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The Role of Inflammasome in Vascular Remodeling Following Injury

Ted Amadi
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THE UNIVERSITY OF SOUTH ALABAMA
COLLEGE OF ALLIED HEALTH PROFESSIONS

THE ROLE OF INFLAMMASOME IN VASCULAR REMODELING FOLLOWING INJURY

BY

Ted Amadi

A Thesis

Submitted to the Honors College of the
University of South Alabama
in partial fulfillment of the
requirements for the degree of

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in

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ABSTRACT

Amadi Ted C., M. S., University of South Alabama, May 2021. The Role of Inflammasome in Vascular Remodeling Following Injury. Chair of Committee: Dr. David Weber, Ph.D.

The purpose of the project presented in this thesis is to assess the contribution of inflammasome activation in mediating vascular wall remodeling that occurs following carotid artery injury due to decreased blood flow. Caspase-1 is involved in promoting inflammation through the recruitment of interleukins. To examine this, we used a mouse model of carotid artery ligation in mice lacking the expression of a key mediator of the inflammasome signaling pathway. Following ligation, we completed a histological assessment to compare the degree of the neointima formation and expression of inflammasome related proteins in both injured and the non-injured contralateral carotid artery in both wild-type (WT) and Caspase-1 knockout mice (Cas1 -/-). Caspase-1 plays a role in promoting inflammation through the recruitment of cytokines. The results of the experiment showed that both the wall thickness and the CSWA of the carotid artery increased following ligation after 7 days in both the WT and Cas1 -/- mice, and the perimeter, or total diameter, of the arteries decreased following ligation in both types of mice. Based on these results, ligation leads to arterial remodeling in both WT and Cas1 -/- mice, but Caspase-1 was not shown to have a significant effect on the change of arterial remodeling compared to wild type mice.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ROS – reactive oxygen species

DAMP – Damage-Associated Molecular Pattern

PAMP – Pathogen-Associated Molecular Pattern

PRR – Pattern Recognition Receptors

IL – Interleukin

NLRC4 – NLR family CARD domain-containing protein 4

NLRP3 – NLR family pyrin domain containing 3

CARD – Caspase recruitment domain

NAIP – neuronal apoptosis inhibitory protein

ASC – Apoptosis-associated speck-like protein

LDL – Low-density lipoprotein

TXNIP – Thioredoxin interacting protein

ICAM – Intercellular adhesion molecule

VCAM – Vascular cell adhesion molecule

PTCA – Percutaneous transluminal coronary artery

OCT – Optical Coherence Tomography

WT – wild-type

Cas – Caspase

-/- – knockout

KO – knockout

SEM – standard error of the mean

CSWA – cross-sectional wall area

CHAPTER I

INTRODUCTION

1.1 Ischemia Reperfusion

Trauma is the condition of serious injury to the body caused by physical force. It is the leading cause of morbidity and mortality in patients under 35, and the sixth most common cause of death (Simon *et al*., 2020). Blunt trauma is a form of trauma caused by physical impact to the body, and it very commonly occurs during motor vehicle accidents and falls. For a motor vehicle accident in particular, the vehicle impact force on a victim can create serious crushing injuries that often limits blood supply to organs. If the accident results in a sustained force being maintained on the victim for a long time, then the trauma can progress to even more serious injuries in the tissue, due to the amount of force being placed on the vulnerable tissue resulting in an extended period of reduced blood flow. There are vital organs that are affected by blunt trauma after a motor vehicle accident or fall. As a result of blunt trauma, ischemia can occur in the organs and disrupt their blood flow.

Ischemia is defined as a condition in which not enough blood flows to a specific region or organ of the body. Due to the reduction in blood flow, oxygen is also reduced in turn, and this is referred to as hypoxia. The tissues that are supposed to receive blood from the artery do not get enough oxygen. This reduced blood flow can happen in many

places in the body and can lead to serious problems, such as heart damage, that occur following a myocardial infarction. The amount of injury in the tissues where ischemia occurs depends on the reduction of the blood flow and oxygen perfusion and the duration of the reduced blood flow (Kalogeris *et al*., 2016). The extent of tissue damage from ischemia can potentially lead to tissues undergoing irreversible damage due to their lack of oxygen. Oxygen perfusion needs to be restored back to its normal levels in order to correct the effects of ischemia on these tissues. If the tissues do not recover fast enough and continue to lack oxygen, they will experience necrosis, or premature death due to the injury.

Reperfusion occurs when blood flows back into the specific part of the body following a period of ischemia. Somewhat ironically, the returning blood flow provides needed oxygen to the damaged tissue, but this oxygen can also produce free radicals leading to lipid peroxidation on those tissues (Malek and Nematbakhsh, 2015), which is a type of sustained oxidative stress. The damaged tissue rapidly produces reactive oxygen species (ROS) that initiate downstream signaling pathways. These pathways often lead to further damages on the tissue. These ROS are formed because of the tissue production of ions that lead to sustained oxidative stress via a gain of oxygen during oxidation. Sustained oxidative stress is the imbalance between the amount of ROS produced versus antioxidant removal (Ray *et al*., 2012).

The initial phase of ischemia causes oxidative phosphorylation in the mitochondria to be inhibited due to the lack of oxygen availability in the affected tissues. A large amount of intercellular ROS is released due to the change in the chemical environment in the mitochondria due to ischemia. Following reperfusion, the residual

ROS, along with other immunocytes, contribute to the initiation of inflammation in tissues damaged from ischemia (Kalogeris *et al*., 2016). Immunocytes are cells that are able to produce an immune response, and they include cells such as macrophages and dendritic cells. Certain kidney regions are very susceptible to ischemia. The nephrons are the most metabolically active and contain the highest requirements for oxygen in the kidney. Surrounding areas such as the more inner medulla have a need for less oxygen. Thus, it is reasonable to presume that the cortical region is impacted more severely than the medullary region by ischemia (Kalogeris *et al*., 2016). Renal ischemia leads to stressed kidneys that are deprived of oxygen, and as a result, those same stressed kidneys release molecules that are known as damage-associated molecular patterns to notify other cells about the ischemia in an attempt to initiate the repair process.

1.2 Damage-Associated Molecular Patterns

Damage-Associated Molecular Patterns (DAMPs) are molecules that are derived from cells and released into the cytoplasm from damaged or dying tissues. Their counterparts are Pathogen-Associated Molecular Patterns (PAMPs) that are derived from invading microorganisms during infections. Both molecules can bind to the same extracellular pattern recognition receptors (PRR) and subsequently activate them. Their activation leads to a downstream response of innate inflammatory responses (Turner *et al*., 2014). DAMPs can be released from macrophages, and the most common PRR that they bind are toll-like receptors. Inflammatory cytokines, such as IL-18 and IL-1β, are released from DAMPs, and these cytokines can lead to other responses such the release of chemokines and induction of apoptosis. A specific kind of apoptosis, named

pyroptosis, is activated from the inflammatory response caused by the release of these DAMPs, which is similar to the response from the release of PAMPs from macrophages.

1.3 Pyroptosis

Pyroptosis is a form of cell death that is similar to apoptosis, except it is proinflammatory. In other words, it can promote inflammation to other cells. It involves the release of proinflammatory cytokines by macrophages to communicate with other cells to extend the effects of the downstream signaling by the cytokines. It does not release cytochrome c, which can induce apoptosis, nor does it involve phagocytosis of cell bodies marked for death as in apoptosis (Bergsbaken *et al*., 2009), but rather pyroptosis is mediated by the activation of the enzyme Caspase-1.

1.4 Caspase-1

Caspase-1 is an inflammatory enzyme that activates the proinflammatory cytokines IL-1 and IL-18 that are subsequently released by macrophages. It is regulated by multiprotein complexes, called inflammasomes, that are activated upon cellular infection to trigger the aforementioned proinflammatory cytokines (Schroder and Tschopp, 2010). It plays an important role in ischemia reperfusion by being a mediator for injuries caused by reperfusion that are dependent on upstream inflammasome activators of Caspase-1 such as NLRC4 and NLRP3 (see Figure 1) (Audia *et al*., 2019). Procaspase-1 is the precursor molecule to caspase-1, indicating that it converts to Caspase-1 upon activation. The interaction stems from a C-terminal Caspase recruitment

domain (CARD) that allows molecules to interact with procaspase-1 to initiate this activation.

1.5 NLRC4

NLRC4 is an inflammasome that triggers the release of cytokines from injured tissues. NLRC4 functions as an upstream mediator of inflammasome assembly and its activation leads to increased Caspase-1 activity. It is specifically activated by proteins in the neuronal apoptosis inhibitory protein (NAIP) family (see Figure 1), and it can directly bind to caspase-1 through its CARD-CARD domain. Therefore, it does not need to go through autoproteolysis, which is the splitting of a protein into smaller molecules (Andrade and Zamboni, 2020). The apoptosis-associated speck-like protein (ASC) in particular, is able to increase the efficiency of Caspase-1 activation by the NLRC4 to enhance the inflammatory response in affected tissues. NLRC4 is a known mediator of the inflammatory response and induces pyroptosis of damaged tissues upon its activation.

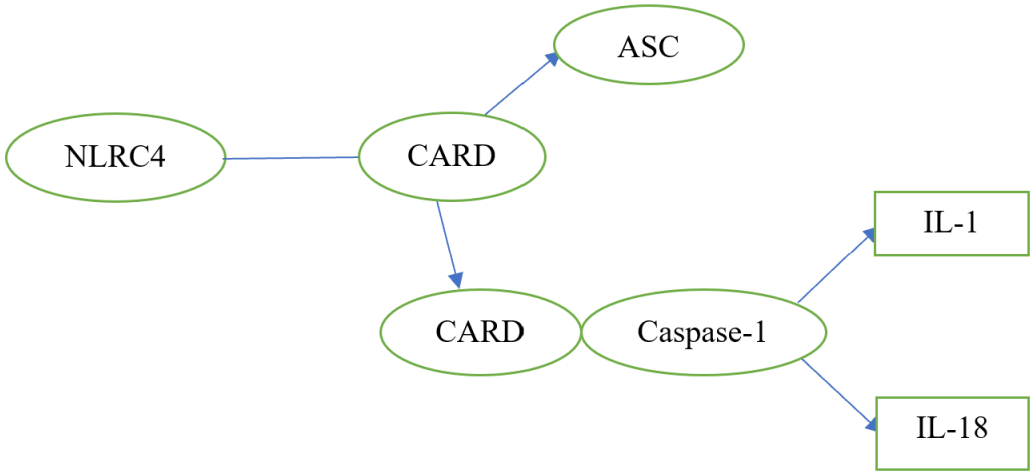

Figure 1: Components of NLRC4 Inflammasome to Activate Caspase-1. An illustration showing how the components of NLRC4 interact with Caspase-1 through CARD-CARD protein interaction to produce inflammation through the secretion of IL-1 and IL-18 (modeled after Andrade and Zamboni, 2020).

1.6 NLRP3

NLRP3 is an additional upstream mediator of inflammasome assembly that has the capability of activating Caspase-1. It plays a role in the inflammatory response by controlling the maturation and release of IL-1 and IL-18 (see Figure 2) via activation of Caspase-1 (Wang *et al*., 2020). When NLRP3 is activated, it oligomerizes and brings in ASC, which leads to the recruitment of pro-caspase-1 through the CARD-CARD domain. It can be activated by multiple mechanisms including the lysosome destabilization

pathway, which works by processing IL-1 and IL-18 to reduce the stability of lysosome; and the release of ROS which leads to oxidative stress that also activates NLRP3 (Wang *et al*., 2020). NLRP3 plays an important role in the development of cardiovascular diseases like atherosclerosis by inflammation.

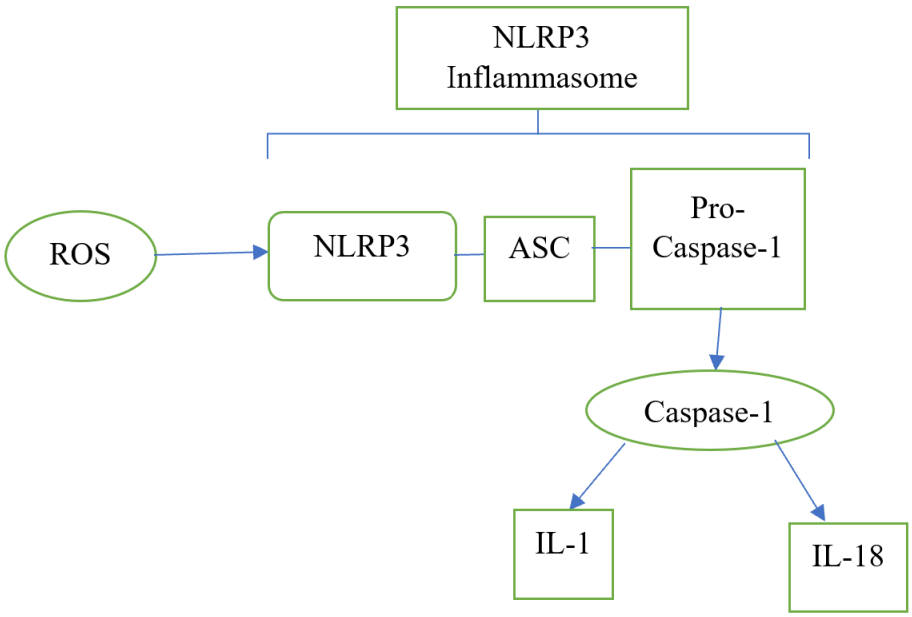

Figure 2: Activation of the NLRP3 Inflammasome. An illustration that shows how the ROS interact with the components of the NLRP3 inflammasome in order to activate Caspase-1 and produce inflammation through the secretion of IL-1 and IL-18. (modeled after Turner *et al*., 2014).

1.7 Atherosclerosis

Atherosclerosis is a cardiovascular inflammatory disease that leads to the accumulation of plaque on walls on large and medium sized arteries, and this build up may obstruct arterial blood flow. It is chronic and is characterized by lipid deposition, leukocyte infiltration, and proliferation of vascular smooth muscle cells (Karasawa and Takahashi, 2017). This pathology is a leading cause of death worldwide among cardiovascular diseases. Cholesterol is potentially a major factor in the activation of inflammasome pathway for this disease. When low-density lipoprotein (LDL) is oxidized, they can be brought in by macrophages to activate the NLRP3 inflammasome by lysosomal damage. That damage comes through the intracellular nucleation of cholesterol crystals (Li *et al*., 2014). Apoptosis occurs as a result of the release of mature IL-1 and IL-18, which contributes to additional harmful effects that atherosclerosis can have beyond the buildup of plaque. Like ischemia from pyroptosis, apoptosis from atherosclerosis results in the accumulation of lipids in the wall of the inside artery, or the tunica intima. Hyperglycemia and hyperinsulinemia are frequently linked to the prevalence of atherosclerosis and can lead to increased expression of thioredoxin interacting protein (TXNIP). This protein can further promote activation of inflammasomes from cholesterol crystals and is involved with the NLRP3 inflammasome.

1.8 Artery Structure

The artery is made up of three different layers: the intima, media, and adventitia (see Figure 3). The intima is the layer that is closest to the arterial lumen, which is the central open area where blood flows, and it is covered by a layer of endothelial cells that act as a barrier between the blood and vessel. The media is the thick middle layer that consists of the internal and external elastic lamina that separates it from the intima and adventitia. It is full of smooth muscle cells and the extracellular matrix, and both of these components allow the media to maintain its elasticity by controlling blood flow during systole and diastole. The presence of smooth muscle cells relates to the thickness of the media. The adventitia is the outer layer of fibrous connective tissue, primarily collagen, that serves as an entrance or exit point for nerves and blood vessels leading to the nourishment of the arterial wall cells (Lilly, 2003).

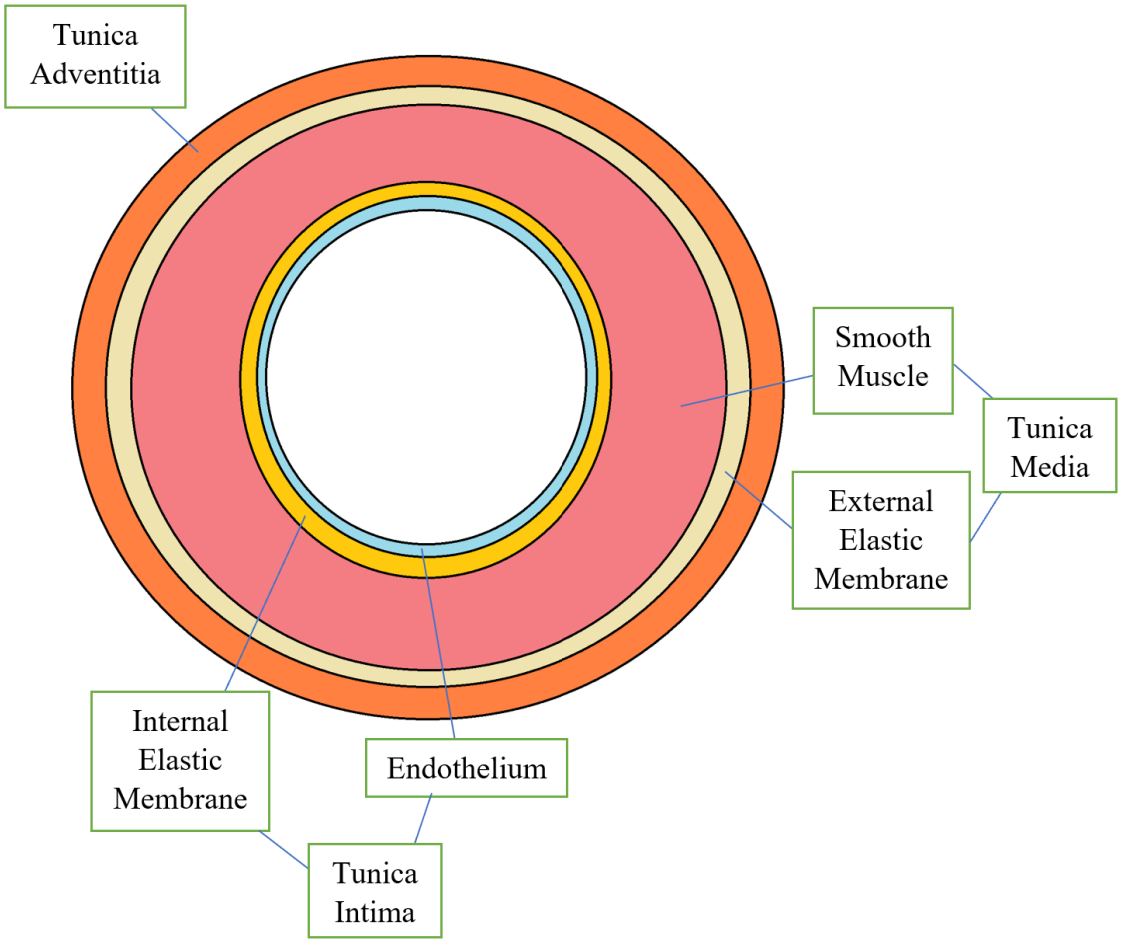

Figure 3: Layers in the Structure of the Artery. An illustration of the cross-sectional view of the structure of an artery. The artery is separated into three main layers with components for each of those layers.

1.9 Artery Injury

The artery can experience a shift in its structure when it is injured. Injury can stem from many different factors, such as exposure to chemicals and physical forces. When those physical and chemical stressors act on the artery, it can lead to inflammation via the release of cytokines and affect the permeability of the endothelial barrier (Lilly, 2003). The internal diameter of the arteries may be affected by substances such as nitric oxide, which may lead to vasodilation on the medial layer of the artery. The formation of thrombosis may be more prevalent after injury due to the inhibition of the immune system that prevents thrombosis. Thrombosis is the event of a blood clot forming in the artery due to damage occurring in the endothelium of the artery. These blood clots, or thrombi, are consisted of platelets that further adhere to the site of damage and lead to the reduction and/or obstruction of blood flow to other tissues being inhibited. It can be caused by any kind of injury to the blood vessel, and it is life threatening because the arteries' role to carry blood from the heart to the tissues in the body is compromised.

1.10 Arterial Remodeling

Arterial remodeling results from the types of injuries that may stem from stressors and diseases like atherosclerosis. When plaque forms on the walls of the artery via atherosclerosis, the structure of the artery may change to accommodate to the pressure of the plaque by increasing the diameter of the arterial lumen to prevent blood flow from being cut off from the accumulation of the plaque (Lilly, 2003). This form of remodeling also functions to bring the shear and wall stress back to normal on the arterial walls.

Shear stress refers to the change of structure in an object when applying pressure parallel to that object. If the shear stress is affected by atherosclerosis, macrophages may be recruited by cell adhesion molecules such as ICAM and VCAM (Ward *et al*., 2000). Atherosclerosis causes the expression of matrix metalloproteinases by hyperlipidemia since it leads to inflammation. If hypercholesterolemia happens from the buildup of plaque, it leads the artery to dilate itself to the point where it may suffer vessel ectasia, or the distention of the lumen.

While our studies do not directly examine for plaque development, we are able to mimic artery remodeling by using the carotid artery ligation. Following complete occlusion induced by surgical suture, blood flow through the carotid artery is blocked. The effect of this is two-fold, in that we have pulsatile pressure leading to changes in flow patterns proximal to the occlusion and a significant inflammatory response in the artery wall. Previous studies suggest that these changes will facilitate significant endothelial damage and activation of smooth muscle cell proliferation and migration leading to significant neointima formation.

1.11 Angioplasty

Angioplasty is a common procedure for patients suffering from atherosclerosis. It is meant to bring back coronary blood flow by opening the lumen of the affected blood vessel. One of the most common examples of angioplasty is percutaneous transluminal coronary angioplasty (PTCA) with stent implantation (Manogue *et al*., 2015). In this noninvasive procedure, a guide wire is placed through the femoral artery into the coronary artery where the plaque is causing blockage. A balloon is then inserted through

the opening via stent into the location of the blockage, and it is inflated to push the plaque against the wall of the artery and open up the lumen area of the coronary artery. A contrast dye is usually used along with an X-ray to check for any further changes on the site where the blockage was. Unfortunately, the effect of angioplasty is often not permanent, and the coronary artery can narrow again over time after treatment. This phenomenon is called restenosis.

1.12 Restenosis

Restenosis can occur as soon as 6 months after treatment via PTCA. It occurs due to the formation of neointimal lesions which stem from smooth muscle cell proliferation and extracellular matrix deposition (Xu, 2004). Due to the lumen area decreasing in the vessel, angina, or chest pain, can occur again as a result. Arterial remodeling is the cause of restenosis as vessel growth occurs on the site of the area where treatment was applied during angioplasty (see Figure 4). With the improvement of the design of stents for angioplasty, restenosis is less common, although the phenomenon can still occur. Historically, restenosis following angioplasty occurred in up to 50 percent of patients after PTCA with balloon alone, but the development of stents reduced the occurrence of restenosis by up to 30% (Moussavian *et al*., 2001), and the development of drug eluting stents has further improved outcomes.

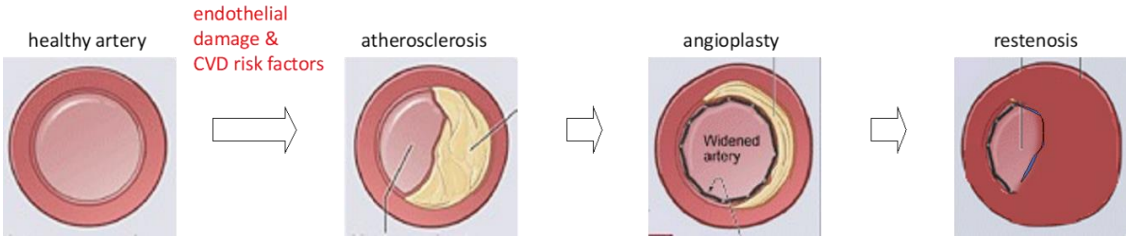

Figure 4: Progression of Atherosclerosis Leading to Angioplasty and Restenosis. An illustration that shows the progression of a healthy artery to an artery affected by restenosis after angioplasty. Endothelial damage and cardiovascular risk factors lead to the formation of plaque on the walls of the artery which causes atherosclerosis. The plaque can be pressed into the walls of the artery via angioplasty, but neointima lesions will form after angioplasty to cause restenosis.

1.13 Further Evaluation of Subjects

Currently, there is a lack of appreciation of how inflammasome activation may contribute to arterial wall remodeling. Although beyond the scope of this project, it is not known how damage-associated molecular patterns released during ischemia following vascular occlusive injury are related to the activation of the NLRC4/Caspase-1 inflammasome, nor is the relationship known between those molecular patterns to localized tissue pyroptosis. Additionally, the generation on DAMPs by the artery wall and their effects on overall cardiovascular health remains an area of active research. It is also not clearly known how inhibiting specific cytokines, such as IL-1 and IL-18, plays a role in the treatment of cardiovascular disease. NLRP3 inflammasomes have recently

been identified as potential contributors to cardiovascular diseases, and thus their overall role and specific mechanisms that leads to those diseases remain poorly defined.

CHAPTER II

HYPOTHESIS AND AIMS

2.1 Hypothesis

We hypothesize that inflammasome activation leads to increased neointima formations following reperfusion after prior carotid artery ligation. We will assess these changes by examining changes in the blood vessel by histological assessment of changes in wall thickness and cross-sectional wall area. We predict that injury would lead to hypertrophy of the blood vessel where the walls will grow in size and thickness, compared to an uninjured blood vessel not changing at all in size. We also predict that the vessel lumen will decrease in size upon hypertrophy of the blood vessel. When Caspase-1 is involved, this will change the extent of the hypertrophy in injured blood vessels, where the injury will lead to even more hypertrophy in the blood vessel. Figure 5 highlights the presumed trend of arterial remodeling in both types of mice. In an uninjured blood vessel, there will still be no change even in the presence of the Caspase-1. The knock-out of Caspase-1 will attenuate vascular hypertrophy, leading to less remodeling following injury in mice lacking Caspase-1 expression.

2.2 Project Goal

The goal of this project is to identify if Caspase-1 activation contributes to artery remodeling.

2.3 Project Aims

To test our hypothesis that inflammasome activation leads to increased neointima formation following carotid artery ligation, we will attempt to complete two aims.

Aim 1: To determine if knock-out mice lacking Caspase-1 demonstrate decreased remodeling following carotid artery ligation injury.

Aim 2: To determine arterial expression of Caspase-1 following carotid ligation injury.

Aim 3: To determine if smooth muscle cell proliferation is limited in mice lacking Caspase-1 expression.

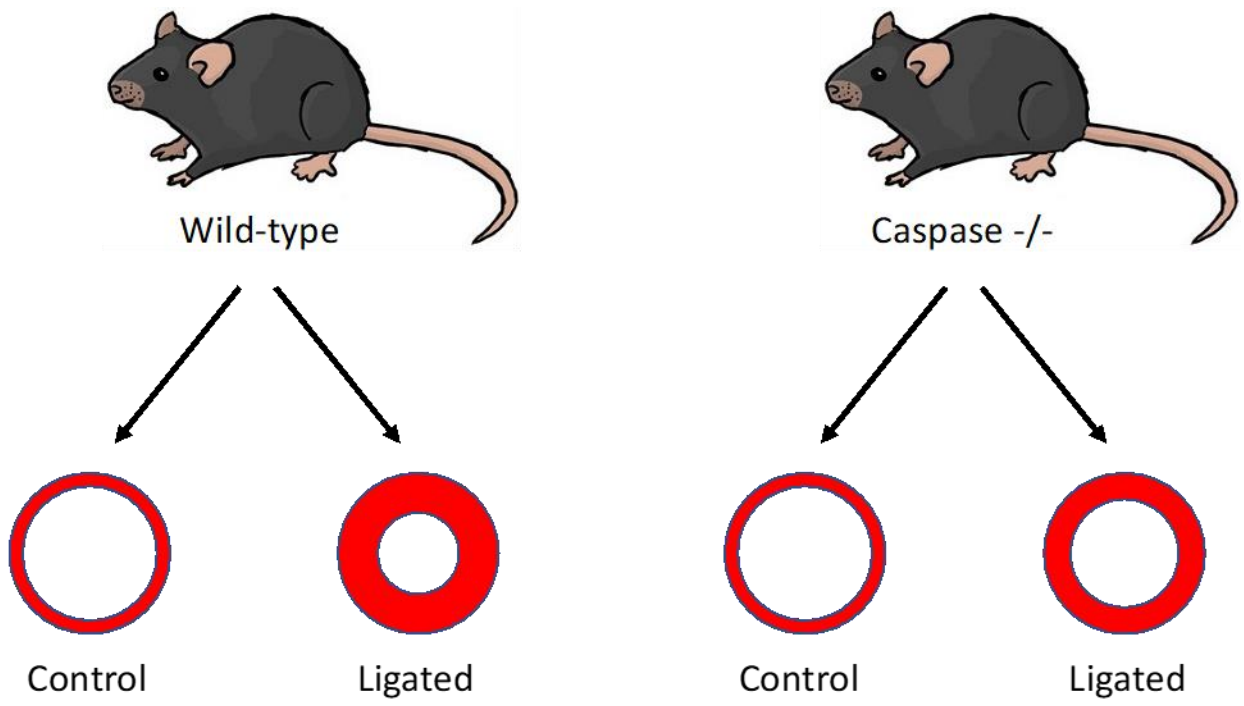

Figure 5: Artery Before and After Ligation for Different Types of Mice. An illustration showing the hypothesis on how size and thickness will be impacted in both control and ligated arteries for both wild type and Caspase-1 -/- mice. The size of the medial layer of the artery is predicted to increase after ligation in both wild-type and Caspase-1 -/- mice, but the extent of arterial remodeling is believed to decrease in the latter group of mice.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Ketamine and xylazine were used as anesthesia. Buprenorphine was used as a narcotic along with the anesthesia. A chlorhexidine solution was used for the cleaning of the skin of the mouse used in the experiment. Eye lubricant was used for the hydration of the cornea of the mouse. Adhesive tape was used for keeping the mouse still during the surgical procedure. A heating pad was used for regulation of the temperature of the mouse after the procedure. A pair of scissors was used for the cutting of the skin from the mouse. Sterilized cotton swabs were used to aid with moving the organs during surgery. A towel was soaked in water and used for the hydration of removed organs of the mouse. Forceps are used to lift the organs of the mouse as needed during the surgical procedure. A 6-0 braided surgical suture was used to induce ligation on the left carotid artery of the mouse. Gel was used for the lubrication of the organs for harvesting. Liquid nitrogen was used to freeze the harvested organs to section them into pieces. A hematoxylin stain was used to stain and visualize arterial tissues in both injured and non-injured arteries.

3.2 Surgical Protocol

The mice were anesthetized with a mixture of ketamine and xylazine (Le Clef *et al*., 2016) via intraperitoneal injection by my mentor. Loss of reflex confirmed that the anesthesia was effective to the point of immobilization of the mouse. The hair of the mouse was removed, skin was cleaned with a diluted chlorhexidine solution, and the mouse was placed on a heated surgical pad. The head and the neck of the mouse were extended for uninhibited airflow (Le Clef *et al*., 2016). The neck had its hair removed with Nair and disinfected with chlorohexidine right after. (Taylor *et al*., 2017). Eye lubricant was used to keep the cornea from drying, and adhesive tape was placed on the upper and lower limbs of the mouse to secure the animal against the heating pad. Buprenorphine was administered occasionally during the surgery to treat pain.

The connective tissues anterior and posterior to the left carotid artery and vein were broken with forceps, and another opening was made under the vessels under the left renal artery and vein. The left carotid artery was isolated by blunt dissection (Taylor *et al*., 2017) and dissected with forceps. On the left common carotid artery, the ligation was completed proximal to the bifurcation with a silk structure to restrict blood flow. Afterwards, the mouse was placed back by myself in their cage on top of a heating pad that maintained its body temperature at 37 °C for the remainder of the protocol.

Both the wild-type and Caspase-1 knockout mice that underwent surgery recovered for 7 days before carotid arteries were harvested. Mice were overdosed with pentobarbital and carotid arteries were removed in place in OCT medium prior to freezing (Manogue *et al*., 2015). After they were fixed, the vessels were cut into multiple 7 μm cross sections by others, and they were then stained with hematoxylin and eosin to

observe the structure of the artery and the damage induced by the left carotid ligation. Digital imaging was used by me to determine the artery wall thickness and cross-sectional wall area (Manogue *et al*., 2015). For the wall thickness, the distance between the luminal border delineated by the internal elastic lamina and the external lamina was taken from nine regions of the artery per sample and averaged to give the result. For the cross-sectional wall area, the area of the outer elastic lamina was calculated while subtracting the area of the lumen to give the result. The methodology used for these measurements are highlighted in Figure 6. Cell signaling technology was used to visualize antibodies that recognize Caspase-1. The left and right carotids were compared to differentiate morphological changes between injured and non-injured arteries.

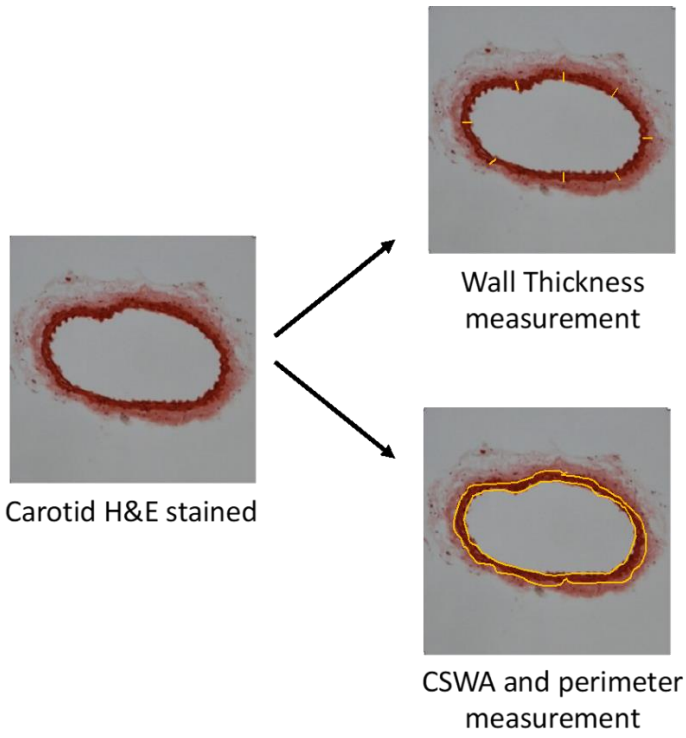

Figure 6: Measurement of CSWA, Wall Thickness, and Perimeter of the Artery in 10x magnification. An illustration of the way that the wall thickness, cross-sectional wall area, and perimeter were measured on the carotid arteries that were stained with hematoxylin and eosin.

3.3 Statistical Analysis

The data in this study is presented as Mean ± SEM. A two-way analysis of variance was performed based on the design. A Tukey's multiple comparisons test was performed after the analysis of variance. A p value < 0.05 was used to show any statistical significance between groups.

CHAPTER IV

RESULTS

Seven days following left carotid ligation injury, the common carotid arteries were harvested from both injured and contralateral control (non-injured from the same mice). Representative histological images that were obtained for analysis of morphometric changes in artery structure in both the wild-type and Caspase-1 knockout mice is shown in Figure 7. Hematoxylin and eosin were used to stain the cross section of the carotid arteries in order to highlight the extent of arterial remodeling in the medial layer of the artery.

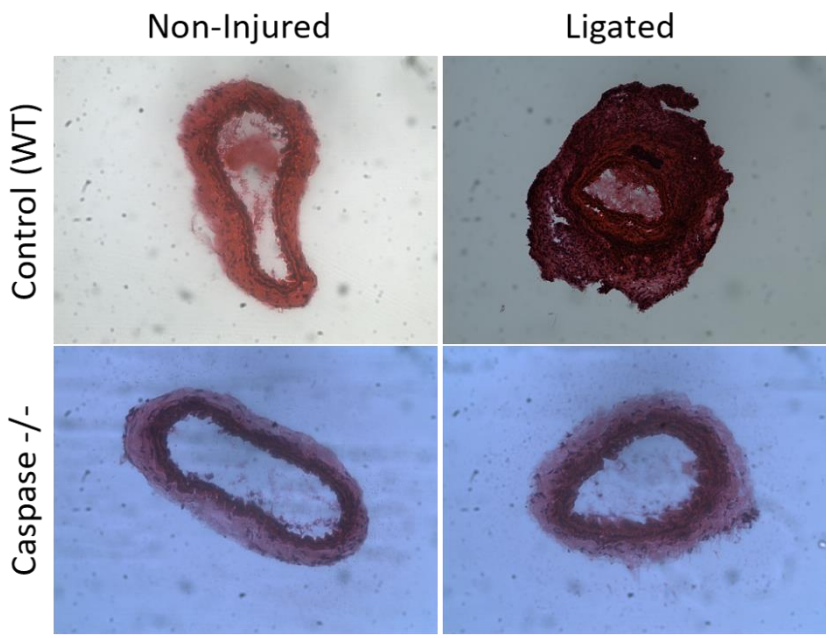

Figure 7: Comparison Between Control and Ligated Arteries for WT and Cas-1 -/- Mice. Images show the wild-type and Caspase-1 knockout carotid arteries before and after injury. Hematoxylin and eosin were used to stain the cross section of the arteries. The carotid arteries were left to progress over a span of 7 days after the ligation. The magnification of the images was 10x with the calibration set to 0.34 μm. The images show evident remodeling of the carotid arteries after injury, including changes in intima and lumen size.

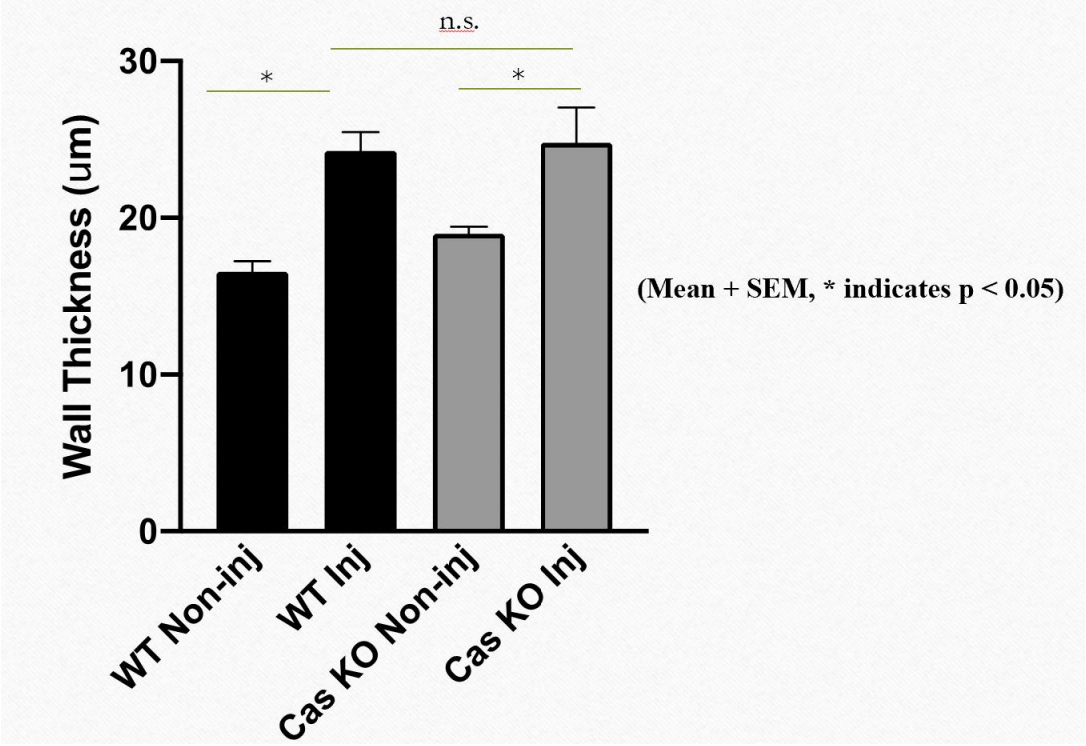

Figure 8: Arterial Wall Thickness After Injury in WT vs Cas-1 -/- Mice. Effect of ligation on wall thickness 7 days after injury in WT and Cas-1 -/- mice. Significant increase in mean wall thickness in WT Non-Inj vs WT Inj ($p < 0.05$). Significant increase in mean wall thickness in Cas-1 -/- Non-Inj vs Cas-1 Inj ($p < 0.05$). No significant difference in mean wall thickness in WT Inj vs Cas-1 -/- Inj. No significant difference in mean wall thickness in WT Non-Inj vs Cas-1 -/- Non-Inj. Values indicate Mean + SEM for 6 animals per group.

To quantify changes in morphology following carotid ligation injury, we assessed multiple parameters including wall thickness, cross-sectional wall area (CSWA), and artery perimeter. Figure 8 shows the mean wall thickness in both the injured and non-injured carotid arteries following left carotid ligation. The carotid arteries were grouped into the types of mice they originated from: Wild-type and Caspase-1 knockout. The extent of the wall thickness change is measured in units of μm. For the non-injured wild-type carotid artery, the mean of the arterial wall thickness is shown as 16.57 ± 0.66 μm, and 7 days following injury, significant increases in wall thickness were observed in the injured wild-type carotid artery (24.25 ± 1.22 μm). The wall thickness of the non-injured Caspase-1 knockout carotid artery was 18.99 ± 0.46 μm, and the average wall thickness following injury was 24.8 ± 2.26 μm.

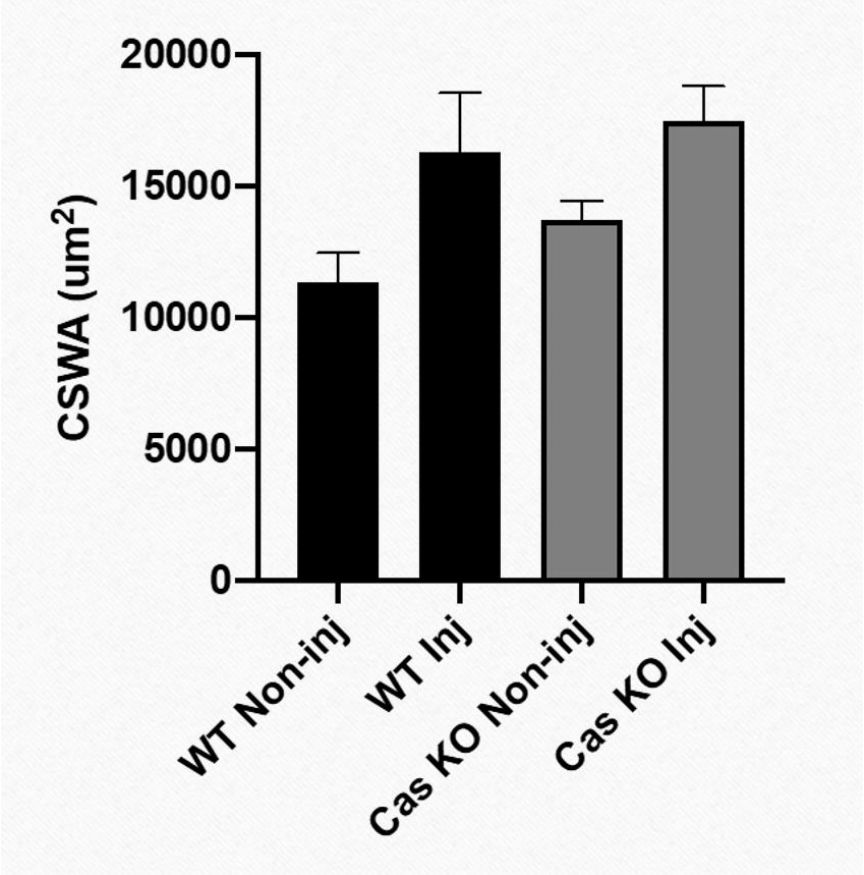

Figure 9: Arterial Cross-Sectional Wall Area After Injury in WT vs Cas-1 -/- Mice. Effect of ligation on cross-sectional wall area 7 days after injury in WT and Cas-1 -/- mice. Significant increase in mean CSWA in WT Non-Inj vs WT Inj ($p < 0.05$). Significant increase in mean CSWA in Cas-1 -/- Non-Inj vs Cas-1 Inj ($p < 0.05$). No significant difference in mean CSWA in WT Inj vs Cas-1 -/- Inj. No significant difference in mean CSWA in WT Non-Inj vs Cas-1 -/- Non-Inj. Values indicate Mean + SEM for 6 animals per group.

Figure 9 shows the mean cross-sectional wall area (CSWA) in both the injured and non-injured carotid arteries following left carotid ligation. The carotid arteries were grouped into the types of mice they originated from: Wild-type and Caspase-1 knockout. The extent of the CSWA change is measured in units of μm^2. For the non-injured wild-type carotid artery, the mean ± SEM of the CWSA is shown as 11337 ± 1112 μm^2. The injured wild-type carotid artery gives the mean of the cross sectional wall area at 16283 ± 2241 μm^2. For the Caspase-1 knockout non-injured carotid artery, the mean of the cross sectional wall area was given as 13697 ± 730 μm^2. The injured carotid artery for the Caspase-1 knockout mice gives its mean of the cross sectional wall area at 17483 ± 1311 μm^2.

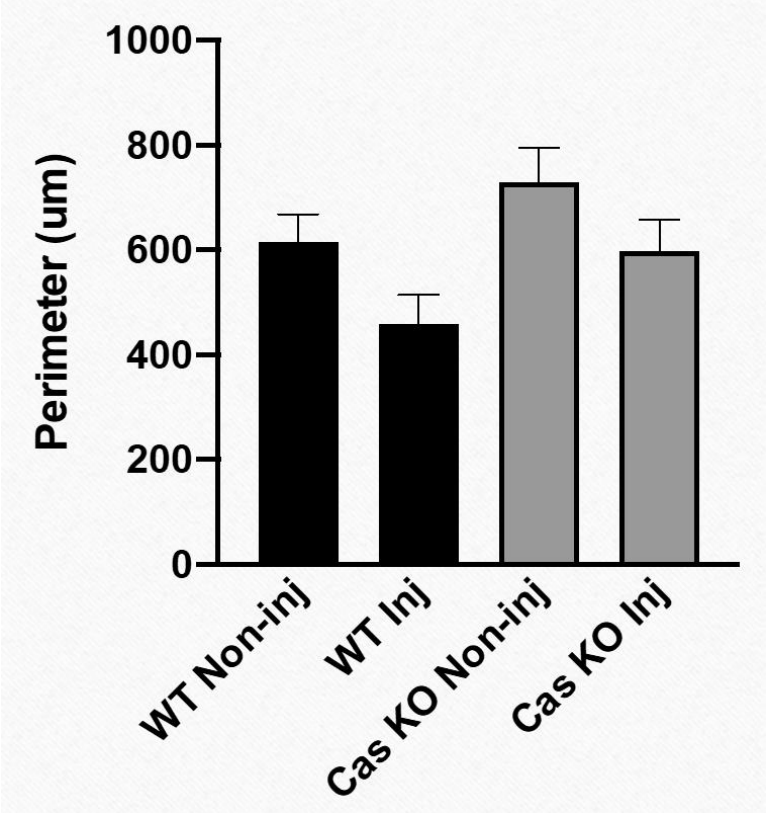

Figure 10: Arterial Perimeter After Injury in WT vs Cas-1 -/- Mice. Effect of ligation on perimeter 7 days after injury in WT and Cas-1 mice. Significant decrease in mean perimeter in WT Non-Inj vs WT Inj ($p < 0.05$). Significant decrease in mean perimeter in Cas-1 -/- Non-Inj vs Cas-1 Inj ($p < 0.05$). No significant difference in mean perimeter in WT Inj vs Cas-1 -/- Inj. No significant difference in mean perimeter in WT Non-Inj vs Cas-1 -/- Non-Inj. Values indicate Mean + SEM for 6 animals per group.

Figure 10 shows the mean perimeter in both the injured and non-injured carotid arteries following left carotid ligation. The carotid arteries were grouped into the types of mice they originated from: Wild-type and Caspase-1 knockout. The extent of the perimeter change is measured in units of μm. For the non-injured wild-type carotid artery, the mean of the perimeter is shown as 616 ± 51 μm. The injured wild-type carotid artery gives the mean at 459 ± 54 μm. For the Caspase-1 knockout non-injured carotid artery, the mean of the carotid artery was given as 728 ± 65 μm. The injured carotid artery for the Caspase-1 knockout mice gives its carotid artery mean at 598 ± 58 μm.

CHAPTER V

DISCUSSION

In these studies, we used both wild-type mice and Caspase-1 knockout mice to assess the effect of the ligation injury on the carotid arteries to test our hypothesis that mice lacking expression of Caspase-1 would have attenuated remodeling following injury. We selected a 7-day timepoint to make these measurements in an effort to examine the impact of inflammasome activation on the early events associated with the injury response.

Control non-injured arteries were similar in size between the two genotypes of mice suggesting that global deletion of Caspase-1 did not impact artery structure. This important observation could be attributed to the fact that Caspase-1 is not a key mediator of artery growth during development, or that the mice activated a compensatory response that masked any contribution of Caspase-1 to artery growth. Clarification of this mechanism was beyond the scope of our current work as we chose to focus on the impact of Caspase-1 in remodeling following ligation injury.

As anticipated, our findings showed that there was a significant increase in the wall thickness of the carotid artery after injury in wild-type mice. This goes with our general hypothesis that ligation injury would lead to hypertrophy of the blood vessel resulting in the increase of its size and shape. There was also an increase (18.99 ± 0.46

μm to 24.8 ± 2.26 μm) in the wall thickness of the injured carotid artery in Caspase-1 knockout mice. There was a tendency for the wall thickness to change somewhat less in the Caspase-1 deficient animals (5.8 ± 2.5 μm vs 7.6 ± 1.5 μm in wild-type mice). This degree of remodeling was not deemed to be statistically significant compared to the aforementioned change seen within the wild-type mice. Thus, based on wall thickness measurements, we conclude that responses to carotid ligation injury were similar in both groups of mice. To further investigate this, future experiments would include longer time points in order to see if there are any differences in changes of the carotid artery following arterial remodeling after 7 days. Intervals of 7 days could be factored in to observe weekly changes to the blood vessel following ligation injury. Different injury models may also be implemented in future experiments to show potential differences in how the arteries respond to injury. Ligation on the external carotid artery instead of the common carotid artery may result in alternative results in the structures of the artery following remodeling. A balloon injury model may serve as a capable alternative to the carotid ligation model in order to observe the effects of restenosis after angioplasty on the arteries over longer time periods.

Although the progression of the wall thickness following ligation was expected, the samples used to calculate the mean had fluctuating measurements in the wall thickness along their artery. There is a reason to consider if this difference may be caused by errors in methodology. There were 9 different measurements of the arterial wall thickness within one mouse, but in an optimal simulation, measurements of all parts of the arterial wall would have been taken which may lead to a more accurate result of the mean, and possibly contribute to the differences between non-injured wild type and

Caspase-1 knockout mice being less prevalent. The wall thickness was not uniform along the whole blood vessel, which is why an increase in the number of measurements taken is needed in future experiments.

The measurements of the cross-sectional wall area of the carotid arteries showed that there was an increase in the mean within wild-type mouse after injury. The interpretation of this result may be that the increase in smooth muscle cell proliferation in the media during injury results in an increase in the size of the neointima after arterial remodeling. This change was expected in the experiment. Within Caspase-1 knockout mice, it showed a similar trend. The CSWA of the Caspase-1 knockout carotid arteries increased after injury, but to a lesser degree than the wild-type carotid arteries. This demonstrates that there was less smooth muscle cell proliferation after injury due to the lack of Caspase-1 on the mice, although the difference between the wild-type and Caspase-1 knockout arteries are not statistically significant. There were some samples included in the data where the lumen area of the vessel was abnormally small. This can be due to possible methodological errors during surgery where the walls of the carotid arteries were not separated properly in order to give as much space to the lumen as possible prior to harvesting. Because of this, those certain arteries had an abnormally small lumen area when observed via microscope. This could have also occurred within the wild-type vessels despite their result matching with our hypothesis that hypertrophy of the arterial walls after injury would lead to a related decrease in the arterial lumen area. For these reasons, an additional measurement in the perimeter of the blood vessels were taken.

The perimeter of the vessels is given as the total distance around the internal lamina of the carotid artery. The way of obtaining this measurement was similar to the method of obtaining the CSWA, and this allowed less limitation to the areas that could be measured within the program. When considering the results observed for the perimeter of the arteries, they matched the expected trend of the lumen area between injured and non-injured arteries. There was a decrease in the perimeter of the carotid arteries after injury for both the wild-type and Caspase-1 knockout mice. Both groups responded to the ligation injury with a similar decrease in perimeter (wild-type: 157 ± 74 μm vs Caspase-1 knockout: 130 ± 63 μm). This supported the idea of inward arterial remodeling that could lead to a decrease in lumen area, although with proper procedure, a stronger relationship would have been seen in terms of lumen area.

Previous studies have shown the extent of hypertrophy in injured blood vessels being increased when reactive oxygen species, a key contributor in the regulation of inflammasomes, were elevated in transgenic mice (Manogue *et al*., 2015). Our current study goal was to show that removing the genes associated with inflammasomes, in this case Caspase-1, would show an opposite effect of decreasing the extent of hypertrophy and arterial remodeling on an injured blood vessel. In this experiment model, Caspase-1 was not shown to have a prevalent effect as an inflammatory mediator on decreasing arterial remodeling, although the extent of its effects on other inflammasomes still have room to be determined. Determining the contribution of the inflammasome could be a promising step to showing how the inflammasome pathway works on atherosclerotic injury, and it may hint towards novel ideas of inhibiting prevalent inflammasomes in

order to hinder the formation of plaque and the secretion of proinflammatory molecules within the body.

In a broader context, our findings could be relevant to understanding arterial remodeling (restenosis) following balloon angioplasty or during the development atherosclerotic plaque formation, as both of these processes involve significant structural changes to arterial walls. Any further understanding of factors contributing to these changes could provide insight for development of therapeutic interventions to treat these prevalent vascular pathologies. Atherosclerosis is a prevalent factor to cardiovascular diseases in various countries due to the buildup of fat on the damaged tunica media of arteries that lead to the restriction blood flow. In order to work around that restriction, the change in structure of those affected arteries is necessary. Thus, determining how the remodeling process is influenced by the activation of the inflammasome is of interest. The observations of our findings in Caspase 1 -/- direct future studies on examining the upstream contributors to include the NLRP3 and NLRC4 inflammasomes, which could lead to a more comprehensive understanding of atherosclerotic related events.

Time was a big limitation to the extent of our studies and experiments, and future studies may additionally target NLRP3 inflammasomes as they are also linked to atherosclerosis and other cardiovascular diseases. Besides possible methodological errors previously mentioned, there is also the possibility of future experiments establishing different time points for the number of days after injury since these experiments analyzed the carotid arteries after 7 days of injury. The additional time points would show the extent of arterial remodeling extends past 7 days after injury, and if those longer periods of time may show differences in hypertrophy and arterial lumen area. Given our results,

the effects of Caspase-1 within non-injured mice may also be analyzed to see if there is an additional pathway that contributes to arterial remodeling in the blood vessels. An experiment that goes deeper into examining the carotid arteries, without ligation involved, following surgery and hematoxylin and eosin staining will allow for a clearer comparison between control mice and Caspase-1 knockout mice. This may be implemented with a wire injury or, even more optimally, a balloon injury model in place of a ligation model. Additional studies to clarify the role of Caspase-1 or other inflammasome related proteins, such as NLRC4 or NLRP3, influence the processes associated with arterial remodeling such as smooth muscle cell proliferation, migration, and apoptosis.

CHAPTER VI

CONCLUSIONS

These experiments showcased a few main points in the study. The wall thickness of the carotid artery after injury increased in both the wild type and Caspase-1 knockout mice. The cross-sectional wall area of the carotid artery became larger after injury within both the wild-type mice and Caspase-1 knockout mice, but in the latter group (Cas 1 -/-), the increase was smaller compared to the former (WT). The perimeter of the carotid artery after injury decreased in both the wild type and Caspase-1 knockout mice. Based on the results, there was not a statistically significant difference between the arteries of the wild-type mice and Caspase-1 knockout mice before or after ligation. This refutes the claim that the knockout of Caspase-1 changes the basilar artery structure and leads to a decrease in arterial remodeling.

The increase in wall thickness of the carotid artery supports the claim that arterial remodeling takes place in response to injury by smooth muscle cell proliferation leading to neointima formation on the injured vessel. The cross-sectional wall area was thought to be a related consequence of the smooth muscle cell proliferation since the neointima forms within the media after injury via ligation, although it may also be related with an increased amount of collagen deposition that strengthens the injury. This was the case for both the wild-type and Caspase-1 knockout mice. The perimeter decreasing with injury

on the wild-type and Caspase-1 knockout mice was the factor that showed the extent of neointima formation on the carotid artery as a result of the ligation.

REFERENCES

Andrade W.A. & Zamboni D.S. NLRC4 biology in immunity and inflammation. J Leukoc Biol. 2020;1–11. https://doi.org/10.1002/JLB.3MR0420-573R

Audia, J.P., Yang, X., Crockett, E.S., Housley, N., Ul Haq, E., O'Donnell, K., Cohen, M.V., Downey, & J.M., Alvarez, D.F. Caspase-1 inhibition by VX-765 administered at reperfusion in P2Y12 receptor antagonist-treated rats provides long-term reduction in myocardial infarct size and preservation of ventricular function. Basic Res Cardiol **113**, 32 (2018). https://doi.org/10.1007/s00395-018-0692-z

Bergsbaken, T., Fink, S.L., & Cookson, B.T. "Pyroptosis: host cell death and inflammation." Nature reviews. Microbiology vol. **7**,2 (2009): 99-109. doi:10.1038/nrmicro2070

Hesketh, E.E., Czopek, A., Clay, M., Borthwick, G., Ferenbach, D., Kluth, D., & Hughes, J. Renal Ischaemia Reperfusion Injury: A Mouse Model of Injury and Regeneration. J. Vis. Exp. (88), e51816, doi:10.3791/51816 (2014).

Kalogeris, T., Baines, C.P., Krenz, M., & Korthuis, R.J. “Ischemia/Reperfusion.” Comprehensive Physiology vol. **7**,1 113-170. 6 Dec. 2016, doi:10.1002/cphy.c160006

Karasawa, Tadayoshi, & Masafumi Takahashi. “Role of NLRP3 Inflammasomes in Atherosclerosis.” Journal of atherosclerosis and thrombosis vol. **24**,5 (2017): 443-451. doi:10.5551/jat.RV17001

Le Clef, N., Verhulst, A., D’Haese, P.C., & Vervaet, B.A. “Unilateral Renal Ischemia-Reperfusion as a Robust Model for Acute to Chronic Kidney Injury in Mice.” PloS one vol. **11**,3 e0152153. 23 Mar. 2016, doi:10.1371/journal.pone.0152153

Li, X., Deroide, N. & Mallat, Z. The role of the inflammasome in cardiovascular diseases. J Mol Med **92**, 307–319 (2014). https://doi.org/10.1007/s00109-014-1144-3

Malek, Maryam, & Mehdi Nematbakhsh. “Renal ischemia/reperfusion injury; from pathophysiology to treatment.” Journal of renal injury prevention vol. **4**,2 20-7. 1 Jun. 2015, doi:10.12861/jrip.2015.06

Manogue M.R., Bennett J.R., Holland D.S., Choi C.S., Drake D.A., Taylor M.S., & Weber D.S. Smooth muscle specific overexpression of p22phox potentiates carotid artery wall thickening in response to injury. Oxid Med Cell Longev. 2015;2015:305686. doi: 10.1155/2015/305686.

Moussavian M., Casterella P.J., & Teirstein P.S. Restenosis after Angioplasty. Curr Treat Options Cardiovasc Med. 2001 Apr;**3**(2):103-113. doi: 10.1007/s11936-001-0066-x. PMID: 11242557.

Ray, P.D., Huang, B., & Tsuji, Y. "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling." Cellular signalling vol. **24**,5 (2012): 981-90. doi:10.1016/j.cellsig.2012.01.008

Schroder K, & Tschopp J. The inflammasomes. Cell. 2010 Mar 19;**140**(6):821-32. doi: 10.1016/j.cell.2010.01.040. PMID: 20303873.

Simon L.V., Lopez R.A., & King K.C. Blunt Force Trauma. 2020 Aug 10. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan–. PMID: 29262209.

Taylor, M.S., Choi, C.S., Bayazid, L., Glosemeyer, K.E., Baker, C.C.P., & Weber, D.S. Changes in vascular reactivity and endothelial Ca2+ dynamics with chronic low flow. Microcirculation. 2017; 24:e12354. https://doi.org/10.1111/micc.12354

Tong, Y., Wang, Z., Cai, L., Lin, L., Lu, J., & Cheng, J. “NLRP3 Inflammasome and Its Central Role in the Cardiovascular Diseases.” Oxidative medicine and cellular longevity vol. 2020 4293206. 14 Apr. 2020, doi:10.1155/2020/4293206

Turner, C.M., Arulkumaran, N., Singer, M., Unwin, R.J., & Tam, F.W.K. Is the inflammasome a potential therapeutic target in renal disease?. BMC Nephrology 2014 15:21.

Ward M.R., Pasterkamp G., Yeung A.C., & Borst C. Arterial remodeling. Mechanisms and clinical implications. Circulation. 2000 Sep 5;**102**(10):1186-91. doi: 10.1161/01.cir.102.10.1186.

Xu Q. Mouse models of arteriosclerosis: from arterial injuries to vascular grafts. Am J Pathol. 2004 Jul;**165**(1):1-10. doi: 10.1016/S0002-9440(10)63270-1. PMID: 15215157; PMCID: PMC2216680.

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