University of Glasgow

Determining the Role of IL-33 and ST2 in Hypertension and Vascular Dysfunction

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BSc (Hons)

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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December 2023

This work was supported by the European Research Council

[grant number: ERC-CoG-726318]

Abstract

Cardiovascular disease (CVD) has long been the leading cause of global morbidity and mortality, largely due to deaths caused by stroke, heart failure (HF), hypertensive heart disease, peripheral arterial disease. Hypertension is defined as levels of blood pressure at which the benefits of treatment unequivocally outweigh the risk of treatment, by which systolic blood pressure values are ≥ 140 mmHg and/or diastolic blood pressure values are 90 ≥ mmHg in younger, middle-aged, and older people. Hypertension is characterised by cardiac and vascular irregularities in structure and function, causing end-organ vascular, heart, kidney, and additional end-organ damage, triggering cardiovascular complications such as myocardial infarction and stroke, heart, and renal failure.

Interleukin-33 (IL-33), a member of the IL-1 cytokine family, is believed to play essential roles in different cardiovascular diseases by binding to its specific receptor suppressor of tumorigenicity 2 (ST2). IL-33, through ST2, has demonstrated a capacity to influence cells of both the vascular and immune systems in healthy conditions and various cardiovascular pathologies, including atherosclerosis. There is curiosity about its potential role in hypertension and how it may be viewed as a potential therapeutic target. While the influence of IL-33 and ST2 has been examined in other aspects of CVD, little is known about its potential role in hypertension pathology. Thus, this thesis aims to test my hypothesis that the IL-33/ST2 axis plays a role in hypertension pathology. I propose that IL-33 is an alarmin molecule upon cell injury and signals the immune system in hypertension and associated target organ damage.

In the context of hypertension, I have demonstrated the upregulation of IL-33 and ST2 in several key organs involved in the disease, particularly the aorta. In addition, I have shown that IL-33 and ST2 are predominantly localised in the vascular endothelium and media of Angiotensin II-induced hypertensive mouse aorta. Furthermore, through the examination of healthy and vascular disease pathology, expression of IL-33 was confined to non-immune cells, and ST2 had key expression in immune cells of the aorta, with marked expression in the ILC2s

(type 2 innate lymphoid cells) and mast cells. These data suggest that IL-33 and ST2 may influence hypertension pathology.

Using an inducible hypertension model in global IL-33 knock-out (IL-$33^{-/-}$) and ST2 knock-out mice ($ST2^{-/-}$) allowed us to assess the functional role of IL-33 and ST2 individually. Upon Ang II-induced hypertension, $ST2^{-/-}$ mice presented protection from periaortic collagen accumulation, a hallmark of hypertension pathology and regulated vascular dysfunction independently of blood pressure. Basally in the heart and in Ang II-induced hypertension, ST2 is crucial in promoting cardioprotective mechanisms. Abolishment of ST2 signalling in mice leads to marked hypertrophy following Ang II hypertension. Conversely, cardiac fibrosis, a common pathophysiological process in hypertension, may be inhibited by IL-33 in our hypertension model. Our results indicate a pleiotropic role of the IL-33/ST2 axis in hypertension.

Single-cell RNA sequencing (scRNA-Seq) data analysis performed in samples of heart transplant patients revealed that IL-33 is principally expressed by the vascular endothelial cells, fibromyocytes and fibroblasts in normal and diseased vessels. Additionally, in the human hypertensive mammary arteries, the expression of IL-33 was localised in endothelial cells. The OLINK dataset in the UK Biobank cohort depicted a positive correlation between plasma ST2 and BP parameters. We, therefore, suggest that sST2 (soluble ST2) could represent a potential risk factor for hypertension and may represent a promising novel marker for the prediction of hypertension and associated cardiovascular damage.

Overall, this study suggests a pleiotropic role for the IL-33/ST2 axis in hypertension. The actions of IL-33 are potentially mediated through non-immune cells, including vascular smooth muscle cells and ST2 through immune cells. This work provides insight into the functional effects of IL-33 and ST2 in hypertension and forms the basis for further work on uncovering its potential as a therapeutic target in hypertension and other vascular disease.

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1. Szczepaniak, P., Siedlinski, M., Hodorowicz-Zaniewska, D., Nosalski, R., Mikolajczyk, T. P., Dobosz, A. M., Dikalova, A., Dikalov, S., Streb, J., Gara, K., Basta, P., Krolczyk, J., Sulicka-Grodzicka, J., Jozefczuk, E., Dziewulska, A., **Saju, B**., Laksa, I., Chen, W., Dormer, J., Tomaszewski, M., Maffia, P., Czesnikiewicz-Guzik, M., Crea, F., Dobrzyn, A., Moslehi, J., Grodzicki, T., Harrison, D. G. & Guzik, T. J. 'Breast Cancer Chemotherapy Induces Vascular Dysfunction And Hypertension Through A Nox4-Dependent Mechanism.' *J Clin Invest, 2022;* 132.

2. Nosalski, R., Mikolajczyk, T., Siedlinski, M., **Saju, B**., Koziol, J., Maffia, P. & Guzik, T. J. Nox1/4 Inhibition Exacerbates Age Dependent Perivascular Inflammation And Fibrosis In A Model Of Spontaneous Hypertension. *Pharmacol Res,2020;* 161**,** 105235.

3. Józefczuk, E., Nosalski, R., **Saju, B**., Crespo, E., Szczepaniak, P., Guzik, T. J. & Siedlinski, M. Cardiovascular Effects Of Pharmacological Targeting Of Sphingosine Kinase 1. *Hypertension, 2020;* 75**,** 383-392

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4. **Saju B**, Nosalski R, Booth E, Maffia P, Guzik T J. The role of IL-33 and ST2 in the modulation of target organ damage in Angiotensin II-dependent hypertension. BAS 2023.

Acknowledgements

There are so many people I would like to thank for their help and support throughout my PhD. I will try my best to remember them all. However, I am sure I might forget a few. To those I may have not mentioned, know that I appreciate all the help you have given me in making my PhD possible and more joyful.

To Professor Tomasz Guzik- I am sincerely thankful for your continuous supervision, support, and direction throughout my PhD, which has been vital in accomplishing much of my academic career. Your constant encouragement, even when things don't turn out the way I had hoped and giving me reassurance and guidance has been a pillar for my accomplishments. Thank you for enabling me to advance in academia through your optimistic outlook on my project, believing in me and giving me confidence that I can approach you with anything. It has been a pleasure, and I look forward to continuing our work together.

To Professor Pasquale Maffia, I immensely enjoyed working together and discussing my work, especially during lab meetings. Your direction has been incredibly beneficial to my project. I look forward to our future collaborations together. Thank you for your supervision throughout my PhD.

I want to acknowledge Doctor Ryszard Nosalski for his fantastic support and input during my project. Thank you for your constant and very direct help and support with many of my experiments, including providing the data for the proteome profiler cytokine assay and analysis, ongoing help with wire myography set-up, mini-pump implantation surgery and more. You are an exceptional academic researcher, diligent and hardworking, and it is a pleasure and honour to learn from you. I can't say how thankful I am for all your scientific mentorship, advice, and encouragement. Furthermore, I would also like to especially thank Professor Mateusz Siedliński for his help in providing the assessments of the UK Biobank OLINK IL-33 and ST2 levels.

Special mention to the whole Guzik Lab group, especially Emma Booth, who has helped me with several aspects of my PhD project, including surgeries, BP monitoring

and more, along with Dr Eva Crespo, for help with single-cell RNA Seq analysis of her data and allowing me to use part of her project's data for my thesis. Moreover, I would like to thank her for keeping me sane in and outside the lab during my PhD's highs and lows; I am incredibly thankful from the bottom of my heart. Eman and Malvika, please know that I am grateful for all the big and small help you guys gave me (of which there are many) throughout my time in the group. To everyone who was with me in the office during the last four years in room 348, specifically Rashida and Eleanor, please know that I am incredibly grateful for all the laughs we shared; it helped me love my PhD more, and I will cherish the memories. To Dr Eleanor Murry, thank you for allowing me to use your clinical samples from the InflamaTENSION project. Also, a huge thank you to the Maffia lab group, especially Dr Lucy McShane for all the help with the single-cell RNA Seq analysis of two different datasets, Dr Danilla Gurgone for all her advice, Shutong Gu, Dr Neil MacRitchie, Asma Oumkaltoum Rezig and all the others for your encouragement and help during the last few years.

While I have worked with an impressive group of people during my PhD, I have also gained remarkable support from my friends and family. I can't forget and thank my friends, especially Preethy, Regina, Steph, Shebin, and Sam, who supported and strengthened me during my PhD, especially while writing my thesis. You all are indeed a gift to me. Thank you to all the youths I mentor, especially Sarah, Ailsah, Joel, Adharsh and Alvin, who have regularly kept me in their thoughts; what a blessing you all are!

To my loving and wonderful parents, I am ever so humbled by all you have done for me throughout the years through everything, providing me with advice and guidance on all matters. To my dear brother, Sam, I will never forget everything you have done for me, making me laugh through every hardship that has come my way. Giving me perspective about why I do what I do and helping me to remember the good times. I am blessed indeed. To my wonderful grandparents and relatives for being near even though we are so far away.

Author's declaration

I declare that, except where reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Name: Blessy Saju

Signature:

Date: 30th November 2023

Abbreviations

ACE	Angiotensin-Converting Enzyme
ACh	Acetylcholine
AdvECs	Adventitial Endothelial Cells
Ang II	Angiotensin II
Apo	Apolipoprotein
ARBs	Angiotensin Receptor Blockers
Areg	Amphiregulin
BAFF-R	B Cell–Activating Factor Receptor
BMP	Bone Morphogenetic Protein
BP	Blood Pressure
CAD	Coronary Artery Disease
C3	Complement Factor 3
CCBs	Calcium Channel Blockers
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COX_{-2}	Cyclooxygenase- 2
CRP	C-Reactive Protein
CVD	Cardiovascular Diseases
DAB	‘3-3’-Diaminobenzidine
DBP	Diastolic Blood Pressure
DC	Dendritic Cells
DOCA	Deoxycorticosterone Acetate
EC	Endothelial Cell
EH	Essential Hypertension
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinase
ET1	Endothelin 1
GVHD	Graft-Versus-Host Disease
HEVs	High Endothelial Venules
HTN	Hypertension

HF	Heart Failure
HRP	Horseradish Peroxidase
IFN-γ	Interferon Gamma
IL-33	Interleukin-33
IL1RL1	Interleukin 1 Receptor Like 1
ILC2s	Type 2 Innate Lymphoid Cells
IMA	Internal Mammary Artery
IPAH	Idiopathic Pulmonary Arterial Hypertension
IRAK	IL-1R-Associated Kinase
IRF	IFN Regulatory Factor
JNK	C-Jun N-Terminal Kinase
KPSS	Potassium Physiological Salt Solution
MAPKs	Mitogen-Activated Protein Kinases
MDSC	Myeloid-Derived Suppressor Cells
MIF	Myocardial Interstitial Fibrosis
MSNA	Muscle Sympathetic Nerve Activity
MyD88	Myeloid Differentiation 88
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor Kappa-B
NK	Natural Killer
NKT	Natural Killer T
NO	Nitric Oxide
Ox-LDL	Oxidised Low-Density Lipoprotein
PBS	Phospho-Buffered Saline
PCA	Principal Component Analysis
PVAT	Perivascular Adipose Tissue
QC	Quality Control
RAAS	Renin-Angiotensin-Aldosterone System
RAG 1	Recombination Activating Gene 1
RIPA	Radioimmunoprecipitation
ROI	Region Of Interest
SBP	Systolic Blood Pressure

scRNA-seq	Single-Cell RNA Sequencing
SEM	Standard Error of The Mean
SNP	Sodium Nitroprusside
SNS	Sympathetic Nervous System
sST2	Soluble ST2
ST2	Suppressor Of Tumorigenicity 2
TAC	Traverse Aortic Constriction
TBHP	Tert-Butyl Hydroperoxide
TBS	Tris-Buffered Saline
Tfh	Follicular Helper T Cells
TGF	Tumour Growth Factor
T_H2	T Helper 2
TNF	Tumour Necrosis Factor
TRAF6	TNFR-Associated Factor 6
Tregs	Regulatory T Cells
UMAP	Uniform Manifold Approximation and Projection
VSMC	Vascular Smooth Muscle Cells
WGA	Wheat Germ Agglutinin
WT	Wild Type
γ/δ	Gamma/Beta
5-HT	Serotonin

Chapter 1 Introduction

1.1 Impact of Cardiovascular Disease

Cardiovascular diseases (CVDs) have collectively remained the leading cause of death worldwide and one of the severe health problems worldwide. CVDs are an umbrella term for disease classes involving the heart and circulatory system. This includes stroke, heart failure (HF), hypertensive heart disease, peripheral arterial disease, and several vascular and cardiac problems (Amini et al., 2021).

More than half a billion people worldwide continue to be affected by cardiovascular diseases, almost doubling from 271 million cases recorded in 1990 to 523 million in 2019 (Roth et al., 2020), and the number of deaths steadily increased from 12.1 million in 1990, reaching 18.6 million by 2019 (Roth et al., 2020). By 2021, heart and circulatory diseases hit an estimated 20.5 million deaths – close to a third of all deaths globally (Vaduganathan et al., 2022). Increases in CVD morbidity and mortality are not equally distributed in all areas across the globe. The burden of CVD falls hardest on middle-income countries, where estimated incidence rates are ~30% higher than high-income countries (Timmis et al., 2022b).

The British Heart Foundation reports that CVD resulted in over 168,000 deaths in 2021 alone in the UK. It is estimated that 7.6 million people, approximately 10 % of the UK population, live with CVD (Timmis et al., 2022a, Timmis et al., 2020). CVD is a substantial economic burden; UK's CVD healthcare costs are calculated at around £9 billion annually (Timmis et al., 2017).

High systolic blood pressure remains the leading modifiable risk factor globally for imputable premature cardiovascular deaths, accounting for 10.8 million cardiovascular deaths and 11.3 million deaths overall in 2021(Razo et al., 2022). In 2021, the all-cause disability-adjusted life years (DALYs) due to high blood pressure were 2,770 per 100,000 (Vaduganathan et al., 2022).

1.2 Introduction to Hypertension

According to the key clinical guidelines 2018/2023, hypertension is defined based on repeated office systolic blood pressure (SBP) values of 140 mmHg and/or diastolic blood pressure (DBP) of 90 mmHg. However, there is a continuous relationship between blood pressure (BP) and cardiovascular or renal morbid or fatal events starting from an office SBP >115 mmHg and a DBP >75 mmHg (Mancia et al., 2023). Hypertension may be accompanied by organ damage in the heart, brain, and kidneys (Wu et al., 2022). The development of this disease is steadily related to structural and functional cardiac and vascular disorders that impair the ability of the heart, vasculature, kidney, brain, and other organs, triggering premature morbidity and mortality (Giles et al., 2005).

Hypertension is a significant risk factor for CVDs. Several observational studies have demonstrated a graded correlation between systolic blood pressure and diastolic BP and the risk of myocardial infarction or stroke (Hinderliter et al., 2021). Globally, high BP is the primary cause of death and disability life years (Lewington et al., 2002); in the United States, high BP accounts for more deaths from CVD than any other modifiable risk factor (Danaei et al., 2009, Rapsomaniki et al., 2014).

There are two broad categories of clinical hypertension, which are grouped into a pathogenesis-based classification. Primary hypertension, formerly known as essential or idiopathic hypertension, is defined as continuously raised blood pressure in which secondary causes such as renal failure, renovascular disease, aldosteronism, or other identified causes of secondary hypertension are not present. Essential hypertension accounts for 95% of all cases of hypertension (Carretero and Oparil, 2000). It is a heterogeneous disorder, with each patient having different factors that lead to high BP.

Conversely, secondary hypertension is elevated blood pressure secondary to an identifiable cause (Rimoldi et al., 2014). It is estimated that 5-10% of hypertensive patients have secondary hypertension. The leading causes of secondary hypertension

are parenchymal renal disease, primary aldosteronism, and renovascular hypertension. However, identification of the aetiology, pathophysiology, and management of secondary hypertension is essential in patients as, in some cases, it can lead to blood pressure control without the need for antihypertensive medications (Rimoldi et al., 2014).

Hypertension constitutes a significant public health burden globally. In 2015, hypertension prevalence in adults was 1.13 billion (Mills et al., 2020, Dzau and Balatbat, 2019), which constitutes around 30 - 45% of the adult population (Chow et al., 2013), and it is predicted that the number of people with hypertension will grow further by 15-20%, affecting up to 1.5 billion, by 2025 (Chockalingam, 2007). Even though hypertension is one of the most preventable causes of premature morbidity and mortality, it is estimated to cause 10 million deaths worldwide and over 200 million disability-adjusted life years (Forouzanfar et al., 2017). Effective treatment and prevention of hypertension are vital in reducing disease burden and promoting longevity (Oparil et al., 2018).

1.3 Pathophysiology of Hypertension

The elusive nature of primary and secondary hypertension and the specific mechanisms underlying elevation of BP, even in experimental models of hypertension, have remained a focus of continuous research for many decades. Early findings by Tigerstedt (Tigerstedt and Bergman, 1898, Luft and Dietz, 1993),and subsequently refined and published by Goldblatt (Goldblatt et al., 1934) presented that the ischaemic or under perfused kidney can release substances with the ability to raise the BP. Experimental observations such as these set the foundation for Dr Irvine Page in 1939 to make the discovery of a potent vasoconstrictor and pro-hypertensive agent isolated from renal extracts that were named hypertension and angiotensin, respectively (Page, 1939b, Page, 1939a).

The Mosaic Theory of Hypertension was proposed by Dr Page in the 1940s, supporting the idea that the pathophysiological processes responsible for hypertension are complex and can involve multiple factors. Dr. Page's Mosaic Theory has formed a framework for future studies of molecular and cellular signals in the context of hypertension. Over the years, studies have begun to elucidate the interactions proposed in this theory at the molecular level. These local events coordinate the actions of several organs, including the brain, the vasculature, and the kidney, to raise blood pressure. The mosaic theory states that several factors, including genetics, environment, adaptive, neural, mechanical, and hormonal perturbations, interdigitate to increase blood pressure. This paradigm has been reformed, adding new concepts such as inflammation, involvement of oxidative stress, genetics, the microbiome, and sodium homeostasis, Figure 1.1 (Harrison, 2013, Harrison et al., 2021a).

Upon ageing, the probability of developing hypertension increases, owing to continuing stiffening of the arterial vasculature caused by slowly developing changes in vascular collagen accumulation and increases in atherosclerosis. Primary hypertension involves multiple types of genes; some allelic gene variants are associated with an increased risk of developing primary hypertension. Genetic

predisposition, along with several environmental factors, such as high Na^+ intake, sleep apnoea, lack of physical activity, excess alcohol intake, and increased mental stress, will contribute to the occurrence of hypertension (Oparil et al., 2018). Immunological factors can also play a crucial part in the pathogenesis of hypertension and related target organ damage. Moreover, genetics and environment contribute to reactive oxygen species (ROS) generation and inflammation (Barrows et al., 2019).

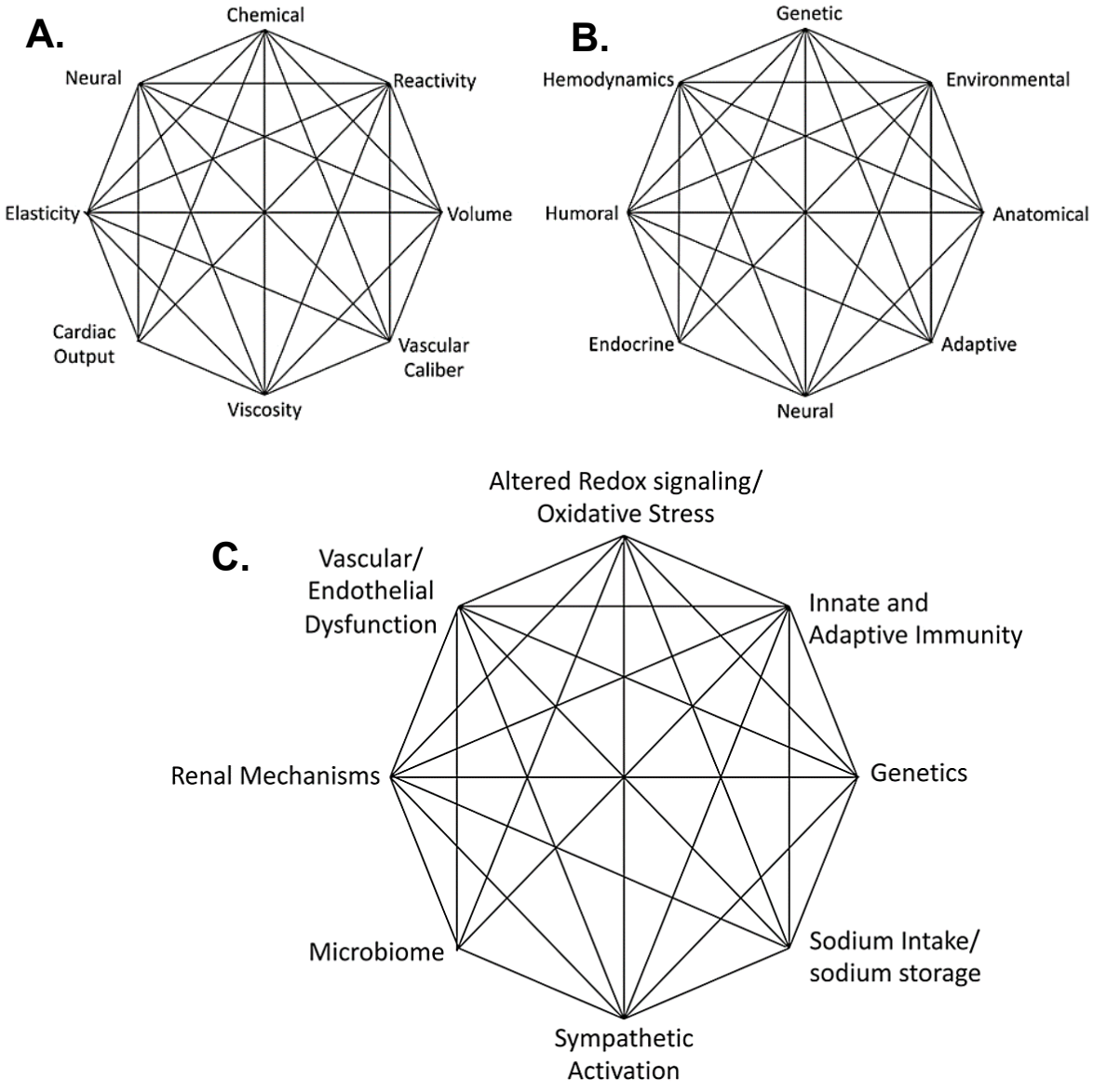

Figure 1.1: Dr Page's Mosaic Theories.

(A) The original and (B) revised versions of the Mosaic Theory. (C) Further revised Mosaic Theory incorporating the new understanding of cellular, environmental, and genetic mechanisms. Figure from the review article 'Pathology of Hypertension- the Mosaic Theory and Beyond' (Harrison et al., 2021a). Reprinted with the permission provided from Wolters Kluwer Health, Inc. and Copyright Clearence Centre.

1.3.1 Management of Hypertension

There are three overarching approaches to diminish BP: modifications in lifestyle, pharmacological treatment and non-pharmacological approaches (e.g. renal denervation or bioelectronic approaches). Behavioural interventions are an essential component of the therapy of hypertension. It has been recommended as the initial treatment strategy for lowering BP in subjects with high normal BP or pre-hypertension (Charchar et al., 2023). These approaches need to also complement other therapeutic modalities in all hypertensive subjects. Adopting the DASH (Dietary Approaches to Stop Hypertension) diet (Appel et al., 1997, Appel et al., 2003, Sacks et al., 2001), reducing dietary sodium intake (Sacks et al., 2001), aerobic exercise, and weight loss can lower blood pressure. It may reduce the need for drug treatment (Bacon et al., 2004). A healthy lifestyle also favourably influences other CVD risk factors, reducing overall cardiovascular risk (Appel et al., 2003). A combined exercise and weight-loss intervention has decreased SBP and DBP by 12.5 and 7.9 mmHg, respectively (Bacon et al., 2004).

Most common medications used in the treatment of hypertension include diuretics, angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), renin inhibitors, beta and alpha-blockers, direct vasodilators, and calcium channel blockers (CCBs) (Nguyen et al., 2010). Some patients will require two or more antihypertensive medications to achieve their BP target, referred to as combination therapy. Avoiding Cardiovascular Events through Combination Therapy in Patients Living with Systolic Hypertension (ACCOMPLISH) trial disclosed that combination drug treatment of benazepril and amlodipine can reduce CV events in high-risk patients with hypertension (Jamerson et al., 2008). Additionally, the HOPE-3 trial (Heart Outcomes Prevention Evaluation-3) exhibited patients with grade 1 hypertension (140–159 mmHg, average 154 mmHg systolic BP) compared with placebo presented a 24% reduction in the risk of cardiovascular outcomes with the initial administration of 2 antihypertensive agents (candesartan + hydrochlorothiazide) (Lonn et al., 2016).

1.3.2 Regulation of Blood Pressure

The effectors that determine one's blood pressure differ in their contributions to the changes in blood pressure depending on age, sex, and situation. Blood pressure is determined by different cardiovascular system parameters, including blood volume, cardiac output, and the regulation of arterial tone, which is affected by both intravascular volume and neurohumoral systems (Oparil et al., 2018). The regulation and maintenance of physiological BP levels involve an intricate interplay of various elements of an integrated neurohumoral system that includes the renin-angiotensin-aldosterone system (RAAS), the involvement of natriuretic peptides, the endothelium, the sympathetic nervous system (SNS) and the role of the immune system (Figure 1.2). Disruption of factors in any of these systems can directly or indirectly lead to increased BP variability and mean BP over a period, resulting in target organ damage such as left ventricular hypertrophy (Oparil et al., 2018).

Vasodilation capacity and intravascular fluid volume can directly affect BP. Vasodilation capacity is affected by vascular elasticity, calibre, and reactivity, which reflects the buffering capacity of vessels against pressure shocks (Ma and Chen, 2022). The poorer the vasodilation capacity, the higher the BP. The volume of intravascular fluid is regulated by the body's intake and elimination of fluid. Once the fluid balance is disturbed, the increase in intravascular fluid can directly increase BP. Therefore, factors that cause increases in blood volume or decrease in vasodilation capacity can lead to hypertension (Ma and Chen, 2022). These factors usually coexist and are intertwined in the occurrence and progress of essential hypertension. The lack of appropriate clinical identification methods currently causes difficulties in making suitable treatment plans for hypertensive patients (Ma and Chen, 2022).

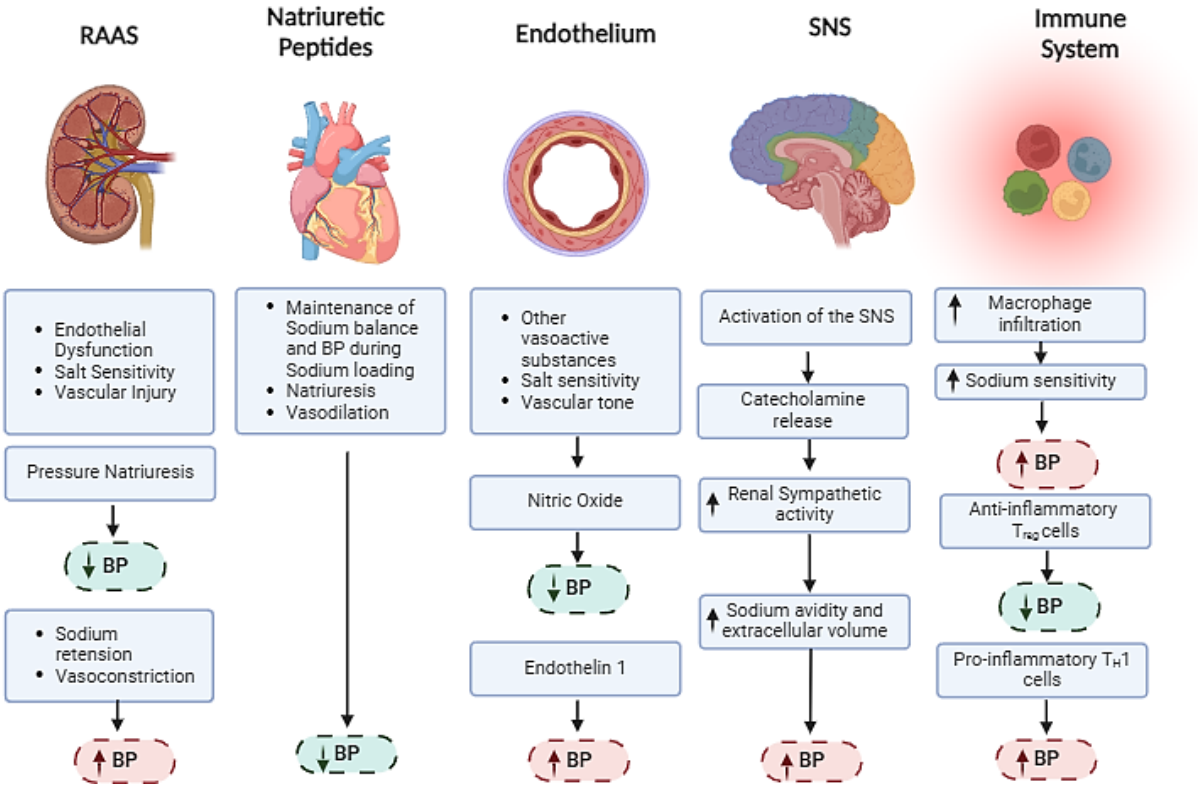

Figure 1.2: The main neuroendocrine systems involved in regulating blood pressure.

Neurohumoral, immune, and organ systems are involved in maintaining blood pressure. BP: Blood pressure, RAAS: renin-angiotensin-aldosterone system adapted from (Oparil et al., 2018).

1.3.3 Renal Mechanisms of Hypertension

The kidney is an organ of "overriding dominance" (Guyton et al., 1972b) in blood pressure regulation; therefore, the development of hypertension is primarily mediated through its intrinsic role in 2 main BP regulatory pathways: the sodium and water homeostasis and the renin-angiotensin system (Tomaszewski et al., 2022). Other significant roles involve modulation of the systemic sympathetic tone by generating reflex signals *via* renal afferent nerves. Lastly, the kidney also serves as a site of immune activation (Harrison et al., 2021a).

Renin is an aspartic protease secreted by juxtaglomerular cells of the kidney; it interacts with a plasma protein substrate to produce a decapeptide prohormone angiotensin I, which in turn, is acted on by the ACE to generate Angiotensin II. Angiotensin II affects vasoconstriction, aldosterone secretion by the adrenal cortex, and sodium retention by the kidneys (Haber, 1979). Renin is the rate-limiting step for activating the circulating RAAS, and its synthesis and secretion by the kidney are well-regulated (Guessoum et al., 2021). It is released to combat the reduction in perfusion pressure, delivery of sodium chloride to macula densa cells, or increase in sympathetic stimulation (Castrop et al., 2010). Moreover, renin is a complement factor 3 (C3) convertase (Perez-Gomez and Ortiz, 2020). It has long been known that circulating plasma levels of C3 are associated with future blood pressure increases and subsequent development of hypertension (Engström et al., 2007), likely by promoting innate and adaptive immune responses (Wenzel et al., 2019).

The kidney's second significant role in hypertension is adjusting the pressure diuresis and natriuresis. Pressure natriuresis (PN) describes increased sodium excretion when the renal perfusion pressure increases (Baek and Kim, 2021). As the renal arterial pressure rises, the kidney increases sodium excretion and reduces the amount of extracellular fluid to maintain normal sodium equilibrium and systemic arterial pressure (Baek and Kim, 2021). As initially described by Guyton et al., a rise in BP causes brisk diuresis and natriuresis, thereby returning BP to normal values (Guyton et al., 1972a). Therefore, the set point of BP is the point at which PN and extracellular fluid volume are in balance. As this balance is disturbed, BP is uncontrolled, making hypertension a disease of the kidney (Baek and Kim, 2021).

The kidneys are densely innervated with renal efferent and afferent nerves to communicate with the central nervous system. In hypertension, the kidneys modulate systemic sympathetic tone by generating reflex signals *via* renal afferent nerves. Approximately 90% of renal nerves are efferent nerves sending sympathetic signals to the kidney, thereby regulating renal blood flow, tubular sodium resorption, renin and prostaglandin release, and vasomotor tone, all of which contribute to cardiovascular and renal regulation (DiBona, 2000, Sata et al., 2018).

Several studies have demonstrated the critical role of the immune system in the pathogenesis of hypertension, mainly through pro-inflammatory mechanisms within the kidney, which eventually drive a myriad of renal and cardiovascular diseases (Benson et al., 2023).

Ang II–induced hypertension activates antigen-presenting dendritic cells in the kidney which migrate to secondary lymphoid organs to activate T cells and promote infiltration. The T cells, in turn returns to the kidney and can lead to subsequent end-organ damage in the development of hypertension (Xiao et al., 2015b). De Miguel et al., observed that T cells invade the kidney of Dahl salt-sensitive rats on a high salt diet, and treatment with the immunosuppressant tacrolimus blunted T cell invasion within the kidney, decreased renal damage, and lowered blood pressure (Miguel et al., 2011).

1.3.4 Vascular Dysfunction in Hypertension

As evident from the above considerations, the pathophysiology of hypertension is multifactorial and associated with vascular dysfunction (Konukoglu and Uzun, 2017). Several nodes in Dr Page's Mosaic diagram refer to potential vascular mechanisms, including vascular calibre, reactivity and elasticity (Harrison et al., 2021b).

Four distinct vascular perturbations can occur in and contribute to hypertension progression. The first is an enhanced milieu of vasoconstrictor hormones involving Ang II, catecholamines, and vasopressin. Often, this is combined with changes in vascular structure and function, promoting vasoconstriction and reduction in vasodilation (Hinderliter et al., 2021).

Hypertension is also associated with impaired vasodilation. For several years, endothelium-dependent vasodilation and nitric oxide (NO) signalling have been recognised to reduce hypertension. Moreover, the vasculature is a source and target of immune activation in hypertension. A crosstalk partly mediates this between the endothelium and immune cells (Harrison et al., 2021a).

Yet another related vascular perturbation likely contributing to hypertension is vascular stiffening of large arteries such as the proximal aorta (Wu et al., 2022). The

aorta stiffens in typical conditions, including aging, diabetes, obesity, and tobacco use (Vaitkevicius et al., 1993, Agnoletti et al., 2013). Hypertension is both a cause and a consequence of artery stiffening, an initiator and indicator of myriad disease conditions. Such artery stiffening results from remodelling of the arterial wall driven by mechanical stimuli and mediated by inflammatory signals, leading to differential gene expression and concomitant changes in extracellular matrix composition and organisation (Humphrey, 2021).

The artery wall from the outside to the inside comprises the tunica adventitia, tunica media, and tunica intima. The tunica adventitia layer contains nerve endings, perivascular adipose tissue, and connective elements like collagen and fibroblasts. This layer is fundamental to vascular development and remodelling (Konukoglu and Uzun, 2017). The second layer consists of the vascular smooth muscle cells, which regulate constriction and dilatation of the blood vessels. Mechanical stimuli, such as shear stress and pressure or pharmacological stimuli, can stimulate the contraction of the vascular smooth muscle cells by increasing the intracellular calcium concentration (Konukoglu and Uzun, 2017).

Tunica intima (or intima), the innermost layer is in contact with the blood (Huang and Niklason, 2014), and consists of a monolayer of endothelial cells (ECs) and a basement membrane composed of a mesh-like substrate of type IV collagen. The ECs play a role in many biological processes, such as coagulation, blood flow regulation, haemostasis, and inflammation (Tennant and McGeachie, 1990). Through control of vascular tone, EC regulates the regional blood flow. They also direct inflammatory cells to foreign materials (Wilson, 2001). Moreover, EC is important in controlling blood fluidity, platelet adhesion and aggregation, leukocyte activation, adhesion, and transmigration (Krüger-Genge et al., 2019).

As mentioned, endothelial cells regulate the vascular tone by synthesising NO, prostaglandins, and another relaxing factors such as endothelium-derived hyperpolarizing factor (EDHF). Moreover, a healthy endothelium provides antiinflammatory and antithrombotic functions that contribute to maintaining blood fluidity (Eelen et al., 2015). Endothelial dysfunction is characterised by a shift of the

actions of a healthy endothelium toward reduced vasodilation, cell proliferation, platelet adhesion and activation and proinflammatory and prothrombotic state (Gallo et al., 2022). The endothelium plays a significant role in several disease pathogeneses; endothelial dysfunction occurs in several CV diseases, including atherosclerosis, systemic and pulmonary hypertension, cardiomyopathies, and vasculitides, contributing to inflammation in the vascular wall, smooth muscle proliferation, extracellular matrix deposition, cell adhesion, and thrombus formation in conducting arteries (Yu et al., 2008, Ross, 1999, Gallo et al., 2022).

Much evidence has shown that reactive oxygen species are involved in endothelium dysregulation. In the vascular system, the primary source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, of which the expression is elevated in hypertensive conditions by several stimuli, including shear stress alterations, RAAS and endothelin activation (Gallo et al., 2022). Increased ROS concentration causes the reduction of NO bioavailability, a central phenotypic characteristic of endothelial dysfunction (Ignarro et al., 1980, Dharmashankar and Widlansky, 2010), and consequently, reduced endothelium-dependent relaxation which in turn contributes further vascular remodelling (Touyz et al., 2020). In hypertension, dysfunction in NO production extends beyond the vasculature and has significant consequences for renal and central nervous system dysfunction (Harrison et al., 2021a).

1.3.5 Cardiac Dysfunction of Hypertension

Extensive cohort studies have demonstrated that high BP is a significant risk factor for heart failure, atrial fibrillation, chronic kidney disease, heart valve disease, aortic syndrome, coronary heart disease, and stroke (Fuchs and Whelton, 2020). Moreover, in the presence of hypertension, cardiac remodelling and left ventricular (LV) hypertrophy develops as an adaptive process that helps to normalise LV and arterial wall stress and compensate for the reduction in myocardial fibre function, allowing the heart to normalise afterload and preserve systolic performance (Mayet and Hughes, 2003, Ganau et al., 1990).

Hypertension is associated with a range of structural changes in the left ventricle. Remodelling is observed in normal ageing, even in healthy individuals

without hypertension (Singam et al., 2020). Observed structural changes likely reflect individual differences in age, myocardial performance, hemodynamics, plasma volume, genetic influence, or neurohormonal status (Roman et al., 2000). Cardiac structure may be influenced by pure hemodynamic pressure and volume overloads (Ganau et al., 1990). The increased pressure load in hypertension is fundamentally caused by the increased resistance, although lowered compliance and adapted magnitude and timing of reflected pressure waves also influence disease progression. Hypertensive remodelling changes can be classified as hypertrophy (increased left ventricular mass, normal left ventricular mass and abnormal relative wall thickness) (Lovic et al., 2017, Heagerty et al., 1993).

The layers of the myocardium are surrounded by an interstitial fibrous network composed primarily of fibrillar collagens. Even though hypertrophy primarily involves myocytes, the interstitial network also changes, causing widespread perivascular and interstitial fibrosis (expansion of the heart's inter-cellular collagen matrix). Myocardial interstitial fibrosis (MIF) is a histological hallmark for several cardiac diseases that enable the alteration of myocardial architecture and function, ultimately associated with progression to HF (Díez et al., 2020). Additionally, elevated intra-cellular collagen is vital in cardiac dysfunction in hypertension patients.

The majority of hypertensive patients have normal LV structure, but left ventricular hypertrophy suggests a poor prognosis (Kannel, 1992). Increased ventricular arrhythmias in hypertension and elevated QT duration and dispersion seen in LV hypertrophy, as well as coronary perfusion, can all account for sudden death (Mayet et al., 1995, Mayet et al., 1996). Heterogeneous conduction in the ventricles can be caused by increased interstitial fibrosis, which can likely cause electrical abnormalities in the heart (Mayet and Hughes, 2003); active relaxation is also weakened in hypertrophy and remodelling. Changes in intracellular calcium handling, ion exchangers, and ion channels can affect altered relaxation. Cardiac hypertrophy is also associated with impaired coronary reserve, which may be caused by endothelial dysfunction, narrowing of tiny arteries, perivascular fibrosis, altered wall

mechanics, and relative myocyte hypertrophy present in hypertension (Mayet and Hughes, 2003).

1.3.6 Neural Mechanism of Hypertension

The sympathetic nervous system are two of the main divisions of the autonomic nervous system; its chief function is to stimulate the body's fight-or-flight response by releasing sympathetic catecholamines such as noradrenaline and adrenaline (Drummond et al., 2019). The other division of the autonomic nervous system, namely, the parasympathetic nervous system, generally opposes the actions of the SNS on peripheral tissues through the release of acetylcholine (Gordan et al., 2015). The SNS receives inputs from the hypothalamus and rostral ventrolateral medulla, and it has projections to all significant blood pressure-regulating organs, including the heart, kidneys, and blood vessels, by which heart rate, vasoconstriction, and sodium and water reabsorption are altered (Drummond et al., 2019).

The SNS plays a central role in the regulation of arterial BP. Contrary to the traditional understanding that the SNS is responsible only for the short-term regulation of BP, results of several studies over the years suggest that sympathetic neural mechanisms play an essential role in the long-term regulation of blood pressure (Joyner et al., 2008, Osborn, 2005). Dysfunction in these pathways may be responsible for the pathophysiology of some forms of "idiopathic" hypertension. Sympathetic neural influences on cardiovascular function can be divided into four main categories: influence on cardiac sympathetic nerves, vascular sympathetic nerves, adrenal medullary influences caused by circulating epinephrine and norepinephrine, and lastly, the sympathetic stimulation of renal juxtaglomerular cells that activate the renin-angiotensin-aldosterone pathway (Charkoudian and Rabbitts, 2009).

Dr Page demonstrated the indirect action of angiotensin II to increase total peripheral resistance and arterial pressure predominantly by sympathetic neural activation (McCubbin et al., 1965). Measures of muscle sympathetic nerve activity, norepinephrine spillover, and heart rate variability have proposed that hypertensive

patients frequently have enhanced sympathetic outflow and heightened catecholamine-mediated vasoconstriction (Head, 2003). Angiotensin II and ROS in the brain stem nuclei raise vascular resistance by inhibiting the microvascular endothelium-dependent hyperpolarising factor (Cao et al., 2020).

Sympathetic tone modulates several mechanisms in hypertension, some of which include enhanced vasoconstriction and vascular remodelling, renal renin production *via* beta-1 adrenergic receptors in the juxtaglomerular apparatus, and improved renal sodium resorption and inflammation (Harrison et al., 2021c). The primary role of the SNS in the cardiovascular system is the maintenance of blood pressure and regulation of blood flow *via* the arterial baroreflex (Charkoudian and Rabbitts, 2009, Fink, 2009).

The sympathetic activation can be targeted by therapeutic strategies that use surgical and pharmacological treatments. ß-adrenergic blockade also appears to have a neutral effect on central sympathetic outflow, mainly when reductions in heart rate are accounted for. ß-adrenergic receptor blockers are widely used to reduce cardiovascular sympathetic tone (Clarke et al., 2010). They perform their antihypertensive functions by obstructing the sympathetic activation from ß-1 adrenergic receptors of the juxtaglomerular renal cells and the myocardium (Wallin et al., 1984). Chronic administration of specific ß-blockers to hypertensive patients lowers muscle sympathetic nerve activity (MSNA) (Wallin et al., 1984), indicating that ß-adrenergic antagonists also possess central sympathoinhibitory effects.

1.4 Hypertension as an Inflammatory Disease

1.4.1 Role of the Immune System in Hypertension

It has been well-documented over the years that hypertension is an inflammatory disease that involves the infiltration and accumulation of various inflammatory cells in the kidneys, central nervous system (CNS), heart and blood vessels of hypertensive humans and in animals with experimental hypertension (Drummond et al., 2019).

In the 1960s, Okuda and Grollman (1967) observed that lymphocyte transfer from rats with unilateral renal infarction triggered hypertension in recipient rats. Moreover, studies led by White and Grollman (1964) demonstrated the ability of BP to be lowered through immunosuppression treatment in rats with partial renal infarction. In 1972, Olsen noted the accumulation of perivascular T cells and monocytes inside the vasculature of patients with different causes of hypertension. Closely after, Ba et al. (1982) explained a significant suppression of blood pressure upon thymus transplant from a Wistar-Kyoto rat to the recipient spontaneously hypertensive rat (SHR). T cells have been observed in the kidneys of hypertensive animal models and humans for several years, and treatment with pharmaceutical drugs such as mycophenolate mofetil has been shown to lower blood pressure in experimental models such as DOCA salt (Rodríguez-Iturbe et al., 2001). In 2007, Guzik et al, presented that recombination-activating gene one deficient mice ($RAG1^{-/-}$), lacking both T and B cells, develop reduced hypertension and have preserved vascular endothelial function when induced with Ang II or after challenge with deoxycorticosterone acetate (DOCA) and salt.

Additionally, the adoptive transfer of T cells restored the vascular dysfunction and hypertensive response observed in WT mice. The deletion of the recombination activating gene (RAG) 1 gene in Dahl salt-sensitive rats caused blunted BP increases upon high-salt feeding and further protected against glomerular and renal damage (Mattson et al., 2013). Consistent with these findings in RAG1-deficient animals, lymphocyte-deficient mice (*SCID*) developed lower hypertension and cardiac hypertrophy during Ang II infusion (Crowley et al., 2010b).

It is now well accepted that essentially every type of immune cell, including those of innate and adaptive immunity, can contribute to hypertension progression. These cells transmigrate and influence hypertension's vascular, renal, cardiac, and neural mechanisms discussed above. Moreover, they contribute to vascular function, structure, and end-organ damage, releasing potent cytokines and promoting oxidative stress through ROS (McMaster et al., 2015, Drummond et al., 2019).

To promote hypertension, the immune system must affect the blood pressure-regulating functions of the blood vessels, kidneys, and heart. Studies have shown

evidence of different immune cell subsets which invade these organs during hypertension. The depletion of selective immune cell subsets protects against hypertension in various animal models (Drummond et al., 2019). Conversely, studies have also addressed mechanistic justification of immune cells altering renal, vascular, autonomic, or cardiac functions to enhance blood pressure (Drummond et al., 2019).

Hypertension pathogenesis is undoubtedly caused by not only the classical non-immune mechanisms such as high salt intake, angiotensin II, aldosterone, and catecholamines but also by several immune mechanisms. Several inflammatory cells and soluble factors interacting with various vital organs impact blood pressure levels (Drummond et al., 2019).

Several immune cells are revealed to participate in the pathogenesis of hypertension, resulting in end-organ damage. Dendritic cells, $CD4^+$ and $CD8^+$ T cells, γ/δ T cells, B cells, and monocyte/macrophages are observed to have prohypertensive properties. Such include endothelial dysfunction, vasoconstriction, resistance and stiffening of arteries; elevated heart rate and stroke volume; amplified sympathetic outflow; increased sodium and water reabsorption and renin release and lastly, activation of the complement system. Regulatory T cells (Tregs), natural killer T (NKT) cells and myeloid-derived suppressor cells (MDSC) also control the development of hypertension by protecting against the prohypertensive properties (Drummond et al., 2019).

1.4.1.1 Dendritic Cells

Antigen-presenting cells such as dendritic cells (DC), which play a pivotal role in adaptive immunity, are potent activators of T lymphocytes, contributing to vascular and kidney injury in hypertension (Caillon et al., 2019a). DCs are mainly derived from hematopoietic bone marrow progenitor cells and exist in an immature state in the blood. DCs of hypertensive mice have been observed to produce increased amounts of ROS, leading to the production of isolevuglandin protein adducts (isoLG). IsoLG are modified proteins that function as a T cell regulator, particularly $CD8^+$ T cell proliferation and cytokine production. When DCs are exposed to an increase in isoLG-modified proteins, it promotes surface expression of CD86 and upregulation of cytokine production, such as IL-6, IL-1 ß, and IL-23. Further, adoptive transfer of the activated DCs into normotensive mice presented enhanced $CD8^+$ proliferation, production of IL-17A, IFN-γ and increased blood pressure (Kirabo et al., 2014, Xiao et al., 2015a).

Treatment with the isoLG scavenger 2-hydroxybenzylamine *in vivo* reduces DC and T cell activation and attenuates Ang II and DOCA-salt-induced hypertension by reducing vascular stiffening. It promotes its action by reducing superoxide production in DC by scavenging reactive isoLG and decreasing the DC activation (Kirabo et al., 2014). Alternatively, the stimulation of VSMCs with Ang II results in the release of thymic stromal lymphopoietin, a key activator of antigen-presenting cells such as DCs, and promotes the expression of cell surface markers such as CD86, CD80, and MHC II, promoting their capacity to activate T cells (Zhao et al., 2012).

1.4.1.2 T Cells

There has been extensive interest in the role of T cells' diverse subtypes in hypertension. The notion that T cells contribute to hypertension gained broad acceptance when Guzik et al. presented that $Rag1^{-/-}$ mice lacking lymphocytes were resilient to Ang II and DOCA-salt-induced hypertension and vascular dysfunction associated with high BP. Further, the adoptive transfer of T cells but not B cells restored the hypertensive response (Guzik et al., 2007) and has been further

developed by multiple other groups in various models modifying T cell biology (Madhur et al., 2020).

Subsets of T lymphocytes are now well-known to influence blood pressure through effects on the local cytokine milieu within key cardiovascular organs (Zhang and Crowley, 2015). Through the expression of fundamental cell surface markers, T lymphocytes can be grouped into distinct subsets with varying functions (Zhu and Paul, 2008). Most cells are either $CD4^+$ T cells, recognised as T helper cells (T_h cells), or $CD8^+$ T cells, considered cytotoxic T cells. The third T cell subset is $CD4^-CD8^-$ (double negative), predominantly consisting of γ/δ T cell subset (McConnell and Hopkins, 1998). $CD4^+$ cells differentiate into diverse subsets, including T helper (Th) 1, Th2, Th9, Th17, Th22, Tregs, and follicular helper T cells (Tfh), all of which are differentiated by different cytokine profiles (Raphael et al., 2015). These different $CD4^+$ subsets play a critical role in T cells' immune and effector response functions (Raphael et al., 2015).

Approximately 10% of $CD4^+$ T cells are Treg cells, which have been shown to modulate hypertension (Norlander et al., 2018) and heart fibrosis. Adoptive transfer of Treg cells into wild-type mice suppressed Ang II-induced hypertension, vascular injury, and immune cell infiltration (Barhoumi et al., 2011). Experimental studies have also observed a reduction in Treg cells in Ang II mice; hypertensive mice injected with Tregs isolated from control mice had significantly reduced macrophage activation, decreased tumour necrosis factor-alpha (TNF-α) release, and overall improved coronary arteriolar endothelium-dependent relaxation (Matrougui et al., 2011). Regulatory T cells, are potent suppressors of effector T cells, which can negatively regulate immune responses (Sakaguchi, 2005). Additionally, several studies suggest that Tregs may modulate the onset and progression of hypertension. Kasal et al. showed a decreased proportion of Tregs in peripheral blood and multiple organs in hypertensive mice and rats even before the onset of hypertension (Kasal et al., 2012). Adoptive transfer of Tregs and administration of an anti-IL-2 monoclonal antibody complex leading to increased circulating Tregs *in vivo* was shown to prevent the progression of hypertension in animal models (Katsuki et al., 2015) (Tang et al., 2023). Depletion of Tregs can advance left ventricle hypertrophy (LVH) in rats

(Katsuki et al., 2015). Conversely, upregulation of Tregs can attenuate the development of LVH in hypertensive animal models (Wang et al., 2016), proposing Tregs ability to negatively modulate hypertension-associated LVH.

There has been increasing interest in the $CD4^+/CD8^+$ ratio as a potential biomarker for hypertension and associated disease. A clinical study by Ni et al (2017) reported a significant trend toward an increase in the percentage of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio in peripheral blood for men and women associated with essential hypertension (Ni et al., 2017). Furthermore, there is strong evidence of the independent association between the ratio of $CD4^+/CD8^+$ T cells and increased risk of coronary artery disease (Gao et al., 2017). $CD8^{-/-}$ mice were found to be protected from hypertension and displayed decreased vascular rarefaction and kidney remodelling, whereas mice lacking $CD4^+$ T cells were not when compared to WT (Norlander et al., 2018). A clinical study by Youn et al (2013) observed the number of "immunosenescent" $CD8^+$ T cells is increased in newly diagnosed hypertensive patients. Furthermore, these cells produced increased Interferon-gamma (IFN-γ), TNF-α, granzyme B and perforin compared with $CD8^+$ T cells compared with normotensive patients (Youn et al., 2013).

Over the years, experimental studies have also demonstrated the importance of gamma/beta (γ/δ) T cells in hypertension. γ/δ T cell-deficient mice exhibit a significantly decreased rise in systolic blood pressure and preserved endothelial T cell activation in response to Ang II infusion (Caillon et al., 2017). Additionally, activated $CD4^+$ T cells expressing the marker CD69 in the spleen and mesenteric arteries were depleted, suggesting that γ/δ T cells might contribute to the development of hypertension (Caillon et al., 2017). These cells are the key source of IL-17A, a prohypertensive cytokine elevating BP and end-organ damage (Saleh et al., 2016).

1.4.1.3 B Cells

The role of B cells has been extensively studied in hypertension. Splenic B cells expressing the activation marker CD86 were demonstrated to be elevated in Ang II-induced mice (Chan et al., 2015). Additionally, the study gave evidence for markedly elevated circulating IgG and its accumulation in aortic adventitia upon hypertension.

In B cell–activating factor receptor—deficient mice (BAFF-$R^{-/-}$ mice), mice lacking mature B cells, there was no evidence of Ang II-induced increase in IgG. Furthermore, the hypertensive response to Ang II was attenuated in BAFF-$R^{-/-}$ mice relative to WT mice, and this response was reduced by B cell adoptive transfer. Hence, these studies demonstrate the significance of B cells in developing hypertension and vessel remodelling (Chan et al., 2015).

1.4.2 Cytokines Contributing to Hypertension

Numerous inflammatory cytokines play a significant part in promoting the progression of hypertension by affecting vascular, cardiac, and renal function. Various cytokines modulate the proliferation and differentiation of immune cells. Sustained activation by multiple cytokines leads to monocyte phenotype transition and activation of matrix metalloproteinases, which can modify interstitial matrix, in turn altering collagen composition, promoting organ remodelling (Nian et al., 2004).

As discussed, numerous T cells and monocytes/macrophages accumulate within vessels in experimental mice model of hypertension. These cells secrete proinflammatory cytokines, impairing organ function, especially the vasculature, heart and kidneys (Zhang et al., 2022)—numerous inflammatory cytokines, including IL-1ß (Rothman et al., 2020b), TNF-α (Mehaffey and Majid, 2017), IL-6 (Chamarthi et al., 2011), Il-17A (Nguyen et al., 2013) and IFN-γ (Benson et al., 2022) have all been implicated to play an essential role in angiotensin II-mediated hypertension.

1.4.2.1 IL-17

IL-17 is a signature cytokine produced by T helper 17 cells, an important subset of $CD4^+$ T cells and includes isoforms such as IL-17A; B cells and $CD8^+$ T cells also stimulate the production of IL-17. IL-17 can also be produced by γ/∂ T cells (O'Brien et al., 2009). The IL-17 family signals *via* the NFκβ, mitogen-activated protein kinases (MAPKs) and C/EBPs (Gu et al., 2013). IL-17 was initially linked to inflammation by the induction of IL-6 production in fibroblasts (Yao et al., 1995). A critical role of IL-17 is the production of pro-inflammatory mediators by cells such as DCs,

macrophages, fibroblasts, and epithelial and endothelial cells (Benedetti and Miossec, 2014). IL-17 can synergise with other pro-inflammatory cytokines, such as IL-1 and TNF, leading to pronounced inflammation (Noack et al., 2019).

Studies have shown that IL-17A, produced by $CD4^+$ T cells, is critical in maintaining angiotensin II-induced hypertension and vascular dysfunction (Madhur et al., 2010a, Nguyen et al., 2013). Mice treated with IL-17 for 1 week showed significantly increased SBP which was associated with decreased aortic NO-dependent relaxation and induced phosphorylation of the endothelial NO synthase (eNOS) on threonine 495 (Nguyen et al., 2013). In humanised mice, infusion of Ang II augmented total human $CD4^+$ T cells in lymph nodes and cell accumulation in the kidneys and aorta (Itani et al., 2016).

Increasing evidence has linked IL-17A and the elevation of blood pressure through multiple mechanisms, including inhibiting endothelial NO production, increasing the formation of reactive oxygen species, promoting vascular fibrosis, and enhancing renal sodium retention (Davis et al., 2021). In animal models of hypertension, IL-17A plasma concentrations are observed at increasing levels. Conversely, lowered oxidative stress and decreased T cell infiltration are observed in IL-$17A^{-/-}$ mice induced with Ang II, as well as mice presenting significantly diminished impairment of endothelial function and systolic BP (Zhang et al., 2022). Treatment with anti-IL-17 antibody further markedly lowers BP and reduces collagen deposition in mice models of hypertension, especially in the heart and kidneys (Zhang et al., 2022). Additionally, Madhur et al. demonstrated IL-$17A^{-/-}$ mice to have blunted hypertension in response to chronic ang II infusion and protection from ang II-induced endothelial dysfunction, vascular superoxide production, and vascular inflammation (Madhur et al., 2010a).

IL-17A acts on endothelial and smooth muscle cells in the vasculature to contribute to the hypertensive phenotype. In endothelial cells, IL-17A activates the expression of endothelial nitric oxide synthase (eNOS) and cyclooxygenase-2 (COX_{-2}), which is associated with angiogenesis (Krstić et al., 2013). In vascular smooth muscle cells, IL-17A induces inflammatory cytokine and chemokine expression. It promotes arterial

stiffening and the accumulation of extracellular matrix, which can contribute to vascular remodelling and can be the cause or consequence of hypertension (Safar et al., 2018).

1.4.2.2 IL-6

IL-6, the major pro-inflammatory cytokine, is produced by activated immune cells and stromal cells, including monocytes, macrophages, and dendritic cells. These stimulate related genes and alter cell proliferation, differentiation, and apoptosis by activating its associated receptors. IL-6 exerts its biological activity through 2 receptors: IL-6R (also known as IL-6R α, gp80, or CD126) and gp130 (also referred to as IL-6R ß, or CD130). Once the receptor recognises the IL-6 signal, downstream activation of the Janus kinases and RAAS-mediated signal pathways are initiated (Heinrich et al., 1998).

Mounting evidence indicates that IL-6 has a crucial role in aspects of the chronic inflammatory response. Reports show that IL-6 contributes to increases in blood pressure, inflammatory cell recruitment, and endothelial dysfunction in the context of Ang II-induced hypertension (Senchenkova et al., 2019). Clinical studies have presented that high BP treatment using irbesartan directly links lowering BP and circulating IL-6 levels in hypertensive patients (Vázquez-Oliva et al., 2005). Furthermore, inhibition of IL-6-related signalling pathways by tocilizumab (an IL-6 inhibitor used in patients with rheumatological disorders) has shown a considerable clinically therapeutic effect on several inflammatory diseases, including rheumatoid and juvenile arthritis (Ogata and Tanaka, 2012). In addition, evidence from *in vitro* experiments indicated that IL-6 may have the ability to facilitate water and sodium retention by promoting the expression and activity of sodium channels in mouse cortical collecting duct cells (Li et al., 2010). Taken together, studies suggest the potential therapeutic role of IL-6 in CVD, especially in hypertension.

1.4.2.3 **IL-1β**

Accumulating evidence suggests that interleukin-1β plays a significant role in the pathogenesis of various types of hypertension (Melton and Qiu, 2021). Preclinical studies indicate that elevated BP is associated with a proinflammatory state mediated, in part, by cytokines such as IL-1β, involved in altering endothelial, immune, and central nervous system responses, potentiating the progression of hypertension (Rothman et al., 2020a).

IL-1β is increased in ang II-induced hypertensive mice kidneys (Crowley et al., 2010a), and the activation of IL-1 receptor 1 (IL-1R1) has been shown to enhance sodium transporter activity leading to salt retention *via* the NKCC2 co-transporter in the nephron (Zhang et al., 2016). Deficiency or physiological blockade of the IL-1 signalling (Ling et al., 2017) and administration of IL-1β neutralising antibody have been demonstrated to limit blood pressure elevation in hypertensive animal models (Ling et al., 2017, Bhaskar et al., 2011). T cell activation, mediated through the central nervous system, vascular inflammation and the involvement of C-reactive protein (CRP) (Vongpatanasin et al., 2007), IL-16, IL-17 (Madhur et al., 2010b) and IL-1 are associated with the development of hypertension (Rothman et al., 2020a). Release of IL-1β is associated with BP elevation, end-organ damage, and hypertension (Rothman et al., 2020a).

In the CANTOS trial, canakinumab, a human monoclonal antibody targeting the IL-1β innate immunity pathway, led to significantly lower rates of recurrent cardiovascular events than placebo, independent of lipid-level lowering (Ridker et al., 2017). All participants had blood pressure systematically measured before randomisation throughout the CANTOS trial and throughout the follow-up (Ridker et al., 2018). Therefore, this trial provided a unique opportunity to test if the inhibition of IL-1β reduces blood pressure, preventing the incidence of hypertension development (Rothman et al., 2020a).

1.4.2.4 TNF- α

TNF- α is a pleiotropic cytokine augmented in chronic inflammatory states such as hypertension and diabetes; it is described to both increase and limit the rise in blood pressure. It is produced by M1 macrophages, lymphocytes, and endothelial and epithelial cells (Arango Duque and Descoteaux, 2014). It has been described to drive renal inflammation, blood pressure and subsequent target organ damage (Justin Rucker and Crowley, 2017).

High TNF-α levels decrease BP, whereas moderate increases in TNF-α is associated with increased NaCl retention and hypertension. However, it has been well documented that elevated TNF-α levels are associated with hypertension development (Ito et al., 2001). An experimental study by Tran et al. described how chronic treatment with etanercept (a chronic recombinant protein consisting of extracellular ligand binding domain for TNF receptor type 2, a TNF blocker) for six weeks prevented the increase in blood pressure in insulin-resistant fructose-fed rats without affecting insulin sensitivity (Tran et al., 2009).

1.4.2.5 IFN- *γ*

IFN-*γ* is produced by T cells, NK cells, macrophages/monocytes, neutrophils and DCs; as discussed previously, each of these immune cells is implicated in the progress of hypertension (Caillon et al., 2019a) and, in particular, modulates immune responses. Global IFN-*γ* deficient mice had blunted BP elevation in the Ang II and DOCA + salt hypertension models. IFN-*γ* can influence its surroundings directly through actions such as local dilation of blood vessels, allowing for immune cells to concentrate at sites of inflammation (Schroder et al., 2004)

Serum IFN-*γ* levels are shown to be a predictor of high systolic BP. With diastolic BP, there was significance correlation with IFN-*γ* and MCP-1 levels after corrections in age, sex, body mass index, smoking, fasting blood glucose and triglycerides (Mirhafez et al., 2014). Within models of hypertension with IFN-*γ* deficiency, studies have demonstrated a reduction in monocyte infiltration within the aorta and a reduction

in MCP-1 and other cytokines such as macrophage inflammatory protein 1α, and P-selectin ligand (Ozawa et al., 2007, Benson et al., 2022). Global KO of IFN-*γ* in hypertensive animal models and the study of related downstream signalling pathways of IFN-*γ* production from $CD8^+$ T cell (CD8T) in the kidney exhibited CD8T-stimulated salt retention *via* renal tubule cells, thereby exacerbating hypertension (Benson et al., 2022).

1.5 Role of IL-33 in Immune Regulation and Cardiovascular Biology

In 2005, Schmitz et al. reported a three-dimensional similarity between the carboxyl-terminal of the human high endothelial venules (HEVs) protein and the IL-1 cytokine family. Based on the observations, they proposed the name interleukin 33 (IL-33) (Schmitz et al., 2005b). Schmitz et al. reported that the new member of the IL-1 family mediates its biological effect *via* the IL-1 receptor ST2 (suppression of tumorigenicity 2; also known as IL1RL1), activating the downstream NF-κB and MAP kinases, driving the production of T_H2-associated cytokines from *in vivo* polarised T_H2 cells. The study also presented the expression of IL-4, IL-5, and IL-13 induced by IL-33, leading to stark pathological changes in mucosal organs (Schmitz et al., 2005b).

The human *IL33* gene (Baekkevold et al., 2003, Schmitz et al., 2005b) encompasses eight exons spanning more than 42 kb of genomic DNA. Single nucleotide polymorphisms linked to asthma susceptibility are identified in intron one and the promotor region (Torgerson et al., 2011). GeneBank entries reveal the existence of two transcripts for mouse *IL33*, *IL33a* and *IL33b* mRNA, with different 5'-untranslated regions, but they encode the same protein (Talabot-Ayer et al., 2012). IL-33 protein comprises two evolutionary conserved domains (the nuclear domain and the IL-1-like cytokine domain) separated by a highly divergent linker region in the central part (the central domain). The crystal structure of the IL-33-ST2 complex and its binding sites are indicated (Figure 1.3). Two individual ST2 binding sites have been identified in IL-33; in site one, acidic residues Glu148 and Asp149, and Glu165 at site 2 have a

crucial role in high-affinity binding by forming specific salt bridge interactions with basic residues of ST2 (Liu et al., 2013).

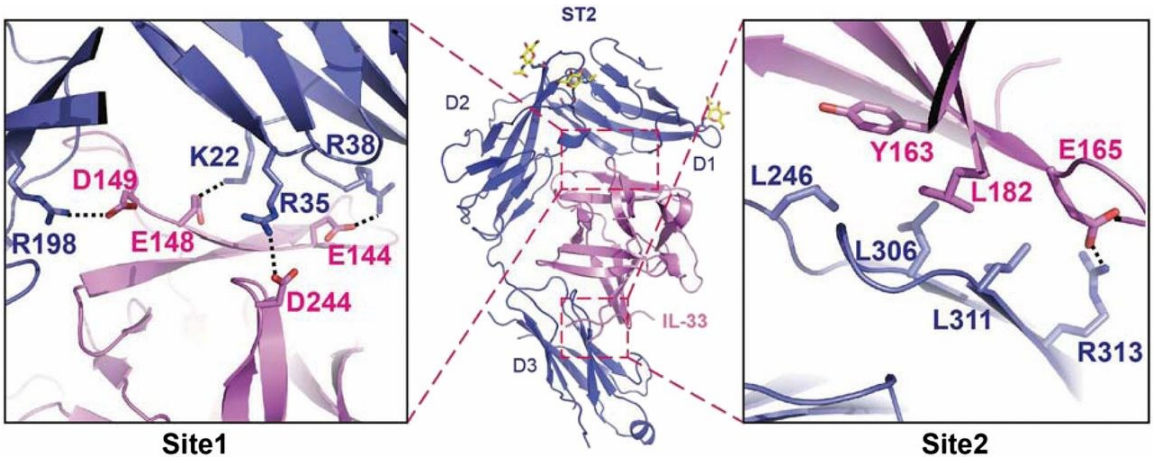

Figure 1.3: IL-33/ST2 interface.

Two distinct binding sites between IL-33 and ST2. At site 1, IL-33 acidic residues Glu144, Glu148, Asp149, and Asp244 form salt-bridge interactions with ST2 basic residues Arg38, Lys22, Arg198, and Arg35, respectively. Glu144 and Asp149 of IL-33 also have hydrogen-bonding interactions with ST2 main chain atoms. At site 2, IL-33 acidic residue Glu165 has salt-bridge interaction with Arg313 of ST2. A significant hydrophobic cluster at site 2 involves residues Tyr163 and Leu182 of IL-33 and Leu246, Leu306, and Leu311 of ST2. Figure from research article 'Structural insights into the interaction of IL-33 with its receptors' (Liu et al., 2013).

IL-33, a tissue-derived nuclear cytokine, is critical in tissue homeostasis and repair, type 2 immunity, viral infection, inflammation and allergy (Gautier et al., 2016a). It is characteristically released by damaged or necrotic barrier cells (such as endothelial and epithelial cells) (Cayrol and Girard, 2014), operating as an alarmin molecule forewarning the immune system upon endothelial and epithelial cell damage and is processed into active forms by various proteases. IL-33 was initially described as an inducer of type 2 immune responses, initiating T helper 2 (T_H2) and mast cells. However, over the years, experimental studies have provided accruing evidence for IL-33 to potentially stimulate type 2 innate lymphoid cells (ILC2s), regulatory T cells, T_H1 cells, $CD8^+$ T cells and natural killer (NK) cells (Liew et al., 2016).

IL-33 is constitutively expressed in the nuclei of producing cells during homeostasis, including endothelial cells from both small and large blood vessels, fibroblastic reticular cells of lymphoid organs, as well as epithelial cells, including epidermal keratinocytes; this further endorses the potential role of IL-33 in immune-inflammatory responses (Nile et al., 2010). Although already present at high levels in the steady state, the expression of IL-33 can be further upregulated during inflammation. For example, levels of nuclear IL-33 are increased in the airway epithelium of patients with chronic obstructive pulmonary disease (COPD) (Kearley et al., 2015, Byers et al., 2013b), in intestinal epithelium from bone marrow transplant recipients suffering graft-versus-host disease (GVHD) (Reichenbach et al., 2015) and in skin keratinocytes and blood vessels from patients with atopic dermatitis (Savinko et al., 2012). Enigmatic nuclear localisation is one of the fundamental properties of the protein detected in all producing cells in human and mouse tissues (Küchler et al., 2008). In inflammatory states, IL-33 is processed in the central activation domain by inflammatory proteases from mast cells and neutrophils, generating mature forms of IL-33 protein with 10 to 30-fold higher biological activity than full-length IL-33 in cellular assays (Lefrançais et al., 2012b).

IL-33 appears to be a chief cytokine in allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, obesity and a diverse range of tissue injury and repair-associated diseases, including myocardial infarction, stroke, microbial infection, hepatic and pulmonary fibrosis, systemic sclerosis, chronic obstructive pulmonary disease, autoimmune diseases and also cancer (Cayrol and Girard, 2014, Molofsky et al., 2015b, Byers et al., 2013b, Gautier et al., 2016a). Given these critical roles of IL-33 in health and disease, a substantial understanding of IL-33 biology and course of action is fundamental. Our knowledge of the role of IL-33 is expected to continue to expand, modulating both protective and pathological immune responses.

1.5.1 Cellular Sources of IL-33

1.5.1.1 Endothelial and Epithelial Cells

IL-33 protein is present in healthy mice and in humans with varying expression, primarily in non-haematopoietic cell nuclei (Schmitz et al., 2005b), with particular abundance in specialised populations of barrier cells (endothelial and epithelial cells) (Moussion et al., 2008a). The endothelium represents one of the primary cellular sources of IL-33 in normal human tissue, including lung, kidney, liver, colon, atherosclerotic plaques, and adipose tissue. A study by Moussion et al. detected ample expression of IL-33 along the vascular tree, specifically in the nuclei of endothelial cells from both large and small blood vessels of healthy tissue *in vivo* (Moussion et al., 2008b). Moreover, endothelial cells are also the chief cellular source of IL-33 during human disease. IL-33 is copiously expressed in blood vessels from chronically inflamed tissues of rheumatoid arthritis and Crohn's disease patients (Carriere et al., 2007) and in peritumoral blood vessels from various human tumour tissues. In addition to the endothelium, IL-33 was found to have constitutive expression in fibroblastic reticular cells of lymphoid tissues and epithelial cells of tissues exposed to the environment (Moussion et al., 2008b).

There are significant species differences between human and mouse IL-33 expression. Although IL-33 is detected in murine vascular adipose tissue, liver, and ovaries (Pichery et al., 2012, Carlock et al., 2014), the protein is not fundamentally expressed along the vascular tree in mouse models at steady state (Pichery et al., 2012). Other experimental studies have revealed that IL-33 is induced in cardiac endothelial cells after myocardial pressure overload, instigating systemic inflammation, and plays a significant role in the heart's response to pressure overload (Chen et al., 2015).

During tissue allergic insults and injury, epithelial cells also release IL-33, likely *via* necrosis and IL-33 expression is further induced (Molofsky et al., 2015a). Humans and mice share IL-33 expression at epithelial surfaces (Moussion et al., 2008a), including skin, stomach, intestine, salivary gland, vagina, and lung, where expression is exceptionally high in alveolar type 2 cells (Schmitz et al., 2005b). The expression

pattern particularly overlaps with other cytokines that target ILC2s, including IL-25, suggesting a potentially shared function (Bulek et al., 2010). In COPD disease models, upon inflammation progression, additional cell populations are presented to express IL-33, including epithelial progenitor cells (Byers et al., 2013a).

1.5.1.2 Fibroblasts

Nuclear expression of IL-33 in fibroblasts is produced by inflammation (Sponheim et al., 2010). Further, experimental wound healing in rat skin has revealed that the induction of IL-33 is associated with an early cell activation state (Sponheim et al., 2010). Depletion of IL-33 has been shown to augment the expression of extracellular matrix components like COL15A1 and TGLN and reduced levels of proinflammatory cytokines such as IL-6 and chemokines CXCL8, CCL7 and CCL8 (Gatti et al., 2021). Inflammation is the main driver for fibrosis, and fibroblast secretion such as CCL8 contributes to fibrosis pathology (Lee et al., 2017b). IL-33 is amply expressed by various fibroblasts, including fibroblastic reticular cells of lymphoid tissues (Moussion et al., 2008b), lung fibroblasts (Adachi et al., 2020), colonic fibroblasts (Waddell et al., 2021) and human cardiac fibroblasts during necrosis (Demyanets et al., 2013a). Proinflammatory cytokines such as TNF-α, IFN-γ and IL-1ß considerably increase both IL-33 protein and mRNA expression in cardiac fibroblast (Sanada et al., 2007c, Demyanets et al., 2013a). In response to biomechanical strain, both cardiomyocyte and cardio fibroblasts produce mature IL-33; however, fibroblasts are the principal cellular source of IL-33 (Sanada et al., 2007c) (Figure 1.4).

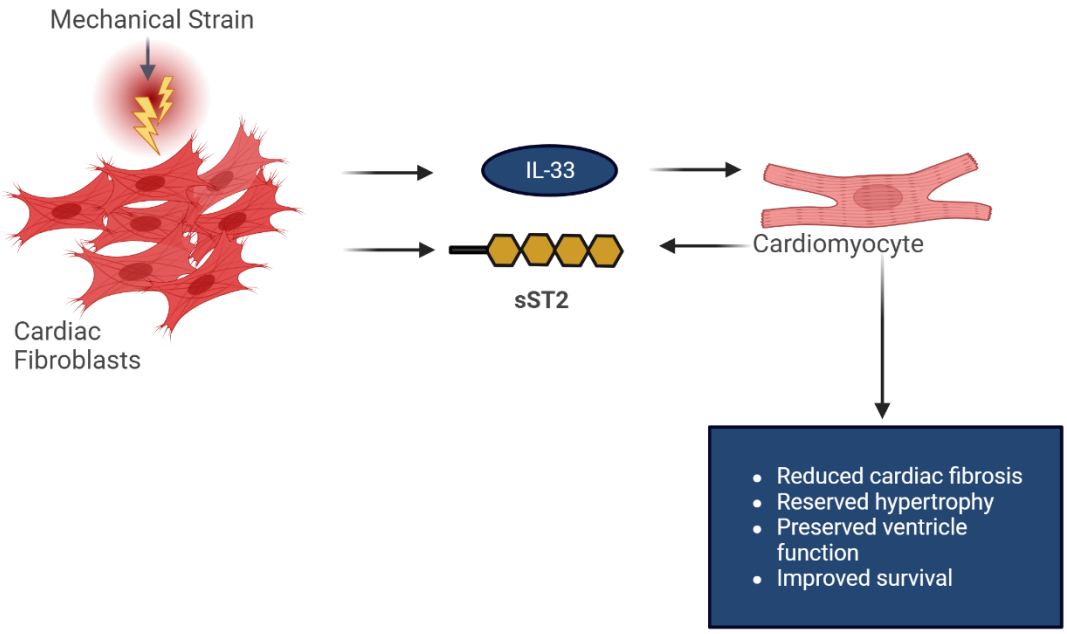

Figure 1.4: IL-33 and ST2 signalling in cardiac fibroblasts.
Disease conditions such as myocardial infarction, hypertension, and valvular diseases result in cardiomyocyte hypertrophy and augmented deposition of extracellular collagen. These responses ultimately lead to clinical heart failure. The IL-33/ST2 axis is emerging as a novel fibroblast-cardiomyocyte communication system that can abrogate maladaptive processes leading to heart failure. In response to biomechanical strain on the heart, cardiac myocytes and fibroblasts produce mature IL-33 and soluble ST2. Inevitable decline of ventricular function and premature mortality observed in the mice subjected to ventricular pressure overload are seen to be diminished upon IL-33 treatment. Figure adapted based on review paper 'The IL-33/ST2 pathway: therapeutic target and novel biomarker' (Kakkar and Lee, 2008).

1.5.1.3 Other Cell Types

Visceral smooth muscle cells, especially of the gastrointestinal and urogenital tracts, are notable cells exhibiting nuclear IL-33 in healthy human tissues (Moussion et al., 2008b). In severe asthma, airway SMCs have been shown to express nuclear IL-33 at low levels compared to epithelial cells and endothelial cells in the same lung tissue (Préfontaine et al., 2009). IL-33 mRNA expression was seen in murine thoracic aorta in normal-fat and ApoE$^{-/-}$ mice fed high-fat diets and augmented in atherosclerotic animals (Demyanets et al., 2011c). Experimental studies have validated the expression of IL-33 and ST2 protein and mRNA levels in human carotid atherosclerotic plaques, where nuclear IL-33 was detected in ECs (Demyanets et al., 2011a). Increased co-localisation of IL-33 with SMCs in atherosclerotic coronary artery sections is present compared to lesion-free human arteries (Cayrol and Girard, 2018).

In several studies, CD45^{+} cells, such as macrophages and mast cells, are declared the primary cellular sources of IL-33 (Tung et al., 2014). A study by Hus et al (2010) established findings that mast cells can produce IL-33 after IgE-mediated activation, and the IL-33/ST2 pathway is critical for the progression of IgE-dependent inflammation. However, conclusive evidence to support these claims has not yet been published.

1.5.2 Mechanism of IL-33 Release and Action

IL-33 is stored as a full-length protein in the cell nucleus under steady-state conditions; however, upon cell injury or necrosis, biologically active IL-33 is released from producing cells into the extracellular space as a full-length protein (IL-33$_{FL}$), which functions as an endogenous danger signal altering the immune system of cell or tissue damage (Cayrol, 2021). Studies have proposed that IL-33$_{FL}$ can be cleaved extracellularly by proteases from environmental allergens and inflammatory cells such as neutrophils or mast cells. IL-33$_{FL}$ is biologically active at high doses but has little activity at low doses. However, the cleavage of IL-33$_{FL}$ results in the generation of hyperactive mature forms exhibiting higher activity than IL-33$_{FL}$. IL-33$_{FL}$ and the hyperactive forms activate various tissue-resident or recruited immune cells

expressing the ST2 receptor to induce type 2 and type 1 inflammatory responses depending on the context (homeostasis, infectious or inflammatory disease) (Cayrol and Girard, 2022).

Viral infection through the respiratory syncytial virus can induce lung epithelium damage, triggering IL-33 release from primary bronchial epithelial cells (Kearley et al., 2015). In subjects with asthma, elevated levels of IL-33 were observed in nasal fluids of rhinovirus infection patients (Bønnelykke et al., 2014). The transient release of endogenous IL-33 has also been reported upon mechanical skin injury, such as tape stripping in mice and scratching on human skin (Galand et al., 2016). Tape stripping caused a significant increase in local and systemic IL-33 levels 1 hour after injury which returned to basal after 6 - 24 hours (Galand et al., 2016). Furthermore, the study by Kakkar et al. proposed the secretion of IL-33 from living cells upon biomechanical strain (1 Hertz, 8% biaxial stretch) in the absence of cellular necrosis (Kakkar et al., 2012). Endogenous IL-33_{FL} is released from endothelial cells after induction of cell necrosis by several cycles of freezing and thawing (Cayrol and Girard, 2018). Luthi et al. (2009) readily observed IL-33 release after cell necrosis induced by hydrogen peroxide, sodium azide or streptolysin O, a bacterial pore-forming toxin. In hypertension, the left ventricle undergoes hypertrophy, a known risk factor for cardiovascular mortality (Levy et al., 1990a). Pressure overload of the mouse heart by transverse aortic construction can induce the expression of IL-33 (Sanada et al., 2007a).

The IL-33 protein must be finely regulated to increase its activity when needed or, on the contrary, to prevent its activity from becoming harmful. Several molecular mechanisms have been described to be involved in regulating its activity. Several mechanisms could limit IL-33 bioactivity following its release. Soluble forms of ST2 have been proposed to function as decoy receptors blocking IL-33 myocardial and vascular benefits in biological fluids (Chen et al., 2015). Moreover, the physical activity of IL-33 is rapidly terminated (~2 h) in the extracellular environment by the formation of disulphide bridges in the IL-1-like cytokine domain, resulting in an extensive conformational change of the ST2 binding site and oxidation of cysteine

residues (Cohen et al., 2015). Inflammatory proteases have also been proposed to have a role in the termination of IL-33 activity following initial activation (Bae et al., 2012).

1.6 ST2

The suppressor of tumorigenicity 2 (ST2) protein is the receptor for IL-33. The ST2 gene is located on chromosome 2q12 in humans and is approximately 40kb long. Homologs for ST2 are found in mouse, rat, and fruit fly genomes. The initial discovery of ST2 in 1989 described a 2.7 kb transcript encoding ~37 kDa unglycosylated secreted protein corresponding to 60-70 kDa glycosylated product, which is now thought to have represented the soluble form of ST2 (Werenskiold, 1992, Kakkar and Lee, 2008). In 1993, a 5 kb transcript was identified with a putative transmembrane motif. The protein product proved to be the now-known transmembrane receptor ST2L (Yanagisawa et al., 1993). The soluble and the transmembrane forms arise from a dual promoter system to drive differential mRNA expression (Kakkar and Lee, 2008).

1.6.1 ST2L

ST2L is a member of the TLR/IL1R superfamily, sharing a common structure with an extracellular domain of three linked immunoglobulin-like motifs, trans-membrane fragments, as well as a cytoplasmic Toll/interleukin-1 receptor (TIR) domain. Multiple cell types (haematopoietic and non-hematopoietic) express ST2L on their surface and can activate IL-33. ST2L is expressed in T and B cells, which can induce the production of Th2 cytokines by T cells and enhance the T cell chemotaxis (Schmitz et al., 2005b, Komai-Koma et al., 2007). Additionally, ST2L is present in several inflammatory cells, particularly in innate helper 2 cells (Neill et al., 2010a), mast cells (Miller and Liew, 2011), and macrophages (Kurowska-Stolarska et al., 2009). Upon binding of IL-33 to the ST2L receptor on the macrophages, it promotes the formation of M2 macrophages and can reduce LDL uptake and increase cholesterol efflux (Miller and Liew, 2011). In endothelial cells, receptor activation can induce IL-6 and IL-8 production, prompt angiogenesis, and improve vascular permeability (Choi

et al., 2009). In epithelial cells, studies have demonstrated the production of innate chemokines and cytokines through the ST2L activation (Yagami et al., 2010). The ST2L receptor is similarly fundamental in regulating apoptosis through cardiomyocytes (Seki et al., 2009b, Weinberg et al., 2002a).

1.6.2 Soluble ST2

The soluble ST2 (sST2) lacks the transmembrane and cytoplasmic domains contained within the structure of ST2L and consists of a unique nine amino-acid C-terminal sequence (Gächter et al., 1996). In *vitro*¸sST2 production is upregulated by pro-inflammatory cytokines, including IL-1ß and TNF-α, in human lung epithelial cells and cardiac myocytes. In humans, soluble ST2 has also been shown to be produced spontaneously by cells in the lungs, kidney, heart, and small intestine. In the experimental model of HF, sST2 is upregulated in the lungs and secreted by pneumocytes in response to strain (Pascual-Figal et al., 2018). Studies have also considered the potential of sST2 production after activation with IL-33 in mast cells (Bandara et al., 2015) or anti-CD3/CD28 in both CD4 and CD8 T cells (Zhang et al., 2015a). The enhanced sST2 presence inhibits the production of type 2 cytokines, including IL-4 and IL-5 (Oshikawa et al., 2002). Soluble ST2 is detected in the serum of patients early after acute myocardial infarction and inversely correlates with ejection fraction (Weinberg et al., 2002a).

1.7 IL33/ ST2 Signalling

The transmembrane form of ST2 enables IL-33's signalling activity, whilst sST2 acts as a decoy receptor binding IL-33 to dampen its effects. Signalling of IL-33 can be activated through nuclear factor kappa-B (NF-κB), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase cascades (Buckley et al., 2019).

Once IL-33 is released from cells by mechanical stress, inflammatory cytokines, or cell necrosis, the full-length biologically active and actively processed IL-33 can bind to the membrane-anchored ST2L (Cayrol, 2021) on ST2L receptor-expressing target cells such as M2 macrophages or NK cells (Miller and Liew, 2011). This binding leads to conformational changing of ST2L, which provokes recruitment of the IL-1RAP subunit, forming a heterodimer complex (Chackerian et al., 2007)—the ligand-mediated heterodimerisation results in the juxtaposing of intracellular TIR domains. Subsequent sequestering of the adaptor proteins, such as myeloid differentiation primary-response protein 88 (MyD88) and MAL, results in modulation of IL-1R-associated kinase (IRAK) family members (such as IRAK1 and 4) mediated TNFR-associated factor 6 (TRAF6) activation and subsequent mitogen-activated protein kinase and IKK/NF- NF- NF-κB or AP1 activation pathway (Akira et al., 2001, Brint et al., 2004). The MAPK pathway is mediated by the activation of the MAPKs extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK) by specific upstream MAPK kinases (MAPKKs). These pathways may synergistically induce gene expression, leading to Th2 cytokine and chemokine synthesis. Soluble ST2 can bind IL-33 directly and act as a decoy receptor to the currently established downstream Th2 protective signalling pathway (Griesenauer and Paczesny, 2017) Figure 1.5.

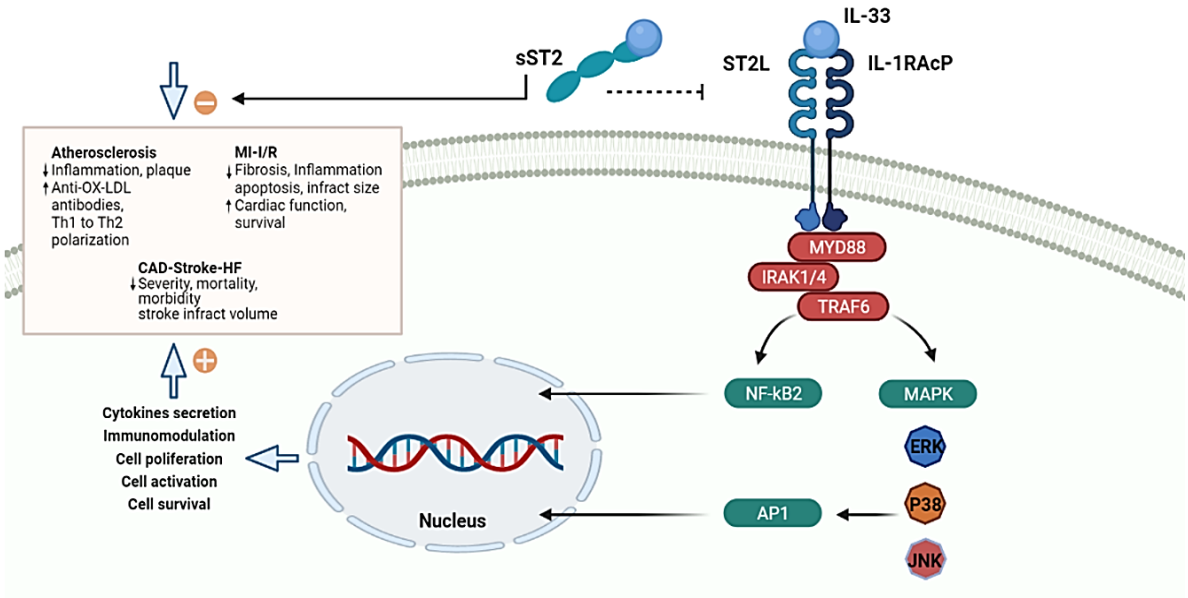

Figure 1.5: Interleukin-33 signalling pathway through ST2L and sST2, the decoy receptor.
Bioactive long-form of IL-33 released due to cell necrosis binds to ST2L/IL-1RAP heterodimer, leading to recruitment of MyD88 protein to the TIR intracellular domain of the IL-1RAP. MyD88 binding leads to IRAK-1/4 and TRAF6 attraction, producing several downstream signalling pathways: NF-κB, p38, and JNK. The same long form-IL-33, however, can also bind to the sST2 decoy receptor, preventing the ST2L/IL-33 actions on the production of Th2 cytokines and chemokines. During apoptosis, cleavage of IL-33 by caspases (-3/7) leads to inactivation of the pro-inflammatory properties. Figure adapted based on the review article 'Conflicting vascular and metabolic impact of the IL-33/sST2 axis' (Altara et al., 2018).

ST2/IL-33 signalling through Tregs promotes Foxp3 and GATA3 expressions and Treg function and expansion through the expansion of TGF-β1-mediated differentiation through a p38-dependent mechanism. It has recently been shown that IFN regulatory factor (IRF) 1, which can be activated through MyD88 signalling, can inhibit Tregs by binding to the Foxp3 promoter and preventing the Foxp3 transcription (Griesenauer and Paczesny, 2017). The dysregulation of IRFs can lead to the development of metabolic and critical cardiovascular diseases, including vascular intimal hyperplasia, cardiac remodelling, and stroke (Zhang et al., 2015b). Modulation of NF-κB activity through the IL-33 signalling pathway is complex. In unstimulated *in vitro* fibroblasts and cardiac myocytes, introducing IL-33 activates the NF-κB pathway. Conversely,

NF-κB activation through mechanical stimuli is attenuated by exposure to IL-33(Sanada et al., 2007a). Funakoshi-Tago et al. observed that IL-33-mediated ERK activation can be independent, although TRAF6 appears to be required for IL-33-mediated NF-κB activation and downstream production of Th2 cytokines (Funakoshi-Tago et al., 2008). Additionally, the AP-1 transcription factor can be independently activated, with its effects on NF-κB (Brint et al., 2002). Before IL-33 can bind to its transmembrane receptor, its actions can be altered by the decoy soluble receptor. sST2 is a variant of the full-length ST2 gene contained within the structure of the transmembrane isoform of the gene (Gächter et al., 1996). The concentration of unbound IL-33 available to bind to ST2L receptors is diminished by the binding of sST2 to IL-33 found in the extracellular environment, thus reducing the biological effects of IL-33 (Sanada et al., 2007a).

1.8 IL-33/ST2 and Immune Cells

Since IL-33 is expressed at barrier tissues, IL-33 and the IL-33/ST2 axis are emerging as an essential immunological pathway implicated in a wide range of immune diseases, activating various innate and adaptive immune systems and functioning as an alarmin molecule (Martin and Martin, 2016). Specific proteases, such as neutrophil and mast cell serine proteases, cathepsin G, chymase, and elastase, cleave IL-33 extracellularly and enhance its activity (Lefrançais et al., 2014, Lefrançais et al., 2012a). IL-33 is the main target of immune cells which consistently express ST2L, including group 2 innate lymphoid cells, mast cells and tissue-resident regulatory T cells. Other targets of IL-33 include dendritic cells, Th2 cells, B cells, macrophages, basophils, eosinophils, and natural killer cells, all expressing ST2L (Martin and Martin, 2016). In some instances, Th1 and $CD8^+$ T cells also show induced expression of ST2L. An experimental study by Dreis et al. demonstrated for the first time that ST2L expression is induced on $CD8^+$ T cells (Dreis et al., 2019). Therefore, IL-33, depending on the circumstances, can either activate and prolong inflammation (through the Th2, mast cells route) (Smith, 2010) or reduce and help protect against inflammatory responses (e.g. *via* ILC2 or tissue-resident regulatory cells).

The IL-33-AREG-EGFR signalling by ILC2 has been shown to mediate protection during intestinal injury by limiting inflammation and/ or promoting epithelial repair (Monticelli et al., 2015). However, through the activation of Th2 cells, IL-33 elicits a type 2 immune response, which, if exuberant, can lead to tissue damage; this likely happens through the activation of mast cells or eosinophils and the development of pathological fibrosis (Liew, Pitman and McInnes, 2010). In this way, IL-33 plays either a protective or harmful role in the pathophysiology of several proinflammatory and autoimmune diseases by acting through different inflammatory cells and pathways. Figure 1.6, adapted from Ghali et al., 2018, summarises various notable actions of IL-33 and its actions in other immune cells, which is relevant in modifying heart functions.

A major part of several cardiovascular diseases, including atherosclerosis, is the chronic inflammation of the artery wall (Jia et al., 2022), which may be caused by the metabolic stress imposed on the endothelium and the resultant excessive production of oxygen free radicals (Ohara et al., 1993, Harrison et al., 1987). Binding of IL-33 to ST2L ameliorates Th1 cytokine production, reducing IFN-γ, IL-2 , TNF-α levels and considerably increases Th2 cytokine production (including IL-4, IL-5, IL-6, IL-8, and IL-13) (Miller et al., 2008). Thus, the IL-33/ST2L axis provokes polarisation from Th1 to Th2 anterior atherosclerotic immune response, inhibiting atherosclerosis progression (Liu et al., 2022, Munjal and Khandia, 2020). In Ang II-infused C57BL/6J mice, INF-γ was elevated in plasma and kidney infiltrating $CD8^+$ T cells but not $CD4^+$ T cells (Saleh et al., 2015). IFN-γ receptor deletion significantly reduces cardiac hypertrophy, macrophage and T cell infiltration and fibrosis initiated by Ang II in 129SV mice (Markó et al., 2012).

ST2L expression by Th2 cells depends on GATA3 signalling and is upregulated by IL-6, IL-1, TNF-α and IL-5 (Guo et al., 2009). Resting Th2 cells express little GATA3, which is upregulated substantially by IL-33 and STAT5 activator, increasing ST2L from its low-level expression on resting Th2 cells (Guo et al., 2009). IL-33 expression of Th2 cells *in vitro* promotes the production of IL-5 and IL-13 (Schmitz et al., 2005a). IL-5 is an anti-inflammatory cytokine that has been demonstrated to play a role in cardiovascular diseases, including aortic aneurysms and heart failure (Ye et al., 2020). IL-13 has also been shown to inhibit the vasculitis response of idiopathic pulmonary artery hypertension (IPAH) by suppressing the proliferation of pulmonary artery smooth muscle cells. IL-13 activates downstream signalling molecules, inducing STAT3 and STAT6 signalling pathways and hinders the migration and proliferation of pulmonary artery smooth muscle cells and the secretion of endothelin-1 (ET-1) by endothelial cells (Wei et al., 2022). By activating Th2 cells, IL-33 stimulates a type 2 immune response, as described previously, if exuberant promotes tissue and organ damage, probable through the activation of mast cells or eosinophils and the development of fibrosis (Gieseck et al., 2018).

Yu et al. recently reported that ILC2s could promote cardiac healing and recovery of ventricular function through the IL-2 axis after myocardial infarction (Yu et al., 2021). IL-33, seen to signal by the ST2 receptor binding, is associated with ILC2 activation and regulates tissue homeostasis and repair following tissue injury in various tissues. Experimental findings indicate the presence of IL-33-responsive ILC2s in cardiac tissue, activation of such ILC2 cells, and expansion provide cardio-protective function *via* ILC2-derived factors (Chen et al., 2021). Chen et al. observed that ILC2s provide protection from cardiac fibrosis and improve myocardial function (Chen et al., 2021).

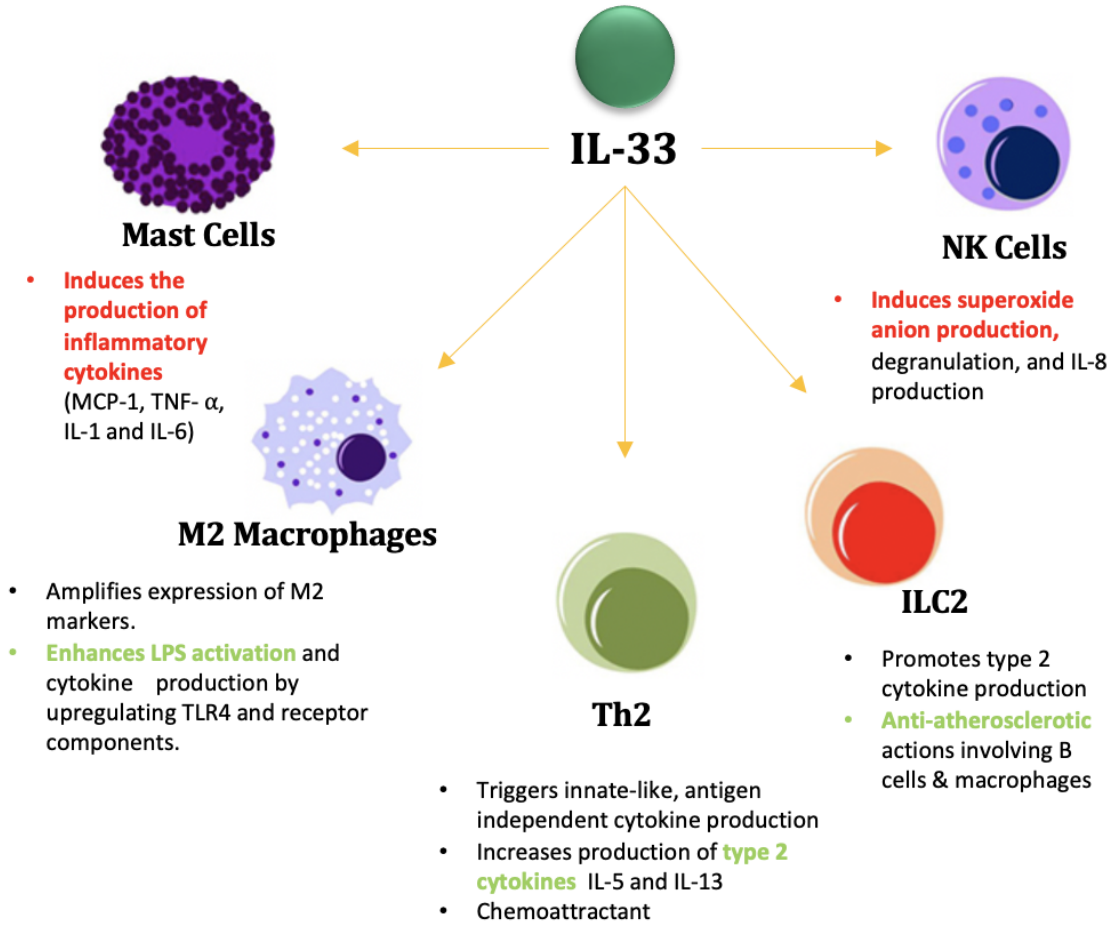

Figure 1.6: Actions of IL-33 signalling on different immune cells.
Principal actions of IL (interleukin)-33 on certain immune cells that are relevant for heart function and remodelling (Ghali et al., 2018). IL33 is predominantly expressed by stromal cells such as epithelial and endothelial cells. Tissue damage and mechanical stress to these cells provoke necrosis and release of IL33. IL-33, when released, have a diverse range of actions on key immune cells, enhancing their function and affecting heart function and remodelling. Figure modified based on the journal article 'IL-33 (Interleukin 33)/sST2 Axis in Hypertension and Heart Failure' (Ghali et al., 2018).

1.9 IL33/ST2 Axis in Cardiovascular Disease

IL-33 plays a role in a diverse range of cardiovascular disorders (Sanada et al., 2007a), either at a genetic level through the regulation of transcription or as a classically active cytokine. The dysregulation of the IL33/ST2L signalling and increased sST2 production is implicated in a variety of different diseases, including cardiac diseases (Caselli, 2014), atherosclerosis (Miller et al., 2008), type 2 diabetes (Miller et al., 2008), inflammatory bowel disease (IBD) (Pastorelli et al., 2010) and graft-versus-host disease (GVHD) (Griesenauer and Paczesny, 2017). IL-33 expression has been reported in coronary artery smooth muscle cells (Schmitz et al., 2005a), coronary artery endothelium (Bartunek et al., 2008), non-HEV endothelial cells (Moussion et al., 2008b, Küchler et al., 2008), and adipocytes (Wood et al., 2009) and further also in cardiac fibroblasts suggesting that IL-33 may play roles in various CV diseases (Miller and Liew, 2011). Stimulation of the IL-33/ST2 axis has been reported to promote the cardioprotective arm in the context of ventricular biomechanical stress (Sanada et al., 2007a, Weinberg et al., 2002b), prevent myocardial apoptosis, and alleviate myocardial fibrosis and myocardial hypertrophy (Seki et al., 2009a). It, therefore, inhibits the progression of atherosclerosis. Conversely, serum IL-33 levels are elevated in heart failure patients and those with stent restenosis after myocardial infarction (Demyanets et al., 2014). Accumulating studies have suggested that sST2 attenuates the cellular and beneficial actions of IL-33 in the cardiovascular system (van den Berg et al., 2022). Due to the involvement of the IL-33/ST2 signalling pathway in several processes, activating this pathway likely has unintended consequences. Inhibition of the IL-33/ST2 system to modulate inflammatory conditions could increase cardiovascular injury in the face of ventricular strain.

1.9.1 Atherosclerosis

Adaptive immune cells are involved in all stages of human atherosclerosis. During atherosclerosis, immune cells such as mast cells, monocytes, and T cells infiltrate the plaques within the tunica intima of the arterial wall (Hansson and Hermansson, 2011). The Th1 immune response drives the disease through the production of cytokines such as IL-2 and IFN-γ, a proatherogenic cytokine able to activate macrophages, inhibit proliferation, and reduce collagen production by smooth muscle cells (Davenport and Tipping, 2003, Gupta et al., 1997). Additionally, studies have established that deficiency of the Th2 cytokine IL-5 reduced the production of atheroprotective ox-LDL antibodies and caused elevation of atherosclerosis in ApoE $^{-/-}$ mice (Binder et al., 2004).

IL-33 and ST2 are present in mice and humans' normal and atherosclerotic vasculature. Miller et al., established that the inducement of IL-33 can reduce the development of atherosclerosis by producing a Th1- to -Th2 switch. Treatment of ApoE$^{-/-}$ mice with recombinant IL-33 administration demonstrated a significant reduction in atherosclerotic lesion size in the aortic sinus and reduced plaque F4/80^{+} macrophage and CD3^{+} T cell content (Miller et al., 2008). Moreover, IL-33 treatment significantly amplified Th2 cytokines IL-4, IL-5, and IL-13 levels in serum and lymph node cells of Apolipoprotein E (ApoE) $^{-/-}$ mice. Furthermore, IL-33-treated ApoE$^{-/-}$ mice produced substantially elevated levels of protective anti-oxidized low-density lipoprotein (ox-LDL) IgM antibodies. All of these are decreased in atherosclerosis and contribute to the disease progression. Conversely, Miller et al. further illustrated the protective role of IL-33 by treating ApoE $^{-/-}$ mice with sST2 through intraperitoneal injections. These mice developed significantly enlarged atherosclerotic plaques and increased IFNγ levels (Miller et al., 2008).

Cell-based experiments have also shown that ST2 is integral to the action of IL-33 on macrophage foam cell formation, providing further evidence for the anti-atherosclerotic effects of IL-33 (McLaren et al., 2010). These studies indicate the protective role of the IL-33/ST2 signalling axis in atherosclerosis.

1.9.2 Myocardial Infarction

IL-33 is known to have a major impact on the process of myocardial infarction (MI) (Chen et al., 2019). After ischaemia–reperfusion, IL-33 treatment decreases fibrosis, infarct size, and apoptosis in the rat, enhancing cardiac function (Seki et al., 2009a). Furthermore, recombinant IL-33 treatment reduced ventricular dilation, improved contractile function, and increased survival following coronary artery ligation in WT but not in $ST2^{-/-}$ mice (Seki et al., 2009a).

IL-33 treatment is linked to a reduction in mast cell density in the infarct area, while an increase in Th2 and a decrease in Th1 genes in the infarct are observed. IL-33 additionally activates mast cells, and a reduction in cardiac mast cells was reported to attenuate myocardial contractility after MI (Ngkelo et al., 2016).

Another study on MI by (Yin et al., 2014) in mice reported similar beneficial effects of post-IL-33 treatment. Treatment with IL-33 substantially heightened cardiac function and structure by augmenting LV function, reducing infarct size and wall thinning, and suppressing myocardial macrophage infiltration and the production of inflammatory cytokines in the myocardium (Yin et al., 2014). MI-induced activation of p38 MAPK and NF-κB pathways were also blocked upon IL-33 administration. A clinical study by Xing et al. observed significant upregulation of sST2 and interleukin-33 levels in patients with acute myocardial infarction compared to normal healthy controls (Xing et al., 2021). IL-33 and sST2 levels were further enhanced in the heart failure group. Multivariate logistic regression analysis demonstrated that interleukin-33 and sST2 could be independent predictors for acute myocardial infarction (Xing et al., 2021).

1.9.3 Hypertension

In chronic hypertension, the LV undergoes hypertrophy, a known risk factor for cardiovascular mortality (Levy et al., 1990b). Pressure overload of mouse heart by TAC has been found to induce IL-33 expression. Moreover, greater adverse remodelling and LV hypertrophy were observed in the global ST2 knock-out mouse (Sanada et al., 2007b). The study reported the ability of IL-33 to antagonise agonist-induced cardiac hypertrophy in neonatal rat cardiomyocytes, an action blocked by sST2. Similarly, treatment with recombinant IL-33 attenuated cardiac hypertrophy and remodelling in WT but not $ST2^{-/-}$ mice (Sanada et al., 2007b).

Endothelial-derived IL-33 facilitated anti-hypertrophic responses in ST2-expressing cardiomyocytes (Chen et al., 2015). Moreover, IL-33 elicits selective inflammatory responses through IL-13 and TGF-ß1. Either endothelial-specific deletion of IL-33 or cardiomyocyte-specific deletion of *ST2* exacerbates cardiac hypertrophy with pressure overload (Chen et al., 2015).

Even though IL-33 exerts cardioprotective functions following cardiac injury, it has been linked to the activation of type 2 immune responses *via* ILC2s. ST2L is present in the cardiac ILC2 population resident in the mouse heart. IL-33 treatment elicits cardiac ILC2 population expansion *via* the ST2 receptor. It provokes protective effects against catecholamine-induced cardiac fibrosis, reducing cardiomyocyte death, immune cell infiltration, cardiac fibroblast activation, fibrotic gene expressions, and improved myocardial function (Chen et al., 2021). The study further informed that ILC2-derived factors such as IL-13, Areg, and bone morphogenetic protein (BMP)-7 concurrently impact IL-33-mediated anti-fibrotic responses following acute cardiac injury promoting cardiac protective functions and the alleviation of cardiac fibrosis (Chen et al., 2021).

Furthermore, mechanical stress in cardiac endothelial cells and fibroblasts during pressure overload can result in the release of active IL-33 without cell death (Kakkar et al., 2012); furthermore, in mice, myocardial overload in hypertension has been shown to cause the release of IL-33 by cardiac endothelial cells with consequent

systemic inflammation (Chen et al., 2015). Enhanced serum concentrations of IL-33 are reported in many diseases, and stimulation of many cell types results in the release of bioactive IL-33.

It has been speculated that when hypertension occurs, the vascular tissue can undergo mechanical stretch, which can cause the release of biologically active IL-33; therefore, IL-33 expression in cells will be enhanced. Yin at al., (2019a) confirmed that stress caused by high blood pressure can stimulate sST2 protein production from cardiac, aortic, and coronary endothelial cells. Expression of IL-33 by qPCR and microarray analysis was significantly increased in the aortas of mice receiving Ang II infusion for 1-7 days compared with control mice. Furthermore, when levels of sST2 and IL-33 were checked in serum and PBMCs of humans, the sST2 levels were markedly elevated in hypertensive patients compared to control patients. They provided the alteration of the IL-33-sST2 pathway in hypertension. Overall, sST2 acts as a risk factor for the occurrence of essential hypertension (EH) and may represent a promising novel marker for EH prediction (Yin et al., 2019a).

Additionally, when ST2L mRNA levels were checked, it showed a marked reduction in expression, indicating that the transcription of ST2L and expression of ST2L on the cell surface was decreased. ST2L/IL-33 binding leads to NF-κB, which can then control the transcriptional activation of genes involved in inflammation, differentiation, proliferation, and apoptosis. Besides, SIGIRR has recently been shown to negatively regulate the IL-33/ST2L signalling (Thomassen et al., 1999). As stated previously, elevation of sST2 can act as a decoy receptor binding to circulating IL-33 in serum and decrease the amount of available IL-33 which can interact with the ST2L receptor, resulting in weakened IL-33/ST2L signalling pathway and therefore reducing the vascular and cardioprotective effect of IL-33 which may promote the development of hypertension.

1.9.3.1 sST2 as a Disease Biomarker

Circulating sST2 levels may have diagnostic and prognostic value in hypertension (Ojji et al., 2014, Ojji et al., 2013). Upregulation of sST2 levels in serum is correlated with modifications of left ventricular geometry (Ojji et al., 2013) and elevated systolic blood pressure, predicting changes in BP typically seen with ageing and progressive arterial stiffness (Ho et al., 2013).

As mentioned above, ST2 is a member of the interleukin-1 receptor family. Several experimental and clinical studies have discussed the likelihood of circulating soluble ST2 concentrations reflecting cardiovascular stress and fibrosis. It influences immunologic processes with consequent cardioprotective effects, including prevention of myocardial hypertrophy and fibrosis, which indicates a predictive potential in hypertensive heart disease. Numerous studies have highlighted the rise in serum sST2 levels as a predictive biomarker for mortality and heart failure in patients with acute myocardial infarction. The primary known source of sST2 is cardiac fibroblasts and cardiomyocytes in response to stress and injury.

Among Framingham Heart Study participants, elevated blood levels of sST2 were associated with hypertension and diabetes mellitus (Hughes et al., 2014); however, in healthy individuals from the general population, sST2 had little predictive value for cardiovascular events (Coglianese et al., 2012). Furthermore, the study by Topf et al. (2021) investigating the potential clinical relevance of sST2 in patients with therapy-resistant arterial hypertension (raHTN) undergoing renal denervation (RD) showed that in patients with raHTN, RD is associated with a significant decrease of sST2 levels, which indicates that sST2 is involved in remodelling processes after RD. Furthermore, baseline sST2 was positively correlated with systolic blood pressure at one month, measured either at the office ($r=0.57$, $p<0.01$) or invasively in the aorta ($r=0.49$, $p=0.03$), indicating a potential predictive value of this biomarker (Topf et al., 2021).

1.10 Hypothesis and Overarching Research Aim

Hypertension has been identified as an inflammatory disease by several experimental and clinical studies (Harrison, 2013). Interleukin-33 acts as an alarmin, and its role has been demonstrated in driving immune regulation and inflammation, influencing cells of both the vascular and immune systems both in steady state and in various cardiovascular pathologies. While the influence of the IL-33/ST2 axis has been examined in other aspects of CVD, the actions of IL-33 in regulating hypertensive pathology are ill-defined.

I propose that IL-33 functions as an alarmin molecule upon cell injury and signals the immune system in hypertension and associated target organ damage. I aimed to define the role of IL-33 and ST2 in the context of the vasculature in hypertension.

To achieve the main objective, three specific aims were addressed:

Aim 1. To characterise the expression of IL-33 and its receptor ST2 in animal models of hypertension and at the cellular level in key organs involved in the development of hypertension, in particular, the vasculature.

Aim 2. To investigate the functional role of IL-33 and ST2 in the pathophysiology of hypertension utilising knockout models, specifically IL-$33^{-/-}$ and $ST2^{-/-}$ animals, to understand the impact of these proteins on hypertensive disease processes.

Aim 3. To explore the function of IL-33 and ST2 in patients with hypertension, aiming to provide translational, clinical context to key pathophysiological findings.

Chapter 2 Chapter 2 Methods

2.1 Animal Models

2.1.1 Mouse Strains

Male C57BL/6J wild-type (WT) 10-week-old mice were obtained from Charles River Laboratories (UK). Breeding pairs of florescent reporter IL-33 $^{Cit/+}$, a global IL-33 knockout BALB/c mouse (also referred to as IL-33$^{-/-}$) (Hardman et al., 2013), and the global ST2-deficient (ST2$^{-/-}$) C57BL/6J mouse strain were obtained from Prof. Padraic Fallon's lab at the Trinity Translational Medicine Institute, Trinity College Dublin, Dublin, Ireland.

The reporter IL-33 $^{Cit/+}$ mouse uses citrine fluorescence as a surrogate for IL-33 mRNA expression with the GFP-derived *Citrine gene* inserted, using gene targeting directly downstream of the ATG start codon of *Il33* (Hardman et al., 2013).

The IL-33 $^{Cit/+}$ and ST2$^{-/-}$ mouse colonies were bred at the Central Research facility, University of Glasgow, Scotland. Mice were housed in ventilated and filtered cages in a pathogen-free facility and were maintained on a 12/12-hour light/dark cycle with free access to food and water. All procedures were performed in accordance with local ethical and UK Home Office regulations under the Animal Scientific Procedure Act 1986 under the licence number PP3412366

2.1.2 Angiotensin II-Induced Model of Hypertension

Randomisation and allocation concealment were performed. Briefly, Male WT, IL-33$^{Cit/+,}$ and ST2$^{-/-}$ mice aged 12 weeks were randomly assigned into treatment groups; mice underwent either sham or Angiotensin II (Ang II) osmotic minipump implantation and were labelled using animal facility labelling system. Operators for all assays were blinded to group allocation during all analytical work.

All twelve-week-old mouse strains used in the project were anaesthetised with Isoflurane 2.5% and osmotic minipumps Alzet Model 2002; Alzet, Cupertino, CA, USA) were implanted to permit subcutaneous infusion of Angiotensin II (Val^5, Sigma-Aldrich; cat# A2900) at a constant rate of 490ng/min/kg for 14-days (Widder et al., 2007), (Guzik et al., 2007). Vehicle-infused animals underwent an identical surgical procedure, except the minipump contained the vehicle for Ang II (0.5M NaCl, 0.01M CH_3COOH in sterile saline). Throughout my thesis the vehicle-infused animals are referred to as "sham", unless otherwise specified.

Following experiment completion, mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation to confirm death; cold phosphate-buffered saline (PBS) was then perfused through the left ventricle of the heart for 3 minutes to remove blood contamination from the organs. The heart/body weight ratio of mice was measured to assess Ang II cardiac hypertrophy. Afterwards, organs were harvested and appropriately stored for experimental studies and further analysis.

2.1.3 Hydralazine/Hydrochlorothiazide Treatment

The combination of hydralazine (320mg/L) and hydrochlorothiazide (60mg/L) was used to treat high blood pressure. In the subset of animals, the vasodilator combination was administered in the drinking water to normalise blood pressure. Mice received Hydralazine and Hydrochlorothiazide combination treatment for two weeks starting the day before osmotic minipump implantation.

2.2 Blood Pressure Measurements

2.2.1 *Tail Cuff Plethysmography*

A BP-2000 Series II Blood Pressure Analysis System (Therassay/Visitec Systems Inc.) was used to measure blood pressure by non-invasive tail-cuff (Wang et al., 2017, Krege et al., 1995).

During Angiotensin II treatment, mice undergoing blood pressure measurement by tail cuff were trained for five days before minipump implantation. BP of mice was monitored each day at the same time with the platform warmed to 37℃. They were handled carefully, causing minimal stress, placed on mouse restrainers, and adjusted carefully. The mice were then secured in place by gently taping down the end of their tails. A total of 10 preliminary and 15 actual measurements in each session were performed at 30-second intervals at a maximum pressure of 200 mmHg. The non-invasive BP measurement was taken every day from day one till day 14 after the surgery when the experiment was terminated. Data were exported in their raw format. Animals were rotated through all the channels daily to avoid channel-to-channel variations.

The Chauvenet's criterion (measurements greater than 2 SD from the mean) was automatically detected by the BP-200 Software. All null values and outliers were also excluded. The mean values of each set of 15 measurements were calculated, and values were used for subsequent analysis.

2.3 Proteome Profiler Mouse XL Cytokine Array

The Mouse XL Cytokine Array Kit (R&D Systems; Cat# ARY028) is a rapid, sensitive, and economical tool to detect cytokine differences between samples simultaneously. The relative expression of 111 soluble mouse proteins was determined through immunoassays (nitrocellulose membrane containing different capture proteins printed in duplicate). Homogenised sham and Ang II tissue lysates of the aorta, kidney, perivascular adipose tissue (PVAT), lymph node, and spleen were generated from C57BL/6J mice.

Provided manufacturer's protocol was carefully followed while preparing all necessary reagents with the materials provided and adhered to throughout the cytokine assay procedure and data analysis.

2.4 Histology

Organs for histological studies were either snap-frozen in OCT, kept at -80°C, or processed and embedded in paraffin and used in separate staining protocols. All paraffin sections used for staining were sectioned using a microtome at 5µm thickness, oven-baked for 1hr at 60 °C, and cooled to room temperature (RT). OCT samples were cut using a cryotome at a thickness of 7µm and stored at -80°C.

2.4.1 IL-33 Immunohistochemistry Using DAB / HRP Staining

2.4.1.1 Mice Samples

In DAB ('3-3'-Diaminobenzidine) / HRP (horseradish peroxidase) staining, DAB is oxidised by hydrogen peroxidase in a reaction typically catalysed by HRP. The oxidised DAB forms a brown precipitate at the location of the HRP, which can be visualised using light microscopy (Török et al., 2020), Figure 2.1.

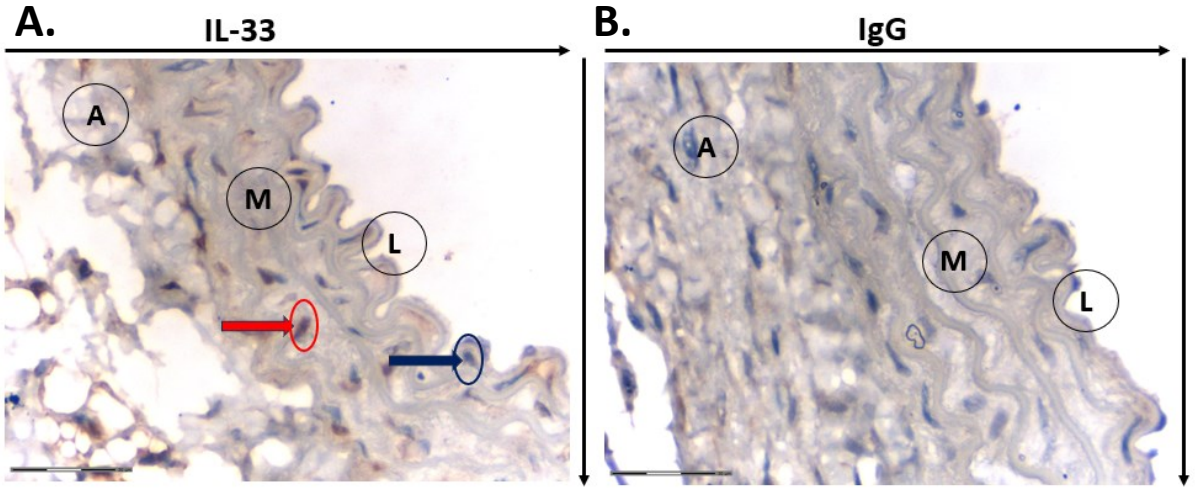

Figure 2.1 DAB/HRP IL-33 representative staining.
Oxidised DAB produces a brown precipitate, at the location of the specific HRP (circled in red), unstained cells circled in blue. (A) IL-33 stained and (B) Rabbit IgG. Scale bar 30 µm. Red arrow marks positive staining and blue arrow marks negative staining. L-lumen, M- media and A- adventitia.

Formalin-fixed and paraffin-embedded 5µm sections were deparaffinised using xylene and several ethanol gradient incubations and washed in dH_20 before antigen retrieval with sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6).

Slides were boiled in a pressure cooker for 10 minutes afterwards left to reach RT, followed by washes in TBS with 0.1% Triton (TBS-T) and TBS. Non-specific antibody binding was blocked with 5% normal goat serum (Vector Laboratories: S-1000) + 1%BSA +0.25% tween diluted in 1xPBS (pH 7.8) at RT for 1 hour. Rabbit polyclonal anti-IL-33 (2.5µg/mL; Thermofisher, PA5-20398) and anti-ST2 (10 µg/mL; Thermofisher, cat# PA5-20077), diluted in 3% normal goat serum with 1%BSA +0.25% tween in 1xPBS, were incubated overnight at 4˚C. After primary antibody incubation, samples were washed and blocked for endogenous peroxidase using 0.3% H_2O_2 at RT for 10, followed by a 1-hour RT incubation of goat anti-rabbit biotinylated secondary antibody (1:500; Dako, UK, E0432). Later, sections were developed with VECASTAIN Elite ABC-HRP solution (Vector Laboratories, UK, cat# PK-6200) for 30 min at RT. HRP was revealed with DAB peroxidase solution (Vector Laboratories; cat# SK-4105) and counterstained with Haematoxylin (Harris) (Sigma-Aldrich; cat# H3136), dehydrated and mounted with DPX-mounting medium (CellPath).

2.4.1.2 Human Samples

Human frozen Internal Mammary Arteries (IMA) of hypertensive patients undergoing coronary artery bypass grafts were obtained as part of a previous study (Sulicka-Grodzicka et al., 2023). IMAs were sectioned at a thickness of 7µm using a cryostat and fixed with 10% formaldehyde for 10 minutes. Non-specific binding was blocked with 0.1% normal serum (Vector Laboratories) diluted in 0.1% Saponin-1xPBS at room temperature (RT) for 1 hour. Primary antibody: Rabbit polyclonal anti-IL-33 (2.5µg/mL; ThermoFisher; cat# PA5-20398), diluted in 1X PBS (pH 7.4) with 0.1% Saponin-1xPBS, was incubated overnight at 4˚C. Afterwards, the previously described protocol for paraffin-embedded mouse aortas was followed.

2.4.2 Picro-Sirius Staining

To visualise collagen accumulation/fibrosis in mouse heart and aorta, frozen OCT sections were air dried for 10mins, incubated for 5 minutes in 1X dPBS (ThermoFisher; cat# 14190-094), and taken down an ethanol gradient, 5mins at each step. Sections were fixed in 10% formalin for 1 hr and washed in running tap water before

proceeding with picrosirius fast green stain (Sigma; cat# CAS 2353-45-9) for 1 hour at room temperature in the dark. Stained sections were then dipped ten times in distilled water (dH_20), 20 seconds in 70% ethanol, 20 dips in 100% ethanol, 2 minutes in 2 changes of xylene, and mounted with DPX mounting media (CellPath).

2.4.2.1 Collagen Quantification

To quantify vascular and cardiac fibrosis, picrosirius green stained sections were imaged using an EVOS microscope, and collagen was stained as deep pink**.** Using ImageJ software, the total fibrotic area of each section for both the aorta and heart was calculated (Schneider et al., 2012), (Awwad et al., 2023). Initially, on ImageJ software, the EVOS-acquired picture was split into separate channels; the green channel was chosen, and the region of interest (ROI) was selected, from which the total tissue area was obtained. A threshold was then set to detect only pixels positive for the collagen staining on the green channel, shown as dark pixels (Figure 2.2.) The area comprising collagen was then quantified and analysed as a percentage (%) for the heart and the total fibrotic cross-sectional area (mm^2) for the aorta.

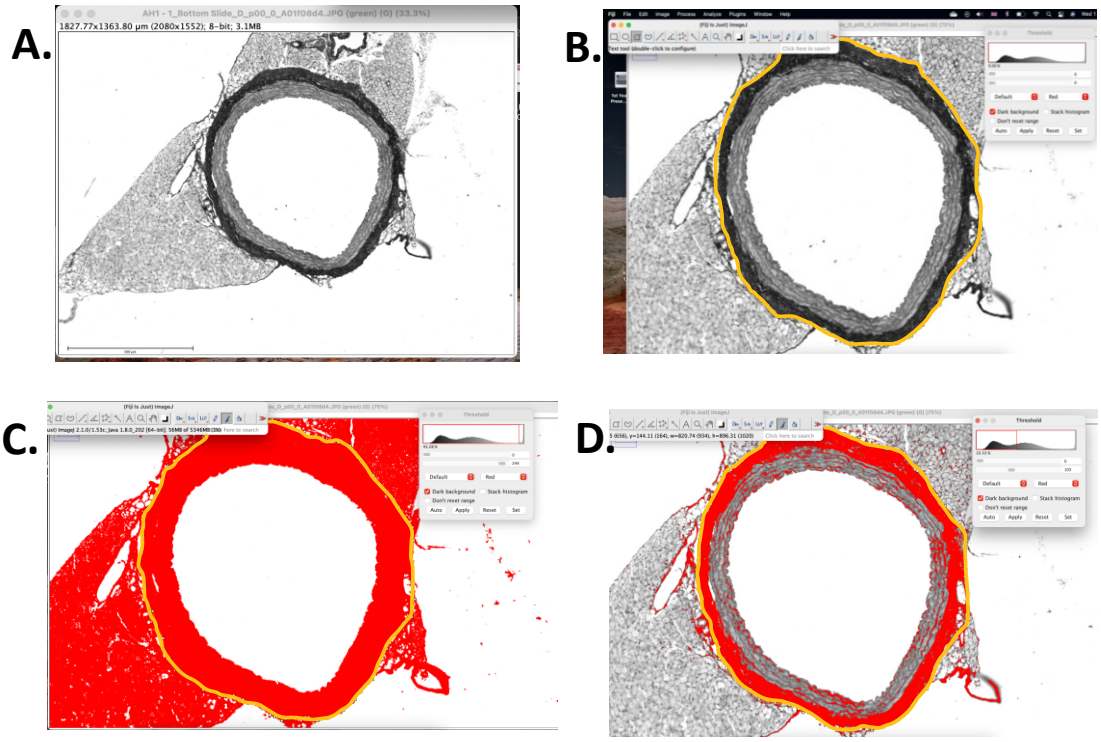

Figure 2.2 Analysis of collagen accumulation in experimental mouse model using ImageJ.
Sections of the mouse thoracic aorta was stained with Picrosirius Green and imaged using a brightfield light microscope. To calculate collagen accumulation, ImageJ software was used and split into different colour channels. Green colour channel was selected (**A**). Region of Interest (ROI) was drawn (highlighted with the yellow line) (**B**), and total area using threshold tool was measured by covering the full ROI of the tissue (**C**). Fibrotic area of the section was measured by readjusting threshold value to cover only the dark area (collagen), shown up as dark/black pixels (**D**). The area comprising of collagen was then quantified and analysed as a percentage (%) for heart and total fibrotic cross-sectional area (μm^2) for the aorta. Scale bar 500 μm.

2.5 Cardiomyocyte Hypertrophy Histological Assessment

2.5.1 Wheat Germ Agglutinin

Cardiac hypertrophy was quantified by wheat germ agglutinin (WGA) staining. Paraffin-embedded heart samples were sectioned at 5µm, baked, and deparaffinised as previously described for the mice HRP/DAP staining protocol. After the antigen retrieval step in 10mM sodium citrate buffer, pH 6 for 10 minutes, heart sections were blocked for 1hr with 1% BSA diluted in 1X dPBS at room temperature to prevent non-specific binding of antibodies to the tissue. Finally, sections were incubated with 10ug/ml WGA (ThermoFisher, WGA Alexa Fluor 594 Conjugate, cat# W11266) in the required buffer. Incubation time was one hour protected from the dark. After two washes in 1X dPBS for 5 minutes each, sections were stained with DAPI (ThermoFisher; Cat #D1306; 1:1000) for 10 minutes and mounted with ProLong Diamond Antifade Mountant (ThermoFisher; cat# p36961).

Slides were analysed with a fluorescence microscope (EVOS). All shown images were taken with 40x objective using the merge function, if not overwise specified.

2.5.1.1 Cardiomyocyte Hypertrophy Quantification

Four distinct regions of each heart section were imaged to quantify cardiac hypertrophy from stained cryosections. Cardiomyocyte hypertrophy was analysed using ImageJ software by quantifying the average cardiomyocyte area (μm^2) of approximately 50-60 different cardiomyocytes from individual imaged sections Figure 2.3.

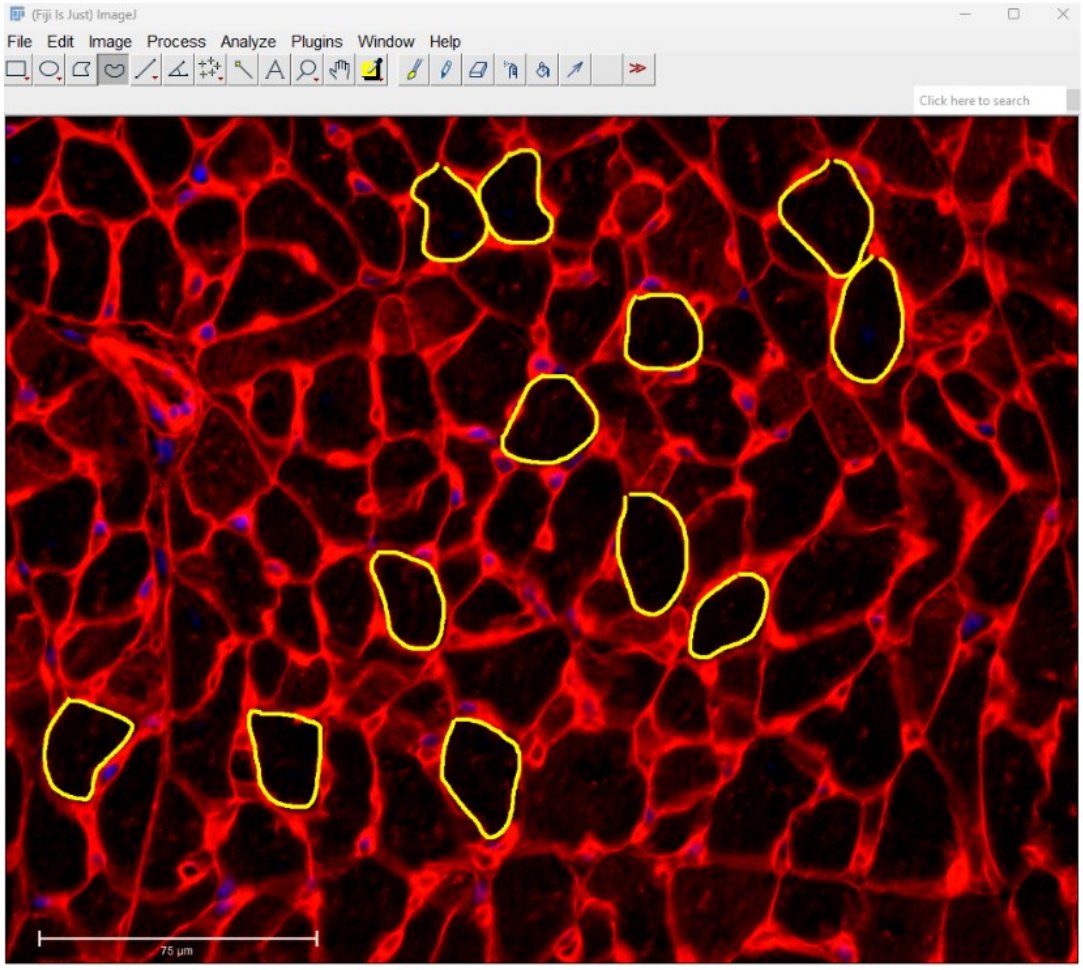

Figure 2.3 Cardiomyocyte hypertrophy quantification.
Yellow outlined cardiomyocytes represent the analysed cardiomyocyte hypertrophy in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ hypertensive mouse models. Investigated from mouse cardiac tissue using ImageJ studio after WGA and DAPI counterstaining. The average cardiomyocyte area from each picture tile was extracted.

2.6 Vascular Studies

2.6.1 Wire Myography

Vessel segments (~2mm in size) from the thoracic aorta were isolated without the Perivascular Adipose Tissue (PVAT) from WT, $ST2^{-/-}$ and IL-33 $^{Cit/+}$ of both sham and Ang II-Induced mice and mounted in organ bath chambers (610M myograph, DMT) filled with PSS buffer (118,99 mmol/l NaCl, 4.69 mmol/l KCl, 1.17 mmol/l $MgSO_4$-$7H_2O$, 1,18 mmol/l KH_2PO_4, 2,5 mmol/L $CaCl_2$-$2H_2O$, 25 mmol/l $NaHCO_3$, 11.1 mmol/l glucose, 0,03 mmol/l EDTA) oxygenated with 95%O_2-5%CO_2). Detailed instructions in LAB-SOP-001 'Procedure for Set-up, Mounting, Equilibration and Normalisation of Tissues in Wire Myography' were followed. Further, aortic rings were left to be equilibrated and stretched as per the 'General Large Artery Wire Myography Protocol.'

After a steady baseline of vessels, two maximal responses by 5mL of 62.5mM High Potassium Physiological Salt Solution (KPSS) were generated and quantified, followed by washes in 5ml warm PSS buffer after each KPSS addition. Relaxation to the endothelium-dependent and -independent vasodilators, acetylcholine (ACh) and sodium nitroprusside (SNP), following preconstriction with serotonin (5-HT), was measured. Table 2.1 provides individual concentration in the bath (M). Before relaxation curves with ACh and SNP, aortic rings were pre-constricted with $1x10^{-5}$M of 5HT in the bath (Figure 2.4 A, B).

2.6.1.1 Isometric Tension Study Analysis

The vascular function of WT, ST2$^{-/-,}$ and IL-33 $^{Cit/+}$ sham and Ang II-Induced mice were analysed using LabChart Reader version 8.1.14.

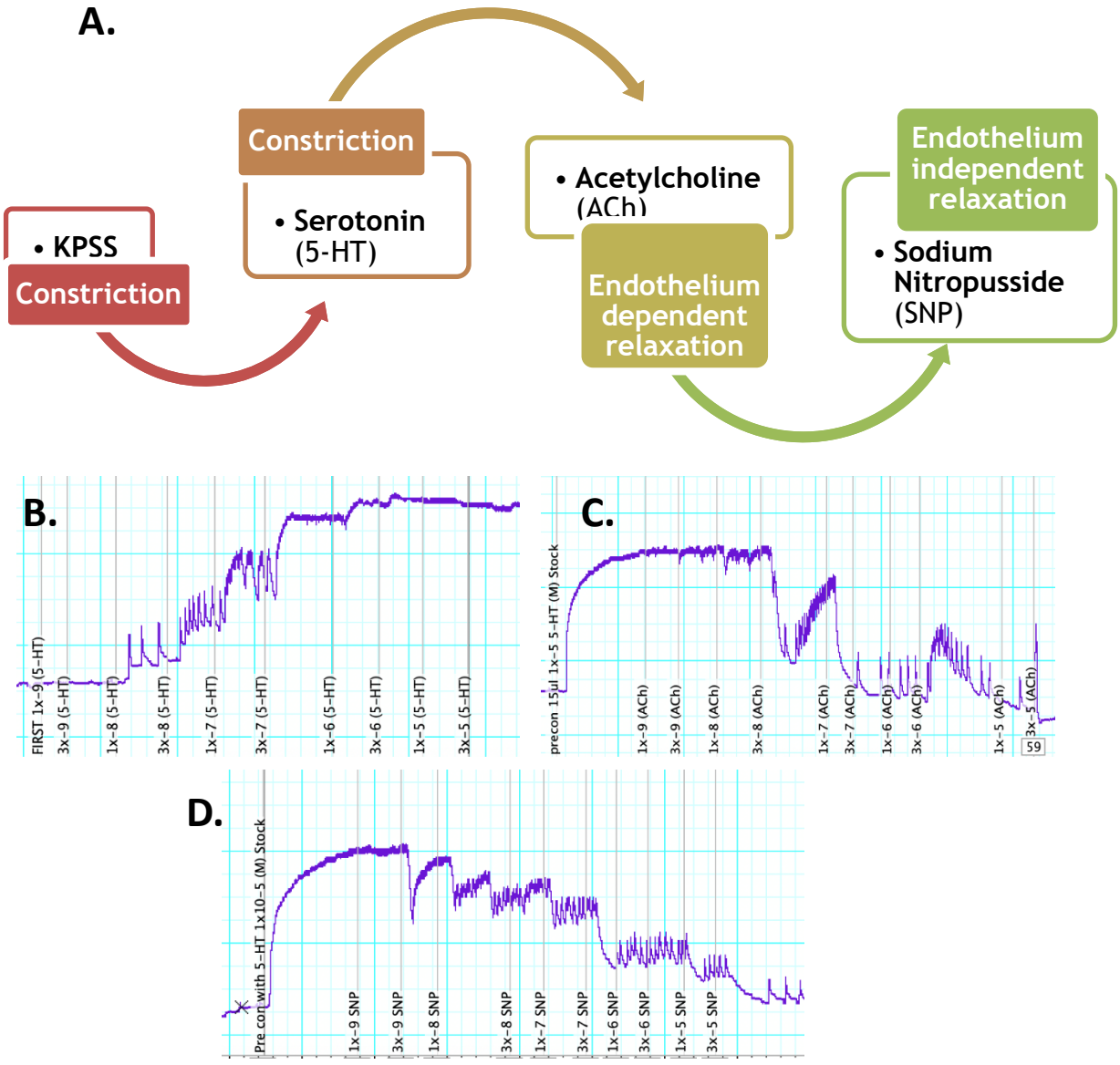

Figure 2.4 Experimental design for wire myography.
General Myography protocol study outline **(A)**. Initial constriction to KPSS was calculated, followed by vascular response check by Serotonin (5HT) from 1x10^{-6} to 1x10^{-2} M stock solution **(B)**. Endothelium-dependent relaxation was checked using Acetylcholine (ACh) **(C)**, and endothelium-independent relaxation was checked using Sodium Nitroprusside (SNP) **(D)**. The vessels were washed with PSS between each curve, and a baseline was achieved before proceeding to the next cumulative dose-response curve.

Volume of Bath = 5mL		
Stock solution (M)	**Volume to add (µL)**	**Concentration In the bath (M)**
$1x10^{-6}$	5	$1x10^{-9}$
$1x10^{-6}$	10	$3x10^{-9}$
$1x10^{-6}$	35	$1x10^{-8}$
$1x10^{-5}$	10	$3x10^{-8}$
$1x10^{-5}$	35	$1x10^{-7}$
$1x10^{-4}$	10	$3x10^{-7}$
$1x10^{-4}$	35	$1x10^{-6}$
$1x10^{-3}$	10	$3x10^{-6}$
$1x10^{-3}$	35	$1x10^{-5}$
$1x10^{-2}$	10	$3x10^{-5}$

Table 2.1 Concentration and volume of 5-HT, ACh and SNP.
The volume of Stock Solution (µL) added to the 5mL final bath volume is shown. The final concentration in the bath (M) is also stated.

2.6.1 Lucigenin Enhanced Chemiluminescence

Superoxide generation was measured in intact thoracic aorta vessels using lucigenin-enhanced chemiluminescence [LGLC]. Harvested blood vessels were cut to expose the endothelial surface, weighed, and placed in Krebs HEPES buffer (37°C) containing 5M lucigenin into a single tube FB12 luminometer (Berthold). Initially, the machine was observed until the background decreased, and the signal was measured once stabilised. Data are expressed as relative light units (RLU) per second per mg of a dry vessel.

2.7 Cell Culture

All cell culture procedures were undertaken in Class II flow hoods (ThermoFisher Scientific, Paisley, UK). Standard aseptic techniques were used, and all solutions and instruments were autoclaved and kept sterile.

2.7.1 Mouse Aortic Smooth Muscle Cell Isolation

Upon isolation of the aorta from the mice, the PVAT was removed and incubated in HBSS. The aorta was placed into a dish with the enzyme mix for digestion, as shown in Table 2.2, and incubated with 5% CO_2 at 37°C in the incubator for 8-10 minutes until the adventitia can be easily removed. The aorta was removed from the enzyme mix solution and placed into a 60mm dish with warmed-up and equilibrated DMEM/F12 media to wash off excess enzymes. At this point, the adventitia was stripped off under the dissection microscope by holding one end of the vessel with one forceps and stripping off the adventitia with another forceps. The aorta was vertically cut open with scissors, and any persisting blood clots were removed. The endothelial layer was then removed by scraping the inside of the vessel with forceps. Afterwards, the cleaned vessel without adventitia and endothelial cells was transferred into a second 60mm dish with equilibrated DMEM/F12 media.

For the final digestion process, the aortas were placed into a new dish with enzyme mix solution and incubated at 37°C in 5% CO_2 in the incubator for around an hour until the vessels looked like they were dissolved and cells seemed to float. After the cells were triturated with a fine polished Pasteur pipette, the dish was washed with 4ml warmed DMEM/F12 media and centrifuged at 1.5 RPM for 5 min, all but 1mL was removed, to which 1.5mL equilibrated DMEM/F12 media was added. 2 aortas were pooled together and plated into three wells of a 48 well dish (0.75mL per well). The cells were left undisturbed for one week to adhere to the well; then, the media was replaced.

Enzyme Mix	Weight (mg)	Catalog number
Collagenase I	18.3 mg	C0130 Sigma Aldrich
Collagenase II	2 mg	C6885 Sigma Aldrich
Collagenase XI	1.56 mg	C9407 Sigma Aldrich
Hyaluronidase	1.33 mg	H3884 Sigma Aldrich

Table 2.2: Digestion Enzyme Mix.
The table provides the enzymes used for the digestion protocol. Each enzyme is diluted in 1ml of RPMI with the correct weight, yielding the final volume of 4mL.

2.7.2 Cell stimulation

Isolated murine vascular smooth muscle cells (mVSMC) were grown in Smooth Muscle Cell Growth Medium 2 (Promo Cell, C-22062) with 1% Penicillin-Streptomycin (ThermoFisher, cat# 15070063). The cells were passaged till passage 7, cell confluency was regularly checked, and media changed every three days. Upon reaching passage 6, the cells were split into 6-well cell culture plates. When the wells reach around 80-90% confluency, the cells were starved for 4 hrs with 1%FBS in media. Afterwards, the starvation medium was removed and changed for the full media containing the cell stimulants and, without any stimulants, set as control samples. The cells were then incubated at 37 ℃ for the stimulation times of 6hrs and 24hrs.

The cells were stimulated by TNF-α (PeproTech; Cat #315-01A; 50ng/mL), IFN-γ (PeproTech; Cat #315-05; 20ng/mL), ET1 (20mm/mL), TGF-β (PeproTech; Cat #100-16A; 20ng/mL (diluted in 1%FBS at 6hrs, 24hrs). Concentration for the stimulation was carefully considered by assessing previously published literature including Kim and Wang et al. (Kim et al., 2019a), (Wang et al., 2020). At the end of the desired time points, cell stimulation was stopped by removing the media and rinsing the wells with 1X sterile dPBS. Later, cells for RNA extraction were collected by adding 700µL

of Qiazol (Qiagen; cat# 79306) to the plate. Cell lysates were kept at -20℃ till the time of use.

2.8 Flow Cytometry

2.8.1 PVAT Digestion

Perivascular adipose tissue was separated from the aorta and digested using the previously mentioned enzyme mix (Table 2.2), dissolved in PBS containing calcium, magnesium, and 20μM HEPES. In a 24-well plate, PVAT was incubated for 45 minutes with 5% CO_2 in 37℃ in the enzyme mix diluted in PBS, with gentle agitation every 15 minutes. The digested tissue was passed through a 70μm sterile cell strainer centrifuged at 400g for 10 minutes at 4℃ to yield a single-cell suspension. Cells were washed and resuspended in FACS buffer (PBS +1%FBS) counted, and 1 million cells were used for staining using monoclonal antibodies and live/dead markers.

2.8.2 Single-Cell Suspension of Vascular Stromal Fraction Staining

After BV510 live/dead (L/D) staining was performed, cells were incubated at RT in the dark for 15 minutes, topped up with FACS Buffer, and centrifuged at 400g for 6mins at 4℃. This was followed by surface marker staining (100μL of antibody mix per sample). Cells were then incubated for 20 minutes on ice, in the dark, and topped up with FACS buffer.

Intracellular staining was performed to evaluate cytokine production. The isolated single-cell suspension was removed, and vortexed; 500μL of Fix and PERM (ThermoFisher; cat number# 00552300) solution was added into the FACS tube with the cells, vortexed, and incubated on ice for 30 minutes in the dark. 2mL of fixation permeabilisation buffer was added to the FACS tubes and centrifuged at 300g for 5 minutes at 4℃. The supernatant was discarded, and 100ul of intracellular antibody mix was added to each sample and incubated in the dark for 30 minutes on ice (Table 2.3). Two wash steps followed this with 2mL of Fixation permeabilisation buffer (1X) (ThermoFisher; cat# 00552300) and centrifuged at 300g, 5 mins at 4℃. Afterwards,

the supernatant was discarded, and cells were resuspended in 200µL FACS Buffer and stored in the fridge covered with aluminium foil till samples were acquired using the BD LSRFortessa™ and analysed using FLOWJo™ v10 Software.

Catalog number	Target	Dye	Supplier
100235	**CD3**	APC	BioLegend
560181	**CD4**	APC/H7	BD Biosciences
551162	**CD8**	PerCP/Cy5.5	BD Biosciences
123116	**F4/80**	APC	BioLegend
101208	**CD11b**	PE	BioLegend
117334	**CD11c**	BV605	BioLegend
741921	**CD44**	BUV805	BD Biosciences
115520	**CD19**	PE/Cy7	BioLegend
563410	**CD45**	BV650	BD Biosciences
108753	**NK 1.1**	BV605	BioLegend

Table 2.3: FACS antibody list.
The antibodies and their dye are used for vascular stromal fraction staining.

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2.9 Western Blotting

Immunoblotting was used to examine the expression of IL-33 (R&D, UK, cat# AF3626, 1:1000 dilution), ST2 (Abcam, UK, cat# ab259877, 1:2000 dilution) and beta-actin (Abcam, cat# ab8227, 1:5000 dilution).

2.9.1 Sample Preparation

Snap-frozen samples (aorta, heart, kidney, PVAT, and spleen) were thawed and homogenised in cold radioimmunoprecipitation (RIPA) lysis buffer (Sigma-Aldrich; cat# R0278) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher; Cat# 78440). Lysate total protein concentrations were determined using the Pierce™ BCA Protein Assay (ThermoFisher; cat#23225). Sample optical density at 595nm wavelength was measured, and each sample's concentration (µg/µL) was calculated according to the standard curve. All samples were prepared with 25µL of 20 ug of protein in dH_2O and 5µL of Gel Loading Buffer (6x concentrated) (Sigma-Aldrich; Cat# G2526) before boiling at 95℃ for 5 minutes.

2.9.2 Gel Electrophoresis and Membrane Transfer

The samples and 5-250 kDa pre-stained protein ladder (ThermoFisher Scientific; cat# 26619) were loaded into a 4-20% Tris-Glycine extended precast gels (Bio-Rad; cat# 4561094) with Tris/Tricine/SDS running buffer (Bio-Rad; Cat#1610744). The gel was run at RT at 150 V for approximately one hour and 15 minutes (or until the sample buffer/ 5kDa ladder marker had reached the bottom of the gel).

After the completion of electrophoresis, the gel and the 0.45 µm Nitrocellulose Membrane (ThermoFisher; cat# 88018) were immersed in the transfer buffer (Bio-Rad; Cat# 1610734) prepared with 20% methanol for 10 minutes before the transfer "sandwich" was prepared. The transfer was run at 100 V for 1 hr at 4℃. After transfer, the membrane was stained with Ponceau to ensure the transfer was successful.

2.9.3 Detection of Proteins of Interest

The membranes were washed in Tris-buffered saline (TBS), and nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline solution containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were then incubated with specific antibodies overnight at 4 °C. (5% BSA TBS-T), washed three times, and incubated with secondary antibodies conjugated with fluorescent IRDyes 800CW (Donkey anti-Goat (Licor, UK; Cat #928-32214; 1:15,000), Donkey anti-Rabbit (Licor, UK; Cat #926-32213;1:15,000) and Goat anti-Mouse (Licor, UK; Cat #926-32210; 1:10,000)) for 1 hour in room temperature. Signals were detected using Odyssey CLx Imaging System (LI-COR) and quantified with Image Studio software (LI-CORE).

2.10 Quantitative PCR

The organs of interest were harvested from the related mouse models and their WT controls. Equal samples of each organ were used throughout and stored in RNA*later*™ Stabilization Solution (ThermoFisher; Cat# AM7021). Total RNA from the organs of interest was extracted using the Direct-zol™ RNA Miniprep Plus kit (Zymo Research; cat R2071). 50 ng/4.5µL of RNA from each sample was converted to complementary deoxyribose nucleic acid (cDNA) using a High-Capacity Reverse Transcription Kit (Applied Biosystems; cat# 43-688-14). A TaqMan Gene expression Assay (Applied Biosystems; cat# 4331182) was then used to determine the expression of several genes, listed in Table 2.3.

Delta cycle threshold (Ct) (ΔCT) was calculated by subtracting the average CT of the housekeeper gene TBP from that of the gene of interest (e.g., IL-33/ST2) for each organ's samples. Average ΔCT was calculated for the sham WT samples and then subtracted from all models to calculate ΔΔCT. 2-ΔΔCT was plotted to visualise data as a fold-change of each gene of interest expression relative to the housekeeper in sham WT versus Ang II.

Gene	Assay ID
Col1a1	Mm00801666_g1
Col3a1	Mm01254476_m1
Col5a1	Mm00489342_m1
Ctgf	mM01192933_g1
Fn1	mM01266744_m1
ST2	Mm00516117_m1
Il33	Mm00505403_m1
Nox4	Mm00479246_m1
Tgfb1	Mm01178820_m1
Housekeeping Gene	
Tbp	Mm01277042_m1

Table 2.4: Gene expression assay TaqMan.
The table provides the TaqMans used for experiments to check expression levels of *IL33*, *ST2* and several fibrotic and collagen markers together with the housekeeping gene, *Tbp*.

2.11 Sc-Seq Dataset Extraction

Online scRNA-Seq datasets of mice (Gu et al., 2019) and human heart failure patients (Wirka et al., 2019) were downloaded from Gene Expression Omnibus. The datasets were analysed using the R package Seurat version 4.3.1. The bioinformatics methodology of the original authors was followed as closely as possible and made available online.
Aortic infiltrating immune cell dataset analysis was conducted as part of Dr Eva Crespo's PhD project (Crespo, 2023).

Firstly, individual samples within larger grouped datasets were subjected to quality control (QC) measures before proceeding with the final analysis. QC is a critical

step in scRNA-seq data analysis. Low-quality cells are removed from the analysis during the QC process to avoid misinterpretation of data.

Genes expressed in less than five cells and cells expressing less than 500 genes were cut to remove potentially low-quality cells or droplets containing free RNA. Further, cells expressing consistently high numbers of genes were also cut to confirm that doublets were not included. The exact cut-off differed for each dataset but ranged from 3000-5000 genes per cell. Another approach to filter out low-quality cells is by looking at the proportion of mitochondrially encoded genes to total genes expressed by an individual cell, also known as the mitochondrial proportion (mtDNA%). A high number of mitochondrial transcripts are indicators of cell stress, and therefore, mtDNA% is a measurement associated with apoptosis, stressed, and low-quality cells (Osorio and Cai, 2021). Furthermore, higher fractions of mitochondrial genes indicate broken cells and loss of cytoplasmic RNA, while mitochondrial RNA is retained and sequenced(Ilicic et al., 2016). Due to this, cells containing a high proportion of mitochondrial genes in relation to total gene expression were excluded. This threshold was set respective to each dataset but typically ranged from >5-10 %.

The gene expression in the datasets was then normalised. Each cell's count for each gene is normalised to the total number of gene counts in that cell, then multiplied by 10,000 and log-transformed. Fifty-two variable features between cells were then determined, and all gene expression data was subjected to linear transformation to give more weight to lower/less often expressed genes in the downstream analysis.

Principal component analysis (PCA) performed a linear dimension reduction on the data. Elbow plots and heatmaps were then utilised to determine the appropriate dimensionality and PC cut-off for each dataset. At this stage, where appropriate, the integration of datasets was performed. Integrated data was then scaled and subjected to PCA linear reduction. After this, clustering analysis was performed and visualised in 2-dimensional space *via* Uniform Manifold Approximation and Projection (UMAP) plots.

Clusters were identified by assessing the markers that define them *via* differential expression analysis using the 'FindAllMarkers' function in Seurat. In most cases, clusters could be determined based on cluster gene markers described in the published studies where the datasets originated.

Finally, IL-33 and ST2 gene expressions were visualised as projections onto the clustered UMAPs or violin plots.

2.12 IL-33 and ST2 LegendPlex

Human blood from normal (n=43) and hypertension participants (n=45) was collected as part of the InflammaTENSION study led by Dr Eleanor Murry, clinical trial ID number: NCT0415635.

Human serum levels of IL-33 and ST2 levels were quantified using a human LegendPlex Assay (Biolegend; IL-33-B9, ST2-A4). Manufacturers protocol was closely followed.

2.12.1 Participant Characteristics

The participants enrolled into the study were aged between 19-55 years old. The average age of participants was 39.2 with an average BMI of 26.2 in normotensive and 28.5 in hypertensive participants. European, Asian, Middle Eastern, Chinese/Japanese, and African descent participants were enrolled. Systolic and diastolic office blood pressure were also acquired. Normotensive mean SBP was observed at 125mmHg and 146mmHg for hypertensive patients. Normotensive mean DBP was observed at 79mmHg and 92mmHg for hypertensive patients.

2.13 UK Biobank Access for Plasma OLINK IL-33 and ST2

Plasma levels in the UK biobank samples were obtained using OLINK Explore. Plasma OLINK IL-33 and ST2 level and BP parameters of 38,000 UK biobank subjects of Caucasian ethnicity were analysed. Beta (mmHg/NPX, 95% Cl) adjusted for BMI, age, sex, smoking status, and alcohol intake frequency were estimated. The research was conducted using the UK Biobank Resource under Application Number 93156.

2.14 Statistical Analysis

Statistical Analysis was performed using GraphPad Prism Version 8.4.1 (GraphPad Prism, California, USA). Data was first tested for normal distribution using the D'Agostino-Pearson normality test. For data determined to be normally distributed, parametric t-tests were used to determine statistical significance when two groups were being compared. Data that were not normally distributed were analysed using a nonparametric Mann-Whitney test. Where appropriate, paired t-tests or two-way ANOVA statistical tests were performed on the data.

Unless otherwise stated, all data values are presented as mean ± standard error of the mean (SEM) of n animals from at least three independent experimental replicates. Levels of statistical significance were evaluated by *p-value*. Differences are regarded as statistically significant at $p<0.05$.

Chapter 3 Expression of IL-33 and ST2 in Hypertensive Animal Model

3.1 Introduction and Aims

Interleukin-33 (IL-33) is a member of the IL-1 superfamily of cytokines and is the ligand for the suppressor of tumorigenicity 2 receptor (ST2, also known as IL-IRL1) (Liew et al., 2010). IL-33 belongs to a group of alarmin molecules, and its release from cells during cell injury instigates an inflammatory tissue damage response (Cayrol and Girard, 2022). Under physiological conditions, IL-33 is expressed in epithelial, endothelial, and fibroblastic cells and locally in the nucleus (Moussion et al., 2008, (Cayrol and Girard, 2022). During inflammation and other types of cell stress, IL-33 is upregulated and released from damaged or necrotic cells (Rider et al., 2017). Further, IL-33 is over-expressed in various human diseases and inflammatory processes. Studies have recognised the same tissue-derived cells expressing nuclear IL-33 at baseline as the significant source of the cytokine during inflammation (Cayrol and Girard, 2022).

Conversely, the ST2 receptor is predominantly expressed by immune cells, including T cells, particularly type 2 T-cells (Löhning et al., 1998), regulatory T cells (Tregs) (Schiering et al., 2014), innate lymphoid cell type 2 (Neill et al., 2010b), M2 macrophages (Kurowska-Stolarska et al., 2009) and others (Griesenauer and Paczesny, 2017). Additionally, the expression of ST2 is documented in cardiomyocytes (Weinberg et al., 2002b). The receptor ST2 is secreted in response to myocardial stress. Thus, there is increased attention to its role in the cardiovascular system. Besides, studies have demonstrated serum concentrations of ST2 (sST2) to be a strong prognostic marker of cardiac disease outcomes (Caselli, 2014, Villacorta and Maisel, 2016).

Accumulating evidence indicates the dysregulation of the IL-33/ST2 signalling axis as an essential immunological pathway implicated in a variety of immune diseases such

as cardiac diseases (Caselli, 2014), atherosclerosis (Miller et al., 2008), type 2 diabetes (Miller et al., 2012), and hepatic diseases (Kotsiou et al., 2018). Activating immune cells such as M2 macrophages, ILC2, and mast cells can alter heart function (Ghali et al., 2018).

The elevated levels of several inflammatory biomarkers, such as C-reactive protein and cytokines, have been detected in patients with hypertension, revealing that the immune system is closely involved in hypertension as a low-grade inflammatory disease. Immune cells can infiltrate multiple organs, thus causing organ dysfunction and leading to elevation of blood pressure (Yin et al., 2019a). Literature speculated that as hypertension develops, the vascular tissue undergoes mechanical stretch, which can release biologically active IL-33, and consequently, IL-33 expression in cells will increase (Yin et al., 2019a).

To date, the only study laying an outline for the role of IL-33 and ST2 in hypertension specifically is by Yin et.al (Yin et al., 2019a). They evaluated the association of IL-33 and ST2 with hypertension in Ang II-induced mice using microarray analysis. Yin et.al did not detail the expression of IL-33 in other organs associated with hypertension or the presence of the cytokine and its receptor, ST2, within the vascular compartment upon hypertension.

3.2 Hypothesis and Chapter Aims

Despite accumulating evidence detailing the expression of IL-33 and ST2 in inflammatory diseases, IL-33/ST2 axis expression in hypertension has not been thoroughly investigated.

Therefore, the main aim of the experimental work in this chapter is to characterise patterns of IL-33 and ST2 expression in hypertension pathology. More specifically, the aims are listed as follows:

1. Determine IL-33 and ST2 mRNA and protein expression in a hypertensive mice model and the related control group.

2. Examine vascular localisation of IL-33 and ST2 in control and upon Angiotensin II treatment.

3. Investigation of publicly available microarray and scRNA-Seq datasets, providing the ability to analyse datasets encompassing various mouse models and cell types in atherosclerosis and hypertension.

4. Identify expression of IL-33 and ST2 in mouse aortic infiltrating leukocytes upon Angiotensin II treatment.

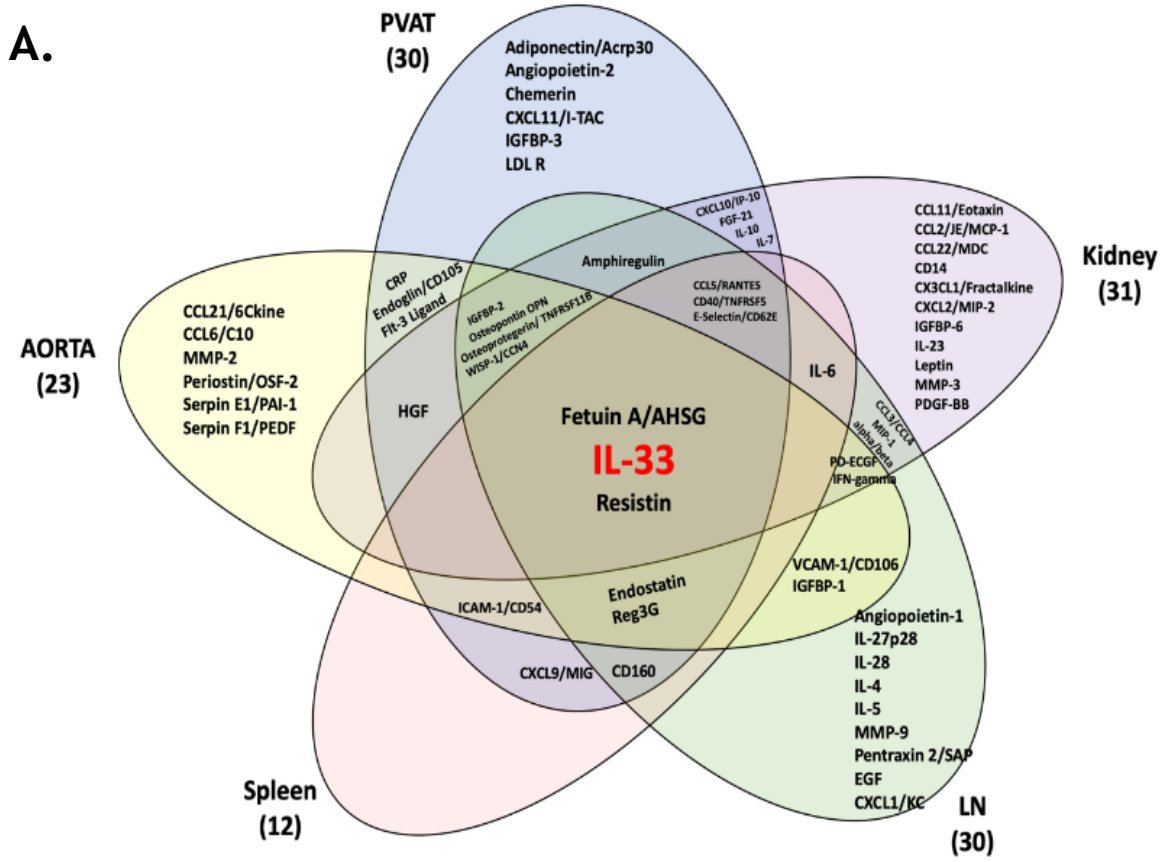
I propose that IL-33 functions as a vascular alarmin molecule upon cell injury to signal the immune system in hypertension and associated target organ damage. Consequently, this chapter is dedicated to characterising IL-33 and ST2 expression in hypertension at the mRNA, protein, and cellular levels across essential organs implicated in hypertension pathogenesis. Additionally, I will elucidate the localisation of IL-33 and ST2 within the mouse vasculature.

3.3 Results

3.3.1 Ang II-Induced Hypertension Upregulates IL-33 and ST2 Expression in WT Mice

To study IL-33 and ST2 expression in hypertensive conditions, 12-week-old male C57BL/6J control mice were infused with Angiotensin II for 2 weeks, resulting in the development of hypertension. Results obtained from the ELISA-Spot indicated the upregulation of IL-33 in essential organs affected by hypertension progression out of proteins within the proteome panel (Figure 3.1 A, B). Conversely, to expand and confirm the initial ELISA-Spot results, mRNA and protein expression of IL-33 and its receptor, ST2, by western blotting was studied. Along with previously studied organs, IL-33 was also quantified in the heart as IL-33 is upregulated in the mouse heart's endothelial (and interstitial) cells after pressure overload stimulation (Chen et al., 2015).

In the aorta and the heart, mRNA IL-33 levels were higher in the Ang II-induced hypertensive model compared to the sham (Figure 3.2A). While in the lymph node, spleen, kidney, and PVAT, there was no notable difference in the level of IL-33 mRNA between the two groups (Figure 3.2A). ST2 mRNA expression was upregulated in the aorta, lymph node, and the spleen. However, in the heart, kidney, or PVAT of Ang II-treated mice, changes in ST2 levels compared with the sham group were not observed (Figure 3.2B).



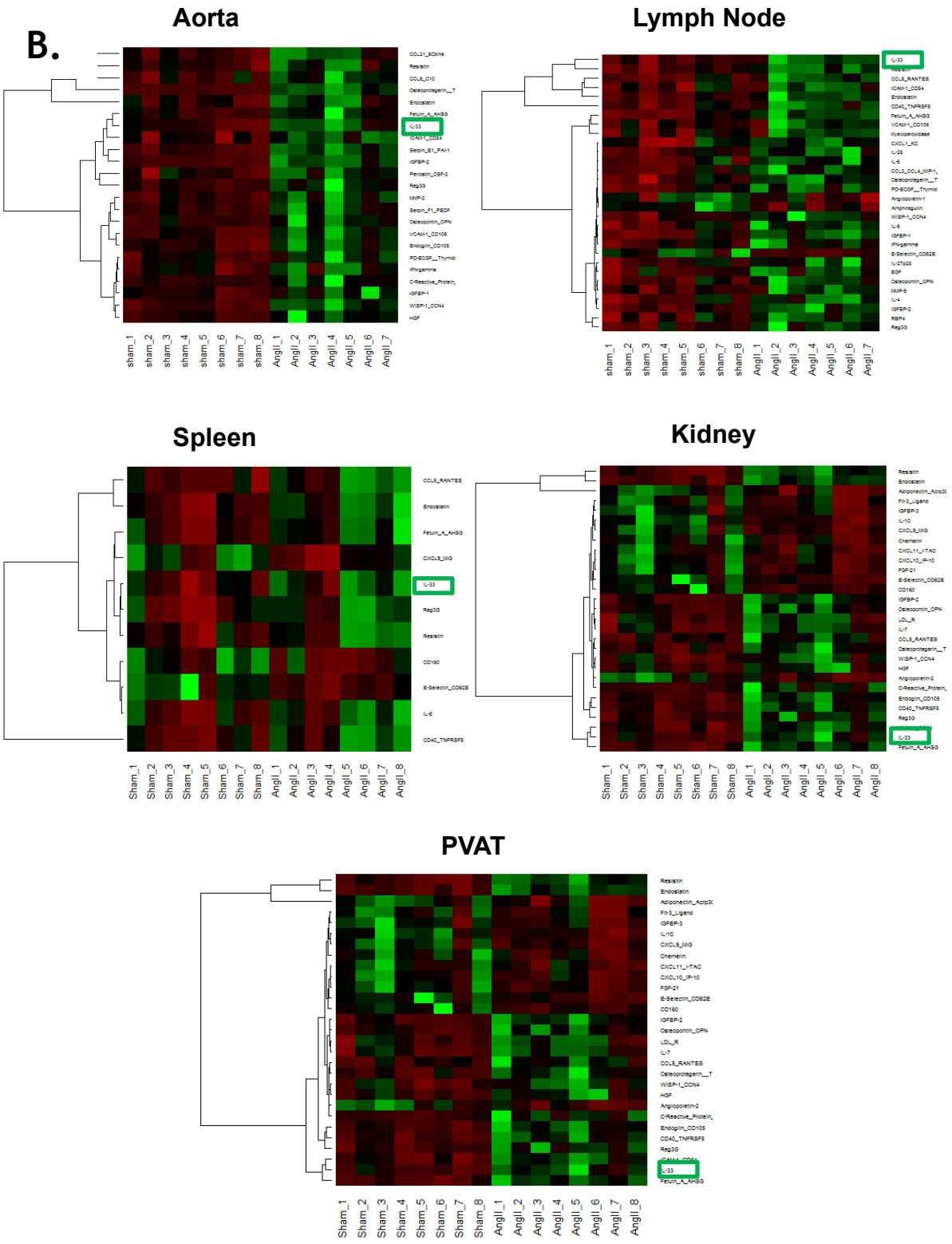


Figure 3.1: Quantification of IL-33 expression in Sham and Ang II mice.
Elisa-Spot results of 12-week-old C57BL/6J (8 per group) mice, which were induced with either Angiotensin II for 14 days before being euthanised or a sham control group. Venn Diagram showing IL-33 upregulation (**A**) and relating heat map expression of IL-33 in the Aorta, Lymph Node, Spleen, Kidney and PVAT (**B**), marked in green.

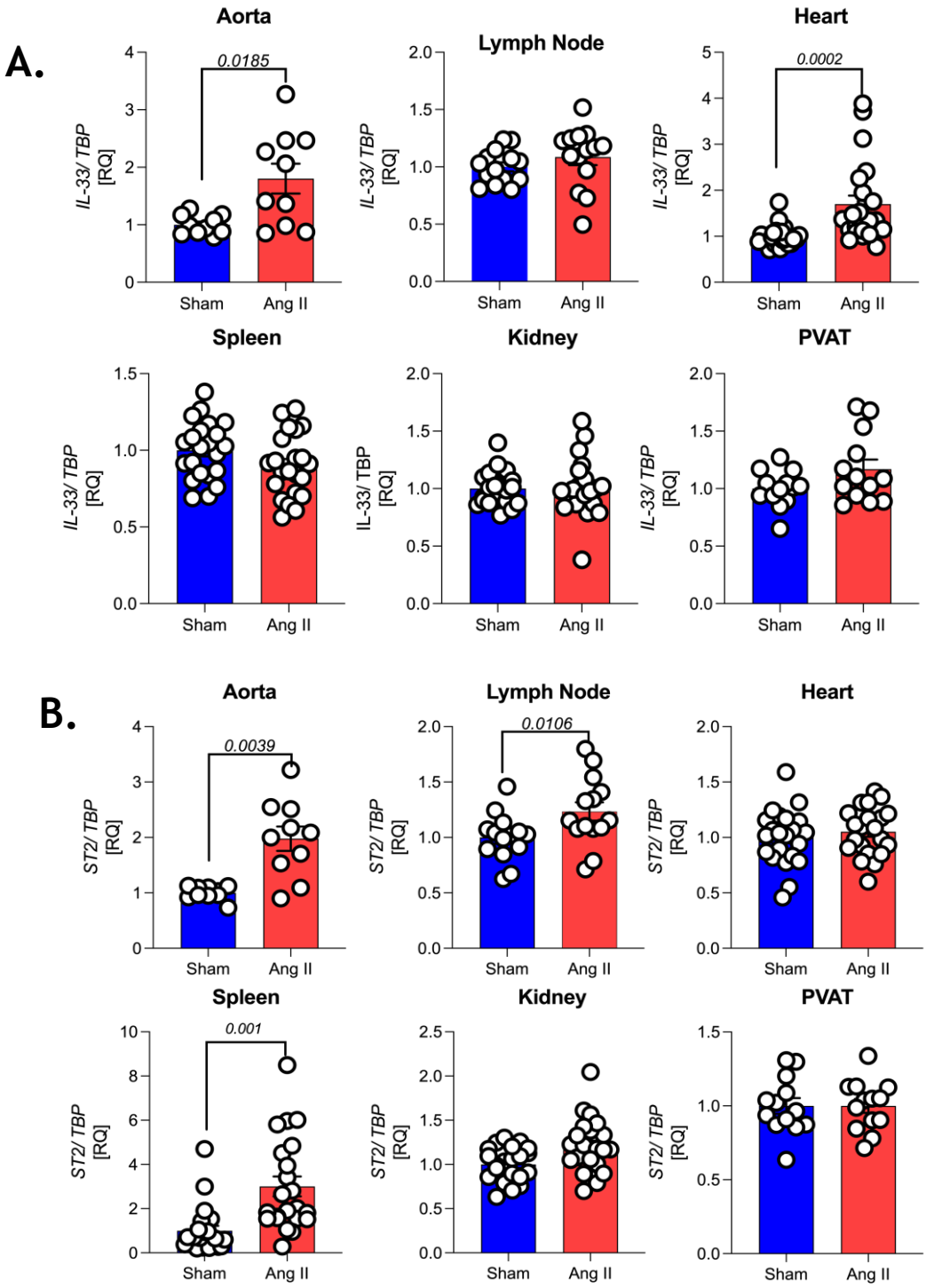


Figure 3.2: Modulation of IL-33 and ST2 mRNA expression in various organs upon Ang II-dependent hypertension.

Expression of IL33 **(A)** and ST2 **(B)** in the aorta, lymph nodes, heart, spleen, kidney, PVAT, mesenteric lymph nodes and mesenteric arteries in Sham and Ang II -treated C57BL/6J mice (490 ng/min/kg for 2 weeks). Data is presented as a relative expression (RQ) to the Sham group with the *tbp* gene as an internal control and analysed using unpaired students t-test, p-values indicated. Biological replicates are represented as mean ± S.E.M (n= 10-24).

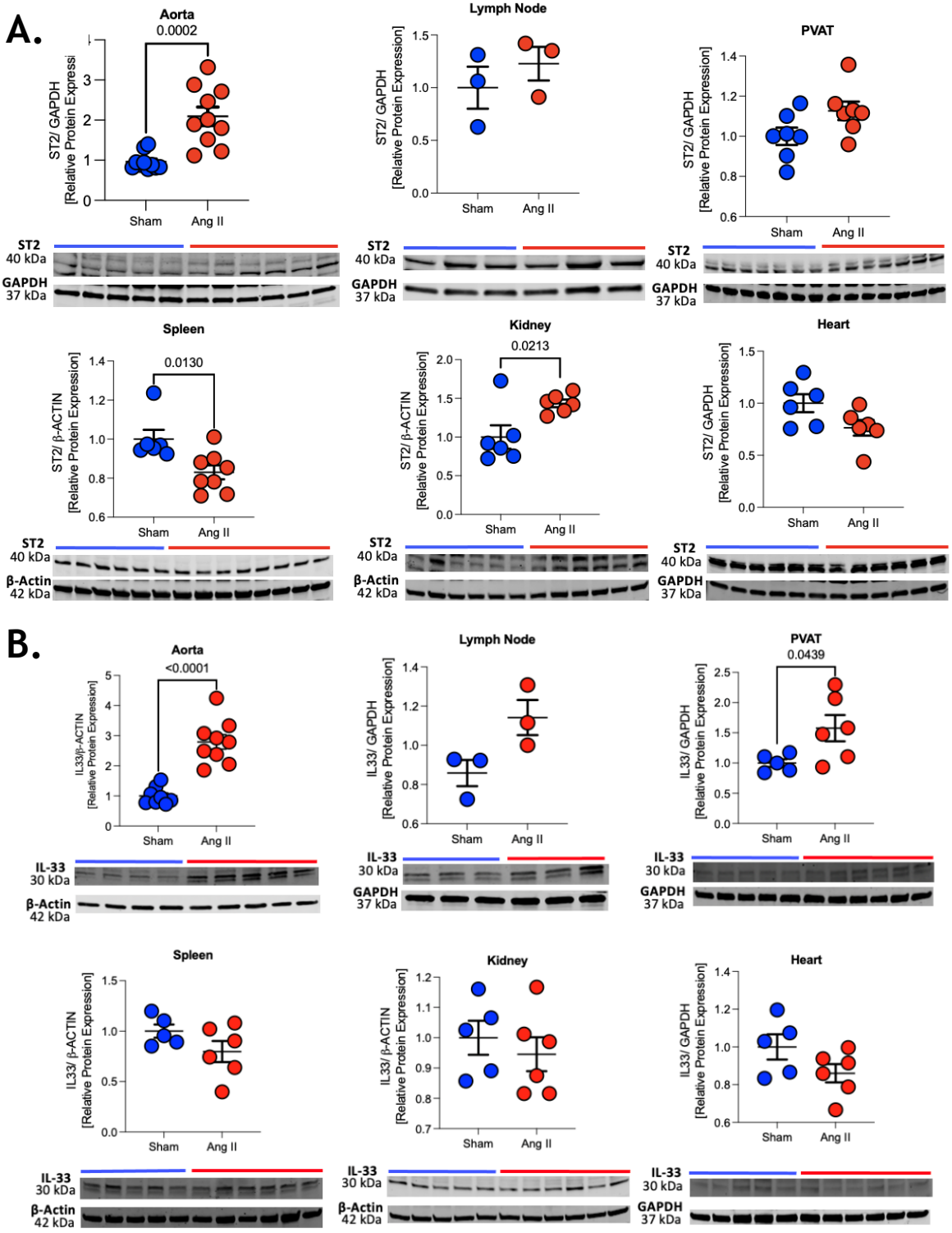


Figure 3.3: Protein expression of IL-33 and ST2 in organs related to hypertension.

Relative protein levels of IL-33 **(A)** and ST2 **(B)** were studied by Western blotting of Sham and Ang II C57BL/6J mice with representative blots. Proteins of interest were normalised to ß-actin or GAPDH. Data analysed using unpaired students t-test, p-values indicated, represented as mean ± S.E.M. n=3-10 per group.

IL-33 and ST2 mRNA elevation upon Ang II treatment compared with sham aligned with the protein level findings using western blotting. IL-33 and ST2 were notably induced in the aorta of hypertensive mice at the protein level (Figure 3.3A, B).

3.3.2 Distribution of IL-33 and ST2 in the Mouse Vasculature

To understand the localisation of IL-33 and ST2 within the mouse vasculature, I performed immunohistochemistry (DAB staining) in thoracic aortic sections from sham and Ang II-infused WT mice. The DAB staining works by hydrogen peroxidase oxidising the DAB in a reaction catalysed by HRP. The oxidised DAB results in the generation of a dark brown deposit.

IL-33 expression was localized within the lumen (consisting of endothelial cells) and the vessel wall's intima-media (consisting of SMCs) in both sham and Ang II animals. However, the level of expression in the Ang II mice aorta was higher compared to the sham (Figure 3. 4A). Additionally, when vascular expression of ST2 was investigated, the localisation was like that of IL-33. ST2 was localised in the lumen and the intima-media (Figure 3. 4B).

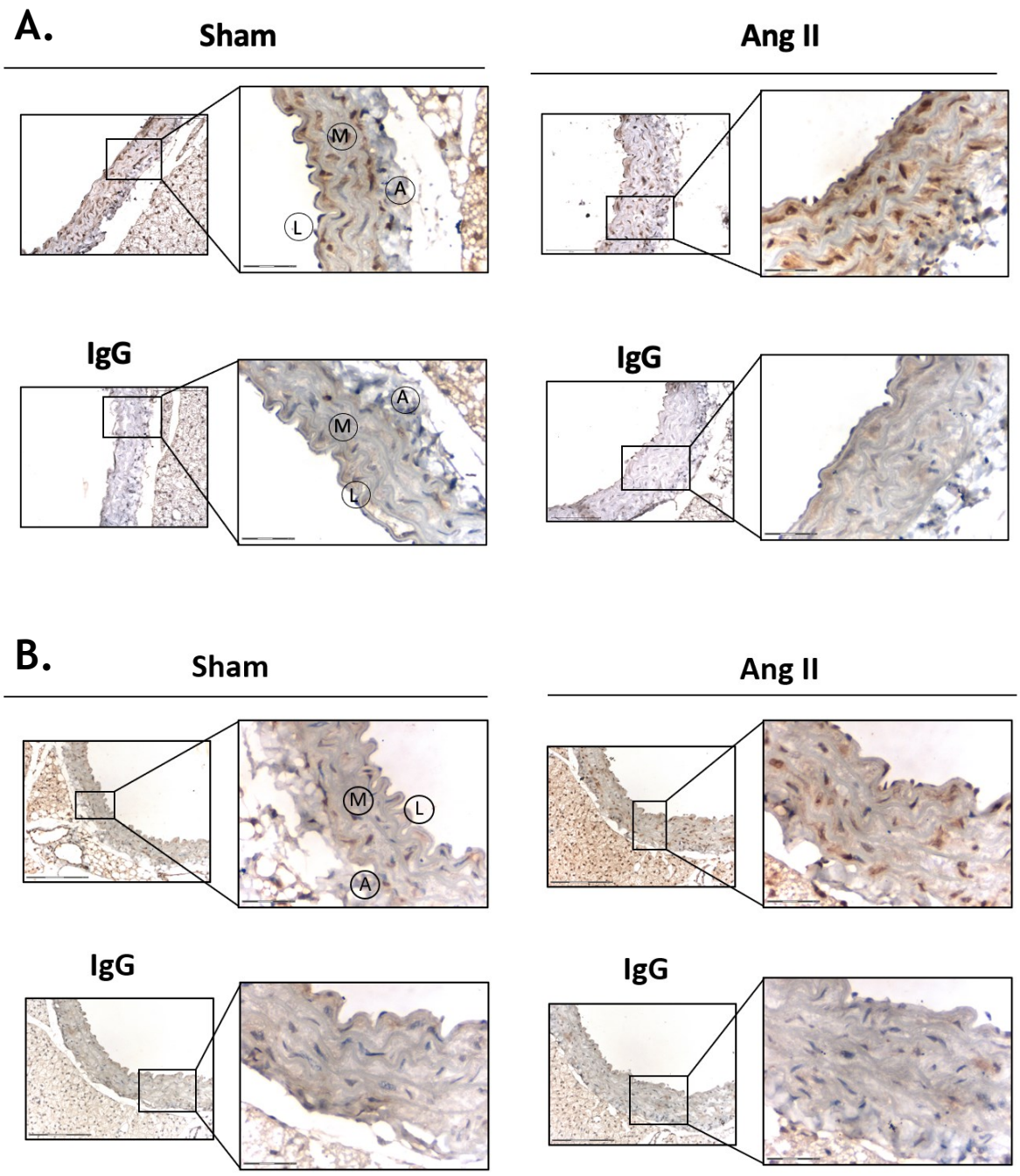


Figure 3.4 Localization of IL-33 and ST2 in the aortas of normotensive and hypertensive animals.
IL-33 **(A)** and ST2 **(B)** levels were evaluated from formalin-fixed paraffin-embedded aortas from Sham and Ang II-induced mice using HRP/DAB staining (n=5-6 in each group). Isotype controls of each are shown under each as IgG. 40x magnification and 62x magnification. Scale bar: 100μm and 30μm representatively **A**, adventitia; **M**, media; **L**, lumen.

3.3.3 IL-33 and ST2 Expression Upon Hydralazine/Hydrochlorothiazide Treatment in Hypertensive Animal Models

To investigate if the induction of IL-33 and ST2 is produced by Ang II or blood pressure elevation, mRNA expression level of *IL-33* was studied on animals receiving hydralazine (320mg/L) and hydrochlorothiazide (60mg/L) in drinking water (Wu et al., 2014). The combination is commonly used in humans to treat high blood pressure. Hydralazine works by relaxing the blood vessels and increasing the supply of blood and oxygen to the heart while reducing the workload.

The animals were split into four groups: sham and Ang II- treated animal groups and sham and Ang II- treated animals, administered the hydralazine and hydrochlorothiazide combination. The initial treatment was on -3 (3 days before Angiotensin II-Infusion), sustained on day 0 (Ang II-Infusion surgery), and the 14 days following until the experiment was terminated.

As previously observed in earlier analysis, Ang II significantly induced *IL-33* expression in the aorta compared to the sham group. Notably, the hydralazine-treated Ang II group had attenuated IL-33 expression (Figure 3.5A) in response to the hydralazine combination upon Ang II- treatment. Furthermore, *ILIRL1* mRNA levels were significantly elevated in the Ang II treated group when compared with the sham; the administration of the vasodilator and diuretic combination treatment reversed this effect in the Ang II- infused group (Figure 3. 5B). Thus, these data indicate that IL-33 and ST2 expression is not produced due to Ang II infusion, instead it is blood pressure dependent.

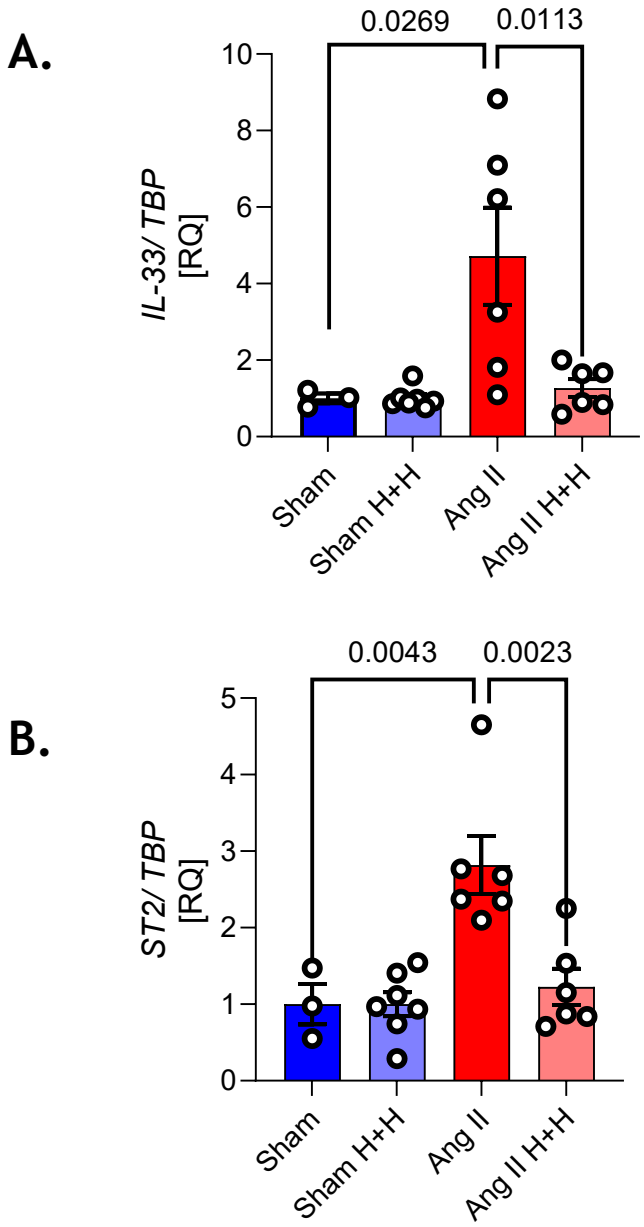


Figure 3.5: Aortic mRNA in Sham and Ang II treated mice without or with additional combined hydralazine/ hydrochlorothiazide treatment.
Expression of IL-33 (**A**) and ST2 (**B**) in the aorta of Sham and Ang II-induced C57BL/6J mice with and without Hydralazine and Hydrochlorothiazide oral administration (Sham H+H and Ang II H+H). Data is presented as a relative expression (RQ) to the Sham group with the *Tbp* gene as an internal control and analysed using two-way ANOVA with multiple comparisons and p-values indicated. Biological replicates are represented as mean ± SEM (n=3-6).

3.3.4 Expression of IL-33 and ST2 by Murine Vascular Smooth Muscle Cells

To investigate the effects of different cytokines associated with hypertension, *IL-33* (Figure 3.6A) and *ST2 mRNA* (Figure 3.6B) mVSMC were stimulated by indicated concentrations for 6 hours. Substantial upregulation of *ST2* mRNA levels was identified upon stimulation by TNF (*p=0.0031)*.

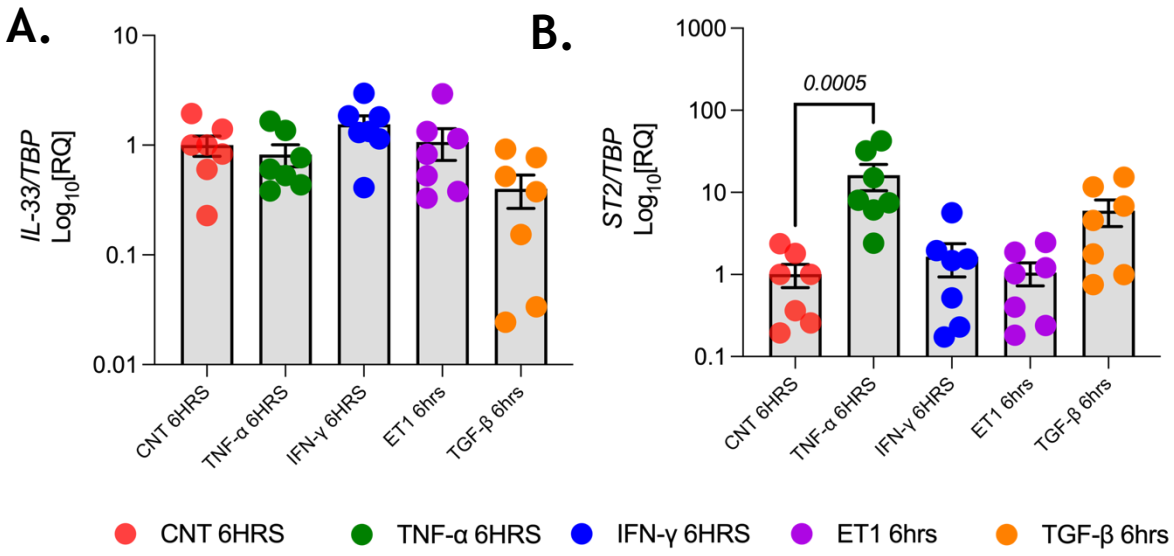


Figure 3.6: Effects of cytokines on *IL-33 and ST2* mRNA in mouse vascular smooth muscle cells.
mVSMC were stimulated at 6hrs in the absence (control) or presence of TNF (50ng/mL), IFN (20ng/mL), ET1 (20μm/mL) and TGF (20ng/mL). mRNA expression levels of *IL-33* **(A)** and *ST2* **(B)** upon stimulation are shown. Data is represented as relative expression (RQ) to the control group, *Tbp* as an internal control. Data presented as mean ± SEM, n=7, individual mice experiments.

3.3.5 IL-33 and ST2 Expression in Immune and Non-Immune Cells from Publicly Available Sc-Seq Datasets

To further establish a broad knowledge of IL-33 and ST2 expression in the mouse vasculature in health and in disease models of cardiovascular disease, available scRNA-Seq dataset from different experimental models of murine atherosclerosis were investigated. This dataset was of interest as the mouse model used partners my study mouse models used in all experiments and analysis. The dataset published includes isolated media and adventitia in healthy and early pathology of atherosclerosis (Gu et al., 2019).

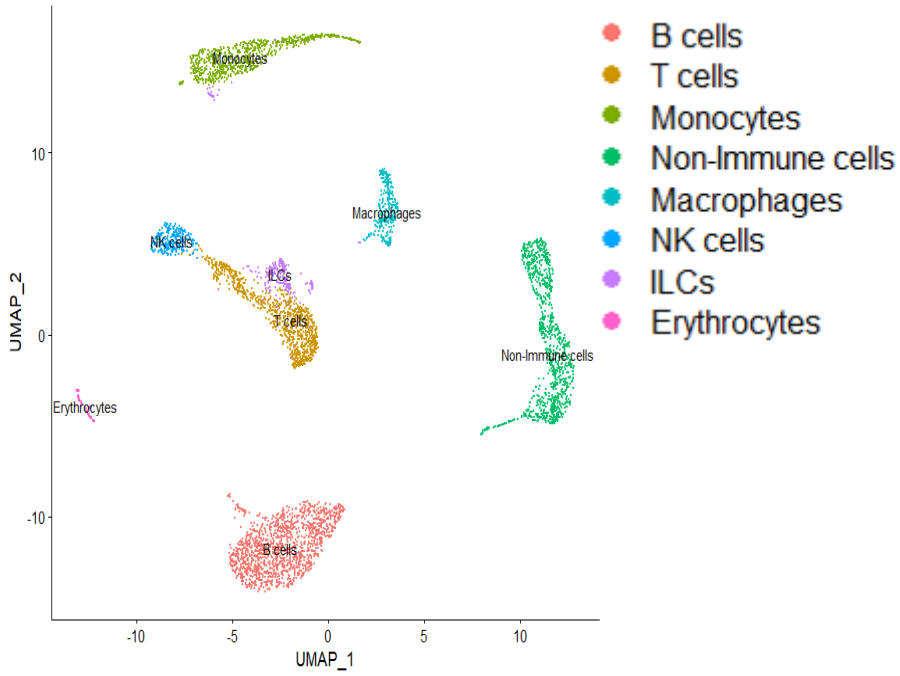
To further characterise the expression of IL-33 and ST2 in the mouse vasculature, I first examined their expression in the adventitial and media-intimal layers of the vessel. For this analysis, I utilised data generated by Gu et al. (2019), which focused on how the adventitial layer of the vessel wall contributes to plaque formation (Gu et al., 2019). To achieve this, 12-week-old male $ApoE^{-/-}$ mice maintained on a chow diet only, with age- and sex-matched C57BL/6J mice as WT controls were used. After harvesting, the adventitia and media-intimal layers were mechanically separated and digested; cells were pooled from 20 mice per group and sequenced (GEO accession: GSE140811).

The adventitial data for both groups was subject to quality control steps before integration, PCA reduction, and clustering analysis. The data was clustered into eight distinct cell populations comprising various immune cells and one group of non-immune cells (Figure 3.7A). Interestingly, expression of IL-33 was not observed in any of the immune cells but localised only in the non-immune cells of the adventitia (Figure 3.7 B, D), whereas ST2 was observed only in ILCs (Figure 3.7 C, E). The non-immune cells expressed markers characteristic of mesenchymal cells.

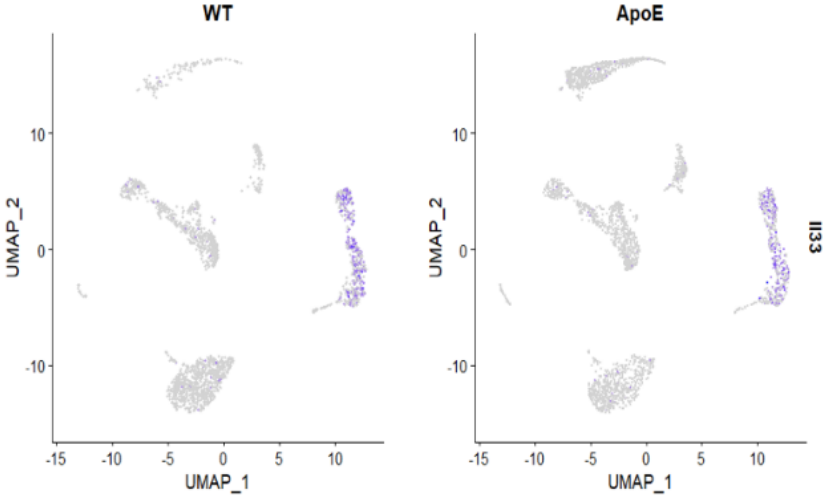
Since IL-33 expression was localised in non-immune cells of the adventitia, further in-depth analysis of the cluster was performed on the dataset. The data clustered into six different cell populations (4 different mesenchymal cells, SMCs, and

adventitial endothelial cells (AdvECs) (Figure 3.8A, B). IL-33 showed expression in all different mesenchyme clusters and SMCs (Figure 3.8C, D).

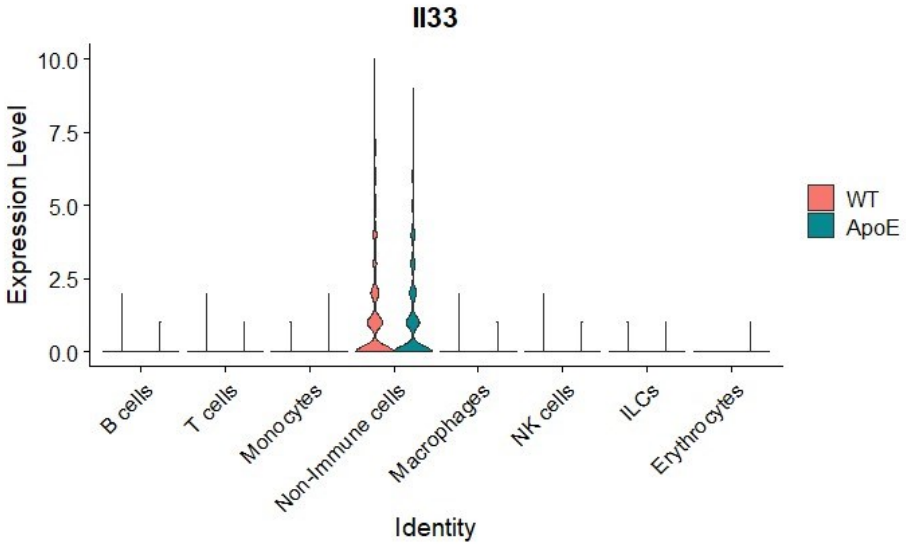
A.


B cells
T cells
Monocytes
Non-Immune cells
Macrophages
NK cells
ILCs
Erythrocytes
Monocytes
Macrophages
NK cells
ILCs
T cells
Non-Immune cells
Erythrocytes
B cells
UMAP_2
UMAP_1
10
0
-10
-10
-5
0
5
10

B.


WT
ApoE
Il33
UMAP_2
UMAP_1
10
0
-10
-15
-10
-5
0
5
10

C.


Il33
Expression Level
10.0
7.5
5.0
2.5
0.0
WT
ApoE
B cells
T cells
Monocytes
Non-Immune cells
Macrophages
NK cells
ILCs
Erythrocytes
Identity

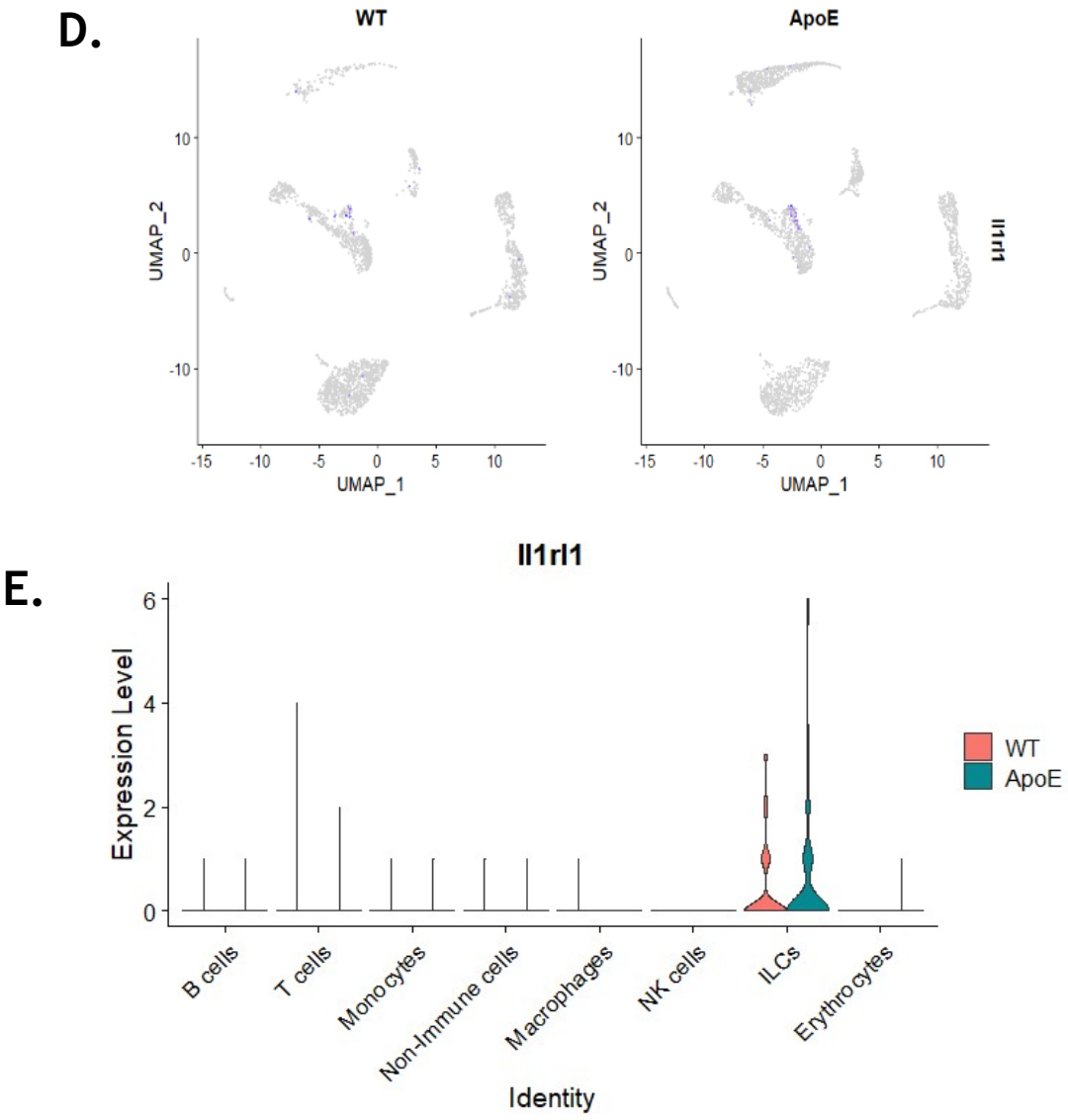


Figure 3.7: IL-33 and ST2 expression in adventitial cells from WT and ApoE$^{-/-}$ mouse aorta.

Analysis of cell clusters present from single-cells extracted from an adventitial layer of the aorta from 8-12-week-old-WT (wild-type) and ApoE $^{-/-}$ chow-fed mice. Data acquired from published single-cell RNA-sequencing database GEO accession: GSE140811 (Gu et al., 2019). **(A)** Uniform Manifold Approximation and Projection (UMAP) representation of aligned gene expression data showing separation into distinct clusters representing different cell populations in the dataset indicated by colour. **(B, D)** Gene expression of **(B)** IL-33 and **(D)** ST2 projected onto UMAP representation of distinct clusters in both WT and ApoE$^{-/-}$ (scale: log-transformed gene expression). Violin plot of **(C)** IL-33 and **(E)** ST2 expression across each distinct cell population cluster for WT (green), ApoE$^{-/-}$ (red) experimental group.

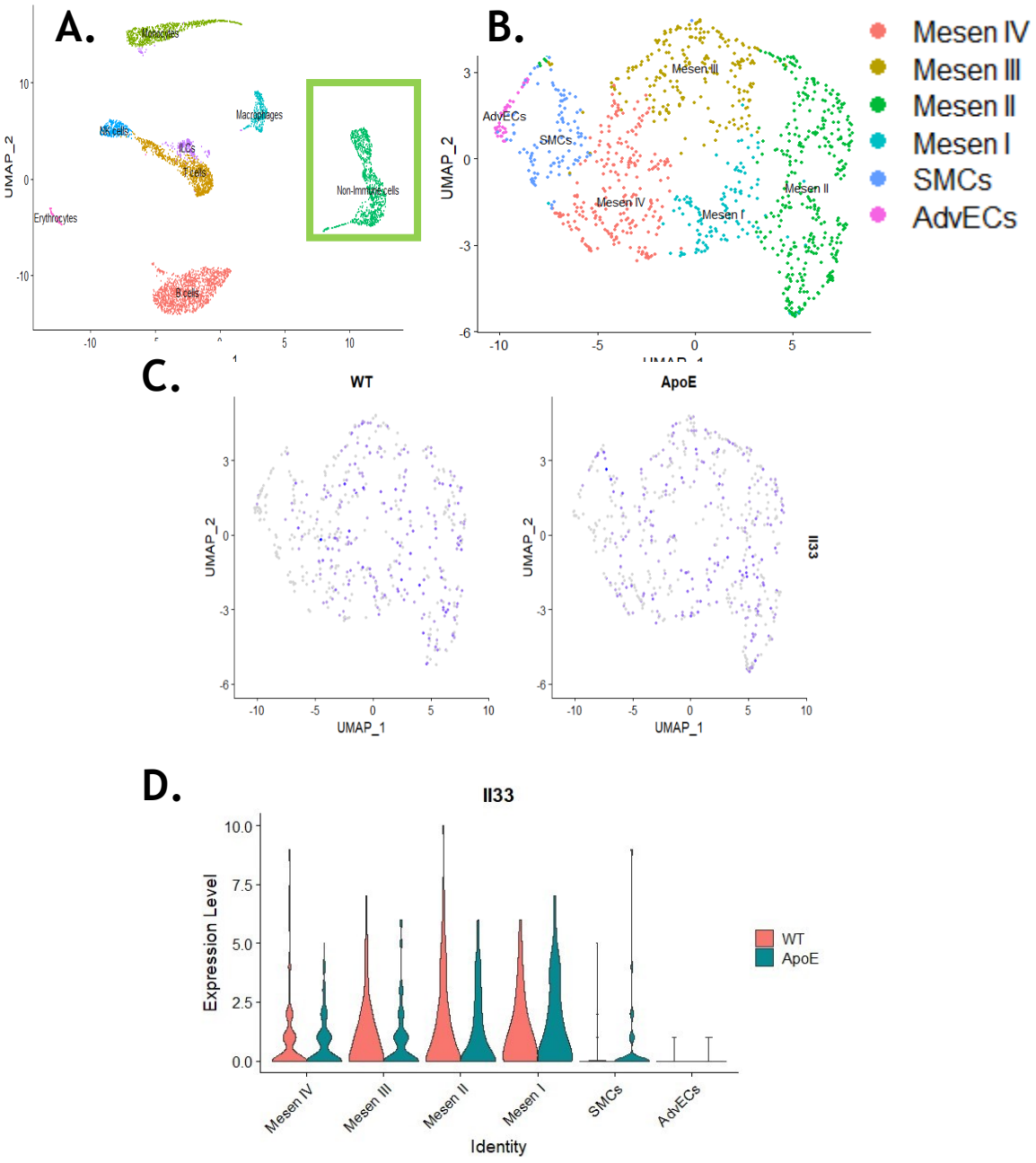


Figure 3.8: IL-33 expression in adventitial non-immune cells from WT and ApoE$^{-/-}$ mouse aorta.

Analysis of cell clusters present from single-cells extracted from an adventitial layer of the aorta from 8-12-week-old-WT and ApoE $^{-/-}$ chow-fed mice. Data acquired from published single-cell RNA-sequencing database GEO accession: GSE140811 (Gu et al., 2019). **(A)** Uniform Manifold Approximation and Projection (UMAP) represents aligned gene expression data and different cell populations in the dataset indicated by colour. **(B, C)** Gene expression of **(B)** IL-33 onto UMAP (scale: log-transformed gene expression). Violin plot of **(D)** IL-33 expression across each distinct cell population cluster for WT (green), ApoE$^{-/-}$ (red) experimental group.

3.3.6 Expression of IL-33 and ST2 in Aortic Infiltrating Leukocytes

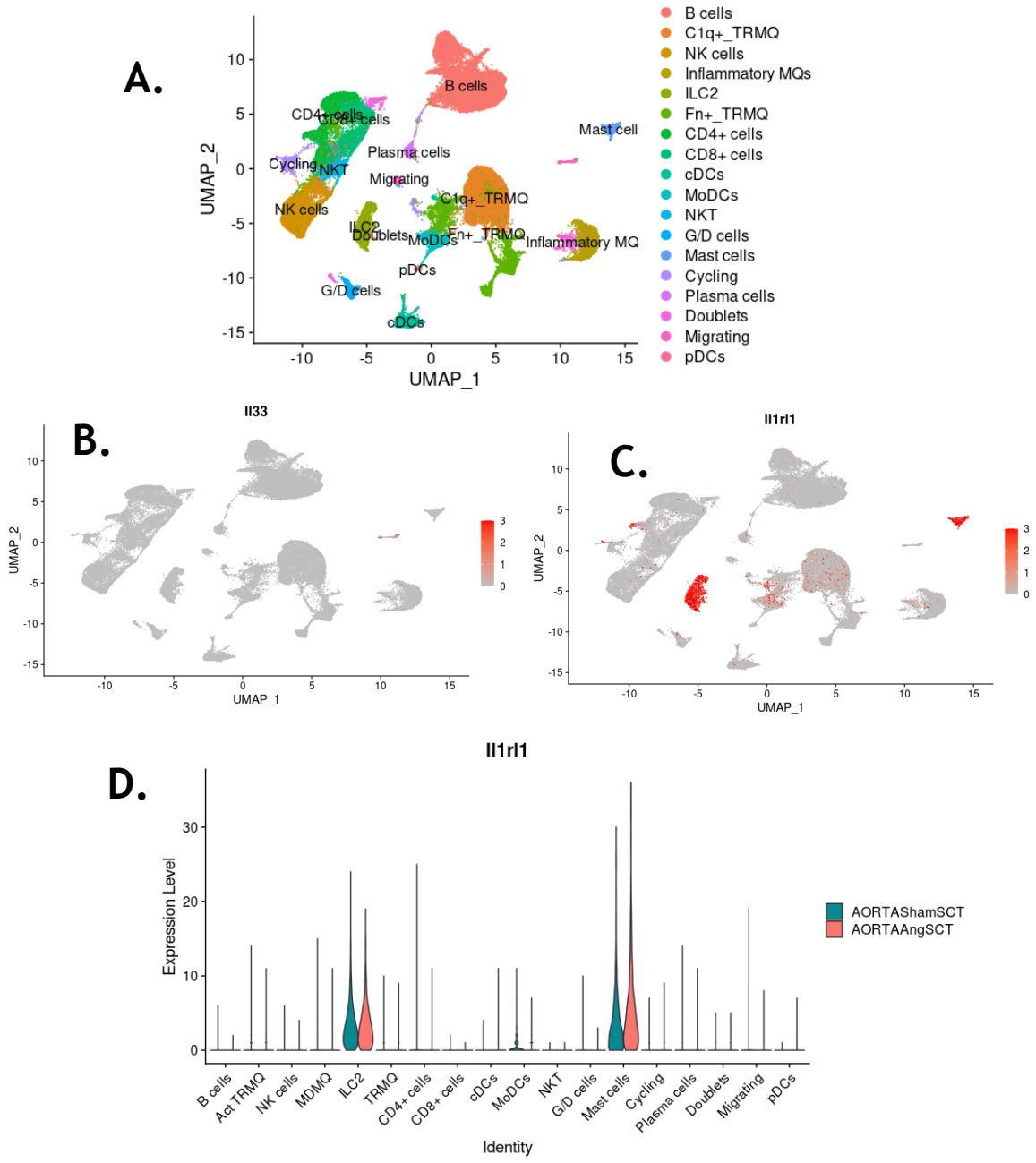


Figure 3.9: IL-33 and ST2 expression in aortic infiltrating leukocytes from WT and Ang II mouse aorta.
Analysis of cell clusters present from single-cells extracted from the aorta of 14-week-old-WT and Ang II mice. Data acquired from previously analysed single-cell RNA-sequencing dataset available in the lab group. (**A**) UMAP represents aligned gene expression data and represents different cell populations in the dataset indicated by colour. Gene expression of **(B)** IL-33 and ST2 (**C**) onto UMAP. Violin plot of **(D)** ST2 expression across each distinct cell population cluster for sham (green), Ang II (red) experimental group.

For clarity on currently gained understanding of the expression of IL-33 in the non-immune cells and ST2 predominantly in immune cells in mouse adventitia of healthy and disease atherosclerotic mice, previously analysed scRNA-Seq dataset available in the lab group was used. The dataset was of 14-week-old mice after sham surgery or Ang II infusion for 14 days. The data includes a wide range of aortic infiltrating leukocytes (Figure 3.9 A). The expression levels of IL-33 and ST2 were investigated in this dataset and have been plotted as feature plots (Figure 3.9 B, C). IL-33 was highlighted only in migrating cells at low levels (Figure 3.8 B). ST2 was present in high levels in both ILC2 and Mast cells (Figure 3.9 C, D).

3.4 Discussion

3.4.1 Chapter Summary

In this chapter, I have characterised the expression of IL-33 and its receptor ST2 in essential organs implicated in hypertension prognosis and elucidated the localisation of IL-33 and ST2 within mouse vasculature. During blood pressure regulation, IL-33 and ST2 are upregulated in several organs, particularly the aorta. Further, this study has established that IL-33 and ST2 are principally localised in ECs and SMCs in sham and Ang II-induced mouse aorta. ST2 mRNA is released from VSMCs in a proinflammatory state through TNF-α stimulation. Lastly, publicly available single-cell RNA seq datasets of mouse aorta identified IL-33 expression in non-immune cells; conversely, its receptor, ST2, was mainly expressed in immune cells such as ILC2s.

3.4.2 IL-33 and ST2 is Induced in Hypertension

Experimental and clinical studies (Yin et al., 2019a) have determined that IL-33 and its known receptor, ST2, have a possible role in hypertension pathology. Previous ELISA-Spot results indicated a potential role of IL-33 in hypertension. IL-33 was upregulated in all organs, including the aorta, lymph node, spleen, kidney, and PVAT in Ang II-infused mice compared to the sham group of proteins within the protein panel. In 2005, Schmitz et al. presented through the analysis of human and mouse cDNA libraries that *IL-33* mRNA is broadly expressed in many tissues, further showing that it is also restricted at the level of cell types. The study revealed high levels of mouse mRNA *IL-33* are observed in the stomach, lungs, spinal cord, brain, and skin. Lower levels of mRNA were seen in lymph tissue, spleen, pancreas, kidney, and heart (Schmitz et al., 2005a). qPCR analysis found that *IL-33* mRNA is upregulated in the aorta and heart of mice receiving Ang II infusion. Furthermore, *ST2* levels were also heightened in the mouse aorta, as well as in the spleen and lymph nodes.

Recently, a 2019 study by Yin et.al. confirmed that IL-33 was significantly increased in the aortas of mice receiving Ang II infusion for 1–7 days. My results support these

findings and report a significant increase in mRNA *IL-33* levels in Ang II-infused mice compared to sham. It is to be noted that observed changes in mRNA *IL-33* levels in the early stages of hypertension (1, 3, and 7 days) (Yin et al., 2019a), while this study focused on a later time of 2 weeks. From this, it can be concluded that mRNA levels of IL-33 surge from the early onset of hypertension, with sustained effects at 14 days, even though relative IL-33 mRNA levels were seen to peek at three days after a gradual reduction was seen by the end of day 7.

IL-33 was initially found in the nucleus of the high endothelial venules of secondary lymphoid tissues (Baekkevold et al., 2003). So far, IL-33 protein is shown to be highly expressed in mouse epithelial barrier tissues, including stratified squamous epithelia from the vagina and skin, as well as cuboidal epithelium from the lung, stomach, and salivary gland (Pichery et al., 2012). Further to this finding, in this study, I observed that protein levels of IL-33 show a 3-fold increase in the aorta of Ang II-induced animals compared to control. It has been speculated that when hypertension occurs, the vasculature undergoes mechanical stretching; thus, the expression of IL-33 in the cells may increase (Yin et al., 2019a). While this increase may protect blood vessels, it could also contribute to endothelial dysfunction, early atherosclerosis, and vascular injury by encouraging angiogenesis development, vascular permeability, and endothelial activation with the upregulation of vascular adhesion molecules (Demyanets et al., 2011a). This could lead to an increased accumulation of immune cells and the development of perivascular inflammation, characteristic of hypertension. Obesity is associated with a chronic inflammatory state of the adipose tissue and increased IL-33 expression (Zeyda et al., 2013). Furthermore, the western blot results show upregulation of IL-33 protein levels in PVAT of hypertensive mouse models. Lymph nodes showed a trend towards upregulation; although not established, results reflect the same trend seen in mRNA.

3.4.3 Elevation of IL-33 and ST2 in Ang II Mice is Blood Pressure Dependent

To confirm that the elevation of *IL-33* and *ST2* mRNA levels was not due to the infusion of angiotensin II alone but rather due to blood pressure elevation, I looked at changes in the mRNA level in the aorta of mice receiving the hypertensive dose of Ang II (490 ng/min/kg) and the Ang II group given the peripheral vasodilator hydralazine. Relating controls were also explored. The data demonstrates that the *IL-33* and *ST2* elevations observed in the aorta are blood pressure-dependent, not due to angiotensin II treatment alone. With the administration of the vasodilator and diuretic combination treatment to the angiotensin II animal group, the elevated levels of *IL-33* and *ST2* after Ang II treatment alone were significantly diminished in the aorta.

3.4.4 IL-33 and ST2 are Predominantly Localised in ECs and SMCs in Ang II- Induced Mouse Aorta

The localisation of IL-33 and its receptor ST2 in hypertensive mouse aorta and control was confirmed by immunohistochemistry. Staining sections using the HRP/DAB method showed IL-33 and ST2 expression throughout both groups' aortic regions: tunica intima, tunica media, and tunica adventitia. In detail, IL-33 expression was more prevalent in the media of the sham and Ang II group and, to a lesser extent, in the adventitia of Ang II. Past studies have discussed the localisation of IL-33 in cardiomyocyte nuclei, cardiac fibroblasts, and smooth muscle cells (Demyanets et al., 2013a). When assessing ST2 localisation, it was found that sham and Ang-II aortic samples exhibit the signal mainly from the media. Interestingly, endothelial cells (present lining the vascular lumen) showed no positive ST2 signal upon Ang II- infusion. This creates a disparity compared to previous studies showing that endothelial cells express the IL-33 receptor, ST2 (Demyanets et al., 2013a). It is to be mentioned that since n=4, further repetition to increase the sample size is necessary to confirm the accuracy of the results that have been obtained. Overall, the presence of IL-33 and ST2 in the controls and a

higher detection in the hypertensive vasculature suggests its potential as a regulator of hypertension under physiological or pathophysiological conditions.

3.4.5 ST2 is Released from Vascular Smooth Muscle Cells Upon TNF Stimulation.

In addition to epithelial and endothelial cells expressing high levels of IL-33, stromal cells, including fibroblasts, are critical sources of IL-33 during inflammation and tissue repair (Cayrol and Girard, 2018). Proinflammatory cytokines like IFN-γ (Seltmann et al., 2013); notch signalling (Sundlisaeter et al., 2012); and mechanical stress (Kakkar et al., 2012) have all been shown to increase IL-33 expression. IL-33 mRNA expression levels were not upregulated in murine vascular smooth muscle cells by various proinflammatory cytokines associated with hypertension.

On the other hand, this experiment highlighted a significant upregulation of *ST2* mRNA expression upon proinflammatory TNF-α stimulation at 6hrs. Literature had previously exhibited elevated *in vitro* ST2 production by pro-inflammatory cytokines (IL-1ß and TNF-α) in human lung epithelial cells and cardiac myocytes (Mildner et al., 2010). Tumour necrosis factor-α is elevated in chronic inflammatory states such as hypertension and other cardiovascular diseases and facilitates increases and decreases in blood pressure (Ramseyer and Garvin, 2013). Thus, my results indicate that the proinflammatory cytokine TNF-α stimulation on murine VSMCs can result in the release of ST2.

3.4.6 IL-33 is Confined to Non-immune Cells and ST2 is Largely Expressed in Immune Cells of Mouse Aorta

Herein, using available scRNA seq datasets, I further investigated the expression of IL-33 and its receptor in different mouse models. Hypertension is considered one of the significant cardiovascular risk factors for the development of atherosclerosis, a

chronic inflammatory disease of the arterial vasculature. An online publicly available sRNA seq dataset generated by Gu et al. was utilised to advance the current understanding of the expression of IL-33 and its receptor in the mouse vasculature. This study examined its expression in the adventitial and media-intimal layers of the healthy and atherosclerotic aorta of the mouse (Gu et al., 2019). Analysis from the adventitial layer showed that IL-33 is only present in the non-immune cell cluster from WT and $ApoE^{-/-}$ mouse aorta. The expression was still pronounced at baseline (WT), although no changes were seen in the disease setting. A recent study showed that IL-33 is highly expressed in adventitial fibroblasts, especially in mouse $CaPO_4$-induced AAA lesions (Li et al., 2019). They also suggested that fibroblasts may release IL-33 and in turn can slow down abdominal aortic aneurysm (AAA) development. Additionally, since the IL-33 expression was localised to the non-immune cells of the adventitia, I performed additional analysis on the same dataset and have shown that the expression was mainly from all the different mesenchyme cell clusters in all the baseline and $ApoE^{-/-}$ mouse and SMCs in the disease mouse model (Li et al., 2019). Perivascular mesenchymal cells include pericytes, adventitial fibroblasts, and mesenchymal stromal cells. They have several roles in specific immune populations' recruitment and activity. Single-cell profiling data of the mouse and human colon identified by Kinchen et al. highlighted that distinct mesenchymal subsets upregulated IL-33 expression in inflammatory settings (Kinchen et al., 2018). Furthermore, IL-33 production by mesenchymal cells has been known to be further upregulated by proinflammatory cytokines such as TNF-α, IL-1B, and IL-17, thus suggesting that there may be a role for perivascular mesenchymal cells through the production of IL-33 in sensing the environment and modulating inflammatory responses in both basal and disease setting (Cayrol, 2021). Unlike IL-33, ST2 was not present in aortic adventitial non-immune cells, but there was a marked expression in the ILC2s cell cluster.

These results were clarified using a separate single-cell dataset, which was more appropriate for my hypertension study of aortic infiltrating leukocytes in both WT and Ang II mouse models. The dataset used was of the same mouse background, age, and hypertension disease pathology as all my other experiments. Expression of the

IL-33 receptor has been reported in many immune cells, including Innate lymphoid cells Type-2, mast cells, basophils, CD4 and $CD8^{+}$T cells, eosinophils, and macrophages (Topczewska et al., 2023).

IL-33 can act on tissue-resident ST2-expressing ILC2s and mast cells and, upon stimulation, can secrete IL-5, IL-13, IL-10, and amphiregulin (Areg) as central effector cytokines to regulate type 2 immune response in tissues. These cells can give feedback on the tissue and might be able to regulate remodelling and limit inflammation by activating progenitor cells (Molofsky et al., 2015b). The dataset analysis revealed that, as observed previously, and indicated in the literature, ST2 was present in high levels in ILC2s and mast cells in both baseline and upon Ang II. Expression of ST2 in mast cells was slightly upregulated when compared with the WT sham group. IL-33 expression, on the other hand, was very low, as expected from my previous analysis and was highlighted only in migrating cells. Through the expression of ST2 by many type-2 effector cells, as mentioned before, IL-33 is thought to play an essential role in triggering type-2 immune responses in mouse models (Topczewska et al., 2023).

3.5 Chapter Conclusion

In this chapter, I have identified the regulation of IL-33 and ST2 expression in key organs upon Ang II-induced hypertension in an animal model. This chapter has uncovered the localisation of IL-33 and ST2 within mouse vasculature in various cell subsets in hypertension and other cardiovascular pathologies. Therefore, I conclude that IL-33 and its known receptor, ST2, have a possible role in hypertension pathology as they are upregulated in hypertension.

Chapter 4 Role of IL-33 and ST2 in Hypertensive Animal Models

4.1 Introduction

Hypertension is the leading cause of global morbidity and mortality (Dai et al., 2021). While blood pressure reduction improves patient disease outcomes, cardiovascular (CV) risks remain elevated, indicating the involvement of several underlying pathological processes mediating adverse clinical effects (Nosalski et al., 2020). Hypertension is a progressive CV disorder promoting several functional and structural vascular abnormalities damaging the vasculature, heart, kidney, and other organs, leading to morbidity (Antonakoudis et al., 2007).

Hypertension is associated with accelerated vascular ageing, observed in both micro and macro circulation (Guzik and Touyz, 2017). It contributes to diverse disease pathophysiological processes, including vascular stiffening, elevated perivascular fibrosis and inflammation, augmented vascular oxidative stress, and endothelial dysfunction, causing a vicious cycle between BP, vascular remodelling, stiffness, and sustained hypertension complications (Guzik and Touyz, 2017, Nosalski et al., 2020).

Cardiac remodelling, such as interstitial fibrosis, cardiomyocyte hypertrophy, and abnormal sympathetic nervous system activity, are pathophysiological processes in many heart diseases, including hypertension (Tomek and Bub, 2017), (Sanada et al., 2007a). Myocardial fibrosis results from pathological processes mediated by neurohormonal and cytokine-activated pathways (Díez, 2007).

Accruing data demonstrate hypertension as a low-grade inflammatory disease (Solak et al., 2016). Immune cells can infiltrate multiple organs, causing dysfunction and leading to elevation of blood pressure. Infiltration of innate (monocyte/macrophages) and adaptive immune cells (T lymphocytes) in the perivascular fat (PVAT), kidney, and myocardium, elevated expression of adhesion molecules and chemokines, cytokine production and ROS generation, are consistent features of models of experimental hypertension (Caillon et al., 2019b, Yin et al.,

2019a). My data indicates a potential role for IL-33 and ST2 in hypertension. I have previously described the upregulation of IL-33 and ST2 in angiotensin II-induced hypertension and its expression in immune and non-immune cells in mouse models.

IL-33 is a profibrogenic and pro-inflammatory cytokine that signals through its receptor, ST2, in many fibrotic disorders. Physiological stretching of the myocardium enables cardiac fibroblasts and cardiomyocytes to release IL-33 into the extracellular space, which binds the ST2L receptor on the cardiomyocyte membrane, promoting cell survival, integrity, and inhibition of pro-fibrotic intracellular signalling (Kotsiou et al., 2018, Millar et al., 2017). Conversely, the IL-33 and ST2L signalling pathway can become detrimental in chronic conditions. Local and neighbouring cells can increase the release of IL-33's decoy receptor, sST2, which can inhibit IL-33 and ST2L binding, inhibiting its cardio protection and promoting tissue fibrosis (Vianello et al., 2019). As a result, IL-33/ST2 signalling has been studied intensively in cardiac fibrosis. However, the role of IL-33 and ST2 in regulating perivascular fibrosis in hypertension has not been investigated.

In hypertension, perivascular fibrosis mainly depends on perivascular inflammation and immune cells (Zhuang et al., 2022). During the development of hypertension, immune cells accumulate in the perivascular fat tissue surrounding vessels such as the aorta and mesenteric arteries (Nosalski and Guzik, 2017). PVAT is the primary site of initial inflammation in hypertension. Furthermore, in hypertension, ROS derived from PVAT can augment endothelial dysfunction (Nosalski and Guzik, 2017). This is induced by endothelial NO scavenging by PVAT-derived ROS or through alteration of perivascular inflammation impacting endothelial function (Ketonen et al., 2010).

4.2 Chapter Aims

The role of IL-33 in cardiovascular diseases such as atherosclerosis, obesity, type 2 diabetes, and cardiac remodelling has been examined (Miller, 2011b). However, an

in-depth study on the mechanisms of action of the IL-33/ST2 axis in hypertension is lacking.

Therefore, the main aim of this chapter is to investigate the role of IL-33 and ST2in hypertension using IL-$33^{-/-}$ and $ST2^{-/-}$ animals. Specific purposes are listed as follows:

1. Investigate the role of IL-33 and ST2 in regulating blood pressure and vascular function in hypertensive mice models with related wild-type control groups.

2. Study the regulation of perivascular fibrosis and inflammation by IL-33 and ST2 upon Angiotensin II treatment in knockout and WT group.

3. Elucidate the function of IL-33 and ST2 in regulating angiotensin II-infused cardiac remodelling.

4.3 Results

4.3.1 Effect of Angiotensin II Infusion on Blood Pressure Regulation in IL-33$^{-/-}$ and ST2$^{-/-}$ mice.

To understand the functional role of IL-33 and ST2 in hypertension, I used IL-33$^{-/-}$ and ST2$^{-/-}$ mice in which I investigated the development of hypertension. *In vivo*, Ang II-infused IL-33$^{-/-}$ and ST2$^{-/-}$ mice did not show altered blood pressure at baseline or throughout the 14-day Ang II infusion evaluated by tail cuff in comparison to WT Ang II-infused animals. (Figure 4.1A and B).

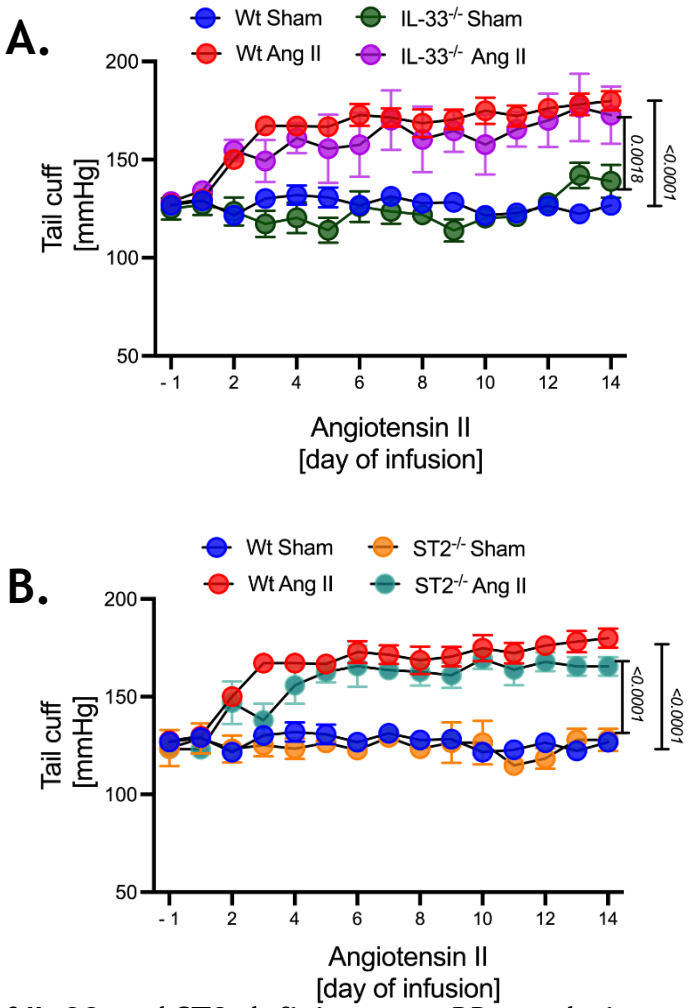


Figure 4.1 Role of IL-33 and ST2 deficiency on BP regulation.

Systolic blood pressure (SBP) in Sham and Ang II-infused IL-33$^{-/-}$ (A), and ST2$^{-/-}$ (B) and WT littermates were measured by Tail cuff at baseline (-1) and for 14 days of Ang II (n=5/group). Data presented as mean ± SEM; repeated measures 2-way ANOVA.

4.3.2 Modulation of Vascular Fibrosis by IL-33 and ST2 in Hypertension.

While Ang II-infused IL-33$^{-/-}$ and ST2$^{-/-}$ mice presented no altered blood pressure differences in comparison to WT Ang II mice, increased levels of these proteins in hypertension warranted further investigation into the role of IL-33 and ST2 in regulating vascular fibrosis.

I measured collagen accumulation/fibrosis by picrosirius staining in mouse thoracic aorta of sham and Ang II-induced IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Figure 4.2A). The fibrotic cross-sectional area was analysed using ImageJ studio. I observed elevated perivascular collagen accumulation upon Ang II infusion in WT mice compared to WT sham. Conversely, there was a significant reduction in perivascular fibrosis in ST2$^{-/-}$ Ang II mice compared to WT Ang II-induced littermates (Figure 4.4 B). IL-33$^{-/-}$ mice presented no substantial changes in fibrotic area compared to the control group (Figure 4.2 C).

I also studied the aortic regulation of fibrotic marker expression in sham buffer and Ang II mice quantified by qPCR in IL-33$^{-/-}$ and receptor knockout mice. Angiotensin II-induced aortic Col1a1, Col3a1, and Col5a1 significantly in wild-type mice in comparison to sham operation. However, this increase was abolished in ST2$^{-/-}$ mice (Figure 4.3 B). Conversely, IL-33$^{-/-}$ littermates highlighted no significant changes in aortic fibrotic marker mRNA expression between WT Ang II and KO Ang II-induced mice (Figure 4.3 A).

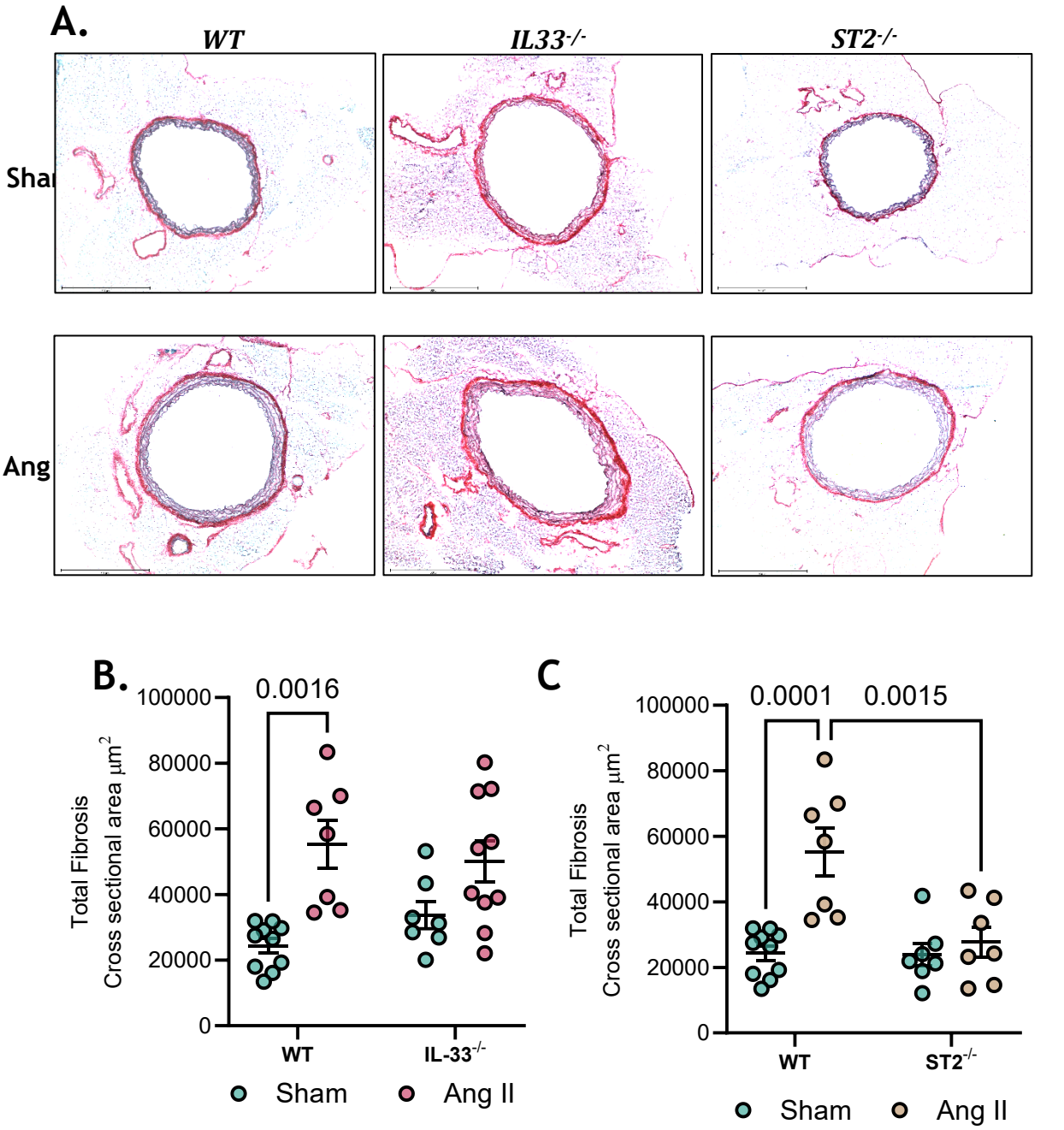


Figure 4.2: Role of IL-33 and ST2 in the regulation of perivascular fibrosis in Ang II-dependent hypertension.

Perivascular collagen accumulation using picrosirius for IL33$^{-/-}$ and ST2$^{-/-}$ mice (A). Quantitative analysis of vascular collagen accumulation by ImageJ studio (Representative of n=7-8; scale bar=500 μm) of IL-33$^{-/-}$ (B) and ST2$^{-/-}$ (C) mice. Data presented as mean ± SEM; 2-way ANOVA.

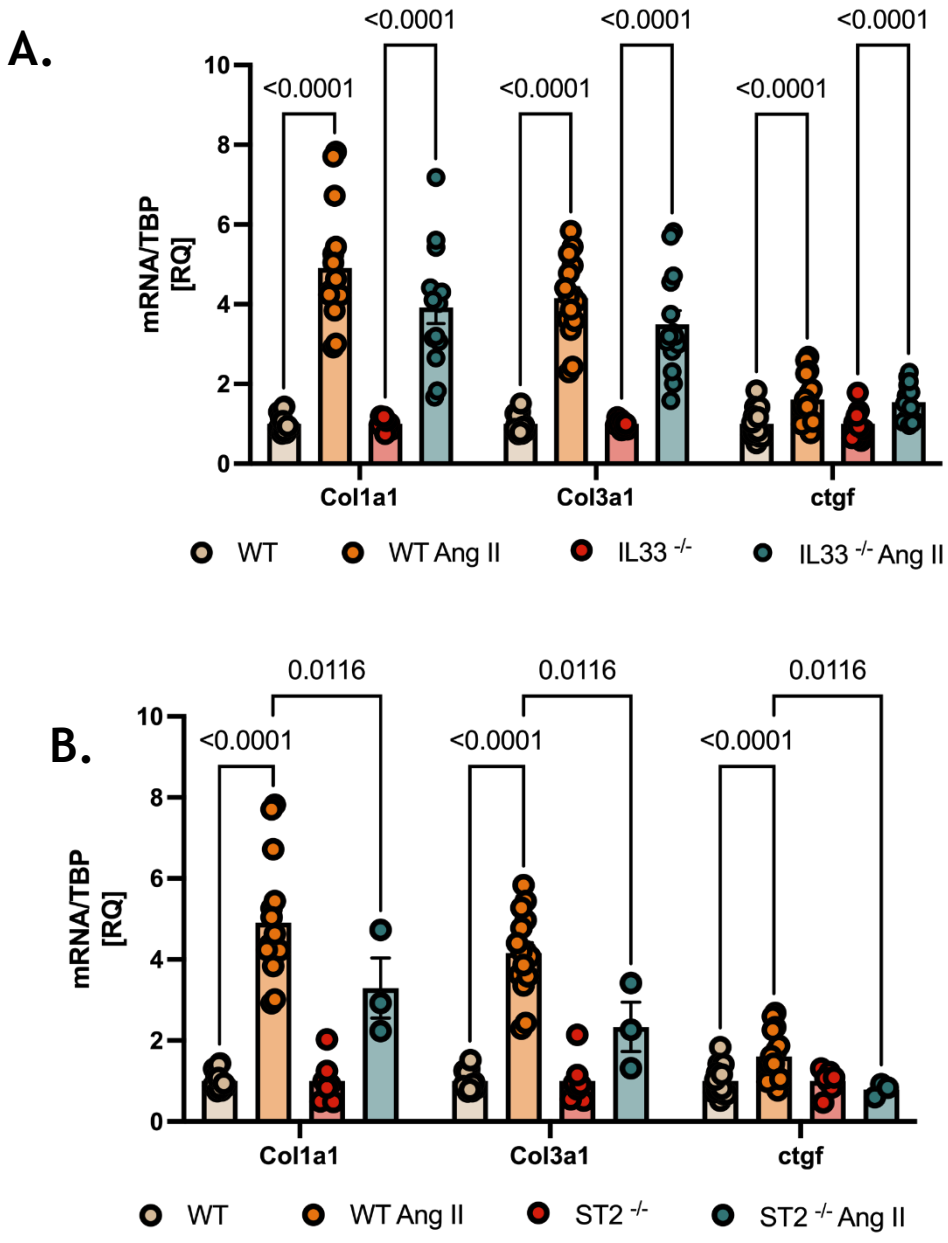


Figure 4.3: IL-33 and ST2 in the regulation fibrotic markers in the vasculature of a mouse with Ang II-dependent hypertension.

Fibrotic markers: collagen 1, 3 and 5 and Ctgf mRNA expression in IL33$^{-/-}$ (A), ST2$^{-/-}$ (B) and WT mice to Sham buffer and Ang II induction. Representative of n= 3-14, data presented as mean ± SEM; 2-way ANOVA with Tukey test.

4.3.3 ST2 Regulates Vascular Dysfunction in Hypertension Independently of Blood Pressure.

Vascular function was studied by wire myography in WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice with sham buffer or Ang II infusion. IL-33$^{-/-}$ mice exhibited no changes in vascular function compared to WT sham and Ang II-infused mice (Figure 4.4A). Conversely, ST2$^{-/-}$ mice infused with Ang II were partially protected from the development of endothelial dysfunction in comparison to WT Ang II mice, revealing a preserved NO-mediated vasodilation despite Ang II-induced hypertension (Figure 4.4B). No difference was observed in non-endothelium-dependent vasorelaxation to sodium nitroprusside between WT, IL-33$^{-/-}$ and ST2$^{-/-}$ sham buffer or Ang II infused mice (Figure 4.4B).

I next studied vascular superoxide production in both IL-33$^{-/-}$ and ST2$^{-/-}$ mice, as oxidative stress is known to mediate endothelial dysfunction in hypertension. IL-33$^{-/-}$ mice indicated no significant alteration in superoxide production compared to WT mice (Figure 4.5A). However, ST2$^{-/-}$ mice displayed reduced superoxide production compared to WT littermates, suggesting decreased vascular oxide stress upon Ang II infusion (Figure 4.5B).

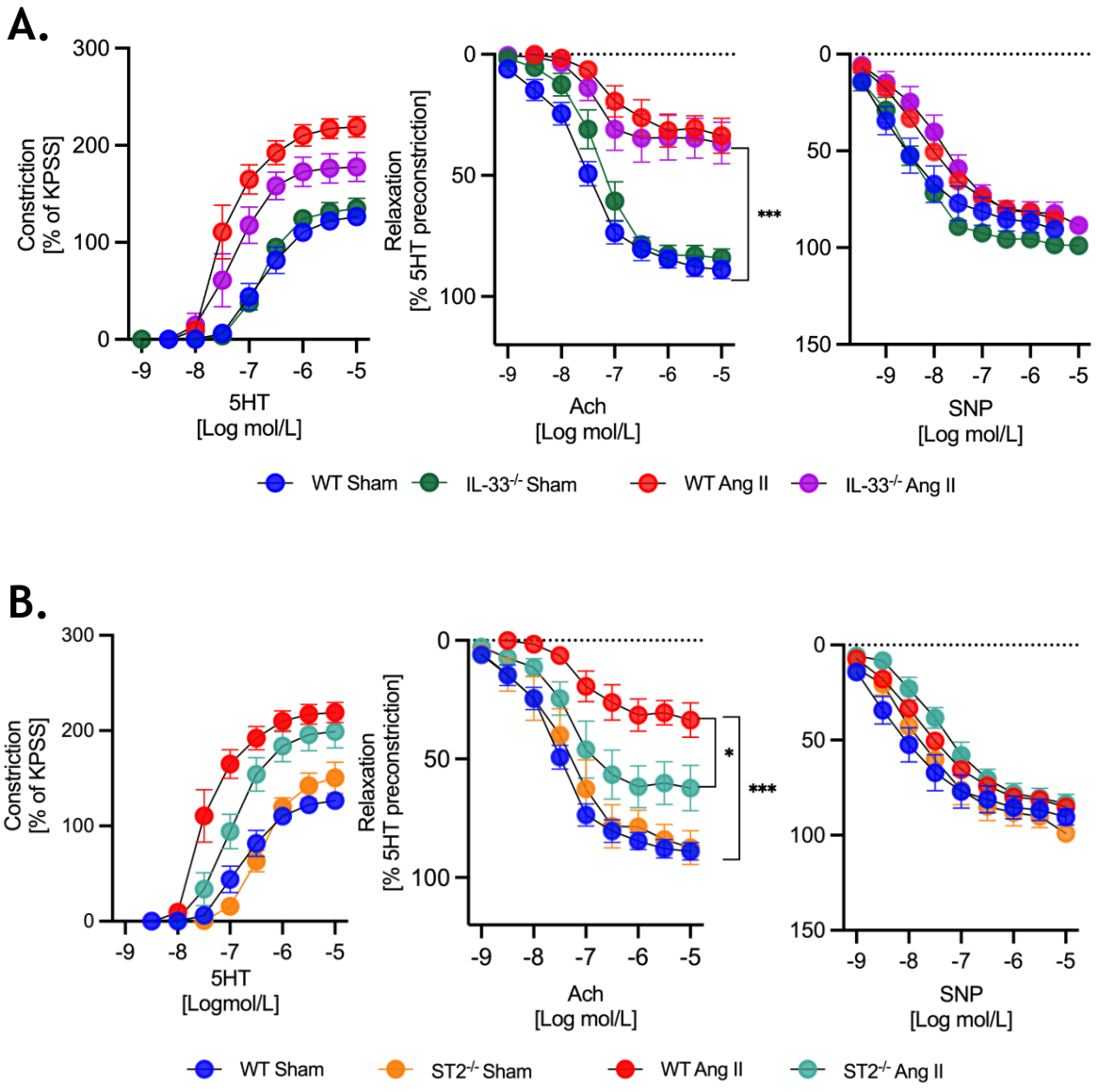


Figure 4.4: Essential role of IL-33 and ST2 in endothelial dysfunction in hypertension.

Isometric tension studies of endothelium-dependent (acetylcholine; Ach) and independent (sodium nitroprusside; SNP) vasorelaxations using wire myography in Sham and Ang II (angiotensin II)-infused (n=3-7/group) WT, IL-33$^{-/-}$ (A) and ST2$^{-/-}$ (B) mice (n=4-6/group). Data are presented as mean ± SEM and analysed by repeated measures ANOVA. *P* values for repeated measures 2-way ANOVA; * = *0.0317*, *** = *0.0004*.

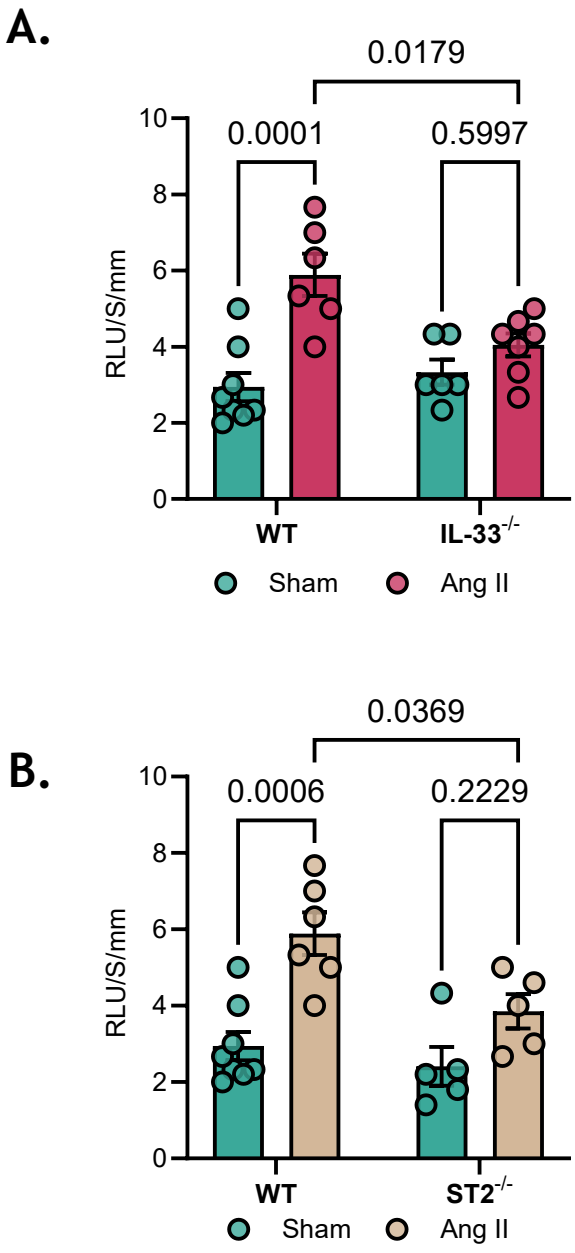


Figure 4.5: Evaluation of vascular superoxide production in aortic mice rings in hypertension.

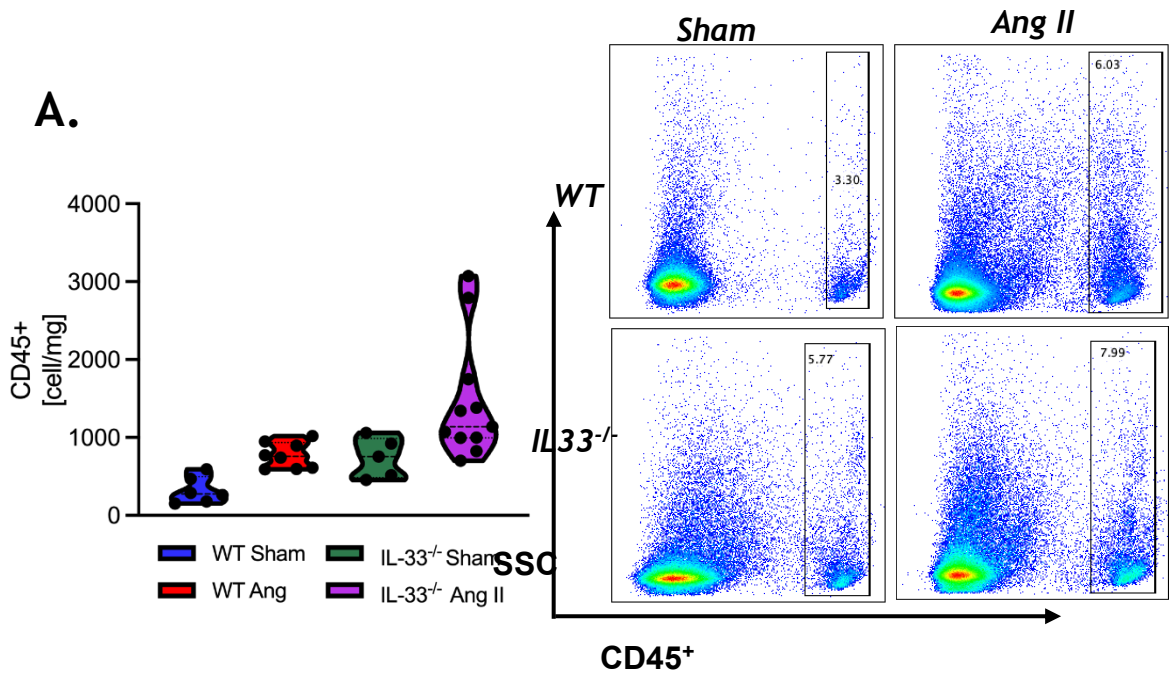
Aortic superoxide production measured by lucigenin (5 μM) enhanced chemiluminescence from WT, IL-33$^{-/-}$ **(A)** and ST2$^{-/-}$ **(B)** littermates upon Sham and Ang II-infusion for 14 days (n=5-8 /group). Data represented as mean ± SEM and analysed using 2-way ANOVA with Tukey post hoc test.

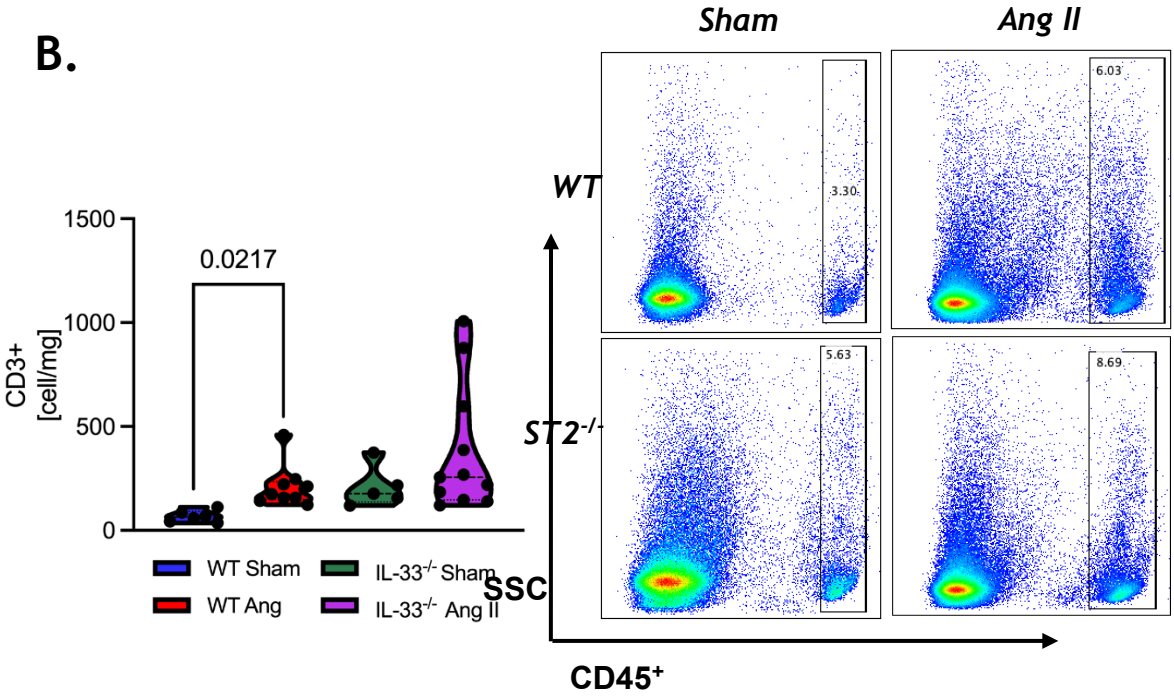
4.3.4 Regulation of Perivascular Inflammation in Hypertension by IL-33 and ST2

To understand the effect of IL-33 and ST2 on vascular inflammation in hypertension, perivascular recruitment of total leukocytes and $CD3^+$T cells in IL-$33^{-/-}$ and $ST2^{-/-}$ hypertensive animals in comparison to hypertensive WT mice were studied by flow cytometry.

In IL-$33^{-/-}$ group experiments alone, no significant changes in the recruitment of total leukocytes was observed in WT and IL-$33^{-/-}$ littermates upon Ang II infusion compared to sham (Figure 4.6A). Total $CD45^+$ cells were upregulated only in the WT Ang II group compared to the sham in the $ST2^{-/-}$ experiment (Figure 4.7A). Ang II infusion significantly elevated the total perivascular $CD3^+$T cells in WT mice in both sham and Ang II mice but not in IL-$33^{-/-}$ or $ST2^{-/-}$ mice (Figure 4.6A, B), (Figure 4.7A, B).

Furthermore, in both experiments, recruitment of NK cells was upregulated in Ang II WT mice compared to WT sham. Still, no changes were observed in IL-$33^{-/-}$ or $ST2^{-/-}$ littermates (Figure 4.6C, 4.7C), and in the $ST2^{-/-}$ experiment, Ang II infusion significantly enhanced the total number of macrophages and dendritic cells ($CD11c^+CD11b^-$) only in WT animals but not in ST2 deficient mice. No significant changes in B cells ($CD19^+$) were noted in the IL-$33^{-/-}$ or $ST2^{-/-}$ study. Conversely, macrophage ($CD11b^+$) expression was significantly increased in IL-$33^{-/-}$ Ang II-induced mice compared to the sham IL-$33^{-/-}$ group.


A.
Sham
Ang II
WT
IL33-/-
CD45+ [cell/mg]
4000
3000
2000
1000
0
WT Sham
WT Ang
IL-33-/- Sham
IL-33-/- Ang II
SSC
CD45+
3.30
6.03
5.77
7.99


B.
Sham
Ang II
WT
ST2-/-
CD3+ [cell/mg]
1500
1000
500
0
0.0217
WT Sham
WT Ang
IL-33-/- Sham
IL-33-/- Ang II
SSC
CD45+
3.30
6.03
5.63
8.69

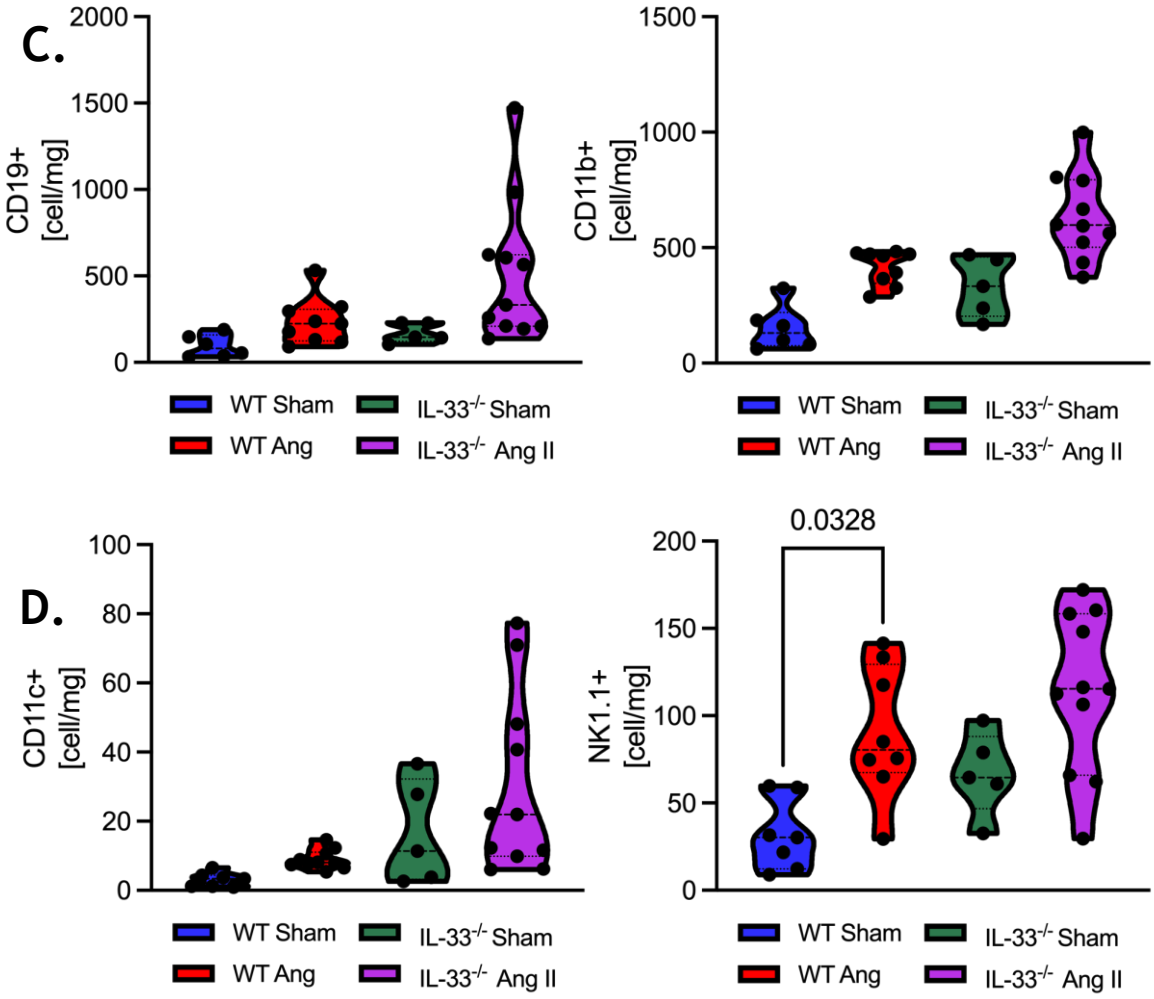
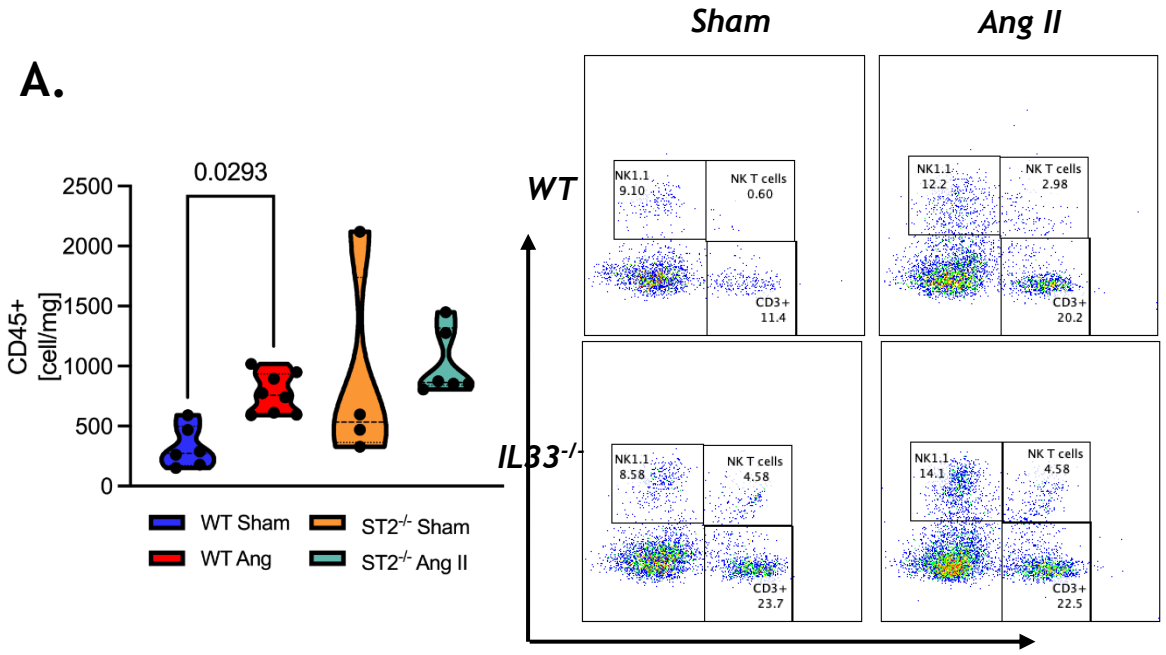
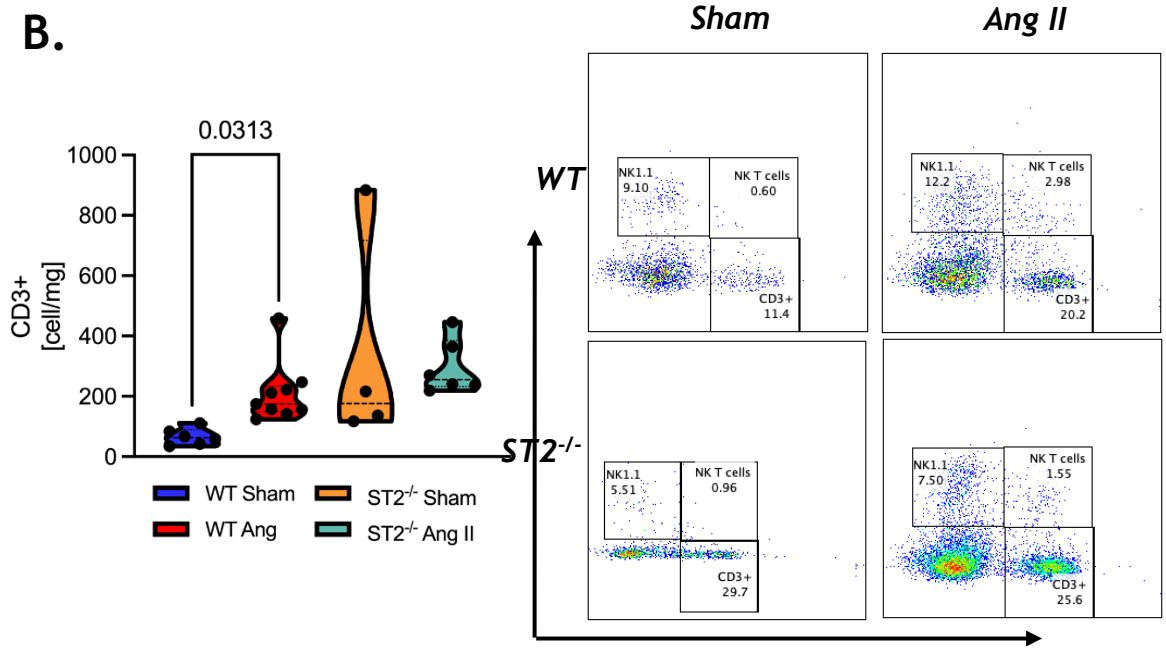


Figure 4.6: Role of IL-33 in the regulation of perivascular inflammation in hypertension.

Total number of leukocytes (**A**) and T cells (**B**) with representative density plot upon Sham and Ang II in WT and IL-33$^{-/-}$ mice (n=5-11). Flow cytometry used to study number of perivascular B cells (CD19^{+}), Macrophage (CD11b^{+}) dendritic cells (CD11c^{+}), NK cells (NK1.1^{+}) (**C**) per mg of tissue. Data presented as mean ± SEM and analysed by the Kruskal-Wallis test.


A.
Sham
Ang II
WT
IL33-/-
0.0293
CD45+ [cell/mg]
2500
2000
1500
1000
500
0
WT Sham
WT Ang
ST2-/- Sham
ST2-/- Ang II
NK1.1 9.10
NK T cells 0.60
CD3+ 11.4
NK1.1 12.2
NK T cells 2.98
CD3+ 20.2
NK1.1 8.58
NK T cells 4.58
CD3+ 23.7
NK1.1 14.1
NK T cells 4.58
CD3+ 22.5


B.
Sham
Ang II
WT
ST2-/-
0.0313
CD3+ [cell/mg]
1000
800
600
400
200
0
WT Sham
WT Ang
ST2-/- Sham
ST2-/- Ang II
NK1.1 9.10
NK T cells 0.60
CD3+ 11.4
NK1.1 12.2
NK T cells 2.98
CD3+ 20.2
NK1.1 5.51
NK T cells 0.96
CD3+ 29.7
NK1.1 7.50
NK T cells 1.55
CD3+ 25.6

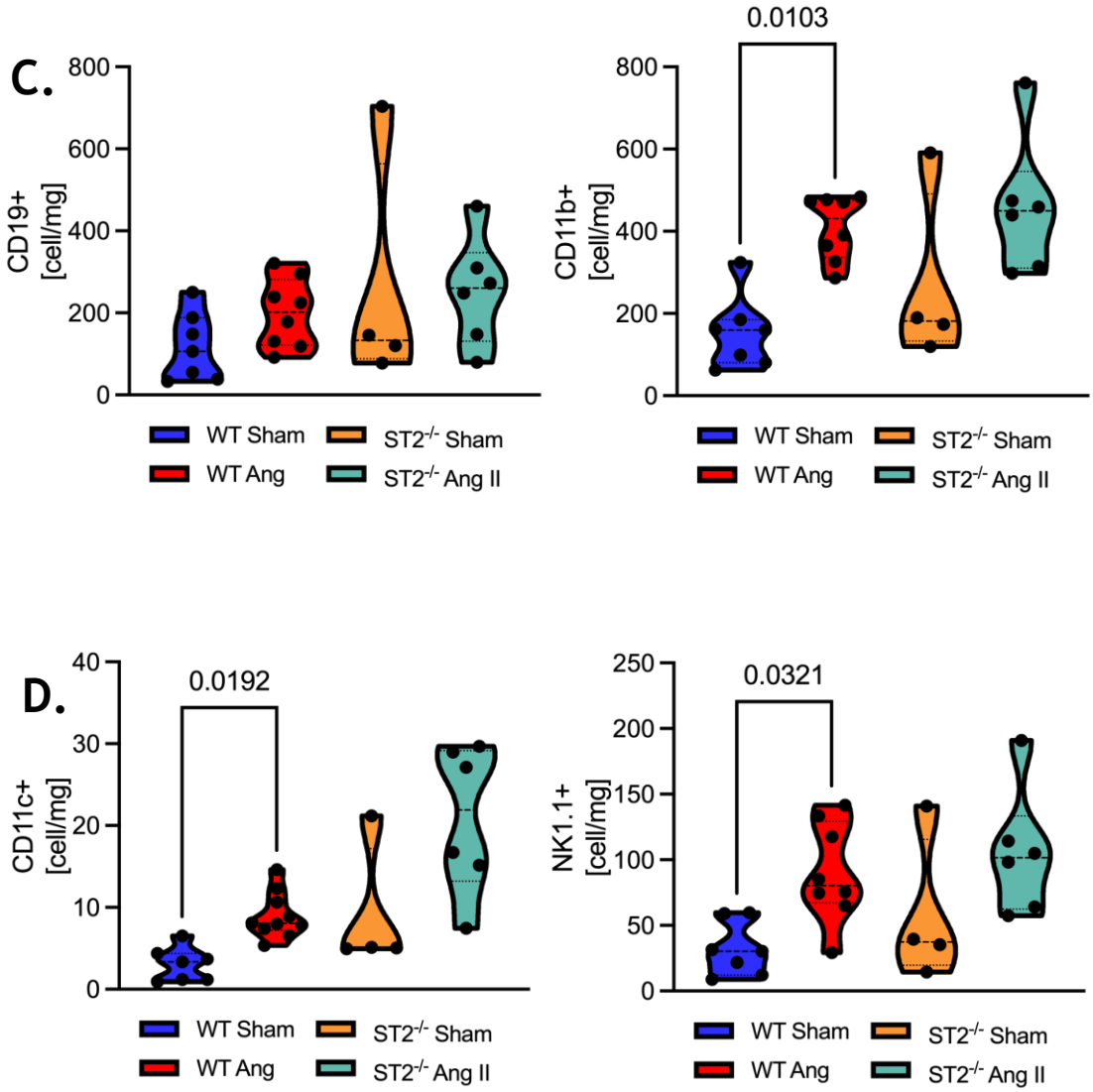


Figure 4.7: Role of ST2 in the regulation of perivascular inflammation in hypertension.

Total number of leukocytes (**A**) and T cells (**B**) with representative density plot upon sham and Ang II in WT and ST2$^{-/-}$ mice (n=4-8). Flow cytometry was used to study a number of perivascular B cells (CD19^{+}), Macrophage (CD11b^{+}) (**C**) dendritic cells (CD11c^{+}), NK cells (NK1.1^{+}) (**D**) per mg of tissue. Data presented as mean ± SEM and analysed by the Kruskal-Wallis test.

4.3.5 IL-33 Regulates Myocardial Fibrosis in Ang II-Dependent Hypertension

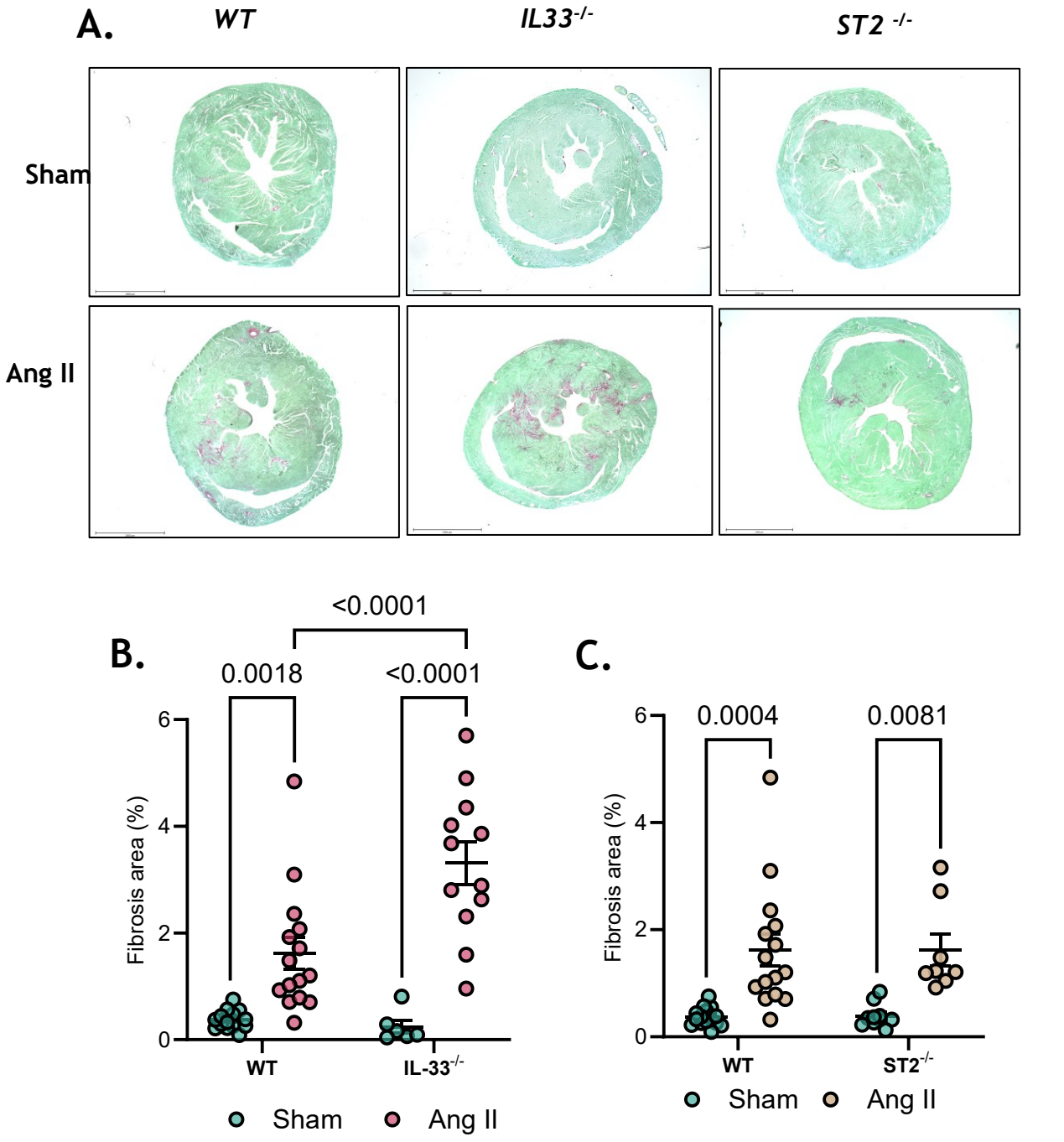


Figure 4.8: Role of IL-33 and ST2 in the regulation of cardiac fibrosis in Ang II - dependent hypertension.
Cardiac collagen deposition visualised by picrosirius green staining for IL33$^{-/-}$ **(A)** and ST2$^{-/-}$ **(B)** quantitative analysis of cardiac collagen deposition (Representative of n=8-12; scale bar=2000 μm. Data presented as mean ± SEM; 2-way ANOVA with Tukey test.

To understand the role of IL-33 and ST2 in regulating cardiac fibrosis, I measured collagen accumulation by picrosirius green staining in mouse hearts of sham buffer and Ang II infused IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Figure 4.8A). The percentage of the fibrotic area was analysed using ImageJ studio. Myocardial fibrosis was significantly upregulated in IL-33$^{-/-}$ mice induced with Angiotensin II compared to WT littermates (Figure 4.8B). ST2$^{-/-}$ did not show any significant changes in the percentage of collagen accumulation compared to WT (Figure 4.8D).

Furthermore, fibrosis marker expression in sham and Ang II hearts was quantified by qPCR in IL-33$^{-/-}$and receptor knockout mice. WT mice, fn1, nox4, Ctgf, Tgfß1, Col1a1, Col3a1, and Col5a1 were significantly upregulated upon Ang II infusion (Figure 4.9A, B). However, in IL-33$^{-/-}$ and ST2$^{-/-}$ littermates, no significant changes in fibrotic marker mRNA levels between WT Ang II and KO Ang II were observed.

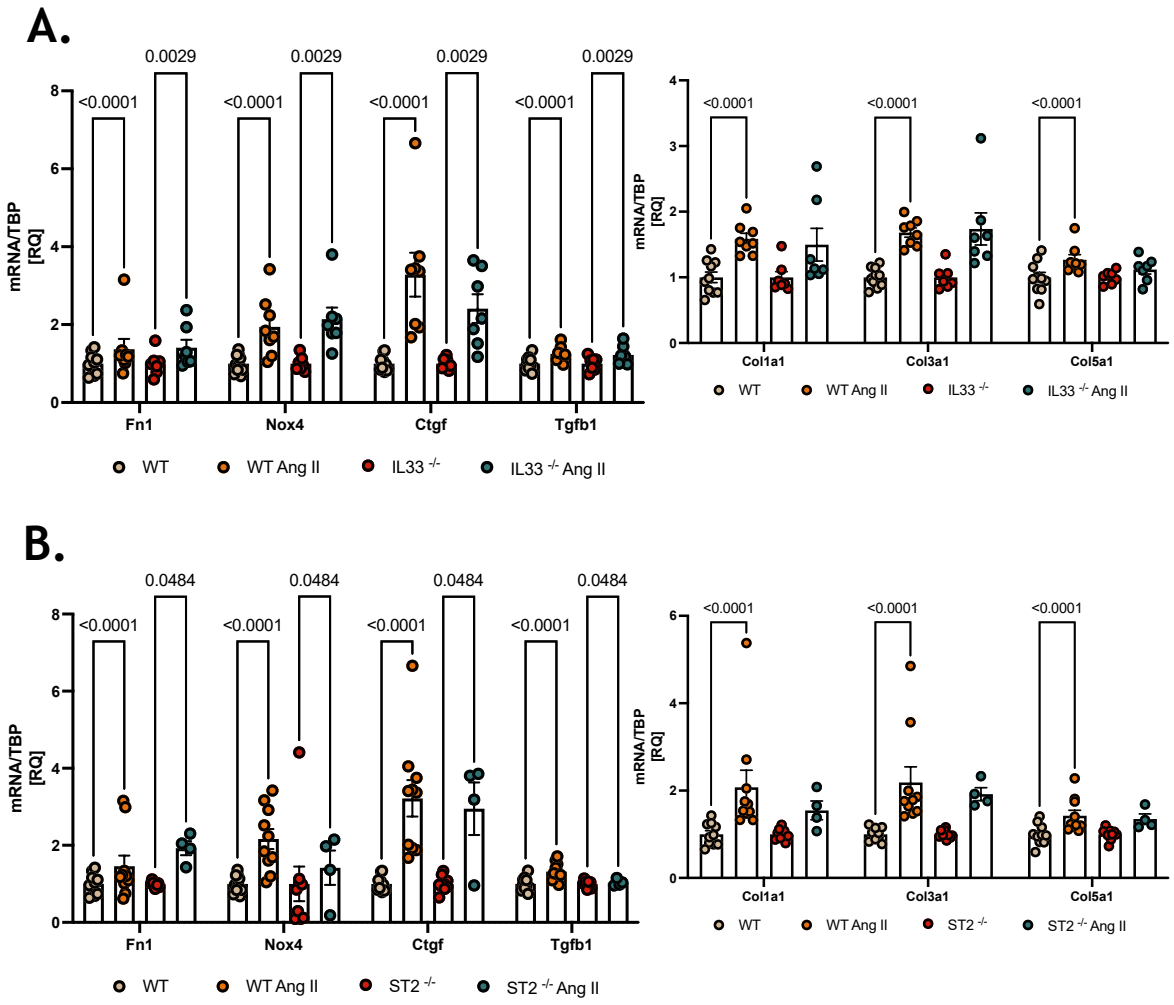


Figure 4.9: IL-33 and ST2 in the regulation fibrotic markers in hearts of mice with Ang II-dependent hypertension.

Fn1, *Nox4*, *Ctgf*, *TgfB1*, *Collagen 1, 3*, and *5* mRNA in IL-33$^{-/-}$ (A), ST2$^{-/-}$ (B) and WT littermates infused with buffer or Ang II (n=4-9/group). Data presented as mean ± SEM; 2-way ANOVA with Tukey test.

4.3.6 Deletion of ST2 Promotes Cardiomyocyte Hypertrophy Following Ang II Induced Hypertension

To investigate if IL-33 and ST2 have a role in heart remodelling and cardiomyocyte hypertrophy, IL-33$^{-/-}$ and ST2$^{-/-}$ mice hearts were stained with wheat germ agglutinin and cardiomyocyte size following Angiotensin II infusion was measured.

In WT littermates, infusion of Ang II significantly increased cardiomyocyte area compared to WT sham. Additionally, no significant change was observed in IL-33$^{-/-}$. However, ST2$^{-/-}$ mice demonstrated increased cardiomyocyte cross-sectional area in both sham and Ang II groups compared with WT littermates (Figure 4.10B, C)

Gross heart/body weight measurements were obtained from IL-33$^{-/-}$ and ST2$^{-/-}$ mice. As expected, the heart/body weight ratio was increased upon Ang II infusion in WT and knockout mice compared to WT sham and KO sham mice. In IL-33$^{-/-}$ mice, a significant increase was observed between the IL-33$^{-/-}$ sham and WT sham group. Furthermore, the ST2$^{-/-}$ mice heart/body weight ratio was significantly increased upon Ang II compared to WT Ang II littermates (Figure 4.11A, B).

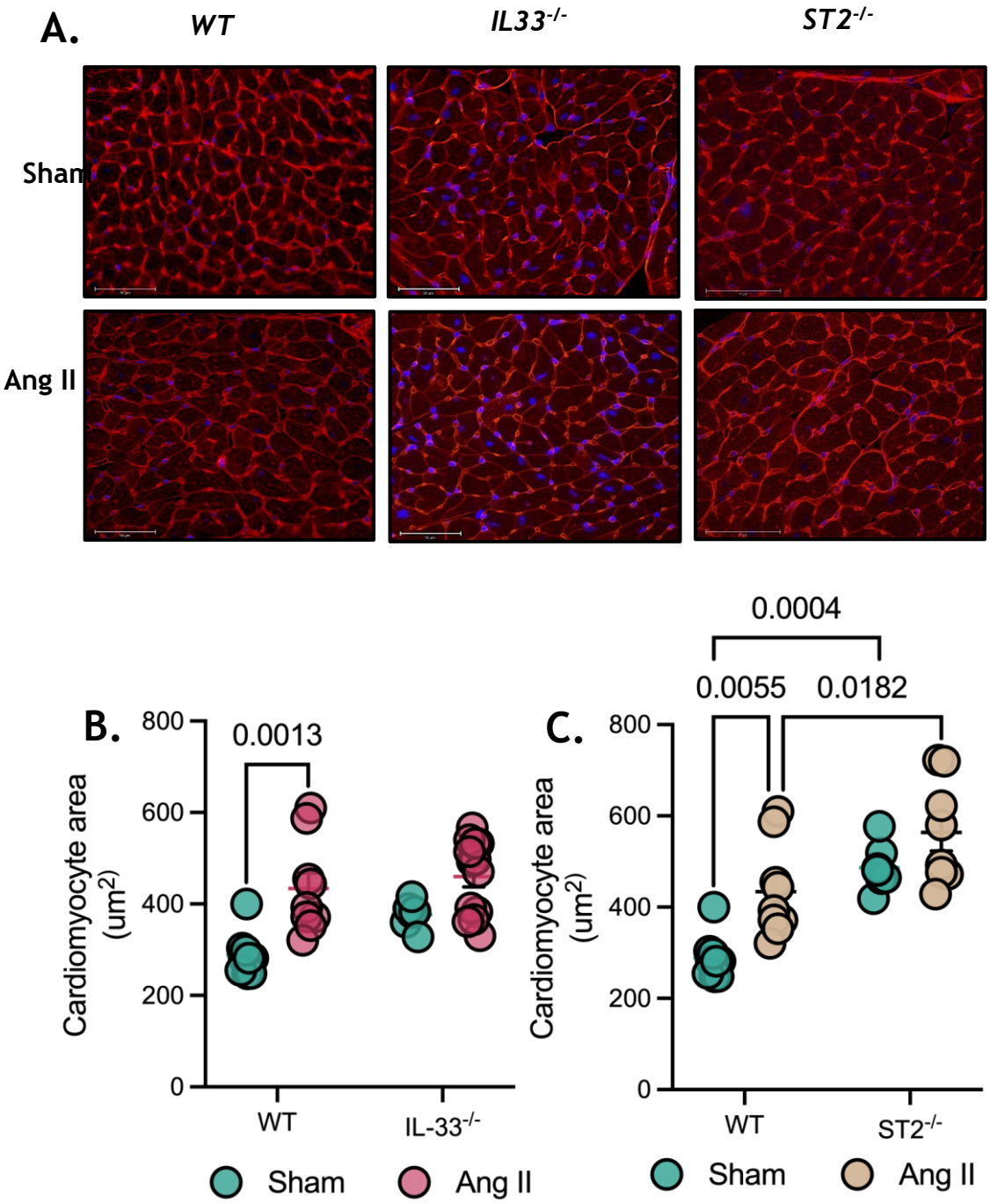


Figure 4.10: Role of IL-33 and ST2 on cardiomyocyte hypertrophy upon Angiotensin II-induced hypertension.

Cardiac hypertrophy in IL-33$^{-/-}$, ST2$^{-/-}$ and WT littermates upon sham or Ang II infusion visualised by WGA staining, overlayed with DAPI nuclei stain **(A)**. Scale bar: 50 μm. Quantification of cardiomyocyte area (μm^2) using ImageJ software for IL-33$^{-/-}$ **(B)** and ST2$^{-/-}$ **(C)** (n= 6-9/group). Data presented as mean ± SEM; 2-way ANOVA.

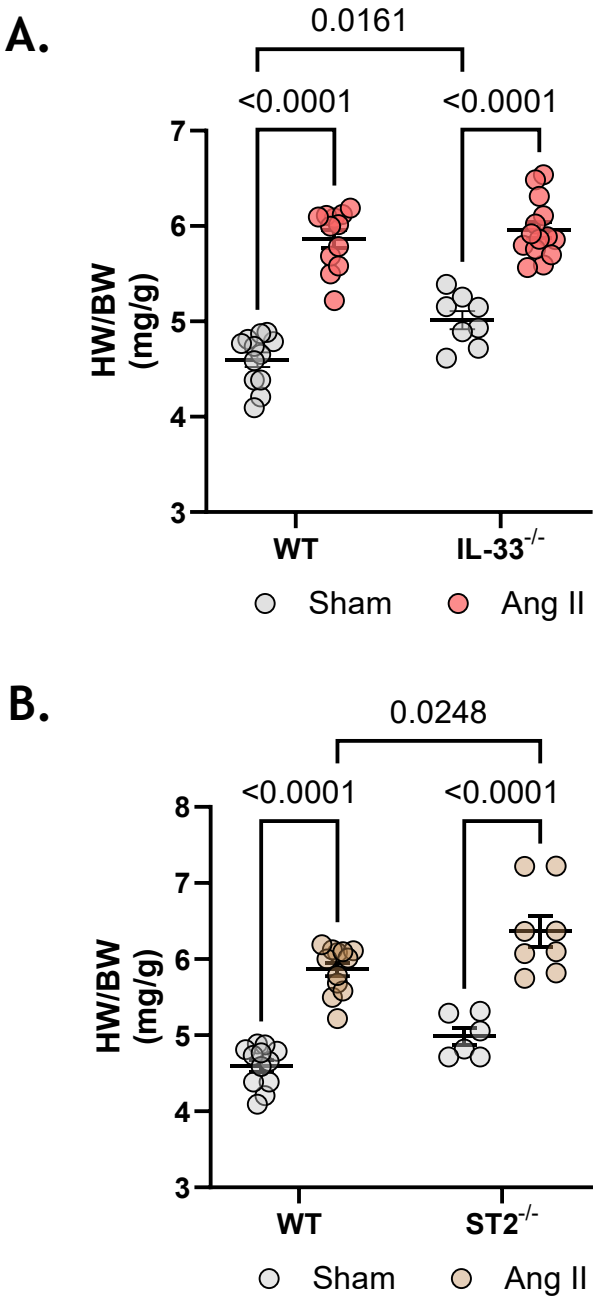


Figure 4.11: Heart/Body weight ratio measurements upon Angiotensin II-induced hypertension.

Gross measurement of heart weight normalised to body weight in IL-33$^{-/-}$, ST2$^{-/-}$ and WT littermates upon sham or Angiotensin II infusion (n=9/group). Data presented as mean ± SEM; 2-way ANOVA.

4.4 Discussion

4.4.1 Chapter Summary

In this chapter, I have demonstrated that both IL-33 and ST2 have a functional role in an inducible hypertension murine model. ST2 regulated vascular dysfunction independently of blood pressure in Ang II-induced hypertension. Interestingly, $ST2^{-/-}$ mice presented protection from hypertension-induced perivascular fibrosis. The changes in vascular function and oxidative stress by ST2 were not accompanied by alteration of perivascular inflammatory cells. Myocardial fibrosis upon Angiotensin II-dependent hypertension was augmented in $IL\text{-}33^{-/-}$ mice. ST2 influenced cardiomyocyte hypertrophy but not IL-33 following hypertension pathogenesis.

4.4.2 ST2 Regulates Vascular Fibrosis in Hypertension Independently of Blood Pressure.

The previous chapter highlighted that both IL-33 and its receptor, ST2, are upregulated in various cell subsets, especially in the mouse vasculature in hypertension. However, no other studies have examined the functional role of IL-33 and ST2 in hypertension induced by Angiotensin II. Therefore, I initially assessed the contribution of IL-33 and its receptor ST2 in hypertension through tail-cuff, and blood pressure measurements were evaluated. The experiment showed that both IL-33 and ST2 knock-out caused no alteration in blood pressure in response to Ang II.

Despite the lack of BP alteration upon Ang II induction in $IL\text{-}33^{-/-}$ and $ST2^{-/-}$ mice, I report that $ST2^{-/-}$ mice are protected from the development of periaortic collagen accumulation/fibrosis in Ang II-induced mice in comparison to sham knockout littermates. However, $IL\text{-}33^{-/-}$ mice presented no changes in vascular collagen accumulation or studied collagen markers in the mouse vasculature. Experimental studies have acknowledged that hypertension induces striking collagen deposition in the vasculature, particularly the aortic adventitia (Wu et al., 2016). The augmentation of the fibrotic process results in the loss of the “Windkessel function”

of the proximal aorta and augments systolic hypertension, resulting in target organ damage (Wu et al., 2016).

At the molecular and cellular levels, hypertension-associated vascular changes are characterised by reduced NO production and augmented oxidative stress through ROS generation, activation of transcription factors, stimulation of proinflammatory and profibrotic signalling pathways, reduced collagen turnover, calcification, increased smooth muscle cell proliferation and ECM remodelling. Circulating soluble ST2 levels are elevated in several cardiovascular and metabolic diseases. The 2023 study by Roy et al. demonstrated that circulating levels of sST2 in metabolic syndrome patients are associated with oxidative stress and inflammation burden and can underlie pathological remodelling and end-organ damage in patients (Roy et al., 2023). For future study directions, it would be critical to investigate the levels of circulating soluble ST2 upon angiotensin II-induced hypertension compared to healthy mice. Agreeing with previous literature, in this study, the inhibition of soluble ST2 through the global ST2 knockout may be producing protection from hypertension-induced perivascular fibrosis.

While this study is the first to link ST2 to vascular dysfunction and perivascular fibrosis, specifically in hypertension, earlier studies, as mentioned above, have described the functional role of IL-33 and ST2 in regulating various fibrosis progressions. The literature suggests a pivotal role of the IL-33/ST2 signalling pathway in its pathogenesis. Such include its role in pulmonary fibrosis (Lee et al., 2017a, Gao et al., 2015b), liver fibrosis (McHedlidze et al., 2013, Sun et al., 2014, Artru et al., 2020), renal fibrosis (Elsherbiny et al., 2020), and heart fibrosis. The pathway can promote inflammation and fibrosis in most fibrotic diseases but simultaneously reduce them in others (Gao et al., 2015a).

Furthermore, the role of ST2 has also been documented in liver fibrosis (Gao et al., 2015a). The study by McHedlidze et al. found that ST2-deficient mice did not increase collagen production when induced with carbon tetrachloride, an organic compound with pro-fibrotic effects (McHedlidze et al., 2013). Similar results are found in my study regarding vascular fibrosis and fibrotic markers involved in regulating collagen

deposition (*Col1a1, Col3a1,* and *Col5a1*) upon Ang II-induced hypertension in $ST2^{-/-}$ mice compared to WT animals. I, therefore, confirm the ability of ST2 to regulate vascular fibrosis in hypertensive animal models. However, understanding the signalling pathway inhibiting vascular collagen accumulation is key for therapeutic targeting.

The precise signalling involved in Ang II-induced vascular fibrosis is, to some extent, determined. However, experimental studies have shown upregulated activity of TGF-ß1 by Ang II (Weigert et al., 2002) TGF-ß is a fundamental cytokine/growth factor which accelerates ECM production in injured tissues as observed in hypertension (Saadat et al., 2020). Additionally, galectin-3 seems to be associated with Ang II-induced vascular fibrosis as cultured fibroblasts exposed to galectn-3 have reduced collagen production and deposition (Yu et al., 2013). Ang II-induced p38 MAPK is also associated with the advancement of fibrosis, observed in hypertension (Wu et al., 2015, Hsieh and Papaconstantinou, 2002). Investigating these pathways through various experimental methods such as western blotting, in future studies might give a better understanding of protection from vascular fibrosis by ST2 in hypertension.

Additionally, IL-33 is shown to stimulate Th2 cytokine production such as IL-4, IL-5 and IL-13 (Benveniste, 2014) which have been closely linked to fibrosis progression (Cayrol and Girard, 2018). Thus, investigating several profibrotic cytokine levels in WT, $IL\text{-}33^{-/-}$ and $ST2^{-/-}$ may also be beneficial in understanding the Th2 induced IL-33/ST2 signalling pathway.

4.4.3 ST2 Regulates Vascular Dysfunction in Hypertension Independently of Blood Pressure.

The endothelium plays a fundamental role in the cardiovascular system by regulating the vascular tone *via* nitric oxide production, prostaglandins, and further relaxing factors. Endothelial dysfunction is characterised by vasoconstriction, cell proliferation, and a shift toward a proinflammatory state(Gallo et al., 2021). Endothelial dysfunction occurs in several cardiovascular diseases, including

hypertension, contributing to inflammation of the vascular wall, of resistance arteries, increased smooth muscle cell proliferation, extracellular matrix deposition, and more (Gallo et al., 2021, Yu et al., 2008).

For the first time, this study has identified that ST2-deficient mice are protected from developing endothelial dysfunction, a hallmark of hypertension. Vascular endothelial function was studied by wire myography. As oxidative stress is known to mediate endothelial dysfunction in hypertension (Nosalski et al., 2020), I also investigated the production of vascular superoxide. To date, no experimental studies have demonstrated the role of either IL-33 or ST2 in the alteration of endothelial function in hypertension. The previous chapter and experimental studies have shown the expression of IL-33 in structural cells of blood vessels such as smooth muscle cells and fibroblasts as well as endothelial (Liu et al., 2018). Thus, IL-33 could have a role in regulating endothelial function.

However, in this study, no changes in vascular endothelial function or production of superoxide were observed in IL-33$^{-/-}$ mice infused with Ang II compared to WT Ang II mice. Conversely, ST2$^{-/-}$ mice were partially protected against Ang II-induced vascular dysfunction compared to the WT Ang II group. These changes in ST2$^{-/-}$ mice were also accompanied by reduced superoxide production. The study by Choi et al.(2009) demonstrated that IL-33 promotes endothelial NO production *via* ST2/TRAF6-mediated activation of phosphoinositide-3-kinase and endothelial NO synthase signalling pathway (Choi et al., 2009). Upon binding to the ST2 receptor, IL-33 promotes the activation of the pathway. However, another study exploring soluble ST2 as a new oxidative stress and inflammatory marker in metabolic syndrome highlighted that higher circulating sST2 levels were significantly associated with oxidative stress and inflammatory markers (Roy et al., 2023). In line with these observations and my studies results, it highlights the disease-specific context in which the IL-33/ST2 pathway can drive opposing responses, presumably due to the activation of different inflammatory cell types by IL-33/ST2 binding.

4.4.4 Regulation of Perivascular Inflammation in Hypertension by IL-33 and ST2

Increasing evidence indicates that hypertension and hypertension induced end-organ damage are not mediated by haemodynamic injury alone, but also through inflammation. Inflammation plays an imperative role in disease pathophysiology and contributes to hypertension deleterious outcomes (Wenzel et al., 2021). Immune cells shown to cause or participate in hypertension pathology are dendritic cells, $CD4^+$ and CD^+T cells, γδ T Cells, B cells and monocyte/macrophages (Wenzel et al., 2021). Study by Mikolajczyk at al. demonstrated the role of perivascular inflammation in promoting hypertension, especially through the elevation of total leukocytes and subsequently macrophages, dendritic cells and in particularly T cells (Mikolajczyk et al., 2016).

In this study, the changes in vascular function and oxidative stress exhibited by $ST2^{-/-}$ mice were not accompanied with reduced perivascular recruitment of total leukocytes and $CD3^+$ T cells in hypertensive animals compared to hypertensive WT mice. Ang II infusion did however significantly elevate the total number of macrophages, dendritic, and NK cells in WT animals compared to sham but not in IL-33 or ST2 deficient mice.

Ang II-induced hypertension stimulates the accumulation of T cells, macrophages, and DCs in the PVAT (Mikolajczyk et al., 2016). The explanation for the preferential effect of hypertension on inflammation in the PVAT is still unclear. It is possible that this is due to catecholamine stimulation in the PVAT. It has been demonstrated that catecholamines can activate resting T cells by stimulating the release of pro-inflammatory mediators (Flierl et al., 2008). Wirsen et al. showed that adipose tissue is highly innervated with sympathetic nerve terminals and proposed that norepinephrine released from periadventitial nerves in vessels could influence adjacent fat (Wirsen, 1964).

This perivascular inflammatory response is accompanied by increased expression of inflammatory cytokines such as IFN-γ or IL-17, which have been implicated in the genesis of hypertension. Studies have demonstrated that WT mice treated with IL-33 have reduced levels of IL-17 and IFN-γ (Jiang et al., 2012), thus implicating a protective role through the over-expression of IL-33. In the $ST2^{-/-}$ mice, there is a possibility of reduced recruitment of IFN-γ-producing cells as a mechanism for the protection from endothelial dysfunction and vascular oxidative stress observed in $ST2^{-/-}$ mice. However, future studies need to address this hypothesis to understand the exact pathway involved in regulating vascular function.

ST2 signals predominantly on immune cells found within tissues, including Treg cells, $CD8^{+}$ T cells, and NK cells (Lei et al., 2022). Thus, the IL-33/ST2 signalling pathway is essential in the immune system. However, my study did not show significant role of IL-33 and ST2 in T lymphocyte migration in response to Ang II infusion in both knockouts. These data need to be interpreted with caution due to the high variability seen among the different replicates, therefore repetition and increased n numbers for these experiments are vital for accurate understanding on the potential of perivascular inflammation regulation by IL-33 and ST2 in Ang II-induced inflammation. Additionally analysing the infiltration of several inflammatory cells such as the γδ T Cells in other tissues including spleen and aorta may propose a pathway connecting the IL-33/ST2 pathway regulating inflammation in hypertension.

4.4.5 IL-33/ST2 Regulates Cardiac Dysfunction in Ang II-Dependent Hypertension

Cardiomyocyte hypertrophy and cardiac fibrosis are common pathophysiological processes in many heart diseases, including myocardial infarction, hypertension, and valvular diseases (Sanada et al., 2007a, Sadoshima and Izumo, 1997). It has been known that ST2 is basally expressed by cardiomyocytes (Sanada et al., 2007a). Data produced by Sandra et al., 2007 showed that IL-33/ST2 signalling was biomechanically activated and influenced cardiomyocyte hypertrophy and cardiac fibrosis. In this study, IL-33 exhibited a cardioprotective role upon Ang II infusion compared to the

WT Ang II group. IL-33$^{-/-}$ mice presented elevated interstitial cardiac fibrosis. Cardiac fibrosis induces pathological processes, which lead to chamber dilatation, muscular hypertrophy, and apoptosis, eventually developing into congestive heart failure (Travers et al., 2016). Cardiac fibrosis pathogenesis is complex and involves several pathways. TGF-β1 is the principle growth factor of TGF-β in the cardiac tissue, which cause Smad2/Smad3 phosphorylation, in turn stimulating cardiac fibrosis (Saadat et al., 2020). The Smad2/Smad3 pathway has been closely associated with IL-33 signalling in other inflammatory diseases such as cancer inducing cell proliferation (Park et al., 2021). Although this study has demonstrated that IL-33 can alter cardiac fibrosis upon Ang II-induced hypertension specifically, future investigations are necessary in understanding the pathways such as the Smad2/3-IL-33 pathway which may be involved in regulating the present findings.

Additionally, this study has shown that basally in the heart and Ang II-induced hypertension, ST2 is crucial in promoting cardioprotective mechanisms. Abolishing ST2 signalling in mice leads to marked hypertrophy following pressure overload by traverse aortic constriction (TAC) (Sanada et al., 2007a). My study illustrates similar results; in the progression of hypertension, ST2$^{-/-}$ mice had elevated cardiomyocyte hypertrophy.

4.4.6 Differences Between IL-33$^{-/-}$ and ST2$^{-/-}$ Hypertension Mouse Model Phenotype

	IL-33 Deficient Mice	ST2 Deficient Mice
Blood Pressure (Tail-cuff)	No Blood pressure regulation	No Blood pressure regulation
Perivascular Fibrosis	No effect on vascular fibrosis and collagen markers	**Protection from hypertension induced development of perivascular fibrosis**
Vascular Dysfunction	No effect on endothelial dysfunction	**Mice protected from development of endothelial dysfunction.**
Vascular Oxidative Stress	No change in superoxide production	**Mice presented reduced vascular superoxide production**
Perivascular Inflammation	No significant regulation of perivascular inflammation in HTN	No significant regulation of perivascular inflammation in HTN
Cardiac Fibrosis	**Augmented myocardial collagen accumulation in IL-33 deficient Ang II mice**	No effect on cardiac fibrosis and fibrotic markers
Cardiac Hypertrophy	No significant cardiac hypertrophy change exhibited in mice upon Ang II-induction	**Promotes cardiomyocyte hypertrophy and elevates heart to body ratio following Ang II hypertension**

Table 4.1: Differences between IL-33 deficient and ST2 deficient hypertension mouse model phenotype from the study.

The IL-33/ST2 axis has apparent pleiotropic functions in many disease models through its critical role in both type 2 and type 1 immunity as well as its action through the ST2L (transmembrane) and sST2 (soluble) receptor. Studies have presented that IL-33 may promote liver fibrosis through generation of profibrotic cytokines from ILC2s and Th2 lymphocytes in chronic inflammation. On the other hand, studies have illustrated the attenuation of cardiac fibrosis induced by IL-33 in cardiovascular pressure overload mice while having no effect on the expression of genes encoding extracellular matrix components (Zhu and Carver, 2012).

Sandra et al. demonstrated the cardioprotective actions of IL-33, preventing the global myocardial fibrosis in pressure overload mouse. Pressure overload has been shown to promote the secretion of IL-33 from endothelial cells, thus suppression of this signalling could promote cardiac hypertrophy and increased global myocardial fibrosis (Sanada et al., 2007d, Garbern et al., 2019). My study in agreement with several others, showing an anti-fibrotic role of IL-33 in the heart of Ang II-induced hypertensive mice model however, with no effect in cardiomyocyte hypertrophy. Mice deficient in ST2 but not IL-33 has been described in the literature by Garbern et al. (2019) as having a significant increase in periarteriolar fibrosis following TAC surgery model compared to WT (Garbern et al., 2019), however my results from this study show the opposing results.

Table 4.1 presents this study's key findings of IL-33 and its receptor ST2 in Ang II-induced hypertension in mouse models. The discrepancies in the results compared to previous literature discussed above provides evidence that the IL-33/ST2 pathway is complex and varies depending on different disease models studied, also the animal models used. In this study I have only studied one model of hypertension, it should be considered that the results obtained may not be the same across other models.

4.5 Chapter Conclusion

In conclusion, this chapter has identified that IL-33 and ST2 are essential regulators of vascular and cardiac fibrosis and dysfunction in hypertension. My results indicate a dual role of IL-33/ST2 in hypertension by demonstrating that pathology is exacerbated or reduced in IL-33$^{-/-}$ and ST2$^{-/-}$ mice.

Chapter 5 Exploring the Expression of IL-33 and ST2 in the Human Vasculature

5.1 Introduction

Blood vessels are composed of (i) an intimal layer of endothelial cells covering the basement membrane, (ii) a media layer composed of circumferentially oriented vascular smooth muscle cells with intervened extracellular matrix, and (iii) an adventitia that consists of extracellular matrix, fat cells, nerve endings, and small arterioles. All three layers are implicated in acute and chronic inflammatory triggers in various cardiovascular diseases (Lamb et al., 2020). Several cytokines, chemokines and inflammatory molecules are expressed in the human vasculature at basal and in disease states (Tedgui and Mallat, 2001).

The localisation of endothelial cells makes them one of the first targets of cytokines circulating in the bloodstream. Studies have over the years confirmed that ECs are not merely targets of cytokines; they also have the capacity to express, generate and secrete cytokines under certain circumstances, such as tissue injury or apoptosis (Mai et al., 2013). In cultured EC, at basal state, pro and anti-inflammatory cytokines, including IL-3, IL-6, IL-7, IL-8, IL-11, IL-15, TNF-α, TGF-β (Davis et al., 2012) and IL-33 (Moussion et al., 2008b), have been detected at the mRNA level. IL-6, an early central regulator of inflammation, is shown to contribute to increased blood pressure, inflammatory cell recruitment and endothelial dysfunction (Brands et al., 2010). Additionally, increased expression of IL-6 has been implicated as a contributing factor for vascular fibrosis (O'Reilly et al., 2014). A study in 1996 by Rus et al. highlighted that IL-6 is present in the atherosclerotic human arterial wall as cellular and extracellular deposits in the connective tissue matrix (Rus et al., 1996).

The study of different isolated human vasculature, such as the human coronary artery (Gibson et al., 1993), internal mammary artery (Sims, 1983) and carotid arteries (Sims, 1983) has revealed important insights into the structural, functional and biochemical features of the normal and diseased cardiovascular systems while also providing molecular understandings of the cells that constitute the arteries and veins

and their interactions. The internal mammary artery has been used to demonstrate restoration of impaired vasodilation in hypertension (Zaabalawi et al., 2022) and other vascular diseases. Furthermore, carotid artery intima-media thickness (IMT) is an early biomarker of atherosclerosis as well as having a close correlation with the early incidence of cardiovascular disease and ischaemic stroke in a wide age range (Polak et al., 2010). Immunohistochemical staining and analysis of atherosclerotic human carotid arteries identified the presence of platelet factor 4 (PF4) in the endothelium, and the levels showed a positive correlation with disease severity (Ma et al., 2022).

Studies in human and animal models suggest a critical role of IL-33 in many key cardiovascular diseases, including atherosclerosis (Miller, 2011a). IL-33 and its receptor ST2 are expressed in human vein endothelial cells and coronary artery endothelium (Demyanets et al., 2011c) have also confirmed strong expression of endogenous nuclear IL-33 in monolayers of primary human endothelial cells (Moussion et al., 2008a). Several studies have demonstrated that IL-33 correlates with ST2 expression in human atherosclerotic tissue at both protein and mRNA levels, proposing that both proteins are highly coregulated in this tissue (Demyanets et al., 2011c). Additionally, ST2L and soluble ST2 are reported to be expressed in human coronary plaques (Demyanets et al., 2011b, Demyanets et al., 2013b). Proteomic analysis indicates that extracellular IL-33 induces the expression of many proteins associated with inflammatory responses and promotes inflammatory activation (Gautier et al., 2016b), (Demyanets et al., 2011c). These include cell adhesion receptors involved in leukocyte/endothelium interactions during inflammation, chemokines and cytokines (such as CXCL6, CCL20, CXCL8/IL-8, EBI3/IL-27beta, Galectin-9) and proteins involved in antigen processing and presentation (Gautier et al., 2016a). As a result, IL-33 may elevate vascular permeability, induce significant production of several inflammatory cytokines, and stimulate angiogenesis (Gautier et al., 2016b), (Choi et al., 2009). Additionally, the NFkB pathway, which plays an important role in the pathogenesis of cardiovascular diseases, including hypertension, is identified as the major cellular pathway activated by extracellular IL-33 cytokine in endothelial cells (Gautier et al., 2016a).

The physical stress of hypertension on the arterial wall results in the aggravation and acceleration of atherosclerosis (Poznyak et al., 2022). Hypertension increases the susceptibility of the small and large arteries to atherosclerosis (Rizzoni et al., 2023). Therefore, hypertensive patients are candidates for both atherosclerosis and hypertensive vascular disease of the coronary and cerebral arteries, resulting in myocardial infarction and stroke (Hollander, 1976). Activated macrophages and T lymphocytes are present during the development of the atherosclerotic lesion and contribute to the proinflammatory milieu that modulates the inflammatory response within the plaque (Rafieian-Kopaei et al., 2014). Chronic inflammation is an independent risk factor for the development of hypertension (Patrick et al., 2021). TNF-α and IL-6 serum concentrations are associated with hypertension in otherwise healthy individuals (Mirhafez et al., 2014). The study by Mirhafez et al. (2014) demonstrated augmented serum concentrations of IL-1α, -2, -8, vascular endothelial growth factor, IFN-γ, TNF-α, and MCP-1; and lower concentrations of the anti-inflammatory cytokine, IL-10 in hypertensive patients compared with healthy groups.

Literature has presented a significant association between the serum concentrations of several cytokines and hypertension (Gordon et al., 2021). The levels of serum ST2 are significantly correlated with the development of several cardiovascular diseases, such as heart failure, coronary atherosclerotic heart disease (CAHD), and type 2 diabetes (Kim et al., 2019b). In chronic and acute decompensated heart failure patients, serum sST2 levels are significantly upregulated and strongly associated with disease severity and mortality (Ky et al., 2011).

5.2 Chapter Aims

IL-33 and ST2 expression has been studied in human atherosclerotic plaques (Demyanets et al., 2011c). However, the localisation and association of IL-33 and ST2 pathways in hypertensive patients have not been investigated.

Therefore, the main aim of this chapter is to study the role of IL-33 and ST2 in hypertension patients. Specific aims include:

1. Examine IL-33 and ST2 expression profiles in human coronary arteries and human internal mammary arteries.

2. Investigate serum IL-33 and sST2 levels in hypertensive patients compared to the control normotensive subjects.

3. Study association between ST2 and IL-33 plasma levels and blood pressure parameters in a large UK Biobank cohort.

5.3 Results

5.3.1 Investigation of IL-33 and ST2 Expression in the Human Coronary Arteries from Publicly Available Sc-RNASeq Datasets

To understand IL-33 and ST2 expression in the human vasculature in disease, I analysed publicly available scRNA-Seq data from diseased human coronary arteries (Wirka et al., 2019) (GEO accession: GSE131778). The human coronary arteries used in the study by Wirka et al. were dissected from heart transplant patients (Wirka et al., 2019).

A total of approximately 120-240 mg atherosclerotic sections were used per patient. Before analysis, data was subject to quality control, integration, PCA-reduction, and clustering analysis. Afterwards, each cluster was labelled according to the markers used by the study's authors (Figure 5.1A).

Due to previously obtained scRNA-Seq expression data from whole mouse aorta and my experimental studies through immunohistochemistry of Sham and Ang II-induced mouse model, I was interested in exploring if IL-33 and ST2 expression patterns translated to the human data. The expression signature of IL-33 was found to closely mimic that of the mouse, with positive signal found throughout the endothelial cells, fibromyocyte (referred to as modulated VSMCs in the mouse dataset), and fibroblast clusters (Figure 5.1A, B, C). Alternatively, ST2 was utterly absent in all the samples analysed and not included in the figures.

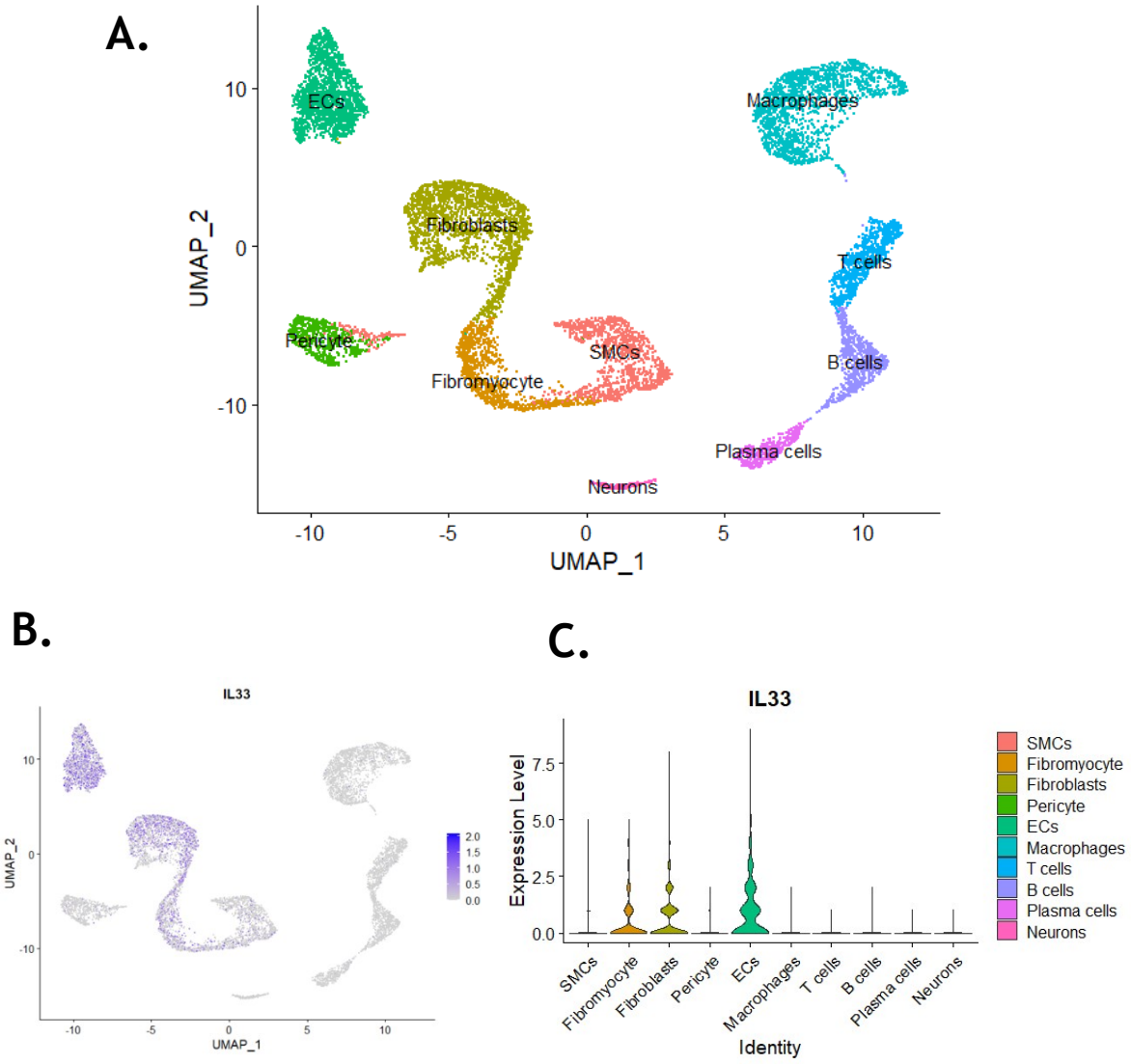


Figure 5.1:IL-33 expression in the human coronary artery.
Analysis of Gene expression data in single-cells extracted from coronary artery samples of explanted hearts (n=4). The proximal mid artery was cleared of periarterial fat, and atherosclerotic lesions were used. A total of approximately 120-240 mg (~3-6 atherosclerotic sections) per patient was used for the dataset. Data acquired from published single-cell RNA-sequencing database GEO accession: GSE131778 (Wirka et al., 2019). **(A)** Uniform Manifold Approximation and Projection (UMAP) representation of aligned gene expression data showing partition into distinct clusters representing different cell populations in the dataset indicated by colour. **(B)** Gene expression of IL-33 was determined using Adaptively-threshold Low-Rank Approximation (ALRA; Linderman et al., 2018) and projected onto UMAP representation of distinct clusters for each time-point (scale: log-transformed gene expression). (**C)** Violin plot of IL-33 expression across each distinct cell population cluster.

5.3.2 Expression of IL-33 in Human Hypertensive Mammary Arteries.

To understand the localisation of IL-33 within the human hypertensive mammary arteries, I performed immunohistochemistry (DAB staining). The DAB staining works by hydrogen peroxidase oxidizing the DAB in a reaction catalysed by HRP—the oxidized DAB generates a dark brown deposit. IL-33 expression was localized within the lumen (consisting of endothelial cells) and the vessel wall's intima-media (consisting of SMCs).

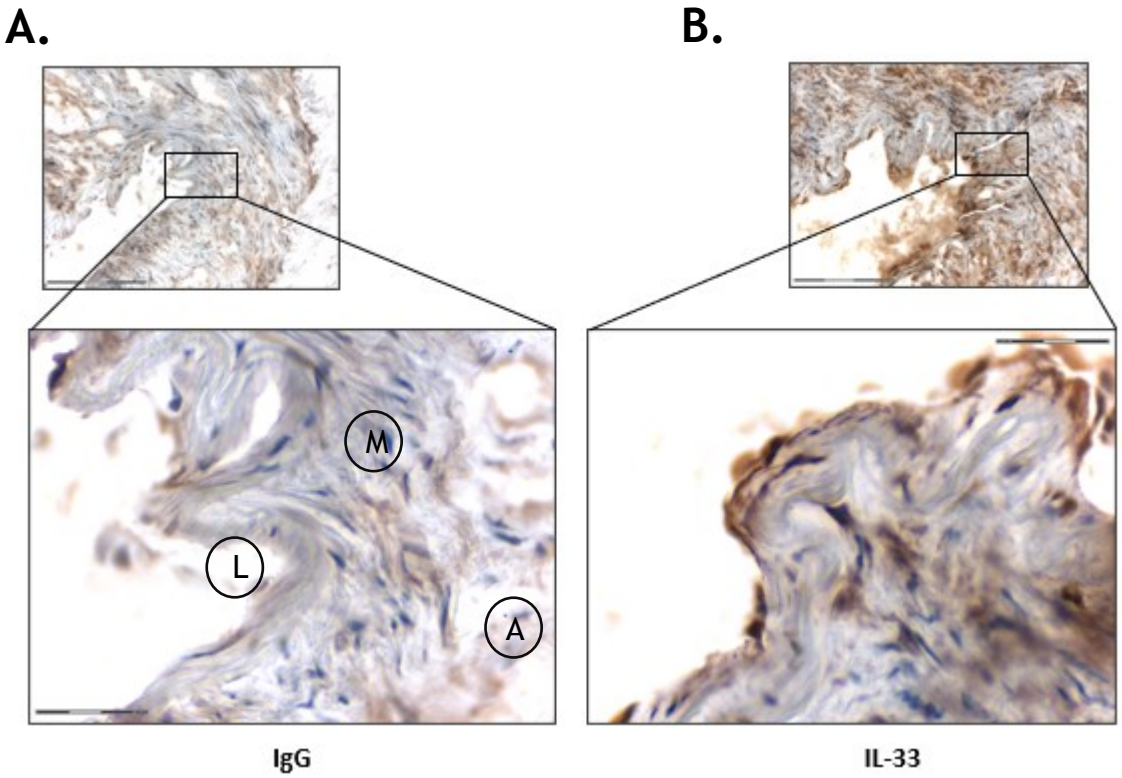


Figure 5.2: Localization of IL-33 in human hypertensive mammary arteries. IL-33 **(B)** localisation was evaluated from OCT-embedded human hypertensive mammary arteries using HRP/DAB staining; each isotype controls (Rabbit IgG) displayed **(A)** n=4**.** Positive signals are stained brown in the vasculature. 20x magnification and 62x magnification. Scale bar: 150μm and 30μm representatively. **A**, adventitia; **M**, media; **L**, lumen.

5.3.3 Serum Levels of IL-33 and ST2 in Hypertensive Patients and Control Groups

I next examined the levels of IL-33 and sST2 in human peripheral blood serum of hypertensive patients and control groups. Multiplex immune assay showed no significant difference in serum IL-33 or sST2 levels between the two groups (IL-33 level, hypertension: median 1.82 pg/ml, Control: median 1.82 pg/ml; Figure 5.3 A), (ST2 level, hypertension: median 2.37 pg/ml, Control: median 1.89 pg/ml; Figure 5.3 A).

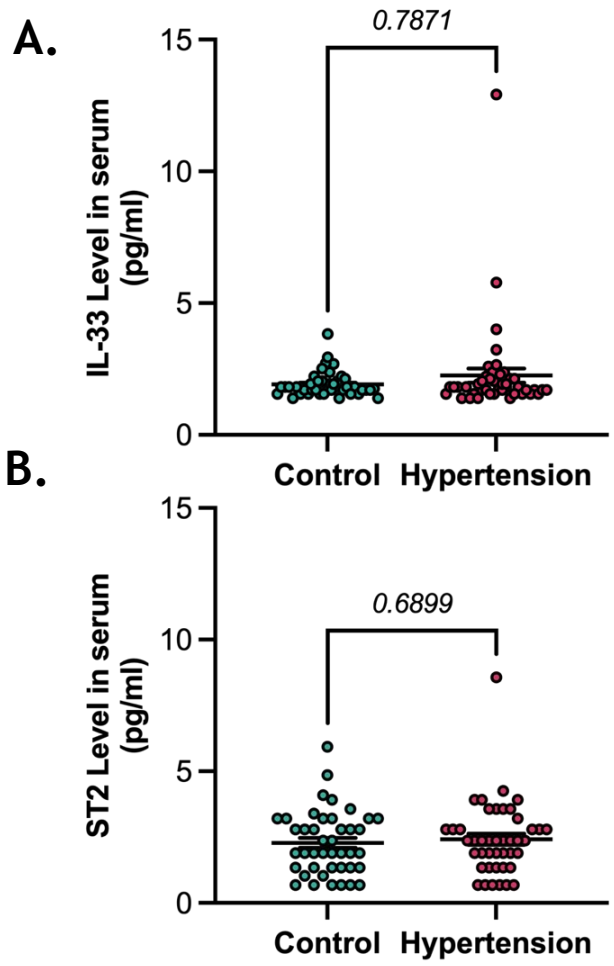


Figure 5.3: Serum levels of IL-33 and ST2 in newly diagnosed uncomplicated, hypertensive patients (n=45) and normotensive (n=43) controls.
Multiplex immune assay was used to quantify IL-33 **(A)** and sST2 **(B)** levels in human serum samples (pg/ml) of hypertensive patients and the control group. Serum samples were collected from 88 patients enrolled in the InflammaTENSION study. Differences between the mean cytokine levels in different subsets of patients were determined using a student two-tailed unpaired *t-test*. Data presented as ± SEM.

5.3.4 ST2 Association Between OLINK Plasma Level and Blood Pressure Parameters

IL-33 OLINK plasma levels are associated negatively with set BP parameters, while ST2 (IL1RL1) levels are associated positively with BP parameters. Furthermore, the results highlight a significant correlation between the two plasma proteins (Pearson r=0.026, p<0.001) acquired from the online OLINK UK Biobank dataset.

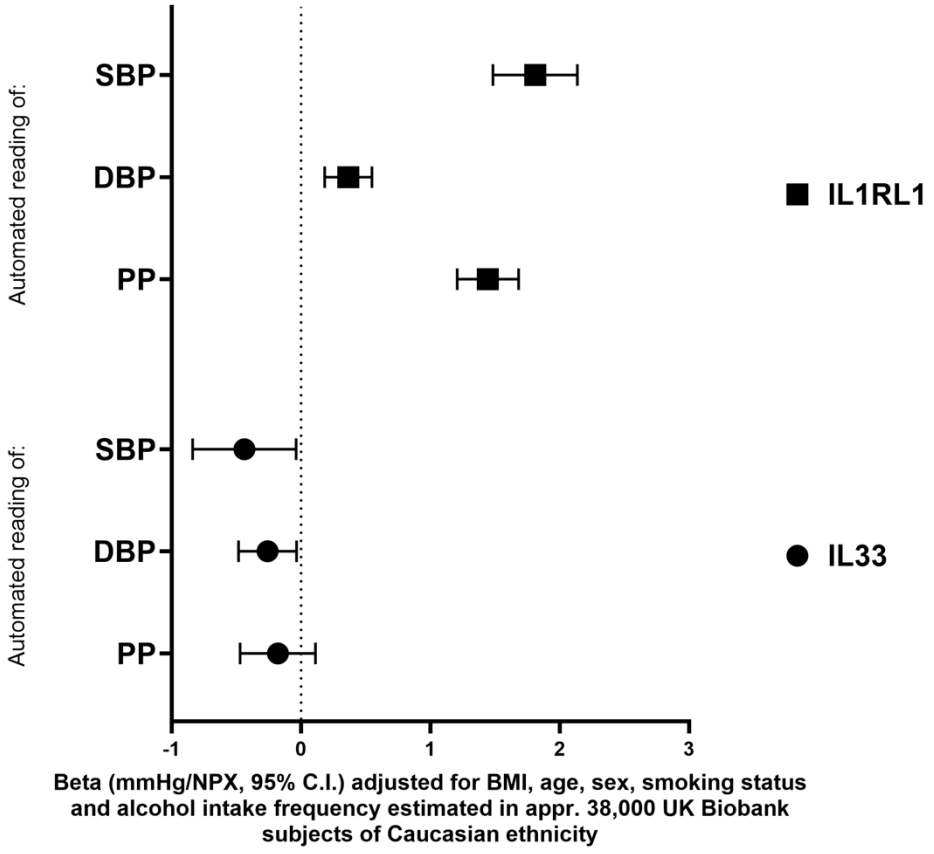


Figure 5.4: Association between plasma OLINK IL-33 and IL1RL1 (ST2) level and BP parameters in the UK Biobank.
A forest plot of multivariate regression analysis of the association between plasma level concentrations and BP parameters was explored from 38,000 UK Biobank participants. Beta (mmHg/NPX, 95% Cl) adjusted for BMI, age, sex, smoking status, and alcohol intake frequency estimated in Caucasian ethnicity. IL-33 and ST2 association: Pearson r=0.026, p<0.001.

5.4 Discussion

5.4.1 Chapter Summary

In this chapter, I have characterised the expression of IL-33 and its receptor ST2 in hypertensive and heart transplant patient tissue; using published scRNA-Seq, I have found that IL-33 appears to be primarily expressed by the vascular endothelial cells, fibromyocytes and fibroblasts in normal and diseased vessels. I also found that IL-33 is expressed in endothelial cells in the human mammary arteries of hypertensive patients. OLINK UK Biobank dataset presented a positive correlation between ST2 and BP parameters. These data support my hypothesis that the IL-33-ST2 axis expression in the vasculature may be necessary in several cardiovascular diseases, especially in hypertension pathology.

5.4.2 IL-33 Expression is Localised in Endothelial Cells of Internal Mammary Arteries of Hypertensive Patients

It is well known that upon inflammation pro-inflammatory cytokines and immune cells can accumulate at the vasculature inducing cell proliferation, differentiation of smooth muscle actin positive cells and thus resulting in severely remodelled arterial wall. Importantly, studies have also observed the accumulation and infiltration of such cells in the adventitial layer (Bennaceur et al., 2006). Perivascular inflammation, a prominent pathogenic feature of hypertension, may be perpetuated by activated adventitial fibroblasts, which through sustained production of pro-inflammatory cytokines and chemokines and adhesion molecules, induce accumulation, retention and further activation of inflammatory and immune cells (Bennaceur et al., 2006).

IL-33 and ST2 are shown to be highly expressed in human vein endothelial cells and coronary artery endothelium (Demyanets et al., 2011c). This study confirmed for the first time by immunohistochemistry the localization of IL-33 in human mammary arteries of patients with high BP.-Stained sections highlighted IL-33 expression in the

human artery's endothelial and smooth muscle cells. Literature has discussed the localisation of IL-33 in the nucleus of cardiac myocytes, cardiac fibroblasts, and smooth muscle cells (Demyanets et al., 2013b). Moreover, in agreement with our observations, Demyanets et al. (2011) demonstrated the detection of IL-33 protein expressed by endothelial cells in human atherosclerotic carotid tissue. Furthermore, in human atherosclerotic plaques, Miller. (2011) highlighted that nuclear IL-33 and membrane-bound ST2 protein were expressed by the same cells, namely endothelial cells, and IL-33 mRNA was significantly correlated with ST2 mRNA expression in carotid atherosclerotic tissue. These results suggest that IL-33 and ST2 proteins may be highly coregulated in the atherosclerotic carotid tissue and hypertensive mammary arteries.

IL-33 and ST2 have also been detected in endothelial cells in human myocardial tissues from patients undergoing heart transplants. Additionally, Demyanets et al. (2013) have observed endothelial cells as the source of sST2 and the target for IL-33 in the cardiovascular system. Abundant nuclear expression of IL-33 has been identified in both large and small blood vessels in most normal human tissues (Moussion et al., 2008b). Moreover, inflammatory activation induces nuclear expression of IL-33 in fibroblasts (Gatti et al., 2021). These findings are consistent with the human coronary arteries of patients undergoing heart transplants, eliciting expression of IL-33 in endothelial cells, fibromyocyte, and fibroblast clusters. My data thus highlights the pivotal role of IL-33 in endothelial cells in cardiovascular disease.

Although IMA has been used to study impaired vasodilation in hypertension (Zaabalawi et al., 2022) and other vascular diseases in particular coronary artery disease (Rustenbach et al., 2022) and atherosclerosis (Bahar and Tuysuz, 2020, Sisto and Isola, 1989), irrespective of their systemic nature, the IMA rarely develops atherosclerotic plaques with prevalence rates of histologically-proven lesions ranging from approximately 3.1–4.2% in unselected individuals (Sisto and Isola, 1989, Kay et al., 1976). Vascular intimal thickening – a preclinical process closely associated with vascular disease including atherosclerosis and hypertension is observed mainly in the left anterior descending coronary artery (Kraler et al., 2021). Moreover, histological

mapping of intimal thickening and advanced atherosclerotic lesions in humans has revealed that the topographical distribution of these two processes is very similar in the coronary arteries (Stary, 1989), the aorta (Movat et al., 1958) and the internal carotid artery (Zarins et al., 1983) . This therefore highlights the need to investigate the functional role and the expression of IL-33 and ST2 in several resistance arteries which are linked to structural changes upon cardiovascular diseases.

5.4.3 Levels of IL-33 and ST2 in Relation to Hypertension

In the human study, the levels of ST2 and IL-33 in serum were not significantly elevated in hypertensive patients compared with the control group. However, the study by Yin et al. in 2019 demonstrated that levels of sST2 in serum and PBMCs were substantially elevated in the hypertensive patient cohort. Further, serum sST2 was positively correlated with systolic and diastolic blood pressure (Yin et al., 2019a). In agreement with this, data extracted from plasma ONLINK UK Biobank presented a positive association between ST2 and BP parameters, including systolic and diastolic blood pressure. Moreover, for the first time, a significant correlation has been identified between the two plasma proteins acquired from the online dataset.

Previous clinical studies have demonstrated the prognostic role of sST2 in many cardiovascular diseases. Levels of sST2 increase immediately after infarction and are independently associated with death (Shimpo et al., 2004, Weinberg et al., 2002b). Soluble ST2 is proposed as a marker for biomechanical strain and, thus, a possible predictor of mortality in patients with chronic heart failure and other cardiovascular diseases such as hypertension. Stress caused by high blood pressure stimulates soluble ST2 secretion from cardiac, aortic, and coronary endothelial cells (Demyanets et al., 2013b). Additionally, clinical studies have demonstrated a correlation between sST2 level and disease severity as a significant predictor of the clinical worsening of pulmonary arterial hypertension in patients and cardiac fibrosis and remodelling (Zheng et al., 2014, Villacorta and Maisel, 2016). Increased sST2 expression can enhance the decoy receptor binding to circulating IL-33 in serum and decrease the amount of IL-33, which binds with the transmembrane ST2 (ST2L) receptor (Thomassen et al., 1999, Yin et al., 2019b). Thus, increased sST2 can deteriorate

the role of the IL-33-ST2L signalling axis and diminish the vascular and cardiac protective effect of IL-33, which may lead to high blood pressure.

5.5 Chapter Conclusion

Given the expression of IL-33 and ST2 and alteration of the IL-33-sST2 axis in hypertension, this chapter and available literature conclude that sST2 could act as a risk factor for hypertension and may represent a promising novel marker for hypertension prediction and potentially relate to target organ damage in hypertension.

Chapter 6 General Discussion

Hypertension is consistently the principal modifiable risk factor for the development of cardiovascular disease. Advancement is persistently made to understand the mechanisms of this disease and consequently improve therapy. Understanding the immune and inflammatory underpinnings of hypertension is crucial because inflammation is increasingly recognised as a pivotal factor in the pathogenesis of hypertension and its complications. Chronic inflammation has been implicated in the dysfunction of vascular endothelium, arterial stiffness, and the subsequent development of high blood pressure. By elucidating the roles of specific inflammatory mediators, such as IL-33 and its receptor ST2, within the cardiovascular system, we can uncover novel pathways that contribute to hypertensive pathology. This knowledge not only deepens the comprehension of hypertension's complex nature but also paves the way for innovative therapeutic strategies targeting inflammation, potentially offering more precise and effective treatments for patients suffering from hypertension. Hence, investigating the immune-inflammatory axis of hypertension is essential for advancing the overall understanding of cardiovascular disease and improving clinical outcomes.

While inflammation plays a pivotal role in the development and maintenance of hypertension, numerous novel discoveries have been made within the past decade, expanding the field and providing new mechanistic insights. Cytokine amplification by the inflammasome causing end-organ dysfunction and immune activation plays a key role in the genesis of hypertension (Patrick et al., 2021).

The IL-33/ST2 signalling axis has been shown to impact the physiology of various diseases, including allergic diseases, cancer and cardiovascular diseases (Cayrol and Girard, 2014, Molofsky et al., 2015b, Byers et al., 2013b, Gautier et al., 2016a). This is a consequence of the ability of IL-33 to bind to its receptor expressed on cardiomyocytes (Weinberg et al., 2002b) and a large variety of immune cells, including T cells, particularly type 2 T cells (Löhning et al., 1998), Tregs (Schiering et al., 2014), ILC2s (Neill et al., 2010b), M2 macrophages (Kurowska-Stolarska et al., 2009) and several others (Griesenauer and Paczesny, 2017). The binding of IL-33 to

the ST2L receptor promotes the activation of a wide range of intracellular signalling networks.

Stimulation of the IL-33/ST2 axis has been reported to promote the cardioprotective arm in the context of ventricular biomechanical stress (Sanada et al., 2007a, Weinberg et al., 2002b), prevent myocardial apoptosis, and alleviate myocardial fibrosis and myocardial hypertrophy (Seki et al., 2009a). It, therefore, inhibits the progression of atherosclerosis and potentially could be beneficial in suppressing hypertension pathology. The transmembrane form of ST2 enables IL-33's signalling activity, whilst sST2 acts as a decoy receptor, binding IL-33 to dampen its effects (Sun et al., 2021). Clinical studies have speculated the elevation of serum soluble ST2 levels in heart failure and myocardial infarction patients with stent restenosis (Demyanets et al., 2014). Accumulating studies have implied that sST2 attenuates the beneficial actions of downstream IL-33/ST2L signalling pathway and functions as a decoy receptor. The elevation of circulating sST2 has shown to lead to disease severity. Thus the soluble ST2 has been identified as a potential biomarker in several cardiovascular disease severity and progression (van den Berg et al., 2022). The inhibition of the IL-33 and ST2L pathway may modulate inflammatory conditions and subsequently cardiovascular injury.

Therefore, the role of IL-33 in cardiovascular disease is controversial in the literature due to its double edge sword type action related to potential binding to either ST2L or sST2 receptor. Specific studies have suggested that its pathological role in other vascular diseases, such as atherosclerosis, would likely alleviate hypertension disease pathology (Choi et al., 2009).

Unfortunately, very few clinical and experimental studies have attempted to determine the role of IL-33 cardiovascular disease, and until now, only one has specifically reviewed hypertension (Yin et al., 2019a). However, the studies are not detailed enough on the hypertension pathology or do not fully account for its ability to affect numerous cell types in the vasculature and other vital organs involved in hypertension. Distinct subpopulations, including SMCs, fibroblasts, endothelial cells, B cells and T cells, are highly regulated during the progression of hypertension (Zhang

et al., 2021) in the arterial blood vessels, heart, kidney and brain (Mensah et al., 2002). It is, therefore, vital to understand the involvement and regulation of IL-33 and ST2 in various organs and identify the expression of the cytokine in different subpopulations, chiefly in the vasculature.

Through this study, I have demonstrated novel findings linking the role of IL-33, ST2 and hypertension. Through the hypertension-induced animal model experiments, we observed augmentation of IL-33 and ST2 expression in critical organs upon hypertension in wild-type mice. The induction observed in the vasculature is dependent on blood pressure increases rather than by the direct actions of Ang II. Localisation of IL-33 and ST2 was identified in ECs and SMCs of Ang II-induced mouse aorta. Using publicly available datasets, single-cell Seq analysis showed a valuable link between the expression of IL-33 in stromal and ST2 expression primarily in immune cells of the mouse aorta, proving the importance of the non-immune to immune cell IL-33/ST2 pathway in cardiovascular diseases, in particular hypertension. Additionally, this study has been the first to demonstrate the regulation of vascular fibrosis and dysfunction by ST2 in hypertension independently of blood pressure. We further report the involvement of the IL-33/ST2 axis in modulating cardiac dysfunction in Ang II-dependent hypertension. It is important to note that, in hypertension patients, IL-33 expression is localised in vascular endothelial cells, like in the animal experimental models. Finally, the levels of sST2 from the OLINK UK Biobank dataset presented a positive correlation to BP parameters. These data reinforce my hypothesis that the IL-33-ST2 axis expression in the vasculature is crucial to hypertension pathology. The possible role of IL-33 and ST2 in hypertension is summarised in figures 6.1 and 6.2.

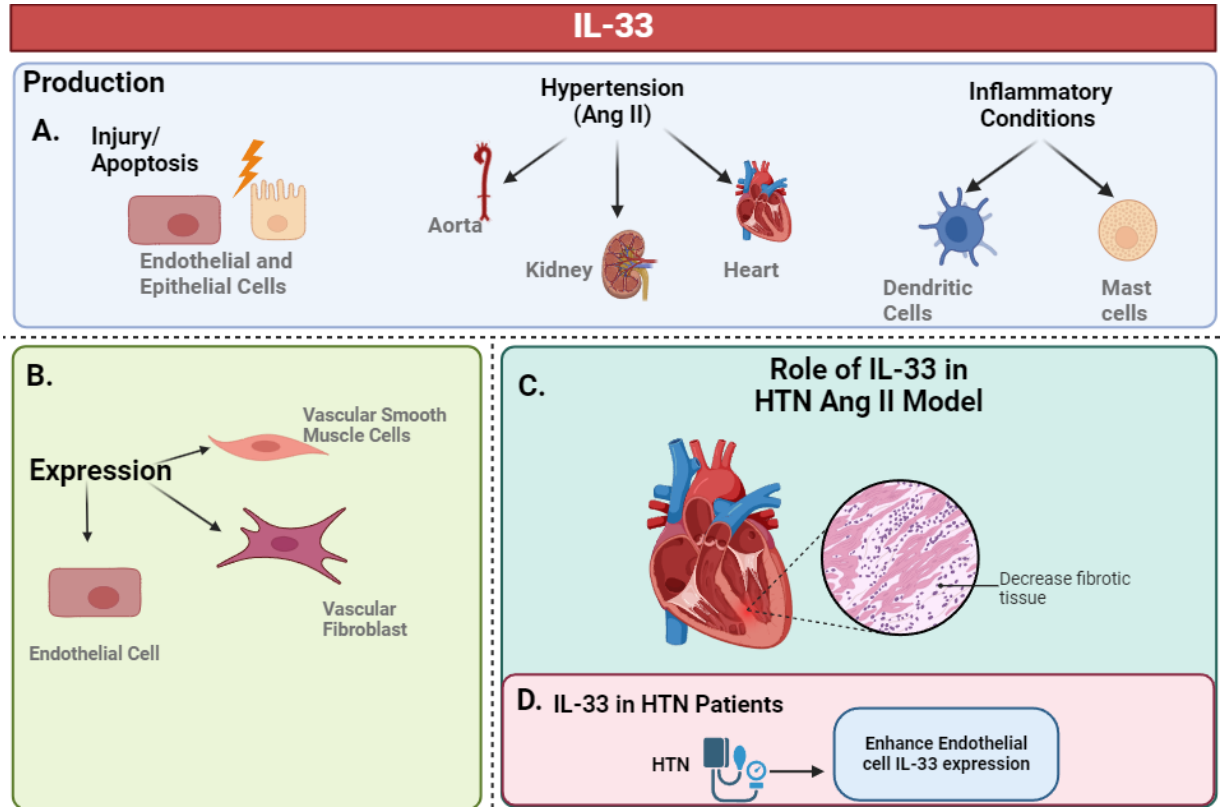


Figure 6.1: Summary of the possible roles of IL-33 in Hypertension.
IL-33 is produced in Ang II-induced hypertension from various organs, including the Heart and Aorta. IL-33 is also produced upon injury by epithelial and endothelial cells (Moussion et al., 2008a) as well as by inflammatory cells such as Dendritic cells and Mast cells (Drake and Kita, 2017) **(A)**. Cellular expression of IL-33 in Ang II hypertension atherosclerosis is observed in vascular endothelial cells, vascular smooth muscle cells and vascular fibroblasts **(B)**. In Ang II hypertension, IL-33 may act to reduce cardiac fibrosis **(C)**. Enhanced vascular expression of IL-33 is observed in internal mammary artery endothelial cells in hypertensive patients **(D)**.

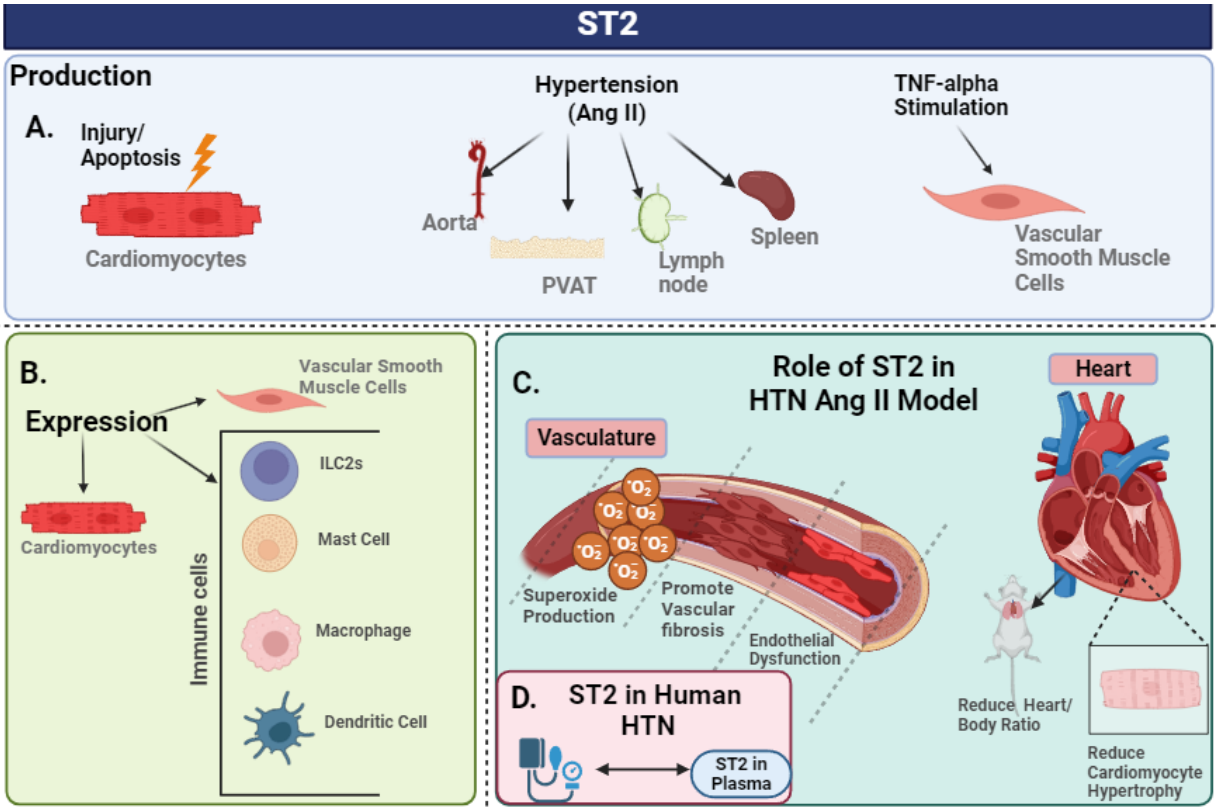


Figure 6.2: Summary of the possible roles of ST2 in Hypertension.
ST2 is produced in Ang II-induced hypertension from various organs, including the Aorta, PVAT, Lymph node and Spleen. ST2 is also produced by vascular smooth muscle cells upon TNF-alpha stimulation. sST2 is produced by cardiomyocytes upon injury (Brunetti et al., 2023) **(A)**. Cellular expression of ST2 in Ang II hypertension atherosclerosis is observed in vascular smooth muscle cells and immune cells, including ILC2s, mast cells, macrophages, and dendritic cells (Turnquist et al., 2008). **(B)**. In Ang II hypertension, ST2 may promote endothelial dysfunction, vascular fibrosis, and superoxide production. In the heart, ST2 may reduce Ang II-induced cardiomyocyte hypertrophy and heart/body weight ratio **(C)**. Elevated plasma ST2 levels correlate with hypertension biomarkers **(D)**.

6.1 IL-33 and ST2 Expression in the Vasculature Provides Understanding of its Potential Role in Hypertension

The thorough examination of the expression of IL-33 and ST2 in the main organs involved in controlling blood pressure was key to understanding where the IL-33/ST2 signalling pathway is most likely to impact hypertension pathology. This stemmed from identifying a disconnect in the knowledge of the role of IL-33 and ST2 in cardiovascular diseases. Despite numerous studies examining the effect of IL-33 in inflammatory diseases such as asthma, rheumatoid arthritis, and some cardiovascular diseases, including atherosclerosis and myocardial infarction, there remains notable gaps in the field of CVDs, predominantly hypertension. The one study investigating the alteration of the IL-33-sST2 pathway in hypertensive and mouse models analysed only the aortic expression of IL-33 and its levels in serum and PBMCs of hypertensive patients compared to control (Yin et al., 2019a).

The idea that the IL-33/ST2 signalling pathway plays a role in several cardiovascular diseases such as diabetes, heart failure, cardiac hypertrophy and cardiomyopathies (Ghali et al., 2018) either at the genetic level through the regulation of transcription as a classically active IL-33 or functions as an “alarmin” or cytokine (Liu et al., 2022) is more apparent. IL-33, bound to the transmembrane receptor ST2L, has been reported to prevent myocardial apoptosis and alleviate myocardial fibrosis and myocardial hypertrophy (Seki et al., 2009a). Further, sST2 is a known biomarker of disease severity and prognosis for most cardiovascular diseases, with many clinical studies describing sST2-induced IL-33 pathway inhibition in the cardiovascular system (van den Berg et al., 2022). However, as discussed previously, these studies presented an absence of information on the effect of IL-33 and ST2 in hypertension pathology.

We believe assessing the role of IL-33 in various organs is essential, particularly in the vasculature and the heart, which are extensively affected by hypertension. This is principally true, since IL-33 expression in vascular endothelial cells and smooth muscle cells has potential consequences in other CVDs including atherosclerosis (Liu et al., 2022, Miller et al., 2008, Miller, 2011b).

To establish a broad knowledge of the involvement of IL-33 and ST2 in the context of hypertension, we first sought to examine their expression in key organs upon Ang II-induced hypertension in mice. IL-33 was upregulated in all organs, including the aorta, lymph node, spleen, kidney, and PVAT in Ang II- compared to the sham group of proteins within the protein panel. Additionally, qPCR analysis exhibited *IL-33* mRNA upregulation in the aorta and heart of mice receiving Ang II infusion. Furthermore, *ST2* levels heightened in mouse aorta, spleen and lymph nodes. Notably, we observed the same expression profile seen in the mouse vasculature in western blot analysis. In addition to highlighting the expression of IL-33 and ST2 in the vasculature, we similarly explored if the increase of IL-33 and ST2 in Ang II mice is blood pressure dependent. Treatment with the vasodilator, hydralazine and the diuretic to the angiotensin II animal group substantially diminished elevated levels of IL-33 and ST2 after Ang II treatment, verifying the expression of IL-33 and ST2 is not due to angiotensin II treatment. However, it would be helpful in the future to perform the same experiments on other organs, including the PVAT and the heart.

In addition to emphasising that IL-33 and ST2 are induced in hypertensive mouse vasculature, we explored the localisation of both in healthy and Ang II-induced mice. Literature has discussed IL-33 expression prevalence chiefly in endothelial cells from both large and small blood vessels (Moussion et al., 2008a), in coronary artery smooth muscle cells, adipocytes (Saidi et al., 2011) and in cardiac fibroblasts (Miller et al., 2008), implying its role in various CV disorders (Sanada et al., 2007a). For the first time, my results showed IL-33 and ST2 localisation in Ang II-induced hypertension was widespread in the media of the sham and Ang II groups, to a lesser extent also in the adventitia of the mouse vasculature. Further repetition to increase sample size is necessary to strengthen the accuracy of current results. Likewise, future studies to perform staining for IL-33 and ST2 in hearts would be essential. Moreover, the presence of IL-33 and ST2 in the healthy and higher detection of the diseased vasculature suggests its possibility as a regulator of hypertension under physiological or pathophysiological conditions.

Moreover, we also explored the expression changes of IL-33 and ST2 in another vascular disease, atherosclerosis, through which we observed expression changes in healthy compared to the diseased aorta. In WT and ApoE$^{-/-}$ atherosclerotic mice aorta, IL-33 is confined to adventitial non-immune cells, and ST2 is predominately expressed in immune cell subsets. Further dataset analysis demonstrated IL-33 expression from distinctive mesenchyme cell clusters at WT and ApoE$^{-/-}$ and SMCs in the disease mice model. Single-cell profiling data of the mice and human colon characterised by Kinchen et al. emphasised distinct mesenchymal subset upregulation of IL-33 expression in inflammatory disease settings (Kinchen et al., 2018). IL-33 receptor was absent in aortic adventitial non-immune cells, but marked expression was observed in the ILC2s cell cluster.

An additional single-cell dataset examining aortic infiltrating leukocytes in WT control and Ang II-induced mice presented high levels of ST2 in ILC2s and mast cells. On the other hand, IL-33 expression was deficient and was found only in migrating cells. These results agree with previous analysis and literature (Topczewska et al., 2023). Experimental studies have presented that ST2 is chiefly present in immune cells, including ILC2s, mast cells, basophils, CD4 and CD8 T cells, eosinophils, and macrophages (Topczewska et al., 2023). My results from this study, therefore, propose crosstalk between non-immune stromal cells and immune cells in the IL-33/ST2 signalling pathway in hypertension. A single-cell seq dataset on the hypertensive heart from a C57BL/6J mouse would be crucial to understanding the production of IL-33 and ST2 in the heart. Furthermore, utilising single-cell RNA-sequencing datasets of adult human hearts across healthy and cardiovascular disease patients could reveal the cellular landscapes of IL-33 and ST2 expression and regulation.

Although interesting, IL-33 and ST2 expression alone in the vasculature does not support or rule out it being harmful or protective in hypertension and other vascular diseases such as atherosclerosis. In several CVDs, IL-33 has demonstrated protective effects by inducing Th2 cytokines and promoting alternative activation through M2 polarisation. However, the soluble ST2 receptor has been shown experimentally and

clinically to exacerbate cardiovascular diseases (Thanikachalam et al., 2023). Therefore, a functional examination of how IL-33-deficiency and ST2-deficiency affect hypertension pathology was necessary to determine its role.

6.2 IL-33 and ST2 has a Fundamental Role in an Inducible Hypertension Murine Model

The identification of ample levels of expression of IL-33 and ST2 in various organs closely involved in hypertension and specifically the vasculature, during homeostasis and disease conditions informed us of the necessity of a global knock-out model to individually assess the functional impact of IL-33 and ST2 on hypertension pathology.

This study was conclusive in finding that both IL-33 and ST2 have a functional role in an inducible hypertension murine model. IL-33-deficient mice exacerbated myocardial fibrosis upon ang II-dependent hypertension. Conversely, $ST2^{-/-}$ mice were protected from vascular dysfunction and perivascular fibrosis in the hypertension animal model.

My study has been the first to link ST2 to vascular fibrosis in hypertension independent of blood pressure. Nevertheless, earlier studies have presented that IL-33 and ST2 regulate various fibrotic diseases, including pulmonary fibrosis (Lee et al., 2017a), (Gao et al., 2015b), liver fibrosis (McHedlidze et al., 2013, Sun et al., 2014, Artru et al., 2020), renal fibrosis (Elsherbiny et al., 2020), and heart fibrosis. My results agree with the 2013 McHedlidze et al. study demonstrating that ST2-deficient mice were protected from collagen accumulation upon carbon tetrachloride induction, an organic compound with pro-fibrotic effects (McHedlidze et al., 2013).

Another fundamental factor in hypertension physiology is the vascular tone, mediated by the endothelium (Wilson et al., 2019). Striking new data generated from this study highlight that $ST2^{-/-}$ mice are protected from the development of endothelial dysfunction despite the similar blood pressure response to Ang II, accompanied by the reduction in superoxide production. This may be regulated by the downstream IL-33/ST2 signalling in the endothelium itself. Still, studies have also proposed that

T-cell infiltration into the vessel wall can induce endothelial dysfunction mainly through IFN-γ-dependent mechanisms (Mikolajczyk et al., 2016). In the $ST2^{-/-}$ mice, there is a possibility of reduced recruitment of IFN- γ-producing cells as a mechanism for the protection from endothelial dysfunction and vascular oxidative stress observed. However, future studies need to address this hypothesis to understand the exact pathway involved in regulating vascular function.

No changes in vascular endothelial function or production of superoxide were observed in IL-33-deficient mice infused with Ang II compared to WT Ang II mice. This does not align with the results presented by the Choi et al. study, which exhibited that IL-33 advances endothelial NO production using ST2/TRAF6-mediated activation of phosphoinositide-3-kinase and endothelial NO synthase signalling pathway (Choi et al., 2009). In future studies, it would be helpful to examine the downstream signalling pathways to understand better the regulatory pathway of IL-33 and ST2 in hypertension.

As well as vascular dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis are common pathophysiological developments in numerous heart-related diseases, including myocardial infarction, hypertension and others (Sanada et al., 2007a), (Sadoshima and Izumo, 1997). In this study, IL-33 exhibited a cardioprotective role in Ang II-induced hypertension; $IL\text{-}33^{-/-}$ mice had elevated interstitial cardiac fibrosis compared to the WT Ang II group. Abolishment of global ST2 signalling in mice induced cardiomyocyte hypertrophy. The study has highlighted that ST2 can have varying roles in different organs; this may be caused by the variable expression of the ST2L receptor on numerous inflammatory cells. Further, it reflects the complexity of the IL-33/ST2 signalling pathway in hypertension and different possible vascular and cardiac pathway regulation at various stages of hypertension. To complement vascular reactivity studies and end-organ damage seen in the heart, forthcoming plans to investigate cardiac function through echocardiography would be essential in control and hypertension-induced $IL\text{-}33^{-/-}$, $ST2^{-/-}$ and WT mice.

In future studies, it may be valuable to carry out the currently presented experimental studies in female WT and Ang II-induced IL-33-deficient and ST2-

deficient mice to assess potential changes in expression levels, organ function and end-organ damage and target any disparities which arise due to sex differences to improve treatment outcomes. Further, understanding if specific collagens are directly affected by IL-33 and ST2 expression upon sex differences may also be necessary. Thousands of published studies describe the occurrence of sex differences in CVDs, including atherosclerotic coronary artery disease, heart failure with preserved ejection fraction and hypertension. High blood pressure affects men and women differently, and understanding these sex differences is necessary for researchers in this field (Olivera and Graham, 2023). In human and experimental model studies, females have been underrepresented (Reue and Wiese, 2022). A recently published review by Olivera and Graham describes the sex differences in preclinical models of hypertension. The most common Ang II infusion models of hypertension are the Sprague-Dawley rat and the C57BL/6J mouse (Olivera and Graham, 2023). Throughout the study, I have used male C57BL/6J mouse, as it is known to be one of the best models to study high blood pressure. Former studies have detailed female-specific protection against increased BP induced by Ang II C57BL/6J mice (Xue et al., 2005, Olivera and Graham, 2023). Nevertheless, we must further the understanding of the mechanisms underlying sex differences to devise optimal preventative and therapeutic approaches for all individuals.

6.3 sST2 Potentially acts as a Risk Factor for Hypertension

The sc-Seq dataset yields incredible data on the characterisation of IL-33 and its receptor ST2 expression in heart transplant patient tissue. I have found that IL-33 is primarily expressed by the vascular endothelial cells, fibromyocytes, and fibroblast cell clusters in normal and diseased vessels from patients undergoing heart transplants. IL-33 and ST2 are shown to be highly expressed in human vein endothelial cells and coronary artery endothelium (Demyanets et al., 2011c) as well as in the nucleus of cardiac myocytes, cardiac fibroblasts, and smooth muscle cells (Demyanets et al., 2013b). This study confirmed the localisation of IL-33 in human mammary arteries of patients with high BP for the first time by immunohistochemistry. Stained sections highlighted IL-33 expression in the human mammary artery's endothelial and smooth muscle cells. In agreement with other

studies, we also found that IL-33 is expressed in endothelial cells in the human mammary arteries of hypertensive patients. Demyanets et al. displayed the detection of IL-33 protein expressed by endothelial cells in human atherosclerotic carotid tissue (Demyanets et al., 2011c). Identifying the localisation of IL-33 and ST2 in normotensive mammary arteries would also be essential for establishing a baseline expression in healthy individuals.

While analysing human serum levels of sST2 and IL-33 exhibited no significance in hypertensive patients compared with the control group, the extensive clinical study by Yin et al. demonstrated that levels of sST2 in serum and PBMCs were significantly elevated in the hypertensive patient cohort (Yin et al., 2019a). This discrepancy in results could be due to its small group and thus underpowered. Repetition of this experiment is crucial for the advancement of this study.

Clinical studies have demonstrated the prognostic role of soluble ST2 in many cardiovascular diseases. A 2018 study led by Jha et al. indicated that elevated levels of sST2 might be a suitable biomarker to elevate the risk of future adverse cardiovascular events in acute coronary syndrome (Jha et al., 2018). Additionally, sST2 was highly correlated with the severity and poor prognosis of acute heart failure (Wang et al., 2022). In accordance with these studies, analysis of the OLINK UK Biobank dataset presented a positive correlation between ST2 and BP parameters, including systolic and diastolic blood pressure and pulse pressure. These results are significant as they potentially support the vascular functional role of ST2. Additionally, in the future, assessing the vascular function of human arteries with the addition of recombinant IL-33 and ST2 will aid currently presented data and provide a significant advancement in whether exogenous IL-33 and ST2 can regulate vascular function. These data so far support my hypothesis that the IL-33-ST2 axis expression in the vasculature may be involved in several cardiovascular diseases, especially sST2 in hypertension pathology.

6.4 Overall, Strengths and Limitations of Approaches Undertaken in the Study

This study design has several strengths, providing valuable results that advance the role of IL-33 and ST2 in hypertension and vascular disease. The integration of animal models with human vascular studies and the analysis of UK Biobank data underlines the study's applicability in a translational setting, helping to bridge the gap between basic research and potential clinical interventions.

The study utilising the Angiotensin II hypertension model represents an excellent approach despite its limitations, such as the short timeframe of development and focus on Ang II-related mechanisms. However, it still offers an extensive understanding of the disease progression. Employing both mice and human scRNA-Seq datasets in this study has allowed for cross-species analysis of gene expression patterns, enhancing the depth of biological insights gained. Moreover, using human blood vessels from patients with comorbidities provides this study with data within a real-world clinical context, advancing the significance of my animal study findings to populations that are affected by hypertension and related complications. The study's design, incorporating both *in vivo* (animal models), *in vitro* (animal cell culture) and human experimental methods, offers a synergistic approach that strengthens the validity of the findings and their potential therapeutic implications.

While we provide a comprehensive assessment of the role of IL-33 and ST2 in regulating vascular function and cardiac damage in hypertension, this study has some limitations that should be addressed in future studies. Due to their unavailability, the study did not employ tissue-specific knock-out models for individual molecules, which could have provided more precise insights into the localised functions of IL-33 and ST2. A model for future studies is proposed where IL-33 is inactivated in smooth muscle cells and ST2 in smooth muscle cells and cardiac fibroblasts. Potential compensatory mechanisms within the IL-1 receptor family may exist. There is a likelihood that other members of the IL-1 receptor family of cytokines might compensate for the lack of IL-33 and ST2, which could mask the true effects of these

proteins in hypertension. Future investigations studying the expression levels of different cytokines such as IL-1α, IL1-ß and IL-18 are necessary.

The study faced difficulties distinguishing the specific actions related to the soluble form of ST2 compared to the transmembrane receptor (ST2L), which might have different roles in the pathophysiology of hypertension. Moreover, the absence of telemetry to measure blood pressure in this study and the use of the Tail-cuff alone could mean that the study underestimated the differences in blood pressure, especially considering that the tail-cuff method showed only a weak lowering of blood pressure. Therefore, telemetry and echocardiography will be essential in future studies to assess blood pressure and cardiac function. Human blood vessels used in the study for immunohistochemistry were obtained from patients with comorbid conditions, which might influence the findings. The lack of availability of vessels from patients without such conditions is a limitation of this study.

6.5 Final Conclusions

This thesis explored the role of IL-33 and its receptor ST2 in hypertension and vascular disease.

1. IL-33 and ST2 expression is expressed in critical organs affected by hypertension at baseline and is further upregulated by Ang II-induced hypertension. The induction noticed in the vasculature is dependent on blood pressure increases rather than caused by the direct actions of Ang II induction.

2. RNA Seq analysis of publicly available datasets confirmed the expression of IL-33 in stromal cells and the ST2 receptor expression principally in immune cells of the mouse aorta, namely ILC2s and mast cells, demonstrating the complexity of the IL-33/ST2 pathway in cardiovascular diseases.

3. IL-33 and ST2 are essential regulators of vascular and cardiac fibrosis and dysfunction observed in hypertension. Results indicate that IL-33 in this hypertension Ang II model may decrease cardiac tissue fibrosis.

4. $ST2^{-/-}$ upon Ang II demonstrated reduced vascular dysfunction, perivascular fibrosis and superoxide production, indicating ST2 dependent adverse vascular role in this hypertension animal model. ST2 dependent cardiomyocyte hypertrophy was also highlighted from this study.

5. The IL-33/ST2 pathway is translationally relevant as IL-33 is expressed in hypertensive human mammary arteries and the plasma sST2 levels appears to be a risk factor for hypertension and may represent a promising novel biomarker for hypertension prediction using OLINK UK Biobank data.

In conclusion, my studie's results indicate both IL-33 and its receptor ST2 are increased in the vascular and perivascular tissue in hypertension. The IL-33/ST2 axis has a definite role in regulating cardiac and vascular function and end-organ damage

in Ang II-dependent hypertension. Lastly, the soluble ST2 receptor may be a potential biomarker for hypertension and other cardiovascular diseases.

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