Title
Role of Interleukin-22 in Tumor Angiogenesis

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Role of Interleukin-22 in Tumor Angiogenesis

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Nicholas James Protopsaltis

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2017
This Dissertation of Nicholas James Protopsaltis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California, San Diego

2017
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<tbody>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AKT</td>
<td>Ak strain transforming (Protein kinase B)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Bcl-(X_L)</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CXC</td>
<td>(\alpha)-chemokine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EBM-2</td>
<td>Endothelial basal medium (2)</td>
</tr>
<tr>
<td>EGM-2</td>
<td>Endothelial growth medium (2)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal–regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus tyrosine kinase</td>
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KO: Knockout
Maf: musculoaponeurotic fibrosarcoma
MVEC: Microvascular endothelial cell
NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells
NP1/NP2: Neuropilin 1 / Neuropilin 2
PBS: Phosphate buffered saline
PTEN: Phosphatase and tensin homolog
RAG: Recombination activating gene
RIPA: Radioimmunoprecipitation assay buffer
RORC: retinoic acid receptor related orphan receptor C
RTK: Receptor tyrosine kinase
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2: Src Homology 2
siRNA: Small interfering ribonucleic acid
Src: sarcoma (kinase)
STAT: signal transducer and activator of transcription
TGF-β: Transforming growth factor Beta
TYK: Tyrosine kinase
VEGF: Vascular endothelial growth factor
WT: Wild type
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ABSTRACT OF THE DISSERTATION

Role of Interleukin-22 in Tumor Angiogenesis

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2017

Professor Napoleone Ferrara, Chair

There is increasing recognition that the immune system plays a crucial role in either the promotion or inhibition of tumor development and growth. Current models of tumorigenesis rely on cells’ ability to evade the immune system. Escaping immune-mediated destruction, however, is only one step in the beginning of tumor growth. While many types of immune cells have been
implicated in cancer progression or suppression, the T\(_h17\) subset of CD4+ helper T cells is of particular interest due to their clinical implication as being a negative prognostic indicator in some types of cancer, and a positive indicator in others. T\(_h17\) cells produce two main cytokines: their hallmark cytokine interleukin-17, and interleukin-22. Interleukin-22 is known for exhibiting a multitude of effects in different types of epithelium, but its role in angiogenesis is only beginning to be elucidated.

This study begins by examining the effects of interleukin-22 on endothelial cells *in-vitro*. It was found that IL-22 stimulates endothelial cell proliferation, survival, and chemotaxis. It also induces activation of the STAT3, MAPK, and AKT pathways in endothelial cells, consistent with its known signaling effects in cells of epithelial origin.

The mouse T cell lymphoma EL4 was used as a tumor model for a number of *in-vivo* studies. It was found that these tumors produce interleukin-22 both *in-vitro* and *in-vivo*, and that blockade of IL-22 *in-vivo* results in a statistically significant decrease in tumor growth in C57BL/6, athymic nude, and Rag 1\(^{-}\) mice. Additionally, it was observed that anti-IL22 antibody therapy decreased blood vessel density within EL4 tumors.

A mouse glioblastoma cell line, GL261, was also investigated to determine the role of interleukin-22 in tumor growth and angiogenesis. IL-22 blockade resulted in decreased tumor growth in both C57BL/6 and athymic nude tumor bearing mice.
In summation, the data observed indicates that interleukin-22 can directly act on endothelial cells to induce angiogenesis. Furthermore, blocking IL-22 \textit{in-vivo} results in a statistically significant reduction in tumor growth using multiple tumor models.
CHAPTER 1.

Introduction to Angiogenesis, Immune Cells in the Tumor Microenvironment, and Interleukin-22

1.1 - Angiogenesis is the formation of new blood vessels.

The term originated in 1935 to describe the formation of blood vessels in the placenta, and it has subsequently been shown to play a role in both normal and pathologic states\(^1\). In the 1960’s, it was demonstrated that solid tumors depend on this process for their own growth, and that a diffusible factor could induce tumor neovascularization\(^2\). While these earlier experiments showed that a “humoral factor” could pass through a membrane and recruit blood vessels, Judah Folkman eventually identified a product secreted from tumor cells, which he called TAF or “tumor-angiogenesis factor,” that was mitogenic on endothelial cells and could induce the formation of new capillaries\(^3\). Furthermore, it was shown that initially slow growing tumors rapidly increased in size after receiving a vascular supply (Figure 1) \(^4\). Additional work revealed that TAF could induce neovascularization in multiple settings, and importantly, that there may be a unique receptor for TAF on endothelial cells which could be blocked as a way of inhibiting tumor angiogenesis\(^5\).
Figure 1: Angiogenesis is a Rate Limiting Step For Tumor Growth. Adapted from Folkman, J 1975.
In 1983, a factor was identified that induced vascular leakage, and was termed vascular permeability factor, or VPF\textsuperscript{6}. VPF was not purified to homogeneity or sequenced at the time, and although the researchers did not know of its mitogenic activity, this same factor was later determined to be vascular endothelial growth factor, or VEGF\textsuperscript{7}. VEGF was identified, isolated, purified, and cloned from bovine pituitary follicular cells in 1989\textsuperscript{8}. cDNA cloning revealed that there are four (major) isoforms of VEGF-A created by splicing, which include VEGF\textsubscript{121}, VEGF\textsubscript{165}, VEGF\textsubscript{189}, and VEGF\textsubscript{206}\textsuperscript{9}.

1.2 - Angiogenesis arises from existing vasculature.

Under normal, physiologic conditions, endothelial cells of the vasculature have very low turnover and rarely undergo mitosis\textsuperscript{10}. While angiogenesis can occur in many settings, the formation of new blood vessels begins with new capillaries arising from the sprouting of small venules. Next, the basement membrane of the parent venule is degraded, and endothelial cells migrate towards the angiogenic stimulus. The migrating endothelial cells elongate and align with each other to form a solid sprout, with a lumen arising within each endothelial cell. Endothelial cell proliferation increases the length of the sprout, and two hollow sprouts merge at the tip to form a loop, which begins the flow of blood. Pericytes next position themselves at the base of the loop, and new sprouts can grow at the apex of the loop to continue the process of angiogenesis\textsuperscript{1,11}. 
1.3 - A number of angiogenic factors have been discovered.

It is now known that a number of growth factors can induce angiogenesis in-vitro and in-vivo. The extent to how potently they can induce effects is highly variable, but some of these factors include bFGF, aFGF, EGF, PDGF, VEGF, Angiogenin, Angiotropin, TGF-β, TNF-α, G-CSF, and GM-CSF. In the setting of cancer, these factors can be produced by the tumor cells themselves, mobilized from the extracellular matrix, or released by macrophages that have infiltrated the tumor. Despite the existence of multiple mechanisms by which a growth factor may induce tumor neovascularization, VEGF is the main factor responsible in primary, solid neoplasms.

1.4 - The VEGF family consists of multiple genes and receptors.

Initial cDNA cloning revealed that there are four (major) isoforms of VEGF created by splicing, which include VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. It is now known that 4 VEGF genes exist: VEGF-A, VEGF-B, VEGF-C, and VEGF-D. There are also 3 different VEGF receptors, named VEGFR-1, VEGFR-2, and VEGFR-3. VEGF-A is the major form of VEGF, and can bind to VEGFR-1 and VEGFR-2, whereas VEGF-B can only bind to VEGFR-1, and VEGF-C as well as VEGF-D can bind to both VEGFR-2 and VEGFR-3. The different members of VEGF and their interactions with VEGF receptors are illustrated in Figure 2.
Figure 2: The Vascular Endothelial Growth Factor Family and its receptors. Adapted from Ferrara *et al* (2003).
The main isoform of VEGF-A is VEGF\textsubscript{165}, which is a 45 kDa homodimeric glycoprotein that exerts the majority of its mitogenic, angiogenic, and permeability enhancing effects through VEGFR-2\textsuperscript{9,16}. The effects of VEGFR-1 signaling are not fully understood, but is believed to play a role in the induction of matrix metalloproteinases and recruitment of macrophages to tumor vasculature which can play a role in the induction of angiogenesis\textsuperscript{9,14,17}. As previously mentioned, VEGFR-1 is the only target for VEGF-B, and therefore it may play a role in these processes, but since it cannot signal through VEGFR-2, it is not responsible for predominant angiogenic effects.

VEGF-C and VEGF-D regulate lymphangiogenesis\textsuperscript{14-15}. VEGFR-1 also exists in a soluble form that may serve as a decoy for VEGF-A, or possibly as a method of VEGF sequestration\textsuperscript{9}. In addition to the ability to bind to VEGFR-1 and VEGFR-2, VEGF-A can also bind to Neuropilin-1 (NP1) and Neuropilin-2 (NP2)\textsuperscript{14}. Binding of VEGF\textsubscript{165} to NP1 has been shown to potentiate VEGFR-2 signaling, while NP1 also has the ability to directly bind VEGFR-1\textsuperscript{17}. This suggests that VEGFR-1 may negatively regulate angiogenesis by competing for NP1 binding\textsuperscript{9,14}. Furthermore, some tumors express NP1 and NP2 on their surface, although in the setting of tumor angiogenesis the significance of the neuropilin, VEGF, VEGF-R signaling pathway is still uncertain\textsuperscript{17}. Of note, the VEGF-A family member VEGF\textsubscript{165} is almost always referred to as simply “VEGF” in literature, and will subsequently
be referred to likewise throughout the rest of the text unless otherwise indicated.

1.5 - VEGFR-2 is activated by VEGF<sub>165</sub> binding.

VEGFR-2 is a receptor tyrosine kinase that consists of seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a tyrosine kinase sequence that is interrupted by a kinase-insert domain<sup>16</sup>. VEGF binds to the second and third Ig-like domains, which induces VEGFR-2 dimerization and tyrosine auto-phosphorylation<sup>9,14</sup>. VEGFR-2 is predominantly expressed on endothelial cells of the vasculature, and while the effects of VEGFR-2 activation are numerous, in endothelial cells it results in the phosphorylation of phospholipase C-y, PI-3 kinase, Ras GTPase-activating protein, and Src family members<sup>14,18</sup>.

1.6 - VEGF expression is regulated through multiple mechanisms.

It was first observed that tumors are often accompanied by extensive vascularity over a hundred years ago<sup>7</sup>. While the explanation for how and why was unknown for most of the 20<sup>th</sup> century, even after the discovery of VEGF it remained to be elucidated what induced its expression. VEGF production was initially observed to be induced by hypoxia and tissue ischemia<sup>19</sup>. The researchers found that in glioblastoma samples from cancer patients, levels of VEGF mRNA were correlated with proximity to centers of necrosis and
ischemia. Furthermore, they demonstrated that growth of glioblastoma cells under hypoxic conditions resulted in a significant increase in VEGF expression, which was reversible upon re-exposure to normal oxygen levels.

In an attempt to elucidate the mechanism by which hypoxia induces VEGF expression, it was found that VEGF shared similar expression activity as erythropoietin\textsuperscript{20}. It was later determined that a common enhancer exists for both VEGF and erythropoietin: a binding site for hypoxia inducible factor-1 (HIF-1)\textsuperscript{21}. Although VEGF expression is regulated by HIF-1, it can be induced independently of low oxygen tension through mutations in PI3K/AKT and PTEN\textsuperscript{22}. This is one mechanism by which tumor cells can hijack normal cellular regulatory mechanisms to produce VEGF and recruit blood vessels.

Cytokines and growth factors that do not directly induce angiogenesis can exert a pro or anti-angiogenic effect by modulating VEGF expression in specific cell types\textsuperscript{23}. For example, FGF-4, PDGF, TNF-α, TGF-β, IL-1β and IL-6 can all induce VEGF production, along with hormones such as TSH and ACTH in responsive tissues\textsuperscript{9,23}. It was previously mentioned that VEGFR-1 can serve as a method of sequestering VEGF, and macrophages have been shown to modulate angiogenesis through production of VEGFR-1\textsuperscript{24}. In contrast, neutrophils have been shown to produce VEGF\textsuperscript{25}. This evidence suggests that the immune system may play an active role in determining the extent to which angiogenesis occurs under both physiologic and pathologic conditions.
1.7 - The tumor microenvironment nurtures tumor growth.

The interaction between a primary tumor and cells of both the innate and adaptive immune system has been shown to play a fundamental role in determining the grade and malignancy of a tumor\textsuperscript{26}. However, cancer cells do not only interact with myeloid and lymphoid cells, but numerous studies have shown they receive strong additional support from interactions with stromal cells, such as fibroblasts and angiogenic vascular cells\textsuperscript{27,28,29}. These other cell types in the tumor microenvironment may play a role in deregulating cellular energetics, sustaining proliferative signaling, evading growth suppressors, inducing angiogenesis, avoiding immune destruction and activating invasion and metastasis\textsuperscript{30}. For example, tumor-associated macrophages have been shown to promote breast cancer metastasis, suppress apoptotic signals, and provide protection from chemotherapeutic agents\textsuperscript{30}. Inflammation, a prominent phenotypic expression of the immune system, has long been known to have an effect on either cancer progression or suppression. Tumor-associated inflammatory cells promote cancer cell proliferation and suppress antitumor immunity\textsuperscript{31}. Inflammation acts as a mechanism for neovascularization, which can provide nutrients and serve as routes for metastasis\textsuperscript{32}. This interaction between the primary tumor and secondary cells types is an area of intense active research.
1.8 - **T-Lymphocytes are actively involved in tumor growth and suppression.**

There is an increasing awareness that T-cells play a major role in the tumor microenvironment, especially the $T_H17$ subset of CD4+ T-cells. These cells are associated with both positive and negative outcomes among different tumor types in humans, including potentially anti-tumor effects in NHBL (Non-Hodgkins B-cell Lymphoma), prostate, ovarian, and breast cancers, while having potentially pro-tumor effect in gastric cancer, hepatocellular carcinoma, NSCLC (Non-Small Cell Lung Cancer), and melanoma$^{33,34}$. Numasaki et al demonstrated that IL-17, the hallmark cytokine for $T_H17$ cells, has a significant effect on angiogenesis, promoting it through CXCR-2 in human NSCLC, while Chung et al showed that in mouse models of lymphoma, IL-17 promotes angiogenesis via a G-CSF, NF-kB, ERK signaling pathway$^{35,34}$. These results demonstrate that IL-17 can promote tumor growth via multiple mechanisms, which could ultimately be the target of specific pharmacotherapy. Furthermore, increased numbers of interleukin-17 producing cells have been correlated with high grade and negative outcomes in breast carcinoma$^{26}$, while tumor infiltrating $T_H17$ cells are associated with poor outcomes in colorectal and lung carcinomas$^{36}$. 
1.9 - \( \text{T}_{\text{H}}17 \) cells mediate effects via two different cytokines: interleukin-17 and interleukin-22.

Previous work has shown that \( \text{Th17} \) cells promote tumor resistance to anti-angiogenic therapy\(^{35} \). The authors postulated that IL-17 mediates this effect, and showed IL-17 inhibition is sufficient to render tumors sensitive\(^{35} \). One group of researchers have suggested that IL-22 stimulates production of VEGF and Bcl-X\( \text{L} \)^{37}. Since \( \text{T}_{\text{H}}17 \) cells produce IL-17 and IL-22, this suggests that they mediate resistance to VEGF inhibition via IL-17, while stimulating VEGF production via IL-22. Furthermore, in cutaneous T-cell lymphoma, IL-22, but not IL-17, dominates within the tumor microenvironment\(^{38} \). Researchers are still uncertain why \( \text{T}_{\text{H}}17 \) cells promote tumor growth in certain tissues, but inhibit it in others. Many tumors do not express the IL-17 receptor, however, and thus IL-17 mediates its effects through other cell types in the tumor microenvironment, or the effects of \( \text{T}_{\text{H}}17 \) cells must be acting through a molecule other than IL-17\(^{39,34} \). Furthermore, \( \text{T}_{\text{H}}17 \) cells have even been found to be associated with both positive and negative outcomes in human breast cancer patients, suggesting that a particular mediator is responsible for this discrepancy\(^{40,26} \). The work that follows in chapters 2-4 arose from the notion that IL-22, the other primary \( \text{T}_{\text{H}}17 \) cell cytokine, could be responsible for the observations that \( \text{T}_{\text{H}}17 \) cells exhibit differential effects on tumor growth.
1.10 - Interleukin-22 is a T cell secreted cytokine in the IL-10 family.

Interleukin-22 is a cytokine that belongs to the IL-10 family. It is a 179 amino acid protein which is secreted as an alpha-helical molecule\textsuperscript{41,42}. It was first discovered and isolated in 2000 from mouse T lymphocytes, and given the tentative name “IL-10 related T cell-derived inducible factor,”\textsuperscript{43}. IL-22 shares 22% sequence homology with IL-10 in mice, and 25% sequence homology with IL-10 in humans\textsuperscript{42,44}.

Interleukin-22 interacts with the IL-22 receptor, a type-II cytokine receptor. The IL-22 receptor is a transmembrane protein dimer, which consists of an IL-22R1 and an IL-10R2 subunit\textsuperscript{21}. The IL-10R2 chain is ubiquitously expressed in human tissues, while the IL-22R1 subunit is primarily localized to tissues of epithelial origin\textsuperscript{45,46}. Although both subunits are capable of ligand binding, neither can transduce IL-22 signaling alone\textsuperscript{46}. The IL-22 receptor complex contains binding sites for Janus tyrosine kinases (JAK), which become activated after ligand binding\textsuperscript{47}. JAK then phosphorylates tyrosine residues of the receptor, which create docking sites for Src homology 2 binding proteins such as STATs\textsuperscript{48}.

IL-22 is largely considered to be a T-cell cytokine, although innate natural killer cells (large granular lymphocytes) can produce modest amounts\textsuperscript{49,45}. Many studies investigating the role of IL-22 have taken place in the context of dermatologic pathologies, and psoriasis was the first disorder associated with abnormal IL-22 production, which derives from CD4+ T\textsubscript{H}1,
TH17, and TH22 cells, whereas in atopic dermatitis IL-22 is derived primarily from TH22 cells and CD8+ IL-22 producing cells\textsuperscript{50}. Not all IL-22 activity is deleterious, however, as TH derived IL-22 has been postulated to play a protective effect in patients with ulcerative colitis and Crohn’s disease\textsuperscript{50,51}.

The source of IL-22 varies among settings, although IL-6 and IL-23 have been shown to promote its production\textsuperscript{52}. In humans, retinoic acid receptor related orphan receptor C (RORC) and aryl hydrocarbon receptor (AHR) are thought to positively regulate IL-22 expression\textsuperscript{53}. In contrast, transforming growth factor Beta (TGF-β) inhibits IL-22 production by acting through the transcription factor c-Maf\textsuperscript{54}. Maximal concentrations, \(\sim 1.2 \text{ ng/mL}\), of IL-22 are produced by CD4+ T-cells when stimulated with IL-6, and IL-23 can also stimulate production of IL-22, but in lower concentrations\textsuperscript{55}.

1.11 - Role of Interleukin-22 in the epithelium and on the endothelium

IL-22’s effect on epithelial cells has been well documented. Rutz et al (2013) and Sabat et al (2014) provide thorough reviews and background on the role of IL-22 in both the normal epithelium and pathologic states\textsuperscript{56,50}. Briefly, its primary physiologic role is to promote barrier defense against pathogens at mucosal surfaces and promote wound healing as well as tissue homeostasis. Overproduction of IL-22 is observed in many human disease states, which is well summarized in Sabat et al (2014).
Investigations into the role of IL-22 on the endothelium, however, are far more limited. IL-22 has been shown to affect endothelial cells at the blood-brain barrier, namely resulting in inflammation\textsuperscript{57}. During the course of the following investigations, a group of researchers showed that IL-22 can inhibit apoptosis in pulmonary microvascular endothelial cells\textsuperscript{58}. Another group demonstrated that IL-22 can promote HUVEC proliferation, and enhance their barrier function by reducing permeability\textsuperscript{59}. As such, it is increasing recognized that interleukin-22 can have a direct effect on the vasculature.

1.1.2 - Angiogenesis plays a major role in tumor growth.

Angiogenesis has long been known to play a major role in cancer growth. Solid tumors can receive nutrients and exchange waste while they are small, but to grow beyond 1-2mm in size, they must recruit new blood vessels for these processes to occur\textsuperscript{5}. Furthermore, many believe it is the rate-limiting factor for rapidly dividing tumor cells\textsuperscript{35}. Numerous studies have shown the link between endothelial stimulation via Vascular Endothelial Growth Factor (VEGF) and cancer progression. Anti-angiogenic therapy, when combined with chemotherapy, has been clinically proven to increase survival in patients with advanced malignancies\textsuperscript{60}.

Although the anti-VEGF monoclonal antibody bevacizumab improves clinical outcome in a number of cancers, patients exhibit a heterogeneous response\textsuperscript{61}. This observation in clinical trials has led to a number of follow-up
investigations into predictive biomarkers, but ultimately none of them have been prospectively validated$^{61,62}$. Many of the clinical trials for which anti-VEGF therapy has shown therapeutic benefit have been in the setting of advanced malignancies. Therefore it is possible that earlier adjuvant therapy may yield valuable information for which patients will receive greatest benefit from anti-angiogenic therapy, as well as lend insight to the mechanisms by which tumors develop resistance$^{63}$. Much work has yet to be done in elucidating interactions within the tumor microenvironment that help or hinder anti-angiogenic treatment, and furthermore, which patients will benefit most from such therapy$^{17}$.

It was previously discussed how the immune system plays an active and important role in the tumor microenvironment. Anti-angiogenic therapy in particular can promote leukocyte infiltration into tumors$^{64}$. In contrast, IL-22 blockade has been shown to reduce granulocyte infiltration$^{65}$. Additionally, IL-22 reduces T$_{h}$2 cell production of IL-4, a potent anti-tumor cytokine, which further suggests that interleukin-22 can exhibit a pro-tumor effect $^{41,66}$. Hence it is possible that IL-22 may play a role in mediating resistance to anti-VEGF therapy. This could arise from directly acting on the endothelium to induce angiogenesis, or perhaps through effects on cells of the innate immune system, with the endothelium as both a mediator and final target in the process. The following chapters attempt to elucidate some of the effects interleukin-22 has in the settings of angiogenesis and tumor growth.
CHAPTER 2.

Effects of Interleukin-22 on Endothelial Cells

2.1 - Introduction

Interleukin-22 is known to exert a direct effect on epithelial cells. IL-22 is unusual among interleukins in this regard, as epithelial cells are major targets of the IL-22 ligand, whereas interleukins traditionally serve as mediators for inducing effects on immune cells$^{50}$. IL-22’s physiological role is in mediating host defense, and overproduction of IL-22 is associated with many disease conditions such as psoriasis, inflammatory bowel disease, and asthma$^{50}$. Although IL-22 has been found to exhibit multiple effects on epithelial cells, hepatocytes, and pancreatic cells, its ability to act on endothelial cells is only beginning to be elucidated$^{56}$. 
2.2 - Results

2.2.1 - Endothelial cells express IL-22 receptor on their cell surface.

Although IL-22 receptor expression has been well documented in epithelial cells, it was necessary to confirm that the available endothelial cells indeed expressed the IL-22 receptor on the cell surface. Utilizing HepG2 as a positive control, a western blot demonstrated positive staining for IL-22 receptor in both HUVEC and MVEC cells (Figure 3). Protein isolation from these cells requires cell lysis, and thus it is conceivable that the cells produce IL-22 receptor, but it is not necessarily transported to and functional on the cellular surface. To address this, HUVEC, MVEC, and HepG2 cells were incubated with a fluorescent antibody against IL-22 receptor or an IgG isotype control antibody. Live cells were then analyzed using flow cytometry (Figure 4). With HepG2 as a positive control, both HUVECs and MVECs demonstrated increased mean fluorescence intensity with IL-22 receptor antibody staining compared to isotype control. This increase in fluorescence signaling was statistically significant across the three cell types analyzed. Although this experiment included an isotype control antibody was as a technical control, HepG2 cells were next incubated with multiple doses of a siRNA against IL-22R to ensure that the observed FACS staining for IL-22R was specific. This specificity could be determined by reduced IL-22 receptor fluorescence staining following targeted knockdown. siRNA treatment reduced surface
staining for IL-22R in HepG2 cells (Figure 5). This served as a final confirmation that the fluorescence staining observed following incubation with anti-IL22 receptor antibody was indeed specific. In summation, these findings suggest that the IL-22R is expressed on the surface of endothelial cells.
Figure 3: Human IL-22 Receptor Expression. Representative Western Blot shows that both HUVEC and MVEC express the IL-22Ra1 subunit.
Figure 4: Human IL-22 Receptor Surface Staining By Flow Cytometry. (error bars indicate standard deviation, n = 2, ** p < .01, * p < .05 by two-way ANOVA with post-hoc Šidák’s correction for multiple comparisons)
Figure 5: siRNA Knockdown of Human IL-22 Receptor. Relative IL-22R fluorescence following single oligonucleotide siRNA knockdown (control siRNA or one targeting IL-22Rα1), assessed by flow cytometry.
2.2.2 - Endothelial Cell numbers are increased following IL-22 treatment.

He et al demonstrated that treating HUVECs with IL-22 caused an increase in proliferation\(^59\). It was independently observed that 3-day incubation of HUVECs with IL-22 results in increased cell numbers compared PBS control (Figure 6). When \(2.5 \times 10^4\) cells were plated, the PBS control resulted in approximately double the number of cells present after 3 days. In contrast, IL-22 treatment resulted in over four times the number of cells present compared to the initial plating. This data demonstrates that IL-22 has a direct effect on increasing the proliferation of HUVECs.

Having observed a proliferation function of IL-22 on HUVECS, it was next examined whether IL-22 treatment could increase HUVECS survival. Increasing doses of IL-22 demonstrated a modest, but statistically significant pro-survival effect on HUVECs (Figure 7).

Having demonstrated that IL-22 could directly act on HUVECs, MVECs were investigated as another endothelial cell line to determine if the effects observed are common amongst different types of endothelial cells. Increasing doses of IL-22 failed to result in increased cell numbers following 3 day incubation (Figure 8). While basal media supplemented with 10\% FBS produced a statistically significant increase in cell numbers compared to PBS control, IL-22 treatment did not. This suggests that in MVEC, IL-22 does not directly promote proliferation as observed in HUVECs.
Ren et al showed that IL-22 increases MVEC survival. A similar observation was found following a 6-day treatment of IL-22 (Figure 9). VEGF and bFGF treatment resulted in cell numbers 10-20 fold higher than media alone or PBS control. IL-22 incubation, in comparison, produced cell numbers approximately 5 fold higher than media alone or PBS treatment. Hence although IL-22 did not increase the number of MVECs in a proliferative fashion, it can increase cell number through survival mechanisms.
Figure 6: IL-22 Promotes HUVEC Proliferation. (error bars indicate standard deviation, n = 18, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 7: IL-22 Promotes HUVEC Survival. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 6-8, * p < .05, ** p < .01, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 8: IL-22 Does Not Promote MVEC Proliferation. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 2, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 9: IL-22 Promotes MVEC Survival. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 8, ** p < .001 by one-way ANOVA with post-hoc Dunnett's test for multiple comparisons)
2.2.3 - IL-22 promotes endothelial cell chemotaxis

Although endothelial cell proliferation and survival play a role in angiogenesis, vessel sprouting is another response that endothelial cells can exhibit in the presence of stimuli. Cell migration across a transwell membrane serves as a functional assay to determine chemoattractant potential of cytokines. To determine if IL-22 could elicit such an effect on endothelial cells, a boyden chamber was utilized as previously described. With HUVECs, compared to PBS, it was observed that IL-22 causes a 50-100% increase in migrated cell number (Figure 10). This suggests that IL-22 can directly promote endothelial cell migration and has a chemoattractant effect on endothelial cells. The role of IL-22 on cellular migration was also investigated with MVEC (Figure 11). Again, IL-22 demonstrated the ability to increase the number of cells that migrate across a transwell membrane. This suggests that IL-22 can directly stimulate endothelial cell chemotaxis, which could play a role in angiogenesis in-vivo.

Another functional angiogenesis assay to investigate the angiogenic potential of a cytokine is the tube formation assay. In this assay, endothelial cells are plated atop a layer of solidified basement membrane extract. Media is added containing cytokines, and at a specified time later, the number and size of tubes that form can be ascertained. Using HUVECs, a low dose of IL-22 (10 ng / mL) increased the relative area of the tubes that formed (Figure 12).
Interestingly, higher doses of IL-22 resulted in smaller tubes, and were not statistically different from PBS control.
Figure 10: IL-22 Promotes HUVEC Migration. Representative of 3 independent experiments (error bars indicate standard deviation, n = 6, * p < .05, ** p < .01, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 11: IL-22 Promotes MVEC Migration. Representative of 2 independent experiments (error bars indicate standard deviation, n = 4, * p < .05, ** p < .01, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 12: IL-22 Induces Tube Formation in HUVEC. (error bars indicate standard deviation, n = 5, * p < .05 by unpaired Welch’s t-test)
2.2.4 - Signaling induced by IL-22 in endothelial cells

Having observed the effects of IL-22 on endothelial cell viability and chemotaxis in-vitro, it was next examined which signaling pathways are activated by IL-22 treatment. In HUVECs, initial experiments revealed that ERK 1/2 had markedly increased phosphorylation following 30 minute incubation with IL-22, with maximal effect between 10 and 25 ng / mL (Figure 13). An increased range of IL-22 concentrations was investigated, and a dose-dependent effect on ERK 1/2 activation was observed up until 100 ng / mL of IL-22 (Figure 14). Repeat examination with doses of IL-22 ranging from 1 to 500 ng / mL demonstrated peak relative ERK 1/2 activation at 25 ng / mL (Figure 15).

IL-22 can signal through the AKT pathway in HUVECs (Figure 16). After 30-minute stimulation, maximal p-AKT was observed with 25 ng/mL IL-22 treatment. A broader range of IL-22 doses were tested, and p-AKT measured following 10 and 30-minute incubations (Figures 17-18). Of note, the levels of activation observed relative to PBS control are lower than those previously observed. It is unclear whether this relates to use of 12-well versus 6-well dishes, or is partially due to the relative variation induced by loading smaller volumes of lysate into gels containing more lanes, and hence lower volumes.

IL-22 stimulation was also found to induce STAT 3 phosphorylation (Figure 19). Quantification of p-STAT 3 revealed increasing activation from 10 to 25 to 50 ng / mL of IL-22. HUVECs were also treated with 25 ng/mL of IL-
22, and cell lysates collected at different time intervals to assess its effect on
STAT 3 phosphorylation over time. It was observed that IL-22 causes
activation at 10 minutes, with relatively stable activation across 30, 60, and
120-minute time points (Figure 20).

In summation, this signaling data suggests that IL-22 appears to
promote a combination of both pro-survival and proliferative pathways in
endothelial cells.
Figure 13: Quantification of p-ERK 1/2 Induced by IL-22. A. Western Blot staining after 10 minute incubation. B. Quantification of p-ERK 1/2 Induced by IL-22.
Figure 14: IL-22 Induces MAPK Activation in HUVEC. A. Western Blot staining after 30 minute incubation. B. Quantification of p-ERK 1/2 compared to total ERK 1/2.
Figure 15: IL-22 Induces ERK 1/2 Activation in HUVEC. A. Western Blot staining after 30 minute incubation. B. Quantification of p-ERK 1/2
Figure 16: IL-22 Induces AKT Activation in HUVEC. A. Western Blot staining after 30 minute incubation. B. Quantification of p-AKT.
Figure 17: IL-22 Induces AKT Activation in HUVEC. A. Western Blot staining after 10 minute incubation. B. Quantification of p-AKT.
Figure 18: IL-22 Induces AKT Activation in HUVEC. A. Western Blot staining after 30 minute incubation. B. Quantification of p-AKT
Figure 19: IL-22 Induces Stat 3 Activation in HUVEC. A. Western Blot staining after 30 minute incubation. B. Quantification of p-Stat 3 Induced by IL-22.
IL-22 Signals through STAT-3 in HUVEC

Figure 20: Stat 3 Activation Over time in HUVEC. Representative Western Blot shows that treatment with IL-22 results in increased p-Stat 3 staining at multiple time points.
2.3 - Discussion

It is well-established in the field of immunology that IL-22 can directly act on epithelial cells, but IL-22’s effects on endothelial cells are only beginning to be investigated. It was found that IL-22 can elicit a proliferative and survival effect in HUVECs. In MVEC, IL-22 can promote survival, but not proliferation. Common to both cell types was the effect of inducing migration in a transwell assay. HUVECs, as their name implies, are derived from the umbilical cord, whereas the MVECs utilized in this study were isolated from adult lungs. It is possible that inherent differences exist in the downstream pathways between these cell types such that HUVECs are more responsive to stimuli compared to MVECs. IL-22 has been shown to be elevated in patients with asthma, and may even have a protective effect in the setting of lung inflammation and injury\textsuperscript{50,70}. If IL-22 is indeed protective in the setting of lung inflammation, it could explain why MVECs exhibit some, but not all of the results observed with HUVECs. Specifically, IL-22 was found to promote MVEC survival but not proliferation, despite the ability of IL-22 to promote both in HUVECs. While VEGF also promoted both survival and proliferation in MVECs, there are numerous human tissues that contain detectable levels of VEGF in normal physiologic, non angiogenic states\textsuperscript{71}. It has been postulated that in these settings, VEGF may contribute to vessel homeostasis, even though VEGF is well-known to potently induce angiogenesis\textsuperscript{14}. Therefore in the
case of lung endothelial cells, it is not unprecedented for a growth factor to promote cell or vessel survival, while not specifically inducing cellular proliferation.

It is also conceivable that as being derived from a specific patient, susceptibility of MVECs to IL-22 varies between different persons, and possibly as a function of age. While an interesting notion, the focus of this study was the role of interleukin-22 in tumor growth and angiogenesis, although it is certainly an avenue for further study.

In the case of HUVECs, it appears that IL-22 promotes its canonical signaling pathways, including but not limited to MAPK, STAT3, and AKT. STAT3 is known to promote growth and survival\(^72\). Hence IL-22 promoting HUVEC proliferation and survival is consistent with the phosphorylation of STAT3 observed following IL-22 treatment. The MAPK and STAT3 pathways are known to act synergistically in the setting of interleukin-22 treatment\(^73\). As a result it can be difficult to discern which downstream effects are due exclusively to one signaling cascade versus another, even in the presence of pharmacologic inhibition. The data observed, however, demonstrates that IL-22 induces known effector signaling pathways in HUVECs, and thus there is likely conservation of many of IL-22’s effects on epithelial cells within the endothelial subset.

VEGFA was used extensively as a positive control for determining the angiogenic effects of interleukin-22. As expected, VEGF potently induced
HUVEC and MVEC proliferation, survival, and migration. VEGFR-2 (Flk-1) is agreed to be the major VEGF receptor responsible for the effects of VEGFA in endothelial cells\textsuperscript{14}. As a receptor tyrosine kinase (RTK), VEGFR-2 has intrinsic kinase activity that results in autophosphorylation and activation after ligand binding and dimerization\textsuperscript{14,74}. RTKs such as VEGFR-2 contain SRC-homology 2 domains that can signal through extensive, often overlapping pathways that include ERKs, AKT, and STATs, among many other intracellular mediators\textsuperscript{75}.

In contrast, the IL-22R is a type-II cytokine receptor that requires dimerization of the IL-22R1 and IL-10R2 subunits after ligand binding. The kinases JAK1 and TYK2 are associated to the cytoplasmic portions of the IL-22R1 and IL-10R2 subunits respectively\textsuperscript{50}. In the case of the IL-22R1 subunit, STAT3 is associated with the cytoplasmic chain before IL-22 ligand binding and receptor dimerization, which allows for rapid phosphorylation by JAK1, P-STAT3 dimerization, and translocation to the nucleus\textsuperscript{47,50}. IL-22 is known to induce STAT, MAPK, and AKT pathways, the activation of which were observed in HUVECS, but importantly, IL-22 has been shown in multiple studies to activate JAK1, but not JAK2\textsuperscript{73,76}. Cytokine receptors are known to exhibit “cross-talk,” and JAK2 in particular has been shown to activate EGFR independently of ligand binding\textsuperscript{77,78}. Given that IL-22 does not activate JAK2, it is possible that IL-22 signaling induces a more narrow range of downstream effectors compared to RTKs such as VEGFR-2. Furthermore, JAK1 is absolutely essential for IL-22 signaling\textsuperscript{73}. This implies that although TYK2 is
activated by IL-22, it is contingent on JAK1, and that subsequent STAT3 phosphorylation is the major mediator of IL-22 signaling. These functional characteristics could explain differences observed between IL-22 and VEGF in both signaling by western blot analysis, and using *in-vitro* assays.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Protopsaltis, N.; Liang, W.; Nudleman, E.; Ferrara, N. The dissertation author was the primary investigator and author of this material.
CHAPTER 3.

Effects of Interleukin-22 in an EL4 Tumor Model

3.1 - Introduction

EL4 cells are a T-cell lymphoma on the C57BL/6 background, and as observed in multiple types of T-cells, EL4 cells are able to produce IL-22\textsuperscript{79}. Multiple conditions were ultimately tested to determine the extent to which EL4 cells can produce IL-22 \textit{in-vitro}, and to confirm previous reports that IL-22 cannot directly affect cells of hematopoietic origin\textsuperscript{56}. A major aim of this investigation was to determine the effects of inhibiting IL-22 on tumor growth. As such, EL4 was an aptly suited model since the cells were postulated to produce IL-22 \textit{in-vivo} as well as not be directly inhibited by IL-22 blockade.
3.2 - Results

3.2.1 - EL4 cells do not express the IL-22 receptor

It has been reported that hematopoetic cells do not express the IL-22Rα1 subunit\textsuperscript{45,56,80}. A western blot confirmed that EL4 cells do not stain for IL-22R (Figure 21). Hep55.1C, a mouse hepatocellular carcinoma cell line, served as a positive control to ensure that this lack of protein staining was not due to decreased species affinity of the antibody, but was indeed a lack of IL-22 receptor expression. It was important to show that EL4 cells lack this receptor, as EL4 expression of the IL-22R could affect cell growth with either addition or blockade of IL-22 \textit{in-vitro}. 
Figure 21: Mouse IL-22 Receptor Expression. Representative Western Blot shows that neither EL4 nor GL261 express the IL-22Rα1 subunit.
3.2.2 - Neither addition nor blockade of IL-22 affects EL4 growth \textit{in-vitro}

To examine the effects of IL-22 in tumor angiogenesis, it was first necessary to ensure that IL-22 did not have a direct effect on EL4 cells themselves. Adding IL-22 to EL4 cells in culture did not result in an increase in cell number after 6 days, determined by an alamar blue cell viability assay (Figure 22). Doses from 5 ng/mL failed to increase cellular proliferation compared to PBS control, whereas cells plated in the presence of 10% FBS resulted in approximately 3 times greater cell number at endpoint compared to PBS control.

Given the observation that increased FBS promoted EL4 cell proliferation, varying doses of IL-22 were added with media containing either 0, 1, or 2% FBS. Regardless of media FBS content, IL-22 concentrations ranging from 5 – 100 ng / mL did not increase cellular proliferation (Figure 23).

The use of EL4 as a model system to investigate the role of IL-22 in tumor growth and angiogenesis ultimately requires administration of an anti-IL22 antibody. Although IL-22 did not increase EL4 proliferation \textit{in-vitro}, it was necessary to ensure that IL-22 antibody blockade would not directly inhibit EL4 cell growth, as this could confound the effects of the antibody blockade on EL4 tumor growth \textit{in-vivo}. Increasing doses of anti-IL-22 antibody did not result in decreased cell growth compared to IgG1A antibody treatment after 6 day incubation (Figure 24).
**Figure 22:** IL-22 Does Not Promote EL4 Proliferation. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 2, *** p < .001 by one-way ANOVA with post-hoc Dunnett's test for multiple comparisons)
Figure 23: Increasing Serum Concentration Does Not Rescue IL-22
Independent EL4 Proliferation. Assessed by alamar blue viability assay. (error bars indicate standard deviation. n = 2, differences not significant by one way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
**Figure 24:** Anti-IL22 Antibody Does not Inhibit EL4 Growth in Vitro. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 3, * p < .05 by two way anova with post-hoc Bonferroni correction for multiple comparisons)
3.2.3 - Anti-IL22 antibody treatment reduces EL4 tumor growth *in-vivo*

After ensuring that the blockade of IL-22 would not directly inhibit the growth of EL4 cells, they were injected into the flanks of C57BL/6 mice. Administration of anti-IL22 antibody resulted in a statistically significant decrease in tumor growth compared to IgG1A isotype control antibody treatment (Figure 25). Repeat study with a wider range of antibody concentrations demonstrated a dose-dependent effect of anti-IL22 antibody treatment on inhibiting EL4 tumor growth in C57BL/6 mice (Figure 26).

After observing the effects of IL-22 on endothelial cells, it was conceivable that IL-22 and VEGF could be acting synergistically to enhance tumor growth. To address this possibility, anti-IL22 and anti-VEGF combination therapy were given to EL4 tumor bearing mice, and it was observed that while combination treatment resulted in the greatest reduction in tumor growth compared to isotype control, the combination therapy did not result in a statistically significant difference compared to either therapy alone (Figure 27). Furthermore, ELISA analysis on these tumors revealed that the mice treated with anti-VEGF monotherapy had higher levels of IL-22 compared to those treated with anti-IL22 monotherapy (Figure 28). While it appears that anti-VEGF therapy increased tumor levels of IL-22, one-way ANOVA lacked the statistical power to find a significant difference.

*Rag1*<sup>−/−</sup> mice lack any functional T-cells, and therefore any significant contribution of IL-22 within the tumor microenvironment would have to be
derived from the syngeneic tumor graft. EL4 cells were injected in Rag1−/− mice, and as previously observed in C57BL/6 mice, anti-IL22 antibody treatment resulted in a statistically significant decrease in tumor growth compared to treatment with isotype control antibody (Figure 29). A repeat study with a larger number of mice in each treatment group yielded the same conclusion (Figure 30). In this second batch of EL4 bearing Rag1−/− mice, tumors were collected at day 11 and those treated with anti-IL22 and anti-VEGF antibody were found to have a statistically significant decrease in weight compared to those treated with isotype control (Figure 31). These same tumors were stained with antibodies against CD31b, and those treated with anti-IL22 antibodies had a significant decrease in both the percent area of the tumor that was positively stained (Figure 32), as well as the number of vessels staining within the tumor (Figure 33).
Figure 25: Anti-IL22 Antibody Treatment Reduces EL4 Tumor Growth in C57BL/6 Mice. (error bars indicate standard deviation, n = 5, ** p < .01 by two-way ANOVA with post-hoc Šidák's correction for multiple comparisons)
Figure 26: Anti-IL22 Antibody Treatment Demonstrates a Dose-Dependent Reduction in EL4 Tumor Growth in C57BL/6 Mice. (error bars indicate standard deviation, n = 5, ns = not significant, * p < .05, ** p < .01 by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 27: Combination Anti-IL22 and Anti-VEGF Antibody Treatment Reduces EL4 Tumor Growth in C57BL/6 Mice. (error bars indicate standard deviation, n = 8, * p < .05, ** p < .01 by two-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 28: Anti-IL22 Treatment Reduces EL4 Tumor IL-22 Levels in C57BL/6 Mice. Assessed by ELISA. (error bars indicate standard deviation, n = 6, ns = not significant, * p < .05 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 29: Anti-IL22 Antibody Treatment Reduces EL4 Tumor Growth in Rag1\textsuperscript{\textasciitilde} Mice. (error bars indicate standard deviation, n = 5, ** p < .01, *** p < .001 by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 30: Anti-IL22 Antibody Treatment Reduces EL4 Tumor Growth in Rag1−/− Mice. (error bars indicate standard deviation, n = 9 - 10, ns = not significant, *** p < .001 by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 31: Anti-IL22 Antibody Treatment Reduces EL4 Tumor Weight at Endpoint in Rag1−/− Mice. (error bars indicate standard deviation, n = 9 - 10, ns = not significant, * p < .05, *** p < .001 by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 32: Anti-IL22 Antibody Treatment Reduces CD31b Staining in EL4 Tumors From Rag1−/− Mice. (error bars indicate standard deviation, n = 5, ns = not significant, * p < .05, ** p < .01 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 33: Anti-IL22 Antibody Treatment Reduces The Number of CD31b Staining Vessels in EL4 Tumors From Rag1^−/− Mice. (error bars indicate standard deviation, n = 5, ns = not significant, *** p < .001 by one-way ANOVA with post-hoc Dunnett's test for multiple comparisons)
3.2.4 - CRISPR-Cas9 knockout of IL-22 ligand in EL4 cells

The primary reason the T-cell lymphoma EL4 was initially chosen to investigate the role of IL-22 in tumor growth and angiogenesis is that T-cells can produce IL-22. It was found that EL4 cells *in-vitro* can produce IL-22 levels over 1 µg/mL when stimulated with IL-6 and TGFβ (Figure 34). Although production is much smaller without any stimulation, hypoxic culture also greatly enhances IL-22 production.

A CRISPR-Cas 9 knockout transfection and sorting yielded a number of viable clones, which were stimulated and assayed by ELISA for IL-22 production (Figure 35). Selected clones were again stimulated and had their conditioned media concentrated 10 fold, which was then assayed by ELISA for IL-22 (Figure 36). The media produced from clones for IL-22 ligand KO numbers 3 and 10 were found to completely lack detectable levels of IL-22. Additionally, while the media used to culture these cells contains 10% FBS, the concentrated media also did not demonstrate any IL-22 reactivity in the ELISA assay. The FBS is bovine in origin, and despite species specific reactivity of the IL-22 ELISA, it is conceivable that there could be some cross-reactivity, especially given the very high protein content in the concentrated media. This experiment, however, assuaged any concerns that the FBS containing media may actually contain IL-22 that could react with the assay.
Figure 34: IL-22 Production by Stimulated EL4 Cells. Assayed by ELISA (error bars indicate standard deviation, n = 2, * p < .05, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 35: IL-22 Production by Stimulated EL4 Cells Following CRISPR Targeted IL-22 Ligand Knockout. Assayed by ELISA. (error bars indicate standard deviation, n = 4 technical replicates for each clone assayed).
**Figure 36:** IL-22 Levels in Concentrated Media Collected from Stimulated CRISPR Knockout EL4 Clones. Assayed by ELISA. (error bars indicate standard deviation, n = 4 technical replicates for each clone assayed)
3.2.5 - Effects of CRISPR-Cas 9 IL-22 ligand knockout \textit{in-vivo}

The aforementioned clones 3 and 10 were injected in nude mice, and found to result in a statistically significant decrease in tumor growth compared to a control KO clone, named control 1, as well as compared to WT cells (Figure 37). An ELISA assay for IL-22 within the homogenized tumors found no detectable levels of IL-22 from the mouse tumors derived from both clones 3 and 10 (Figure 38). This suggests that in athymic nude mice, any IL-22 in the tumors is derived from the EL4 cells themselves, and not from infiltrating immune cells.

Since the control 1 clone actually grew most quickly in the nude mice, the decision was made to switch to another ligand knockout clone as a control. Ligand knockout clone 6 was chosen, as it had undergone the CRISPR-Cas9 transfection procedure and sorting, but was found on ELISA to actually produce detectable levels of IL-22. This clone, alone with clones 3, 10, and wild type EL4 cells were injected in athymic nude mice and treated with either anti-VEGF antibody or isotype control antibody. In the animals treated with IgG2A isotype control, the clone subtype had no statistically significant effect on tumor growth (Figure 39). In those treated with anti-VEGF antibody, however, clones 6 and 10 had a statistically significant reduction in tumor growth (Figure 40). While clone 3 had produced a statistically significant decrease in tumor growth compared to wild type in the untreated animals, it failed to repeat the same effect in this experiment. As such, it appears that
despite all of the clones being derived from the same batch of wild-type cells, there is inherent variability in their cell growth \textit{in-vivo}.

ELISA assays for IL-22, IL-17, and VEGF were conducted on mice from both IgG2A and anti-VEGF treatment groups (Figures 41-49). As previously observed, clones 3 and 10 in both treatment groups lacked detectable levels of IL-22 (Figure 41). Interestingly, clone 10 had statistically elevated levels of IL-17 production (Figure 42). Of note, clone 10 produced the smallest tumors at endpoint in both nude mice studies conducted with CRISPR knockout EL4 clones. While a previous study had shown that IL-17 can mediate resistance to VEGF, when examining IL-17 levels in both isotype treated animals (Figure 48) and those treated with anti-VEGF (Figure 49), the IL-17 levels in both groups were significantly higher in tumors derived from clone 10. The mice treated with anti-VEGF therapy had higher levels of IL-17 regardless of the clone used, although the levels of clone 10 were greater, relatively, among this treatment group.

Among EL4 clones in both treatment groups, there was no difference in VEGF levels (Figure 43). The animals treated with anti-VEGF therapy, however, had higher levels of VEGF within their tumors compared to those treated with isotype control antibody (Figures 46-47). As previous studies have suggested, this is could be due either to sequestration of VEGF within the tumors by the antibody, or biological upregulation of VEGF production either by the tumor cells themselves or the stroma\textsuperscript{81}.
Figure 37: EL4 Tumor Growth in Athymic Nude Mice. (error bars indicate standard deviation, n = 5, * p < .05, **** p < .001 by two way ANOVA with post-hoc Tukey's test for multiple comparisons)
**Figure 38:** Tumor IL-22 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 39: EL4 CRISPR Clone Growth in Athymic Nude Mice Treated with IgG2A Isotype Antibody. (error bars indicate standard deviation, n = 5, ns = not significant by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 40: Mouse EL4 CRISPR Clone Growth in Athymic Nude Mice Treated with Anti-VEGF Antibody. (error bars indicate standard deviation, n = 5, ns = not significant, *** p < .001 by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 41: Tumor IL-22 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice. Assayed by ELISA. (error bars indicate standard deviation, n = 10, ns = not significant, ** p < .01, *** p < .001 by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 42: Tumor IL-17 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice. Assayed by ELISA. (error bars indicate standard deviation, \( n = 10 \), *** \( p < .001 \) by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 43: Tumor VEGF Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice. Assayed by ELISA, (error bars indicate standard deviation, n = 10, ns = not significant by one-way ANOVA with Tukey’s test for multiple comparisons)
Figure 44: Tumor IL-22 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with IgG2A Isotype Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant. * p < .05, ** p < .01 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 45: Tumor IL-22 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with Anti-VEGF Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant, *** p < .001 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 46: Tumor VEGF Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with IgG2A Isotype Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, * p < .05 by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 47: Tumor VEGF Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with Anti-VEGF Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
**Figure 48:** Tumor IL-17 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with IgG2A Isotype Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant, *** p < .001 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 49: Tumor IL-17 Levels in EL4 CRISPR Clone Bearing Athymic Nude Mice Treated with Anti-VEGF Antibody. Assayed by ELISA. (error bars indicate standard deviation, n = 5, ns = not significant, *** p < .001 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
3.3 - Discussion

This investigation yielded several important findings. First, it was confirmed that EL4 cells can produce detectable levels of interleukin-22 \textit{in-vitro}, and that higher levels are produced in the setting of hypoxia than normoxia. Tumors are known to have decreased oxygen saturation, which is a major mechanism by which they can induce angiogenesis. The observation that decreased oxygen levels also increase IL-22 production appears consistent with the potential for IL-22 to mediate tumor growth through a paracrine network in an EL4 model. It was also confirmed that neither IL-22 addition nor blockade affected the growth of EL4 cells \textit{in-vitro}. Blocking IL-22 reduced tumor growth in "wild type" C57BL/6 mice, immunodeficient athymic nude mice lacking functional CD4+ T-cells, as well as in Rag1\textsuperscript{−/−} mice lacking any functional adaptive immune cells. Interestingly, the C57BL/6 mice had the lowest tumor levels of IL-22, despite having the most active immune system of the three mouse models investigated. A CRISPR-Cas9 knockout was ultimately not able to provide conclusive evidence that EL4 cells that lack IL-22 production would grow more slowly than wild-type EL4 cells \textit{in-vivo}. It was found, however, that the tumors isolated from athymic nude mice bearing EL4 clones that did not produce any IL-22 \textit{in-vitro} also lacked detectable levels of IL-22 by ELISA. This strongly suggests the IL-22 measured within EL4 tumors is derived from the EL4 cells themselves, and not from infiltrating lymphocytes.
or monocytes. While more work is needed to elucidate the particular paracrine mechanisms by which inhibiting IL-22 can reduce tumor growth, it is evident across multiple murine models that IL-22 blockade can consistently inhibit tumor growth *in-vivo*.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Protopsaltis, N.; Liang, W.; Nudleman, E.; Ferrara, N. The dissertation author was the primary investigator and author of this material.
CHAPTER 4.

Effects of IL-22 in a GL261 Tumor Model

4.1 - Introduction

Use of an EL4 model demonstrated that blocking IL-22 can reduce tumor growth \textit{in-vivo}. While EL4 cells produce IL-22 \textit{in-vitro} and \textit{in-vivo}, the glioblastoma tumor model GL261 was chosen as an additional model for its predicted lack of endogenous IL-22 production, and was postulated to lack IL-22 receptor expression based on tissue-specific expression studies\textsuperscript{45}. Glioblastomas are highly vascularized, and as a result, glioblastoma multiforme is one of the cancers for which the anti-VEGF antibody bevacizumab has been FDA approved. This chapter examines the role of IL-22 on GL261 tumor cells both \textit{in-vitro} and \textit{in-vivo}. 
4.2 - Results

4.2.1 - GL261 does not express IL-22 receptor

Simultaneous investigation of GL261 along EL4 demonstrated that GL261 cells do not express the IL-22Rα1 subunit (Figure 21). It was important to show that GL261 cells lack this receptor, as GL261 expression of the IL-22R could affect cell growth with either addition or blockade of IL-22 in-vitro.

4.2.2 - Neither addition nor blockade of IL-22 affects GL261 growth in-vitro

To examine the effects of IL-22 in tumor angiogenesis, it was first necessary to ensure that IL-22 did not have a direct effect on GL261 cells themselves. Adding IL-22 to GL261 cells in culture did not result in an increase in cell number after 6 days, determined by an alamar blue cell viability assay (Figure 50). Doses from 5 to 100 ng IL-22 / mL failed to increase cellular proliferation compared to PBS control, whereas cells plated in the presence of 10% FBS resulted in a cell number approximately 3 fold greater at endpoint compared to PBS control.

The use of GL261 as a model system to investigate the role of IL-22 in tumor growth and angiogenesis ultimately requires administration of an anti-IL22 antibody. Although IL-22 did not increase GL261 proliferation in-vitro, it was necessary to ensure that IL-22 antibody blockade would not directly inhibit
GL261 cell growth, as this could confound the effects of the antibody blockade on GL261 tumor growth *in-vivo*. Increasing doses of anti-IL-22 antibody did not result in decreased cell growth compared to IgG1A antibody treatment after 6 day incubation (Figure 51). Not only was there no decrease in cell growth between anti-IL22 antibody and IgG1A control, but there was no effect of antibody dose from 0.5 to 50 µg / mL for either antibody added *in-vitro*. Anti-VEGF antibody serves as a control and comparison for *in-vivo* studies, and was also investigated for its ability to inhibit GL261 growth *in-vitro*. 50 µg / mL of anti-VEGF antibody did not inhibit GL261 growth compared to media alone (Figure 52). In summation, the data observed indicates that neither IL-22 addition nor blockade has a direct effect on GL261 cells growth *in-vitro*. 
**Figure 50**: IL-22 Does Not Promote GL261 Proliferation. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 2, *** p < .001 by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
Figure 51: Anti-IL22 Antibody Does not Inhibit GL261 Growth in Vitro. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 3, ns = not significant by two-way ANOVA with post-hoc Šidák’s correction for multiple comparisons)
Figure 52: Anti-VEGF Antibody Does not Inhibit GL261 Growth in Vitro. Assessed by alamar blue viability assay. (error bars indicate standard deviation, n = 6, ns = not significant by Welch’s unpaired t-test)
4.2.3 - GL261 cells do not produce IL-22 in-vitro

While EL4 was chosen for its ability to produce IL-22, it was postulated that as a glioblastoma, it was unlikely GL261 cells would produce IL-22. Indeed, GL261 conditioned media did not contain detectable levels of IL-22 by ELISA (Figure 53). As a control, recombinant murine IL-22 was added, along with the anti-IL22 antibody. IL-22 addition alone demonstrated readout levels beyond the functional range of the ELISA assay, but addition of anti-IL22 antibody reduced the detected levels. Antibody was also added to the DMEM media alone, as well as to the GL261 conditioned media, neither of which demonstrated detectable IL-22 levels in the ELISA assay. Not only did these controls reveal that the anti-IL22 antibody can sequester and reduce detectable IL-22 in the ELISA assay, but that the antibody in and of itself can not react with the ELISA assay to give false-positive results.
Figure 53: IL-22 Levels in GL261 Tumor Conditioned Media. (error bars indicate standard deviation, n = 2 technical replicates, *** p < .001 by one-way ANOVA with post-hoc Tukey's test for multiple comparisons)
4.2.4 - Blocking IL-22 reduces GL261 tumor growth in-vivo

After ensuring that the blockade of IL-22 would not directly inhibit the growth of GL261 cells, they were injected into the flanks of C57BL/6 mice. Administration of anti-VEGF antibody resulted in a statistically significant decrease in tumor growth compared to IgG1A isotype control antibody treatment (Figure 54). Anti-IL22 antibody treatment also resulted in a significant decrease compared to isotype control, and the effect was statistically equivalent to the anti-VEGF therapy. ELISA analysis revealed an increase of intra-tumor IL-22 in the mice treated with anti-VEGF but not anti-IL22 antibody (Figure 55). Interestingly, despite not producing detectable levels of IL-22 in-vitro, it appears that GL261 tumors contain IL-22 in-vivo.

Given the effects of IL-22 on endothelial cells, it was conceivable that IL-22 and VEGF could be acting synergistically to enhance tumor growth. Although studies in EL4 tumor bearing mice failed to demonstrate a greater reduction for combination antibody treatment than either therapy alone, to address this possibility with a GL261 model, anti-IL22 and anti-VEGF combination therapy were given to GL261 tumor bearing athymic nude mice (Figure 56). Having observed intra-tumor IL-22 in the GL261 tumors from C57BL/6 mice, nude mice were chosen as an attempt to eliminate T-cell sources of IL-22 and ascertain if the IL-22 was indeed derived from the glioblastoma tumors themselves. Both anti-IL22 and anti-VEGF monotherapy resulted in a significant reduction in tumor growth compared to isotype control
treatment. While Combination anti-IL22 and anti-VEGF treatment resulted in the greatest reduction in tumor growth compared to isotype control, the combination therapy did not result in a statistically significant difference compared to either therapy alone. Of note, this was the same effect that was observed in an EL4 tumor model.

ELISA analysis on these tumors revealed that the mice treated with anti-VEGF monotherapy, but not anti-IL22 treatment, had higher levels of IL-22 compared to those treated with isotype control antibody (Figure 57). Those treated with combination anti-IL22 and anti-VEGF therapy had the highest levels of intratumor IL-22.

CD31b staining on GL261 tumors from C57BL/6 mice revealed no difference in the percent area that was positively stained (Figure 58).
Figure 54: Anti-IL22 Antibody Treatment Reduces GL261 Tumor Growth in C57BL/6 Mice. (error bars indicate standard deviation, n = 9 - 10, * p < .05, ** p < .01 by two-way ANOVA with post-hoc Tukey's test for multiple comparisons)
Figure 55: GL261 Tumor IL-22 Levels in C57BL/6 Mice. Assessed by ELISA. (error bars indicate standard deviation, n = 8 - 10, ns = not significant, * p < .05 by Welch’s unpaired t-test)
Figure 56: Anti-IL22 Antibody Treatment Reduces GL261 Tumor Growth in Athymic Nude Mice. (error bars indicate standard deviation, n = 10, *** p < .001 by two-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 57: GL261 Tumor IL-22 Levels in Athymic Nude Mice. Assessed by ELISA. (error bars indicate standard deviation, n = 10, ns = not significant, ** p < .01, *** p < .001 by one-way ANOVA with post-hoc Tukey’s test for multiple comparisons)
Figure 58: Neither Anti-IL22 nor Anti-VEGF Antibody Treatment Reduces The Percent Area Stained for CD31b in GL281 Tumors From C57BL/6 Mice. (error bars indicate standard deviation, n = 2-5, ns = not significant by one-way ANOVA with post-hoc Dunnett’s test for multiple comparisons)
4.3 - Discussion

It was found that IL-22 does not directly stimulate GL261 cell growth \textit{in-vitro}, which contrasts a report published during the process of this study\textsuperscript{82}. While the researchers measured IL-22 binding protein in mouse tumors, the data does not indicate that they directly examined IL-22R. Having demonstrated that GL261 cells lack IL-22 receptor expression, it seems unlikely that IL-22 could directly stimulate GL261 growth \textit{in-vitro}, which is in accordance with the data reported here. The same researchers found that IL-22 can promote GL261 growth \textit{in-vivo} through combination of IL-22 knockout and directly injecting IL-22 into the brains of mice with GL261 tumors. While GL261 was studied subcutaneously in this work, it was found that the tumors endogenously contained detectable levels of IL-22, despite the observation that IL-22 was undetectable by ELISA \textit{in-vitro}. During initial characterization of IL-22, it was observed that murine spleen cells did not produce IL-22 when stimulated \textit{in-vitro}, yet demonstrated IL-22 production \textit{in-vivo}\textsuperscript{42}. The investigators postulated that this discrepancy could arise from differential or indirect mechanisms of IL-22 induction, and hence it is possible that the same effects are true regarding GL261 production of IL-22 \textit{in-vivo}, and lack thereof \textit{in-vitro}. 
It was found that IL-22 levels in mice treated with anti-VEGF therapy were significantly higher than those treated with isotype antibody. This suggests that IL-22 may be upregulated in GL261 tumors \textit{in-vivo} as a method of escaping anti-angiogenic pharmacological inhibition. Despite the tumor inhibition observed \textit{in-vivo} following anti-IL22 and anti-VEGF therapy, staining for CD31b did not demonstrate a statistically significant difference in blood vessel density for treated GL261 tumor bearing mice.

The anti-angiogenic effect of a therapy, however, is more complicated than simply the density of blood vessels within the tumor\textsuperscript{83}. Some studies have shown that angiogenesis inhibitors can actually increase tumor blood flow and oxygenation during the initial few weeks of treatment, and furthermore, that some benefit of anti-angiogenic therapy may arise from reducing interstitial pressure\textsuperscript{84,85,13}. Additionally, while it is well accepted that VEGF can promote tumor growth and neovascularization, paradoxically, pharmacologic inhibitors do not always allow for understanding the contribution of VEGF to angiogenesis\textsuperscript{86,87}.

In the setting of GL261 bearing mice, anti-IL22 and anti-VEGF therapy both resulted in a statistically significant decrease in tumor growth, but not blood vessel density. It is possible that they may slow down the initial process of angiogenesis, i.e. vessel sprouting, but as the tumor secretes increasing quantities of IL-22, among other growth factors, the pharmacologic inhibition fails to suppress new vessel formation. As such, perhaps immunofluorescence
staining at an earlier time point during tumor growth would reveal that the treatment does in fact reduce tumor blood vessel density. Given that anti-VEGF therapy is well-known to reduce angiogenesis, it could be argued that the lack of significant difference observed in treated GL261 bearing mice suggests that staining for microvessel density in GL261 tumors at the end point chosen is not indicative for the ability of a treatment to reduce to angiogenesis. In other words, the lack of reduction in CD31b staining in GL261 bearing mice treated with anti-IL22 antibody does not necessarily mean that blocking IL-22 cannot reduce tumor angiogenesis. This is especially plausible given the results observed on endothelial cells in-vitro, the reduction in tumor growth by anti-IL22 treatment in both an EL4 and GL261 model, and the reduction in vessel density observed in the EL4 model.

Furthermore, the GL261 tumors demonstrated significant necrosis and fluid accumulation. Staining for CD31b is contingent on having solid tumor with enough cross sectional area for quantification. It is possible that the anti-VEGF and anti-IL22 treatment reduced tumor growth, but that there was consistent vessel density along the tumor periphery, i.e. non-necrotic portion. As such, there may be systemic error inherent from needing to measure vessel density in portions of tumor amenable to staining, even if the treatments did in fact reduce neovascularization resulting in tumor hypoxia, necrosis, and reduction of growth. Further studies using in-vivo live imaging of vessel parameters are
a potentially worthwhile future endeavor that would bypass this limitation, and would provide valuable insight to the effects of anti-angiogenic therapy.

In summation, IL-22 blockade results in a reduction of GL261 growth in-vivo. These findings, taken in combination with the effects observed on endothelial cells by IL-22, suggests that IL-22 may promote angiogenesis in glioblastoma, although further work is needed to definitively elucidate the mechanisms observed.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Protopsaltis, N.; Liang, W.; Nudleman, E.; Ferrara, N. The dissertation author was the primary investigator and author of this material.
CHAPTER 5.

Materials and Methods

Materials

Mice

Female (6-8 week) C57BL/6 and Rag1/-/- mice were purchased from Jackson Laboratories and were allowed 5 days to acclimate following arrival. Female (6-8 week) athymic nude mice were purchased from the University of California, San Diego. Animals were housed in clean cages and experimental procedures were carried out under pathogen-free conditions in accordance with established standards of care and approved protocols from the Animal Care and Use Committee of the University of California, San Diego. Prior to tissue harvest, mice were euthanized by CO$_2$ inhalation followed by cervical dislocation.

Cells

Primary human umbilical vein endothelial cells (HUVEC, passage 4–8) were purchased from Lonza and cultured on 0.1% gelatin-coated plates in EGM-2 endothelial cell growth media. Primary human lung microvascular
endothelial cells (MVEC, passage 3-6) were purchased from Lonza and cultured on 0.1% gelatin coated plates in EGM-2 microvascular cell growth media. EL4 cells were purchased from ATCC and maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). GL261 cells were a gift from Dr. Santosh Kesari and maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Reagents

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Method Details

Conditioned Media Preparation and IL-22 ELISA

One million EL4 cells in 25 cm$^2$-flasks were stimulated with cytokines as noted. Cells were grown in a 37°C humidified atmosphere with ambient O$_2$ in the setting of normoxia, or a 37°C humidified atmosphere with 1% O$_2$ in the setting of hypoxia. After 6 days, media were collected. IL-22 concentrations were measured using a murine R&D systems IL-22 Quantikine ELISA kit according to the manufacturer’s instructions.

Western Blots

HUVECs between passages 6 and 8 were seeded onto 6 or 12-well dishes pretreated with a 1% gelatin solution. The cells were starved overnight in EBM-2 solution supplemented with 2% FBS. The next morning, cells were rinsed with EBM-2 media, and starved in EBM-2 media containing 0.1% FBS for 4 hours. The media was then refreshed again containing cytokines of interest and stimulated for the time indicated. The cells were then rinsed with ice-cold PBS, lysed with RIPA buffer supplemented with 1% phosphatase/protease inhibitor, and collected with a cell scraper. The lysate was placed on a rotating inverter for 10 minutes, centrifuged for 30 minutes at 14000 x g, and the supernatant collected by pipette. Lysates were diluted with 4X running buffer and 10% β-Mercaptoethanol, heated for at least 5 minutes
at 95°C, and run by SDS-PAGE. Protein was transferred to nitrocellulose membranes for antibody staining and quantification as indicated.

**Endothelial Cell Proliferation Assays**

HUVECs between passages 6 and 8 were seeded onto 12-well dishes pretreated with a 0.1% gelatin solution. The cells were starved overnight in EBM-2 solution supplemented with 2% FBS. The next morning, cells were rinsed with EBM-2 media, and refreshed with EBM-2 solution supplemented with 10% FBS containing cytokines of interest and incubated for 3 days.

MVECs between passages 3 and 6 were starved overnight in EBM-2 solution supplemented with 2% FBS. The next morning, cells were rinsed with EBM-2 media and seeded with EBM-2 solution supplemented with 2% FBS and cytokines of interest onto 96-well dishes pretreated with a 0.1% gelatin solution.

**Endothelial Cell Survival Assays**

HUVECs between passages 6 and 8 were seeded onto 96-well dishes pretreated with a 0.1% gelatin solution. The cells were grown in EBM-2 solution supplemented with 2% FBS and cytokines of interest, then incubated for 3 days. On day 4, cells were incubated with Alamar Blue for 8h.
Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength.

MVECs between passages 6 and 8 were seeded onto 96-well dishes pretreated with a 0.1% gelatin solution. The cells were grown in EBM-2 solution supplemented with 2% FBS and cytokines of interest, then incubated for 3 days. After 3 days, the media was refreshed by adding 100 µL EBM-2 solution supplemented with 2% FBS and cytokines of interest, and incubated for 3 additional days. On day 7, cells were incubated with Alamar Blue for 8h. Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength.

**Tumor Cell IL-22 Proliferation Assays**

1x10³ GL261 or 2.5x10³ EL4 cells were seeded onto 96-well dishes containing DMEM supplemented with 0,1, or 2% FBS, 1% antimycobacteria/antibacterial solution, and cytokines of interest. After 6 days, cells were incubated with Alamar Blue for 8h. Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength.
Tumor Cell Antibody Treatment Assays

$1 \times 10^3$ GL261 or $2.5 \times 10^3$ EL4 cells were seeded onto 96-well dishes containing DMEM supplemented with 10% FBS, 1% antimycobacteria/antibacterial solution, and antibodies of interest. After 6 days, cells were incubated with Alamar Blue for 8h. Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength.

Migration Assay

HUVECs (passage 6-8) were cultured and serum-starved as described in “Western blots.” The cells (10000 cells) in 150 µl of EBM-2 medium were then added to the upper chamber of 24 well 8 µm pore size cell culture inserts (Falcon) coated with 0.1% gelatin. The lower compartment was filled with 600 µL EBM-2 medium containing 1 µL/mL PBS with 0.1% BSA, 10 ng/mL IL-22, 25 ng/mL IL-22, 50 ng/mL IL-22, 100 ng/mL IL-22, or 50 ng/ml VEGF$_{165}$. The plates were incubated at 37°C to allow migration. After 4h, cells were fixed with 4% PFA for 30 min and then stained with crystal violet for 30 min at RT. Migrated cells on the bottom side of the insert membrane were quantified by counting whole area of the insert at 20X magnification. The experiments were carried out in quadruplicate and repeated two times. For MVEC, the migration assays were carried out as described for HUVECs, with the only alteration being an 8h incubation time.
EL4 in-vivo studies

Mice were injected subcutaneously with $1 \times 10^6$ cells in a 1:1 mixture of reduced growth factor basement membrane extract and sterile PBS. 5 days post injection, mice were sorted into groups based on weight and tumor size. They were given antibody treatment by intraperitoneal injection. Mice were again treated and tumor size measured every other day until conclusion of the study.

GL261 in-vivo studies

Mice were injected subcutaneously with $1 \times 10^6$ cells in a 1:1 mixture of reduced growth factor basement membrane extract and sterile PBS. For C57BL/6 mice, 13 days post injection they were sorted into groups based on weight and tumor size. On day 16, tumor size was measured and mice were given antibody treatment by intraperitoneal injection. Mice were again treated and tumor size measured every other day until conclusion of the study. For athymic nude mice: 5 days post injection they were sorted into groups based on weight and tumor size. They were given antibody treatment by intraperitoneal injection. Mice were again treated and tumor size measured every other day until conclusion of the study.
Quantification and Statistical Analysis

All image analysis was done using ImageJ. Cell counting was done manually using the cell counter function.

Statistical parameters, including the value of n, are indicated in the figure legends. All statistical analysis was conducted in Graphpad Prism software. One-way ANOVA, Two-way ANOVA, and Unpaired t-test with Welch’s correction were used as statistical tests. All statistical tests use post-hoc analysis when appropriate to account for multiple comparisons. Test details are included in figure legends. Data are considered significant when p < 0.05. Significant p values are represented in the figures as follows: ***p < 0.001, **p < 0.01, *p < 0.05.
References


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