# Retinoic Acid Inducible Gene-I (RIG-I) Modulates Endothelial Cell Function

By

Adrian M. Baris

DISSERTATION

Presented to the Department of Cell, Developmental and Cancer Biology
and the Oregon Health & Science University
School of Medicine in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

January 2024

School of Medicine

Oregon Health & Science University

---

CERTIFICATE OF APPROVAL

---

This is to certify that the PhD dissertation of Adrian M. Baris has been approved .

Sudarshan Anand, PhD

Jonathan Brody, PhD

Amy Moran, PhD

Anusha Mishra, PhD

Owen McCarty, PhD

Jonathan Nelson, PhD

## Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| EC | Endothelial Cell |
| VEGFR2 | Vascular Endothelial Growth Factor Receptor-2 |
| VEGF | Vascular Endothelial Growth Factor |
| SHH | Sonic Hedgehog |
| PDGF-B | Platelet-Derived Growth Factor B |
| TGF-β | Transforming Growth Factor β |
| DLL4 | Delta-Like Ligand 4 |
| Notch | Notch Receptor |
| JAG-1 | Jagged-1 |
| ECM | Extracellular Matrix |
| HLA | Human Leukocyte Antigen |
| VCAM1 | Vascular Cell Adhesion Molecule 1 |
| ICAM1 | Intercellular Adhesion Molecule 1 |
| MHC-II | Major Histocompatibility Complex Class II |
| HLA-DR | Human Leukocyte Antigen - DR isotype |
| HEVs | High Endothelial Venules |
| DAMPs | Danger-Associated Molecular Patterns |
| EndoMT | Endothelial-to-Mesenchymal Transition |
| RA | Rheumatoid Arthritis |
| ROS | Reactive Oxygen Species |
| DNA | Deoxyribonucleic Acid |
| VEGF-A | Vascular Endothelial Growth Factor-A |
| FDA | Food and Drug Administration |

| | |
|---|---|
| HEV | High Endothelial Venule |
| PDL1 | Programmed Death Ligand 1 |
| TLR7 | Toll-like Receptor 7 |
| RLR | Retinoic Acid Inducible Gene-I like Receptor |
| MDA5 | Melanoma Differentiation-Associated protein 5 |
| LGP2 | Laboratory of Genetics and Physiology gene 2 |
| CTD | C-terminal Domain |
| HD | Helicase Domain |
| CARD | Caspase Activating and Recruiting Domain |
| IFN-I | Type 1 Interferon |
| MAVS | Mitochondrial Antiviral Signaling Proteins |
| dsRNA | Double-Stranded RNA |
| EMP | Endothelial Microparticle |
| HCAEC | Human Coronary Endothelial Cells |
| EPC | Endothelial Progenitor Cells |
| TRIM25 | Tripartite Motif 25 |
| MAVS | Mitochondrial Antiviral Signaling Proteins |
| TRAFs | Tumor Necrosis Factor Receptor Associated Factors |
| IRF3, IRF7 | Interferon Regulatory Factors 3 and 7 |
| NF-kB | Nuclear Factor-kappa B |
| lncRNAs | Long Non-Coding RNAs |
| miRNA | MicroRNA |
| HDACs | Histone Deacetylases |
| PRR | Pattern Recognition Receptor |
| PAMP | Pathogen Associated Molecular Patern |

| | |
|---|---|
| RIG-I | Retinoic Acid Inducible Gene-I |
| TYMP | Thymidine Phosphorylase |
| TPI | Tipiracil |
| RNA | Ribonucleic Acid |
| TME | Tumor Microenvironment |
| ICI | Immune Checkpoint Inhibitor |

**Acknowledgements**

I would like to thank my supervisor Sudarshan Anand for his guidance and support throughout my dissertation. I would also like to thank everyone in the Anand lab, past and present, for their constant support, assistance, and friendship. I would especially like to thank Rebecca Ruhl and Sokchea Khou, without whom the completion of this thesis would not have been possible. I would like to thank the members of my Dissertation Advisory Committee: Jonathan Brody, Amy Moran, Anusha Mishra, and Owen McCarty. Each member of my committee went above and beyond to provide critical guidance for this project. I would also like to thank my external reader Jonathan Nelson for his time both in reading this and for all of his guidance and friendship. I would like to thank my T32 program and the Knight Cancer Institute for their generous funding and support. I would like to thank the following OHSU Cores for their assistance with these experiments: The Flow Cytometry Core, The Advanced Light Microscopy Core, and the Genomics Core. I would also like to thank everyone who has been a part of or supported the Graduate Researchers United for showing me what a supportive and caring community can accomplish.

I would like to thank all of the wonderful people who have supported me on this journey. I would like to thank my parent, who have always believed I would be and do anything I wanted. I want to thank the rest of my family for always supporting me. I also want to thank all of the friends I have made over the years. I would like to thank my suite fam/social blood for keeping me sane, and I want to thank my wonderful friends in my cohort for going through this process with me. I would especially like to thank my amazing partner Michael for being with me through it all, and for letting me ask him 100 times a day if he thinks I will pass my defense. I would also like to thank my wonderful pets, my dog JD (RIP), and my cats Lireal and Liam, for providing endless joy.

**Abstract**

Activation of nucleic acid sensors in endothelial cells (ECs) has been shown to drive inflammation across pathologies including cancer, atherosclerosis and obesity. Here we show that activation of a cytosolic RNA sensor, Retinoic acid Induced Gene 1 (RIG-I) diminishes EC survival, angiogenesis and triggers tissue specific gene expression programs. We discovered a RIG-I dependent 7 gene signature that affects angiogenesis, inflammation and coagulation. Among these, we identified the thymidine phosphorylase TYMP as a key mediator of RIG-I induced EC dysfunction via its regulation of a subset of interferon stimulated genes. Our RIG-I induced gene signature was also conserved in the context of human diseases – in lung cancer vasculature and herpesvirus infection of lung endothelial cells. Pharmacological or genetic inhibition of TYMP rescues RIG-I induced EC death, migration arrest and restores sprouting angiogenesis. Interestingly, using RNAseq we identified a gene expression program that was RIG-I induced but TYMP dependent. Analysis of this dataset indicated that IRF1 and IRF8 dependent transcription is diminished in RIG-I activated cells when TYMP is inhibited. Functional RNAi screen of our TYMP dependent EC genes, we found that a group of 5 genes - Flot1, Ccl5, Vars2, Samd9l and Ube2l6 are critical for endothelial cell death mediated by RIG-I activation. Additionally, we identified a role for RIG-I depletion in vascular function. Analysis of RIG-I (-/-) tumors and lungs showed a significant decrease in blood vessels, accompanied by an increase in VegfA, Ccl5, and myeloid cells in the tumor. We also see an increase in Vegfr1 in lung ECs in RIG-I (-/-) mice. RNAseq identified changes in membrane and transport function between WT and RIG-I (-/-) tumors, leading to a working hypothesis that RIG-I depletion leads to membrane/transport dysfunction. This causes EC dysfunction, leading to a feedback loop causing an increase in angiogenic chemokines and myeloid cells. This informs our overall model that homeostatic RIG-I signaling is critical for vascular function. This work identified a novel function for RIG-I in ECs, as well as describes a method of using RIG-I both as an immunotherapy and as a therapy for vascular normalization.

## Chapter 1- Prologue

## Blood vessel formation and function

*Developmental Angiogenesis*

The cardiovascular system is the first functional organ system that develops in vertebrates, as embryonic growth and differentiation require the transport of nutrients and waste (Risau and Flamme 1995). Understanding how and why blood vessels form is critical to understanding how they can become dysfunctional in pathogenesis. Vasculogenesis is the formation of new blood vessels *de novo* (Drake 2003). Vasculogenesis is stimulated by the rapid growth of new tissues and organs and involves the release of angiogenic growth factors and the degradation of extracellular matrices (Patan 2004; Risau and Flamme 1995).

This process begins in the yolk sack, in which blood islands containing hemangioblasts, the precursor cells to hematopoietic and endothelial cells (ECs), form within the embryonic and extraembryonic mesoderm (Risau and Flamme 1995; Kolte, McClung, and Aronow 2016). Hemangioblasts give rise to angioblasts, which express VEGFR2, and eventually transform into ECs that line blood vessels (Risau and Flamme 1995; Eichmann et al. 2002). A primordial vascular network is formed by connecting the blood islands via migrating angioblasts (Risau and Flamme 1995). These networks connect during development to form a simple vascular network, which is later remodeled through sprouting angiogenesis, which creates arteries and veins (Czirok and Little 2012). This process involves the formation of new vessel branches, the pruning of excess branches, and the enlargement and fusion of endothelial tubes to form major vessels (Risau and Flamme 1995). Critical to this phase is the recruitment of supporting cells such as pericytes and smooth muscle cells. These cells stabilize the vascular network and are necessary for the structural integrity of the vasculature (Risau and Flamme 1995).

*Signaling pathways in vasculogenesis*

The formation of new blood vessels is a tightly controlled process involving a multitude of factors, and it is critical for nearly every organ system. Some of the major signaling pathways that govern blood vessel development are reviewed in detail elsewhere (Risau and Flamme 1995; Goldie, Nix, and Hirschi 2008).

Hemangioblasts are marked by vascular endothelial growth factor receptor-2 (VEGFR-2), the major receptor for VEGF-A (Chung and Ferrara 2011; Melincovici et al. 2018). Angioblasts, in a process regulated by VEGF-A, Sonic hedgehog (SHH), and Notch signaling, aggregate directly into the dorsal aorta or cardinal vein (Coultas, Chawengsaksophak, and Rossant 2005). Platelet-derived growth factor B (PDGF-B), angiopoietins, and transforming growth factor β (TGF-β) signaling facilitate the remodeling and stabilization of the nascent vessels through the recruitment of mural cells and pericytes (Jain 2003; Chung and Ferrara 2011).

*Sprouting Angiogenesis*

In contrast to developmental angiogenesis, sprouting angiogenesis is the formation of blood vessels from a preexisting capillary bed, and it is just as complex and critical as vasculogenesis (Folkman 1984). Sprouting angiogenesis typically occurs following hypoxia, injury, or due to signaling from angiogenic growth factors due to oncogenesis (Folkman 1984; Chung and Ferrara 2011). Sprouting angiogenesis typically follows tightly regulated steps: 1) the basement membrane is locally degraded 2) ECs migrate in parallel, forming a slit-like lumen and sealed by intact inter-endothelial junctions 3) the basement membrane is deposited continuously by polarized ECs so only the tip of the growing capillary bed lacks a basement membrane 4) proliferating pericytes migrate along the capillary bed, completely covering the new vessel **(Figure 1)** (Folkman 1984; Laschke, Gu, and Menger 2022).

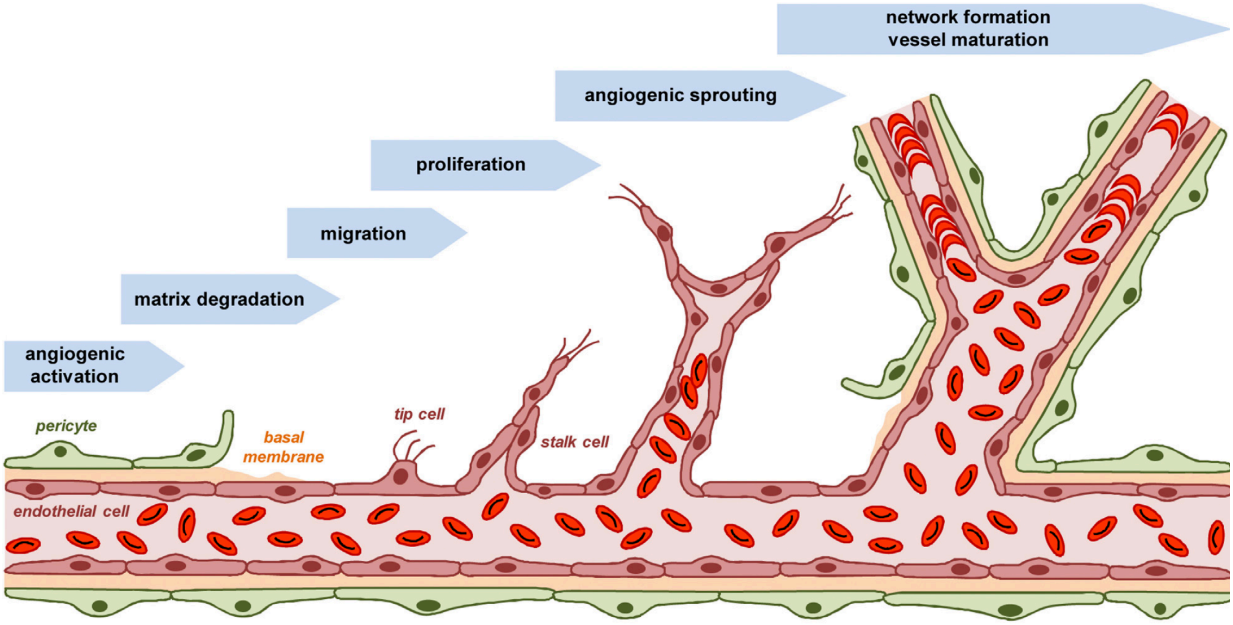



**Figure 1. The process of angiogenesis.** The process of angiogenesis can be subdivided in several well-characterized steps, which involve i) the angiogenic activation of micro vessels by growth factors, ii) the detachment of stabilizing pericytes and the degradation of the basal membrane by MMPs, iii) the migration of endothelial tip cells towards an angiogenic stimulus and iv) the proliferation of following endothelial stalk cells, which results in v) the formation of angiogenic sprouts. These sprouts develop a lumen and vi) finally interconnect with each other to new blood-perfused microvascular networks, which are stabilized by the formation of a new basement membrane and the recruitment of perivascular cells. From Laschke et al, Frontiers 2022. Open access distributed under Creative Commons Attribution License

*Signaling pathways in sprouting angiogenesis*

The VEGF signaling pathway is a master regulator of angiogenesis (**Figure 2)**. ECs express Vascular endothelial growth factor 2 (VEGFR2), a tyrosine kinase receptor that positively drives the mitogenic and chemotactic responses of ECs to Vascular endothelial growth factor (VEGF) (Risau 1997; Ferrara, Gerber, and LeCouter 2003; Leung et al. 1989). The expression of VEGF induces sprouting angiogenesis and promotes EC proliferation and migration through the activation of VEGF receptors on ECs (Risau 1997; Ferrara, Gerber, and LeCouter 2003; Leung et al. 1989). VEGF supports most of the steps of angiogenesis and it has concentration-dependent activity to induce EC proliferation, as well as gradient-dependent activity to promote migration (Ferrara, Gerber, and LeCouter 2003; Takahashi and Shibuya 2005). During this process, specialized ECs called pathfinding tip cells use metabolic angiogenic factors and proteolytic features to dissolve the ECM (Risau 1997). Pathfinding tip cells use specialized polarized membrane protrusions called dactylopodida and filopodia to assist with sprouting angiogenesis (Risau 1997). Initially blood vessels are leaky and immature but will eventually deposit a new extracellular matrix (ECM) that attracts pericytes to stabilize them. (Risau 1997)

On the other ‘side’ of the endothelial sprouts are the perivascular cells aka pericytes and smooth muscle cells that support the nascent blood vessels. It has been proposed that Angiopoietin signaling pathway is important in stabilizing nascent blood vessels (Augustin et al. 2009). Ang1 stabilizes vessels induced by VEGF by recruiting pericytes. In contrast, Ang2 blocks Ang1 and destabilizes vessels. The balance between Ang1 and Ang2 and its receptors (Tie-1 and Tie2) is a critical mediator of vascular maturation and sprouting angiogenesis (Fagiani and Christofori 2013).

Platelet-derived growth factor (PDGF) signaling is critical for pericyte and smooth muscle cell proliferation and migration (Hellberg, Östman, and Heldin 2010). Arterial and capillary

endothelial cells secrete PDGF-B, while smooth muscle cells and pericytes express its receptor PDGFR-B (Betsholtz, Karlsson, and Lindahl 2001). Growth of the developing vessel relies on reciprocal interactions between endothelium and support cells(Gerhardt and Betsholtz 2003).

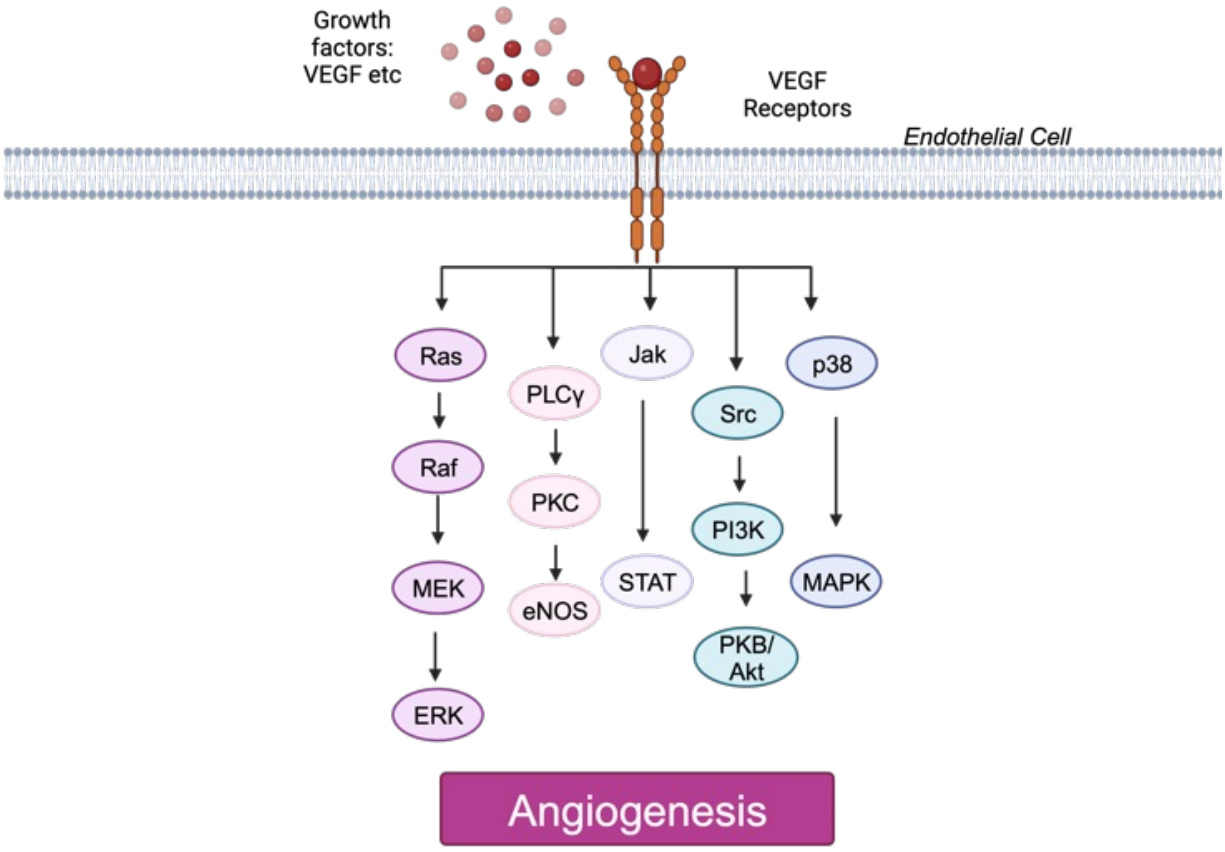



**Figure 2. Schematic diagram showing the VEGF/VEGF receptor pathway** Ligand binding to the extracellular domain induces dimerization and autophosphorylation of VEGFR2, activating downstream pathways and inducing angiogenesis. Created with biorender.

Outside of the receptor tyrosine kinases VEGFR, Tie-1 family and the Notch receptors, interactions with the ECM also contributes to sprouting angiogenesis. For instance, the integrin family is an extensive group or ECM proteins that promote intracellular signal transduction, cell migration, and survival. A number of EC integrins, including α1β1, α2β1, α4β1, α5β1, α9β1,

αVβ3, αVβ5 and α6β4 have been implicated in several specific functions in developmental and pathological angiogenesis (Avraamides, Garmy-Susini, and Varner 2008; Brooks 1996).

Signaling in angiogenesis is an intricate dance between a wide variety of growth factor ligands (VEGF, FGF) guidance receptors (Notch, Semaphorins) ECM-integrin interactions, mechanical forces/shear stress and interactions with perivascular cells (Weis and Cheresh 2011; G. Wang et al. 2020). The tiniest shift in any of these proteins, or the external forces that drive expression of these proteins, can impact vascular function and lead to pathologies.

*ECs have distinct tissue specific homeostasis roles*

After formation, ECs continue to play critical and active roles in organismal health. Blood vessels supply nutrients, oxygen, and other critical molecules to the entire body, which requires ECs to maintain tightly regulated pathways in diverse tissue contexts. For instance, in the brain, ECs form a highly selective barrier composed of specialized tight junctions to limit toxic molecules from entering the brain(Goldstein and Betz 1986). In the lungs, EC differentiate alongside epithelial cells to form gas exchange units that are in contact with the environment, requiring a rapid immune response from ECs(Niethamer et al. 2020; Gebb and Stevens 2004). In the heart, ECs are specialized to ensure a ready supply of fatty acids to cardiomyocytes, which rely on them as fuel necessary for cardiac contraction(Hsieh et al. 2006; Lother et al. 2018). In the liver, specialized ECs known as liver sinusoidal endothelial cells (lsecs) form a permeable barrier with high endocytotic capability. Lsecs regulate hepatic vascular tone and maintain hepatic stellate cell quiescence (Poisson et al. 2017; Sørensen et al. 2015). In the kidneys, multiple specialized ECs support glomerular filtration, as well as metabolite secretion and reabsorption(S.-J. Chen et al. 2020). This highlights the highly specialized nature of ECs in various tissues. Given the variety of signaling pathways described earlier, the tissue specific specializations of ECs add another layer of complexity to vascular function. Therefore,

understanding the specific signaling pathways involved as well as the tissue specific context of EC roles will help us understand vascular dysfunction and pathologies.

ECs play a wide variety of roles in development, homeostasis, and disease. ECs are responsible for oxygenating tissue, regulating vascular tone, and controlling the transfer of molecules between circulation and tissue. In addition to these functions, ECs play several immune functions including acting as gatekeepers for immune and inflammatory cells, recruiting effector immune cells, and in some instances, present antigen. In this next section, I will focus on the immune and inflammatory roles of ECs.

## Vasculature as an immune gatekeeper

*Endothelial Activation and inflammation*

ECs become activated following inflammatory or growth signals, such as under the stimulation by interleukin 1, tumor necrosis factor (TNF), or other cytokines (Pober 2002). Activated ECs experience a loss of vascular integrity, expression of leukocyte adhesion molecules, prothrombotic phenotypes, cytokine production, and upregulation of HLA molecules(Hunt and Jurd 1998).

There are two stages of EC activation. The first phase involves EC stimulation or E activation type I. This phase includes the retraction of ECs, the expression of P selectin, and the release of von Willebrand factor, the glycoprotein involved in blood and EC homeostasis (Bach et al. 1995). This phase occurs very rapidly. The second phase, EC activation type II, causes selective upregulation of inflammatory gene transcription programs and protein synthesis of adhesion molecules and cytokines (Hunt and Jurd 1998). This phase occurs more slowly, as it requires time for the stimulating agent to induce transcription and protein synthesis (Hunt and Jurd 1998).

*ECs as regulators of immune cell trafficking*

ECs have been known to modulate inflammation by regulating immune cell trafficking, activation, and function(Rita and Young 2012). ECs orchestrate the migration of immune cells between circulation and tissue. For example, ECs are critical for the movement of neutrophils, T-cells, and dendritic cells (DCs) during immune surveillance (Wilhelmsen et al. 2012; Salsman et al. 2011). ECs have been proposed to be innate immune cells due to expression of features typical of immune cells. These features include the expression of co-stimulatory and co-inhibitory receptors, the capacity to induce apoptosis in other cells, and the secretion of cytokines (Pober and Sessa 2007). ECs can control tissue and lymph node inflammation through differential expression of adhesion molecules (VCAM1), selectins, and chemokines (Pober and Sessa 2007). During immune homeostasis, ECs allow patrolling immune cells to extravasate into tissue (Langer and Chavakis 2009). During inflammation, ECs become activated and can recruit effector immune cells (Pober and Sessa 2007; Hunt and Jurd 1998). Some EC subtypes are also considered semi-professional antigen presenting cells (APCs) as they express genes involved in antigen capture, processing, and presentation. ECs can express the MHC-II molecule HLA-DR, which allows them to present antigens to CD4+ T cells (Hirschberg, Bergh, and Thorsby 1980). However, unlike professional APCs, ECs generally to not express the surface receptors CD80 and CD86 (Vandenberghe et al. 1993). ECs are exposed to shear stress, often higher in sprouting blood vessels such as the tumor vasculature(Givens and Tzima 2016), which increases the expression of ICAM1(Chiu et al. 2004). ICAM1 binds to T cell integrins, which increases T cell receptor signaling(Jankowska et al. 2018).

*Endothelial dysfunction and immune mediated pathologies*

Endothelial dysfunction is a functional and reversible alteration of ECs that leads to a shift in properties of ECs towards reduced vasodilation, a pro-inflammatory state, and proliferative and prothrombotic properties(Endemann and Schiffrin 2004).

Atherosclerosis:

Chronic inflammation and immune dysregulation play an important role in the development of atherosclerosis (Q. Huang et al. 2021; Mussbacher et al. 2022). Specifically, disturbed endothelial homeostasis facilitates the permeation and trapping of lipoproteins in the subendothelial space, which then is sensed as danger-associated molecular patterns (DAMPs) (Mussbacher et al. 2022). Some ECs may undergo the EndoMT process and differentiate into mesenchymal cells as well (Q. Huang et al. 2021). This leads to a loss of cell-cell contacts and the deposition of extracellular matrix (ECM) (Q. Huang et al. 2021). This leads to cell death and necrotic core formation, which thickens the vascular wall and limits blood flow and oxygen supply. Consequently, constrictions of the arterial wall cause turbulent flow and EC activation. Lipid-rich plaques are prone to rupture, which exposes thrombogenic material to circulation and initiates atherothrombotic occlusion, which is potentially life-threatening (Q. Huang et al. 2021; Mussbacher et al. 2022).

Arthritis:

Cytokines synthesized by ECs including TNF-α, IL-1, and IL-6 have all been implicated in the progression of Rheumatoid Arthritis (RA) (X. Yang, Chang, and Wei 2016). VEGF has also been found to be highly expressed in RA patients (Taylor 2002). These cytokines can induce the VEGF receptors or directly bind VEGF receptors resulting in angiogenesis at the site of inflammation (Taylor 2002). Newly formed vessels offer entrance for inflammatory cells into the synovial membrane, perpetuating inflammation and leading to eventual cartilage and bone destruction (Taylor 2002). Despite new vessel formation, high levels of proliferation lead to the

synovial joint being hypoxic. This causes a cycle of hypoxia, inflammation, and tissue destruction (X. Yang, Chang, and Wei 2016; Taylor 2002).

## Tumors as a model ecosystem for aberrant angiogenesis

In order to support the high proliferation rate of cancer cells, tumors need to rapidly develop new vascular networks (Folkman 1971; Folkman et al. 1971). However, the abnormal levels of angiogenic growth factors such as VEGF leads to immature and poorly formed blood vessels (Lugano, Ramachandran, and Dimberg 2020). Because of this abnormal growth, tumor vasculature is characterized by disorganized and immature vessels that lead to excessive permeability, poor perfusion, increased hypoxia that often drives tumor cells to acquire more drug resistant and metastatic phenotypes (Baluk, Hashizume, and McDonald 2005) **(Figure 3).** Tumors also display high levels of vascular heterogeneity, with regions of both hypervascularization and hypovascularization highlighting the challenges of understanding the role of angiogenesis in tumor progression (Lugano, Ramachandran, and Dimberg 2020).

### *Alternative Methods of Tumor Neovascularization*

Even though sprouting angiogenesis is the primary mean of new vessel formation, tumors exploit alternative mechanisms to produce a vascular network (Lugano, Ramachandran, and Dimberg 2020). One of these, intussusceptive angiogenesis, allows new vessels to generate more quickly and with fewer metabolic needs (Burri and Tarek 1990; Wilting et al. 1996). Intussusceptive angiogenesis, or splitting angiogenesis, is characterized by the insertion of interstitial tissue pillars into the lumen of preexisting vessels that split them into two new

vessels. A large variety of tumors use intussusception to grow (Burri and Tarek 1990; Wilting et al. 1996).

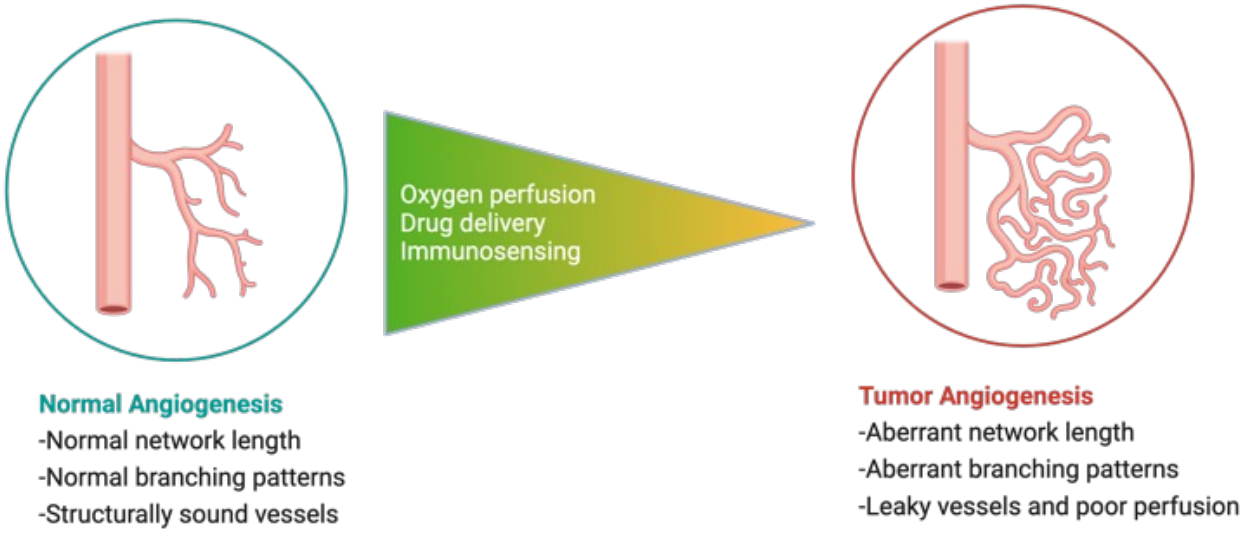



**Figure 3. Normal vs tumor angiogenesis.** Normal angiogenesis involves vessels that are structurally sound, well organized, and generally lined with pericytes. In contrast tumor angiogenesis involves aberrant, tortuous vessels that are leaky and lack pericyte coverage. This leads to a decrease in oxygen perfusion, drug delivery and immunosensing. Created with biorender

Another alternative method for tumors to form vessels is vasculogenic mimicry, which refers to tumor cells that behave like ECs (Upile et al. 2011). Vasculogenic mimicry is characterized by tumor cells co-expressing endothelial and tumor markers and forming perfused channel structures (Fausto 2000). These tumor cells can express vascular markers such as VE-cadherin, which maintains channel integrity through the establishment of cell-cell connections (Fausto 2000; Folberg and Maniotis 2004). Tumor cells can also be integrated into the walls of tumor-associated vessels, forming mosaic vessels (Fausto 2000; Folberg and Maniotis 2004;

Maniotis et al. 1999). Tumor cells lining the inner surface of channels are directly exposed to blood flow, which can lead to the detachment of tumor cells lining these channels and their circulation into the bloodstream (Maniotis et al. 1999). Cancer stem cells (CSCs), which possess the capacity to self-renew, can express endothelial markers and participate in vasculogenic mimicry (Bussolati et al. 2009).

Tumor cells can also grow without neovascularization by hijacking preexisting vessels in the surrounding normal tissues. Cancer cells grow alongside existing vessels and integrate them into the tumor (Winkler 2017).

*Vessel Permeability*

Tumor blood vessels are often much more permeable than “normal” blood vessels (Lugano, Ramachandran, and Dimberg 2020). This is in part due to impaired connection between perivascular cells, the basement membrane, and ECs. Tumor vessels display decreased pericyte density as well as abnormal pericyte morphology(Morikawa et al. 2002; Bergers and Song 2005). Increased EC proliferation, along with dysfunctional pericyte coverage and function, lead to vessel wall instability(Greenberg et al. 2008). Barrier integrity is also disrupted by loss of VE-cadherin function. Tumor cells release proteolytic enzymes such as MMPs to promote VE-cadherin cleavage(Dejana, Tournier-Lasserve, and Weinstein 2009). These factors also alter the immune engagement with the tumors by allowing more immune cell infiltration into tumors as well as tumor cell extravasation at distal sites (Gerhardt and Betsholtz 2003).

*Poor Perfusion*

Following the uncontrolled cell growth of tumors, surrounding host tissue restricts tumor expansion. This in turn compresses and collapses intratumoral vessels, suppressing blood flow (Jain 1987). Increased vessel permeability reduces perfusion even further by causing blood to

leak in upstream vessels and causing an increase in fluid flux from the vascular to the interstitial space (Jain 1987; Jain, Martin, and Stylianopoulos 2014). These factors all limit perfusion, limiting drug delivery and decreasing oxygen and nutrient supply (Hobbs et al. 1998). This leads to a hypoxic microenvironment, which causes selection for tumor cells that can resist the hypoxic conditions(Jain, Martin, and Stylianopoulos 2014).

*Hypoxia*

Hypoxic tumor cells are often more aggressive and migratory, activating oncogenes and undergoing the "epithelial to mesenchymal transition" (EMT), which increases metastatic potential (Reymond, d'Água, and Ridley 2013). Under normal oxygen levels, prolyl hydroxylase domain protein 2 (PDH2) uses oxygen to hydrolyze hypoxia inducible factors (HIF), which targets them for ubiquitination and leads to their degradation by the proteosome (Giaccia, Simon, and Johnson 2004; Mazzone et al. 2009). However, during hypoxia, PHD2 becomes inactive. This allows HIF1 to escape degradation. Increased levels of HIF proteins lead to the transcription of genes involved in cellular adaptation against hypoxia(Marin-Hernandez et al. 2009).

Thus, altered permeability, adaptation to poor perfusion and resultant hypoxia act as a deadly trifecta that drives tumor progression. However, while immune cell infiltration is heterogenous across tumor types, there is a strong correlation with the vascular density that could be exploited for immunotherapies.

*Dysfunctional tumor vasculature as a barrier to treatment responses*

Immature blood vessels can have a direct impact on anticancer treatment efficacy. Radiotherapy exposes cancer cells to ionizing radiation, leading to the generation of reactive oxygen species (ROS) in an aerobic microenvironment. This ultimately leads to DNA damage and tumor cell death (Ahmad et al. 2012). However, radiation sensitivity is correlated with

oxygen levels, and treatment efficacy is lowered in hypoxic conditions(Horsman and Overgaard 2016). Chemotherapy is also affected by tumor vessel immaturity, as poor perfusion and vessel collapse can prevent chemotherapeutic drugs from reaching large regions of tumors (Jain, Martin, and Stylianopoulos 2014). Hypoxic cells are also often quiescent, making chemotherapeutics that target proliferating cells ineffective. As many chemotherapeutics require oxygen for their tumor cell-killing, these drugs will lose efficacy in hypoxic regions of the tumor (Cosse and Michiels 2008).

Immunotherapies, which uses the host immune system to target and kill tumor cells, also show decreased therapeutic efficiency because of poor vessel perfusion (Y. Huang et al. 2013; Emblem et al. 2013). Dysfunctional tumor vessels can prevent immune cells from infiltrating the tumor (Y. Huang et al. 2013). In addition, suppressive immune cells have been shown to preferentially accumulate in tumors (Curiel et al. 2004). This may be due to the fact that tissue-resident macrophages migrate to hypoxic and necrotic tumor areas, where they switch to a tumor-associated macrophage (TAM) phenotype (Curiel et al. 2004). In addition, regulatory T cells (Treg), which are immune-suppressive, are preferentially recruited to tumors via the expression of chemotactic factors in hypoxic areas of the tumor (Curiel et al. 2004). Altogether, impaired tumor vessel function contributes to an abnormal and immunosuppressed tumor microenvironment, which is a major hurdle to successful immunotherapy treatments(Y. Huang et al. 2013; Curiel et al. 2004).

*Anti-Angiogenic Therapy* The notion of anti-angiogenic cancer therapy relies on the idea that removing tumor vasculature will prevent nutrients from entering the tumor, leading to an effective therapy. Conventional anti-angiogenic drugs often target agents that promote blood vessel formation and are overexpressed in tumors, such as VEGF-A. Bevacizumab, a humanized anti-VEGF-A antibody, is approved by the Food and Drug Administration (FDA) to target neovascularization(Crawford and Ferrara 2009; Leung et al. 1989; Senger et al. 1983).

However, anti-angiogenesis treatment cannot eradicate the tumor on its own. The use of chemotherapy or immunotherapy in conjunction with anti-angiogenic treatment can provide a more effective strategy. Additive or synergistic effects of bevacizumab with conventional therapies has been evaluated in a variety of tumor types(Jayson et al. 2016). In patients with gastric cancer, the combination of bevacizumab with chemotherapy significantly improves progression-free survival and overall response (Ohtsu et al. 2011). Patients with non-small cell lung cancer or metastatic breast cancer also see positive results when bevacizumab is combined with conventional chemotherapeutics(Miller et al. 2007; Reck et al. 2009). In addition, inhibitions targeting VEGFRs, including sunitinib or sorafenib, are approved by the FDA for the treatment of renal cell carcinoma (Escudier et al. 2007). The efficacy of VEGF blockade is dependent on tumor type. Prostate and pancreatic cancers are less sensitive to angiogenesis modulators, where renal-cell carcinoma is more sensitive (Jayson et al. 2016). Studies have shown that chronic exposure of tumor cells to VEGF inhibitors can increase the expression of VEGF molecules, leading to an increase in tumor cell migration, invasion, and metastatic potential(Fan et al. 2011). Though anti-VEGF therapies are generally well tolerated, complication such as venous thromboembolism are among the adverse effects(Widakowich et al. 2007). The several complications with anti-VEGF therapies have lead to the concept of alternative methods of targeting tumor angiogenesis.

*Vascular normalization*

The concept of vascular normalization suggests that VEGF inhibitors can lead to maturation of tumor blood vessels. As tumor cells are immature and lack pericyte coverage, careful dosage of VEGF inhibitors can normalize levels of angiogenic signals. This is thought to decrease vessel permeability through the tightening of cell-cell junctions and pericyte recruitment(Tolaney et al. 2015). However, a major challenge with using VEGF inhibitors to obtain vascular normalization is achieving the “window of opportunity”, which is the time frame

and dosage of VEGF inhibitor administration required to normalize tumor blood vessels. The "window of opportunity" is narrow and depends on tumor type, schedule, and type of VEGF inhibitor used(Jain 2014). Normalization is typically short-lived (7-10 days) but can last for up to four months depending on the drug used and tumor type(Batchelor et al. 2007). High doses or prolonged exposure to VEGF inhibitors can cause vascular normalization to be replaced by vascular regression, leading to an increase of tumor hypoxia(Graeber et al. 1996; Ebos and Kerbel 2011). As prolonged maintenance of vascular normalization is necessary to enhance the therapeutic index of radiotherapy, chemotherapy, and immunotherapy, it is critical to identify strategies that lead to long-term stabilization of tumor blood vessels(Y. Huang et al. 2012).

*Alternative methods to restore vascular normalization*

There are several problems with conventional anti-VEGF therapies: 1) risk of hemorrhage or venous thromboembolism are increased with conventional anti-angiogenic treatments (Nalluri et al. 2008; Hapani et al. 2010), 2) severe vascular regression is associated with an increased risk of tumor metastasis(Graeber et al. 1996), 3) anti-VEGF therapies exhibit transient benefits and are often followed by an increase in tumor growth due to adaptive resistance (Bergers and Hanahan 2008). For these reasons, alternative methods of normalize tumor blood vessels is necessary to improve therapeutic efficacy.

One of the alternative methods for vessel normalization is the improvement of pericyte coverage. Several pathways can be exploited to modulate pericyte coverage. One of these, angiopoietins, play a critical role in the formation and remodeling of blood vessels. Angiopoietins act by binding to Tie2, a tyrosine kinase receptor expressed on ECs and subsets of macrophages(Thomas and Augustin 2009). The angiopoietin Ang1, which is produced by perivascular cells, promotes vessel maturation. Ang2, which is produced by ECs, is an antagonist for Tie2 activity and is involved in the destabilization of the EC/pericyte association(Augustin et al. 2009). Therefore, Ang2 is an interesting therapeutic target for vessel

normalization. Several inhibitors targeting the angiopoietin pathway have been developed and are being investigated in preclinical and clinical studies (Holopainen et al. 2012; Leow et al. 2012). The anti-Ang2 inhibitor MEDI3617 has been shown to reduce tumor angiogenesis, improve chemotherapy, and decrease lung metastasis in several preclinical models (Monk et al. 2016). Ang2 inhibition has also been shown to reduce vascular leakage by enhancing cell-cell junction integrity. The combined inhibition of VEGFR and Ang2 has been shown to improve survival in murine glioblastoma models through vascular normalization and macrophage reprogramming (Monk et al. 2016). In a phase 3 clinical trial, an inhibitor of the Angiopoietin-Tie2 interaction (trebananib) was shown to improve progression-free survival in epithelial ovarian cancer (Monk et al. 2016). Activation of Tie2 using a phosphatase inhibitor VE-PTP increased pericyte coverage, vessel diameter, vascular density, and tumor perfusion as well as reducing metastasis in breast cancer(Monk et al. 2016).

Another pathway that modulates pericyte coverage is the PDGF axis. ECs, particularly tip cells, secrete high levels of PDGF-B, which binds to PDGFRA/B and promotes pericyte recruitment (Heldin 2013). Studies have shown that anti-angiogenic resistance is due to active proliferation of mural cells and high PDGF-B expression (J. Huang et al. 2004). This shows that VEGF inhibition may be more efficient in combination with agents blocking the PDGF pathway. One such drug, SU6668, has been shown to have a synergistic effect with a VEGFR inhibitor and enhanced efficacy of local irradiation (Bergers et al. 2003; Erber et al. 2004; Timke et al. 2008). However, loss of perivascular coverage caused by such inhibitors increases vessel immaturity and enhanced metastasis (J. Kim et al. 2016). This led to the notion that promoting PDGF signaling may achieve vascular normalization. PDGF-B overexpression was shown to inhibit tumor growth in preclinical colorectal and pancreatic cancer models(McCarty et al. 2007). While PDGF-B overexpression appears to promote tumor growth, its use in conjunction with chemotherapy may enhance drug delivery and efficacy (J. Liu et al. 2011).

Enhancing cell-cell junctions is another method of normalizing tumor vessels. Sac-1004 is a compound that strengthens the endothelial barrier through upregulation of adherens junctions such as VE-cadherin (Maharjan et al. 2013). In preclinical models, SAC-1004 reduced vascular leakage through increasing junctional integrity. SAC-1004 reduced metastasis and tumor hypoxia, and synergizes with cisplatin (Maharjan et al. 2013; K. Lee et al. 2014).

*Immunotherapy and ECs*

Treatment of tumors using immunotherapies and anti-VEGF therapy is a potentially useful way to normalize vasculature and promote antitumor immunity. VEGF can have immunosuppressive functions. Through the inhibition of the NF-kB signaling pathway and the upregulation of programmed death-protein ligand 1 (PD-L1), VEGF can inhibit dendritic cell (DC) antigen-presentation function which further suppresses the activation and expansion of T-cells(Oyama et al. 1998; Dikov et al. 2005; Gabrilovich et al. 1996). In addition, VEGF can also induce the upregulation of immune checkpoint molecules including PD-1, TIM-3, and CTLA-4, leading to cytotoxic T-cell exhaustion. At the same time, VEGF promotes the proliferation of regulatory T-cells (Tregs), which inhibit anti-tumor immunity(Wada et al. 2009).

Based on the role VEGF plays in the tumor microenvironment (TME), inhibition of VEGF-induced signaling, or other angiogenic signaling, is a promising avenue to suppress tumor growth through dual mechanisms: suppressing angiogenesis and through promoting anti-tumor immunity (Gibson et al. 2007; Oestreich et al. 2008; Voron et al. 2015; Lapeyre-Prost et al. 2017). Several studies are addressing the effect of combination anti-VEGF therapy with immune checkpoint inhibitors (ICI) (**Table 1).**

| NCT Number | Study Status | Conditions | Interventions | Start Date | Completion Date |
|---|---|---|---|---|---|
| NCT04137588 | UNKNOWN | Advanced Non-squamous Non-small Cell Lung Cancer | Antiangiogenesis Agents, Immune checkpoint inhibitor | 3/3/21 | 6/1/22 |
| NCT05688046 | COMPLETED | NSCLC | Immune checkpoint inhibitors plus angiogenesis inhibitors | 1/1/19 | 12/31/22 |
| NCT03890952 | ACTIVE_NOT_RECRUITING | Recurrent Adult Brain Tumor | Nivolumab, Bevacizumab | 10/1/18 | 8/1/23 |
| NCT04670107 | COMPLETED | NSCLC | Anlotinib in combination with Immune checkpoint inhibitors | 6/1/18 | 11/1/20 |
| NCT04393506 | COMPLETED | Oral Cancer | Camrelizumab\| Apatinib | 4/23/20 | 11/10/23 |
| NCT04872582 | ACTIVE_NOT_RECRUITING | Metastatic Nasopharyngeal Carcinoma | PD-1 Immune Checkpoint Inhibitor Combined With Bevacizumab | 7/29/21 | 2024-10 |
| NCT05273814 | NOT_YET_RECRUITING | Non-Small Cell Lung Cancer | Tislelizumab\|: Pemetrexed\|: Bevacizumab | 8/1/22 | 2/1/24 |
| NCT05193188 | RECRUITING | Chondrosarcoma | Anlotinib hydrochloride\| PD-1 inhibitor | 2/6/23 | 3/31/26 |
| NCT04493203 | RECRUITING | Advanced Melanoma | Nivolumab, Axitinib | 12/18/20 | 2025-12 |

**Table 1. Immunotherapy + antiangiogenic therapy clinical trials.**

Status according to https://clincialtrials.gov/, accessed on 1 December 2023. Immunotherapy drugs: Anti-PD-1: Pembrolizumab, nivolumab, sinitilimab; anti PD-L1: Atezolizumab, avelumab; anti-CTL4: Ipilimumab. Anti-angiogenic therapies: Anti-VEGF: Bevacizumab; anti-VEGFRs: Axitinib, sunitinib, regorafenib, cabozanitib

## Vascular contributions to pathology in Infectious diseases

*Adapted from:* ***Baris, A.M****.; Fraile-Bethencourt, E.; Anand, S. Nucleic Acid Sensing in the Tumor Vasculature. Cancers 2021, 13, 4452. https://doi.org/10.3390/cancers13174452*

Vascular endothelial cells line the inner surface of blood vessels and provide a barrier between organ systems and blood vessels. This makes them critical during viral infections. Viral infection of endothelial cells gives the virus an opportunity to disperse to other organs, as well as a reservoir for long-term persistence. In addition, viral replication and the immune response in the endothelium leads to an increased tissue permeability as well as inflammation. Altogether these changes drive vascular and pulmonary disease that further exacerbates the viral disease(Goodrum and Bughio 2015). Endothelial activation and dysfunction have been shown to serve a necessary mechanistic role in the pathology of severe influenza(Iwasaki and Pillai 2014; Armstrong, Darwish, and Lee 2013). For example, RIG-I like receptors (RLRs) and Toll-like-receptor 7 (TLR7) signaling have been shown to be necessary for cell survival and for restricting virus growth in mice(Koyama et al. 2007). RLRs, as well as other aspects of the innate immune system, were found to induce an antiviral innate and adaptive immune response. Interestingly, in some cases influenza virus has been shown to co-opt signaling of TLR7 and RIG-I(Pang, Pillai, and Iwasaki 2013).

Other viruses rely on endothelial cells for their replication and host response. Particularly relevant of late, endothelial cells have hypothesized to be essential mediators of pathology in SARS-CoV-2 infections(Teuwen et al. 2020). After the initial phase of infection, some patients experience an overactive inflammatory response which leads to lung damage and increased disease severity. It was proposed that SARS-CoV-2 may cause pulmonary vascular changes based on clinical observations. Thus, endothelial cell injury and dysfunction caused by SARS-CoV-2 likely contributes to COVID-19 life-threatening complications (Huertas et al. 2020). SARS-CoV-2 infection of pericytes is also a likely cause of vascular injury. As SARS-CoV-2 co-

opts the ACE2 receptor, which is highly expressed on pericytes, and pericytes are critical for vascular stabilization and permeability, pericyte infection is likely to cause vascular damage (McQuaid and Montagne 2022). While the mechanistic role of nucleic acid sensors in the vascular pathologies induced by SARS-CoV-2 remain to be elucidated, they have been well characterized in the vascular inflammatory response to other infectious pathogens.

*ECs as sentinels for sensing danger and activating immune responses*

In order to protect itself from outside pathogens and other agents, organisms have developed two interlinked forms of defense systems: an innate and an acquired immunity. The innate immune response reacts rapidly to an infection, which can often exponentially multiply long before the adaptive immune response is able to take effect(Alberts et al. 2002). These responses should be tightly regulated to prevent dysfunction and damage to the host. The innate immune system responds to Damage Associated Molecular Patterns (DAMPs), which are recognized by Pattern Recognition Receptors (PRRs). Activation of PRRs typically induces a downstream type 1 interferon (IFN-I) and cytokine response **(Figure 4).**

Nucleic acid sensors are a specific type of PRRs that recognize pathogen-derived cytosolic nucleic acids and activate downstream signaling cascades, which produces a proinflammatory response(Ori, Murase, and Kawai 2017). This response is critical to the ability of nucleic acid sensors to stop pathogens in their tracks. Nucleic acid sensors are unique in that they can recognize and differentiate exogenous and endogenous nucleic acids(Takeuchi and Akira 2010). A number of nucleic acid sensors (NAS) play a prominent role in inflammation(Shahanshah Khan, Godfrey, and Zaki 2019). Recent studies have shown that NAS play a large role in endothelial function and dysfunction(Bhagwani, Thompson, and Farkas 2020). Endothelium regulates vascular tone and growth(Lüscher and Tanner 1993), and is critical in pathologies such as viral infections, cardiovascular disease, and cancer. A better understanding of the role NAS play in endothelial function is key to improving our knowledge of

tumor angiogenesis, as well as cardiovascular and infectious diseases. Several outstanding reviews discuss the sensors outlined in **Table 2**

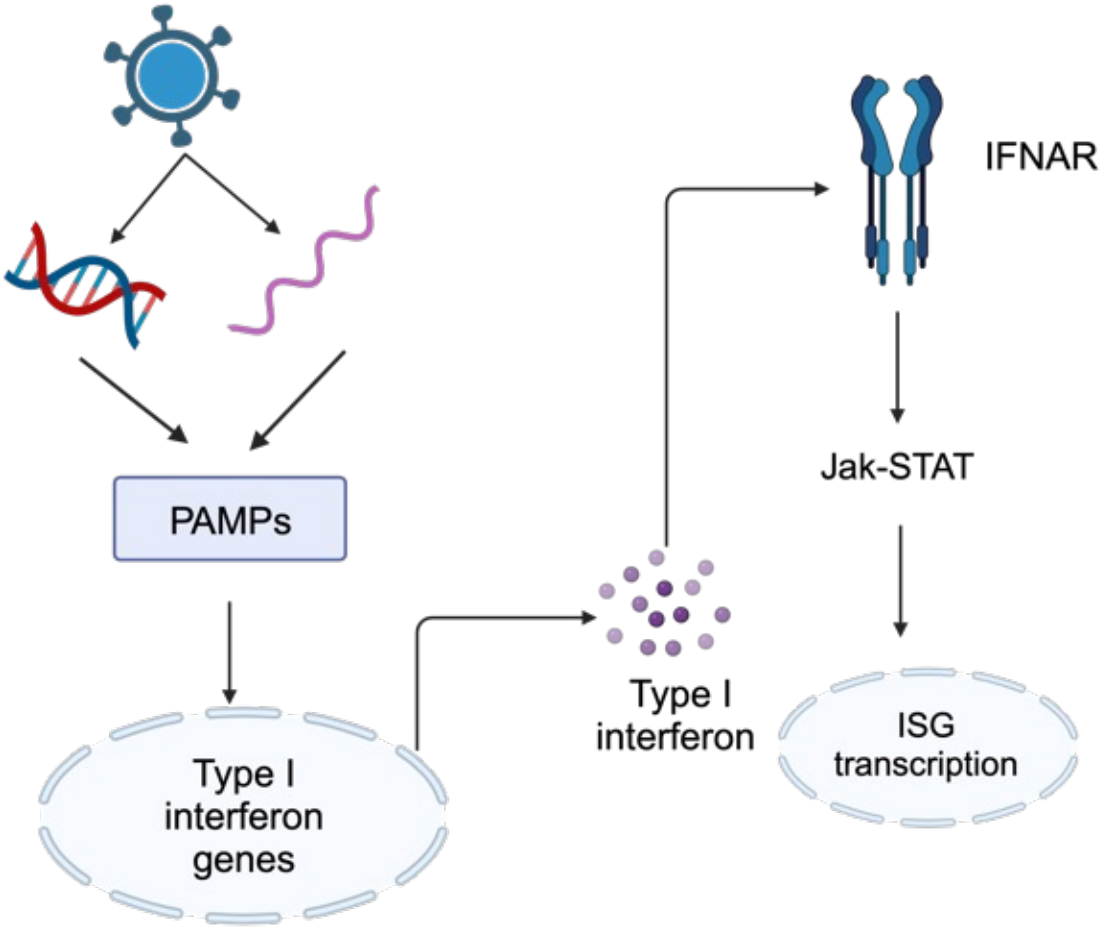



**Figure 4. Nucleic acid sensors of the innate immune system recognize unusual DNA and RNA molecules, for example, viral genomes.** This results in the triggering of an intracellular signaling cascade that transcriptionally induces the genes encoding type I interferons, which are subsequently secreted. Type I interferons act by binding to their receptor, interferon-α/β receptor (IFNAR), which activates the Janus kinases (JAKs). These activate the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2, leading to expression of interferon-stimulated genes (ISGs). Created with Biorender

(Takeuchi and Akira 2010; Emming and Schroder 2019; Vanpouille-Box, Hoffmann, and Galluzzi 2019; Jiao et al. 2020; Schlee and Hartmann 2016). This introduction will focus on the viral RNA sensor Retinoic Acid Inducible Gene I (RIG-I).

In addition to DNA, PRRs can also recognize RNA in the cytoplasm. The RIG-I like receptor family (RLR) contains three RNA sensors: Retinoic Acid Inducible Gene-I (RIG-I), Melanoma Differentiation-Associated protein 5 (MDA5) and Laboratory of Genetics and Physiology gene 2 (LGP2). These three proteins share a C-terminal domain (CTD) and a helicase domain (HD), but only RIG-I and MDA5 bear the effector domain known as caspase activating and recruiting domain (CARD) in the N-terminal. Therefore, only RIG-I and MDA5 trigger the IFN-I response through interactions with mitochondrial antiviral signaling proteins (MAVS) **(Figure 5)** (Brisse and Ly 2019; Kell and Gale 2015; B. Wu and Hur 2015)**.**

It has been shown that endothelial RIG-I activation leads to endothelial dysfunction. In wild-type mice, activation of RIG-I leads to endothelial stress, damage, and vessel impairment. After injection with a RIG-I agonist (dsRNA with a triphosphate at the 5' end), mice experienced an increase in endothelial cell dysfunction, as seen by an increase in vascular oxidative stress and circulating EMP numbers. In addition, after stimulation with a RIG-I agonist, both human coronary endothelial cells (HCAEC) and endothelial progenitor cells (EPC) showed increased reactive oxygen species formation, and HCAES increased production of proinflammatory cytokines (Asdonk et al. 2012). Similarly, stimulation of MDA5 led to endothelial apoptosis, formation of reactive oxygen species, and the release of pro-inflammatory cytokines. MDA5 activation in mice similar to RIG-I, leads to vascular oxidative stress and an increase in circulating endothelial microparticles and endothelial progenitor cells. In addition, chronic MDA5 stimulation exacerbated atherosclerosis (Asdonk et al. 2016). Similar to these pathways, the

activation of another RNA sensor, Toll-Like Receptor 7 also leads to vascular inflammation and impaired vascular growth(Krogmann et al. 2020).

| Gene | Protein | Substrate | Function |
|---|---|---|---|
| *Cgas* | cGAS | dsDNA | Apoptosis |
| *TLR9* | TLR9 | RNA-DNA hybrid | Inflammation |
| *TREX1* | TREX1 | dsDNA | Immune suppression |
| *AIM2* | AIM2 | dsDNA | Pyroptosis |
| *IFI16* | IFI16 | dsDNA | Pyroptosis |
| *IFIH1* | MDA5 | dsRNA | Apoptosis |
| *DDX58* | RIG-I | dsRNA | Inflammation |
| *TLR3* | TLR3 | dsRNA | Nectroptosis |
| *ZBP1* | ZBP1 | B-DNA, Z-DNA | Necroptosis |

**Table 2. Summary of nucleic acid sensor substrates and functions.**

Several genes that directly recognize nucleic acids have been identified and characterized in the human genome. A few well-characterized genes, their substrates and the mechanisms of action due to activation with ligands in cells are shown. Adapted from Baris et al Cancers 2021.

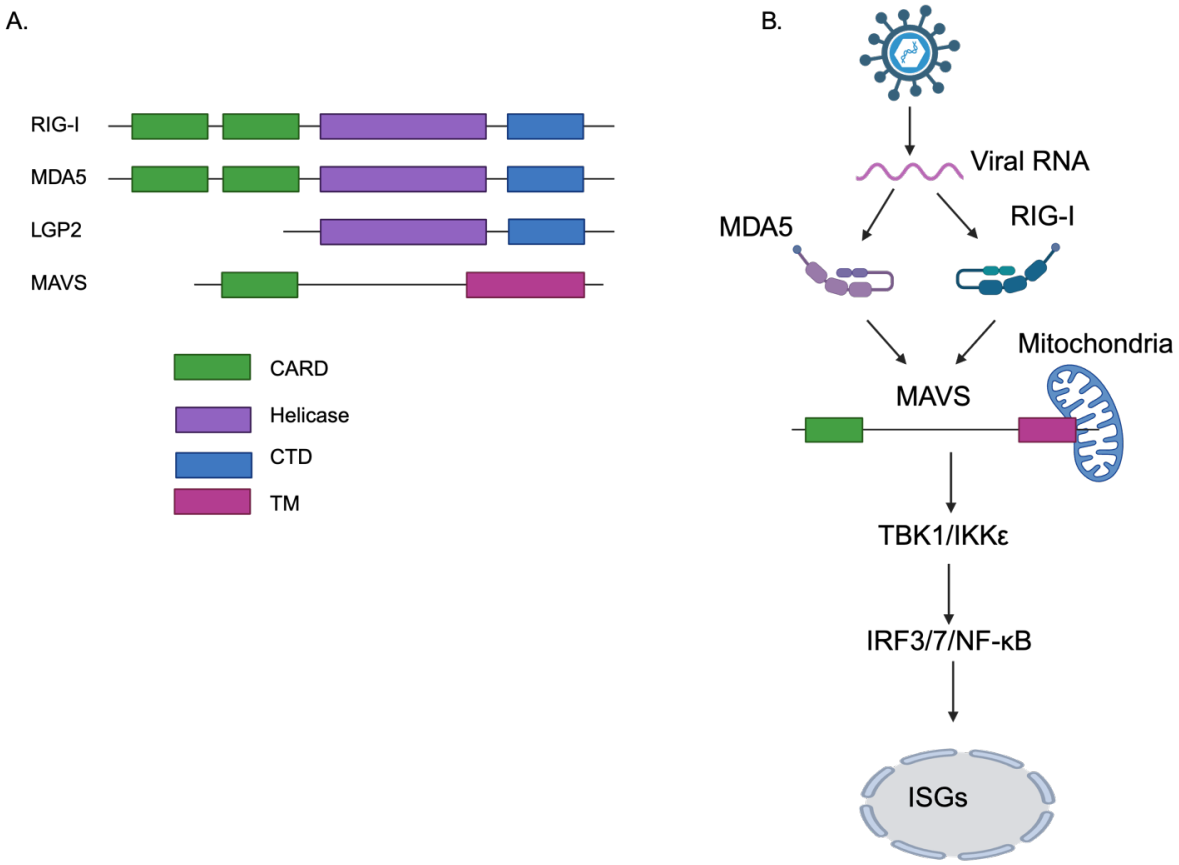



**Figure 5. RIG-I family receptors. a** ┼Domain architecture of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and mitochondrial antiviral-signalling protein (MAVS). The three members of the RLR family have a central helicase. Another RNA-binding fold, called the carboxy-terminal domain (CTD), is also found in all RLRs. RIG-I and melanoma differentiation-associated protein 5 (MDA5) contain two caspase activation and recruitment domains (CARDs). The signalling adaptor protein MAVS also has a CARD as well as a C-terminal transmembrane domain (TM). **B** RLR signalling pathway. RIG-I and MDA5 are activated by viral RNAs. They then undergo conformational changes which allows homotypic CARD–CARD interactions with MAVS. MAVS is anchored with its Tm to the the mitochondria and relays the signal to TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε). These activate interferon regulatory factor 3 (IRF3) and IRF7, which together with the transcription factor nuclear factor-κB (NF-κB) induce the expression of type I interferons and other genes. Created with Biorender

*RIG-I in ECs*

It has been shown that endothelial RIG-I activation leads to endothelial dysfunction. In wild-type mice, activation of RIG-I leads to endothelial stress, damage, and vessel impairment. After injection with a RIG-I agonist (dsRNA with a triphosphate at the 5’ end), mice experienced

vascular oxidative stress and increased circulating endothelial microparticle (EMP) numbers, indicating endothelial dysfunction. In addition, after stimulation with a RIG-I agonist, both human coronary endothelial cells (HCAEC) and endothelial progenitor cells (EPC) showed increased reactive oxygen species (ROS) formation, and HCAES increased production of proinflammatory cytokines (Asdonk et al. 2012). Similarly, stimulation of MDA5 led to endothelial apoptosis, cytokine production, and formation of ROS. MDA5 activation in mice similar to RIG-I, leads to vascular oxidative stress and an increase in circulating EMPs and endothelial progenitor cells. In addition, chronic MDA5 stimulation exacerbated atherosclerosis (Asdonk et al. 2016). Similar to these pathways, the activation of another RNA sensor, Toll-Like Receptor 7 also leads to vascular inflammation and impaired vascular growth (Krogmann et al. 2020).

*Structure and function of RIG-I*

RLRs are activated by cytosolic RNA. RIG-I and MDA5 are similar in structure and function, but they recognize different RNA structures. RIG-I is preferentially activated by blunt-ended 5'ppp short RNAs which bind to the CTD (Kolakofsky, Kowalinski, and Cusack 2012; Stok, Vega Quiroz, and van der Veen 2020). The CTD of RIG-I has a pocket that specifically binds either a 5'-PPP or a 5'-PP. In normal conditions, CARD domain is bound to the HD in a repressing form. Upon RNA recognition, base-paired region of RNA complexes with the HD of RIG-I, releasing the CARD domain. Thus, stable RNA-RIG-I interaction displaces CARDs, which causes multiple RIG-I proteins to oligomerize and become accessible for MAVS signaling. One main player in this process is the E3 ubiquitin ligase TRIM25. TRIM25 ubiquitination is critical to release RIG-I from autorepression. To interact with MAVS in the mitochondria, the RIG-I complex, RIG-I/14-3-3ε/TRIM25, mediates the redistribution or "translocation" of RIG-I from the cytosol to the intracellular membrane compartments. There, RIG-I binds to MAVS through homotypic CARD-CARD interaction(Rehwinkel and Gack 2020). Once activated, MAVS recruits the tumor necrosis factor receptor associated factors (TRAFs), which are essential to activate

interferon regulatory factors 3 and 7 (IRF3, IRF7) and NF-kB mediated response. Finally, activation of RIG-I results in the expression of cytokines and IFN-I genes, which recruits innate and eventually adaptive immune cells **(Figure 6).**

RLRs are able to recognize self-derived RNAs, leading to either enhanced or depleted IFN response in a context dependent manner(Stok, Vega Quiroz, and van der Veen 2020). A recent study showed that mitochondrial DNA double stranded breaks release mitochondrial RNA into the cytoplasm, triggering the RLR dependent immune response. Moreover, following cellular irradiation, mitochondrial DNA breaks synergize with nuclear DNA to promote the immune response(Tigano et al. 2021). These emerging studies highlight the potential of RIG-I activation without extrinsic pathogens and could potentially explain the ‘sterile inflammation’ in tissues.

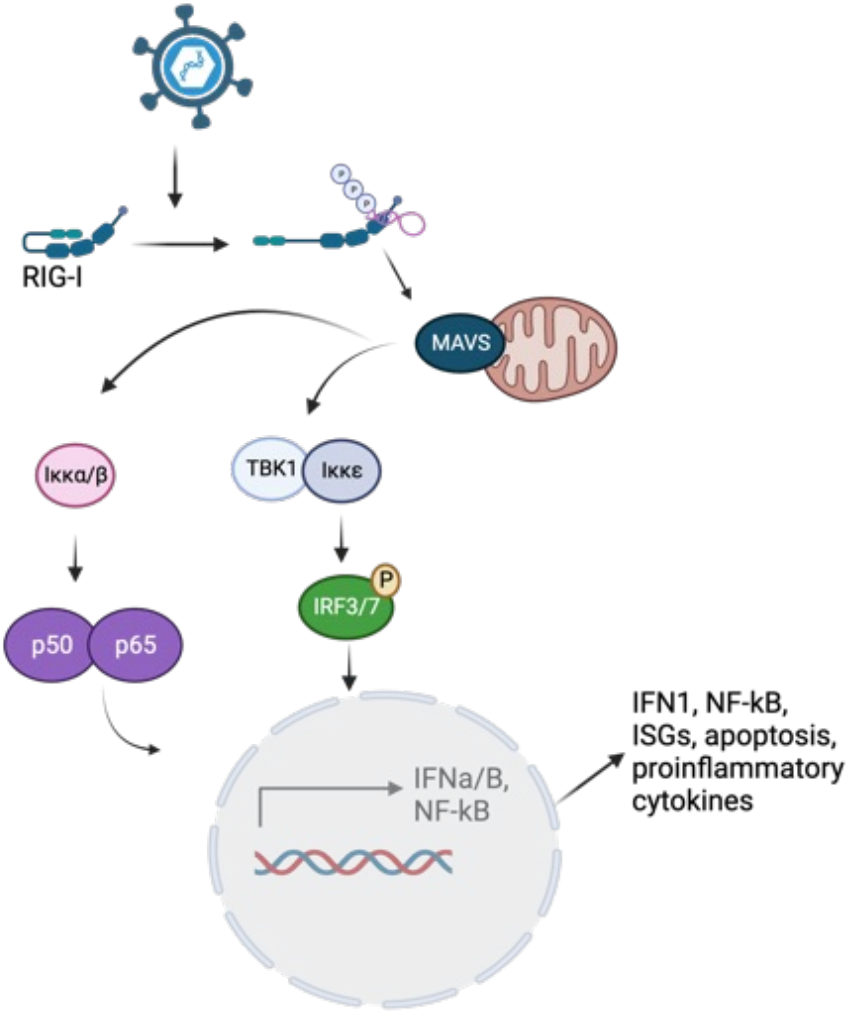



**Figure 6. RIG-I signaling pathway.** RIG-I is activated by viral RNA. Activated RIG binds to mitochondrial antiviral-signaling protein(MAVS). This leads to the downstream activation of interferons and NF-kB, inducing an immune response. Created with biorender.

While RIG-I and MDA5 drive very similar signaling pathways, they do differentially induce type 1 IFN response to different pathogens(Stok, Vega Quiroz, and van der Veen 2020). For example, while RIG-I is activated most potently in response to negative-strand viruses such as the influenza virus(Weber-Gerlach and Weber 2016), MDA5 is activated in response to positive-strand viruses such as the hepatitis D virus(Z. Zhang et al. 2018). In addition, animal models show that RIG-I and MDA5 have functional differences *in vivo*, as well as distinct molecular immune functions(Brisse and Ly 2019).

RIG-I knockout mice show a colitis-like phenotype, reduced Peyer's patches, and show increased effector T cells and decreased naïve T cells ( Y. Wang et al. 2007). MAVS and MDA5 knockout mice lose type 1 interferon production and suffer early mortality in response to infection with Coxsackie B virus (CVB), which has been associated with myocarditis(J. P. Wang et al. 2010). In a study of RLRs in West Nile Virus, RIG-I x MDA5 double knockout mice lacked the innate immune response against the virus infection. Surprisingly, they did not suffer severe pathological damage in tissues during infection, which was similar to animals lacking MAVS(Errett et al. 2013).

Singleton-Merten syndrome (SMS) is an autosomal-dominant disorder characterized by aortic calcification, skeletal abnormalities, psoriasis, as well as other conditions. Jang et al performed exome sequencing and found gain-of-function mutations in DDX58, the gene which encodes the RIG-I protein, leads to variable manifestation of SMS, often without the typical dental anomalies(Jang et al. 2015). In addition, gain-of-function mutations of MDA5 have been found in SMS patients with upregulated interferon signature genes. The sustained signaling of MDA5 and RIG-I in SMS patients in possibly due to an increase of protein levels, to the recognition of self-RNA or both. It is believed that excess IFN-I and other inflammatory cytokines in the endothelial cells in aortic and mitral valves are critical for the SMS development (Lu and MacDougall 2017).

**Conclusion and Gaps in Knowledge**

The cardiovascular system is a broadly studied organ system that is well understood, however, there are still several key questions that have gone unanswered in the field. In particular, the role of the immune system in vascular function is still poorly understood. While it is known that ECs are active in the immune response, it is not well understood how critical EC activation and immune status is for vascular function and immune function. One clear example of this is how poorly understood endothelial activation and inflammation status affect vascular function in infectious disease. While there are several studies that have shown that infectious disease leads to vascular dysfunction, further studies into how endothelial activation or inflammation may contribute to disease state are necessary. We also see the lack of knowledge on immune-EC interactions with cardiovascular disease. While several cardiovascular diseases are known to lead to chronic inflammation, whether EC activation or EC-immune interactions contribute to inflammation or vascular dysfunction is still unclear.

We also clearly see a need to understand the EC-immune interactions in the context of cancer. It is abundantly clear that vascular function plays a major role in tumor development and response to therapy. However, treatments focusing on targeting tumor vasculature have stalled due to limited efficacy. Though tumor therapies targeting the immune system have shown immense promise, many patients fail to respond to immunotherapy. As is clear through numerous studies, the vascular system and immune system are intertwined, and understanding how to improve response to one type of therapy will very likely require understanding how it reacts with the other. For example, how immune-checkpoint blockade(ICB) alters tumor vasculature is still poorly understood. Whether (ICB) activates tumor ECs and promotes poor perfusion, therefore leading to hypoxia and limited drug delivery, is still unclear. On the other hand, ICB may normalize tumor ECs and lead to improved therapeutic outcomes due to both improved immune infiltration and normalized vasculature. However, a more likely scenario is

that a combination of ICB and vascular normalization will provide improved therapeutic outcomes. Despite the wealth of knowledge on the cardiovascular system and on RIG-I, there is still very little known about how they interact with one another. While there is some evidence that RIG-I leads to increased oxidative stress in ECs, the majority of RIG-I studies have focused on its role in immunity and in tumor progression. As discussed above, one cannot look at the immune system or the tumor without also understanding how vasculature is affected. Therefore, it is critical to understand how RIG-I affects normal and pathological ECs. I hypothesize that RIG-I signaling plays a critical role in endothelial function and angiogenesis, and that understanding the role of RIG-I in ECs will improve our perspective on the bigger picture role of how ECs sense inflammatory stimuli and what prevents EC activation and immune function in cancers. Such understanding is critical to appreciate the pathology of several infectious and autoimmune pathologies including viral infections such as COVID.

## Chapter 2: Materials and Methods

### Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) (Cat: C-2519, Lonza) and dermal human microvascular endothelial cells (HMVECs) (Cat: CC-2543, Lonza) were cultured in EBM-2 media (Cat: NC1447083, Fischer) supplemented with bullet kit and 10% fetal bovine serum (Cat: S11550H, Biotechne). Normal human lung fibroblasts (Cat: CC-2512, Lonza) were cultured in FBM media (Cat: CC3131, Lonza) and supplemented with bullet kit and 10% fetal bovine serum. All cells were maintained at 37 °C and 5% $CO_2$. Cells used for experiments were low passage number between 2–8. Tipiracil (TPI) was purchased from Sigma (Cat: SML1552-10MG) and dissolved in PBS. Cell lines were authenticated with STR profiling if maintained in the lab (not purchased). All cell lines were routinely tested and found negative for mycoplasma contamination before use in the assays as described.

### Cell transfection

HUVECs (70% confluence) were transfected with 0.1–1 ug/mL RIG-I or control agonist RNA (Cat: tlrl-hprna-100, Invivogen) according to manufacturers' instructions. Specifically, The RIG-I or control agonist was diluted in Lyovec transfection reagent (Cat: lyec-12 Invivogen).

### Cell viability and apoptosis assays

Viability and apoptosis were assessed using the Cell-Titer Glo and Caspase-Glo kits, respectively (Cat: G9242, G8091, Promega). White walled, 96-well tissue culture microplates (Corning) were used for luciferase-based assays. Luminescence was measured using a Promega GloMax instrument using a 0.5 s integration time.

RNA sequencing and gene expression

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 the RANAseq pipeline and web interface with differential expression comparisons through DESeq2 and Wald's test for significance calculations (Prieto and Barrios 2020; Wen 2017). Reverse transcription was preformed using High-Capacity cDNA Reverse Transcription Kit (Cat: 4368814, Applied Biosystems) according to manufacturer's instructions. Gene expression was measured using real-time quantitative PCR (qRT-PCR) with TaqMan Master Mix II no UNG (Cat: 4440048, Thermofisher Scientific) with the following primers: human TYMP (Cat: 4453320, Assay ID: Hs00157317_m1), human FLT1 (Cat: 4453320, Assay ID: Hs01052961_m1), human CX3CL1 (Cat: 4448892, Assay ID: Hs01011407_m1), human IL6 (Cat: 4448892, Assay ID: Hs03929033_u1), human PROCR (Cat: 4448892, Assay ID: Hs00941183_g1), human SERPINE1 (Cat: 4453320, Assay ID: Hs00167155_m1), human PLAU (Cat: 4448892, Assay ID: Hs01547050_m1), human CXCL10 (Cat: 4331182 Assay ID: Hs00171042_m1), human MX1 (Cat: 4331182 Assay ID: Hs00895608_m1), and human GAPDH (Cat: 4331182, Assay ID: Hs02758991_g1), mouse Vegf(Cat: 4331182, Assay ID: Mm00437306_m1), mouse Ccl5 (Cat: 4331182, Assay ID: Mm01302427_m1), mouse Gapdh (Cat: 4331182, Assay ID: Mm99999915_g1) according to manufacturer's instructions.

Silencer Select Pre designed siRNA siRNA ID: s4434, s4392420, or Silencer™ Select Negative Control No. 1 siRNA were purchased from Qiagen. Endothelial cell biology

angiogenesis gene array was assessed according to manufacturer's instructions (Cat: 330231 PAMM-015ZE-4, Qiagen). Specifically, the SYBR Green qRT-PCR assays were conducted using PowerUP SYBR Green Master Mix with predetermined primers in the array. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method relative to an internal control (GAPDH).

### Western and simple western blots

HUVECs were seeded in six well plates (2,000,000 cells/well) and transfected as described above. In some cases, after 24 h, cells were treated with 10 nmol TPI for 24 h. After treatment, media was aspirated and cells were washed in ice cold PBS and lysed directly in the plate in RIPA buffer (Cat: PI89900, Fischer) containing Protease Inhibitor Mini Tablets (1/10 mL RIPA buffer, Cat: 50-720-4060, Fisher) with phosphatase inhibitor cocktail 2 and 3 (1:1000, P5726-1ML, P0044-1ML, Sigma). Lysates were centrifuges for 12,000 x g and 4 °C for 20 min. The supernatant was collected, and protein concentration was determined using the Pierce BCA Protein assay kit (Cat: 23227, Thermofisher).

For western blot, samples were mixed with 4x Protein Sample Loading Buffer (Cat: 928-40004, Li-Cor) supplemented with 5% of beta-mercaptoethanol, denaturalized and loaded in 4–20% precast polyacrylamide gels (Cat: 456-1094, BioRad). Electrophoresis was done in 1x Tris/Glycine/SDS at 200 V for 30 min. Trans-blot Turbo Transfer system (Biorad) was used to transfer the proteins to a PVDF membrane. Blocking and antibody dilutions were done in Intercept blocking buffer (Cat: P/N 927-60001, Licor). The membranes were developed using Li-Cor Odyssey Clx imaging system. For simple western blot, the samples were diluted with 1x sample buffer (Cat: DM-001,

ProteinSimple). Protein quantification was performed using a 12-230 kDA 25 lane plate (Cat: SM-W004, ProteinSimple) in a ProteinSimple Wes Capillary Western Blot analyzer according to the manufacturer's instructions. Anti-RIG-I (#3743), Anti- ISG15 (#2743), Anti-TYMP (#4307), Anti-IRF8 (#98344) and Anti- GAPDH (#5174) were all purchased from Cell Signaling. Anti-IRF1 (PA5-64093) was purchased from ThermoFisher.

### Scratch assay

HUVECs were plated on a 12 well plate at 100% confluency and the monolayer was scratched with a pipette tip. At 0, 4, and 8 h, the scratch area was visualized using brightfield microscopy in an Incucyte Live Cell Analysis Platform. Area of scratch was quantified using Image J.

### 3D angiogenic sprouting assay

HUVECs transfected for 24 h and were coated on cytodex-3 beads at a density of 1 million cells per 25 uL beads and incubated in suspension for 3–4 h with gentle mixing every hour. In some cases, TPI was added during the incubation. Beads were then plated on TC treated 24 well dishes overnight and resuspended in 2 mg/ml fibrin gel with 200,000 fibroblasts. The gel was allowed to polymerize and complete EGM-2 media was added. Sprouts were visualized on day 4 via confocal imaging following a 4-h incubated with 1:200 fluorescein isothiocyanate (FITC)-labelled *Ulex europaeus* lectin (Vector labs).

### In vivo methods

All animal work was approved by the OHSU Institutional Animal Use and Care Committee. WT male and female C57Bl/6 N and Ddx58 (−/−) 8–10-week-old mice were

purchased from Jackson Labs and injected subcutaneously with Growth factor reduced (Matrigel BD) with 400 ng $mL^{-1}$ recombinant human bFGF (Millipore). One-week later Matrigel plugs, as well as lung, liver, and heart, were harvested from mice and RNA was isolated using the Eurx RNA purification kit according to manufacturer's instructions. Matrigel plugs were homogenized and analyzed for hemoglobin content using a colorimetric assay kit (Sigma). In addition, RNA from tissue was used to analyze endothelial activity using the Qiagen endothelial cell activity qRT-PCR array according to manufacturer's instructions.

For tumor experiments, WT male and female C57Bl/6 N and Ddx58 (−/−) 8–10-week-old mice were purchased from Jackson Labs and injected subcutaneously with 200,000 mycoplasma negative MC38 tumor cells in Geltrex (Thermofisher). Tumor growth was measured using calipers, with volume computed as ½ x Length x Width^2. At 15 days following injection, mice were treated with isolectin B4 and FITC-Dextran intravenously (100 uL of 1/200 dilution in PBS). Mice were euthanized 1 hours post injection and tumors and lungs were harvested for histology, flow and RNA analysis.

Immunofluorescence

In some experiments, tumors and lungs were harvested from euthanized mice and directly frozen in OCT. Tissue was sectioned into slides by the OHSU histology core. Slides were thawed in a humid chamber and blocked in blocking buffer for 30 minutes (10% normal goat serum, 2% BSA, 0.05% saponin in PBS) Endothelial cells were analyzed directly via isolectin 649 (I.v. injected prior to euthanasia). VEGFR1 was imaged using a VEGF Receptor 1 Rabbit monoclonal antibody (Thermofisher MA5-32045) at a 1:100 dilution in 2%BSA, 0.05% saponin in PBS overnight. Slides were

washed in PBS 3 times for 30 seconds. Secondary staining was performed using goat anti rabbit IRdye 800CW (Li-Cor, 926-32211) at a 1:200 dilution in 2%BSA, 0.05% saponin in PBS for 1 hour. Slides were then washed 3 times for 30 seconds each and mounted using mounting medium with DAPI (Vectorlabs H-1200-10). Imaging was performed either by the microscopy core using the LSM 880 Zeiss Laser-Scanning Confocal Fast Airy, or using the Apotome3-Zeiss Microscope with Grid-Based Optical Sectioning. Analysis was done using Zen analysis software.

Flow Cytometry

In some experiments, tumors were harvested and digested into a single cell suspension as described previously (Nelson et al paper). Cells were frozen in 10% FBS, 10% DMSO, 80%DMEM. Cells were later thawed and checked for viability. Cells were then split into 3 and plated on a 3 96 well plates and blocked with Fc-Block (BD Bioscience, cas # 553142) 1:200 in FACs buffer and Live/Death NIR reagent solution 1:500 in FACs buffer for 25 minutes on ice. Subsequently cells were stained with an antibody cocktail mix containing the following antibodies with the indicated flourophores diluted in FACs buffer (**Tables 3-5**) for extracellular markers. Following staining, cells were fixed with Cytofix Buffer (BD Bioscience, cat # 554655). In some instances, cells were then stained with an intracellular antibody cocktail mix containing antibodies with the corresponding flourophores for intracellular markers. Cells were then washed and resuspended in 200 uL of FACS buffer and stored at 4° protected from light until analysis in a Cytek Aurora cell analyzer. Data was analyzed with FlowJo v10.8.0 software.

| Laser Channels | Filters | Fluorochrome | Marker | 1X dilution |
|---|---|---|---|---|
| 405-1 | 450/50 | BV421 | MHCII | 1/1000 |
| 405-2 | 515/20 | BV510 | L/D Aqua | 1/1000 |
| 405-3 | 605/30 | BV605 | NK1.1 | 1/100 |
| 405-4 | 660/20 | BV650 | CD11c | 1/100 |
| 405-5 | 710/50 | BV711 | CD24 | 1/200 |
| 405-6 | 780/60 | BV785 | F4/80 | 1/100 |
| | | | | |
| 488-1 | 525/50 | FITC | Ly6G | 1/400 |
| 488-4 | 710/50 | PerCPCy55 | CD19 | 1/200 |
| | | | | |
| 561-1 | 582/15 | PE | PDL1 | 1/200 |
| 561-2 | 610/20 | PE-CF594 | Ly6C | 1/200 |
| 561-3 | 670/30 | | | |
| 561-4 | 780/60 | PE-Cy7 | CD11b | 1/400 |
| | | | | |
| 640-1 | 670/30 | APC | CD45 | 1/500 |
| 640-2 | 730/45 | APCR700 | CD103 | 1/100 |
| 640-3 | 780/60 | APCCy7 | Siglec F | 1/100 |

**Table 3.** Myeloid flow panel for tumor experiments

| Laser Channels | Filters | Fluorochrome | Marker | 1X dilution |
|---|---|---|---|---|
| 405-1 | 450/50 | BV421 | CD3e | 1/200 |
| 405-2 | 515/20 | BV510 | CD45 | 1/500 |
| 405-3 | 605/30 | BV605 | | |
| 405-4 | 660/20 | BV650 | | |
| 405-5 | 710/50 | BV711 | CD8a | 1/200 |
| 405-6 | 780/60 | BV785 | | |
| | | | | |
| 488-1 | 525/50 | FITC | Ki67 | 1/400 |
| 488-4 | 710/50 | PerCPCy55 | | |
| | | | | |
| 561-1 | 582/15 | PE | PD1 | 1/200 |
| 561-2 | 610/20 | PE-Dazzle | Lag3 | 1/200 |
| 561-3 | 670/30 | | | |
| 561-4 | 780/60 | PE-Cy7 | CD44 | 1/600 |
| | | | | |
| 640-1 | 670/30 | APC | CD4 | 1/200 |
| 640-2 | 730/45 | APCR700 | CD62L | 1/400 |
| 640-3 | 780/60 | APCCy7 | LD NIR | 1/1000 |

**Table 4.** Lymphoid flow panel for tumor experiments

| Laser Channels | Filters | Fluorochrome | Marker | 1X dilution |
|---|---|---|---|---|
| 405-1 | 450/50 | BV421 | MHCII | 1/1000 |
| 405-2 | 515/20 | BV510 | CD45 | 1/1000 |
| 405-3 | 605/30 | BV605 | NK1.1 | 1/100 |
| 405-4 | 660/20 | BV650 | CD11c | 1/100 |
| 405-5 | 710/50 | BV711 | CD24 | 1/200 |
| 405-6 | 780/60 | BV785 | F4/80 | 1/100 |
| | | | | |
| 488-1 | 525/50 | FITC | Ly6G | 1/400 |
| 488-4 | 710/50 | PerCPCy55 | CD19 | 1/200 |
| | | | | |
| 561-1 | 582/15 | PE | VEGFR-2 | 1/200 |
| 561-2 | 610/20 | PE-CF594 | Ly6C | 1/200 |
| 561-3 | 670/30 | | | |
| 561-4 | 780/60 | PE-Cy7 | CD11b | 1/400 |
| | | | | |
| 640-1 | 670/30 | APC | CD31 | 1/500 |
| 640-2 | 730/45 | APCR700 | CD103 | 1/100 |
| 640-3 | 780/60 | APCCy7 | LD NIR | 1/1000 |

**Table 5**. Flow panel for lung experiments

Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA). In vitro experiments were performed in biological replicates (typically $N = 3$ unless stated otherwise in the legends). No statistical methods were used to predetermine sample size for mouse studies. No animals were excluded from analysis. No blinding or randomization was used in the studies. Differences between pairs of groups were analyzed by Student's *t*-test. Variance was similar between groups. Comparison among multiple groups was performed by

one-way ANOVA followed by a post hoc test (Tukey's or Holm-Sidak). In the absence of multiple comparisons, Fisher's LSD test was used. Values of *n* refer to the number of experiments used to obtain each value. For mouse studies where the data was not normally distributed, we used two-tailed Mann–Whitney *U* test. Values of $p \leq 0.05$ were considered significant.

## Data availability

RNAseq datasets are available from GEO under the accession number GSE204809.

# Chapter 3:

## Thymidine Phosphorylase Facilitates RIG-I induced EC dysfunction

Reproduced in its entirety from: **Baris, A**., Fraile-Bethencourt, E., Eubanks, J. et al. Thymidine phosphorylase facilitates retinoic acid inducible gene-I induced endothelial dysfunction. *Cell Death Dis.* 2023

### Introduction

The vascular endothelium is an incredibly complex, dynamic system with a variety of functions in cardiovascular health. As such, endothelial cells (ECs) can face numerous injuries that lead to pathogenesis, disease, and dysfunction. Understanding how ECs undergo dysfunction and methods to target that dysfunction are critical to understanding and improving cardiovascular disease treatment. There is emerging evidence that nucleic acid sensors (NAS)—one of the fundamental arms of innate immunity—play a critical role in endothelial cell function (Asdonk et al. 2012; Chelvanambi et al. 2021; Jeong et al. 2021; S. J. Lee et al. 2021; Y. Liu et al. 2014; Pallarés et al. 2022; da Conceição et al. 2013; Ma et al. 2011; Moser et al. 2016; Opitz et al. 2009).

Among NAS, RIG-I recognizes RNA cytosolic 5’ppp short RNAs. Upon recognition of RNA, the base-paired region of RNA complexes with the HD of RIG-I, released the CARD, which is normally bound to the HD in a repressing form. This interaction displaces CARDs, which causes several RIG-I proteins to oligomerize and become accessible for MAVS signaling. RIG-I binds to MAVS through homotypic CARD-CARD interactions. Once activated, MAVS recruits the tumor necrosis factor receptor associated factors, which activate interferon regulatory factors 3 and 7, and the NF-kB mediated pathway. This results in the expression of cytokines and IFN-I genes, which recruit innate and adaptive immune cells (Kolakofsky, Kowalinski, and Cusack 2012; Rehwinkel and Gack 2020).

It has been shown that RIG plays a role in EC dysfunction and inflammation(Asdonk et al. 2012). In addition, it is well known that ECs are the site of cytokine storms following infection, and that inflammation in ECs from innate immune sensing can impact tumor growth(Teijaro et al. 2011). Here we show that RIG-I activation using a small 5'ppp RNA diminishes EC survival and causes EC dysfunction. We have discovered a RIG-I dependent 7 gene signature that affects angiogenesis, inflammation and coagulation. Among these, we identified the thymidine phosphorylase TYMP as a key mediator of RIG-I induced EC dysfunction via its regulation of a subset of interferon stimulated genes. Inhibition of TYMP with a small molecule drug or siRNA rescues RIG-I induced EC death, migration arrest and restores sprouting angiogenesis likely via decrease of IRF1 dependent transcription. Our observations identify mechanisms by which RIG-I drives EC dysfunction and define pathways that can be pharmacologically targeted to ameliorate RIG-I induced vascular inflammation. Our studies identify novel mechanisms by which RIG drives endothelial dysfunction and highlights potential strategies for mitigating inflammation caused by RNA sensing.

## Results

It is well understood that RIG-I can be robustly activated using a small hairpin triphosphate containing RNAs. We have previously established a 89 base-paired RNA agonist from the influenza virus is a potent activator of RIG-I signaling (*15*). To determine the effect of RIG-I activation on ECs, we treated HUVECs and HMVECs with a control agonist (14 base paired ds RNA) or a RIG-I agonist, validated the increase in interferon stimulated genes (Fig 7**)** and evaluated EC health. We found the RIG-I agonist significantly decreased proliferation and increased cell death in a dose responsive manner (Fig8A-8C). To further understand the functional consequences of RIG-I agonist treatment, we used a scratch assay. We identified significant delays in wound healing upon treatment with a RIG-I agonist (Fig 8D). Similarly, we found that upon transfection of the RIG-I agonist, endothelial cells were unable to form

angiogenic sprouts in a 3D fibrin bead assay (Fig 8E). These observations suggest that activation of RIG-I impacts EC proliferation, survival, and fundamental functional characteristics such as migration and sprouting angiogenesis.

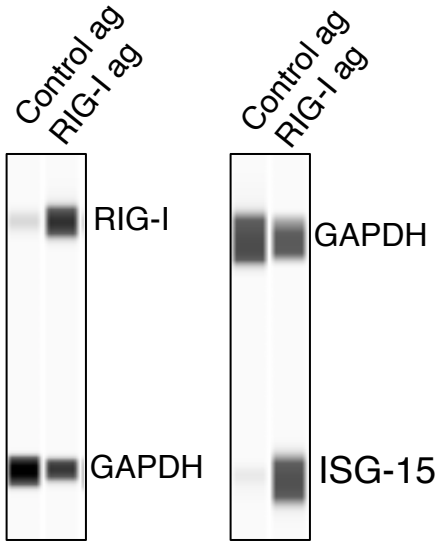



**Figure 7. RIG-I agonist induces activation of ISG expression**. HUVECs were treated with control agonist or RIG-I for 24h. Cell lysates were evaluated for RIG-I and ISG-15 expression using Simple Western Capillary based western blot system (Wes). One of two independent experiments.

To identify transcriptional changes involved in RIG-I activation, preformed RNA sequencing from control or RIG-I activated HUVECs and HMVECs 24 hours post treatment. We found that RIG-I activation with this agonist led to a robust interferon response, including an upregulation of genes such as MX1, OAS1, and CXCL10. In addition, we also found that DDX58, the gene that encodes RIG-I, is upregulated following RIG-I activation, indicating a feed-forward loop (**Fig 9A,9B**). We performed a pathway enrichment analysis using Enrichr(Kuleshov et al. 2016; Xie et al. 2021) and found that type I interferon signaling is highly enriched (**Fig 9C,9D**). While pathway analysis inferred interferon gamma response, endothelial cells do not make interferon gamma. The Enrichr annotation for this pathway consists of 39 genes including Akt1, IL-1B, IRF1, JAK1, STAT1, STAT3, several MAP kinases that are common to other pathways induced by RIG-I activation. In addition to these canonical pathways, we observed pathways that are important for vascular function such as cholesterol homeostasis, coagulation and hypoxia were modulated by RIG-I activation (Fig 9C,9D).

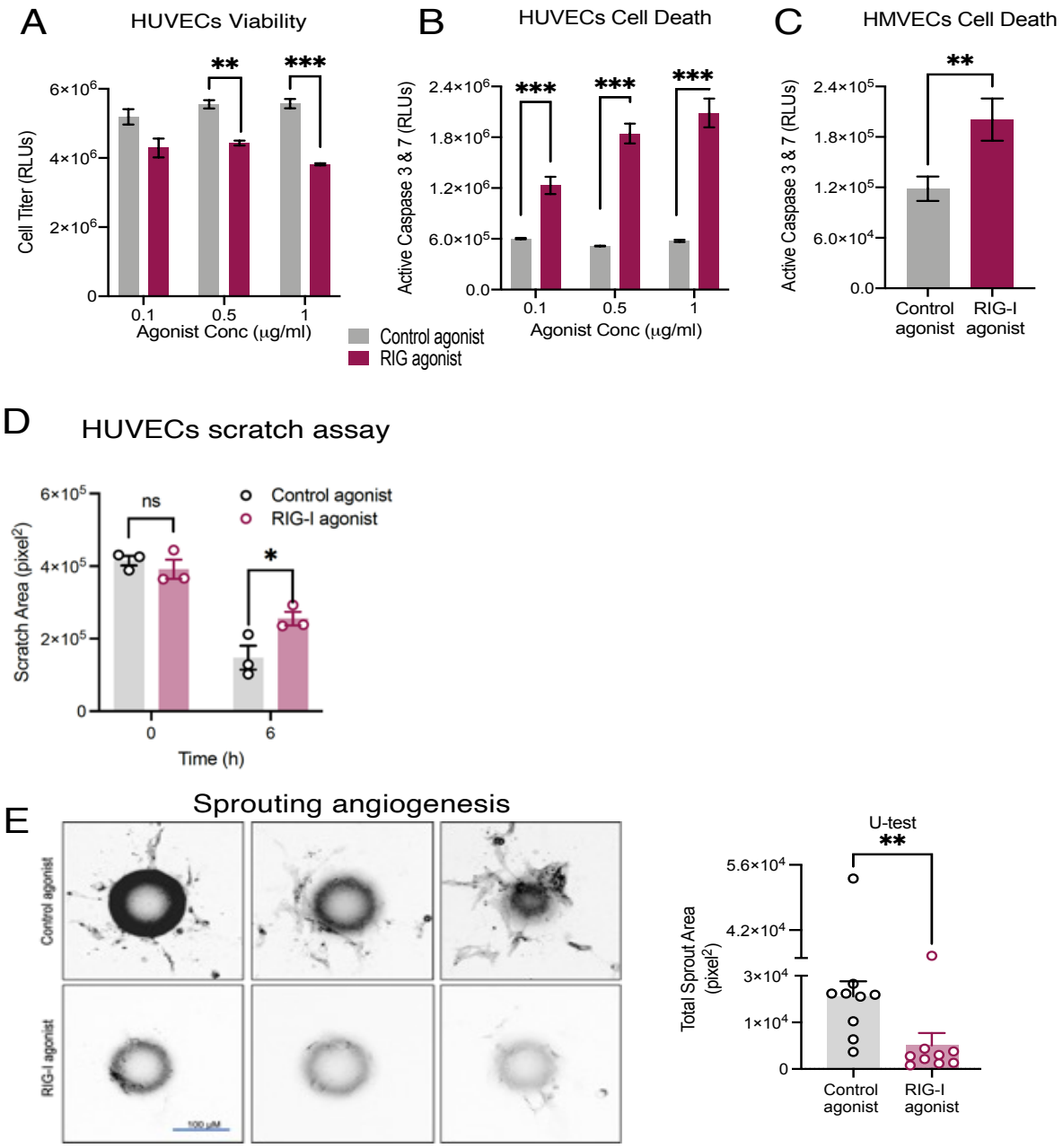
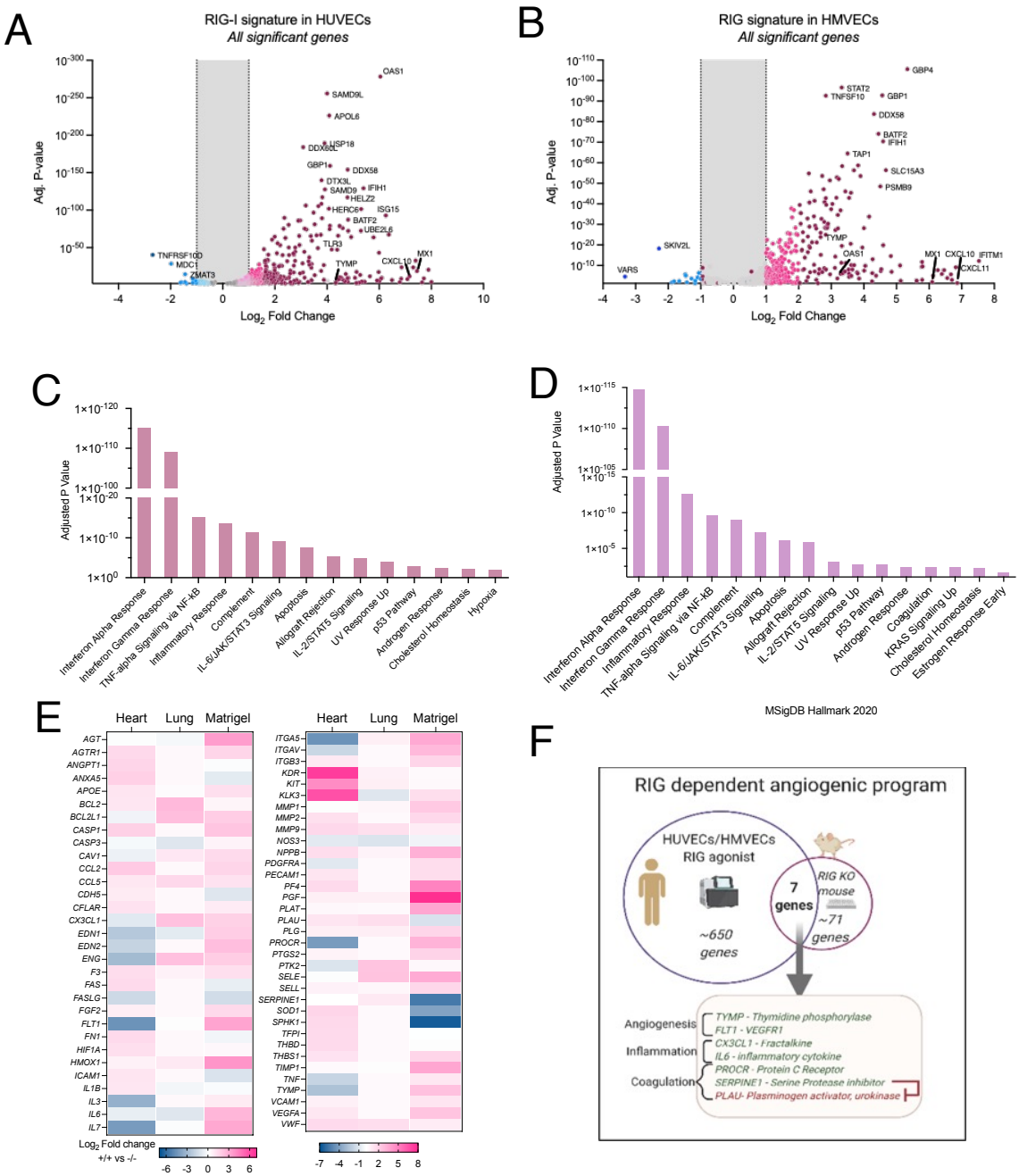



**Figure 8. RIG-I activation increases EC death and decreases angiogenesis.** HUVECs or HMVECS were treated with either control agonists or RIG-I agonists (1 ug/ml) (A-E). Viability was measured using Cell Titer glo (**A**) and Cell Death was measured using Caspase 3&7 glo (**B**–**C**) assays. Panel C used 0.1 ug/mL of agonist. **D** Migration was measured using a scratch assay. **E** Sprouting angiogenesis was measured using a 3D tube formation assay in a fibrin gel. Scale bar = 100 μm. Image depicts 3 frames along the same bead. Quantification of sprout area stained using *U.europaeus* lectin. Dots represent individual beads. $^{**}P < 0.01$, $^{***}P < 0.005$ using ANOVA or two-tailed Student's *T*-test and Mann–Whitney *U*-test for panel **E**. Bars represent mean ± SEM of independent replicates.


A
RIG-I signature in HUVECs
All significant genes
Adj. P-value
Log2 Fold Change
OAS1
SAMD9L
APOL6
USP18
DDX60L
GBP1
DDX58
DTX3L
SAMD9
IFIH1
HELZ2
HERC6
ISG15
BATF2
UBE2L6
TLR3
TYMP
CXCL10
MX1
TNFRSF10D
MDC1
ZMAT3
B
RIG signature in HMVECs
All significant genes
Adj. P-value
Log2 Fold Change
GBP4
STAT2
TNFSF10
GBP1
DDX58
BATF2
IFIH1
TAP1
SLC15A3
PSMB9
SKIV2L
TYMP
OAS1
MX1
CXCL10
IFITM1
CXCL11
VARS
C
Adjusted P Value
Interferon Alpha Response
Interferon Gamma Response
TNF-alpha Signaling via NF-kB
Inflammatory Response
Complement
IL-6/JAK/STAT3 Signaling
Apoptosis
Allograft Rejection
IL-2/STAT5 Signaling
UV Response Up
p53 Pathway
Androgen Response
Cholesterol Homeostasis
Hypoxia
D
Adjusted P Value
Interferon Alpha Response
Interferon Gamma Response
Inflammatory Response
TNF-alpha Signaling via NF-kB
IL-6/JAK/STAT3 Signaling
Complement
Apoptosis
Allograft Rejection
IL-2/STAT5 Signaling
UV Response Up
p53 Pathway
Androgen Response
Coagulation
KRAS Signaling Up
Cholesterol Homeostasis
Estrogen Response Early
MSigDB Hallmark 2020
E
Heart
Lung
Matrigel
AGT
AGTR1
ANGPT1
ANXA5
APOE
BCL2
BCL2L1
CASP1
CASP3
CAV1
CCL2
CCL5
CDH5
CFLAR
CX3CL1
EDN1
EDN2
ENG
F3
FAS
FASLG
FGF2
FLT1
FN1
HIF1A
HMOX1
ICAM1
IL1B
IL3
IL6
IL7
Log2 Fold change
+/+ vs -/-
ITGA5
ITGAV
ITGB3
KDR
KIT
KLK3
MMP1
MMP2
MMP9
NOS3
NPPB
PDGFRA
PECAM1
PF4
PGF
PLAT
PLAU
PLG
PROCR
PTGS2
PTK2
SELE
SELL
SERPINE1
SOD1
SPHK1
TFPI
THBD
THBS1
TIMP1
TNF
TYMP
VCAM1
VEGFA
VWF
F
RIG dependent angiogenic program
HUVECs/HMVECs
RIG agonist
~650
genes
7
genes
RIG KO
mouse
~71
genes
Angiogenesis
Inflammation
Coagulation
TYMP - Thymidine phosphorylase
FLT1 - VEGFR1
CX3CL1 - Fractalkine
IL6 - inflammatory cytokine
PROCR - Protein C Receptor
SERPINE1 - Serine Protease inhibitor
PLAU- Plasminogen activator, urokinase

G

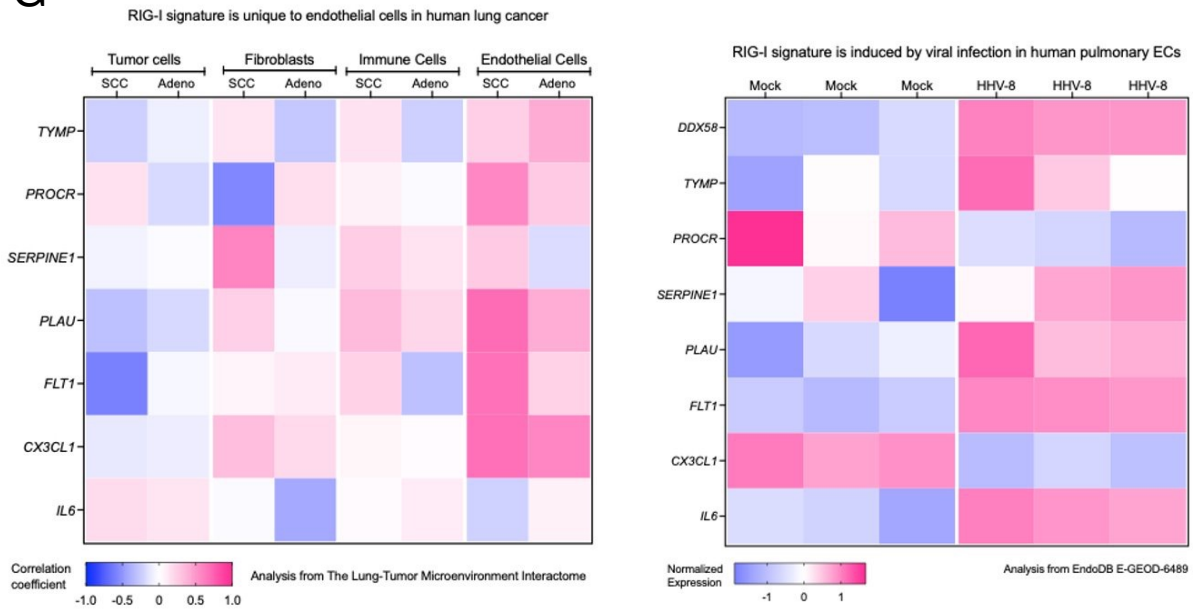



**Figure 9. RIG-I activation induces an angiogenic-specific gene signature in ECs. A**–**C** HUVECs or **B**–**D** HMVECs were treated with RIG agonists or control agonist. 24 h later RNA was extracted and RNA sequencing was performed. Data was analyzed using RaNAseq pipeline as described in Prieto & Barrios, *Bioinformatics* 2020. All genes with adjusted *P*-values <0.05 are plotted and critical IFN responsive genes are colored and labeled. **C**–**D** Gene set enrichment analysis using Enrichr. **E** Heatmap depicting gene expression changes in indicated tissues from WT vs RIG-I −/− mice ($n = 2$) from an angiogenesis qRT-PCR array. **F** Schematic depicting a 7 gene RIG-I dependent angiogenic signature. **G** Heatmap depicting enrichment of endothelial RIG-I signature from F in public datasets. Left panel depicts correlation coefficients of RIG-I with the 7 genes across four major cell types from the lung-tumor microenvironment interactome, a dataset of scRNA-seq from patients with squamous cell carcinoma or adenocarcinoma of the lung. Right panel depicts normalized expression scores of RIG-I and the 7 gene signature in human pulmonary ECs during infection with human herpesvirus 8 (HHV-8) plotted from EndoDB dataset E-GEOD-6489.

To complement our gain of function studies in human cell lines, we performed a targeted angiogenesis gene signature evaluation from RIG-I deficient mice. We obtained a RIG-I gene edited mouse (**Figure 10)** While these mice are viable and fertile, we observed that they had more vascular leak in a bFGF Matrigel plug model (**Figure 10**). To identify transcriptional changes in these mice, we harvested the Matrigel, heart and lungs from RIG-I (+/+) and (-/-) mice. Using a qPCR angiogenesis array, we found several organ-specific angiogenic changes

in RIG-I (-/-) mice (**Fig 9E**), which is indicative of RIG-I's role in angiogenesis. Finally, we compared our human gain of function and mouse loss of function datasets and discovered a unique gene signature that consists of 7 genes that are commonly regulated in both human and mouse (**Fig 9F**). We validated our 7 gene signature using independent qPCR assays (**Figure 11**). These 7 genes are known to play a role in angiogenesis and coagulation. We observed that our gene signature is also highly correlated with RIG-I expression only in the endothelial cells in squamous cell carcinomas and adenocarcinomas of the lung based on a dataset of single cell RNAseq from human lung cancer patients(Gentles et al. 2020) (The Lung-Tumor Microenvironment Interactome) (**Fig 9G**). Similarly, analysis of a public dataset of human Herpesvirus 8 infected pulmonary endothelial cells from EndoDB(Shawez Khan et al. 2019) also revealed high normalized expression of RIG-I as well as the 7 signature genes. One difference between our data and the public datasets was that the expression of PLAU – we observed PLAU was downregulated with RIG-I activation whereas it was inversely correlated only in the lung adenocarcinoma endothelial cells.

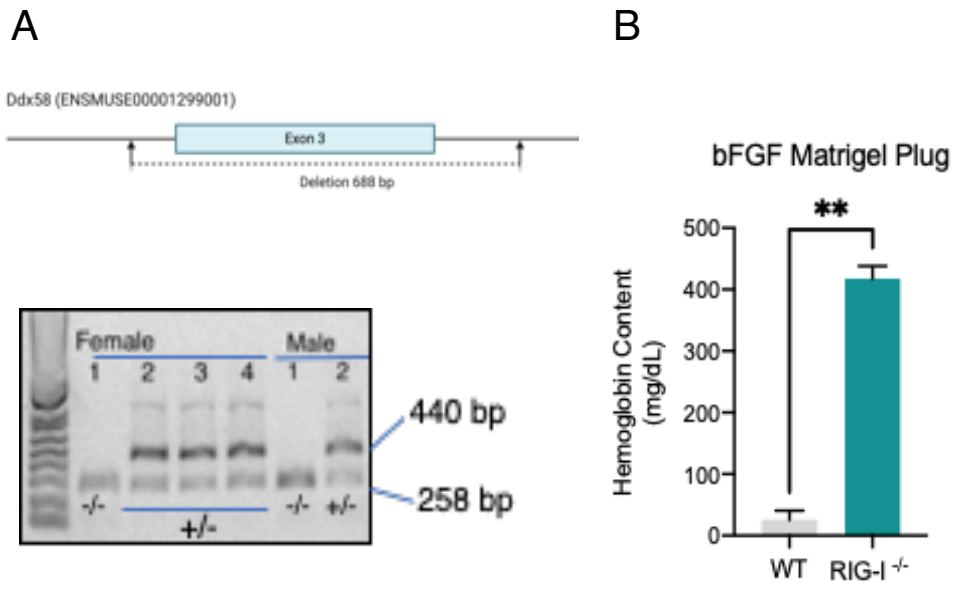



**Figure 10. Increased hemoglobin content in subcutaneous Matrigel plugs in RIG-I -/- mice.** A) Design of 688 bp exon 3 deletion to generate CRISPR edited RIG-I-/- mice and validation of genotypes using tail PCR. B) Hemoglobin content of bFGF containing Matrigel plugs from WT and RIG-I -/- mice on day 7 after subcutaneous implantation (n=4 plugs per group).

Thymidine phosphorylase (TYMP) and VEGFR1 are known regulators of angiogenesis(W. Li et al. 2014; Shibuya 2006). Fractalkine and interleukin-6 and heavily involved in inflammation(Jeong et al. 2021; Fonseca et al. 2009). A recent proteomics study showed that patients with fatal COVID19 have enhanced expression of both TYMP and RIG-I in lung parenchyma with TYMP being the most significantly induced gene(Russell et al. 2022). In addition, Protein C Receptor and Serine Protease inhibitor are regulators of coagulation(Dennis et al. 2012; Pavet et al. 2014). Interestingly, Plasminogen activator urokinase is negatively

regulated by SERPINE1, and is downregulated by RIG-I activation. We chose to focus on TYMP due to its high expression in human ECs following RIG-I activation.

We identified TYMP as being the most upregulated gene by RIG-I activation in HUVECs. Notably, TYMP is strongly upregulated at the mRNA and protein levels in RIG-I activated HUVECs (**Fig 11A** and **Figure 12A**). In addition, high levels of TYMP in tumor endothelium are linked to increased angiogenesis and a poor prognosis(Uchimiya et al. 2002; Kikuno et al. 2003; X. Zhang et al. 2014; S. Li et al. 2020). The TYMP inhibitor Tipiracil (TPI), is commonly used in anti-cancer treatments to prevent the degradation of 5-FU and has been shown to prevent thrombosis(Belcher et al. 2021).

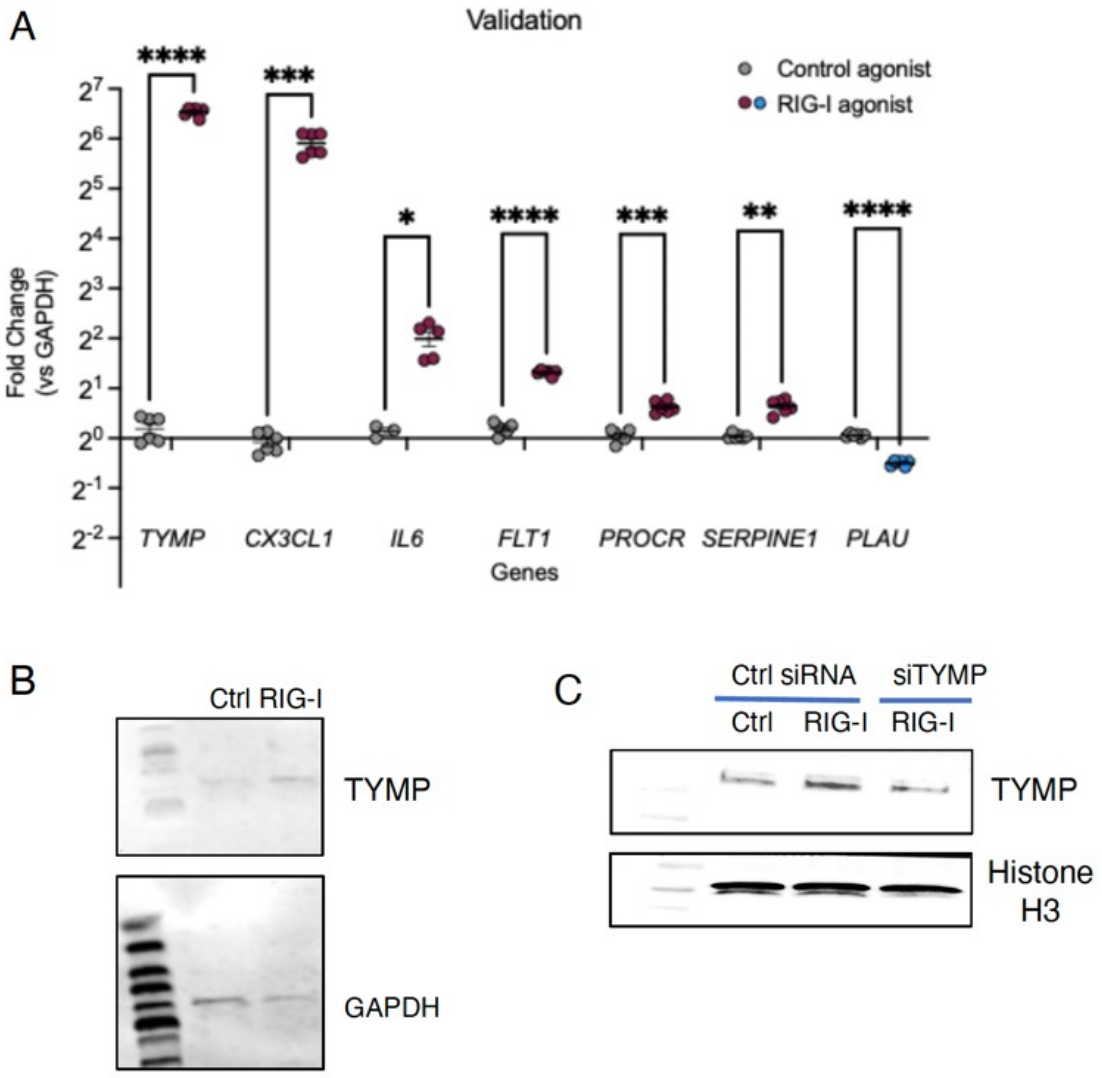
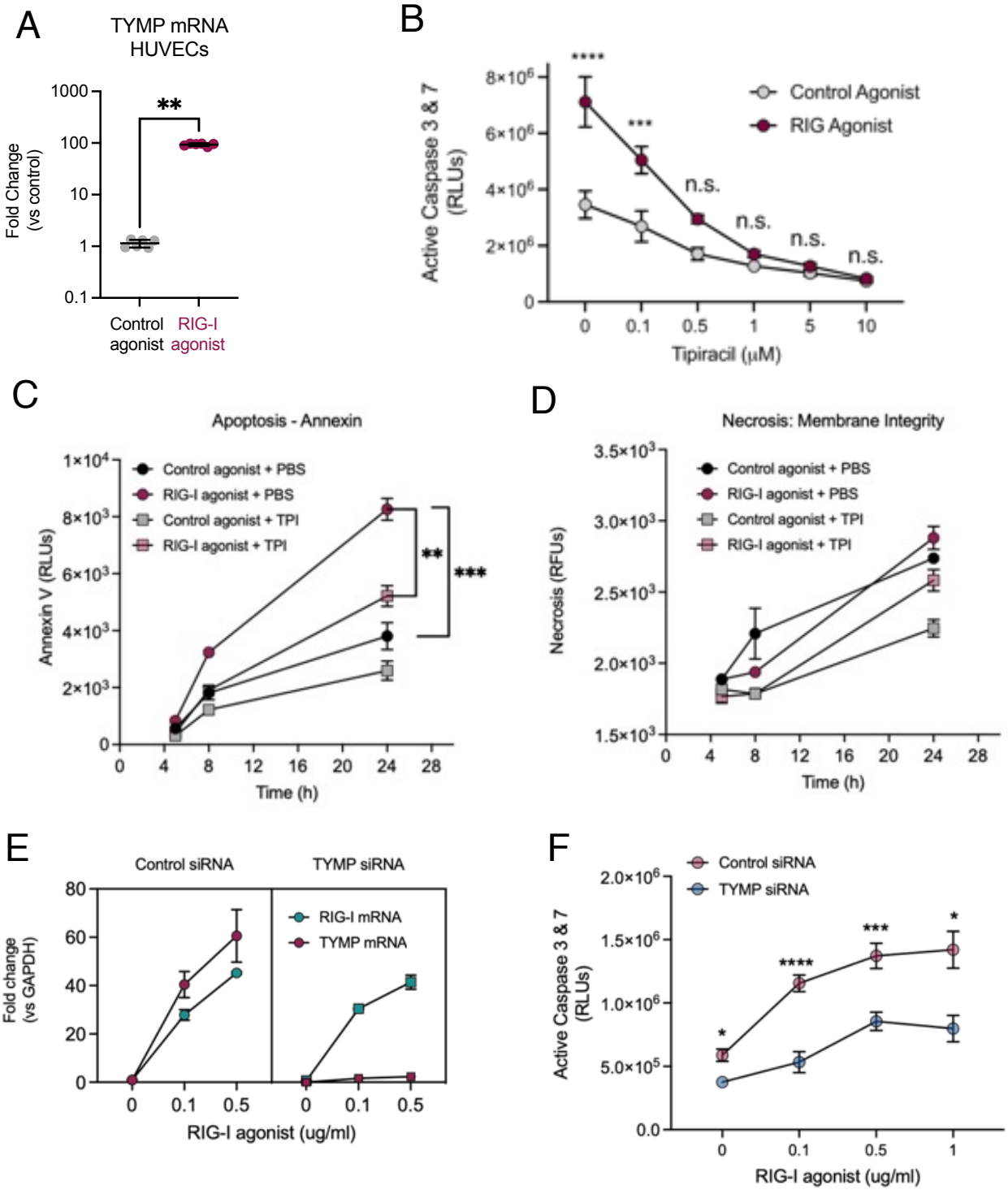



**Figure 11. Validation of TYMP as a RIG-I induced gene in ECs.** A) Gene expression changes of indicated genes 24h after RIG-I agonist or control agonist treatment in HUVECs as measured by qRT-PCR. B) Western blot depicting increase in TYMP protein levels after RIG-I agonist treatment.C. Western blod depicting siTYMP mediated decrease in RIG-I agonist induced TYMP expression

To evaluate the role of TYMP in RIG-I induced EC dysfunction, we tested the impact of TPI treatment on RIG-I induced phenotypes. We found that TPI prevents RIG-I associated apoptosis in a dose-dependent manner (**Fig 12B**). We observed increase in Annexin V staining as early as 8h post RIG-I activation. This increase in Annexin staining was also diminished by TPI treatment (**Fig 12C**). In contrast to the caspase and Annexin readouts of apoptosis, we did not observe any appreciable effect of RIG-I on necrosis (**Fig 12D**). To complement the effects of pharmacological TYMP inhibition, we used siRNA to knockdown TYMP. We first confirmed that siRNA was able to diminish TYMP levels even during the robust induction with the RIG-I agonist (**Fig 13**). Similar to our TPI studies, TYMP siRNA decreased the induction of caspases 3 & 7 (**Fig 12F**). In addition, we found that TPI reverses RIG-I induced migration arrest (**Fig 12G-H**). This indicates that TYMP may have specific roles in disrupting EC viability, and migration downstream of RIG-I activation. Therefore, pharmacological inhibition of TYMP might mitigate endothelial dysfunction.


A
TYMP mRNA
HUVECs
Fold Change
(vs control)
1000
100
10
1
0.1
**
Control
agonist
RIG-I
agonist
B
Active Caspase 3 & 7
(RLUs)
8×10^6
6×10^6
4×10^6
2×10^6
0
****
***
n.s.
n.s.
n.s.
n.s.
Control Agonist
RIG Agonist
0
0.1
0.5
1
5
10
Tipiracil (μM)
C
Apoptosis - Annexin
Annexin V (RLUs)
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
0
Control agonist + PBS
RIG-I agonist + PBS
Control agonist + TPI
RIG-I agonist + TPI
**
***
0
4
8
12
16
20
24
28
Time (h)
D
Necrosis: Membrane Integrity
Necrosis (RFUs)
3.5×10^3
3.0×10^3
2.5×10^3
2.0×10^3
1.5×10^3
Control agonist + PBS
RIG-I agonist + PBS
Control agonist + TPI
RIG-I agonist + TPI
0
4
8
12
16
20
24
28
Time (h)
E
Control siRNA
TYMP siRNA
Fold change
(vs GAPDH)
80
60
40
20
0
RIG-I mRNA
TYMP mRNA
0
0.1
0.5
0
0.1
0.5
RIG-I agonist (ug/ml)
F
Control siRNA
TYMP siRNA
Active Caspase 3 & 7
(RLUs)
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
*
****
***
*
0
0.1
0.5
1
RIG-I agonist (ug/ml)

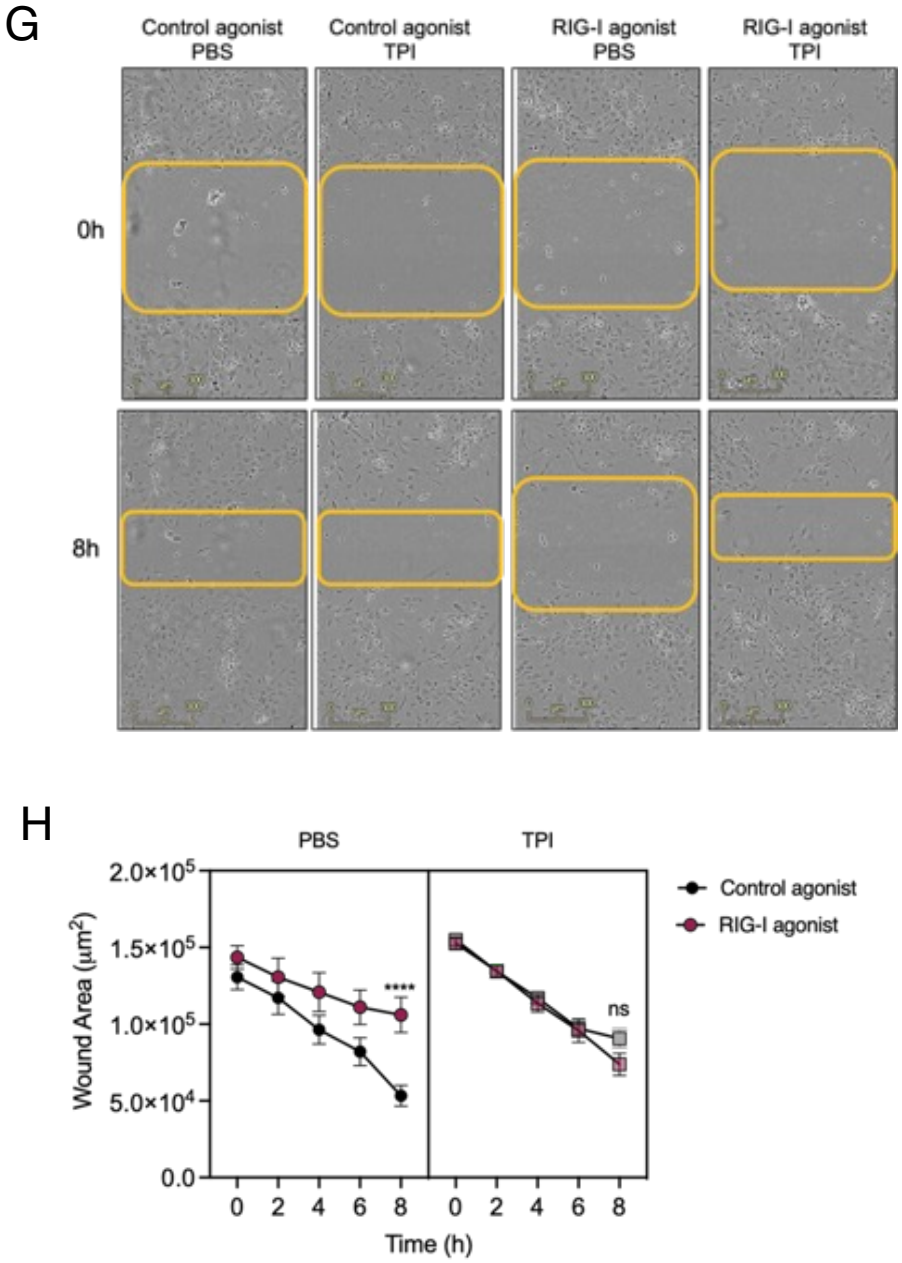



**Figure 12. TYMP inhibition reverses RIG-I induced phenotypes A** TYMP induction was measured using qRT-PCR. **B** HUVECs were treated with a control or RIG-I agonist in combination with Tipiracil. **B** Cell Death was assessed using a Caspase Glo assay (**C**) Annexin V luminescence assay and (**D**) Necrosis assay. **E**–**F** HUVECs were transfected with either a control siRNA or TYMP siRNA and treated with a control agonist or RIG-I agonist at the indicated doses. **E** mRNA levels showing efficient knockdown of TYMP 24 h post transfection. **F** Cell death was measured using Caspase-Glo assay. **G**–**H** Migration was assessed using a scratch assay in a 6-well TC plate. Scale bar = 100 uM. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001 using ANOVA or two-tailed Student's *T*-test. Bars represent mean ± SEM of independent replicates. One of two independent experiments.

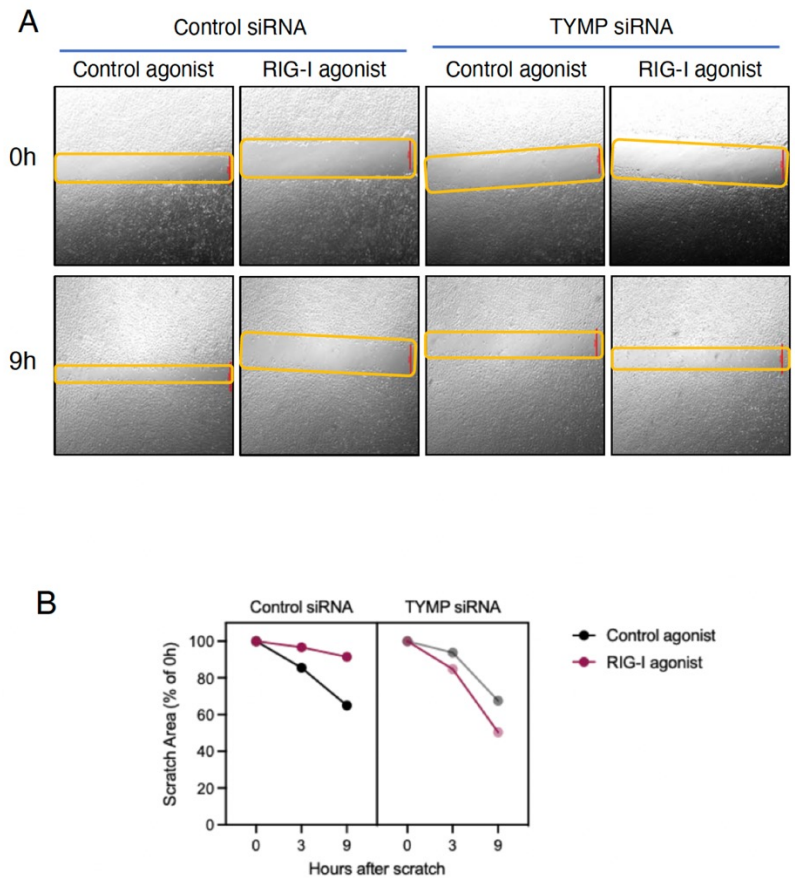



**Figure 13**. **Silencing of TYMP with siRNA partially rescues RIG-I induced migration defects in HUVECs**. A) HUVECs were transfected with siRNAs as indicated. 16h later, HUVECs were stimulated with either a control agonist or RIG-I agonist as described in Fig 3. Migration was assessed using a scratch assay in a 6-well TC plate. Scale bar = 500 uM. B) Scratch area from images were quantified using Image J. Representative images from one of two independent experiments is shown.

**Identification of putative TYMP dependent genes and pathways that regulate RIG-I function**

To identify the mechanism behind TPIs reversal of RIG-I activation phenotype in HUVECs, we performed RNA-seq on control and RIG-I activated HUVECs with and without TPI treatment (**Fig 14A-B**). We identified 19 genes that were differentially regulated (at least 2-fold) in TPI RIG-I activated HUVECs compared to RIG-I activation alone (**Fig 14C-D**). We further evaluated the transcription factor motif enrichment in our RNAseq data and found that IRF1 and IRF8 motifs were diminished in the RIG-I agonist + TPI treated cells compared to the RIG-I agonist treatment alone (**Fig 14E**). We evaluated whether the gene signatures were representative of a direct effect on IRF levels. We observed that RIG-I treatment led to increased expression of IRF1 and not IRF8 in HUVECs (**Fig 15**). The induction of IRF1 was lost upon TPI treatment. We assayed for the expression of three known IRF1 target genes – CXCL9, CXCL10 and CXCL11. While TPI decreased RIG-I induced CXCL9 and CXCL11, there was a robust increase in the levels of CXCL10 upon RIG-I activation combined with TPI treatment. These observations suggest that although IRF1 is induced by RIG-I and suppressed by TPI, there are complex interactions either in terms of nuclear localization, engagement with other transcription factors/co-factors etc. that dictate which subset of IRF1 transcribed genes are RIG-I/TYMP dependent.

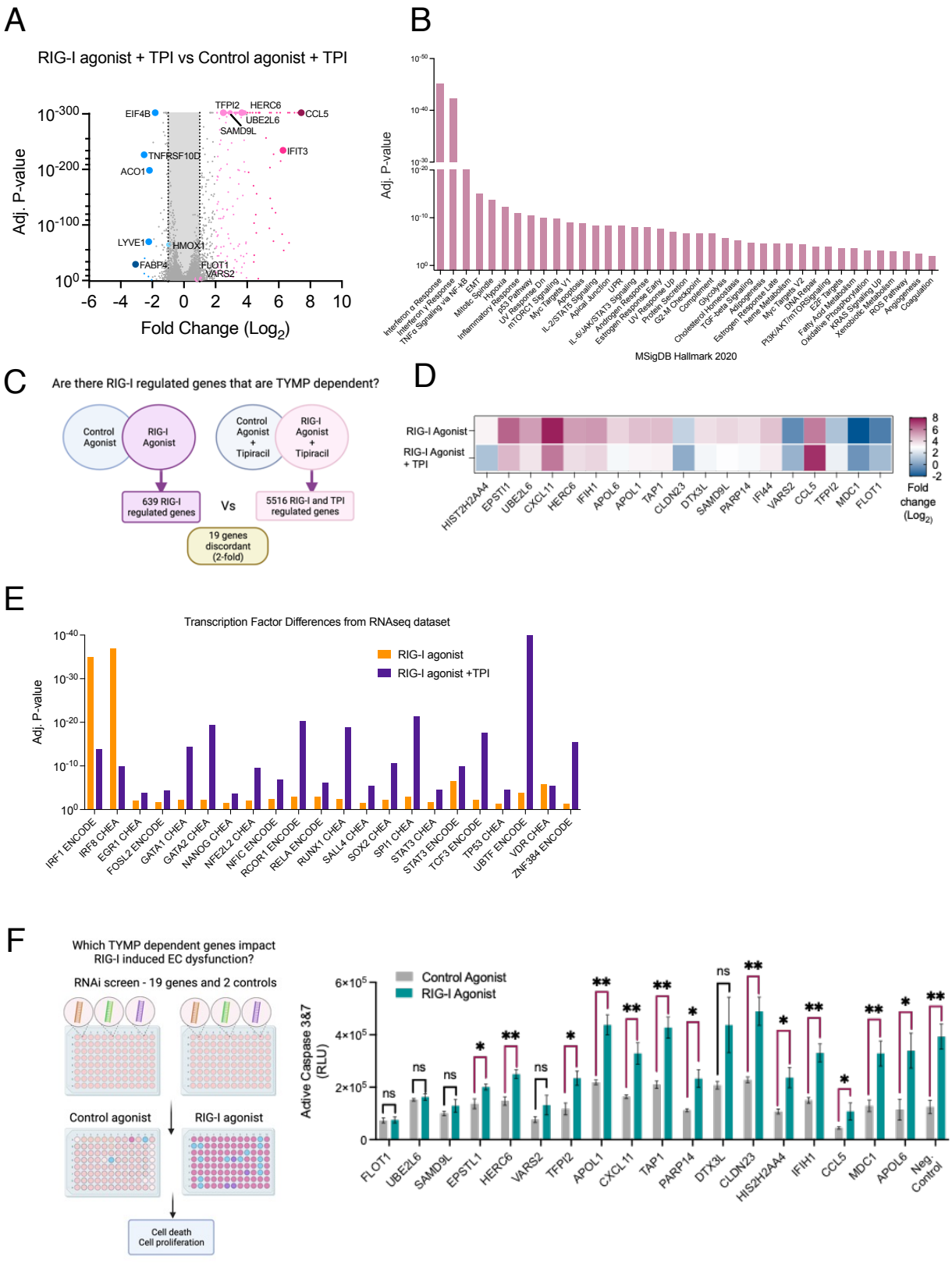
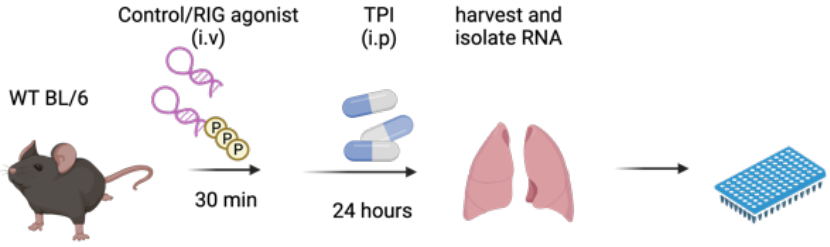

A
RIG-I agonist + TPI vs Control agonist + TPI
Adj. P-value
Fold Change (Log2)
EIF4B
TFPI2
HERC6
UBE2L6
CCL5
SAMD9L
IFIT3
TNFRSF10D
ACO1
LYVE1
HMOX1
FABP4
PLOT1
VARS2
B
Adj. P-value
MSigDB Hallmark 2020
C
Are there RIG-I regulated genes that are TYMP dependent?
Control Agonist
RIG-I Agonist
Control Agonist + Tipiracil
RIG-I Agonist + Tipiracil
639 RIG-I regulated genes
Vs
5516 RIG-I and TPI regulated genes
19 genes discordant (2-fold)
D
RIG-I Agonist
RIG-I Agonist + TPI
Fold change (Log2)
HIST2H2AA4
EPSTI1
UBE2L6
CXCL11
HERC6
IFIH1
APOL6
APOL1
TAP1
CLDN23
DTX3L
SAMD9L
PARP14
IFI44
VARS2
CCL5
TFPI2
MDC1
FLOT1
E
Transcription Factor Differences from RNAseq dataset
Adj. P-value
RIG-I agonist
RIG-I agonist +TPI
IRF1 ENCODE
IRF8 CHEA
EGR1 CHEA
FOSL2 ENCODE
GATA1 CHEA
GATA2 CHEA
NANOG CHEA
NFE2L2 CHEA
NFIC ENCODE
RCOR1 ENCODE
RELA ENCODE
RUNX1 CHEA
SALL4 CHEA
SOX2 CHEA
SPI1 CHEA
STAT3 CHEA
STAT3 ENCODE
TCF3 ENCODE
TP53 CHEA
UBTF ENCODE
VDR CHEA
ZNF384 ENCODE
F
Which TYMP dependent genes impact RIG-I induced EC dysfunction?
RNAi screen - 19 genes and 2 controls
Control agonist
RIG-I agonist
Cell death
Cell proliferation
Adive Caspase 3&7 (RLU)
Control Agonist
RIG-I Agonist
FLOT1
UBE2L6
SAMD9L
EPSTL1
HERC6
VARS2
TFPI2
APOL1
CXCL11
TAP1
PARP14
DTX3L
CLDN23
HIS2H2AA4
IFIH1
CCL5
MDC1
APOL6
Neg. Control

**Figure 14 TYMP inhibition induces specific gene expression patters in RIG-I activated ECs. A**, **B** HUVECs were treated with control agonist or RIG-I agonist in combination with Tipiracil or vehicle (PBS). RNAseq was performed 24 h after treatment. **A** Volcano plot depicting differentially expressed genes (**B**) Gene set enrichment analysis (Enrichr) is depicted. **C**, **D** 19 genes were identified as differentially expressed (ie up with RIG-I but down with RIG-I + TPI or vice-versa). **E** Consensus transcription factor analysis from ENCODE and ChEA (Enrichr) (**F**) HUVECs were transfected with 4 pooled siRNAs against each of the 19 genes. 24 h later cells were treated with control or RIG-I agonist and cell death was measured. Loss of significance in bar graph indicate the siRNAs that prevent RIG-I induced EC death. One of two independent experiments.

To evaluate the functional significance of our 19--gene--signature, we then performed a limited siRNA screen in control or RIG-I activated conditions. We sought to test which siRNAs diminished the ability of RIG-I to drive apoptosis in HUVECs. (**Fig 13F**). We found siRNAs targeting 4 TPI regulated genes – FLOT1, UBE2L6, SAMD9L, VARS2 diminished RIG-I induced cell death. It is possible that the interference of ISG signaling, due to the downregulation of these genes via decreased IRF1 or IRF8 is responsible for TPI's ability to reverse the RIG-I phenotype.

To evaluate the role of RIG-I/TYMP *in vivo,* we treated WT mice with a Control or RIG-I agonist via I.V. injection, and then treated mice with either PBS or TPI via I.P. injection. Lungs were harvested 24 hours later, and RNA was analyzed on an endothelial cell biology microarray. Unsurprisingly, RIG-I activation broadly activated interferon and apoptosis associated genes. More interestingly, TPI suppressed a broad array of interferon associated genes, both with and without RIG-I activation. Many of these genes are regulated by NFKB suggesting that TPI's activity is caused by differential NFKB regulation (**Fig 15).**

A




B

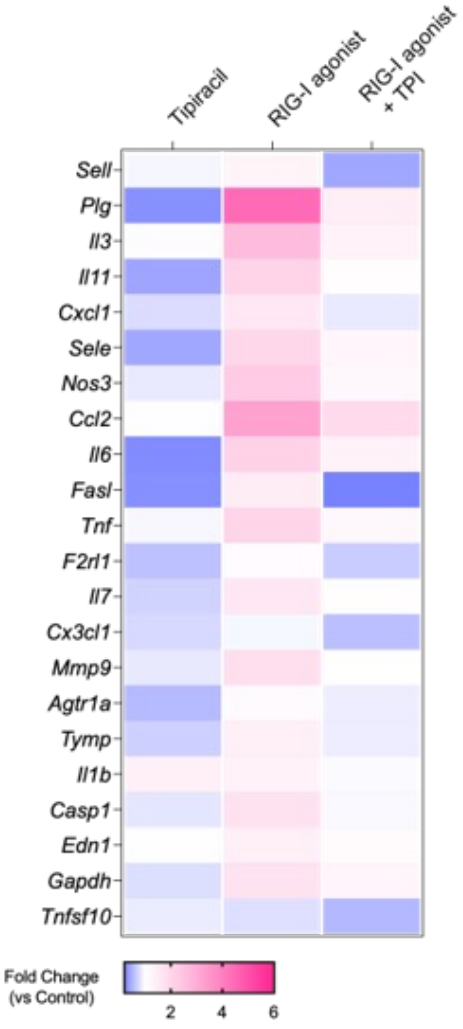



**Figure 15. Identification of differentially expressed genes following RIG + TPI treatment *in vivo.*** A. Workflow of experiment. Wild-type mice were injected with a control or RIG-I agonist and subsequently injected with TPI or PBS. 24 hours later lungs were harvested and analyzed on an angiogenesis qPCR array. B. Fold change of most differentially expressed genes (TPI vs Control).

## Discussion

Studies have shown that chronic activation of the immune system, such as from pattern recognition receptors (PRRs) can lead to adverse changes in vascular structure and function(Ehrentraut et al. 2012). There is evidence that activation of the viral RNA sensor RIG-I induces endothelial dysfunction, vascular oxidative stress, and the recruitment of innate immune cells. RIG-I activation has been observed in several inflammatory and autoimmune disorders. A clear example of this is in Singleton-Merten Syndrome, which is caused by a mutation leading to constitutively active RIG-I(Russell et al. 2022; Lässig et al. 2018). Singleton Merton Syndrome leads to several vascular defects such as aortic calcification and skeletal abnormalities. While there are pathological consequences associated with RIG-I signaling, activation of RIG-I is also being explored as a promising therapeutic strategy in cancer. Therefore, understanding how RIG-I causes EC dysfunction and approaches to mitigate the vascular pathologies associated with RIG-I activation become important.

Our work determined that RIG-I activation can induce angiogenic defects in endothelial cells. Specifically, we observed both HUVECS and HMVECs had significant apoptosis but not necrosis in response to our RIG-I agonist and decreased migration, sprouting angiogenesis. Asdonk et al(Asdonk et al. 2012) have also found that RIG-I activation leads to an increase in vascular oxidative stress and reactive oxygen species in vivo and in vitro. While Asdonk et al identified vascular dysfunction in human coronary endothelial cells (HCAEC) and endothelial progenitor cells (EPC), our studies highlight that this affect occurs across a wide range of endothelial cell types and that RIG-I activation induces a wide range of phenotypes associated with EC dysfunction. While our in vivo studies in the knockout mice demonstrate an increase in hemoglobin content, this could potentially be due to increased vascular leak or increased angiogenesis or a decrease in coagulation/thrombosis. These observations establish RIG-I activation as a robust driver of EC dysfunction.

To understand the biological and mechanistic basis for RIG-I activation induced phenotypes, we performed RNAseq experiments from our cell culture models and an angiogenesis focused qRT-PCR array from Matrigel plugs from our RIG-I−/− mice. Using gene set enrichment analysis tools, we found significant enrichment of interferon signaling pathways, as well as cholesterol homeostasis, coagulation and hypoxia pathways in both HUVECs and HMVECs. Many of these pathways are directly relevant to the phenotypes we observed in Figure 8. Comparing the human EC gain of function and the mouse tissue EC's loss of function signatures, we deduced a unique 7 gene signature of RIG-I activation. Our signature appears to be relevant in human disease pathologies. For example, we found that our 7 genes showed high positive correlations with RIG-I expression in the endothelial cells from lung cancer, both squamous cell carcinomas and adenocarcinomas(Gentles et al. 2020). Similarly, we analyzed a publicly available dataset and observed that RIG-I as well as the 7 gene signature was upregulated in lung endothelial cells in response to HHV-8 infection. TYMP and RIG-I were recently identified as part of a 22-protein signature associated with fatal COVID-19 in a proteomics study(Russell et al. 2022).

Thymidine phosphorylase (TYMP) is abundantly expressed in platelets and induces EC migration, inhibits apoptosis, and has been shown to stimulate pro-angiogenic factors such as VEGF in a context-dependent manner. Till date there is some evidence indicating TYMP plays a pro-angiogenic role in endothelial cells and endothelial progenitor cells(Pula et al. 2009). It has been shown that loss of TYMP leads to mitochondrial neurogastrointestinal encephalopathy(Ronchi et al. 2020) and TYMP interacts with VEGF to promote the breakdown of the blood-brain barrier(Chapouly et al. 2015). TYMP inhibition using the pharmacological inhibitor tipiracil (TPI) is used in a clinical setting and has been shown to reverse thrombosis(Belcher et al. 2021). We found that TPI is able to rescue HUVECs from RIG-I induced cell death and restore the migration abilities of RIG-I activated HUVECs. An siRNA

recapitulated the phenotype by protecting HUVECs from RIG-I induced cell death. These observations indicate that TYMP is a positive regulator of RIG-I induced EC dysfunction. Indeed, proteomics studies have identified TYMP and RIG-I as being highly upregulated in lungs of patients with lethal COVID infections(Russell et al. 2022). Our RNAseq data from RIG-I activated ECs with and without TPI identified a robust group of 19 genes that were RIG-I induced and TYMP dependent. Our subsequent RNAi experiment highlighted five of these genes as being functionally relevant for RIG-I induced cell death. Two of these genes, UBE2L6 and SAMD9L, are associated with the ISG signaling pathway(Orfali et al. 2020; Allenspach et al. 2021). VARS2 is a mitochondrial gene whose mutation is linked to mitochondrial encephalopathies. The gene FLOT1 is implicated in proliferation and tumorigenicity(C. Lin et al. 2011). CCL5 is a chemokine involved in inflammatory responses(Marques et al. 2013). As we observed in our gene set enrichment analysis, IRF1 and IRF8 transcription factor motifs appear to be diminished during TPI treatment. While we do observe IRF1 induction with RIG-I and loss with TPU treatment, it remains to be seen if these transcription factors are directly responsible for the induction of RIG-I induced, TYMP dependent ISGs that lead to endothelial cell death. One limitation of our data is that these observations of TYMP dependent gene expression pathways are correlative. Additional functional studies are necessary to elucidate the mechanisms by which TYMP and RIG-I regulate EC dysfunction.

Our work here indicates that pharmacological inhibition of TYMP using TPI reverses a subset of RIG-I induced genes and phenotypes in cultured ECs, creating a protective effect. We have also identified a subset of ISG-related genes that are inhibited by TPI in a RIG-I activated context, identifying a potential mechanism for TYMP's reversal of the RIG-I activated angiogenic defects. Taken together, our findings highlight novel pathways that drive RIG-I induced EC dysfunction and provide potential druggable targets that can mitigate some of the cardiovascular pathologies associated with RIG-I activation.

# Chapter 4

# RIG-I inhibition induces EC dysfunction

## Introduction

To complement the gain-of-function studies presented in chapter 3, we looked to understand the effect of RIG-I depletion on angiogenesis. Though there are currently no known angiogenic phenotypes associated with RIG-I loss in vivo, there is some data to indicate depletion of nucleic acid sensors modulates angiogenesis. Some studies have shown that inhibition of the DNA sensor STING, as well as the Toll Like Receptor 2 (TLR2) promote angiogenesis (X. Chen et al. 2021; Wagner et al. 2013).Though STING knockout mice show a decrease in acute pancreatic and liver injury, there is no current knowledge on vascular phenotypes. TLR knockout mice have also been described as having neuroprotective effects but there is no currently known phenotypes in these mice. In contrast, other studies show that activation of STING and TLR2 can promote angiogenesis as well(S. J. Lee et al. 2021; H. Yang et al. 2019; Xu et al. 2013). These studies highlight the fact that the inhibition of NAS can have varied effects on the vasculature, are more research is needed to fully uncover the role of NAS in the endothelium.

The known phenotypes in RIG-I depletion are primarily immune. RIG-I (-/-) mice have a colon inflammation as well as fewer peyer's patches(Y. Wang et al. 2007). RIG-I (-/-) mice lose the ability to control viral infection(Errett et al. 2013). Interestingly, a recent study showed that RIG-I depletion restrains tumor growth through T-cell intrinsic activity (Jiang et al. 2023). Overall, there is still very little known about how RIG-I depletion affects normal or pathological angiogenesis.

## Results

### siRNA mediated RIG-I silencing increases EC death and type I interferon signaling

Based on our RIG-I activation studies, we *hypothesized that RIG-I knockdown promotes endothelial cell growth and decreased interferon response*. To investigate the role of RIG-I depletion on angiogenesis, we first used siRNA to knock down *DDX58* (gene encoding RIG-I protein) in HUVEC cells. Surprisingly, we found that RIG-I depleted cells had increased caspase 3&7 activity, as well as increased interferon signaling as measured by qRT-PCR of MX1 and CXCL10, despite a decrease in DDX58. (**Fig 16).** Studies have shown siRNAs can directly impact cell viability and EC activation by inducing TLR signaling (Mansoori et al. 2016). Avoid activating other NAS when using siRNAs , we moved to an *in vivo* system to analyze the role of RIG-I depletion and used our RIG-I (-/-) mouse model for the following studies.

To identify the role of RIG-I loss on angiogenesis *in vivo,* we used a bFGF Matrigel plug that was implanted into wild-type and RIG-I (-/-) mice, as described in chapter 3. We found that that while plugs implanted in RIG-I (-/-) mice had increased hemoglobin content (**Fig 10**) the plugs also had decreased endothelial cells (**Fig 16),** indicating the possibility of leaky or dysfunctional blood vessels. Additionally, angiogenic-specific transcriptional changes in RIG-I (-/-) mice were identified using an angiogenic gene signature microarray (**Fig 9)**. We identified tissue specific changes in vascular gene expression in the RIG-I (-/-) mice compared to the WT mice, indicating that RIG-I depletion causes angiogenic-specific transcriptional changes.

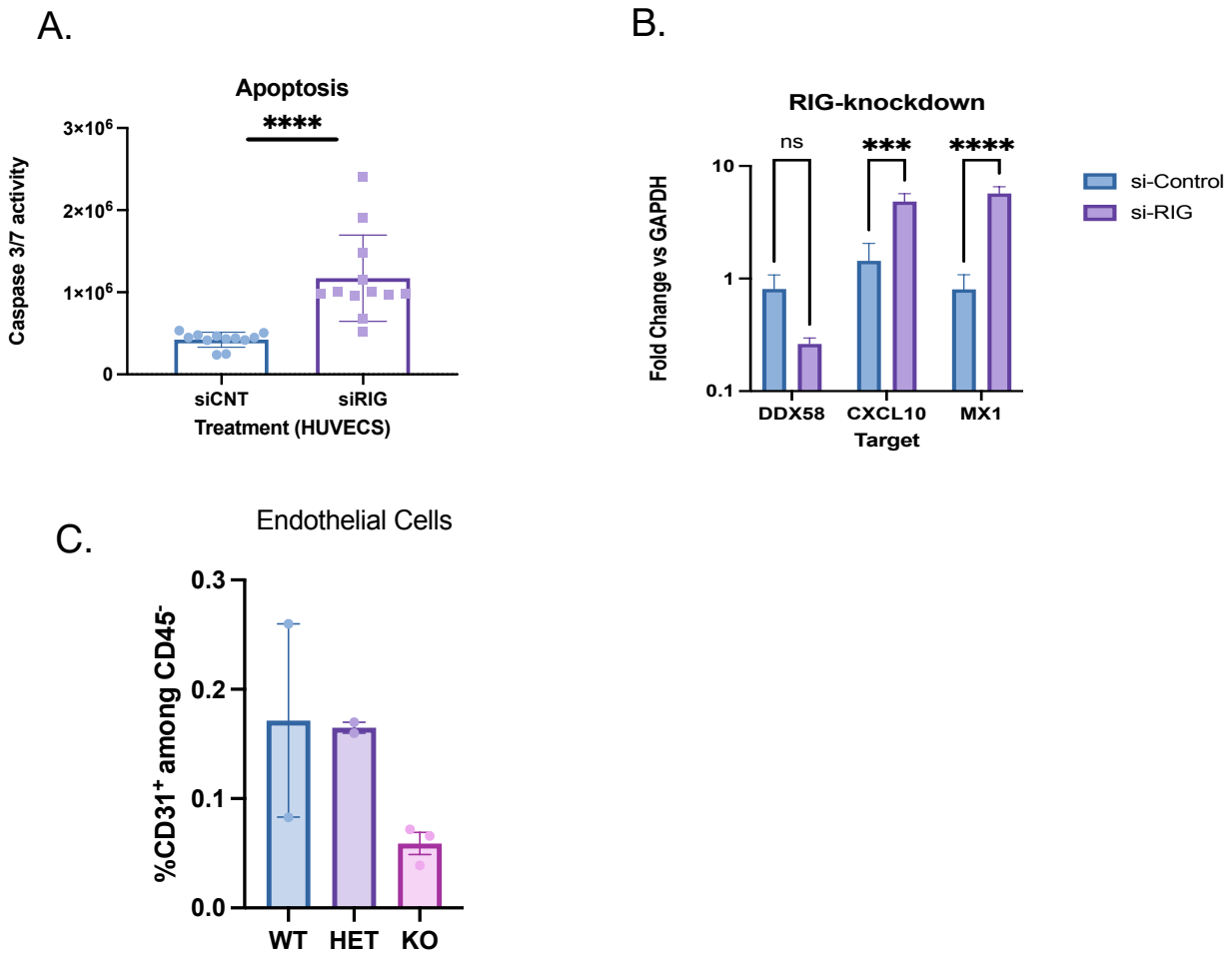



**Figure 16. RIG-I depletion leads to increased cell death + interferon response *in vitro*.** siRNA targeting RIG-I (or vehicle control, 100 nM) A. Cell Death was measured using Caspase 3&7 glo assay. ****p<0.0001 using two-tailed Student's T-test. B. Induction of DDX58, CXCL10, and MX1 were measured using qRT-PCR (n=3). ***p<0.005, ****p<0.0001 using ANOVA. C. CD31+CD45- cells in RIG-I wt vs RIG-I (-/-) Matrigel plugs. There was no significant difference between groups.

**Loss of RIG-I in vivo impacts tumor angiogenesis and tumor growth**

To further understand the role of RIG-I depletion in pathological angiogenesis, we compared the growth of MC38 colorectal cancer cells in WT C57BL/6 and RIG-I (-/-) mice over the course of 15 days. We found that RIG-I (-/-) mice grew larger tumors, only occurred in male mice (**Fig 17A,B**). Based on my *in vitro* data in which RIG-I activation caused EC death, I hypothesized that the loss of RIG-I led to hypervascularization of the tumors, which caused aggressive tumor growth. However, immunofluorescence of tumor vasculature using isolectin staining showed that tumors from RIG-I (-/-) mice showed decreased vasculature compared to WT mice **(Fig 17C-E).**

Transcriptomic analysis of tumors showed an increase in *VegfA*, and *Ccl5* **(Fig 18).** *VegfA* is a growth factor that is induced in the presence of hypoxia to promote angiogenesis (Claesson-Welsh and Welsh 2013). In addition, *Ccl5* promotes *Vegf* expression and induces angiogenesis (S.-W. Wang et al. 2015). Therefore, increases in both cytokines would be characteristic of a tumor lacking in angiogenesis. However, the increase in angiogenic chemokines following RIG-I ablation does not explain the hypovascularization phenotype; rather the increase of these factors is likely due to feedback induction in response to the loss of endothelial cells.

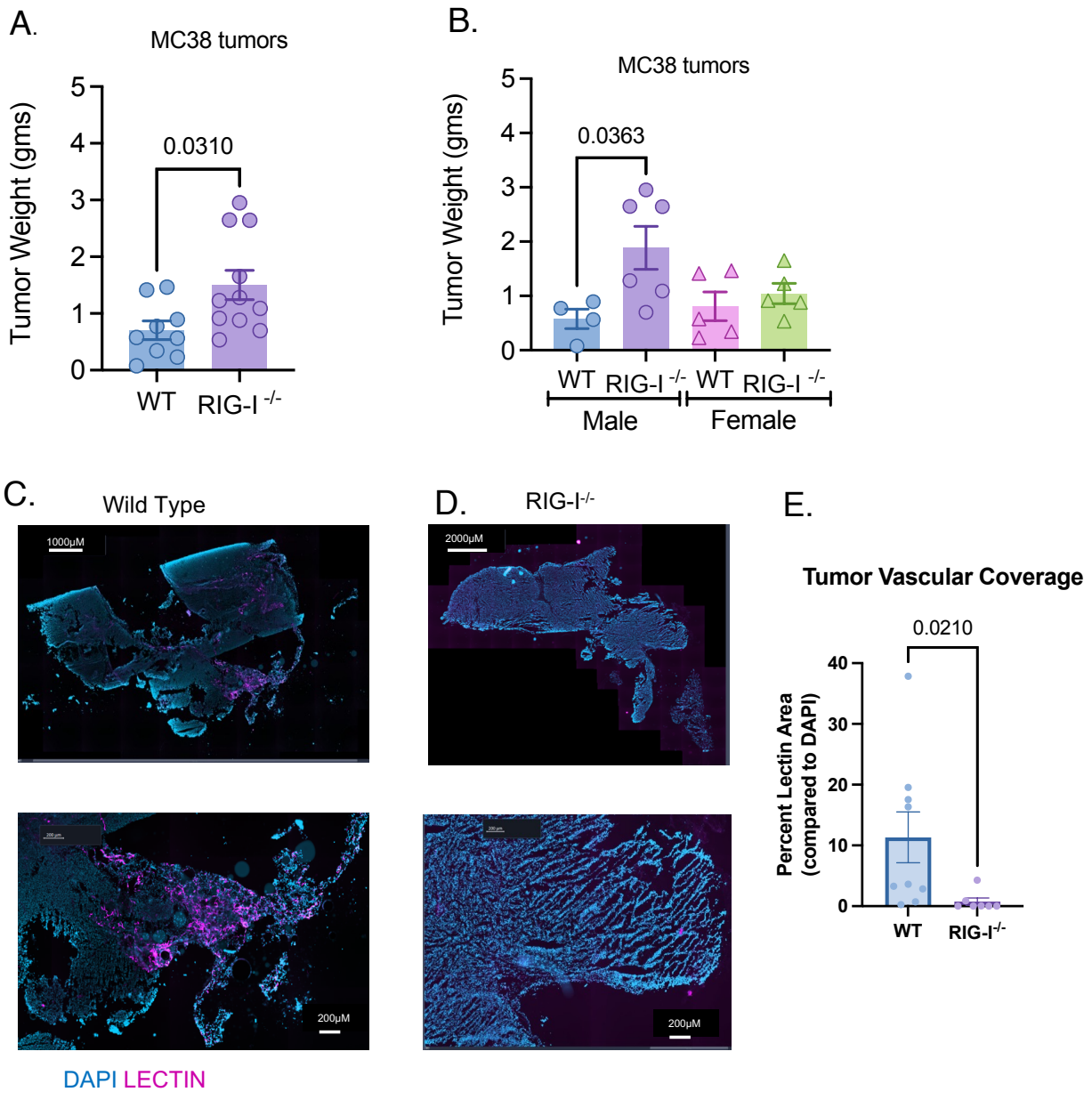



**Figure 17. RIG-I (-/-) mice show increased tumor size, decreased tumor vasculature**. A,B. tumor growth was measured using calipers. C,D. Images of tumors analyzed using immunofluorescence microscopy. Perfused vessels are stained with isolectin (pink) and nuclei are stained with DAPI (blue). Magnifications to depict locations of interest are included below. E Percent lectin area compared to DAPI. Statistical significance analyzed using Two-tailed Student's T test.

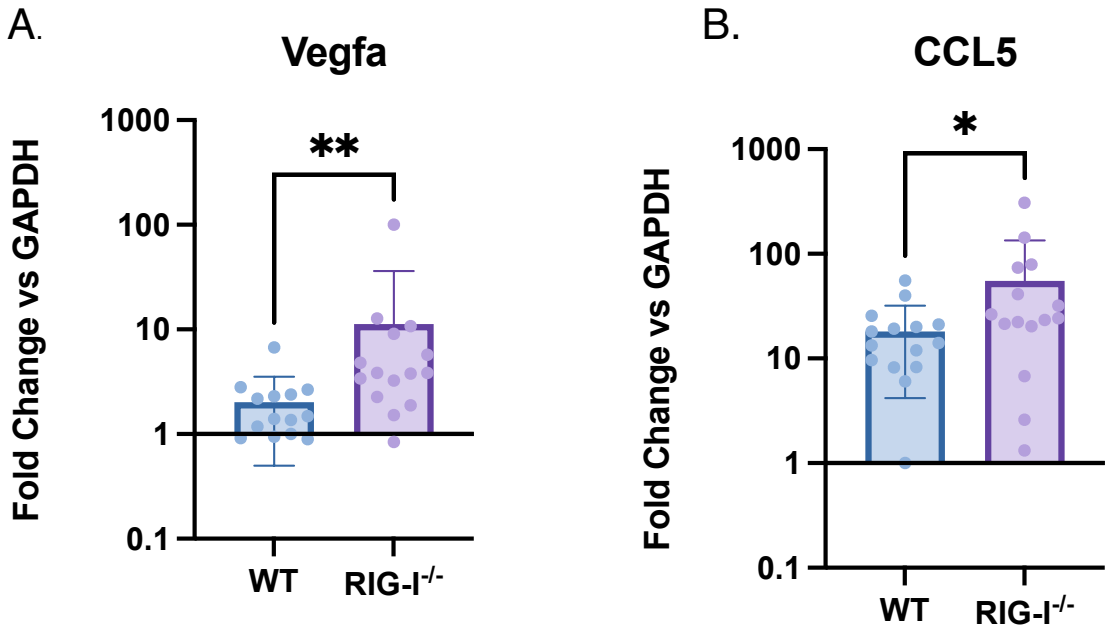
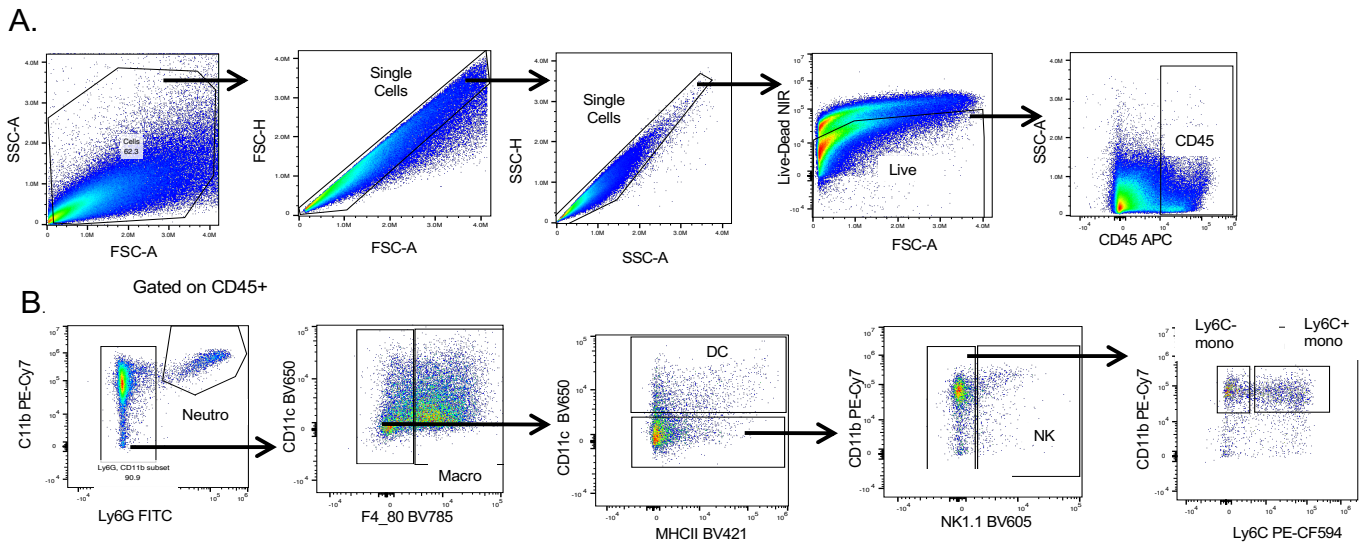
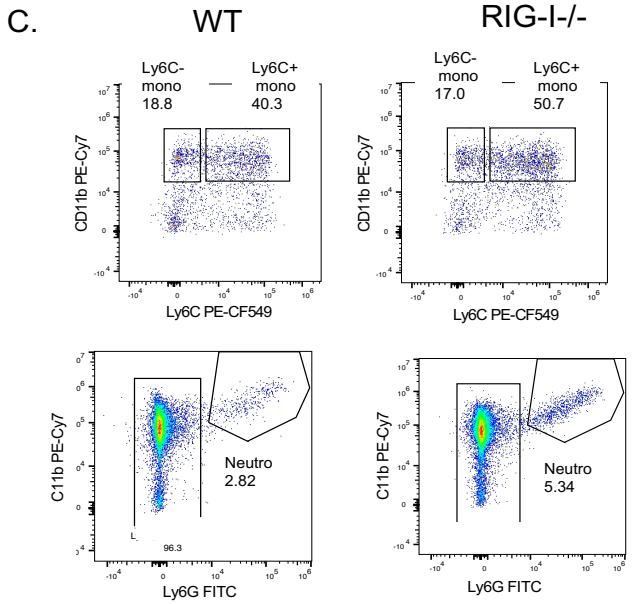
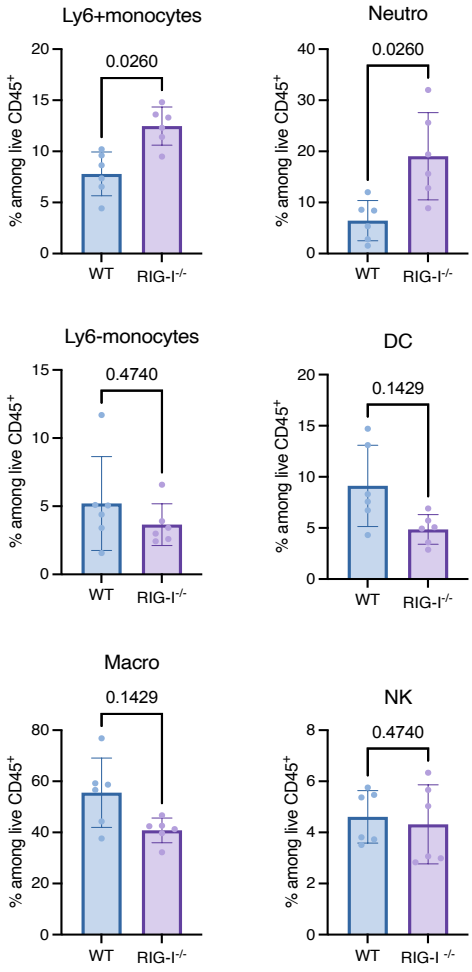


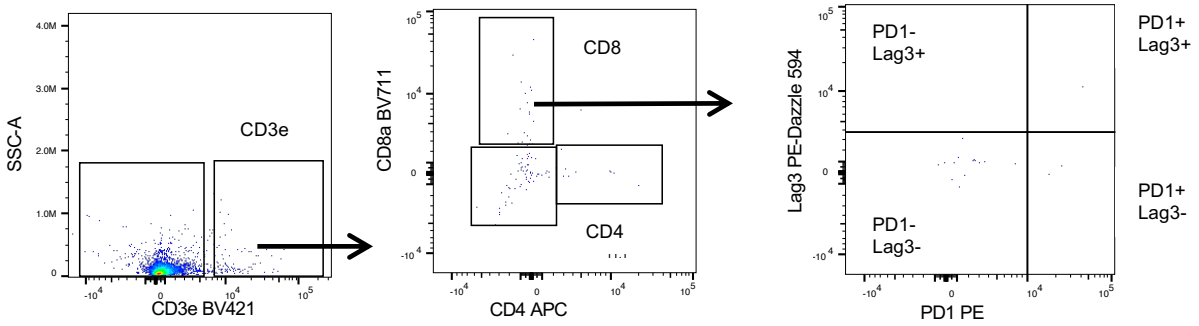

**Figure 18. Angiogenic chemokine induction in RIG-I$^{-/-}$tumors**
Induction of Vegfa(a) and Ccl5 (b) in tumors from WT and RIG-I (-/-) mice measured using qRT-PCR. *p<0.05, **p<0.001 using two-tailed Student's T-test


A.
SSC-A
FSC-A
Single Cells
FSC-H
FSC-A
Single Cells
SSC-H
SSC-A
Live-Dead NIR
Live
FSC-A
SSC-A
CD45
CD45 APC
B.
Gated on CD45+
C11b PE-Cy7
Neutro
Ly6G FITC
CD11c BV650
Macro
F4_80 BV785
CD11c BV650
DC
MHCII BV421
CD11b PE-Cy7
NK
NK1.1 BV605
Ly6C- mono
Ly6C+ mono
CD11b PE-Cy7
Ly6C PE-CF594


C.
WT
RIG-I-/-
Ly6C- mono 18.8
Ly6C+ mono 40.3
Ly6C- mono 17.0
Ly6C+ mono 50.7
CD11b PE-Cy7
Ly6C PE-CF549
Neutro 2.82
Neutro 5.34
Ly6G FITC


D.
Ly6+monocytes
Neutro
Ly6-monocytes
DC
Macro
NK
0.0260
0.4740
0.1429
% among live CD45+
WT
RIG-I-/-

E.


Gated on CD45
SSC-A
CD3e
CD3e BV421
CD8a BV711
CD8
CD4
CD4 APC
Lag3 PE-Dazzle 594
PD1-
Lag3+
PD1+
Lag3+
PD1-
Lag3-
PD1+
Lag3-
PD1 PE

F.

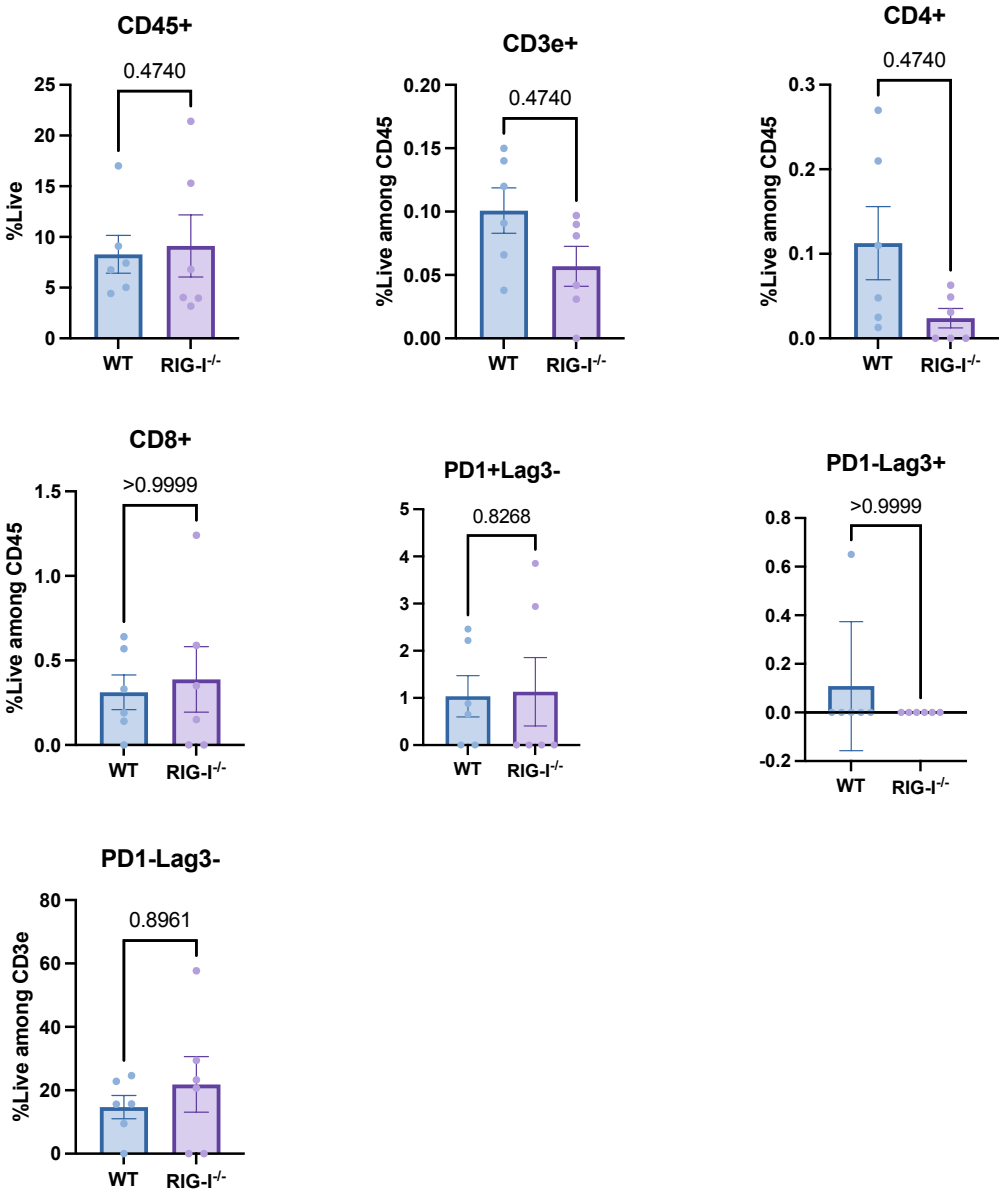

CD45+
0.4740
%Live
CD3e+
0.4740
%Live among CD45
CD4+
0.4740
%Live among CD45
CD8+
>0.9999
%Live among CD45
PD1+Lag3-
0.8268
PD1-Lag3+
>0.9999
PD1-Lag3-
0.8961
%Live among CD3e
WT
RIG-I-/-

**Figure 19. Flow cytometry analysis of WT vs RIG-I (-/-) tumors**. A, B. Gating strategy for myeloid panel. C. Representative images of Ly6C high monocytes and neutrophils in WT and RIG-I (-/-) tumors. D. Quantification of myeloid cells using Students Two tailed T test. E. Gating strategy for T cell panel. F. Quantification of T Cell panel using two-tailed Student's t-test.

As outlined earlier, RIG-I knockout mice have been shown to have immune phenotypes including colitis and a decrease in Peyer's patches (Y. Wang et al. 2007). Therefore, we speculated the loss of tumor ECs could potentially be due to an immune mediated mechanism. To establish immune microenvironment changes I stained the tumors from the WT and RIG-I-/- mice with a panel of immune cell phenotyping markers for monocytes, macrophages, NK cells, neutrophils, T-cells, DCs, and markers for T-cell phenotypes (**Table3-5, see methods)** and performed multi-color flow cytometry. Analysis of the immune compartment revealed few changes with the exception of an increase in neutrophils and Ly6C high monocytes **(Fig 19).** Infiltrating neutrophils have been shown to mediate the angiogenic switch in tumors, and are correlated with resistance to anti-VEGF therapy (Liang and Ferrara 2016; Nozawa, Chiu, and Hanahan 2006) Inflammatory monocytes can increase VEGF expression, leading to an increase in tumor angiogenesis(Shi et al. 2016). The lack of major immune changes in the RIG-I (-/-) tumors lead to the hypothesis that the hypovascularization phenotype may be *EC intrinsic* rather than immune or TME mediated. Further tumor studies likely with EC specific or immune-specific RIG-I knockout mice will be necessary to evaluate EC intrinsic phenotypes due to the loss of RIG-I.

To identify whether loss of vascularization is unique to the tumor, I harvested lungs from the tumor bearing mice (WT and RIG-I (-/-)) and stained them for ECs using isolectin, as well as for VEGFR1. Interestingly, I found a similar decrease in ECs in the lungs that was seen in the tumor, however some ECs remained(**Fig 20A-C).** Further analysis showed that lungs from RIG-I (-/-) lungs have an increased expression of VEGFR1 (**Fig 20D-F).** There was no significant

difference identified between WT and RIG-I (-/-) VEGFR2 expression via flow cytometry **(Fig 20G-F).** This data suggests that the decrease in angiogenesis in the RIG-I -/- background is not unique to the TME but also present in normal lung tissue. This is surprising because the mice appear developmentally normal and we have not observed significant differences in fertility or perinatal mortality suggesting the developmental angiogenesis may not be severely compromised. More studies across tissues and across the lifespan of the mouse would be needed to clarify the extent of vascular and angiogenic abnormalities in the RIG-I -/- mouse.

To understand global changes in the tumors in the RIG-I -/- host, I turned to RNA sequencing to identify transcriptional changes between WT and RIG-I (-/-) tumors. Interestingly, tumors from RIG-I (-/-) mice showed dysregulation in pathways involved in membrane function, synaptic transport, and metabolism **(Fig 21**). Given the tumor cells are WT RIG-I and only the host cells are RIG-I -/- our observations are limited to evaluating the impact of host RIG-I on the tumor transcriptional programs. We believe the lack of host RIG-I whether in the immune compartment or vasculature leads to changes in membrane transport, VEGF signaling and transport function that may contribute to tumor progression (**Fig 23).**

To complement my tumor studies, I analyzed the transcriptome of lung ECs from WT and RIG-I (-/-) mice. I identified several heat shock proteins and associated genes as being downregulated in RIG-I (-/-) ECs, as well as a decrease in immune genes including MARCO, interleukin genes, and CXCR1(**Fig 22a).** Gene set enrichment analysis (GSEA) also identified suppression of myeloid and immune pathways (**Fig 22b).** Heat shock proteins have been associated with angiogenesis, and inhibition of heat shock proteins has been implicated as a possible anti-angiogenic therapy(Sun and Liao 2004; Keezer et al. 2003; Staufer and Stoeltzing 2010; T.-K. Kim et al. 2016). In addition, GSEA analysis identifies several myeloid cell pathways as being downregulated in RIG-I (-/-) lungs. While we do not see a subsequent change in myeloid cells at the protein level, it is possible that downstream signaling or function of these

cells is dysregulated in a RIG-I (-/-) context. Based on this data, I propose a working hypothesis that RIG-I loss is ECs leads to loss of Hsp and myeloid function, contributing to vessel regression (**Figure 24).**

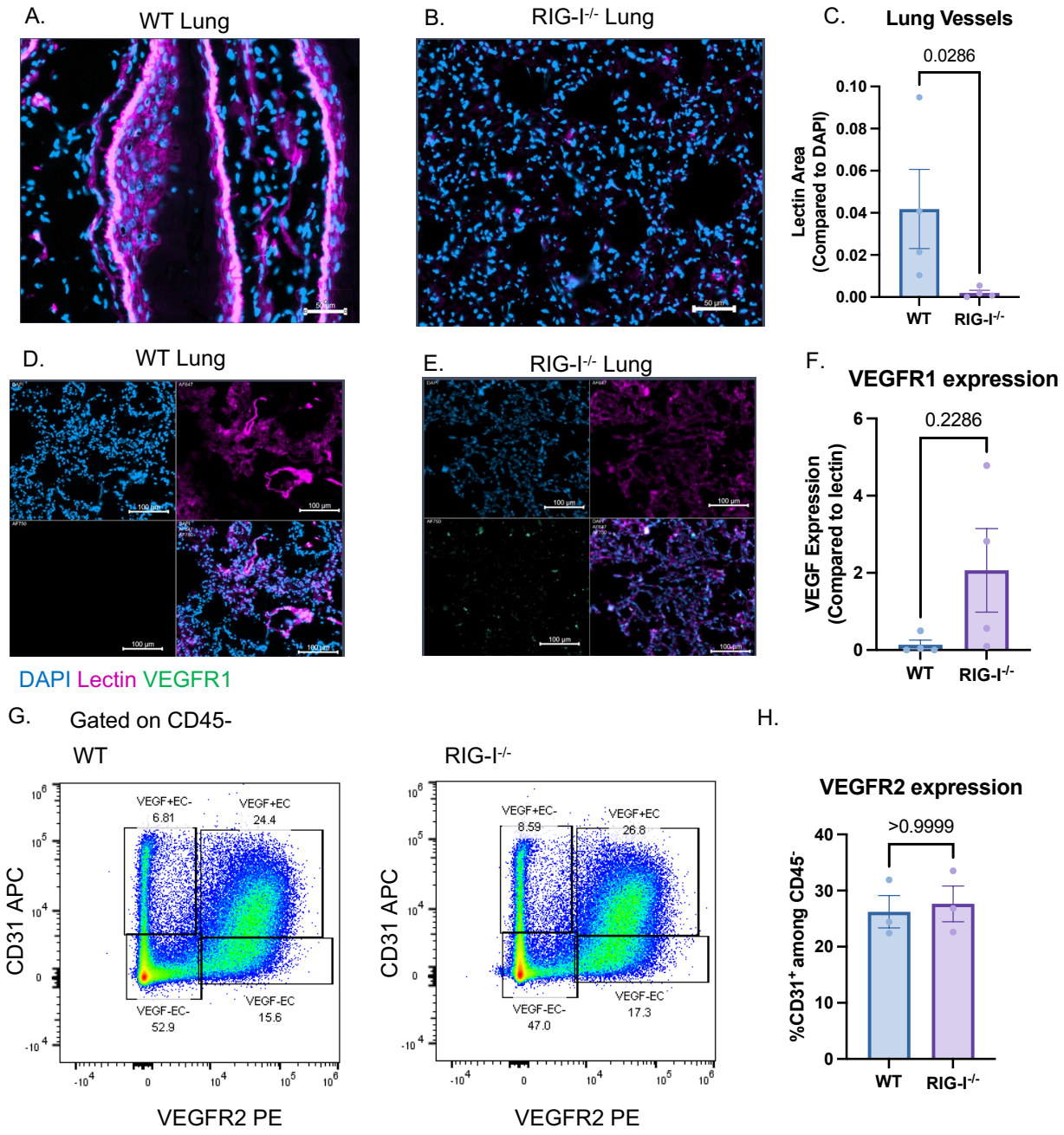
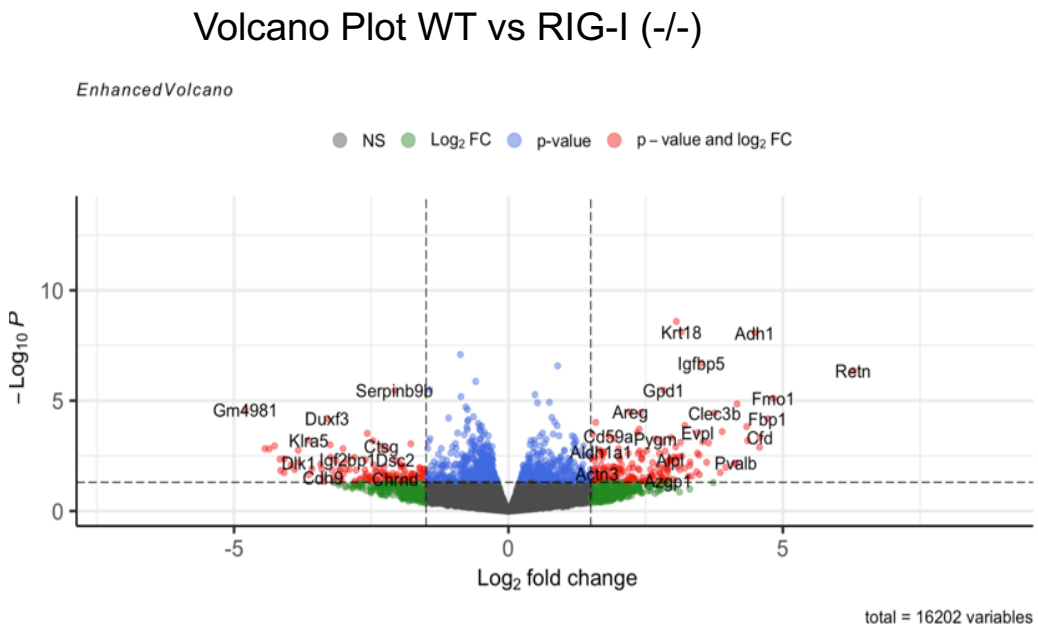



**Figure 20. Lungs from RIG-I (-/-) mice show decreased vessels**. A, B. Immunoflourescence from lungs harvested from C57Bl/6 WT and RIG-I (-/-) mice. Endothelial cells were stained with isolectin (pink) and nuclei were stained with DAPI (blue). Images were taking at 20x magnification. C. Analysis of lectin area using two-tailed Student's T-test. , D,E. Immunoflourescence from lungs harvested from C57Bl/6 WT and RIG-I (-/-) mice. Endothelial cells were stained with isolectin (pink), VEGFR1. F. Analysis of VEGFR1 area using Student's two-tailed T test.- G.Representative gating of VEGFR2+CD31+ (ECs) from WT and RIG-I (-/-) lungs analyzed via flow cytometry. H. Analysis of data from (G) using two-tailed Student's T-test

A




B

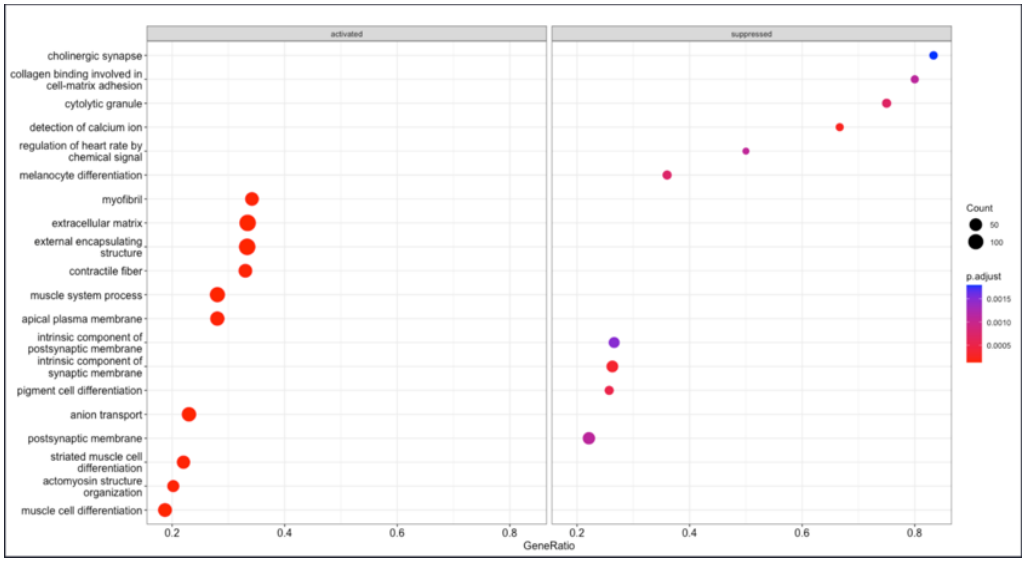
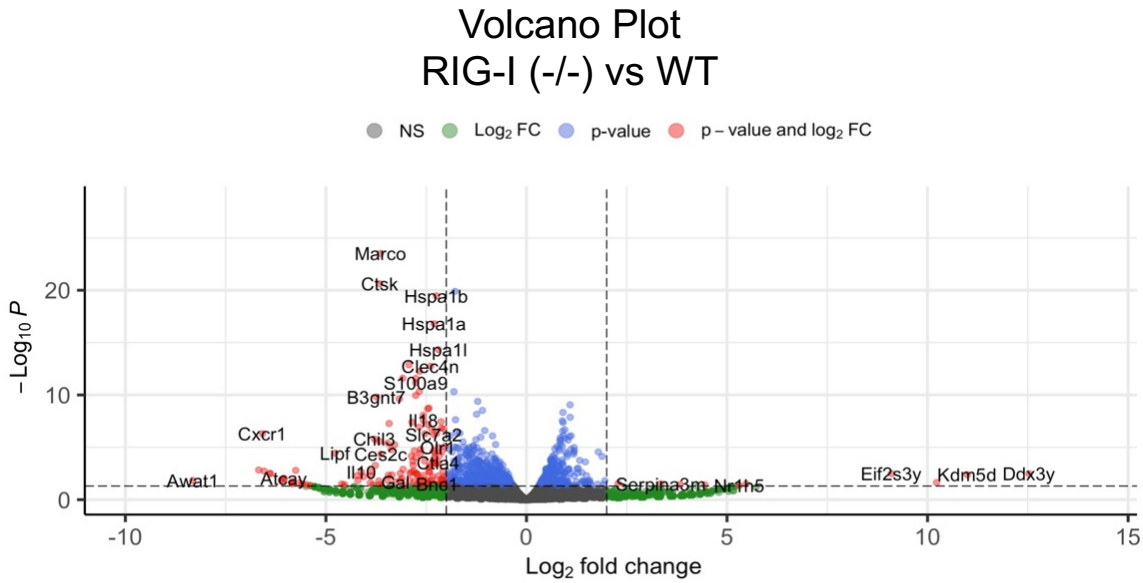



**Fig 21. RNAsequencing from WT vs RIG-I (-/-) tumors**. A. Volcano plot displaying differentially expressed genes between WT and RIG-I (-/-). B. Gene Set Enrichment Analysis (GSEA) plot highlighting pathways activated or suppressed in RIG-I (-/-) vs WT tumors. Sequencing performed by Azenta Genewiz and analysis performed using standard DESeq2 pipeline (n=3).

A.




B.

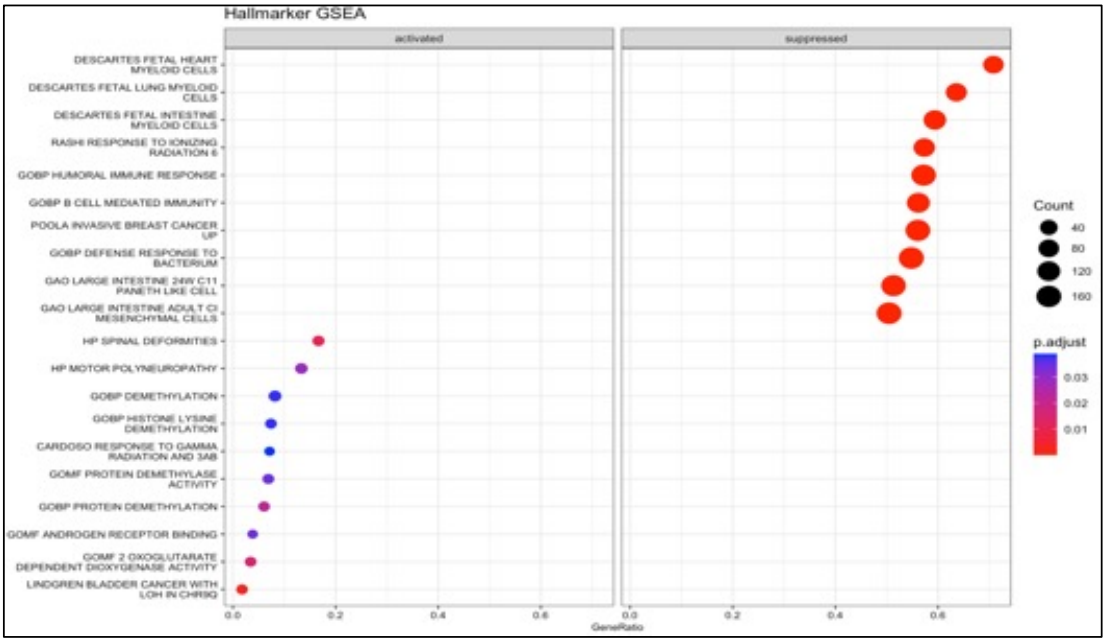



**Figure 22. RNAseq analysis of ECs isolated from WT and RIG-I (-/-) lungs** A. Volcano plot depicting up and downregulated genes in RIG-I (-/-) lung ECs compared to WT. B. Gene set enrichment analysis of ECs identifying activated and suppressed pathways. Analysis performed using standard Deseq2 pipeline (n=2).

This analysis of RIG-I (-/-) mice shows a significant increase in tumor growth compared to WT mice, which contradicts some of the current literature on loss of RIG-I and tumor burden. Specifically, a recent study describes RIG-I loss as decreasing tumor burden in multiple tumor models, including MC38 subcutaneous models (Jiang et al. 2023). One likely cause for this discrepancy is that Jiang use younger mice that used in the studies described here, as they did argue that young mice look as healthy as WT littermates, with similar spleen sizes and apoptotic/activates levels of splenic $CD8^+$ T cells (Jiang et al. 2023). We did not identify any differences in health or spleen size between WT and RIG-I (-/-) mice at 10 weeks, the age of our mice for these experiments, however we did not analyze splenic T cells. It is possible that our 10-week-old RIG-I (-/-) mice exhibit loss of splenic T cell function, causing the increased tumor growth these studies describe. In addition, Jiang used a RIG-I (-/-) mouse in which exons 4-8 were deleted (Jiang et al. 2023; Y. Wang et al. 2007), while we used a RIG-I (-/-) mouse in which exon 3 was deleted (Johnson et al. 2021). Previous studies have shown that different approaches to generating RIG-I (-/-) have incredibly different outcomes, including embryonic lethality (Kato et al. 2005). Even though all of these mutations should result in a non-functional protein, they may result in different functional consequences. It remains to be determined whether the deletion of specific exons results in aberrant translation of truncated RIG-I proteins that might function as dominant negative to other CARD domain containing proteins and can lead to confounding results.

We also see a decrease in tumor blood vessels in the RIG-I (-/-) mice, as well as a loss of blood vessel architecture in normal tissue. There are several potential reasons for this phenotype. One is that vessels in RIG-I (-/-) mice are not decreased but simply poorly perfused, ie constricted in a way that prevents sufficient isolectin from reaching the tissue. Another possible cause is that RIG-I loss causes a downregulation of the glycoproteins on ECs that bind to isolectin. These could be validated by staining slides post-mortem instead of using perfusion-

based staining, and by using additional EC markers such as CD31 and VE-Cadherin. Notably CD31 staining in our flow cytometry panel from the lungs did not show a dramatic decrease in cell numbers between WT and RIG-I -/- mice. I could also use a label independent approach such as doppler ultrasound to identify if blood flow is affected in superficial tissues including heart, spleen or lungs in mice.

The mechanism for RIG-I's role in vascular function is still unclear, though RNA analysis leads to a possible role for Hsp or myeloid function as a cause. Heat shock proteins are induced during stress and are critical for many functions in the cell (Sharp, Massa, and Swanson 1999). Heat shock proteins have been discovered to play roles in angiogenesis and tumor angiogenesis (T.-K. Kim et al. 2016; Staufer and Stoeltzing 2010; Sun and Liao 2004). Specifically, HSP90 has been shown to be anti-angiogenic by affecting PI-3K/Akt/eNOS signaling, and inhibitors that bind to HSP90 are being tested as anti-angiogenic therapies (Staufer and Stoeltzing 2010). HSP 70-1A has been shown to stimulate angiogenesis by activating ERK (T.-K. Kim et al. 2016). HSPs 27 and 47 have also been shown to promote angiogenesis (Keezer et al. 2003; Z. B. Wu et al. 2016). Additionally, myeloid cell function is important for driving angiogenesis (Chambers et al. 2013; Murdoch et al. 2008). Neutrophils and myeloid derived suppressor cells can drive angiogenesis by producing VEGF and MMP-9 (Rivera and Bergers 2015; L. Yang et al. 2004; Pan et al. 2008). Macrophages also produce VEGF, which facilitates angiogenesis in some breast cancer models (E. Y. Lin et al. 2006). Further studies to determine if loss of Hsp or myeloid function is causing the hypovascularization phenotype, and how loss of RIG-I prevents Hsp or myeloid function leading to vascular dysfunction are critical to understanding this phenotype.

In addition, the phenotype seen in tumors from RIG-I (-/-) bearing mice is opposite what would be expected. Despite apparent loss of angiogenesis, tumors grow much larger than WT. This is surprising as these tumors should lack nutrients to grow to these sizes. One potential

cause is that tumor cells from RIG-I (-/-) mice show dysregulation in transport function. This may allow tumors to receive nutrients without the use of blood vessels. It is also possible that these tumors experienced hypoxic tumor growth due to the decrease in perfusion.

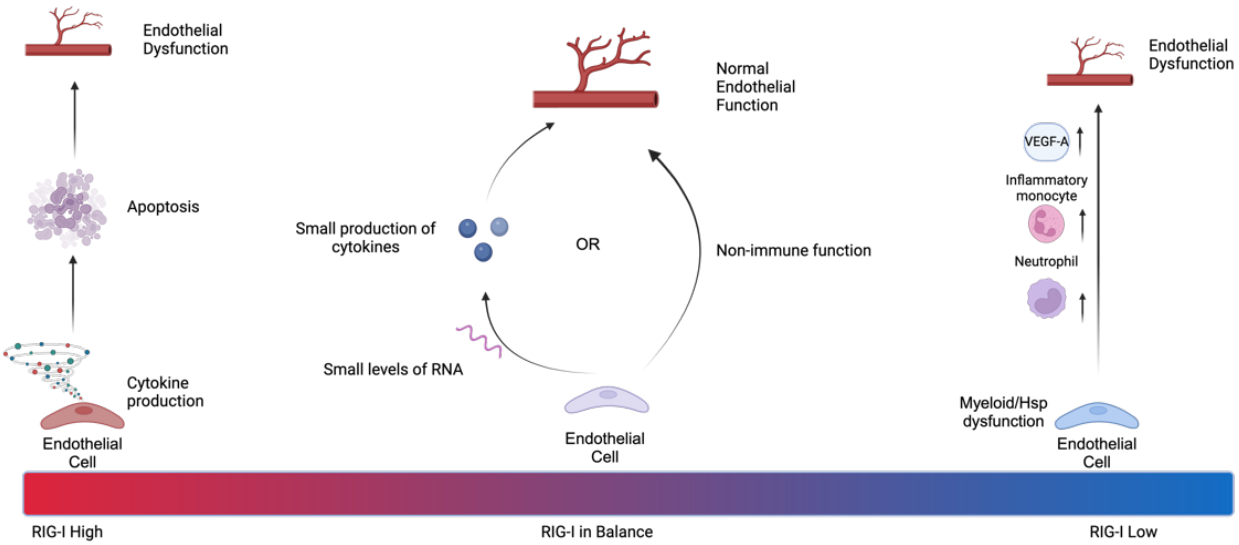



**Figure 23. Working hypothesis for mechanism behind RIG-I signaling in ECs.** High levels of RIG-I lead to increased cytokine production and EC apoptosis, causes EC dysfunction. Low levels of RIG-I potentially lead to myeloid or HSP dysfunction, also leading to EC dysfunction. This also leads to an increase in myeloid cells and VEGF. Therefore, RIG-I needs to be in balance for functional ECs. This homeostatic function of RIG-I may be due to chronic activation with small amounts of endogenous RNA, or due to yet to be discovered non-immune function of RIG-I. Created with Biorender.

## Discussion

We initially hypothesized that depletion of RIG-I in mice would prevent VEGF induction due to loss of transcriptional programs that lead to the induction of VEGF. As several immune cell subsets including monocytes can secrete VEGFA, we hypothesized that loss of the innate immune sensor would decrease myeloid cell number, potentially due to low-levels of RIG-I being required for normal myeloid function, therefor preventing VEGFA production. However, we found that tumor cells had increased levels of VEGFA. We hypothesize that this is likely due to hypoxia, which caused an increase in VEGFA production from tumor associated monocytes or neutrophils that were elevated in our immune profiling data (**Fig 19**). Despite the apparent increase in VEGFA, there is no consequential increase in blood vessels at the endpoint of the tumor studies. We are limited by the experimental time point at which we observed the increase in VEGFA given the tumors in the RIG-I knockout mice grow more rapidly and therefore we were unable to extend to a later time point. Whether this decrease in perfused vessels and increase in VEGF is due to other defects in endothelial cell biology, or if we are able to evaluate later time points, tumors will eventually compensate for the loss of ECs is still unclear.

C-C Motif Chemokine Ligand 5 (CCL5) is another chemokine that was found to be upregulated in tumors from RIG-I (-/-) mice. Interestingly, CCL5 is a known angiogenic chemokine that can cause VEGF-induced angiogenesis(Suffee et al. 2011; S.-W. Wang et al. 2015). CCL5 is most commonly expressed on monocytes and T-cells. CCL5 has also been reported to support tumor progression and metastasis (Marques et al. 2013), possibly leading to the phenotype we see of increased tumor size we see in RIG-I (-/-) mice.

Our data indicates that monocytes, particularly Ly6Chigh monocytes, are upregulated in RIG-I (-/-) tumors. These monocytes are typically considered proinflammatory and are recruited to tissue following injury(S. L. Lin et al. 2009). Ly6Chigh monocytes have been shown to produce VEGF and promote injury in the lung(Shi et al. 2016), possibly contributing to the VEGF

production seen in RIG-I (-/-) tumors. Notably, Ly6C High monocytes have been found to seed the inflamed colon and promote colon inflammation. As colon inflammation is typical of RIG-I (-/-) mice, overproduction of Ly6Chigh monocytes may be contributors.

One of the most altered pathways in RIG-I (-/-) tumors are pathways associated with transmembrane signaling and membrane function. This is apparent in Gene Set Enrichment Analysis (GSEA) that indicates activation and suppression of synapse pathways, cell-matrix adhesion pathways, and transport pathways. These pathways are critical for angiogenic function as membrane function and transport are necessary for angiogenic molecules, including VEGF, to signal to ECs that angiogenesis is needed (Rivera and Bergers 2015). Not only that, but membrane function is necessary for proper blood vessel development.

In contrast to the tumor transcriptomes, the most altered genes in lung EC transcriptomes were transcripts of heat shock proteins (HSPs). HSPs are stress response proteins that play many roles in the cell including protein chaperoning, cell cycle signaling, and immune function(Z. Li and Srivastava 2003). HSPs have also been shown to be angiogenic, so their suppression may point to a mechanism behind hypovascularization in RIG-I (-/-) mice. In addition, several pathways involved in myeloid cells are suppressed in RIG-I (-/-) lungs, despite no apparent decreases in myeloid cells via flow cytometry. It is possible that downstream signaling in myeloid cells is dysfunctional, leading to hypovascularization.

Overall, we have found that RIG-I depletion leads to global loss of blood vessels. Despite this, we see an increase in VEGFA and VEGFR1, possibly due to an increase in inflammatory monocytes. It has been shown that tumor associated macrophages, neutrophils, and myeloid derived suppressor cells produce VEGF in response to a hypoxic microenvironment (Ding et al. 2014; Rivera and Bergers 2015; L. Yang et al. 2004; Nozawa, Chiu, and Hanahan 2006). We also see an increase in tumor growth, possibly also due to an increase in VEGF or inflammatory monocytes. If monocyte/VEGF production is caused by

hypoxia is likely but unknown. RNA analysis shows that several pathways involved in membrane function transport are disrupted in RIG-I (-/-), tumors hinting at a possible mechanism causing loss of ECs in RIG-I (-/-) mice. We also found that ECs isolated from RIG-I (-/-) lungs showed a decrease in Hsp genes and pathways involving myeloid cells, possibly hinting at another mechanism. Confirming this mechanism is critical to understanding the role of RIG-I in EC function, as is better understanding the downstream consequences of RIG-I induced loss of ECs.

# Chapter 5

## Epilogue

As outlined earlier, endothelial cells are underappreciated gatekeepers of immune responses. I sought to understand how sensing of nucleic acids, particularly RNA by RIG-I, impacts EC function. In Chapter 2, we identified a novel role for RIG-I signaling in endothelial function. In Part I, we demonstrate that RIG-I activation causes EC death and loss of angiogenic function. I also identify a gene signature specific to RIG-I activation in ECs that is relevant *in vitro, in vivo,* and in human disease based on my analysis of publicly available datasets. These genes included Flt1 (VEGFR1), a tyrosine kinase that is critical for angiogenesis (Melincovici et al. 2018). Interestingly, VEGFR1 can act as a decoy receptor for VEGFA, therefor VEGFR1's activation can actually act as a negative regulator for angiogenesis (Boucher et al. 2017). CX3Cl1, or Fractalkine, is a multifunctional inflammatory chemokine (Conroy and Lysaght 2020). CX3CL1 contributes to tumor angiogenesis in multiple tumor models through the recruitment of pro-angiogenic tumor associated macrophages, as well as directly upregulating endothelial cell tube formation (Schmall et al. 2015; J. Zhang et al. 2012; Conroy and Lysaght 2020). Interleukin 6 (IL6) is an inflammatory cytokine and plays several roles in autoimmune disease and chronic inflammatory diseases (Hirano 1998). IL6 stimulation has been shown to induce dysfunctional angiogenesis in *ex vivo* models, as well as increasing VEGF and tumor angiogenesis (S.-P. Huang et al. 2004; Gopinathan et al. 2015). Protein C Receptor (PROCR) is a transmembrane glycoprotein that is present on the surface of ECs, and is necessary for anticoagulation function (Dennis et al. 2012; Laszik et al. 1997). Plasminogen Activator Inhibitor-1 (PAI-1) is a serine protease inhibitor, whose function is inhibited by SERPINE1. PAI-1 is mainly produced by ECs and is a regulator of fibrinolysis as well as inflammation, wound healing, and cell adhesion (Morrow and Mutch 2023; Pavet et al. 2014). Thymidine phosphorylase (TYMP) is an enzyme involved in nucleoside metabolism and is critical to the pyrimidine pathway. TYMP catalyzes the conversion of thymidine to thymine and 2-deoxy-a-D-

ribose-1-phosphate, as well as the phosphorolysis of deoxyuridine to uracil and 2-deoxy-α-D-ribose-1-phosphate (2dDR) (Elamin et al. 2016). 2dDR is understood to upregulate VEGF in ECs and stimulate VEGF in tissue engineering models (X. Zhang et al. 2014). High levels of TYMP have been found in the plasma of cancer patients relative to healthy volunteers, and TYMP has been shown to be anti-apoptotic in some cancer types (Uchimiya et al. 2002). Interestingly some studies suggest the TYMP overexpression leads to the accumulation of ROS, due to the accumulation of 2dDR, which causes oxidative stress (Tabata et al. 2012).

Among these genes, I identified thymidine phosphorylase (TYMP) as a regulator of RIG-I signaling. Importantly, pharmacologic and genetic inhibition of TYMP rescued several RIG-I induced phenotypes in ECs in vitro. Despite the fact that TYMP is considered pro-angiogenic, TYMP can have many functions in the cell that can attribute to this phenotype. Our data describes a relationship between TYMP and inflammatory genes such as UBE2L6 NFkB genes. In addition, TYMP may be upregulating ROS, leading to increased cell death. In addition, as TYMP directly regulates nucleotide synthesis, it is possible that TYMP inhibition prevents the synthesis of apoptotic and inflammatory genes.

In Chapter 3, we characterized the role of RIG-I depletion in normal and pathological angiogenesis. We found that tumor-bearing RIG-I (-/-) show increased tumor size, which is more profound in male mice. Due to the increased tumor size in RIG-I (-/-) mice, as well as our *in vitro* data, we hypothesized that RIG-I depletion would cause a hypervascularization phenotype. However, we found that tumors from RIG-I (-/-) mice almost universally lack perfused blood vessels. Despite this, tumors from RIG-I (-/-) mice show increased levels of angiogenic chemokines. Tumors also did not show major changes in immune cell number or types except for an increase in neutrophils and monocytes. I subsequently evaluated whether the vascular and immune phenotypes were unique to the tumor microenvironment or were a feature of tissue angiogenesis in the absence of RIG-I. To answer this question, I evaluated lungs from the same

mice and found that they show a similar decrease in blood vessels. Further analysis showed that VEGFR1 was also overexpressed in RIG-I (-/-) lungs compared to WT. While the cause of the hypovascularization is still unknown, analysis of the tumor transcriptome points to a dysregulation of membrane and transport function. It is important to note that the tumor cells were WT for RIG-I expression and only the host mice lacked RIG-I. In addition, analysis of ECs from WT and RIG-I (-/-) lungs showed a decrease in Hsp genes and myeloid immune pathways. We also see a suppression of several immune-associated genes including Marco and Cxcr1. Combined, these results suggest that RIG-I signaling must be in homeostasis for functional vascular formation.

Though it may be surprising that both RIG-I activation and inhibition causes EC death, the activation of NAS have been reported to cause a wide variety of effects on the endothelium. Activation of TLRs has been shown to be pro-angiogenic following injury[1]. In addition, STING activation has been shown to 'normalize' tumor vasculature (S. J. Lee et al. 2021; H. Yang et al. 2019). In contrast to these observations, my work identifies RIG-I activation can have anti-angiogenic properties. This highlights the varied and complex nature of NAS and of the innate immune system affects the vascular system.

While these studies are critical to understanding the effect of RIG-I on ECs, a major limitation is the lack of study of RIG-I activation on wound healing, developmental angiogenesis, or pathological angiogenesis *in vivo.* Improving and validating delivery of RIG-I agonists to various tissues is necessary to study these various angiogenic processes. In addition, more detailed study on the effects of PAMPs from influenza or coronaviruses on endothelial function are critical. As these PAMPs would not only activate RIG-I, but other NAS, they could have more complex effects on the endothelium.

Our identification of Tipiracil (TPI) as a novel modulator of RIG-I induced EC dysfunction may have wide-reaching implications for vascular dysfunction. As (TPI) is an FDA approved

drug, its use to mitigate EC death following inflammation is promising. Additionally, TYMP expression is highly upregulated following infection from several viruses including coronavirus and Zika, implicating it as a possible therapeutic target (Pula et al. 2009; W. Li and Yue 2021). Several studies have also shown that TPI may bind to uridine sites on SARS-CoV-2 and prevent endoribonuclease activity (Y. Kim et al. 2021). Further study into TPI as a novel protectant for vascular function, such as whether it works in vivo following viral infections and as a protectant of normal vasculature for RNA drugs targeting tumors is warranted.

My further work in RIG-I (-/-) mice showed that tumor bearing male RIG-I (-/-) mice showed an increase in tumor size. This may be due to a number of factors including potential loss of immune infiltration into the tumor, decrease in anti-tumor immune responses, increase in hypoxia leading to a more aggressive tumor growth. Interestingly, this is an opposite phenotype to that seen by Jiang et al, who describe restrained tumor growth in RIG-I (-/-) mice (Jiang et al. 2023). As outlined above, this may be due to a number of factors. Jiang et al specifically used 6-week-old mice as these mice to not display the autoimmune diseases seen in older mice (Jiang et al. 2023). Jiang et al also describe T cell specific phenomena, while our studies do not identify any major changes in T cells. Performing a more comprehensive immune phenotyping analysis at different points during tumor growth in tumors from WT and RIG-I (-/-) mice, as well as the spleen and tumor draining lymph node, would allow me to identify T-cell specific changes. These analyses would include analysis of exhaustion, proliferation, production if IFN-y, granzyme A, TNF-$\alpha$, and other markers of T-cell function. Further analysis to identify whether RIG-I derived from immune cells or non-immune cells (ECs, stroma) is critical for the vascular phenotype may be necessary to unravel these discrepancies.

My work shows a sex-specific difference in RIG-I (-/-) mice. Specifically, male RIG-I (-/-) show increased tumor growth, while there is no difference between male and female WT mice. One possible reason for this difference in pattern recognition receptors between male and

female sexes. TLR-7, a nucleic acid sensor, is encoded on the X-chromosome, and may escape X-inactivation. This can cause an increase in TLR7 expression in females compared to males (Klein and Flanagan 2016; Pisitkun et al. 2006). In addition, following virus challenge or vaccination, females show an increase in the expression of TLR-pathway genes, as well as other genes in the innate immune response pathways, compared to males (Klein, Jedlicka, and Pekosz 2010; Hannah, Bajic, and Klein 2008). This may cause an increased immune response, potentially compensating for the lack of RIG-I, in response to the tumor specifically in female mice. Another possible cause for this discrepancy is the tumor model used. I used MC38 colorectal tumor cells, originally derived from female mice as a model system for many of the experiments in this study. Colorectal cancer occurs more often in males than females (S.-E. Kim et al. 2015; Murphy et al. 2011). Females over 65 years old show higher mortality, as well as a lower 5-year survival rate of colorectal cancer compared to age matched counterparts, confounding the data on sex-disparities (S.-E. Kim et al. 2015). However, our female mice (wild type and RIG-I knockout) developed tumors at the same rate as male wild-type tumors, so we eliminated the possibilities that the sex differences were due to the tumor implantations alone. Expansion of our study with more cell lines from colorectal cancer and other tumor types will address whether the sex differences we see are unique to this model or across other tumors. Other potential approaches such as crossing RIG-I-/- mice with genetically engineered spontaneous tumor models such as the PyMT mammary carcinoma model or the KPC pancreatic tumor model or chemical carcinogenesis with mutagens (eg. Diethyl nitrosamine) will help us evaluate whether RIG-I -/- male mice are a more permissive host for tumorigenesis or tumor progression.

One major limitation of this work is the use of whole-body knockouts of RIG-I, rather than endothelial-specific knockouts. While this is a useful model for determining the role of RIG-I loss in any cell on endothelial function, it does not determine whether ECs require RIG-I for their

normal function. An endothelial-specific knockout of RIG-I will help us determine whether ECs specifically require RIG-I for proper function, or if other cell types such as immune cells require RIG-I for cross-talk with ECs. This question can also be answered using a CRISPR or RNAi based knockout of RIG-I *in vitro* to determine if loss of RIG-I prevents EC function. Another limitation of this work is we have not evaluated any potential compensation mechanisms for the loss of RIG-I. Other NAS such as MDA5, MAVS or TLRs could potentially compensate for the loss of RIG-I, confounding our results. This could be analyzed by challenging WT vs RIG-I KO mice with RNA ligands targeting other pathways in comparison with RIG-I agonists and determining if other pathway ligands induce a more robust immune response as measured by the production of ISGs etc.

There are several other outstanding questions to come from this work. One is whether decrease in vascular integrity that is seen in the lungs is seen in other tissues, and whether that decrease affects the fitness of the animal under physiological and pathological conditions such as aging, exercise, or infection. It is also critical to determine if overexpression or overactivation of RIG-I *in vivo* produces the same phenotype that is seen *in vitro.* In addition, as this work describes a homeostatic role for RIG-I signaling in ECs, identifying whether there is an optimal 'dose' of RIG-I that can normalize pathological vasculature will be critical. This may be similar to VEGF inhibitors, in which inhibition rather than complete ablation can normalize tumor vasculature and improve efficacy of anticancer therapeutics (Y. Huang et al. 2013). It is possible that either subtle activation or inhibition or RIG-I will lead to vascular normalization and improve efficacy of immunotherapies or other anticancer therapies. As RIG-I activation is being considered an immunotherapy adjuvant by itself, RIG-I modulation being used for vascular normalization may provide an extra therapeutic benefit. Understanding this optimal dose for vascular homeostasis will enable the development of RIG-I targeted agents as dual purpose agents - immune adjuvants as well as anti-angiogenic therapeutics.

Overall, this work identifies a novel interaction between the RNA sensor RIG-I and the vascular system. Understanding this interaction is critical as RIG-I agonists are currently being tested clinically as anti-cancer therapeutics, however their effects on both normal and tumor vasculature are presently unknown. We must have a better understanding of how these treatments will affect normal vasculature, and how they will affect tumor angiogenesis, in order to determine their efficacy as a therapeutic agent. While this research focuses on RIG-I in the vasculature, it highlights the need to elucidate the interactions between the immune system and the vascular system in a broader context. Endothelium affects a wide variety of tumor behaviors including migration, drug delivery, and immunosensing. How new and emerging therapies impact the vascular endothelium is a critical question that must continue to be addressed to mitigate the cardiovascular impacts while amplifying the suppression of pathological angiogenesis.

## References


Ahmad, Saif S., Simon Duke, Rajesh Jena, Michael V. Williams, and Neil G. Burnet. 2012. "Advances in Radiotherapy." *BMJ* 345 (December): e7765. https://doi.org/10.1136/bmj.e7765.

Alberts, Bruce, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. 2002. "Innate Immunity." In *Molecular Biology of the Cell. 4th Edition*. Garland Science. https://www.ncbi.nlm.nih.gov/books/NBK26846/.

Allenspach, Eric J., Frank Soveg, Laura S. Finn, Lomon So, Jacquelyn A. Gorman, Aaron B. I. Rosen, Suzanne Skoda-Smith, et al. 2021. "Germline SAMD9L Truncation Variants Trigger Global Translational Repression." *The Journal of Experimental Medicine* 218 (5): e20201195. https://doi.org/10.1084/jem.20201195.

Armstrong, Susan M, Ilyse Darwish, and Warren L Lee. 2013. "Endothelial Activation and Dysfunction in the Pathogenesis of Influenza A Virus Infection." *Virulence* 4 (6): 537–42. https://doi.org/10.4161/viru.25779.

Asdonk, Tobias, Inga Motz, Nikos Werner, Christoph Coch, Winfried Barchet, Gunther Hartmann, Georg Nickenig, and Sebastian Zimmer. 2012. "Endothelial RIG-I Activation Impairs Endothelial Function." *Biochemical and Biophysical Research Communications* 420 (1): 66–71. https://doi.org/10.1016/j.bbrc.2012.02.116.

Asdonk, Tobias, Martin Steinmetz, Alexander Krogmann, Christine Ströcker, Catharina Lahrmann, Inga Motz, Kathrin Paul-Krahe, et al. 2016. "MDA-5 Activation by Cytoplasmic Double-Stranded RNA Impairs Endothelial Function and Aggravates Atherosclerosis." *Journal of Cellular and Molecular Medicine* 20 (9): 1696–1705. https://doi.org/10.1111/jcmm.12864.

Augustin, Hellmut G., Gou Young Koh, Gavin Thurston, and Kari Alitalo. 2009. “Control of Vascular Morphogenesis and Homeostasis through the Angiopoietin–Tie System.” *Nature Reviews Molecular Cell Biology* 10 (3): 165–77. https://doi.org/10.1038/nrm2639.

Avraamides, Christie J., Barbara Garmy-Susini, and Judith A. Varner. 2008. “Integrins in Angiogenesis and Lymphangiogenesis.” *Nature Reviews Cancer* 8 (8): 604–17. https://doi.org/10.1038/nrc2353.

Bach, F. H., S. C. Robson, H. Winkler, C. Ferran, K. M. Stuhlmeier, C. J. Wrighton, and W. W. Hancock. 1995. “Barriers to Xenotransplantation.” *Nature Medicine* 1 (9): 869–73. https://doi.org/10.1038/nm0995-869.

Baluk, Peter, Hiroya Hashizume, and Donald M. McDonald. 2005. “Cellular Abnormalities of Blood Vessels as Targets in Cancer.” *Current Opinion in Genetics & Development* 15 (1): 102–11. https://doi.org/10.1016/j.gde.2004.12.005.

Batchelor, Tracy T., A. Gregory Sorensen, Emmanuelle di Tomaso, Wei-Ting Zhang, Dan G. Duda, Kenneth S. Cohen, Kevin R. Kozak, et al. 2007. “AZD2171, a Pan-VEGF Receptor Tyrosine Kinase Inhibitor, Normalizes Tumor Vasculature and Alleviates Edema in Glioblastoma Patients.” *Cancer Cell* 11 (1): 83–95. https://doi.org/10.1016/j.ccr.2006.11.021.

Belcher, Adam, Abu Hasanat Md Zulfiker, Oliver Qiyue Li, Hong Yue, Anirban Sen Gupta, and Wei Li. 2021. “Targeting Thymidine Phosphorylase With Tipiracil Hydrochloride Attenuates Thrombosis Without Increasing Risk of Bleeding in Mice.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 41 (2): 668–82. https://doi.org/10.1161/ATVBAHA.120.315109.

Bergers, Gabriele, and Douglas Hanahan. 2008. “Modes of Resistance to Anti-Angiogenic Therapy.” *Nature Reviews. Cancer* 8 (8): 592–603. https://doi.org/10.1038/nrc2442.

Bergers, Gabriele, and Steven Song. 2005. “The Role of Pericytes in Blood-Vessel Formation and Maintenance.” *Neuro-Oncology* 7 (4): 452–64. https://doi.org/10.1215/S1152851705000232.

Bergers, Gabriele, Steven Song, Nicole Meyer-Morse, Emily Bergsland, and Douglas Hanahan. 2003. “Benefits of Targeting Both Pericytes and Endothelial Cells in the Tumor Vasculature with Kinase Inhibitors.” *The Journal of Clinical Investigation* 111 (9): 1287–95. https://doi.org/10.1172/JCI17929.

Betsholtz, Christer, Linda Karlsson, and Per Lindahl. 2001. “Developmental Roles of Platelet-Derived Growth Factors.” *BioEssays* 23 (6): 494–507. https://doi.org/10.1002/bies.1069.

Bhagwani, Aneel, A. A. Roger Thompson, and Laszlo Farkas. 2020. “When Innate Immunity Meets Angiogenesis—The Role of Toll-Like Receptors in Endothelial Cells and Pulmonary Hypertension.” *Frontiers in Medicine* 7. https://www.frontiersin.org/articles/10.3389/fmed.2020.00352.

Boucher, Joshua M., Ryan P. Clark, Diana C. Chong, Kathryn M. Citrin, Lyndsay A. Wylie, and Victoria L. Bautch. 2017. “Dynamic Alterations in Decoy VEGF Receptor-1 Stability Regulate Angiogenesis.” *Nature Communications* 8 (1): 15699. https://doi.org/10.1038/ncomms15699.

Brisse, Morgan, and Hinh Ly. 2019. “Comparative Structure and Function Analysis of the RIG-I-Like Receptors: RIG-I and MDA5.” *Frontiers in Immunology* 10. https://www.frontiersin.org/articles/10.3389/fimmu.2019.01586.

Brooks, P. C. 1996. “Role of Integrins in Angiogenesis.” *European Journal of Cancer*, Basic and Clinical Research on Angiogenesis, 32 (14): 2423–29. https://doi.org/10.1016/S0959-8049(96)00381-4.

Burri, Peter H., and Mojmir R. Tarek. 1990. “A novel mechanism of capillary growth in the rat pulmonary microcirculation.” *The Anatomical Record* 228 (1): 35–45. https://doi.org/10.1002/ar.1092280107.

Bussolati, Benedetta, Cristina Grange, Anna Sapino, and Giovanni Camussi. 2009. “Endothelial Cell Differentiation of Human Breast Tumour Stem/Progenitor Cells.” *Journal of Cellular and Molecular Medicine* 13 (2): 309–19. https://doi.org/10.1111/j.1582-4934.2008.00338.x.

Chambers, Sarah E. J., Christina L. O’Neill, T. Michelle O’Doherty, Reinhold J. Medina, and Alan W. Stitt. 2013. “The Role of Immune-Related Myeloid Cells in Angiogenesis.” *Immunobiology*, Special Issue: EMDS 2012, 218 (11): 1370–75. https://doi.org/10.1016/j.imbio.2013.06.010.

Chapouly, Candice, Azeb Tadesse Argaw, Sam Horng, Kamilah Castro, Jingya Zhang, Linnea Asp, Hannah Loo, et al. 2015. “Astrocytic TYMP and VEGFA Drive Blood–Brain Barrier Opening in Inflammatory Central Nervous System Lesions.” *Brain* 138 (6): 1548–67. https://doi.org/10.1093/brain/awv077.

Chelvanambi, Manoj, Ronald J. Fecek, Jennifer L. Taylor, and Walter J. Storkus. 2021. “STING Agonist-Based Treatment Promotes Vascular Normalization and Tertiary Lymphoid Structure Formation in the Therapeutic Melanoma Microenvironment.” *Journal for Immunotherapy of Cancer* 9 (2): e001906. https://doi.org/10.1136/jitc-2020-001906.

Chen, Si-Jie, Lin-Li Lv, Bi-Cheng Liu, and Ri-Ning Tang. 2020. “Crosstalk between Tubular Epithelial Cells and Glomerular Endothelial Cells in Diabetic Kidney Disease.” *Cell Proliferation* 53 (3): e12763. https://doi.org/10.1111/cpr.12763.

Chen, Xiaojun, Wenxin He, Mengzhe Sun, Yingjie Yan, Yichuan Pang, and Gang Chai. 2021. “STING Inhibition Accelerates the Bone Healing Process While Enhancing Type H Vessel Formation.” *The FASEB Journal* 35 (11): e21964. https://doi.org/10.1096/fj.202100069RR.

Chiu, Jeng-Jiann, Pei-Ling Lee, Cheng-Nan Chen, Chih-I Lee, Shun-Fu Chang, Li-Jing Chen, Sheng-Chieh Lien, Ya-Chen Ko, Shunichi Usami, and Shu Chien. 2004. “Shear Stress Increases ICAM-1 and Decreases VCAM-1 and E-Selectin Expressions Induced by Tumor Necrosis Factor-α in Endothelial Cells.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 24 (1): 73–79. https://doi.org/10.1161/01.ATV.0000106321.63667.24.

Chung, Alicia S., and Napoleone Ferrara. 2011. “Developmental and Pathological Angiogenesis.” *Annual Review of Cell and Developmental Biology* 27 (1): 563–84. https://doi.org/10.1146/annurev-cellbio-092910-154002.

Claesson-Welsh, L., and M. Welsh. 2013. “VEGFA and Tumour Angiogenesis.” *Journal of Internal Medicine* 273 (2): 114–27. https://doi.org/10.1111/joim.12019.

Conceição, Thaís Moraes da, Naiara Miranda Rust, Ana Carolina Egypto Rosa Berbel, Nathalia Balthazar Martins, Carlos Antonio do Nascimento Santos, Andrea Thompson Da Poian, and Luciana Barros de Arruda. 2013. “Essential Role of RIG-I in the Activation of Endothelial Cells by Dengue Virus.” *Virology* 435 (2): 281–92. https://doi.org/10.1016/j.virol.2012.09.038.

Conroy, Melissa J., and Joanne Lysaght. 2020. “CX3CL1 Signaling in the Tumor Microenvironment.” *Advances in Experimental Medicine and Biology* 1231: 1–12. https://doi.org/10.1007/978-3-030-36667-4_1.

Cosse, Jean-Philippe, and Carine Michiels. 2008. “Tumour Hypoxia Affects the Responsiveness of Cancer Cells to Chemotherapy and Promotes Cancer Progression.” *Anti-Cancer Agents in Medicinal Chemistry- Anti-Cancer Agents)* 8 (7): 790–97. https://doi.org/10.2174/187152008785914798.

Coultas, Leigh, Kallayanee Chawengsaksophak, and Janet Rossant. 2005. “Endothelial Cells and VEGF in Vascular Development.” *Nature* 438 (7070): 937–45. https://doi.org/10.1038/nature04479.

Crawford, Yongping, and Napoleone Ferrara. 2009. “VEGF Inhibition: Insights from Preclinical and Clinical Studies.” *Cell and Tissue Research* 335 (1): 261–69. https://doi.org/10.1007/s00441-008-0675-8.

Curiel, Tyler J., George Coukos, Linhua Zou, Xavier Alvarez, Pui Cheng, Peter Mottram, Melina Evdemon-Hogan, et al. 2004. “Specific Recruitment of Regulatory T Cells in Ovarian Carcinoma Fosters Immune Privilege and Predicts Reduced Survival.” *Nature Medicine* 10 (9): 942–49. https://doi.org/10.1038/nm1093.

Czirok, Andras, and Charles D. Little. 2012. “Pattern Formation during Vasculogenesis.” *Birth Defects Research. Part C, Embryo Today: Reviews* 96 (2): 153–62. https://doi.org/10.1002/bdrc.21010.

Dejana, Elisabetta, Elisabeth Tournier-Lasserve, and Brant M. Weinstein. 2009. “The Control of Vascular Integrity by Endothelial Cell Junctions: Molecular Basis and Pathological

Implications." *Developmental Cell* 16 (2): 209–21. https://doi.org/10.1016/j.devcel.2009.01.004.

Dennis, Jessica, Candice Y. Johnson, Adeniyi Samuel Adediran, Mariza de Andrade, John A. Heit, Pierre-Emmanuel Morange, David-Alexandre Trégouët, and France Gagnon. 2012. "The Endothelial Protein C Receptor (PROCR) Ser219Gly Variant and Risk of Common Thrombotic Disorders: A HuGE Review and Meta-Analysis of Evidence from Observational Studies." *Blood* 119 (10): 2392–2400. https://doi.org/10.1182/blood-2011-10-383448.

Dikov, Mikhail M., Joyce E. Ohm, Neelanjan Ray, Elena E. Tchekneva, Jared Burlison, Drew Moghanaki, Sorena Nadaf, and David P. Carbone. 2005. "Differential Roles of Vascular Endothelial Growth Factor Receptors 1 and 2 in Dendritic Cell Differentiation1." *The Journal of Immunology* 174 (1): 215–22. https://doi.org/10.4049/jimmunol.174.1.215.

Ding, Peng, Weimin Wang, Jinkun Wang, Zhiyong Yang, and Liping Xue. 2014. "Expression of Tumor-Associated Macrophage in Progression of Human Glioma." *Cell Biochemistry and Biophysics* 70 (3): 1625–31. https://doi.org/10.1007/s12013-014-0105-3.

Drake, Christopher J. 2003. "Embryonic and Adult Vasculogenesis." *Birth Defects Research Part C: Embryo Today: Reviews* 69 (1): 73–82. https://doi.org/10.1002/bdrc.10003.

Ebos, John M. L., and Robert S. Kerbel. 2011. "Antiangiogenic Therapy: Impact on Invasion, Disease Progression, and Metastasis." *Nature Reviews. Clinical Oncology* 8 (4): 210–21. https://doi.org/10.1038/nrclinonc.2011.21.

Ehrentraut, Stefan Felix, Anne Dörr, Heidi Ehrentraut, Ralph Lohner, Sun-Hee Lee, Andreas Hoeft, Georg Baumgarten, Pascal Knuefermann, Olaf Boehm, and Rainer Meyer. 2012.

"Vascular Dysfunction Following Polymicrobial Sepsis: Role of Pattern Recognition Receptors." *PloS One* 7 (9): e44531. https://doi.org/10.1371/journal.pone.0044531.

Eichmann, Anne, Luc Pardanaud, Li Yuan, and Delphine Moyon. 2002. "Vasculogenesis and the Search for the Hemangioblast." *Journal of Hematotherapy & Stem Cell Research* 11 (2): 207–14. https://doi.org/10.1089/152581602753658411.

Elamin, Yasir Y., Shereen Rafee, Nemer Osman, Kenneth J. O Byrne, and Kathy Gately. 2016. "Thymidine Phosphorylase in Cancer; Enemy or Friend?" *Cancer Microenvironment: Official Journal of the International Cancer Microenvironment Society* 9 (1): 33–43. https://doi.org/10.1007/s12307-015-0173-y.

Emblem, Kyrre E., Kim Mouridsen, Atle Bjornerud, Christian T. Farrar, Dominique Jennings, Ronald J. H. Borra, Patrick Y. Wen, et al. 2013. "Vessel Architectural Imaging Identifies Cancer Patient Responders to Anti-Angiogenic Therapy." *Nature Medicine* 19 (9): 1178–83. https://doi.org/10.1038/nm.3289.

Emming, Stefan, and Kate Schroder. 2019. "Tiered DNA Sensors for Escalating Responses." *Science* 365 (6460): 1375–76. https://doi.org/10.1126/science.aay2701.

Endemann, Dierk H., and Ernesto L. Schiffrin. 2004. "Endothelial Dysfunction." *Journal of the American Society of Nephrology* 15 (8): 1983. https://doi.org/10.1097/01.ASN.0000132474.50966.DA.

Erber, Ralf, Andreas Thurnher, Alice D. Katsen, Gesine Groth, Heinz Kerger, Hans-Peter Hammes, Michael D. Menger, Axel Ullrich, and Peter Vajkoczy. 2004. "Combined Inhibition of VEGF and PDGF Signaling Enforces Tumor Vessel Regression by Interfering with Pericyte-Mediated Endothelial Cell Survival Mechanisms." *FASEB*

*Journal: Official Publication of the Federation of American Societies for Experimental Biology* 18 (2): 338–40. https://doi.org/10.1096/fj.03-0271fje.

Errett, John S., Mehul S. Suthar, Aimee McMillan, Michael S. Diamond, and Michael Gale. 2013. "The Essential, Nonredundant Roles of RIG-I and MDA5 in Detecting and Controlling West Nile Virus Infection." *Journal of Virology* 87 (21): 11416–25. https://doi.org/10.1128/jvi.01488-13.

Escudier, Bernard, Tim Eisen, Walter M. Stadler, Cezary Szczylik, Stéphane Oudard, Michael Siebels, Sylvie Negrier, et al. 2007. "Sorafenib in Advanced Clear-Cell Renal-Cell Carcinoma." *The New England Journal of Medicine* 356 (2): 125–34. https://doi.org/10.1056/NEJMoa060655.

Fagiani, Ernesta, and Gerhard Christofori. 2013. "Angiopoietins in Angiogenesis." *Cancer Letters* 328 (1): 18–26. https://doi.org/10.1016/j.canlet.2012.08.018.

Fan, F., S. Samuel, P. Gaur, J. Lu, N. A. Dallas, L. Xia, D. Bose, V. Ramachandran, and L. M. Ellis. 2011. "Chronic Exposure of Colorectal Cancer Cells to Bevacizumab Promotes Compensatory Pathways That Mediate Tumour Cell Migration." *British Journal of Cancer* 104 (8): 1270–77. https://doi.org/10.1038/bjc.2011.81.

Fausto, Nelson. 2000. "Vasculogenic Mimicry in Tumors." *The American Journal of Pathology* 156 (2): 359.

Ferrara, Napoleone, Hans-Peter Gerber, and Jennifer LeCouter. 2003. "The Biology of VEGF and Its Receptors." *Nature Medicine* 9 (6): 669–76. https://doi.org/10.1038/nm0603-669.

Folberg, Robert, and Andrew J. Maniotis. 2004. "Vasculogenic Mimicry." *APMIS* 112 (7–8): 508–25. https://doi.org/10.1111/j.1600-0463.2004.apm11207-0810.x.

Folkman, Judah. 1971. “Tumor Angiogenesis: Therapeutic Implications.” *New England Journal of Medicine* 285 (21): 1182–86. https://doi.org/10.1056/NEJM197111182852108.

———. 1984. “Angiogenesis.” In *Biology of Endothelial Cells*, edited by Eric A. Jaffe, 412–28. Developments in Cardiovascular Medicine. Boston, MA: Springer US. https://doi.org/10.1007/978-1-4613-2825-4_42.

Folkman, Judah, Ezio Merler, Charles Abernathy, and Gretchen Williams. 1971. “ISOLATION OF A TUMOR FACTOR RESPONSIBLE FOR ANGIOGENESIS.” *The Journal of Experimental Medicine* 133 (2): 275–88.

Fonseca, J. E., M. J. Santos, H. Canhão, and E. Choy. 2009. “Interleukin-6 as a Key Player in Systemic Inflammation and Joint Destruction.” *Autoimmunity Reviews* 8 (7): 538–42. https://doi.org/10.1016/j.autrev.2009.01.012.

Gabrilovich, D.I., H.L. Chen, K.R. Girgis, H.T. Cunningham, G.M. Meny, S. Nadaf, D. Kavanaugh, and D.P. Carbone. 1996. “Production of Vascular Endothelial Growth Factor by Human Tumors Inhibits the Functional Maturation of Dendritic Cells.” *Nature Medicine* 2 (10): 1096–1103. https://doi.org/10.1038/nm1096-1096.

Gebb, Sarah, and Troy Stevens. 2004. “On Lung Endothelial Cell Heterogeneity.” *Microvascular Research* 68 (1): 1–12. https://doi.org/10.1016/j.mvr.2004.02.002.

Gentles, Andrew J., Angela Bik-Yu Hui, Weiguo Feng, Armon Azizi, Ramesh V. Nair, Gina Bouchard, David A. Knowles, et al. 2020. “A Human Lung Tumor Microenvironment Interactome Identifies Clinically Relevant Cell-Type Cross-Talk.” *Genome Biology* 21 (1): 107. https://doi.org/10.1186/s13059-020-02019-x.

Gerhardt, Holger, and Christer Betsholtz. 2003. “Endothelial-Pericyte Interactions in Angiogenesis.” *Cell and Tissue Research* 314 (1): 15–23. https://doi.org/10.1007/s00441-003-0745-x.

Giaccia, Amato J., M. Celeste Simon, and Randall Johnson. 2004. “The Biology of Hypoxia: The Role of Oxygen Sensing in Development, Normal Function, and Disease.” *Genes & Development* 18 (18): 2183–94. https://doi.org/10.1101/gad.1243304.

Gibson, Heather M., Carrie J. Hedgcock, Barbara M. Aufiero, Adam J. Wilson, Mikehl S. Hafner, George C. Tsokos, and Henry K. Wong. 2007. “Induction of the CTLA-4 Gene in Human Lymphocytes Is Dependent on NFAT Binding the Proximal Promoter1.” *The Journal of Immunology* 179 (6): 3831–40. https://doi.org/10.4049/jimmunol.179.6.3831.

Givens, Chris, and Ellie Tzima. 2016. “Endothelial Mechanosignaling: Does One Sensor Fit All?” *Antioxidants & Redox Signaling* 25 (7): 373–88. https://doi.org/10.1089/ars.2015.6493.

Goldie, Lauren C., Melissa K. Nix, and Karen K. Hirschi. 2008. “Embryonic Vasculogenesis and Hematopoietic Specification.” *Organogenesis* 4 (4): 257–63. https://doi.org/10.4161/org.4.4.7416.

Goldstein, Gary W., and A. Lorris Betz. 1986. “The Blood-Brain Barrier.” *Scientific American* 255 (3): 74–83.

Goodrum, Felicia, and Farah Bughio. 2015. “Viral Infection at the Endothelium.” *Oncotarget* 6 (29): 26541–42.

Gopinathan, Ganga, Carla Milagre, Oliver M.T. Pearce, Louise E. Reynolds, Kairbaan Hodivala-Dilke, David A. Leinster, Haihong Zhong, et al. 2015. “Interleukin-6 Stimulates Defective

Angiogenesis." *Cancer Research* 75 (15): 3098–3107. https://doi.org/10.1158/0008-5472.CAN-15-1227.

Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe, and A. J. Giaccia. 1996. "Hypoxia-Mediated Selection of Cells with Diminished Apoptotic Potential in Solid Tumours." *Nature* 379 (6560): 88–91. https://doi.org/10.1038/379088a0.

Greenberg, Joshua I., David J. Shields, Samuel G. Barillas, Lisette M. Acevedo, Eric Murphy, Jianhua Huang, Lea Scheppke, et al. 2008. "A Role for VEGF as a Negative Regulator of Pericyte Function and Vessel Maturation." *Nature* 456 (7223): 809–13. https://doi.org/10.1038/nature07424.

Grote, Karsten, Harald Schütt, and Bernhard Schieffer. NaN/NaN/NaN. "Toll-Like Receptors in Angiogenesis." *The Scientific World Journal* 11: 981–91. https://doi.org/10.1100/tsw.2011.92.

Hannah, Michele F., Vladimir B. Bajic, and Sabra L. Klein. 2008. "Sex Differences in the Recognition of and Innate Antiviral Responses to Seoul Virus in Norway Rats." *Brain, Behavior, and Immunity* 22 (4): 503–16. https://doi.org/10.1016/j.bbi.2007.10.005.

Hapani, Sanjaykumar, Amna Sher, David Chu, and Shenhong Wu. 2010. "Increased Risk of Serious Hemorrhage with Bevacizumab in Cancer Patients: A Meta-Analysis." *Oncology* 79 (1–2): 27–38. https://doi.org/10.1159/000314980.

Heldin, Carl-Henrik. 2013. "Targeting the PDGF Signaling Pathway in Tumor Treatment." *Cell Communication and Signaling: CCS* 11 (December): 97. https://doi.org/10.1186/1478-811X-11-97.

Hellberg, Carina, Arne Östman, and C.-H. Heldin. 2010. “PDGF and Vessel Maturation.” In *Angiogenesis Inhibition*, edited by Rüdiger Liersch, Wolfgang E. Berdel, and Torsten Kessler, 103–14. Recent Results in Cancer Research. Berlin, Heidelberg: Springer. https://doi.org/10.1007/978-3-540-78281-0_7.

Hirano, T. 1998. “Interleukin 6 and Its Receptor: Ten Years Later.” *International Reviews of Immunology* 16 (3–4): 249–84. https://doi.org/10.3109/08830189809042997.

Hirschberg, H., O. J. Bergh, and E. Thorsby. 1980. “Antigen-Presenting Properties of Human Vascular Endothelial Cells.” *The Journal of Experimental Medicine* 152 (2 Pt 2): 249s–55.

Hobbs, Susan K., Wayne L. Monsky, Fan Yuan, W. Gregory Roberts, Linda Griffith, Vladimir P. Torchilin, and Rakesh K. Jain. 1998. “Regulation of Transport Pathways in Tumor Vessels: Role of Tumor Type and Microenvironment.” *Proceedings of the National Academy of Sciences* 95 (8): 4607–12. https://doi.org/10.1073/pnas.95.8.4607.

Holopainen, Tanja, Pipsa Saharinen, Gabriela D’Amico, Anita Lampinen, Lauri Eklund, Raija Sormunen, Andrey Anisimov, et al. 2012. “Effects of Angiopoietin-2-Blocking Antibody on Endothelial Cell-Cell Junctions and Lung Metastasis.” *Journal of the National Cancer Institute* 104 (6): 461–75. https://doi.org/10.1093/jnci/djs009.

Horsman, Michael R., and Jens Overgaard. 2016. “The Impact of Hypoxia and Its Modification of the Outcome of Radiotherapy.” *Journal of Radiation Research* 57 (S1): i90–98. https://doi.org/10.1093/jrr/rrw007.

Hsieh, Patrick C.H., Michael E. Davis, Laura K. Lisowski, and Richard T. Lee. 2006. “Endothelial-Cardiomyocyte Interactions in Cardiac Development and Repair.” *Annual*

*Review of Physiology* 68: 51–66. https://doi.org/10.1146/annurev.physiol.68.040104.124629.

Huang, Jianzhong, Samuel Z. Soffer, Eugene S. Kim, Kimberly W. McCrudden, Joe Huang, Tamara New, Christina A. Manley, et al. 2004. "Vascular Remodeling Marks Tumors That Recur during Chronic Suppression of Angiogenesis." *Molecular Cancer Research: MCR* 2 (1): 36–42.

Huang, Qingyan, Yuhong Gan, Zhikang Yu, Heming Wu, and Zhixiong Zhong. 2021. "Endothelial to Mesenchymal Transition: An Insight in Atherosclerosis." *Frontiers in Cardiovascular Medicine* 8: 734550. https://doi.org/10.3389/fcvm.2021.734550.

Huang, Shih-Pei, Ming-Shiang Wu, Chia-Tung Shun, Hsiu-Po Wang, Ming-Tsan Lin, Min-Liang Kuo, and Jaw-Town Lin. 2004. "Interleukin-6 Increases Vascular Endothelial Growth Factor and Angiogenesis in Gastric Carcinoma." *Journal of Biomedical Science* 11 (4): 517–27. https://doi.org/10.1159/000077902.

Huang, Yuhui, Shom Goel, Dan G. Duda, Dai Fukumura, and Rakesh K. Jain. 2013. "Vascular Normalization as an Emerging Strategy to Enhance Cancer Immunotherapy." *Cancer Research* 73 (10): 2943–48. https://doi.org/10.1158/0008-5472.CAN-12-4354.

Huang, Yuhui, Jianping Yuan, Elda Righi, Walid S. Kamoun, Marek Ancukiewicz, Jean Nezivar, Michael Santosuosso, et al. 2012. "Vascular Normalizing Doses of Antiangiogenic Treatment Reprogram the Immunosuppressive Tumor Microenvironment and Enhance Immunotherapy." *Proceedings of the National Academy of Sciences of the United States of America* 109 (43): 17561–66. https://doi.org/10.1073/pnas.1215397109.

Huertas, Alice, David Montani, Laurent Savale, Jérémie Pichon, Ly Tu, Florence Parent, Christophe Guignabert, and Marc Humbert. 2020. "Endothelial Cell Dysfunction: A Major

Player in SARS-CoV-2 Infection (COVID-19)?" *European Respiratory Journal* 56 (1). https://doi.org/10.1183/13993003.01634-2020.

Hunt, Beverley J., and Karen M. Jurd. 1998. "Endothelial Cell Activation: A Central Pathophysiological Process." *BMJ* 316 (7141): 1328. https://doi.org/10.1136/bmj.316.7141.1328.

Iwasaki, Akiko, and Padmini S. Pillai. 2014. "Innate Immunity to Influenza Virus Infection." *Nature Reviews Immunology* 14 (5): 315–28. https://doi.org/10.1038/nri3665.

Jain, Rakesh K. 1987. "Transport of Molecules across Tumor Vasculature." *Cancer and Metastasis Reviews* 6 (4): 559–93. https://doi.org/10.1007/BF00047468.

———. 2003. "Molecular Regulation of Vessel Maturation." *Nature Medicine* 9 (6): 685–93. https://doi.org/10.1038/nm0603-685.

———. 2014. "Antiangiogenesis Strategies Revisited: From Starving Tumors to Alleviating Hypoxia." *Cancer Cell* 26 (5): 605–22. https://doi.org/10.1016/j.ccell.2014.10.006.

Jain, Rakesh K., John D. Martin, and Triantafyllos Stylianopoulos. 2014. "The Role of Mechanical Forces in Tumor Growth and Therapy." *Annual Review of Biomedical Engineering* 16 (1): 321–46. https://doi.org/10.1146/annurev-bioeng-071813-105259.

Jang, Mi-Ae, Eun Kyoung Kim, Hesung Now, Nhung T. H. Nguyen, Woo-Jong Kim, Joo-Yeon Yoo, Jinhyuk Lee, et al. 2015. "Mutations in DDX58, Which Encodes RIG-I, Cause Atypical Singleton-Merten Syndrome." *The American Journal of Human Genetics* 96 (2): 266–74. https://doi.org/10.1016/j.ajhg.2014.11.019.

Jankowska, Katarzyna I., Edward K. Williamson, Nathan H. Roy, Daniel Blumenthal, Vidhi Chandra, Tobias Baumgart, and Janis K. Burkhardt. 2018. "Integrins Modulate T Cell

Receptor Signaling by Constraining Actin Flow at the Immunological Synapse." *Frontiers in Immunology* 9. https://www.frontiersin.org/articles/10.3389/fimmu.2018.00025.

Jayson, Gordon C., Robert Kerbel, Lee M. Ellis, and Adrian L. Harris. 2016. "Antiangiogenic Therapy in Oncology: Current Status and Future Directions." *The Lancet* 388 (10043): 518–29. https://doi.org/10.1016/S0140-6736(15)01088-0.

Jeong, Seung-Hwan, Myung Jin Yang, Seunghyeok Choi, JungMo Kim, and Gou Young Koh. 2021. "Refractoriness of STING Therapy Is Relieved by AKT Inhibitor through Effective Vascular Disruption in Tumour." *Nature Communications* 12 (1): 4405. https://doi.org/10.1038/s41467-021-24603-w.

Jiang, Xinyi, Jian Lin, Chengfang Shangguan, Xiaoyao Wang, Bin Xiang, Juan Chen, Hezhou Guo, et al. 2023. "Intrinsic RIG-I Restrains STAT5 Activation to Modulate Antitumor Activity of $CD8^+$ T Cells." *The Journal of Clinical Investigation* 133 (9). https://doi.org/10.1172/JCI160790.

Jiao, Huipeng, Laurens Wachsmuth, Snehlata Kumari, Robin Schwarzer, Juan Lin, Remzi Onur Eren, Amanda Fisher, et al. 2020. "Z-Nucleic-Acid Sensing Triggers ZBP1-Dependent Necroptosis and Inflammation." *Nature* 580 (7803): 391–95. https://doi.org/10.1038/s41586-020-2129-8.

Johnson, Lexus R., Daniel Y. Lee, Jacqueline S. Eacret, Darwin Ye, Carl H. June, and Andy J. Minn. 2021. "The Immunostimulatory RNA RN7SL1 Enables CAR-T Cells to Enhance Autonomous and Endogenous Immune Function." *Cell* 184 (19): 4981-4995.e14. https://doi.org/10.1016/j.cell.2021.08.004.

Kato, Hiroki, Shintaro Sato, Mitsutoshi Yoneyama, Masahiro Yamamoto, Satoshi Uematsu, Kosuke Matsui, Tohru Tsujimura, et al. 2005. "Cell Type-Specific Involvement of RIG-I in

Antiviral Response." *Immunity* 23 (1): 19–28. https://doi.org/10.1016/j.immuni.2005.04.010.

Keezer, Susan M., Susan E. Ivie, Henry C. Krutzsch, Anita Tandle, Steven K. Libutti, and David D. Roberts. 2003. "Angiogenesis Inhibitors Target the Endothelial Cell Cytoskeleton through Altered Regulation of Heat Shock Protein 27 and Cofilin1." *Cancer Research* 63 (19): 6405–12.

Kell, Alison M., and Michael Gale. 2015. "RIG-I in RNA Virus Recognition." *Virology*, 60th Anniversary Issue, 479–480 (May): 110–21. https://doi.org/10.1016/j.virol.2015.02.017.

Khan, Shahanshah, Victoria Godfrey, and Md. Hasan Zaki. 2019. "Chapter Seven - Cytosolic Nucleic Acid Sensors in Inflammatory and Autoimmune Disorders." In *International Review of Cell and Molecular Biology*, edited by Claire Vanpouille-Box and Lorenzo Galluzzi, 344:215–53. Nucleic Acid Sensing and Immunity, Part A. Academic Press. https://doi.org/10.1016/bs.ircmb.2018.10.002.

Khan, Shawez, Federico Taverna, Katerina Rohlenova, Lucas Treps, Vincent Geldhof, Laura de Rooij, Liliana Sokol, et al. 2019. "EndoDB: A Database of Endothelial Cell Transcriptomics Data." *Nucleic Acids Research* 47 (D1): D736–44. https://doi.org/10.1093/nar/gky997.

Kikuno, Nobuyuki, Tateki Yoshino, Shinji Urakami, Kazushi Shigeno, Hirofumi Kishi, Kohkichi Hata, Hiroaki Shiina, and Mikio Igawa. 2003. "The Role of Thymidine Phosphorylase (TP) mRNA Expression in Angiogenesis of Prostate Cancer." *Anticancer Research* 23 (2B): 1305–12.

Kim, Jiha, Pedro Correa de Sampaio, Donna Marie Lundy, Qian Peng, Kurt W. Evans, Hikaru Sugimoto, Mihai Gagea, et al. 2016. "Heterogeneous Perivascular Cell Coverage Affects

Breast Cancer Metastasis and Response to Chemotherapy." *JCI Insight* 1 (21): e90733. https://doi.org/10.1172/jci.insight.90733.

Kim, Sung-Eun, Hee Young Paik, Hyuk Yoon, Jung Eun Lee, Nayoung Kim, and Mi-Kyung Sung. 2015. "Sex- and Gender-Specific Disparities in Colorectal Cancer Risk." *World Journal of Gastroenterology* 21 (17): 5167–75. https://doi.org/10.3748/wjg.v21.i17.5167.

Kim, Taek-Keun, Hee Jun Na, Woo Ran Lee, Mee Hyun Jeoung, and Sukmook Lee. 2016. "Heat Shock Protein 70-1A Is a Novel Angiogenic Regulator." *Biochemical and Biophysical Research Communications* 469 (2): 222–28. https://doi.org/10.1016/j.bbrc.2015.11.125.

Kim, Youngchang, Jacek Wower, Natalia Maltseva, Changsoo Chang, Robert Jedrzejczak, Mateusz Wilamowski, Soowon Kang, et al. 2021. "Tipiracil Binds to Uridine Site and Inhibits Nsp15 Endoribonuclease NendoU from SARS-CoV-2." *Communications Biology* 4 (1): 1–11. https://doi.org/10.1038/s42003-021-01735-9.

Klein, Sabra L., and Katie L. Flanagan. 2016. "Sex Differences in Immune Responses." *Nature Reviews Immunology* 16 (10): 626–38. https://doi.org/10.1038/nri.2016.90.

Klein, Sabra L., Anne Jedlicka, and Andrew Pekosz. 2010. "The Xs and Y of Immune Responses to Viral Vaccines." *The Lancet. Infectious Diseases* 10 (5): 338–49. https://doi.org/10.1016/S1473-3099(10)70049-9.

Kolakofsky, Daniel, Eva Kowalinski, and Stephen Cusack. 2012. "A Structure-Based Model of RIG-I Activation." *RNA* 18 (12): 2118–27. https://doi.org/10.1261/rna.035949.112.

Kolte, Dhaval, John Arthur McClung, and Wilbert S. Aronow. 2016. "Chapter 6 - Vasculogenesis and Angiogenesis." In *Translational Research in Coronary Artery Disease*, edited by

Wilbert S. Aronow and John Arthur McClung, 49–65. Boston: Academic Press. https://doi.org/10.1016/B978-0-12-802385-3.00006-1.

Koyama, Shohei, Ken J. Ishii, Himanshu Kumar, Takeshi Tanimoto, Cevayir Coban, Satoshi Uematsu, Taro Kawai, and Shizuo Akira. 2007. “Differential Role of TLR- and RLR-Signaling in the Immune Responses to Influenza A Virus Infection and Vaccination.” *The Journal of Immunology* 179 (7): 4711–20. https://doi.org/10.4049/jimmunol.179.7.4711.

Krogmann, Alexander O., Enzo Lüsebrink, Catharina Lahrmann, Anna Flender, Georg Nickenig, and Sebastian Zimmer. 2020. “Toll-Like Receptor 7 Stimulation Promotes the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice.” *International Heart Journal* 61 (2): 364–72. https://doi.org/10.1536/ihj.19-365.

Kuleshov, Maxim V., Matthew R. Jones, Andrew D. Rouillard, Nicolas F. Fernandez, Qiaonan Duan, Zichen Wang, Simon Koplev, et al. 2016. “Enrichr: A Comprehensive Gene Set Enrichment Analysis Web Server 2016 Update.” *Nucleic Acids Research* 44 (W1): W90-97. https://doi.org/10.1093/nar/gkw377.

Langer, Harald F., and Triantafyllos Chavakis. 2009. “Leukocyte – Endothelial Interactions in Inflammation.” *Journal of Cellular and Molecular Medicine* 13 (7): 1211–20. https://doi.org/10.1111/j.1582-4934.2009.00811.x.

Lapeyre-Prost, A., M. Terme, S. Pernot, A. -L. Pointet, T. Voron, E. Tartour, and J. Taieb. 2017. “Chapter Seven - Immunomodulatory Activity of VEGF in Cancer.” In *International Review of Cell and Molecular Biology*, edited by Lorenzo Galluzzi, 330:295–342. Academic Press. https://doi.org/10.1016/bs.ircmb.2016.09.007.

Laschke, Matthias W., Yuan Gu, and Michael D. Menger. 2022. “Replacement in Angiogenesis Research: Studying Mechanisms of Blood Vessel Development by Animal-Free in Vitro,

in Vivo and in Silico Approaches." *Frontiers in Physiology* 13. https://www.frontiersin.org/articles/10.3389/fphys.2022.981161.

Lässig, Charlotte, Katja Lammens, Jacob Lucián Gorenflos López, Sebastian Michalski, Olga Fettscher, and Karl-Peter Hopfner. 2018. "Unified Mechanisms for Self-RNA Recognition by RIG-I Singleton-Merten Syndrome Variants." *eLife* 7 (July): e38958. https://doi.org/10.7554/eLife.38958.

Laszik, Zoltan, Alexander Mitro, Fletcher B. Taylor, Gary Ferrell, and Charles T. Esmon. 1997. "Human Protein C Receptor Is Present Primarily on Endothelium of Large Blood Vessels." *Circulation* 96 (10): 3633–40. https://doi.org/10.1161/01.CIR.96.10.3633.

Lee, Keunho, Vijayendra Agrawal, Kyeojin Kim, Jihye Kim, Hyojin Park, Sungwoon Lee, Young-Myeong Kim, Young-Ger Suh, and Young-Guen Kwon. 2014. "Combined Effect of Vascular-Leakage-Blocker Sac-1004 and Antiangiogenic Drug Sunitinib on Tumor Angiogenesis." *Biochemical and Biophysical Research Communications* 450 (4): 1320–26. https://doi.org/10.1016/j.bbrc.2014.06.139.

Lee, Seung Joon, Hannah Yang, Woo Ram Kim, Yu Seong Lee, Won Suk Lee, So Jung Kong, Hye Jin Lee, et al. 2021. "STING Activation Normalizes the Intraperitoneal Vascular-Immune Microenvironment and Suppresses Peritoneal Carcinomatosis of Colon Cancer." *Journal for Immunotherapy of Cancer* 9 (6): e002195. https://doi.org/10.1136/jitc-2020-002195.

Leow, Ching Ching, Karen Coffman, Ivan Inigo, Shannon Breen, Meggan Czapiga, Serguei Soukharev, Neill Gingles, et al. 2012. "MEDI3617, a Human Anti-Angiopoietin 2 Monoclonal Antibody, Inhibits Angiogenesis and Tumor Growth in Human Tumor

Xenograft Models." *International Journal of Oncology* 40 (5): 1321–30. https://doi.org/10.3892/ijo.2012.1366.

Leung, David W., George Cachianes, Wun-Jing Kuang, David V. Goeddel, and Napoleone Ferrara. 1989. "Vascular Endothelial Growth Factor Is a Secreted Angiogenic Mitogen." *Science* 246 (4935): 1306–9. https://doi.org/10.1126/science.2479986.

Li, Shuangling, Hongli Yang, Kun Li, Guiling Fan, Li Deng, and Changqing Xu. 2020. "Thymidine Phosphorylase Promotes Angiogenesis and Tumour Growth in Intrahepatic Cholangiocarcinoma." *Cell Biochemistry and Function* 38 (6): 743–52. https://doi.org/10.1002/cbf.3541.

Li, Wei, Alba Gigante, Maria-Jesus Perez-Perez, Hong Yue, Michio Hirano, Thomas McIntyre, and Roy L Silverstein. 2014. "Thymidine Phosphorylase Participates in Platelet Signaling and Promotes Thrombosis." *Circulation Research* 115 (12): 997–1006. https://doi.org/10.1161/CIRCRESAHA.115.304591.

Li, Wei, and Hong Yue. 2021. "Thymidine Phosphorylase Is Increased in COVID-19 Patients in an Acuity-Dependent Manner." *Frontiers in Medicine* 8. https://www.frontiersin.org/articles/10.3389/fmed.2021.653773.

Li, Zihai, and Pramod Srivastava. 2003. "Heat-Shock Proteins." *Current Protocols in Immunology* 58 (1): A.1T.1-A.1T.6. https://doi.org/10.1002/0471142735.ima01ts58.

Liang, Wei, and Napoleone Ferrara. 2016. "The Complex Role of Neutrophils in Tumor Angiogenesis and Metastasis." *Cancer Immunology Research* 4 (2): 83–91. https://doi.org/10.1158/2326-6066.CIR-15-0313.

Lin, Chuyong, Zhiqiang Wu, Xi Lin, Chunping Yu, Tingting Shi, Yong Zeng, Xi Wang, Jun Li, and Libing Song. 2011. “Knockdown of FLOT1 Impairs Cell Proliferation and Tumorigenicity in Breast Cancer through Upregulation of FOXO3a.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 17 (10): 3089–99. https://doi.org/10.1158/1078-0432.CCR-10-3068.

Lin, Elaine Y., Jiu-Feng Li, Leoid Gnatovskiy, Yan Deng, Liyin Zhu, Dustin A. Grzesik, Hong Qian, Xiao-nan Xue, and Jeffrey W. Pollard. 2006. “Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer.” *Cancer Research* 66 (23): 11238–46. https://doi.org/10.1158/0008-5472.CAN-06-1278.

Lin, Shuei Liong, Ana P. Castaño, Brian T. Nowlin, Mark L. Lupher Jr., and Jeremy S. Duffield. 2009. “Bone Marrow Ly6Chigh Monocytes Are Selectively Recruited to Injured Kidney and Differentiate into Functionally Distinct Populations1.” *The Journal of Immunology* 183 (10): 6733–43. https://doi.org/10.4049/jimmunol.0901473.

Liu, Jieqiong, Shan Liao, Yuhui Huang, Rekha Samuel, Tony Shi, Kamila Naxerova, Peigen Huang, et al. 2011. “PDGF-D Improves Drug Delivery and Efficacy via Vascular Normalization, but Promotes Lymphatic Metastasis by Activating CXCR4 in Breast Cancer.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 17 (11): 3638–48. https://doi.org/10.1158/1078-0432.CCR-10-2456.

Liu, Y., A. A. Jesus, B. Marrero, D. Yang, S. E. Ramsey, G. A. Montealegre Sanchez, K. Tenbrock, et al. 2014. “Activated STING in a Vascular and Pulmonary Syndrome.” *The New England Journal of Medicine* 371 (6): 507–18. https://doi.org/10.1056/NEJMoa1312625.

Lother, Achim, Stella Bergemann, Lisa Deng, Martin Moser, Christoph Bode, and Lutz Hein. 2018. “Cardiac Endothelial Cell Transcriptome.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 38 (3): 566–74. https://doi.org/10.1161/ATVBAHA.117.310549.

Lu, Changming, and Mary MacDougall. 2017. “RIG-I-Like Receptor Signaling in Singleton-Merten Syndrome.” *Frontiers in Genetics* 8. https://www.frontiersin.org/articles/10.3389/fgene.2017.00118.

Lugano, Roberta, Mohanraj Ramachandran, and Anna Dimberg. 2020. “Tumor Angiogenesis: Causes, Consequences, Challenges and Opportunities.” *Cellular and Molecular Life Sciences* 77 (9): 1745–70. https://doi.org/10.1007/s00018-019-03351-7.

Lüscher, Thomas F., and Felix C. Tanner. 1993. “Endothelial Regulation of Vascular Tone and Growth.” *American Journal of Hypertension* 6 (7_Pt_2): 283S-293S. https://doi.org/10.1093/ajh/6.7.283S.

Ma, Bing, Charles S. Dela Cruz, Dominik Hartl, Min-Jong Kang, Shervin Takyar, Robert J. Homer, Chun Geun Lee, and Jack A. Elias. 2011. “RIG-like Helicase Innate Immunity Inhibits Vascular Endothelial Growth Factor Tissue Responses via a Type I IFN-Dependent Mechanism.” *American Journal of Respiratory and Critical Care Medicine* 183 (10): 1322–35. https://doi.org/10.1164/rccm.201008-1276OC.

Maharjan, Sony, Kyeojin Kim, Vijayendra Agrawal, Hyun-Jung Choi, Nam-Jung Kim, Young-Myeong Kim, Young-Ger Suh, and Young-Guen Kwon. 2013. “Sac-1004, a Novel Vascular Leakage Blocker, Enhances Endothelial Barrier through the cAMP/Rac/Cortactin Pathway.” *Biochemical and Biophysical Research Communications* 435 (3): 420–27. https://doi.org/10.1016/j.bbrc.2013.04.104.

Maniotis, Andrew J., Robert Folberg, Angela Hess, Elisabeth A. Seftor, Lynn M. G. Gardner, Jacob Pe'er, Jeffrey M. Trent, Paul S. Meltzer, and Mary J. C. Hendrix. 1999. "Vascular Channel Formation by Human Melanoma Cells in Vivo and in Vitro: Vasculogenic Mimicry." *The American Journal of Pathology* 155 (3): 739–52. https://doi.org/10.1016/S0002-9440(10)65173-5.

Mansoori, Behzad, Ali Mohammadi, Solmaz Shir Jang, and Behzad Baradaran. 2016. "Mechanisms of Immune System Activation in Mammalians by Small Interfering RNA (siRNA)." *Artificial Cells, Nanomedicine, and Biotechnology* 44 (7): 1589–96. https://doi.org/10.3109/21691401.2015.1102738.

Marin-Hernandez, Alvaro, Juan C. Gallardo-Perez, Stephen J. Ralph, Sara Rodriguez-Enriquez, and Rafael Moreno-Sanchez. 2009. "HIF-1α Modulates Energy Metabolism in Cancer Cells by Inducing Over-Expression of Specific Glycolytic Isoforms." *Mini Reviews in Medicinal Chemistry* 9 (9): 1084–1101. https://doi.org/10.2174/138955709788922610.

Marques, Rafael Elias, Rodrigo Guabiraba, Remo Castro Russo, and Mauro Martins Teixeira. 2013. "Targeting CCL5 in Inflammation." *Expert Opinion on Therapeutic Targets* 17 (12): 1439–60. https://doi.org/10.1517/14728222.2013.837886.

Mazzone, Massimiliano, Daniela Dettori, Rodrigo Leite de Oliveira, Sonja Loges, Thomas Schmidt, Bart Jonckx, Ya-Min Tian, et al. 2009. "Heterozygous Deficiency of PHD2 Restores Tumor Oxygenation and Inhibits Metastasis via Endothelial Normalization." *Cell* 136 (5): 839–51. https://doi.org/10.1016/j.cell.2009.01.020.

McCarty, Marya F., Ray J. Somcio, Oliver Stoeltzing, Jane Wey, Fan Fan, Wenbiao Liu, Corazon Bucana, and Lee M. Ellis. 2007. "Overexpression of PDGF-BB Decreases

Colorectal and Pancreatic Cancer Growth by Increasing Tumor Pericyte Content." *The Journal of Clinical Investigation* 117 (8): 2114–22. https://doi.org/10.1172/JCI31334.

McCoy, Michael G., Daniel W. Nascimento, Manoj Veleeparambil, Rakhylia Murtazina, Detao Gao, Svyatoslav Tkachenko, Eugene Podrez, and Tatiana V. Byzova. 2021. "Endothelial TLR2 Promotes Proangiogenic Immune Cell Recruitment and Tumor Angiogenesis." *Science Signaling* 14 (666): eabc5371. https://doi.org/10.1126/scisignal.abc5371.

McQuaid, Conor, and Axel Montagne. 2022. "SARS-CoV-2 and Vascular Dysfunction: A Growing Role for Pericytes." *Cardiovascular Research*, September, cvac143. https://doi.org/10.1093/cvr/cvac143.

Melincovici, Carmen Stanca, Adina Bianca Boşca, Sergiu Şuşman, Mariana Mărginean, Carina Mihu, Mihnea Istrate, Ioana Maria Moldovan, Alexandra Livia Roman, and Carmen Mihaela Mihu. 2018. "Vascular Endothelial Growth Factor (VEGF) - Key Factor in Normal and Pathological Angiogenesis." *Romanian Journal of Morphology and Embryology = Revue Roumaine De Morphologie Et Embryologie* 59 (2): 455–67.

Miller, Kathy, Molin Wang, Julie Gralow, Maura Dickler, Melody Cobleigh, Edith A. Perez, Tamara Shenkier, David Cella, and Nancy E. Davidson. 2007. "Paclitaxel plus Bevacizumab versus Paclitaxel Alone for Metastatic Breast Cancer." *The New England Journal of Medicine* 357 (26): 2666–76. https://doi.org/10.1056/NEJMoa072113.

Monk, Bradley J., Andrés Poveda, Ignace Vergote, Francesco Raspagliesi, Keiichi Fujiwara, Duk-Soo Bae, Ana Oaknin, et al. 2016. "Final Results of a Phase 3 Study of Trebananib plus Weekly Paclitaxel in Recurrent Ovarian Cancer (TRINOVA-1): Long-Term Survival, Impact of Ascites, and Progression-Free Survival-2." *Gynecologic Oncology* 143 (1): 27–34. https://doi.org/10.1016/j.ygyno.2016.07.112.

Morikawa, Shunichi, Peter Baluk, Toshiyuki Kaidoh, Amy Haskell, Rakesh K. Jain, and Donald M. McDonald. 2002. “Abnormalities in Pericytes on Blood Vessels and Endothelial Sprouts in Tumors.” *The American Journal of Pathology* 160 (3): 985–1000. https://doi.org/10.1016/S0002-9440(10)64920-6.

Morrow, Gael B., and Nicola J. Mutch. 2023. “Past, Present, and Future Perspectives of Plasminogen Activator Inhibitor 1 (PAI-1).” *Seminars in Thrombosis and Hemostasis* 49 (3): 305–13. https://doi.org/10.1055/s-0042-1758791.

Moser, Jill, Peter Heeringa, Rianne M. Jongman, Peter J. Zwiers, Anita E. Niemarkt, Rui Yan, Inge A. de Graaf, et al. 2016. “Intracellular RIG-I Signaling Regulates TLR4-Independent Endothelial Inflammatory Responses to Endotoxin.” *Journal of Immunology (Baltimore, Md.: 1950)* 196 (11): 4681–91. https://doi.org/10.4049/jimmunol.1501819.

Murdoch, Craig, Munitta Muthana, Seth B. Coffelt, and Claire E. Lewis. 2008. “The Role of Myeloid Cells in the Promotion of Tumour Angiogenesis.” *Nature Reviews Cancer* 8 (8): 618–31. https://doi.org/10.1038/nrc2444.

Murphy, Gwen, Susan S. Devesa, Amanda J. Cross, Peter D. Inskip, Katherine A. McGlynn, and Michael B. Cook. 2011. “Sex Disparities in Colorectal Cancer Incidence by Anatomic Subsite, Race and Age.” *International Journal of Cancer* 128 (7): 1668–75. https://doi.org/10.1002/ijc.25481.

Mussbacher, Marion, Klaudia Schossleitner, Julia B. Kral-Pointner, Manuel Salzmann, Astrid Schrammel, and Johannes A. Schmid. 2022. “More than Just a Monolayer: The Multifaceted Role of Endothelial Cells in the Pathophysiology of Atherosclerosis.” *Current Atherosclerosis Reports* 24 (6): 483–92. https://doi.org/10.1007/s11883-022-01023-9.

Nalluri, Shobha Rani, David Chu, Roger Keresztes, Xiaolei Zhu, and Shenhong Wu. 2008. “Risk of Venous Thromboembolism with the Angiogenesis Inhibitor Bevacizumab in Cancer Patients: A Meta-Analysis.” *JAMA* 300 (19): 2277–85. https://doi.org/10.1001/jama.2008.656.

Niethamer, Terren K, Collin T Stabler, John P Leach, Jarod A Zepp, Michael P Morley, Apoorva Babu, Su Zhou, and Edward E Morrisey. 2020. “Defining the Role of Pulmonary Endothelial Cell Heterogeneity in the Response to Acute Lung Injury.” Edited by Gordana Vunjak-Novakovic and Didier YR Stainier. *eLife* 9 (February): e53072. https://doi.org/10.7554/eLife.53072.

Nozawa, Hiroaki, Christopher Chiu, and Douglas Hanahan. 2006. “Infiltrating Neutrophils Mediate the Initial Angiogenic Switch in a Mouse Model of Multistage Carcinogenesis.” *Proceedings of the National Academy of Sciences of the United States of America* 103 (33): 12493–98. https://doi.org/10.1073/pnas.0601807103.

Oestreich, Kenneth J., Hyesuk Yoon, Rafi Ahmed, and Jeremy M. Boss. 2008. “NFATc1 Regulates PD-1 Expression upon T Cell Activation1.” *The Journal of Immunology* 181 (7): 4832–39. https://doi.org/10.4049/jimmunol.181.7.4832.

Ohtsu, Atsushi, Manish A. Shah, Eric Van Cutsem, Sun Young Rha, Akira Sawaki, Sook Ryun Park, Ho Yeong Lim, et al. 2011. “Bevacizumab in Combination with Chemotherapy as First-Line Therapy in Advanced Gastric Cancer: A Randomized, Double-Blind, Placebo-Controlled Phase III Study.” *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 29 (30): 3968–76. https://doi.org/10.1200/JCO.2011.36.2236.

Opitz, Bastian, Julia Eitel, Karolin Meixenberger, and Norbert Suttorp. 2009. “Role of Toll-like Receptors, NOD-like Receptors and RIG-I-like Receptors in Endothelial Cells and Systemic Infections.” *Thrombosis and Haemostasis* 102 (6): 1103–9. https://doi.org/10.1160/TH09-05-0323.

Orfali, Nina, Deborah Shan-Krauer, Tracey R. O’Donovan, Nigel P. Mongan, Lorraine J. Gudas, Mary R. Cahill, Mario P. Tschan, and Sharon L. McKenna. 2020. “Inhibition of UBE2L6 Attenuates ISGylation and Impedes ATRA-Induced Differentiation of Leukemic Cells.” *Molecular Oncology* 14 (6): 1297–1309. https://doi.org/10.1002/1878-0261.12614.

Ori, Daisuke, Motoya Murase, and Taro Kawai. 2017. “Cytosolic Nucleic Acid Sensors and Innate Immune Regulation.” *International Reviews of Immunology* 36 (2): 74–88. https://doi.org/10.1080/08830185.2017.1298749.

Oyama, Tsunehiro, Sophia Ran, Tadao Ishida, Sorena Nadaf, Lawrence Kerr, David P. Carbone, and Dmitry I. Gabrilovich. 1998. “Vascular Endothelial Growth Factor Affects Dendritic Cell Maturation Through the Inhibition of Nuclear Factor-κB Activation in Hemopoietic Progenitor Cells1.” *The Journal of Immunology* 160 (3): 1224–32. https://doi.org/10.4049/jimmunol.160.3.1224.

Pallarés, Judith, Núria Torreguitart, Gloria Arqué, Manuel Portero-Otin, and Francisco Purroy. 2022. “Human Atheromatous Plaques Expressed Sensing Adaptor STING, a Potential Role in Vascular Inflammation Pathogenesis.” *Thrombosis and Haemostasis* 122 (9): 1621–24. https://doi.org/10.1055/a-1772-1192.

Pan, Ping-Ying, George X. Wang, Bingjiao Yin, Junko Ozao, Teresa Ku, Celia M. Divino, and Shu-Hsia Chen. 2008. “Reversion of Immune Tolerance in Advanced Malignancy:

Modulation of Myeloid-Derived Suppressor Cell Development by Blockade of Stem-Cell Factor Function." *Blood* 111 (1): 219–28. https://doi.org/10.1182/blood-2007-04-086835.

Pang, Iris K., Padmini S. Pillai, and Akiko Iwasaki. 2013. "Efficient Influenza A Virus Replication in the Respiratory Tract Requires Signals from TLR7 and RIG-I." *Proceedings of the National Academy of Sciences* 110 (34): 13910–15. https://doi.org/10.1073/pnas.1303275110.

Patan, Sybill. 2004. "Vasculogenesis and Angiogenesis." *Cancer Treatment and Research* 117: 3–32. https://doi.org/10.1007/978-1-4419-8871-3_1.

Pavet, V., Y. Shlyakhtina, T. He, D. G. Ceschin, P. Kohonen, M. Perälä, O. Kallioniemi, and H. Gronemeyer. 2014. "Plasminogen Activator Urokinase Expression Reveals TRAIL Responsiveness and Supports Fractional Survival of Cancer Cells." *Cell Death & Disease* 5 (1): e1043. https://doi.org/10.1038/cddis.2014.5.

Pisitkun, Prapaporn, Jonathan A. Deane, Michael J. Difilippantonio, Tatyana Tarasenko, Anne B. Satterthwaite, and Silvia Bolland. 2006. "Autoreactive B Cell Responses to RNA-Related Antigens Due to TLR7 Gene Duplication." *Science (New York, N.Y.)* 312 (5780): 1669–72. https://doi.org/10.1126/science.1124978.

Pober, Jordan S. 2002. "Endothelial Activation: Intracellular Signaling Pathways." *Arthritis Research & Therapy* 4 (3): S109. https://doi.org/10.1186/ar576.

Pober, Jordan S., and William C. Sessa. 2007. "Evolving Functions of Endothelial Cells in Inflammation." *Nature Reviews Immunology* 7 (10): 803–15. https://doi.org/10.1038/nri2171.

Poisson, Johanne, Sara Lemoinne, Chantal Boulanger, François Durand, Richard Moreau, Dominique Valla, and Pierre-Emmanuel Rautou. 2017. “Liver Sinusoidal Endothelial Cells: Physiology and Role in Liver Diseases.” *Journal of Hepatology* 66 (1): 212–27. https://doi.org/10.1016/j.jhep.2016.07.009.

Prieto, Carlos, and David Barrios. 2020. “RaNA-Seq: Interactive RNA-Seq Analysis from FASTQ Files to Functional Analysis.” *Bioinformatics* 36 (6): 1955–56. https://doi.org/10.1093/bioinformatics/btz854.

Pula, Giordano, Ursula Mayr, Colin Evans, Marianna Prokopi, Dina S. Vara, Xiaoke Yin, Zoe Astroulakis, et al. 2009. “Proteomics Identifies Thymidine Phosphorylase as a Key Regulator of the Angiogenic Potential of Colony-Forming Units and Endothelial Progenitor Cell Cultures.” *Circulation Research* 104 (1): 32–40. https://doi.org/10.1161/CIRCRESAHA.108.182261.

“RaNA-Seq: Interactive RNA-Seq Analysis from FASTQ Files to Functional Analysis | Bioinformatics | Oxford Academic.” n.d. Accessed December 6, 2023. https://academic.oup.com/bioinformatics/article/36/6/1955/5626181.

Reck, Martin, Joachim von Pawel, Petr Zatloukal, Rodryg Ramlau, Vera Gorbounova, Vera Hirsh, Natasha Leighl, et al. 2009. “Phase III Trial of Cisplatin plus Gemcitabine with Either Placebo or Bevacizumab as First-Line Therapy for Nonsquamous Non-Small-Cell Lung Cancer: AVAil.” *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 27 (8): 1227–34. https://doi.org/10.1200/JCO.2007.14.5466.

Rehwinkel, Jan, and Michaela U. Gack. 2020. “RIG-I-like Receptors: Their Regulation and Roles in RNA Sensing.” *Nature Reviews Immunology* 20 (9): 537–51. https://doi.org/10.1038/s41577-020-0288-3.

Reymond, Nicolas, Bárbara Borda d'Água, and Anne J. Ridley. 2013. "Crossing the Endothelial Barrier during Metastasis." *Nature Reviews Cancer* 13 (12): 858–70. https://doi.org/10.1038/nrc3628.

Risau, Werner. 1997. "Mechanisms of Angiogenesis." *Nature* 386 (6626): 671–74. https://doi.org/10.1038/386671a0.

Risau, Werner, and Ingo Flamme. 1995. "Vasculogenesis." *Annual Review of Cell and Developmental Biology* 11 (1): 73–91. https://doi.org/10.1146/annurev.cb.11.110195.000445.

Rita, M., and I. Young. 2012. "Endothelial Cells in the Eyes of an Immunologist." *Cancer Immunology, Immunotherapy : CII* 61 (10): 1609–16. https://doi.org/10.1007/s00262-012-1335-0.

Rivera, Lee B., and Gabriele Bergers. 2015. "Myeloid Cell-Driven Angiogenesis and Immune Regulation in Tumors." *Trends in Immunology* 36 (4): 240–49. https://doi.org/10.1016/j.it.2015.02.005.

Ronchi, Dario, Leonardo Caporali, Giulia Francesca Manenti, Megi Meneri, Susan Mohamed, Andreina Bordoni, Francesca Tagliavini, et al. 2020. "TYMP Variants Result in Late-Onset Mitochondrial Myopathy With Altered Muscle Mitochondrial DNA Homeostasis." *Frontiers in Genetics* 11: 860. https://doi.org/10.3389/fgene.2020.00860.

Russell, Clark D., Asta Valanciute, Naomi N. Gachanja, Jillian Stephen, Rebekah Penrice-Randal, Stuart D. Armstrong, Sara Clohisey, et al. 2022. "Tissue Proteomic Analysis Identifies Mechanisms and Stages of Immunopathology in Fatal COVID-19." *American Journal of Respiratory Cell and Molecular Biology* 66 (2): 196–205. https://doi.org/10.1165/rcmb.2021-0358OC.

Salsman, Vita S., Kevin K. H. Chow, Donald R. Shaffer, Huseyin Kadikoy, Xiao-Nan Li, Claudia Gerken, Laszlo Perlaky, et al. 2011. “Crosstalk between Medulloblastoma Cells and Endothelium Triggers a Strong Chemotactic Signal Recruiting T Lymphocytes to the Tumor Microenvironment.” *PloS One* 6 (5): e20267. https://doi.org/10.1371/journal.pone.0020267.

Schlee, Martin, and Gunther Hartmann. 2016. “Discriminating Self from Non-Self in Nucleic Acid Sensing.” *Nature Reviews Immunology* 16 (9): 566–80. https://doi.org/10.1038/nri.2016.78.

Schmall, Anja, Hamza M. Al-Tamari, Susanne Herold, Marian Kampschulte, Andreas Weigert, Astrid Wietelmann, Natasha Vipotnik, et al. 2015. “Macrophage and Cancer Cell Cross-Talk via CCR2 and CX3CR1 Is a Fundamental Mechanism Driving Lung Cancer.” *American Journal of Respiratory and Critical Care Medicine* 191 (4): 437–47. https://doi.org/10.1164/rccm.201406-1137OC.

Senger, Donald R., Stephen J. Galli, Ann M. Dvorak, Carole A. Perruzzi, V. Susan Harvey, and Harold F. Dvorak. 1983. “Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid.” *Science* 219 (4587): 983–85. https://doi.org/10.1126/science.6823562.

Sharp, Frank R., Stephen M. Massa, and Raymond A. Swanson. 1999. “Heat-Shock Protein Protection.” *Trends in Neurosciences* 22 (3): 97–99. https://doi.org/10.1016/S0166-2236(98)01392-7.

Shi, Chung-Sheng, Tzu-Hsiung Huang, Chin-Kuo Lin, Jhy-Ming Li, Mei-Hsin Chen, Mei-Ling Tsai, and Chih-Ching Chang. 2016. “VEGF Production by Ly6C+high Monocytes

Contributes to Ventilator-Induced Lung Injury." *PLOS ONE* 11 (10): e0165317. https://doi.org/10.1371/journal.pone.0165317.

Shibuya, Masaubmi. 2006. "Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1/Flt-1): A Dual Regulator for Angiogenesis." *Angiogenesis* 9 (4): 225–30; discussion 231. https://doi.org/10.1007/s10456-006-9055-8.

Sørensen, Karen Kristine, Jaione Simon-Santamaria, Robert S. McCuskey, and Bård Smedsrød. 2015. "Liver Sinusoidal Endothelial Cells." In *Comprehensive Physiology*, 1751–74. John Wiley & Sons, Ltd. https://doi.org/10.1002/cphy.c140078.

Staufer, K., and O. Stoeltzing. 2010. "Implication of Heat Shock Protein 90 (HSP90) in Tumor Angiogenesis: A Molecular Target for Anti-Angiogenic Therapy?" *Current Cancer Drug Targets* 10 (8): 890–97. https://doi.org/10.2174/156800910793357934.

Stok, Jorn E., M. Eloisa Vega Quiroz, and Annemarthe G. van der Veen. 2020. "Self RNA Sensing by RIG-I–like Receptors in Viral Infection and Sterile Inflammation." *The Journal of Immunology* 205 (4): 883–91. https://doi.org/10.4049/jimmunol.2000488.

Suffee, Nadine, Benjamin Richard, Hanna Hlawaty, Olivier Oudar, Nathalie Charnaux, and Angela Sutton. 2011. "Angiogenic Properties of the Chemokine RANTES/CCL5." *Biochemical Society Transactions* 39 (6): 1649–53. https://doi.org/10.1042/BST20110651.

Sun, Jianxin, and James K. Liao. 2004. "Induction of Angiogenesis by Heat Shock Protein 90 Mediated by Protein Kinase Akt and Endothelial Nitric Oxide Synthase." *Arteriosclerosis, Thrombosis, and Vascular Biology* 24 (12): 2238–44. https://doi.org/10.1161/01.ATV.0000147894.22300.4c.

Tabata, Sho, Ryuji Ikeda, Masatatu Yamamoto, Tatsuhiko Furukawa, Takuya Kuramoto, Yasuo Takeda, Katsushi Yamada, et al. 2012. “Thymidine Phosphorylase Enhances Reactive Oxygen Species Generation and Interleukin-8 Expression in Human Cancer Cells.” *Oncology Reports* 28 (3): 895–902. https://doi.org/10.3892/or.2012.1887.

Takahashi, Hiroyuki, and Masabumi Shibuya. 2005. “The Vascular Endothelial Growth Factor (VEGF)/VEGF Receptor System and Its Role under Physiological and Pathological Conditions.” *Clinical Science* 109 (3): 227–41. https://doi.org/10.1042/CS20040370.

Takeuchi, Osamu, and Shizuo Akira. 2010. “Pattern Recognition Receptors and Inflammation.” *Cell* 140 (6): 805–20. https://doi.org/10.1016/j.cell.2010.01.022.

Taylor, Peter C. 2002. “VEGF and Imaging of Vessels in Rheumatoid Arthritis.” *Arthritis Research & Therapy* 4 (3): S99. https://doi.org/10.1186/ar582.

Teijaro, John R., Kevin B. Walsh, Stuart Cahalan, Daniel M. Fremgen, Edward Roberts, Fiona Scott, Esther Martinborough, Robert Peach, Michael B. A. Oldstone, and Hugh Rosen. 2011. “Endothelial Cells Are Central Orchestrators of Cytokine Amplification during Influenza Virus Infection.” *Cell* 146 (6): 980–91. https://doi.org/10.1016/j.cell.2011.08.015.

Teuwen, Laure-Anne, Vincent Geldhof, Alessandra Pasut, and Peter Carmeliet. 2020. “COVID-19: The Vasculature Unleashed.” *Nature Reviews Immunology* 20 (7): 389–91. https://doi.org/10.1038/s41577-020-0343-0.

Thomas, Markus, and Hellmut G. Augustin. 2009. “The Role of the Angiopoietins in Vascular Morphogenesis.” *Angiogenesis* 12 (2): 125–37. https://doi.org/10.1007/s10456-009-9147-3.

Tigano, Marco, Danielle C. Vargas, Samuel Tremblay-Belzile, Yi Fu, and Agnel Sfeir. 2021. "Nuclear Sensing of Breaks in Mitochondrial DNA Enhances Immune Surveillance." *Nature* 591 (7850): 477–81. https://doi.org/10.1038/s41586-021-03269-w.

Timke, Carmen, Heike Zieher, Alexandra Roth, Kai Hauser, Kenneth E. Lipson, Klaus J. Weber, Jürgen Debus, Amir Abdollahi, and Peter E. Huber. 2008. "Combination of Vascular Endothelial Growth Factor Receptor/Platelet-Derived Growth Factor Receptor Inhibition Markedly Improves Radiation Tumor Therapy." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 14 (7): 2210–19. https://doi.org/10.1158/1078-0432.CCR-07-1893.

Tolaney, Sara M., Yves Boucher, Dan G. Duda, John D. Martin, Giorgio Seano, Marek Ancukiewicz, William T. Barry, et al. 2015. "Role of Vascular Density and Normalization in Response to Neoadjuvant Bevacizumab and Chemotherapy in Breast Cancer Patients." *Proceedings of the National Academy of Sciences of the United States of America* 112 (46): 14325–30. https://doi.org/10.1073/pnas.1518808112.

Uchimiya, Hiroshi, Tatsuhiko Furukawa, Masahiro Okamoto, Yuichi Nakajima, Shigeto Matsushita, Ryuji Ikeda, Takenari Gotanda, et al. 2002. "Suppression of Thymidine Phosphorylase-Mediated Angiogenesis and Tumor Growth by 2-Deoxy-L-Ribose." *Cancer Research* 62 (10): 2834–39.

Upile, Tahwinder, Waseem Jerjes, Hani Radhi, Mohammed Al-Khawalde, Panagiotis Kafas, Seyed Nouraei, and Holger Sudhoff. 2011. "Vascular Mimicry in Cultured Head and Neck Tumour Cell Lines." *Head & Neck Oncology* 3 (1): 55. https://doi.org/10.1186/1758-3284-3-55.

Vandenberghe, Peter, Jan Delabie, Mark de Boer, Chris De Wolf-Peeters, and Jan L. Ceuppens. 1993. “In Situ Expression of B7/BB1 on Antigenpresenting Cells and Activated B Cells: An Immunohistochemical Study.” *International Immunology* 5 (3): 317–21. https://doi.org/10.1093/intimm/5.3.317.

Vanpouille-Box, Claire, Jules A. Hoffmann, and Lorenzo Galluzzi. 2019. “Pharmacological Modulation of Nucleic Acid Sensors — Therapeutic Potential and Persisting Obstacles.” *Nature Reviews Drug Discovery* 18 (11): 845–67. https://doi.org/10.1038/s41573-019-0043-2.

Voron, T., O. Colussi, E. Marcheteau, S. Pernot, M. Nizard, A.-L. Pointet, S. Latreche, et al. 2015. “VEGF-A Modulates Expression of Inhibitory Checkpoints on CD8++ T Cells in Tumors.” *Journal of Experimental Medicine* 212 (2): 139–48. https://doi.org/10.1084/jem.20140559.

Wada, J., H. Suzuki, R. Fuchino, A. Yamasaki, S. Nagai, K. Yanai, K. Koga, et al. 2009. “The Contribution of Vascular Endothelial Growth Factor to the Induction of Regulatory T-Cells in Malignant Effusions.” *Anticancer Research* 29 (3): 881–88.

Wagner, Nana-Maria, Laura Bierhansl, Gabriele Nöldge-Schomburg, Brigitte Vollmar, and Jan Patrick Roesner. 2013. “Toll-Like Receptor 2–Blocking Antibodies Promote Angiogenesis and Induce ERK1/2 and AKT Signaling via CXCR4 in Endothelial Cells.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 33 (8): 1943–51. https://doi.org/10.1161/ATVBAHA.113.301783.

Wang, Gangqi, Sarantos Kostidis, Gesa L. Tiemeier, Wendy M. P. J. Sol, Margreet R. de Vries, Martin Giera, Peter Carmeliet, Bernard M. van den Berg, and Ton J. Rabelink. 2020. “Shear Stress Regulation of Endothelial Glycocalyx Structure Is Determined by

Glucobiosynthesis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 40 (2): 350–64. https://doi.org/10.1161/ATVBAHA.119.313399.

Wang, Jennifer P., Anna Cerny, Damon R. Asher, Evelyn A. Kurt-Jones, Roderick T. Bronson, and Robert W. Finberg. 2010. "MDA5 and MAVS Mediate Type I Interferon Responses to Coxsackie B Virus." *Journal of Virology* 84 (1): 254–60. https://doi.org/10.1128/jvi.00631-09.

Wang, Shih-Wei, Shih-Chia Liu, Hui-Lung Sun, Te-Yang Huang, Chia-Han Chan, Chen-Yu Yang, Hung-I Yeh, et al. 2015. "CCL5/CCR5 Axis Induces Vascular Endothelial Growth Factor-Mediated Tumor Angiogenesis in Human Osteosarcoma Microenvironment." *Carcinogenesis* 36 (1): 104–14. https://doi.org/10.1093/carcin/bgu218.

Wang, Yi, Hong-Xin Zhang, Yue-Ping Sun, Zi-Xing Liu, Xue-Song Liu, Long Wang, Shun-Yuan Lu, et al. 2007. "Rig-I−/− Mice Develop Colitis Associated with Downregulation of Gαi2." *Cell Research* 17 (10): 858–68. https://doi.org/10.1038/cr.2007.81.

Weber-Gerlach, Michaela, and Friedemann Weber. 2016. "Standing on Three Legs: Antiviral Activities of RIG-I against Influenza Viruses." *Current Opinion in Immunology*, Allergy and hypersensitivity * Host pathogens, 42 (October): 71–75. https://doi.org/10.1016/j.coi.2016.05.016.

Weis, Sara M., and David A. Cheresh. 2011. "Tumor Angiogenesis: Molecular Pathways and Therapeutic Targets." *Nature Medicine* 17 (11): 1359–70. https://doi.org/10.1038/nm.2537.

Wen, Guangzheng. 2017. "A Simple Process of RNA-Sequence Analyses by Hisat2, Htseq and DESeq2." In *Proceedings of the 2017 International Conference on Biomedical*

*Engineering and Bioinformatics*, 11–15. ICBEB 2017. New York, NY, USA: Association for Computing Machinery. https://doi.org/10.1145/3143344.3143354.

Widakowich, Christian, Gilberto de Castro, Evandro de Azambuja, Phuong Dinh, and Ahmad Awada. 2007. "Review: Side Effects of Approved Molecular Targeted Therapies in Solid Cancers." *The Oncologist* 12 (12): 1443–55. https://doi.org/10.1634/theoncologist.12-12-1443.

Wilhelmsen, Kevin, Kailin R. Mesa, Arun Prakash, Fengyun Xu, and Judith Hellman. 2012. "Activation of Endothelial TLR2 by Bacterial Lipoprotein Upregulates Proteins Specific for the Neutrophil Response." *Innate Immunity* 18 (4): 602–16. https://doi.org/10.1177/1753425911429336.

Wilting, Jörg, Ralf Birkenhäger, Anne Eichmann, Haymo Kurz, Georg Martiny-Baron, Dieter Marmé, John E. G. McCarthy, Bodo Christ, and Herbert A. Weich. 1996. "VEGF121Induces Proliferation of Vascular Endothelial Cells and Expression Offlk-1without Affecting Lymphatic Vessels of the Chorioallantoic Membrane." *Developmental Biology* 176 (1): 76–85. https://doi.org/10.1006/dbio.1996.9993.

Winkler, Frank. 2017. "Hostile Takeover: How Tumours Hijack Pre-Existing Vascular Environments to Thrive." *The Journal of Pathology* 242 (3): 267–72. https://doi.org/10.1002/path.4904.

Wu, Bin, and Sun Hur. 2015. "How RIG-I like Receptors Activate MAVS." *Current Opinion in Virology*, Antiviral strategies • Virus structure and expression, 12 (June): 91–98. https://doi.org/10.1016/j.coviro.2015.04.004.

Wu, Zhe Bao, Lin Cai, Shao Jian Lin, Zhi Gen Leng, Yu Hang Guo, Wen Lei Yang, Yi Wei Chu, Shao-Hua Yang, and Wei Guo Zhao. 2016. “Heat Shock Protein 47 Promotes Glioma Angiogenesis.” *Brain Pathology* 26 (1): 31–42. https://doi.org/10.1111/bpa.12256.

Xie, Zhuorui, Allison Bailey, Maxim V. Kuleshov, Daniel J. B. Clarke, John E. Evangelista, Sherry L. Jenkins, Alexander Lachmann, et al. 2021. “Gene Set Knowledge Discovery with Enrichr.” *Current Protocols* 1 (3): e90. https://doi.org/10.1002/cpz1.90.

Xu, Yifei, Ying Zhou, Haiyan Lin, Haiyang Hu, Yuxing Wang, and Geng Xu. 2013. “Toll-like Receptor 2 in Promoting Angiogenesis after Acute Ischemic Injury.” *International Journal of Molecular Medicine* 31 (3): 555–60. https://doi.org/10.3892/ijmm.2013.1240.

Yang, Hannah, Won Suk Lee, So Jung Kong, Chang Gon Kim, Joo Hoon Kim, Sei Kyung Chang, Sewha Kim, Gwangil Kim, Hong Jae Chon, and Chan Kim. 2019. “STING Activation Reprograms Tumor Vasculatures and Synergizes with VEGFR2 Blockade.” *The Journal of Clinical Investigation* 129 (10): 4350–64. https://doi.org/10.1172/JCI125413.

Yang, Li, Laura M. DeBusk, Koari Fukuda, Barbara Fingleton, Brenda Green-Jarvis, Yu Shyr, Lynn M. Matrisian, David P. Carbone, and P. Charles Lin. 2004. “Expansion of Myeloid Immune Suppressor Gr+CD11b+ Cells in Tumor-Bearing Host Directly Promotes Tumor Angiogenesis.” *Cancer Cell* 6 (4): 409–21. https://doi.org/10.1016/j.ccr.2004.08.031.

Yang, XueZhi, Yan Chang, and Wei Wei. 2016. “Endothelial Dysfunction and Inflammation: Immunity in Rheumatoid Arthritis.” *Mediators of Inflammation* 2016 (March): e6813016. https://doi.org/10.1155/2016/6813016.

Zhang, Junlan, Wenli Yang, Bao Luo, Bingqian Hu, Akhil Maheshwari, and Michael B. Fallon. 2012. “The Role of CX3CL1/CX3CR1 in Pulmonary Angiogenesis and Intravascular

Monocyte Accumulation in Rat Experimental Hepatopulmonary Syndrome." *Journal of Hepatology* 57 (4): 752–58. https://doi.org/10.1016/j.jhep.2012.05.014.

Zhang, Xianglan, Zhenlong Zheng, You Keun Shin, Ki-Yeol Kim, Sun Young Rha, Sung Hoon Noh, Hyun Cheol Chung, and Hei-Cheul Jeung. 2014. "Angiogenic Factor Thymidine Phosphorylase Associates with Angiogenesis and Lymphangiogenesis in the Intestinal-Type Gastric Cancer." *Pathology* 46 (4): 316–24. https://doi.org/10.1097/PAT.0000000000000094.

Zhang, Zhenfeng, Christina Filzmayer, Yi Ni, Holger Sültmann, Pascal Mutz, Marie-Sophie Hiet, Florian W. R. Vondran, Ralf Bartenschlager, and Stephan Urban. 2018. "Hepatitis D Virus Replication Is Sensed by MDA5 and Induces IFN-β/λ Responses in Hepatocytes." *Journal of Hepatology* 69 (1): 25–35. https://doi.org/10.1016/j.jhep.2018.02.021.