Sex Differences in IgG1-Mediated Inhibition of Choroidal Neovascularization

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Dedication and Acknowledgements

"Accomplishments will prove to be a journey, not a destination." – Dwight D. Eisenhower

First and foremost, I dedicate this dissertation to my Mom. She has sacrificed so much for me to reach this point. As a single parent, she had to be mom and dad and she succeeded. She taught me the value of hard work, creativity, the importance of having faith, and never giving up. My mom might not have understood what I was studying or what I was working on, but she always was a shoulder to cry on if things were not going well or be my biggest cheering section when things were going great. Her unwavering support was one of the things that kept me going to pursue this degree. Thank you, Mom, for everything.

A second dedication goes to Dr. Patricio Meneses. He was the first mentor post college who motivated me to pursue my Ph.D. He made me feel that not only I could achieve more, but I had to achieve more. He was the first principal investigator I worked for that saw my passion for science and thought of me as more than a technician. He always wanted me to think outside the box when it came to my project. He always wanted me to ask questions and to be the expert. Thank you, Dr. Meneses, for helping me become the scientist that I am today.

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These three individuals have provided me with the skills and mental strength for me to get to this point of presenting my research and earning my Ph.D. in Experimental Pathology from the University of Virginia. The impact they have had on me in the present time, in my career, and will have on my future cannot be stressed enough. I cannot thank them enough for what they have given me throughout the years.

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The team assembled in the Gelfand Lab, past and present, - Ryan Makin, Joe Olivieri, Reesheda Jones, Hannah Hall, Ivana Apicella, Grace Pradhan, and Anna Steenson- have helped in more ways than I can count. They made my lab experience a positive and productive one which cannot be repaid. Thank you for making me feel a part of the team. You all made it a pleasure for me to come into the lab, whether it was the morning or a weekend and for that I will always be grateful.

Dissertation Abstract

Sex differences significantly impact diseases involving angiogenesis, such as age-related macular degeneration (AMD), which is more prevalent in females. The basis for this sexual dimorphism is unknown. Using AMD as a disease and pathological angiogenesis model, this project explores the mechanisms underlying these contrasts, focusing on human IgG1, an endogenous anti-angiogenic protein, which is a treatment option for those experiencing various types of conditions classified as pathological angiogenesis and the influence of sex chromosomes and hormones on its activity. Human IgG1s possess intrinsic anti-angiogenic activity by suppressing macrophage chemotaxis, independent of their ability to bind antigens. Human and mouse models reveal that males exhibit stronger IgG1-induced chemotaxis inhibition and anti-angiogenic effects in choroidal neovascularization (CNV).

The Y chromosome, particularly the DEAD-Box Helicase 3 Y-Linked (DDX3Y) gene, has been identified as crucial for these effects in males through gene and RNAi screening. The loss of this gene has been demonstrated to affect the response to IgG1 treatments in vitro and in vivo. Male mice and cells lacking Ddx3y exhibited blunted IgG1 responses in CNV and macrophage chemotaxis matching the responses observed in females.

Estrogen is found to suppress IgG1-mediated angiogenesis inhibition, providing another possible explanation for the sexual dimorphic responses that have been observed

in several models and systems. Removing estrogens or using estrogen receptor inhibitors enhance IgG1 responses in females, suggesting hormonal modulation as a therapeutic strategy.

Additionally, voluntary exercise is shown to reduce CNV in mice, with a 41% decrease observed in trained mice, in comparison to sedentary mice highlighting the potential of lifestyle interventions as a cost-effective treatment option or prevention medicine. These findings provide new insights into sex-specific angiogenic mechanisms, with implications for treating AMD and other sex-related disorders.

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Abbreviations

ADCAI- antibody-dependent cell-mediated angioinhibition

Ala- alanine

AMD- age-related macular degeneration

Akt- Ak strain transforming

ANOVA- analysis of variance

ARPE-19- adult retinal pigment epithelial cell line-19

Asp- aspartic acid

BMDM- bone marrow derived macrophage

cDNA- complementary deoxyribonucleic acid

CD14- cluster of differentiation 14

CD16- cluster of differentiation 16

CD64- cluster of differentiation 64

CNV- choroidal neovascularization

CI- confidence interval

CRISPR- clustered regularly interspaced short palindromic repeats

CVD- cardiovascular disease

DAPI- 4'6'-diamidino-2-phenlindole

DDX3X- DEAD-box helicase 3 X-linked

DDX3Y- DEAD-box helicase 3 Y-linked

DMEM- Dulbecco's modified eagle medium

D1pas1- DNA segment, Chr 1, Pasteur Institute 1

DR- diabetic retinopathy

E1- estrone

E2- estradiol

E3- estriol

E4- esterol

EC- endothelial cell

ECM- extracellular matrix

EG-VEGF- endocrine gland-derived vascular endothelial growths factor

ERα- estrogen receptor alpha

ERβ- estrogen receptor beta

ERE- estrogen response element

ERK- extracellular signal-regulated kinase

FBS- fetal bovine serum

FCG- Four Core Genotypes

FcγR1- Fc gamma receptor 1

FFA- fundus fluorescein angiography

FITC- fluorescein isothiocyanate

Flk-1- fetal liver kinase 1

Flt-1- fms related receptor tyrosine kinase 1

Flt-4- fms related receptor tyrosine kinase 4

GA- geographic atrophy

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

Glu- glutamic acid

GPER/GP30- G protein-coupled estrogen receptor

HIF- hypoxia inducible factor

HIF-1α- hypoxia inducible factor 1 alpha

HL-60- human leukemia-60

HRE- hypoxia- responsive element

hIgG1- human immunoglobulin G1

HUVEC- human umbilical vein endothelial cells

IgG1- immunoglobulin G1

IgG2- immunoglobulin G2

IL-12- interleukin 12

IFNγ- interferon gamma

IMDM- Iscove's modified Dulbecco's medium

IP- intraperitoneal

kDa- kilo Dalton

KDM5D- lysine-specific demethylase 5D

KDR- kinase insert domain receptor

LDL- low-density lipoprotein

Mϕ- macrophage

M-CSF- macrophage colony stimulating factor

MAPK- mitogen-activated protein kinase

mLOY- mosaic loss of Y chromosome

MSY- male specific region of the Y chromosome

nAMD- neovascular age-related macular degeneration

NF-κB- nuclear factor kappa-light-chain-enhancer of activated B cells

NIH- National Institutes of Health

NSCLC- non-small cell lung cancer

OCT- optical coherence tomography

PAD- peripheral artery disease

PMA- phorbol-12-myristate-13-acetate

PBMC- peripheral blood mononuclear cell

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PDGF- platelet-derived growth factor

PDT- photodynamic therapy

PI3K- phosphoinositide 3-kinase

PlGF- placenta growth factor

qRT-PCR- real-time polymerase chain reaction

Rbm31y- RNA binding motif 31, Y-linked

RNA- ribonucleic acid

RPE- retinal pigment epithelial

RPMI- Roswell Park Memorial Institute

RT- room temperature

RT-PCR- reverse transcription polymerase chain reaction

SABV- sex as a biological variable

SC- subcutaneous

SERD- selective estrogen receptor degrader

SERM- selective estrogen receptor modulator

sFlt-1- soluble fms-like tyrosine kinase-1

siRNA- small interfering ribonucleic acid

Ssty1- Y-linked testis-specific protein 1

Ssty2- Y-linked testis-specific protein 2

THP-1- Tohoku Hospital Pediatrics-1

USP9Y- ubiquitin specific peptidase 9 Y-linked

UTY- ubiquitously transcribed tetratricopeptide repeat containing, Y-linked

VEGF- vascular endothelial growth factor

VEGF-A- vascular endothelial growth factor A

VEGF-B- vascular endothelial growth factor B

VEGF-C- vascular endothelial growth factor C

VEGF-D- vascular endothelial growth factor D

VEGF-E- vascular endothelial growth factor E

VEGF-F- vascular endothelial growths factor F

VEGFR1- vascular endothelial growth factor receptor 1

VEGFR2- vascular endothelial growth factor receptor 2

VEGFR3- vascular endothelial growth factor receptor 3

Zfy1- zinc finger protein 1, Y-linked

Keywords: sexual dimorphism, IgG1, neovascularization, DDX3Y, estrogen, exercise

Preface

Over the past six years, I have had the opportunity to work in a lab at the University of Virginia that allowed me to inquire about different aspects of age-related macular degeneration. Some of those questions included why IgG1 treatments have different potencies between males and females and can exercise be a form of therapy that can help those with age-related macular degeneration or help in preventing others from that diagnosis later in life. This dissertation addresses those questions and provides some data as to why 1) the Y chromosome, specifically the DDX3Y gene, affects the male response to IgG1 treatment, 2) how estrogen may be a hindrance to allowing IgG1 to work efficiently in treating pathological angiogenesis, and 3) how exercise can be a positive supplement in the treatment of choroidal neovascularization.

In this dissertation, first I will present some background information that will set up the impending manuscripts on DDX3Y and estrogen. Following the introduction, there will be a section on pathological angiogenesis and how age-related macular degeneration is an example of this abnormality. The next section focuses on a main pro-angiogenic factor, the vascular endothelial growth factor, its family of receptors, and how it is treated in cases of pathological angiogenesis. The third section provides some background on the published manuscript on exercise and its association with choroidal neovascularization. The following section identifies different models used when researching sex differences and their utilization in this project. The final two background sections focus on the main

points of the impending manuscripts. The first is on DDX3Y and the second is on estrogen.

After the background sections, there are the two imminent manuscripts that will be submitted to two different journals. The first is on DDX3Y and the second is on estrogen. Both manuscripts contain specific methodologies, ideas, and data that paint a picture about the sex differences observed over various in vitro and in vivo assays. Because the starting point for both DDX3Y and estrogen are the same, some data will be repeated. To avoid any confusion, some data will be referred as “Argyle et al. 2024 Figure X”. These manuscripts are followed by the published paper, “Voluntary exercise suppresses choroidal neovascularization in mice”, from IOVS. This dissertation is wrapped up with a summary and future directions section. All references from the different sections and manuscripts are at the end.

Introduction

Sex and gender are major determinants of health that affect patterns of disease and injury. Historically, there has been a bias across many disciplines within biomedical research heavily dominated by male models and research subjects despite widespread recognition of conditions that are more prevalent in women than in males and vice versa[1,2]. Including sex as a research variable increases rigor, promotes discovery, and expands the relevance of biomedical research[3]. Two mechanisms that influence these effects are the sex chromosome complement and sex hormones. Both mechanisms can act through several cellular and molecular processes that can alter function of different procedures and structures such as the immune system. These differences in immunity can affect disease pathogenesis and an individual's response to treatments such as immunomodulators, vaccines, and checkpoint blockade therapy. An area of disease where this is evident is in pathological angiogenesis.

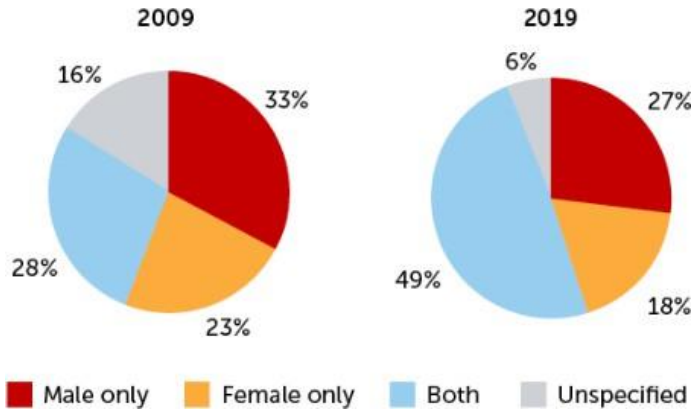


Figure 1: Sex of subjects in biomedical studies (2009 and 2019). Over a 10-year period, there was an increase in the use of both sexes in biomedical studies from 28% to 49%. There was also a decrease in studies involving one sex and studies where sex was not specified. From Science News *Biomedical studies are including more female subjects (finally)* June 2020- Bethany Brookshire

Angiogenesis is a normal physiological process where new blood vessels are formed from the existing vasculature. The system itself is controlled through the balance of pro- and anti-angiogenic factors. These factors are important for processes such as wound healing, inflammation, and vascular permeability. There are instances where the balance of these components is disturbed, resulting in insufficient or excessive angiogenesis. Examples of the former include peripheral artery disease (PAD)[4,5], and heart failure[6]. Examples of the latter include cancer[4,5], rheumatoid arthritis [5,7] and nAMD[8]. Besides the differences due to the balance of angiogenic factors, there is also a difference in how women are affected in conditions of pathological angiogenesis such as nAMD[9,10], PAD[11,12], and stroke[13,14]. It is also known that in cardiovascular disease (CVD), genetics, epigenetics, and sex hormone signaling are instrumental to the biological mechanisms of sex differences in CVD[15]. Because of these issues, modulating angiogenesis is a broadly applicable therapeutic goal. By researching the pathways and mechanisms that may play a role in modulating angiogenesis, new therapeutics can be designed and used that could prolong lives, prevent limb amputations, reverse vision, and improve general health.

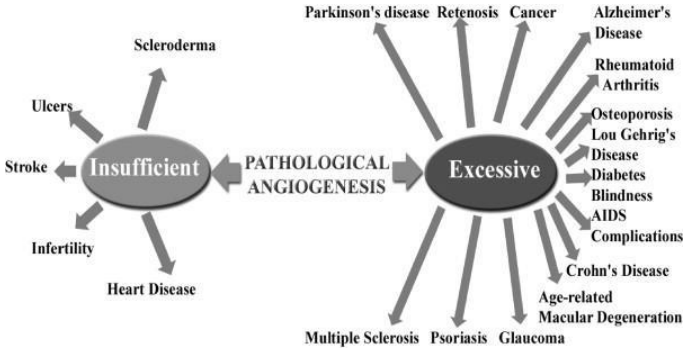


Figure 2: Examples of conditions that occur when there is insufficient or excessive angiogenesis. From Trends in Food Science & Technology *Targeting excessive angiogenesis with functional foods and nutraceuticals* November 2003- JN Lasso

Our lab has contributed to the discovery of a novel form of anti-angiogenic immune-vascular crosstalk, identifying human IgG1, and the murine equivalents IgG2a and IgG2c, as endogenous negative regulators of VEGF signaling and neovascularization[16]. We discovered that antigen-independent binding of monomeric human IgG1 or murine IgG2a/2c antibodies via the constant Fc domain to the high affinity receptor Fc gamma receptor 1 (FcγR1) on the surface of macrophages[17], impairs VEGF-mediated chemotaxis and restricts angiogenesis. This activity is referred to as "antibody-dependent cell-mediated angioinhibition" (ADCAI). ADCAI is active in models of angiogenesis induced by suture-induced corneal injury, laser-induced choroidal neovascularization, femoral artery ligation, tumor xenograft, and developmental retinal neovascularization[16,18]. Observing this activity in several models illustrates that ADCAI is a fundamental process that affects vascular remodeling in multiple tissue beds and physiologic states.

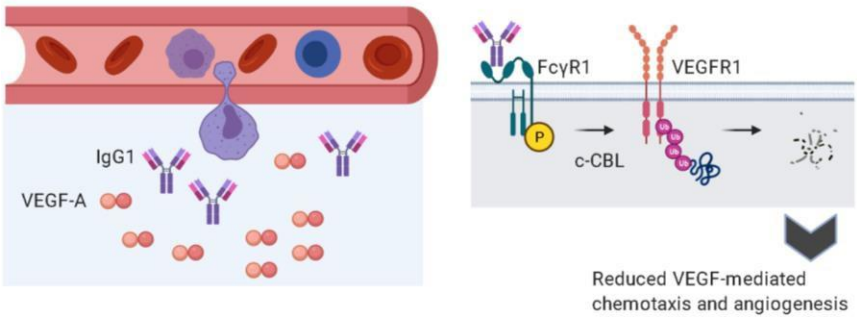


Figure 3: Proposed pathway of antibody-dependent cell mediated angioinhibition (ADCAI). IgG1 antibodies inhibit VEGF-mediated chemotaxis and reduces angiogenesis. This pathway was identified in Bogdanovich et al. 2016. IgG1 binds to FcγRI on the surface of macrophages, resulting in phosphorylation of the ITAM domain and phosphorylation and recruitment of the ubiquitin ligase c-CBL. c-CBL then ubiquitinates VEGFR1, leading to its proteolysis. This impairs macrophage chemotaxis towards a VEGF-A gradient, thereby restricting angiogenesis.

Due to the sexual dimorphism observed in the immune biology of males and females, it was important to quantify the effect of sex on ADCAI. The original research that first discussed ADCAI involved findings using predominantly male mice and cell models. The addition of female counterparts builds on the foundation of the initial project. This was important because it could possibly provide clues as to why certain pathological angiogenic disorders occur with different prevalence or manifestations between sexes and help in our understanding the role of sex in immune-vascular crosstalk. This work also has potential practical clinical implications for many diseases of vascular remodeling.

Using AMD as an example of pathological angiogenesis, laser choroidal neovascularization as an in vivo model for the disease which illustrates its hallmark pathology and characteristics, and macrophages as a vehicle for measuring the response of IgG1 treatments, this project has provided some answers to questions about sexual dimorphic responses to therapeutics and treatments while providing a different perspective in the fields of vascular biology and immunology. Also using these same parameters, this project was able to have 2 different focuses of investigation: the Y chromosome with an emphasis on the DDX3Y/Ddx3y gene and sex hormones, prioritizing estrogen. By exploring both possibilities, there is an opportunity to see how much each aspect contributes to the sexual dimorphic ADCAI response observed regarding IgG1 treatment. With both studies, there are opportunities to design experiments that involve knockdown/knockout models, use of human cell lines, primary cells from humans and mice, and have extensive studies that utilize in vitro and in vivo

techniques. The results obtained from these experiments illustrate the importance of understanding patients, their histories, and mechanisms of therapeutics used. Having these aspects will allow for the best treatment options for patients with an emphasis on sex.

Pathological Angiogenesis and AMD

Angiogenesis is the natural development of new blood vessels from pre-existing vessels in the vascular system. This process is one that begins in utero and continues throughout one's lifetime. There are two main forms of angiogenesis: sprouting and splitting (also known as intussusceptive angiogenesis)[19,20]. Sprouting occurs when new endothelial cell growth emerges from an existing blood vessel. The growths are directed toward a chemical stimulus such as vascular endothelial growth factor (VEGF). These sprouts develop into blood vessels that reach tissue areas lacking blood supply. The second type of angiogenesis, known as splitting, occurs when a capillary forms without sprouting. In this process, a blood vessel splits into two, forming a new capillary. Endothelial cells move into the vessel's opening, creating pillars that lead to the formation of new capillary tubes. Physiological angiogenesis is associated with processes such as wound healing, female menstrual cycle, and development. These processes are regulated by maintaining a balance between pro- and anti-angiogenic factors. A disruption in this balance can result in pathological angiogenesis.

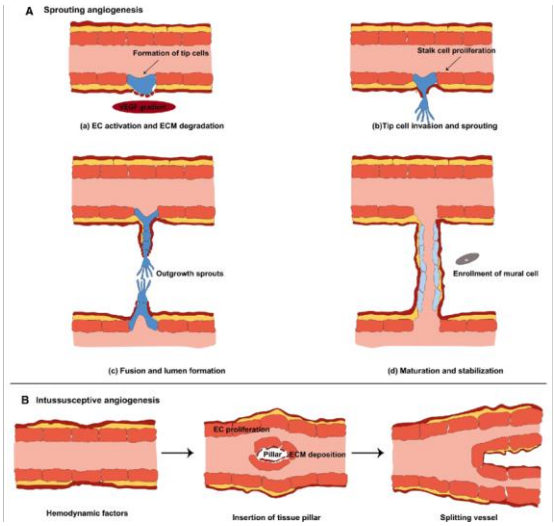


Figure 4: Illustration of the angiogenic processes of sprouting and splitting in the formation of new blood vessels. From *Tumor cells derived-exosomes as angiogenic agents: possible therapeutic implications* Journal of Translational Medicine June 2020- Ahmadi & Rezaie

Pathological angiogenesis is marked by the atypical growth of new blood vessels from existing ones. This process is initiated when cells in hypoxic tissues secrete pro-angiogenic growth factors (i.e. VEGF) to meet their metabolic needs[20]. Another element that creates a pathological angiogenic environment is inflammation. Angiogenic chemokines can facilitate the formation of new blood vessels and attract inflammatory cells[21,22]. The inflammatory cells that can be recruited include neutrophils, lymphocytes, mast cells, eosinophils, and macrophages (which will be discussed in detail)[21–23]. This process also involves the breakdown of endothelial cell (EC) junctions. The blood vessels that are formed can be described as irregularly shaped, enlarged, tortuous, and leaky.

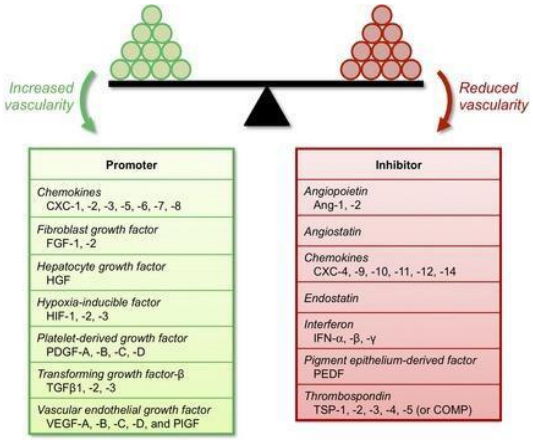


Figure 5: Pro- and anti-angiogenic factors that are involved in physiological and pathological angiogenesis. A balance of these factors is necessary for homeostasis. Disease characterized by hypervascularization include cancer and AMD. Increased inhibitor expression is observed in ischemic diseases- From *A systems biology view of blood vessel growth and remodelling* Journal of Cellular and Molecular Medicine (2013)- Logsdon et al.

A condition that is an example of pathological angiogenesis is AMD. AMD is the primary cause of legal blindness in developed countries. It is projected that

by 2040, 300 million people worldwide will be diagnosed with AMD[24]. It is also estimated that by 2050, 5.4 million Americans will be told that they have this condition[25]. The disease is characterized by the accumulation of extracellular deposit clusters called drusen, along with the progressive degeneration of photoreceptors and surrounding tissues. This degeneration is brought forth through senescent retinal pigment epithelial (RPE) cells triggering the immune system to produce VEGF[26,27]. This then leads to blood vessel production in Bruch's membrane which is caused by calcification, rupturing, and phagocytosis in the RPE[26,28,29]. Factors such as aging, environment, smoking, diet/obesity, CVD, sex and genetics play a role in AMD. The complexity of this disease is not only observed through its risk factors, but also in its pathogenesis. There are a few key factors that are involved in the pathogenesis of AMD. These factors include and are not limited to oxidative stress and an impaired extracellular matrix (ECM). With multiple factors and characteristics in its pathogenesis, there are two main forms of AMD that is observed in patients. The first is known as dry AMD or geographic atrophy (GA) and the second is wet AMD or neovascular AMD (nAMD)[30,31]. The former affects approximately 85-90% of those diagnosed with AMD while the latter, the more severe form, is observed in approximately 10-15% of those diagnosed with AMD[31].

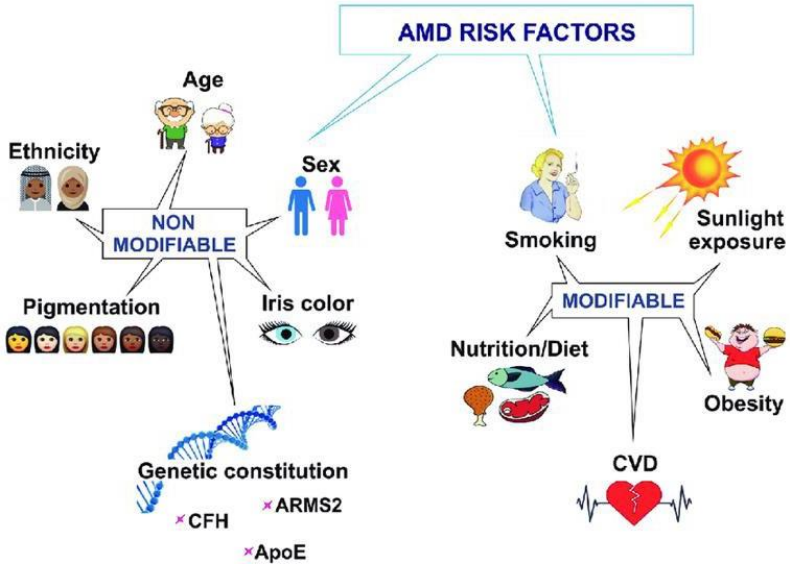


Figure 6: AMD risk factors. AMD is a multifactorial disease with several genetic and environmental factors important for its pathogenesis. Several widely acknowledged risk factors for AMD include advanced age, Caucasian descent, female gender, lighter skin tone, eye pigmentation, blue iris, high-fat diet, smoking, exposure to UV and/or blue light, obesity, and CVD. Additionally, genetic mutations in complement factor H (CFH), age-related maculopathy susceptibility (ARMS2), and apolipoprotein E (ApoE) have been linked to disease. From Acta biochimica Polonica 66 *Can vitamin D protect against age-related macular degeneration or slow its progression?* June 2019- Kaarniranta et. al.

Patients usually show signs of having dry AMD first and can progress to wet or nAMD. For this to occur, there are four defined stages that a patient experiences with each characterized by different symptoms. The four stages of AMD are classified as subclinical, early, intermediate, and late[32–34]. During subclinical AMD, the earliest symptom is difficulty seeing at night or in the dark. This manifestation is usually accompanied by difficulty seeing at night or reading in low light and is characterized by impaired dark adaptation. At this stage, there is typically no pigmentary abnormalities and if drusen are present they are less than 63 μm in size[33,34]. Early AMD, which is known as the second stage, is characterized by the appearance of medium-sized drusen deposits (63-125 μm in size) and increased impaired dark adaptation[33,34]. In individuals with intermediate AMD, symptoms can vary. Some individuals may notice a blurred spot

in their central vision, others might struggle with contrast sensitivity, some can have pigmentary changes, and at least one drusen deposit may be larger than 125 μm[33,34]. At the intermediate stage of AMD, additional symptoms may include visual distortions, blurry text, reduced color intensity, difficulty seeing in low light or at night, and restricted central vision[32]. In the final stage, advanced AMD, vision deteriorates significantly due to either geographic atrophy or choroidal neovascularization. During this phase, individuals may experience trouble recognizing faces, further decline in visual acuity, distorted vision, and loss to central vision.

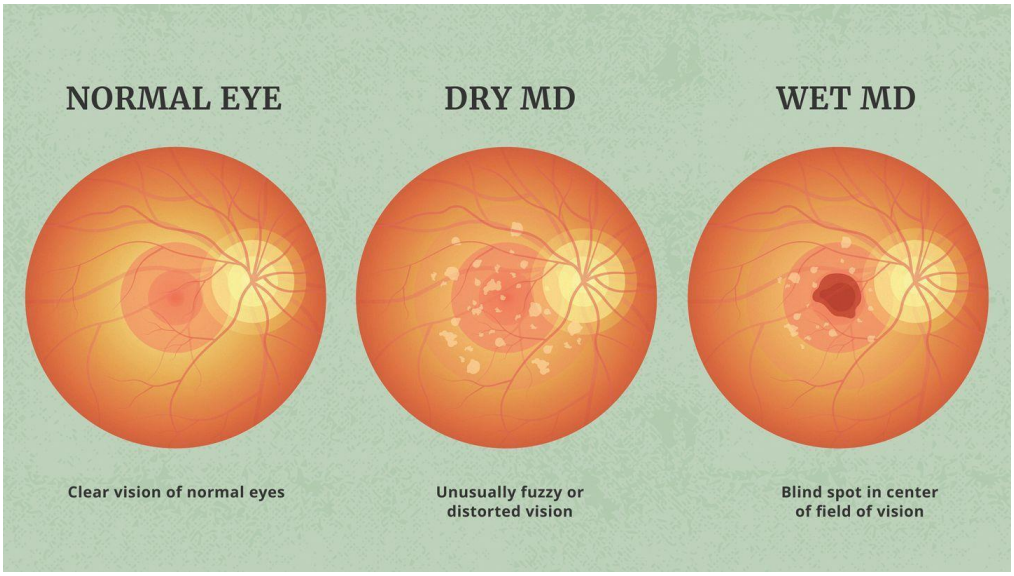


Figure 7: The physiological changes that occur in patients that are diagnosed with dry macular degeneration or wet macular degeneration. From Thomas Eye Group

Before drusen deposits become visible, cholesterol can accumulate between the RPE and Bruch's membrane. This buildup leads to oxidative stress and inflammation, disrupting nutrient transport to the photoreceptor cells. Consequently, the photoreceptors die, resulting in impaired dark adaptation.

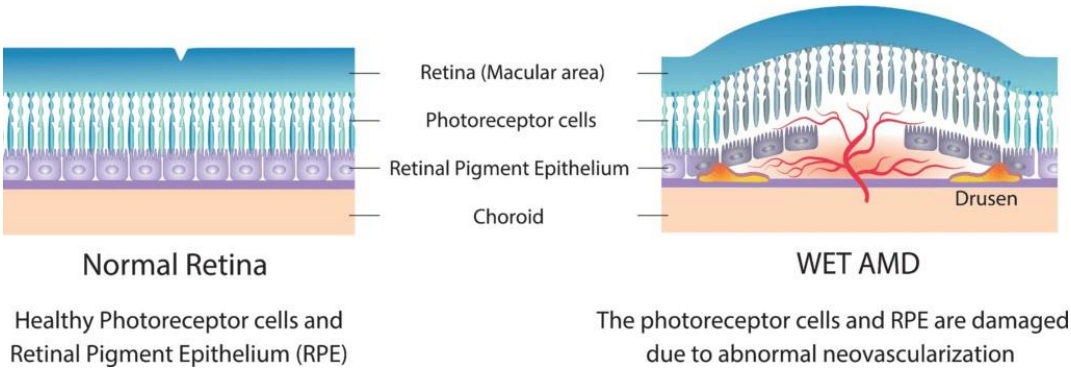


Figure 8- Graphical depiction of the differences in the photoreceptor cells and retinal pigment epithelium in normal eyes and those that have been diagnosed with wet age-macular degeneration. From American Macular Degeneration Foundation

There are various tests used to determine if a patient is suffering from AMD. The initial test typically conducted is a visual acuity test, commonly known as the eye chart test, which assesses an individual's vision capability at different distances[35]. A second test involves pupil dilation where eyedrops are used to widen the pupil, enabling a detailed examination of the retina. A third and more specific test is fundus fluorescein angiography (FFA)[36]. This diagnostic test involves injecting fluorescein, a fluorescent dye, into a vein in the arm. Images are captured as dye moves through the blood vessels of the retina. These pictures are evaluated by a doctor by checking for leakage in the blood vessels and if there is leakage, whether that leakage can be treated. A fourth test that may be used is the Amsler grid[37]. This exam uses a checkerboard-like grid to check if the straight lines in the pattern appear wavy or missing to the patient[37]. The third and fourth exams mentioned are more used for detecting the wet form of AMD. Along with the visual acuity and pupil dilation, an exam that can be used if GA or dry AMD is suspected is optical coherence tomography (OCT)[36,38]. With this test, the doctor will use a

special machine and light to scan the eye and take high-resolution infrared images of the macula. The exam itself is painless, can detect fluid, detect changes associated with AMD, and can be used to monitor the condition over time. Autofluorescence is another technique that can be utilized for detection and diagnosis. Like FFA, a doctor would use photos to study the retina and measure the progression of geographic atrophy. This technique allows for the doctor to monitor the RPE, which are naturally auto fluorescent due to lipofuscin accumulation, without the use of a dye injection.

Treatment options for AMD depend on whether a person has been diagnosed with dry AMD/GA or wet AMD/nAMD. With the latter, the culprit or driver for progression is usually VEGF and there are several therapeutics that can be used to address this issue. This will be discussed in the section on VEGF, its receptors and its therapeutics. As for the former, treatment options have been difficult to develop due to the various mechanisms that have been proposed for its progression. Some proposed mechanisms include activation of complement pathway, formation of a membrane attack complex, and death of retinal cells[39–41]. To address this, there are several clinical trial investigations ongoing to find an effective treatment for dry AMD. Some of these potential treatments fall into groups that include complement inhibitors, retinoid cycle modulators, reduction by toxic products, antioxidant therapy, neuroprotectants, laser therapy, surgical options, gene therapy, stem cell therapy, and other miscellaneous treatments[40]. The goals of these therapies are reducing the progression of GA and restoration of retinal function[40].

As mentioned, treatments and therapeutics for nAMD usually target VEGF. The most common treatments used are intraocular injections of anti-VEGF medications such

as bevacizumab (Avastin) (discussed in more detail in the section on VEGF, its receptors and therapeutics), ranibizumab (Lucentis), and aflibercept (Eylea)[42]. There are pros and cons with each of these treatments with bevacizumab being the focus of this research project[42]. These pros and cons will also be addressed in a later section. Outside of anti-VEGF treatments, there are some other options that are less common, but available if anti-VEGF treatments are ineffective in patients. One option is photodynamic therapy (PDT) which involves using a photosensitive dye to convert light into chemical energy[43–45]. This process generates free radicals that cause damage to abnormal blood vessels, which leads to the closure of these vessels and a reduction in leakage and bleeding while minimizing damage to surrounding tissues[43–45]. PDT involves intravenously injecting verteporfin (the photosensitive dye) and then applying a low power, long-duration infrared laser to the eye[43,45]. This treatment induces occlusion of abnormal blood vessels in choroidal neovascular membranes and choroidal tumors. Nutritional supplementation has also been viewed as a form of therapy for wet AMD patients. Antioxidants such as vitamins C, and E, copper, lutein, zeaxanthin, and zinc have been shown to slow the progression of wet AMD[46]. A newer treatment option has been gene therapy. The goal of gene therapy is to enable the eye to produce its own anti-VEGF treatment, effectively providing a continuous delivery of antiangiogenic proteins[47,48]. With this approach, gene therapy utilizes an empty vector to carry and deliver a gene containing specific instructions for producing a protein. One of the goals of gene therapy is for this approach to be “one and done” where there can be savings in the cost for the patient and reduced injection burden[47,48]. Despite the potential benefits, there are several challenges

associated with this option, including determining the best treatment targets, choosing the appropriate routes of administration, and addressing potential safety concerns.

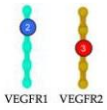
	Ranibizumab (Lucentis)	Aflibercept (Eylea)	Bevacizumab (Avastin)
Company	NOVARTIS Genentech	BAYER REGENERON	Genentech Roche
Mechanism of Action	Anti-VEGF-A Antibody fragment Inhibits all the VEGF-A isoforms	Anti-VEGF-A/PIGF/VEGF-B Fusion protein Inhibits all the VEGF-A, VEGF-B and PIGF isoforms	Anti-VEGF-A Full antibody Inhibits all the VEGF-A isoforms
Molecular Weight	48kDa	97-115 kDa	149 kDa
Structure			

Figure 9: Anti-VEGF treatment options for patients diagnosed with wet AMD. From Lanier Biotherapeutics

With AMD being a multifaceted condition, there are various areas that researchers target to gain a better understanding of disease pathology and options for therapeutics.

For this project, two areas of interest are the infiltrating macrophages that have been implicated in nAMD progression and IgG1 antibodies as a possible treatment. The latter is discussed in detail in the section on VEGF and anti-VEGF treatments. Most commonly, when studying angiogenesis, endothelial cells are used, but in this case, we chose to focus on macrophages. The reasons for using macrophages in this project are the following: 1) macrophage behaviors and their interactions with vascular cells play a vital role in regulating angiogenesis; 2) different subtypes of macrophages (M1 & M2) promote pro- and anti-angiogenic cytokines; 3) macrophages have a widespread presence in normal and inflamed tissues, and 4) macrophages can become activated when exposed to the right stimuli. More specifically, the type of macrophages used in our in vitro model

are bone marrow derived macrophages (BMDMs). The justification to using such a model is that monocyte derived macrophages have been shown to be essential in the development and progression to AMD[49–52].

Infiltrating macrophages are the primary inflammatory cells in AMD lesions contributing to inflammation and angiogenesis. Macrophages have been found to promote the development and progression of CNV through several mechanisms including their role as pro-inflammatory and angiogenic mediators, modulating the ECM, recruitment and activation of other immune cells, polarization and functional plasticity, interaction with the RPE, and oxidative stress[51,53,54]. In AMD patients, macrophages are located near thin and perforated areas of Bruch's membrane and help break down its outer collagenous zone, which aids in the subretinal infiltration of CNV[55,56]. Quantitative flow cytometric analysis revealed that macrophages, which had gathered at the injury site, migrated from the retina and peripheral blood before the RPE cells showed increased VEGF expression[55,57–59]. Due to their role in the immune system, macrophages can protect and/or damage local tissues in AMD. It is this dynamic that adds to the need for understanding their role in AMD and their response to IgG1 treatment. Because of their prominent role in this disease, macrophages were chosen as the vehicle to better understand the sexual dimorphic response observed in the response to IgG1 treatment.

In the laser CNV model also known as a macrophage-dependent angiogenesis model, macrophages begin to invade the laser injury site within one day of photocoagulation, with their infiltration continuing and peaking at three days[56,60]. Reducing macrophage levels in this model has been shown to decrease the size and

leakage of laser-induced CNV and is associated with lower levels of macrophage infiltration and VEGF protein. The chemokines and cytokines secreted by macrophages can provide a signature as to what type of AMD a patient has. Molecular pathology techniques such as microdissection and immunochemistry, have identified a pathological imbalance in macrophage polarization within AMD lesions. There are higher M1 (pro-inflammatory) infiltration in those with GA and higher M2 (anti-inflammatory) infiltration observed with those with nAMD[61,62]. These findings suggest that macrophage polarization (highly controversial with the idea of M1 and M2 not being sufficient classifications) and plasticity can play a role in the development and progression of AMD. The findings also indicate that M1 macrophages, which are pro-inflammatory, might be involved in the early stages of CNV, whereas M2 macrophages, which are anti-inflammatory and pro-angiogenic, could play a crucial role in the middle and advanced stages of CNV development and remodeling[27,62–64].

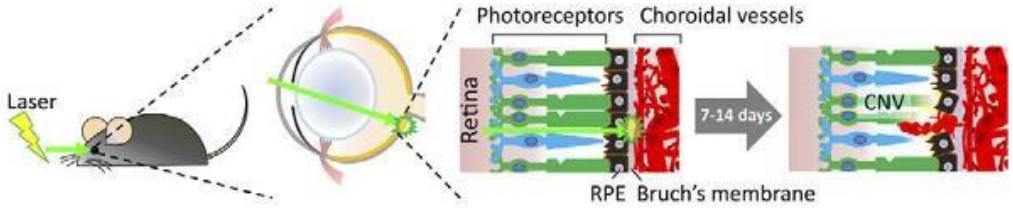


Figure 10: Graphical depiction of laser choroidal neovascular model that illustrates that hallmark pathogenesis associated with wet AMD. Laser-induced choroidal neovascularization model of AMD illustrating the target of the laser to induce the angiogenesis that is the hallmark pathology is observed in neovascular AMD (wet AMD). The laser targets the retinal pigment epithelial (RPE) and Bruch's membrane to cause injury. From FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology July 2017

Vascular Endothelial Growth Factor (VEGF), VEGF Receptors (VEGFRs) and anti-VEGF Therapy

Angiogenesis is a complex and multifaceted process regulated by balanced pro and anti-angiogenic factors, enabling the formation of new blood vessels from the existing vasculature. These factors exist in a dynamic equilibrium for physiological angiogenesis, but that balance is lost in conditions of pathological angiogenesis. It is for this reason why targeting these factors is a choice for treatments and therapeutics.

A potent pro-angiogenic factor is vascular endothelial growth factor (VEGF). VEGF is a group of signaling proteins produced by various cells that promote the formation of blood vessels. VEGF is involved in normal development and growth, wound healing, and tissues, but it can also contribute to various pathological conditions, such as cancer, AMD, and diabetic retinopathy (DR) when not kept in check. VEGF is also known as a vascular permeability factor because of its ability to destabilize endothelial junctions and induce vascular leakage[65,66]. VEGF signaling is regarded as a crucial, rate-determining factor in physiological angiogenesis. VEGF is a 40-kDa heterodimeric glycoprotein that features a cysteine-knot motif, which is defined by the arrangement of specific disulfide bridges in its structure[67–69]. In humans, the VEGF-A gene is located on the 6p21.3 chromosome and it is organized as eight exons that is separated by seven introns[65,70,71] and is a member of the VEGF/platelet-derived growth factor (PDGF) gene family. The family itself contains VEGF-A with its various isoforms (the member that is predominately discussed in this paper), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral VEFG), VEGF-F (snake venom VEGF), endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and the placenta growth factor (PlGF)[68,72–74].

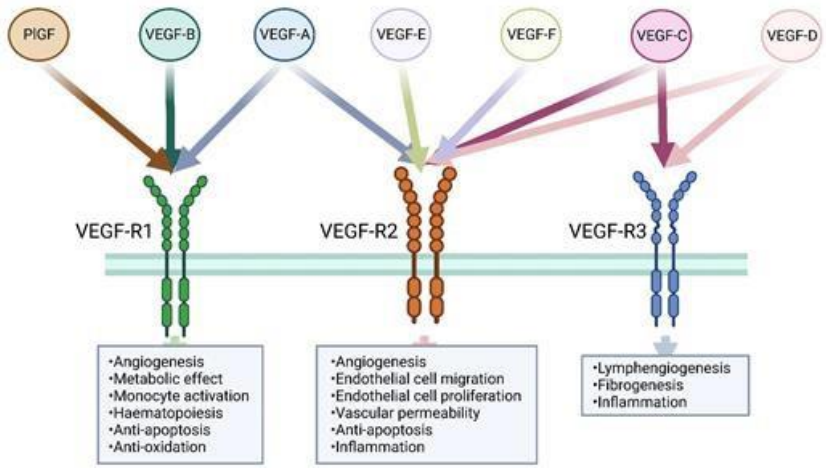


Figure 11: Family of Vascular Endothelial Growth Factor Receptors, their physiological functions, and VEGF factors that they interact with. From *Therapeutic Potential of VEGF-B in Coronary Heart Disease and Heart Failure: Dream or Visions* **Cells 2022- Mallick & YIä-Herttuala**

VEGF binds to specific receptors which have been termed VEGF receptor 1 (VEGFR-1/Flt-1), VEGF receptor 2 (VEGFR-2/KDR/Flk-1) and VEGF receptor 3 (VEGFR-3/Flt-4). These are tyrosine kinase receptors which contain three domains: 1) an extracellular domain for VEGF binding, 2) a transmembrane domain and 3) an intracellular domain with tyrosine kinase activity. These receptors can form homodimers, heterodimers, and interact with other proteins such as neuropilins, integrins, cadherins, and heparan sulfate proteoglycans which can enhance or modify the activities of the receptors and add specificity to their binding capacities[75].

The expression of VEGFR-1 is influenced by the microenvironment, which varies under hypoxic and inflammatory conditions[76]. Its activation can affect vascular permeability and stimulate macrophages and microglia to release proinflammatory and proangiogenic factors[57]. It binds with VEGF-A, VEGF-B, and PlGF. It is also different from the other members of its family in that it splices into two mRNA isoforms, which are a full-length receptor and another for a soluble shorter protein soluble VEGFR-1 (sFlt-1). The latter version has been observed to behave like a decoy receptor for VEGF. It competes with membrane-bound VEGFR-1 and other VEGF receptors for binding and

is therefore able to sequester VEGF and prevent it from binding to its membrane-bound receptors. Through this action, sFlt-1 can regulate the bioavailability of VEGF in the extracellular environment and modulate VEGF-mediated angiogenesis[77]. This protein has been involved in a range of physiological and pathological processes such as pregnancy, cancer, and cardiovascular diseases. Its levels can be altered under various conditions allowing it to influence angiogenesis and vascular function. VEGFR-1 has a higher binding affinity for VEGF-A than VEGFR-2 but has a weaker kinase activity in comparison to VEGFR-2[77]. Its role in pathological angiogenesis is also less clear than that of VEGFR-2.

In recent years, VEGF and its receptors have become therapeutic targets in the treatment of various cancers and other conditions of pathological angiogenesis. When used for cancer treatment, most times VEGF therapies are used in combination with other treatments such as chemotherapy. Some examples of VEGF treatments include bevacizumab (commercial name- Avastin) and ranibizumab (commercial name- Lucentis) which are humanized monoclonal antibodies; aflibercept (commercial name Eyelea) which is a VEGF-Trap and fusion protein composed of VEGFR-1 and VEGFR-2 components, and brolucizumab (commercial name Beovu) which is a humanized monoclonal single-chain variable fragment (scFv). Other treatments such as sorafenib and sunitinib target kinase activity.

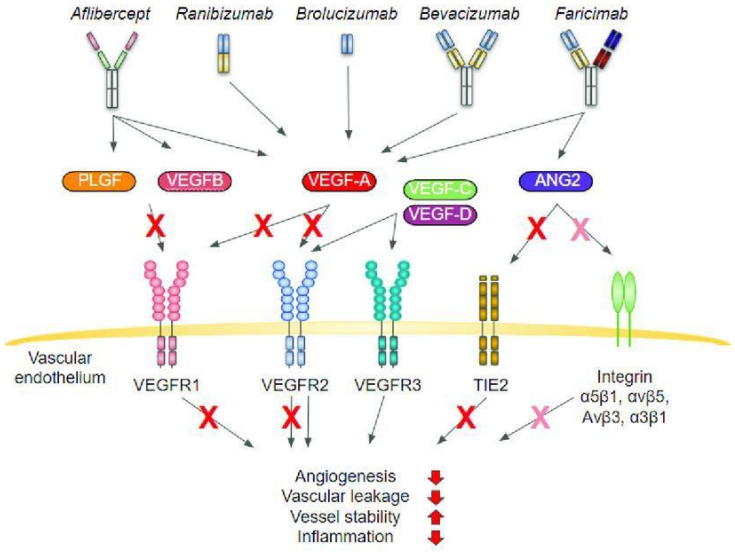


Figure 12: Anti VEGF treatments and mechanisms used to treat age-related macular degeneration. All these agents have VEGF-A as a main target. Aflibercept, a fusion protein, also binds to PLGF and VEGF-B. Faricimab, a bi-specific antibody binds to ANG2 and VEGF-A. From Cells November 2022 *A Linkage between Angiogenesis and Inflammation in Neovascular Age-Related Macular Degeneration*- Heloterä & Kaarniranta

For this project, bevacizumab was the focus of the treatment for several reasons. In 2016, our lab demonstrated that IgG1 antibodies regardless of their intended target can inhibit angiogenesis in in vitro and in vivo experiments using human cells and rodent models[16,18]. This was significant because bevacizumab only has an affinity for human VEGF-A, but not for mouse or rat VEGF-A[78–89]. It was determined that the Fc portion of IgG1 antibodies suppresses angiogenesis in several models such as choroidal neovascularization (the in vivo model that illustrates the hallmark pathology of wet AMD), suture-induced corneal injury, femoral artery ligation, tumor xenograft, and developmental retinal neovascularization. This suppression of angiogenesis occurs through the binding of the Fc portion of the IgG1s with Fc gamma receptor 1 (FcγRI/CD64) on the surface of macrophages, which diminishes VEGF-mediated chemotaxis and restricts angiogenesis through VEGFR-1[16]. This activity was termed antibody-dependent cell-mediated angioinhibition (ADCAI). It is important to note that it is the Fc region which possesses this capability since when ranibizumab (a Fab fragment

antibody lacking the Fc domain) or enzymatically cleaved bevacizumab where the Fc and Fab portions were separated, the Fab portions did not show any anti-angiogenic activity while the Fc region did[16]. As an off-label therapeutic in the treatment of AMD, the cost is key. Bevacizumab costs $60 per dose versus aflibercept which costs $1850 per dose and ranibizumab which costs $1170 per dose[90]. Because of this difference in cost, bevacizumab is usually the first line of treatment used by many ophthalmologists. Also of note, bevacizumab was used only in mouse or rat experiments while a generic human IgG1 isolated from the plasma of a myeloma patient was used in human studies. The use of the latter was to ensure that the response being measured was that of angiogenic suppression and not direct VEGF-A inhibition.

Bevacizumab is a recombinant monoclonal antibody comprised of human (94%) and murine (6%) components. It works by binding and neutralizing all circulating VEGF-A isoforms allowing for inhibition of its biological activity through its two binding sites. By blocking all VEGF-A isoforms from binding to their receptors, there is a reduced VEGF activity, leading to an inhibition of angiogenesis and vascular permeability.

Its development began at Genentech in 1993, where scientists created a mouse-anti-human VEGF monoclonal antibody (A.4.6.1)[91]. This antibody showed the capability of inhibiting the growth of different tumor cells in nude mice, making it a candidate for cancer treatment. Four years later in 1997, a humanized version of the antibody was completed. This antibody demonstrated similar ligand-binding affinity to its mouse counterpart and was able to bind and neutralize all human VEGF-A isoforms[91]. Using human cell lines, one that expresses VEGFR-1 and VEGFR-2 (human umbilical vein

endothelial cells- HUVEC) and one that only expresses VEGF-R1 (THP-1 monocytes), bevacizumab inhibited VEGFR-1/Flt-1 mediated signaling[92,93].

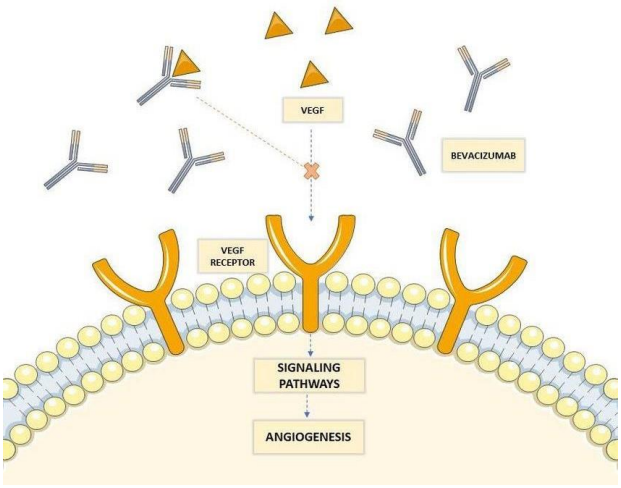


Figure 13: Mode of action of bevacizumab. VEGF is captured by bevacizumab preventing it from interacting with its receptor. From World Scientific News 2018

When used in the context of wet AMD, bevacizumab is administered through intravitreous injection (directly into the vitreous chamber of the eye) to inhibit VEGF-A in the eye thereby reducing the growth of abnormal blood vessels and decreasing fluid leakage. Typically, a patient receives injections every 4-8 weeks depending on the patient's response and the severity of the condition. When given by intravitreal injection, the half-life of bevacizumab in the bloodstream is 4.32 days in rabbits, and 3.59-5.05 days in mice[94]. In the bloodstream, the half-life in humans is 20 days[94]. The removal of bevacizumab from the aqueous humor and serum followed a pattern like that observed in the vitreous humor, with half-lives of 4.88 days and 6.86 days, respectively[94]. These measurements have never considered the sex of the patients or models which might have slight differences.

Exercise- A Cost-Effective AMD Treatment

Another proposed treatment option for those diagnosed with nAMD is exercise. It has been suggested that exercise can help in protecting the eye against an abnormal

growth of blood vessels and that physical activity can reduce the severity of AMD[95–98]. Because of its effect on blood flow and nutrition, exercise can change the basic molecular metabolism of cells. Exercise can be thought of as a cost-effective form of therapeutics and preventative medicine. Physical activity is beneficial for eye health by reducing the risk of central retinal vein occlusion and nAMD through better management of systemic hypertension and diabetes[96]. Because AMD is a multi-faceted disease, it has been linked to several chronic conditions such as high blood pressure, heart disease, and hyperglycemia. One way to control these problems is through regular exercise. Through regular physical activity, one can lower their cholesterol, blood pressure and blood sugar.

The positive effects of exercise have been demonstrated in mouse models such as the laser choroidal neovascularization model (CNV) model and the light induced retinal degeneration model[97,99–101]. The findings of how exercise can positively affect the laser CNV model will be addressed in this paper in Chapter IV. In the light induced retinal degeneration model, scientists demonstrated that aerobic exercise could protect retinal function and structure using BALB/c mice that were subjected to treadmill training[101]. This study demonstrated that aerobic exercise can be a direct benefit to degenerating photoreceptors in this model and that this treatment can be used as a noninvasive, inexpensive rehabilitative therapy for patients.

Human studies have also shown that exercise can be beneficial for patients diagnosed with AMD. Middle-aged adults that participated in higher levels of vigorous exercise and people over the age of 75 that engaged in a moderate level of activity had

lower incidence rates of AMD[102]. The Beaver Dam Study which included close to 4,000 men and women between 43-86 years old investigated the relationship between AMD and exercise[103]. There was an evaluation every 5 years for 15 years and the scientists observed that those who walked more than 12 blocks per day decreased their incidence of exudative AMD by 30% over the 15-year period[103].

Exercise and some physical activity help in reducing the kind of inflammation and blood vessel changes that are observed in wet AMD. In terms of the types of exercise, cardiovascular activity such as walking or running increase the flow of blood to the optic nerve and the retina. Exercise can also be beneficial to the prevention of dry AMD. In dry AMD/GA, the retina does not receive the necessary amount of oxygen for normal function or survival which leads to the death of cells in the macula. Exercise can be a way to improve the cardiovascular health leading to a better flow of blood. By increasing one's blood circulation to the retina, there will be better delivery of vital nutrients, oxygen, and antioxidants that are necessary for peak retinal function. Sufficient blood flow is important for the removal of waste products and preventing the accumulation of damaging substances in the macula.

Physical activity is also beneficial to reducing oxidative stress which is a known factor in the development and progression of AMD. Exercise can stimulate the production of antioxidants which can neutralize harmful free radicals[104], protect retinal cells from oxidative damage and amplify the retina's ability to self-repair.

Exercise may not replace anti-VEGF injections as the primary form of treatment for AMD, but it can possibly be a supplement to the injections. This is a therapeutic that patients can control themselves and this may be an indirect positive as well. When used in conjunction with other lifestyle adjustments such as a healthy diet, maintaining a healthy weight, and not smoking, the positive effects of physical activity would be enhanced and have a more protective effect against AMD. The next step would be identifying the mechanisms by which physical activity and AMD are connected.

Sex differences, disease, and AMD

Biological sex differences are important factors in disease. These variations can be influenced by the sex chromosome complement (XY or XX), reproductive organs or gonads (testes or ovaries) and levels of sex steroids (testosterone, estrogen and progesterone). These differences can affect rates of disease incidence, symptoms, and age of onset. Despite these differences being recognized, it is an area that has been underappreciated and underexplored until recently. Growing evidence indicates that sex chromosomes and sex hormones may affect both the number and function of immune cells[105–114]. Sex differences affect the innate and adaptive immune responses which can explain the differences in disease onset, disease incidence, and symptoms. The variations seen in innate and adaptive immunity contribute to higher prevalence of autoimmunity in females and elevate the likelihood of organ rejection after transplantation[115–117]. There is still much to be discovered about the mechanisms linking sex differences with immune-mediated conditions. Gaining a deeper understanding of these processes and associations

could lead to more targeted patient care and potentially the creation of sex-specific approaches for disease screening and treatment.

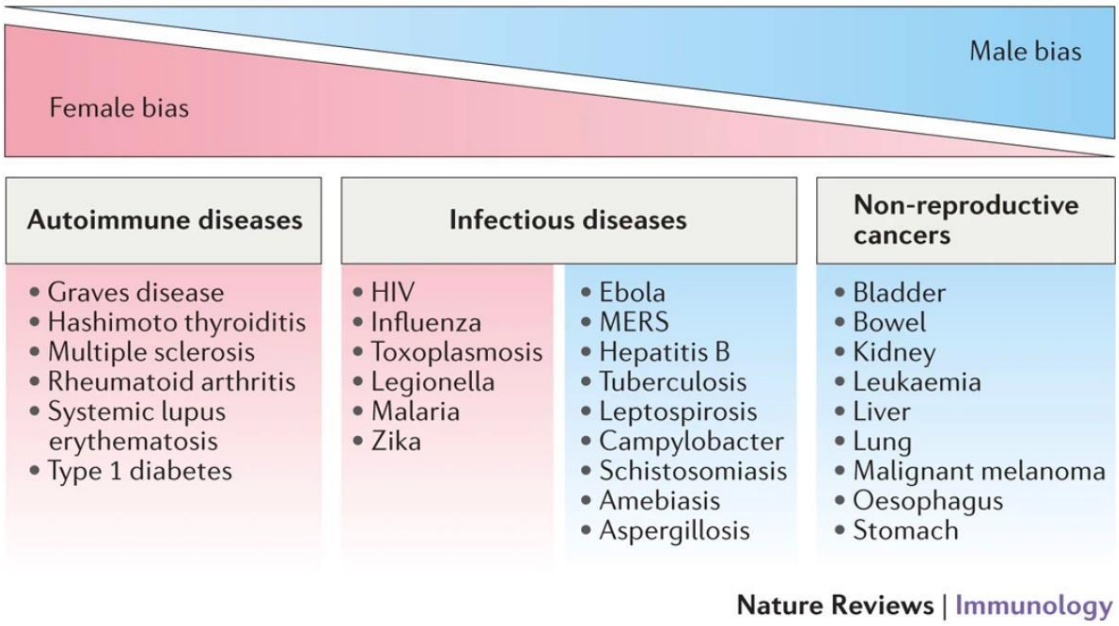


Figure 14: Graphical display of how sex bias exists in infectious diseases, inflammatory diseases, and cancers. From *Sex differences in immune responses* **Nature Reviews Immunology August 2016- SL Klein**

Sex-based immunological differences contribute to variations in the rates of autoimmune diseases and cancers, susceptibility to infections, and responses to vaccines between males and females[105,109,117–120]. In humans, females have shown lower infection rates than males for various viral, bacterial, and parasitic pathogens, while also experiencing a higher incidence of autoimmune disorders compared to males. Males are more prone to infections and malignancies. Because of this, females tend to have a heightened immune reaction to both self and non-self-molecular patterns[109]. Sex-specific variations in the host microbiome also play a role in immune responses. Differences in pharmacokinetics and pharmacodynamics between sexes suggest that the same plasma drug concentration may not produce identical pharmacological effects[121,122]. When put

together, all this suggests that sex has distinct influences on immune responses, which shape the pathophysiology of health and disease. The molecular mechanisms that drive such sexual dimorphic immune responses are not fully understood.

A disease where sexual dimorphism is apparent is AMD. The most frequently diagnosed eye diseases in women are AMD, glaucoma, and cataracts[123,124]. Due to their longer life expectancy, women tend to experience a higher burden of these age-related diseases, including AMD[125,126]. In addition to aging, fluctuations in circulating sex hormone levels are believed to affect retinal and ocular physiology. Even accounting for age, clinical studies have consistently identified female sex as an independent risk factor for neovascular AMD[10,127–129]. The underlying causes for the elevated risk to females is not known, and the influence of hormonal changes during female lifespan and AMD development are complex and often contradictory[124,130]. Experimentally, mouse studies of choroidal neovascularization demonstrate that as animals age, a greater sex difference is observed, with female animals consistently developing greater CNV.

A study in 2017 was published where researchers studied the effect of sex steroid hormone fluctuations in the pathophysiology of male RPE cells[131]. To study this effect, the cell line ARPE-19 (arising retinal epithelium) was subjected to various concentrations of estradiol and progesterone challenges. What was discovered was that exposure to estradiol promoted the proliferation of RPE cells, whereas progesterone counteracted the effects of estradiol on cell growth[131]. When there was a chronic exposure to estradiol, the cells experienced induced cell death via necrosis, due possibly to the activation of chronic inflammatory pathways[131]. On the other hand, progesterone activated homeostatic

mechanisms (i.e. apoptosis) which helped to restore normal cellular function[131]. This study revealed potential effects of sex steroid hormone fluctuations on eye physiology that can occur during aging in males. Even though estrogen levels are usually associated with women, it has been demonstrated that estrogen levels increase as men age since testosterone is converted to estrogen through aromatase activity[131]. A 2003 study demonstrated that estrogens might affect RPE function through estrogen receptors, and that either a deficiency or excess estrogen could lead to dysregulation of molecules that impact ECM turnover in Bruch's membrane, which is linked to AMD[132]. Estrogen may also be a separate risk factor for AMD, but some studies suggest that estrogen exposure may be associated with a lower prevalence of AMD.

In another published report from 1997, scientists in Australia demonstrated that females over the age of 75 had a higher risk for AMD[133]. They also found that a significant reduction in early AMD with a longer duration from menarche to menopause, supporting the notion that a shorter period of estrogen production may be a risk factor for AMD[133]. These studies illustrate the possibility that hormones can be drivers of epigenetic change that contribute to the sexual dimorphism observed in AMD.

Another hypothesis for sex-related differences in AMD progression could be related to the number of X-linked genes that might influence cellular functions involved in the onset and progression of AMD[134,135]. Although no AMD variants have been identified in X-linked genes so far, epigenetic mechanisms may play a role in AMD pathogenesis by affecting these X-linked genes. A 2013 paper proposed that epigenetic changes affecting the inactivation centers of the X chromosome (or X-chromosome

skewing) are linked not only to aging but could also be a novel factor that makes women more susceptible to AMD compared to men[136]. Genes linked to innate immunity molecules, which are found on the X chromosome, may also contribute to the observed differences between sexes in this condition. Besides investigating the X chromosome, in recent years there has been research linking the Y chromosome and AMD in males. Data published in 2022 suggested that the mosaic loss of the Y chromosome (mLOY) in peripheral blood cells is associated with AMD[137]. Peripheral monocytes and macrophages that have lost the Y chromosome might contribute to AMD by altering immune responses and inflammation, leading to inadequate retinal repair, excess neovascular growth, or increased retinal damage[137–139].

One of the key components of AMD is the infiltration of macrophages in the eye. In advanced AMD, macrophages are localized to degraded areas of Bruch's membrane and are more numerous in the choroid of affected eyes[62,140–142]. Macrophages have exhibited the ability to express pro-angiogenic cytokines which indicate that these types of cells might directly encourage abnormal endothelial cell growth in CNV[51]. This is significant because one study found that certain macrophage and monocyte populations show higher activation and phagocytic activity in female mice compared to male mice[143,144]. Studies of drusen composition presented MHC class II antigens in their deposits. Along with the antigens being present in these drusen compositions, a 2018 paper mentioned that in women they observed elevated levels of total cholesterol and low-density lipoprotein (LDL) cholesterol were linked to a higher risk of developing AMD[145]. The presence of these type of deposits suggests that cells of monocytic origin are involved in the development of drusen[51,142]. Macrophages also have a role in the

turnover of the ECM and express several matrix metalloproteinases that could lead to the breakdown of Bruch's membrane[51,146,147]. Macrophages can release growth factors, cytokines, and reactive species, potentially causing harmful effects on the choriocapillaris and RPE involved in AMD[30,36,110,116,117]. These characteristics indicate a possible explanation for why AMD is observed more in males and females from a mechanistic perspective.

Studying Sex Differences Using Mouse Models

The integration of both sexes in biomedical and clinical research has highlighted the need for improved models to explore sexual dimorphism across various medical disciplines. These models enable scientists and researchers to investigate how disease mechanisms and treatment responses differ between sexes. Prominent models in recent years, particularly utilized in this project, are the Four Core Genotypes model and the XY* model[150]. These models are instrumental in discerning whether sex differences in phenotypes arise from components that are hormonal, chromosomal, or a blending of the two. When used together, these mouse models can further distinguish the specific effects of X or Y chromosome genes on sex differences in phenotypes. Since their adaptation for studying sexual dimorphism, these models have been pivotal in uncovering the role of sex chromosomes in several areas, which included and not limited to immunity, metabolism, and behavioral sciences[151]. Additionally, they have shed light on how certain X or Y chromosome genes may protect against or worsen diseases and how sex chromosome and hormonal factors interact, influencing each other's effects in various conditions.

The Four Core Genotypes (FCG) mouse model was developed to distinguish the influences of sex chromosomes (XX/XY) from those of gonadal hormones (testes/ovaries) in various biological processes and diseases. In this model, the Sry gene, which determines the development of testes in mammals, is transferred from the Y chromosome to an autosome (chromosome 3; a non-sex chromosome)[152]. This manipulation allows for the creation of four different genotypes (see chart below). There are advantages and disadvantages to using this model. Some advantages include the ability to isolate genetic and hormonal effects and versatility where these models can be used in a wide range of biomedical research fields. A limitation with this model is the complexity of breeding.

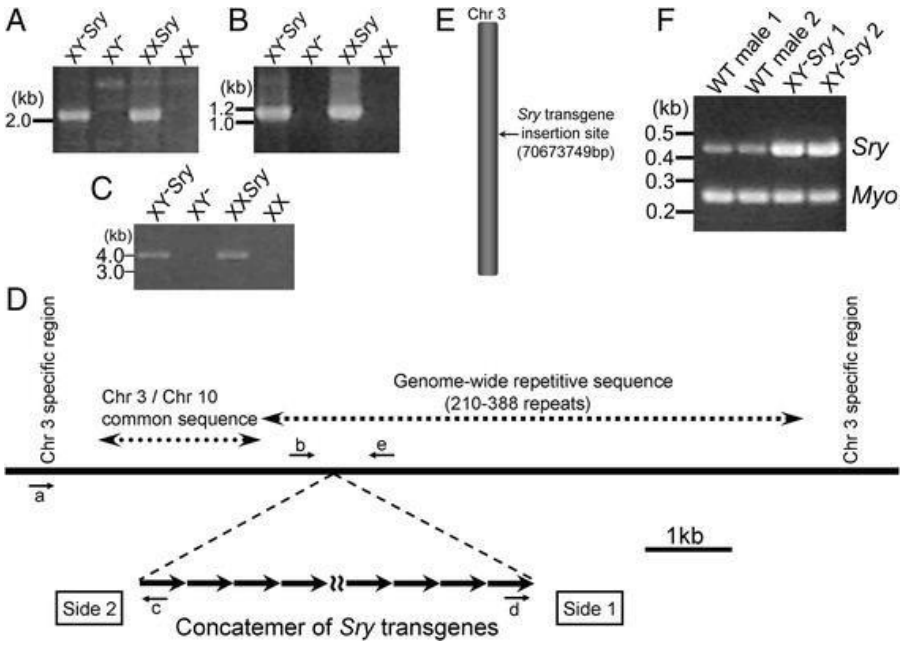


Figure 15: Location of the *Sry* transgene in four core genotypes mouse model. Samples from each genotype are shown here. Figures A and B show the PCR amplification between the Sry transgene and the surrounding repetitive sequence. Figures C and D show the Sry transgene integration site. Figure E shows the 74 base pair deletion involved in the intrgration of Sry on chromosome 3. Figure F confirms the Sry transgene band in the FCG mice versus that in the WT. *Four Core Genotypes mouse model: localization of the Sry transgene and bioassay for testicular hormone levels* **From BMC Research Notes 2015- Itoh et al.**

With this model, there are four different genotypes that can be produced. These genotypes are 1) XX mice with ovaries (typical female genotype and phenotype); 2) XY mice with testes (typical male genotype and phenotype); 3) XX mice with testes (genetically female but with male gonads due to the presence of the Sry gene on an autosome); 4) XY mice with ovaries (genetically male but with female gonads due to the absence of the Sry gene on the Y chromosome). Fields that have been able to utilize these mice include neuroscience, endocrinology, immunology, and behavioral sciences[151].

This model can also be used as an alternative to gonadectomy surgery (ovariectomy in females; castration in males) to study the impact of sex chromosomes independently from the effects of gonadal hormones. The FCG model allows for the separation of sex chromosomes (XX vs. XY) from the influence of gonadal hormones (estrogens produced by ovaries; androgens produced by testes). Using this model as a substitute to gonadectomy surgery, there are some benefits that researchers can take advantage of. These advantages include 1) the ability to study animals with their gonads intact and preserve their normal hormonal environment; 2) there is a reduced stress on the animals from surgery and recovery and the elimination of variability that can be introduced in surgical procedure and recovery; 3) with intact gonads, researchers can conduct longitudinal studies where the impact of sex chromosomes and gonadal hormones can be studied over an animal's lifespan. Despite these advantages, there are also some limitations when compared with gonadectomy surgery. These limitations include 1) certain hormonal manipulations cannot be studied and 2) they cannot be used for acute hormonal studies where there is a need for the precise control of hormone levels

at specific times, surgical removal and controlled hormone administration may be preferred.

A second mouse model that was utilized in this project was the XY* mouse model. This model is another important tool used in genetic and biomedical research, particularly for studying the effects of sex chromosomes on development, physiology, and disease. This model involves the manipulation of the Y chromosome to create mice with different combinations of sex chromosomes.

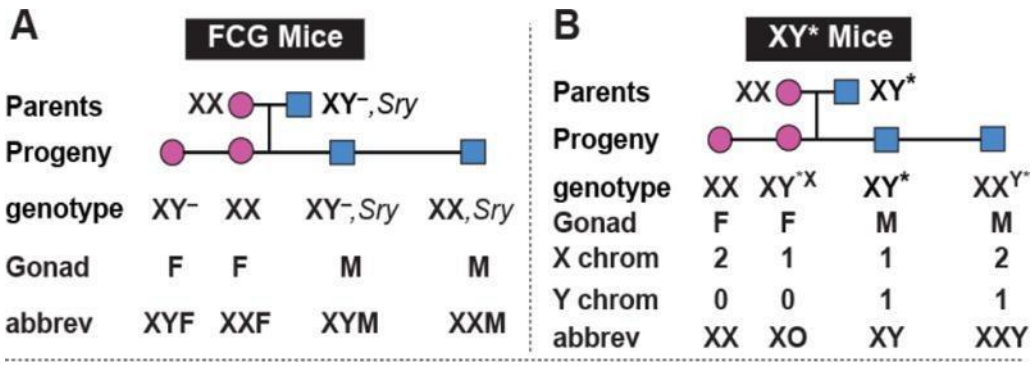


Figure 16: Pedigree depicting the offspring that can be produced using the FCG and XY* mouse models. Each model produces four different genotypes that can be dependent on the presence or absence of the Sry gene or a sex chromosome. From *Sex Hormones and Sex Chromosomes Cause Sex Differences in the Development of Cardiovascular Diseases* **Arteriosclerosis, Thrombosis, and Vascular Biology (2017)- Arnold et al.**

The XY* mouse model includes various modifications of the Y chromosome to produce different karyotypes. These modifications can result in a range of genotypes, each with distinct characteristics, allowing researchers to dissect the roles of sex chromosomes. There are four types of offspring that can be produced. They are as follows:

1. XY male (XY)- These mice have a normal Y chromosome with the Sry gene intact, leading to typical male development.
2. XO mice (XO)- These mice have only one X chromosome and lack a Y chromosome. There is typical female development, but only one X chromosome that allows for the studying of the effects of one X chromosome without the influence of Y-linked genes. These mice can be compared to Turner Syndrome in humans.
3. XY-female (XY)- These mice have a Y chromosome with a mutation or deletion that prevents the expression of the Sry gene that leads to the development of female characteristics despite the presence of a Y chromosome.
4. XXY mice (XXY)- These offspring have an extra X chromosome and a Y chromosome, which can be used to study conditions like Klinefelter syndrome in humans.

These various genotypes allow for several applications of this model. Some include sex determination and differentiation, gonadal development, genetic disorders (particularly using the XO and XXY mice), behavioral studies, and comparative physiology. As with all animal models, there are pros and cons. Some advantages include versatility that allows for a wide range of genotypes for studying the impact of sex chromosomes on various biological processes, and specific genetic insights which allows for the study of specific genes located on the Y chromosome and their roles in development and disease. The cons or limitations of such a model include the breeding and maintenance which require

sophisticated breeding strategies and genetic tools, and phenotypic variability where the phenotypes can be variable and unpredictable which require careful experimental design and interpretation.

Sexual Dimorphism Explanation #1- DDX3Y

Sex and gender are major determinants of health that affect patterns of disease and injury[13,15,118–120,124,153–156]. Historically, there has been a bias across many disciplines within biomedical research heavily dominated by male models and research subjects despite widespread recognition of conditions that are more prevalent in women than males and vice versa[1–3]. Including sex as a research variable increases rigor, promotes discovery, and expands the relevance of biomedical research[1–3].

This inclusion of both sexes has allowed for the discovery of many sexually dimorphic conditions, diseases, and treatments. These sex biased responses can be the result of the sex chromosome complement- are the differences due to the presence of an extra X chromosome in females or the presence of a Y chromosome in males? Or can it be the result due to the presence of different sex hormones- estrogen and progesterone in females, testosterone in males? This project has allowed for the exploration of both options.

Much is unknown regarding the impact of the Y chromosome on human health and disease. Although the Human Genome Project was “completed” in 2003, complete sequencing of the human Y chromosome was only published in 2023[157]. Similarly, there

is much more published research on the X chromosome than its Y counterpart. Some of the disadvantages of studying the Y chromosome are the presence of several pseudogenes, long non-coding regions, and several repeated sequences. The male specific region of the Y chromosome (MSY) is the area that contains the protein coding genes of the Y chromosome. For a long time, it was believed that these genes were only important for fertility, spermatogenesis, male sex determination, and male development. Using the C57BL/6J mouse as a reference, thirteen protein coding genes were found on the Y chromosome[158]. Most of these genes had counterparts on the X chromosome which share evolutionary history and strong amino acid homology. Some have similar functions to their X counterparts and vary in which tissues and parts of the body where they are expressed.

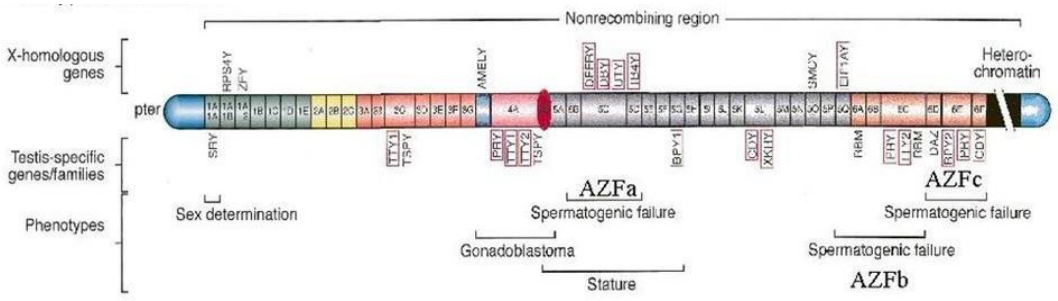


Figure 17: Functional map of the human Y chromosome. This map breaks down the Y chromosome into various regions and identifies all the protein coding genes. From *Functional coherence of the human Y chromosome* **Science 1997- Lahn & Page**

A gene of interest that could possibly explain the sexually dimorphic responses observed in the IgG1 response is the DEAD-box helicase 3 Y-linked (DDX3Y). The protein produced by this gene is distinguished by nine conserved motifs, including a conserved Asp-Glu-Ala-Asp (DEAD) motif[159]. These motifs are believed to play roles in ATP binding and

hydrolysis, RNA binding, and the formation of intramolecular interactions[159]. Due to alternative splicing, DDX3Y has four different isoforms, with one of those isoforms being exclusive to the testes[160]. Although DDX3Y mRNA translation is ubiquitous throughout the body, it was thought until recently that the protein (73 kDa) was only produced in the testes[161,162]. Recent research has illustrated that this protein has been detected in certain B cell lymphomas[160,163–165] and in some cell lines. Also since this protein is an RNA helicase, it may play a role in rearranging RNA secondary structure and be involved in RNA metabolism. This protein has a 92% homology with DDX3X located on the X chromosome. Because of this, the loss of DDX3X, can be partially compensated for by DDX3Y. Only partial because DDX3Y encodes a polypeptide with different biochemical activity[160,162,165–167]. Mutations in this gene can result in male infertility, reduced germ cell counts, and Sertoli-cell only syndrome. Beyond these associations, the functions of DDX3Y are not well understood. One of the difficulties of studying this gene that was discovered during this project was that there are no reliable commercial antibodies for the detection of DDX3Y along with other Y chromosome proteins via Western blot or other expression assays on the market[168]. This has been an obstacle to researching genes on this chromosome and gaining a better understanding of their function outside of fertility. Besides the similarity with DDX3X structure and being an RNA helicase, DDX3Y/Ddx3y was the highest expressed transcript in primary BMDMs when the initial screening for Y chromosome genes was conducted.

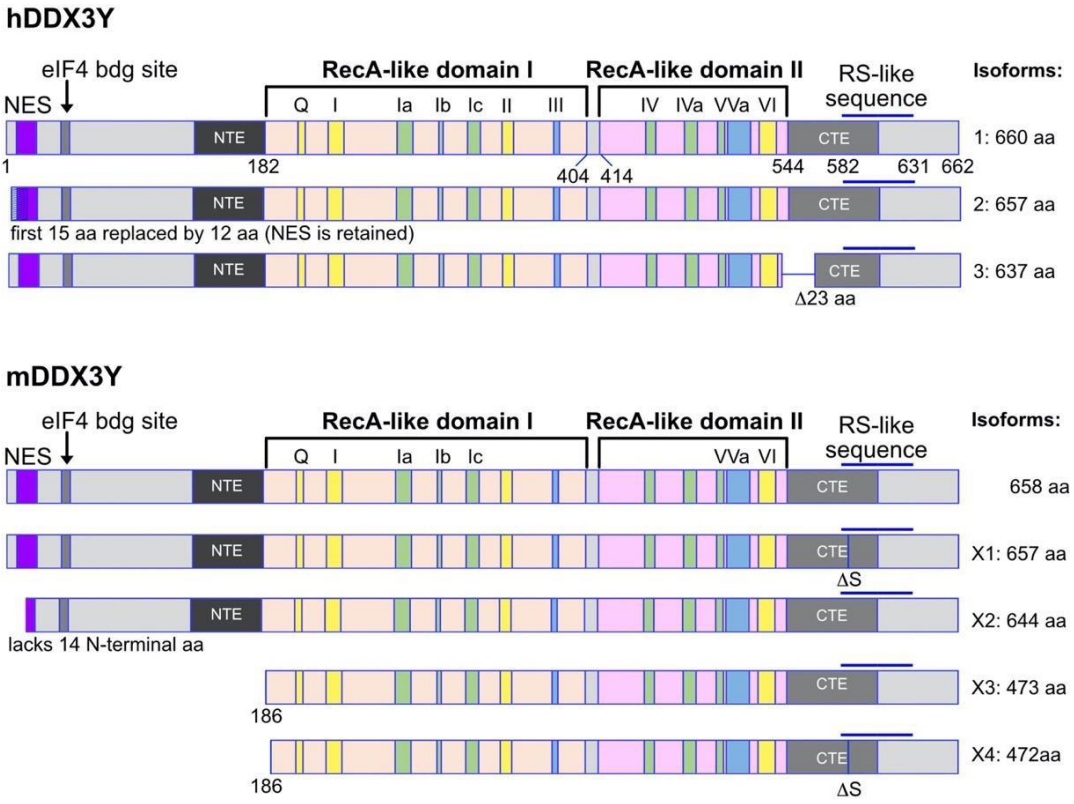


Figure 18: Human and murine DDX3Y isoforms. Isoforms X2 and X4 in the mouse lack a functional nuclear export sequence (NES). Because of this, mouse DDX3Y isoforms may be localized in the nucleus while human isoforms are likely to be mostly cytoplasmic. From *The RNA helicase DDX3 and its role in c-MYC driven germinal center-derived B-cell lymphoma* **Frontiers in Oncology 2023- Lacroix et al.**

DDX3X, on the other hand, has an association with the innate immune system by influencing the NF-κB pathway where the production of various inflammatory cytokines such as IL-12 and IFNγ are affected[169–171]. Because of this connection to the immune system, it is possible that there is a link between DDX3Y and the immune system that has yet to be uncovered. Using macrophages as a vehicle, along with in vitro and in vivo assays, this project allows for the exploration of DDX3Y and possible role in ADCAI and IgG1 treatment.

It is possible that there is a connection between DDX3Y, a Y chromosome gene, and VEGF which could provide an explanation for the ADCAI differences observed

when there is a loss of this gene through either a CRISPR-Cas9 knockout model or a transient loss using siRNA. Even though DDX3Y is being singled out and the focus of this project, it is noted that the other Y chromosome genes may indirectly affect angiogenesis or the vascular system[172–174]. These effects are usually mediated through broader physiological pathways (i.e. FcgR1/VegfR1 signaling pathway) rather than direct regulation of VEGF genes.

Since DDX3Y can possibly affect the IgG1 response indirectly possibly through the FcγR1/VegfR1 pathway, there are some theories that should be kept at the forefront that could piece together the puzzle of the sexual dimorphic ADCAI that has been observed through several models and cell lines. Some of these theories include the following:

1. Gene expression- this can be in the form of regulatory genes or epigenetic modifications. The Y chromosome carries genes that can regulate the expression of other genes involved in drug metabolism and response. There is also a possibility that epigenetic changes can affect gene expression potentially altering drug response.
2. Immune system- there is a chance that DDX3Y may be involved in immune functions like its paralog, DDX3X. Since the immune system can influence the effectiveness and side effects of medications, Y-linked genetic variations can lead to differences in immune responses to drugs. Males and females also exhibit different susceptibilities to autoimmune diseases and inflammatory conditions, partly due to genetic differences on the Y chromosome. Such

conditions can impact how individuals respond to certain medications, especially immunosuppressants and anti-inflammatory drugs.

3. Pharmacogenomics- With genetic variants on the Y chromosome, there can be an effect on the metabolism of drugs. The effects of Y chromosome genes on drug response can interact with other genetic factors, including those on the X chromosome and autosomes, leading to complex patterns of drug response.

Sexual Dimorphism Explanation #2: Estrogen

The mammalian immune system exhibits sexual dimorphism through the outcomes of various diseases. Generally, females are healthier than males because they tend to have better outcomes to illnesses caused by infectious diseases, trauma, sepsis, or injury[109,111,119,120,144,153,175–177]. At the molecular level, the female immune system exhibits greater responsiveness compared to males, attributed to the higher levels of immunoglobulins, IgM and IgG, as well as more robust humoral and cell-mediated immunity[107,178–180]. There is also a sex bias observed in various types of cancer and response to viral vaccines, and transplantations[109,115,120,154,177,181,182]. Reasons for the sex differences that is observed can be due to the action of sex hormones (estrogen and progesterone in females; testosterone in males) and the sex chromosome complement (unequal number of X and Y chromosomes in females and males). This project has provided an opportunity to explore both possible explanations in the different responses observed between males and females when treated with IgG1 antibodies for conditions of pathological angiogenesis.

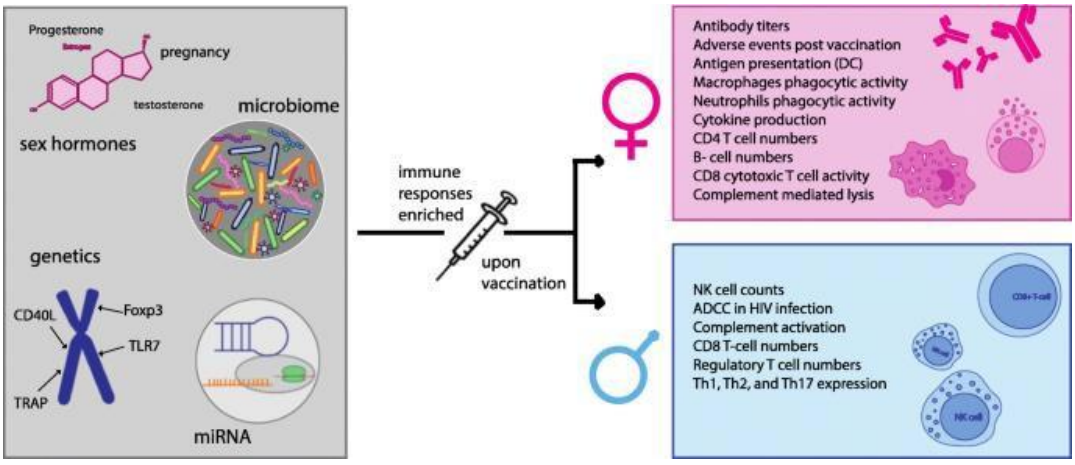


Figure 19: Factors that influence sex-specific humoral immunity to vaccination. The immune responses in males and females exhibit differences with females generally showing higher antibody levels, more adverse reactions, greater B cell frequencies, and increased phagocytic activity of innate immune cells. Males, on the other hand, tend to have higher numbers of NK cells and stronger type-1 immune responses. Factors such as hormone levels, mRNA expression, genetic chromosomal differenes, sex hormones, and gender-specific variations in the microbiome all contribute to the differences in humoral immunity following vaccination. From Seminars in Immunopathology *Sex differences in vaccine-induced humoral immunity* December 2018- Fischinger et al.

Sex hormones can have profound impacts on the immune system[105,106,113,114,117,178,183]. In females, the major sex hormones that can influence the immune system are estrogen and progesterone. Pregnancy, age, and menopause are some examples of dynamic changes that can influence estrogen and progesterone concentrations[120,184–186]. These fluctuations in hormone concentrations can lead to changes in disease pathology and disease treatment responses[114,131,185,187–189]. Estrogens are a group of hormones crucial for normal sexual and reproduction development in females. They are primarily made in the ovaries, but a small portion are made in the adrenal glands and fat cells and is released into the bloodstream. There are four major forms of estrogen. Those forms are estrone (E1) which is the primary form of estrogen made after menopause, estradiol (E2) which is the form in the body produced during an individual's reproductive years and it is the most dominant form of estrogen, estriol (E3) which is the primary form generated during pregnancy and esterol (E4) which is

developed in the fetal liver and is only detectable during pregnancy[190]. The levels of E4 are relatively high in the fetus and at lower levels in the maternal circulation.

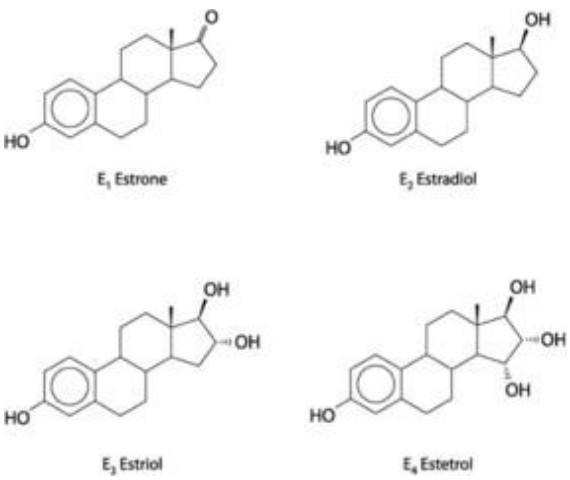


Figure 20: Chemical structures of major endogenous estrogens. From Wikimedia Commons, the free media repository

When researching estrogen and its effects, generally experiments use estradiol (E2) in the form of β-estradiol. Estrogen is known to either enhance or suppress immune responses depending on the ligand concentration, receptor type, and type of cell involved[191,192, 109,193,194]. Estrogen can directly modulate angiogenesis via effects on various types of cells. The mechanisms by which estrogen can regulate angiogenesis under physiological and pathological circumstances have not been defined[195]. The connection between estrogen, angiogenesis, and macrophages is also unknown. Gaining a better understanding of this relationship can shed light on the role that estrogen plays in immune-vascular crosstalk and provide clues about the best treatment options during a particular stage in life such as pre- versus post-pregnancy or pre- versus post-menopause.

Estrogen binds to specific receptors to exert its effects on various types of cells. The two most predominant receptors for estrogen are estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ)[190,196,197]. A third receptor is the G protein-coupled estrogen receptor (GPER/GP30). ERα is primarily expressed in reproductive tissues such as the ovaries, uterus, and mammary glands. It is also found in other tissues and cells such as

the liver, bone, and macrophages[190,196,197]. This receptor is known to play a critical role in the regulation of reproductive function, bone density, cardiovascular health, and cognitive function[190,196,197]. ERβ is another receptor that is expressed in a variety of tissues including the ovaries, prostate, bladder, gastrointestinal tract, bone, brain, and the cardiovascular system[190,196,197]. It has been implicated in the regulation of prostate and colorectal cancer along with the modulation of mood and cognitive function. The GPER is a membrane-bound receptor that is widely expressed throughout the body including the brain, cardiovascular system, reproductive organs, and other various tissues[198,199]. The role of GPER is to mediate rapid estrogen signaling pathways, often leading to non-genomic effects[198,199]. It is also involved in diverse physiological processes such as vasodilation, cell proliferation, and neurotransmitter release[198,199]. It is important to note that the expression of estrogen receptors can vary depending on factors such as sex, age, hormonal status, and tissue type. Additionally, estrogen receptors can also be expressed in non-classical tissues and cell types, contributing to the diverse effects of estrogen throughout the body.

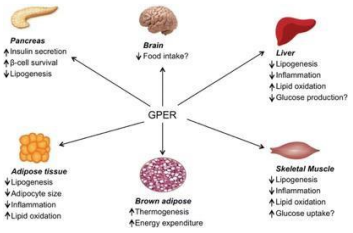


Figure 21: Schematic representation of the metabolic roles of GPER. This diagram illustrates the different roles GPER is involved in throughout the human body. From *G-Protein-Couples Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis* **Advances in Experimental Biology and Biology 2017- Sharma & Prossnitz**

ERα and ERβ are known to be expressed by macrophages and have different roles. ERα activation in macrophages can modulate immune responses and inflammatory processes[191]. ERβ, on the other hand, has its expression dependent on the context of inflammation[191]. When this receptor is activated, it has been associated with anti-

inflammatory effects and modulation of macrophage polarization. Because of the presence of estrogen receptors on macrophages, it is possible that estrogen can directly influence immune responses and inflammation processes via the regulation of macrophage function. This can have implications for various physiological and pathological conditions where estrogen levels fluctuate, such as during one's menstrual cycle, pregnancy, and menopause as well as in diseases influenced by inflammation, such as autoimmune disorders.

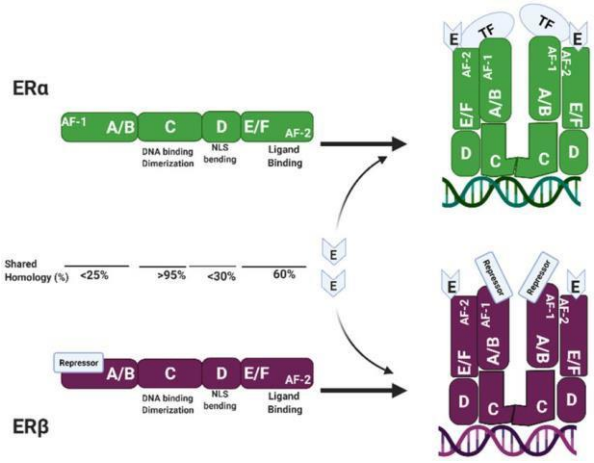


Figure 22: Structure of estrogen receptors α and β. Both contain 6 domains with a shared homology with the DNA binding domain being the most highly conserved. Similarities include the D domain where the nuclear localization signal (NLS) is located and a hinge for the protein to fold. Differences are in the A/B domain where the ERα has a ligand dependent activator factor (AF-1) and ERβ has a repressor site. With estrogen binding, there is a confirmational change and a folding of the receptor at the D domain, bringing the C-terminal close to the N-terminal and attracting transcription factors in ERα.
From *Estrogen Signaling in Bone* **Applied Sciences 2021- Lara-Castillo**

In the context of AMD, estrogen receptors may have an important role in its pathology, incidence, and severity. ERs are present in various tissues of the eye, which include the conjunctiva, cornea, lacrimal glands, and meibomian glands[200]. ER expression can be affected by age and gender. For instance, the protein for ERα can be found in the retina and RPE of younger women, but not in men or postmenopausal women[201]. Estrogen also has a role in how moist or dry eyes feel suggesting that the presence of ERs may indicate some involvement in the development of some eye diseases and conditions[200–202]. Another influence of estrogen in the condition of AMD is

in idiopathic full-thickness macular holes[201,203,204]. Idiopathic full-thickness macular holes which are defects in the central fovea of the retina that are known to occur spontaneously due to pathological changes, are more common in women than in men.

To study the effect that estrogen has on the immune system, there are several tools and reagents that can be utilized to answer the questions about estrogen, its effects on the immune system, and angiogenesis using macrophages as a vehicle. Estrogen can be eliminated from in vitro studies using charcoal stripped media and fetal bovine serum (FBS) and the elimination of phenol red media since it acts like a weak estrogen in cell culture[205,206]. The inhibition of estrogen can occur through drug intervention using estrogen receptor antagonists where the drug binds to the ER and inhibits the actions of estrogen without activating the receptor or there can be a degradation of the ERs. Examples used in the treatment of various conditions, such as breast cancer, include fulvestrant, a selective estrogen receptor degrader (SERD), and tamoxifen, a selective estrogen receptor modulator (SERM)[207,208]. Mouse models where ERα and ERβ have been knocked out can also be utilized to gain more knowledge about estrogen effects[209]. These models are known to have limited or inhibited fertility but may be useful in understanding how estrogen and receptors affect ADCAI. BMDMs can be obtained from these mice and used in a chemotaxis assay where the question can be asked about the loss of estrogen receptors and ADCAI or laser CNV experiments can be performed investigating if the lack of estrogen receptors can affect the response to IgG1 treatments. A fourth way of studying the effect of estrogen through inhibition would be using siRNAs targeting the estrogen receptors that would allow for a temporary/transient

silencing of the messenger RNA needed to produce those proteins or CRISPR-Cas9 where the receptor can be permanently edited out. With animal models for in vivo experiments, ovariectomies are the gold standard for experiments where estrogen is a factor that can be studied. An alternative model that can possibly be more accurate in understanding the effects of estrogen along with its natural changing levels would be VCD menopause mouse model[210].

Class of Drug	Mechanism of Action	Drugs Approved for Clinical Use	Drugs in Clinical Development
Selective estrogen receptor modulators (SERMs)	Suppression of E2-regulated gene expression by enhancing corepressor recruitment to ERα [56]	Tamoxifen, Toremifene, Raloxifene [62]	Bazedoxifene [62,63]
Selective estrogen receptor downregulators (SERDs)	SERDs disrupt ER dimerization and DNA binding and aid premature proteosomal degradation of the receptor [64]	Fulvestrant [57,65,66]	Elacestrant (RAD1901), AZD-9496, GDC-0927, LSZ102, SAR439859, G1T48 [66–70]
Aromatase inhibitors (AIs)	AIs prevent aromatase-mediated synthesis of estrogens from androgens. Thereby, decreasing circulating estrogen levels [71]	Exemestane (steroidal), Letrozole, Anastrozole (nonsteroidal) [72]	

Table 1 (Figure 23): Table of drugs used in breast cancer treatment to address estrogen in different ways. SERMs prevent ER signaling by binding to ERs and creating an inactive complex; SERDs prevent ER signaling through the degradation of ERs; aromatase inhibitors prevent signaling by inhibiting estradiol synthesis. From *Nexus between PI3K/AKT and Estrogen Receptor Signaling in Breast Cancer* Cancers 2021- Khatpe et al.

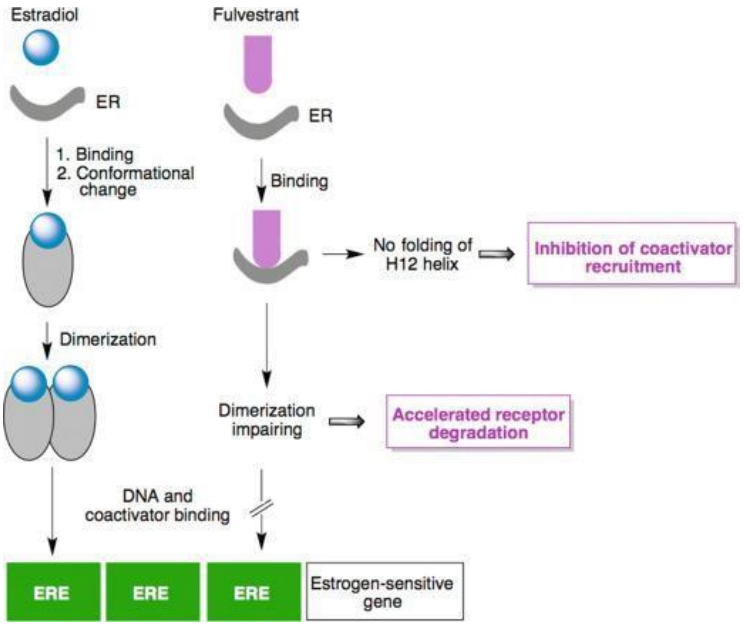


Figure 24: Mechanisms by how estradiol (E2) and fulvestrant work. Fulvestrant is an estrogen receptor antagonist that works as an inhibitor and blocks estrogen from binding to its receptor. From ScienceDirect- Fulvestrant

If estrogen experiments include the addition of estrogen in vivo (knock in/overexpression experiments), its administration would have to be taken into consideration. Depending on the research question being asked, mode of administration can be critical to the outcome of the experiment. For instance, estrogen can be given orally (mixture with food or water), intraperitoneal (IP) or subcutaneous (SC) injections (best administration options when using the laser CNV model that is utilized in this project), implants (subcutaneous pellets or osmotic pumps for sustained release), or topical (application to the skin for local effects)[211,212]. Besides the modes of administration, dosage will also be an important factor (i.e should estrogen levels be at physiological levels or how often the animals will receive estrogen).

In investigating the role that estrogen can play in the sexual dimorphic response to IgG1 treatments, there has been some research that suggest that it can be a hindrance to its effectiveness. Previous research has demonstrated that estrogen promotes increased myeloid infiltration and resistance to bevacizumab in murine models of non-small cell lung cancer (NSCLC)[213]. This finding suggests that personalized treatment plans where

hormone levels and receptor status may be important for optimizing therapeutic outcomes in patients where IgG1 treatment is an option. Other findings have also suggested that estrogen is involved in the proliferation of cells which is a key factor for angiogenesis. Estrogen induces endothelial cell proliferation and migration[214–217]. Because of its involvement in these processes, estrogen can induce angiogenic factors such as VEGF, which is another reason to study this hormone and investigate if it affects the sexual dimorphic effect observed in IgG1 treatment response. The challenge is that molecular mechanisms by which estrogen stimulates the expression of these angiogenic factors are not well understood. Because of this, there are several avenues that can be utilized to investigate this question and then connect it with the sexual dimorphic ADCAI response. Some possible mechanisms by which VEGF is induced by estrogen are the following:

1) Estrogen receptor (ER) activation- upon the binding between estrogen and its receptor, there is a conformational change that allows for the binding of estrogen response elements (EREs) located in the promoter regions of target genes, including VEGF.
2) Genomic mechanisms -direct transcriptional activation or the use of co-activators and co-repressors
3) Signaling Pathways- PI3K/Akt pathway, MAPK/ERK pathway, etc.
4) Hypoxia-Inducible Factor (HIF) pathway- under hypoxic conditions, estrogen can increase the stabilization and activity of Hypoxia-Inducible Factor 1-alpha (HIF-1α) which is a key transcription factor for VEGF expression. Also, estrogen and hypoxia can synergistically enhance VEGF expression with

estrogen promoting the accumulation and activity of HIF-1α, which then binds to the hypoxia-responsive elements (HREs) in the VEGF promoter.

5) Crosstalk with other signaling molecules- this includes communication between estrogen and growth factors, cytokines, and microRNAs.

It is possible that one or more than one of these mechanisms can explain the sexual dimorphic ADCAI response. It is also possible that the connection between the IgG1 response, estrogen and VEGF is something different and not listed above. For instance, there might be an element in the FcgR1/VegfR1 pathway that is affected by estrogen that is worth investigating further. The preliminary results from experiments focused on estrogen has started a conversation that can lead to better choices for therapeutics in conditions of pathological angiogenesis.

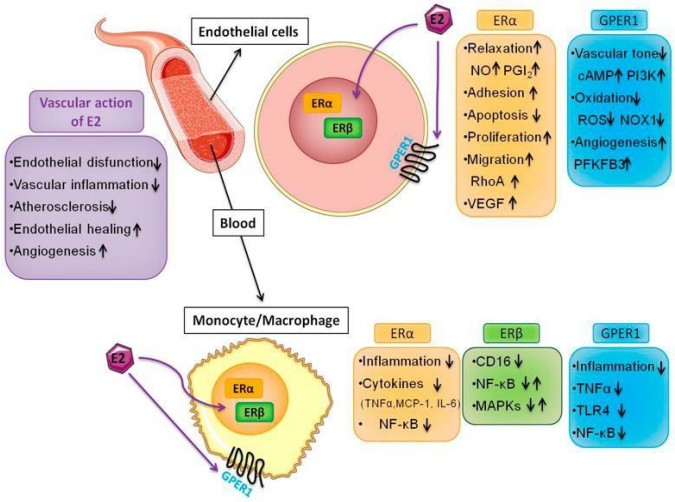


Figure 25: Multiple effects of E2 in endothelial cells and macrophages. E2 promotes endothelial healing and angiogenesis through several pathways exerting itself on the cardiovascular system. E2 is also involved in the regulation of chemokines and cytokines, affecting macrophages and immune phenotypes. From *Estrogen, Angiogenesis, Immunity and Cell Metabolism: Solving the Puzzle* **International Journal of Molecular Sciences 2018- Trenti et al.**

II. Sex differences in IgG1-mediated angiogenesis inhibition depends on Y chromosome-encoded DDX3Y

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(Manuscript to be submitted to *Nature Communications*)

Abstract

Prevalent diseases of angiogenesis such as age-related macular degeneration (AMD), exhibit sex biases, with neovascular AMD being more prevalent in females. The basis for these sex differences is unknown. Here, we quantify the impact of sex on aberrant choroidal angiogenesis regulation by human IgG1, an abundant endogenous anti-angiogenic protein. Males exhibited significantly more robust human IgG1-induced chemotaxis inhibition than females in primary human and mouse macrophages and anti-angiogenic activity in mouse laser-induced choroidal neovascularization (CNV). Four core genotypes and XY* Turner Syndrome mouse models revealed the Y chromosome as a sex biasing factor for IgG1 responses. Gene and RNAi screening identified DEAD-Box Helicase 3 Y-Linked (DDX3Y) as necessary for the robust effects of IgG1 in males. Male mice and cells lacking Ddx3y exhibited blunted IgG1 responses in CNV and macrophage chemotaxis, resembling those of females. These results unveil a novel mechanism through which sex chromosome complement differences impact key processes involved in AMD, carrying implications for various sex-related disorders and conditions involving angiogenesis.

Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness with an estimated worldwide prevalence of approximately 2.5%[52]. Neovascular AMD is an

advanced disease stage that accounts for approximately 90% of legal blindness due to AMD[218]. Neovascular AMD is caused by choroidal neovascularization (CNV) in which pathological neovessels invade the outer retina, which is normally avascular. These fragile neovessels cause vision threatening processes including hemorrhage, retinal edema, and fibrosis, compromising vision.

Established risk factors for AMD include age, genetics, tobacco consumption, and female sex[218]. Compared to males, females are at a significantly increased risk of developing neovascular AMD[10,127–129]. However, the mechanistic basis for female predisposition to neovascular AMD remains unclear. Associations between AMD and menarche and menopause ages and duration, and hormone replacement therapy are variable and inconsistent (reviewed in[124]). Mouse studies of experimental CNV report that sex does not affect CNV in relatively young animals, though middle-aged females develop greater pathology than males[130,219,220]. Though some evidence suggests that estrogens may play a role[130], the mechanistic basis of such differences have not been thoroughly investigated.

Human immunoglobulin 1 (hIgG1) and its murine counterparts, IgG2a and IgG2c, inhibit angiogenesis independently of antigen binding, an activity termed "antibody-dependent cell-mediated angiogenesis inhibition" (ADCAI). This inhibition arises from the interaction between the IgG1 Fc domain and macrophage-expressed Fc gamma receptor 1 (FcγR1), resulting in reduced VEGFR1 expression, blunted macrophage chemotaxis, and reduced angiogenesis[16].

Here we report robust sex differences in the inhibitory activity of IgG1 for human and mouse macrophage chemotaxis and CNV in young mice, with IgG1 eliciting substantially more angiogenesis inhibition in males compared to females. Using genetic models, we identified sex chromosome complement as a contributing sex biasing factor to this difference. Using genetic and functional screens, we identified the Y chromosome-encoded gene DDX3Y as a critical mediator of ADCAI. Together, our findings suggest that greater female susceptibility to neovascular AMD may arise due to reduced angioregulatory activity of IgG1 antibodies, which is mediated in part by the Y chromosome gene DDX3Y. This activity could also contribute to sex differences in other diseases of angiogenesis as well.

Methods

Mice

All animal experiments were approved by the University of Virginia's Institutional Animal Care and Use Committee. Male and female mice between 4 and 6 weeks of age were used in the study. WT C57BL/6J, *Fcgr1*$^{-/-}$, (Strain No. 000664), B6D2F1/J (Strain No. 100006), and BALB/cJ (Strain No. 000651) were purchased from Jackson Laboratory. B6.Cg-Tg (Sry) 2Ei Sry^{dl1Rlb}/ArnoJ mice were previously described[151,222]. Turner syndrome model XY* mice were previously described[151,223].

Cell Culture

THP-1 cells were cultured in RPMI-1640 media (ThermoFisher No. 118754119) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HL-60 cells were cultured in IMDM media (ThermoFisher No. 12440061) supplemented with 20% FBS and 1% penicillin-streptomycin. RAW 264.7 and J774 cells were cultured in DMEM media (ThermoFisher No. 12430062) supplemented with 10% FBS and 1% penicillin-streptomycin. Primary BMDMs were isolated from 4-6-week-old mice as previously described[224] and cultured in IMDM media with 10% FBS and 30% L929 supernatants, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and antibiotics. All cells were maintained at 37°C in a 5% CO_2 environment.

Primary Human Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS density gradient (Cytiva) from the buffy coats of healthy donors. To maximize the number of monocytes collected, RosetteSep® was added to the buffy coat for 20 minutes with incubation at room temperature. The sample was then diluted with PBS + 2% FBS (Stemcell) at 2 times the initial volume then mixed. The diluted sample is added to the Ficoll® and spun for 20 minutes at 1200 x g at room temperature with the brake off. Cells were washed with PBS + 2% FBS and collected. CD14+ cells were then selected for using the EasySep™ HLA Chimerism Buffy Coat CD14 Positive Selection Kit (Stemcell) following the manufacturer's instructions. Cells were grown in DMEM/F12 media + 10% human serum + 1% penicillin-streptomycin + M-CSF for 5-7 days for macrophage differentiation and maintained at 37°C in a 5% CO_2.

Real-time PCR

Total RNA was purified from cells with either TRIzol reagent (Invitrogen) or RNeasy Micro Kit (Qiagen) according to the manufacturer's recommendations and reverse transcribed with a QuantiTect Reverse Transcription kit (Qiagen). The RT products (cDNA) were amplified by real-time quantitative PCR (Applied Biosystems 7900 HT Fast Real-Time PCR System) with Power SYBR Green Master Mix. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method, with Beta-Actin, 18S, or GAPDH used as an internal control. All primers used can be found in the Supplementary Data section.

Macrophage Migration Assay

Primary mouse macrophages, primary human macrophages, differentiated THP-1 and HL-60 cells, and RAW 264.7, and J774 cells were treated with trypsin, suspended in 2% BMDM medium and seeded onto the upper chamber of an 8 μm polycarbonate filter such that $5x10^3$ cells were seeded onto a 24-well or 2×10^4 cells were seeded onto a 12-well format filter. Medium containing bevacizumab (0.1 mg/ml), human IgG1 purified from human myeloma plasma (0.1 mg/ml; Athens Research), or human IgG2 purified from human myeloma plasma (0.1 mg/ml; Athens Research) was placed in the lower chamber for 4 hours prior to the addition of recombinant mouse VEGF 164 (50 ng/ml; R&D Systems) or recombinant human VEGF 165 (50 ng/ml; R&D Systems). After allowing cell migration for 12-16 hours, cells were removed from the upper side of the membranes, and nuclei of migratory cells on the lower side of the membrane was stained

with DAPI (ThermoFisher No. 36935). The entire filter was imaged with a Nikon A1R epifluorescent microscope and the number of migratory cells were quantified using ImageJ.

Western blotting

Cells were lysed in RIPA lysis buffer (ThermoFisher) supplemented with a combined protease and phosphatase cocktail inhibitor and homogenized by sonication. Equal amounts of protein (25-30 μg) were prepared in 4x Laemmli buffer with β-mercaptoethanol and resolved by SDS-PAGE on Novex® Tris-Glycine Gels (ThermoFisher) and transferred onto Immobilon-FL PVDF membranes (EMD Millipore). The transferred membranes were blocked for 1 -2 hr at RT using 5% milk, 5% BSA or Licor PBS Blocking Buffer, dependent on the primary antibody then incubated with antibodies against Vegfr1 (1:500; Abcam), phospho-c-Cbl (1:1000; ThermoFisher), and FcγR1/CD64 (1:500; BioLegend). The immunoreactive bands were developed using fluorescent secondary antibodies and the Odyssey Clx system. Protein loading was assessed by western blotting using antibodies against α-tubulin (1:1000; Sigma Aldrich) or Vinculin (1:1000; ThermoFisher). Densitometry of immunofluorescent bands was quantified using the Image Studio™ software analysis tool (Licor). Fold change in the bands of interest was calculated after normalizing the signal intensity with loading controls.

Ubiquitination Experiments

BMDM from C57BL/6J male, B6/D2 *Ddx3y*$^{Y+}$ male, B6/D2 female and DDX3Y KO mice were plated and cultured in reduced 2% BMDM media overnight. The following

day, designated cells were treated with bevacizumab (0.1 mg/mL) and MG 132 (5 nM, Invivogen) for 0, 60 minute, and 120-minute timepoints. Cells were lysed in IP lysis buffer (ThermoFisher) supplemented with a combined protease and phosphatase cocktail inhibitor and homogenized by sonication. For the detection of ubiquitination, lysates were prepared using the UBI-QAPTURE-Q Kit (Enzo Life Sciences) following the manufacturer's instructions. Ubiquitination was then detected by Western blotting using an anti-ubiquitin antibody (1:5000 ThermoFisher).

Laser photocoagulation-induced choroidal neovascularization

As previously described[97], laser photocoagulation (532 nm, 180 mW, 100 ms, 75 μm) (OcuLight GL; IRIDEX Corp., Mountain View, CA, USA) was performed bilaterally (four spots per eye) on day 0 to induce CNV in 6-week-old mice. 25 μg in 1 μL of bevacizumab or an equivalent volume of PBS were injected into the vitreous humor of mice using a 33-guage double-caliber needle once, immediately after laser injury. Where indicated, cell-permeable cholesterol conjugated siRNA targeting Ddx3y or scrambled control was administered via intravitreous injection. 7 days after injury, mice were euthanized, and eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4ºC. Eyecups were incubated with fluorescein isothiocyanate (FITC)-isolectin B4 (Vector Laboratories, Burlingame, CA, USA). CNV volume was visualized by scanning laser confocal microscopy (TCS, SP, Leica). Volumes were quantified using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) as previously reported[97].

siRNA transfection

siRNA transfection was performed using DharmaFECT 4 (Dharmacon) according to the manufacturer's instructions. MISSION siRNA Universal Negative Control #1 (Sigma) and Control siRNA-A (Santa Cruz) were used as negative controls. Target siRNA for Ddx3y/DDX3Y, Uty/UTY, Kdm5d/KDM5D, and Rbm31y were manufactured by Dharmacon. Sequences are available in the Supplementary data. Knockdown of targets was assessed by RT-PCR.

Statistical Analysis

Statistical tests, analyses and graph generation were performed with GraphPad Prism 10 software. Outliers were assessed by Grubb's test. Comparisons between two groups were performed with unpaired two tail t-tests for normally distributed datasets, or Mann-Whitney U tests for CNV volumes which are not normally distributed[97]. Comparisons between more than two groups were performed using ANOVA or the Kruskal-Wallis test for CNV volumes with Tukey correction for multiple comparisons. P values < 0.05 were considered statistically significant.

Results

Human IgG1 elicits a sex-specific anti-angiogenic effect

Human IgG1 (hIgG1) suppresses macrophage migration chemotaxis toward a Vegfa gradient, which is an essential pro-angiogenic process[56,225,226]. This activity occurs

independently of antigen binding, but instead requires monomeric IgG1 to bind its high affinity receptor FcγR1/CD64, which suppresses the expression of the Vegfa receptor VEGFR[16].

Bevacizumab is a full-length humanized (94% human, 6% murine) IgG1 antibody that neutralizes human VEGFA but has essentially no affinity for Vegfa from mice due to amino acid differences between mouse and human in the antibody-VEGFA binding region[16,227,228]. Thus, in mouse studies, bevacizumab can be used to evoke antigen-independent hIgG1 activities[16]. Consistent with this model, bevacizumab treatment inhibited mouse Vegfa-induced chemotaxis in bone marrow derived macrophages (BMDMs) from male wild type (C57BL/6J) mice (**Figure 1a**) but did not inhibit chemotaxis of BMDMs from male *Fcgr1*$^{-/-}$ mice (**Figure 1b**). Compared to male wild-type cells, cells isolated from female C57BL/6J mice exhibited significantly less chemotaxis inhibition following hIgG1 treatment (**Figure 1a).** Female *Fcgr1*$^{-/-}$ cells were also unaffected by hIgG1 treatment **(Figure 1b)**.

In primary BMDMs isolated from male and female BALB/c mice, hIgG1 exhibited a more potent inhibitory effect on male compared to female-derived cells **(Figure 1c)**, suggesting the sex difference is not unique to the C57BL/6J mouse strain.

Recruitment of angiogenic macrophages is essential for pathological lesion development in choroidal neovascularization (CNV)[56]. Therefore, we hypothesized that hIgG1 would exhibit a greater anti-angiogenic effect in male than in female mice. Indeed,

bevacizumab treatment reduced CNV volume in male, but not female C57BL/6J mice **(Figure 1d)**.

Collectively, we interpret these findings to indicate that IgG1/ FcγR1-induced chemotaxis and angiogenesis inhibition exhibits a robust sex difference in mice, where males are more susceptible to hIgG1-induced anti-angiogenic responses.

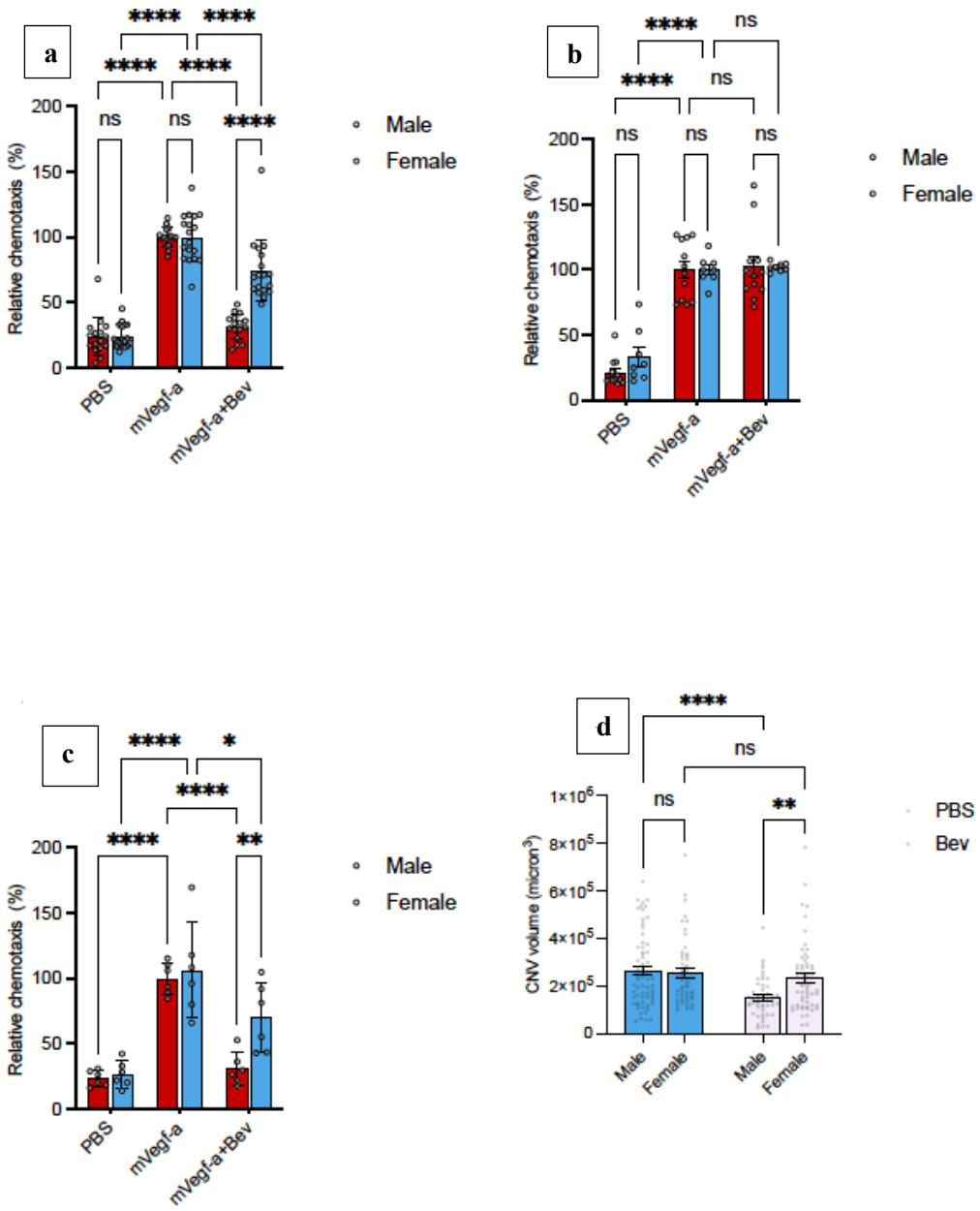


Figure 1 Human IgG1 elicits a sex-specific anti-angiogenic response in in vitro and in vivo mouse models

(a) Chemotaxis assay of WT male and female BMDMs stimulated with mouse VegfA and treated with bevacizumab. PBS is used as a negative control. (b) Chemotaxis assay of *Fcgr1*$^{-/-}$ male and female BMDMs stimulated with mouse VegfA and treated with bevacizumab. (c) Chemotaxis assay of Balb/C male and female BMDMs stimulated with mouse VegfA and treated with bevacizumab. (d) Laser CNV experiment using WT male and female mice whose eyes were treated with PBS or bevacizumab. In a-c, each data point represents an individual transwell. In a-c, red represents males and blue represents females. In d, each point represents a CNV lesion in the eye. Blue represents PBS and white represents bevacizumab. Statistical analysis was performed using 2-way ANOVA testing (a-c) and Mann-Whitney u testing (d). Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

Sex differences in ADCAI responses in human cells

Antigen-independent hIgG1 suppression of angiogenic processes is also observed in human peripheral blood mononuclear cell (PBMC)-derived macrophages[16]. The prior studies investigated pooled (a presumed mixture of male and female) human PBMC and immortalized male THP-1 cells. Thus, to test whether the dimorphism identified in mouse BMDM and CNV models was also present in primary human macrophages, we measured the IgG1 inhibition in single donor primary $CD14^+$, $CD16^-$ PBMCs differentiated into macrophages by M-CSF. Instead of bevacizumab, which potently neutralizes human VEGF-A[91], we used human IgG1 purified from the plasma of a donor with multiple myeloma (Athens Research and Technology). In contrast to hIgG1, human IgG2 (hIgG2) does not bind to FcγR1,[229] and does not inhibit angiogenesis[16]. Therefore, we also treated cells with hIgG2, also purified from the plasma of a donor with multiple myeloma, as a negative control. This was tested successfully using THP-1 cells (**Supplementary Figure 1a).** The effect of donor sex and treatment on macrophage chemotaxis are displayed in **Figure 2**. A two-way ANOVA was conducted that examined the effect of donor sex and IgG treatment on chemotaxis. There was a statistically significant interaction between the effects of sex and treatment on chemotaxis, $F = 7.255$, $p < 0.001$. Consistent with the dimorphism observed in mouse models, IgG1-induced chemotaxis was more robust in macrophages from male compared to female donors compared to IgG2 treatment. Similarly, human IgG1 suppressed chemotaxis towards a VEGF-A gradient to greater degree in THP-1, immortalized from a male donor, compared to HL-60, immortalized from a female donor **(Supplementary Figure 1b)**. Together, these data support that

male-specific IgG1 anti-angiogenic responses are conserved between human cells and mice.

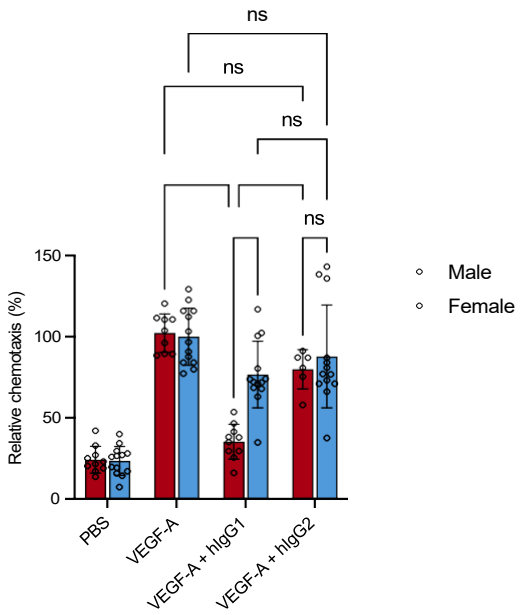


Figure 2 Sex differences in ADCAI responses in human cells
Chemotaxis assay using primary macrophages isolated from single human donors. Recombinant human VEGFA is used to stimulate cells along with treatment with human IgG1 or human IgG2. PBS is used as a negative control. Males are represented by blue, and females are represented in red. Each point represents a result from a single transwell. Statistical analysis was performed using 2-way ANOVA testing. Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

Sexual dimorphism in IgG1 angiogenesis inhibition is mediated in part by the Y chromosome

We next investigated the biasing factors responsible for these sex differences in hIgG1-induced angiogenesis inhibition by evaluating this process in the four core genotypes (FCG) model system, which isolates effects of gonadal secretions and sex chromosome complement on sexually dimorphic phenotypes[151,230]. We performed laser-induced CNV in FCG mice and analyzed the effect of bevacizumab treatment on angiogenesis. As in wild-type mice, bevacizumab significantly reduced CNV volume in XYM (mice with XY chromosomes and testes) but not XXF (mice with XX chromosomes and ovaries) (**Figure 3a**). Bevacizumab did not significantly reduce CNV

in either XYF or XXM mice, implying interacting contributions between sex chromosome complement and gonadal secretions as contributing to sex differences in hIgG1-mediated angiogenesis inhibition.

We next quantified hIgG1-induced chemotaxis inhibition in macrophages isolated from the FCG mice. As in laser CNV, macrophages from XYM were susceptible and macrophages from XXF were resistant to bevacizumab-induced chemotaxis inhibition (**Figure 3b**). Interestingly, while XXM macrophages were resistant, XYF macrophages were susceptible to bevacizumab-induced chemotaxis suppression **(Figure 3b)**. These findings indicate that differences in sex chromosome complement (XX vs. XY) determine susceptibility to hIgG1-induced chemotaxis inhibition.

Sex differences that depend on chromosome complement may arise due to the presence of a Y chromosome in males vs. the absence of a Y chromosome in females, or the presence of two X chromosomes in females vs. one X chromosome in males. To establish which of these is responsible for sex differences in hIgG1 chemotaxis inhibition, we isolated macrophages from the XY* (Turner syndrome) mouse model[230], and compared BMDMs isolated from wild-type male (XY), female (XX), and female XO mice, which have neither a Y chromosome nor a second X chromosome. Like macrophages from XX mice, XO cells were resistant to bevacizumab-induced chemotaxis suppression **(Figure 3c)**. Because the chemotaxis inhibition responses in XO cells were significantly diminished compared to XY cells, we concluded that the presence of the Y chromosome contributes to male-skewed hIgG1 chemotaxis inhibition susceptibility**.**

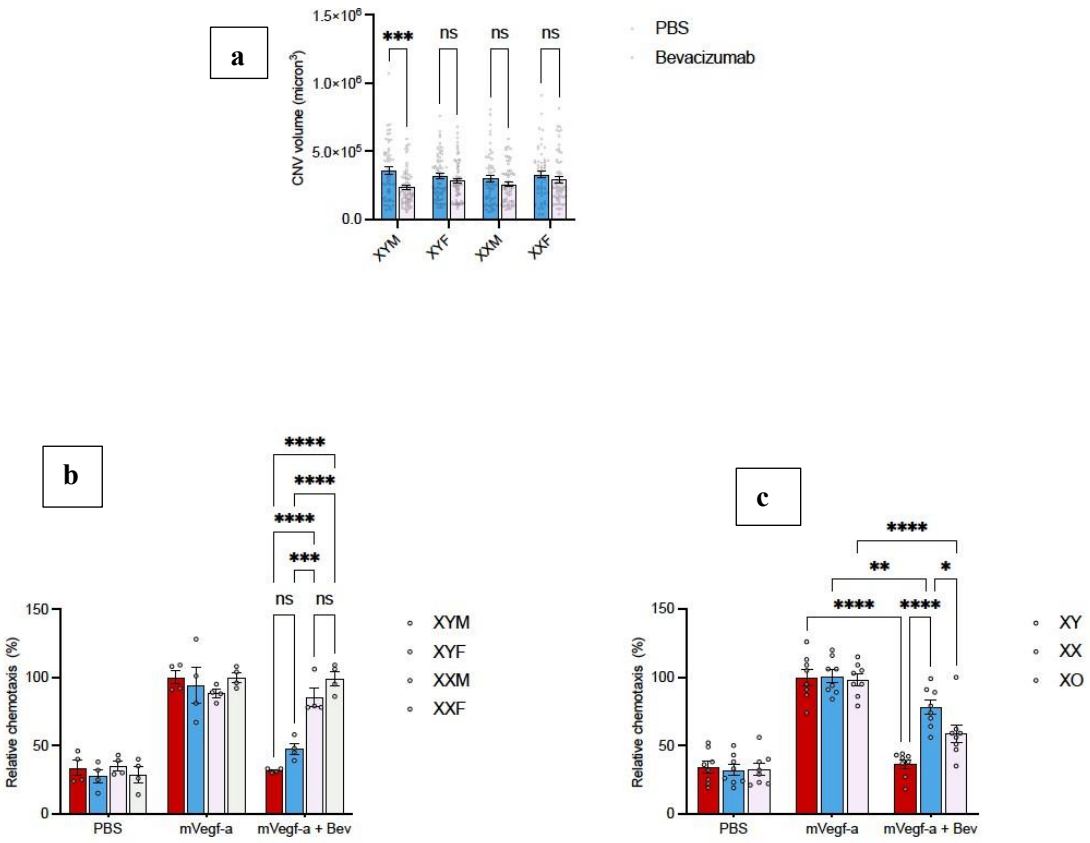


Figure 3 Sexual dimorphism in IgG1 angiogenesis inhibition is mediated in part by the Y chromosome
Manipulation of sex chromosomes using the FCG model (a- laser CNV; b- chemotaxis assay), and the XY* model (c- chemotaxis assay) illustrates how IgG1 angiogenesis inhibition can be affected by the Y chromosome. Bevacizumab was used as a treatment with PBS as a negative control in all experiments. In the chemotaxis assays, recombinant mVegfa was used as the stimulant and positive control. Statistical analysis was performed using Kruskal-Wallis testing (a). Statistical analysis was performed using 2-way ANOVA testing (b,c). Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

Sexual dimorphism in IgG1 chemotaxis inhibition depends on Y chromosome-encoded Ddx3y

We next sought to identify whether individual Y chromosome-encoded genes contributed to IgG1 responses. We performed a transcriptomic screen of the mouse Y chromosome, which contains just 13 known protein-coding genes[158]. Using qRT-PCR of wild-type male BMDM, we detected expression of 8 out of 13 genes **(Figure 4a)**. Among

these, we performed an initial siRNA-based screen of four genes (Ddx3y, Kdm5d, Rbm31y, Uty), while 4 other genes (Zfy1, Ssty1, Ssty2, Usp9y) were poor candidates for reliable siRNA design due to excess homology to other genes or internally repetitive sequences[231]. Using human and mouse macrophages, we identified DDX3Y/Ddx3y and UTY/Uty targeted siRNAs as able to abrogate hIgG1 responses in human and mouse macrophages **(Figure 4b)**. DDX3Y/Ddx3y was chosen as the focus due to its high expression level in BMDMs, its functions as an RNA helicase, and its paralog, DDX3X/Ddx3x, being involved in the innate immune system. *Ddx3y* transcripts were abundant in BMDMs from male mice but undetectable in female BMDMs, validating the specificity of the RT-PCR assay **(Figure 4a)**. Transcripts of the related mouse-specific paralogous gene D1pas1, encoded on somatic chromosome 1 and which may possess overlapping functions in spermatogenesis[232], were undetectable in male BMDM. DDX3Y is a male-specific paralog of DDX3X, a gene that is critical for macrophage hematopoiesis and innate immune responses[170,233]. siRNAs targeting Ddx3y did not affect expression of Ddx3x, and siRNAs targeting Ddx3x did not silence Ddx3y or affect male macrophage responses **(Supplementary Figure 2a)**. Ddx3y-targeted siRNAs did not affect chemotaxis in female cells, supporting the specificity of these reagents (**Supplementary Figure 2b**).

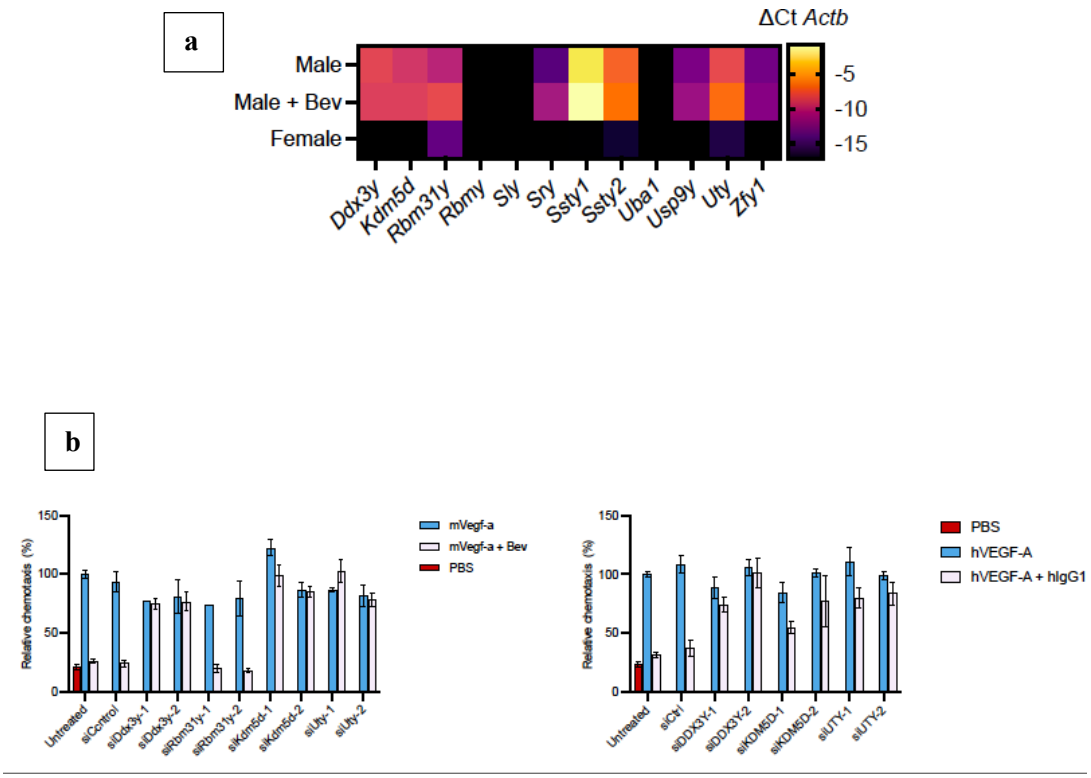
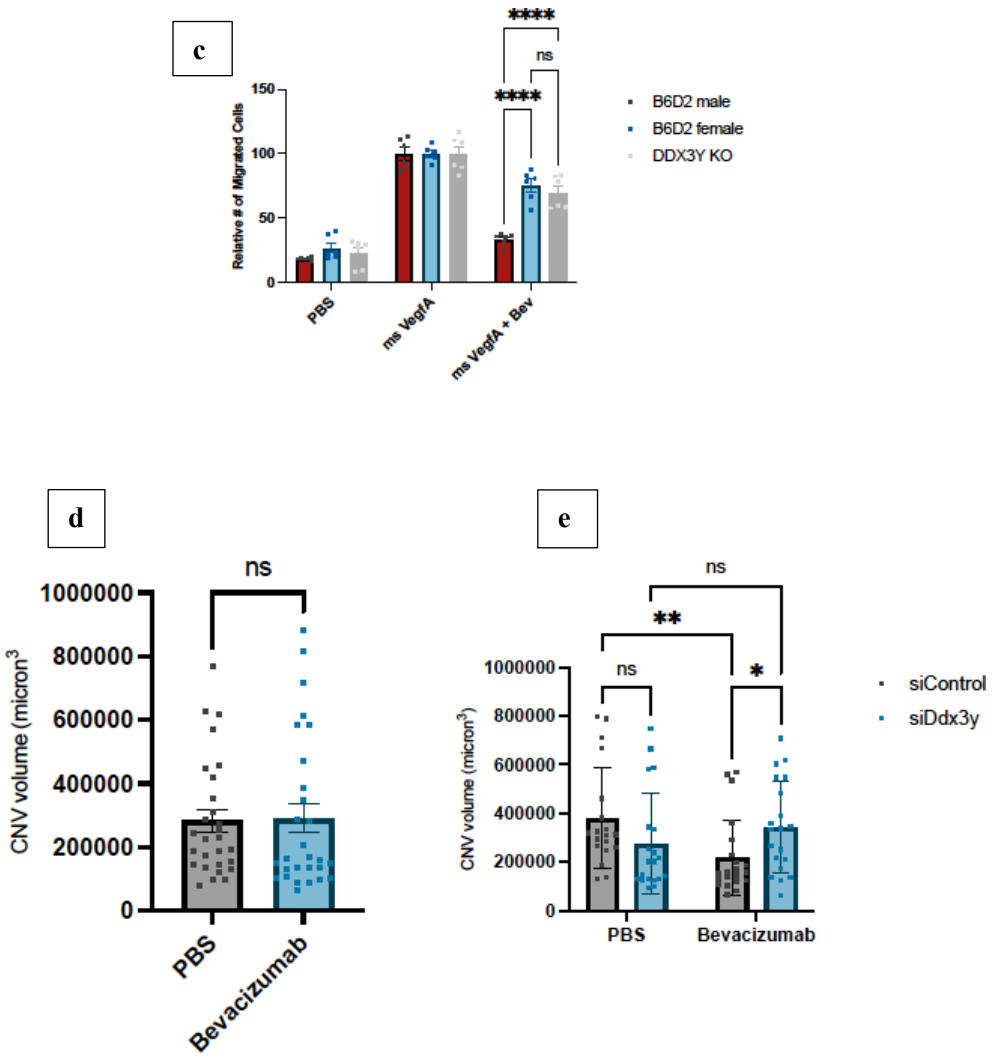


Figure 4a and 4b Y chromosome gene expression and siRNA testing using BMDMs and THP-1 cells
(a) 8 out of 13 protein coding genes expressed in the Y chromosome were expressed in WT male BMDMs and none were detected in WT female BMDMs **(b)** siRNAs were designed for 4 out of the 8 genes to test if their loss affects ADCAI in males using male BMDMs (left) and THP-1 (right). A scrambled sequence was used as a control and the chemotaxis assay was used as a vehicle of testing.



Figures 4c-e The loss of DDX3Y through CRISPR or RNA interference affects ADCAI in male mouse cells c) DDX3Y KO cells exhibit lower chemotactic activity in the presence of IgG1, comparable to female cells; d) Bevacizumab treatment had no effect on DDX3Y KO cells in the laser CNV model; e) Silencing Ddx3y using siRNA demonstrated a reduced response to bevacizumab treatment. Statistical analysis was performed using 2-way ANOVA testing (c). Statistical analysis was performed using Mann-Whitney testing (d and e). Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

To validate findings from the siRNA screen, we next isolated macrophages from a *Ddx3y*$^{Y-}$ mouse line, originally created by CRISPR-mediated targeting of exon 4 of Ddx3y[232], as well as female littermates and wild-type B6/D2 *Ddx3y*$^{Y+}$ male mice. *Ddx3y*$^{Y-}$ exhibited a 10-fold reduction in *Ddx3y* mRNA compared to *Ddx3y*$^{Y+}$ male BMDM by qRT-PCR **(Supplementary Figure- Table 1)**, and *Uty* mRNA abundance was

unchanged compared to B6/D2 *Ddx3y*$^{Y+}$ male macrophages. The *Ddx3y*$^{Y-}$ created contain a mutant sequence that causes a frameshift mutation that produces a stop codon prior to the RNA helicase domain[232]. Because of this, the protein produced is non-functional and without a translatable RNA helicase domain[232]. Bevacizumab inhibited chemotaxis in BMDM from *Ddx3y*$^{Y+}$ male mice, but *Ddx3y*$^{Y-}$ and female macrophages were relatively resistant **(Figure 4c)**. This experiment also demonstrated that there was no difference in the response to bevacizumab between female and *Ddx3y*$^{Y-}$ cells **(Figure 4c)**. In addition, *Ddx3y*$^{Y-}$ mice were resistant to the inhibitory effect of bevacizumab on laser-induced CNV **(Figure 4d)**. Similarly, administration of a cell-permeable cholesterol-conjugated *Ddx3y*-targeted siRNA likewise abrogated bevacizumab responses in wild-type C57BL/6J male mice **(Figure 4e)**. Together, these findings implicate Y chromosome-encoded Ddx3y as contributing to sex differences in IgG1-induced macrophage chemotaxis and angiogenesis suppression.

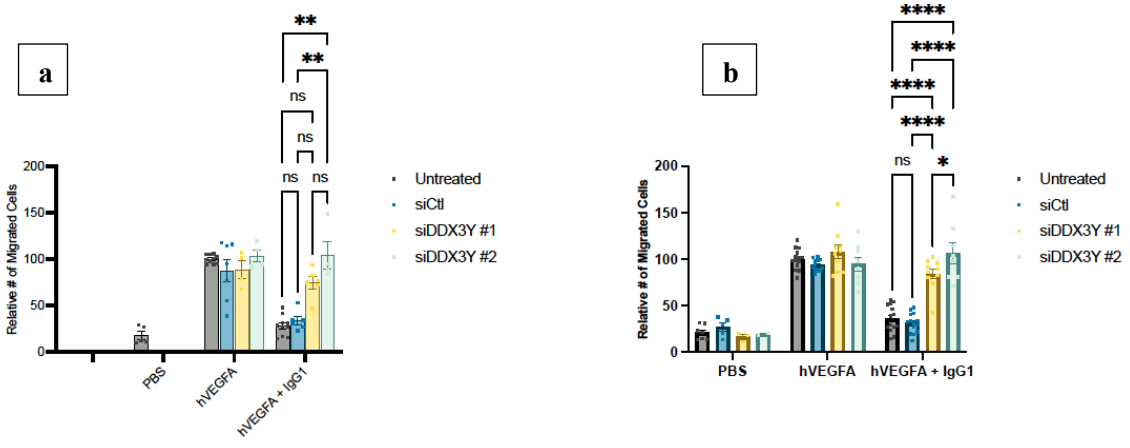


Figure 5 Sexual dimorphism in IgG1 chemotaxis inhibition depends on Y chromosome-encoded Ddx3y The loss of DDX3Y with siRNAs negatively affects ADCAI in THP-1 (a) and primary human macrophages (b). A generic IgG1 was used as a treatment, PBS was used as a negative control. Recombinant hVEGFA was used as a positive control and stimulant for the chemotaxis assay. Statistical analysis was performed using 2-way ANOVA testing (a,b). Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

DDX3Y mediates human macrophage sexual dimorphism in IgG1 chemotaxis suppression

Human *DDX3Y* mRNA transcripts were detected in human male THP-1 cells, but not in human female HL-60 cells. DDX3Y silencing by either of two independent siRNAs significantly blunted the ability of IgG1 to inhibit chemotaxis of THP-1 **(Figure 5a)**. Likewise, siRNAs targeting human DDX3Y reduced IgG1-induced chemotaxis suppression in primary male macrophages derived from healthy donors **(Figure 5b)**. These findings suggest that DDX3Y-dependent hIgG1 responses are conserved in human male macrophages.

Mechanism of DDX3Y- Western blot results using Ddx3y KO mice/B6D2 (M/F)

hIgG1 induces antigen-independent angiogenesis inhibition by inducing ubiquitin-mediated degradation of VEGF receptor 1 (VEGFR1) in macrophages[16]. Consistent with this model, treatment of male BMDM from C57BL/6J mice with bevacizumab reduced VEGFR1 abundance within 2 hours but remained stable despite bevacizumab treatment in BMDM from female wild-type mice (**Figure 6a**). VEGFR1 levels were resistant to bevacizumab treatment in BMDM from male and female *Fcgr1*$^{-/-}$ mice **(Figure 6b)**.

We sought to determine how DDX3Y affects FcγR1/VegfR1 signaling. Angiogenesis inhibition by human IgG1 depends on its binding to macrophage FcγR1,

resulting in c-Cbl activation and ubiquitination and proteolytic degradation of VEGFR[16]. FcγRI abundance was not diminished in male or female WT mice (**Figure 6c**). Similarly, IgG1-induced c-Cbl phosphorylation was unchanged in female and male *Ddx3y*$^{Y-}$ macrophages compared to wild-type male macrophages **(Figure 6d)**. Conversely, both female and *Ddx3y*$^{Y-}$ macrophages exhibited significantly reduced Vegfr1 degradation compared to *Ddx3y*$^{Y+}$ macrophages following 2-hours of hIgG1 treatment **(Figure 6e).** These results suggest that the lack of Ddx3y affects the ability of IgG1 to stimulate Vegfr1 degradation.

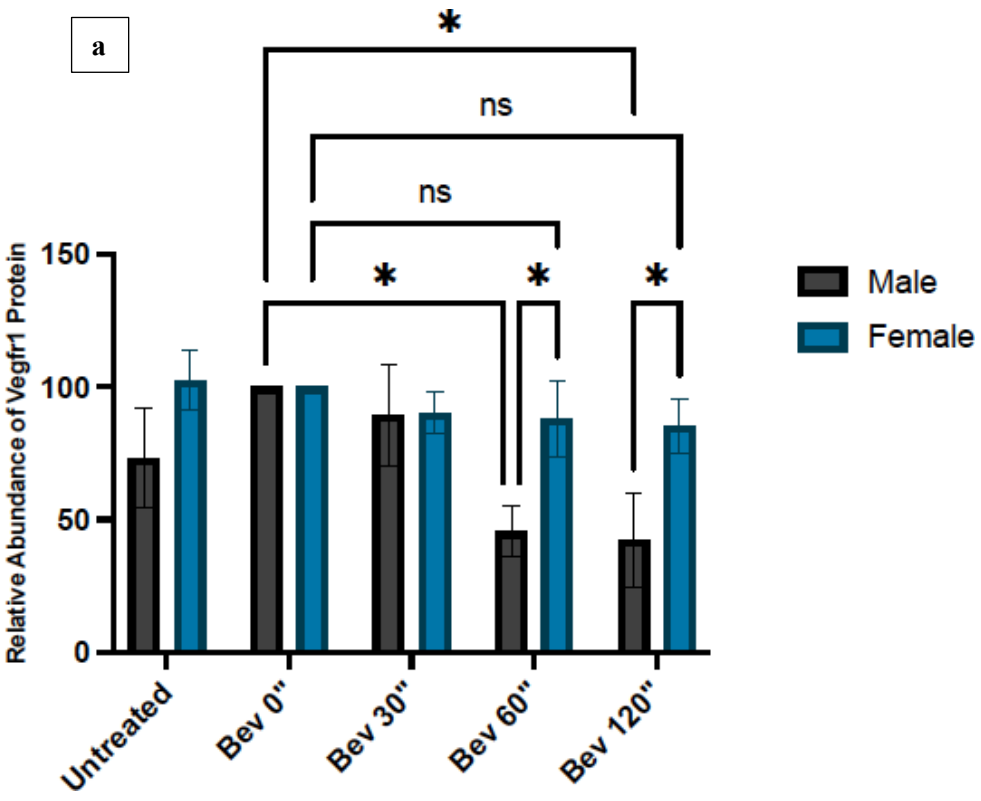


Figure 6a VegfR1 degradation WT male vs WT female Over 120 minutes WT male BMDM lysates exhibited a more robust response to bevacizumab treatment when measuring VegfR1 expression. The results shown are from 5 independent experiments with normalization to Bev 0" samples. Statistical analysis was performed using 2-way ANOVA testing.

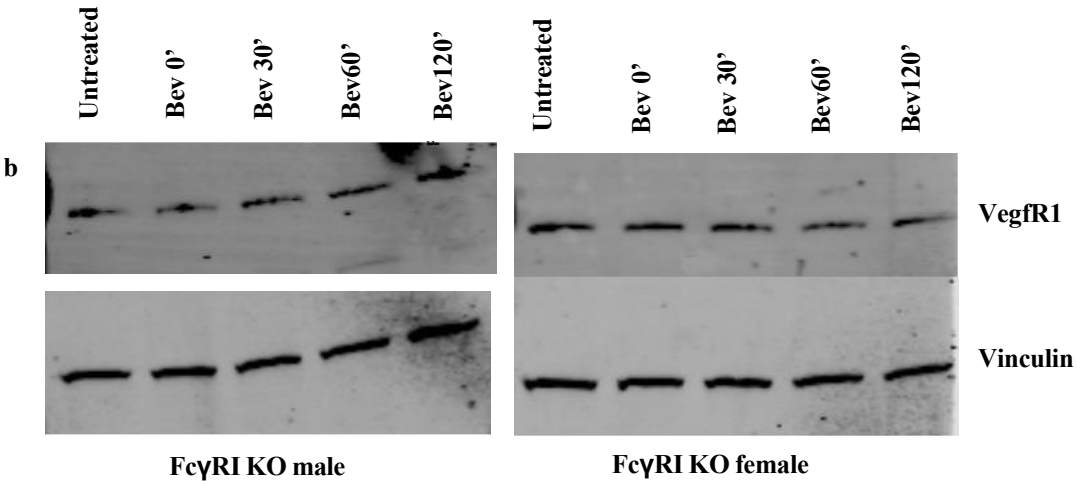


Figure 6b VegfR1 degradation does not occur in FcγRI KO mice treated with bevacizumab Lysates from male and female FcγRI KO mice are treated with bevacizumab up to 2 hours. The expression of VegfR1 is unchanged over the 2-hour period. Vinculin is used as the loading control.

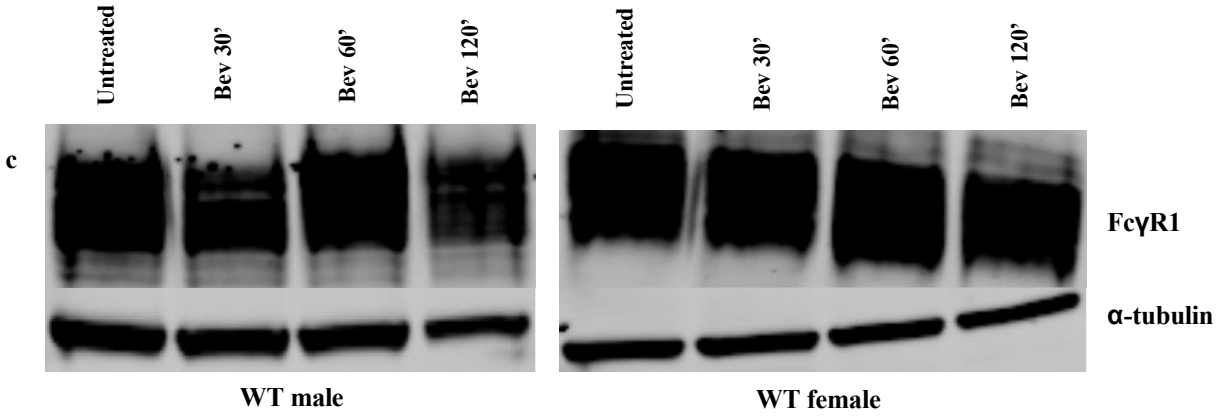


Figure 6c FcγR1 abundance is not affected by sex, or the presence of bevacizumab WT male (left) and WT female (right) lysates are treated with bevacizumab up to 2 hours. FcγR1 expression is measured via immunoblotting. α-tubulin is used as the loading control.

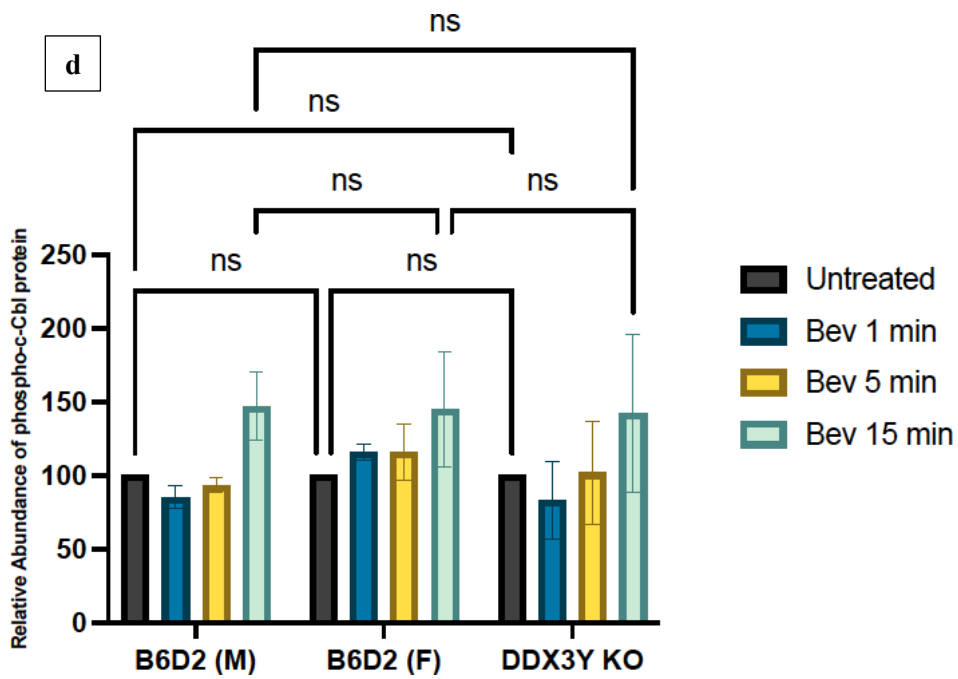


Figure 6d The phosphorylation of c-Cbl is not affected by sex or the loss of Ddx3y Lysates of *Ddx3y*$^{Y+}$ male (left), B6D2 female (center), and *Ddx3y*$^{Y-}$ male (right) were treated with bevacizumab up to 15 minutes. The densitometry shows no significant changes among the three groups whether investigating sex (male v female) or the loss of Ddx3y in the presence of bevacizumab. Statistical analysis was performed using 2-way ANOVA testing.

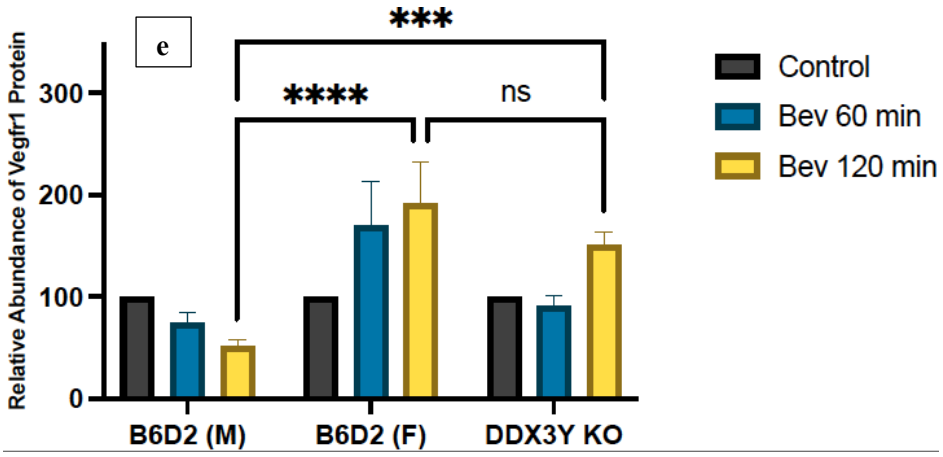


Figure 6e VegfR1 degradation is affected by the loss of Ddx3y Lysates of *Ddx3y*$^{Y+}$ male, B6D2 female, and *Ddx3y*$^{Y-}$ male were treated with bevacizumab up to 120 minutes. There was a significant difference between *Ddx3y*$^{Y+}$ male and B6D2 female cells, as well as between *Ddx3y*$^{Y+}$ male and *Ddx3y*$^{Y-}$ male cells. There was no significant difference between B6D2 female and *Ddx3y*$^{Y-}$ male cells. This result is from 5 independent experiments. Statistical analysis was performed using 2-way ANOVA testing

DDX3Y influences ubiquitination of Vegfr1

To investigate the possible role of Ddx3y in the ubiquitination of VegfR1, cell lysates were collected from the BMDMs of B6D2 female, *Ddx3y*$^{Y+}$ male, and *Ddx3y*$^{Y-}$ mice that were treated with MG-132. MG-132 is a proteasome inhibitor that stops the breakdown of ubiquitin-conjugated proteins. Cells were treated with the inhibitor along with bevacizumab up to 2 hours which is the time frame when Vegfr1 degradation occurs. MG-132 treatment allowed for the observation of ubiquitination patterns in the three strains of interest in coordination with Vegfr1 degradation (**Figure 7a**). Global ubiquitination patterns differ between the groups suggesting that female and male *Ddx3y*$^{Y-}$ cells have some difference to males in the process of ubiquitination. We interpret the findings to indicate that Ddx3y is a key mediator of hIgG1-induced VEGFR1 ubiquitin-mediated proteasomal degradation.

a

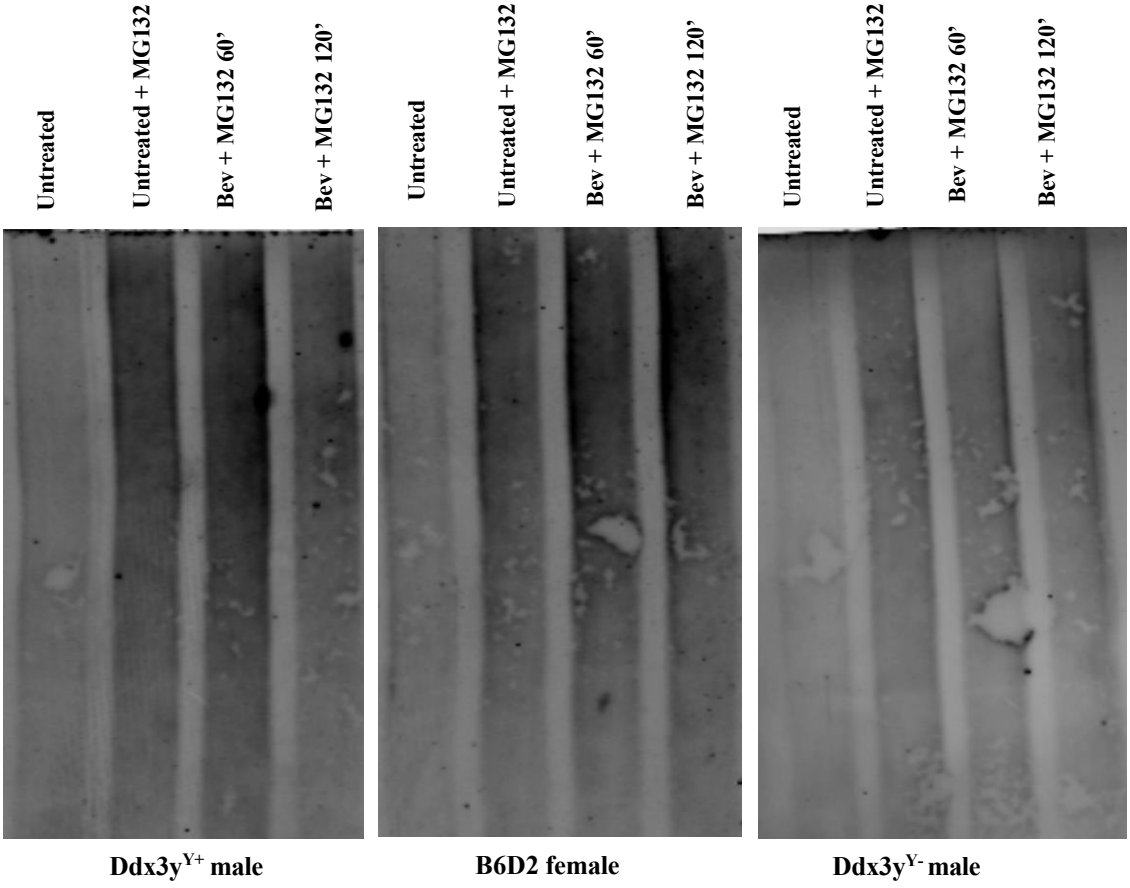


Figure 7a There is a difference in the global ubiquitination patterns in males, females, and mice lacking Ddx3y Using lysates that were treated with MG132 and bevacizumab up to 2 hours, ubiquitination was measured via immunoblotting.

To ensure that MG132 did not affect abundance of VegfR1 protein, *Ddx3y*$^{Y+}$ male cells, B6D2 female cells, and *Ddx3y*$^{Y-}$ male cells were treated with MG132 and bevacizumab. The cell lysates of all 3 groups were collected with the expression of VegfR1 checked via immunoblotting and analyzed for any differences in expression due to the addition of MG132 (**Figure 7b)**. It was determined that the addition of the proteasome inhibitor, MG132, did not affect the abundance of VegfR1. Cells from WT male mice were also treated with bevacizumab to illustrate that VegfR1 degradation

occurs without the addition of MG132 and without bevacizumab treatment VegfR1 protein abundance at baseline is not affected by MG132 treatment (**Figure 7c).**

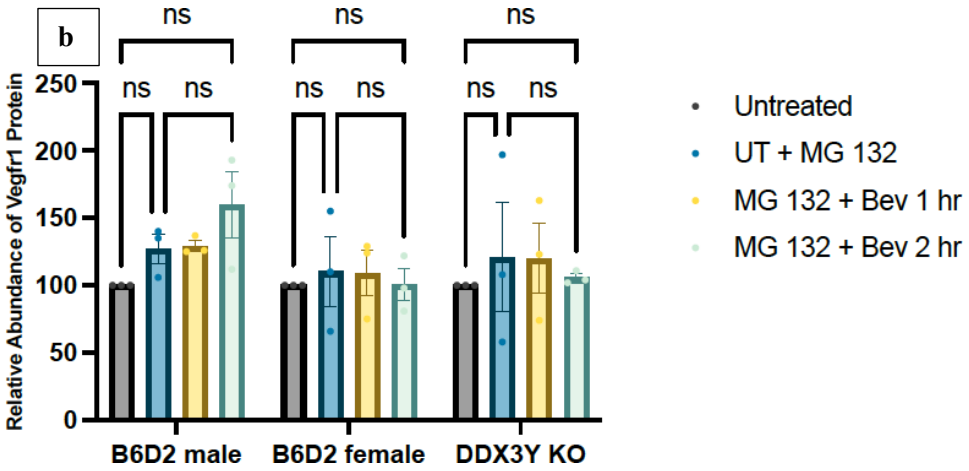


Figure 7b MG132 does not alter baseline VegfR1 abundance *Ddx3y*$^{Y+}$ male lysates, B6D2 female lysates, and *Ddx3y*$^{Y-}$ male lysates were treated with MG132 and bevacizumab for up to 2 hours. Vinculin was used as a loading control. Statistical analysis was performed using 2-way ANOVA testing.

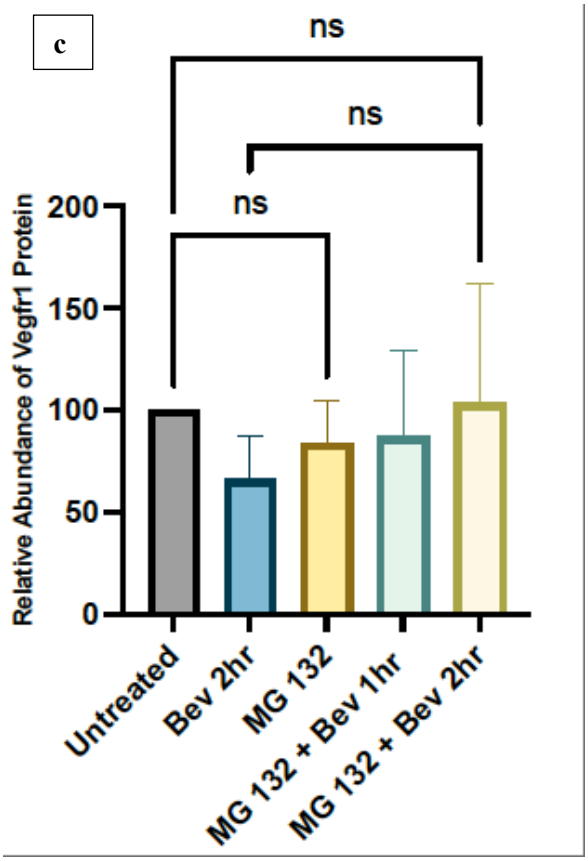


Figure 7c MG132 abrogates bevacizumab-induced VEGFR1 degradation WT male lysates were treated with bevacizumab without and with MG 132 up to 2 hours to determine if MG 132 affects the abundance of VegfR1 and its degradation

Discussion

Sex and gender are major determinants of health that affect patterns of disease and injury[13,15,109,110,183,234]. Historically, there has been a bias across many disciplines within biomedical research heavily dominated by male models and research subjects despite widespread recognition of conditions that are more prevalent in women than males and vice versa[1,2]. Including sex as a research variable increases rigor, promotes discovery, and expands the relevance of biomedical research[3].

Angiogenesis, the growth of new blood vessels from the existing vasculature is a key feature of several diseases that affect males and females differently such as AMD[9,10], peripheral artery disease (PAD)[11,12] and stroke[13,235]. In cardiovascular disease (CVD), genetics, epigenetics, and sex hormone signaling are factors to the biological mechanism of sex differences in CVD[15].

The Four Core Genotype and XY* mouse models enable researchers to assess whether the varied responses are related to the chromosome complement, gonadal differences, or interactions thereof[151,230]. Results from these models suggest that sex chromosomes influence IgG1 responses. With expression and knockdown profiling of Y chromosome-encoded genes, we were able to identify DDX3Y as a mediator of the IgG1 response in macrophages. DDX3Y is a gene that is ubiquitously expressed throughout the body that has been mostly associated with spermiogenesis and infertility[161]. Its female-specific paralog, DDX3X, has been associated with macrophage hematopoiesis and innate immune responses[170,237]. We have demonstrated that the loss of DDX3Y, whether transient (siRNA) or permanent (CRISPR), diminishes the effectiveness of an IgG1 antibody's ability to reduce chemotaxis and angiogenesis. DDX3Y affects ADCAI in its latter stages by altering the efficiency of Vegfr1 ubiquitination and degradation.

These studies support the growing notion that Y chromosome genes may possess functional activities beyond those of reproductive tissues. For example, DDX3Y has been associated with B cell lymphoma[163], neuronal differentiation[238] and graft versus host disease[164]. The extent to which DDX3Y mediates these activities via its protein remains a

difficult question to study given the intrinsic challenges associated with protein-based analyses of Y chromosome encoded genes.

These findings support that Y chromosome genes may be a possible therapeutic target in the treatment of pathological angiogenic conditions[183,242–247]. There has been emerging evidence that suggests that the loss the of the Y chromosome that occur in human leukocytes during aging can lead to several changes in males. Also, with the completion of the sequencing of the human Y chromosome further research into DDX3Y along with other Y chromosome genes and their association with other diseases and conditions is required.

Supplementary Data

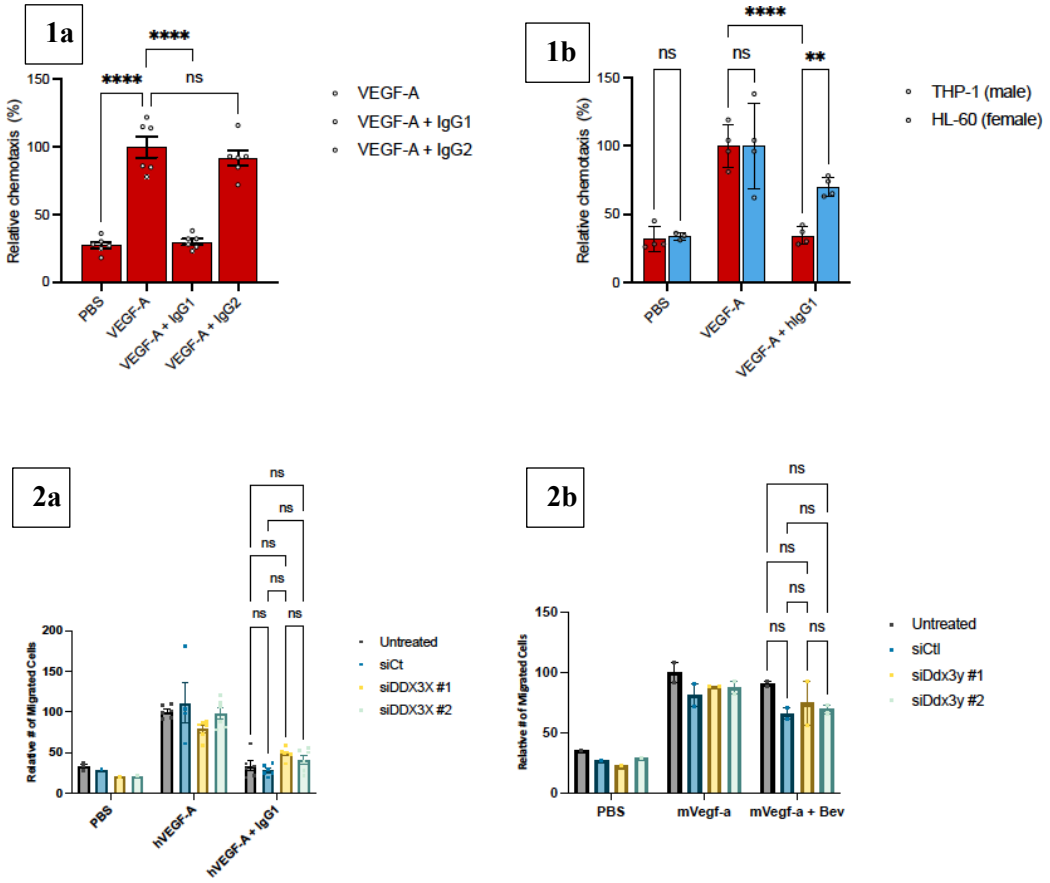


Table 1- Ddx3y mRNA expression

Strain	β- actin Ct value	Ddx3y Ct value	ΔCt
B6D2 male	23.141	26.435	-3.294
B6D2 female	23.391		
DDX3Y KO	22.942	29.839	-6.897

Supplementary Figures 1a) Chemotaxis assay of THP-1 cells treated with human IgG1 and human IgG2 with hVEGF-A as a stimulant and PBS as a negative control. 1b) Chemotaxis assay of THP-1 cells (male) and HL-60 cells (female) treated with human IgG1 and stimulated with hVEGF-A. 2a) Chemotaxis assay using THP-1 cells treated with siRNAs targeting DDX3X. Stimulation with hVEGF-A and PBS as a negative control. 2b) WT female BMDMs treated with siRNAs targeting Ddx3y and treated with bevacizumab. Stimulation with mVegf-A and PBS as a negative control. Table 1) qpcr data showing the difference in mRNA expression of Ddx3y in B6D2 male, B6D2 female, and DDX3Y KO mice. Beta-actin was used the housekeeping gene. Statistical analysis was performed using 2-way ANOVA testing. Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

List of Primers

Primers: Human
DDX3Y F- 5'GCCATACCGAGGCT 3'
DDX3Y R- 5'ATATAGCGCCCTTTGCTCGC 3'

KDM5D F- 5'AGCCATCTGCAAGGATCGTC 3'
KDM5D R- 5' TGTAGGCTCTGGATCAGGCT 3'

UTY F- 5' CTGTAGCATTTGTGAGGTGGAG 3'
UTY R- 5' GTGCAGAAATTTCCTGAAGAGC 3'

Beta Actin F- 5' GATTCCTATGTGGGCGACGA 3'
Beta Actin R- 5' AGGTCTACATGATCTGGGT 3'

DDX3Y transcript variant (TV) 3 F-5' GCGTGCTTCTCTTGACCCT 3'
DDX3Y transcript variant (TV) 3 R- 5' TCTTGCTCCAAGCGTTCACT 3'

DDX3Y transcript variant (TV) 4 F- 5' ATGGAGACCGGTCACAGAGA 3'
DDX3Y transcript variant (TV) 4 R- 5' TGTTTAGCTTCCAGGTTTCCTACA 3'

DDX3Y transcript variant (TV) 1 F- 5' TGCGGACCTGTTCTTTCACC 3'
DDX3Y transcript variant (TV) 1 R- 5' TATATAGCGCCCTTTGCTCGC 3'

Primers: Mouse
Zfy 1 F- 5' TCTGGGTTTTCAGGCGTTCT 3'
Zfy 1 R- 5' GTCAGCTCCAGACCAGTGTA 3'

Ssty 1 F- 5' GCCTAGAAGGTGTGTCCACT 3'
Ssty 1 R- 5' ATATTGCCCTCTCTTGTACCTGC 3'

Ssty 2 F- 5' TCTATGGGAGCTCAAATCTGTTT 3'
Ssty 2 R- 5' GGGACTGAAGATCTTGCCCG 3'

Kdm5d F- 5' CGCGTTTTGAGCGAACATGA 3'
Kdm5d R- 5' GTCTGGGCCTCCAGTTCATTTA 3'

Uty F- 5' AGAAAATGGCGGCGGAAAAG 3'
Uty R- 5' GAATTGCCTTGTTCAACAGCGT 3'

Ddx3y F- 5' GTGGCTGTTCCGTGAGAAGT 3'
Ddx3y R- 5' GCGCCCTTTGCTCTCTGTAT 3'

Usp9y F- 5' ATGGCAGGTTGCACATTCAC 3'
Usp9y R- 5' CCAAGCCATTCCATAGCCCA 3'

Rbm31y F- 5' GTATGCTTTGGCAGCGTACC 3'
Rbm31y R- 5' TCCCAGAAGCATATTGGCCG 3'

D1Pas1 #1 F- 5'CACGAGAGCCAAGTCCAGTT 3'
D1Pas1 #1 R- 5' TCGACCAGTCATCCTCGTCT 3'

D1Pas1 #2 F- 5’ TCGACCAGTCATCCTCGTCT 3’
D1Pas1 #2 R- 5’ TCGACCAGTCATCCTCGTCT 3’

D1Pas1 #3 F- 5’ TCGACCAGTCATCCTCGTCT 3’
D1Pas1 #3 R- 5’ GCAGTTATTCCCTGTCGCCT 3’

Actin F- 5’ CGGTTCCGATGCCCTGAGGCTCTT 3’
Actin R- 5’ CGTCACACTTCATGATGGAATTGA 3’

Gapdh F- 5’ CGGCCGCATCTTCTTGTG 3’
Gapdh R- 5’ ACCGACCTTCACCATTTTGTCT 3’

18S F- 5’ AATGCTGTGGCGGATTCCT 3’
18S R- 5’ CCTCTCCCTTTGTCTTCAAGTTG 3’

siRNA

Human
DDX3Y #1 S 5’-P-GCAGAUUCGGGACUUAGAA-dtdt
AS- UUCUAAGUCCCGAAUCUGC-dtdt

DDX3Y #2 S 5’-P-GACGUCUAGUGGAUAUGAU-dtdt
AS- AUCAUAUCCACUAGACGUC-dtdt

KDM5D #1 S 5’-P-GCUCUAGUAGCCGACAGUA-dtdt
AS- UACUCUCGGCUACUAGAGC-dtdt

KDM5D #2 S 5’-P-GGGUAGAAACGUUGAGAAU-dtdt
AS- AUUCUCAACGUUUCUACCC-dtdt

UTY #1 S 5’-P-GGUUCGUGAGGCUUCAUGA-dtdt
AS- UCAUGAAGCCUCACGAAC-dtdt

UTY #2 S 5-P-GGAGGCAUAUGAACAACUU-dtdt
AS- AAGUUGUUCAUAUGCCUCC-dtdt

Mouse
Ddx3y #1 S 5’P-GGAGCUGUAGUAAAGAUAA-dtdt
AS- UUAUCUUUACUACAGCUCC-dtdt

Ddx3y #2 S 5’ P-GGAGCUGUAGUAAAGAUAA-dtdt
AS- UUAUCUUUACUACAGCUCC-dtdt

Kdm5d #1 S 5’-P-GAUUUACUCCUCGAAUUCA-dtdt
AS- UGAAUUCGAGGAGUAAAUC-dtdt

Kdm5d #2 S 5’-P-GCUGCAGUGAUAAUUACCA-dtdt
AS- UGGUAAUUAUCACUGCAGC-dtdt

Uty #1 S- 5’-P-GUCACUUCAAUCUCUUACU-dtdt
AS- AGUAAGAGAUUGAAGUGAC-dtdt

Uty #2 S 5’-P-GUCACUUCAAUCUCUUACU-dtdt
AS- AGUAAGAGAUUGAAGUGAC-dtdt

Rbm31y #1 S 5’-P-GAGCUUUCGGCUUUAUCAA-dtdt
AS- UUGAUAAAGCCGAAAGCUC-dtdt

Rbm31y #2 S 5’-P-GGGUAAGCCACUAUACUCU-dtdt
AS- AGAGUAUAGUGGCUUACCC-dtdt

III. The Anti-angiogenic effects of IgG1 antibodies is negatively influence by estrogen

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(Manuscript to be submitted to *Biology of Sex Differences*)

Abstract

Background: Human IgG1s possess intrinsic anti-angiogenic activity that suppresses macrophage chemotaxis, independent of their ability to bind antigens. This activity is sex-dependent, with more profound anti-angiogenic activity being observed in males than in females. This study sought to determine the influence of estrogens on this sex difference.

Methods: Estrogen-dependent mouse and human macrophage chemotaxis was assessed in a modified Boyden chamber by charcoal stripping with or without exogenous 17-β-estradiol or treatment with the estrogen receptor inhibitor fulvestrant. Laser photocoagulation-induced choroidal neovascularization was performed in mice with or without intravitreous injection of human IgG1 as a model of macrophage-dependent angiogenesis. Ovariectomy was performed to determine the effect of gonadal secretions on angiogenesis inhibition by human IgG1.

Results: Inhibiting estrogen signaling by removing estrogens or treatment with fulvestrant significantly increased IgG1-mediated chemotaxis inhibition in female cells. The elimination of estrogen via ovariectomy did not exhibit a significant difference versus mice that had intact ovaries.

Conclusions: Estrogen suppresses IgG1-mediated angiogenesis inhibition to some extent, which may contribute to sex differences in the anti-angiogenic activity of IgG1 proteins

observed in in vitro studies. These findings have implications for conditions of angiogenesis that exhibit sex differences. Further study is required to understand the mechanism that allows for this sexual dimorphic response utilizing in vitro and in vivo assays.

Introduction

Sexual dimorphism is observed in immune responses and disease susceptibility. In general, females mount stronger innate and adaptive responses than males[109,112]. This allows for females to be more efficient at clearing pathogens but also makes them more susceptible to autoimmune and inflammatory diseases[118,119,176]. One of the factors for this difference is sex hormone production. Females primarily produce estrogen and progesterone while males are the primary producers of testosterone.

Sex hormones can have profound impacts on immune function[106,113,249]. Pregnancy, age, and menopause are some examples of the dynamic changes that can influence estrogen and progesterone concentrations[120,184,185]. These fluctuations in hormone concentrations can lead to changes in disease pathology and disease treatment responses[105,108,114,120,185,188]. Because of their role as modulators of sex differences and their influence on immunology, it is important to determine which signaling pathways they affect to better treat diseases and conditions.

Previous work revealed that human IgG1 and its murine equivalents, IgG2a and IgG2c, independently inhibit angiogenesis without antigen binding, an activity termed "antibody-dependent cell-mediated angiogenesis inhibition" (ADCAI)[16]. This inhibition occurs through the interaction between the IgG1 Fc domain and the Fc domain gamma receptor 1 (FcγR1) on macrophages[17]. This interaction results in reduced chemotaxis and angiogenesis. We sought to study the effect that sex hormones, specifically estrogen, have on the IgG1 response and whether that effect promoted or inhibited ADCAI. The results of this study could shed light on the role that estrogen plays in immune-vascular crosstalk and provide clues about the best treatment options during a particular stage in life such as pre- versus post-menopausal or pre- or post-pregnancy.

Plain English Language

The biological differences that exist between males and females such as sex chromosomes (XX and XY) and hormone levels (estrogen, progesterone, and testosterone) can impact health and disease. Previous research demonstrated that the family of IgG1 antibodies can stop the abnormal blood vessel growth without a specific target. This activity is called "antibody-dependent cell-mediated angiogenesis inhibition" (ADCAI), and it occurs when the Fc region of the antibody binds with Fc gamma receptor 1 (FcγR1) on immune cells, in this case macrophages.

Most previous studies used male subjects and cell lines. This current study explores how estrogen, a hormone that is known to be at higher concentrations in females

than males, influences ADCAI. In vitro experiments presented here utilized specific reagents and treatments that remove external influences such as hormones or specific receptors that can affect studies where sex is a factor. In vivo experiments allowed for the removal of sex organs to investigate their importance and the hormones that they produce. The results suggest that sex hormones, specifically estrogen, can affect how the body responds to treatments with antibodies from the IgG1 subclass. These findings might change how men and women are treated for conditions involving abnormal blood vessel growth in the future along with understanding how sex differences can affect different mechanisms involved in immunity and vascular biology.

Highlights

- IgG1 antibodies suppress the abnormal growth of blood vessels independent of their antigens. This activity is called antibody-dependent cell-mediated angiogenesis inhibition (ADCAI).
- ADCAI responses are sexually dimorphic with males having more robust responses to IgG1 treatment compared to females.
- Conditions of reduced estrogen enabled females to have a greater ADCAI response, comparable to what was observed in males.
- The addition of estrogen reduced the IgG1 response, illustrated by an increase in macrophage chemotaxis.
- The reduction of estrogen binding using fulvestrant enabled a more potent response to IgG1 treatment in females.

- A better understanding of how sex hormones such as estrogen can influence therapeutics for pathological angiogenic conditions can lead to better diagnoses and treatment options for patients based on sex.

Materials and Methods

Mice

All animal experiments were approved by the University of Virginia's Institutional Animal Care and Use Committee. Male and female mice between 4 and 6 weeks of age were used in the study. C57BL/6J (Strain No. 000664) and Balb/c (Strain No. 000651) were purchased from Jackson Laboratory.

Cell Culture

THP-1 cells were cultured in RPMI-1640 media (ThermoFisher No. 118754119) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HL-60 cells were cultured in IMDM media (ThermoFisher No. 12440061) supplemented with 20% FBS and 1% penicillin-streptomycin. Primary BMDMs were isolated as previously described[224] and cultured in IMDM media with 10% FBS and 30% L929 supernatants, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and antibiotics. For sex hormone free conditions, HL-60 cells were cultured in phenol red free IMDM media (ThermoFisher No. 21056023) supplemented with 20% charcoal stripped FBS (Sigma F6765-100ML) and 1% penicillin-streptomycin. Primary BMDMs were isolated as previously described[224]

and cultured in phenol red free IMDM media (ThermoFisher No. 21056023) with 10% FBS (Sigma F6765-100ML) and 30% L929 supernatants (also cultured in phenol red free IMDM media), nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and antibiotics. For β-estradiol treated cells, addition to cell culture for 7 days at a 1nM concentration to match physiological conditions. All cells were maintained at 37ºC in a 5% CO_2 environment.

Macrophage Migration Assay

Freshly isolated bone marrow cells were differentiated in vitro into macrophages as described earlier[250] from C57BL/6J (wild type) and Balb/c mice. 5 x 10^3 or 2 x 10^4 cells were treated with trypsin, suspended in 2% BMDM medium and seeded onto the upper chamber of an 8 μm polycarbonate filter (12 well or 24 well transwell format). THP-1, HL-60 cells and primary human macrophages were also used for this procedure. Medium containing bevacizumab (0.1 mg/ml) or human IgG1 (0.1 mg/ml; Athens Research) was placed in the lower chamber for 4 hours prior to addition of mouse or human Vegfa (50 ng/ml; BioLegend). After allowing cell migration for 12-16 hours, cells were removed from the upper side of the membranes, and nuclei of migratory cells on the lower side of the membrane was stained with DAPI. The number of migratory cells was determined by fluorescence microscopy from the entire 12 mm or 6.5 mm diameter acquired using Nikon software. Cell numbers were acquired using ImageJ (http://imagej.nih.gov/ij).

Fulvestrant Treatment

Primary BMDMs were isolated as previously described[224] and cultured in either normal BMDM media (base IMDM media containing phenol red) or charcoal stripped media (base IMDM media without phenol red). Some cells cultured in charcoal stripped media received exogenous estrogen in the form of β-estradiol at a physiological concentration (1nM) for one week. After 6 days when the cells had differentiated into macrophages, fresh cell culture media was added and fulvestrant was added to some cells at a 100nM concentration overnight prior to the chemotaxis assay. Some cells also received fulvestrant combined with estrogen overnight.

Western blotting

Cells were lysed in RIPA buffer (ThermoFisher) supplemented with a combined protease and phosphatase cocktail inhibitor and homogenized by sonication. Equal amounts of protein (25-30 μg) were prepared in 4x Laemmli buffer with β-mercaptoethanol and resolved by SDS-PAGE on Novex® Tris-Glycine Gels (ThermoFisher) and transferred onto Immobilon-FL PVDF membranes (EMD Millipore). The transferred membranes were blocked 1-2 hr at RT using Licor PBS Blocking Buffer. The primary antibody, ERα (1:500 Genetex) was incubated overnight at 4^0C. The immunoreactive bands were developed using a fluorescent secondary antibody and the Odyssey Clx system. Protein loading was assessed by western blotting using an antibody again Vinculin (1:1000; ThermoFisher).

Ovariectomy and sham surgery

Ovariectomy and sham surgeries were performed on 5-week-old C57BL/6J female mice at Jackson Laboratory. Mice were given a recovery time of 28 days prior to laser photocoagulation-induced choroidal neovascularization.

Laser photocoagulation-induced choroidal neovascularization

As previously described[97], laser photocoagulation (532 nm, 180 mW, 100 ms, 75 μm) (OcuLight GL; IRIDEX Corp., Mountain View, CA, USA) was performed bilaterally (four spots per eye) on day 0 to induce CNV in 8-week-old mice. 25 μg in 1 μL of bevacizumab or an equivalent volume of PBS were injected into the vitreous humor of mice using a 33-guage double-caliber needle once, immediately after laser injury. 7 days after injury, mice were euthanized, and eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4ºC. Eyecups were incubated with fluorescein isothiocyanate (FITC)-isolectin B4 (Vector Laboratories, Burlingame, CA, USA). CNV volume was visualized by scanning laser confocal microscopy (TCS, SP, Leica). Volumes were quantified using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) as previously reported[97].

Statistical Analysis

Statistical tests, analyses and graph generation were performed with GraphPad Prism 10 software. Comparisons between two groups were performed with unpaired t-test or Mann-Whitney U test for CNV volumes which are not normally distributed[97].

Comparisons between more than two groups were performed using ANOVA or the Kruskal-Wallis test for CNV volumes. A P-value < 0.05 was considered statistically significant.

Results

ADCAI responses are sex dependent

Bevacizumab is a full-length humanized (94% human, 6% murine) IgG1 antibody that primarily targets human VEGFA[91], with little affinity for mouse[16,82,227,228] or rat Vegfa[85]. Its inhibitory effect relies on the IgG1 Fc domain binding to the high affinity receptor FcγR1/CD64[16]. In experiments utilizing bone marrow derived macrophages (BMDMs), bevacizumab inhibited chemotaxis in wild type C57BL/6J mice, but not in *Fcgr1*$^{-/-}$ mice (Argyle et. al, 2024, **Figures 1a, b**). This established the importance of the FcγRI in the angiogenesis inhibition in ADCAI. Notably, cells isolated from female C57BL/6J mice exhibited significantly less chemotaxis inhibition following bevacizumab treatment (Argyle et, al, 2024, **Figure 1a).** Female *Fcgr1*$^{-/-}$ cells were also unaffected by bevacizumab treatment **(**Argyle et. al, 2024, **Figure 1b)**. The use of BMDMs to model ADCAI signaling is justified because macrophages are known to be critical contributors to angiogenesis[226,251,252].

To assess whether this dimorphism was specific to the C57BL/6J mouse strain, BMDMs were isolated from male and female BALB/c mice. Similar results were

obtained with the BMDMs from BALB/c mice **(**Argyle et. al, 2024, **Figure 1c**) indicating a sex-dependent response to bevacizumab.

An in vivo model to induce angiogenesis is the laser choroidal neovascularization model (CNV). This model induces angiogenesis by targeting the retinal pigment epithelium (RPE) and Bruch's membrane with a laser to create an injury that models the hallmark pathology observed in neovascular/wet AMD[253]. Recruitment of angiogenic macrophages is essential for pathological lesion development in choroidal neovascularization[56]. In male mice, intraocular administration of bevacizumab inhibits CNV volume in an FcγRI-dependent manner. Bevacizumab treatment resulted in reduced CNV volume in males, while female C57BL/6J mice exhibited reduced responsiveness **(**Argyle et. al, 2024, **Figure 1d)**. These findings suggest a sexual dimorphism in IgG1/ FcγR1-induced chemotaxis and angiogenesis inhibition, with males showing more robust responses.

Sexual dimorphism in ADCAI responses in humans

To investigate whether the sexual dimorphism observed in mouse models and cells is also present in primary human macrophages, we evaluated ADCAI in human peripheral blood mononuclear cells (PBMCs) differentiated into macrophages. Utilizing single donor primary CD14+, CD16- cells that were differentiated using macrophage colony stimulating factor (M-CSF), we employed human IgG1 purified from a multiple myeloma donor's plasma as a substitute for bevacizumab which is

known to neutralize human VEGF-A[228,254,255]. As a negative control, we used human IgG2 also obtained from the plasma of a multiple myeloma donor, which lacks binding affinity to FcγR1[229,256] and does not inhibit angiogenesis.

In alignment with the dimorphism observed in mouse models, IgG1-induced chemotaxis exhibited greater potency in macrophages derived from male donors compared to females. Conversely, IgG2 failed to inhibit chemotaxis in cells of either sex **(**Argyle et. al, 2024, **Figure 2)**. Consistent with these results, immortalized human cell lines, THP-1 and HL-60, differentiated into macrophages with phorbol-12-myristate-13-acetate (PMA)[257,258], demonstrated a similar pattern. Human IgG1 more effectively suppressed chemotaxis towards a VEGF-A gradient in male THP-1 cells than in female HL-60 cells **(**Argyle et. al, 2024, **Supplementary Figure 1b)**. These findings further support the presence of a sexual dimorphism in IgG1/ FcγR1-induced chemotaxis, extending the observation from mouse models to primary human macrophages and human cell lines.

Female cells are capable of ADCAI in estrogen depleted media

After observing more robust responses in male cells to IgG1 treatment, we sought to find out if sex hormones, specifically estrogen, can be a factor in the limited response by female macrophages. To test this, BMDMs were isolated from age-matched female mice with one set of cells grown in normal media and a second set of cells from the same mouse grown in a sex hormone free media. For the latter, cells

were grown in IMDM media that lacked phenol red due to its ability to act like a weak estrogen[205,206] and charcoal stripped fetal bovine serum (FBS) which removes nonpolar molecules such as hormones without removing nutrients such as glucose, amino acids, and salts. After being cultured in their media for 7 days, the cells were used in a chemotaxis assay moving towards a Vegfa gradient in the presence of bevacizumab. Female cells that were grown in the charcoal stripped media showed a robust response to bevacizumab, comparable to results observed in male cells (**Figure 1a)**. Charcoal stripped media did not have any effect on the ADCAI in male cells, as they had a robust response to bevacizumab treatment that is like what is observed in normal media **(Figure 1a)**.

After observing this response in mouse cells, we wanted to investigate whether this effect is observed in human female cells. To answer this question, we evaluated ADCAI in HL-60 cells that were differentiated into macrophages after treatment with PMA in a chemotaxis assay. Again, cells were grown in normal media and charcoal stripped media with cells moving towards a VEGF-A gradient with a human IgG1 presence. Mirroring what was observed in mouse cells, HL-60 cells cultured in the charcoal stripped media had a more potent response to IgG1 treatment compared to those cells cultured in normal media **(Figure 1b).** These findings indicate that there is a factor in female cells that prevents IgG1- FcγR1-induced chemotaxis from being more efficient and robust.

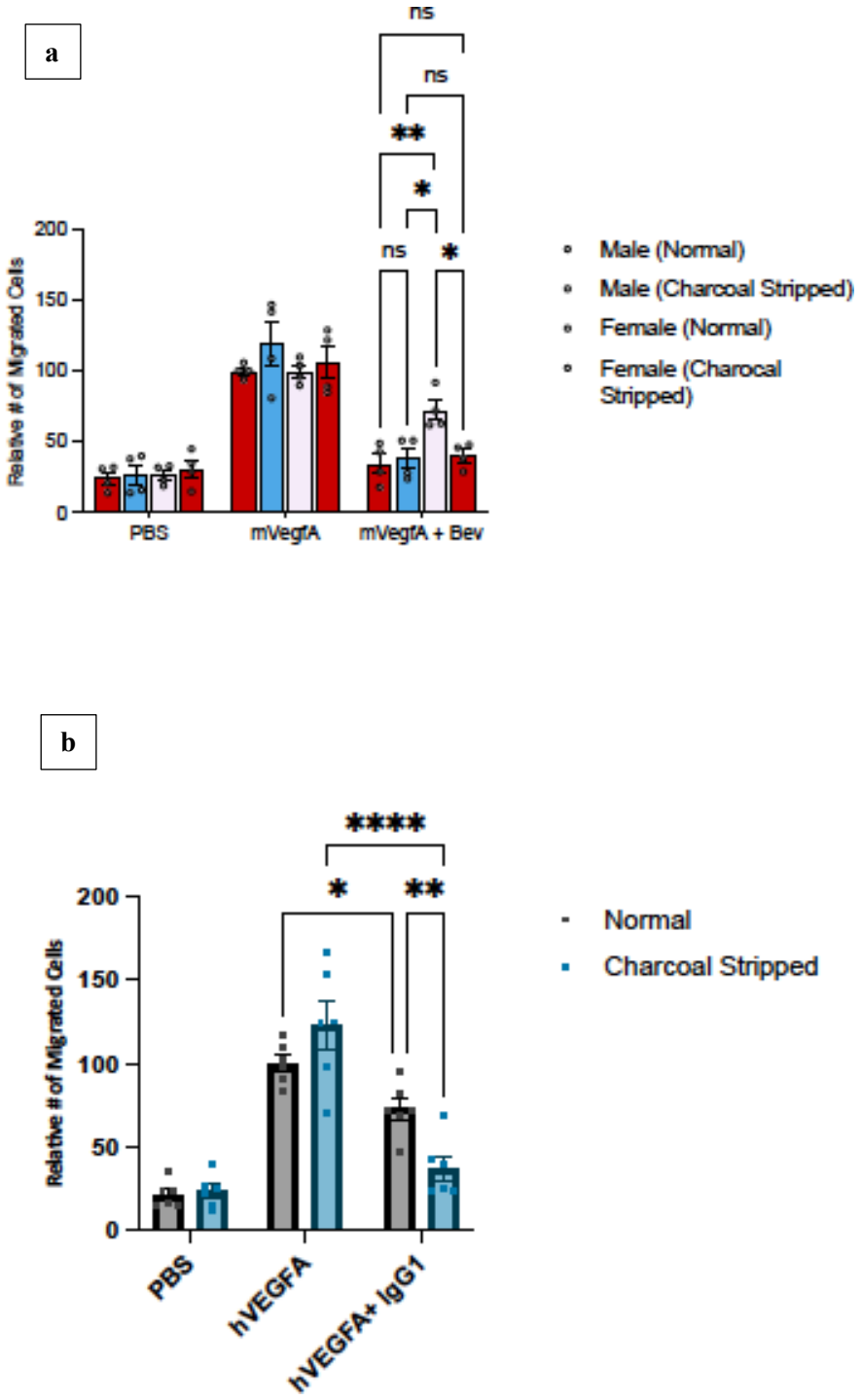


Figure 1 Charcoal stripped media potentiates ADCAI in female cells Chemotaxis assays using WT BMDMs (male and female) (a) and human HL-60 cells (b) utilizing charcoal stripped media. Experiments demonstrate that female cells ADCAI response is more robust in a charcoal stripped environment. PBS was used as a negative control. Stimulant was a recombinant mVegfA (a) or hVEGFA (b). Treatment was bevacizumab (a) or human IgG1 (b). Statistical analysis was performed using 2-way ANOVA testing. Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

The stark differences observed in female cells cultured in normal and charcoal stripped media prompted us to identify hormonal factors that can alter the IgG1 response. Estrogen is a hormone that has a higher concentration in females and has been associated with anti-inflammatory characteristics[186,195,259], breast cancer[260,261], endometriosis[262–264], and insulin resistance[265,266]. Because of this, estrogen can act as a positive or negative modulator depending on the situation and environment. To measure the effect that estrogen has on ADCAI in female cells, we cultured isolated BMDMs from female mice in charcoal stripped media in the presence of physiological concentrations (1 nM) of β-estradiol to the cells for 7 days. BMDM cells were also cultured in normal media and charcoal stripped media without β-estradiol. The IgG1 effect on ADCAI in the presence of β-estradiol was analyzed using a chemotaxis assay with a Vegfa gradient. Cells cultured with β-estradiol exhibited reduced sensitivity in response to IgG1 treatment **(Figure 2a)**. These results suggest that estrogen negatively affects ADCAI in female cells.

With the response observed in female BMDMs, we wanted to know if the addition of estrogen in the form of β-estradiol would have a similar effect on human cells. HL-60 cells (human female in origin) were grown in different conditions over a 7-day period to acclimate the cells to the environment prior to any experiments. The cells were grown in normal media (IMDM containing phenol red, FBS, and penicillin-streptomycin), charcoal stripped media (IMDM without phenol red, charcoal stripped FBS, and penicillin-streptomycin), and charcoal stripped media (same as mentioned prior with the addition of 1 nM concentration of β-estradiol every

2 days). After 7 days, the cells were then treated with PMA to differentiate into macrophages for use in a chemotaxis assay for treatment with IgG1 towards a VEGFA gradient. When used in a chemotaxis assay, we observed that cells showed an insensitivity in the normal media that was reduced in the charcoal stripped media. When estrogen was added in the form of β-estradiol, the insensitivity was rescued **(Figure 2b)**.

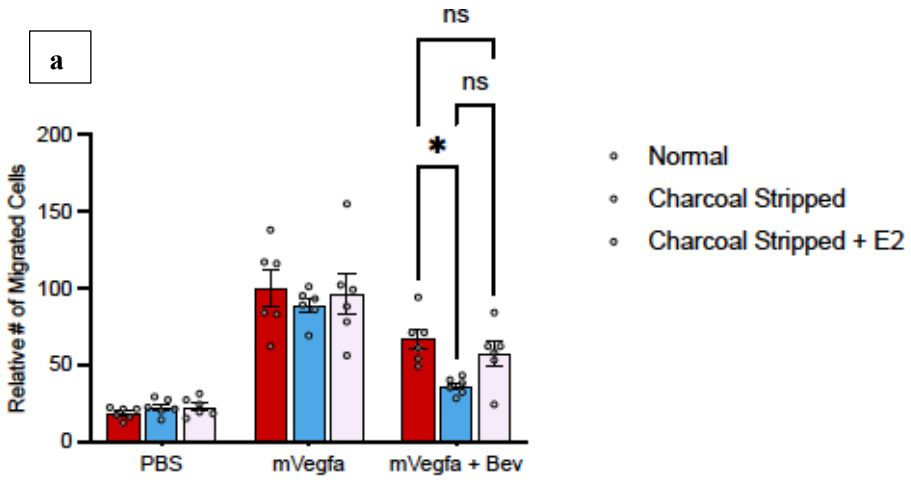
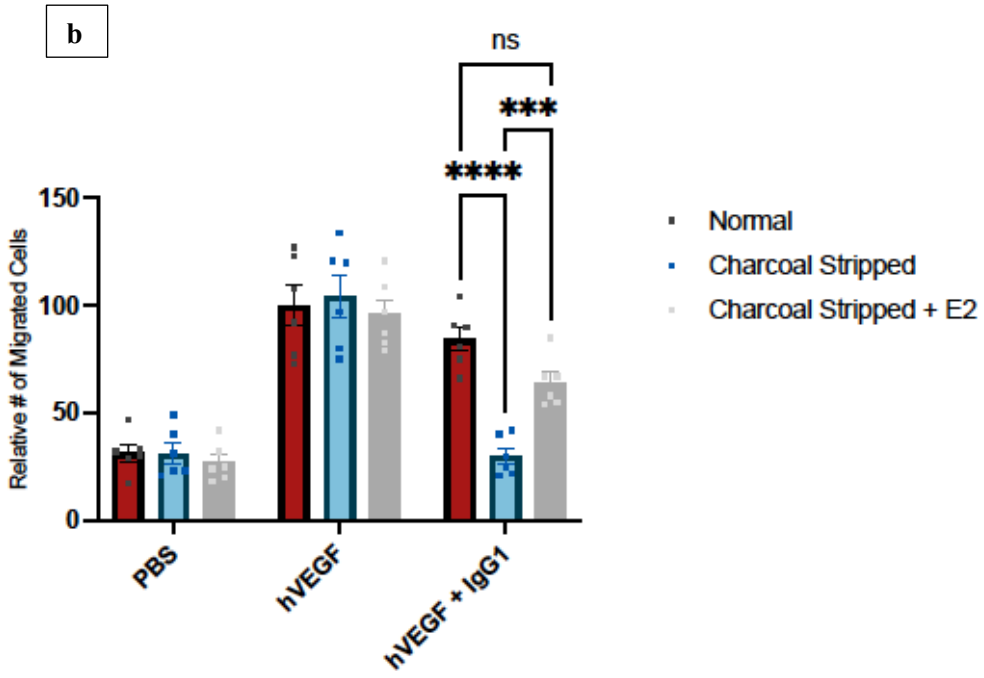


Figure 2 β-estradiol antagonizes the ADCAI response in female cells The removal of estrogen (E2) from the environment of female cells (a- WT female BMDMs; b- HL-60 cells), resulted in a potent response to bevacizumab/IgG1, comparable to male cells. The re-introduction of E2 resulted in an increased sensitivity to the treatment. PBS was used as a negative control.
Statistical analysis was performed using 2-way ANOVA testing. Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001



Estrogen signaling inhibition enhances response to IgG1

To further investigate the role that estrogen plays in the IgG1 response, we interfered with estrogen signaling pharmacologically. Fulvestrant, a selective estrogen receptor downregulator (SERD), was added to the media of female BMDMs overnight and the expression of estrogen receptor alpha (ERα) was checked via Western blot **(Figure 3)**. Once it was confirmed that fulvestrant degrades the estrogen receptors expressed in macrophages, macrophages from females (mouse and human) were used in a chemotaxis assay to determine if the degradation of estrogen receptors by fulvestrant affected the IgG1 response.

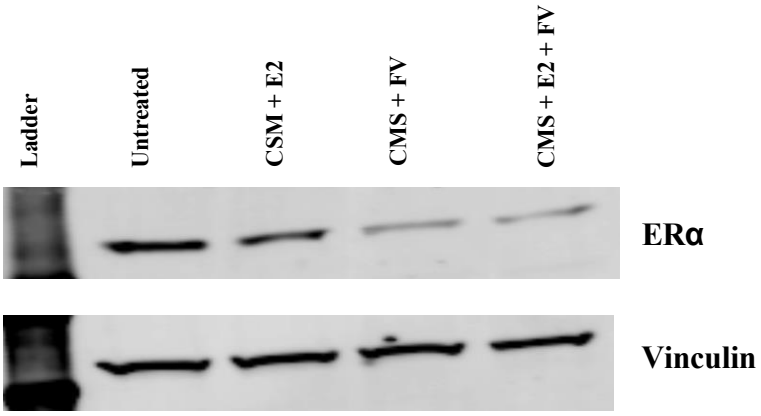
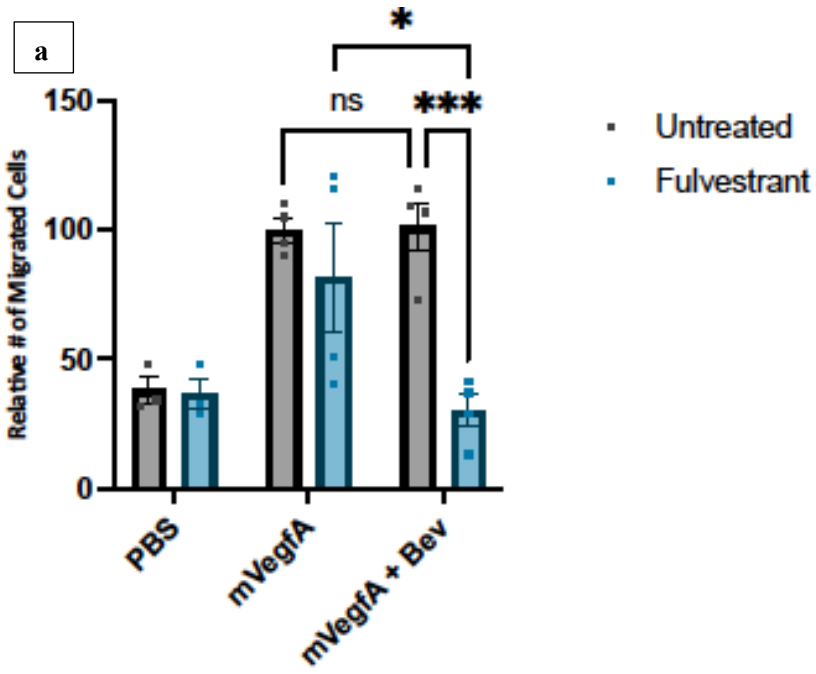


Figure 3 Fulvestrant reduces ERα abundance WT female BMDMs treated with fulvestrant for 24hr demonstrated a decreased expression of the estrogen receptor. Vinculin was used as a loading control.

Cells treated with fulvestrant responded more robustly compared to those cultured in normal media or charcoal stripped media with the addition of estrogen **(Figures 4a-c)**. This was the case using primary female cells (WT female BMDMs) **(Figures 4a and 4c)** and a human cell line (HL-60 cells) **(Figure 4b)**. These results suggest

that with the degradation of estrogen receptors due to fulvestrant binding, there are fewer receptors for estrogen to bind to on macrophages which allows for ADCAI to occur in females more efficiently.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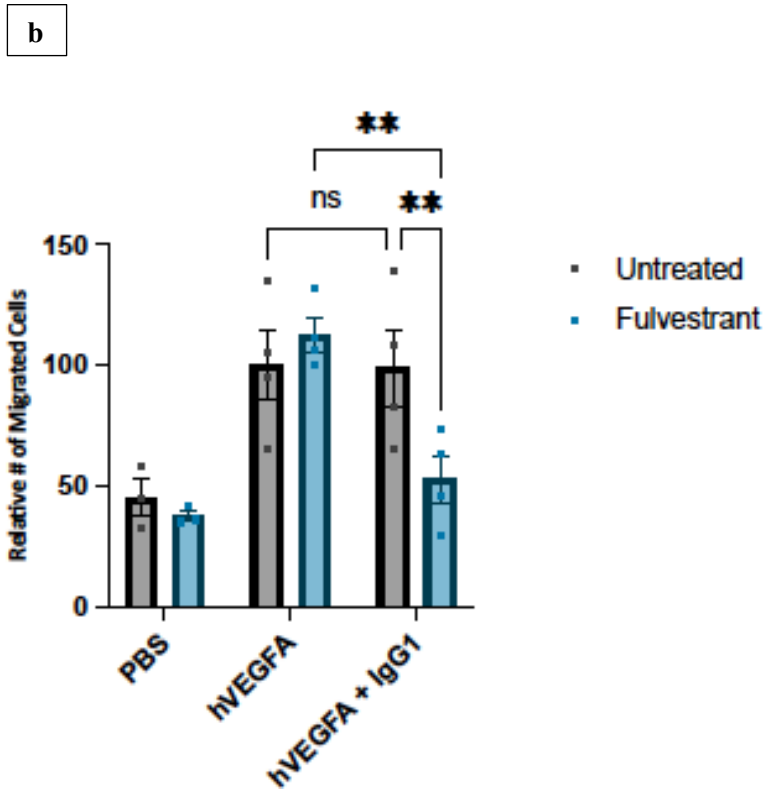
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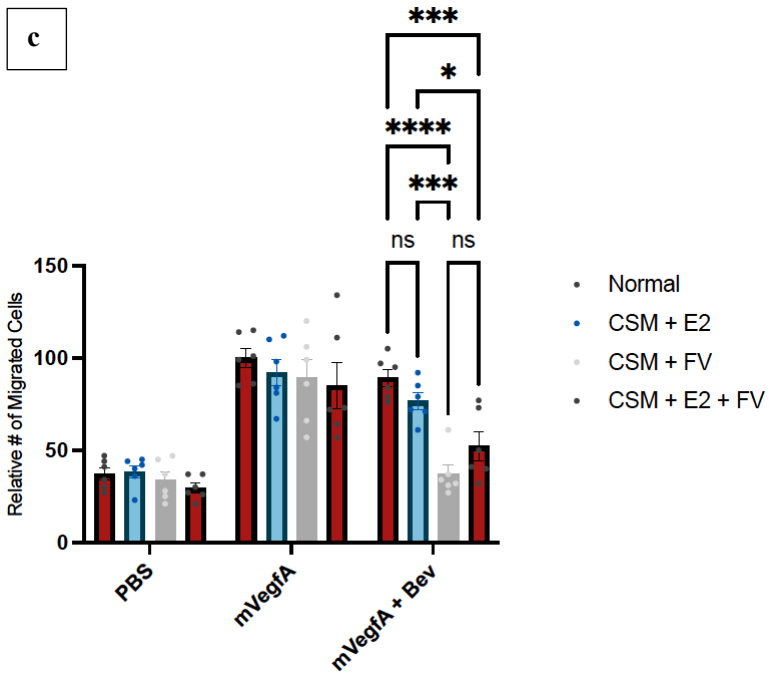

Figure 4 Fulvestrant enhances ADCAI in female cells Using primary WT female BMDMs (a,c) and HL-60 cells (b), there was a positive response to bevacizumab/IgG1 treatment after 24hr of fulvestrant treatment in normal media conditions. Using CSM to reduce estrogen prior to fulvestrant treatment, WT female BMDMs demonstrated in robust response to bevacizumab treatment. Statistical analysis was performed using 2-way ANOVA testing. Data are mean ± SEM. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001

After observing such stark differences in vitro with the removal of estrogen either using charcoal stripped reagents or fulvestrant, we sought to determine whether similar effects would be conserved in mice. To investigate this, female mice underwent either an ovariectomy or sham surgery followed by laser choroidal neovascularization (CNV) after a surgical recovery period of 21-28 days. Mice were injected with either PBS (control) or bevacizumab (treatment) at equal volumes with lesions created through photocoagulation disruption of Bruch's membrane. After 7 days, the eyes are collected with CNV volume measured to determine the amount of angiogenesis that occurred. No significant difference was observed in baseline CNV

between animals that received an ovariectomy or sham surgery **(Figure 5)**. In addition, though a trend toward lower CNV was observed in ovariectomized mice following bevacizumab treatment, there was no statistically significant effect of ovariectomy on bevacizumab induced CNV inhibition.

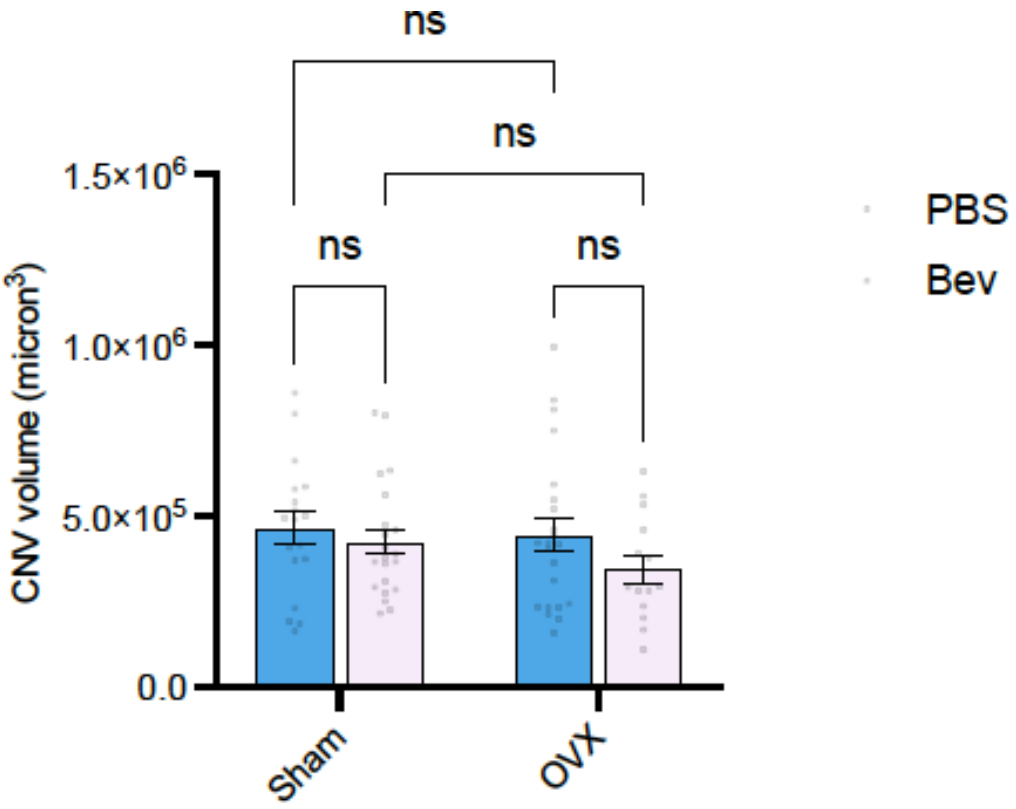

Figure 5 Ovariectomy (OVX) does not significantly increase IgG1-mediated CNV inhibition in mice Ovariectomized mice had small but not statistically significant improvement in CNV following bevacizumab treatment compared to sham-operated mice. Each point represents an individual CNV lesion. Statistical analysis was performed using 2-way ANOVA testing.

Discussion

Biological sex is a factor known to affect a person's susceptibility to a specific disease, its pathology, and its treatment. One of the mechanisms that affects biological sex is the production of sex hormones. Evidence suggests that sex chromosomes and

gonadal hormones can influence the function of immune cells and systems[112,178]. Focusing on the latter, women produce higher levels of estrogen and progesterone, whereas men produce higher levels of testosterone, all of which can be anti-inflammatory[186,195,259,267,268] and can impact various responses of the immune system and signaling pathways[105,108,113,189,269]. Understanding the mechanism by which any of these hormones can affect these systems or pathways would be beneficial in a person's diagnosis and treatment and would allow for improved disease outcomes in all sexes.

An area of disease where the impact of biological sex is apparent is pathological angiogenesis. Conditions of insufficient or excessive angiogenesis affect males and females differently. Some examples include rheumatoid arthritis[155,156,182,270], cancer[154,181], and peripheral artery disease (PAD)[11,12]. With so many diseases and conditions associated with pathological angiogenesis, modulating it is an applicable therapeutic goal. Some conditions where anti-angiogenic therapies have been successful include some cancers[271,272], AMD[234,273,274], and diabetic retinopathy[66,275].

Previous work identified human IgG1, and the murine equivalents IgG2a and IgG2c, as endogenous negative regulators of VEGF signaling and neovascularization[16]. We found that the antigen-independent binding of monomeric human IgG1 or murine IgG2a/2c antibodies via the constant Fc domain to the high affinity receptor FcγR1 on the surface of macrophages, impairs VEGF-mediated chemotaxis and restricts angiogenesis[16]. We called this activity "antibody-dependent

cell-mediated angioinhibition" (ADCAI)[16]. We found ADCAI to be active in models of angiogenesis induced by suture-induced corneal injury, laser-induced choroidal neovascularization, femoral artery ligation, and tumor xenograft, as well as in developmental retinal neovascularization[16,18]. Thus, ADCAI is a fundamental process that affects vascular remodeling in multiple tissue beds and physiologic states.

Given the broadly acknowledged differences in the immune biology of males and females, we sought to quantify the effect of sex on ADCAI, which had been discovered in studies of predominately male mice and cell models. Utilizing previous experimental methods and techniques, we recognized that the differences in the responses suggested a sexual dependence. Female cells and models showed an insensitivity to IgG1 treatment when compared to males. Since gonadal hormones play a key role in regulating sex differences in immune responses[276,277], we decided to investigate if the presence or absence of sex hormones, specifically estrogen in the form of estradiol influences ADCAI. Previous research also demonstrated that estrogen promoted resistance to bevacizumab in mouse models of non-small cell lung cancer[213] when used as a treatment option, suggesting that estrogen may influence the efficiency by which anti-VEGF therapies work.

Our findings suggest that estrogen can have an inhibitory impact on ADCAI. Estrogen can either enhance or suppress immune responses, depending on the concentration of the ligand, receptor, and type of cell involved[109]. In its presence, female cells have shown resistance to IgG1 treatment while males, which have a

naturally lower level of estrogen, have a more robust response to IgG1 treatment. This has been illustrated by a reduced chemotactic response in males demonstrated by a reduced macrophage migration in males when compared to females. When the in vitro environment had reduced or eliminated sources of estrogen, there was a more robust and efficient response to the treatment in the chemotaxis assay in females, while in males the results were unchanged. When estrogen was re-introduced to the cultured cells, we observed a reduced ADCAI response. Using an in vivo laser CNV model, there was not a significant difference in response to treatment in comparison to female mice that received a sham compared to ovariectomy surgeries. This differs from the cell culture studies. One possible explanation for this discrepancy is that ovarian secretions other than estrogen (such as progesterone) may influence ADCAI as well, but more research is needed in this area.

Further research involving in vivo models would be beneficial in gaining a better understanding of the underlying mechanisms of where and how estrogen negatively affects the IgG1- FcγR1 pathway and ADCAI. Using fulvestrant in an in vivo setting such as the laser CNV model would provide beneficial information about the relationship between IgG1 treatment and angiogenesis in females. Tools that can expand on these findings include animal models such as the four core genotypes mice (FCG) and XY* mice[151] where researchers can assess the effect that sex hormones, sex chromosomes and/or their interactions have on the ADCAI response. Since there are fluctuations in hormone levels during the female life cycle, utilizing the VCD mouse model of menopause[210] would create a more accurate picture of the influence

that estrogen concentration can have on IgG1 treatment in conditions of pathological angiogenesis.

Voluntary exercise suppresses choroidal neovascularization in mice

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Author contributions

S.H., M.Z., Y.N., R.M., D.A., and B.D.G. performed experiments or analyzed data. B.D.G. conceived and directed the project. B.D.G., R.M., and D.A. interpreted the findings and wrote the paper with assistance from Z.Y., N.K., and J.A. All authors had the opportunity to discuss the results and comment on the manuscript.

Abstract

Purpose: To determine the effect of voluntary exercise on choroidal neovascularization (CNV) in mice.

Methods: Age- and sex-matched wild-type C57BL/6J mice were housed in cages equipped with or without running wheels. After four weeks of voluntary running or sedentariness, mice were subjected to laser injury to induce CNV. Following surgical recovery, mice were placed back in cages with or without exercise wheels for 7 days. CNV lesion volumes were measured by confocal microscopy. The effect of wheel running only in the 7 days following injury was also evaluated. Macrophage abundance and cytokine expression were quantified.

Results: In the first study, exercise-trained mice exhibited a 45% reduction in CNV volume compared to sedentary mice. In the replication study, a 32% reduction in CNV volume in exercise-trained mice was observed (P=0.029). Combining these two studies, voluntary exercise was found to reduce CNV by 41% (P=0.0005). Exercise-trained male and female mice had similar CNV volumes (P=0.76). The daily running distance did not correlate with CNV lesion size. Exercise only after the laser injury without a preconditioning period did not reduce CNV size (P=0.41). CNV lesions of exercise-trained mice also exhibited significantly lower F4/80+ macrophage staining and *Vegfa* and *Ccl2* mRNA expression.

Conclusions: These findings provide the first experimental evidence that voluntary exercise improves CNV outcomes.

Pathological neovascularization underlies dozens of vision-threatening diseases including age-related macular degeneration (AMD), corneal neovascularization, glaucoma, diabetic retinopathy, and retinopathy of prematurity. Although intraocular anti-VEGFA therapies are a clinical success, they are not a panacea. For example, 12-25% of neovascular AMD patients, representing hundreds of thousands of individuals in the U.S. [278], have 20/200 vision or worse despite treatment[279–281]. Prolonged exposure to anti-VEGFA is accompanied by loss of initial visual acuity gains[282–285], and a significant portion of anti-VEGFA-exposed eyes develop untreatable central retinal atrophy[281,286]. Moreover, between 2013 and 2015, 3.75 million doses of FDA approved anti-VEGFA drugs were administered in the U.S., costing patients, taxpayers, insurers, and providers approximately $7.5B[287]. Thus, there is a compelling need for new, inexpensive anti-angiogenic strategies that can target the molecular drivers of neovascularization.

Physical activity is a non-invasive, patient-controlled, and inexpensive intervention that improves numerous health outcomes both in healthy people and in those suffering from diverse clinical conditions (systematically reviewed in[288,289]. In contrast to prevalent conditions, such as diabetes[290], cardiovascular disease[291,292], and neurocognitive disease[293], the relationship between exercise and AMD is far less established. Numerous epidemiological studies have attempted to characterize this impact, with the majority reporting a positive influence of physical activity on AMD and related outcomes (e.g. large macular drusen[103,294–309]. A recent systematic meta-analysis of nine studies on exercise and AMD in White subjects found that physical activity was associated with modest reduction in early AMD (odds ratio 0.92; 95% confidence interval (CI), 0.86-0.98) and dramatic

reduction in late AMD (odds ratio 0.59; CI, 0.49-0.72)[310]. Together, these findings suggest that physical activity may represent a significant modifiable risk factor for AMD.

Here, we sought to examine the effects of voluntary wheel running, a physiological model of endurance exercise in mice, on laser photocoagulation-induced CNV in a rigorous and controlled experimental setting.

Materials and Methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Animal studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male and female C57BL/6J mice were housed in temperature-controlled (21°C) cages in a pathogen-free room with a 12:12-h light-dark cycle and free access to water and normal chow.

Voluntary running

Voluntary wheel running has been widely used to induce physiological adaptations including muscle fiber transformation, angiogenesis, mitochondrial biogenesis, and mitophagy with significantly improved physiological and metabolic functions and protection against chronic diseases[311–317]. A voluntary regimen allows mice to exercise during their normal active dark cycle[318], which would be disrupted by forced exercise regimens, such as treadmill running and swimming[319–321]. Forced exercise is reported to cause acute and chronic stress responses that can manifest systemically[322–325] and may

confound results. Finally, perhaps because of these issues, direct comparison of voluntary and forced exercises has found voluntary exercise superior in improving other pathological phenotypes[326–328]. Voluntary running was conducted as established previously[311]. Briefly, mice in the exercise group were housed individually in cages equipped with running wheels and sedentary mice were housed in cages not equipped with running wheels. Daily running was recorded via a computerized monitoring system, as described in previous studies[312,313].

Laser photocoagulation induced choroidal neovascularization

Laser photocoagulation (532 nm, 180 mW, 100 ms, 75 μm) (OcuLight GL; IRIDEX Corp., Mountain View, CA, USA) was performed bilaterally (4 spots per eye) on day 0 to induce CNV as previously described[250]. Irrespective of the exercise protocol, mice were 3 months old at the time of laser injury.

CNV volume and F4/80 labeling

Following laser injury, mice were euthanized, eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Eyecups were incubated with 0.7% FITC-isolectin B4 (Vector Laboratories, Burlingame, CA, USA), and R-phycoerythrin-conjugated anti-F4/80 (Bio-Rad, Hercules, CA, USA) and the flat mounts of RPE-choroid-sclera were mounted in antifade medium (Immu-Mount Vectashield Mounting Medium; Vector Laboratories). CNV volume was visualized using a scanning laser confocal microscope (Nikon AR1, Nikon Instruments). Volumes were quantified using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes

of Health, Bethesda, MD, USA) as previously reported[250]. F4/80 labeling was quantified by densitometry of the F4/80 signal in the maximum z-projection of the CNV lesion.

Fluorescent in situ hybridization

Enucleated mouse eyes were embedded in optimal cutting temperature (OCT) medium (Sakura Finetek USA, Torrance, CA) and snap frozen in liquid nitrogen-supercooled isopentane. 7 µm-thick sections were hybridized with RNAscope probes for Ccl2 (ID: 311791), Il6 (ID: 315891), and Vegfa (ID: 412261) according to manufacturer's instructions (ACDBio, Newark, CA). Sections were mounted in Invitrogen™ ProLong™ Gold Antifade Mountant with DAPI (Thermo Scientific, Waltham, MA) and imaged on a Nikon A1R inverted confocal microscope (Nikon Instruments Inc., Melville, NY). Quantification of absolute transcripts was performed in ImageJ. The integrated density of an individual punctum was measured as the first peak in the intensity histogram of each 8-bit greyscale image, thresholded to reduce background. Then, the following equation was used to calculate the total number of transcripts: $\frac{\sum integrated\ density}{average\ intensity\ of\ single\ dot}\ x\ area\ of\ image\ x\ section\ thickness.$

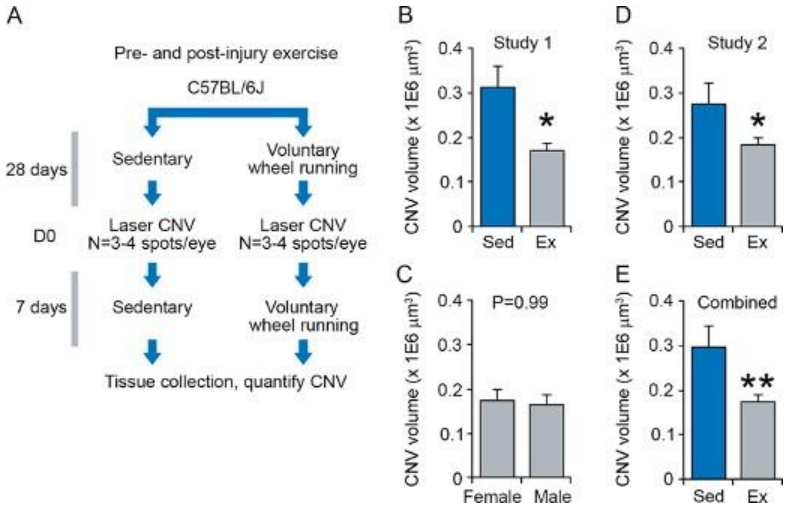
RESULTS

Effect of voluntary exercise on CNV in mice

The first study design is depicted in **Fig. 1A**. Age- and sex-matched wild-type C57BL/6J mice were singly housed in cages equipped with or without running wheels (N=3 male sedentary, N=3 female sedentary, N=3 male exercise, N=3 female exercise).

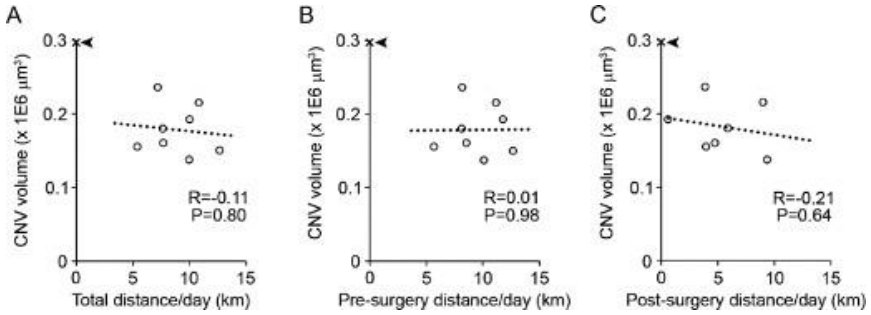
After four weeks of voluntary running, mice were anesthetized and subjected to laser injury to induce CNV. Following surgical recovery, mice were placed back in cages with or without exercise wheels for seven days, at which time animals were euthanized and CNV lesions were analyzed. Seven days after injury was selected as an endpoint because the lesion is sufficiently large to measure accurately and the lesion is actively expanding, with a peak volume occurring at 14 days[329], allowing us to quantify pathology in a state that is both established and expanding.

In total, N=40 CNV lesions from sedentary and N=48 lesions from exercise-trained mice were included for analysis in Study 1. One mouse in the sedentary group was excluded because the procedure failed, possibly due to its poor health. We observed a 45% reduction in CNV volume in exercise-trained mice compared to sedentary mice (P=0.017 by two-tailed Mann-Whitney U test, **Fig. 1B**). Exercise-trained male and female mice had similar CNV volumes (P=0.99, **Fig. 1C**). We did not find a significant difference in body weights of exercise or sedentary mice (25.1 ± 0.8 g vs. 25.5 ± 1.1 g, P=0.76). We conducted a replication study of similar design, with the exception that sedentary mice were not individually housed, as CNV volumes from individually housed mice were not significantly different from group-housed mice as established in prior studies. In the replication study, an additional N=25 CNV lesions from sedentary mice and N=19 CNV lesions from exercise-trained mice were analyzed. In this second study, we again observed a reduction in CNV volumes in exercise-trained compared to sedentary mice (P=0.029, **Fig. 1D**). Combining these two studies, voluntary exercise was found to reduce CNV by 41% (P=0.0005, **Fig. 1E**).



Dose effect of wheel running on CNV

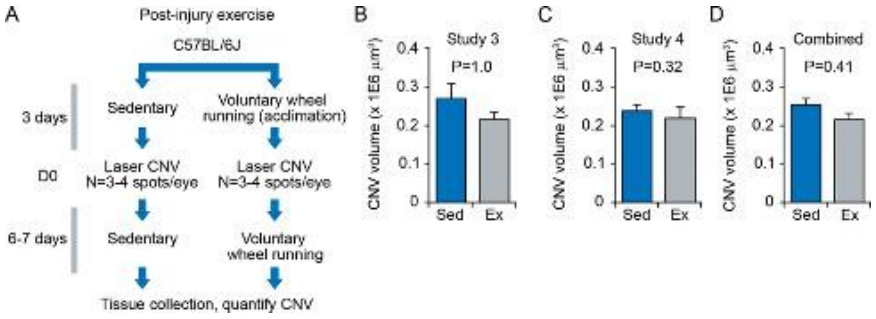
Throughout both studies, mice with exercise wheels traveled an average of 8.2 km/day, comparable to C57BL/6J in previous studies[130,318]. Quantifying the relationship between running activity and CNV volume in individual mice, the average daily distance traveled did not correlate strongly with CNV volume (R=-0.11, P=0.80, **Fig. 2A**). Daily distance traveled was significantly greater in mice prior to laser photocoagulation surgery (P=0.03 by two-tailed paired Student's t-test). Neither the daily distance traveled prior to nor after surgery significantly correlated with CNV volume (**Fig. 2B**, **C**), though there was a slight negative relationship between post-surgery run distance and CNV volume that did not reach statistical significance (R=-0.21, P=0.64).



Effect of post-injury voluntary exercise on CNV in mice

We sought to determine whether exercise undertaken concurrent with pathology, without pre-injury preconditioning, was sufficient to improve CNV outcomes. To isolate the effects of post-injury exercise, a second study design was conducted as depicted in **Fig. 3A**. Here, mice were allowed a brief three-day acclimation period with the exercise wheel, followed by laser injury to induce CNV, and then permitted to exercise throughout the recovery period with or without exercise wheels. Once again, a replication study of similar design was performed. In the first of two independent post-injury exercise trials (Study 3), a total of N=44 CNV lesions from sedentary and N=45 lesions from post-injury exercise-trained mice were included for analysis. We observed a 21% reduction in CNV volume in exercise-trained mice compared to sedentary mice, though this effect did not achieve statistical significance (P=1.0 by two-tailed Mann-Whitney U test, **Fig. 3B**). In a replication study of similar design (Study 4), an additional N=54 CNV lesions from sedentary mice and N=23 CNV lesions from exercise-trained mice were analyzed. In this

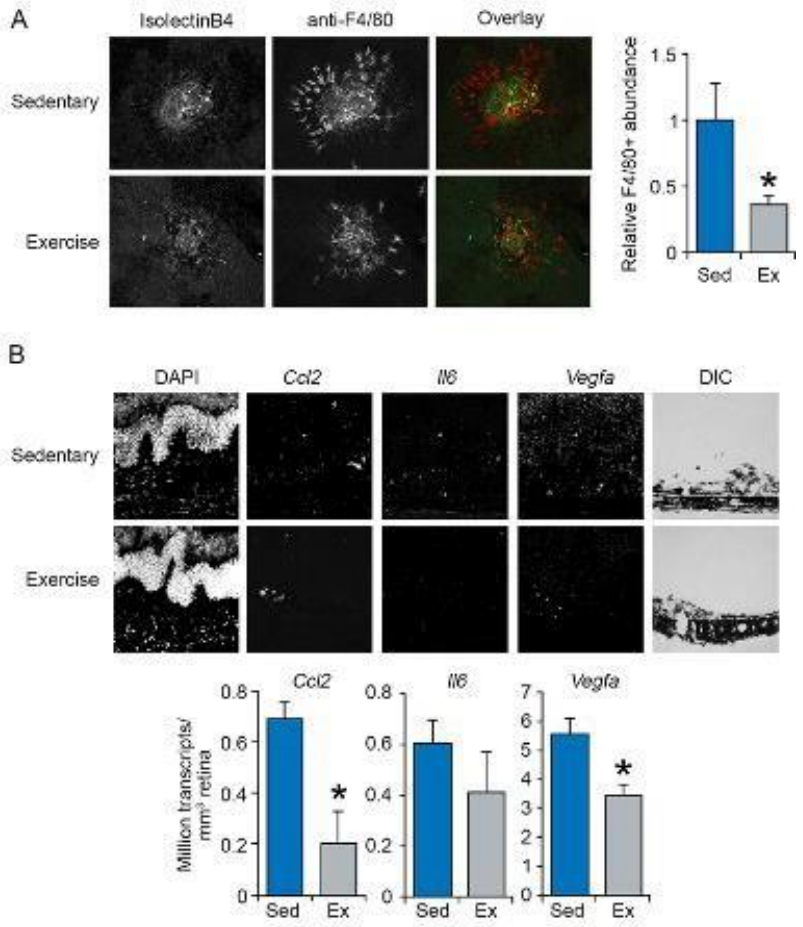
second study, we again observed a non-significant reduction in CNV volumes in exercise-trained compared to sedentary mice (8% reduction, P=0.32, **Fig. 3C**). Combining these two studies, post-injury exercise did not significantly reduce CNV (P=0.41, **Fig. 3D**).



Reduced F4/80+ cells and cytokine transcription in CNV in exercise-trained mice

Immune cells, including macrophages (Mϕ), are prevalent in human CNV[55,330–332] and critically contribute to experimental CNV[56,333,334]. We quantified the effect of exercise training on immune cell infiltration in CNV seven days after injury by measuring F4/80 immunolabeling in RPE/CNV whole mounts. In mice undergoing pre- and post-injury exercise, we observed a dramatic 72% reduction in F4/80 positive staining in the CNV lesions of exercise-trained mice compared with sedentary mice (P=0.037, **Fig. 4A**). Additionally, we utilized *in situ* hybridization to quantify the absolute number of transcripts of angiogenic cytokines in CNV lesions of exercise-trained and sedentary mice. In exercise trained eyes, we observed a 38% reduction in *Vegfa* mRNA (P=0.012 by two tailed t-test)

and 71% reduction in *Ccl2* mRNA (P=0.021) in CNV lesions of exercise-trained eyes compared to lesions from sedentary mice (**Fig. 4B**). We also observed a 32% reduction in *Il6* mRNA in lesions of exercise-trained eyes, though this was not statistically significant (P=0.18).



DISCUSSION

This study provides the first experimental evidence on the influence of physical activity on CNV, supporting the findings of epidemiologic studies reporting beneficial

effects of exercise on AMD-related pathologies. The dose effect of exercise was modest and did not achieve statistical significance. We interpret these findings to mean that the amount of exercise undertaken in this experimental design exceeded the threshold to achieve the maximal effect. Limiting exercise training to the CNV lesion growth period did not significantly reduce lesion size. We interpret this finding to mean that exercise preconditioning prior to the initiation of CNV is necessary to achieve a salutary effect.

In contrast to CNV, prior studies in mice report that exercise promotes angiogenesis and vascularity in skeletal muscle[307], brain[330], and subcutaneous adipose tissue[332]. It appears that the mechanisms by which exercise affects blood vessel homeostasis in these tissues may differ from CNV. We observed that lesions of exercise-trained mice exhibited reduced F4/80+ labeling and cytokine expression, suggesting that exercise may impart immunomodulatory effects. Indeed, exercise has been shown to ameliorate macrophage mobilization in a murine aging model[335], and in high-fat diet-induced inflammation[336–338]. Whether reduced immune cell recruitment is a driver of the beneficial effects of voluntary exercise on CNV is an important avenue of future study, as is identifying molecular intermediates of this effect.

Voluntary exercise induces a variety of systemic changes that may modulate CNV size, including food consumption and plasma cholesterol. Interestingly, short-term voluntary exercise is reported to induce an anorexic effect in mice, with reduced food consumption[339,340] while prolonged voluntary wheel running increases food consumption[341,342]. Voluntary exercise is reported to lower plasma triglycerides in humans[343] and triglycerides and cholesterol turnover in mice[344].

Prior studies have found that voluntary exercise does not affect fasting blood glucose levels in normal, non-diabetic mice[345,346]. We found no significant difference in body weights of exercise and sedentary mice. Therefore, we find it unlikely that blood glucose or body weight per se are responsible for the effect of exercise on CNV we observed. The extent to which these exercise-modifiable biomarkers correlate with CNV lesion size is an important avenue of future study.

Apart from our findings in CNV, exercise has also been reported to prevent retinal degeneration in normal aged mice[347,348], in light-induced retinal degeneration[349], and in a light injury model of retinitis pigmentosa[101]. Thus, the beneficial effects of exercise on the retina may extend beyond suppressing pathological angiogenesis.

A recent study found that Korean men, but not women, self-reporting five or more sessions of vigorous exercise per week were significantly more likely to develop neovascular AMD (hazard ratio, 1.54; CI, 1.15-2.06)[350]. However, limitations in the methodology of this study include survival bias of the low physical activity cohort ('left truncation'), and potential disproportionate underreporting of neovascular AMD in the non-active group[351]. Other studies have also reported marginal positive associations between physical activity and risk of developing AMD[352,353]. It should also be noted that the definitions of "adequate", "moderate", "strenuous" and "vigorous" physical activity are non-uniform between studies. In general, it is challenging to draw conclusions from epidemiologic studies of this nature due to the potential unreliability of questionnaire-based

data[354] and the confounding effects that vision loss may have on the amount and type of exercise an individual undertakes[355]. Thus, the continued study of exercise on AMD-relevant phenotypes in experimental models may provide clarity as to the nature of the effect and mechanistic drivers of physical activity in this condition.

Physical activity may be a low-cost, effective, and non-invasive treatment option in the prevention with a number of eye diseases, including AMD. Identifying the molecular mediators that couple physical activity and CNV is an important avenue of research to understand the relationship between this complex modifiable risk factor and retinal disease. This study presents an experimental platform from which such investigations may be undertaken in future studies. The translational relevance of this study must be considered in the context of the limitations of mouse voluntary wheel running as a model for human exercise and laser photocoagulation as a model of CNV in human patients. Ultimately, the extent to which exercise proves beneficial for humans suffering with or at risk of developing CNV must be tested in the context of controlled, prospective clinical trials.

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J.A. is a co-founder of iVeena Holdings, iVeena Delivery Systems and Inflammasome Therapeutics, and has been a consultant for Allergan, Biogen, Boehringer-Ingelheim, Immunovant, Janssen, Olix Pharmaceuticals, Retinal Solutions, and Saksin LifeSciences unrelated to this work. J.A., B.D.G., and N.K. are named as inventors on patent applications on macular degeneration filed by the University of Virginia or the University of Kentucky.

FIGURE LEGENDS

Figure 1. Exercise-trained mice develop less choroidal neovascularization than sedentary mice, independent of sex. (A) Study design for Studies 1 and 2: C57BL/6J mice were housed with (voluntary wheel running) or without (sedentary) an exercise wheel for 28 days. After laser photocoagulation on day 29, mice were returned to their respective cages for seven days. (**B**) CNV volume in sedentary and exercise-trained mice in Study 1. (**C**) CNV volume in exercise-trained male and female mice (P=0.99, Mann-Whitney U test). (**D**) CNV volume in sedentary and exercise-trained mice in Study 2. (**E**) CNV volumes from Studies 1 and 2 combined. N=65 sedentary, N=67 exercise. *P<0.05, **P<0.01

Figure 2. CNV volume and average distance traveled. (**A**) The average CNV volume of each mouse plotted against its average distance traveled throughout the duration of the experiment. Arrow on y-axis denotes the average CNV volume in sedentary mice. (**B**) The average CNV volume of each mouse plotted against its average distance traveled prior to laser photocoagulation surgery. (**C**) The average CNV volume of each mouse plotted against its average distance traveled after laser photocoagulation surgery.

Figure 3. Post-injury exercise and choroidal neovascularization. (**A**) Study design for Studies 3 and 4: C57BL/6J mice were housed with or without an exercise wheel for 3 days to acclimate. Then, mice were subjected to laser photocoagulation surgery, and returned to their respective cages for seven (Study 3) or six days (Study 4). (**B**) CNV volume in sedentary and post-injury, exercise-trained mice in Study 3. N=44 sedentary, N=45 post-injury, exercise-trained, P=1.0 by Mann-Whitney U test. (**C**) CNV volume in sedentary and post-injury, exercise-trained mice in Study 4. N=54 sedentary, N=23 post-injury, exercise-trained. P=0.32 by Mann-Whitney U test. (**D**) CNV volumes from Studies 3 and 4 combined. P=0.41 by Mann-Whitney U test.

Figure 4. Voluntary exercised-trained mice and macrophage infiltration in CNV. (**A**) Fluorescent micrographs of choroid-RPE sclera flat mounts from sedentary (top) and exercise-trained (bottom) mice seven days after laser injury. Isolectin-B4 depicted in green in overlay and F4/80 depicted in red in overlay. Quantification of F4/80+ staining depicted at right. N=14 lesions from sedentary and N=15 from exercise-trained mice (following the protocol in Figure 1A). (**B**) Representative images (top) and quantification (bottom) of *in*

situ hybridization of mRNA in retina of sedentary and exercise-trained mice (following the protocol in Figure 1A) seven days after laser injury. N=3 lesions per condition. *P<0.05 by two-tailed student's t-test.

V. Summary and Future Directions

This project explored how sex chromosomes and sex hormones may influence the response to IgG1 on pathological angiogenic conditions. The introduction of male and female models, cells and systems allowed for a more balanced and thorough study. There are other experiments that would provide more evidence for the findings about how genes on the Y chromosome and estrogen can exert their effects on the response to IgG1 treatment in the face of pathological angiogenesis. Also, we observed how exercise can be a noninvasive and cost-effective treatment for AMD and to help in the protection against AMD.

The Y chromosome has been overlooked for many years in research, but with the recent completion of its sequencing, new opportunities and questions can be brought forth about this chromosome and its genes. In trying to understand why the ADCAI response observed was different between males and females, we decided to investigate if any of its genes can provide an explanation. DDX3Y emerged as a candidate as we observed its loss whether transient (siRNA) or permanent (CRISPR-Cas9) can have a negative effect on ADCAI. The loss of DDX3Y caused male responses to be more like female responses indicating a sex difference to IgG1 treatment. DDX3Y exerts its effect in certain areas of the FcγRI/VegfR1 pathway as this gene affected VegfR1 degradation and ubiquitination of the receptor.

Estrogen is a hormone that can have enhanced or suppressive type effects. It is also a hormone that fluctuates throughout a woman's lifetime and is influenced by dynamic events such as pregnancy and menopause. Observing a sex difference in the response to IgG1 treatments, estrogen became a possible risk factor of interest to explain what was observed in cell and animal experiments. Having the ability to remove estrogen using charcoal stripped and phenol red free reagents as well as using a SERD like fulvestrant, there was a more efficient and robust response to the IgG1 treatments, with results comparable to what was noticed in males. When estrogen was put back into the system, the inhibitory effects were re-introduced. The in vivo experiments did not produce the same results, but more exploration needs to be done to reconcile these findings.

With so many members of the world population being diagnosed with AMD, there is a need to find new and innovative ways to treat people and to prevent its diagnosis. Physical activity has been associated with many benefits which include lowering cholesterol, lowering blood pressure, and better blood flow- all of which can be positive for one's ocular health. Exercise can be seen as a noninvasive and cost effective therapeutic for ocular health. In our study, mice that voluntarily ran on a treadmill had a lower incidence of CNV compared to sedentary mice.

To take a closer look at DDX3Y, a revealing experiment would be to overexpress this gene in a female cell line such as HL60 to then determine if DDX3Y is sufficient to induce a male-like response in female cells to IgG1 treatment. The difficulty in this is

how to add this gene to a macrophage type cell or cell line. Typical means such as transfection with plasmids expressing DDX3Y and nucleofection/electroporation with the plasmid has proven to be very challenging. Possible uses of a lentivirus or AAV technology might be more applicable for this to work. A second experiment that would further assess the importance of DDX3Y would be a rescue experiment. In this scenario, we would be able to investigate if reintroducing DDX3Y back into a system after its loss, would reestablish ADCAI. Further investigation of the other Y chromosome genes of KDM5D and UTY and the role they may play is also another avenue that should be examined. These two genes did have the same effect on IgG1 treatment in male macrophages that was observed with DDX3Y using primary male BMDMs. Confirming these findings using primary male macrophages from healthy donors could be a next step in studying these genes. There is a possible connection between DDX3Y and UTY due to their proximity to each other on the Y chromosome which is worth further exploration. The use of a female mouse model where DDX3Y is expressed would allow us to investigate how influential the DDX3Y gene is in the IgG1 response and if its presence would be affected by the presence of estrogen.

There is also something to be said about investigating the Y chromosome. My project really probed the properties of one specific gene, DDX3Y. Several of the experiments discussed here focused on the results due to the loss of DDX3Y, but with the loss of the Y chromosome (LOY) there is an association between that and different types of cancers in men[242]. There has also been an association between LOY and AMD[138,139,356] and LOY leading to cardiac fibrosis and reduced heart function in mice. Gaining a better understanding of how the Y chromosome and its genes affect different

instances and pathological angiogenesis and how it can affect the immune-vascular crosstalk would be valuable in understanding a possible mechanism and knowing the best route to take with patients regarding treatments and therapeutics.

Experiments where we can learn more about estrogen and its effects include the silencing of mRNA that directs the production of estrogen receptors on macrophages, investigating the effect that IgG1 treatment has on female cells at different times in a female's life cycle such as pre- versus post-menopause, after hormone replacement, while using birth control, or using CRISPR technology to edit estrogen receptors prior to IgG1 treatment and investigating the effect it has on the IgG1 response. Despite these exciting findings, there are some limitations to this aspect of the study that need to be addressed in the future. The female menstrual cycle is full of changes and fluctuations. Samples of PBMCs from healthy women from all stages of life would allow for a more accurate picture of what is occurring over time and provide us with more information about the role estrogen plays at different physiological levels. Having samples from women who are pre-menopause, post-menopause, pregnant, and in their childbearing years would be a good start since these are all stages where estrogen is at different levels. For mouse studies, utilizing the VCD mouse model of menopause should be an option. Ovariectomies are the gold standard when we want to reduce the influence of estrogen, but this model allows for researchers to study estrogen depletion in a more gradual and natural progression through an ovary intact animal with follicle depletion.

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* Please note that the following reference numbers are not used due to technical issues: 221, 236, 239, 241, and 248