‘RIG’-ING TUMOR BLOOD VESSELS: RIG-1 TREATMENT ON PROLIFERATING, SENESCENT, AND QUIESCENT ENDOTHELIAL CELLS

by

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The complex nature of a tumor is derived from its composition of both normal and malignant cells. Selectively targeting the malignant cells while healthy tissues are preserved is the primary objective of many cancer therapies. RIG-1 is an RNA-sensing protein that causes inflammation to induce cell-programmed death. It can become activated by RNA from different viruses and the cell's own RNA to protect against cell damage. Our research attempts to use this molecule as a treatment method in conjunction with other existing cancer treatments, like radiation, while utilizing different cell stages to kill tumor blood vessels. We hope to use this molecule to build the immune system's ability to differentiate between normal and cancer cells. If we can target the tumor blood vessels, we could potentially make other cancer treatments more effective by starving solid tumors while protecting the surrounding healthy tissue.

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List of Abbreviations

RIG-1: Retinoic Acid Inducible Gene 1
HUVECs: human umbilical vein endothelial cells
HMVECs: human microvascular endothelial cells
RLRs: RIG-1 like receptors
CARD: Caspase Recruiting Domains
ISGs: Interferon-stimulating genes
Cas9: Caspase 9
ASC: Inflammasome Adaptor
VEGF: Vascular Endothelial Growth Factor
TGFβ: Transforming Growth Factor Beta
CAFs: Cancer-Associated Fibroblasts
TNFα: Tumor Necrosis Factor-alpha

Introduction

Cancer cells are proliferating cells that are growing and mutating out of control. As they continue to grow, they start to take over other nutrients and space required for organs and cells to function properly. Cancerous endothelial cells, which are the cells that make up blood vessels, behave similarly and are unable to stop dividing, destroying everything in its path.[1] The goal of our studies is to target and kill the cancerous endothelial cells while preventing any damage to surrounding healthy endothelial cells.

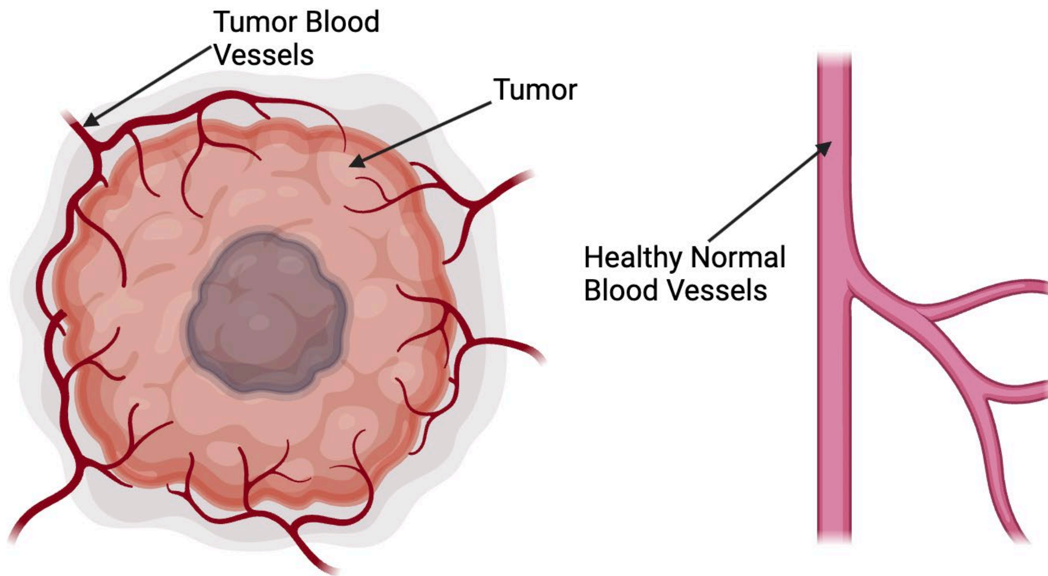


Figure 1: Tumor Blood Vessels vs Normal Blood Vessels

The tumor blood vessels are a part of the tumor or feeding solid tumors and are growing out of control, these are our targets. We are trying to protect the normal blood vessels in other locations of the body.

RIG-1 is an RNA-sensing protein found within the cytoplasm that activates inflammation to induce cell apoptosis, also known as cell death. It can be activated by the presence of DNA but is primarily activated by viral RNA along with endogenous self-RNA to protect against viral invasion or DNA damage.[1] Radiation has been shown to increase nuclear and mitochondrial DNA in the cytoplasm.[2] Endothelial cells are sensitive to radiation therapy and are involved in

radiation-induced pathology in cancerous and normal tissues.[3] Furthermore, growing blood vessels in tumors play an important role in tumor growth and are targeted in various cancer therapies. However, an additional goal of these cancer therapies is to inflict as little damage as possible to healthy surrounding blood vessels.[4] By increasing DNA damage with radiation, we hypothesized that the activation of RIG-1 in endothelial cells with radiation therapy would result in more cell death. We researched how proliferating human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HMVECs) responded to a combination of RIG-1 treatment and irradiation and recorded the changes in apoptosis. We found no synergy between RIG-1 and radiation, with the higher levels of radiation having observably less cell apoptosis.

Following these results, we wanted to determine how RIG-1 impacted endothelial cells in different cell cycle stages. Healthy adult endothelial cells are primarily in the quiescence stage (G0), while aged endothelial cells are in the senescence stage and remain in cell cycle arrest.[5,6] Proliferating endothelial cells represent endothelial cell regrowth and migration, which is physiologically associated with growth, wound healing, or pathological conditions such as cancer. Previous studies have shown that RIG-1 is involved in antiviral signaling pathways and plays a crucial role in cardiovascular diseases and specific cancers.[1,7] We compared how different stages of the cell cycle impact RIG-1 activity and the immune cell response. We observed differential gene expression of inflammatory, immune, and vascular genes (TNFα, Cas8, VEGF, ISG15, and IL6) triggered by the RIG-1 pathway in endothelial cells in different cell cycle stages. The differences of RIG-1 signaling on proliferating, senescent, and quiescent endothelial cells have the potential to shed light on its role and utility regarding cancer and cardiovascular diseases.

One method of tumor control is an attempt to target tumor cells and tumor endothelial cells, utilizing RIG-1, that are dividing and migrating out of control while preserving surrounding healthy endothelial cells in the quiescence stage. The various cell stages represent tumor endothelial cells, healthy endothelial cells, and old endothelial cells. Therapeutic approaches to activate RIG-1 and kill tumor cells are currently in advanced preclinical and clinical trials. However, if RIG-1 activation also impacts quiescent endothelial cells, it can lead to possible toxicities.[7] We are interested in investigating how RIG-1 and radiation at different cell stages impact endothelial cell apoptosis and proliferation levels to potentially improve tumor control while also providing insight into how it could impact other cardiovascular and immune system diseases. Furthermore, the proliferating endothelial cells, or cells that could be cancerous, are impacted by these tumor control methods. Alternatively, the senescent cells provide information on how older cells that are permanently in cell cycle arrest would react to this treatment, which is important for understanding how this method of tumor control would be further impacted among different age cohorts. RIG-1 provides a possible treatment method to utilize the immune system to target and induce programmed cell death in both tumor endothelial cells and tumor cells.

Purpose: To induce the innate immune system through activation of RIG-1 in endothelial cells, in combination with radiation or the cell cycle stage, to reduce tumor growth while mitigating the effects of RIG-1 on the surrounding endothelial cells.

Questions:

1. Will radiation increase RIG-1 activation?
2. Will a combination of RIG-1 and radiation have a synergistic effect and induce more endothelial cell apoptosis?

3. Do endothelial cells in different stages of the cell cycle activate different levels of inflammation and immune responses with RIG-1 treatment?
4. Do senescent endothelial cells in vitro and in vivo react to RIG-1 treatment?

Hypotheses:

1. A combination of RIG-1 agonist and radiation will increase endothelial cell apoptosis.
2. Endothelial cells in quiescence will be less reactive to RIG-1-induced cell death than proliferating endothelial cells.
3. Endothelial cells in senescence will be less reactive to RIG-1-induced cell death than both proliferating and quiescent endothelial cells.

Background

RIG-1 and Radiation

Within all vertebrates, there are two immune systems that protect the body against an invasion: the innate immune system, which is a more general defense system and the first line of attack, and then the adaptive immune system, which is more specialized to the virus or infection invading the body.[8] When a virus attempts to hijack host cells, it can inject pieces of its double-stranded DNA or double-stranded RNA into the host. As the virus' genome is replicated by the host and the various proteins the virus requires to survive are produced, the virus can grow.[9] A conserved defense mechanism in cells is the ability to identify DNA and RNA, which could potentially be a viral attack in the cytoplasm, and instigate an inflammatory and antiviral immune response to protect the host.[2]

In the innate immune system, positive and negative strand viral RNA activates a cytoplasmic RNA sensor protein called retinoic acid-inducible gene-1 (RIG-1) to induce inflammation and cell apoptosis.[1,8] However, previous research has also shown that RIG-1 can additionally be activated by endogenous RNA and cause similar immune and inflammatory action.[1] The presence of endogenous RNA or DNA often signals to the organism that a particular cell is damaged, its cellular processes are malfunctioning, or there is an issue during cell division.[10] This induces programmed cell death with the goal of protecting the organism. Radiation has the capability to cause breaks in nuclear and mitochondrial DNA, leading to higher levels of endogenous DNA floating in the cytoplasm of radiation-treated cells.[2] The increase in endogenous DNA within subsets of radiation-treated cells has the potential to increase RIG-1 activation and cause a targeted innate immune system response.

RIG-1 plays a key part in the innate immune system along with MADA5 and LGP2, which are other RIG-1-like receptors (RLRs).[11] RIG-1 is structured with two N-terminal caspase recruiting domains (CARD), two central Rec A-like RNA helicases, and a C-terminal domain that have been highly conserved in RLRs among vertebrates throughout evolution (Figure 2).[11,12] When bound by nucleic acids, RIG-1 exposes its CARD domain and mediates antiviral signaling by activating the mitochondrial antiviral signaling protein (MAVs), which then binds to IRF3 and NFkB.[13] This induces a type-I interferon response (IFN1) leading to cell inflammation, which causes further downstream signaling and activates many interferon-stimulating genes (ISGs) (Figure 3). ISGs ultimately force these target cells into cell cycle arrest, leading to apoptosis.[14] Previous studies have shown that RLRs are responsible for recognizing major viral families, playing a significant role in the immune system.[1]

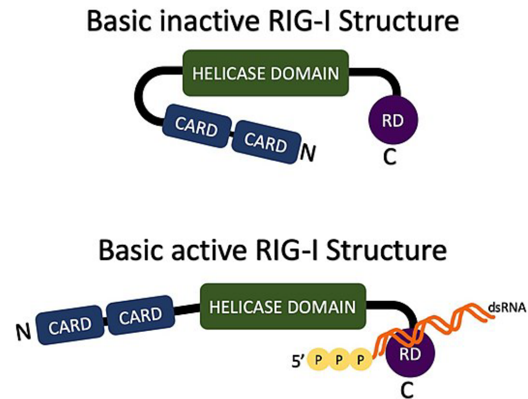


Figure 2: RIG-1 Molecule Structure

The top figure represents RIG-1's structure when RNA is not bound while the bottom figure represents how the double stranded RNA will bind to the C terminal domain and cause a change in formation resulting in the active structure.

RIG-1's pathway has been shown to instigate inflammation and cell death by activating the innate immune response to target specific cells within the organism via intrinsic apoptosis, extrinsic apoptosis, or pyroptosis. In intrinsic apoptosis, RIG-1 activation turns on pro-apoptotic genes and releases cytochrome c from the mitochondria to stimulate caspase 9 (Cas9), which then induces caspase 3, 6, and 7. This ultimately turns on the intrinsic cell apoptosis pathway. Extrinsic apoptosis is caused when RIG-1 signaling leads to binding a death ligand to the cell

membrane, inducing the caspase pathway and causing cell death. The alternative way the RIG-1 pathway can induce cell death is via pyroptosis, which recruits the inflammasome adaptor protein (ASC) and caspase 1. This cleaves gasdermin D, which then translocates to the plasma membrane, causing inflammation and cell death.[15] RIG-1 plays a diverse role in the immune pathway, with multiple routes to induce cell-programmed death and defend the body against viral infections, cellular dysfunctions, and cancer.

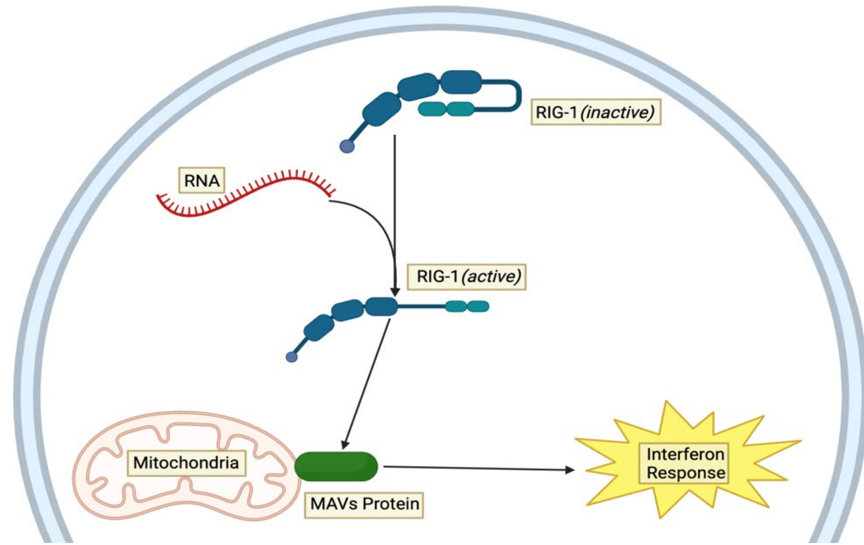


Figure 3: RIG-1 MAVs Pathway

This diagram shows how RIG-1 can become activated and ultimately cause an interferon response which results in cell death

Endothelial Cells and Vasculature System

The vascular system is crucial for delivering oxygen and nutrients throughout the body while cycling lymph fluid alongside the lymphatic system. At the cellular level, the vascular system is made up of endothelial cells, which form a single layer of cells that line the inside of blood vessels (Figure 4).[16] These cells are responsible for transporting oxygen, nutrients, and hormones between the blood and surrounding tissues by acting as a semipermeable membrane. Endothelial cells promote the growth of surrounding tissue by regulating nutrient transportation

to specific areas.[17] Additionally, these cells defend against harmful viruses or pathogens by releasing cytokines and chemokines. The release of cytokines and chemokines activates an interferon response, which causes the cell to become inflamed, triggering an early innate immune system response. This particular response notifies the body of an invasion while signaling other immune molecules to migrate toward the targeted area of the body.[18] Endothelial cells play a crucial role in the vascular system's ability to defend against foreign invaders that are a threat to the host while simultaneously directing the flow of important compounds and waste.

Endothelial cell proliferation and migration to create new blood vessels, in a process defined as angiogenesis, is present when the endothelium is damaged or injured. In this situation, endothelial cells will divide and travel to specific areas to rebuild blood vessels and tissues.[19] Angiogenesis is also present in fetuses and infants who are actively growing blood vessels and expanding their vascular system to supply blood to new tissues in the body.[20] However, angiogenesis also plays a crucial part in tumor growth. At the early stages of tumor growth, many solid tumors take advantage of blood vessels supplying surrounding tissues with nutrients and oxygen. Over time, the tumor can utilize angiogenesis to redirect new blood vessel growth directly toward itself, contributing significantly to the survival and growth of the tumor.[17] A key part of endothelial cells' role in tumor growth and proliferation includes vascular endothelial growth factors (VEGF), which are upregulated in tumor cells, inducing further

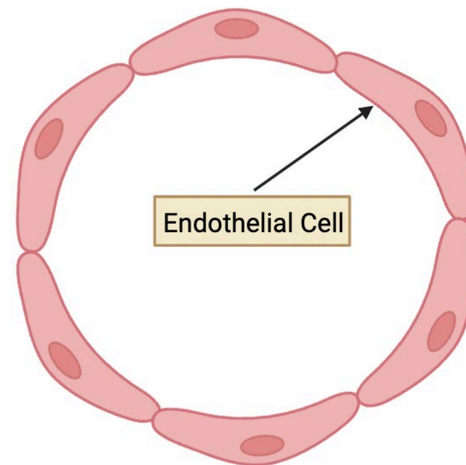


Figure 4: Endothelial Cell Diagram Surrounding Blood Vessels

Represents the inner layer of endothelial cells that make up the blood vessels.

angiogenesis to sustain these cancerous cells.[21] When VEGF is decreased, it activates transforming growth factor beta (TGFβ), a tumor suppressor cytokine, which inhibits cell growth and induces cell apoptosis. When the VEGF pathway is activated, the signaling loop promotes endothelial cell growth, increasing inflammation, hypertension, obesity, and even autoimmune disorders.[22]

Moreover, tumor angiogenesis is closely correlated with metastasis, as tumor cells can travel from one location through the vasculature to form a tumor in a new location.[23] Metastatic cancer is extremely deadly. A recent study over a 28-year period evaluated over a million metastatic cancer survivors and found the median survival rate of patients with cancer metastasis was 10 months. This particular study ended in 2019, and while the five-year survival rate varies depending on the type of cancer and extent of metastasis, the lethality of metastatic cancer has remained consistent over the past 28 years.[24]

Endothelial cells that comprise the vascular structure are critical in promoting and allowing tumor growth and metastasis.[23] They provide nutrients and oxygen to tumors, remove waste, encourage tumor endothelial cell growth, secrete molecules that defend the tumor from the immune system and cancer treatments, and simultaneously present an extensive route for cancer cells to travel and metastasize. The various roles of endothelial cells regarding tumor survival are notable, and they have the potential to be a successful target in cancer therapeutics.

Quiescence, Senescence, and Proliferating Endothelial Cells

Our research compared proliferating, quiescent, and senescent endothelial cells. Quiescent cells are in the G0 state and have very little growth or migration with a low metabolic rate, yet they are alive and still require oxygen and energy. This stage of reversible growth arrest

allows quiescent cells to be sustained in an unchanging state while maintaining homeostasis. Healthy adult endothelial cells are primarily in the quiescence stage when there is no longer a need to rapidly divide and create new blood vessel networks.[25] This stage represents normal and healthy endothelial cells that can carry out their multitude of functions within the body.

In contrast, proliferating endothelial cells consist of cells in the cell cycle that are constantly dividing and growing. Proliferation can be present due to a variety of reasons. Infants have normal proliferating endothelial cells because their bodies are growing, and angiogenesis is present to extend the vascular system to ensure proper nutrient and oxygen delivery to develop

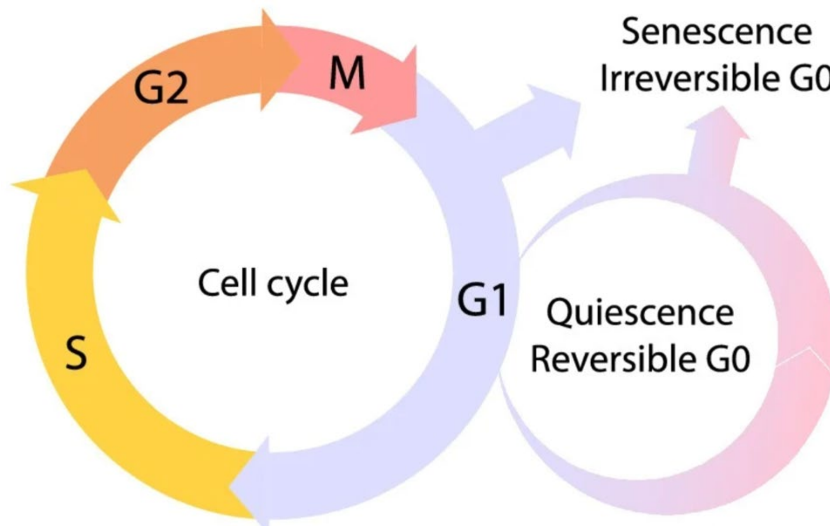


Figure 5: Cell Cycle

Locates where proliferating (in the cell cycle), quiescent (reversibly removed from the cell cycle) and senescent cells (irreversibly removed from the cell cycle) exist within the cell cycle

and expand tissues and organs. Angiogenesis is also present in the event of an injury, which would cause endothelial cells to migrate and regrow broken blood vessels and support tissue healing. However, proliferating endothelial cells could also be tumor endothelial cells that are

dividing to support tumor growth. Higher levels of angiogenesis would provide the tumor with more nutrients and oxygen, allowing it to cause even more damage.[19]

Senescent cells are old endothelial cells that have been irreversibly removed from the cell cycle. This permanent growth arrest can be activated by aging, replication, mitochondrial damage, oxidative stress, or DNA damage like irradiation.[6] However, previous research has shown that these cells are relatively stable and currently metabolically active while in senescence. These senescent cells have altered cell communication and even resist apoptosis, preventing RLRs like RIG-1 from having as large or effective of an antiviral signaling response.[8]

The quiescent, proliferating, and senescent cell stages further represent different age cohorts and physiological states such as normal tissues and tumors. Understanding the differences between these cell stages and how they react to inflammation and RIG-1-induced cell apoptosis could shed light on various populations and the differences in cell programmed death. Furthermore, we could attempt to target proliferating endothelial cells, which are either cancerous endothelial cells or endothelial cells feeding tumors, while saving the normal endothelial cells surrounding it as a potential cancer therapeutic.

Methods

Adapted from Anand Lab Protocols

Growing HUVECs and HMVECs

We thawed our human umbilical vein endothelial cells (HUVECs) (Cat: C-2519, Lonza) and human microvascular endothelial cells (HMVECs) (Cat: CC-2543, Lonza) and allowed them to grow in an endothelial cell EBM-2 media (Cat: NC1447083, Fischer) with 10% FBS and growth factors. All cells were held in an incubator with 5% CO_2 at 37 °C. Each time we split our cells, we removed the media, washed them with PBS, and added trypsin to break up the cells and remove them from the plate. We allowed the cells to incubate for three minutes and then added media to neutralize the trypsin. Then, we placed these cells into a new plate with new media. All cells are checked every six months for mycoplasma contamination.

Ensuring Quiescence

We first split our HUVECs with normal endothelial cell media: EBM-2 with 10% FBS and growth factors. After 24 hours, when the cells settled on the bottom of the plate, we removed the media, washed with PBS, and then added EBM media without growth factors or FBS. Then, to ensure that the cells were quiescent, we did flow cytometry to ensure that the cells had low pyronin y and dapi compared to the proliferating cells we tested.

Ensuring Senescence

We split our HUVECs 20 times. Each time, we removed the media, washed them with PBS, added trypsin, and placed them into a new plate with new EBM-2 media, 10% FBS, and growth factors. Previous research has stated that senescence is often present between passages 18 and

22.[27] We then ensured senescence by staining the cells with a beta-galactosidase biomarker and observed that over 90% of the cells stained blue, proving they were senescent.

Ensuring Proliferation

We ensured that our HUVECs were recently thawed and split less than five times. They grew in normal EBM-2 media with 10% fetal bovine serum, and proliferation was ensured with flow cytometry.

RIG-1 Treatment

The cells were treated with 0.1µg/µL RIG-1 diluted with lyovec transfection reagent or 0.1µg/µL of control agonist diluted with lyovec transfection reagent (Cat: tlrl-hprna-100, InvivoGen). We added the RIG-1 or control treatment to the cell media after the cells were settled on the bottom of plate. All samples were left to incubate for 24 hours post RIG-1 treatment.

RNA Isolation and qPCR Process

We first extracted RNA from the cells using the manufacturer's instructions from the Zymo Research Total RNA Purification Kit. Then, we measured the nanograms per microliter of RNA using the Nanodrop Microvolume Spectrophotometer. We created a master mix with reverse transcriptase, primers, buffers, water, RNAse, dNTPS, and our RNA following the manufacturer instructions in the High-Capacity cDNA Reverse Transcription Kit (Cat: 4368814, Applied Biosystems). We mixed 10 µL of each sample of RNA with 10 µL of the master mix and placed it in the thermocycler PCR machine to reverse-transcriptase the RNA to cDNA. Then we created a master mix for each sample by adding the master mix, water, and cDNA. In a 96 well plate we placed 4 µL of the probe we were testing for and the master mix cDNA sample. Then we pipetted 3 replicates of each sample in a 136 well plate and ran this under sybr green in the

qPCR. We utilized excel to calculate the dCT, ddCT, and fold change for samples that had received different radiation.

Cas Glo and Cell Titer

We utilized Promega Caspase-Glo and Cell-Titer Glo kits to measure cell death and proliferation, respectively, and followed the manual's instructions (Cat: G9242, G8091, Promega). All assays were carried out in clear-bottom white 96-well plates and measured in a Promega GloMax machine with a 0.5s integration time.

Elisa

We utilized the Invitrogen GDF-15 Elisa kit and the IL15 Elisa kit and followed the manuals instructions. Absorbance levels were measured in a Promega GloMax machine with a 0.5s integration time.

Mouse Harvest

All animal work was approved by the OHSU Institutional Animal Use and Care Committee. We carried out our RIG-1 in vivo experiment on six female mice that were all over 1 year old and senescent. We injected three mice via IV through the tail with 100 μL of RIG-1 treatment and the other three with a control treatment. We harvested their lungs and isolated the endothelial cells. We then carried out RNA isolation and the qPCR process.

RNA Sequencing

Total mRNA was isolated from cells and tissues using the Eurx RNA isolation kit (Cat: E3598-02, EURX). RNA sequencing was performed using the Oregon Health and Sciences University Massively Parallel Shared Sequencing Resource (MPSSR) and analyzed using standard DeSEQ pipeline. We confirmed that mx1 was upregulated as our positive control (Figure 14E). Delta CT

values from experimental targets were subtracted from control target genes. Then CT values of treated samples were subtracted from control samples. Finally, this value was log-transformed to obtain fold change and is represented on a graph.

Statistical Analysis

Analyzed data using GraphPad Prism Software (GraphPad Software, San Diego, CA). We carried out an ANOVA analysis when measuring the significance between multiple variables and a T-test analysis when comparing two variables. A p-value less than 0.05 was considered significant and represented on the graph with one star (*), a p-value less than 0.01 is two stars (**), and a p-value less than 0.001 is three stars (***).

Results

Radiation and RIG-1 Treatment

We wanted to observe whether radiation increased RIG-1 levels in HUVECs and HMVECs. We measured each sample for the presence of DDX58, the gene that encodes RIG-1, compared to our control (Figure 6A, 6B). In HUVECs, at 2 Gy radiation, there was a slight visible increase in RIG-1 expression; however, at 5 Gy and 10 Gy of radiation, RIG-1 expression significantly decreased (Figure 6A). At all levels of radiation in HMVECs, there was no change in RIG-1 expression (Figure 6B). This supported the null hypothesis that radiation did not increase RIG-1 expression and showed that it even had the potential to decrease RIG-1 expression at higher radiation levels. Following these results, we predicted that the radiation could possibly be killing our cells and, therefore, preventing RIG-1 expression.

We carried out a Caspase-Glo cell viability assay to measure cell apoptosis and a Cell-Titer Glo assay to measure proliferation in HUVECs and HMVECs. We observed that among HUVECs, there was no significant decrease in apoptosis levels with the combination of 8 Gy radiation treatment and RIG-1 treatment (Figure 6C). Additionally, there was no significant change in HUVEC cell proliferation with the combination of radiation and RIG-1 treatment (Figure 6D). However, radiation without RIG-1 led to a decrease in cell proliferation and RIG-1-treated HUVECs that did not receive radiation showed a significant decrease in cell proliferation as well (Figure 6D). This supported the prediction that RIG-1 was able to decrease cell proliferation levels and interfere with cell division. When we carried out this experiment with HMVECs, we instead used 2 Gy radiation due to their increased sensitivity to radiation

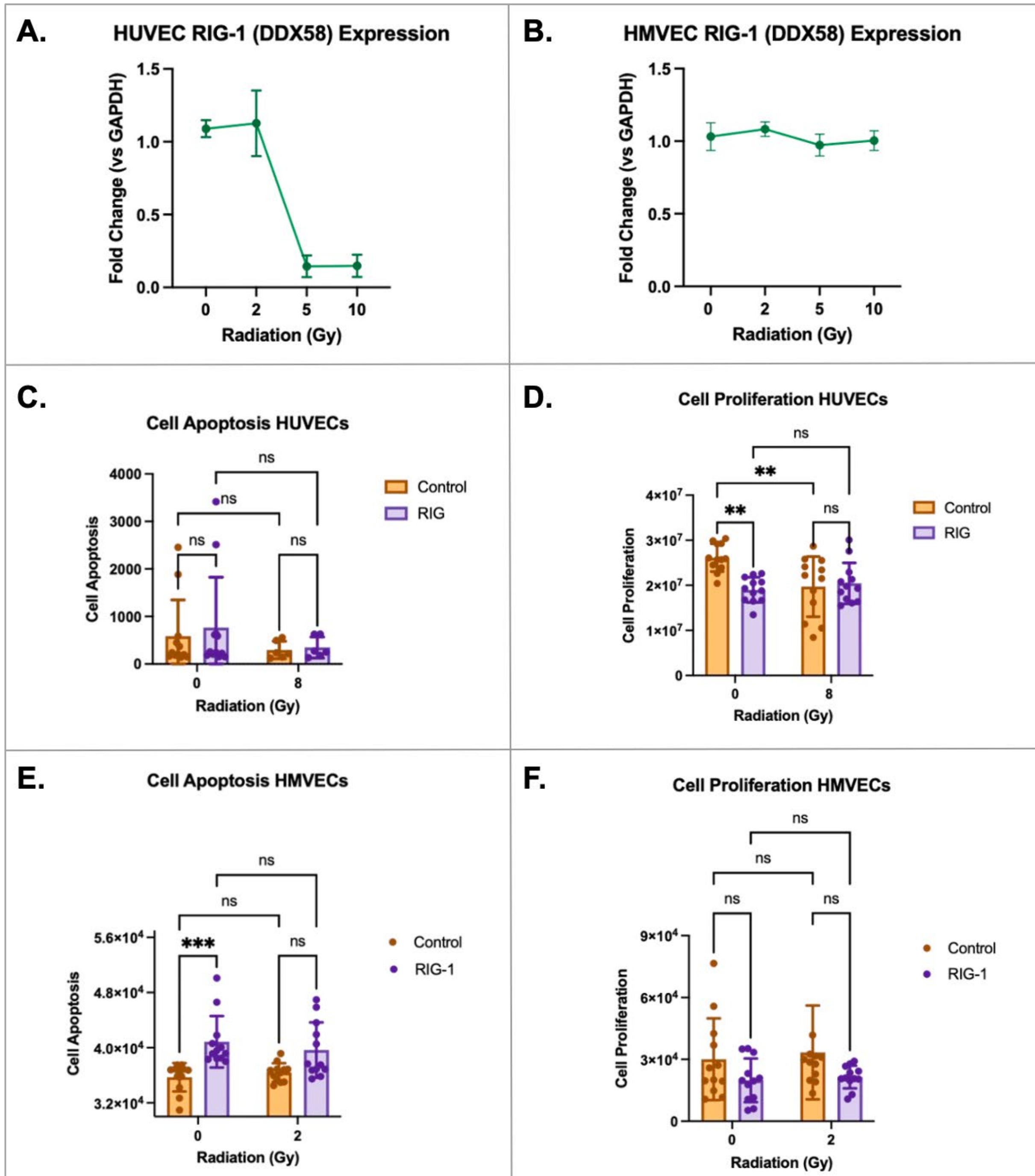


Figure 6: Radiation and RIG-1 Treatment in HUVECs and HMVECS

A) DDX58 fold change in HUVECs compared to control at 0, 2, 5, and 10 Gy radiation. B) DDX58 fold change in HMVECs compared to control at 0, 2, 5, and 10 Gy radiation. C) Cell apoptosis levels in HUVECs with RIG-1 treatment, irradiation at 8 Gy, or a combination of these treatments. D) Measured cell proliferation levels in HUVECs with RIG-1 treatment, irradiation at 8 Gy, or a combination of these treatments. E) Cell apoptosis levels in HMVECs with RIG-1 treatment, irradiation at 2 Gy, or a combination of these treatments. F) Cell proliferation levels in HMVECs with RIG-1 treatment, irradiation at 2 Gy, or a combination of these treatments. Significance determined via ANOVA for panels C/D/E/F.

(Figure 6E). We found that there was no significant change in cell apoptosis when the cells were treated with radiation and RIG-1, however there was a significant increase in apoptosis with RIG-1 activation and no radiation (Figure 6E). Then, we carried out a cell proliferation assay looking at RIG-1 and radiation treatment with HMVECs and recorded no significant difference in cell proliferation with the combination of these treatments (Figure 6F).

Next, we wanted to see if incubation time post-irradiation impacted induced cell apoptosis levels measured by Caspase 3 & 7. We found that with no RIG-1 treatment, regardless of the presence of radiation therapy, the activated Caspase 3 & 7 levels remained relatively constant over 24-, 48-, and 72-hour time points. However, in our samples with RIG-1 agonist treatment and no radiation, there was a visible increase in cell death at 48 hours post-irradiation (Figure 6). There was also a visible increase in activated Caspase 3 & 7 when the HMVECs were treated with RIG-1 compared to the control treatment. Furthermore, cell apoptosis levels were higher at 24 and 48-hour time points with RIG-1 and no radiation compared to the combination of RIG-1 activation and radiation (Figure 7). At 72 hours post-irradiation in all trials, there was a significant decrease in cell apoptosis levels (Figure 7). Following these results, we could see that RIG-1 played a role in inducing apoptosis in endothelial cells. However, we noticed that the combination of RIG-1 and radiation did not increase apoptosis of HMVECs or HUVECs like we

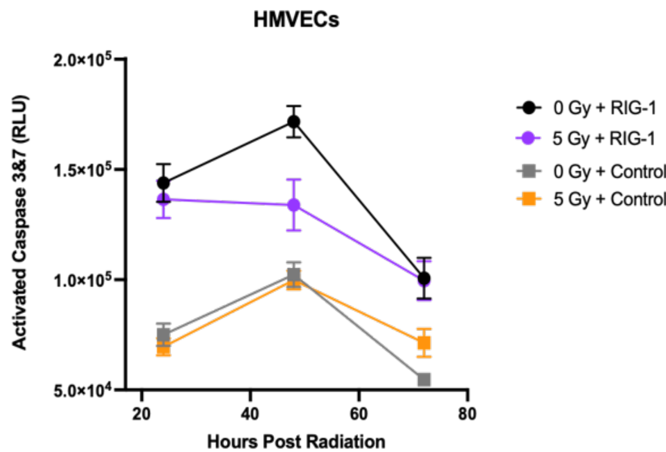


Figure 7: Apoptosis of HMVECs with RIG-1 and radiation at 5 Gy.

Presence of activated Caspase 3 & 7 at 24-, 48-, and 72-hours post-irradiation.

originally hypothesized. The effect of RIG-1-induced cell apoptosis as measured by Caspase 3 & 7 appeared to be lost when radiation treatment was combined with RIG-1 activation. This led us to hypothesize that the radiation could possibly act as a protective factor and reduce the impact of the RIG-1 agonist. Based on the results in Figure 7, we began to suspect that the radiation was inhibiting cell cycle progression and removing the cells from the cell cycle and into senescence. Furthermore, previous studies have shown that radiation can force cells into other cell stages, like quiescence or senescence, which could possibly impact cell survival rate, cell division, and other cellular functions.[6]

RIG-1 Treatment at Proliferating, Quiescent, and Senescent Cell Stages

To better understand how endothelial cells in different cell stages responded to RIG-1, we induced HUVECs into quiescence and treated them with a RIG-1 agonist. We measured levels of apoptosis to see if there was a difference between cells that were in quiescent compared to cells that were proliferating (Figure 9B). First, we ensured that our cells were in quiescence (Figure 9A). Then, we compared the RIG-1-treated proliferating cells against the proliferating control-treated cells and the RIG-1-treated quiescent cells against the quiescent control-treated cells. We found that cell apoptosis increased in both cell stages with RIG-1 (Figure 9B). However, our results revealed that RIG-1-induced cell apoptosis was higher in the proliferating cells compared to the quiescent cells (Figure 9B). This supported the prediction that the radiation could be forcing the cells into different cell

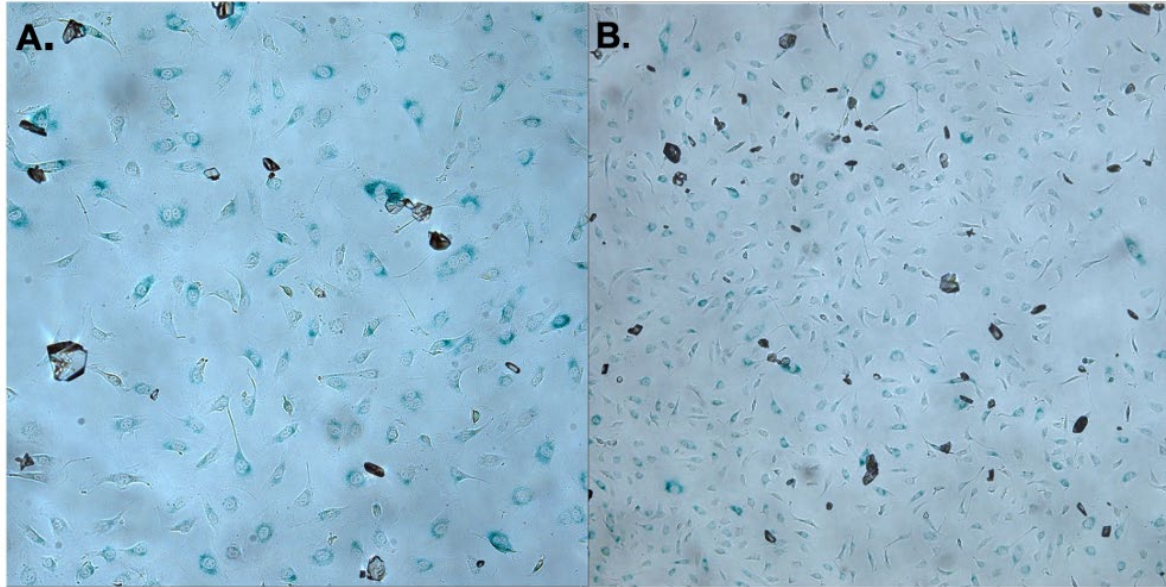


Figure 8: Beta-Galactosidase Senescent Marker Staining

HUVECs at passage 20 stained with beta-galactosidase senescent marker at pH 6; the blue staining surrounding the nuclei confirms senescence. A)10X image. B) 20X image.

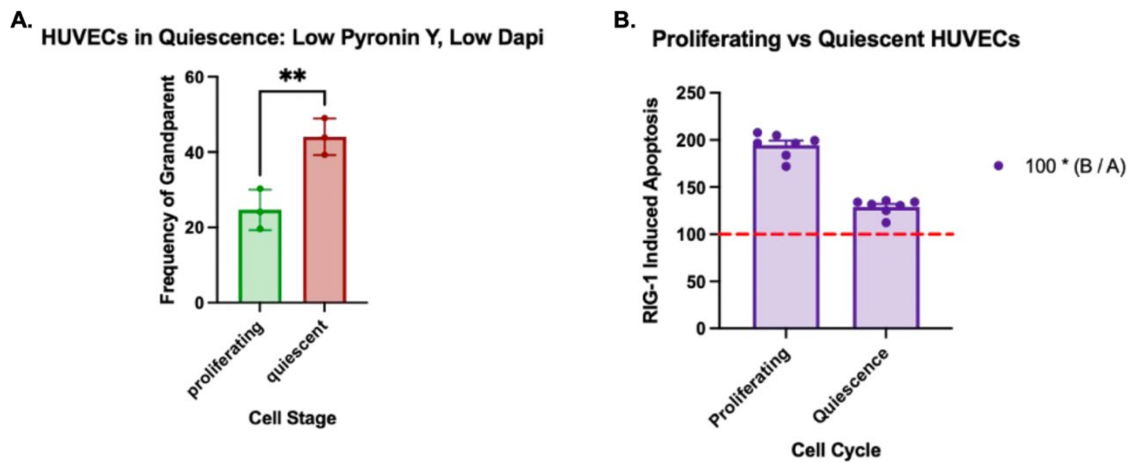
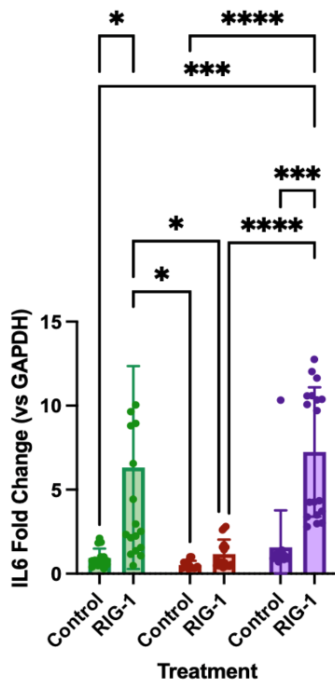
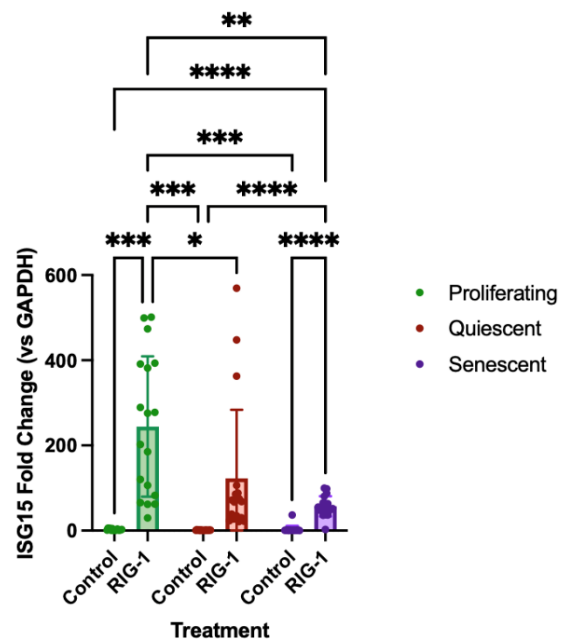


Figure 9: Quiescent Endothelial Cell Apoptosis

A) Difference in frequency of grandparent with low pyronin y and low dapi in proliferating and quiescent cells. Significance determined with t-test. B) RIG-1 agonist-induced cell apoptosis compared to its control (marked at 100) for proliferating HUVECs and quiescent HUVECs.





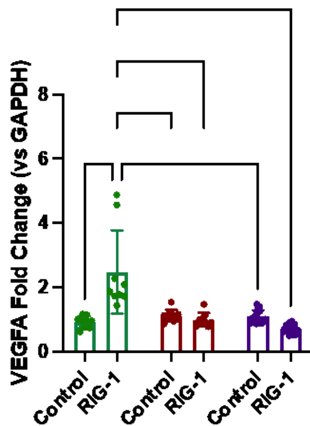


Figure 10: Proliferating, Quiescent, and Senescent IL6, ISG15 and VEGFA Expression

A) IL6 expression of RIG-1 treated proliferating, quiescent, or senescent HUVECs. B) ISG15 expression of RIG-1 treated proliferating, quiescent, or senescent HUVECs. C) VEGFA expression of RIG-1 treated proliferating, quiescent, or senescent HUVECs. ANOVA analysis for panels A/B/C.

stages and that these different cell stages reacted differently to RIG-1 treatment (Figure 9B). This revealed that proliferating tumor blood vessels could be more sensitive to RIG-1 treatment than quiescent cells, which would allow the healthy non-growing blood vessels within the body to survive while RIG-1 treatment induced an immune response in tumor cells. Following these results, we wanted to observe the differences in the innate immune system and angiogenesis activation, particularly IL6, ISG15, and VEGFA gene expression, in proliferating, senescent, and quiescent

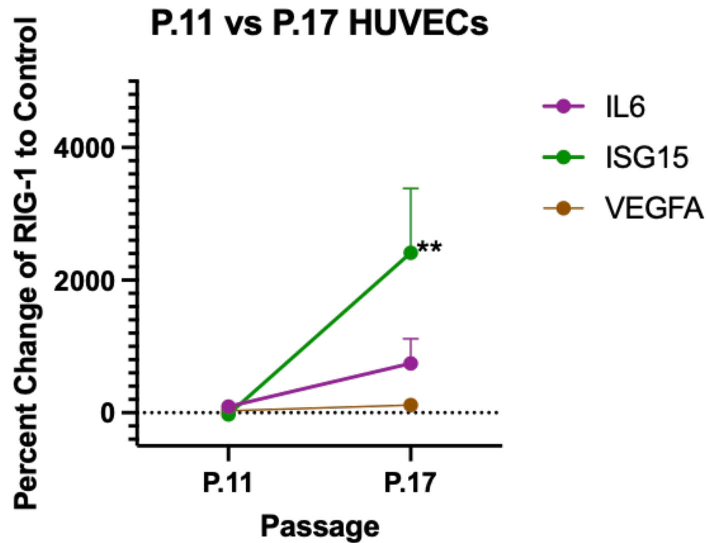


Figure 11: ISG15, VEGFA, IL6 Expression with RIG-1 as HUVECs Approached Senescence

Measured IL6, ISG15 and VEGFA at passage 11 and passage 17. ISG15 significantly increased from passage 11 HUVECs to passage 17 HUVECs with RIG-1 treatment. Significance determined with ANOVA analysis.

HUVECs with RIG-1 activation. We ensured that our cells were in senescence by utilizing a beta-galactosidase marker (Figure 8). IL6 induces chronic inflammation, which is involved in autoimmune diseases and cancer.[24] Figure 10A shows proliferating and senescent HUVECs treated with RIG-1 had higher levels of IL6 expression than their respective controls. Furthermore, these quiescent cells showed no significant change in IL6 expression with RIG-1 compared to the control. Figure 10B shows the expression of ISG15 in proliferating, quiescent, and senescent HUVECs with RIG-1 agonist treatment. ISG15 has a multitude of functions. Previous research has shown that it is released by the innate immune system and is upregulated by type 1 interferons. For example, in breast cancer, ISG15 inhibits cancer growth by upregulating NK cells and activating the immune system, while in liver and cervical cancer, it can inhibit growth and induce apoptosis, emphasizing its broad role depending on the specific cancer and location.[28] We found significantly higher ISG15 fold change with RIG-1 treated

HUVECs in proliferating and senescent cells, but no significant change in quiescent cells compared to its respective control (Figure 10B). The RIG-1-treated proliferating cells expressed more ISG15 than RIG-1-treated quiescent cells and RIG-1-treated senescent cells (Figure 10B). Then, we measured how VEGFA expression was influenced by RIG-1 expression among different cell stages (Figure 10C). VEGFA is a molecule involved in angiogenesis, proliferation, and cell survival.[21] We found that there was a significant increase in VEGFA expression in proliferating cells treated with RIG-1 (Figure 10C). RIG-1 treatment did not influence quiescent and senescent VEGFA expression, and both were significantly lower than VEGFA expression in proliferating RIG-1-treated cells (Figure 10C). Additionally, we compared the levels of ISG15, IL6, and VEGFA expression in HUVECs at passages 11 and 17 to record how expression changed as the HUVECs approached senescence (Figure 11). In passage 11, there was almost no change in IL6, VEGFA, or ISG15 expression when treated with RIG-1 compared to the control (Figure 11). However, at passage 17, there was a significant increase in ISG15 expression (Figure 11). Compared to the experiments in Figure 10, this reveals that the increase in ISG15

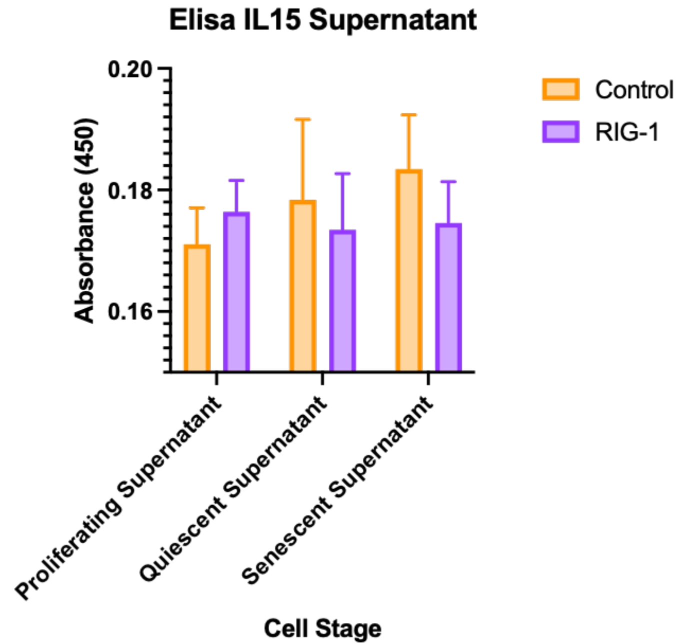


Figure 12: IL15 Elisa

Elisa of GDF-15 with RIG-1 treatment compared to its control. Absorbance is measured in the proliferating, quiescent, or senescent supernatant.

among senescent cells is already present at passage 17, but that the IL6 increase in expression among senescent cells is not yet present at this particular point.

Then, we measured the expression of GDF-15 and IL15 for the proliferating, quiescent, and senescent HUVECs. IL15 is a cytokine that increases T-cell production and induces inflammation.[29] We found no significant changes in IL15 expression when treated with RIG-1 agonist (Figure 12). However, we noticed a slight visible decrease in IL15 in the senescent RIG-1 treated cells compared to the senescent control cells (Figure 12). GDF15 is an angiogenic cytokine that promotes blood vessel growth.[30] We utilized two biological replicates of the proliferating and quiescent cells (proliferating 1 and proliferating 2). We found that there was no significant difference between proliferating, quiescent, or senescent GDF15 presence when treated with RIG-1 (Figure 13).

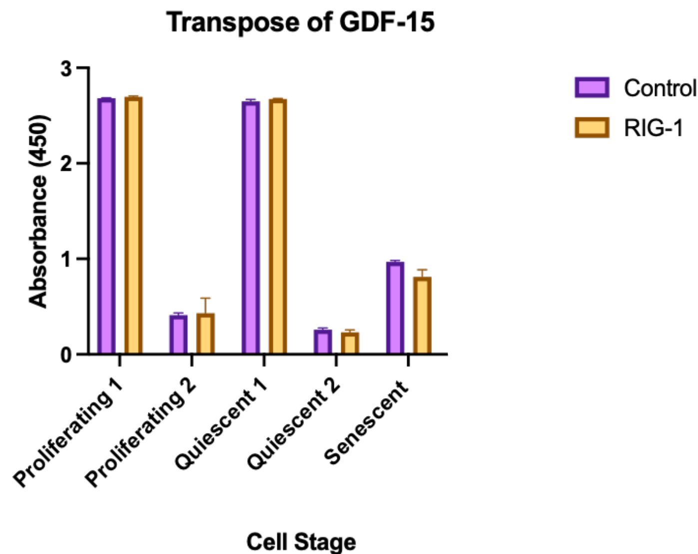


Figure 13: GDF15 Elisa

Elisa of GDF-15 with RIG-1 treatment. Absorbance is measured in the proliferating, quiescent, or senescent supernatant.

Senescent Mouse In Vivo with RIG-1 Treatment

Then, to observe how RIG-1 impacts both the innate and adaptive immune system in vivo, we injected mice with RIG-1 agonist. We designed a volcano plot to identify which genes were being expressed and repressed with RIG-1 treatment (Figure 14E). We found that ISG15,

IL6, and GDF15 increased compared to their respective controls (Figure 14A). Furthermore, there was only a slight increase in VEGFA and no change in IL15 expression with RIG-1 treatment. Figure 14A depicts an increase in ISG15 and IL6 expression and a non-significant change in VEGFA, which matches the results we found in our in-vitro experiments in Figure 9. The lack of change in IL15 expression matches the results we collected in Figure 13, which revealed that there was no significant difference in IL15 expression between senescent endothelial cells treated with RIG-1. Then, we observed the expression and repression of various caspase genes, that induce cell apoptosis, to locate the particular apoptosis pathway RIG-1 treatment induces in senescent cells in-vivo. We found that Caspase 4 increased with RIG-1 treatment while Caspase 3, 6, 7, and 8 decreased with RIG-1 treatment (Figure 14B). Caspase 4 promotes tumor necrosis factor-alpha (TNFα), which induces apoptosis by pyroptosis.[16, 31]

Then we identified which genes were most expressed with RIG-1 and found Alox15, Tnfs11, Mx1, Tiam2, and Crnde (Figure 14C). Alox15 is a lipoxygenase that is present in mice and humans. Previous research has shown that the overexpression of Alox15 activates apoptosis and inhibits tumor metastasis.[32] The Tnfs11 gene is part of the TNF superfamily. It is present in mice, with an orthologous gene in humans, and codes for a cytokine protein involved in the pro-inflammatory response.[33] Tiam2 is important for endothelial cell-to-cell contact while mediating cell migration and proliferation.[34] Crnde has a multifaceted role depending on the particular tumor it resides in and is often upregulated in various cancers (Figure 14C).[35] In Figure 13D, we saw that Mcm10, Ska2, Kif2c, Prc1, and Foxm1 were repressed with RIG-1 treatment. Mcm10 induces cell cycle progression and promotes DNA replication.[36] Ska3 promotes tumor progression

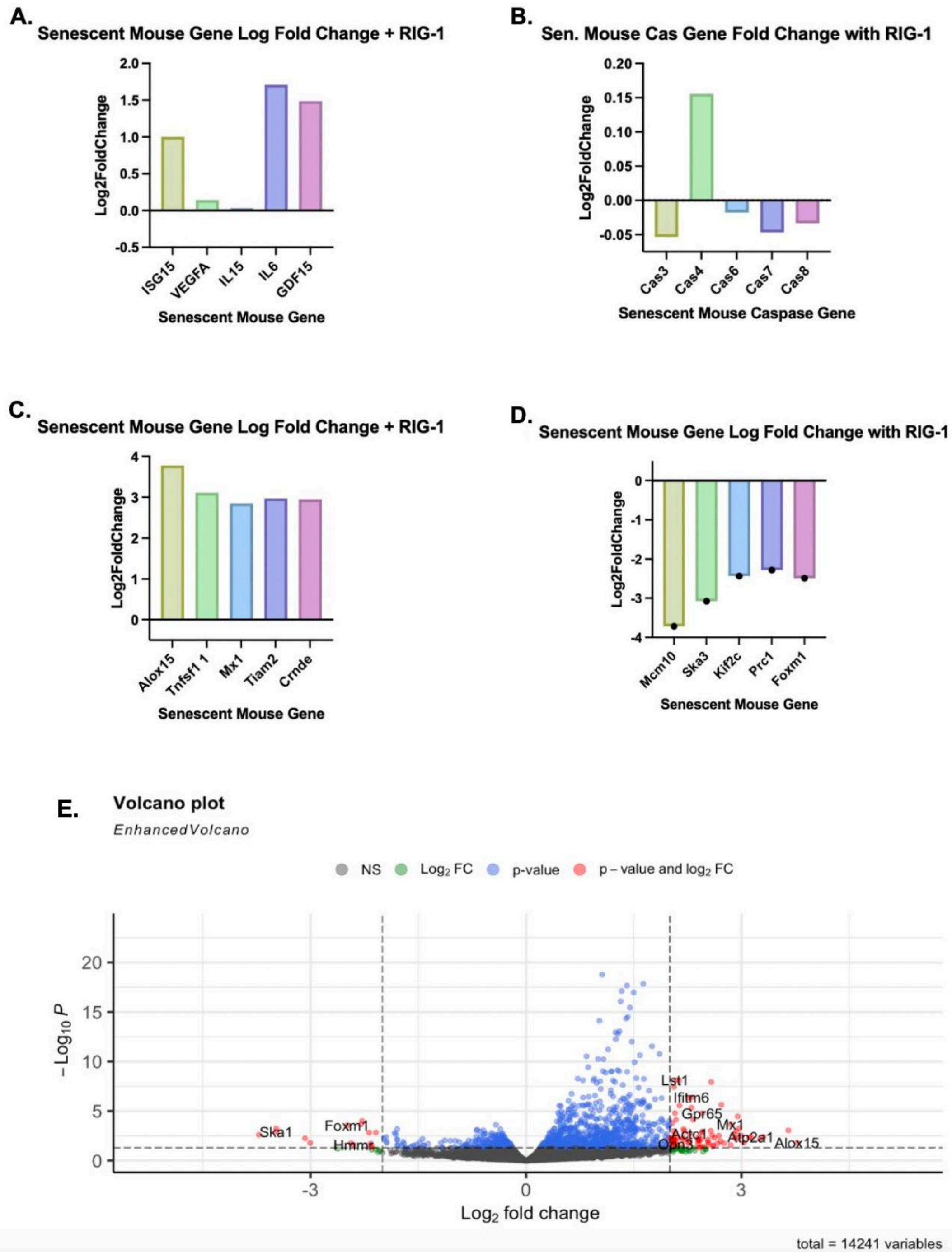


Figure 14: Gene Expression in Senescent Mouse Treated with RIG-1

A) Measured Log2Fold Change of genes examined in previous in-vitro experiments: VEGFA, IL6, ISG15, IL6, and GDF15. B) Measured Log2Fold Change of Cas3, Cas4, Cas6, Cas7, and Cas8 genes. C) Identified genes significantly expressed with RIG-1 and measured Log2Fold Change: ALOX6, TNFS 1 1, Mx1, Tiam2, and Crnde. D) Identified and measured Log2Fold Change of the genes that were being actively repressed with RIG-1 treatment: Mcm10, Ska2, Kif2c, Prc1, and Foxm1. E) Volcano plot of RNA sequencing results made with the assistance of Dr. Adrian Baris in RStudio.

among multiple types of cancers while utilizing the cell cycle. Furthermore, Ska3 is utilized as a cancer prognostic biomarker (Figure 14D).[7, 37] Previous research has found that the gene Kif2c induces tumor progression.[38] Finally, Foxm1, an oncogenic transcription factor, and Prc1 are known to activate tumorigenesis, cell proliferation, and cell cycle upregulation (Figure 14D).[39, 40]

Discussion

Tumor endothelial cells and solid tumors can shield themselves from the immune system while stimulating blood vessel growth to support tumor migration throughout the body. RIG-1 is an RNA sensor protein that can cause inflammation and notifies the body of a foreign invader, leading a targeted immune attack. We hypothesized that a combination of RIG-1 treatment and radiation would increase tumor endothelial cell death, that normal adult endothelial cells would have less RIG-1-induced cell death than tumor-like endothelial cells, and that old endothelial cells would have the least amount RIG-1-induced cell death compared to tumor and normal endothelial cells. Utilizing these different cell cycle stages could reveal how RIG-1 impacts different age cohorts by providing insight into how RIG-1 treatment would impact tumor blood vessels, normal endothelial cells, and older endothelial cells.

Our studies showed that radiation alone did not increase RIG-1 expression when it increased the amount of RNA and DNA present within endothelial cells. Furthermore, combining RIG-1 activation and radiation therapy did not kill more cells, and instead protected the tumor-like endothelial cells from death, supporting the prediction that radiation could drive the endothelial cells into other cell stages like senescence. We found that tumor-like blood vessels reacted more to RIG-1-induced death than normal blood vessels. Furthermore, we observed that RIG-1 increased the expression of various inflammatory molecules in both tumor-like and old endothelial cells while increasing immune and cell growth factors only in tumor-like endothelial cells. However, we also saw that RIG-1 could decrease endothelial cell death in tumor-like cells, increasing the tumor's chance of survival. This resistance to cell death is also present among other cancer therapeutics like radiation and chemotherapy, which both have the possibility of increasing tumor resistance to treatment.[41] This represents the diverse pathways

that RIG-1 can activate in cancer-like endothelial cells. Furthermore, this supports that RIG-1 is not inducing as strong of an attack on normal blood vessels compared to tumor blood vessels. Nevertheless, old blood vessels are still targeted by RIG-1 activation even though they are not constantly dividing like tumor cells. This could reveal potential implications for older populations with more old endothelial cells.

Our studies in a mouse model identified specific genes that caused an immune response that was activated in RIG-1-treated old mice. We also found that various cell proliferation factors were being heavily repressed with RIG-1 treatment, which revealed that RIG-1 could decrease tumor growth and division. However, RIG-1 has the potential to increase endothelial cell growth factors, emphasizing the multifaceted and diverse function of RIG-1 in senescent endothelial cells in a mouse model. These results further reveal targets of interest that contribute to immune system activation in old mice. Furthermore, it shows the effectiveness of this treatment in older populations and cancer patients.

RIG-1 has an incredibly diverse role with the potential to be utilized as a cancer therapeutic in conjunction with other cancer treatment methods. Our findings help shed light on the specific immune and tumor growth factors that are turned on or off in blood vessel cells. Furthermore, our studies reveal potential pathways RIG-1 influences regarding cell targeted death by creating a larger inflammatory response in tumor-like cells. Additionally, RIG-1 signals the immune system of an invader and the need to protect the body and simultaneously is providing information about specific characteristics of these invading tumor cells. As the body receives information about these tumor endothelial cells it memorizes these tumor characteristics, and then learns to better differentiate between tumor blood vessels and healthy blood vessels with the goal of preventing tumor survival, growth, and metastasis. This would

accomplish the overarching goal of killing tumor blood vessels feeding tumors or inside tumors by starving the tumor of oxygen and nutrients. Ultimately, by utilizing the immune system to destroy harmful cells, we could aid the body in identifying and killing tumor endothelial cells in a less toxic manner than current cancer treatments. These findings reveal the potential impact that RIG-1 could have on various types of cancers and age cohorts and its possible success as a cancer therapeutic.

Future Work

Based on our results, future experiments could include irradiating HUVECs and HMVECs and identifying the number of cells that are proliferating, quiescent, or senescent. This would allow us to test our prediction that radiation forces proliferating endothelial cells into senescence. Following this, we could carry out an experiment by first treating HUVECs and HMVECs with RIG-1 agonist and then irradiating them to see if the order of this combination therapy influences various inflammatory and immune responses among proliferating, quiescent, and senescent endothelial cells. Additionally, we could refer to our results in Figure 13 and identify downstream molecules expressed by these specific genes that are playing a crucial role in blocking or stimulating endothelial cell growth.

Furthermore, we could conduct an RNA Sequencing analysis of young adult mice treated with RIG-1 and a control treatment. We could compare this to our senescent mouse sequencing results. Younger mice would have higher amounts of quiescent endothelial cells compared to the senescent mice, allowing us to compare how the presence of the innate and adaptive immunity of quiescent cells in the mouse model would react to RIG-1. We could further compare the specific immune and angiogenesis-related genes being activated with the RIG-1 treatment in senescent mice compared to young adult mice. Then, we could investigate how RIG-1 treatment influences an immune response in senescent mice with solid tumors and in young adult mice with solid tumors. These tumors will contain higher amounts of proliferating endothelial cells. We could conduct an RNA sequencing analysis from endothelial cells within the tumor microenvironment and from a separate location within the same mouse. This would provide information about which genes are expressed in endothelial cells in the tumor microenvironment and in endothelial cells outside of the tumor microenvironment.

In addition to its role in inducing tumor endothelial cell death, RIG-1 activation influences the progression and development of cardiovascular diseases. Future experiments could identify how RIG-1 activation, with the primary goal of targeting tumor blood vessels, could interfere with the cardiovascular system and the development of other diseases within the body.

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