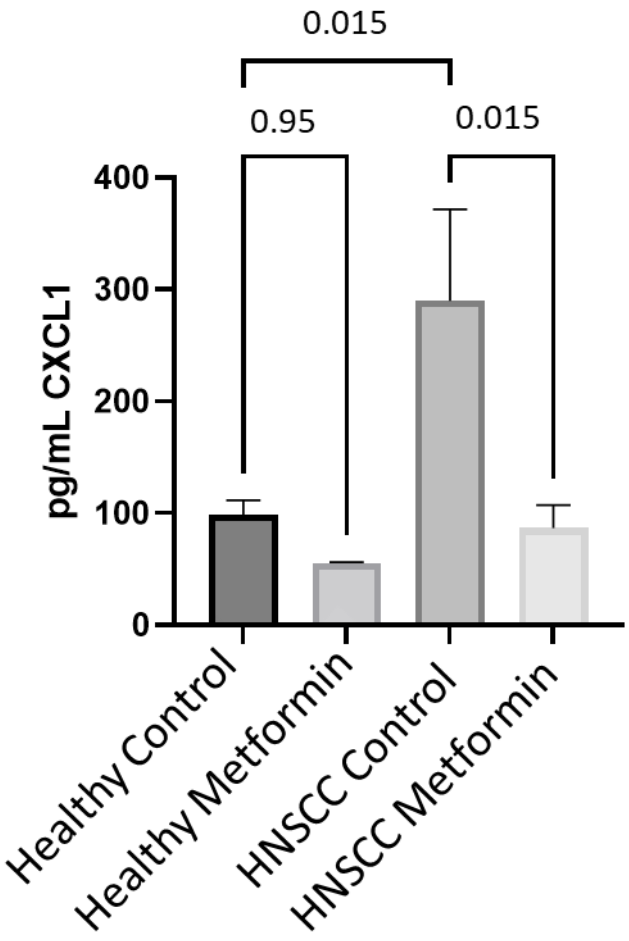

Figure 3.2: Head and neck squamous cell carcinoma (HNSCC) natural killer cells (NK) cells secrete more CXCL1. Healthy donor and HNSCC NK cells were isolated from PBMCs and were treated for 24hrs with vehicle or metformin. Supernatant was collected and analyzed by ELISA. Analyzed by one way ANOVA. n = 10.

Understanding that HNSCC NK cells had higher levels of CXCL1 led us to investigate how CXCL1 may cause dysregulation in NK Cells. We treated HNSCC patient-derived NK cells with exogenous CXCL1 and an inhibitor of the CXCL1 receptor, CXCR2, or metformin. We exposed patient-derived NK cells to Cal27 cells for 4 hours and collected supernatant for perforin analysis by ELISA. Although metformin increased perforin as expected, CXCL1 reduced NK cell perforin production. Metformin could not completely reverse CXCL1 suppression alone but the CXCR2 inhibitor alone and in combination with metformin reversed CXCL1 mediated inhibition (**Figure 3.3**).

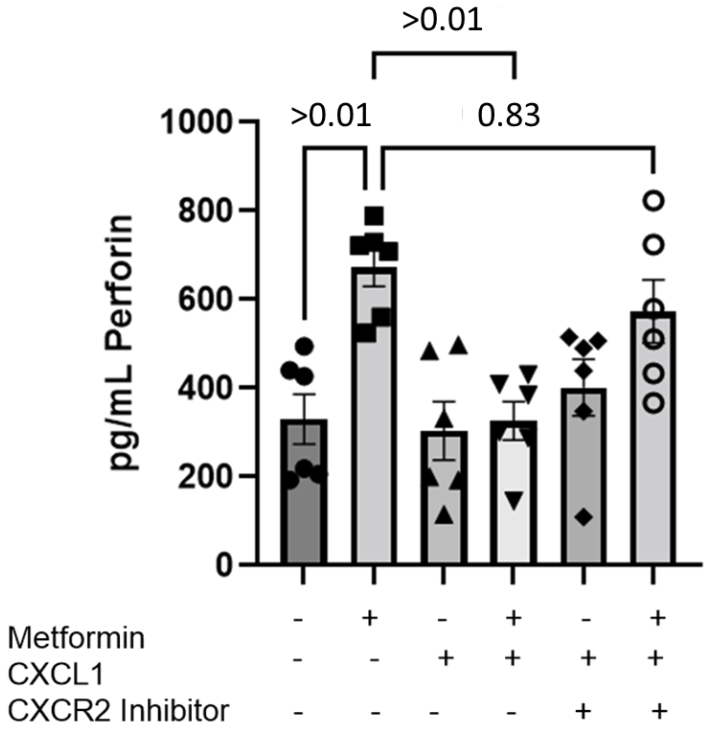

Figure 3.3: Exogenous CXCL1 reverses metformin activation. Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells derived from PBMCs were treated with 50ng CXCL1, 12mM metformin, or 10nM CXCR2i (navarixin) and subjected to an NKCA against Cal27 target cells. Cells were analyzed by flow and analyzed by one way ANOVA. n = 8.

Metformin and CXCL1 could be affecting NK cells in opposing pathways. To determine what those pathways could be, we investigated pathways known to be affected by both metformin and CXCL1. pSTAT3 and mTOR are known to be increased by activation of the CXCR2 receptor, and mTOR activation can inhibit pSTAT1 expression. Metformin is also hypothesized to inhibit pSTAT3 and mTOR in NK cells. Therefore, we collected HNSCC patient-derived NK cells treated with metformin, CXCL1, or CXCR2i (navaxarin) and analyzed by western blot analysis (**Figure 3.4**). We observed an increase in pSTAT1 with metformin treatment, but CXCL1 did not reverse pSTAT1 expression with metformin or change it from baseline. However, metformin reduction of pSTAT3 was reversed by exogenous CXCL1.

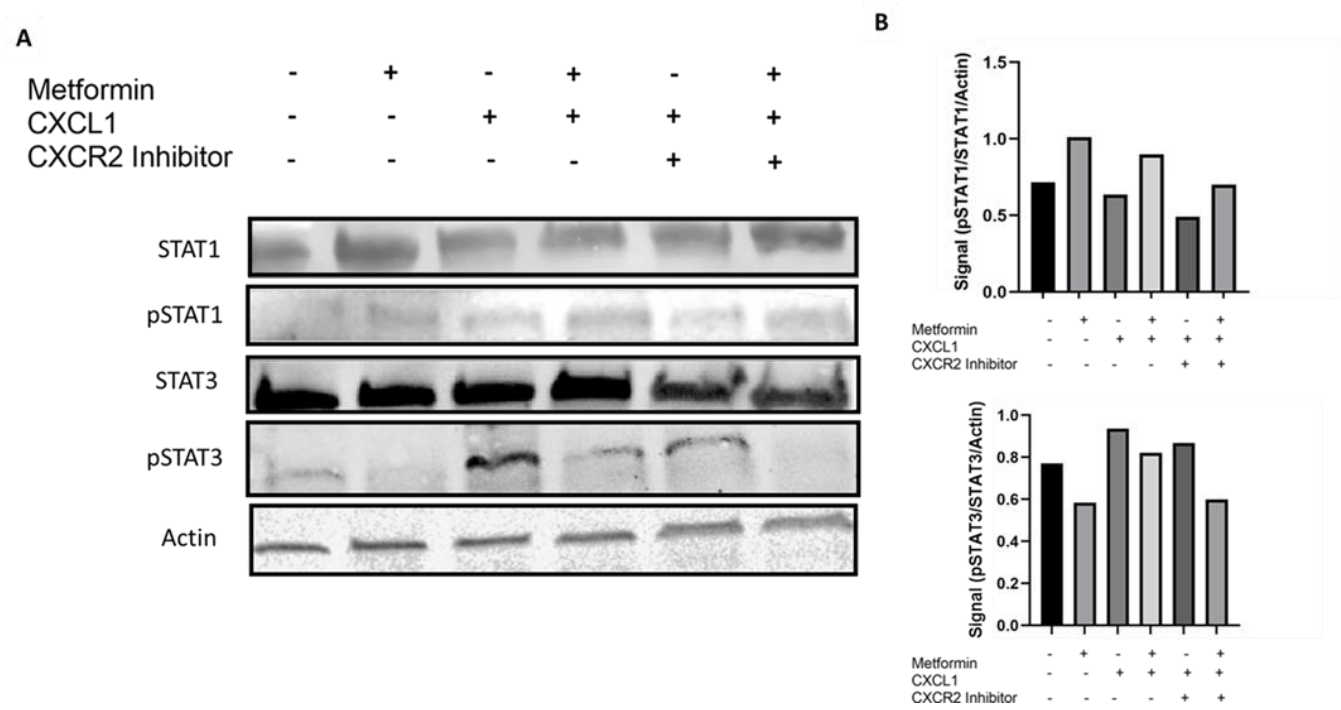

Figure 3.4: Metformin increases pSTAT1 and decreases pSTAT3 while CXCL1 increases pSTAT3. (A) Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells were treated with metformin, 50ng CXCL1, or 10uM CXCR2i (navarixin) for 24hrs and collected for lysates analyzed by western blot analysis. (B) Quantification of western bands.

We hypothesized that metformin increases pSTAT1 and reduces pSTAT3, while exogenous CXCL1 overcomes pSTAT3 inhibition. This increase in pSTAT3 could lead to NK cell dysfunction. We also hypothesized that CXCL1 was increasing mTOR, increasing CXCL1 production and reducing pSTAT1**. Figure 3.5** is a visual of our proposed pathway.

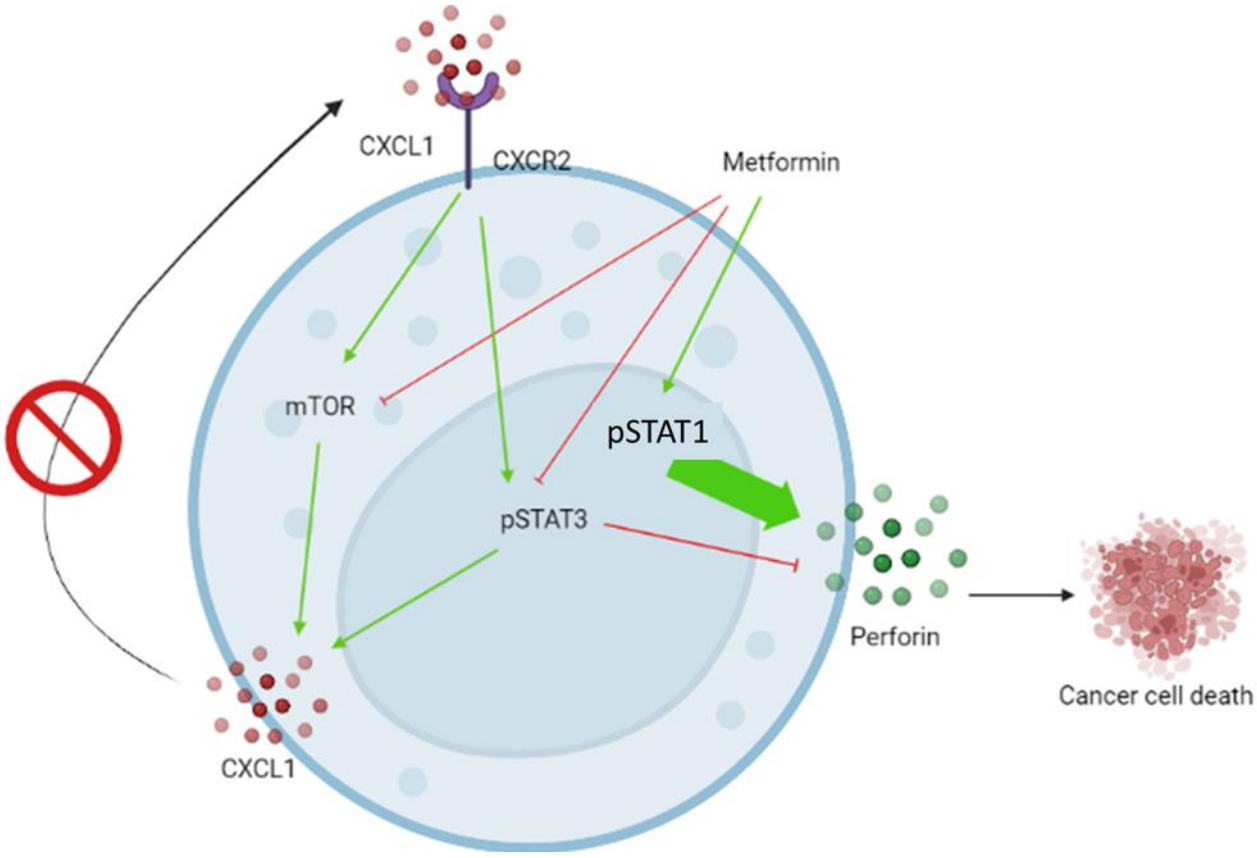

Figure 3.5: Proposed mechanism of action for metformin. Metformin increases pSTAT1, which in turn increases perforin production. mTOR and pSTAT3 inhibition by metformin decrease negative effects on natural killer (NK) cells and breaks the autocrine loop of CXCL1 secretion. Figure created in biorender.

AMPK is known to be upregulated in response to metformin, but studies have indicated that NK cells may be activated independent of AMPK with metformin treatment.[131] To determine if this trend is followed in HNSCC NK cells, we treated HNSCC patient-derived NK cells with vehicle, metformin, or AMPK inhibitor dorsomorphin and analyzed perforin production after 4 hours of

exposure to Cal27 cells. (**Figure 3.6**). Inhibiting AMPK did not change basal perforin or perforin increased by metformin.

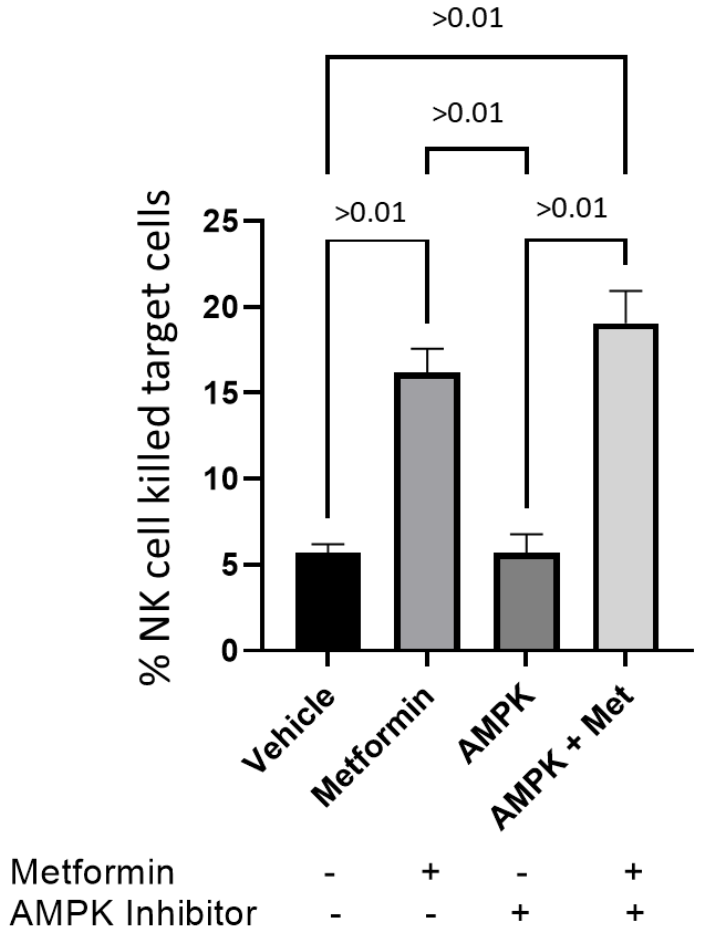

Figure 3.6: Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells do not require AMPK activation for metformin induced perforin production. HNSCC NK cells were treated with vehicle, metformin, or 10uM AMPK inhibitor (Dosomorphin) for 24hrs, washed, and co-cultured with Cal27 cells for 4hrs. Supernatant was collected for ELISA and analyzed by one way ANOVA. n = 5.

AMPK activation in turn inhibits mTOR although metformin can also inhibit mTOR directy. Given AMPK did not impact metformin induced perforin, we determined if mTOR inhibition was necessary. We treated HNSCC patient-derived NK cells with an mTOR inhibitor (everolimus) or

activator (MHY 1485), exposed cells to Cal27s for 4 hours,. Inhibiting mTOR enhanced perforin production similar to metformin (**Figure 3.7**) but the combination did not increase perforin over either agent alone. Interestingly, mTOR activation did not result in suppression of perforin.

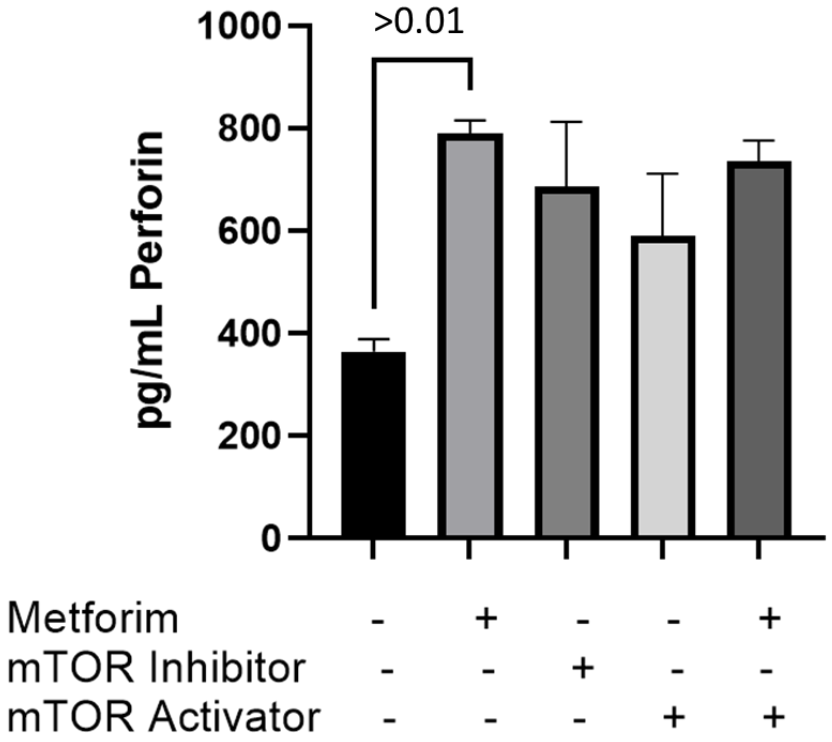

Figure 3.7: mTOR inhibition enhances perforin production. Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells were treated with vehicle, metformin, 10uM mTOR inhibitor (Everolimus), or 10uM mTOR activator (MHY1485) for 24hrs, washed, and co-cultured with Cal27 cells for 4hrs. Supernatant was collected for ELISA and analyzed by one way ANOVA. n =5.

Now that we had investigated two pathways popularly explored by metformin studies, we turned to downstream pathways affected by metformin observed in our western blot analysis (Figure 3.4). As pSTAT1 can modulate perforin production, we first treated HNSCC patient-derived NK cells with vehicle, 12nM Metformin, 50ng CXCL1, or fludarabine which at 10nM is a pSTAT1 inhibitor.[161] When co-cultured with Cal27 cells, pSTAT1-inhibited NK cells had lower perforin that could be rescued to baseline by metformin (**Figure 3.8**).

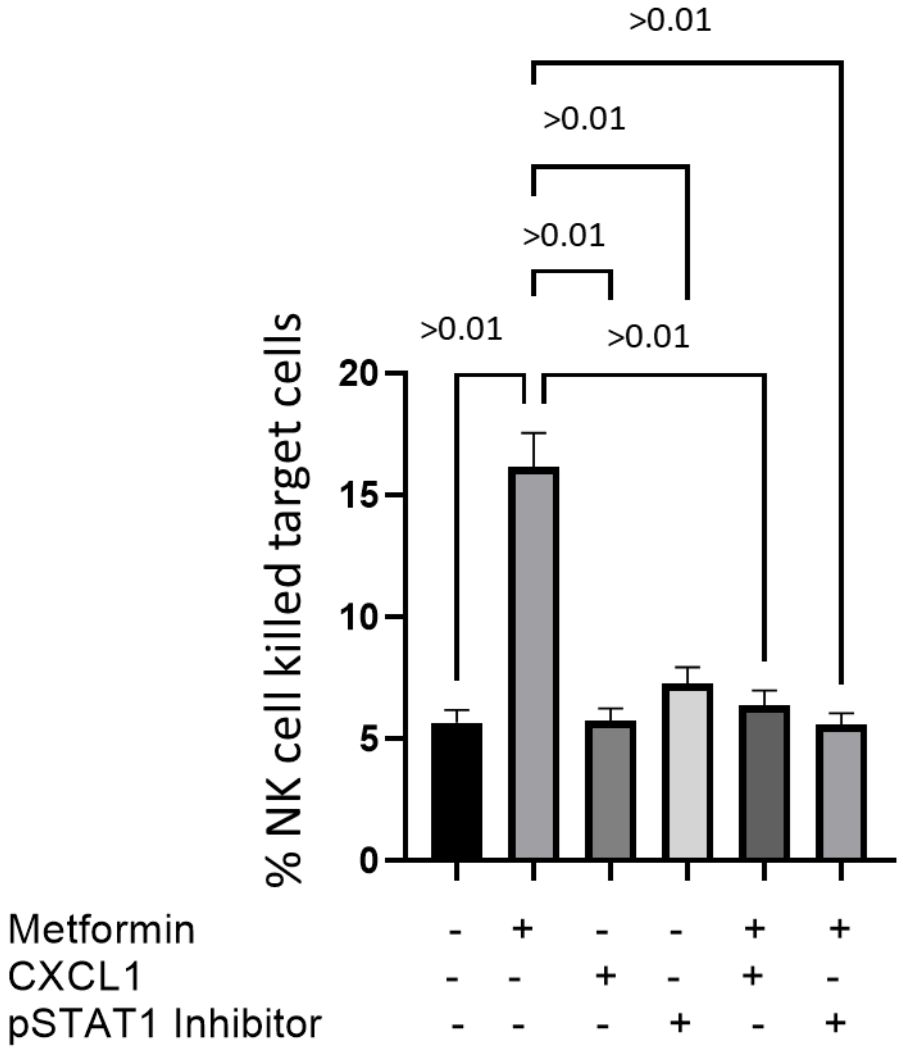

Figure 3.8 pSTAT1 is involved in metformin induced perforin production. Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells were treated with vehicle, metformin, or 10uM pSTAT1i Fludarabine) for 24hrs, washed, and co-cultured with Cal27 cells for 4hrs. Supernatant was collected for ELISA and analyzed by one way ANOVA. n = 5.

We next determined a potential role for pSTAT3 inhibition for metformin-mediated activation and CXCL1 suppression of perforin. HNSCC NK cells were treated with vehicle, 12mM metformin, 50ng CXCL1, or 10uM pSTAT3 inhibitor BP-1012 and co-cultured with Cal27 cells. NK cells treated with a pSTAT3 inhibitor had no effect on perforin at baseline, but STAT3 inhibition was

able to rescue CXCL1 mediated suppression of perforin both alone or in presence of metformin (**Figure 3.9**).

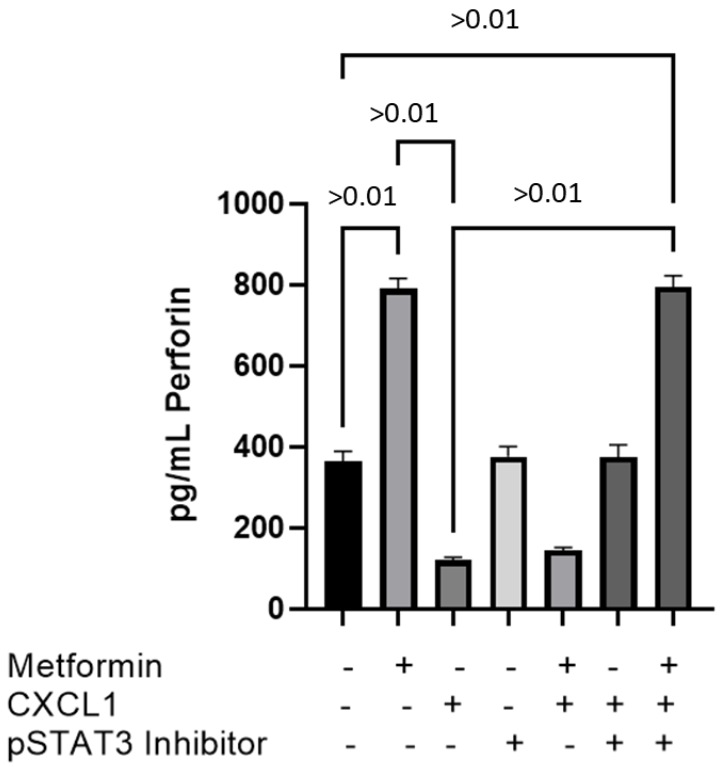

Figure 3.9: pSTAT3 is involved in CXCL1 overcoming metformin induced perforin production. Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) were treated with vehicle, metformin, or 10uM pSTAT3i 24hrs, washed, and co-cultured with Cal27 cells for 4hrs. Supernatant was collected for ELISA and analyzed by one way ANOVA. n = 5.

Now that we had established supporting evidence for our proposed interaction of metformin with CXCL1, we asked how metformin was reducing CXCL1 secretion. We hypothesized that inhibiting mTOR and pSTAT3 in combination would result in a similar reduction of CXCL1 as metformin. We treated HNSCC NK cells with vehicle, 12mM metformin, 50ng CXCL1, 10uM everolimus, or 10uM BP-1-102 for 24hr, washed the cells, and collected supernatant after 24 additional hours. pSTAT3 or mTOR inhibition alone did not reduce CXCL1,

but a combination decreased levels down to those similar of metformin even in the presence of CXCL1 (**Figure 3.10**).

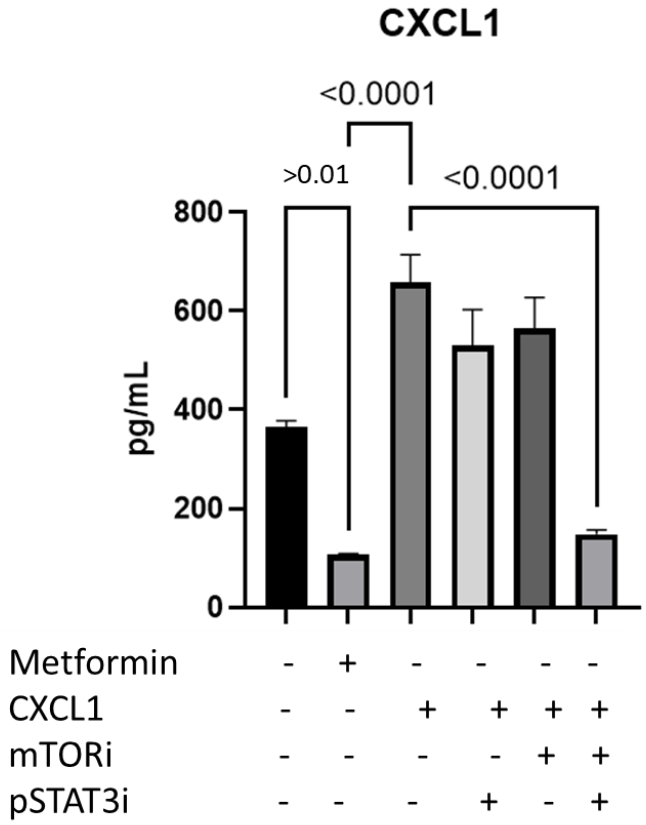

Figure 3.10: Metformin reduces CXCL1 through mTOR and pSTAT3. Head and neck squamous cell carcinoma (HNSCC) natural killer (NK) cells were isolated from PBMCs and treated with CXCL1, metformin, 10uM mTORi (everolimus), or 10uM pSTAT3i (BP-1-102) for 24hrs, washed, and plated in fresh media. After 24hrs, supernatant was collected for ELISA. Analyzed by one way ANOVA. n = 5.

Now that we had mechanistic data, we asked how this could be translated into patients. We took plasma from patients enrolled in the clinical trial “Combining pembrolizumab and metformin in metastatic head and neck cancer patients” (NCT04414540) and probed for CXCL1 by ELISA (**Figure 3.11**). Samples were collected from patients at 3 time points: (1) pre-treatment, (2) post-metformin only, and (3) post-metformin and pembrolizumab combination (week 4). Metformin

treatment reduced circulating CXCL1 as expected, but this decrease was not retained through pembrolizumab treatment.

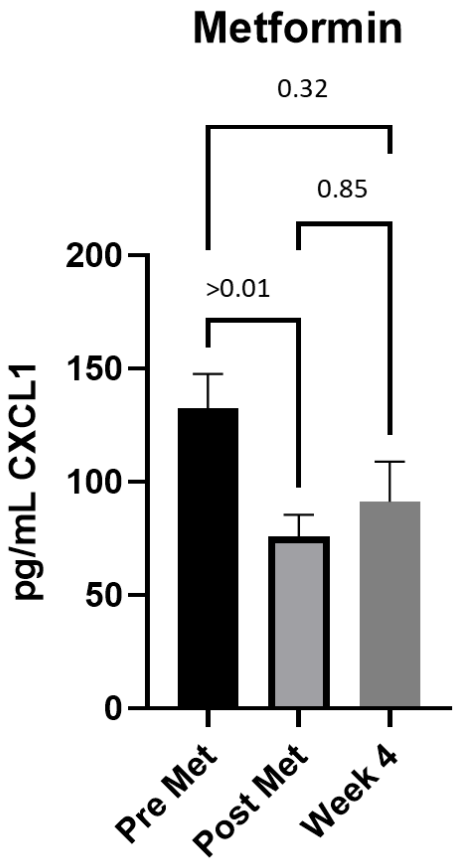

Figure 3.11: CXCL1 is decreased in metformin treated patients. Patient plasma pre and post metformin and post pembrolizumab plus metformin (Week 4) were subjected to CXCL1 ELISA. Analyzed by paired One-Way ANOVA. n = 5.

After observing this change with pembrolizumab treatment, we hypothesized that pembrolizumab increased CXCL1 in patients. We took plasma from the clinical trial “Adjuvant Cisplatin and radiation with pembrolizumab in resected head and neck squamous cell carcinoma” (NCT02641093) and probed for CXCL1 by ELISA (**Figure 3.12**). There was a significant increase in CXCL1 post-pembrolizumab, but when we divided patients out into

responders and non-responders, non-responders accounted for the increase. Pre-treatment CXCL1 had no prognostic value (data not shown).

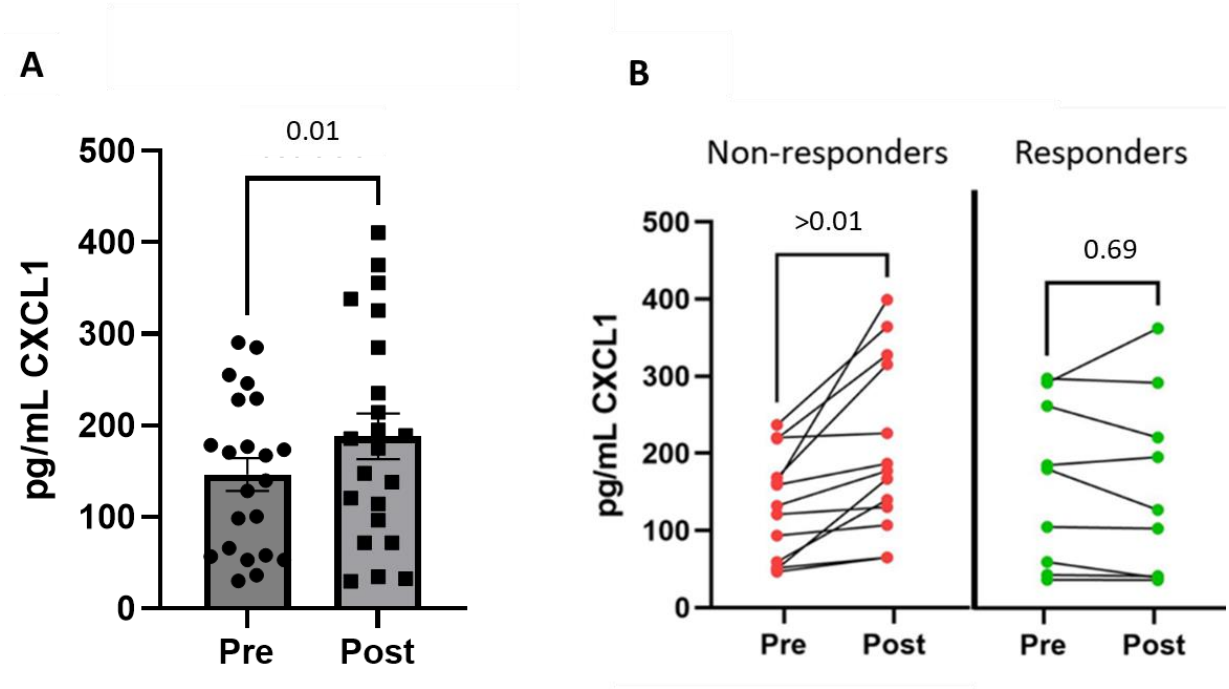

Figure 3.12: CXCL1 is increased with Pembrolizumab. (A) patient plasma pre and post pembrolizumab was subjected to CXCL1 ELISA. (B) Plasma data from (A) divided into responder and non-responder. (A) and (B) were analyzed by paired student t test. n = 10 responders and 10 non-responders.

Section 3.4 – Summary

CXCL1 is a neutrophil recruiting cytokine that we identified as being significantly downregulated by metformin. We determined that CXCL1 is nearly 3-fold higher in HNSCC patients than age matched patients, but that metformin could bring CXCL1 levels down to normal baseline. We found that exogenous CXCL1 can reverse metformin activation, but addition of a CXCR2 inhibitor rescued metformin's effects. We chose to investigate STATs, which have diverse effects on NK cell function. Metformin increases pSTAT1 and decreases pSTAT3, while CXCL1 increased pSTAT3. Exogenous CXCL1 was capable of overcoming metformin's ability to decrease pSTAT3. Utilizing a series of inhibitors and measuring cytotoxicity and perforin production, we found that metformin induced perforin secretion is pSTAT1 dependent while CXCL1 ability to overcome metformin induced perforin is pSTAT3 and mTOR dependent. We combined pSTAT3 and mTOR inhibitors to determine if this inhibition by metformin is what causes CXCL1 reduction, and indeed observed a significant decrease in CXCL1 in response to mTOR and pSTAT3 inhibition in the presence of CXCL1. Our experiments support that metformin activates NK cells by activating pSTAT1 and inhibiting pSTAT3, which in turn reduces CXCL1. CXCL1 is also capable of causing an autocrine loop, and metformin can reduce this CXCL1 production and loop by inhibiting both pSTAT3 and mTOR.

Finally, we used patient plasma from two clinical trials: NCT04414540 and NCT02641093. NCT02641093 samples were obtained from patients pre- and post-pembrolizumab treatment. Although there was no significant difference pre- and post-treatment in CXCL1 plasma levels, there was a significant increase in CXCL1 post-treatment in pembrolizumab non-responders. NCT0441540 collected samples pre-treatment, post-metformin treatment, and post-metformin plus pembrolizumab treatment. CXCL1 was decreased by metformin as expected, but pembrolizumab began to increase plasma levels of CXCL1. Therefore, it might not be beneficial to add metformin alone to pembrolizumab treatment to

increase responses. However, this study only has a small sample size (n = 4) at the time of writing. Metformin was an excellent tool to elucidate new ways to activate NK cells. Potential new therapeutics proposed from this study will be discussed in Chapter 4.

Chapter IV: Discussion and Future Directions

Section 4.1 – Summary

The clinical trial first cited in this thesis explored metformin as a potential therapeutic in HNSCC, and here we have found that NK cells are significant contributors to immune-oncologic activity. NK cells were more dramatically impacted than T cells outside of $T_{(EM)}$ cells, therefore we chose to further explore NK cell activation by metformin. Restoration of NK cell function is of emerging interest in cancer immunotherapy.[162] Patients with HNSCC often have impaired tumor immune surveillance, highlighted by increased quantities of regulatory T cells (T_{reg}) in the TME and impaired functions of T cells, NK cells, and NKT cells.[103] NK cells play a pivotal role in the anti-tumor innate immune response requiring strong stimulatory signaling by means of activating receptors that recognize stress-induced ligands on the surface of target cells. Immunotherapies designed to increase NK cell functionality have had mixed results, including an antibody to target the killer immunoglobulin-like receptor (KIR).[163] Here we report that patients with HNSCC have lower circulating subpopulations of NK cells with reduced functional capacity and expression of NKG2D receptors, which can be partially restored by metformin treatment. Importantly, circulating NK and NKT cells were reduced in our cohort of HNSCC patients. These NK cells exhibited reduced IFN-γ secretion, indicative of suppressed functionality. While insufficient NK cell activity in cancer is thought to be modulated by immunosuppressive mediators such as activation of certain STAT pathways, the correct balance of pathway activation has not yet been elucidated.[164] What is clear is that a reduction in NK cells is an indicator of poor survival in patients with advanced stage HNSCC, and future therapeutics

should target NK cells both directly and indirectly by impacting cytokine and chemokine balance.[87]

Metformin has been observed to be directly toxic to tumor cells mostly through AMPK activation and mTOR inhibition, but few studies have evaluated the anti-tumorigenic immune response to metformin in solid tumors in patients from clinical trials.[137] In our study, although there is no overall difference in white blood cell count (WBC) or absolute lymphocyte count (ALC) after metformin treatment, there was a larger population of T_{EM} cells after metformin treatment. Metformin also partially restored circulating NK and NKT cell populations with increasing expression of NKG2D back to levels seen in healthy controls. Analysis of the cytokine-profiles of patient serum suggested activated anti-tumor activity, highlighted by increasing IL-2 and TNF-α. However, given the paucity of patient samples and that most patients did well on clinical trial NCT02325401 (1-year OS and PFS were 90%) and survival data are not yet available for NCT02083692, we were unable to elaborate on whether metformin induced effects are correlated with improved survival in this study.[138]

Metformin activates AMPK which in turn reduces mTOR, a pathway upregulated and targeted in cancer cells but also important to maturation and function of immune cells.[137] Metformin has also been identified as a direct inhibitor of mTOR and pSTAT3. pSTAT3 upregulation is a negative prognostic factor in many solid tumors, and negatively regulates NK cell functions.[164,165] A recent publication indicated that mTORC1 inhibition by everolimus decreases NK cell maturation in peripheral NK cells in breast cancer.[166] The impact on tumor infiltrating cells was not explored and they found that despite lower maturation, NK cells had increased cytotoxic activity when mTOR is inhibited. Here, we also show in an *ex vivo* setting, metformin can rescue cytokine release and cytotoxicity of suppressed PBMCs and tumor infiltrating NK cells and that metformin mediated NK cellular cytotoxicity is dependent on mTOR inhibition but independent of AMPK.

RNA-seq analysis of metformin treated HNSCC patient-derived NK cells revealed potential alterations of pathways outside of AMPK. Interestingly, metformin significantly downregulated CXCL1, which is normally activated by pSTAT3 as well as NFκB and mTOR, in NK cells.[167] CXCL1 is a neutrophil recruiting chemokine that has been implicated as a negative prognosis factor in many cancers and is highly expressed by CD56dim NK cells. CXCL1 may recruit NK cells to a tumor site, but continued exposure results in increased pSTAT3 and NFκB, which can ultimately lead to exhaustion of NK cells.[94] As expected, metformin treatment inhibited pSTAT3 but also activated pSTAT1. Although increased pSTAT3 can lead to exhaustion of NK cells, exhaustion can be reversed by pSTAT1 activation.[94] Addition of exogenous CXCL1 reversed metformin mediated pSTAT3 inhibition and ablated metformin induced cytotoxicity supporting a role for CXCL1 as an important inhibitor of NK cell cytotoxicity through pSTAT3. Given metformin induced NK cellular cytotoxicity was mTOR dependent, it is possible that pSTAT1 activation occurs indirectly by mTOR inhibition or possibly directly by activating a STAT1 phosphorylase, as metformin is known to directly inhibit mTOR independent of AMPK.[94,168] CXCL1 inhibition by metformin could be reversed by both mTOR and pSTAT3 inhibition, indicating those pathways as related to possible NK cell dysfunction. Inhibiting these pathways at the source of CXCL1 activation using a CXCR2 inhibitor recovered metformin induced activation, indicating CXCR2 inhibitors could help activate dysfunctional cells.

Section 4.2 – Strengths and Weaknesses

The studies in this thesis are unique in several ways. RNA-seq with metformin treat NK cells identified a unique potential pathway of metformin, CXCL1. We used several inhibitors to support a previously undiscovered mechanism of action of metformin in HNSCC. We also had use of patient samples from clinical trials.

This study does have some limitations. NK cytotoxicity in response to metformin, CXCL1, and CXCR2 inhibitors has not yet been analyzed in a systemic context but was achieved as closely as possible with the *ex vivo* tumor infiltrating NK cells and tumor cell co-culture models. HPV status was not always matched when combining patient NK cells and HNSCC cell lines but given NK cells do not rely on MHC-1 and it has been previously shown there is no discernable phenotypical difference between HPV+ and HPV- NK cells, and we have shown matched patient NK cells and tumor cells produced similar results, matching likely was not a factor in cytotoxicity results. Patient numbers were low in some studies due to availability of samples, but use of primary NK cells for *ex vivo* NK studies with metformin is unique.[185] HNSCC patients are not homozygous and do not follow a set, normalized trend. This can cause a small sample size potentially represent a small portion of HNSCC patients, and not the overall cohort. It would be more relevant to use knockout models of our pathways to confirm their role, but knockouts in primary NK cells are difficult and still a work in progress in our lab. Although we used perforin as a measure of cytotoxicity mechanism, there are several ways NK cells can exert cytotoxicity that were not explored here. Future studies should look at NKG2D, a marker of activation, and Fas, a ligand for cytotoxicity.

Section 4.3 – Future Studies and Directions

Section 4.3.1 – CXCR2 inhibitors and Immunotherapy

Our study has shown that CXCL1 negatively impacts NK cell activation by metformin in HNSCC. We propose that inhibiting the receptor of CXCL1, CXCR2, could aid in recovering NK cell activity. Both inhibiting and activating CXCR2 and CXCR1 have been proposed and studied in solid tumors. CXCR2 and CXCR1 are migration activating receptors, therefore it has been debated whether increasing these receptors will inhibit activation or positively increase migration to the tumor.[151]

CXCR2 inhibition has been studied in lung, prostate, ovarian, and breast cancer models. In lung cancer mouse models, systemic CXCR2 inhibition decreased angiogenesis and tumor growth, but did not improve survival.[169,170] In prostate and ovarian cancers, VEGF and vascularization was decreased.[170,171] In pancreatic cancer mouse models, systemic inhibition of CXCR2 or CXCR2 combined with PD-1 antibodies decreased metastasis but did not significantly increase survival.[172],[173] In breast cancer, CXCR2 small molecule inhibitors reduced growth of mammospheres derived from patients in combination with immunotherapy and radiation.[174] HNSCC and renal cancer cells also had decreased proliferation when exposed to a dual CXCR1/CXCR2 inhibitor, and a mouse model showed decreased macrophage trafficking that improved NK cell transfer.[175,176]

CXCR2 inhibition has been less interrogated in patients. CXCR2 plays a critical role in wound healing, and inhibitors have been implicated in decreasing spinal injury healing and recovery from lung infections.[155,177] One inhibitor, AZD5068, is in clinical trials for prostate cancer (NCT03177187). In breast cancer, a window of opportunity trial utilized a dual CXCR2/CXCR1 inhibitor and found it safe, but outcome-based trials have not been proposed.[178] CXCR2 inhibition has not been studied in head and neck cancer, but clinical trials adoptively transferring T cells, stem cells, and NK cells with increased CXCR2 receptors are in progress

within melanoma and leukemia.[151,179] Migration to the tumor seems to be improved in early studies, but these knock-ins lose their CXCR2 receptors once in the tumor.[179] Therefore, CXCR2 may initially help with migration but negatively affect activity once migration occurs. No adoptive transfer studies of these cells have been completed in HNSCC.

Since NK cells have an autocrine loop of CXCL1, it may be beneficial to simply adoptively transfer healthy NK cells which do not produce high levels of CXCL1 compared to HNSCC NK cells. NK cells have garnered interest for adoptive transfer because they seemly do not need HLA matched and can have 'off the shelf' potential.[180,181] Cells could be expanded from a healthy donor, frozen, and reanimated for patient transfer when needed. These healthy transfers have been successful in leukemia, and clinical trials are active for solid tumors.[181] Additionally, NK immortal cell line NK92 has been evaluated for safety for adoptive transfer.[182,183] These cells have been deemed safe by a phase I clinical trial and further studies are in progress.[183] However, it is unclear how long these unaltered cells can last in patients and if they will become dysfunctional once exposed to the tumor environment.

Knock down CXCR2 NK cells have not been reported in literature at the time of this dissertation. We believe studying CXCR2 specific knockdown NK cells in primary and mouse models will help us further elucidate how this pathway could be therapeutic for patients.

Section 4.3.2 – Basic Science Directions

There is still much unknown about the mechanism of CXCL1/CXCR2 in NK cells. It is not clear why CXCL1 is the most significantly downregulated cytokine by metformin when many are affected by the JAK/STAT and mTOR axis. It could be that CXCR2 receptor activation is subsequently promoting other pathways involved in NK cell activation that overlap with metformin. Deep analysis, such as RNA-seq on NK cells treated with CXCR2 inhibition or that have CXCR2 knocked down, could reveal additional pathways CXCR2 impacts in the specific cell type.

CXCR2 has several ligands. CXCL2, 3, 5, and 6 and 8 are all ligands of CXCR2. All of these are increased on an RNA level in HNSCC tumors and 1,2,3, and 8 were all poor prognosis factors.[159] How these are altered in patients and if they change NK cell activation was not addressed in this thesis. Continued research on if these ligands also change NK cell response to the tumor could be valuable, especially in bolstering support for inhibiting the CXCR2 receptor instead of individual chemokines.

There has not been a CXCR2 knockout mouse model for head and neck cancer. It is unclear how a systemic knockout versus an NK cell specific knockout could change response to the tumor. A partner lab has an established HNSCC mouse model that utilizes MOC-1 tumor cells in either the flank or cheek of B6/C57 mice.[184] We have reported that these models do have elevated CXCL1 (**Appendix Figure 3**). These could be sufficient models to study CXCL1 in HNSCC. Future studies will establish a mouse model to test CXCL1 inhibition in preclinical mouse models and help determine how it could benefit the outcome for HNSCC.

Section 5.3.4 - Clinical Directions

We have concerns about CXCR2 inhibitor's impact on overall safety for patients. HNSCC patients are immunocompromised and impairing their ability to clear lung infections could be deadly. In addition, these patients are older on average and more prone to injuries. It would not be beneficial to inhibit neutrophil trafficking with this risk.

Before going through effort to target CXCR2, we will first need to determine if normal NK cell transfer in patients would be sufficient. These NK cells do not secrete CXCL1 in the abundance that HNSCC patients do, so they could prevent activation of the CXCL1 autocrine loop. However, HNSCC cancer cells do secrete CXCL1. It is unclear if normal NK cells would become dysfunctional once transferred to the patient. In future studies, we will expose normal patient NK cells to CXCL1 for an extended time and test functionality and CXCL1 secretion to determine if these cells become dysfunctional and if they begin the autocrine loop. We will also co-culture the cells with HNSCC tumor cell lines to determine if exposure to tumor cells can cause these normal cells to behave like HNSCC NK cells. We will analyze functionality by NKCA, IFNy, and perforin release.

Genetic alteration of NK cells is gaining popularity. CAR-NK cells are in production in many labs and there are 42 active studies on ClinicalTrials.gov at the time of writing determining the safety of these genetically mutated cells. In preliminary data, we have knocked out CXCR2 using CRISPR/Cas9 technology in normal NK primary cells. Confirmation of this knockdown and data can be found in the appendix (**Appendix Figures 4,5**).

Exposing normal cells to CXCL1 for 5 days slightly reduced activity. Knocking down CXCR2 in these cells protected them from this dysfunction. Interestingly, CXCR2 knockdown cells were significantly more active than their scrambled counterparts. We plan to repeat this experiment with more normal patients and complete the knockdown in HNSCC primary cells. We could eventually use expanded normal patient NK cells with CXCR2 KO to transfer into

patients, allowing for an ‘off the shelf’ NK cell therapy. To potentially avoid HLA mismatch, which has not been implicated as a problem in NK cell transfer, we also want to knockdown HNSCC cells to determine if they can recover to normal cell activity.

We also found that in patient plasma, pembrolizumab non-responders had higher CXCL1 post-treatment. Metformin did not reverse this increase. It could be possible to pair these CXCR2 KO NK cells with pembrolizumab to increase response rates if adoptive therapy is not enough on its own.

Section 4.3 – Conclusions

Metformin has proven to be a powerful tool for elucidating how NK cells can be activated by balancing several deactivating and activating signals within the cell. NK targeted studies have failed to achieve high and sustained NK activity.[91] We have determined that NK cell activation can be achieved by pSTAT1 activation by metformin, but can be reversed by errant pSTAT3 and mTOR, both of which are activated by NFkB dependent cytokines such as CXCL1. Many immunotherapies increase these NFkB dependent cytokines, and in turn increase both pSTAT3 and pSTAT1. Further studies are needed to determine how pSTAT1 can be increased independent of pSTAT3, and how this balance impacts NK cell functionality and vitality especially given the variable impact of metformin in different cell types and other systemic consequences.[137] Importantly, blocking the CXCL1 receptor, CXCR2, restores NK cell activity. Combining a CXCR2 inhibitor, which would reduce activity of pathways that inhibit NK cell activity, with immunotherapies that increase positive pathways could be useful for future head and neck cancer treatments.

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Addendum – COVID-19 and Immunotherapy in Cancer

Section A.1 – Reasoning for this addendum

The following work is adapted from a manuscript in submission. It is outside the main dissertation's scope, so it has been included as an addendum for more insight into a full project outside the thesis.

Section A.2 - Background

In early 2020 the SARS-CoV-2 virus, which causes COVID-19, became a pandemic of global concern. As of July 2022, there have been 582,600,000 cases worldwide, 92 million of those in the US [1]. COVID-19 is a respiratory disease that can present with either no symptoms or a wide range of mild to severe symptoms and has caused high rates of mortality [2]. Patients with a history of and those with active cancer infected with SARS-CoV-2 have worse disease severity and higher mortality rates than non-cancer patients [3,30-35]. Importantly, cancer patients are at particular risk for infection and death from SARS-CoV-2 due to frequent clinic visits, immunosuppression, and existing inflammation from cancer [4]. In addition, immunotherapy may potentiate immune hyperactivation leading to cytokine storm [4,30].

Cytokine storm is a disorder in which overproduction of cytokines, such as IL-6 and IL-10, cause immune-related tissue injury and sometimes death. Importantly, cytokine storm has been observed in COVID-19 patients [5,30]. Immune checkpoint inhibitors (ICIs), often used in several types of solid tumors, result in cytotoxic immune cell activation but have a low baseline incidence of cytokine storm (58 cases out of 80,000 patients) [6]. Activation of the NFκβ pathway, a key transcription factor responsible for inflammation in cancer, can increase the risk of cytokine storm [7]. NFκβ is increased in immune cells, such as T-cells and NK cells exposed to the SARS-CoV-2 spike (S) protein [8]. S protein facilitates viral fusion by binding the ACE2 receptor on the host cell and is found on the surface of the SARs-CoV-2 virus [8]. Pembrolizumab, a PD-1 inhibitor, is also known to increase NFκβ in T-cells in several types of

cancer [15]. Additionally, patients who were recently treated for cancer, regardless of therapy, had higher mortality than those who were untreated [31]. Therefore, we hypothesize that continuing ICIs during COVID-19 disease could lead to an increased risk of cytokine storm and mortality (**Fig A.1**).

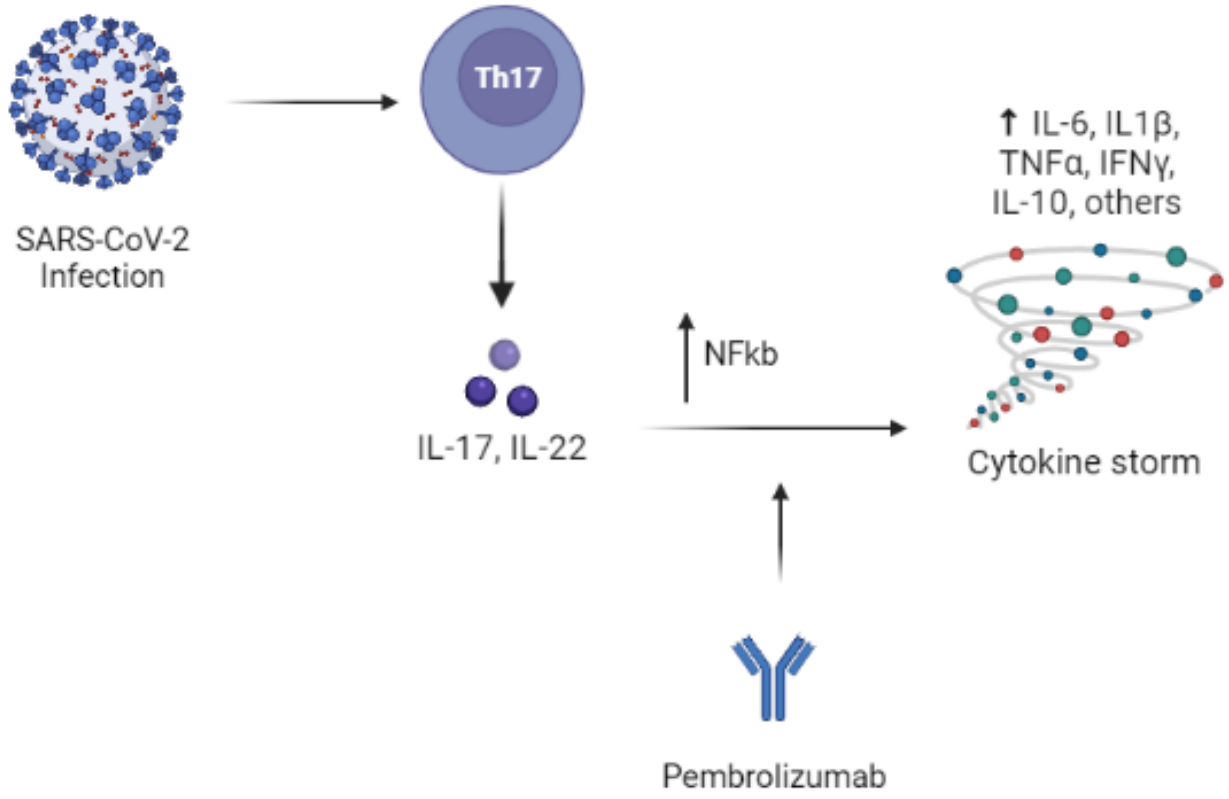

Figure A.1: Cytokine Storm, COVID-19 disease, and Immunotherapy. Many immunotherapies increase production of cytokine storm inducing cytokines, such as IL-6 and IL-10.

Although early observational studies linked ICIs to increased COVID-19 disease severity in cancer patients, subsequent investigations demonstrated that ICIs do not necessarily result in increased COVID-19 disease severity independently, and death outcomes for cancer patients can be dependent on a number of factors [9-14, 30-35, 37, 38]. However, the mechanism and direct effect of ICI treatment on PBMC cytokine release in patients with COVID-19 has only

recently been investigated for use in non-cancer patients to restore exhausted T-cells [36]. Given that ICIs are now commonly used in oncology practice, understanding any potential risk of ICIs exacerbating COVID-19 related cytokine storm would better guide treatment in this population.

Section A.3 - Materials and methods

Section A.3.1 Human samples

Peripheral blood and plasma were obtained from adult patients from the University of Cincinnati Biorepository (UCB). Additional *ex vivo* studies were performed on age matched peripheral blood obtained from IRB approved studies UCCI-UMB-14-01 (IRB #2014-4755), Cincinnati COVID Biorepository #2020-0329 and general specimen collection protocol (IRB #2017-2137). Adult human subjects who presented to the University of Cincinnati Medical Center between April 2020 and December 2020 with symptoms indicative of COVID-19 disease (shortness of breath, fever and/or cough) and found to be SARS-CoV-2 PCR positive were approached and those willing provided written informed consent. Samples were collected from consented patients with mild (outpatients or patients seen in the ER and sent home), moderate (admitted to the floor), or severe (admitted to the medical ICU) COVID-19 disease within 10 days of their acute presentation. The studies were approved by the Institutional Review Board at the University of Cincinnati and were conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was received from all participating patients prior to enrollment. Additional de-identified plasma samples were obtained from Case Western Reserve's COVID-19 Biorepository under IRB STUDY20200517. Disease severity for COVID-19 is defined as follows: 0 = Outpatient, 1 = Hospitalization, 2 = Oxygen, 3 = Intubation, 4 = ICU, 5 = Death. This scale is referenced from the COVID-19 and Cancer Consortium [10].

Section A.3.2 Peripheral blood mononuclear cell (PBMC) isolation and storage

Peripheral blood was received in ethylenediaminetetraacetic acid (EDTA) tubes and serum separator (SST) tubes. EDTA samples were processed to isolate PBMCs using Ficoll-Paque PLUS (GE-Healthcare Life Sciences) density gradient centrifugation. PBMCs were cryopreserved in Cryostor CS10 (Stem Cell Technologies) or Fetal Bovine serum (FBS) + 10% dimethyl sulfoxide (DMSO). SST samples were spun at 1200xg for 15 min and plasma was collected into microcentrifuge tubes stored at -80°C. Samples received from Case Western were stored at -80°C until use. All sample preparation was done within 24hrs of sample collection.

Section A.3.3 Flow cytometry for cell markers

Flow cytometry was performed on PBMCs that were thawed quickly for 2min in a 37°C water bath. Cells were then washed in PBS, stained for viability with fixable viability dye (Invitrogen), then washed with flow buffer (FB) (1x phosphate buffered saline (PBS) + 2% FBS) and fixed for 1hr at room temperature (RT) in 2% paraformaldehyde (PFA), (Electron Microscopy Sciences, Hatfield, PA) in PBS. Cells were then washed and stained with the appropriate antibodies for 15min at 4°C in the dark, washed in FB, and fixed in 2% PFA in PBS and kept at 4°C in the dark until analyzed by flow cytometry. Flow cytometry was performed using a BD Fortessa. Data were analyzed with FlowJo V10. The following antibodies were used: CD19 (HIB19, Biolegend), CD56 (5.1H11, Biolegend), CD16 (3G8, Biolegend), Live/dead (L34989,Invitrogen), CD8 (SK1, Biolegend), CD45 (HI30, Biolegend), and CD4 (A161A1, Biolegend). Populations are defined as follows: Cells were gated into live/dead and gated for CD45+. CD45+ cells were divided into CD3+/- and CD19+/-. CD3+ were gated into CD4+ and CD8+. CD3- were gated into CD16+CD56+dim, and CD16-CD56+ bright.

Section A.3.4. ELISAs

ELISA kits for IL-6, IL-8, IL-4, TNF□, and INF□ were obtained from R&D Systems. ELISA plates were coated by using mid-adhesion 96-well plates and dilution of capture antibody indicated by lot specific dilution. Coating was allowed to adhere overnight. Capture antibody was removed and washed with PBS containing 0.05% Tween-20. Blocking buffer (PBS with 1% bovine serum albumin [ThermoFisher]) was added to wells for 1hr and washed. Samples (plasma or supernatant, undiluted) and standards diluted per lot specifications were added to wells and incubated for 2hrs at RT. Samples were washed and detection antibody diluted per lot specifications was added to each well for 2hr. Detection antibody was washed away and streptavidin diluted per lot specifications was added to wells for 20min. Streptavidin was washed off and replaced with TMB ELISA (ThermoFisher) for 10min. Stop solution of 2N sulfuric acid [Selleck Chem] was added and plate was read at 420nm wavelength.

Section A.3.5 Bulk Cytokine Analysis

Plasma or supernatant were thawed on ice and spun at 300xg for 5min. 11uL were loaded into individual wells of IsoPlexis Codeplex chips (IsoPlexis, Branford, CT) and loaded onto the Isolight machine for analysis of cytokine levels including GM-CSF, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, IL-17A, IP-10, MCP-1, MIP-1α, MIP-1β, Perforin, and TNF-α.

Section A.3.6 Spike Protein Experiments

PBMCs from Head and Neck Cancer (HNC) patients or healthy donors were thawed in 37°C water bath for 2min and added to 5mL RPMI with 10% FBS and 1% Pen/Strep. Samples were spun for 5min at 400xg and resuspend in complete RPMI with 100U/mL IL-2 (Peprotech, 200-02). Cells were then incubated overnight. Spike protein (Acros biosystems, SARS-CoV-2(COVID-19) S protein (R683A,R685A), and His Tag 100ug, Cat# SPN-C52H4, Lot# 3534b-2042F2-RA) were stored at -80°C and thawed on ice. PBMCs were exposed to 10nM spike protein at a density of 200,000 cell/mL for 20min at 37°C. Spike protein was removed by

washing, cells were resuspended in complete RPMI containing 100U/mL IL-2 and vehicle or pembrolizumab at a concentration of 20ng/mL for 24hrs. Supernatant was collected for ELISA analysis and cells were collected for western blot analysis. In experiments where SARS-CoV-2 peptide is indicated as used, the same protocol was followed with peptide from Miltenyi mimicking the alpha variant (Catalogue #130-127-844).

Section A.3.7 Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with 1x protease inhibitor (Thermo Scientific, Cat #78440) for 10min and analyzed for protein content by bicinchoninic acid (BCA) kit (Thermo Fisher Cat #23225). 50ug protein was loaded onto Biorad pre-cast gels and ran for 1.5hrs at 85V, transferred for 1hr at 100V, and blocked in 5% BSA in Tris-buffered saline (TBS) for 1hr. Blots were washed with TBS-T (Tris Buffered Saline + Tween 20) and NFκβ (Cell Signaling, Ca t#6956), pNFKB (Cell Signaling, Cat# 3033) and Actin (Cell signaling, Cat #4970) diluted 1:1000 were added to blots overnight. Blots were washed in TBS. Licor Secondary (Cat #926-32213 and 926-68072) was added for 1hr and washed. Blots were imaged Biorad Chemidoc apparatus. Signal was obtained in Image J.

Section A.3.8 Immunotherapy treatment

PBMCs from the Cincinnati COVID-19 Repository (CCR) were thawed, washed in complete RPMI, and incubated overnight in complete RPMI with 100U/mL IL-2. 100,000 cells were plated in 100uL RPMI with 100U/mL IL-2 and 20ng/mL pembrolizumab or vehicle for 24hrs. Supernatant was collected for bulk cytokine analysis and cells were collected for flow cytometry.

Section A.3.9 Ethics statement

Samples were collected under IRB approved studies UCCI-UMB-14-01 (IRB #2014-4755), Cincinnati COVID Biorepository #2020-0329 and general specimen collection protocol (IRB #2017-2137). The studies were approved by the Institutional Review Board at the University of

Cincinnati and were conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Additional de-identified plasma samples were obtained from Case Western Reserve's COVID-19 Biorepository under IRB STUDY20200517. Written informed consent was received from all participating patients prior to enrollment.

Section A.3.10 Statistical analysis

All data was analyzed in GraphPad Prism V9. One-way ANOVA was utilized were multiple data sets were used and unpaired students t-test for two data sets.

Section A.4 – Results and discussion

Section A.4.1 Cancer patients with COVID-19 disease produce inflammatory cytokines associated with cytokine storm.

Many patients with solid tumors have high levels of plasma inflammatory cytokines that promote tumor growth. For example, high levels of IL-6 have been correlated with a poor prognosis [16,17]. Cancer patients are uniquely susceptible to COVID-19, with high mortality rates, but it is unclear if pro-tumorigenic and inflammatory cytokines are altered upon infection with SARS-CoV-2. We used bulk cytokine analysis to investigate cytokine levels directly in the plasma from normal aged matched healthy patients, non-cancer patients with COVID-19, mixed cohort cancer patients, and mixed cohort cancer patients with COVID-19 (**Fig A.2**). IL-4, a cytokine with a role in T-cell and T-reg regulation, was decreased in COVID-19 and cancer patients compared to normal healthy patients ($p = 0.043$) [18]. Most other cytokines were not significantly changed. However, IL-2, IL-10 and IL-15, inflammatory cytokines involved in cytokine storm, were significantly increased in patients with COVID-19 disease and cancer compared to healthy controls [19]. Interestingly, IL-10 levels were increased in those with both cancer and COVID-19, while IL-15 was increased in COVID-19 disease only and COVID-19 and cancer.

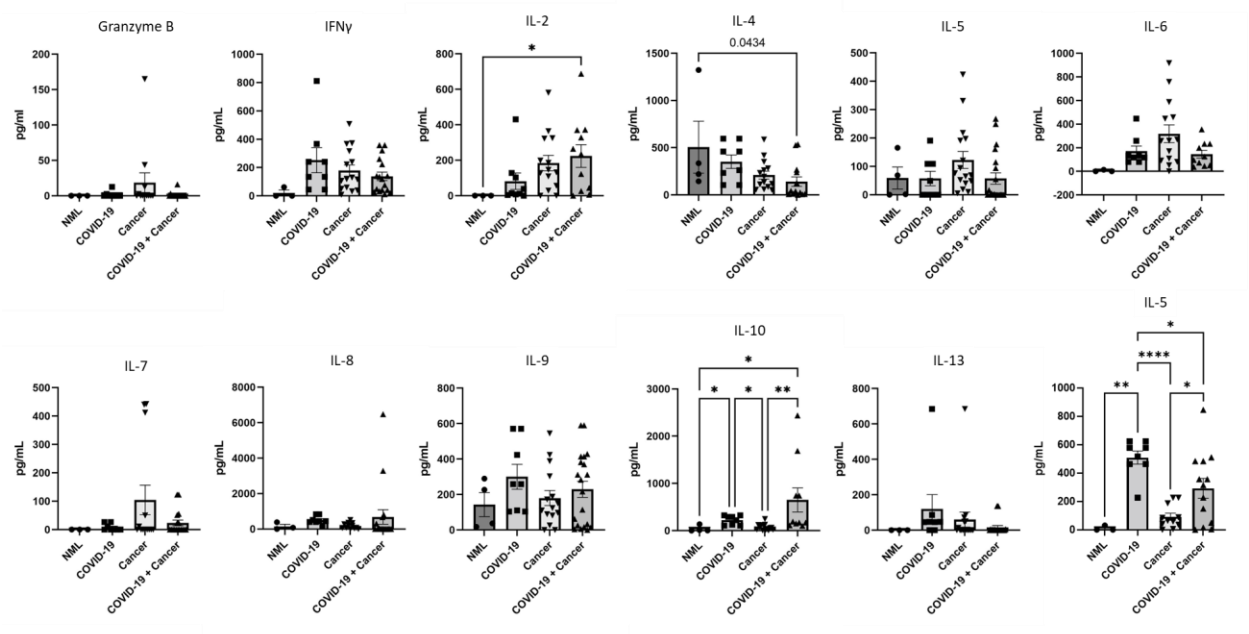

Figure A.2: Bulk analysis of plasma inflammatory cytokines. Healthy donor (n=5), COVID-19 only (n=3), mixed cohort cancer patients (n=16) and mixed cohort cancer patients with COVID-19 (n=17) plasma were analyzed by bulk cytokine analysis. Significance is P = 0.05 in a one-way ANOVA.

Section A.4.2 Pembrolizumab enhances spike protein mediated cytokine release from head and neck cancer but not healthy patient PBMCs *ex vivo*.

Although cytokines appear to be increased in patients with COVID-19 disease and cancer, it remained unclear the effect, if any, of systemic therapy on harmful cytokine production. Due to concerns that ICIs might enhance cytokine release in patients with COVID-19 and cancer, we exposed PBMCs derived from aged-matched healthy donor and head and neck cancer (HNC) patients with vehicle, spike protein (a capsid protein of SARs-CoV-2 that allows virus cellular entry by binding the ACE-2 receptor), pembrolizumab (PD-1 inhibitor), or the combination for 24, 48 or 72hrs *ex vivo*. Supernatant was collected to evaluate cytokine release (**Fig A.3**). Similar to detection in plasma from mixed cancer cohort and COVID-19 patients, spike protein alone did result in induction of IL-2 release from HNC patient PBMCs at 24hrs (**Fig A.3**). However, pembrolizumab treatment of HNC cancer patient PBMCs in combination with spike protein resulted in significantly enhanced cytokine release in several cytokines observed at various

timepoints including IL-2, IL -4, IL -6, IL -8 and IL -10 compared to control, but other cytokines were relatively unaffected. IL-8 had a significant increase upon exposure to spike protein in healthy PBMCs, but no other changes in cytokines were observed.

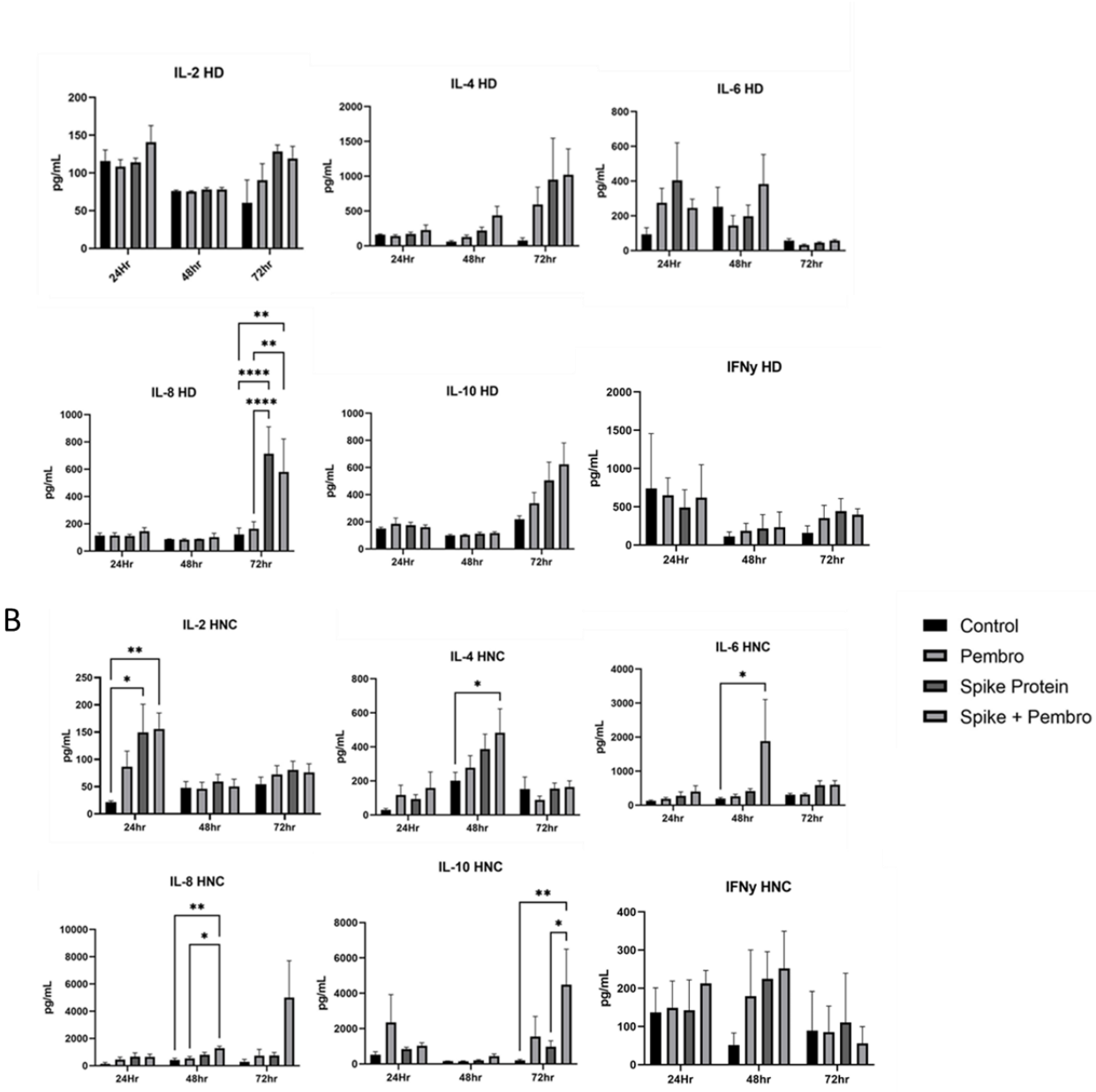

Figure A.3: Spike protein and pembrolizumab effect inflammatory cytokine release. HNC (A) and healthy (B) PBMCs were treated with vehicle, 20ng/mL pembrolizumab, 10ng spike protein, or both for 24, 48 or 72hrs. Supernatant was collected and analyzed by ELISA. N = 10 for HNC and n = 5 for NML. Data was analyzed by two-way ANOVA.

Section A.4.3 NFkB phosphorylation is enhanced in the presence of spike protein and pembrolizumab in HNC PBMCs.

NFκβ is a regulator of many inflammatory cytokines and activation of NFκβ causes the production of cytokines such as TNF-α, IL-1β, IL-6, IL-8, and MCP-1, all of which can exacerbate cytokine storm [24,25]. Spike protein has been known to increase NFκβ activation [20,21]. Pembrolizumab has also been implicated in enhanced NFκβ activation due to immune activation [15]. We exposed HNC and aged matched normal patient PBMCs to vehicle, spike protein, pembrolizumab, or the combination for 24hrs and analyzed NFκβ protein levels. HNC samples exposed to both pembrolizumab and spike protein had a higher pNFκβ/NFκβ ratio compared to vehicle alone, but spike protein alone did not increase NFκβ compared to vehicle. Pembrolizumab, but not spike protein, increased pNFκβ in normal patient PBMCs (**Fig A.4**).

HNC patient PBMCs also had higher pNFκβ at baseline

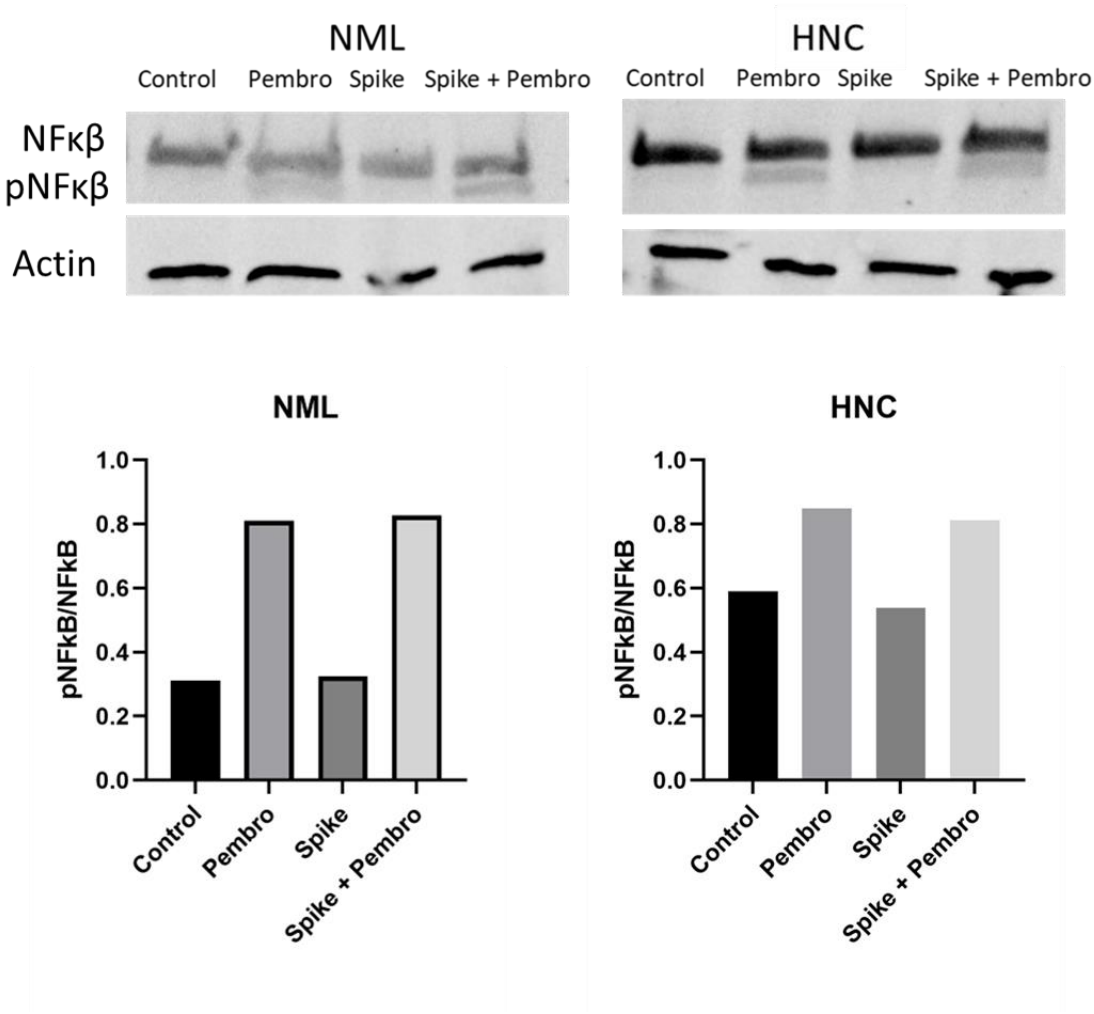

Figure A.4: Evaluation of NFkB levels upon treatment with spike protein and pembrolizumab. Healthy normal and HNC PBMCs were treated with vehicle, 20ng/mL pembrolizumab, 10ng spike protein, or combination for 24hrs and collected and analyzed by Western Blot analysis.

Section A.4.4 Patients with cancer and COVID-19 have increased cytokine producing NK cells which are not changed by pembrolizumab treatment.

Given the minimal change in cytokine release detected in patients treated with PD-1 inhibitors *ex vivo*, we also explored the composition of peripheral immune cells in patients with mixed cancer cohorts and COVID-19 and potential change upon pembrolizumab treatment. Interestingly, we found that mixed cohort cancer patients with COVID-19 (n=3) have significantly more cytokine producing NK cells ($CD56^{bright}$ CD16-) than non-cancer age matched COVID-19

patients (**Fig A.5**). Treatment with 20ng pembrolizumab for 24hrs *ex vivo* did not change the distribution of NK cells. Of the patients included in this study, those who died from COVID-19 complications also had, on average, higher percentages of cytokine producing NK cells (**Fig A.5**). No change in T cell populations was observed including no change in cytotoxic and helper T-cells. However, it was not clear if cytokine-producing NK cells were responsible for the release of cytokines observed in patient plasma. To simulate infection *ex vivo*, we subjected healthy or HNC PBMCs to spike protein, pembrolizumab or combination for 24 hours and determined cytokine expression in immune cell subsets by flow cytometry.

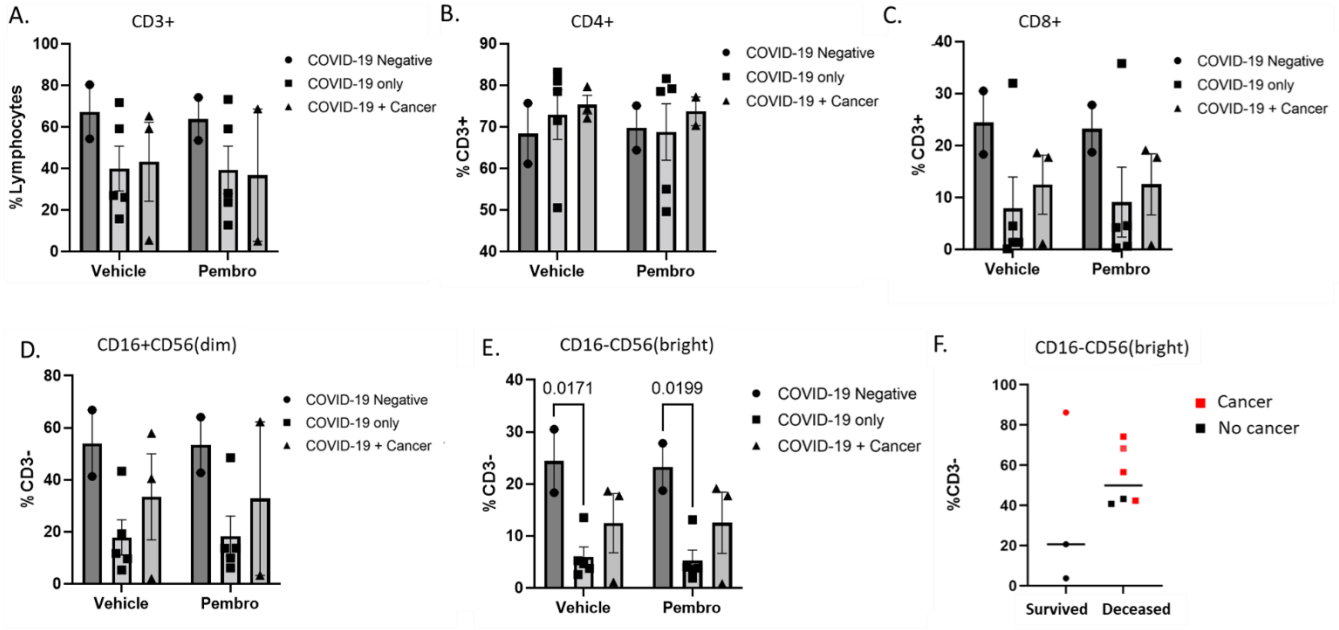

Figure A.5: Distribution of peripheral immune cell populations. PBMCs from healthy controls (n=2), COVID-19 only (n=5) or mixed cohort cancer and COVID-19 (n=3) patients were treated with vehicle or 20ng/mL Pembrolizumab for 24hrs and collected for flow cytometry (**A)** Percentage of total T cells. **(B)** Percentage of CD+4 T cells. **(C)** Percentage of CD8+ T cells. **(D)** Percentage of CD16+ NK cells. **(E)** Percentage of CD16- NK cells. **(F)** Percentage of CD16- NK cells divided by survival. Data (**A-E**) analyzed by one way ANOVA and (**F**) analyzed by student's t-test.

To determine if pembrolizumab would increase cytokine production in PBMCs collected from patients with COVID-19 disease, PBMCs from a mixed cohort of patients with COVID-19 with or without cancer were treated with vehicle or pembrolizumab and supernatant was analyzed by bulk cytokine analysis. There was no significant change in cytokine production of inflammatory cytokines INF□, TNF□, perforin, IL-6, IL-9 and IL-10 upon pembrolizumab treatment (**Fig A.6**). Other cytokines observed in patient plasma were below the level of detection in the supernatant.

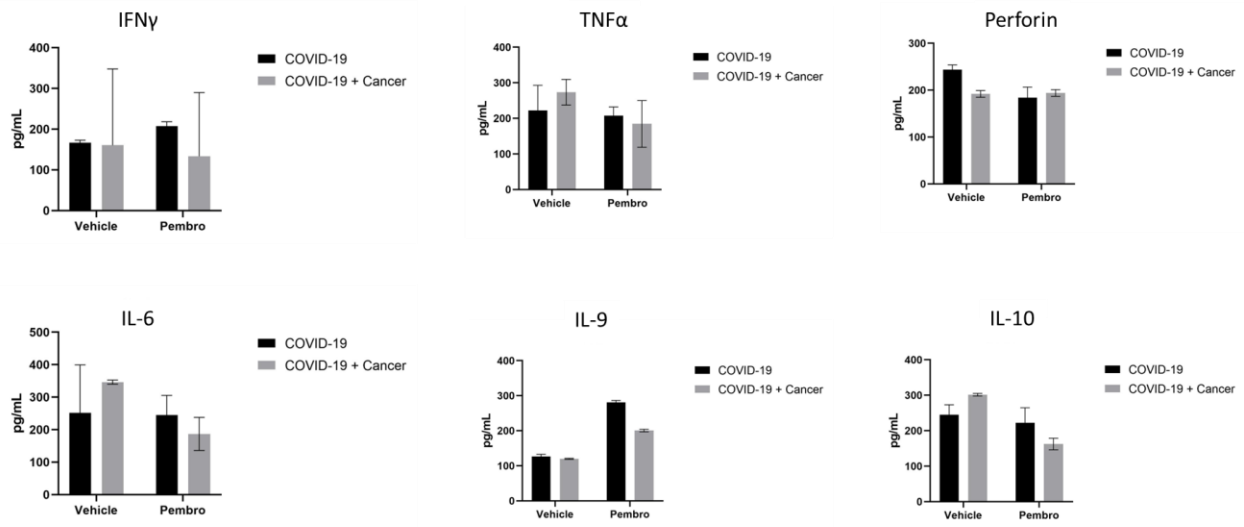

Figure A.6: PBMC Cytokine Production upon pembrolizumab treatment. PBMCs from COVID-19 patients +/- mixed cohort cancer were treated with vehicle or 20ng/mL pembrolizumab for 24hrs and collected for cytokine analysis. n=4 for COVID-19 and n=3 for cancer + COVID-19. Samples were analyzed by paired students t-test when analyzing vehicle vs pembrolizumab and unpaired students t-test when analyzing COVID-19 vs cancer + COVID-19 disease.

Given no observable changes of cytokine release with pembrolizumab treatment of PBMCs *ex vivo*, we then analyzed patient plasma amongst a mixed cohort of cancer patients with COVID-19 disease to determine if those on ICIs had increased inflammatory cytokines compared to those on immunosuppressive or no therapy (**Fig A.7**). There was no significant change in cytokines or disease severity (see Methods), however, analysis was limited by low numbers of patients.

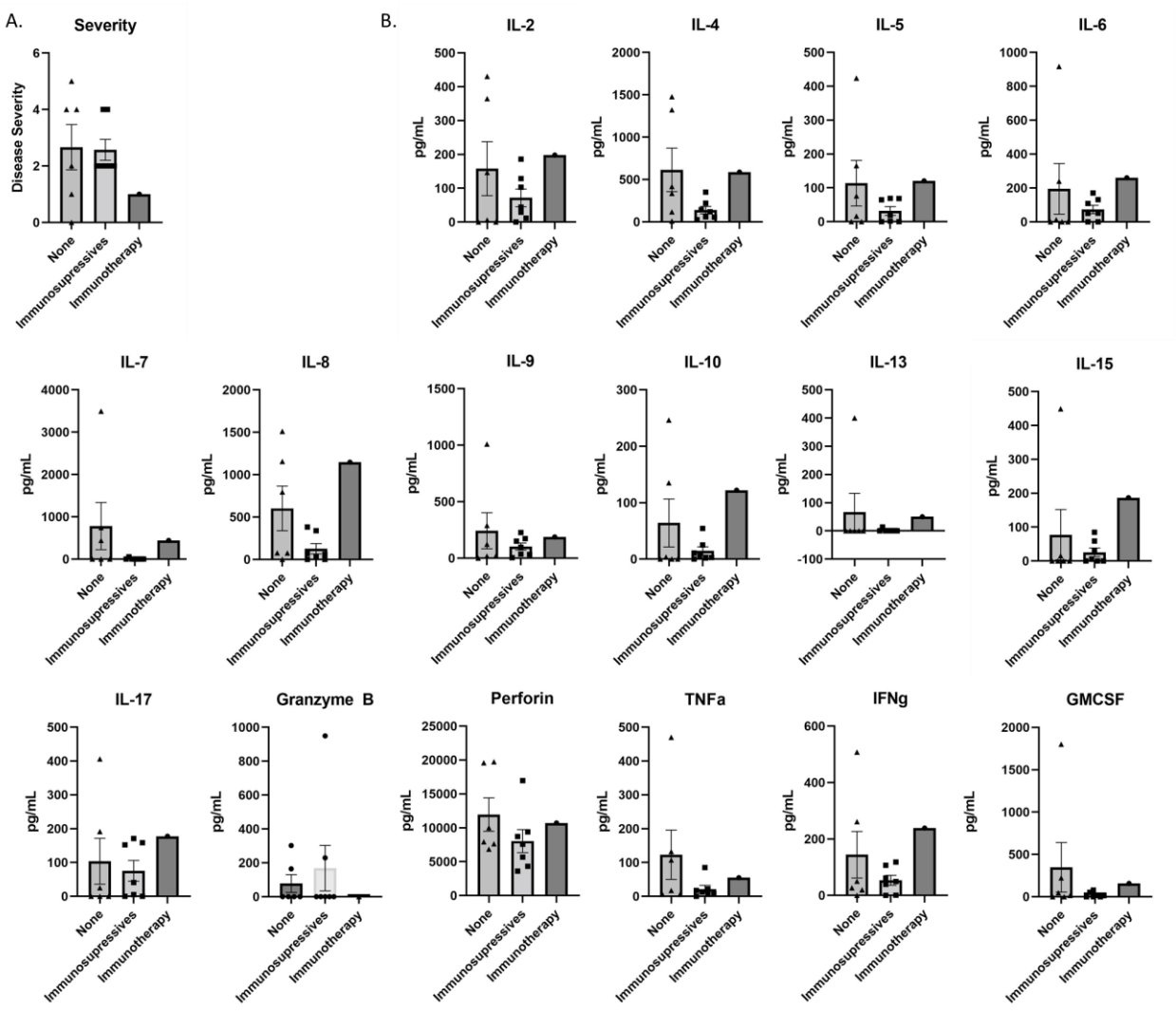

Figure A.7: Cytokine secretion within cancer patients with COVID-19 disease. (A) Disease severity (0 = Outpatient, 1 = Hospitalization, 2 = Oxygen, 3 = Intubation, 4 = ICU, 5 = Death) from Cancer and COVID-19 patients. (B) Plasma cytokine levels from mixed cohort of cancer and COVID-19 patients. Patients included were those on immunotherapy (n=1), those on immunosuppressive treatment (n=7), and those receiving neither (n=6). (A, B) data analyzed by one way ANOVA.

Section A.5 – Summary

Even with increased vaccination rates, COVID-19 remains a highly transmissible disease that could prove deadly to immunosuppressed persons such as those with cancer. In addition, many anti-cancer treatments alter the immune response. Therefore, evaluating how cancer treatment could affect COVID-19 disease severity in patients with cancer is relevant.

In general, we observed higher inflammatory plasma cytokines in patients with COVID-19 disease (**Fig A.2**). IL-2, IL-10 and IL-15 were significantly higher in COVID-19+ cancer patients, and anti-inflammatory cytokine IL-4 was significantly reduced. However, other cytokines commonly associated with cytokine storm (IL-6, IL-17, IFN-□) were not higher in patients with cancer and COVID-19 (**Fig. A.2**), suggesting that although cancer patients may have worse outcomes in general with COVID-19, they do not seem to be at particular risk for cytokine storm compared to patients without cancer.

We also investigated if inflammation and subsequent cytokine release can be exacerbated by the common immune checkpoint inhibitor (ICI), pembrolizumab. Pembrolizumab, when combined with SARS-CoV-2 spike protein *ex vivo,* did result in enhanced release of IL-2, IL-6, IL-8 and IL-10, major players in cytokine storm, from HNC PBMCs compared to healthy PBMCs [18]. However, pembrolizumab treated PBMCs from patients with COVID-19 disease, with and without cancer, did not result in increased cytokine release (**Fig. A.6**). Although a key mediator in inflammation and cytokine storm, NFκβ, was upregulated upon *ex vivo* treatment with spike protein of HNC PBMCs, and pembrolizumab did not further enhance this effect (**Fig. A.4**). Overall, this compilation of data supports previous clinical reports that cancer patients with COVID-19 are not at increased risk of cytokine storm and enhanced inflammation upon treatment with ICIs [26,27].

Cancer patients have been reported to have lower levels of circulating NK cells, which correlates with a poor prognosis [28]. Interestingly, COVID-19 patients had lower levels of NK

cells in general, but those with mixed cohorts of cancer and COVID-19, had a higher proportion of $CD56^{bright}$ CD16- cytokine producing NK cells than COVID-19 only patients (**Fig A.5**). In addition, those with COVID-19 that succumbed to the disease had the highest levels of cytokine producing NK cells (**Fig A.5**). Importantly, it was previously reported that the more cytokine producing NK cells observed in patients during early COVID-19 disease, the more likely they were to have severe disease or death.[19] However, our data suggests that although cytokine producing NK cells were higher in patients with cancer and COVID-19 disease, these cells were not necessarily responsible for observed changes in cytokine release. It is plausible they may be influencing other immune cell types, causing enhanced cytokine production. Immunotherapy has also been observed to increase all NK cell types, including cytokine producing NK cells [20]. However, we did not observe any change in the cytokine producing NK cells in the 24 hours following treatment with pembrolizumab again supporting that pembrolizumab does not contribute to poor prognostic factors of COVID-19 disease.

Our study does have limitations. While the spike protein model we used is a safe and easily utilized model for COVID-19, it cannot completely mimic a systemic SARS-CoV-2 infection. Our *ex vivo* model may not be representative of all patients with cancer. For example, COVID-19 disease is often more severe in lung and breast cancers than HNC, which was our predominant *ex vivo* model [30-32]. Due to small numbers of available patient samples, we could not control for several demographic factors including potential previous therapy which likely resulted in variability. Due to collection limitations, some samples were collected across different cohorts that could add to pre-analytical changes caused by differences in PBMC processing and collections at various points in COVID-19 disease progression. However, despite these limitations, pembrolizumab consistently did not worsen correlates of cytokine storm and, therefore, worse COVID-19 outcomes are not expected in cancer patients treated with ICIs, consistent with current available clinical information.

Section A.5 – Addendum References

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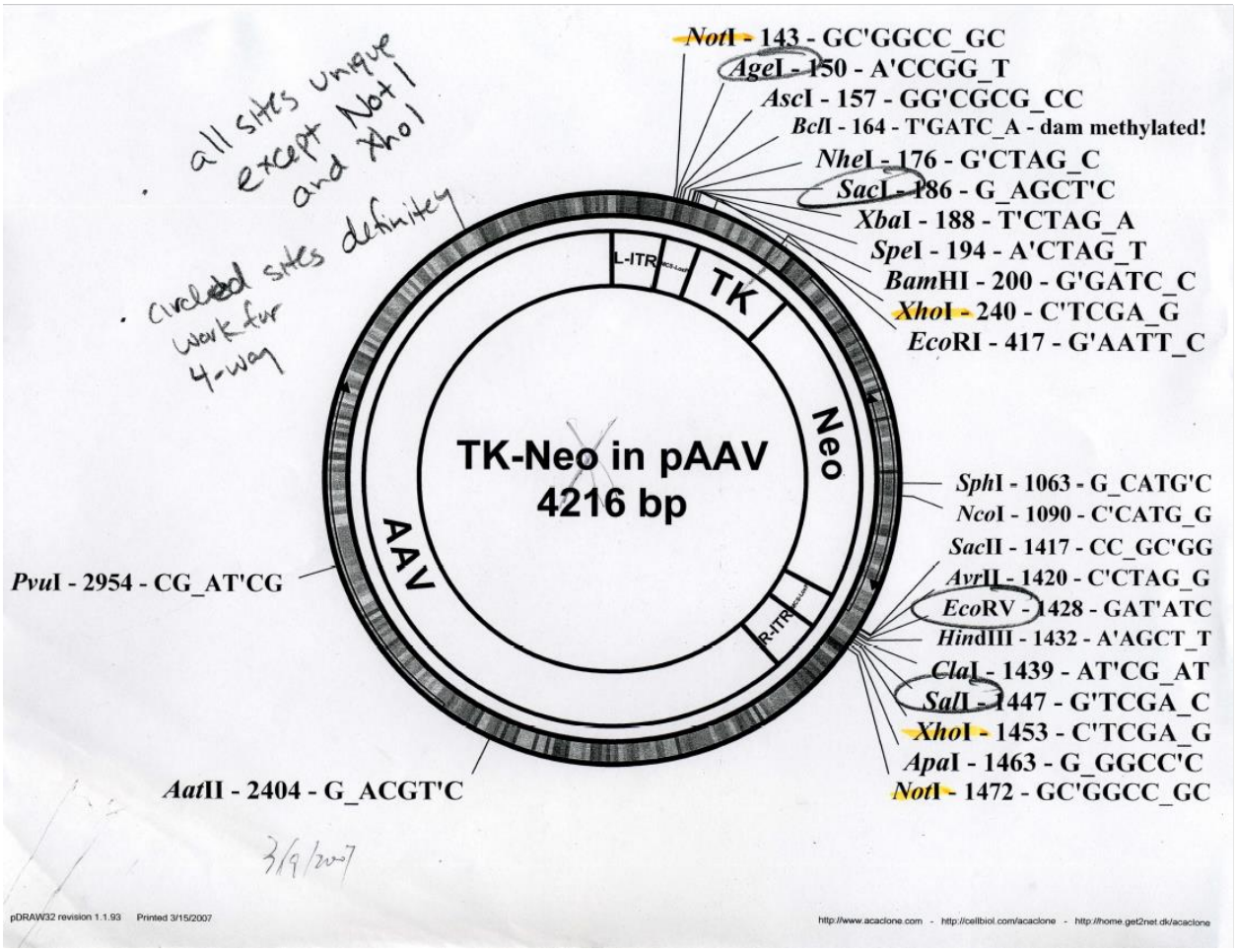
Appendix

Appendix Table 1: Demographics for clinical trial NCT02325401

Pt. No	Sex	Age	Race	TNM	Stage	Site	Outcome	Outcome-2
1	M	53	Caucasian	T3N1M0	III	Larynx	Alive	No progression
3	M	46	Caucasian	T1N2bM0	IVA	Oropharynx	Alive	No progression
5	M	47	Caucasian	T1N2bM0	IVA	Oropharynx	Alive	No progression
7	M	62	Caucasian	TN2bM0	IVA	Oropharynx	Alive	No progression
8	M	61	Caucasian	T2N2cM0	IVA	Oropharynx	Alive	No progression
10	M	65	Caucasian	T4aN2M0	IVA	Oropharynx	Alive	No progression
12	M	55	Caucasian	T1N2bM0	IVA	Larynx	Alive	Progressed
13	M	59	AF	T4aN0M0	IVA	Oropharynx	Alive	No progression
15	F	47	AF	T2N2cM0	IVA	Larynx	Alive	No progression
16	F	34	Caucasian	T2N2bM0	IVA	Oropharynx	Alive	No progression
17	M	64	Caucasian	T3N3M0	IVB	Oropharynx	Alive	No progression
18	M	54	Caucasian	T4aN2bM0	IVA	Oropharynx	Alive	No progression
20	M	58	Caucasian	T4aN3M0	IVB	Oropharynx	Alive	No progression
21	M	57	Caucasian	T4aN2cM0	IVA	Oropharynx	Alive	No progression

Appendix Table 2: Demographic information for clinical trial NCT02083692

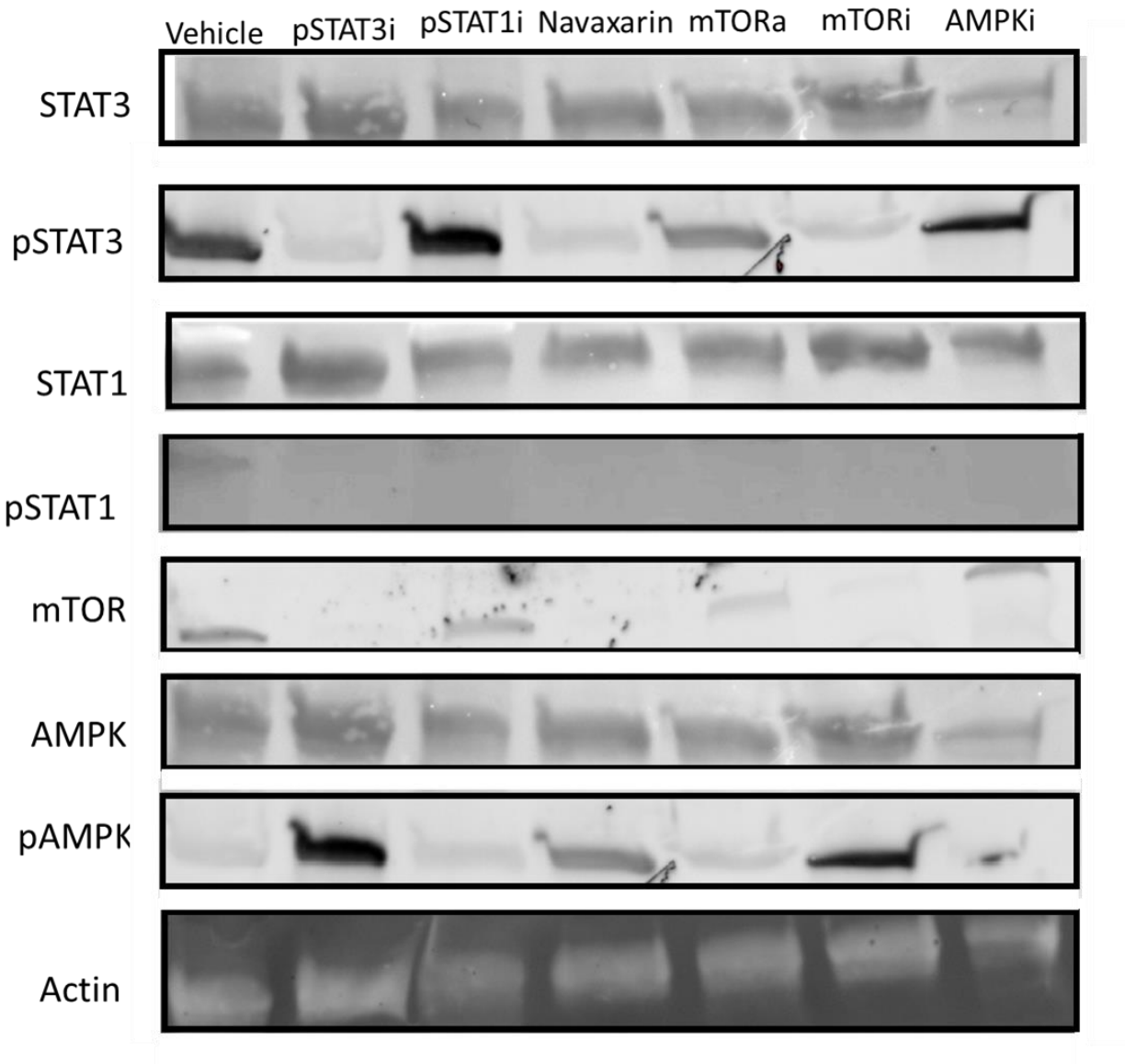
Pt. No	Sex	Age	TNM	Stage	Site	HPV Status
33	Male	49	T3N2aM0	II	Oropharynx	Positive
34	Male	61	T3N2bM0	III	Oropharynx	Positve
36	Male	54	T4aN0M0	II	Larnyx	Not Assesed
39	Male	59	T1N1M0	II	Oral Cavity	Not Assesed
40	Male	52	T1N0M0	II	Larnyx	Not Assesed
45	Female	66	T4aN2bM0	II	Oral Cavity	Not Assesed
46	Male	59	T1N3M0	III	Oropharynx	Not Assesed
48	Female	60	T1N0M0	II	Larnyx	Not Assesed
50	Male	60	T2N2bM0	II	Oral Cavity	Not Assesed



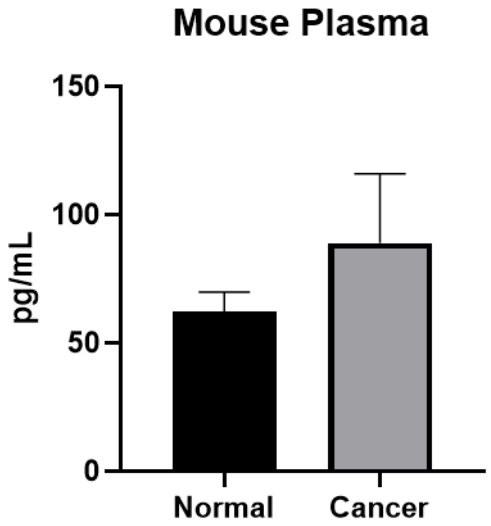
Appendix Figure 1: Vector map for pSTAT3 knockout

Appendix Table 3: List of non-adjusted p-value genes from RNA-seq

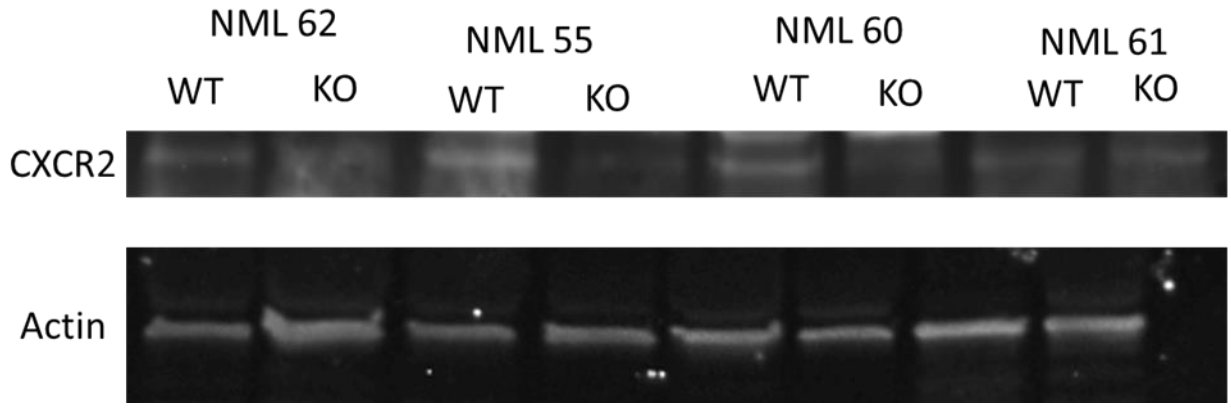
geneid	symbol	name	baseMean	baseMeanC	baseMeanM	foldChange	log2FoldChange	pval	padj
2919	CXCL1	C-X-C motif chemokine ligand 1	131.85	255.32	8.38	0.02	-5.40	1.12E-08	0.00017
10628	TXNIP	thioredoxin interacting protein	3260.46	4333.93	2186.99	0.50	-1.00	2.89E-06	0.02202
1890	TYMP	thymidine phosphorylase	8224.79	10723.30	5726.29	0.54	-0.88	2.54E-05	0.11552
6369	CCL24	C-C motif chemokine ligand 24	714.37	1238.87	189.88	0.15	-2.69	3.04E-05	0.11552
94240	EPSTI1	epithelial stromal interaction 1	1842.49	2401.38	1283.60	0.54	-0.88	5.88E-05	0.13964
83463	MXD3	MAX dimerization protein 3	353.73	178.22	529.25	2.94	1.55	6.21E-05	0.13964
1.01E+08	SMG1P7	SMG1 pseudogene 7	373.92	218.06	529.79	2.41	1.27	8.26E-05	0.13964
4496	MT1H	metallothionein 1H	56.95	6.82	107.08	15.04	3.91	8.57E-05	0.13964
1286	COL4A4	collagen type IV alpha 4 chain	48.91	7.18	90.63	12.74	3.67	9.78E-05	0.13964
4495	MT1G	metallothionein 1G	156.19	49.69	262.69	5.40	2.43	9.93E-05	0.13964
79622	SNRNP25	small nuclear ribonucleoprotein U11/U12 subunit 25	634.78	860.96	408.59	0.47	-1.09	1.01E-04	0.13964
3437	IFIT3	interferon induced protein with tetratricopeptide repeats 3	1662.25	2175.73	1148.77	0.52	-0.93	1.16E-04	0.14709
27074	LAMP3	lysosomal associated membrane protein 3	1250.60	1613.17	888.03	0.55	-0.87	1.46E-04	0.16678
5265	SERPINA1	serpin family A member 1	1043.03	289.14	1796.93	5.97	2.58	1.53E-04	0.16678
55008	HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	1361.99	1723.46	1000.51	0.58	-0.79	1.93E-04	0.19626
128506	OCSTAMP	osteoclast stimulatory transmembrane protein	58.97	108.60	9.34	0.09	-3.49	2.40E-04	0.22385
900	CCNG1	cyclin G1	2235.80	1613.72	2857.89	1.76	0.82	2.75E-04	0.22385
9975	NR1D2	nuclear receptor subfamily 1 group D member 2	1350.49	991.36	1709.62	1.72	0.79	2.77E-04	0.22385
6556	SLC11A1	solute carrier family 11 member 1	111.04	178.90	43.17	0.17	-2.54	2.79E-04	0.22385
7140	TNNT3	troponin T3, fast skeletal type	115.16	29.51	200.80	6.67	2.74	2.99E-04	0.22756
440836	ODF3B	outer dense fiber of sperm tails 3B	462.34	639.18	285.49	0.43	-1.21	3.42E-04	0.23224
10347	ABCA7	ATP binding cassette subfamily A member 7	4332.92	2911.93	5753.92	1.97	0.97	3.48E-04	0.23224
4599	MX1	MX dynamin like GTPase 1	2605.66	3332.77	1878.54	0.56	-0.82	3.75E-04	0.23224
130557	ZNF513	zinc finger protein 513	541.12	722.19	360.04	0.49	-1.02	3.80E-04	0.23224
2537	IFI6	interferon alpha inducible protein 6	1914.07	2386.14	1441.99	0.60	-0.73	3.81E-04	0.23224
51175	TUBE1	tubulin epsilon 1	738.96	515.55	962.37	1.88	0.91	4.34E-04	0.25392
283870	BRICD5	BRICHOS domain containing 5	183.21	96.89	269.53	2.79	1.48	5.07E-04	0.28611
4940	OAS3	2'-5'-oligoadenylate synthetase 3	3545.29	4388.22	2702.36	0.61	-0.71	5.40E-04	0.29384
9636	ISG15	ISG15 ubiquitin like modifier	1366.40	1684.07	1048.74	0.62	-0.69	6.13E-04	0.32161
64333	ARHGAP9	Rho GTPase activating protein 9	3620.14	2607.96	4632.33	1.77	0.82	6.40E-04	0.32502
91543	RSAD2	radical S-adenosyl methionine domain containing 2	1116.73	1459.76	773.69	0.54	-0.90	6.79E-04	0.33327
3669	ISG20	interferon stimulated exonuclease gene 20	2598.21	3213.78	1982.64	0.61	-0.72	7.33E-04	0.33921
684	BST2	bone marrow stromal cell antigen 2	3450.95	4238.39	2663.51	0.62	-0.68	7.35E-04	0.33921
129607	CMPK2	cytidine/uridine monophosphate kinase 2	640.05	830.38	449.72	0.52	-0.94	8.57E-04	0.37773
23129	PLXND1	plexin D1	1405.51	974.35	1836.67	1.87	0.91	8.68E-04	0.37773



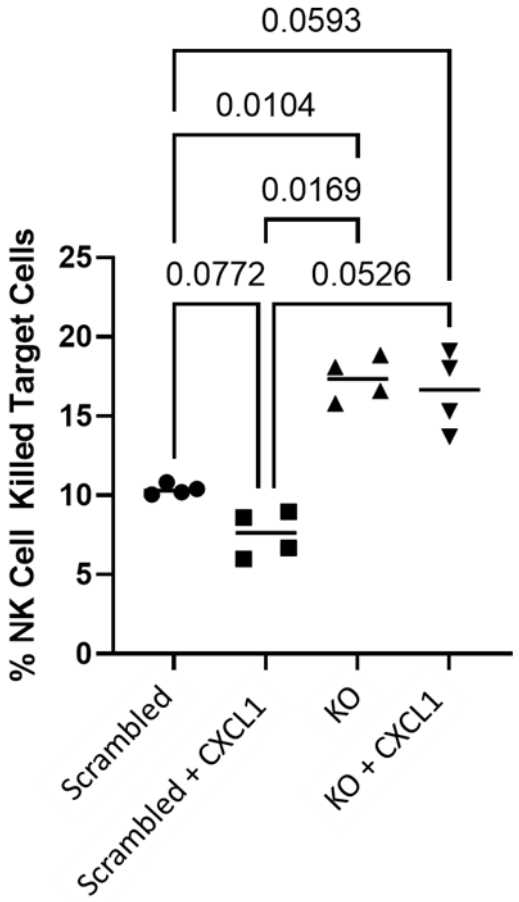
Appendix Figure 2: Inhibitor confirmation western blot analysis. Head and neck squamous cell peripheral blood mononuclear cells were treated with 10uM of indicated drug for 24 hours and subjected to western blot.



Appendix Figure 3: Head and neck squamous cell carcinoma mouse model has elevated CXCL1. Tail vein blood from normal B6/C57 mice and B6/C57 mice with MOC-1 tumors in either the flank or cheek was collected in EDTA tubes. Samples were spun for plasma and analyzed by mouse CXCL1 ELISA. Analyzed by student's t-test. n = 3 per group.



Appendix Figure 4: CXCR2 knockdown confirmation western blot analysis. Head and neck squamous cell peripheral blood mononuclear cells were treated with 10uM of indicated drug for 24 hours and subjected to western blot.



Appendix Figure 5: CXCR2 knockdown NK cells have higher cytotoxic activity. Primary NK cells from healthy patients were isolated from peripheral blood and expanded in 1000U/mL IL-2 for 10 days. Cells were counted and nucleofected with CRISPR/Cas9 system for CXCR2 knockdown or scrambled guide. Cells were placed back in 1000U/mL IL-2 without 50ng CXCL1 for 5 days. Cells were collected, washed, and analyzed by NKCA. Data analyzed by one way ANOVA. n = 4.