Aging Impairs VEGF-A mRNA Stability and Consequent Inflammatory Arteriogenesis

By

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BS, Fu Foundation School of Engineering and Applied Science, Columbia University, 2017

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Graduate Program of Biotechnology at Brown University

PROVIDENCE, RHODE ISLAND

May 2024

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Acknowledgements

I would like to thank all my professors at Brown who made learning a joy and inspired me to continue my research trajectory in unique ways. I want to extend a special thank you to my program director, Dr. Jacquelyn Schell, for supporting me in multiple ways throughout the past two years including teaching one of the most insightful classes I've ever taken and granting me the Biotechnology Graduate Program Sc.M. Fellowship scholarship. The Biotechnology master's program is the most supportive educational environment I've ever experienced, and it is largely due to Dr. Schell's kindness and commitment to student excellence. I also want to thank Dr. Anita Zimmerman for introducing me to Dr. Morrison and supporting me in my goal of pursuing a Masters.

I would also like to thank the American Indian Science and Engineering Society, Native Forward Scholars, and the Rhode Island Indian Council for supporting my studies through their scholarship programs.

I want to thank my family for their endless support and constant encouragement, especially my mother, sister, and partner. I would be nowhere without you.

I consider it a great privilege to have Dr. Mantsounga and Dr. Oancea as my thesis readers and committee members. They are two of the best teachers I have had during my time at Brown and have made me a better student and researcher. I would also like to acknowledge the entire Morrison Lab for all their help in learning new skills along with their general kindness and camaraderie.

Finally, I would like to thank my advisor and mentor, Dr. Alan R. Morrison for going above and beyond what is expected of any advisor by helping me to secure grant funding for my education. I am endlessly grateful for the multiple opportunities I have been given under his

mentorship as well as the dedicated time to shape me into a better scientist. My experience here has been transformative and launched me into my dream career of being a research scientist.

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Abstract

Abstract: Aging impairs VEGF-A mRNA stability and consequent inflammatory arteriogenesis, by Olivya Caballero, Sc.M, Brown University, May, 2024.

Objective: Aging is associated with impaired inflammatory arteriogenesis in response to vascular injury. Following inflammation and ischemia, macrophage recruitment and early macrophage pro-angiogenic VEGF-A isoform (VEGF-A_{165a}) expression contribute to setting the stage for post developmental arteriogenesis. Preliminary data demonstrated reduced expression of proangiogenic VEGF-A levels by both mRNA and protein by bone marrow-derived macrophages (BMDMs) from aged (52-week-old) mice when compared to young (12-week-old) mice. We sought to determine the age-related mechanisms which contribute to the reduction of pro-angiogenic VEGF-A expression and consequent impaired inflammatory-mediated arteriogenesis.

Methods: We used aged (52-week-old) and young (12-week-old) C57BL/6 mice as well as mouse strains with tamoxifen-inducible, myeloid-specific knockouts and knock-ins of Dicer1. A hind limb ischemia procedure was used to mimic the effects of lost blood flow due to peripheral artery disease. Subsequent blood flow recovery was measured using laser Doppler imaging of the rear paws in addition to microCT angiography. These findings were validated with RT-qPCR of lysed muscle tissue on day 3 post HLI. In addition, BMDMs were collected and analyzed using immunoblot, RT-qPCR, and ELISA.

Results: In an experimental model of hindlimb ischemia, we found decreased blood flow recovery in aged mice and significant reductions in new small arterial development by microCT angiography consistent with decreased arteriogenesis. Reduced arteriogenesis was associated with decreased muscle tissue VEGF-A and VEGF-A_{165a} levels during early inflammation 3 days after induction of hindlimb ischemia despite adequate macrophage recruitment to the muscle

tissue. BMDMs from aged mice demonstrated increased levels of DNA methylation. BMDMs from aged mice also revealed decreased VEGF-A_{165a} mRNA half-life along with decreased association of VEGF-A with the RNA-stabilizing protein, HuR. Treatment of BMDMs with a DNA methyltransferase inhibitor led to improved association of HuR with VEGF-A mRNA and improved blood flow recovery during hindlimb ischemia, indicating that age associated increases in DNA methylation impact HuR-mediated VEGF-A mRNA stability and consequent inflammation-driven arteriogenesis.

Conclusions: Understanding and reversing the mechanisms driving age-related impairments in vascular healing may pave the way for new therapeutic strategies to improve vascular healing in the elderly.

Chapter 1: Introduction

Disease Background

Peripheral Artery Disease (PAD) is characterized by reduced blood flow to the arterial vessels furthest away from the heart, with the most prevalent type occurring in the lower extremities: the legs and feet. This decreased blood flow is a result of plaque buildup in the arteries, known as atherosclerosis, leading to narrowed vessels. Risk factors include increased age, family history of stroke, heart disease, or vasculitis. Other medical conditions such as diabetes, chronic kidney disease, fibromuscular dysplasia, preeclampsia, and unhealthy blood cholesterol increase the risk for developing PAD (Farber et al., 2016, Barrett et al., 2016). Age remains one of the highest risk factors for PAD deaths, potentially due to impaired inflammatory angiogenesis response to injury (Van Loo et al., 2012, Jain et al., 2011). There is a negative correlation between increased age and the rate of angiogenesis-dependent wound healing. This leads to chronic wounds, increased bruising, and a higher rate of both amputations and mortality for the elderly (Jinfeng et al., 2022). The mechanisms responsible for impairing angiogenesis later in life are not well understood and could provide promising therapeutics.

Currently over 8 million people in the U.S. and 200 million people worldwide suffer from PAD (Rudan et al., 2013). Furthermore, a PAD prognosis comes with decreased quality of life, increased mortality, and greater likelihood of cardiovascular and limb events (Jinfeng et al., 2022). Barnes et al. found that "[t]he 5-year mortality associated with chronic limb-threatening ischemia without amputation is estimated to be 55 to 65%, which exceeds the 5-year mortality of many malignancies". PAD has also been found to affect females more than males worldwide with higher diagnoses but similar mortality rates (Jinfeng et al., 2022). More specifically, Indigenous women in the U.S. are known to have a higher risk of PAD due to a variety of

reasons including having the highest rate of Diabetes Mellitus and limited access to adequate healthcare (Barnes et al, 2020). Similarly, PAD has a higher prevalence in African Americans compared to other ethnicities and has higher rates of complications, including amputation and impaired walking. Barnes et al emphasize that, "African American and Native-American patients, and those of low socioeconomic status have the highest risk of amputation. These findings call for improved treatments.".

Additionally, the financial burden of a PAD diagnosis is costly. It is estimated that in the U.S. the average expenditure of patients with PAD is $7,334 more than those without (Nguyen et al., 2018). This cost goes up in those requiring major amputation with severe PAD. Managing PAD presents other hidden costs as well such as changes in lifestyle, endovascular repair, lost wages/opportunities due to decreased mobility, medical management, and family care (Jinfeng et al., 2022).

Treatment options depend on the severity of symptoms and vary from lifestyle changes to medication, and even more intensive surgical interventions. Lifestyle changes that might be recommended include regular physical activity, tobacco cessation, and the adoption of a heart-healthy diet (Barrett et al., 2016). Medications such as antiplatelets, statins, and ACE inhibitors can be taken to treat PAD, however these medications only treat prevention of blockages and do not stimulate angiogenesis (Annex et al., 2017). For serious blockages, surgical bypass or angioplasty are the only options and revascularization is often incomplete due to the complexity of the lesions and the severity and burden of the lesions (Shishehbor et al., 2016).

Angiogenesis, VEGF-A, and Aging

Angiogenesis, the creation of new blood vessels from pre-existing vessels, is an important therapeutic concept for future PAD treatment, yet to date it has fallen short of its potential in clinical studies (Lyer and Annex, 2017). What makes angiogenesis a unique and challenging model for therapeutic intervention is the existence of a preformed vessel. Several in vivo and in vitro models exist for understanding simple vessel formation from embryonic development or endothelial cell precursors. The de novo formation of vessels is known as vasculogenesis. Angiogenesis is more complex and characterized by two specific markers: intussusceptive microvascular growth and endothelial sprouting. Intussusceptive microvascular growth involves the spread of new capillary networks created through the division of lumen from existing vessels. This division is achieved via the insertion of transcapillary pillars (Zucchelli et al., 2019). Endothelial sprouting is described as the movement of endothelial cells into connective tissues while simultaneously breaking down the existing basement lamina to form new capillaries. Vascular endothelial growth factor (VEGF-A), remains one of the most influential in the family of signal proteins for angiogenesis and wound healing. VEGF-A is a cystine-knot growth factor from the platelet-derived growth factor family. VEGF-A binds to tyrosine kinase receptors, VEGFR-1 and VEGFR-2, resulting in dimerization and subsequent activation by transphosphorylation. Some of the functions of VEGF-A include the creation of blood vessel lumen through endothelial cell sprouting, increasing migration and mitosis of endothelial cells, acting as a chemotactic for macrophages, and creation of fenestrations (Bautch, 2012). While VEGF-A is most widely associated with endothelial cells, VEGF-A and VEGF receptors 1 and/or 2 are found on many cell types, most notably, macrophages. Macrophages and monocytes have been found to be vital for angiogenesis and a major source of VEGF-A production following ischemic injury (Guo et al., 2018, Morrison 2014, Takeda 2011).

Inflammatory macrophages produce excess VEGF-A in response to injury through a series of signaling actions starting with CCR2 stimulation by CCL2 in conjunction with ICAM-1 adhesion to activated β2 integrins (Kuziel et al., 1997; Lu et al., 1998; Gerszten et al., 1999, Morrison et al., 2014). C-C motif chemokine receptor 2 (CCR2) is a Gαq GPCR that is used to characterize inflammatory macrophages when highly expressed (Willenborg et al., 2012). C-C motif chemokine ligand 2 (CCL2) is a key monocyte chemokine that binds to the extracellular portion of CCR2 and acts as a known β2 integrin activator (Carr et al., 1996; Ito et al., 1997; van Royen et al., 2003). Intercellular adhesion molecule 1 (ICAM-1) is an adhesion receptor and cell surface glycoprotein that is highly expressed in epithelial cells in response to inflammation (Sumagin et al., 2020). It is well known for its role in connecting epithelial and immune cells in response to injury/inflammation following binding to an activated β2 integrin. β2 integrins are heterodimeric surface receptors that are exclusively expressed in leukocytes (Arnaout, 2016). When macrophages are exposed to CCL2 stimulation and allowed to adhere to ICAM-1, a downstream cascade is triggered to activate the Rac2 and Myosin IIA signaling complex, resulting in the translocation of HuR from the nucleus to the cytoplasm (Morrison et al., 2014). Human antigen R (HuR) is an RNA binding protein responsible for transporting and protecting various mRNAs, including VEGF-A mRNA (Cho et al., 2019). Following translocation to the cytoplasm, HuR binds to a non-coding region of VEGF-A mRNA, thereby stabilizing it (Li et al., 2013). Given the impaired inflammatory response observed in elderly populations, we sought to determine if macrophage HuR-mediated VEGF-A stabilization and consequent inflammatory angiogenesis was negatively affected by aging.

Aging Models

Models of aging typically include advanced age mice that are 104 weeks old; however, when hindlimb ischemia experiments were done on these mice, the angiogenic response was so

impaired that tissue would become necrotic and unsuitable for complete characterization and molecular study (Silver et al., 1999). The Morrison laboratory uses middle-aged (52 weeks) mice as they exhibit delayed wound healing when compared to 12-week-old mice and without necrosis. To further probe the relationship between HuR expression, VEGF-A mRNA, and aging it was found that Dicer1 expression affects the mRNA expression of several mRNAs including VEGF-A mRNA (Chang et al., 2013). The relationship between Dicer1-dependent microRNAs and VEGF-A was previously demonstrated to be an antagonistic one, including microRNAs that help suppress VEGF-A expression (Chang et al., 2013). It is also known that Dicer1 expression can be influenced by aging resulting in changed cellular phenotypes (Reis et al., 2016, Bhattacharya et al., 2015). To further explore these mice with inducible myeloid-specific Dicer1-deletion were explored. These mice exhibited similar responses to wound healing as the aged mice and have a significantly reduced lifespan. Because of this, *CSF1R*^{mcm}*Dicer1*$^{fl/fl}$ were chosen as another model for studying the effects of aging on VEGF-A production and angiogenesis following injury.

Chapter 2: Materials and Methods

Animals

The Providence VA Medical Center Institutional Animal Care and Use (IACUC) approved all experiments using animals. 12- and 52-week-old C57BL/6 mice were purchased from The Jackson Laboratory. *Dicer1* knock-in mice were created in our lab with a constitutively active promoter under regulation of LoxP flanked Stop codon sequence in the ROSA locus for conditional overexpression of Dicer using CRISPR/CAS9 technology, *(CSF1R^{mcm}DicerKI^{+})* Csf1rmercremer (Cat# 019098) (Qian et al., 2011). Inducible myeloid-specific *Dicer1*-deletion were also created in the lab using CRISPR/CAS9 technology, *CSF1R^{mcm}Dicer1$^{fl/fl}$* , Csf1rmercremer (Cat# 019098) (Qian et al., 2011). Mice were injected with 2 mg of tamoxifen intraperitoneally for 10 days leading up to experiments to ensure either gene deletion or overexpression.

Hind limb ischemia

First, mice were anesthetized by inhaled isoflurane (2%) in an induction chamber before being transferred to a nose cone on a surgical plate. The femoral artery was ligated at two positions spaced 5 mm apart, one just below the inguinal ligament and the second distal to superficial epigastric artery. All branches between the two ligatures were ligated and the femoral artery segment was cut.

BMDM primary cell culture

BMDMs were obtained by in vitro differentiation of primary femur and tibia BM cells using an established protocol (Davies and Gordon, 2005). Femurs and tibias were dissected, cleaned, disinfected in 70% ethanol, and washed with fully supplemented RPMI 1640 medium, (10% fetal bovine serum, 10 mmol/L HEPES at pH 7.4, 2 mmol/L L-glutamine, 100 units/mL penicillin, 10 μg/mL streptomycin, and 50 μM 2-ME [2-mercaptoethanol]). Red blood cells were

removed by ammonium-chloride-potassium lysis buffer and subsequent centrifugation. Remaining BM cells were then cultured at a density of 3.5×10^6 cells/10 cm Petri dish in fully supplemented RPMI with 30% (vol/vol) L929 cell-conditioned medium. Cells were matured to phenotypic macrophages over 6–8 d. Nonadherent cells were washed away with PBS. Adherent cells were recovered with gentle pipetting in PBS with 1 mM EDTA.

ICAM adhesion and CCL2 stimulation

Petri dishes or glass coverslips were coated with 10 μg/ml goat anti–human IgG, Fcγ fragment-specific, in 50 mM Tris-HCl, pH 9.5, for 1 h at room temperature. Blocking was performed for 1 h at room temperature with PBS containing 2% (wt/vol) BSA. Dishes or coverslips were then incubated with 100 ng/ml human or mouse rICAM-1-Fcγ fragment chimeric protein or, as a negative control, 100 ng/ml Fcγ alone overnight at 4°C. Where appropriate, cells were resuspended in fully supplemented RPMI 1640 with or without 50 ng/ml mouse recombinant CCL2 at the time of adhesion.

RT-qPCR

Total RNA from ischemic muscle tissue (100-150 mg) was extracted using the Trizol kit (Fisher 15596026). Total RNA from BMDMs (1.5million cells/mL) was extracted from RLT Lysis buffer containing 2-bME (QIAGEN; RNeasy Kit cat.74106) according to the manufacturer's protocol. RNA concentration was measured by Nanodrop One (Thermo Fisher). First-strand complementary DNA was synthesized with iScript cDNA synthesis Kit (Bio-Rad; 1708891). Equal amounts of RNA (200 ng/ml) were used as templates in each reaction. Quantitative RT-PCR was performed with SSoAdvanved UnivSYBR Supermix (Bio-Rad, 1725274) on the StepOne Plus PCR machine (AB Applied Biosystems) using primers. All

measurements were made in triplicate and then averaged, using HPRT as the housekeeping gene for BMDMs and GAPDH as the housekeeping gene for muscle tissue lysates.

VEGF Elisa

Conditioned media from BMDMs were used to evaluate VEGF-A (R&D Systems, DY493) protein concentrations, according to the manufacturer's instructions. Each animal measurement reflects the average of experimental triplicates.

Immunoblotting

Proteins from ischemic muscle tissue (100-150 mg) were extracted using Trizol kit (Fisher 15596026). Proteins from BMDMs (1.5million/mL) were collected by using Laemmli sample buffer (Bio-Rad, cat.1610737) containing 2-bME; then boiled at 95C and aliquoted. Aliquots from tissue lysates or cell lysates were separated by Tris/Glycine/SDS–PAGE (Bio-Rad 1610732) and blotted into PVDF membranes. Membranes were blocked in fluorescent blocking buffer (Rockland Immunochemicals, cat. MB070) and probed at 4C overnight with antibodies against VEGF-A (1:1000; Sigma SAB2502119); cleaved mature IL-1b (1:500; Cell Signaling Technology 52718); b-Actin (1:1000, sc-47778). After multiple washes, membranes were incubated with fluorescent secondary antibodies (Thermo Fisher A21088, 1:5000; Thermo Fisher A10043, 1:5000; and LI-COR 926-32212, 1:5000). Protein bands were quantified by densitometry, using the LI-COR Imager (Odyssey CLx) and normalization to b-Actin.

HuR Translocation assay

Plated cells were incubated for 30 min at 37°C, washed with PBS, and then fixed with 4% PFA for 15 min at room temperature. Cells were then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS, followed by blocking with 5% goat serum for 60 min at room temperature. Immunostaining with 1/250 dilution of anti-HuR antibody was performed overnight at 4°C. Cells were incubated with Alexa Fluor–conjugated secondary antibodies for 2 h in the dark at room temperature. Counterstaining was performed with phalloidin and 0.005% DAPI as per manufacturer protocols. Confocal microscopy was performed on a five-laser inverted microscope (Ti-E Eclipse; Nikon) equipped with a Perfect Focus, motorized XY stage on a Nano Focusing Piezo Stage. Oil immersion objective lenses (60 and 100×; CFI Plan Apochromat VC Series; Nikon) with a numerical aperture of 1.4 were used. Samples were imaged at 21°C. Fluorochromes included Alexa Fluor 488, 568, and 647 (Invitrogen). Cells were mounted in Fluorogel with Tris Buffer (Electron Microscopy Sciences). Images were taken with the UltraVIEW VoX confocal imaging system (Perkin Elmer) using Volocity 6.2.1 (Perkin Elmer) acquisition software. We have defined an algorithm (MATLAB-based) to quantify the relative translocation of HuR in each cell. Background subtracted cell images were identified and segmented using the watershed algorithm and then tracked across the z-plane by using the centroid of the nucleus. From this point, the relative translocation of HuR was calculated by multiplying the relative external (to nucleus) intensity with the relative external (to nucleus) volume. Thresholds for the quantification of cells with a robust translocation in a 30-min period are based on a minimum intensity-volume product of 0.25.

RNA decay curve

After cells were adhered for 30 min, transcriptional arrest was imparted by 10 μg/ml actinomycin D (time 0 in assay). RNA was harvested at 20-min intervals for 60 min mRNA expression was analyzed by RT-qPCR as previously described (Wang et al., 2006). VEGF-A mRNA levels were normalized to HPRT mRNA.

Laser Doppler

Flow images of the foot were acquired using a Moor Laser Doppler Imager (Moor Instruments) at 37 ± 0.50°C under inhaled isoflurane (2%) in an induction chamber before being transferred to a nose cone. The data were analyzed with moorLDI image processing software (V5.3; Moor instruments) and reported as the ratio of flow in the ischemic relative to non-ischemic contralateral control hind limbs (Tirziu et al., 2005, 2012; Lanahan et al., 2010).

Micro-CT analysis

Quantitative micro-CT was performed after bismuth-gelatin perfusion of small arteries by the Yale Translational Research Imaging Center. Ischemic hind limbs were staged in a high-resolution micro-CT scanner (GE eXplore Locus SP; GE Healthcare) with a cone beam filtered back projection algorithm, set to an 8-μm effective detector pixel size. Micro-CT was operated with 80-kVp x-ray tube voltage, 80-mA tube current, 3,000-millisecond per frame, 1 × 1 detector binning model, 360° angle, and 0.5° increments per view.

Chapter 3: Results

There is reduced blood flow recovery in aged mice following hind limb ischemia.

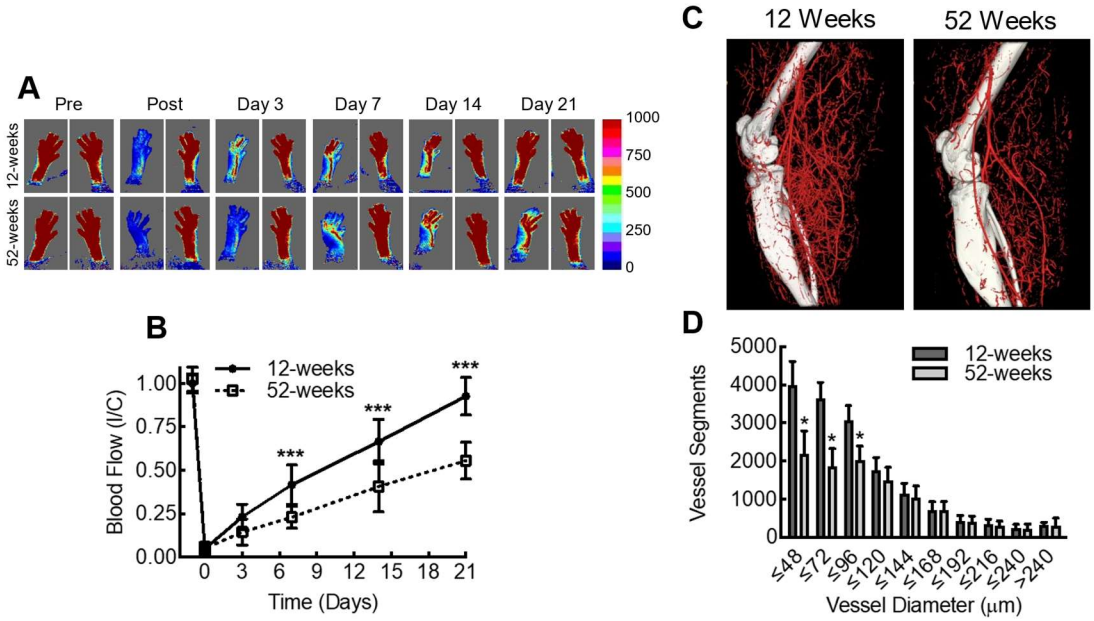

Figure 1: There is reduced blood flow recovery in aged mice following hind limb ischemia. (A) Laser Doppler scans of flow in the ischemic (I) and contralateral control (C) hind limb of mice aged 52 and 12 weeks at indicated time points before and after femoral artery ligation with quantitative blood flow analysis (B; ***, $P < 0.0001$; n = 10: 5 male & 5 female). (C) MicroCT arteriogram of new artery growth 10 Days post femoral artery ligation in 12- and 52-week-old mice along with vessel quantitation in gastrocnemius (calf) muscle tissue (D) (*, $P<0.05$, n=6: 3 male and 3 female). Bar, 1000 microns.

To determine if there was a significant difference in blood flow recovery and angiogenesis due to age, male and female mice young (12-week-old) and aged (52-week-old) underwent hind limb ischemia and their subsequent blood flow recovery was monitored using laser Doppler imaging as well as microCT quantification of vasculature. Over the course of three weeks, young mice showed greater blood flow recovery as early as 7 days following HLI and achieved full recovery by 21 days (Figure 1 A-B). In comparison, the aged cohort had reduced blood flow at every time point and only achieved about 50% blood flow recovery by day 21 (Figure 1 A-B). To confirm these results, bismuth gelatin perfusion of the ischemic limb in conjunction with microCT imaging showed that young mice had a greater number of arterioles

(Figure 1C). Due to the viscous nature of the gelatin-bismuth solution and the arterial infusion site, the dye was only able to permeate arteries, associated arterioles, and capillaries, meaning no venous vasculature was included. These results indicate there is faster blood flow recovery and angiogenic activity in younger mice following injury. However, the question of what mechanisms govern this response remains.

There is reduced VEGF-A Expression in Aged Mice.

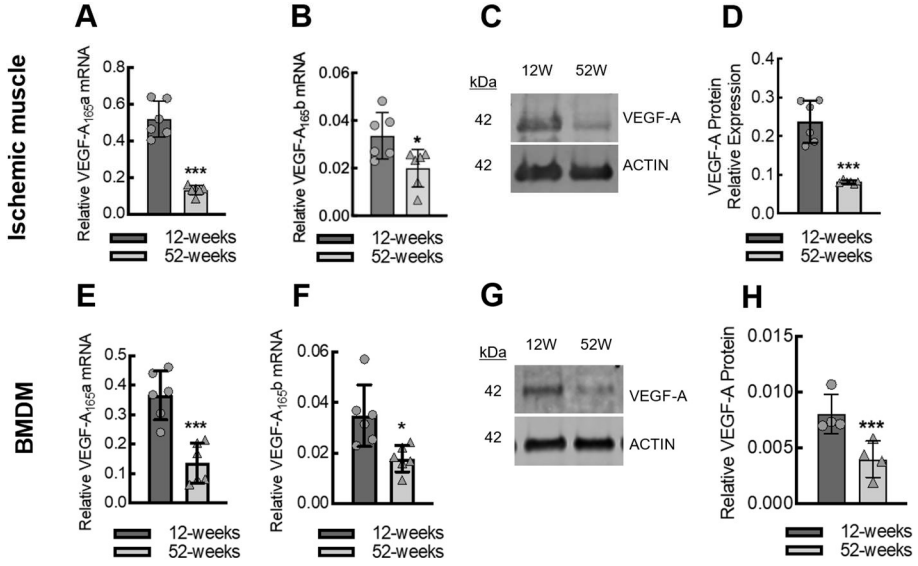

Figure 2: There is reduced VEGF-A Expression in Aged Mice. (A, B) RT-PCR quantification of relative (to GAPDH) VEGF-A_{165}a, and VEGF-A_{165}b from ischemic muscle tissue at day 3 post femoral artery ligation of mice aged 12 and 52 weeks (***, P<0.0001, *, P<0.05 n=6: 3 male & 3 female). (C) Representative VEGF-A immunoblots from ischemic muscle tissue at day 3 post femoral artery ligation from 12- and 52-week-old mice with each lane containing muscle lysate from a separate animal along with quantification (D) of b-actin-normalized protein (***, P<0.0001, n=6: 3 male & 3 female). (E, F) RT-PCR quantification of relative (to HPRT) VEGF-A_{165}a, and VEGF-A_{165}b mRNA from BMDMs of mice aged 52 and 12 weeks (***, P<0.0001, *, P<0.05, n=6: 3 male & 3 female). (G) Representative VEGF-A immunoblots from BMDMs of mice aged 52 and 12 weeks with each lane containing cell supernatant from a separate animal along with quantification (H) of b-actin-normalized protein (***, P < 0.0001 by t test; n =4 mice, two males and two females).

One of the potential mechanisms of action that we suspected could be a factor in reduced blood flow recovery was VEGF-A expression. To assess this, we harvested muscle tissue from

the calf of the ischemic limb on day 3 following Hind limb ischemia. Day 3 post HLI was chosen as the time point for study because it exhibited the greatest difference in macrophage activity following HLI (Morrison et al., 2014, Mantsounga et al., 2022). A horizontal section of muscle tissue was taken from the midpoint of the calf to ensure accurate, even representation of angiogenesis in the calf tissue. After isolating RNA and protein from the segment, RT-qPCR and VEGF-A immunoblots were used to determine the relative amount of VEGF-A expression. The results confirmed that both VEGF-A mRNA and VEGF-A protein were significantly reduced in the muscle tissue from aged mice (Figure 2 B-D). What's more, the pro-angiogenic isoform of VEGF-A, VEGF-A_{165}a, was significantly reduced as well (Figure 2A). This evidence indicates that VEGF-A is one of the mechanisms negatively affected by age in angiogenesis. However, because there are multiple cell types found in muscle tissue, we sought to determine if the reduction in VEGF-A expression could be directly tied to an impaired inflammatory response. It was previously established that macrophages and monocytes are a large source of VEGF-A production and essential to blood flow recovery, (Morrison et al., 2014, Mantsounga et al., 2022). To test this, bone marrow derived macrophages were harvested from young and aged mice. Their relative amounts of VEGF-A were quantified using RT-qPCR and VEGF-A immunoblots. The results confirmed that macrophage-based VEGF-A is reduced in aged mice for both mRNA and protein expression (Figure 2 F-H). Also, the macrophages mirrored the reduced pro-angiogenic isoform VEGF-A_{165}a expression seen in the muscle lysate (Figure 2E). Taken together, these findings establish a link between impaired angiogenesis and an impaired immune response in aged mice. Without injury-based stimulation, aged mice have reduced VEGF-A expression in their macrophages. This means that when injury does occur, aged mice are at a distinct disadvantage for recovery.

Aging does not change macrophage HuR expression and its translocation.

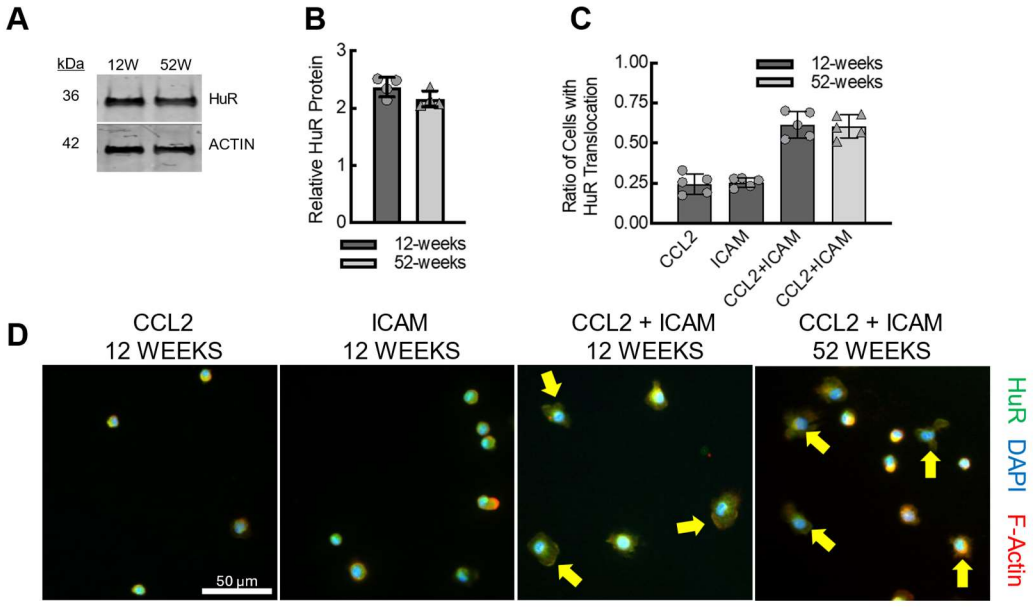

Figure 3: Aging does not change macrophage HuR expression and its translocation. (A) Representative HuR immunoblots from BMDMs of mice aged 52 and 12 weeks with each lane containing cell lysate from a separate animal along with quantification (B) of b-actin-normalized protein ($P > 0.05$ by t test; n =4 mice, two males and two females). (D) Widefield micrographs of BMDMs from mice aged 52 and 12 weeks adhered to immobilized ICAM-1 or control Fc fragment in the presence or absence of 50 ng/ml CCL2 for 30 min, after which HuR was immunostained to detect induced translocation. (C) Quantification of cells in A with nuclear-to-cytosolic HuR translocation, using automated confocal tracking and threshold based, algorithmic analysis. ($P > 0.05$, n = 5).

Given that VEGF-A expression in macrophages is negatively affected by aging, we wondered if other proteins associated with it had a similar response. Based on previous studies we know that VEGF-A mRNA stability is dependent on HuR translocation in macrophages (Morrison et al., 2014). To investigate whether HuR expression was affected by aging, BMDMs from young and aged mice were examined for HuR protein expression by immunoblot. The results indicated that there was no significant difference in HuR protein expression between the two groups (Figure 3A-B). However, it's entirely possible that even though there is no difference in expression there could be a difference in HuR activity. The hallmark of HuR mRNA stabilizing activity is nuclear to cytosolic translocation (Morrison et al., 2014). Prior studies found this activity is observable in macrophages following both CLL2 stimulation and ICAM

adhesion. To determine if HuR translocation activity was affected by aging, BMDMs were harvested from young and aged mice, subjected to CCL2 stimulation, ICAM adhesion, or both, and then stained using immunofluorescence markers for HuR and the nucleus. HuR translocation was established if co-localization of the two markers was not observed. Surprisingly, HuR translocation activity is unaffected in macrophages from aged mice (Figure 3C-D). Collectively these results show that neither HuR activity nor expression is changed as a function of aging. Given these results the next question is if HuR binding to VEGF-A mRNA and subsequent stabilization is affected in aged mice.

HuR Binding to VEGF-A mRNA and VEGF-A mRNA half-life decreased in aged models.

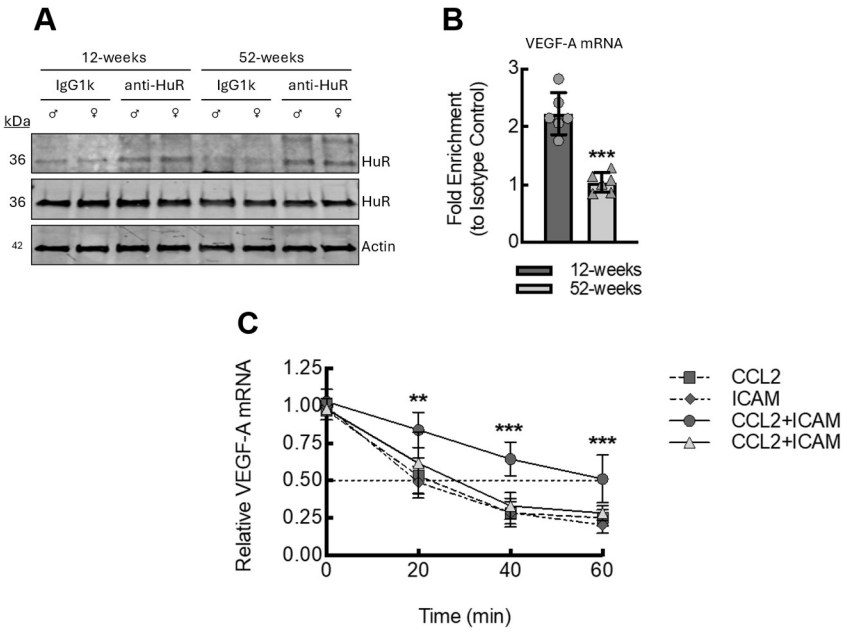

Figure 4: HuR Binding to VEGF-A mRNA and VEGF-A mRNA half-life decreased in aged models. BMDMs lysates from mice, aged 12 weeks and 52 weeks underwent immunoprecipitation, using anti-HuR or isotype (IgG1k) control antibodies. (A) Representative HuR or isotype (IgG1k) control immunoblots from BMDMs of mice aged 12 and 52 weeks with each lane containing cell lysate from a separate animal. (B) Following this RNA was isolated for qRT-PCR of VEGF-A (***, P<0.001, n=6: 3 male & 3 female). (C) VEGF-A mRNA decay assay, using the indicated BMDMs, adhered to immobilized ICAM-1 in the presence CCL2 for 30 min, after which transcription was arrested with 10 µg/ml actinomycin D at time 0, and RNA collected at the noted time points for qRT-PCR (***, P<.0001, **, P<0.001, n=4: 2 male & 2 female)

To assess if HuR binding to VEGF-A mRNA was a possible mechanism affected by aging we conducted an immunoprecipitation pulldown assay for HuR protein in BMDMs from young and old mice. Again, we found no significant difference in the relative amount of HuR present (Figure 4A-B). However, when isolating RNA from the immunoprecipitated sample and then quantifying for VEGF-A mRNA using RT-qPCR, it was discovered that there is a significant decrease in VEGF-A mRNA bonded to HuR in the aged macrophages (Figure 4C). Since HuR binding to mRNA stabilizes it, we sought to determine if the half-life of VEGF-A mRNA was similarly reduced in aged macrophages. To do this, BMDMs were harvested from 12- and 52-week-old mice, stimulated with CCL2, ICAM adhesion, or both, to promote HuR translocation for thirty minutes. Directly following this, actinomycin-D was added to halt any further transcription and allow direct observation of only RNA that was currently present at the indicated 20-minute time intervals. We found that indeed the half-life of VEGF-A mRNA was significantly reduced in aged models with a half-life of about 20 mins. In comparison, the VEGF-A mRNA from the young mice had a half-life over 60 mins, more than twice that of the aged (Figure 4D). Stabilizing VEGF-A mRNA is vital to produce excess VEGF-A as it reduces the extra time for transcription (Van Kouwenhove et al., 2011). This results in VEGF-A mRNA that is readily available for translation in response to injury leading to both greater amounts of VEGF-A and faster rates of angiogenesis. Although this establishes a clear mechanism involved in reduced inflammatory angiogenesis, it does not answer what is causing the impaired binding between HuR and VEGF-A mRNA in aged mice.

Increased DNA methylation and decreased VEGF-A are associated with aged mice.

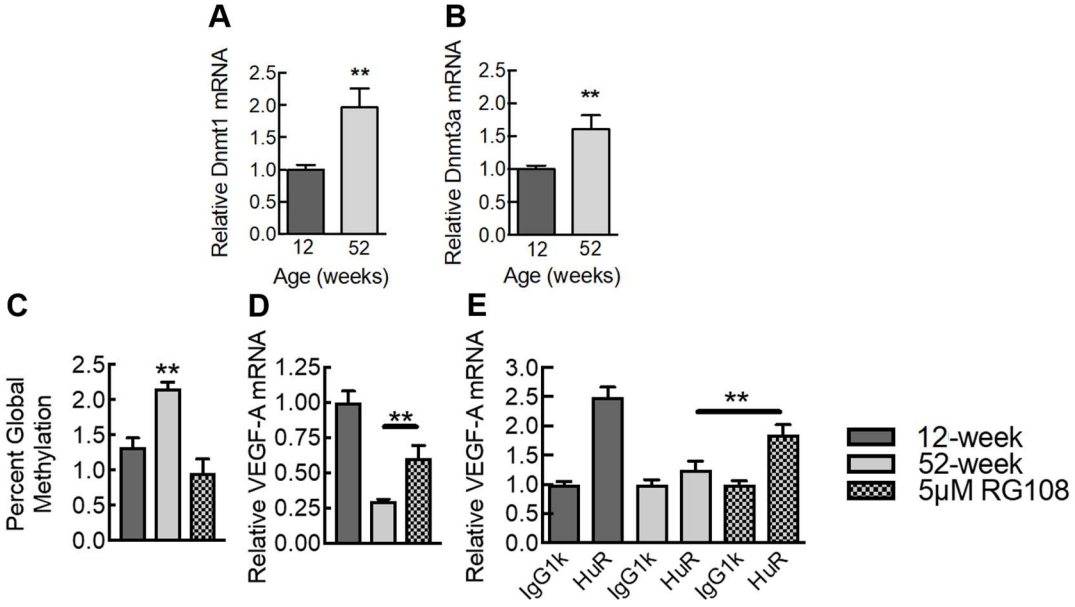

Figure 5: Increased DNA methylation and decreased VEGF-A are associated with aged mice. (A, B) RT-qPCR quantification of DNMT1 and DNMT3A mRNA from BMDMs from mice aged 12 and 52 weeks (**, P<0.001, n=4: 2 male and 2 female). (C) Percent of global DNA methylation in BMDMs from mice aged 12 and 52 weeks, and 52-week BMDMs treated with RG108 (**, P<0.001, n=4: 2 male and 2 female). (D) RT-qPCR of VEGF-A mRNA from BMDMs as in C (**, P<0.001, n=4: 2 male and 2 female). (E). RT-qPCR quantification of VEGF-A mRNA from HuR Co-IP of BMDM lysates treated as in C (**, P<0.001, n=4: 2 male and 2 female).

When considering potential causes for impaired binding of HuR to VEGF-A mRNA we considered looking at DNA methylation as a possible source for this discrepancy between young and old mice. Change in DNA methylation is strongly correlated with aging (Johnson et al., 2012). To determine if this was reflected in aged macrophages, we examined the relative amount of DNA methyltransferase activity in BMDMs from young and old mice using RT-qPCR. We found there was indeed increased activity in aged mice (Figure 5 A-B). Building on this discovery, we next examined what the percent of global methylation was between these two groups and if it could be reversed using RG108, a DNA methylation inhibitor. As expected, aged macrophages exposed to RG108 had reduced methylation levels in the presence of RG108 as well as increased VEGF-A mRNA levels, though not as high as those from 12-week-old

macrophages (Figure 5 C-D). To determine whether DNA methylation also affects the binding and stability of VEGF-A mRNA bound to activated HuR, the immunoprecipitation pulldown assay was repeated but this time macrophages from aged mice were treated with RG108. RT-qPCR on RNA pulled down with the different experimental groups reveals that inhibiting DNA methylation corresponds to an increase in VEGF-A mRNA. The VEGF-A mRNA from the aged, methylation inhibited group was greater than those from the young macrophages (Figure 5E). This indicates that DNA methylation plays a part in VEGF-A mRNA binding to HuR but is not completely responsible for the reduction in binding. Because DNA methylation encompasses several possibilities further models need to be explored to explain the differences seen in young and old mice regarding immune mediated angiogenic responses.

Validation of Macrophage specific DICER1 KO.

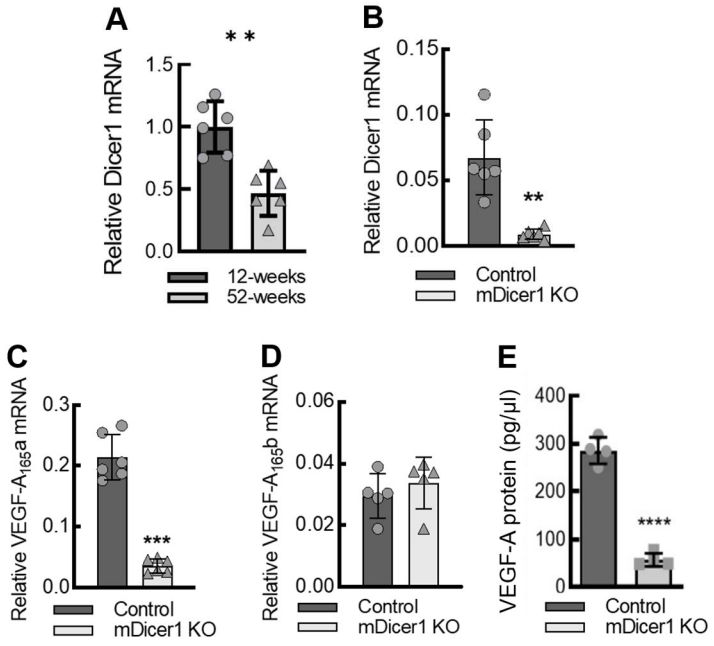

Figure 6: Validation of Macrophage specific DICER1 KO. (A) RT-qPCR quantification of relative (to HPRT) Dicer1 mRNA from BMDMs of mice aged 52 and 12 weeks (**, P<0.001, n=6: 3 male & 3 female). (B, C, D) RT-qPCR quantification of relative (to HPRT) Dicer1, VEGF-A_{165}a, and VEGF-A_{165}b mRNA from BMDMs of mDICER1 KO mice aged 12 weeks (***, P<0.0001, **, P<0.001 n=6: 3 male & 3 female). (E) ELISA on culture supernatant for

secreted VEGF-A from BMDMs from control or mDICER1 KO mice aged 12 weeks (****, P<0.00001 n=4: 2 male & 2 female).

When considering what other factors are reduced in BMDMs we came across DICER1 KO as a potential model for aged mice. Upon further examination it was found that in BMDMs from 52-week-old mice there was a marked reduction in DICER1 as shown by RT-qPCR (Figure 6A). BMDMs from mDicer1 KO mice were tested to confirm there was a significant loss in Dicer1 expression to move forward with the model (Figure 6B). To validate that the macrophage specific DICER1 knockout could be an accurate model for aging, BMDMs were examined for relative amounts of VEGF-A isoforms, VEGF-A_{165}a, and VEGF-A_{165}b mRNA using RT-qPCR. Results indicate that the macrophages have reduced levels of VEGF-A_{165}a mRNA but no significant change in the anti-angiogenic isoform, VEGF-A_{165}b (Figure 6 C-D). Finally, secreted VEGF-A protein in BMDMs was assessed using ELISA. Results indicate comparable losses in VEGF-A protein to that of aged mice (Figure 6E). Given the similarity in VEGF-A levels, we sought to determine if mDICER1KO mice also exhibited reduced blood flow recovery.

Myeloid Dicer1-deletion (mDicer1KO) leads to reduced blood flow recovery and reduced ischemic muscle tissue VEGF-A.

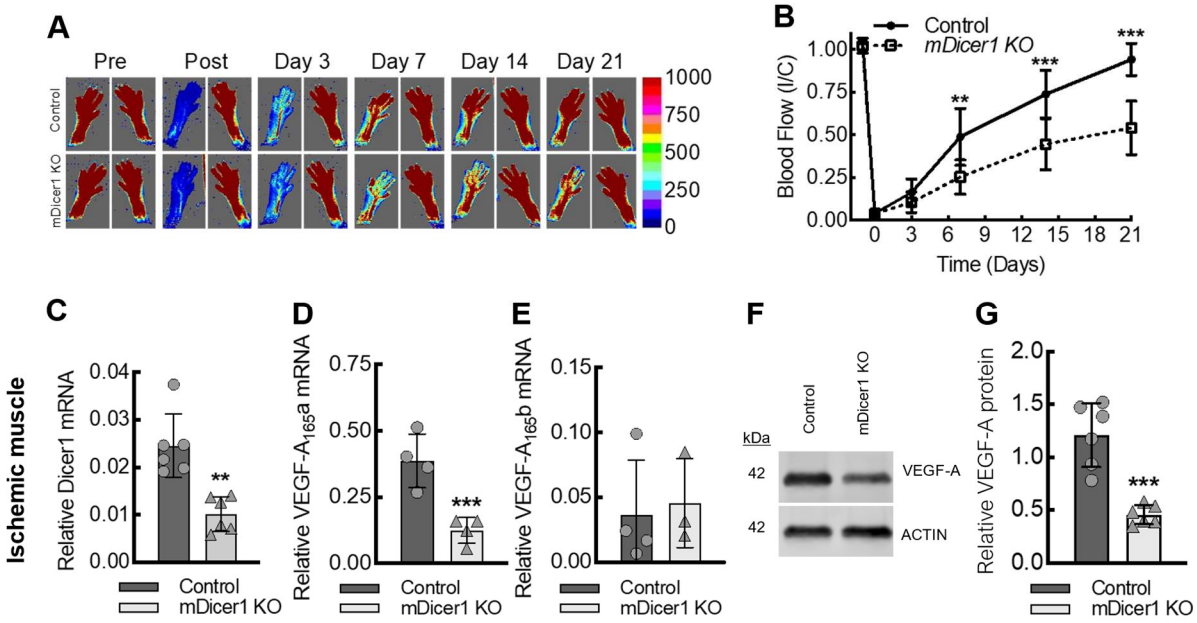

Figure 7: Myeloid Dicer1-deletion (mDicer1KO) leads to reduced blood flow recovery and reduced ischemic muscle tissue VEGF-A. (A) Laser Doppler flow scans in the ischemic (I) and contralateral control (C) hindlimb of myeloid Dicer1-deletion (mDicer1KO) and wildtype (Control) mice at indicated times after femoral artery ligation with quantitative blood flow analysis (B; **, P<0.001; n=10: 5 male & 5 female). (C, D, E) RT-qPCR quantification of relative (to GAPDH) Dicer1, VEGF-A_{165}a, and VEGF-A_{165}b mRNA from ischemic muscle tissue at day 3 post femoral artery ligation of myeloid Dicer1-deletion (mDicer1KO) and wildtype (Control) mice (***, P<0.0001, **, P<0.001, n=4: 2 male & 2 female). (F) VEGF-A and b-actin immunoblots from ischemic muscle tissue on day 3 after femoral artery ligation with each lane from a separate animal. (G) Quantification of relative (b-actin) VEGF-A expression in A (***, P=0.0001, n=4: 2 male & 2 female).

Furthermore, we tested if they have the same reaction to hind limb ischemia and exhibit reduced blood flow recovery. To determine this the mDICER1 KO mice were subjected to HLI with subsequent laser doppler imaging. The results confirm that these mice show reduced blood recovery and angiogenesis (Figure 7 A-B). Additionally, day 3 muscle tissue following HLI confirms there is a reduced amount of Dicer1, VEGF-A_{165}a, and VEGF-A_{165}b mRNA and reduced VEGF-A protein expression using RT-qPCR and immunoblots, respectively (Figure 7

C-G). These results suggest that there may be a connection between VEGF-A and DICER1 expression for an inflammatory angiogenesis response.

HuR Binding to VEGF-A mRNA and VEGF-A mRNA half-life decreased in mDicer1 KO model.

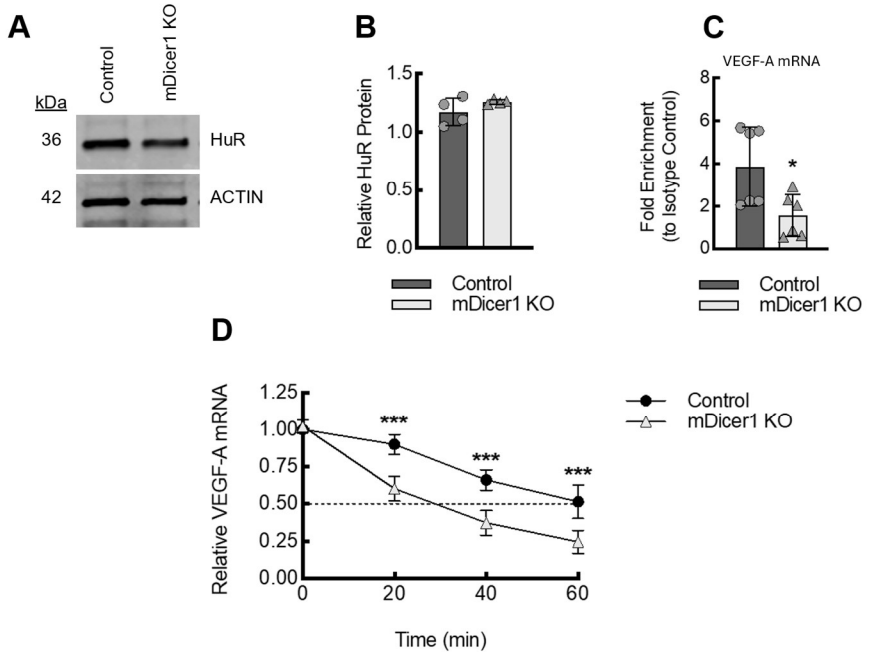

Figure 8: HuR Binding to VEGF-A mRNA and VEGF-A mRNA half-life decreased in mDicer1 KO model. BMDMs lysates from myeloid Dicer1-deletion (mDicer1KO) and wildtype (Control) mice underwent immunoprecipitation, using anti-HuR or isotype (IgG1k) control antibodies. (A) Representative HuR or isotype (IgG1k) control immunoblots from BMDMs of mDICER1 KO mice aged 12 weeks with each lane containing cell lysate from a separate animal along with quantification (B) of b-actin-normalized protein ($P > 0.05$ by t test; n = 4 mice, two males and two females). Following this RNA was isolated for qRT-PCR of VEGF-A (*, $P<0.05$, n=6: 3 male & 3 female). (D) VEGF-A mRNA decay assay, using the indicated BMDMs, adhered to immobilized ICAM-1 in the presence CCL2 for 30 min, after which transcription was arrested with 10 µg/ml actinomycin D at time 0, and RNA collected at the noted time points for qRT-PCR (***, $P<0.001$, n=4: 2 male & 2 female).

As an additional means of verifying the involvement of Dicer1 with the stabilization of VEGF-A mRNA by binding with Hur, we repeated the immunoprecipitation experiments conducted on the aged mice with the mDicer1 KO mice. The findings were in line with what was

seen with the aged mice where HuR protein expression was unaffected in the mDicer1 KO mice (Figure 8 A-B). Like the aged mice the amount of VEGF-A mRNA pulled down with HuR was also reduced by nearly half (Figure 8C). VEGF-A mRNA taken from these experiments showed a similarly reduced half life of about 30 minutes compared to the nearly double average half-life of 60 minutes seen in the control mice. Altogether these results indicate that Dicer1 expression is reduced with age and contributes to the loss of VEGF-A mRNA stability.

Preliminary data suggests increased DICER1 expression could increase blood flow recovery following HLI.

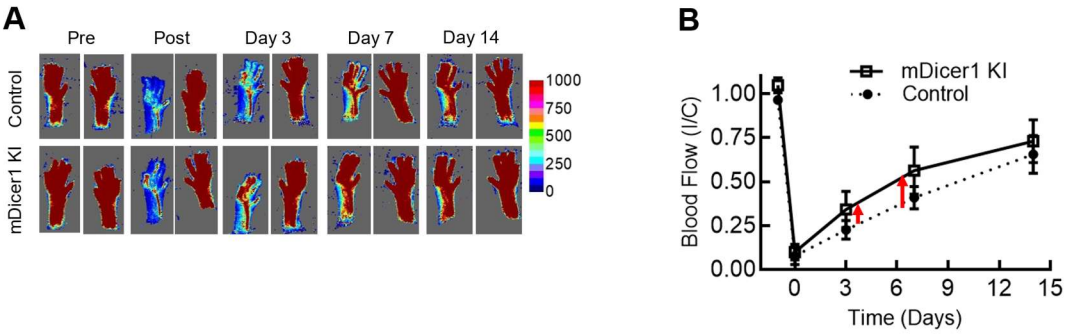

Figure 8: Preliminary data suggests increased DICER1 expression could increase blood flow recovery following HLI. (A) Laser Doppler flow scans in the ischemic (I) and contralateral control (C) hindlimb of myeloid Dicer1-Knock In (mDicer1KI) and wildtype (Control) mice at indicated times after femoral artery ligation with quantitative blood flow analysis (B), (P> 0.05; n=11: 5 male & 6 female).

If DICER1 KO models have a similar phenotype of aged mice, could this phenotype be reversed with a DICER1 knock in model? To investigate this, mDICER1 KI mice were injected with tamoxifen for 10 before HLI to activate increased DICER1 expression. Laser doppler was used to monitor blood recovery and preliminary data indicates that the knock in mice have similar, slightly faster blood flow recovery compared to their wild type counterparts (Figure 8 A-

B). If possible, this could provide an interesting therapeutic for on demand blood flow recovery. However, further data must be collected.

Chapter 4: Discussion and Conclusions

In summary, these results reveal a novel mechanism involving VEGF-A, HuR and DICER1 in the complex process for angiogenesis. Within the aged model used, arteriogenesis is reduced as a response to vascular injury and is most likely even more severe in mice aged greater than a year. This response is reflected in both the muscle tissue and macrophages from aged mice with an additional reduction in VEGF-A and DICER1 expression. We have determined that HuR remains unchanged in both young and aged mice and that further study must be dedicated to understanding what else binds to the VEGF-A mRNA/HuR complex to either promote or destabilize VEGF-A expression. We have shown that there is a relationship between macrophage DICER1 and VEGF-A expression regarding aging and each other. The details of this relationship remain unclear, but the impact DICER1 has on both VEGF-A expression and arteriogenesis is irrefutable. A limitation of this study is the use of middle-aged mice, 52-weeks old as opposed to advanced age mice, 104 weeks old. It's entirely possible that the expression of VEGF-A and Dicer1 could be even further reduced or have an entirely different expression profile. Further studies should examine the expression of VEGF-A and DICER1 in BMDMs from advanced age mice to determine if they have a similar profile. It would be interesting to complete an RNA sequencing profile of the advanced age mice to see how it compares to the middle-aged mice and determine if there are other immune mediated mechanisms affected by aging.

Another limitation of this study is the interrogation of the role of Dicer1 in aging. It's counterintuitive that a reduction in Dicer1 expression would correspond with a reduction in VEGF-A expression. Dicer1 is known to cleave several different RNAs into micro-RNAs with most of them resulting in silencing micro-RNAs (Chang et al., 2013). It's possible that the micro-RNAs produced by DICER1 activity are silencing the activity of RNases that destabilize

VEGF-A mRNA. Albeit non-canonical, it's also possible that the MicroRNAs created by DICER1 have a stabilizing effect on VEGF-A mRNA. Further study is needed to determine the expression profiles of microRNAs that are found in both aged and DICER1 KO mice and compare it to that of young mice. If a specific microRNA is found that is more robustly expressed in young mice compared to aged and DICER KO mice, a follow up experiment of silencing their expression should be conducted to see if it has a similar, negative impact on angiogenesis and VEGF-A/Dicer1 expression in young mice. If a specific microRNA is found to be more robustly expressed in aged mice, it would be interesting to see if a rescue model could be created where the expressed microRNA is silenced to achieve a younger phenotype of angiogenesis in the aged models. It would also be worthwhile to determine if other RNA cleaving proteins are similarly downregulated with age or if this effect is unique to Dicer1. Other RNA cleaving enzymes include the Drosha family of enzymes. Although Drosha is largely known for its activity in the nucleus, c-Drosha has been shown to have activity in the cytoplasm similar to Dicer1, but its activity has not been well characterized and could be an interesting subject of study (Dai et al., 2016, Link et al., 2016).

A large amount of vascular research focuses on the contribution endothelial cells play in angiogenesis, but our work shows clearly that macrophages and immune mediated responses are critical for vessel regrowth and understanding the complex signaling involved. A potential limitation of this study is that only one cell type is examined. It would be meaningful to not only examine other immune cells such as lymphocytes and neutrophils but also endothelial cells. Because there is a dynamic relationship between macrophages and endothelial cells in the formation of new vessels, it would be intriguing to determine if VEGF-A and Dicer1 is also down-regulated in endothelial cells from aged mice.

Many unanswered questions remain such as what other post-translational modifications are occurring with VEGF-A mRNA and are these modifications also happening with the pro and anti-angiogenic isoforms, 165a and 165b. The dynamic relationship between the two isoforms may account for the changes in angiogenesis due to aging. It's possible that the pro-angiogenic isoform is downregulated because of the change in Dicer1 expression resulting in less overall VEGF-A expression and reduced angiogenesis. To determine if this is true, the relative ratio of both variants that are pulled down with HuR following CCL2 stimulation and ICAM1 adhesion should be determined.

It also remains unclear where exactly the DNA methylation is occurring that accounts for the inhibited binding of VEGF-A mRNA to HuR. Moving forward a methylated DNA chromatin immunoprecipitation kit (MeDIP ChIP) could be implemented in examining the DNA from BMDMs of 12- and 52-week-old mice to determine where these potential sites of increased DNA methylation may be coming from and if they are related to VEGF-A, Dicer1, or HuR activity and expression.

Finally, what is most exciting is further testing on the mDICER1 KI mice to characterize how aged mice from this group will respond to HLI and if the increased blood flow recovery will be even more exaggerated than in the control aged mice. As well, it needs to be determined if all the experiments in this paper can be replicated with the Dicer1 knock-in mice to further elucidate the function of Dicer1 in aging and angiogenesis. Future studies will be focused on how changes in DICER1 affect the HuR, VEGF-A mRNA complex and how it can be manipulated to potentially provide useful therapeutics.

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