The Role of IL-18 in Abdominal Aortic Aneurysm Formation

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I. *Statement of Authorship*

I, Ashleigh-Georgia Anne Sherriff, declare that this thesis is an original piece of research based on experiments conducted primarily by myself under the supervision of my supervisors Dr. Antony Vinh and Dr Quynh Nhu Dinh. Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

The following individuals also contributed to the work in my thesis:

Name	Nature of contribution
Dr Chrishan Samuel	Assisted with gelatin zymography of MMP-2 and MMP-9
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II.   Acknowledgments

III. List of figures

IV.   List of Abbreviations

(1K/DOCA/salt) 1 kidney deoxycorticosterone acetate salt

(AAA) Abdominal aortic aneurysms

(AP-1) Activating-Protein 1

(Ang II) Angiotensin II

(ApoE$^{-/-}$) Apolipoprotein E-deficient

(ASC) Apoptosis-Associated Speck-Like Protein Containing CARD

(CaCl) Calcium chloride

(DAMP) Danger-Associated Molecular Pattern

(DOCA) Deoxycorticosterone acetate

(EVAR) Endovascular AAA repair

(ELISA) Enzyme-linked immunosorbent assay

(ECM) Extracellular Matrix

(FAK) Focal Adhesion Kinase

(IFN) Interferon

(IL-18) Interleukin-18

(IL-18BP) Interleukin-18 Binding protein

(IL-18R) Interleukin-18 Receptor

(IL-37) Interleukin-37

(ILT) Intraluminal thrombus

(LRR's) Leucine-Rich Repeat

(LPS) Lipopolysaccharide

(LOX) Lysyl oxidase

(MMP) Matrix Metalloproteases

(NCC) NaCl co-transporter

(NK) Natural Killer cells

(NO) Nitric Oxide

(NLR) NOD-Like Receptor

(NLRP3) NOD-like receptor protein 3

(NA) Non-aneurysmal

(NF-kB) Nuclear Factor Kappa-light-chain-enhancer of activated B cells

(NACHT) Nucleotide-Binding and Oligomerization Domain

(OAR) Open AAA repair

(OPN) Osteopontin

(PPE) pancreatic elastase

(PAMP) Pattern-Associated molecular pattern

(PRR) Pattern-Recognition receptor

(PWS) Peak wall stress

(PBS) Phosphate Buffered Saline

(PYD) Pyrin-Domain

(ROS) Reactive Oxygen Species

(RAG1$^{-/-}$) Recombination activation gene 1$^{-/-}$

(Treg) Regulatory T cells

(SBP) Systolic blood pressure

(Th) T Helper Cells

(TIMPS) Tissue inhibitors of metalloproteinases

(TIR) Toll-IL-1 receptor

(TLR) Toll-Like Receptor

(TNF-α) Tumour necrosis factor-α

(VSMCs) Vascular smooth muscle cells

(WT) Wildtype

(BAPN) β-aminopropionitrile

V. Abstract

Abdominal aortic aneurysms (AAA) are characterised by weakening of the vascular wall resulting in the ballooning of the aorta which if ruptured often results mortality. The aetiology of AAA formation remains unclear, although it is known to have increased immune cell infiltration. IL-18 binds to its receptor IL-18R to produce a powerful inflammatory response. IL-37 can also bind to IL-18R and when bound causes an anti-inflammatory response. The aim of this study was to understand the potential roles of IL-18 and IL-37 on AAA formation using a mouse model of AAA formation. Using a gold standard angiotensin (Ang) II-infused model of AAA formation, C57BL6 (Wildtype; WT), IL-18-deficient (IL-18$^{-/-}$), IL-18Rα-deficient (IL-18Rα$^{-/-}$) and human recombinant IL-37 overexpressing transgenic (IL-37Tg) mice aged 10-12 weeks were infused with Ang II (1000ng/kg/min, *S.C.*) for 28 days via osmotic minipump. All mice receiving Ang II exhibited significant elevations in BP (~40mmHg), but no differences in BP were observed between the strains. AAA incidence (including deaths due to AAA rupture) were completely abolished in the IL-18$^{-/-}$ mice compared to Ang II treated WT (0% vs 52%; n=20-23). Conversely, AAA incidence was augmented in IL-18Rα$^{-/-}$ mice (88% vs 52% n=23-25). Analysis of ultrasound images of abdominal aorta at day 7 showed IL-18Rα$^{-/-}$ mice had significantly larger aortic diameters compared to Ang II treated WT (1.49 mm ± 0.13 mm vs 1.15 mm ± 0.05 mm; $P<0.05$; n=23-25). Interestingly, IL-37Tg mice showed decreased incidence of AAA compared to Ang II treated WT (37% vs 52%; n=23-24) with less premature deaths due to rupture. Furthermore, it was found via ELISA that IL-37Tg mice had a 2-fold increase in the amount of plasma IL-18 compared to Ang II treated WT. In summary, IL-18 may play a significant detrimental role in the development of AAA, however, therapies aimed at inhibiting the IL-18Rα may not represent a viable strategy to prevent/treated AAA due to its potential role in IL-37-mediated protection.

Chapter 1: General Introduction

1.1 Introduction to Abdominal Aortic Aneurysms

An aneurysm is defined as a permanent ballooning of a blood vessel that occurs in cerebral vessels, thoracic aorta but most commonly in the abdominal aorta [1,2,3]. Aneurysms can be benign in some patients, while others can grow to become haemorrhagic and rupture. The aorta is the largest artery in the body and is the primary blood source to the abdomen and lower limbs (Figure 1.1). Hence, when an abdominal aortic aneurysm (AAA) ruptures, it is often catastrophic with a mortality rate of 70-90% [4]. AAA risk increases with age and occurs more commonly in males aged above 65 years and to a lesser extent in females (4-7% AAA incidence vs 1-2% respectively) [5,6]. Currently, there are no pharmacological treatments to halt the progression of AAA. Instead, patients rely on surgical intervention, which presents significant risks of post-operative complications [7,8]. While risk factors for AAA have been identified, the mechanisms resulting in aortic ballooning remain unclear. However, aneurysms are associated with profound infiltration of immune cells, which are thought to cause degradation of the aortic wall [9–11]. Hence, the role of inflammation and the immune response in AAA development is a topical research area. A particularly emerging area of immunity research focuses on the inflammasome, specifically the NOD-like receptor protein 3 (NLRP3) inflammasome, a prime activator of innate immunity. The NLRP3 inflammasome is a significant source of pro-inflammatory cytokines, interleukin (IL)-1β and IL-18. There has been limited research on the role of IL-1β and IL-18 in AAA formation. However, circulating IL-18 is reportedly increased in patients with AAA [12]. This warrants further investigation into the role of IL-18 in the inflammatory response associated with AAA. Thus, this thesis will explore the role of IL-18 and its associated receptor targets with aneurysm formation. There are no pharmacological

treatments for patients diagnosed with AAA, and the hope is that the findings from this thesis will present a novel therapeutic target.

Healthy Aorta   **Abdominal Aortic Aneurysm**

Superior mesenteric artery:

Intestines (lower part), duodenum

(2/3rds) transverse colon and

pancreas

Renal arteries:

Kidneys

Inferior mesenteric artery:

Colon, and rectum

L. & R. common iliac arteries:

Lower limbs
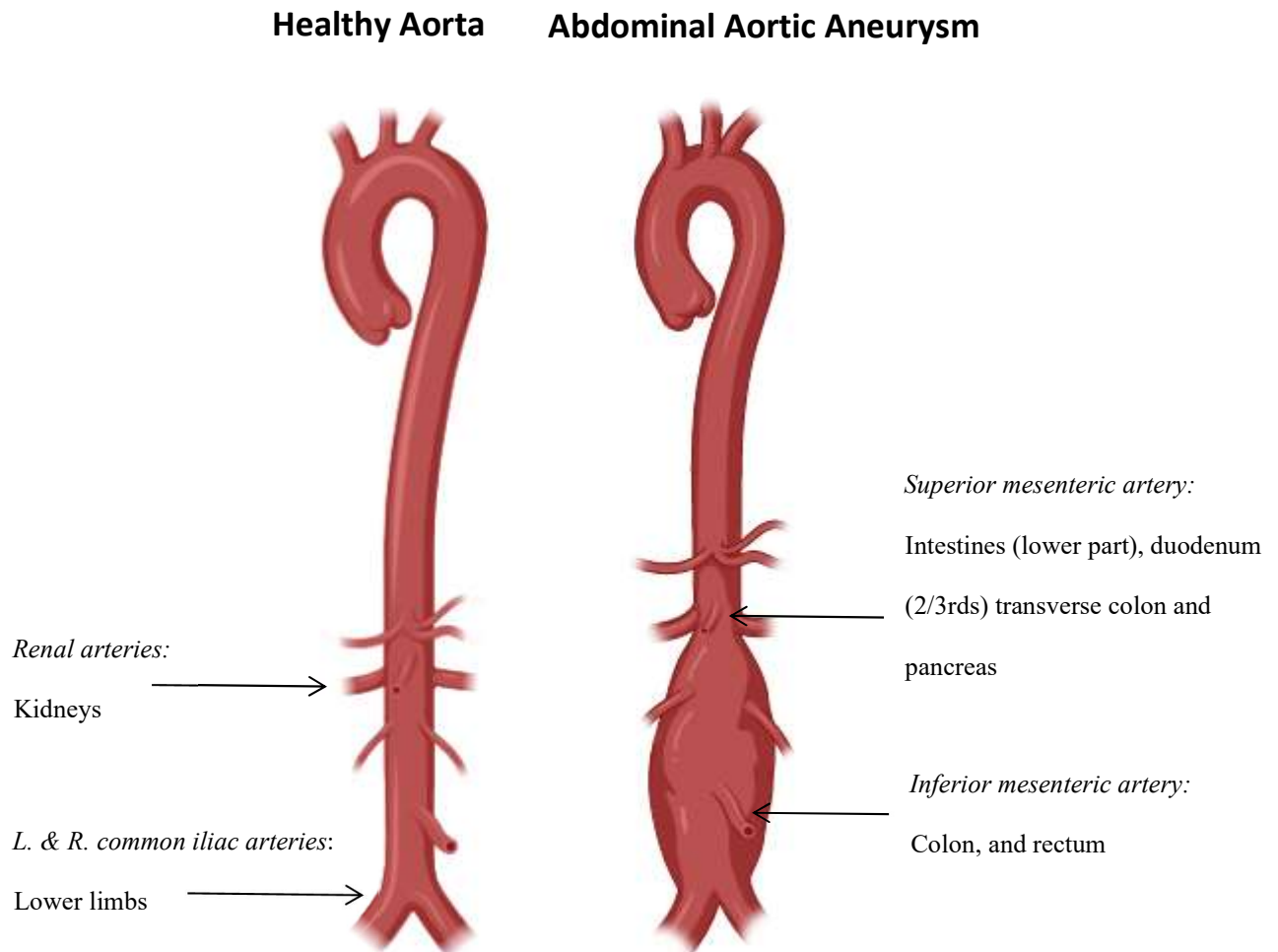
Figure 1.1 A schematic representation of a healthy aorta and an abdominal aortic aneurysm. An illustration of the gross morphological difference between a healthy aorta and an abdominal aortic aneurysm. Branching arteries and structures supplied are labelled. *Image by Sherriff, A. using BioRender (unpublished).*

1.2   Aetiology and prevalence of AAA

Diagnosis of AAA can often be complicated due to the fact the disease naturally presents asymptomatically in most patients. However, some patients can experience pain in the chest, abdomen and lower back, pulsating sensations in the abdomen, or a black/blue painful foot if the AAA is overly large [13]. Due to AAA commonly being a silent disease, the most common form of diagnosis is preventative screening. To confirm the presence of an AAA, patients undergo an ultrasound imaging of the abdomen, computed tomography scanning or magnetic resonance imaging. Males aged over 65 should present for a single ultrasound screening to assess the risk of AAA. Note it is only males that need to present for this screening as they are more likely to develop an AAA than women (4-7% in men vs 1-2% in women)[14,15]. Preventative screening is in place in many countries, including the United States of America (USA), United Kingdom (UK), and Europe, but no formal program currently exists in Australia [16,17]. Recommended once-off screening of 65-year-old asymptomatic men has resulted in a statistically significant reduction in AAA-related mortality and rupture in Canada [18]. Following the initial screening, follow-up screening results in a risk reduction of 42% at 5 years and 21% at 10 years [19,20]. In addition to lowering the risk of death, pre-emptive screening has further assisted in understanding the disease. For example, it has provided greater clarity on disease prevalence in the USA, UK and Australia (2.2%, 1.5% and 6.7% respectively) [21,22]. Information derived from screening has identified risk factors such as aging [23,24] and gender [25,26] as strong predictors of AAA incidence. As previously noted men are more likely to develop an AAA; however, women are likely to have a fatal outcome if diagnosed [14,15]. People aged 75 years or more account for 66% of the total diagnosed aneurysms, demonstrating the significance of age as a risk factor [7,23]. Additionally, the disease has a higher incidence in Caucasians compared to any other race [27]. Other risk factors include atherosclerosis and pre-existing hypertension [5,28,29] and environmental factors such as smoking [28,30–32].

1.3   The current methods of treatment for diagnosed AAA

Aneurysm size, risk of rupture and patient-specific factors such as comorbidities that affect life expectancy and operative risk can influence the treatment strategies [33]. While an AAA is not reversible early detection through screening enables patients to seek treatments and intercept disease progression. Early detection through the previously discussed screening allows interception of the disease progression. This is done through lifestyle changes and treatment of comorbidities that collectively improve a surgical intervention's success.

1.3.1   Lifestyle Interventions

Lifestyle interventions target patients with a small AAA (diameter: 4.0 - 5.5 cm) intending to slow the expansion rate and provide better survival outcomes post-surgery [34]. Many lifestyle factors contribute to an increased risk of AAA. Once diagnosed, cessation of smoking is one of the most important lifestyle changes to implement. Smokers have a higher AAA expansion rate than non-smokers (2.56   mm/year vs 2.1 mm/year respectively) [35,36]. Exercise is an additional lifestyle factor that can be adjusted to help treat comorbidities after diagnosis. A group of researchers considered different intensities of exercises and their effect on patients with a small AAA. The study concluded that high-intensity interval training reduced the rate of expansion compared to the non-rehabilitation group (2.1 ± 3.0 versus 4.5 ± 4.0 mm/y) [37]. The most common comorbidities are cardiovascular complications, including hypertension and coronary artery disease [38]. There is limited research into dietary changes and AAA. However, patients with cardiovascular disease have better outcomes when avoiding traditional Western diets. This is achieved through reducing salt intake and eating foods low in saturated fats and

cholesterol. Interestingly, these dietary changes have shown to decrease growth rates in mouse models of AAA [39,40].

1.3.2 Prescription Therapies

The use of prescription medication for the treatment of AAA patients targets the comorbidities such as hypertension which often accompany the disease. Pharmacotherapies administered are patient-specific and can often involve anti-hypertensives and statins [41].

The search for effective drug therapies to treat AAA is ongoing, and many clinical trials have not been successful. Previous clinical trials have used propranolol (anti-hypertensive: beta-blocker), doxycycline (antibiotic) and amlodipine (anti-hypertensive: Ca^{2+} channel blocker) over a 6-24-month period and all studies reported no benefit in halting the growth of AAA [41–43]. Metformin is a drug commonly prescribed for patients with type 2 diabetes and is currently undergoing clinical trial as a therapeutic agent for AAA (metformin therapy). Its use has shown that high blood glucose levels increase AAA diagnosis risk, whereas patients already diagnosed with diabetes and on metformin have a lower risk of AAA [44]. However, there is also conflicting evidence suggesting that metformin has no long term protective effects on AAA [45]. Taken together, it is clear that novel treatments are required for AAA.

1.3.3 Surgical Intervention

The use of surgical intervention is critical for patients with large AAA (diameter: ≥5.5cm) survival, although no long-term survival benefits have been reported for small AAA [46–48]. The Royal Australian College of General Practice states that surgery should be considered if: an aneurysm is greater than 5.5cm for men and 5cm for women; if there is rapid growth (>1.0cm/year); or if symptoms such as abdominal/back pain and tenderness appear. The two

most common surgical repair types for an intact AAA are the open AAA repair (OAR) and the endovascular AAA repair (EVAR) [33,49]. OAR involves an incision from the breastbone to the navel. From there the aorta is clamped while the aneurysm is cut, and a graft is sewn to connect the two ends of the aorta [49]. EVAR patients are placed under local anaesthetic, and a small incision is made near the groin [50]. A camera and needle are threaded through the femoral artery to attach a stent-graft to the aortic wall. The use of EVAR Surgery has increased with 43-74% of AAA patients opt for this approach over OAR due to the less-invasive nature of the surgery [49]. However, OAR surgery is still used due to it being more cost-effective, while offering a lower risk of complication while also being than EVAR surgery[51]. When comparing OAR's survival rates to EVAR, they are similar 69.9% vs 68.9%, respectively [52]. Ruptured AAA is particularly fatal, resulting in a mortality rate of 90%, where only one in three patients will reach the hospital alive [8]. While it is possible to perform reparative surgery, it reduces the risk of mortality to 40% [8]. Given the high mortality rates associated with rupture and post-surgery, the need for alternative pharmacological therapy is of great clinical significance.

1.4   Pathophysiology of AAA

Human AAA is broadly described as either a true aneurysm where each layer of the blood vessel has expanded or a false aneurysm where there is an enlargement of a single layer [53,54]. A true aneurysm can either be fusiform (symmetrical) or saccular (asymmetrical). Simultaneously, a pseudoaneurysm can be either a hematoma (leakage of blood) or arterial dissection (splitting of the vessel) (Figure 1.2) [55–57]. It is unknown why aneurysms form, but a pseudoaneurysm is a complication of trauma inflicted on the vessel and can occur post-surgery [53,58]. Fusiform is the more common type of true aneurysm, although saccular aneurysms have a higher risk of rupture [55]. It is important to note that in humans, AAA commonly occurs

infrarenal with a regular abdominal aorta diameter of 1.5-1.7cm [7]. Studying human samples is limited because they can only be obtained from surgery or death, which occurs at a later stage of AAA formation. Therefore, to understand the entire pathogenesis of AAA, animal models are necessary.
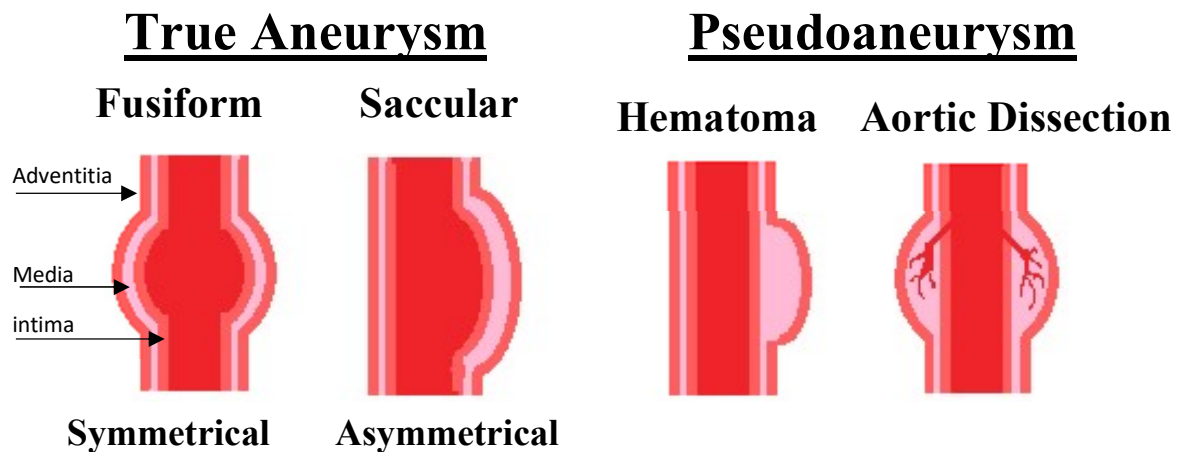
Figure 1.2 An image representing the different types of aneurysms. A true aneurysm occurs when there is expansion of all three layers of the aorta. A true aneurysm can either be "fusiform" (the ballooning occurs on both sides and is thus symmetrical) or "saccular" (ballooning occurs on one side and is asymmetrical). A pseudoaneurysm occurs when there is expansion in only one layer of the aorta. a pseudoaneurysm can either be a "hematoma" (blood leaks into the media and pools) or an aortic dissection (where there is a tear in the aortic wall causing a leakage). *Image created by Sherriff, A. unpublished, using Biorender*

1.5 Animal Models of Abdominal Aortic Aneurysm

Studying human AAA samples provides population statistics, including incidence and demographics. However, clinical studies are limited to identifying associative evidence, hence the need for animal models of AAA. Animal models have uncovered potential pathways involved in AAA formation, which has resulted in interventional strategies and drug therapy research. Animal models also pose limitations, predominantly a lack of translatability demonstrated in previous drug therapy studies. (Table 1) [10]. Nonetheless, the use of animal models has been quintessential for uncovering current mechanisms associated with AAA. Current models are discussed below and summarised in Table 1.

1.5.1 Elastase perfusion model

The elastase perfusion model involves the pressurised infusion of porcine pancreatic elastase (PPE) into an aorta segment for up to two hours [59]. Induction of PPE leads to elastin breakdown and infiltration of leukocytes in the early stages. As the AAA progresses, it begins to develop the characteristic intraluminal thrombus (ILT), also present in AAAs human pathology [60]. Additionally, the increases in diameter match human AAA progression as aneurysm growth is nonlinear in both [61]. The length of infusion/treatment strongly influences the mortality rate due to hind limb ischemia [62]. The model's significant limitations include the need for microsurgical expertise, and the duration is limited to 8 weeks, limiting the potential to examine long-term prolonged drugs effects [62,63].

1.5.2   Calcium chloride model

The calcium chloride ($CaCl_2$) model involves a laparotomy and application of cotton gauze pre-soaked in $CaCl_2$ to the aortic adventitial surface. Surprisingly, the exact pathophysiology of AAA formation remains unclear in this model, it is thought that the calcium ions which are known to have a high affinity for elastin, influences elastin fibre breakdown [1,64]. This model presents very few similarities to human pathophysiology, where only medial degeneration and leukocyte infiltration are observed [1,60,62,64].

1.5.3   1 Kidney/Deoxycorticosterone acetate/salt (1K/DOCA/salt)model

The 1K/DOCA/salt model is an invasive mouse model whereby mice are placed under anaesthesia and undergo uninephrectomy while a deoxycorticosterone acetate (DOCA) pellet is implanted, and drinking water is replaced with 0.9% saline [65,66]. It is traditionally a volume-dependent, low circulating renin model of hypertension. The 1K/DOCA/salt model is a less characterised AAA model and more commonly used as a model of hypertension or chronic kidney disease. Nonetheless, AAA incidence (exclusive of ruptures) is 60%, while ruptures are much lower at 18% [67]. Studies that modify this model and only use the DOCA pellet and saline present a similar incidence (68.7%) and rupture (12.5%) rate [68]. Similar to the other models discussed, elastin degradation is a crucial part of this model, with other pathologies such as inflammatory cell infiltration and oxidative stress being present as well [69].

1.5.4 Angiotensin II-infused model of AAA

The angiotensin (Ang) II-infused mouse model of AAA was first characterised by Alan Daugherty [70]. This model involves subcutaneous implantation of an osmotic minipump that delivers Ang II (0.7 to 1.4 mg/kg/day) over a 28 day period[1–3,71]. The Ang II infusion model of AAA traditionally utilises hypercholesterolemic mice such as the apolipoprotein E-deficient (ApoE$^{-/-}$) mouse. Studies using this strain have previously demonstrated exacerbated incidence of atherosclerotic lesion formation [71–74]. However, atherosclerosis is not a prerequisite for AAA formation, as demonstrated by C57Bl6 mice (WT) also developing AAA following Ang II infusion [75]. However, while mice without hypercholesterolemia present with a lower incidence of AAA than ApoE$^{-/-}$ mice (45% vs 80% respectively), they allow studying AAA formation in genetic strains of mice without the need to crossbreed with hypercholesterolemic strains of mice [76]. While the Ang II and 1K/DOCA/salt models also induce hypertension, it has been shown that AAA formation is not dependent on elevated blood pressure [77,78]. Like the 1K/DOCA/salt model, AAA pathology in the Ang II-infusion model is linked to inflammation. It displays aortic elastin degeneration, macrophage infiltration, aneurysm wall remodelling and aortic rupture [70,71]. The Ang II model can also be modified to include the co-administration of β-aminopropionitrile (BAPN). BAPN is known to exert an inhibitory effect on lysyl oxidase (LOX), which plays a role in maintaining homeostasis of the elastic lamina [79]. While LOX activity is essential for normal vascular function, inhibition of LOX activity decreases the stability of the vessel wall [80]. This reduced stability is why AAA incidence can be as high as 90% in this model and is defined as a chronic-advanced stage of AAA [81].

Table 1. Models of AAA formation

Model	Severity	Rate of growth	Similarities to human AAA	Differences to human AAA	References
Angiotensin II-infusion	Moderate-severe (dose dependent)	AAA present as early as day 3	Atherosclerosis (only in hypercholesterolemic mice), wall disruption, medial degeneration, leukocyte infiltration and risk of rupture	No ILT present or persistent growth, aneurysms occur suprarenal, and occasionally thoracic	1,10,60
1Kidney/ DOCA/Salt	Moderate	AAA present as early as day 3	Elastin degradation, MMP activation, smooth muscle cell degeneration and oxidative stress, risk or rupture	Aneurysms occur suprarenal and thoracic	65,67–69
Elastase perfusion	Moderate	AAA present at day 14	Occurs infrarenal, ILT present medial degeneration and leukocyte infiltration present	No rupture unless very early on, no wall dissection or continued growth. No atherosclerosis	1,60,82,83
Calcium Chloride	Mild	AAA present at day 14	Medial degeneration and leukocyte infiltration are present within the AAA.	No rupture, wall disruption, persistent growth, ILT, atherosclerosis	1,10,60,84

1.6　Extracellular remodelling of the aorta

To date the complete pathophysiology of AAA remains unclear, but it is understood that the aortic wall undergoes extensive remodelling in response to various stressors, including hypertension. More specifically, "remodelling" of the aorta refers to excess alterations of the structure, contributing to the pathophysiology of many cardiovascular diseases [85]. The aortic wall structure is comprised of three distinct layers; the intima, media and adventitia (figure 1.3). The intima is the innermost layer of the vessel wall and consists of a monolayer of endothelial cells and the basement membrane. The media is made up of layers of vascular smooth muscle cells (VSMCs) that are separated by elastin and collagen. The adventitia is the outermost layer and is comprised of an intricate network of extracellular matrix (ECM) proteins, including collagen and elastin as well as vasa vasorum (tiny blood vessels) [7]. Elastin is an insoluble and hydrophobic protein produced by VSMCs in the media and fibroblasts in the adventitia [86]. Elastin is also the dominant protein in the vascular wall making up 50% of its dry weight [87]. The role of elastin is to provide structure to the vascular wall and act as a store for recoil energy, making it a key component of the aortic wall that provides elasticity [87]. Collagen is the predominant connective element and is synthesised by smooth muscle cells in and fibroblasts [86]. Collagen is stiff and rigid and has a range of subtypes where type-I and type-III collagen are the most relevant to the vascular wall [88]. Collagens are organised within the vessel wall to maintain the vascular structure and strength. As seen in AAA, excess collagen development leads to vascular fibrosis and increased stiffening, resulting in an inability to expand and contract [87]. Many factors play a role in this remodelling, but some of the most important are matrix metalloproteases (MMPs) activity and inflammation of the aorta.

Adventitia: extracellular matrix proteins (collagen and elastin), vasa vasorum

Media: extracellular matrix proteins (collagen and elastin) vascular smooth muscle cells

Intima: endothelial cells, basement membrane
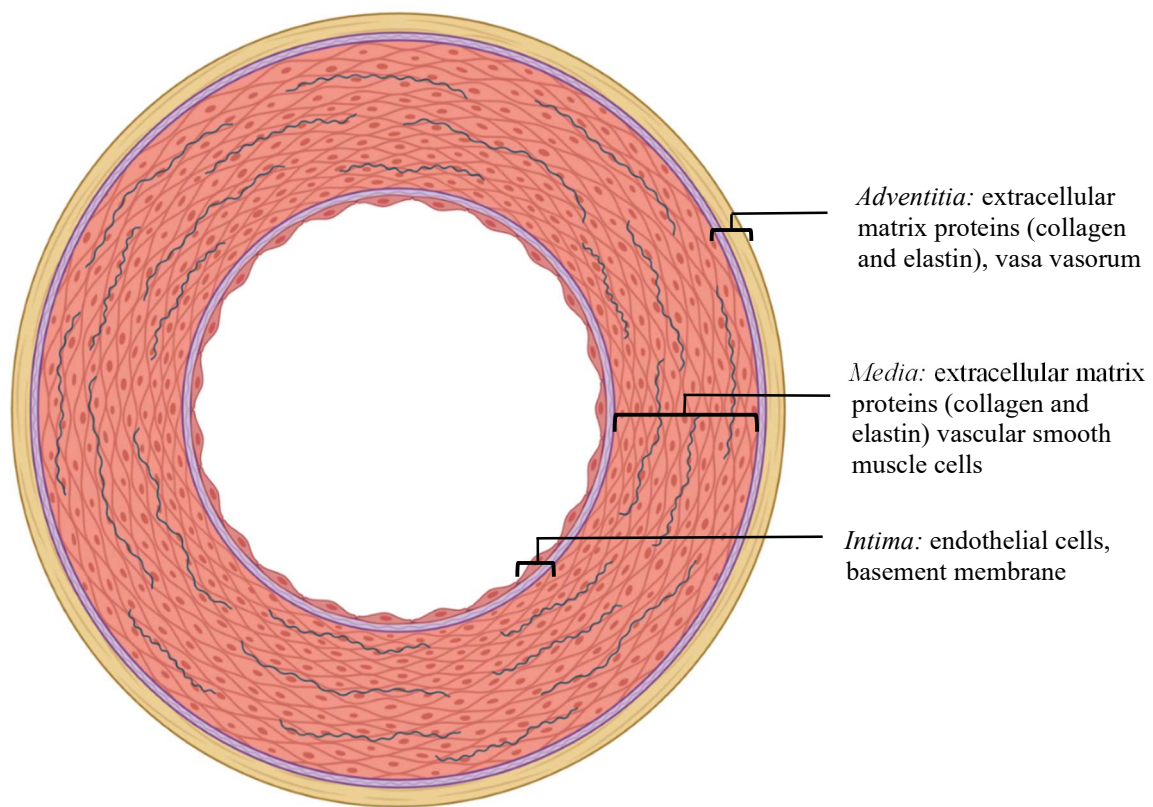
Figure 1.3 A schematic diagram of the various layers within a healthy aorta. A cross-sectional image depicts the layers of the aortic wall. The external layer, the adventitia, comprises extracellular matrix proteins like collagen and elastin and vasa vasorum (tiny blood vessels). The middle layer, the media, comprises vascular smooth muscle cells, elastin and collagen. The innermost layer, the intima, comprises endothelial cells and the basement membrane. *Image created by Sherriff, A. using Biorender*

1.6.1 Matrix metalloproteinases and AAA

MMPs are a family of enzymes that are involved in modulating ECM composition [89]. MMPs are generally expressed at a low level and regulated by cytokines, growth factors and tissue inhibitors of metalloproteinases (TIMPS) [90]. The ratio of MMP to TIMPS is known to be greater in AAA as compared to a healthy aorta [56], where MMPs are upregulated, causing an imbalance in the degradation rate and regeneration of ECM factors [56]. Several MMPs have been implicated in ECM degradation during the development of AAA [56]. For example, MMP-2 and MMP-9 are known to weaken the aortic wall, leading to its eventual ballooning. MMP-2- and MMP-9-deficient mice have been reported to be protected from AAA formation [91]. In a healthy aorta, MMP-2 is lowly expressed within the media, but it is upregulated in AAA patients (particularly small and medium-sized AAA) [92]. A decrease in collagen causes a weakening of the aortic wall, with an increase in susceptibility to ballooning due to forces exerted on it by blood flow [93]. Increased MMP-9 causes an increase in elastin degradation within the media, which is a hallmark of AAA growth [92]. Elastin degradation correlates with increased aortic wall distensibility, which is believed to cause the characteristic ballooning seen in AAA [86]. Increased wall distensibility describes an increase in the arterial swelling as blood flow forces exert pressure on the vascular wall [94]. The extensive degradation of elastin and collagen by MMP leaves the aortic wall-less structurally secure, resulting in the vessel's ballooning.

While MMP-2 and MMP-9 have the most significant role in AAA development, there are other MMPs involved. Neutrophils can secrete ECM degrading factors such as neutrophil collagenase (MMP-8) to trigger the structural remodelling within the aorta [47]. Neutrophil collagenase breaks down vascular collagen while neutrophil protease breaks down elastin, and together they contribute to the weakening of the vessel wall [95]. MMP-8 is increased in AAA

compared to healthy tissue, but more strikingly, it is significantly upregulated in ruptured tissue obtained in biopsies compared to matches AAA tissue [96,97].

1.6.2 The development of an intraluminal thrombus (ILT)

ILTs are present in 70%-80% of all AAA cases, especially in large aneurysms, and are made up of a complex mixture of activated platelets, leukocytes, thick fibrin mesh and entrapped erythrocytes [98,9998,99]. While it is unclear why an ILT forms, some theories suggest it to be based-on platelet accumulation [100], biochemical changes [101] or biomechanical modifications [94]. Of the three, biomechanical changes have been suggested to be the most important as this incorporates the changes in peak wall stress (PWS). PWS describes the amount of stress which can be exerted on the aortic wall before it exceeds the mechanical strength of the tissue [94]. When the pressure on the aorta exceeds the wall strength, it causes a rupture of the AAA [94]. AAA is associated with increased PWS, and the dense fibrin network found within the ILT alleviates any increases in pressure [94]. Eased pressure results in a decrease in PWS, meaning an increase in blood flow and protection from rupture due to stress [102]. However, as the ILT grows, it is thought to become more detrimental than beneficial. Thus, the size of the ILT is related to the risk of AAA rupture [103]. AAA rupture occurs as large ILT become hypoxic, negatively affecting the adjacent vascular wall [57,99]. The hypoxia can further weaken the vessel wall by the degradation of ECM factors and increased cell inflammation [57,99]. The loss of ECM factors causes a profound decrease in wall stability and has greatly attributed to the pathogenesis of AAAs.

1.7   Current theories of AAA pathogenesis

There are currently two main theories regarding AAA pathogenesis that are associated with the development of atherosclerosis/thrombosis and/or immune driven destruction of the aortic wall. This thesis will provide a summary of both theories while focusing on the latter.

1.7.1   Atherosclerosis is a driver of AAA formation

Atherosclerosis is defined as an inflammatory condition that is associated with an accumulation of lipids and cholesterol in the arterial wall that can obstruct blood flow. Atherosclerosis is also one of the leading causes of cardiovascular disease, an umbrella term inclusive of AAA [104]. This theory states that AAA develops as a pathological response to aortic atherosclerosis [105][105][105][105][82,105,106]. Furthermore, while atherosclerosis is a risk factor for AAA, it is also present in 95% of cases [107]. As a result of the obstructed blood flow, it is thought that aortic wall expansion acts as a compensatory mechanism [41]. Changes in haemodynamic factors, such as shear stress, stimulate the positive remodelling through activations of MMPs [82,107]. An excess of this positive remodelling is what leads to the vessel instability and the characteristic ballooning of an AAA. Hypercholesterolemia and atherosclerosis can exacerbate AAA incidence in mouse models of AAA and until recently, studies often used strains that are more susceptible to spontaneous atherosclerosis development. However, mice with healthy cholesterol (125 to 200mg/dL) are generally resistant to atherosclerosis, are still capable of developing AAA [104,108]. This suggests that other mechanisms promote AAA formation independent of lipid deposition [108], hence, atherosclerosis is not a prerequisite for AAA formation in preclinical models.

1.7.2 AAA is a result of immune-driven degradation of the aortic wall.

While it is accepted that inflammation plays a role in AAA pathogenesis, the precise inflammatory mechanisms lead to AAA is a topical area of research. Inflammatory AAA is characterised by thickening of the aneurysm wall due to inflammation, peri-aneurysmal fibrosis and marked adhesion to surrounding structures [105]. Additionally, due to the complex characteristics of inflammatory AAA, there is often an increased risk when these patients undergo surgical intervention [109]. Evidence of inflammation being the causation of AAA is that ubiquitous inflammatory cell infiltration has been demonstrated in all AAA, irrespective of atherosclerosis (Figure 1.4) [109]. Cell infiltrates include neutrophils, macrophages and T cells which will be further described later. Post-surgical biopsies harbour numerous immune cell infiltrates within the ILT and the aortic wall [110–113]. Furthermore, AAA models consistently describe an influx of immune cells to the aortic wall [71,114–116].

1.8 Inflammation is a crucial driver of aneurysm formation

While the exact process leading to the pathophysiology is unknown, AAAs are strongly associated with the accumulation of highly active immune cells predominantly found in the adventitial layer and ILT. The increase of AAA inflammatory cells is a stark contrast to healthy aortas that present with very few inflammatory cells [6]. Histological staining of AAA samples from human and animal models have revealed that the most prominent inflammatory cells infiltrating the aneurysm are macrophages, neutrophils and $CD4^+$ T cells [6].
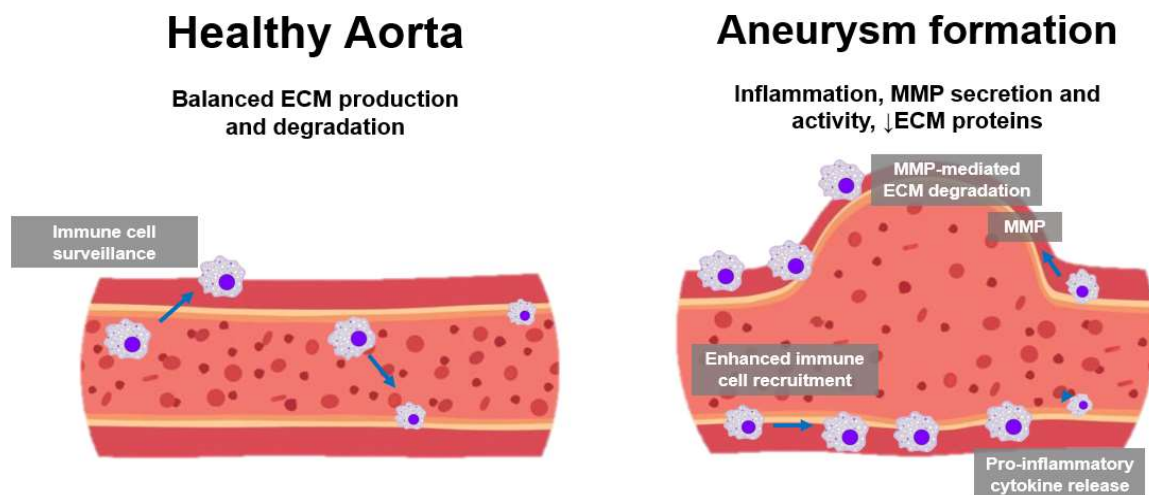
Figure 1.4: **A schematic diagram of immune cells within a healthy aorta vs an aorta with a developed aneurysm.** The healthy aorta is defined by its ability to produce extracellular matrix proteins (ECM) in accordance with its degradation to keep a healthy balance. Furthermore, there is constant immune cell surveillance for foreign pathogens. This is opposed to the aneurysm where there is a decrease in ECM factors due to increased inflammation as well as MMP expression and activation. Furthermore, immune cells such as macrophages accumulate in the vascular wall and to release pro-inflammatory cytokine release in addition to MMPs. *Image created by Sherriff, A. unpublished using Biorender*

1.8.1 Neutrophils

Neutrophils are one of the first immune cells to be recruited to the inflammatory site and play an essential role in AAA pathology [117]. Once at the site of inflammation, neutrophils can produce reactive oxygen species (ROS), bioactive lipid mediators, and various proteases (cathepsin, neutrophil elastase and myeloperoxidase) to exert their effects [118]. Neutrophils have been linked to oxidative stress, proteolytic degradation of the media, inflammation of the adventitia, angiogenesis and ILT development [119,120]. As previously discussed, neutrophil collagenase MMP-8 plays a vital role in the aortic ballooning and is upregulated in AAA

ruptures [96,121]. The importance of neutrophils in AAA formation has been confirmed as neutrophil depletion significantly decreased the AAA frequency and size in an elastin perfusion model of AAA [121]. As such, neutrophils have been identified as significant contributors to AAA development and subsequent rupture.

1.8.2 T cells

T cells are a part of the adaptive immune response and have been implicated in experimental and human AAA. A recent study demonstrated that T cells might be the most dominant immune cell population in human AAA [122]. During the immediate response to vascular injury, CD8$^+$ T cells will produce additional TNF-α as well as IFN-γ, contributing to the already present inflammation [123]. More specifically, IFN-γ producing CD8$^+$ T cells enhance cellular apoptosis and MMP activity, promoting AAA formation [124]. This was demonstrated through antagonism of IFN-γ in elastase treated WT mice, causing an absence of AAA [124]. While CD8$^+$ T cells and CD4$^+$ T cells are upregulated, the latter displays more activity within an AAA [125].

CD4$^+$ T cells are essential for fighting infection and they assist in coordinating immune responses by stimulating other immune cells such as macrophages, B lymphocytes and the aforementioned CD8$^+$ T cells [126]. CD4$^+$ T cells are known to be markedly increased in serum of patients at the end stage of AAA [127]. Animal-based research often utilises recombination activation gene 1$^{-/-}$ (*Rag1$^{-/-}$*) mice, which lack mature B and T lymphocytes. Studies using this strain have shown them to be protected from AAA formation [126,128]. Further research has demonstrated when human CD4$^+$ T cells are added to the *Rag1$^{-/-}$* mice, there is a marked increase in diameter [128]. This response to CD4$^+$ T cells could suggest a possible role in increasing AAA severity.

T regulatory (Treg) cells are another subtype of T cells and are essential for preventing chronic inflammation, which in turn suppresses AAA. Treg cells release the anti-inflammatory cytokines such as IL-10 within an AAA, which causes a reduction in cell death, macrophage function and an overall reduction in inflammatory cell recruitment [129–132]. It has also been demonstrated that larger AAA have significantly fewer Treg cells than smaller AAA, suggesting that when the diameter reaches a threshold, differentiation to Tregs may be reduced which may further promote AAA-associated inflammation [111].

1.8.3 Macrophages

Macrophages are an innate immune cell and are the most abundant inflammatory cell in an AAA in both human and animal models [108]. Macrophages accumulate in the adventitia and are thought to contribute to the destruction of the ECM [133,134]. The phenotype and function of macrophages are primarily dependent on the local cytokine milieu that can be broadly divided into M1 pro-inflammatory macrophages (e.g. in the presence of interferon-gamma (IFN)-γ) and M2 anti-inflammatory macrophages (e.g. in the presence of IL-4 and IL-10) [135,136]. M1 macrophages are characterised by their ability to produce ROS which destroy infectious organisms and tumour cells [30,136]. M1 macrophages also release multiple pro-inflammatory cytokines such as IL-6, tumour necrosis factor-α (TNF-α) and IL-1β [137]. Additionally, M1 macrophages cause an increase in the secretion of monocyte chemoattractant protein-1 (CCL2; formerly MCP-1) by VSMCs [138]. CCL2 recruits immune cells which cause an additional increase in MMP activity and serine proteases which assist in the breakdown of ECM factors in aneurysms [139]. M1 macrophages have been linked to MMP production, and are thus considered to play a crucial role in vascular remodelling and more specifically weakening the vessel wall during AAA formation [134].

Interestingly, the cellular density of M2 macrophages is more significant in the aortic wall of an AAA then M1 macrophages [140]. However, M1 macrophages are located within the adventitia while the M2 macrophages are primarily found within the ILT [140]. While there is varying quantities of M1 and M2 macrophages in human AAA samples, dependent on complexity of the patients disease , M2 have a higher concentration then M1[140]. The primary function of M2 macrophages is to facilitate the repair and wound healing and is upregulated as a compensatory mechanism during AAA development [136,141]. M2 macrophages improve survival, decrease aortic dilation and preserve elastin within an elastase perfusion model of AAA [128].

1.9   The role of the Inflammasome in AAA

 The inflammasome regulates immune responses and tissue homeostasis, as such, their upregulation is a possible cause for the destructive immune response in AAA [142]. Inflammasomes are a group of pattern-recognition receptors (PRRs). PRRs include toll-like receptors (TLR), which scan the endosomal compartments for pattern-associated molecular patterns (PAMP's) [143]. PRRs also include NOD-like receptors (NLRs), which distinguish host-derived or pathogenic signals called danger-associated molecular patterns (DAMPs) and PAMPs [143]. The inflammasome is a unique set of PRRs as they have a high molecular weight and are platforms for caspase-1 activation and activity [143].The most studied inflammasome to date is the NLRP3 inflammasome.. It's structure consists of the NLRP3 domain, an adaptor protein called Apoptosis-Associated Speck-Like Protein Containing CARD (ASC), and a pro-caspase-1 portion [143,144].

1.9.1 NLRP3 "Priming" and "Activation"

Inflammasome activity consists of two steps that include a priming followed by an activation step. The priming sequence occurs first where TLRs recognise a PAMP, leading to signalling downstream to NF-κB and AP-1, which then upregulate genes encoding the subunits of NLRP3, pro-caspase-1, pro-IL-1β and pro-IL-18. Secondly, activation of NLRP3 can result from many stimuli, including increased ROS, an influx of ATP, and cathepsin signalling [145–150]. Alternatively, cathepsin B has been shown to activate inflammasomes through the cytosolic release of phagocytosed microcrystals [151]. Extracellular ATP, which acts as a danger signal to the immune system, is another well-described stimulus for NLRP3 activation [144]. NLRP3 inflammasome activation leads to NLRP3 oligomerisation, causing PYD clustering and interaction with CARD-containing adaptor ASC to recruit pro-caspase-1. This clustering allows for the auto-cleavage of pro-caspase-1 into two subunits, p10 (kDa) and p20 (kDa), which heterodimerise to form an active caspase-1. Caspase-1 then cleaves pro-IL-1β and pro-IL-18 into their respective active forms, IL-1β and IL-18 [143]. This process is summarised in *Figure 1.5*. The central focus of this thesis is the inflammasome-derived cytokine, IL-18, its receptor targets and its potential role in AAA pathology.
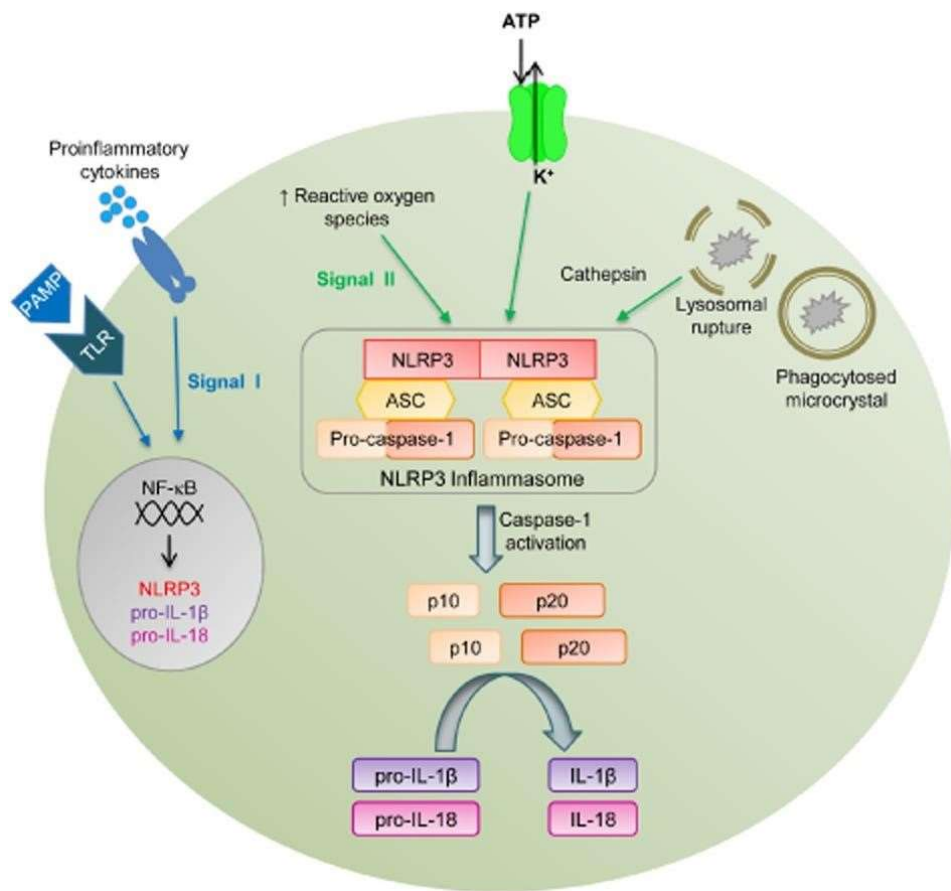
Figure 1.5: A schematic diagram of the priming and activation of inflammasomes. A schematic diagram representing the "priming"/signal 1 sequence which consists of Toll-like receptor (TLR) and receptors for cytokines utilising NF-kB to upregulate Nod-like receptor3 (NLRP3), ASC and pro-caspase-1. Following this "activation"/signal 2 will occur where a number of signals represented by green arrows will act on the NLRP3 inflammasome causing it to activate leading to caspase-1 activation through autocleavage and the conversion of pro-IL-1β and pro-IL-18 to IL-1β and IL18 respectively. The diagram demonstrates the three components of the NLRP3 inflammasome; the receptor recognition site (NLRP3 domain), the adaptor protein (ASC) and pro-caspase 1. Image adapted from Krishnan *et al* [144].

1.10 Interleukin 18 signalling

IL-1β and IL-18 both induce inflammatory responses to further the progression and development of an aneurysm. Of the two inflammasome-derived cytokines, much research has focused on the role of IL-1β and AAA formation. Genetic deletion of IL-1β or the pharmacological blockade of its receptor, IL-1R, significantly suppressed AAA development in elastase perfusion and Ang II models of AAA. [106,152]. At the commencement of this thesis, the role of IL-18 in AAA formation had not been explored even though IL-18 has a range of pro-inflammatory effects in the human body. These include macrophage activation, T cell activation and MMP induction [153]. These pro-inflammatory effects are also involved in psoriasis, acute kidney injury and myocardial function [154–157]. IL-18, is produced from macrophages, endothelial cells, keratinocytes and intestinal epithelial cells [158]. Due to its potent inflammatory effects, IL-18 production is tightly regulated and requires the priming and activation sequence described previously [159]. IL-18 selectively binds to the IL-18 receptor (IL-18R) that is comprised of two main subunits; IL-18Rα and IL-18Rβ. IL-18 binds with low affinity to IL-18Rα and requires IL-18Rβ to act as a co-receptor with IL-18Rα to create a high-affinity complex [158]. Binding results in MyD88 recruitment and phosphorylation of the four IRAKs and TRAF-6, leading to the activation of NF-ĶB and release of pro-inflammatory cytokines, including IFN-γ [158]. Thus, when IL-18 was first isolated in mouse serum it was originally described as "IFN-γ inducing factor" [154].

The potent IL-18-dependent inflammatory response is regulated by an endogenous protein called IL-18 binding protein (IL-18BP), which binds to IL-18 with high affinity (400 pM) and prevents binding to the IL-18R. IL-18BP is constitutively expressed and exists in high concentrations that are up to 20-fold greater than IL-18 [160,161].

1.10.1 The natural inhibitor of IL-18, IL-37

While IL-18 requires the IL-18Rα to recruit the accessory protein IL-18Rβ to cause pro-inflammatory actions, another cytokine known as IL-37 can also bind with IL-18Rα and recruit an alternative accessory recruitment protein, IL-1R8. Once attached, it is known that human IL-37 causes MyD88 inhibition, the opposite effect of IL-18, leading to an anti-inflammatory effect [162]. IL-37, an anti-inflammatory cytokine, has exclusively been found in humans tissue types such as liver, thymus, lung, and bone marrow at low levels in healthy humans [163,162]. Expression of IL-37 occurs almost exclusively in response to severe inflammatory conditions. A study conducted by Nold et al. [164] found that transgenic mice expressing human IL-37 (IL-37Tg) were protected against lipopolysaccharide (LPS)-induced inflammation compared to WT mice. IL-37Tg mice exhibited decreases in hypothermia, acidosis, and hepatitis and decreased circulating IFN-γ levels [164]. Furthermore, IL-37Tg mice exhibited elevated expression of anti-inflammatory cytokines IL-10 and IL-13, but also reduced expression of IL-1α and IL-8 by up to 88% [165]. As depicted in *Figure 1.6B*, IL-37 binds to the IL-18Rα and IL-1R8, producing an anti-inflammatory response and blocks MyD88 [162]. Notably, while a mouse homologue of IL-37 has yet to be identified, these studies support an anti-inflammatory pathway sensitive to human IL-37.

Interestingly, given that both IL-18 and IL-37 require IL-18Rα to promote pro-inflammatory and anti-inflammatory effects, deletion of the IL-18Rα in mice could induce variable impacts of the pathogenesis of inflammatory disorders. In mouse models of lupus erythematosus, IL-18-deficiency protects against renal damage, which can be recapitulated in IL-18Rα -deficient mice [155]. Conversely, deletion of IL-18Rα in splenocytes, macrophages results in 2-3 greater

fold expression of pro-inflammatory cytokines such as IFN-γ, macrophage inflammatory protein 1 and 2 (MIP1 and MIP2) and TNF-[661] α. This finding suggested that deletion of IL-18Rα abrogated the anti-inflammatory effects of a yet to be identified mouse IL-37-like pathway. Thus, it would be interesting to determine the impact of IL-18Rα-deficiency in the setting of AAA.
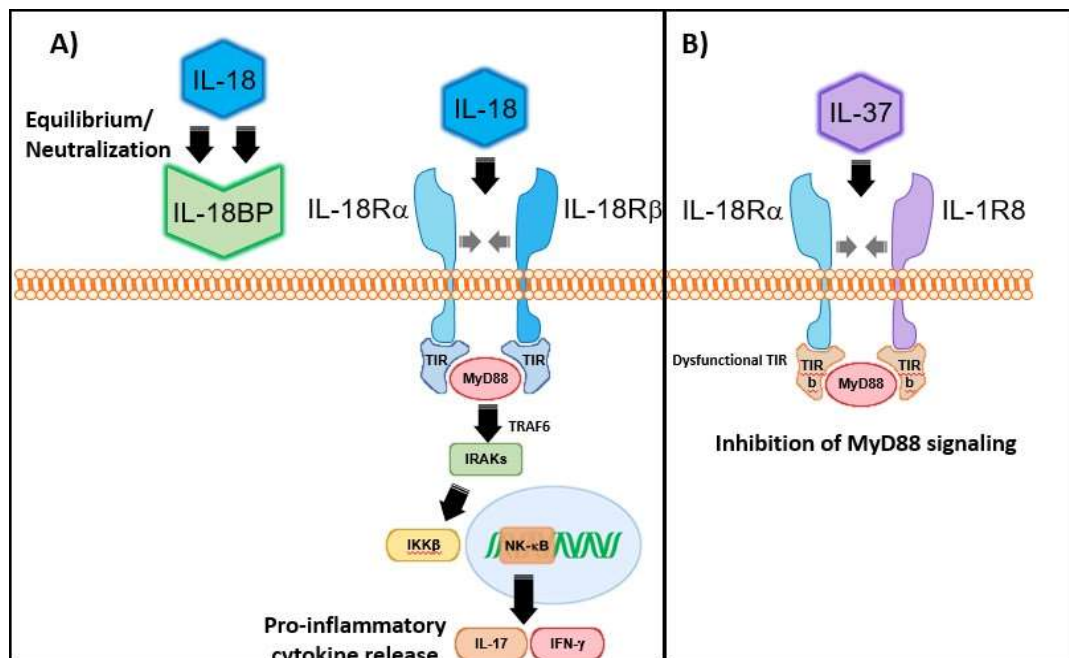
Figure 1.6: A schematic representation of IL-18 and IL-37 binding to IL-18R receptors and the subsequent signalling effects. A) A representation of the interaction of IL-18 and its natural antagonist IL-18BP. It also shows the signalling cascade following the attachment of IL-18 to IL-18R, which recruits MyD88 and signals TRAF6 to IRAKs and IKKβ, causing NK-kB upregulation transcription of proinflammatory cytokines. B) The binding of IL-37 to the IL-18R and the consequential inhibition of the MyD88 signal. *Image by Vinh et al (unpublished).*

1.11 The role of the NLRP3 inflammasome and IL-18 in AAA formation

While there are clear links between inflammation and AAA, the relationship between inflammasomes, IL-18 and AAA is less defined. There is emerging evidence for an essential role of inflammasomes in AAA formation. NLRP3^{-/-} and Caspase-1^{-/-} mice are protected from Ang II-induced AAA as demonstrated by reduced aortic diameters compared to the WT mouse [167]. Our group has recently shown that treatment with MCC950, an NLRP3 inflammasome inhibitor, effectively decreases blood pressure and renal inflammation in 1K/DOCA/salt mice. Relevant to this project, MCC950 also abolished mortality associated with AAA formation and rupture [168]. Human studies by *Wortmann et al,* have reported inflammasome expression and activation in circulating blood mononuclear cells of AAA patients [169]. Additionally, NLRP3 was found to be overexpressed in the aorta of patients diagnosed with AAA and aortic dissection compared to healthy donors [170]. This study correlated with the known gender-associated risk factor where men are more likely to experience an AAA since women had significantly lower inflammasome-related mRNA levels [169]. One study suggests increased NLRP3 is partially due to the previously discussed SMC apoptosis within the aorta [171].

There is less evidence for the role of IL-18 in AAA. However, highly relevant to the current study, IL-18 levels are elevated in plasma from patients with acute aortic dissection [141]. Clinical studies have also shown increased expression of IL-18 in the aorta of patients suffering from AAA [141]. Interestingly, IL-18 is known to increase the expression and activity of several MMPs which are strongly associated with AAA formation, including MMP-2, MMP-3 and MMP-9 [172,173]. Therefore, in light of the preclinical and clinical evidence that supports a role for the

NLRP3 inflammasome and IL-18, it's speculated that IL-18 may be a strong promoter of AAA formation.

1.12 Summary

To summarise the introduction, chronic inflammation is a significant factor in AAA pathophysiology. Immune cells such as neutrophils, T cells and macrophages are drivers of this immune response, while MMP-2 and MMP-9 primarily undertake extracellular remodelling. The NLRP3 inflammasome, which induces a powerful innate immune response, has been suggested to have a role in AAA development. This thesis focuses on IL-18, a pro-inflammatory cytokine derived from NLRP3. While there are various AAA models, the gold standard is the Ang II-induced hypertension model, which will be used in this study. The research conducted was to combat the crucial need for AAA pharmacological therapies, as the current and only method of treatment is surgical intervention.

1.13   Hypothesis and Aims

Since IL-18 has potent pro-inflammatory effects and IL-37 act to oppose this by producing an anti-inflammatory effect, we hypothesised that IL-18 promotes AAA formation while IL-37 acts to suppress AAA formation.

Aim 1: To determine the effect of IL-18- and IL-18R-deficiency on the incidence and progression of AAA formation in the Ang II model of AAA.

Aim 2: To determine if human IL-37 overexpression causes a reduction on the incidence and progression of AAA formation in the Ang II model of AAA.

The outcomes of this study may validate targeting the IL-18/IL-18R signalling axis as a novel therapeutic approach to controlling AAA progression.

Chapter 2: General Methods

2.1   Ethics

All experimental procedures were approved by the La Trobe University Animal Ethics Committee (Project number: AEC 16-93). All operations were conducted following the Australian Code for The Care and Use of Animals for Scientific Purposes (8th ed).

2.2   Mice

Male C57Bl/6 (wild-type) (n = 34), IL-18$^{-/-}$ (n = 22), IL-18R$\alpha^{-/-}$ (n = 25) and IL-37Tg (n = 24) mice (total n = 101) aged 10-14 weeks were bred at AgriBio (Bundoora, Australia) before transfer to the La Trobe Animal Research and Training Facility (LARTF). They were kept in individually ventilated cages (Techniplast, Australia) at a maximum of four mice per cage. Mice were kept under a 12-hour light-dark cycle, with access to food and water *ad libitum*.

2.3   Minipump surgery

WT mice were randomly assigned by a colleague to receive either Ang II (n=23) (1.44 mg/kg/day) or vehicle (n=11; containing the Ang II diluent (0.5M NaCl, 0.01% acetic acid in dH$_2$O)). Ang II or vehicle was continuously infused subcutaneously via an osmotic minipump (Alzet Model 2004; Durect; USA) for a 28-day treatment period. Mice were anaesthetised with inhaled isoflurane (2% at 0.4 L/min). Once unconscious, mice were injected subcutaneously with carprofen (5 mg/kg) and bupivacaine (2.5 mg/kg) for analgesia. The mice were prepared for surgery by shaving the subscapular region and sterilising with 4% chlorhexidine scrub

followed by 2% chlorhexidine (in 70% isopropyl alcohol). A transverse incision was made using scissors at the nape, and a subcutaneous pocket was created using blunt dissection with haemostats, where an osmotic minipump was inserted. The wound was closed using sterile sutures (5-0 nylon silk, Daclon SMI; Belgium). Carprofen (5 mg/kg) was injected subcutaneously once a day for two days post-surgery. Mice were monitored twice daily for two days, then once daily for the following 7 days, and then once daily every second day until the 28 days had passed.

2.4   Systolic Blood Pressure Detection

Systolic blood pressure (SBP) was measured via tail-cuff plethysmography using an MC4000 Multichannel blood pressure analysis system (Hatteras Instruments, USA). Mice were briefly placed in a restraint on a heated platform (40°C), their tails were threaded through an inflatable tail-cuff, and their pulse was detected using a light-emitting diode. SBP was measured when the blood flow was occluded as a result of the cuff inflation. Mice were acclimatised to SBP measurements prior to the commencement of this study. Baseline measurements (day 0) were taken before the minipump surgery. Following minipump surgery, SBP was measured at days 7, 14, 21 and 28. During each time point, 40 SBP measurements were recorded per mouse. The average of the 40 readings was calculated to determine the SBP for each timepoint.

2.5   Ultrasound Imaging

Ultrasound images were taken of the abdominal aorta on days 0, 7, 14, 21 and 28 post-surgery using The Vevo 2100 (VisualSonics; FUJIFILM; Canada). Mice were anaesthetised using inhaled isoflurane (2% at 0.4 L/min) and laid supine on a heated platform. Mouse paws were

lubricated with electrode gel (Sigma Gel, Sigma-Aldrich, Germany) before being restrained using surgical tape. Abdominal fur was removed using depilatory cream (Nair Hair Removal cream), and ultrasound transmission gel (Aquasonics, USA) was applied to the abdomen directly below the sternum. An MS-400 ultrasound transducer was placed on the chest's centre below the sternum and adjusted until the aorta was located. Pulse-Wave Doppler images were obtained from longitudinal sections of the abdominal aortas (suprarenal) in B-mode. Data were analysed using the VevoLab and VevoVasc software (FUJIFILM Visualsonics Inc. Canada) to measure the external aortic diameter (expressed in millimetres).

2.6   Post-mortem and AAA classification

At the endpoint of the experiment (day 28), mice were killed using CO_2 asphyxiation. Blood from the inferior vena cava was collected using a 27-gauge needle and placed in lithium heparin blood collection tubes (1.3 ml, Sarstedt, Australia) to prevent coagulation. The right atrium was cut to allow expulsion of perfusate while the left ventricle was pierced using a 27-gauge needle and then perfused with phosphate-buffered saline (PBS; Sigma-Aldrich, USA) containing clexane (400 IU, Sanofi Aventis, France) using a peristaltic perfusion pump (Cole-Palmer Instruments, USA). The whole aorta was dissected up to the bifurcation of the common iliac arteries. The aorta was then cleared of any peri-aortic fat deposits, and gross morphology images were captured using an Olympus BX52 for AAA classification. AAA was classified using Daugherty's scale of severity. The original scale was as follows; type I- dilated lumen in the aorta's supra-renal region with no thrombus, Type II – remodelled tissue in the supra-renal area that frequently contains a thrombus[174], Type III- a pronounced bulbous form of type II that includes a thrombus[174] and Type IV- a state in which there are multiple aneurysms containing thrombus in the supra-renal region[174]. We further modified the scale to have a type 0; no

aneurysm present, as well as a type 5; ruptured AAA, to obtain a more inclusive image of our data set.

The aorta was then sectioned into three parts; the thoracic aorta, superior abdominal aorta and inferior abdominal aorta. Both the thoracic aorta and superior abdominal aorta were snap-frozen in separate Eppendorf tubes. At the same time, the inferior abdominal aorta was frozen in Tissue-Tek® optimal cutting temperature compound (OCT compound, Tissue-Tek, Australia). These samples were stored in a -80°C freezer.

2.7 Enzyme-linked immunosorbent assay (ELISA)

The blood taken from the mice was centrifuged at 10000 RPM for 10 minutes at 4°C in the lithium heparin tubes to isolate the plasma, which was then snap-frozen. Plasma levels of IL-18 were quantified using a mouse IL-18/IL-1F4 ELISA kit (Thermofisher®, USA, analytical specificity = 19.0 pg/mL) per the manufacturer's instructions. A concentration range of serially diluted standards (supplied) and the undiluted mouse plasma samples were loaded onto a microtiter plate's wells in duplicates. The plate was then sealed and incubated at room temperature for 1 hour. Following incubation, the plate was washed four times with saline before a biotin-conjugated anti-mouse IL-18 secondary detection antibody was added and incubated for 2 hours at room temperature. The washing step was repeated, followed by the addition of streptavidin horseradish peroxidase with a final incubation period of 20 minutes. A final wash step was performed, and 3,3',5,5'-tetramethylbenzidine (TMB) was added, and the plate was placed in a dark room and checked every 5 minutes until a colour change occurred. 0.5 M of H_2SO_4 was added to stop the reaction, and the absorbance was measured at 450 nm

using a Clariostar Plus Microplate reader (BMG; Labtech, Australia). The amount of IL-18 per sample was then estimated from the standard curve.

2.8 Gelatin Zymography

Gelatin zymography analysing MMP-2 and MMP-9 activity was completed by Prof. Chrishan Samuel (Department of Pharmacology, Monash University). Snap frozen abdominal aortic samples taken from the mice were thawed and incubated with 5 mM 4-amino-phenyl mercuric acetate (APMA; Sigma-Aldrich) for 6 hours at $37°C$. The samples were then mixed with sample loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol and 0.01% (w/v) bromophenol blue; (Bio-Rad, USA) in a volumetric ratio of 1:3 (sample loading buffer: sample) for 1 hour at room temperature. Equal volumes of APMA-treated conditioned media (4-30 µl) from each sample were loaded onto 7.5% acrylamide separating gels (containing 1mg/l porcine skin gelatin (Sigma-Aldrich) and 3.75% acrylamide stacking gel). They were then electrophoresed at 150V until the bromophenol blue marker dye reached the bottom of the gel. It was then washed twice with 0.25% (w/v) Triton X-100 while being gently oscillated for 15 minutes at room temperature. Gels were incubated with an incubation buffer containing 50mM Tris-HCl (pH 7.5), 10mM $CaCl_2$, 1% (w/v) Triton X-100, 0.02% (W/V) NaN_3 and 1µM $ZnCl_2$) overnight at $37°C$. After decanting the incubation buffer, gels were stained with 0.01% (w/v) Coomassie blue R-250 (Sigma-Aldrich) in 40% isopropanol for 1 hour. They were then de-stained in 20% methanol containing 7% acetic acid for up to an hour to reveal clear, discrete bands of gelatin lysis by MMP-2 and MMP-9. A pre-stained kaleidoscope standard marker (Bio-Rad) was used as a molecular weight marker. Densitometry of MMP bands was performed with a GS710 Calibrated Imaging Densitometer (Bio-Rad) and Quantity-one software (Bio-Rad). The density (OD reading) of each MMP measured with each

treated group was expressed as the relative ratio of the untreated control group's values, which was expressed as 1.

2.9 Statistical analysis

Unless otherwise stated, data are expressed as mean ± standard error of the mean (SEM) with a significance level of $P > 0.05$. GraphPad Prism v8 (GraphPad Software Inc., USA) or SPSS Statistics software v26 (IBM, USA) was used to perform all statistical analyses. SBP and diameter size data were analysed using a two-way repeated-measures ANOVA with Bonferroni post hoc test (a two-way ANOVA was performed without repeated measures with relevant post hoc analysis). Analysis of ELISA experiments were performed using a one-way ANOVA with Bonferroni's post hoc test. A chi-squared test was performed to analyse the AAA incidence data. A Log-Rank test was used to compare Kaplan-Meier survival curves, and a non-parametric Kruskal-Wallis analysis was used to analyse AAA severity data.

Chapter 3: Results

3.1   Pilot Ang II Dosing Experiments

Pilot experiments were performed to establish the required dose of Ang II needed to induce a AAA incidence of 40-60% (inclusive of deaths due to aneurysm rupture) in WT mice. We observed that the dose of 1.44 mg/kg/day for 28 days, induced an optimal AAA incidence of 60%, and thus, this dose was used in all subsequent experiments (Figure 3.1).

3.2   Ang II increased SBP in all strains

In all mice that survived the treatment protocol, Ang II increased SBP by ~40 mmHg compared to baseline measurements in all mice strains (Figure 3.2). No changes in SBP were observed in vehicle-treated WT mice over the 28 days. Interestingly, no differences in pressor responses to Ang II was found between any of the mouse strains. In order to prevent selection bias for survivors, an analysis was also performed on SBP data from all mice prior to death due to rupture (Appendix 5.1). Consistent with the results observed in surviving mice only, no differences in pressor responses to Ang II were observed between all strains when including data from mice that died prematurely.

3.3   Incidence of AAA

AAA incidence that also included deaths due to AAA rupture was recorded and quantified over 28 days (Figure 3.3). As expected, AAA formation was not observed in the vehicle-treated WT mice while treatment with Ang II caused AAA formation in 55% of WT mice ($P<0.05$). Strikingly, the incidence of AAA was absent in IL-18$^{-/-}$ mice. In contrast, AAA incidence was

significantly augmented in Ang II-infused IL-18Rα$^{-/-}$ mice compared to WT mice (88% vs 53%, P<0.05). Interestingly, the IL-37Tg mice had an AAA incidence of 38%, which was not statistically significant from the Ang II-treated WT mice (P>0.05).

3.4 Ang II-treated WT and IL-18Rα$^{-/-}$ mice but not IL-37Tg mice experienced premature death due to AAA rupture.

Mice that died prematurely during the 28-day Ang II infusion underwent an autopsy to confirm that death was due to AAA rupture. A Kaplan-Meier survival curve was used to compare the mortality rate between all strains throughout the 28-day study period (Figure 3.4). As expected, no deaths were observed in the mice strains that did not develop aneurysms (vehicle-treated WT and IL-18$^{-/-}$ mice). Interestingly, it was found that approximately 1-week after surgery, mice that developed AAA were susceptible to AAA rupture (Ang II-infused WT mice, IL-37Tg and IL-18Rα$^{-/-}$ mice). While the Ang II-infused WT and IL-37Tg demonstrated a similar decline in survival, the survival of IL-18Rα$^{-/-}$ mice declined at an augmented rate (P<0.02).

3.5 Ultrasound imaging: temporal changes in aortic diameter

Ultrasound imaging of the abdominal aorta was performed weekly to monitor the progression of aneurysm formation (Figure 3.5). Ultrasound images were captured at days 0, 7, 14, 21 and 28 from which external diameters were analysed using the VevoSonics software. (Figure 3.6.a). An increase in diameter was only observed in mouse strains that developed AAA; Ang II-treated WT, IL-18Ra$^{-/-}$ and IL-37Tg. From day 14 onwards, the Ang II-treated WT mice's aortic diameter was statistically greater than the vehicle-treated WT mice and Ang II-treated IL-18$^{-/-}$ mice (1.40 ± 0.05 mm vs 1.09 ± 0.02 mm vs 1.12 ± 0.02 mm respectively; P<0.05).

However, no differences in the aortic diameters were observed between the Ang II-treated WT, IL-18Rα$^{-/-}$ and the IL-37Tg mice throughout the study (1.40 ± 0.05 mm vs 1.27 ± 0.08 mm vs 1.27 ± 0.06 mm respectively; P>0.05). At day 28, the aortic diameter of the Ang II-treated WT mice was statistically greater than vehicle-treated WT mice as well as the Ang II-treated IL-18$^{-/-}$ mice (1.46 ± 0.07 vs 1.09 ± 0.02 vs 1.13 ±0.02; P<0.05). Again, there were no differences in the aortic diameters observed between the Ang II-treated WT, IL-18Rα$^{-/-}$ and the IL-37Tg mice at day 28 (1.46 ± 0.04 vs ± 1.55 ± 0.07 vs 1.36 ± 0.07). The aforementioned analysis of aortic diameters was performed only on mice that survived the entire 28-day treatment period and excluded aortic diameters from mice that died prematurely To account for selection bias, a separate analysis was also performed for all mice (Appendix 6.2). The results were similar at day 28 where the aortic diameter of the Ang II-treated WT mice was statistically greater than the vehicle-treated WT mice and the Ang II-treated IL-18$^{-/-}$ mice (1.46 ± 0.07 mm vs 1.09 ± 0.02 mm vs 1.13 ± 0.02 mm respectively; P<0.05). However, at day 28 no differences in the aortic diameters were observed between the Ang II-treated WT, IL-18Rα$^{-/-}$ and the IL-37Tg mice throughout the study (1.46 ± 0.07 mm vs 1.55 ± 0.07 mm vs 1.36 ± 0.06 mm respectively). Interestingly, when analysing data for mice that died prematurely (Figure 3.6.b), a larger increase in aortic diameter was observed in IL-18Rα$^{-/-}$ mice only at day 7 compared to the aortic diameter of the WT mice (1.426 ± 0.133 mm vs 1.164 ± 0.054 mm; P<0.05).

3.6 AAA severity

After the mice were killed, the aortae were cleaned of all fat and images were captured and scored based on a scale of severity previously developed by Alan Daugherty, with the addition of a grade 0 and 5 to represent no AAA and ruptured AAA/death respectively (Figure 3.7.b) [174]. As predicted, all vehicle-treated WT mice and Ang II-treated IL-18$^{-/-}$ mice had a median

score of 0 as neither group developed any aneurysms throughout the course of the study. Furthermore, Ang II-treated WT mice displayed a significant increase in severity compared to the vehicle-treated WT (median: 2 vs 0, P<0.05). The severity of IL-18Rα$^{-/-}$ was statistically higher than the IL-37Tg mice (median: 3 vs 0, P<0.05).

3.7   Plasma IL-18 levels are elevated in IL-37Tg mice

Plasma IL-18 was quantified using an ELISA kit (Figure 3.8). In WT mice, Ang II infusion did not influence plasma IL-18 levels compared to vehicle-treated WT (594 ± 114 pg/ml vs 755.5 ± 145 pg/ml; P>0.05). As expected, plasma IL-18 was undetectable in the IL-18$^{-/-}$ mice. Plasma IL-18 levels were similar in IL-18R$^{-/-}$α compared to the Ang II-treated WT mice (608 ± 129 pg/ml vs 594 ±114 pg/ml). However, plasma IL-18 was significantly greater in IL-37Tg mice compared to Ang II-treated WT (1314 ± 227 pg/ml vs 594 ± 114 pg/ml; P<0.05).

3.8 MMP-2 activity is elevated in IL-18Rα$^{-/-}$ mice

Abdominal aortae from all groups were collected to detect MMP-2 and MMP-9 activity using gelatin zymography (Figure 3.9). No differences in MMP-9 activity were found between all mouse groups; Ang II-treated WT, IL-18$^{-/-}$, IL-18Rα$^{-/-}$ and IL-37Tg (1.28 ± 0.37 OD vs 1.52 ± 0.62 OD ± 1.36 ± 0.37 OD vs 1.00 ± 0.16 OD respectively, P>0.05). Similarly, MMP-2 activity was not different between Ang II-treated WT, IL-18$^{-/-}$ and IL-37Tg mice (0.80 ± 0.22 OD vs 1.11 ± 0.15 OD vs 0.5553 ± 0.15 OD respectively, P>0.05). However, MMP-2 activity was significantly higher in IL-18Rα$^{-/-}$ mice than Ang II-treated WT (2.54 ± 0.83 OD vs 0.80 ± 0.22 OD, P<0.05).
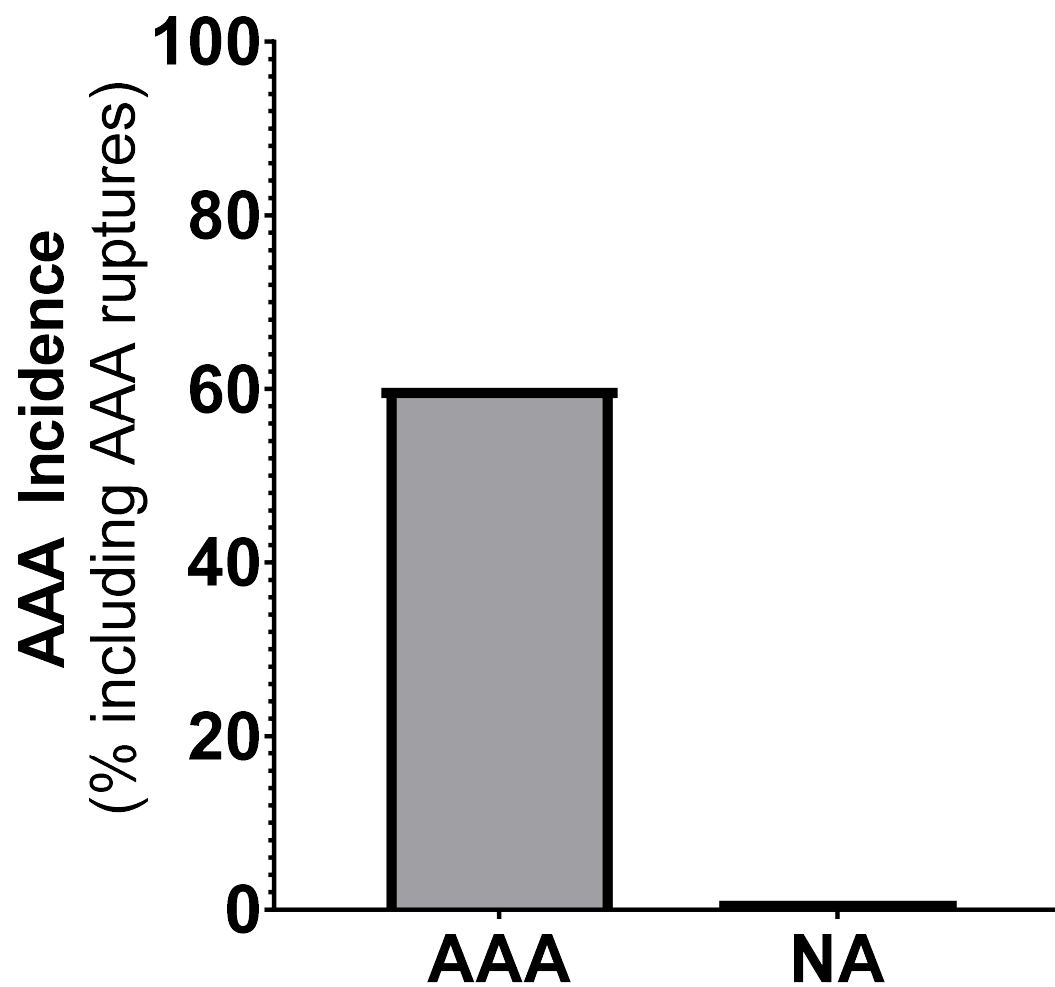
Figure 3.1. Pilot study to determine the incidence of AAA in WT (including premature deaths due to rupture) after a 28-day period of Ang II infusion (1.44 mg/kg/day). N = 10. Abdominal aortic aneurysm (AAA) and no aneurysm (NA)
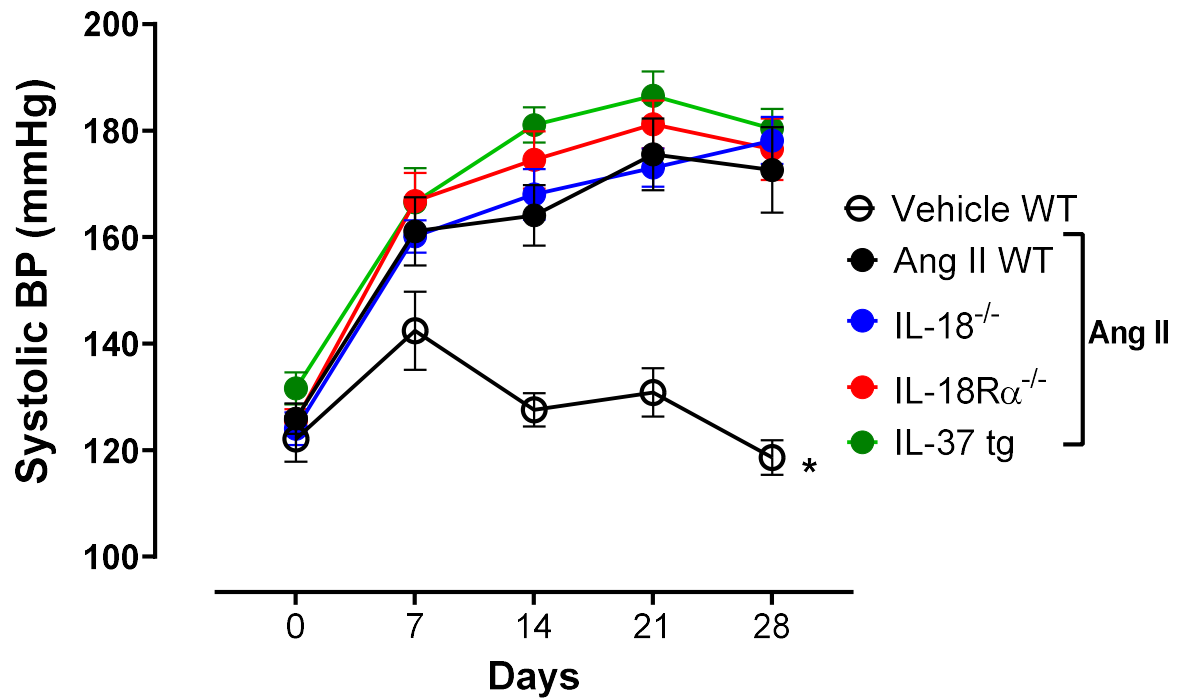
Figure 3.2. Ang II infusion increases blood pressure in all mouse strains. SBP measured by tail-cuff plethysmography of vehicle-treated wild type (WT) mice (n=11) and Ang II-treated WT (n=20), IL-18$^{-/-}$ (n=22), IL-18R$\alpha^{-/-}$ (n=25) and IL-37Tg mice (n=23) over a 28-day period. Data are expressed as mean ± SEM. *P<0.05 vehicle-treated WT vs Ang II-treated WT. Two-way repeated ANOVA with Tukey's multiple comparisons test.
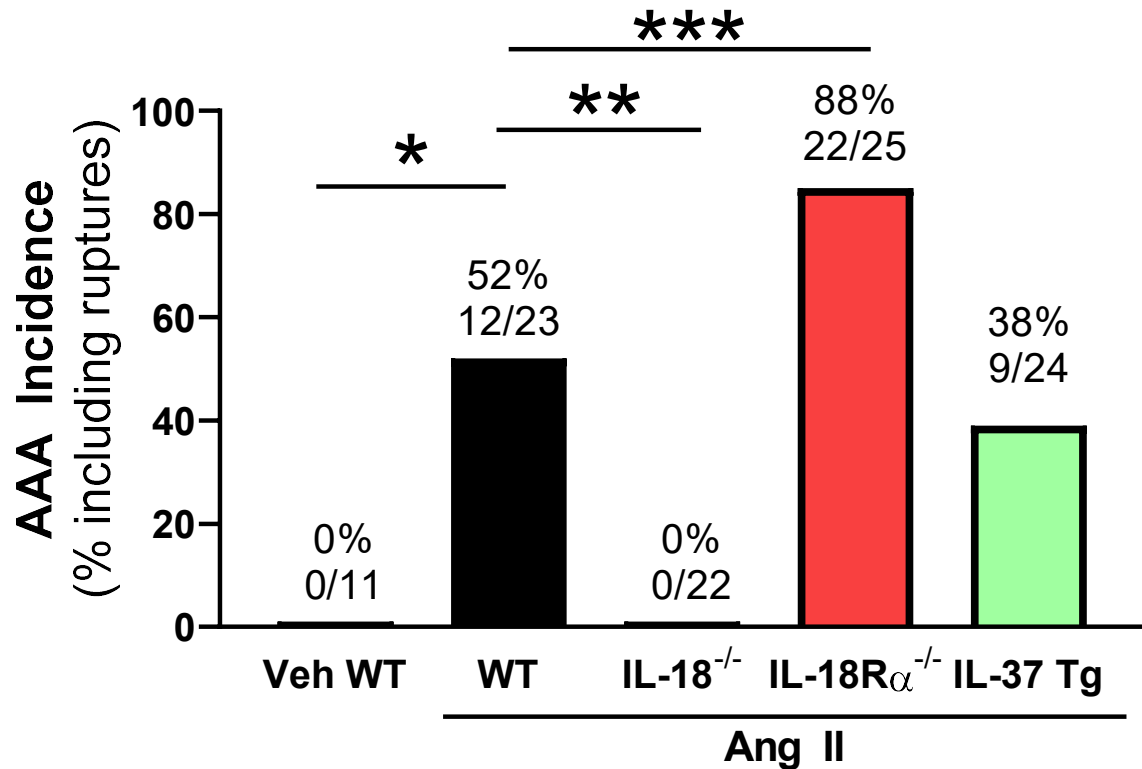
Figure 3.3. IL-18Rα deficiency elevated AAA incidence, while IL-18 deficiency demonstrated complete protection from AAA incidence and overexpression of IL-37 had no change in AAA incidence. AAA incidence in vehicle-treated WT, and Ang II-treated WT, IL-18^{-/-}, IL-18Rα^{-/-} and IL-37Tg mice (N=7-22). Data are expressed as a percentage. *$P<0.05$ vehicle-treated wild type (WT) vs Ang II-treated WT. **$P<0.001$ Ang II-treated WT vs IL-18^{-/-} mice. ***$P<0.005$ Ang II-treated WT vs IL-18Rα^{-/-} mice. Chi-squared analysis test**.**
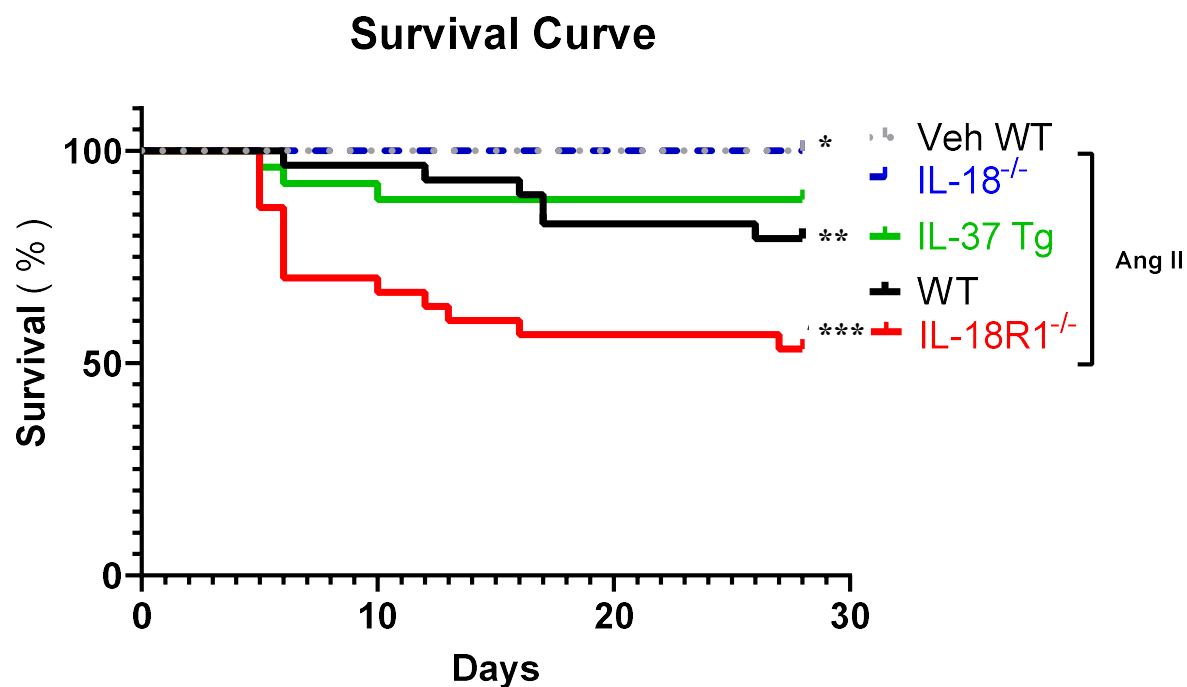
Figure 3.4. IL-18Rα$^{-/-}$ mice have increased mortality due to AAA rupture in an Ang II-induced AAA model. The mortality rate in vehicle-treated WT, and Ang II-treated WT, IL-18$^{-/-}$, IL-18Rα$^{-/-}$ and IL-37Tg mice (N=7-22). Data are expressed as a % of survival. *P<0.05 Ang- II-induced WT vs vehicle-induced WT. **P<0.001 Ang-II induced WT vs IL-18$^{-/-}$. ***P<0.05 Ang II-treated WT v IL-18Rα$^{-/-}$. Log-rank test.
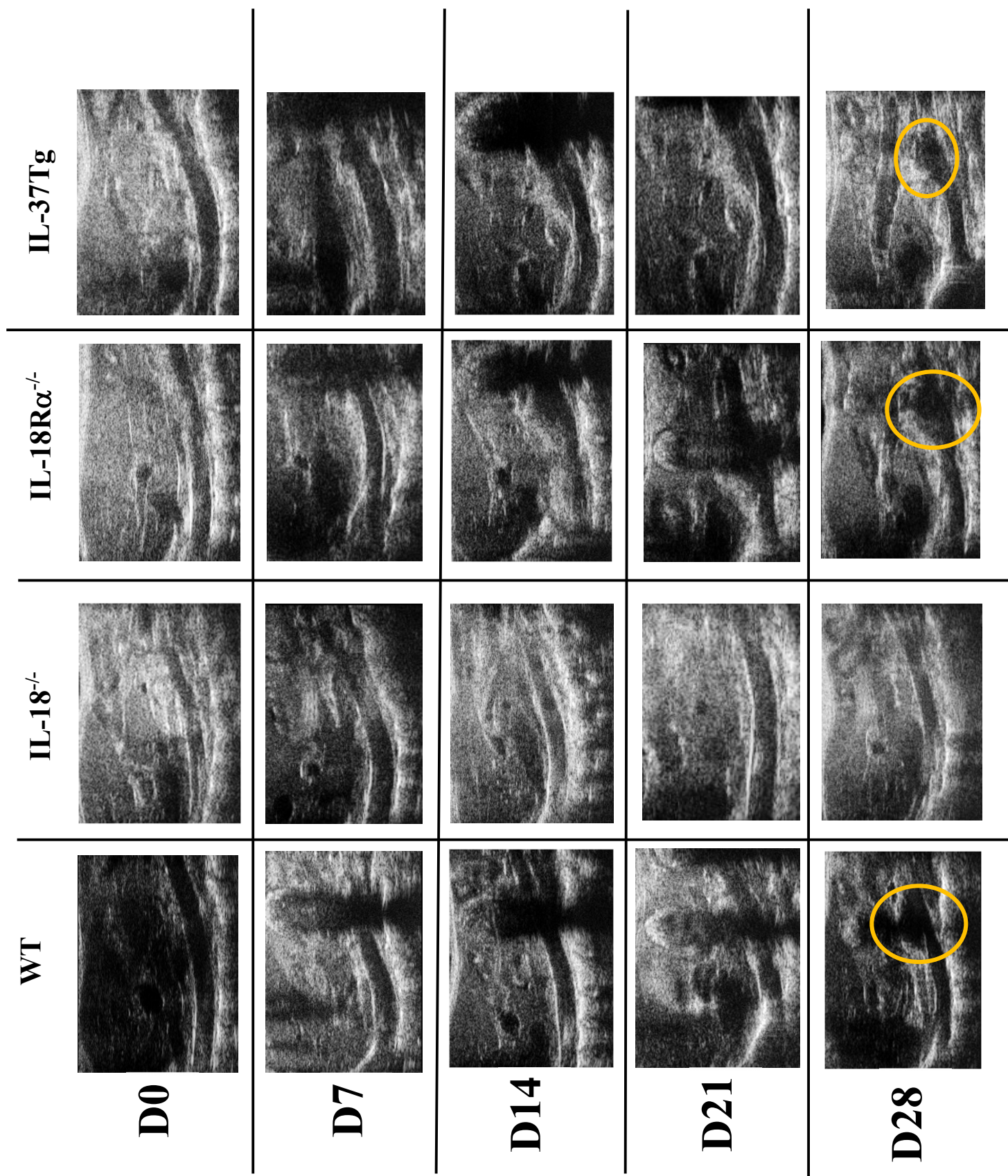
Figure 3.5 Ang II increases aortic diameter in all strains except IL-18$^{-/-}$. Representative ultrasounds images of the abdominal aorta from vehicle-treated wild type (WT) mice as well as angiotensin II-treated WT, IL-18$^{-/-}$, IL-18R$\alpha^{-/-}$ and IL-37Tg mice over a 28-day period via 2100 Vevosonics ultrasound machine. The yellow circles indicate a AAA at day 28.
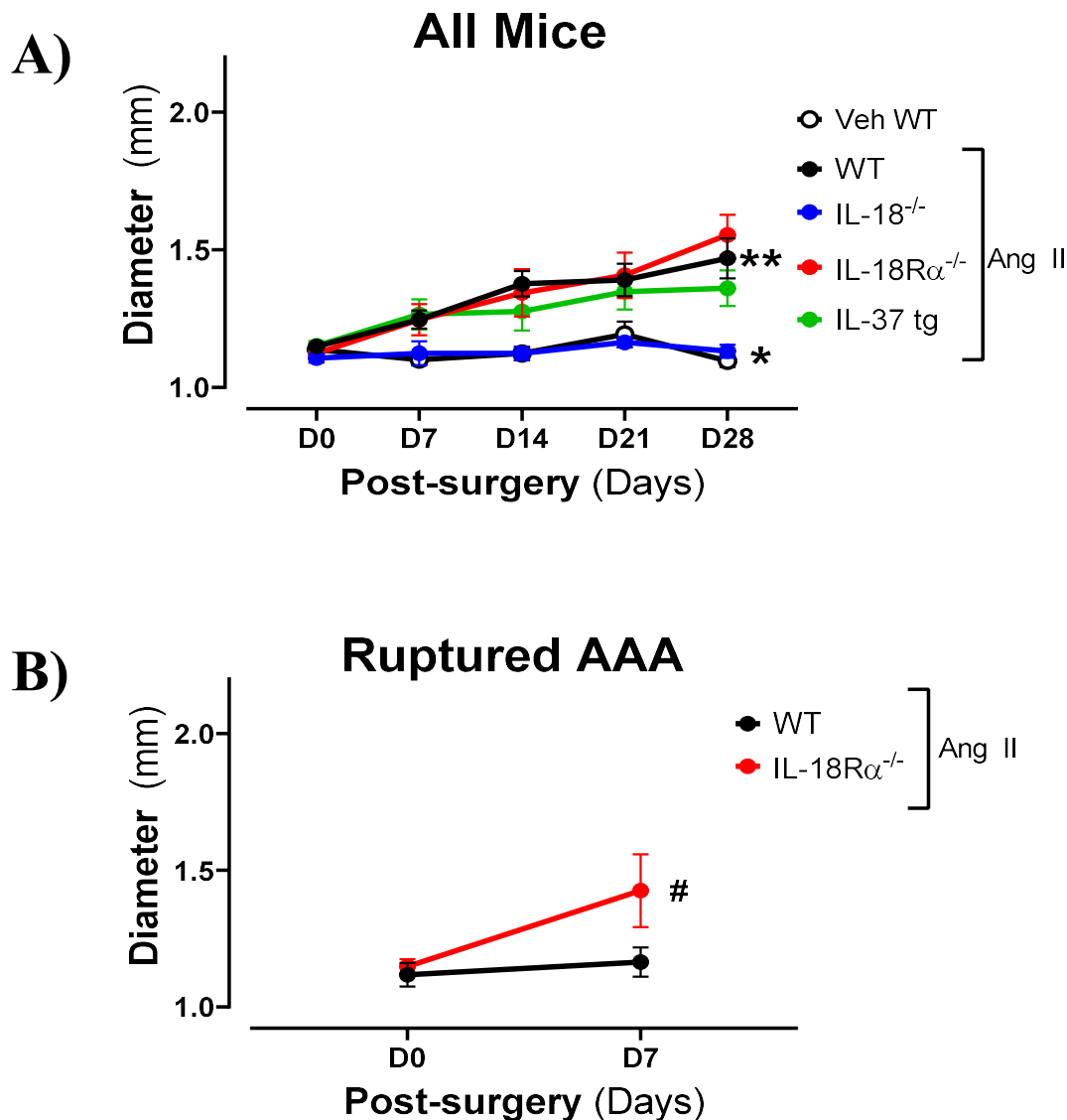
Figure 3.6 AAA growth rate and mortality due to rupture. A) Diameters of abdominal aorta of all mice over a 28-day period as measured by ultrasound (N=7-22). B) Diameters of abdominal aorta of mice that died from AAA rupture over a 7-day period as measured by ultrasound (N=8-14). Data are expressed as mean ± SEM. *p<0.05 vehicle-treated wild type (WT) vs ang II-treated WT.**P<0.05 IL-18Rα$^{-/-}$ vs ang II-treated WT. Two-way repeated ANOVA with Tukey's multiple comparisons test.

Vehicle WT Ang II WT IL-18$^{-/-}$ IL-18Rα-/- IL-37Tg

A)

B)

Daugherty scale of severity

5

4

3

2

1

0

WTVeh WT IL-18$^{-/-}$ IL-18Rα$^{-/-}$ IL-37Tg

*

**

Ang II
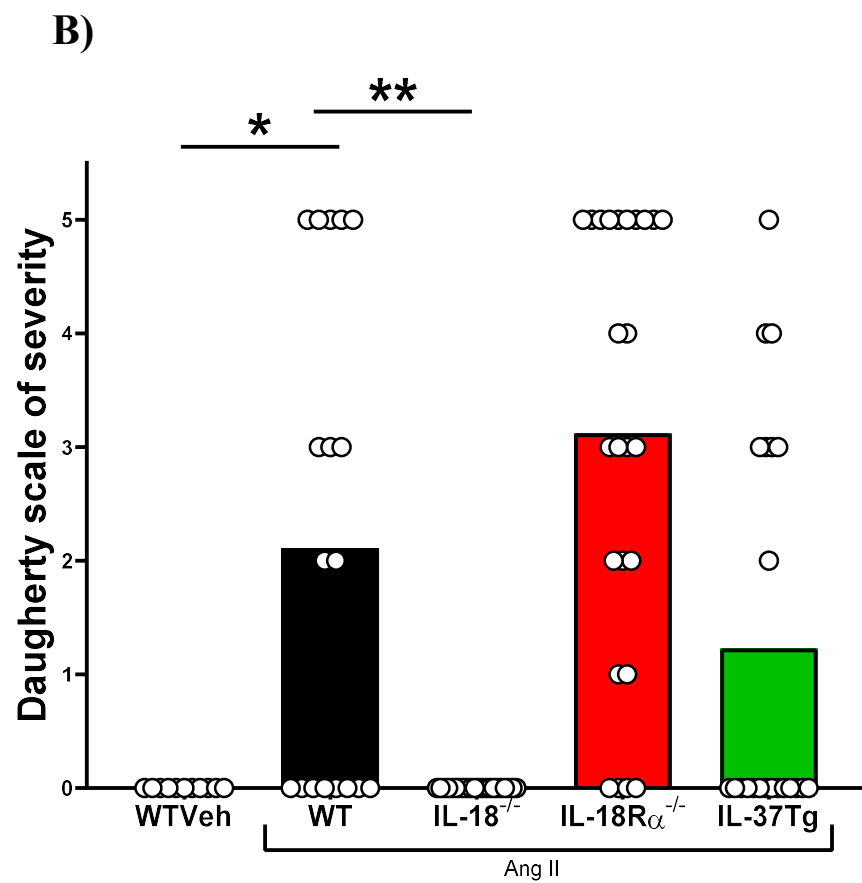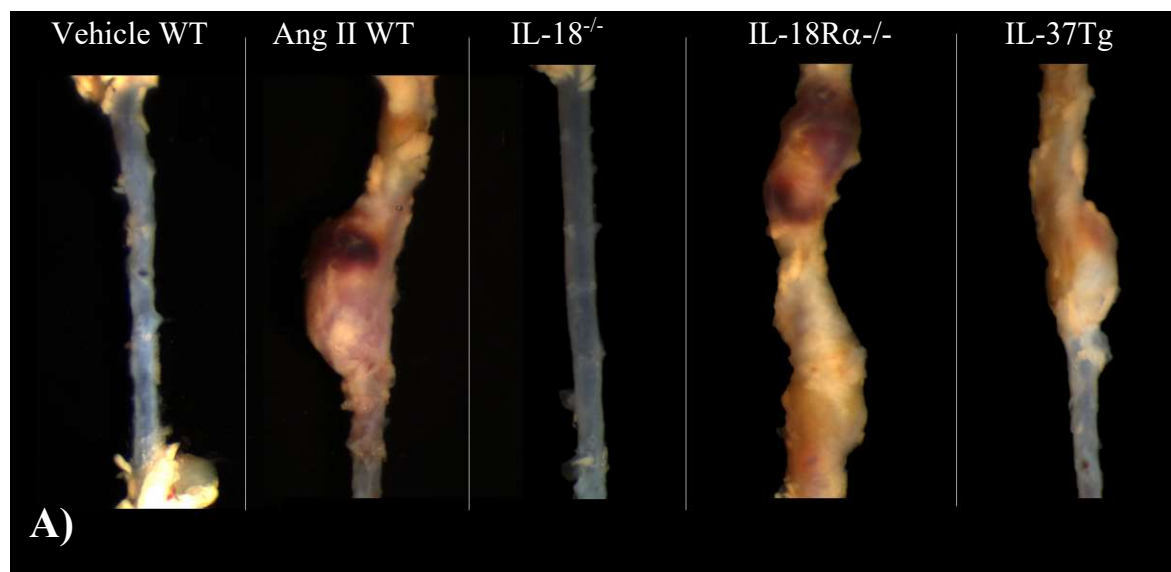
Figure 3.7 Daugherty's scale of severity based on representative images.

A) Representative images of the abdominal aorta from each mouse strain taken post-mortem after dissection and cleaning of fat tissue around the aorta. B) AAA severity was graded using the pre-established scale of severity known as Daugherty's scale in the abdominal aorta of vehicle-treated wild type (WT) mice (n=11) as well as angiotensin II-treated WT (n=20), IL-18$^{-/-}$ (n=22), IL-18R$\alpha^{-/-}$ (n=25) and IL-37Tg (n=23) after a 28-day period. Type 0: no aneurysm was present. Type 1: dilation of the lumen in the suprarenal region of the aorta but no thrombus is present. Type 2: is equivalent to remodelling of the tissue in the suprarenal area that often contains a thrombus. Type 3: has a pronounced bulbous form of type 2 that includes a bulbous. Type 4: multiple aneurysms are apparent and contain thrombus, some overlapping in the suprarenal area Type 5: ruptured AAA. Data are expressed as mean (n=7-22). *$P<0.05$ Ang II-treated WT vs vehicle-treated WT. **$P<0.0001$ Ang II-treated WT vs IL-18$^{-/-}$. ***$P<0.05$ Ang II-treated WT vs IL-37Tg. Two-way ANOVA with Dunn's multiple comparisons test.
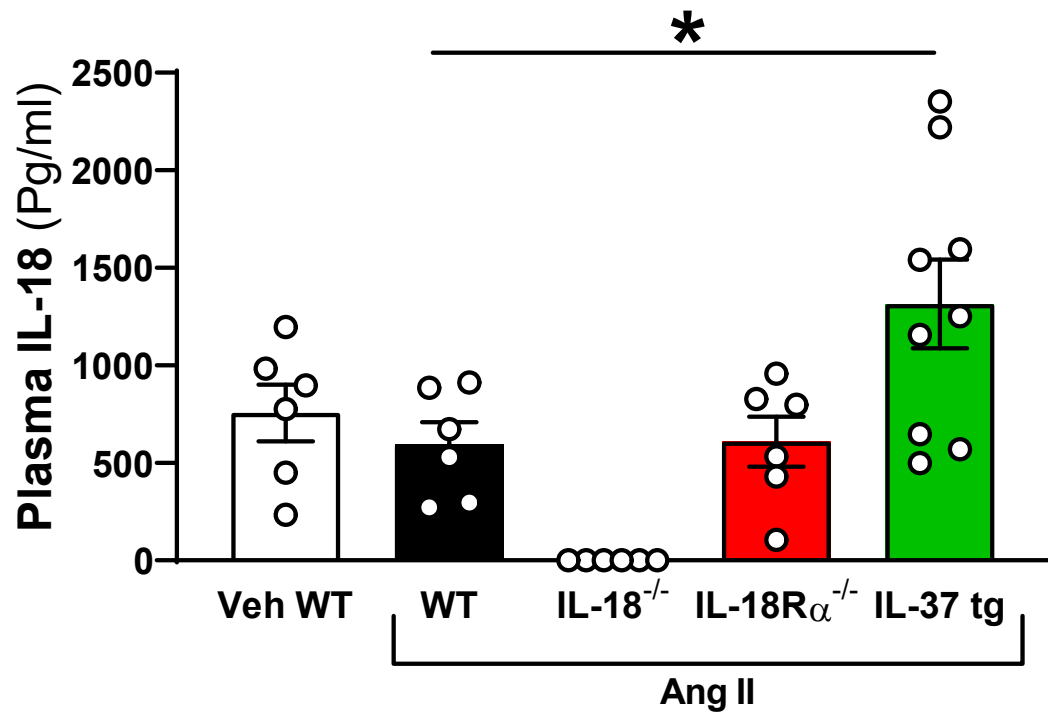
Figure 3.8. Plasma IL-18 levels are increased in IL-37Tg mice. Plasma concentrations of IL-18 were measured in vehicle-treated wild type (WT) mice (n=11) mice as well as Ang II-treated WT (n=20), IL-18$^{-/-}$ (n=22), IL-18R$\alpha^{-/-}$ (n=25) and IL-37Tg (n=23) mice after a 28-day treatment period. Data are expressed as mean ± SEM. *P<0.05 Ang II-treated WT mice vs IL-37Tg mice. One-way ANOVA with Tukey's multiple comparisons test.
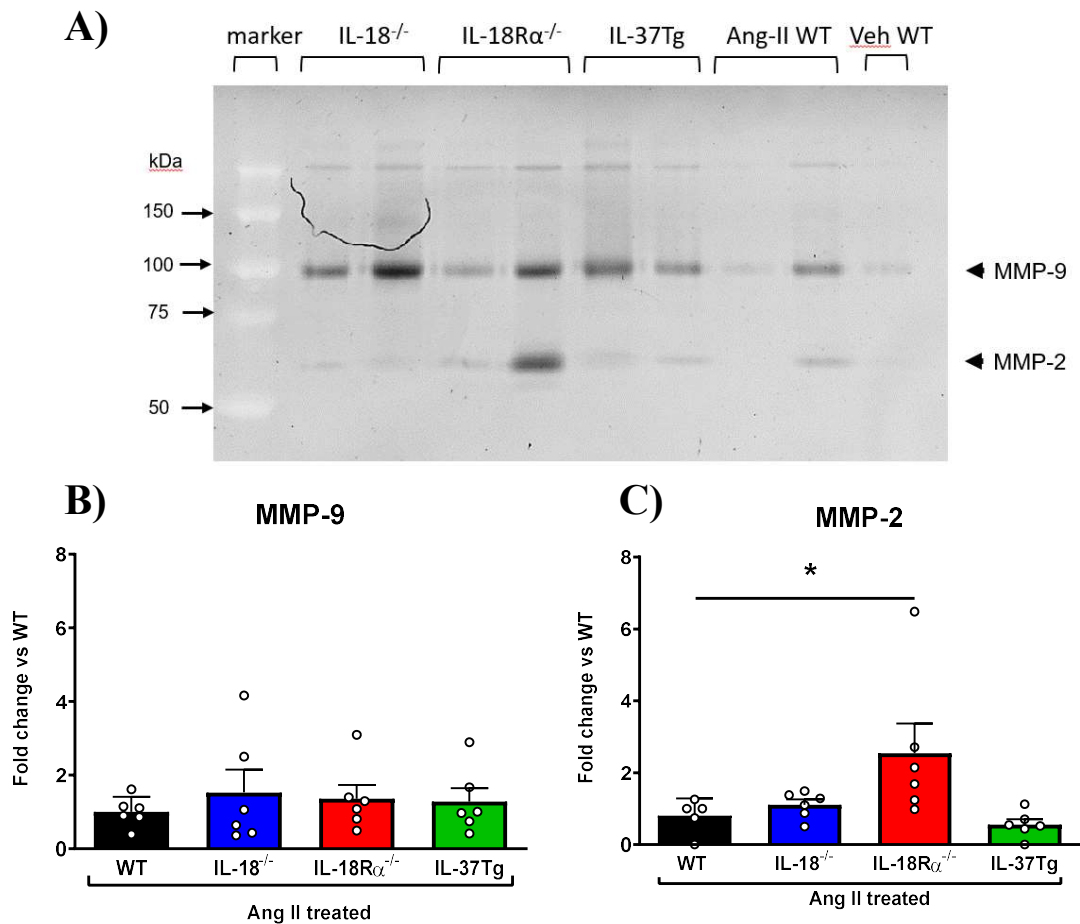
Figure 3.9 Matrix metalloproteinase-2 activity is increased in IL-18Rα⁻/⁻ mice. (A) Zuni graph images of gel zymography. Zymography testing quantified activity of (B) MMP-9 and (C) MMP-2 in Ang II-treated WT (n=20), IL-18⁻/⁻ (n=22), IL-18Rα⁻/⁻ (n=25) and IL-37Tg (n=23) mice after a 28-day treatment period. Data are expressed as mean ± SEM. *$P<0.05$ Ang II-treated WT mice vs IL-18Rα⁻/⁻ mice. One-way ANOVA with Tukey's multiple comparisons test.

Chapter 4: General Discussion

This study was conducted to investigate the potential roles of IL-18, IL-18Rα, and IL-37(human) in AAA development and progression. The significant findings of the study were that: (1) IL-18$^{-/-}$ mice were entirely protected from Ang II-induced AAA formation; (2) genetic ablation of the IL-18Rα receptor for which IL-18 binds, augmented AAA incidence, severity and mortality associated with AAA when compared to WT mice infused with Ang II; and (3) IL-37Tg mice displayed a trend towards a reduction in AAA incidence and severity compared to Ang II-treated WT mice.

4.1   IL-18 is crucial for AAA formation

In the current study using a gold-standard model of AAA [71], AAA formation was completely abolished in IL-18$^{-/-}$ mice. Despite significant elevations in SBP, AAA formation was independent of hypertension, which supports the current literature [74,175]. During this Masters project, Suehiro et al. published a study showing a reduction in AAA incidence in IL-18$^{-/-}$ mice compared to WT (15% vs 58%) and mortality due to rupture (4% vs 15%) [176]. These findings are consistent with that of the current study though a notable difference is that we observed complete protection from AAA formation (0% AAA incidence). A possible reason for this difference may be attributable to the difference in the model used in each study. While both studies infused Ang II at the same dose of 1.44 mg/kg/day over a four-week period, Suehiro et al. also co-administered BAPN orally for the first 2 weeks of Ang II-infusion. Hence, the co-administration of BAPN with Ang II significantly increases AAA formation and risk of rupture [80]. Thus, the increased severity of the model may explain the slight difference in AAA incidence. Nonetheless, our findings are consistent with the Suehiro et al. study, which strongly supports a significant role for IL-18 in AAA development.

IL-18 acts via the receptor subunits, IL-18Rα and IL-18Rβ, to produce a pro-inflammatory response. As previously discussed, IL-18 $^{-/-}$ results in a reduction of AAA frequency; thus, it was hypothesised that the genetic ablation of the IL-18Rα receptor subunit would induce similar levels of protection. Contrary to this, IL-18R$^{-/-}$α mice had augmented AAA incidence compared to Ang II-treated WT mice (88% vs 53%, respectively). Strikingly, almost half of all IL-18Rα$^{-/-}$ mice infused with Ang II died due to AAA rupture before the end of the 28 days, with the highest mortality occurring between day 7 and day 21. This data suggests that IL-18Rα$^{-/-}$ mice exhibit accelerated AAA growth rate, as the rate of expansion is strongly correlated to an increase in mortality due to rupture [177].

Ultrasound imaging tracked AAA formation and progression by measuring diameters (in systole) of the aorta at weekly time points. Interestingly, we observed no differences in the mean aortic diameters from all mice strains that survived the 28-day Ang II infusion. This information would suggest that if an AAA were to develop, it would progress at the same rate regardless of strain. However, we hypothesised that the analysis of only mice that survived whilst excluding mice that died due to AAA rupture could mask the severity of AAA development, particularly in IL-18R$^{-/-}$α mice that had a severely reduced survival rate. In support of this hypothesis, when analysing diameters of mice that died prematurely due to AAA rupture, we observed significantly greater aortic diameters in IL-18R$^{-/-}$α mice after 7 days of Ang II-infusion than Ang II-treated WT mice. Note there was an insufficient sample size to incorporate IL-37Tg mice at this time point. Hence, collectively our data suggests that IL-18R$^{-/-}$α results in accelerated AAA formation, subsequently increasing the risk of rupture, resulting in a greater AAA incidence.

During this project, another study was published which investigated the role of IL-18R-deficiency in AAA. Liu et al. crossbred ApoE$^{-/-}$ hypercholesterolemic mice, IL-18R$^{-/-}$ mice and ob/ob, used for obesity studies, to determine the link between IL-18 and obesity in AAA [178]. This significant finding of this study was that AAA that occurred in Ang II-treated IL-18R$^{-/-}$ mice demonstrated a reduction in AAA pathology [178]. More strikingly, this strain experienced blunted activity of MMP-2 and MMP-9 [178]. Additional results did contrast to our study; despite no reductions in mortality (~30%), Liu et al. reported that Ang II-treated IL-18R$^{-/-}$ mice resulted in reductions in aortic diameter and macrophage infiltration [178]. A disadvantage of the above-mentioned study was a lack of reporting on AAA incidence, and as such direct comparison with our findings is difficult. Nonetheless, the contrasting results between Liu et al and our study could be potentially attributed to different models and differing mice strains (hypercholesterolemic ApoE$^{-/-}$ mice vs C57Bl6 mice). Liu et al, also initially used the Ang II model, but due to high mortality also incorporated the CaCl$_2$ model, however, those results were not compared [178]. The authors did acknowledge that their small sample size (6 mice per group vs 20 mice per group in our study) could be statistically underpowered and noted a larger sample size is needed to make a conclusive result.

A reduction in AAA pathology in IL-18R$^{-/-}$ mice[178] suggests that IL-18 plays a crucial role in AAA development by acting on the IL-18R. In our results, the augmented AAA pathology in IL-18R$^{-/-}$α mice may be difficult to interpret since IL-18-deficiency resulted in complete ablation of AAA formation. However, when interpreting this result, we must also consider the results of the IL-37Tg mice. IL-37 is a potent inhibitor of the innate immune response that also binds to the IL-18Rα but requires a different receptor subunit – IL-1R8 – compared to IL-18, which requires IL-18R[461] β. IL-37 is known to act as a negative feedback regulator of

inflammatory responses [164]. Studies have demonstrated that reducing th/e synthesis of IL-37 results in an increased production of pro-inflammatory mediators [164]. Our original hypothesis was that overexpression of human IL-37 would result in protection against Ang II-induced AAA formation. To our knowledge, the current study is the first to examine IL-37Tg mice in a model of AAA formation. This strain has previously been utilised to study transgenic overexpression of human IL-37 in ApoE$^{-/-}$ mice. These studies found human IL-37 promoted protection against atherosclerosis and increased plaque stability [179]. Ji et al. also reported that administering recombinant IL-37 in ApoE$^{-/-}$ mice also decreased atherosclerotic plaque development compared to vehicle control [180]. Furthermore, neutralisation of IL-18Rα using monoclonal antibodies has been shown to reduce IL-37-mediated anti-inflammatory effects in ApoE$^{-/-}$ mice [180]. Atherosclerosis is commonly found in patients who suffer from AAA, and there is indeed a strong link between atherosclerotic plaque development and AAA formation [6]. Although not statistically significant, we found that the IL-37Tg mice exhibited a trend towards reducing AAA incidence than the WT (35% vs 57%). As mentioned above, IL-37 binds to the IL-18Rα to exert anti-inflammatory actions. Thus, we speculate that genetic deletion of IL-18Rα not only removes the detrimental effects of IL-18 on AAA formation but also prohibits the activity of a yet to be identified IL-37 homologue in mice [164] (Figure 4.1). In support of this hypothesis, there is strong evidence that IL-18Rα deletion in splenocytes, macrophages result in 2-3 greater fold expression of pro-inflammatory cytokines such as IFN-γ, macrophage inflammatory protein 1 and 2 (MIP1 and MIP2) and TNF-.[661] α Of relevance to the current study, TNF-α has been implicated in AAA development as a promoter of MMP-9 expression and macrophage infiltration [181]. Nold-Petry et al. also showed that deletion of IL-18Rα in mouse embryonic fibroblasts results in phosphorylation of several pro-inflammatory genes leading to elevated pro-inflammatory cytokine release [182]. The authors suggested a yet to be identified ligand for the IL-18Rα existed and mediated the anti-inflammatory effects. 54

However, it must be noted that there are indeed several studies in other disease models that oppose our hypothesis where deletion of IL-18Rα recapitulates the protective effects of IL-18-deficiency [155,183].

Our results suggested that human IL-37 does not protect from the development of AAA, as the incidence of AAA was not significantly reduced. Curiously, plasma IL-18 was increased in IL-37Tg mice compared to WT mice. Our analysis of plasma IL-18 levels showed that IL-37Tg mice showed a 2-fold increase in the levels of IL-18 compared to all other mice. This increase in IL-18 may promote the pro-inflammatory pathway via IL-18Rα, leading to a higher AAA formation degree. Thus, it is tempting to speculate that the elevated IL-18 may have competed with the human IL-37 to mask any protection that it may have had. Nonetheless, while not significant, the trend towards reducing AAA incidence would undoubtedly suggest overexpression of human IL-37 may influence AAA formation, but importantly, our results highlight a potential complex interplay between IL-18, IL-37 and their shared receptor subunit, IL-18Rα.

An alternative to the hypothesis above could be that IL-18 could also be acting on another IL-18R-independent site to promote AAA formation. Recent studies have reported that IL-18 can also act at another site to exert pro-inflammatory effects. Wang et al. showed that IL-18 directly interacts with the NaCl co-transporter (NCC). NCC is a transmembrane-domain ion-transporter and vital for sodium/H_2O absorption[184]. Moreover, the study mentioned earlier by Liu et al. reported that IL-18 interacts with NCC to promote AAA formation independent of IL-18R [178]. The authors suggested that genetically deletion of both IL-18R and NCC, but not individual

deletion, is required to abrogate the pro-inflammatory effects of IL-18 [178,185]. Thus, based on this evidence, a revision to our first hypothesis is that IL-18Rα-deficiency not only removes the IL-37-like pathway, but IL-18 can also still act on NCC – unopposed by IL-37 - to promote AAA formation resulting in the augmented AAA formation observed in IL-18Rα^{-/-} mice. Thus, to summarise each strain's effects on AAA formation, we hypothesise that IL-18^{-/-} mice lack the pro-inflammatory stimuli mediated via IL-18Rα and NCC. They may also have unopposed activation of the anti-inflammatory pathway associated with a yet to be identified IL-37 homologue in mice, leading to complete protection from AAA formation (Figure 4.1A). Conversely, while IL-18Rα-deficiency prevents IL-18-mediated effects through its cognate receptor, it also abrogates the IL-37-like pathway in addition to IL-18 freely acting on NCC, resulting in augmented AAA formation (Figure 4.1B). Finally, the overexpression of IL-37 confers a trend towards a reduction in AAA, which may attribute to IL-37 acting on IL-18R(αFigure 4.1C). However, as IL-18 is still present in these mice and plasma levels were elevated in these mice. Thus, IL-18 may be functionally antagonising the effects of elevated human IL-37, resulting in no added protection.
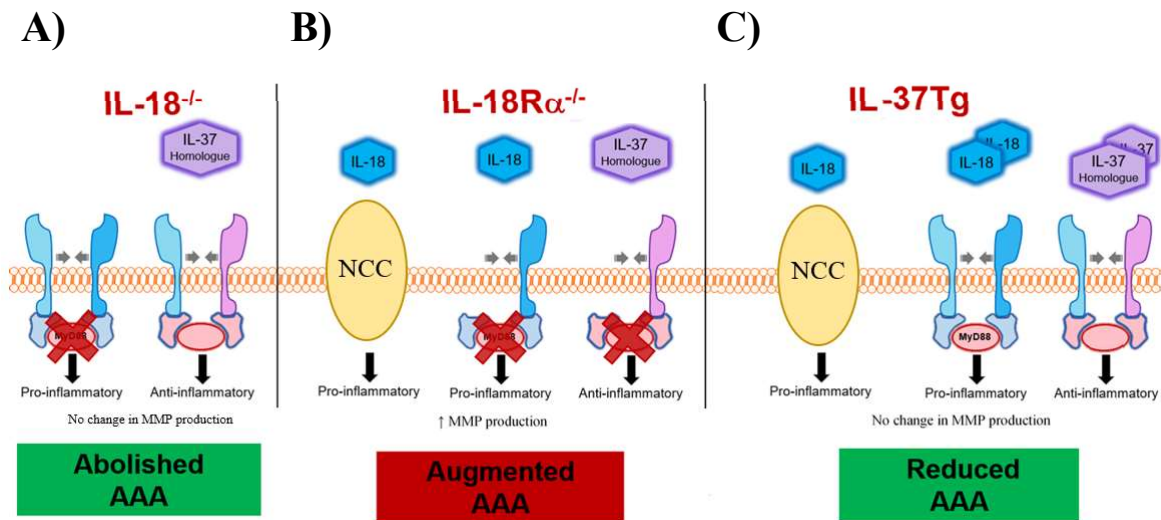
Figure 4.1 IL-18 promotes aneurysm formation. (A) Genetic deletion of IL-18 prevents binding to the IL-18R receptor, effectively inhibiting the pro-inflammatory pathway's activation. While IL-37 mouse homologue can act unopposed on the IL-18R inducing an anti-inflammatory response. (B) Genetic deletion of IL-18R prevents the binding of IL-18 and IL-37 mouse homologue, but IL-18 can still interact with NaCl co-transporter (NCC) to induce a pro-inflammatory response. (C) Overexpression of human IL-37 in transgenic mice results in increased production of IL-18, whereby both can act on the IL-18R receptor, but IL-18 also interacts with NCC to produce a pro-inflammatory response. *Image created by Sherriff. A, (unpublished)*

4.2   Mechanisms of IL-18 and IL-37 effects on AAA

Since IL-18 plays a role in AAA formation, we sought to identify potential mechanisms associated with this protective effect. The activity of MMP-2 and MMP-9 was measured in the tissue samples of our mice through gelatin zymography. No differences in MMP-9 activity were observed between any of the Ang II-treated strains. Similar results for MMP-2 were found, except IL-18Rα$^{-/-}$ mice had a significant increase in MMP-2 activity than Ang II-treated WT. Studies have suggested IL-18 induces MMP-2 activity without direct effects on MMP-9 [186].

Suehiro et al. reported that IL-18-deficiency resulted in attenuation of macrophage infiltration and MMP-2 and MMP-9 activity in the abdominal aorta[176]. While we did not observe effects on IL-18-deficiency on MMP2 activity, the increase in MMP-2 activity in the IL-18Rα$^{-/-}$ mice in our study may be due to the lack of IL-37 activated anti-inflammatory pathway as mentioned above. Jiang and colleagues reported that IL-37 overexpression significantly inhibited MMP-2 activity [179]. However, an important potential confounder to the elevated MMP2 activity is the fact that nearly all aortic samples used for MMP analyses in IL-18Rα$^{-/-}$ had an AAA present. Thus, compared to other strains where AAA were infrequent, it is not surprising that MMP-2 activity is elevated in the strain with the highest percentage of AAAs. Therefore, to prevent potential confounding factors, future studies must separate aortas into non-aneurysmal and AAA. A key consideration that could also be investigated is the level of TIMP activity in these mice. Currently, there have been no reports exploring the relationship between IL-37 or IL-18 and TIMPs.

4.3   Limitations

Although we have extensive physiological data to support the hypotheses, there is an apparent lack of mechanistic data. To validate the findings of this study we would need to determine if they are replicable in different mouse breeds, to determine if results are limited to breeder genetics. We were also unable to determine if the IL-18$^{-/-}$ or IL-18R1$^{-/-}$ mice demonstrate any genetic compensatory mechanisms in the form of upregulation or downregulating other genes, which could have impacted on the findings. The increased sample sizes for each group planned for this year would have further consolidate the findings of the zymography data, allowing for zymography data to be separated into non-aneurysmal (NA) and AAA groups. This would have counteracted any bias that may be present due to AAA having higher amounts of MMP activity as they have already undergone extensive remodelling compared to NA.

Another limitation of this study was that quantification of plasma IL-18 by ELISA alone does not give an accurate indication of IL-18 levels. While it did provide insight into the relationship between IL-37 and IL-18, it is vital to note that some IL-18 could have been undetected. Simultaneous quantification of IL-18BP – which binds to IL-18 inhibiting detection during the ELISA assay - would need to be done to measure freely active IL-18 levels [158]. Thus, measuring plasma IL-18BP levels would validate our findings associated with IL-37Tg mice. Due to IL-18BP being upregulated in inflammatory disorders and in conjunction with its higher affinity to IL-18 than IL-18R, we would expect to see increased amounts in our Ang II-treated WT, IL-18Rα$^{-/-}$ and possibly even in the IL-37Tg mice [153]. Furthermore, a study analysing human AD has demonstrated that IL-18BP is significantly upregulated compared to human non-aneurysm aorta[141]

A future direction would be to analyse potential pathways involved with each strain, it would be essential to validate these hypotheses by examining molecular mechanisms involved, including macrophage infiltration (immunolocalisation and flow cytometry) and elastin degradation (Van Gieson's staining). Moreover, studying the molecular pathways activated within the vessels from each strain will confirm whether there are indeed pro-inflammatory or anti-inflammatory pathways. This would highlight which pathways are preferentially activated in different strains leading to the resulting AAA incidence. An attempt was made to conduct staining experiments, such as Van Geison and macrophage immunofluorescence. Due to time constraints and limited access to the lab due to the COVID-19 pandemic, these staining experiments could not be completed. Another important future direction would be to support the current study's findings by using pharmacological agents to inhibit or neutralise IL-18 or IL-18Ra and measure AAA incidence. Intervention studies where pharmacological agents administered to Ang II-infused WT mice with established AAAs could determine whether neutralising IL-18 or IL-18R could stabilise and prevent rupture of AAA. This would mimic a clinical scenario where patients with established AAA receive pharmacological treatment. Furthermore, antibodies that target the accessory proteins required for IL-18 function (IL-18Rb) or IL-37 (IL-1R8) could also be studied—determining whether the pro-inflammatory pathway or the anti-inflammatory pathway is playing a more significant role in AAA formation. Additionally, a time-course study that culls mice at day 7, day 14 and day 21 would be crucial to understand the pathophysiology further. It would demonstrate when specific inflammatory processes occur, such as ECM degradation, collagen deposition and the development of a fibrin mesh [86,187].

We have had access to a recent experiment performed in our group using single-cell transcriptomic (scRNA-seq) analysis of vehicle- and Ang II-infused mouse aorta (Appendix 5.3). This study demonstrated that in Ang II-treated WT mice, IL-18 is increased in fibroblasts and macrophages. Interestingly, the scRNA-seq also found upregulation of MMP-12 within the same macrophage clusters (Mac3 subset). MMP-12 is best known for its ability to cleave elastin; previous research has already demonstrated it to be upregulated in AAA [188]. Indeed, our scRNA-seq showed a marked increase in MMP-12 in Ang II-treated mouse aorta, predominantly being expressed by a discrete population of macrophages. This cell-specificity was expected as MMP-12 is also known as macrophage elastase due to the fact that macrophages are its principal cellular source [189]. Further analysis of the literature found no research has been conducted for the implication of IL-18, IL-37 or even the NLRP3 inflammasome in MMP-12 activation or upregulation. Future research investigating the role of MMP-12 and IL-18/IL-18Rα/IL-37 may be warranted.

4.4   Summary and conclusion

This study aimed to determine the roles of IL-18, IL-18Rα and IL-37 in the development of AAA, with the prediction that IL-18 will induce AAA formation and IL-37 will inhibit AAA. There are no pharmacological treatments available for AAA, and this study has identified potential therapeutic targets for AAAs. Specifically, this study's findings demonstrated that IL-18 plays an essential role in the initial development of AAA and pharmacological inhibition of IL-18 represents a promising strategy to treat/control AAA formation/progression. Surprisingly, targeting IL-18Rα is not a feasible preventative solution for AAA since it may interfere with IL-37 activity leading to exacerbation of AAA formation. Thus, therapies aimed

at targeting IL-18, but not the IL-18R, may represent a novel potential approach to halting the progression of AAA.

Chapter 5: Appendix

Appendix 5.1
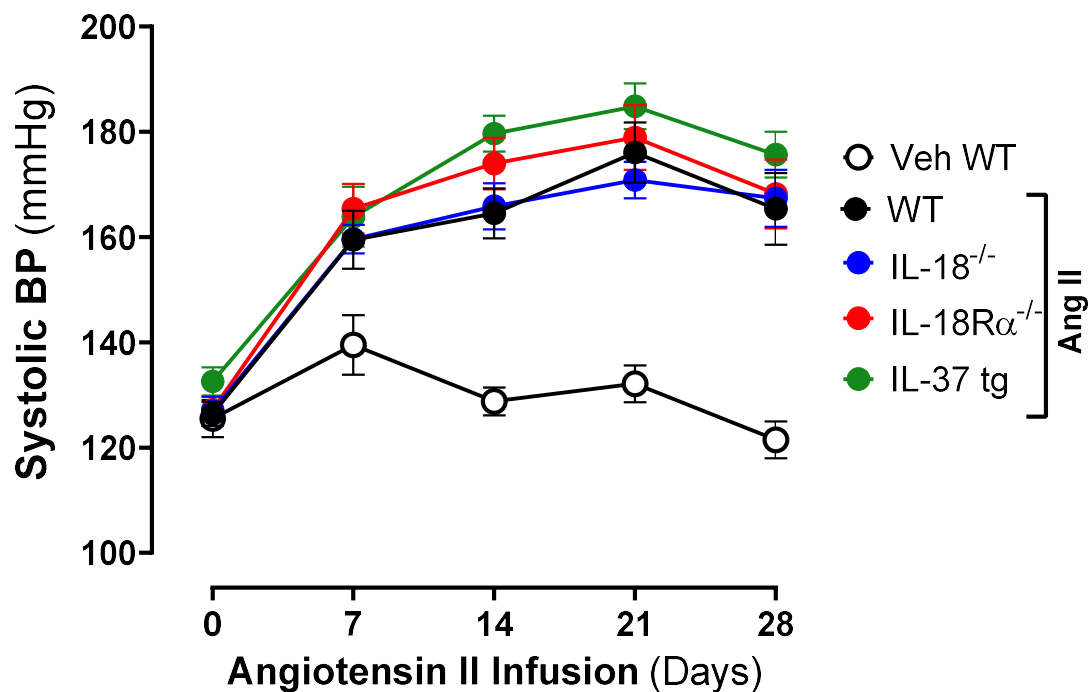
Figure 5.1. Angiotensin-II induced hypertension across all mouse strains. Systolic BP measurements of vehicle-treated WT mice (n=7), Angiotensin II-treated WT (n=19), IL-18-/- (n=20), IL-18Rα-/- (n=22) and IL-37Tg mice (n=20) over a 28-day period via tail-cuff plethysmography. Data are expressed as mean ± SEM. *P<0.05 vs Ang II-treated WT. Two-way repeated ANOVA with Tukey's multiple comparisons test.
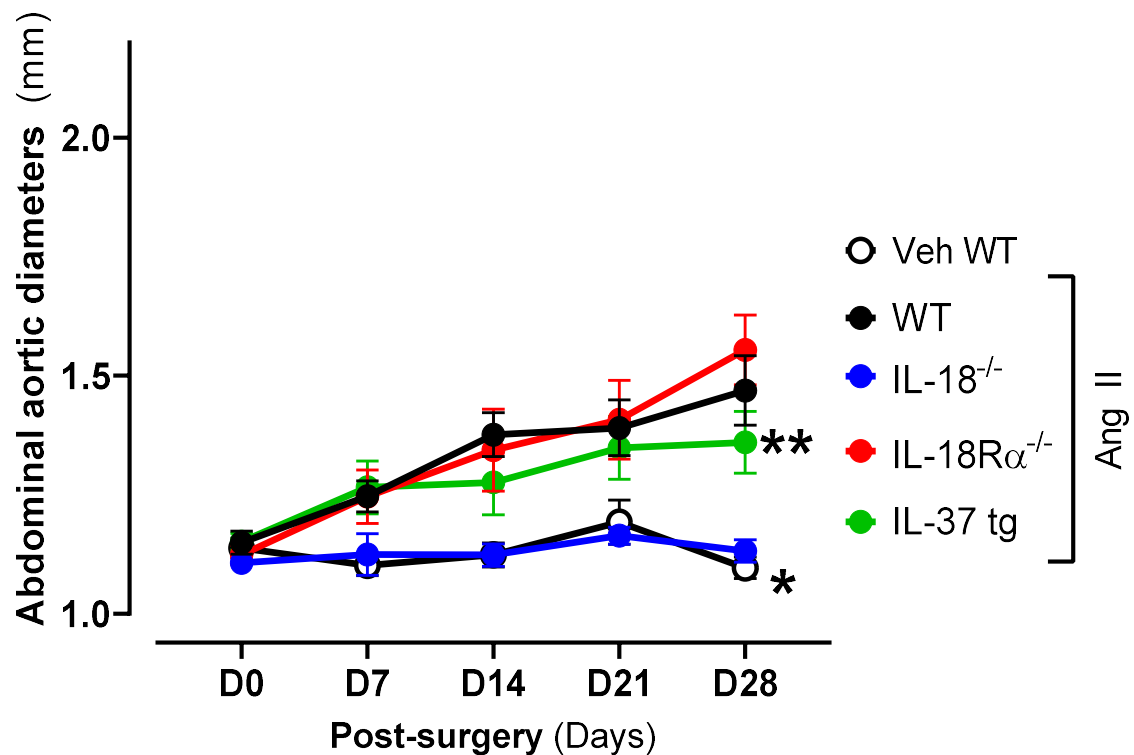
Figure 5.2. Change in aortic diameters during Ang II infusion A: Diameters of abdominal aorta of all vehicle-treated WT (n=7), Angiotensin II-treated WT (n=19), IL-18-/- (n=20), IL-18Rα-/- (n=22) and IL-37Tg mice (n=20) over a 28-day period as measured by ultrasound. Data are expressed as mean ± SEM. *P<00.5 Ang II-treated WT vs vehicle-treated WT. **P<0.05 Ang II-treated WT vs IL-18-/-. Two-way repeated ANOVA with Tukey's multiple comparisons test.

A

B

C
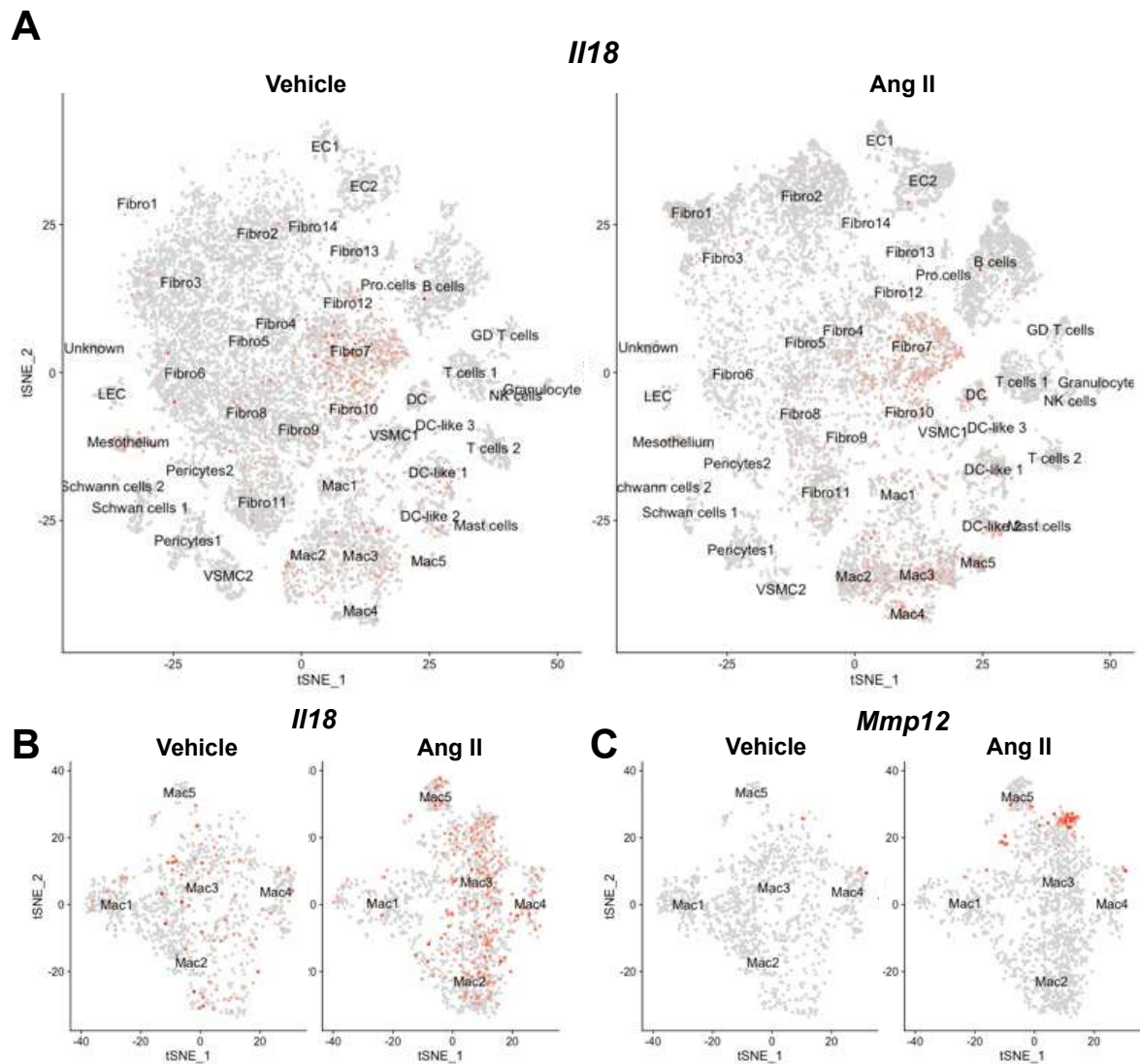
Figure 5.3. Single-cell transcriptomic analysis of vehicle- and Ang II-treated mouse aorta. A) Feature t-SNE projection of vehicle- and Ang II-treated mouse. Each dot represents a cell, and cells coloured in red denote IL-18 gene expression. The intensity of red colouring denotes relative level of expression. B) tSNE projection of macrophage subclusters in the aorta. Red denotes IL-18 expression. C) tSNE projection of macrophage subclusters in the aorta. Red denotes MMP-12 expression

6. References

1.   Daugherty A, Cassis LA. Mouse Models of Abdominal Aortic Aneurysms. *Arterioscler Thromb Vasc Biol*. 2004;24:429–434.

2.   Bridge K, Revill C, Macrae F, Bailey M, Yuldasheva N, Wheatcroft S, Butlin R, Foster R, Scott DJ, Gils A, Ariëns R. Inhibition of plasmin-mediated TAFI activation may affect development but not progression of abdominal aortic aneurysms. *PLoS One*. 2017;12:1–15.

3.   Cao RY, St. Amand T, Ford MD, Piomelli U, Funk CD. The murine angiotensin II-induced abdominal aortic aneurysm model: Rupture risk and inflammatory progression patterns. *Front Pharmacol*. 2010;JUL:1–7.

4.   Qiu Y, Yuan D, Wen J, Fan Y, Zheng T. Numerical identification of the rupture locations in patient-specific abdominal aortic aneurysmsusing hemodynamic parameters. *Comput Methods Biomech Biomed Engin*. 2018;21:1–12.

5.   Vardulaki KA, Walker NM, Day NE, Duffy SW, Ashton HA, Scott RAP. Quantifying the risks of hypertension, age, sex and smoking in patients with abdominal aortic aneurysm. *Br J Surg*. 2000;87:195–200.

6.   Lu HS, Davis FM, Daugherty A. Animal Studies Updates of Recent Aortic Aneurysm Research Recent Highlights of ATVB. *Arter Thromb Vasc Biol*. 2019;39:83–90.

7.   Upchurch GR, Schaub TA. Abdominal Aortic Aneurysm. *Am.fam.physician*. 2006;1:73:1191–204.

8.   Tambyraja AL, Chalmers RTA. Aortic aneurysms. *Surg - Oxford Int Ed*. 2009;27:342–345.

9.   Walker DI, Bloor K, Williams G, Gillie I. Inflammatory aneurysms of the abdominal aorta. *Br J Surg*. 1972;59:609–614.

10.   Golledge J. Abdominal aortic aneurysm: update on pathogenesis and medical treatments. Nat. Rev. Cardiol. 2019;16:225–242.

11.   Juvonen J, Surcel HM, Satta J, Teppo AM, Bloigu A, Syrjälä H, Airaksinen J, Leinonen M, Saikku P, Juvonen T. Elevated circulating levels of inflammatory cytokines in patients with abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. 1997;17:2843–2847.

12.   Chang CH, Fan PC, Lin CY, Yang CH, Chen YT, Chang SW, Yang HY, Jenq CC, Hung CC, Yang CW, Chen YC. Elevation of interleukin-18 correlates with cardiovascular, cerebrovascular, and peripheral vascular events. *Med (United States)*. 2015;94:e1836.

13.   Ullery BW, Hallett RL, Fleischmann D. Epidemiology and contemporary management of abdominal aortic aneurysms. *Abdom Radiol*. 2018;43:1032–1043.

14.   Lo RC, Schermerhorn ML. Abdominal aortic aneurysms in women. J. Vasc. Surg. 2016;63:839–844.

15.   Ashton HA, Gao L, Kim LG, Druce PS, Thompson SG, Scott RAP. Fifteen-year follow-up of a randomized clinical trial of ultrasonographic screening for abdominal

aortic aneurysms. *Br J Surg*. 2007;94:696–701.

16. Owens DK, Davidson KW, Krist AH, Barry MJ, Cabana M, Caughey AB, Doubeni CA, Epling JW, Kubik M, Landefeld CS, Mangione CM, Pbert L, Silverstein M, Simon MA, Tseng CW, Wong JB. Screening for Abdominal Aortic Aneurysm: US Preventive Services Task Force Recommendation Statement. JAMA - J. Am. Med. Assoc. 2019;322:2211–2218.

17. Stather PW, Dattani N, Bown MJ, Earnshaw JJ, Lees TA. International variations in AAA screening. *Eur J Vasc Endovasc Surg*. 2013;45:231–234.

18. Ali MU, Fitzpatrick-Lewis D, Miller J, Warren R, Kenny M, Sherifali D, Raina P. Screening for abdominal aortic aneurysm in asymptomatic adults. J. Vasc. Surg. 2016;64:1855–1868.

19. Scott RAP, Wilson NM, Ashton HA, Kay DN. Influence of screening on the incidence of ruptured abdominal aortic aneurysm: 5-year results of a randomized controlled study. *Br J Surg*. 1995;82:1066–1070.

20. Vardulaki KA, Walker NM, Couto E, Day NE, Thompson SG, Ashton HA, Scott RAP. Late results concerning feasibility and compliance from a randomized trial of ultrasonographic screening for abdominal aortic aneurysm. *Br J Surg*. 2002;89:861–864.

21. Li X, Zhao G, Zhang J, Duan Z, Xin S. Prevalence and trends of the abdominal aortic aneurysms epidemic in general population-a meta-analysis. *PLoS One*. 2013;8.

22. Benson RA, Poole R, Murray S, Moxey P, Loftus IM. Screening results from a large United Kingdom abdominal aortic aneurysm screening center in the context of optimizing United Kingdom National Abdominal Aortic Aneurysm Screening Programme protocols. *J Vasc Surg*. 2016;63:301–304.

23. Howard DPJ, Banerjee A, Fairhead JF, Handa A, Silver LE, Rothwell PM. Age-specific incidence, risk factors and outcome of acute abdominal aortic aneurysms in a defined population. *Br J Surg*. 2015;102:907–915.

24. Summers KL, Kerut EK, Sheahan CM, Sheahan MG. Evaluating the Prevalence of Abdominal Aortic Aneurysms in the United States through a National Screening Database. *J Vasc Surg*. 2020;

25. Lindquist Liljeqvist M, Hultgren R, Siika A, Gasser TC, Roy J. Gender, smoking, body size, and aneurysm geometry influence the biomechanical rupture risk of abdominal aortic aneurysms as estimated by finite element analysis. *J Vasc Surg*. 2017;65:1014-1021.e4.

26. Siika AK, Mattila R, Wahlberg B, Roy J. PC136 An Optimal Gender-Specific Treatment Policy for Abdominal Aortic Aneurysms Constructed Using a Markov Decision Process Model. *J Vasc Surg*. 2017;65:175S.

27. Deery SE, O'Donnell TFX, Shean KE, Darling JD, Soden PA, Hughes K, Wang GJ, Schermerhorn ML. Racial disparities in outcomes after intact abdominal aortic aneurysm repair. *J Vasc Surg*. 2018;67:1059–1067.

28. Baba T, Ohki T, Kanaoka Y, Maeda K, Ito E, Shukuzawa K, Momose M, Hara M. Risk Factor Analyses of Abdominal Aortic Aneurysms Growth in Japanese Patients. *Ann Vasc Surg*. 2019;55:196–202.

29. Kobeissi E, Hibino M, Pan H, Aune D. Blood pressure, hypertension and the risk of abdominal aortic aneurysms: a systematic review and meta-analysis of cohort studies. Eur. J. Epidemiol. 2019;34:547–555.

30. Björck M, Wanhainen A. Pathophysiology of AAA: Heredity vs Environment. 2013;

31. Wilmink ABM, Quick CRG. Epidemiology and potential for prevention of abdominal aortic aneurysm. *Br J Surg*. 1998;85:155–162.

32. Makrygiannis G, Mourmoura E, Spanos K, Roussas N, Kuivaniemi H, Sakalihasan N, Tsezou A, Giannoukas A. Risk Factor Assessment in a Greek Cohort of Patients With Large Abdominal Aortic Aneurysms. *Angiology*. 2019;70:35–40.

33. Chaikof EL, Dalman RL, Eskandari MK, Jackson BM, Lee WA, Mansour MA, Mastracci TM, Mell M, Murad MH, Nguyen LL, Oderich GS, Patel MS, Schermerhorn ML, Starnes BW. The Society for Vascular Surgery practice guidelines on the care of patients with an abdominal aortic aneurysm. *J Vasc Surg*. 2018;67:2-77.e2.

34. Carino D, Sarac TP, Ziganshin BA, Elefteriades JA. Abdominal Aortic Aneurysm: Evolving Controversies and Uncertainties. Int. J. Angiol. 2018;27:58–80.

35. Sweeting MJ, Thompson SG, Brown LC, Powell JT. Meta-analysis of individual patient data to examine factors affecting growth and rupture of small abdominal aortic aneurysms. *Br J Surg*. 2012;99:655–665.

36. R U, HA U, J W. The Aging Population and Research into Treatments for Abdominal Aortic Aneurysms. *Acta Med Okayama*. 2019;73.

37. Nakayama A, Morita H, Nagayama M, Hoshina K, Uemura Y, Tomoike H, Komuro I. Cardiac rehabilitation protects against the expansion of abdominal aortic aneurysm. *J Am Heart Assoc*. 2018;7.

38. Müller V, Miszczuk M, Althoff CE, Stroux A, Greiner A, Kuivaniemi H, Hinterseher I. Comorbidities Associated with Large Abdominal Aortic Aneurysms. *AORTA*. 2019;07:108–114.

39. Nordkvist S, Sonestedt E, Acosta S. Adherence to diet recommendations and risk of abdominal aortic aneurysm in the Malmö Diet and Cancer Study. *Sci Rep*. 2018;8.

40. Liu J, Sawada H, Howatt DA, Moorleghen JJ, Vsevolozhskaya O, Daugherty A, Lu HS. Hypercholesterolemia accelerates both the initiation and progression of angiotensin II-induced abdominal aortic aneurysms. bioRxiv. 2020;6.

41. Golledge J, Norman PE, Murphy MP, Dalman RL. Challenges and opportunities in limiting abdominal aortic aneurysm growth. J. Vasc. Surg. 2017;65:225–233.

42. Baxter BT, Matsumura J, Curci J, McBride R, Blackwelder WC, Liu X, Larson LA, Terrin ML. Non-invasive Treatment of Abdominal Aortic Aneurysm Clinical Trial (N-TA3CT): Design of a Phase IIb, placebo-controlled, double-blind, randomized clinical trial of doxycycline for the reduction of growth of small abdominal aortic aneurysm. *Contemp Clin Trials*. 2016;48:91–98.

43. Laupacis A, Barber G, Hajjar G, Hoey L, MacPhail N, Harris K, Lovell M, Jameison WG, DeRose G, Douville Y, Lortie M, Labbé R, Noël HP, Rouleau C, Dion YM, Bowyer B, Kucey D, Maggisano R, Clarke K, Etchells E, Kalman P, Nishikawa J,

Taylor L, Doobay B, Doris I, Doré C, Miller, Obrand, Poloni S, Victor G, Wellington J, Hill A, Morin JF, Verreault L, Steinmetz O, Larocque B, Redford L, Scott MK, Bisaillon S, Ghosn P, Ameli FM, Aro L, Lossing A, Cole W, Hsiang Y, Kassen B, Kaulon G, Barban K, Fratesi S, Lee H, McLean S, Deschalet P, Nault P, Kraft L, O'Brien B, Patil H, Mahon J, Powell A, Hill G, Moher D, Gray A, Moens E, Tran ML, Yetisir E, Hebert P, VanWalraven C, Ballard D, Gent M, Hallett J. Propranolol for small abdominal aortic aneurysms: Results of a randomized trial. *J Vasc Surg*. 2002;35:72–79.

44. Morris DR, Sherliker P, Clack R, Lam H, Carter J, Halliday A, Bulbulia R, Lewington S. P3230The association of blood glucose and diabetes with peripheral arterial disease involving different vascular territories: results from 628 246 people who attended vascular screening. *Eur Heart J*. 2017;38.

45. Kristensen KL, Pottegård A, Hallas J, Rasmussen LM, Lindholt JS. Metformin treatment does not affect the risk of ruptured abdominal aortic aneurysms. *J Vasc Surg*. 2017;66:768-774.e2.

46. Brady AR, Brown LC, Fowkes FGR, Greenhalgh RM, Powell JT, Ruckley C V., Thompson SG, Horrocks M, Budd J, Baird RN, Lamont P, Wilkins DC, Ashley S, Flowerdew K, Baker A, Earnshaw J, Heather B, Gibbons C, Blackett RL, Parvin SD, Harvey DR, Hedges R, Finch D, Hocken DB, Morris GE, Shearman CP, Lear P, Lewis P, Clarke RJ, Ruckley C V., Jenkins AM, Cooper GG, Engeset J, Naylor R, Stewart G, Cumming J, McCormick J, Howd A, Turner A, Harper DR, Smith RC, Chamberlain J, Jones AG, Wyatt MG, McKay AJ, Forrester JC, McCollum P, Stonebridge PA, Davidson AIG, Baker R, Forsythe JLR, Lambert D, Duncan JL, Bell PRF, Ratliff D, Callum KG, Nash JR, McPherson DS, Jenner RE, Stewart R, Armitstead PR, Barrie WW, Hamer DB, Powis S, Coen LD, Michaels J, Welsh CL, Hopkinson BR, Wenham PW, Beard J, Auckland A, Black J, Downing R, Hickey NC, Greenhalgh RM, Davies AH, Nott D, May ARL, McFarland R, Taylor P, Bradley JWP, Paes T, Cameron AEP, McIrvine A, Negus D, Taylor PR, Butler CM, Hoile RW, Pardy B, Ackroyd J, Hamilton G, Lane R, Giddings AEB, Dormandy J, Taylor R, Thomas M, Burnand KJ, Adiseshiah M, et al. Long-term outcomes of immediate repair compared with surveillance of small abdominal aortic aneurysms. *N Engl J Med*. 2002;346:1445–1452.

47. Lederle FA, Wilson SE, Johnson GR, Reinke DB, Littooy FN, Acher CW, Ballard DJ, Messina LM, Gordon IL, Chute EP, Krupski WC, Busuttil SJ, Barone GW, Sparks S, Graham LM, Rapp JH, Makaroun MS, Moneta GL, Cambria RA, Makhoul RG, Eton D, Ansel HJ, Freischlag JA, Bandyk D. Immediate repair compared with surveillance of small abdominal aortic aneurysms. *N Engl J Med*. 2002;346:1437–1444.

48. Cao P, De Rango P, Verzini F, Parlani G, Romano L, Cieri E. Comparison of surveillance versus aortic endografting for small aneurysm repair (CAESAR): Results from a randomised trial. *Eur J Vasc Endovasc Surg*. 2011;41:13–25.

49. Desai SS, Upchurch G, Vogel TR, Kray JE, Kruse RL, Khan SM, Nazzal M, Zelenock G, Qu W, Aplin B, Baldawi M, Arishi A, Stoller J. A Nationwide Comparison of Early Outcomes After Carotid Endarterectomy and Carotid Artery Stenting. Vascular; 2015.

50. Purkiss S, Keegel T, Vally H, Wollersheim D. Long-term survival following successful abdominal aortic aneurysm repair evaluated using Australian administrative data. *ANZ J Surg*. 2019;ans.15598.

51. Stroupe KT, Lederle FA, Matsumura JS, Kyriakides TC, Jonk YC, Ge L, Freischlag JA. Cost-effectiveness of open versus endovascular repair of abdominal aortic aneurysm in the OVER trial. *J Vasc Surg*. 2012;56:901-909.e2.

52. Joseph P. Long-term outcome of open or endovascular repair of abdominal aortic aneurysm. *Vasc Med*. 2010;15:515–516.

53. Malvindi PG, Van Putte BP, Heijmen RH, Schepens MAAM, Morshuis WJ. Reoperations for aortic false aneurysms after cardiac surgery. *Ann Thorac Surg*. 2010;90:1437–1443.

54. Manning MW, Cassis LA, Huang J, Szilvassy SJ, Daugherty A. Abdominal aortic aneurysms: Fresh insights from a novel animal model of the disease. *Vasc Med*. 2002;7:45–54.

55. Pappu S, Dardik A, Tagare H, Gusberg RJ. Beyond Fusiform and Saccular: A Novel Quantitative Tortuosity Index May Help Classify Aneurysm Shape and Predict Aneurysm Rupture Potential. *Ann Vasc Surg*. 2008;22:88–97.

56. Maguire EM, Pearce SWA, Xiao R, Oo AY, Xiao Q. Matrix metalloproteinase in abdominal aortic aneurysm and aortic dissection. Pharmaceuticals. 2019;12.

57. Piechota-Polanczyk A, Jozkowicz A, Nowak W, Eilenberg W, Neumayer C, Malinski T, Huk I, Brostjan C. The Abdominal Aortic Aneurysm and Intraluminal Thrombus: Current Concepts of Development and Treatment. *Front Cardiovasc Med*. 2015;2.

58. Sobocinski J, Lombardi J V., Dias N V., Berger L, Zhou Q, Jia F, Resch T, Haulon S. Volume analysis of true and false lumens in acute complicated type B aortic dissections after thoracic endovascular aortic repair with stent grafts alone or with a composite device design. *J Vasc Surg*. 2016;63:1216–1224.

59. Delbosc S, Rouer M, Alsac JM, Louedec L, Philippe M, Meilhac O, Whatling C, Michel JB. Elastase inhibitor AZD9668 treatment prevented progression of experimental abdominal aortic aneurysms. *J Vasc Surg*. 2016;63:486-492.e1.

60. Sénémaud J, Caligiuri G, Etienne H, Delbosc S, Michel J-B, Coscas R. Translational Relevance and Recent Advances of Animal Models of Abdominal Aortic Aneurysm. *Arterioscler Thromb Vasc Biol*. 2017;37:401–410.

61. Kurvers H, Veith FJ, Lipsitz EC, Ohki T, Gargiulo NJ, Cayne NS, Suggs WD, Timaran CH, Kwon GY, Rhee SJ, Santiago C. Discontinuous, staccato growth of abdominal aortic aneurysms. *J Am Coll Surg*. 2004;199:709–715.

62. Tanaka A, Hasegawa T, Chen Z, Okita Y, Okada K. A novel rat model of abdominal aortic aneurysm using a combination of intraluminal elastase infusion and extraluminal calcium chloride exposure. *J Vasc Surg*. 2009;50:1423–1432.

63. Anidjar S, Salzmann JL, Gentric D, Lagneau P, Camilleri JP, Michel JB. Elastase-induced experimental aneurysms in rats. *Circulation*. 1990;82:973–981.

64. Wang Y, Krishna S, Golledge J. The calcium chloride-induced rodent model of abdominal aortic aneurysm. Atherosclerosis. 2013;226:29–39.

65. Ling YH, Krishnan SM, Chan CT, Diep H, Ferens D, Chin-Dusting J, Kemp-Harper BK, Samuel CS, Hewitson TD, Latz E, Mansell A, Sobey CG, Drummond GR. Anakinra reduces blood pressure and renal fibrosis in one kidney/DOCA/salt-induced

hypertension. *Pharmacol Res*. 2017;116:77–86.

66. Krishnan SM, Dowling JK, Ling YH, Diep H, Chan CT, Ferens D, Kett MM, Pinar A, Samuel CS, Vinh A, Arumugam T V., Hewitson TD, Kemp-Harper BK, Robertson AAB, Cooper MA, Latz E, Mansell A, Sobey CG, Drummond GR. Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice. *Br J Pharmacol*. 2016;173:752–765.

67. C. Gong M, Liu S, Guo Z. A New Mouse Model of Aortic Aneurysm Induced by Deoxycorticosterone Acetate or Aldosterone in the Presence of High Salt. In: Aortic Aneurysm and Aortic Dissection. IntechOpen; 2020.

68. Lutshumba J, Liu S, Zhong Y, Hou T, Daugherty A, Lu H, Guo Z, Gong MC. Deletion of BMAL1 in smooth muscle cells protects mice from abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2018;38:1063–1075.

69. Liu S, Xie Z, Daugherty A, Cassis LA, Pearson KJ, Gong MC, Guo Z. Mineralocorticoid receptor agonists induce mouse aortic aneurysm formation and rupture in the presence of high salt. *Arterioscler Thromb Vasc Biol*. 2013;33:1568–1579.

70. Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic Dissection Precedes Formation of Aneurysms and Atherosclerosis in Angiotensin II-Infused, Apolipoprotein E-Deficient Mice. *Arterioscler Thromb Vasc Biol*. 2003;23:1621–1626.

71. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–12.

72. Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in ApoE-deficient mice. *Circulation*. 2001;103:448–454.

73. Guzik B, Sagan A, Ludew D, Mrowiecki W, Chwała M, Bujak-Gizycka B, Filip G, Grudzien G, Kapelak B, Zmudka K, Mrowiecki T, Sadowski J, Korbut R, Guzik TJ. Mechanisms of oxidative stress in human aortic aneurysms - Association with clinical risk factors for atherosclerosis and disease severity. *Int J Cardiol*. 2013;168:2389–2396.

74. Cassis LA, Gupte M, Thayer S, Zhang X, Charnigo R, Howatt DA, Rateri DL, Daugherty A. ANG II infusion promotes abdominal aortic aneurysms independent of increased blood pressure in hypercholesterolemic mice. *Am J Physiol Circ Physiol*. 2009;296:H1660–H1665.

75. Tilson MD. Decline of the atherogenic theory of the etiology of the abdominal aortic aneurysm and rise of the autoimmune hypothesis. *J Vasc Surg*. 2016;64:1523–1525.

76. U H, Howatt DA, Dajee M, Xie ; Xiaojie, Moorleghen J, Rateri DL, Balakrishnan A, Valdeci ;, Cunha D, Johns DG, Gutstein DE, Daugherty A. Relaxin and Matrix Metalloproteinase-9 in Angiotensin II-Induced Abdominal Aortic Aneurysms. *Circ J*. 2017;81:888–890.

77. Cassis LA, Gupte M, Thayer S, Zhang X, Charnigo R, Howatt DA, Rateri DL, Daugherty A. ANG II infusion promotes abdominal aortic aneurysms independent of

increased blood pressure in hypercholesterolemic mice. *Am J Physiol Heart Circ Physiol*. 2009;296:H1660-5.

78. Cassis LA, Helton MJ, Howatt DA, King VL, Daugherty A. Aldosterone does not mediate angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. *Br J Pharmacol*. 2005;144:443–448.

79. Rodríguez C, Martínez-González J, Raposo B, Alcudia JF, Guadall A, Badimon L. Regulation of lysyl oxidase in vascular cells: Lysyl oxidase as a new player in cardiovascular diseases. Cardiovasc. Res. 2008;79:7–13.

80. Eberson LS, Sanchez PA, Majeed BA, Tawinwung S, Secomb TW, Larson DF. Effect of lysyl oxidase inhibition on angiotensin ii-induced arterial hypertension, remodeling, and stiffness. *PLoS One*. 2015;10.

81. Remus EW, O'Donnell RE, Rafferty K, Weiss D, Joseph G, Csiszar K, Fong SFT, Taylor WR. The role of lysyl oxidase family members in the stabilization of abdominal aortic aneurysms. *Am J Physiol - Hear Circ Physiol*. 2012;303.

82. Golledge J, Norman PE. Atherosclerosis and abdominal aortic aneurysm: Cause, response, or common risk factors? Arterioscler. Thromb. Vasc. Biol. 2010;30:1075–1077.

83. Anidjar S, Salzmann JL, Gentric D, Lagneau P, Camilleri JP, Michel JB. Elastase-induced experimental aneurysms in rats. *Circulation*. 1990;82:973–981.

84. Chiou AC, Chiu B, Pearce WH. Murine Aortic Aneurysm Produced by Periarterial Application of Calcium Chloride. *J Surg Res*. 2001;99:371–376.

85. Renna NF, Garcia R, Ramirez J, Miatello RM. Vascular Repair and Remodeling: A Review. In: Physiologic and Pathologic Angiogenesis - Signaling Mechanisms and Targeted Therapy. InTech; 2017.

86. Carmo M, Colombo L, Bruno A, Corsi FRM, Roncoroni L, Cuttin MS, Radice F, Mussini E, Settembrini PG. Alteration of elastin, collagen and their cross-links in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg*. 2002;23:543–549.

87. Xu J, Shi GP. Vascular wall extracellular matrix proteins and vascular diseases. Biochim. Biophys. Acta - Mol. Basis Dis. 2014;1842:2106–2119.

88. Van Vlijmen-Van Keulen CJ, Pals G, Rauwerda JA. Familial Abdominal Aortic Aneurysm: a Systematic Review of a Genetic Background. 2002;

89. Jabłońska-Trypuć A, Matejczyk M, Rosochacki S. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem*. 2016;31:177–183.

90. Nagase H, Woessner JF. Matrix metalloproteinases. *J Biol Chem*. 1999;274:21491–4.

91. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest*. 2002;110:625–632.

92. Li Y, Wang W, Li L, Khalil RA. MMPs and ADAMs/ADAMTS inhibition therapy of abdominal aortic aneurysm. Life Sci. 2020;253:117659.

93. Stevens RRF, Grytsan A, Biasetti J, Roy J, Lindquist Liljeqvist M, Gasser TC.

Biomechanical changes during abdominal aortic aneurysm growth. *PLoS One*. 2017;12:e0187421.

94.   Jalalahmadi G, Helguera M, Mix DS, Hodis S, Richards MS, Stoner MC, Linte CA. Peak wall stress as an indicator of abdominal aortic aneurysm severity. In: IEEE Western New York Image and Signal Processing Workshop, WNYISPW 2018. Institute of Electrical and Electronics Engineers Inc.; 2018.

95.   Yan H, Zhou HF, Akk A, Hu Y, Springer LE, Ennis TL, Pham CTN. Neutrophil Proteases Promote Experimental Abdominal Aortic Aneurysm via Extracellular Trap Release and Plasmacytoid Dendritic Cell Activation. *Arterioscler Thromb Vasc Biol*. 2016;36:1660–1669.

96.   Wilson WRW, Anderton M, Schwalbe EC, Jones JL, Furness PN, Bell PRF, Thompson MM. Matrix metalloproteinase-8 and -9 are increased at the site of abdominal aortic aneurysm rupture. *Circulation*. 2006;113:438–445.

97.   Wilson WRW, Schwalbe EC, Jones JL, Bell PRF, Thompson MM. Matrix metalloproteinase 8 (neutrophil collagenase) in the pathogenesis of abdominal aortic aneurysm. *Br J Surg*. 2005;92:828–833.

98.   Cameron SJ, Russell HM, Phillip Owens A. Antithrombotic therapy in abdominal aortic aneurysm: Beneficial or detrimental? Blood. 2018;132:2619–2628.

99.   Zambrano BA, Gharahi H, Lim CY, Jaberi FA, Choi J, Lee W, Baek S. Association of Intraluminal Thrombus, Hemodynamic Forces, and Abdominal Aortic Aneurysm Expansion Using Longitudinal CT Images. *Ann Biomed Eng*. 2016;44:1502–1514.

100. Chateau H, Robin D, Uzel S, Holden L, Pourcelot P, Falala S, Estoup P, Valette J-P, Denoix J-M, Crevier-Denoix N. Biomechanical analysis of hoof landing in horses trotting at high speed and the effects of ground surfaces. *Comput Methods Biomech Biomed Engin*. 2009;12:73–75.

101. Gimbrone MA, Anderson KR, Topper James N. The Critical Role of Mechanical Forces in Blood Vessel Development, Physiology and Pathology. *J Vasc Surg Off Publ Soc Vasc Surg [and] Int Soc Cardiovasc Surgery, North Am Chapter*. 1999;29:1104–1151.

102. Riveros F, Martufi G, Gasser TC, Rodriguez-Matas JF. On the Impact of Intraluminal Thrombus Mechanical Behavior in AAA Passive Mechanics. *Ann Biomed Eng*. 2015;43:2253–2264.

103. Hans SS, Jareunpoon O, Balasubramaniam M, Zelenock GB. Size and location of thrombus in intact and ruptured abdominal aortic aneurysms. *J Vasc Surg*. 2005;41:584–588.

104. Torres N, Guevara-Cruz M, Velázquez-Villegas LA, Tovar AR. Nutrition and Atherosclerosis. Arch. Med. Res. 2015;46:408–426.

105. Sasaki S, Yasuda K, Takigami K, Yamauchi H, Shiiya N, Sakurna M. Inflammatory Abdominal Aortic Aneurysms and Atherosclerotic Abdominal Aortic Aneurysms. *Jpn Circ J*. 1997;61:231–235.

106. Peshkova IO, Schaefer G, Koltsova EK. Atherosclerosis and aortic aneurysm – is inflammation a common denominator? *FEBS J*. 2016;283:1636–1652.

107. Kasashima S, Kawashima A, Zen Y, Ozaki S, Kasashima F, Endo M, Matsumoto Y, Kawakami K. Upregulated interleukins (IL-6, IL-10, and IL-13) in immunoglobulin G4-related aortic aneurysm patients. *J Vasc Surg*. 2018;67:1248–1262.

108. Dale MA, Ruhlman MK, Baxter BT. Inflammatory Cell Phenotypes in AAAs. *Arterioscler Thromb Vasc Biol*. 2015;35:1746–1755.

109. Tang T, Boyle JR, Dixon AK, Varty K. Inflammatory abdominal aortic aneurysms. Eur. J. Vasc. Endovasc. Surg. 2005;29:353–362.

110. Furusho A, Aoki H, Ohno-Urabe S, Nishihara M, Hirakata S, Nishida N, Ito S, Hayashi M, Imaizumi T, Hiromatsu S, Akashi H, Tanaka H, Fukumoto Y. Involvement of B cells, immunoglobulins, and syk in the pathogenesis of abdominal aortic aneurysm. *J Am Heart Assoc*. 2018;7.

111. Zhou Y, Wu W, Lindholt JS, Sukhova GK, Libby P, Yu X, Shi GP. Regulatory T cells in human and angiotensin II-induced mouse abdominal aortic aneurysms. *Cardiovasc Res*. 2015;107:98–107.

112. Tsuruda T, Kato J, Hatakeyama K, Kojima K, Yano M, Yano Y, Nakamura K, Nakamura-Uchiyama F, Matsushima Y, Imamura T, Onitsuka T, Asada Y, Nawa Y, Eto T, Kitamura K. Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm. *Circ Res*. 2008;102:1368–1377.

113. Lindeman JHN, Rabelink TJ, Van Bockel JH. Immunosuppression and the abdominal aortic aneurysm: Doctor jekyll or mister hyde? *Circulation*. 2011;124.

114. Alsiraj Y, Thatcher SE, Charnigo R, Chen K, Blalock E, Daugherty A, Cassis LA. Female mice with an XY sex chromosome complement develop severe Angiotensin II-induced abdominal aortic aneurysms. *Circulation*. 2017;135:379–391.

115. Isoda K, Akita K, Kitamura K, Sato-Okabayashi Y, Kadoguchi T, Isobe S, Ohtomo F, Sano M, Shimada K, Iwakura Y, Daida H. Inhibition of interleukin-1 suppresses angiotensin II-induced aortic inflammation and aneurysm formation. *Int J Cardiol*. 2018;270:221–227.

116. Ramella M, Bernardi P, Fusaro L, Manfredi M, Casella F, Porta CM, Nicolai L, Galeazzi E, Boldorini R, Settembrini AM, Settembrini P, Marengo E, Cannas M, Boccafoschi F. Relevance of inflammation and matrix remodeling in abdominal aortic aneurysm (AAA) and popliteal artery aneurysm (PAA) progression. *Am J Transl Res*. 2018;10:3265–3275.

117. Nagashima H, Aoka Y, Sakomura Y, Uto K, Sakuta A, Aomi S, Kurosawa H, Hagiwara N, Kawana M, Kasanuki H. Matrix metalloproteinase 2 is suppressed by trapidil, a CD40-CD40 ligand pathway inhibitor, in human abdominal aortic aneurysm wall. *J Vasc Surg*. 2004;39:447–453.

118. Silvestre-Roig C, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. Nat. Rev. Cardiol. 2020;17:327–340.

119. Plana E, Oto J, Medina P, Fernández-Pardo Á, Miralles M. Novel contributions of neutrophils in the pathogenesis of abdominal aortic aneurysm, the role of neutrophil extracellular traps: A systematic review. Thromb. Res. 2020;194:200–208.

120. Michel JB, Martin-Ventura JL, Egido J, Sakalihasan N, Treska V, Lindholt J, Allaire E, Thorsteinsdottir U, Cockerill G, Swedenborg J. Novel aspects of the pathogenesis of

aneurysms of the abdominal aorta in humans. Cardiovasc. Res. 2011;90:18–27.

121. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, Roelofs KJ, Woodrum DT, Ennis TL, Henke PK, Stanley JC, Thompson RW, Upchurch GR. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. *Circulation*. 2005;112:232–40.

122. Sagan A, Mikolajczyk TP, Mrowiecki W, MacRitchie N, Daly K, Meldrum A, Migliarino S, Delles C, Urbanski K, Filip G, Kapelak B, Maffia P, Touyz R, Guzik TJ. T Cells Are Dominant Population in Human Abdominal Aortic Aneurysms and Their Infiltration in the Perivascular Tissue Correlates With Disease Severity. *Front Immunol*. 2019;10.

123. Sagan A, Mikolajczyk TP, Mrowiecki W, MacRitchie N, Daly K, Meldrum A, Migliarino S, Delles C, Urbanski K, Filip G, Kapelak B, Maffia P, Touyz R, Guzik TJ. T Cells Are Dominant Population in Human Abdominal Aortic Aneurysms and Their Infiltration in the Perivascular Tissue Correlates With Disease Severity. *Front Immunol*. 2019;10.

124. Zhou H, Yan H, Cannon JL, Springer LE, Green JM, Pham CTN. CD43-Mediated IFN-γ Production by CD8 + T Cells Promotes Abdominal Aortic Aneurysm in Mice . *J Immunol*. 2013;190:5078–5085.

125. Berger A. Th1 and Th2 responses: What are they? *BMJ*. 2000;321:424.

126. Xiong W, Zhao Y, Prall A, Greiner TC, Baxter BT. Key Roles of CD4 + T Cells and IFN-γ in the Development of Abdominal Aortic Aneurysms in a Murine Model . *J Immunol*. 2004;172:2607–2612.

127. Téo FH, De Oliveira RTD, Villarejos L, Mamoni RL, Altemani A, Menezes FH, Blotta MHSL. Characterization of CD4 + T cell subsets in patients with abdominal aortic aneurysms. *Mediators Inflamm*. 2018;2018.

128. Dale MA, Xiong W, Carson JS, Suh MK, Karpisek AD, Meisinger TM, Casale GP, Baxter BT. Elastin-Derived Peptides Promote Abdominal Aortic Aneurysm Formation by Modulating M1/M2 Macrophage Polarization. *J Immunol*. 2016;196:4536–4543.

129. Wang Y, Ait-Oufella H, Herbin O, Bonnin P, Ramkhelawon B, Taleb S, Huang J, Offenstadt G, Combadière C, Rénia L, Johnson JL, Tharaux PL, Tedgui A, Mallat Z. TGF-β activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II-infused mice. *J Clin Invest*. 2010;120:422–432.

130. Yin M, Zhang J, Wang Y, Wang S, Böckler D, Duan Z, Xin S. Deficient CD4+CD25+ T regulatory cell function in patients with abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2010;30:1825–1831.

131. Hayashi T, Sasaki N, Yamashita T, Mizoguchi T, Emoto T, Amin HZ, Yodoi K, Matsumoto T, Kasahara K, Yoshida N, Tabata T, Kitano N, Fukunaga A, Nishigori C, Rikitake Y, Hirata K ichi. Ultraviolet B exposure inhibits angiotensin II-induced abdominal aortic aneurysm formation in mice by expanding CD4+Foxp3+ regulatory T cells. *J Am Heart Assoc*. 2017;6.

132. Yodoi K, Yamashita T, Sasaki N, Kasahara K, Emoto T, Matsumoto T, Kita T, Sasaki Y, Mizoguchi T, Sparwasser T, Hirata KI. Foxp3+ regulatory T cells play a protective role in Angiotensin II-induced aortic aneurysm formation in mice. *Hypertension*.

2015;65:889–895.

133. Harada T, Yoshimura K, Yamashita O, Ueda K, Morikage N, Sawada Y, Hamano K. Focal Adhesion Kinase Promotes the Progression of Aortic Aneurysm by Modulating Macrophage Behavior. *Arterioscler Thromb Vasc Biol*. 2017;37:156–165.

134. Raffort J, Lareyre F, Clément M, Hassen-Khodja R, Chinetti G, Mallat Z. Monocytes and macrophages in abdominal aortic aneurysm. *Nat Rev Cardiol*. 2017;14:457–471.

135. Liu Y, Gao X, Miao Y, Wang Y, Wang H, Cheng Z, Wang X, Jing X, Jia L, Dai L, Liu M, An L. NLRP3 regulates macrophage M2 polarization through up-regulation of IL-4 in asthma. *Biochem J*. 2018;475:1995–2008.

136. Ley K. M1 Means Kill; M2 Means Heal. *J Immunol*. 2017;199:2191–2193.

137. Chen L, Gao B, Zhang Y, Lu H, Li X, Pan L, Yin L, Zhi X. PAR2 promotes M1 macrophage polarization and inflammation via FOXO1 pathway. *J Cell Biochem*. 2019;120:9799–9809.

138. Li H, Bai S, Ao Q, Wang X, Tian X, Li X, Tong H, Hou W, Fan J. Modulation of Immune-Inflammatory Responses in Abdominal Aortic Aneurysm: Emerging Molecular Targets. 2018;

139. McCormick ML, Gavrila D, Weintraub NL. Role of Oxidative Stress in the Pathogenesis of Abdominal Aortic Aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:461–469.

140. Boytard L, Spear R, Chinetti-Gbaguidi G, Acosta-Martin AE, Vanhoutte J, Lamblin N, Staels B, Amouyel P, Haulon S, Pinet F. Role of proinflammatory CD68+ mannose receptor-macrophages in peroxiredoxin-1 expression and in abdominal aortic aneurysms in humans. *Arterioscler Thromb Vasc Biol*. 2013;33:431–438.

141. Hu H, Zhang G, Hu H, Liu W, Liu J, Xin S, Zhao X, Han L, Duan L, Huang X, Chang C. Interleukin-18 Expression Increases in the Aorta and Plasma of Patients with Acute Aortic Dissection. *Mediators Inflamm*. 2019;2019:1–10.

142. Wilson JS, Virag L, Di Achille P, Karšaj I, Humphrey JD. Biochemomechanics of intraluminal thrombus in abdominal aortic aneurysms. J. Biomech. Eng. 2013;135:0210111.

143. Schroder K, Tschopp J. The Inflammasomes. Cell. 2010;140:821–832.

144. Krishnan SM, Sobey CG, Latz E, Mansell A, Drummond GR. IL-1β and IL-18: Inflammatory markers or mediators of hypertension? *Br J Pharmacol*. 2014;171:5589–5602.

145. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature*. 2009;459:996–999.

146. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A*. 2008;105:9035–40.

147. Cruz CM, Rinna A, Forman HJ, Ventura ALM, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion

of proinflammatory cytokines in macrophages. *J Biol Chem*. 2007;282:2871–9.

148. Pétrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ*. 2007;14:1583–1589.

149. Gross O, Poeck H, Bscheider M, Dostert C, Hannesschläger N, Endres S, Hartmann G, Tardivel A, Schweighoffer E, Tybulewicz V, Mocsai A, Tschopp J, Ruland J. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. 2009;459:433–436.

150. Tiemi Shio M, Eisenbarth SC, Savaria M, Vinet AF, Bellemare M-J, Harder KW, Sutterwala FS, Bohle DS, Descoteaux A, Flavell RA, Olivier M. Malarial Hemozoin Activates the NLRP3 Inflammasome through Lyn and Syk Kinases. *PLoS Pathog*. 2009;5:e1000559.

151. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT. The NALP3 inflammasome is involved in the innate immune response to amyloid-β. *Nat Immunol*. 2008;9:857–865.

152. Meher AK, Johnston WF, Lu G, Pope NH, Bhamidipati CM, Harmon DB, Su G, Zhao Y, McNamara CA, Upchurch GR, Ailawadi G. B2 cells suppress experimental abdominal aortic aneurysms. *Am J Pathol*. 2014;184:3130–3141.

153. Mühl H, Bachmann M. IL-18/IL-18BP and IL-22/IL-22BP: Two interrelated couples with therapeutic potential. Cell. Signal. 2019;63:109388.

154. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. *Front Immunol*. 2013;4:1–10.

155. Schirmer B, Wedekind D, Glage S, Neumann D. Deletion of IL-18 expression ameliorates spontaneous kidney failure in MRLlpr Mice. *PLoS One*. 2015;10.

156. Lieben L. Free IL-18 causes macrophage activation syndrome. *Nat Rev Rheumatol*. 2018;

157. Crayne CB, Albeituni S, Nichols KE, Cron RQ. The immunology of macrophage activation syndrome. Front. Immunol. 2019;10:119.

158. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. Front. Immunol. 2013;4.

159. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. 2009;183:787–91.

160. Kim S-H, Eisenstein M, Reznikov L, Fantuzzi G, Novick D, Rubinstein M, Dinarello CA. Structural Requirements of Six Naturally Occurring Isoforms of the IL-18 Binding Protein to Inhibit IL-18. 2000.

161. Novick D, Schwartsburd B, Pinkus R, Suissa D, Belzer I, Sthoeger Z, Keane WF, Chvatchko Y, Kim SH, Fantuzzi G, Dinarello CA, Rubinstein M. A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18. *Cytokine*. 2001;14:334–342.

162. Dinarello CA, Bufler P. Interleukin-37. *Semin Immunol*. 2013;25:466–468.

163. Gao W, Kumar S, Lotze MT, Hanning C, Robbins PD, Gambotto A. Innate immunity mediated by the cytokine IL-1 homologue 4 (IL-1H4/IL-1F7) induces IL-12-dependent adaptive and profound antitumor immunity. *J Immunol*. 2003;170:107–13.

164. Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. *Nat Publ Gr*. 2010;11.

165. Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol*. 2010;11:1014–1022.

166. Lewis EC, Dinarello CA. Responses of IL-18- and IL-18 receptor-deficient pancreatic islets with convergence of positive and negative signals for the IL-18 receptor. *Proc Natl Acad Sci U S A*. 2006;103:16852–16857.

167. Wu D, Ren P, Zheng Y, Zhang L, Xu G, Xie W, Lloyd EE, Zhang S, Zhang Q, Curci JA, Coselli JS, Milewicz DM, Shen YH, LeMaire SA. NLRP3 (Nucleotide oligomerization domain-like receptor family, pyrin domain containing 3)-caspase-1 inflammasome degrades contractile proteins implications for aortic biomechanical dysfunction and aneurysm and dissection formation. *Arterioscler Thromb Vasc Biol*. 2017;37:694–706.

168. Krishnan SM, Ling YH, Huuskes BM, Ferens DM, Saini N, Chan CT, Diep H, Kett MM, Samuel CS, Kemp-Harper BK, Robertson AAB, Cooper MA, Peter K, Latz E, Mansell AS, Sobey CG, Drummond GR, Vinh A. Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage, and dysfunction in salt-sensitive hypertension. *Cardiovasc Res*. 2018;61.

169. Xiaoyu Wu SC, Markus Wortmann, Maani Hakimi JZ, Dittmar Böckler and Susanne Dihlmann. Sex- and Disease-Specific Inflammasome Signatures in Circulating Blood Leukocytes of Patients with Abdominal Aortic Aneurysm. *Mol Med*. 2016;22:508–518.

170. Gonzalez-Hidalgo C, De Haro J, Bleda S, Cañibano C, Michel I, Acin F. Differential mRNA expression of inflammasome genes NLRP1 and NLRP3 in abdominal aneurysmal and occlusive aortic disease. *Ther Adv Cardiovasc Dis*. 2018;12:123–129.

171. Wortmann M, Skorubskaya E, Peters AS, Hakimi M, Böckler D, Dihlmann S. Necrotic cell debris induces a NF-κB-driven inflammasome response in vascular smooth muscle cells derived from abdominal aortic aneurysms (AAA-SMC). *Biochem Biophys Res Commun*. 2019;511:343–349.

172. Wang F, Guan M, Wei L, Yan H. IL-18 promotes the secretion of matrix metalloproteinases in human periodontal ligament fibroblasts by activating NF-κB signaling. *Mol Med Rep*. 2019;19:703–710.

173. Wang F, Guan M, Wei L, Yan H. IL-18 promotes the secretion of matrix metalloproteinases in human periodontal ligament fibroblasts by activating NF-κB signaling. *Mol Med Rep*. 2019;19:703–710.

174. Daugherty A, Manning MW, Cassis LA. Antagonism of AT2 receptors augments Angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. *Br J Pharmacol*. 2001;134:865–870.

175. Takagi H, Umemoto T, ALICE (All-Literature Investigation of Cardiovascular

Evidence) Group. Association of Hypertension with Abdominal Aortic Aneurysm Expansion. *Surgery*. 2017;39:74–89.

176. Suehiro C, Suzuki J, Hamaguchi M, Takahashi K, Nagao T, Sakaue T, Uetani T, Aono J, Ikeda S, Okura T, Okamura H, Yamaguchi O. Deletion of interleukin-18 attenuates abdominal aortic aneurysm formation. *Atherosclerosis*. 2019;289:14–20.

177. Van Kuijk JP, Flu WJ, Witteveen OP, Voute M, Bax JJ, Poldermans D. The influence of statins on the expansion rate and rupture risk of abdominal aortic aneurysms. J. Cardiovasc. Surg. (Torino). 2009;50:599–609.

178. Liu CL, Ren J, Wang Y, Zhang X, Sukhova GK, Liao M, Santos M, Luo S, Yang D, Xia M, Inouye K, Hotamisligil GS, Lu G, Upchurch GR, Libby P, Guo J, Zhang J, Shi GP. Adipocytes promote interleukin-18 binding to its receptors during abdominal aortic aneurysm formation in mice. *Eur Heart J*. 2020;41:2456–2468.

179. Liu J, Lin J, He S, Wu C, Wang B, Liu J, Duan Y, Liu T, Shan S, Yang K, Dong N, Ji Q, Huang K, Li D. Transgenic Overexpression of IL-37 Protects Against Atherosclerosis and Strengthens Plaque Stability. *Cell Physiol Biochem*. 2018;45:1034–1050.

180. Ji Q, Meng K, Yu K, Huang S, Huang Y, Min X, Zhong Y, Wu B, Liu Y, Nie S, Zhang J, Zhou Y, Zeng Q. Exogenous interleukin 37 ameliorates atherosclerosis via inducing the Treg response in ApoE-deficient mice. *Sci Rep*. 2017;7.

181. Xiong W, MacTaggart J, Knispel R, Worth J, Persidsky Y, Baxter BT. Blocking TNF-alpha attenuates aneurysm formation in a murine model. *J Immunol*. 2009;183:2741–6.

182. Nold-Petry CA, Nold MF, Nielsen JW, Bustamante A, Zepp JA, Storm KA, Hong JW, Kim SH, Dinarello CA. Increased cytokine productionin interleukin-18 receptor α-deficient cells is associated with dysregulation of suppressors of cytokine signaling. *J Biol Chem*. 2009;284:25900–25911.

183. Sugiyama M, Kinoshita K, Kishimoto K, Shimazu H, Nozaki Y, Ikoma S, Funauchi M. Deletion of IL-18 receptor ameliorates renal injury in bovine serum albumin-induced glomerulonephritis. *Clin Immunol*. 2008;128:103–108.

184. Wang J, Sun C, Gerdes N, Liu C, Liao M, Liu J, Shi MA, He A, Zhou Y, Sukhova GK, Chen H, Cheng XW, Kuzuya M, Murohara T, Zhang J, Cheng X, Jiang M, Shull GE, Rogers S, Yang CL, Ke Q, Jelen S, Bindels R, Ellison DH, Jarolim P, Libby P, Shi GP. Interleukin 18 function in atherosclerosis is mediated by the interleukin 18 receptor and the Na-Cl co-transporter. *Nat Med*. 2015;21:820–826.

185. Xie S-L, Chen Y-Y, Zhang H-F, Deng B-Q, Shu X-R, Su Z-Z, Lin Y-Q, Nie R-Q, Wang J-F. Interleukin 18 and extracellular matrix metalloproteinase inducer cross-regulation: implications in acute myocardial infarction. *Transl Res*. 2015;165:387–95.

186. Abraham M, Shapiro S, Lahat N, Miller A. The role of IL-18 and IL-12 in the modulation of matrix metalloproteinases and their tissue inhibitors in monocytic cells. Int. Immunol. 2002;14:1449–1457.

187. Fontaine V, Jacob MP, Houard X, Rossignol P, Plissonnier D, Angles-Cano E, Michel JB. Involvement of the mural thrombus as a site of protease release and activation in human aortic aneurysms. *Am J Pathol*. 2002;161:1701–1710.

188. Longo GM, Buda SJ, Fiotta N, Xiong W, Griener T, Shapiro S, Baxter BT. MMP-12

has a role in abdominal aortic aneurysms in mice. *Surgery*. 2005;137:457–462.

189. Curci JA, Liao S, Huffman MD, Shapiro SD, Thompson RW. Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. *J Clin Invest*. 1998;102:1900–1910.