The Role of P2RX7 in Angiotensin II-induced Hypertension and Cardiovascular Disease

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Montréal, QC, Canada
August 2022

A thesis submitted to McGill University
in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Table of Contents

Abstract

Hypertension affects more than one billion people worldwide and is the leading risk factor for burden of disease and mortality. It is associated with chronic low-grade inflammation, and targets to diminish this low-grade inflammation are of interest. The purinergic receptor P2X7 (P2RX7) is activated by extracellular ATP and is a key regulator of inflammatory signalling, cellular death, and T lymphocyte development and function. Elevated plasma ATP levels have been observed in hypertensive patients, providing a potential mechanism for P2RX7 activation. Additionally, a hypomorphic polymorphism for P2X7 is correlated with a decreased risk for essential hypertension in Chinese post-menopausal women. The work in this thesis tests the hypothesis that P2RX7 mediates angiotensin (Ang) II-induced blood pressure elevation and cardiovascular injury by promoting the activation of innate and adaptive immune cells.

In the first study, we assessed the contribution of P2RX7 to immune activation in hypertension and cardiovascular injury. Both *P2rx7* knockout and P2RX7 antagonism attenuated Ang II (1000ng/kg/min) induced hypertension and small artery endothelial dysfunction. In addition, we found decreased infiltration of activated T cells into the aortic perivascular adipose tissue (PVAT) of Ang II-treated compared to wild-type (WT) control mice. Furthermore, WT mice treated with Ang II had an expansion of $CD4^+$ and γδ T effector memory (T_{EM}) cells in the spleen and aortic PVAT along with increased $CD8^+$ T_{EM} in the bone marrow. These changes were abrogated in *P2rx7*$^{-/-}$ mice or mice treated with a P2RX7 antagonist suggesting decreased immune activation in these mice. Neither *P2rx7*$^{-/-}$ mice nor P2RX7 antagonist treated mice infused with Ang II were protected from cardiac dysfunction. In fact, Ang II-treated *P2rx7*$^{-/-}$ mice had reduced left ventricle fractional shortening and a dilated left ventricle compared to Ang II-treated WT mice, suggesting enhanced cardiac dysfunction. This study highlighted that *P2rx7* knockout or P2RX7 antagonism attenuated Ang II-induced immune activation, and subsequent hypertension and vascular injury, but not cardiac dysfunction.

In the second study, we sought to tease out the relative contribution of P2RX7 and an inflammasome component, NLRP3 (NOD-like receptor family, pyrin domain containing 3), in Ang II-induced hypertension and vascular injury. Both *P2rx7*$^{-/-}$and *Nlrp3*$^{-/-}$ mice were protected from Ang II (490ng/kg/min) induced small artery endothelial dysfunction and had attenuated T cell activation. However, only *P2rx7*$^{-/-}$ mice were protected from Ang II induced systolic blood

pressure elevation. This study suggested that P2RX7-NLRP3 signalling is important for Ang II-induced T cell activation and endothelial dysfunction, but primarily P2RX7 contributes to Ang II-induced blood pressure elevation.

The third study is a brief letter identifying a memory T lymphocyte subset with an unknown role in the context of hypertension. $CD4^+$, $CD8^+$ and γδ tissue-resident memory (T_{RM}) cells increased up to 12-fold in the aortic PVAT of WT mice treated with Ang II. The expansion of T_{RM} cells was inhibited by the genetic knockout or pharmacologic blockade of P2RX7. T_{RM} cells are known to be potent pro-inflammatory cells in the context of infection, and thus may contribute to vascular dysfunction and remodelling in hypertension.

In summary, the P2RX7-NLRP3 signalling pathway contributes to hypertension-associated vascular dysfunction, but only P2RX7 contributes to blood pressure elevation. P2RX7 antagonism attenuates Ang II-induced blood pressure elevation, vascular dysfunction, and immune activation without exacerbating cardiac dysfunction as in *P2rx7*$^{-/-}$ mice. Finally, *P2rx7* knockout and P2RX7 antagonism limit the development of pro-inflammatory T_{EM} and T_{RM} cells in Ang II-induced hypertension. Taken together, these results provide evidence that P2RX7 may be a viable target for the treatment of hypertension and associated vascular dysfunction via suppressing immune activation.

Résumé

L'hypertension touche plus d'un milliard de personnes dans le monde et constitue le principal facteur de risque de maladie et mortalité. Elle est associée à une inflammation chronique de bas niveau, et il pourrait être intéressant d'identifier des cibles diminuant cette inflammation. Le récepteur purinergique P2X7 (P2RX7) est un régulateur clé de la signalisation inflammatoire, de la mort cellulaire et du développement et de la fonction des lymphocytes T. Des niveaux élevés d'ATP plasmatique ont été observés chez des patients hypertendus, fournissant un mécanisme potentiel d'activation de P2RX7. De plus, un polymorphisme hypomorphe pour P2X7 est corrélé à une diminution du risque d'hypertension essentielle chez les femmes ménopausées chinoises. Les travaux rapportés dans cette thèse testent l'hypothèse selon laquelle P2RX7 médie l'élévation de la tension artérielle et les lésions cardiovasculaires induites par l'angiotensine (Ang) II via l'activation des cellules immunitaires innées et adaptatives.

Dans la première étude, nous avons évalué la contribution de P2RX7 dans l'activation immunitaire dans l'hypertension et les lésions cardiovasculaires. L'inactivation de *P2rx7* et l'antagonisme de P2RX7 ont réduit l'élévation de la tension artérielle et le dysfonctionnement endothélial des petites artères induits par l'Ang II (1000 ng/kg/min). De plus, nous avons constaté une diminution de l'infiltration des lymphocytes T activés dans le tissu adipeux périvasculaire (TAPV) aortique des souris traitées avec l'Ang II par rapport aux souris sauvages contrôles. En plus, les souris sauvages traitées avec l'Ang II présentaient une expansion des cellules T mémoire effectrices (T_{EM}) $CD4^+$ et γδ dans la rate et le TAPV aortique, ainsi qu'une augmentation des cellules T_{EM} $CD8^+$ dans la moelle osseuse. Ces augmentations ont été abrogées chez les souris *P2rx7*$^{-/-}$ ou les souris traitées avec un antagoniste P2RX7 suggérant une diminution de l'activation immunitaire chez ces souris. Ni les souris *P2rx7*$^{-/-}$ ni les souris traitées avec un antagoniste P2RX7 infusées avec l'Ang II n'étaient protégées contre le dysfonctionnement cardiaque. En fait, les souris *P2rx7*$^{-/-}$ traitées avec l'Ang II présentaient une diminution de la fraction de raccourcissement du ventricule gauche et un ventricule gauche dilaté comparativement aux souris sauvages traitées avec l'Ang II suggérant une dysfonction cardiaque exacerbée. Cette étude a mis en évidence que le knock-out de *P2rx7*ou l'antagonisme de P2RX7 atténuait l'activation immunitaire induite par l'Ang II et l'hypertension et les lésions vasculaires qui en résultaient, mais pas le dysfonctionnement cardiaque.

Dans la deuxième étude, nous avons cherché à déterminer les contributions relatives de P2RX7 et d'une composante de l'inflammasome, NLRP3 ("NOD-like receptor family, pyrin domain containing 3"), dans l'hypertension et les lésions vasculaires induites par l'Ang II. Les souris *P2rx7*$^{-/-}$ et *Nlrp3*$^{-/-}$ étaient toutes deux protégées du dysfonctionnement endothélial des petites artères et présentaient une activation atténuée des lymphocytes T induite par l'Ang II (490 ng/kg/min). Cependant, seules les souris *P2rx7*$^{-/-}$ étaient protégées contre l'élévation de la tension artérielle systolique induite par l'Ang II. Cette étude suggère que la signalisation P2RX7-NLRP3 est importante pour l'activation des lymphocytes T induite par l'Ang II et le dysfonctionnement endothélial, mais seul P2RX7 contribue à l'élévation de la tension artérielle induite par l'Ang II.

La troisième étude a déterminé le rôle de P2RX7 dans le développement des lymphocytes T mémoire dans le TAPV aortique dans le contexte de l'hypertension. Les cellules T mémoire résidentes (T_{RM}) $CD4^+$, $CD8^+$ et γδ étaient augmentés jusqu'à 12 fois dans le TAPV aortique des souris sauvages traitées avec l'Ang II. L'expansion des cellules T_{RM} était inhibée par le knock-out génétique ou l'inhibition pharmacologique de P2RX7. Les cellules T_{RM} sont connues pour être de puissantes cellules pro-inflammatoires dans le contexte de l'infection, et peuvent donc contribuer au dysfonctionnement vasculaire et au remodelage dans l'hypertension.

En résumé, la voie de signalisation P2RX7-NLRP3 contribue au dysfonctionnement vasculaire associé à l'hypertension, mais seul P2RX7 contribue à l'élévation de la pression artérielle. L'antagonisme de P2RX7 atténue l'élévation de la pression artérielle, le dysfonctionnement vasculaire et l'activation immunitaire induite par l'Ang II sans exacerber le dysfonctionnement cardiaque observé chez les souris *P2rx7*$^{-/-}$. Enfin, le knock-out de *P2rx7*$^{-/-}$ et l'antagonisme de P2RX7 limitent le développement des cellules pro-inflammatoires T_{EM} et T_{RM} dans l'hypertension induite par l'Ang II. Pris ensemble, ces résultats fournissent la preuve que P2RX7 peut être une cible viable pour le traitement de l'hypertension et du dysfonctionnement vasculaire via la suppression de l'activation immunitaire.

Acknowledgments

Throughout my doctoral journey I have had the support of many fantastic people, without whom I would not have been able to succeed.

Firstly, I would like to express my gratitude to my supervisor Dr. Ernesto L. Schiffrin, who provided an exceptional environment to grow and mature as a young researcher. His passion and dedication to his research, his students, and his patients have inspired me to pursue a career as a physician-scientist.

I would also like to extend my thanks to Dr. Pierre Paradis, who was always available to provide input on experimental designs. His insightful feedback honed my critical thinking and pushed me to work at my highest level.

To all my colleagues in Dr. Schiffrin's laboratory, I would personally like to thank them for their help, friendship, and support along the way. To Kevin Comeau for all the technical help with flow cytometry and the countless hours prepping tissues. For the great conversations around the coffee machine, often at the expense of getting work done, and for making the lab a fun place to be. To Dr. Kugeng Huo, who provided me with technical and career advice over rounds of golf. To Dr. Olga Berillo, who introduced me to my love/hate relationship with myography. She has provided sound guidance and technical support throughout the course of my Ph.D., for which I am grateful. Thank you to Dr. Nathanne Ferreira for sharing my passion for the NLRP3 inflammasome and love of Brazilian food and music. To the many others who I was fortunate enough to work with, Dr. Akinori Higaki, Dr. Ahmad Mahmoud, Dr. Nada Mahjoub, Bianca Dancose-Giambattisto and Dr. Suellen Coelho. I would like to especially thank Dr. Antoine Caillon, who was my mentor and guide as I ventured into the, unbeknownst to me, exceedingly complicated world of immunology. Our late-night discussions over a cold pint helped steer the course of this Ph.D. project. I would additionally like to thank Kevin and Antoine, who volunteered for the daunting task of proofreading sections of this thesis.

I would like to thank Adriana Cristina Ene, Veronique Michaud, Isabelle Miguel, Christian Young, Yvhans Chéry, and Kathy Forner for their excellent technical support within the Lady Davis Institute over the years. A special thanks to Christian Young for providing astute support in flow cytometry and Veronique Michaud for not only the fantastic technical support in telemetry

surgeries but for also providing exceptional conversations and many laughs in the surgery room of the animal quarters.

To the members of my thesis committees: Dr. Stefanie Lehoux, Dr. Marc Servant, Dr. Stella Daskalopoulou, Dr. Maya Saleh, Dr. Lorraine Chalifour, and Dr. Brett Mitchell who never failed to provide insightful feedback to push my project forward. To my academic advisor, Dr. Ivan Topisirovic, who always offered me a firm voice of support when I needed it during my annual committee meetings.

I must also take this opportunity to acknowledge the financial support I have received during my Ph.D., without which this would not have been possible. To the Fonds de recherche Santé Quebec (FRQS) bourse 289184, Lady Davis Institute/TD Bank Studentship award and the Canadian Institutes of Health Research Canada Graduate Scholarship, I am extremely grateful.

I would be remiss not to thank my girlfriend, Leticia Goedert, for putting up with my incessant discussions about my project. For putting up with the late nights and absent weekends. For providing a comforting shoulder when needed. For sharing in the highs and lows of my journey.

To my family who have always been very supportive of me throughout my academic journey, even from afar.

Finally, I would like to thank the many lifelong friends I have made during my time in Montréal. They provided me with the occasional escape from the lab and helped me achieve that much sought-after work/life balance.

Abbreviations

Acta1	Actin alpha 1 skeletal muscle
ACE	Angiotensin converting enzyme
AF	Alexa-flour
Ang	Angiotensin
ANOVA	Analysis of variance
APC	Allophycocyanin
ARB	Angiotensin receptor blocker
ARTC2.2	ADP-ribosyltransferase C2.2
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
AT1R	Angiotensin I type 1 receptor
ATP	Adenosine triphosphate
AZ106	AZ10606120 dihydrochloride
BA	Baroreceptor activation
BD	Becton Dickinson
BMDM	Bone-marrow-derived macrophages
BMDC	Bone marrow-derived dendritic cells
BP	Blood pressure
BUV	Brilliant ultraviolet
BV	Brilliant violet
CANTOS	Canakinumab anti-inflammatory thrombosis outcome study
CCR7	C-C chemokine receptor 7
CD	Cluster of differentiation
CD103	Integrin alpha E
CD62L	L-selectin
CF594	Cyanine-based fluorescent dye 594
CNS	Central nervous system
CO	Cardiac output
Col1a1	Collagen type I alpha 1 chain

Col3a1	Collagen type III alpha 1 chain
CSD	Carotid sinus nerve denervation
CVD	Cardiovascular disease
Cy	Cyanine
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DBP	Diastolic blood pressure
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium/nutrient mixture
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DUB	Deubiquitinase
EF450	Eflour 450
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
FS	Fractional shortening
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GFR	Glomerular filtration rate
GSDM-D	Gasdermin-D
HR	Heart rate
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KCl	Potassium chloride
KO	Knockout
I/R	Ischemia-reperfusion
L-NAME	N^{ω}-nitro-L-arginine methyl ester

LPS	Lipopolysaccharide
LV	Left ventricle
LVIDd	Left ventricle internal diameter in diastole
LVOT	Left ventricle outflow tract
MΦ	Macrophage
MAP	Mean arterial pressure
M-CSF	Macrophage-colony stimulating factor
M/L	Media/lumen
MAPK	Mitogen-activated protein kinases
MCSA	Media cross-sectional area
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMF	Mycophenolate mofetil
Mono	Monocyte
Myh7	Myosin heavy chain 7 cardiac muscle beta
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLRP3	NLRP3 nucleotide-binding oligomerization domain like receptor family pyrin domain containing 3
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
Nppa	Natriuretic peptide type A, also known as atrial natriuretic peptide
Nppb	Natriuretic peptide type B, also known as brain natriuretic peptide
OVLT	Organ vasculosum of the lamina terminalis
P2RX7	P2X7 receptor

PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDL1	Programmed death-ligand 1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PMA	phorbol 12-myristate 13-acetate
PNS	Parasympathetic nervous system
PS	Phosphatidylserine
PVAT	Perivascular adipose tissue
PVN	Paraventricular nucleus
PWV	Pulse wave velocity
RAAS	Renin-angiotensin-aldosterone system
RAG1	Recombination-activating gene 1
RFU	Relative fluorescent units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rps	Ribosomal protein
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RV	Right ventricle
SBP	Systolic blood pressure
SC	Subcutaneous
SGK1	Serum/glucocorticoid kinase1
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SSC-A	Side scatter area
SSC-H	Side scatter height
SOD	Superoxide dismutase
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SV	Stroke volume

T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TGF	Transforming growth factor
Th	Helper T cells
TNF	Tumour necrosis factor
TLR	Toll-like receptor
Treg	T regulatory cell
T_{RM}	Resident memory T cell
USP	Ubiquitin-specific peptidase
VSMC	Vascular smooth muscle cell
VTI	Velocity time integral
WT	Wild-type

List of Tables

List of Figures

Statement of Originality and Contribution to Original Knowledge

In accordance with the guidelines of the Faculty of Graduate and Postdoctoral Studies of McGill University, this thesis is presented in a manuscript-based format. The research presented in this thesis constitutes original work, and collaboration from co-authors in the manuscripts are detailed in the section *Contribution of Authors*.

Contributions to original knowledge:

- We provided the first study that demonstrated that genetic knockout or pharmaceutical antagonism of P2RX7 attenuated Ang II-induced BP elevation, immune activation, vascular remodelling, and endothelial dysfunction.
- We are the first group to present the differential effects of genetic knockout versus pharmaceutical antagonism of P2RX7 in hypertension. In particular, *P2rx7* knockout mice develop exacerbated cardiac dysfunction and hypertrophy, whereas mice receiving a P2RX7 antagonist do not. As well, there are differences in large and small arterial remodelling, and we report differences in immune cell populations, activation states and effector cytokine production between *P2rx7* knockout mice and mice receiving a P2RX7 antagonist.
- We present the first evidence that P2RX7 contributes to the development of effector memory T cells in the context of hypertension.
- We are the first to report on the development of γδ effector memory T cells in hypertension.
- This thesis provides the first study to report the development of resident memory T cells in the context of hypertension.

- We are the first group to directly compare P2RX7 versus NLRP3 signalling in hypertension.

Contribution of Authors

All chapters of this thesis were written by Brandon Shokoples and edited by Dr. Ernesto L. Schiffrin. The body of this thesis, Chapters 2, 4, 5, and 6, were prepared in manuscript format, and thus, the contributions of collaborating authors are detailed below.

Chapter 2: Literature Review: P2X7: An untapped target for the management of cardiovascular disease

- Brandon Shokoples: Conducted the literature review, drafted the manuscript and figures, and responded to revision requests.
- Pierre Paradis: Contributed to the design of the figures.
- Ernesto L Schiffrin: Contributed to the design and revisions of the manuscript.

Chapter 4: P2RX7 Contributes to Angiotensin II-induced Hypertension, Vascular Injury, and T cell Activation, But Its Involvement in Cardiac Dysfunction Is Unclear

- Brandon Shokoples: Design of experiments, technical setup for the experiments, collection of samples, myography, surgeries for mice, flow cytometry, blood pressure analysis, echocardiography analysis, immunofluorescence and immunohistochemistry, plasma ATP analysis, cell culture experiments, enzyme-linked immunosorbent assays, statistical analysis, writing the manuscript and making the figures.
- Olga Berillo: Preparation of tissues for immunofluorescence and immunohistochemistry, myography (partial), design of PCR primers and running of RT-qPCR.
- Kevin Comeau: Collection of tissues (partial), preparation of samples for flow cytometry and technical input into flow cytometry panels and analysis.
- Hao Yu: Analysis of the UK Biobank and generation of the figures pertaining to the UK Biobank data.
- Akinori Higaki: Collection of samples (partial), myography (partial).
- Antoine Caillon: Collection of samples (partial), and technical advice for flow cytometry.
- Nathanne Dos Santos Ferrai: Collection of samples (partial), myography (partial), cell culture (partial).
- Pierre Paradis: Design of experiments, supervision of work and evaluation of raw data, input to the drafting of the manuscript and figures.
- George Thanassoulis: Supervision and technical input into the analysis of the UK Biobank and generation of figures pertinent to the data obtained from the UK Biobank.
- Jamie Engert: Supervision and technical input into the analysis of the UK Biobank and generation of figures pertinent to the data obtained from the UK Biobank.
- Ernesto L Schiffrin: Originated the study as the principal investigator of a CIHR grant, funding of studies, design of experiments, supervision and evaluation of raw data, and manuscript revision.

Chapter 5: The Role of P2RX7 and the Inflammasome in Angiotensin II-Induced Hypertension

- Brandon Shokoples: Design of experiments, technical setup for the experiments, collection of samples, myography, surgeries for mice (partial), flow cytometry (partial), blood pressure analysis, statistical analysis, writing the manuscript and making the figures.
- Antoine Caillon: Contributed to the design of experiments, collection of samples (partial), surgeries for mice (partial), flow cytometry (partial), technical advice for flow cytometry and editing of the manuscript and figures.
- Bianca Dancose-Giambattisto: Contributed to the design of experiments, collection of samples (partial), surgeries for mice (partial), and myography (partial).
- Kevin Comeau: Collection of tissues (partial), and preparation of samples for flow cytometry (partial).
- Akinori Higaki: Collection of samples (partial), and myography (partial).
- Nathanne Dos Santos Ferrai: Collection of samples (partial), and myography (partial).
- Pierre Paradis: Design of experiments, supervision of work and evaluation of raw data, input to the drafting of the manuscript and figures.
- Ernesto L Schiffrin: Originated the study as the principal investigator of a CIHR grant, funding of studies, design of experiments, supervision and evaluation of raw data, and manuscript revision.

Chapter 6: Resident Memory T Cells: An Avenue of Interest for Future Hypertension Research

- Brandon Shokoples: Design of experiments, technical setup for the experiments, collection of samples, surgeries for mice, flow cytometry, and writing the manuscript and making the figures.
- Kevin Comeau: Preparation of samples for flow cytometry and technical input into flow cytometry panels and analysis.
- Pierre Paradis: Design of experiments, supervision of work and evaluation of raw data, input to the drafting of the manuscript and figures.
- Ernesto L Schiffrin: Originated the study as the principal investigator of a CIHR grant, funding of studies, design of experiments, supervision and evaluation of raw data, and manuscript revision.

Chapter 1: Introduction

Hypertension is estimated to affect 1.4 billion people and is the leading risk factor for mortality worldwide.(1) In fact, in 2017 high systolic blood pressure (SBP) was estimated to account for 10.4 million deaths and 218 million disability-adjusted life years (sum of years lived with disability and years of life lost).(2) In Canada, 42.4% of the adult population is estimated to have hypertension when using the American Heart Association's criteria for hypertension (BP> 130/80 mmHg)(3), with an estimated attributable cost of $13.9 billion or ~10% of national health care spending as of 2010.(4) The past several decades have seen successful campaigns launched in Canada to bring BP control significantly up from 16% in the late 1980's.(5) However, despite intensive efforts, the percentage of hypertension patients meeting a BP target of <130/80 mmHg was only 44% as of 2015(3), with an estimated 10-30% of hypertension patients with resistant hypertension (patients treated with 3 or more anti-hypertensives who still do not have well-controlled BP).(6)

The difficulty in adequately managing patient BP is partly due to the complex and varied pathophysiology involved, with ~90% of cases representing an idiopathic origin.(7) In recent years, evidence has emerged for a prominent role of low-grade inflammation in the development of hypertension.(8) Targeting inflammation and the immune axis has proven successful in pre-clinical rodent models for reducing BP and end-organ damage, but studies in human populations are still in their infancy. In patients suffering from psoriasis, treatment with the immunosuppressive agent mycophenolate mofetil (MMF) resulted in a significant reduction in BP after three months of administration.(9) Removal of MMF resulted in a gradual increase in BP again. A recent study including 811 patients with systemic lupus erythematosus showed that administration of the immunosuppressive drugs MMF or hydroxychloroquine resulted in a significant reduction in BP.(10) These studies gives support to the idea that targeting the immune axis may be beneficial for hypertension management. However, as widespread immunosuppression is not optimal for treating the majority of uncomplicated hypertension cases, a more targeted approach should be favoured. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), involving over 10 000 patients, demonstrated that the risk for recurrent cardiovascular disease (CVD) could be decreased by targeting a single cytokine, interleukin (IL)-1β, (with the monoclonal antibody Canakinumab) without lowering systemic lipid

levels.(11) However, a secondary analysis investigating the effect of IL-1β inhibition on BP saw no reduction in SBP or incident hypertension in the patients receiving Canakinumab, challenging the concept that targeting just IL-1β can lower BP.(12) As targeting broad immune activation with MMF, but not specific targeting of IL-1β, proved effective at lowering BP we wanted to try and identify a specific immune target that still had potential for lowering BP. Upstream of IL-1β signaling is the P2X7 receptor (P2RX7), which besides IL-1β activation, can also influence innate and adaptive immune activation through other mechanisms. This thesis sought to illuminate the role of P2RX7 in mediating immune activation, cardiovascular damage and regulating BP in an angiotensin (Ang) II-induced mouse model of hypertension.

In this chapter, a brief overview of BP regulation will be given, followed by a discussion of the pathophysiology of hypertension centred around elements of Irving Page's mosaic theory of hypertension pertinent to this thesis. Particularly, the involvement of inflammation and the innate and adaptive immune system in hypertension will be discussed in detail. The concept of purinergic signalling will be introduced, and the structural and functional roles of P2RX7 will be addressed. The literature review will include a comprehensive investigation into the involvement of P2RX7 signalling in CVD, including hypertension, atherosclerosis, myocardial ischemic injury, heart failure and stroke. The literature review will be followed by two original research articles and a brief research letter demonstrating a central role of P2RX7 signalling in Ang II-induced immune activation, hypertension, and vascular dysfunction, but not cardiac dysfunction or hypertrophy. Finally, in Chapter 7, P2RX7 mediated immune activation regarding hypertension, vascular injury, and cardiac dysfunction will be discussed, linking to and going beyond the context of the research presented in this thesis.

1.1 Brief overview of Blood Pressure Regulation

Maintaining adequate BP is critical for maintaining consistent blood flow to tissues. If BP is too low, tissues can become hypoxic, whereas prolonged periods of elevated BP result in vascular and cardiac maladaptations. Blood pressure is a reflection of how much blood is pumped from the heart (cardiac output, CO) and the resistance facing the blood (peripheral vascular resistance, PVR). Cardiac output is regulated through changes in heart rate (HR) and stroke volume (SV). SV is affected by preload (venous return and stretching of sarcomeres), contractile force (Frank-Starling Forces) and afterload (pressure needed to expel blood from the heart). Both SV and HR can be controlled by sympathetic and parasympathetic autonomic regulation, vasoactive peptides (such

as atrial natriuretic peptide or adenosine) and gases (nitric oxide [NO], hydrogen disulfide). Peripheral vascular resistance is regulated through vasodilation or vasoconstriction of peripheral arteries, which is also controlled by the autonomic nervous system or through vasoactive peptides. Finally, the kidneys play a critical role in the long-term maintenance of BP via the regulation of sodium and water through a phenomenon known as pressure natriuresis. When BP is high, sodium and water are excreted, lowering extracellular fluid volume and thus BP. Conversely, when BP is low, the renin-angiotensin-aldosterone system (RAAS) is engaged, suppressing pressure natriuresis. This results in the kidneys secreting potassium while preserving sodium and water, thus raising BP.

1.2 Pathophysiology of Hypertension

The origin of hypertension is known in less than 10% of patients, highlighting the complex and varied nature of the disease.(7) In 1949, the "Mosiac Theory" of hypertension was proposed (13) that suggested multiple systems, cardiovascular, nervous, endocrine and renal, were involved in the development of hypertension. This "mosaic" has been expanded several times since reflecting our growing knowledge of the pathology. Recently an updated mosaic was published in the *Canadian Journal of Cardiology* and is presented in Figure 1.1.(7) Of particular relevance to this thesis are the elements of renal participation, neural regulation, oxidative stress, vascular structure, mechanics, and function, cardiovascular participation, and inflammation and will be touched upon in this section.

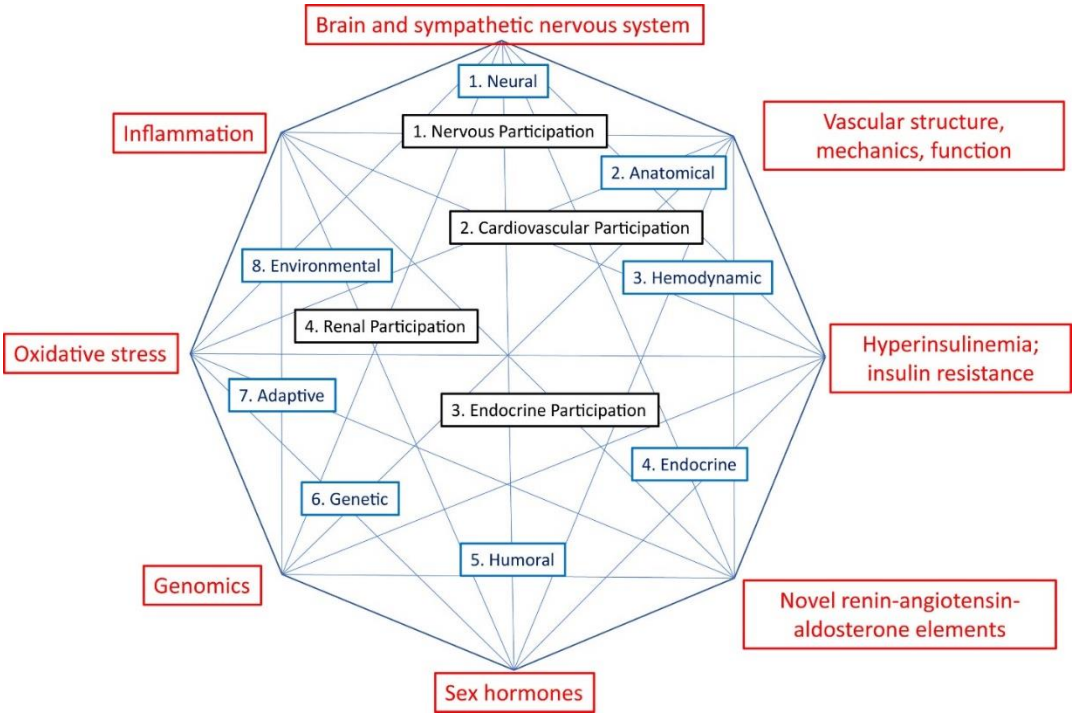

Figure 1.1: An expanded Mosiac Theory of Hypertension. Adapted with permission from Touyz et al. (7). The original 4 elements of Irving Page's mosaic theory of hypertension are presented in black text, with the blue and red elements representing expansions of the mosaic in 1982 and 2020, respectively.

1.2.1 The Kidney

The kidneys play a dominant role in long term BP regulation, as depicted in one of the original nodes in the Mosaic theory, mainly through maintaining water and electrolyte homeostasis. One well-described mechanism through which the kidney maintains BP is through a process referred to as pressure natriuresis. Pressure natriuresis describes the phenomenon in which increases in BP promote renal sodium and water excretion to lower extracellular fluid volume and subsequently BP.(14) In this manner, the kidneys can maintain a relatively constant BP by appropriately adjusting sodium and water excretion according to alterations in BP. It was initially speculated that the increased sodium excretion observed with increases in BP was strictly due to increased renal perfusion pressure. However, even when renal perfusion pressure is experimentally maintained, sodium excretion increases as BP does.(15) Therefore, there must be another mechanism for the increased sodium excretion, and indeed the RAAS can help account for this regulation of sodium homeostasis. This section will briefly describe the RAAS, discuss how the

RAAS can alter pressure natriuresis in hypertension, and then discuss the long-term regulation of BP by the kidney, focusing on sodium and potassium homeostasis.

The RAAS is activated in instances of low BP to enhance sodium and water retention and increase peripheral vascular resistance to raise BP to appropriate levels. Renin production/secretion is the rate-limiting step in the RAAS and is regulated through several mechanisms.(16) Low perfusion pressure of the afferent arterioles, sympathetic nerve stimulation or signalling from macula densa cells in response to low sodium concentrations in the distal tubule, stimulate renin production and release from a set of specialized cells referred to as juxtaglomerular cells. Renin then cleaves angiotensinogen produced primarily from the liver into angiotensin (Ang) I, which is subsequently cleaved by angiotensin-converting enzyme (ACE) expressed on endothelial cells mainly in the lungs but also in peripheral tissues, to the main effector of the renin-angiotensin system, Ang II. Ang II has a plethora of pro-hypertensive actions that contribute to hypertension, including peripheral vasoconstriction, enhanced sympathetic nervous system activity, immune activation and modulation of renal function. Focusing on renal function, Ang II favours sodium and water retention through several mechanisms. Ang II causes vasoconstriction of the afferent and efferent arterioles, reducing renal blood flow, and favouring sodium reabsorption. In addition, Ang II directly promotes sodium reuptake by upregulating the expression of sodium-hydrogen exchangers in the proximal convoluted tubule. The release of anti-diuretic hormone from the pituitary gland is also triggered by Ang II, resulting in increased aquaporin expression in the collecting ducts of the kidney, and increased water reabsorption. As well, Ang II triggers aldosterone synthesis and release from the adrenal glands. In the kidney, aldosterone promotes sodium uptake and potassium excretion through modulating sodium and potassium channel expression.(17) Together, these actions facilitate sodium and water retention contributing to increased extracellular fluid volume and BP.(16)

A central tenet of pressure natriuresis is that as BP is elevated, sodium excretion increases to normalize BP. However, many hypertensive patients without overt renal dysfunction do not have increased sodium excretion despite elevated BP. This observation led to the generation of the theory that hypertension may result from abnormal pressure natriuresis.(14) However, whether abnormal pressure natriuresis is a cause or a consequence of hypertension is an area of debate.(14, 18) Regardless, in instances of chronically elevated BP, such as hypertension, the RAAS should be suppressed, and sodium and water excretion should continue to increase until BP is normalized.

However, RAAS activity is increased in many hypertensive patients, and inhibition of RAAS, either through ACE inhibitors and/or angiotensin receptor blockers (ARBs), decreases BP even in patients without increased RAAS activity.(17) These observations suggest that a dysregulated RAAS may suppress pressure natriuresis and contribute to hypertension. Indeed, endogenous renal production of Ang II is augmented in chronic hypertension (19), and Ang II makes the pressure natriuresis curve much steeper, meaning that a greater rise in BP is needed to maintain appropriate levels of sodium excretion.(20) Experiments conducted by Hall *et al.* assessing the BP and renal response of dogs exposed to sodium challenges, showed that the BP of dogs receiving exogenous Ang II needed to increase by ~40-50 mmHg to maintain sodium balance with non-Ang II-treated dogs.(20) Importantly, administration of the ACE inhibitor captopril (formerly called SQ 14,225), "reset" the pressure natriuresis curve, lowering the BP needed to increase sodium excretion. The rise in the steepness of the pressure natriuresis curve from Ang II is most likely due to the direct (i.e. increased sodium-hydrogen exchanger expression) and indirect (i.e. aldosterone production, inflammation, etc.) effects that Ang II has on sodium retention. Therefore, the importance of the RAAS in regulating BP has led the RAAS axis to become a primary target for anti-hypertensive agents, with ACE inhibitors and ARBs recommended as a first line of treatment in Canada and around the world.(21)

Appropriately balancing electrolytes and extracellular fluid volume is critical for the long-term maintenance of normal BP, and any disturbances in renal function can upset this delicate balance. In fact, disturbances in renal function are common in almost all cases of hypertension, with a decreasing glomerular filtration rate (GFR) correlated with an increased incidence of hypertension.(22) This correlation most likely accounts for the fact that ~90% of patients suffering from chronic kidney disease also are hypertensive.(22, 23) However, abnormal sodium retention, despite a normal GFR, can still lead to hypertension due to an increased extracellular fluid volume. Almost all monogenic forms of hypertension are due to abnormal renal sodium handling.(24, 25) For example, Liddle's syndrome, characterized by early-onset hypertension, can be caused by a single mutation of epithelial sodium channels leading to sodium retention and hypokalemia.(26) Excessive accumulation of sodium can have profound effects on BP and is a reason why dietary interventions to limit sodium intake are recommended to virtually all patients with hypertension, prehypertension or conditions that put them at risk of developing hypertension (i.e. chronic kidney disease, or Liddle's syndrome). Furthermore, it has been estimated that approximately 25% of

people with normal BP and 50% of hypertensive individuals are deemed salt sensitive.(27) Salt sensitivity is defined as abnormal changes in BP in response to high dietary sodium intake, meaning individuals with salt sensitivity have BP that is abnormally reliant on their dietary sodium intake. Dietary interventions focusing on sodium restrictions have been proven effective at reducing BP. For example, in the Dietary Approaches to Stop Hypertension (DASH) trial, switching from the high sodium control diet (~3.5g of sodium/day) to the low sodium diet (~1.2g/day) resulted in an 11.5 mmHg reduction in SBP in hypertensive patients.(28) Interestingly, in normotensive people, switching from the high to low sodium diet also reduced SBP by 7.1 mmHg, highlighting the critical role of sodium in influencing systemic BP.

Although most dietary guidelines mainly focus on sodium, potassium also has a critical role in renal function and BP management. In contrast to sodium, potassium supplementation favours a decrease in BP. Stroke-prone spontaneously hypertensive or Dahl salt-sensitive rats supplemented with potassium had reductions of SBP between 12-16 mmHg.(29) More strikingly, rats supplemented with potassium had a 98% and 93% reduction in mortality (spontaneously hypertensive and Dahl salt-sensitive rats, respectively), with fewer lesions in cerebral arteries even when BP was eliminated as a protective factor. Similar observations have been made in humans with a reported 40% reduction in the risk of stroke-associated mortality with each 10 mmol (~391 mg) increase in daily potassium intake.(30) Furthermore, a recent study carried out in China found that substituting normal salt (sodium chloride) with a potassium salt substitute (25% potassium, 75% sodium chloride) reduced BP by an average of 3 mmHg and significantly reduced the risk of stroke, major cardiovascular events and death.(31) The protective effect of potassium supplementation may be mediated through its reported ability to promote natriuresis through downregulation of the sodium chloride cotransporter.(32) This potassium-mediated natriuresis can still occur even in individuals with elevated aldosterone, which should typically favour sodium retention. These observations may explain why the protective effect of potassium supplementation is more pronounced in individuals with a high salt diet.(32) Another mechanism through which potassium supplementation is thought to be cardioprotective is by reducing vascular dysfunction potentially through interacting with vascular potassium channels.(25) Due to the protective nature of potassium, and detrimental cardiovascular effects of sodium, a dietary sodium/potassium ratio of <1 is recommended and is predictive of better cardiovascular outcomes.(32) However, as mentioned a modern western diet is often rich in sodium and poor in potassium and thus potassium

supplementation may be needed. A concern raised around potassium supplementation is the risk of hyperkalemia, which can be cardiotoxic. However, hyperkalemia is most likely only a concern in patients with severe CKD or those receiving aldosterone antagonists or high doses of other RAAS blockers. For the majority of people with essential hypertension and normal renal function, the risk would be very low. Therefore potassium supplementation, along with sodium restriction provides an effective dietary tool to lower BP and cardiovascular risk.

1.2.2 Neural Regulation

The exact manner through which long-term regulation of BP is coordinated within the nervous system is still an area of investigation, but several regions within the brain are known to contribute to BP control. An intact area postrema appears necessary for the development of chronic, but not acute, hypertension.(33) Ang II-infusion in rats with the area postrema ablated resulted in a similar immediate increase in BP compared to control rats. However, by the second day, rats with an ablated area postrema had a steady decline in BP until the end of the treatment. Regions within the brain are also sensitive to changes in blood osmolality. Salt-sensitive neurons are located within the subfornical organ (SFO) of the hypothalamus and the organ vasculosum of the lamina terminalis (OVLT). The SFO and OVLT regulate BP by suppressing sympathetic nervous system (SNS) activity through the paraventricular nucleus (PVN) and rostral ventrolateral medulla when sodium concentrations are high, leading to lower BP.(34) In addition, humoral stimulation, including from elements of the RAAS, can influence neural regulation of BP. Injection of an Ang II receptor antagonist into the PVN prevented acute BP increases to sodium challenges by decreasing renal sympathetic activity.(35) Furthermore, Ang II can also activate the SFO in the brain via reactive oxygen species (ROS) signalling, amplifying SNS activity.(36, 37) Together, these regions of the brain monitor incoming afferent signals from baroreceptors (discussed later), respond to humoral stimulation (RAAS signals) and/or directly sense the osmolality of the blood and adjust sympathetic and parasympathetic neuronal signalling to the heart, peripheral vasculature and the kidney as required to moderate BP.

A model has been proposed for long-term BP regulation in which there is a central nervous system (CNS)-mean arterial pressure (MAP) set-point.(38, 39) This theory posits that the CNS adjusts sympathetic and parasympathetic signalling as needed to maintain an appropriate MAP for cerebral and coronary perfusion. In hypertension, it is thought that this CNS-MAP set-point is reset to a higher BP threshold, resulting in enhanced SNS activity. In support of this theory, hypertensive

patients often display enhanced SNS activity.(40) At the level of the heart, enhanced SNS activity increases HR and SV. In the periphery, this enhanced SNS activity can result in vasoconstriction and increased peripheral vascular resistance. Whereas in the kidneys, increased SNS stimulation induces afferent arteriole vasoconstriction, reducing GFR and promoting renal tubular reabsorption and sodium retention. The SNS can also engage the RAAS by causing renin release from juxtaglomerular cells, thereby promoting downstream hypertensive responses through Ang II.(41) In addition, Ang II triggers a positive feedback loop by stimulating the SFO, further amplifying SNS activity.(36, 37) As SNS activation of the kidney is a critical element in BP regulation, there have been numerous clinical trials investigating the effectiveness of renal denervation in resistant hypertension with generally favourable results in BP reduction.(42) A recent review article summarized long-term findings in resistant hypertensive patients. Twelve months after renal denervation, a median office SBP reduction of 12.3-23.6 mmHg was reported across nine trials; after 24 months, a median reduction of ~12-30 mmHg across 4 trials, and after 36 months, ~15-30 mmHg across two trials, suggesting long-term effectiveness for reduction of SBP in resistant hypertensive patients.(43)

It is also necessary to mention the role of baroreceptors and peripheral chemoreceptors in the regulation of BP. Acutely, blood pressure is regulated through the baroreflex, which works to minimize fluctuations in BP. In response to high BP, specialized neurons called baroreceptors in the wall of the aortic arch and carotid sinus stretch, inducing increased firing of action potentials down the vagus nerve and the carotid sinus nerve (a branch of the glossopharyngeal nerve), respectively to the nucleus of the tractus solitarius in the brainstem. The result is suppression of the SNS and activation of the parasympathetic nervous system (PNS), leading to acetylcholine (Ach) release. In the periphery, Ach induces vasodilation, whereas in the heart, Ach decreases HR and SV and concomitantly, BP. Conversely, low BP induces the opposite effect, enhancing SNS activity while reducing PNS activity. The SNS releases norepinephrine (NE), causing peripheral vasoconstriction, increasing HR and SV, thus increasing blood pressure. Peripheral chemoreceptors are present in the aortic and carotid bodies monitoring blood oxygen, carbon dioxide and pH. Decreased blood oxygen, pH and/or increased carbon dioxide trigger sympathetic activation in a similar manner to the baroreceptor response to low BP, working to increase SV, HR and BP. The baroreceptors and chemoreceptors work in conjunction to maintain BP and blood oxygenation to ensure adequate tissue perfusion.

Early studies investigating baroreceptor denervation suggested that baroreceptors play a role in acute but not chronic BP management. Baroreceptor denervation of healthy dogs resulted in a temporary increase in BP, with BP values gradually returning to normal.(44) Furthermore, electrical baroreceptor activation (BA) in dogs lowered plasma NE but failed to enhance renal secretion of sodium and water, thus failing to lower BP in Ang II treated dogs.(45) Therefore, it has been suggested that RAAS inhibition may be a prerequisite for robust antihypertensive responses to BA.(46) However, numerous studies have since emerged validating that baroreceptors do in fact play a role in chronic BP management and can present a viable target in hypertension.(34, 46) Chronic electrical stimulation of the baroreceptors (triggering suppression of the SNS), has shown success in clinical hypertension.(46) Three major clinical trials have been completed investigating the effectiveness of BA in resistant hypertension: the US Rheos Feasibility Trial(47), the DEBuT-HT Trial(48), and the Rheos Pivotal Trial.(49) From these trials, long-term follow-up data were available for over 100 patients and were recently analyzed.(50) In the 143 patients who completed 5 years of follow-ups, a sustained reduction in SBP of greater than 30 mmHg with chronic BA was evident. In addition, 58 patients had follow-up data available for 6 years, and these patients displayed an average SBP reduction of 35 mmHg.(50) A second-generation BA device was recently tested in the Barostim trial and showed comparable results with an average SBP reduction of 26 mmHg after 6 months.(51) Six of the patients from the Barostim trial had previously undergone renal denervation therapy. SBP decreased by an average of 22 mmHg in these patients, suggesting that BA suppresses BP independently from decreasing renal SNS activation. An important observation from these trials was that when the BA devices were turned off during visits, a rapid rise in BP occurred in the patients, suggesting continual BA is needed for an anti-hypertensive effect.(48)

It has been suggested that there is resetting of baroreceptors in hypertension, whereby the baroreceptors adapt to the higher BP and fail to suppress SNS activity.(34) Baroreceptor resetting could account for the continual BA required to suppress BP in the patients from the DEBuT-HT Trial. Interestingly, baroreceptor resetting may be in part mediated through chemoreceptors. In spontaneously hypertensive rats, the chemoreceptors in the carotid body are tonically active, leading to enhanced sympathetic activation and higher BP.(52) Attenuating chemoreceptor signalling by carotid sinus nerve denervation (CSD) resulted in robust BP decreases. Baroreceptor sensitivity increased after CSD, reflecting a left-ward shift in the sympathetic activity/MAP curve.

In simpler terms, baroreceptor-mediated suppression of sympathetic activity was enhanced at a lower MAP, resulting in a restored baroreceptor reflex. Importantly CSD in normotensive Wistar rats did not affect baroreceptor sensitivity, strongly suggesting that chemoreceptors contribute to baroreceptor resetting only in hypertensive states. A further finding of the study was that renal sympathetic activity and T cell infiltration were also suppressed with CSD. This suggests that altered CSD responses may have wide-reaching effects in hypertension, contributing to enhanced sympathetic activity and BP through several mechanisms. A safety and feasibility trial of unilateral carotid body ablation was recently conducted in 15 patients with resistant hypertension.(53) Carotid body ablation successfully lowered ambulatory BP by >10mmHg (average of 23mmHg) after 3 months in 8 (53.3%) of the patients, with a sustained average decrease in BP of 12 mmHg after 12 months. Decreased sympathetic activity (measured by muscle sympathetic nerve activity in the arm) was observed only in the responders to the therapy but not in the non-responders. Similarly, baroreflex responses were improved in responders 6 months after carotid body resection. This early trial suggests carotid body ablation may be a more beneficial therapy for treating resistant hypertension than BA. A limitation of BA therapies is the need for chronic electrical stimulation and the replacement of the batteries in the devices (~3 years in early systems). In contrast, chemoreceptor ablation is a one-time intervention that seems to improve the baroreceptor reflex as well as lowering BP.

1.2.3 Oxidative Stress

ROS forms as a natural by-product of metabolism, is used as a cellular signalling molecule inducing reversible oxidation of targets, and is also an essential component of cellular defence against pathogens. In normal physiology, the body has antioxidant molecules such as superoxide dismutase (SOD), which scavenge excess ROS preventing the damaging effects of unchecked ROS. The oxidative stress theory of disease suggests that increased levels of ROS (superoxide anion, peroxynitrite, hydrogen peroxide, etc.) induces cellular damage and death by adversely affecting DNA, RNA, proteins, lipids and carbohydrates essential for cell functioning.(54) As early as the 1990's, a predominant role of ROS in the pathogenesis of hypertension was evident. Rajagopalan *et al.*(55) demonstrated that Ang II-infusion, but not NE, induced ROS generation through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), promoting endothelial dysfunction. Treatment with losartan (an AT1R antagonist) or SOD diminished Ang II-induced endothelial dysfunction. Similarly, high salt intake also augments ROS production, with

a high salt diet resulting in increased NOX and decreased SOD activity, leading to an unfavourable prooxidant/antioxidant profile.(56) Numerous studies since have demonstrated that various NOXes contribute to ROS mediated endothelial damage, vascular dysfunction, cardiovascular remodelling, renal dysfunction, SNS excitation, immune cell activation, and systemic inflammation, contributing to the pathogenesis of hypertension.(57)

Enhanced activity of NOXes is not the only means of ROS production in the context of hypertension. NO synthase (NOS) is the primary generator of NO in endothelial cells, which under normal conditions favours vascular dilatation. In the presence of oxidative stress, such as in hypertension, endothelial NOS (eNOS) can become uncoupled, leading to the production of O_2^- radicals.(57) Other significant sources of ROS in hypertension include endoplasmic reticulum (ER) stress, xanthine oxidase and mitochondrial dysfunction, with targeting these sources of ROS showing variable efficacy in suppressing hypertension and end-organ damage.(57) Targeting oxidative stress in general has had mixed results in clinical trials, but recent compounds such as Resveratrol which enhance the expression of endogenous antioxidants (SOD, catalase, and glutathione peroxidase), improved vascular function in hypertensives(58) and reduced blood pressure when combined with standard therapy in hypertensive patients.(59) Many of the anti-hypertensives in the clinic, including renin inhibitors, ACEi, AT1R inhibitors, and aldosterone inhibitors (eplerenone), increase eNOS expression while simultaneously decreasing NOX expression, normalizing NOS activity (i.e. decreasing NOS uncoupling).(60) As ROS plays a prominent role in most aspects regarding the pathology of hypertension, it will be brought up throughout the subsequent sections of the thesis.

1.2.4 Vascular Structure, Mechanics, and Function

The vasculature plays a vital role in regulating blood pressure passively through elastic compliance of the vessels and actively through vasoconstriction and vasodilation. Both the large conduit arteries and the small resistance arteries contribute significantly to peripheral vascular resistance and thus BP.(61) According to Poiseuille's law, which states that pressure is inversely proportional to the fourth power of the vessel radius, even small changes in luminal diameter can contribute substantially to changes in BP. Hypertension is often associated with arterial stiffening (loss of elastic compliance), hypercontractility and impaired vasodilation (endothelial dysfunction).(16) In the large conduit arteries, maladaptive vascular remodelling in hypertension leads to medial thickening, often accompanied by deposition of collagen and/or fibronectin in the medial and

adventitial layers contributing to vascular stiffening. Pulse pressure increases as the artery's ability to distend and dampen the pulsatile flow diminishes. As such, the forward pulse wave extends deeper into the microvasculature contributing to endothelial injury and target organ damage.(62) An intact endothelium is required for proper vasodilation responses(63), and as such endothelial dysfunction emerges as a result contributing to increased peripheral resistance.(62)

Myogenic tone is a feature of resistance arteries that preserve the blood vessel's internal diameter at ~50-80% of their maximal passive diameter to allow the vessel to vasodilate or vasoconstrict in response to stimuli to maintain adequate blood flow.(64) In response to acute increases in BP, vascular smooth muscle cells are stretched, resulting in a vasoconstrictor response increasing peripheral resistance. There is evidence of enhanced myogenic tone in hypertension, contributing to increased systemic BP and vascular remodelling.(61) When BP is elevated chronically, blood vessels undergo maladaptive vascular remodelling in the form of increased vascular wall thickness or decreased lumen diameter to maintain appropriate wall tension according to the Law of LaPlace.(65, 66) Vascular remodelling can be either eutrophic or hypertrophic. Eutrophic remodelling occurs when there is an equal combination of cell growth and apoptosis, leading to an increased media/lumen ratio but preserved cross-sectional area (CSA).(61) Whereas in hypertrophic remodelling, cellular growth outpaces cellular apoptosis resulting in the growth of the media and a narrowing of the lumen leading to an increased M/L and CSA. Hypertrophic remodelling is associated with severe hypertension, whereas eutrophic remodelling is often associated with stage 1 or less severe hypertension. Further, hypertrophy of resistance arteries is associated with an increased risk of adverse cardiovascular events in hypertensive individuals.(67)

As discussed above, multiple systems regulate vascular tone. The CNS regulates vascular tone via sympathetic or parasympathetic signalling (i.e. NE or Ach release). Elements of the RAAS axis, such as Ang II, act as potent vasoconstrictors.(16) Often vascular dysfunction is accompanied by immune infiltration (discussed below) to the perivascular adipose tissue (PVAT) and contributes to an inflammatory milieu, including ROS production that further exacerbates vascular dysfunction and remodelling, leading to increased peripheral vascular resistance and subsequently BP.

1.2.5 Cardiac Participation

CO, a determinant of SV and HR, is fundamental in maintaining BP. Some instances of secondary hypertension (hypertension due to an identifiable underlying condition) derive from a pathologic excess CO. Some of the more common causes of secondary hypertension resulting from increased CO will be briefly discussed below. Primary aldosteronism is the most common form of secondary hypertension, accounting for >5% of hypertension.(68) In primary aldosteronism, there is increased adrenal aldosterone secretion, along with suppressed renin, resulting in volume overload. This volume overload leads to an increased venous return to the heart and subsequently increased CO and BP. In fact, treated patients with primary aldosteronism, have 8% higher CO than treated patients with essential hypertension, potentially accounting for the higher BP observed in patients with primary aldosteronism.(69) Similarly, the presence of an arteriovenous fistula in patients can lead to increased venous return, with a subsequent increase in CO and BP. Anemia is another condition that can lead to increased CO and subsequently hypertension.(70) Patients with anemia often experience hypoxia, and in order to increase tissue perfusion, peripheral vasodilation occurs, decreasing cardiac afterload, along with tachycardia, resulting in increased CO. Hyperthyroidism can lead to isolated systolic hypertension by increasing CO through an increase in HR and cardiac contractility, with a simultaneous decrease in peripheral vascular resistance.(71) Other conditions such as aortic regurgitation cause cardiac adaptations to increase SV and CO to overcome the regurgitant blood flow, and when unaddressed, can lead to hypertension.(72) One last example is pheochromocytoma, which accounts for ~0.2-0.6% of hypertension.(73) Pheochromocytoma is a condition where patients present with a tumour of chromaffin cells from the adrenal gland resulting in excess production of catecholamines, especially NE, and more rarely epinephrine. The excess production of epinephrine, when this is the major catecholamine produced in some cases of pheochromocytoma, increases cardiac contractility, and induces tachycardia, as well as causes peripheral vasoconstriction, all contributing to the development of hypertension. In summary, conditions that induce chronic cardiovascular adaptations leading to increased CO, can contribute to the development of secondary hypertension. However, typically in pheochromocytoma when norepinephrine is the major catecholamine produced in the majority of cases, intense vasoconstriction leads to reduced blood volume, which can result in severe orthostatic hypotension associated with supine hypertension.

Most often, cardiac maladaptation appears as an effect of prolonged hypertension, rather than a causal agent. In healthy vasculature, a reflected pulse wave occurs when the forward pulse wave reaches a point of changing impedance (i.e. a transition to a vessel with a smaller diameter) and returns to the heart typically during early diastole. The return of the waveform during early diastole causes a favourable rise in diastolic pressure allowing for adequate perfusion of the coronary arteries.(62) As arteries stiffen, the reflected pulse wave arrives earlier at the heart during systole, amplifying SBP, diminishing coronary perfusion, and increasing the pulse pressure. As afterload increases, the left ventricle (LV) must work harder to eject blood from the heart, with less coronary perfusion to satiate the increased demand. The result is maladaptive LV hypertrophy, often accompanied by cardiac fibrosis. LV hypertrophy is a characteristic feature of hypertension(74) and is an independent risk factor for cardiovascular events and mortality in hypertensive subjects.(75)

Enhanced cardiac sympathetic activity has been demonstrated in hypertensive subjects with LV hypertrophy, potentially contributing to hypertension in these subjects.(76) Clinical trials have shown that RAAS blockade by losartan, an Ang II receptor antagonist (77), or by ACE inhibitors (78) results in regression of LV hypertrophy in patients with essential hypertension. However, even if blood pressure is normalized, well-managed hypertensives still have a 50% increased risk for any adverse cardiovascular event, highlighting the need for better cardioprotective interventions.(79)

1.2.6 Inflammation

Inflammation is a critical element of wound healing and the body's defence against pathogens. However, it is now well accepted that chronic inflammation contributes to CVD and hypertension development.(80) Increased inflammasome gene expression and circulating IL-1β in subjects over the age of 60 are strongly associated with increased risk for hypertension and vascular dysfunction, as well as all-cause mortality.(81) Many different groups since 1976 using different approaches and different models of hypertension have repeatedly shown the critical role of immune cells as modulators of hypertension development and end-organ damage.(82, 83) This section will focus on elements of the innate and adaptive immune system pertinent to the development of hypertension.

1.2.6.1 Innate Immunity

The innate immune system is considered in many organisms as the first line of defence against pathogens. It is composed of various cell types, of which monocytes/macrophages and dendritic cells (DCs) have garnered particular interest concerning hypertension. Innate immune cells possess robust immune sensors activated by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs and DAMPs trigger innate immune activation through pattern recognition receptors such as toll-like receptors (TLRs), and including inflammasomes, both of which have been reported to contribute to hypertension development.(84, 85) Inflammasomes are a family of multi-protein complexes assembled by pattern recognition receptors in response to PAMPs or DAMPs. The NLRP3 inflammasome is perhaps the best characterized of the known inflammasomes and will be discussed in section 1.4.4, whereas TLRs will be discussed below.

1.2.6.1.1 Toll-Like Receptors

There are currently 11 TLRs documented in humans (TLR1-11), which respond to varied PAMPs or DAMPs (i.e. single-stranded or double-stranded RNA or DNA), triggering an inflammatory response.(86) TLR3, TLR6 and TLR9 have all been reported to contribute to hypertension or cardiovascular disease, but the most extensively studied TLR regarding hypertension and cardiovascular injury is TLR4.(86) TLR4 responds to PAMPs such as extracellular lipopolysaccharides (LPS) and DAMPs such as high mobility group box 1 protein or heat shock proteins, triggering the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway which upregulates pro-inflammatory elements such as NLRP3, caspase-1 and pro-IL-1β (discussed later). A recent review article summarized the major findings regarding TLR4 pertinent to hypertension, identifying eight studies that reported a decrease in BP with TLR4 antagonism or KO and three studies that did not.(87) Regardless of affecting BP or not, all of the studies reported that TLR4 promoted oxidative stress and, therefore could contribute to the pathogenesis of hypertension. In fact, it has been shown that TLR4 can directly engage NOX4 to facilitate ROS production.(88) Further, TLR4 mediated ROS generation has been linked to increased vascular contractility and endothelial dysfunction, which could be reversed with TLR4 antagonism (89).

1.2.6.1.2 Monocytes/Macrophages

Monocytes are innate immune cells of a hematopoietic origin that typically patrol the blood watching for infection or tissue damage. Upon encountering sites of inflammation, monocytes can egress into tissues where they differentiate into macrophages or dendritic cells. Macrophages are typically classified as either pro-inflammatory M1 macrophages secreting cytokines such as IL-1β, IL-23, and TNF-α, or M2 macrophages secreting IL-10 favouring inflammation resolution and tissue repair. In 2005, our laboratory demonstrated that mice devoid of macrophages (macrophage colony-stimulating factor [M-CSF] deficient mice) were protected against Ang II-induced BP elevation, vascular remodelling and endothelial dysfunction.(90) This was the first demonstration of involvement of the innate immune system in an experimental model of hypertension. Similar findings were later reported in M-CSF deficient mice in a deoxycorticosterone acetate (DOCA) salt model of hypertension.(91) Furthermore, depletion of lysozyme M-positive monocytes was also demonstrated to be protective against the development of Ang II-induced hypertension and vascular injury,(92) reinforcing the notion that monocytes/macrophages contribute to the development of hypertension.

Interactions with endothelial cells may in part mediate the activation of monocytes in hypertension. Stretching of endothelial cells promotes activation of monocytes with increased production of the pro-inflammatory cytokines IL-6, IL-1β, IL-23 and TNF-α.(93) The authors found decreased NO production of the endothelial cells coupled with increased hydrogen peroxide production, suggesting NOS uncoupling. Scavenging hydrogen peroxide or blocking IL-6 could prevent monocyte activation, suggesting endothelial ROS and IL-6 signalling in response to stretching can activate monocytes/macrophages in hypertension. Macrophages may also play a critical role in salt-sensitive hypertension. High sodium chloride concentrations can promote activation of murine bone-marrow-derived macrophages (BMDM), triggering inflammasome-dependent IL-1β signalling.(94) Macrophages also have a prominent role in extrarenal sodium handling. The skin has recently been identified to contribute to extrarenal sodium handling, providing a storage site of osmotically inactive sodium (95), with macrophages playing a pivotal role in maintaining interstitial sodium and water homeostasis.(96) Depletion of macrophages in the skin led to impaired electrolyte and water clearance through the lymphatic system and promoted the development of salt-sensitive hypertension.(97) Therefore, it is unclear if widespread

targeting of monocytes/macrophages would be effective in treating hypertension, particularly in salt-sensitive hypertension, and instead, a more selective approach may be desired.

1.2.6.1.3 Dendritic Cells

DCs have also been demonstrated to play a critical role in hypertension development. DCs are potent producers of IL-1β and IL-23 and are professional antigen-presenting cells, playing a key role in activating the adaptive immune system. Fms-like tyrosine kinase 3 ligand knockout (KO) mice are depleted of classical DCs and have reduced blood pressure elevation and renal inflammation after 4 weeks of Ang II infusion.(98) In another study, depletion of $CD11c^+$ DCs blunted the development of hypertension in response to Ang II plus a high salt diet.(99) Excessive extracellular salt is thought to be a key activator of DCs in the context of hypertension. Mice lacking the salt sensing serum/glucocorticoid kinase1 (SGK1) in $CD11c^+$ DCs prevented the development of hypertension and endothelial dysfunction in a L-NAME/high salt model of hypertension.(100) In addition, extracellular sodium can act on DCs through amiloride-sensitive epithelial sodium channels and sodium hydrogen exchangers to facilitate ROS production.(101) The resulting ROS generation then leads to the production of isolevuglandins, which are isoketal adducts, that are subsequently presented by DCs to T lymphocytes, driving T cell activation. Further studies have shown that B7 ligand (CD80/CD86) interactions with CD28 on T-cells is critical for DC-T-Cell priming in hypertension. Genetic KO of B7 ligands or use of a B7 ligand inhibitor, abatacept (CTLA4-Ig), resulted in diminished T cell activation and blunted Ang II-induced hypertension and vascular inflammation.(102) In addition, CD86-CD28 interactions facilitate activation of IFN-γ producing $CD8^+$ T cells in hypertension, and this interaction was shown to occur in a P2RX7 dependent manner(discussed in detail later).(103) In summary, DCs contribute to hypertension and end-organ damage by promoting activation of the adaptive immune system through co-stimulatory molecule interactions and proinflammatory cytokine signalling.

1.2.6.1.4 Neutrophils

Neutrophils are the most abundant granulocyte and are often one of the first cells recruited to sites of injury or inflammation. Direct evidence for the involvement of neutrophils in the pathogenesis of hypertension is currently scarce. Increased circulating levels of neutrophils were found to be correlated with an increased risk of developing hypertension, particularly among women, in a Japanese cohort of patients.(104) The authors speculated neutrophils could augment hypertension through ROS generation and subsequent vascular dysfunction. In support of this, neutrophil

extracellular trap (NET) production can be induced by ROS and by the plasma from hypertensive patients.(105) NETs are extracellular web-like structures containing decondensed chromatin and granule proteins including myeloperoxidase, that are normally used to trap and neutralize pathogens. However, NETs have been also demonstrated to induce vascular injury,(106, 107) and the observation that NETs accumulate within the kidneys and aortas of hypertensive patients supports the notion that NETs can contribute to end-organ damage in hypertension.(105) Furthermore, isolevuglandins (discussed above) can induce NET production in hypertension,(108) and NETs have been found to induce NLRP3 inflammasome activation in a P2RX7 dependent fashion.(109) Thus, it is likely neutrophils contribute to the inflammation and subsequent vascular and kidney damage in hypertension. However, adoptive transfer of neutrophils alone into mice devoid of myeloid cells failed to recapitulate hypertensive responses to Ang II suggesting neutrophils work in concert with other myeloid cells to promote hypertension development.(92)

1.2.6.1.5 Natural Killer Cells

Natural killer (NK) cells are cytotoxic lymphocytes of a common origin of T and B cells, but classified as innate immune cells, that respond rapidly to clear infected host cells or cancer cells. In 2013, Kossmann *et al.*(110) showed that depletion of NK cells prevented vascular dysfunction in an Ang II model of hypertension. In particular, they demonstrated that NK cells are major producers of IFN-γ that in turn contributed to vascular dysfunction. However, studies using *CD1d*$^{-/-}$ mice, mice devoid of NK cells, or *Ja18*$^{-/-}$ mice, mice devoid of invariant NKT cells, demonstrated that NK cell depletion was insufficient to lower BP in Ang II-induced models of hypertension.(111) Furthermore, Wang *et al.*(112) demonstrated that *CD1d*$^{-/-}$ mice had exacerbated Ang II-induced cardiac dysfunction due to decreased IL-10 producing NKT cells. Therefore it appears NK cells have a dichotomous role in hypertension, with IFN-γ producing NK cells contributing to vascular dysfunction and IL-10 producing NK cells attenuating cardiac dysfunction.

1.2.6.1.6 Myeloid-Derived Suppressor Cells

A less understood innate cell type, in regard to their role in hypertension, are myeloid derived suppressor cells (MDSCs). MDSCs work to reign in the adaptive immune response, supressing T cell activation and subsequently inflammation.(113) Using three separate models of hypertension (Ang II, L-NAME and high salt models) Shah *et al.* (114) demonstrated that the number MDSCs increase during hypertensive insults, possibly in an attempt to restrain excessive inflammation.

Depletion of MDSCs with a chemotherapeutic agent, Gemcitabine, resulted in an exacerbated BP increase in response to Ang II-infusion, which could be abrogated by adoptively transferring back MDSCs.(114) This study suggests that MDSCs play a role in suppressing inflammation in the context of hypertension, but further studies are needed to elucidate a therapeutic role for these cells.

1.2.6.2 Adaptive Immunity

A pivotal paper by Guzik *et al*. in 2007 unleased a slew of research into the role of adaptive immunity in hypertension.(115) Using recombination-activating gene 1 (*Rag1*) KO mice, mice devoid of B and T lymphocytes, they demonstrated that these mice had blunted BP elevation and endothelial dysfunction in response to Ang II infusion or DOCA salt treatment. Adoptive transfer of T cells, but not B cells into the *Rag1* KO mice restored the BP response and endothelial dysfunction associated with Ang II or DOCA salt treatments, suggesting that T-lymphocytes were a predominant driver of BP increase and endothelial dysfunction observed in hypertension. These results have come under scrutiny as other studies have failed to establish robust protective effects of *Rag1*$^{-/-}$ mice in Ang II or high salt diet hypertensive models.(116-119) Differences in results are speculated to be due to an unknown genetic drift in the colony provided by Jackson Laboratories (United States).(116) Although many questions remain surrounding the phenotypic change of the *Rag1* KO mice from Jackson Laboratories, the role of T cells in hypertension remains unchallenged as many studies have since emerged cementing their role in the development of hypertension.

T lymphocytes are generally categorized by their T cell receptor (TCR) subunit, with $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells representing a greater proportion than T cells bearing a $\gamma\delta$ TCR.(120) Each abovementioned subset of T cells has been shown to play a part in the development of hypertension and will be discussed in the subsequent sections.

1.2.6.2.1 IL-17 Producing $CD4^+$ T Cells

$CD4^+$ T cells are subdivided into helper (Th) and regulatory (Treg) subsets with distinct roles in the pathophysiology of hypertension. IL-17A producing $CD4^+$ T cells (Th17) are increased in blood samples collected from hypertensive patients versus normotensive controls(121), and a positive correlation has been found between serum IL-17A concentration and duration of hypertension in a group of patients with uncontrolled hypertension.(122) IL-17A KO mice do not

sustain increases in blood pressure in response to 3 weeks of Ang II infusion compared to WT mice, although blood pressure did remain elevated compared to sham.(123) Antibodies targeting IL-17A and IL-17RA yielded a similar decrease in blood pressure to IL-17A KO animals in response to Ang II infusion.(124) A culmination of the literature suggests IL-17 contributes to hypertension by promoting superoxide production while inhibiting NO production, contributing to aortic stiffening by enhancing collagen synthesis, favouring hypertrophic remodelling of resistance arteries and promoting renal sodium transporter abundance and activity.(80)

1.2.6.2.2 $CD8^+$ T Cells

Although Th17 cells have a clear involvement in the pathogenesis of hypertension, they may not be essential. In 2014, Trott *et al*.(125) showed that mice devoid of $CD8^+$ T cells were protected against Ang II-induced hypertension and vascular dysfunction, whereas mice devoid of $CD4^+$ T cells were not. Furthermore, adoptive transfer of $CD8^+$, but not $CD4^+CD25^-$ T cells into *Rag1*$^{-/-}$ mice restored hypertensive responses to Ang II. The authors also noted that in response to a sodium/volume challenge, *CD4*$^{-/-}$ mice infused with Ang II retained water and sodium, whereas *CD8*$^{-/-}$ mice did not, suggesting a role for $CD8^+$ T cells in regulating kidney function during hypertensive challenges. In favour of this, $CD8^+$ T cells have been demonstrated to increase the activity of the Na-Cl transporter in the distal tubule of the kidney through a ROS-mediated mechanism.(126) This increased activity leads to retention of Na^+, promoting salt-sensitive hypertension.

$CD8^+$ T cells are also major producers of IFN-γ, ($CD4^+$ Th1 and γδT1 cells also produce IFN-γ) which in of itself has been demonstrated to contribute to hypertension. Mice overexpressing IFN-γ develop endothelial dysfunction in the absence of Ang II, and *IFN-γ*$^{-/-}$ mice infused with Ang II were largely protected from Ang II-induced vascular dysfunction.(110) Although the authors of this study did not report on differences in blood pressure between WT and *IFNγ*$^{-/-}$ mice, other work done by Kamat *et al*.(127) found decreased SBP in *IFNγ*$^{-/-}$ mice infused with Ang II in comparison to Ang II-infused WT mice. IFN-γ production from $CD8^+$ T cells in the kidney also promotes upregulation of MHC-1 and programmed death-ligand 1 (PDL1) in distal tubule cells, leading to the recruitment of more T cells to the kidney and upregulation of Na-Cl transporters as discussed above.(128) Hypertensive humans have increased circulating levels of IFN-γ producing senescent $CD8^+$ T cells compared to normotensive controls.(129) Pan *et al.* demonstrated that adoptive transfer of aged (senescent) T cells into *Rag1*$^{-/-}$ mice accelerated Ang II-induced

cardiovascular and renal fibrosis by promoting IFN-γ and superoxide production.(130) Adoptive transfer of senescent T cells from *IFNγ*$^{-/-}$ mice displayed attenuated renal, cardiovascular and vascular fibrosis and dysfunction. This suggests that as T cells become senescent, they produce more IFN-γ contributing to target organ damage. This could help explain in part why hypertension associated target organ damage is more prevalent in geriatric populations, although this remains to be investigated.

1.2.6.2.3 T Regulatory Cells

Counterbalancing the pro-inflammatory immune cells are the anti-inflammatory Tregs. Tregs are a subset of CD4^{+} T cells, characterized by expression of the markers CD25 and forkhead box P3 (FOXP3), that suppress immune effector functions primarily through IL-10 secretion. Our lab in 2011 demonstrated that hypertension induced by 2-weeks of Ang II-infusion reduced the frequency of Foxp3^{+} Tregs in the renal cortex of mice, and adoptive transfer of Tregs attenuated Ang II-induced increases in BP, ROS, endothelial dysfunction and vascular remodelling.(131) The role of Tregs in hypertension was further refined using T cells from Scurfy mice, which have a mutation in the FOXP3 gene and are devoid of T regulatory cells. Adoptively transferring Scurfy T cells into Ang II-infused *Rag1*$^{-/-}$ mice resulted in hypertension and endothelial dysfunction, which could be attenuated by co-injecting WT Tregs with the scurfy T cells.(118)

That the protective effect of Tregs is mediated through IL-10 is evident from several studies. First, *IL-10*$^{-/-}$ mice have deteriorated endothelial dysfunction compared to WT mice in response to Ang II treatment, which could be reduced by scavenging ROS, suggesting IL-10 mitigates ROS accumulation.(132) Adoptive transfer of WT Tregs, but not Tregs from *IL-10*$^{-/-}$ mice, reduced Ang II-induced SBP elevation, endothelial dysfunction and NOX activity.(133) Furthermore, infusion of IL-10 alone blunts Ang II-induced BP elevation in mice.(133, 134) Taken together, these studies illustrate that Tregs play a protective role in mitigating inflammation and oxidative stress in hypertension, primarily through IL-10 signalling.

1.2.6.2.4 γδ T cells

γδ T cells are a small subset of T cells (~1-10% of circulating immune cells in mice and humans) expressing γδ T cell receptors (TCR) instead of conventional αβ TCRs. γδ T cells are unconventional, “innate-like” lymphocytes that can respond rapidly to proinflammatory stimuli without requiring antigen processing or MHC presentation, triggering cytokine production and

cytotoxic activity.(135) In a multi-ethnic study of atherosclerosis including 1195 patients, a one standard deviation elevation in circulating γδ T cells correlated with a 2.4mmHg increase in SBP, suggesting a role for γδ T cells in human hypertension.(136) Supporting this, γδ T cells were found to be the primary producers of IL-17A in a murine model of Ang II-induced hypertension and depletion of γδ T cells protected against Ang II-induced cardiac fibrosis.(137) We have further shown the importance of γδ T cells in the progression of hypertension.(138) Ang II treatment caused an increase in the number and activation of γδ T cells in the spleen after 7 and 14 days of Ang II infusion. Deficiency in γδ T cells due to *Tcrδ* KO or injection of γδ T cell-depleting antibodies, prevented Ang II-induced BP elevation, small artery endothelial dysfunction and activation of $CD4^+$ and $CD8^+$ T cells in the mesenteric PVAT. Therefore, although only representing a small fraction of total T cells, γδ T cells appear to play a critical role in the pathogenesis of hypertension and end-organ damage.

1.2.6.2.5 B Cells

Despite the paper by Guzik *et al.* in 2007(115) that suggested B cells do not have a role in hypertension, several groups have since shown that B cells may contribute to hypertension development. B-cell–activating factor receptor-deficient (*Baff-r*$^{-/-}$) mice are devoid of B cells and have a modest decrease in SBP compared to WT mice treated with Ang II.(139) These mice were also protected from aortic stiffening and had decreased IgG, macrophage, TGF-β and collagen accumulation in the aorta. These protective effects could be reversed by adoptively transferring B cells back to the *Baff-r*$^{-/-}$mice. Similarly, depletion of B cells with anti-CD20 antibodies recapitulated the results observed in *Baff-r*$^{-/-}$ mice.(139) Another mouse line with diminished B cells, hypomorphic corresponding nuclear transcription factor (*c-Myb*$^{h/h}$) mice, demonstrated lower basal SBP and DBP and displayed attenuated hypertensive responses to DOCA-salt treatment.(140) Although *c-Myb*$^{h/h}$ mice are known to have deficiencies in other immune cell subsets. To further validate a role of B cells, the authors also used J_HT mice, mice that fail to develop immature or mature B cells and found similar reduced basal BP. Interestingly both *c-Myb*$^{h/h}$ and J_HT mice had increased 24-hour urine output and reduced vasopressin 2 receptor expression compared to WT mice. These results suggest B cells may contribute to renal function in maintaining blood pressure homeostasis. However, more work is still needed to identify a role for B cells in the pathogenesis of hypertension.

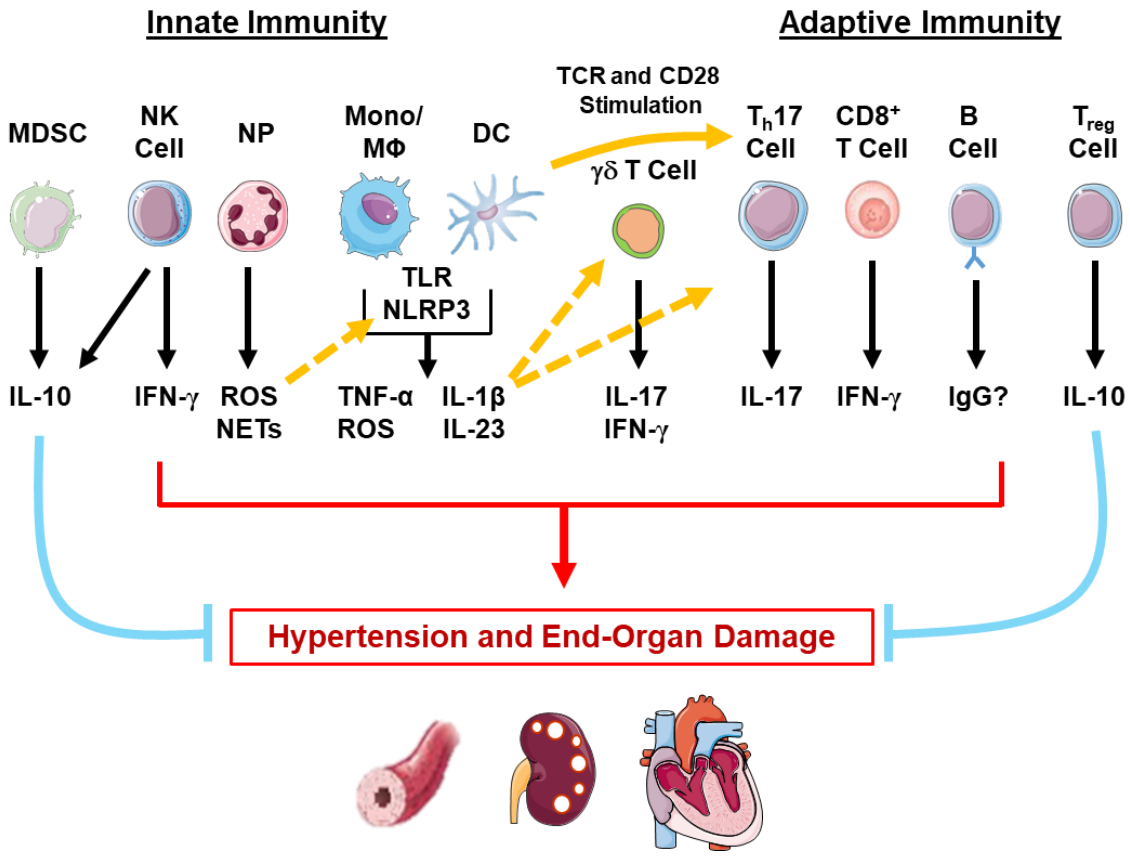

Figure 1.2: A simplified sketch of how the innate and adaptive immune systems contribute to hypertension. Neutrophils (NPs) are activated through reactive oxygen species (ROS) or isolevuglanids to produce neutrophil extracellular traps (NETs) and more ROS that contribute to vascular dysfunction and to NLRP3 activation. Monocytes (Mono), Macrophages (MΦ) and dendritic cells (DC) are stimulated through pattern recognition receptors like toll-like receptors (TLRs) and the NLRP3 inflammasome resulting in the production of interleukin (IL)-1β, IL-23, tumour necrosis factor-alpha (TNF-α), and ROS. These cytokines and ROS can directly contribute to hypertension and end-organ damage or can trigger activation of innate-like γδ T cells, IL-17 producing helper T (Th17) cells, and interferon-gamma (IFN-γ) producing $CD8^+$ T cells. Dendritic cells can also activate $CD4^+$ and $CD8^+$ T cells through direct stimulation of the T cell receptor (TCR) and CD28. IFN-γ and IL-17 production from these T cell subsets promote hypertension and end-organ damage. B cells are activated through an unknown mechansim in hypertension, but it is speculated that immunoglobin G (IgG) production from B cells may contribute to end-organ damage. Regulatory T (Treg) cells and myeloid derived suppressor cells (MDSCs) can suppress immune cell activation and end-organ damage by releasing the anti-inflammatory cytokine IL-10, although their activity is often suppressed during hypertension. Natural Killer (NK) cells can both contribute to (through release of IFN-γ) and attenuate hypertension (by releasing IL-10) and end-

organ damage. The figure was created using images from smart.servier.com and is licenced under a creative commons attribution 4.0 licence (CC BY 4.0).

1.2.6.3 Immune Memory

A vital feature of the adaptive immune system is its ability to form long-lasting immune memory cells after antigenic stimulation.(141) Memory T cells respond faster and more potently in response to re-exposure with their cognate antigen. Memory T cells are typically categorized into three broad categories based on the expression of different cell surface markers (Figure 1.3).(141) Central memory T (T_{CM}) cells are long-lived, recirculate between the blood and secondary lymphoid organs, and are characterized by expression of the lymphoid homing receptors C-C chemokine receptor 7 (CCR7) and L-selectin (CD62L). Effector memory T (T_{EM}) cells can recirculate through blood, secondary lymphoid organs and non-lymph tissues and can be differentiated by the lack of expression of CCR7 and L-selectin. Distinct from other memory T cell subsets, resident memory (T_{RM}) cells were only recently characterized as they do not recirculate and instead permanently reside in their location of activation.(142) They can be identified by the expression of the tissue retention markers CD69 and integrin alpha E (CD103) and the lack of expression of CCR7 and CD62L. All memory T cell subsets express the hyaluronic acid receptor and activation marker, CD44, which is upregulated upon antigen encounter and stays expressed in memory T cells.

Hypertensive insults result in the formation of memory T lymphocytes that predisposes mice to exacerbated hypertension and end-organ damage in response to further, mild hypertensive challenges (Figure 1.3).(143) The co-stimulatory molecule CD70, present on both DCs and macrophages, was indispensable to the development of these memory T lymphocytes, as these memory cells did not develop in its absence. Furthermore, sympathetic nerves were found to contribute to the homing and survival of hypertension-specific T_{EM} cells, particularly $CD8^+$ T_{EM} cells, in the bone marrow.(144) Global inhibition of sympathetic outflow or β2 adrenergic receptor antagonism attenuated the development of $CD8^+$ T_{EM} in the bone marrow after hypertensive insults and reduced the hypertensive response to a subsequent exposure to subpressor amounts of Ang II. As hypertension is a chronic disease, the role of long-lived pro-inflammatory memory T cells in human populations will be an interesting avenue of future research.

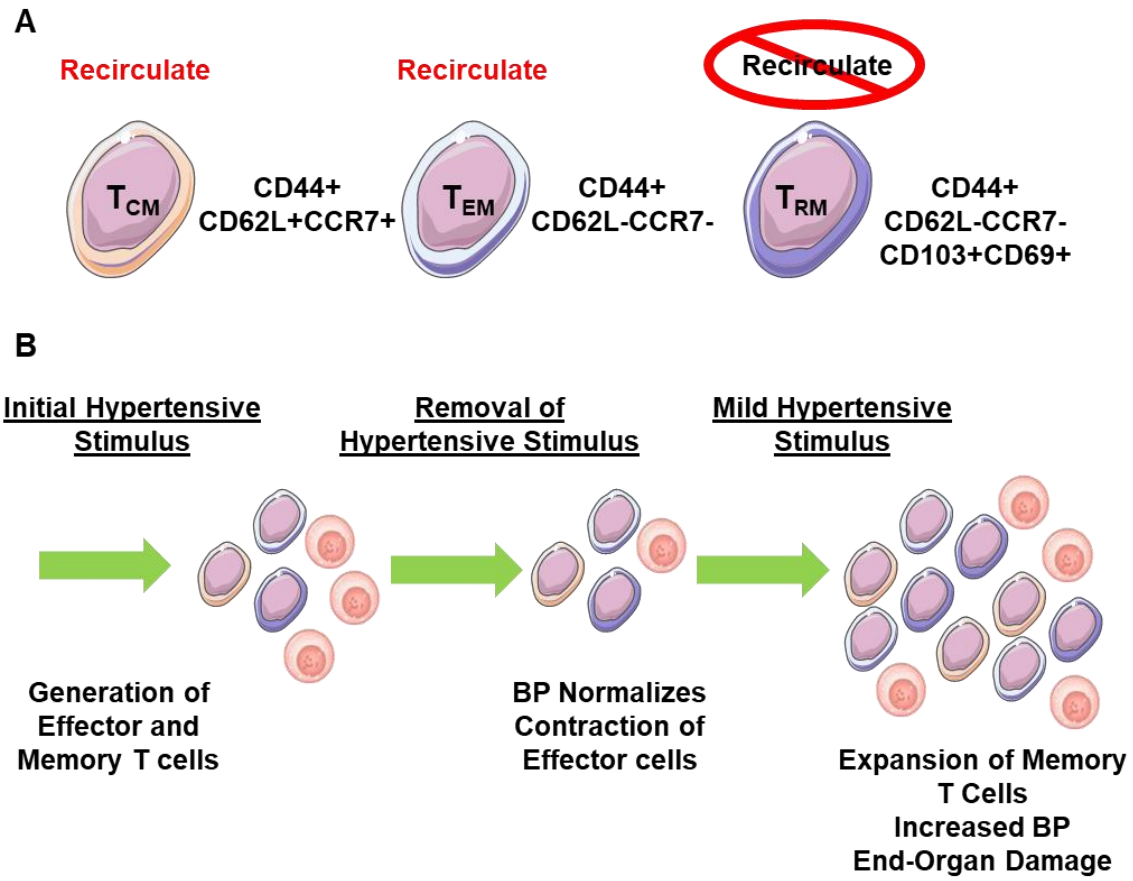

Figure 1.3: Memory T cell populations. Markers used to define different populations of memory T cells (**A**). Schematic representing how memory T cells are generated after hypertensive stimuli, remain after removal of hypertensive stimuli, and then rapidly expand upon reexposure to hypertensive insults resulting in elevated blood pressure and end-organ damage (B). CCR7, C-C chemokine receptor 7; CD, cluster of differentiation; CD103, integrin alpha E; CD62L, L-selectin.

1.3 A Brief Overview of Purinergic Receptors

As early as 1929, the importance of purine nucleotides in cardiovascular disease was being established when Drury and colleagues demonstrated that adenosine injection into cardiac tissue induced bradycardia.(145) Identifying the receptors that purines acted upon however, would take several decades. Geoffrey Burnstock first put forward the term "purinergic" in 1971 as a candidate name for a class of nerves that were neither adrenergic nor cholinergic but instead seemed to respond to purine nucleotides.(146) Purinergic receptors were first described in 1976,(147) with functional classes (P1 vs P2) being proposed in 1978(148) and P2Y vs P2X classifications described in 1985.(149) Now, it is appreciated that purinergic receptors make up several classes of receptors expressed ubiquitously throughout the body that responds to extracellular purines

(adenosine, ATP, ADP, UTP and UDP).(150) There are two broad classes of purinergic receptors, P1 and P2 receptors. P1 receptors are made up of 4 subtypes (A_1, A_{2A}, A_{2B}, A_3) that are activated primarily by extracellular adenosine. P2 receptors are subdivided into 2 categories, metabotropic G protein-coupled P2Y receptors and ligand-gated ionotropic P2X receptors. P2Y receptors have varied biological functions depending on the subtype expressed ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) and the tissue they are expressed in.(151) P2X receptors (P2X1-7) act as functional trimers and when stimulated by ATP, they serve as non-specific cation ion channels, particularly for K^+, Na^+, and Ca^{2+}. P2X receptors form homo- or heterotrimers, adding further diversity to their functional roles.(152) It is now widely appreciated that purinergic receptors contribute to a wide variety of pathologies, including cardiovascular disease and hypertension, with P2X7 garnishing particular interest.(153, 154)

1.4 The P2X7 Receptor

P2X7 was originally termed P2Z as it possesses properties unique to other members of the P2X family, namely its long carboxylic tail, cytotoxic effect and the belief at that time that it was only expressed on lymphocytes.(155) P2Z was conclusively described as belonging to the P2X family and given the name P2X7 by Surprenant *et al.* when they successfully isolated and cloned it in 1996.(156) Like other purinergic receptors, the principle agonist for P2RX7 is ATP. However, in most instances, P2RX7 requires near millimolar concentrations of ATP for activation rather than micromolar or nanomolar concentrations necessary for other P2X receptors.(156) With transient stimulation, the P2X7 receptor acts as a non-specific cation channel facilitating Na^+ and Ca^{2+} influx and K^+ efflux, and prolonged stimulation with high concentrations of ATP favouring cellular death.(157) P2RX7 is expressed near ubiquitously throughout the body, but its expression is generally highest in immune cells. As such, P2RX7 is thought to contribute to numerous pathologies involving inflammation. This section will introduce the structure and function of P2X7 receptors and describe their role in modulating immune cell function.

1.4.1 Receptor Structure and Channel Properties

The structural formation of P2RX7 resembles that of a dolphin (Figure 1.4), with two trans-membrane α-helices representing the tail and 14 β-strands representing the body.(158) The extracellular ATP-binding pocket is composed of hydrophobic amino-acid residues, accounting for the low ATP affinity of P2RX7. ATP binding in the pocket causes an outward flexing of the extracellular domain, in turn pulling on transmembrane domain 2, forming the characteristic

pore.(159) A unique feature of P2RX7 compared to the other P2X channels is its long carboxylic tail (~240 amino acids), which was long thought to facilitate P2RX7's ability to form a macropore permeable up to 900Da.(156, 160) However, recently this was challenged. A group out of Cornell University demonstrated that P2RX7 macropore formation was independent of the C-terminal domain but instead dependent on the membrane lipid composition.(161) Membrane cholesterol attenuated P2RX7 pore-forming ability, particularly in C-terminal truncated P2RX7 variants, while palmitoylated cysteines near the pore-lining helix facilitated pore opening. Another interesting point was that physiologic amounts of cholesterol in the cell membrane (~25%) were sufficient to prevent pore formation in C-truncated P2RX7 variants. These observations may account for studies in the past suggesting a full-length C-terminal (156) or pannexin-1 co-expression was essential for P2RX7's macropore forming ability.(162) The C-terminal domain is also essential for proper P2RX7 cellular trafficking to the cell surface.(160, 163) Point mutations in the C-terminal domain, particularly in dibasic amino acids, significantly impair P2RX7 trafficking to the cell surface.(163) The C-terminus of P2RX7 is also responsible for the unique feature that P2RX7 does not desensitize after prolonged stimulation.(164) Cysteine residues at the end of the transmembrane domain anchor the pore-lining helix with palmitoyl groups in the plasma membrane, locking the pore open as long as ATP is bound.(159) Deleting the cysteine anchor or palmitoylatable residues resulted in a loss of the desensitization capability of P2RX7. Thus, many of the unique properties of P2RX7 are mediated by its long C-terminal domain.

As discussed above, P2X receptors are trimers with P2RX7 formed primarily of homomeric subunits. However, the trimers can be comprised of various splice variants that may alter the function of the receptor (discussed in section 1.4.3). In addition, some publications have suggested the possibility of heteromeric P2X4/P2X7 ion channels.(165-167) The role and even existence of these heteromeric channels is still an area of debate. One study showed that P2X4 KO in a mouse macrophage cell line suppressed P2RX7 mediated IL-1β release but promoted P2RX7 mediated cellular death (autophagy).(166) This would suggest that heteromeric P2X4/P2X7 may favour inflammation pathways over cellular death. However, studies by other groups have suggested that heteromeric P2X4/P2X7 have similar electrophysiologic properties to their respective homomeric channels.(165, 167) As these studies only measured ion currents, they can not exclude that downstream responses such as IL-1β signalling were not modulated. The role of

homo vs heteromeric P2RX7 channels in disease pathologies remains unclear and is an area of active investigation.

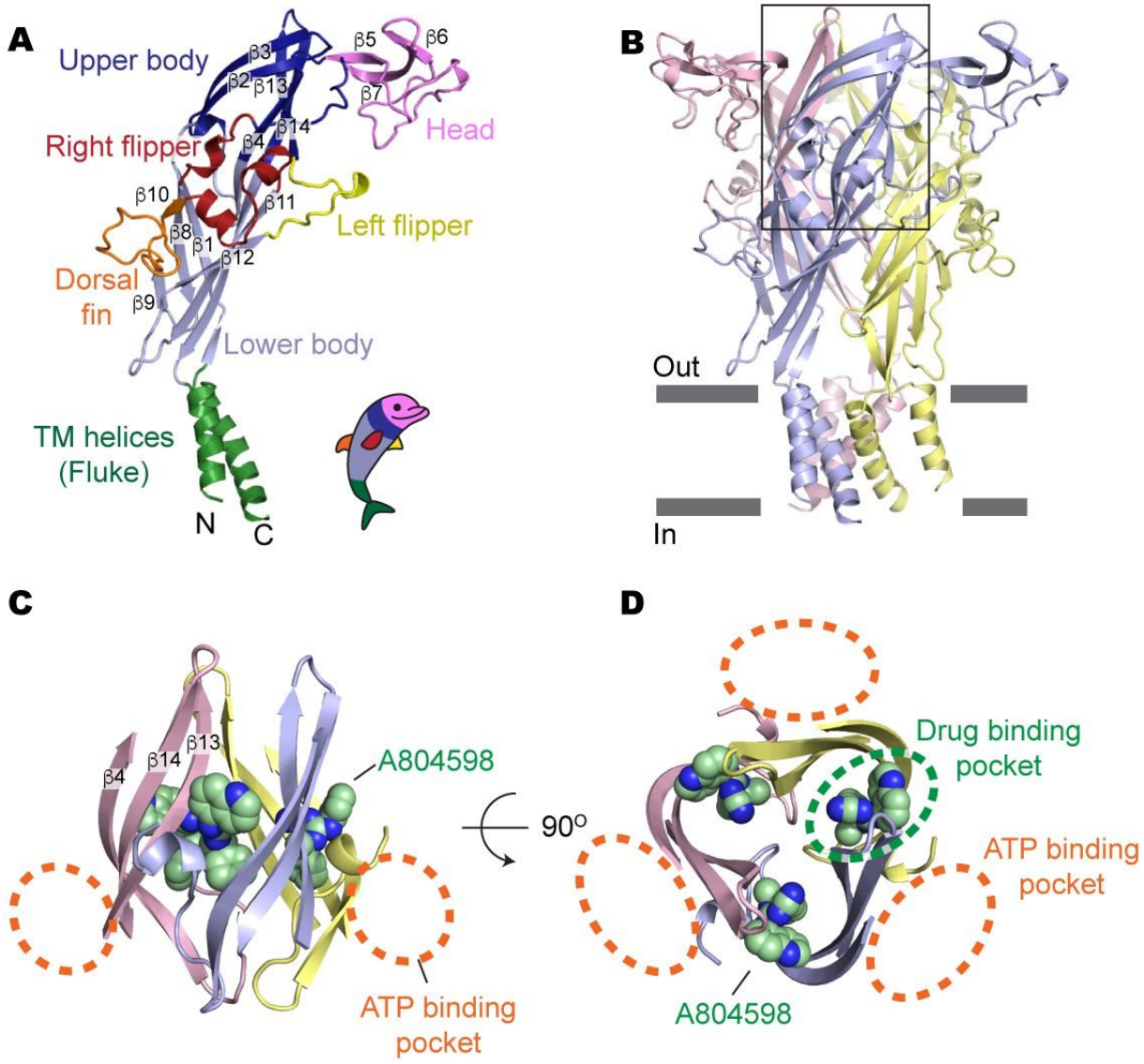

Figure 1.4: Drug-binding pocket of the P2X7 receptor. Figure from Karasawa *et al.*(158) and used under Creative Commons Attribution license 4.0 (CC BY 4.0).

1.4.2 Alternative Agonists for P2RX7

Several alternative agonists for P2RX7 have been proposed besides ATP. In murine lymphocytes (nicotinamide adenine dinucleotide) NAD^+ acts as a potent activator for the P2RX7 variant K (discussed in section 1.4.3). The plasma membrane enzyme ADP-ribosyltransferase (ARTC2.2, not encoded in humans) catalyzes the transfer of an ADP-ribose moiety from NAD^+ to arginine 125, close to the ATP-binding pocket of P2X7R.(168, 169) It is debated whether this bonding

enacts sustained activation or simply lowers the ATP threshold for activation of P2RX7.(170) Either way, stimulation with NAD^+ provokes long-lasting activation of P2RX7 which can activate a P2RX7 mediated cell death pathway within an hour.(168) Besides ATP and NAD^+ other agonists for P2RX7 have been identified recently, including cathelicidin (LL37; a potent antimicrobial peptide)(171), amyloid β (172), serum amyloid A (173), and Alu-RNA.(174) In addition, numerous modulators of P2RX7 activity have been cited and are reviewed by Stokes *et al.*(175) In general, divalent cations such as Mg^{2+} decrease P2RX7 activity by altering the cation flux induced by P2RX7 activation, whereas positive modulators for P2RX7 are diverse in nature and can not be easily categorized but include Ginsenoside CK, Clemastine and Polymyxin B, to name a few.(175, 176) These modulators of P2RX7 activity are currently being investigated for use in infectious diseases and cancer treatment but could have wide reaching impact for the management of other conditions.(175)

1.4.3 P2RX7 Splice Variants

There are currently 11 human splice variants for P2RX7 that have been identified and at least partially characterized, given the names P2X7A to P2X7L (excluding P2X7K, which is only in mice).(177-181) P2X7A represents the full-length isoform and is responsible for the typical responses associated with P2RX7 activation, such as inflammasome activation and pore formation. Isoform B (P2X7B) is also widely expressed throughout the body but has a truncated carboxy-terminal which impairs the receptor's pore-forming ability.(177) The channel activity of P2X7B is retained, and its stimulation can have trophic effects.(182) Furthermore, P2X7B variants can form functional heteromeric channels with P2X7A potentiating responses in comparison to homomeric P2X7A channels. P2X7E is similar to P2X7B in that it has a truncated C-terminal (177), however, P2X7E is not expressed on the cell surface (verified by flow cytometry) and thus is presumed to be not functional.(181, 183) The absence of surface expression of P2RX7E is most likely due to a loss of amino acids in the C-terminal that are important for the cellular trafficking of P2RX7. Isoforms P2X7C, and P2X7G, also have truncated carboxy terminals inhibiting their pore-forming ability, but their functional role is unclear, while the P2X7I isoform results in loss of function of the receptor.(177, 180, 181, 184) P2X7H lacks transmembrane 1 of the receptor and is most likely not functional.(177) P2X7J lacks the entire intracellular C-terminus, transmembrane 2, and the distal third of the extracellular loop (184, 185) but can form a heterotrimer with P2X7A inhibiting receptor function.(184) Finally, the most recently characterized human variant is

P2X7L.(181) P2X7L has a mutated ATP binding site, and P2X7L transfected HEK 293 cells lack channel function, membrane blebbing and pore-forming capabilities. When co-transfected with P2X7A, heteromeric P2X7A/L channels form, resulting in reduced channel function compared to homomeric P2X7A channels. An additional P2X7 variant exists in rodents, P2X7K, which is exclusively found in T cells and is ~8times more sensitive to P2RX7 agonists.(186). P2X7K mediates T-cell responses to ATP and NAD^+, facilitating T-cell class switching through CD62L and CD27 cleavage and cellular death via externalization of phosphatidylserine (PS).(168, 178, 187, 188) However, the relevance of P2X7K for human pathology is uncertain, as a human homologue has yet to be identified.

1.4.4 NLRP3 Inflammasome, IL-1β and IL-18

One of the best characterized cellular roles of P2RX7 is the activation of the NLRP3 inflammasome. Inflammasomes are multiprotein complexes critical in innate immunity host defence. NLRP3 is one of a family of five well-documented inflammasomes and consists of the NLRP3 protein, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase 1.(189) NLRP3 inflammasome activation can occur via 3 different routes: the canonical, non-canonical or alternative pathways.(190) Traditionally, NLRP3 activation has been considered a "2 hit" system. As IL-1β and IL-18 are potent pro-inflammatory cytokines, this 2-hit system was thought to evolve to protect against unwanted activation and excessive inflammation. The first "hit" is a priming event initiated by diverse stimuli, including bacterial associated LPS stimulating TLR 4 or other inflammatory stimuli that upregulate NF-κB expression (Figure 5).(191) This priming event creates the machinery for the NLRP3 inflammasome complex, as well as pro-IL-1β and IL-18. The second "hit" is triggered by the recognition of DAMPs. The best described of these DAMPs is ATP, although uric acid (192), cholesterol crystals (193, 194), and oxidized low-density lipoproteins(195, 196) can also trigger NLRP3 inflammasome activation. In terms of ATP, K^+ efflux through P2RX7 was suggested as the most likely stimulus for NRLP3 oligomerization and activation.(197) Recently, how K^+ efflux triggers NLRP3 activation was further delineated. Wang *et al*. demonstrated that K^+ triggers phosphorylation of an adaptor protein, Paxillin, which brings together a P2RX7-Paxillin-ubiquitin-specific peptidase 13 (USP13)-NLRP3 complex (Figure 1.5).(198) USP13 then acts to deubiquitinate NLRP3, allowing oligomerization of the NLRP3 inflammasome. This deubiquitination step of NLRP3 is critical for NLRP3 activation, and other deubiquitinases

(DUBs) also play a role, including BRCC3 and other USPs.(199-201) Although NLRP3 deubiquitination can occur in an ATP-independent manner, ATP stimulation potentiates the signal.(200) The activated NLRP3 inflammasome complex then cleaves pro-caspase 1 into its active subunits, which subsequently cleaves pro-IL-1β and pro-IL-18 to their active forms.(191) Gasdermin-D (GSDM-D) can also be cleaved by caspase-1, which then oligomerizes and forms a pore in the cell membrane.(202, 203) It is hypothesized that this pore provides a mechanism for IL-1β and IL-18 release and also promotion of pyroptosis. Interestingly it was recently demonstrated that microparticles containing the machinery of P2RX7 activation (Caspase-1, pro-Il-1β and GSDM-D) can be released upon LPS stimulation.(204, 205) These microparticles can then respond to extracellular ATP causing the cleavage of IL-1β and GSDM-D, releasing their inflammatory contents proximal to the source of ATP.

The non-canonical and the alternative pathways are primarily independent of P2RX7 signalling but will be touched on briefly. The non-canonical pathway responds to LPS independently of TLR signalling, activating caspase 11 (caspase 4/5 in humans) to cause cleavage of GSDM-D and membrane localization of pannexin-1, inducing pyroptosis.(206-209) Pannexin-1 can then facilitate ATP release, inducing activation of the canonical pathway (described above).(209, 210) The alternative pathway was identified in human monocytes(211) (and later murine dendritic cells(212)) where NLRP3 activation was observed through TLR signalling (LPS) without a second stimulus (211), resulting in caspase-1 activation and IL-1β cleavage independent of K^+ efflux and not leading to pyroptosis.

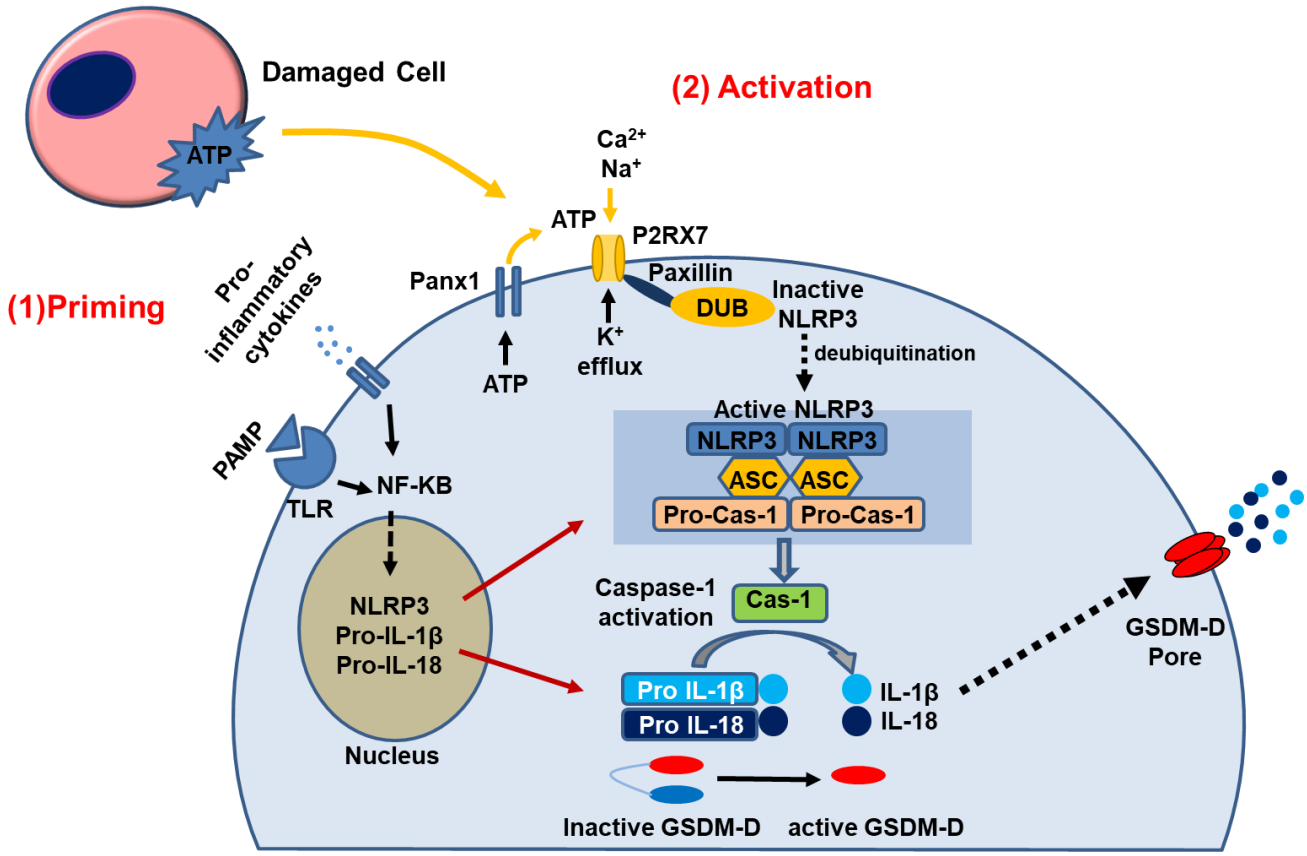

Figure 1.5: P2RX7 Dependent Activation of the Inflammasome. Priming is the first step and involves activation of the NF-κB signalling pathway either through toll-like receptor (TLR) or other cytokine stimulation, resulting in the synthesis of components of the NLRP3 inflammasome (NLRP3, adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), pro-caspase (Cas)-1), pro-interleukin (IL)-1β, pro-IL-18, and inactive gasdermin D (GSDM-D). Activation occurs when sufficient ATP concentrations trigger P2RX7 activation, and ion flux ensues. Upon P2RX7 activation, pannexin 1 (Panx1) is also recruited to P2RX7, facilitating ATP release from the cell and propagating a feed-forward signalling pathway. K^+ efflux then activates deubiquitinases (DUBs) which deubiquitinate NLRP3, triggering its activation and Cas-1 cleavage. Cas-1 subsequently cleaves IL-1β, IL-18 and GSDM-D into their active forms. GSDM-D then forms a pore in the membrane, which is thought to facilitate the release of IL-1β and IL-18 from the cell.

1.4.5 Other Cellular Roles of P2RX7

Several prominent signalling pathways besides NLRP3 inflammasome activation have also been linked to P2RX7 activation (summarized in Figure 1.6). As P2RX7 facilitates intracellular Ca^{2+} accumulation, many Ca^{2+} signalling pathways can be stimulated by P2RX7. Among these pathways include Ca^{2+}/calmodulin-dependent protein kinase II(213), calcineurin, protein tyrosine

phosphorylation, nuclear factor of activated T cells (NFAT)(214, 215) and various mitogen-activated protein kinases (MAPK) such as MEK, ERK(216) and JNK(217). The diverse signalling pathways triggered by P2RX7 activation may account for the various roles P2RX7 plays in other tissues and organ systems, such as nociception, osteoclast activity, and glucose uptake.(218-222) Two additional cellular roles that will be briefly discussed are P2RX7's role in cellular death and mitochondrial function.

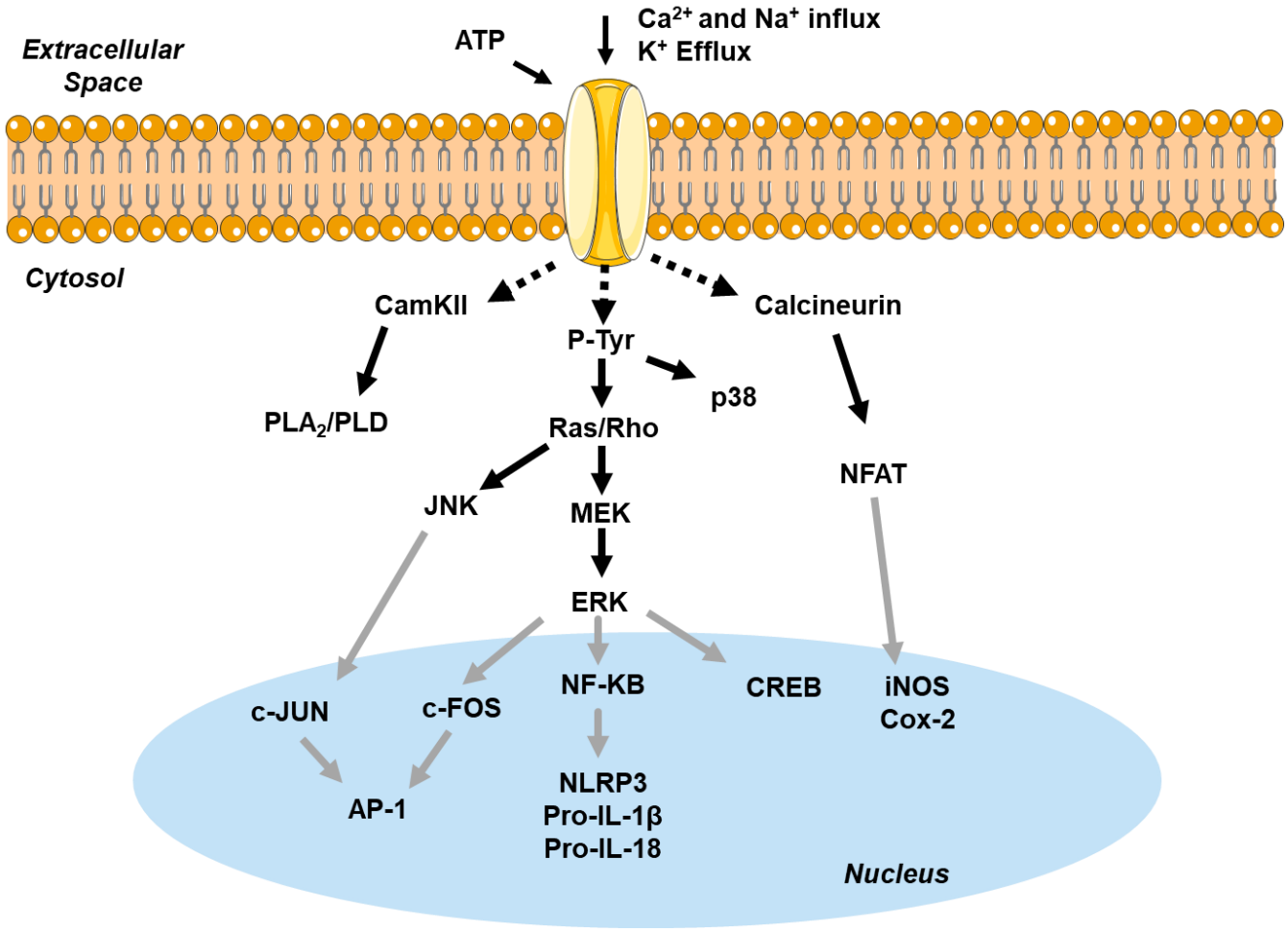

Figure 1.6: Alternative pathways activated by P2RX7. AP-1, activator protein-1; CamKII, Ca^{2+}/calmodulin-dependent protein kinase; Cox-2, cyclooxygenase-2; CREB, cyclic adenosine monophosphate response element-binding protein; ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal Kinase; MEK, mitogen protein activate kinsase kinase; NFAT, nuclear factor of activated T cells; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; PLA_2, phospholipase A2; PLD, phospholipase D; p-Tyr,

phosphorylated tyrosine. The figure was created using images from smart.servier.com and is licenced under a creative commons attribution 4.0 licence (CC BY 4.0).

1.4.5.1 Cell death

Rapidly upon stimulation, P2RX7 mediates cellular changes mimicking apoptosis, a process referred to by Mackenzie *et al.* as "pseudoapoptosis."(223) The authors found that within 5-10s of ATP exposure, there is PS exposure on the outer leaflet, mitochondrial swelling, mitochondrial membrane potential collapse, and actin filament and microtubule disruptions. Within 60 seconds of stimulation, membrane blebbing begins to occur. This pseudoapoptosis is calcium-dependent and reversible if stimulation is removed within 20-30 minutes. Prolonged P2RX7 activation was found to lead to calcium-independent activation of the "traditional apoptosis" ROCK-1 dependent pathway, cytochrome C release and cellular death. Khadra *et al.* recently clarified the stimulus for cellular death versus ion flux in P2RX7 activation, describing a "dual gating mechanism."(157) The authors found that when only one or two of the ATP binding sites (there are three binding sites total, one for each P2RX7 subunit of the trimer) of P2RX7 are engaged (representing low ATP concentration), a slow desensitizing monophasic current ensues, along with internalization of P2RX7, before changes in cellular morphology occur. When all three binding sites were occupied (high ATP concentration), pore formation occurred, with cellular death following. This study suggests that not only the duration of P2RX7 stimulation is important for P2RX7 mediated cellular death but also ATP concentration. In summary, transient P2RX7 stimulation promotes ion flux and a "pseudoapoptosis," especially in low ATP concentrations. In contrast, prolonged stimulation of P2RX7 with high concentrations of ATP lead to macropore formation and cellular death.

1.4.5.2 Mitochondrial Energetics

In contrast to promoting cellular death, P2RX7 activation can also have a trophic effect.(224) The survival-promoting effects of P2RX7 agonism are likely due to its capacity to support oxidative phosphorylation and glycolysis and thus enhance intracellular ATP synthesis.(224, 225) P2RX7 activation appears to be able to modulate key components of aerobic glycolysis within the cell, including glycolytic enzymes and glucose transporters, that allow cells to adapt to adverse environmental conditions such as low glucose conditions.(225) HEK293 cells (normally lacking P2RX7 expression) stably transfected with P2X7 have a higher basal mitochondrial potential, intra-mitochondrial calcium levels and cellular ATP stores and can grow in serum-free

conditions.(224) P2RX7 KO in cell lines that normally express P2RX7, decreased resting mitochondrial potential, basal respiratory rate, ATP-coupled respiration, maximal uncoupled respiration, resting mitochondrial potential, and mitochondrial matrix Ca^{2+} levels.(226) Along these lines, stimulation of P2RX7 by daily injections of non-hydrolyzable ATP (2'(3')O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate [Bz-ATP]) enhances energy metabolism (metabolic rate and oxygen consumption) in mice.(227) Concomitantly, *P2rx7*$^{-/-}$ mice or mice treated with a P2RX7 antagonist have decreased whole-body energy expenditure and increased respiratory exchange ratio, indicating a greater carbohydrate to fat oxidation ratio.(228) Relative sparing of fatty acids and decreased energy expenditure possibly account for the adipocyte hyperplasia and weight gain of *P2rx7*$^{-/-}$ mice despite comparable food consumption with WT mice.(228, 229) Recently, P2RX7 expression on the outer mitochondrial membrane was discovered, although its function there is as of yet unclear. P2RX7 might play a role in Ca^{2+} migration across the membrane or in mitochondrial fission/fusion as P2RX7 activity increases mitochondrial size and thickness of the mitochondrial network.(226) Taken together, these studies show that P2RX7 can regulate the mitochondria's ability to adapt to environmental stressors, however how the balance between enhancing mitochondrial energetics and mitochondrial collapse is maintained remains to be addressed.

1.4.6 P2RX7 and Innate Cells

The most prominent role of P2RX7 in innate cells is inflammasome activation, with IL-1β and IL-18 mediating a myriad of downstream effects. For instance, P2RX7 mediated IL-1β and IL-18 release from DCs has a hand in the priming of IFN-γ producing $CD8^+$ T cells.(230) In fact, P2RX7 signalling in innate cells serves as an important mediator for several pathways of T cell activation. Extracellular ATP induces CD86 expression, an important co-stimulatory molecule for T cell activation, on DCs and human monocytes, which can be inhibited by P2RX7 antagonism.(103, 231) Furthermore, P2RX7 has been shown to promote differentiation of Th17 cells through mediating IL-23 release from DCs in a process that can be inhibited through P2RX7 antagonism.(232) However, P2RX7 activation serves several other key roles in innate immune cells besides inflammasome activation and T cell priming. P2RX7 activation in macrophages promotes ROS production and autophagy (166), while in DCs, P2RX7 activation is required to facilitate cytoskeletal rearrangements necessary for fast migration.(233) Activation of P2RX7 in DCs also facilitates the release of microparticles with Tissue Factor, a glycoprotein acting as an

essential cofactor of activated factor VII, triggering blood coagulation.(234) In summary, P2RX7 contributes to diverse cellular roles within the innate immune system, contributing to their proper functioning and activation.

1.4.7 P2RX7 and T cells

P2RX7 activation in T cells serves diverse roles. P2RX7 plays an important role in regulating T cell development, function and activation. However, P2RX7 activation can also trigger T cell apoptosis.(235) P2RX7 activation promotes γδ lineage choice in the thymus.(188) In *P2rx7*$^{-/-}$ mice or mice receiving a P2RX7 antagonist, immature γδTCR-expressing cells were diverted to an αβ lineage in the thymus during development resulting in an altered γδ variant expression in the periphery, with increased innate-like γδNK1.1-expressing cells. In CD4^{+} T cells, P2RX7 activation controls the balance between Th17 and Tregs.(236) Tregs highly express P2RX7 and thus are sensitive to changes in extracellular ATP. ATP-P2RX7 signalling downregulates FOXP3 expression and upregulates RORγT, signifying a conversion from anti-inflammatory Tregs to pro-inflammatory Th17 cells. In order to limit the extent of P2RX7 activation, Tregs have high expression of ectonucleotidases such as CD39, which breaks down ATP to AMP.(237) In the absence of P2RX7 signalling, TCR stimulation of naïve CD4^{+} T cells promoted Treg lineage choice.(236) Furthermore, P2RX7 KO resulted in an increased number of Tregs in mesenteric lymph nodes, which were more effective IL-10 producers than their WT counterparts.(238)

P2RX7 also has a vital role in regulating T cell death. For example, P2RX7 activation may restrain aberrant effector T cell accumulation.(239) In a model of systemic lupus erythematosus, P2RX7 activation restricted the expansion of aberrant T follicular helper cells and the generation of self-reactive antibodies. P2RX7 signalling may also promote host-microbiota mutualism as ATP released from the gut microbiota restricts T cell populations in the intestine, suppressing immune activation via P2RX7 signalling.(240, 241) This may explain why *P2rx7*$^{-/-}$ mice have substantially more T cells in the intestine, likely as a result of decreased ATP-P2RX7 mediated T cell apoptosis.(241, 242) The sensitivity of T cells to P2RX7 mediated cellular death may be conferred by the unique P2RX7 variant, P2X7K, expressed on murine T cells. As discussed above, P2X7K is sensitive to activation by ADP-ribosylation(243) with NAD^{+} inducing CD62L shedding, cell shrinkage, pore formation and PS exposure, which if sustained, leads to cell death.(168) The relevance of this pathway is unclear in humans however, as they lack ART2.2 and P2X7K variant expression.

P2RX7 signalling also contributes to promoting T cell activation. TCR stimulation rapidly releases ATP (<100uM) and upregulates P2RX7 gene expression.(244) The extracellular ATP then mediates calcium flux through P2RX7 channels activating the NFAT signalling pathway. Removal of extracellular ATP by apyrase or alkaline phosphatase treatment, inhibition of ATP release with the maxi-anion channel blocker gadolinium chloride, or siRNA silencing of P2X7 receptors blocks calcium entry and impaired T-cell activation. P2RX7 mediated Ca^{2+} influx in T cells also leads to L-selectin (CD62L) shedding through ROS-dependent activation of the metalloproteinases ADAM10 and ADAM17.(245) CD62L is a lymphoid homing marker, and its downregulation is an important step in T cell activation, allowing egress to target tissues. In chemoresistant patients, reduced P2RX7 expression was associated with decreased CD62L shedding and IFN-γ secretion from $CD8^+$ T cells.(246) Similarly, P2RX7 antagonism resulted in fewer IFN-γ producing $CD8^+$ T cells following vaccine antigen incubation.(247) In a T cell migratory assay, the authors also demonstrated that $P2X7R^+CD8^+$ T cells migrate more efficiently and produce more IFN-γ/IL-17 when challenged with islet peptides compared with P2X7R-CD8+ T cells, suggesting P2RX7 is vital for $CD8^+$ T cell motility and cytokine production. Interestingly, one study found that P2RX7 mediated T cell activation versus cellular death was dictated by the levels of mitochondrial ROS, with elevated ROS favouring P2RX7 mediated T cell death.(245)

1.4.8 P2RX7 and Immune Memory

P2RX7 is highly expressed on T_{RM} and T_{CM} cells and to a lesser extent, T_{EM} cells suggesting a potential role for P2RX7 in regulating their function.(248) Following infection with acute lymphocytic choriomeningitis virus, *P2rx7*$^{-/-}$ T cells showed normal production of effector cells, but had a major defect in the establishment of T_{CM} and T_{RM}, and a slight deficiency in T_{EM} cells. In particular, memory cell retention was severely affected. By 8 days post-infection, *P2rx7*$^{-/-}$ T cells in the memory precursor phase already displayed mitochondrial dysfunction with reduced mitochondrial mass and spare respiratory capacity. As metabolic reprogramming occurs during the transition from effector to memory cells, it appears mitochondrial P2RX7 signalling is required to maintain the compatibility of memory cells. Indeed, a study by Tezza *et al*. showed that $P2RX7^+CD8^+$ T cells had enhanced glycolytic capacity and higher maximal respiration than $P2RX7^-CD8^+$ T cells suggesting that some level of P2RX7 signalling is necessary for proper mitochondrial function in memory T cells.(247)

In T_{RM} cells, P2RX7 plays a key role in controlling the expansion/contraction of specific T_{RM} lineages. Due to the high expression of P2RX7 and particularly the variant P2RX7K, T_{RM} cells are susceptible to NAD^+-P2RX7 mediated cell death.(249) As a survival mechanism, T_{RM} cells downregulate surface P2RX7 rapidly upon cognate antigen recognition and TCR stimulation. This resulting cell death of non-stimulated T_{RM} cells, but survival of stimulated T_{RM} cells most likely allows for fine-tuned amplification of pathogen-specific T_{RM} populations. As a final note, P2RX7 also appears to be important for T_{RM} effector functions, as T_{RM} cells from mice genetically devoid of P2RX7 produced less IFN-γ and granzyme B, and had reduced capacity to activate neighbouring $CD8^+$ T cells and DCs.(248)

Preface to Chapter 2: Literature Review

This chapter provides context for the role of P2RX7 in cardiovascular disease including in the context of hypertension. The review article provides evidence from animal models for a role of P2RX7 in hypertension, atherosclerosis, myocardial ischemic injury, heart failure and stroke. Previous review articles existed on the topic of P2RX7 and cardiovascular disease, but this review was updated and presented in a more comprehensive and complete manner, including discussions on available human data. The article discussed ongoing clinical trials for P2RX7 antagonists in other pathologies and suggested that P2RX7 could be a viable target for the treatment of various cardiovascular pathologies.

The review article entitled "P2X7: An untapped target for the management of cardiovascular disease" was published in the January 2021 issue of Arteriosclerosis, Thrombosis, and Vascular Biology.

Chapter 2: Literature Review

P2X7: An untapped target for the management of cardiovascular disease

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Running Title: The Role of P2X7 in Cardiovascular Disease

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Key Words: P2X7, Inflammation, ATP, Cardiovascular disease, NLRP3, IL-1β, IL-18

Subject Terms: Hypertension, Atherosclerosis, Vascular Disease, Myocardial Infarction

TOC: Basic

TOC Subcategory: Arteriosclerosis, Thrombosis and Vascular Biology

2.1 Abstract

Chronic low-grade inflammation contributes to the development of several diseases, including cardiovascular disease. Adequate strategies to target inflammation in cardiovascular disease are in their infancy, and remain an avenue of great interest. The purinergic receptor P2X7 is a ubiquitously expressed receptor that predominately mediates inflammation and cellular death. P2X7 is a ligand-gated cation channel that is activated in response to high concentrations of extracellular adenosine triphosphate, triggering the assembly and activation of the NLRP3 inflammasome and subsequent release of pro-inflammatory cytokines IL-1β and IL-18. Increased P2X7 activation and IL-1β and IL-18 concentrations have been implicated in the development of many cardiovascular conditions including hypertension, atherosclerosis, ischemia reperfusion injury and heart failure. P2X7 receptor knockout mice exhibit a significant attenuation of the inflammatory response, which corresponds with reduced disease severity. P2X7 antagonism blunts blood pressure elevation in hypertension and progression of atherosclerosis in animal models. IL-1β and IL-18 inhibition have shown efficacy in clinical trials reducing major adverse cardiac events, including myocardial infarction, and heart failure. With several P2X7 antagonists available with proven safety margins, P2X7 antagonism could represent an untapped potential for therapeutic intervention in cardiovascular disorders.

2.2 Abbreviations

Ang II, angiotensin II; ATP, adenosine triphosphate; BP, blood pressure; DOCA, deoxycorticosterone acetate; IL, Interleukin; KO, Knockout; I/R, ischemia-reperfusion; MI, myocardial infarction; NLRP3, nuclear oligomerization domain like receptor family pyrin domain containing 3; NO, nitric oxide; ROS, reactive oxygen species; Tregs, T regulatory cells.

2.3 Highlights

- Accumulating evidence demonstrates that P2X7 plays a prominent role in chronic inflammatory conditions, including cardiovascular disease.
- P2X7 activation contributes to the development of hypertension through promotion of renal and vascular dysfunction.
- P2X7-mediated endothelial dysfunction and inflammation directs atherosclerotic plaque formation and rupture.
- In ischemic injury in the heart, P2X7 activation promotes cardiomyocyte death, and enhances inflammation leading to cardiovascular dysfunction.
- P2X7 inhibitors may provide a new avenue for treatment in cardiovascular disorders.

2.4 Introduction

Cardiovascular disease is the leading cause of mortality worldwide, representing up to 31% of annual global deaths.(1) Despite its widespread prevalence, there remain inadequate treatment options for a large proportion of patients, in part due to the complex and varied pathophysiology involved in cardiovascular disease. In recent years the role of inflammation in cardiovascular disease has been garnishing a lot of attention. Numerous studies have indicated a prominent role for low-grade inflammation in the development of hypertension, atherosclerosis, myocardial ischemic injury and heart failure. Consequently, targeting the source of inflammation in these conditions remains a tantalizing yet elusive option. One such target that has shown promising results is the purinergic receptor P2X7.

P2X7 belongs to a family of purinergic receptors divided into two classes: metabotropic G protein-coupled P2Y receptors and ligand-gated ion channel P2X receptors. P2X receptors are primarily activated by extracellular adenosine triphosphate (ATP), with P2X7 being distinct from the other receptors due to its low affinity for ATP. P2X7 requires 100-1000x physiologic concentrations of extracellular ATP for its activation with a reported EC_{50} of ~100 μM.(2) With transient stimulation, the P2X7 receptor acts as a non-specific cation channel facilitating Na^+ and Ca^{2+} influx and K^+ efflux, resulting in the activation of numerous downstream signaling complexes in a cell type-dependent manner (reviewed by(3, 4)). The most prominent downstream effector of P2X7 activation is the nuclear oligomerization domain like receptor family pyrin domain containing 3 (NLRP3) inflammasome. The NLRP3 inflammasome cleaves and activates caspase-1, which subsequently cleaves the pro-inflammatory cytokines pro-interleukin (IL)-1β and pro IL-18 into their mature, active forms. Prolonged stimulation of P2X7 with ATP promotes the formation of macropores in the cell membrane, resulting in an inflammatory cell death program termed ‘pyroptosis’.(2, 5-7) IL-1β and IL-18 are the primary mediators of P2X7-induced inflammation, which facilitate immune cell recruitment and inflammation, endothelial dysfunction, plaque formation and cardiac dysfunction (reviewed by(8-10)).

Beyond the predominant role of P2X7 in triggering inflammation and cellular death, it has been implicated in numerous other functions including nociception, vascular function, glucose uptake, and paradoxically, promoting cellular survival (interested readers are directed to the following excellent articles(11-15)). The pleiotropic effect of P2X7 is in part cell type dependent and in part dependent on the isoform of P2X7 expressed. Ten human splice variants have been

identified, named P2X7A to P2X7J.(16-19) Isoform A (P2X7A), the full length receptor, responds in a biphasic manner, with tonic activation by low concentrations of ATP promoting cellular proliferation, and high concentrations of ATP promoting the typical responses of P2X7 activation, such as inflammasome activation and pore formation.(16, 20, 21) P2X7B has a truncated carboxy terminal, impairing its pore forming ability.(20) It has been demonstrated to have an anti-apoptotic effect in numerous cell types.(14, 20-26) Isoforms P2X7C, P2X7E, P2X7G and P2X7J also have a truncated carboxy terminal inhibiting their pore forming ability, but their functional role is unclear, while the P2X7I isoform result in loss of function of the receptor.(16, 19, 27, 28) In rodent T cells, an additional variant, P2X7K, mediates T cell responses to ATP and NAD^+, facilitating T cell class switching through CD62L and CD27 cleavage and cellular death via externalization of phosphatidyl serine.(17, 29-31) To date, a human homologue of P2X7K has yet to be identified and its relevance in human pathology is unclear. Finally, P2X7 variants may be preferentially expressed in different cell types, and are known to have varying affinities to ATP (P2X7K>A>B), which may help account for the cell type-dependent P2X7 responses.(17, 18, 21, 32) Despite the work done so far to delineate the function of P2X7 variants, much remains unknown with regard to their role in disease progression, particularly in cardiovascular disease.

P2X7 receptor activation has been implicated in the progression of many chronic inflammatory diseases. In animal models, P2X7 inhibition has proven to be an effective treatment strategy for many chronic inflammatory disorders including arthritis, Duchenne's muscular dystrophy, multiple sclerosis, Alzheimer's disease, chronic pain and cardiovascular disease.(33) However, the functional role of P2X7 outside of inflammation remains largely uncharacterized, and the pleiotropic nature of P2X7 function raises the question of the feasibility of P2X7 as a therapeutic target.

This review will focus on the role of P2X7 in the cardiovascular system and postulate on its utility as a target for treatment and management of cardiovascular conditions.

2.5 Hypertension

Hypertension affects approximately 1.13 billion people worldwide and is the largest cause of burden of disease worldwide, and the most important risk factor for the development of cardiovascular disease.(1) Current studies investigating the role of P2X7 in hypertension are limited. However, available studies point to a role of P2X7 in regulating inflammation, as well as

vascular and renal function in response to hypertensive challenges. The single nucleotide polymorphism (rs598174) for P2X7 was strongly associated with both systolic and diastolic ambulatory blood pressure (BP) in a Caucasian population.(34) In a Chinese population of post-menopausal women, a hypomorphic single nucleotide polymorphism (rs3751143) for P2X7 was associated with a decreased risk of primary (formerly called essential) hypertension.(35) Increased inflammasome expression and circulating IL-1β in subjects over the age of 60 years was strongly associated with increased risk for hypertension and vascular dysfunction, as well as all-cause mortality.(36) Furthermore, elevated plasma ATP levels have been observed in hypertensive patients in comparison to normotensive controls or patients with controlled hypertension, leading to heightened T cell responses in a P2X7-dependent manner.(37)

2.5.1 P2X7, IL-1β and Hypertension

There is accumulating evidence that P2X7 contributes to the connection between low-grade chronic inflammation and hypertension.(8) Macrophages isolated from Dahl salt-sensitive rats produced more IL-1β in response to ATP than normotensive Lewis rats, highlighting heightened inflammasome responses in rats genetically predisposed to hypertension.(38) NLRP3 inflammasome proteins in mice and IL-1β in humans, have been reported to be elevated in hypertension, and directly antagonizing the NLRP3 inflammasome has modestly reduced BP in various animal models of hypertension.(39-42) Targeting IL-1β using Anakinra, an IL-1 receptor antagonist, significantly reduced BP in a one kidney deoxycorticosterone acetate (DOCA)-salt model of hypertension.(43) However, the efficacy of targeting IL-1β in human hypertensive patients is unclear. In the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), patients given an anti-IL-1β monoclonal antibody (canakinumab) had no reduction in BP at 3, 6 or 12 months follow up and there was no reduction in incident hypertension in the cohort.(44, 45) Despite a lack in reduction of BP, patients with elevated systolic BP (130-140 mmHg) or hypertension (systolic BP>140 mmHg) treated with canakinumab had a significant reduction in composite end-points (myocardial infarction, stroke or cardiovascular mortality).(44, 45) The CANTOS trial suggests that blocking IL-1β alone may be insufficient to reduce BP, and therefore targeting upstream of IL-1β, at the P2X7 receptor, may provide a more attractive target for BP management as it could antagonize additional downstream effects of P2X7 activation outside of IL-1β production (discussed below).

2.5.2 P2X7 and Kidney Function

Under non-pathological conditions, there is sparse P2X7 expression throughout the kidney. However, P2X7 expression is significantly increased in hypertensive states.(46) Transgenic rats expressing the mouse *Ren-2* gene have an overactivated renin-angiotensin-aldosterone system and develop severe hypertension that can be attenuated with angiotensin converting enzyme inhibitors.(47, 48) These transgenic rats have increased P2X7 expression in the glomeruli in comparison to normotensive rats.(46) Other hypertensive models demonstrate similar results, with P2X7 expression significantly increased in the kidney in angiotensin II (Ang II) and DOCA-salt induced hypertensive rodents, as well as in Dahl salt-sensitive rats.(38, 49-51) P2X7 receptor silencing decreased renin activity and angiotensin converting enzyme 1 and 2 expression in the renal cortex, preventing renal dysfunction in a model of diabetic nephropathy.(52) In addition, P2X7 antagonism may also reduce the pro-hypertensive effects of Ang II. Ang II acts as a potent vasoconstrictor of the renal vasculature, and it can alter renal sodium and water handling through increased aldosterone release.(53) In rodent models, P2X7 antagonism reduced renal vascular resistance, and increased medullary perfusion resulting in enhanced pressure natriuresis.(49, 50, 54) Menzies et al. reported a six-fold increase in sodium excretion with P2X7 antagonism, blunting Ang II-induced BP elevation in rats.(49) In addition, ATP promotes transepithelial sodium transport through epithelial sodium channels, which can be attenuated by brilliant blue G, a P2X7 antagonist.(55) This, along with increased pressure natriuresis, may account for the increased Na^+ excretion associated with P2X7 antagonism.(49, 50) However, another study found that P2X7 antagonism had no effect on Ang II-induced BP elevation in rats, although the authors used a 10-fold higher dose of Ang II which may account for the differences observed.(50) Overall these studies provide evidence for a role of P2X7 in the regulation of kidney responses to hypertensive stimuli and support P2X7 as a novel anti-hypertensive target.

Further supporting the beneficial effects of inhibiting P2X7, activation of the receptor itself exerts pro-hypertensive effects in the kidney. Ang II and aldosterone both increase renal ATP concentrations, with the concentration of renal interstitial ATP strongly correlated with BP increase.(56, 57) P2X7 activation on the renal vasculature, by elevated ATP, appears to exert a tonic vasoconstrictive effect.(49) In addition, P2X7 mediated vasoconstriction of the medullary microcirculation has been shown to cause regional hypoxia promoting vascular hypertrophy, and renal inflammation.(49) Prolonged exposure to elevated extracellular ATP results in P2X7-

mediated mesangial, fibroblast, endothelial, and renal tubular cell death, contributing to renal inflammation and fibrosis as well as promoting endothelial dysfunction.(58-62) P2X7 antagonism results in a partially nitric oxide (NO)-dependent vasodilation of the afferent, efferent, and renal arteries, increasing renal perfusion and reducing renal inflammation and fibrosis.(49, 50, 52, 54) P2X7 knockout (KO) or antagonism has also proved effective in preventing renal fibrosis, renal immune cell infiltration and lowering BP and albuminuria in Dahl-salt sensitive rats, and in a DOCA-salt model of hypertension.(38, 51) In summary, continuous P2X7 activation leads to microvascular dysfunction and regional hypoxia. This promotes renal inflammation and renal fibrosis, leading to a decline in renal function that contributes to hypertension.

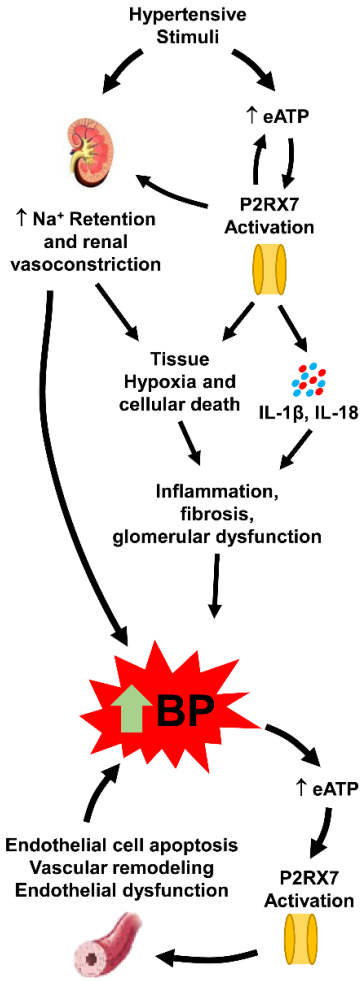

Figure 2.1: P2X7 and Hypertension. Hypertensive stimuli induce an upregulation of P2X7 receptor surface expression as well as directly and indirectly cause increases in extracellular ATP in the renal interstitial fluid. Elevated ATP activates P2X7 receptors promoting cellular death, causing

the release of pro-inflammatory cytokines, inducing renal vasoconstriction, and promoting sodium retention. P2X7-induced renal vasoconstriction causes tissue hypoxia, where along with inflammatory cytokines and reactive oxygen species, it causes inflammation, fibrosis and glomerular dysfunction. Together, renal fibrosis, increased sodium retention and renal vasoconstriction promote a rise in blood pressure that can increase systemic circulating ATP concentrations. The resulting P2X7 activation promotes endothelial cell apoptosis, vascular remodeling and ultimately endothelial dysfunction, which further exacerbates the increase in blood pressure. Abbreviations: BP, blood pressure; eATP, extracellular adenosine triphosphate; IL, Interleukin; P2RX7, P2X7 receptor; TNF-α = tumor necrosis factor alpha.

2.5.3 P2X7 and Systemic Vasculature

P2X7 expression has been reported in the endothelium and the smooth muscle layer of most of the systemic arterial and venous circulation in human and animal tissues.(63-66) In the microvasculature, P2X7 activation has been shown to promote vascular dysfunction through increased oxidative stress, and increased endothelial cell permeability and apoptosis. In a rat model of type 1 diabetes, P2X7 expression was found to be elevated in the retinal microvasculature, contributing to increased microvasculature permeability, whereas in human retinal epithelial cells P2X7 activation induced endothelial cell death.(67, 68) In both experiments microvasculature dysfunction could be reversed by a P2X7 inhibitor. Further it was demonstrated that P2X7 vasotoxicity was mediated through P2X7-dependent pore formation as well as NADPH oxidase-dependent ROS generation.(69) In addition, surgical stretch of human saphenous veins prepared for coronary artery bypass grafts caused P2X7 activation inducing apoptosis resulting in vascular dysfunction.(60) P2X7 activation can also induce constriction of the retinal and renal microvasculature as well as of large veins, which could lead to increased systemic vascular resistance.(49, 50, 63, 70) In diabetic rats P2X7 antagonism improved endothelium-dependent relaxation, and decreased constrictor responses to phenylephrine in the aorta.(71) A model investigating vascular surgical stretch injury demonstrated that P2X7 activation diminished endothelium-dependent relaxation through decreased NO production.(62) The resulting vascular dysfunction and remodeling can contribute to increased systemic vascular resistance and the development of hypertension (reviewed in(72, 73)).

However, conflicting results suggest P2X7 activation may also play a role in vasodilatation. P2X7 activation on murine mesenteric artery endothelial cells resulted in enhanced NO production.(74) In addition, P2X7-mediated responses to lipopolysaccharides have been reported

to cause hyporeactivity of the thoracic aorta in mice, leading to P2X7-mediated hypotension in an IL-1β- and NO-dependent manner.(75, 76) These studies highlight critical differences in the role of P2X7 responses in different tissues and different disease states. It remains unclear, if in a chronic disease setting such as hypertension, P2X7 antagonism could be beneficial or detrimental to vascular function and mechanics, and the area warrants further investigation.

2.6 Atherosclerosis

Atherosclerosis is a common comorbidity with hypertension and presents similar features, such as endothelial dysfunction and low-grade inflammation.(77) Immune cell recruitment and activation at the site of plaques is required for the development of atherosclerotic lesions, and P2X7-directed inflammation could play a central role in plaque formation and promoting plaque rupture. In human carotid arteries presenting with atherosclerotic plaques, there is increased P2X7 expression in plaque-rich areas compared to regions devoid of plaques.(66, 78) In addition, the expression of P2X7 mRNA in circulating mononuclear cells significantly correlates with the degree of coronary artery stenosis.(79) ATP accumulates in atherosclerotic vessels as compared to non-atherosclerotic ones, and elements of the inflammasome (NLRP3, caspase-1 and IL-1β) are increased in plaque-rich regions, providing an indication of P2X7 activation.(66, 80, 81) Together, these studies provide support for an involvement of P2X7 in the development of atherosclerosis, and there are several potential mechanisms for P2X7 activation in atherosclerosis.

P2X7 activation in atherosclerosis may be initiated through alterations in blood flow (turbulent blood flow) or as a result of a secondary metabolic disorder. At sites with turbulent blood flow, there is a dramatic elevation in local extracellular ATP.(82, 83) The increase in ATP is driven through decreased ATPase (CD39) expression and an enhanced release of ATP from endothelial cells in regions rich with caveolin.(84-86) These sites of turbulent blood flow have increased P2X7 expression, which can co-localize with Caveolin-1, placing P2X7 receptors proximal to sites of ATP release.(87-91) These P2X7 receptors expressed in caveolin-1-rich domains have been shown to be non-pore forming, and instead facilitate intracellular Ca^{2+} accumulation, leading to p38 MAP kinase phosphorylation, and subsequent up-regulation of surface adhesion molecules in plaque prone regions.(87, 88, 92) In addition, exposure of endothelial cells and human fibroblasts to high concentrations of glucose or palmitate, such as in diabetes, causes extracellular ATP release and the formation of P2X7 aggregates near the cell periphery.(93-95) These P2X7 aggregates have a

lowered threshold for activation to ATP and mediate endothelial dysfunction through elevated ROS generation, increased cell permeability, and expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1.(78, 93, 94, 96, 97) Furthermore, oxidized low density lipoproteins and cholesterol crystals, common elements in atherosclerosis, can activate the NLRP3 inflammasome resulting in the release of IL-1β and IL-18.(98-101) Consequently, factors common to atherosclerosis development, hyperglycemia, hyperlipidemia, oxidized low density lipoproteins, cholesterol crystals and turbulent blood flow have been shown to influence P2X7 activation.

P2X7 activation on endothelial cells at sites prone to development of atherosclerosis promotes leukocyte recruitment, adhesion and transmigration into the developing plaque through the production of pro-inflammatory cytokines, ROS generation, and increased cellular adhesion molecules on endothelial cells.(78, 80, 87, 93) The subsequent tissue damage amplifies extracellular ATP concentrations and facilitates P2X7-mediated IL-1β secretion from smooth muscle cells, and infiltrating leukocytes.(78, 80, 102) Secreted IL-β then triggers the release of matrix metalloprotease 9 from vascular smooth muscle cells and leukocytes, which destabilizes the plaque and renders it vulnerable and prone to rupture.(80, 102-104) Furthermore, P2X7 facilitates thrombosis at the site of the ruptured plaque. When exposed to elevated circulating ATP, myeloid and smooth muscle cells release tissue factor in a P2X7/ROS-dependent manner which triggers thrombus formation, and can lead to coronary obstruction and sudden death.(105, 106)

Strategies targeting P2X7 or its downstream effectors have proven efficacious in preventing atherosclerosis progression in several pre-clinical and clinical models. P2X7 KO mice present with lower blood cholesterol than wild-type mice, and in atherosclerotic animal models have decreased plaque size.(78, 107) The reduction in lesion size appears to be the result of decreased leukocyte recruitment and macrophage infiltration in P2X7 KO animals or after P2X7 antagonism.(78, 87) The attenuated immune infiltration was associated with decreased adhesion molecule expression on endothelial cells, with decreased caspase-1 activation and pro-inflammatory cytokine release.(78, 87) Decreased cholesterol levels in P2X7 KO mice may also play a role in decreasing inflammation, as oxidized low density lipoproteins and cholesterol crystals have been shown to induce inflammasome activation that promotes atherosclerosis.(98, 99, 101, 107) In addition, P2X7 receptor targeting or IL-1β blockade increased plaque stability through inhibition of matrix metalloprotease 9 release.(80, 104) In the CANTOS trial, IL-1β blockade resulted in a reduction in all cardiovascular events, including coronary revascularization and myocardial infarction (MI),

without lowering systemic lipid levels.(44) This reduction in adverse cardiovascular events was comparable to the effects of lipid lowering by proprotein convertase subtilisin-kexin type 9 inhibitors.(44, 108, 109) Whether P2X7 antagonism rather than P2X7 KO also reduces blood cholesterol has yet to be determined. In summary, targeting downstream P2X7 effector molecules or P2X7 receptors prevents leukocyte recruitment and inflammation in plaques, prevents plaque rupture and may have lipid lowering and anti-thrombotic effects, making P2X7 a potential target in managing atherosclerosis.

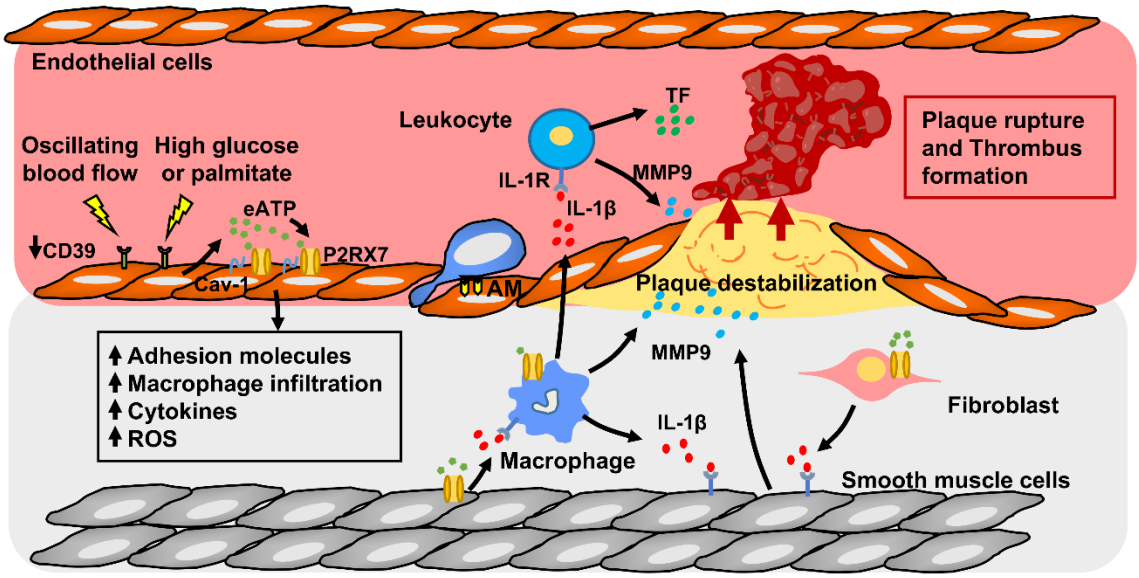

Figure 2.2: P2X7 and Atherosclerosis. Oscillating flow or high glucose or palmitate promote P2X7 surface expression, elevate extracellular ATP and decrease ATPase (CD39) expression in the endothelium at sites prone to develop atherosclerosis, creating an environment suitable for enhanced P2X7 activation. Endothelial P2X7 activation promotes leukocyte recruitment, adhesion and transmigration into the developing plaque through production of inflammatory cytokines and increased adhesion molecule expression on endothelial cells. P2X7 dependent IL-1β production from vascular smooth muscle cells (VSMCs), macrophages, and fibroblasts promotes MMP9 release from macrophages and VSMCs. MMP9 destabilizes the plaque, making it vulnerable to rupture, whereas P2X7 activation on myeloid cells induces the release of tissue factor promoting thrombus formation. Abbreviations: AM, adhesion molecules; Cav-1, Caveolin-1; eATP, extracellular ATP; IL-1β, Interleukin-1β; IL-1R= Interleukin-1 Receptor; MMP9, Matrix Metalloprotease 9; P2RX7, P2X7 receptor; TF, Tissue Factor.

2.7 Heart Disease

Heart disease encompasses a wide variety of conditions with inflammation being the primary driver of many non-congenital conditions.(110) IL-1β and IL-18, downstream effectors of P2X7, have been repeatedly identified as mediators to this inflammatory response.(111, 112) A loss of function P2X7 variant, rs3751143, was significantly associated with a decreased risk of ischemic heart disease and stroke, especially in individuals with hypertension.(113) However, the contribution of P2X7 to heart disease has still yet to be fully elucidated.

2.7.1 Myocardial Ischemic Injury

During cardiac ischemia there is an interruption of blood flow to coronary tissue that can disrupt cardiac function and damage surrounding tissues, resulting in a substantial release of ATP.(114, 115) The rise of ATP following ischemia/reperfusion (I/R) activates surrounding cardiac fibroblasts, stimulating P2X7-mediated release of IL-1β, IL-18 and ROS that can lead to the recruitment of leukocytes to the hypoxic region.(116-118) The recruited leukocytes then contribute to amplify inflammation through P2X7-mediated activation and release of IL-1β and IL18, thus promoting myocardial damage and cardiac fibrosis leading to declining cardiac function.(118-120) Inhibition of IL-1β, IL-18 or caspase-1 significantly decreased infarct size and improved contractile function of the heart.(118, 119) However, whether P2X7 antagonism alone in I/R in the heart would also be protective is unclear.

Paradoxically, preconditioning cardiac tissue with short bouts of ischemia/reperfusion has shown to protect from I/R injury through an ATP-driven mechanism.(121) Cardiac protection was facilitated through the release of sphingosine-I-phosphate and adenosine via P2X7/pannexin-1 pores, occurring pre-ischemia and post-reperfusion.(121-123) Inhibition of pannexin-1 or P2X7 abrogated the protective effect of I/R conditioning and resulted in increased infarct sizes.(121) The difference between protection and harm associated with P2X7 activation may be the result of P2X7 splice variants. Splice variants of P2X7 are known to have varying affinities for ATP and can elicit different responses.(17) Further strengthening this hypothesis, P2X7 functional coupling with pannexin-1 was found to be dependent on the P2X7 isoform expressed, specifically to a common allelic mutation resulting in a proline to leucine mutation at amino acid 451 in the P2X7A variant.(17) This same mutation, was found to result in a decreased sensitivity to ATP (~10-fold).(124) In addition, activation of P2X7A with low concentrations of ATP has been demonstrated to have growth promoting effects.(21) Since P2X7-mediated protection from I/R was

dependent on pannexin-1 coupling, it is possible that the differing effects of P2X7 in I/R are dependent on the isoform of P2X7 expressed. Whether the protective effect of P2X7 activation during I/R is mediated through one of these splice variants has yet to be shown, but if this is the case, this could provide a selective target in order to protect the heart during I/R without the accompanying inflammation.

2.7.2 Angina Pectoris

Angina is a common symptom in many patients suffering from coronary ischemia, and P2X7 appears to play an important role in persistent angina symptoms post MI. After acute MI, P2X7 mRNA and protein were upregulated in the superior cervical ganglia and in cardiac sympathetic afferents of rats.(125-127) P2X7 dependent transmission of nociception down these cardiac afferents has been demonstrated, along with activated cardiac sympathetic efferent nerves, leading to increased BP, heart rate and circulating pro-inflammatory cytokines (TNF-α and IL-6). P2X7 antagonism post MI attenuates sympathetic stimulation of cardiac tissue, reducing tachycardia, blood pressure, myocardial injury and nociception signaling, ultimately alleviating symptoms of angina.

2.7.3 Myocardial Infarction and Heart Failure

MI is a life-threatening condition caused by obstruction of blood flow to cardiac tissue. Following an acute MI there is a substantial increase in extracellular ATP released from damaged cells. This rising extracellular ATP promotes P2X7-mediated inflammasome formation and activation around the border of the infarct in surrounding fibroblasts, cardiomyocytes and invading leukocytes, leading to elevated IL-1β and IL-18.(128-130) P2X7 activation in cardiomyocytes promotes caspase-dependent apoptosis, which contributes to cardiac dysfunction.(128, 131, 132) During acute MI, epicardium derived cells are also directed to the infarct region.(120) Epicardium derived cells give rise to various cardiovascular cells and migrate to injured myocardium to initiate tissue repair.(133-136) However, during ischemia, invading epicardium derived cells can also promote further inflammation by secreting ATP, NAD, and tenascin-C.(120) Tenascin-C can prime the NLRP3 inflammasome via toll like receptor 4 activation, and coupled with elevated ATP, can activate the inflammasome in infiltrating leukocytes, further amplifying inflammation.(118, 120) Elevated IL-1β and IL-18 contribute to cardiac enlargement, cardiac fibrosis and a deterioration of heart function post MI leading to heart failure.(119, 128, 137, 138) Additionally, the NAD released by epicardium derived cells can cause P2X7-mediated phosphatidyl serine exposure on the outer

leaflet of T regulatory cells (Tregs) leading to their death.(29, 30, 139, 140) Tregs normally increase in ischemic tissue 3-7 days after reperfusion, and contribute to resolution of inflammation and promote tissue repair.(141) P2X7 activation may lead to a decreased presence of anti-inflammatory Tregs in ischemic tissue, and indeed, P2X7 antagonism in a kidney I/R model resulted in a significant increase of infiltrating Tregs and improved tissue recovery.(142)

Antagonizing or knocking out P2X7 or its downstream effectors, caspase-1 or NLRP3, in animal models decreased infarct size, improved cardiac function and enhanced survival post MI via reduced IL-1β and IL-18 levels in the heart.(128, 130, 137, 143) Targeting IL-1β directly has also proven effective in reducing cardiac dysfunction and promoting survival post MI in animal models and in clinical trials.(44, 138, 144, 145) The protective effect of P2X7 antagonism in ischemia and acute MI appears to be due to decreased inflammation through decreased pro-inflammatory cytokine production and increased anti-inflammatory Tregs. Therefore, targeting P2X7-mediated inflammation post MI may provide a therapeutic avenue for improved cardiac function and survival in patients. Indeed, circulating P2X7 mRNA expression is predictive of prognosis in acute MI, with elevated P2X7 expression correlating with worse patient outcomes.(79)

2.8 Cerebral Ischemic Injury

P2X7 activation has also been implicated in cerebral ischemic injury (ischemic stroke). In a permanent focal cerebral ischemia model, P2X7 expression was up-regulated on neuronal and glial cells post-ischemia, and was particularly associated with apoptotic cells.(146) P2X7 antagonism in rat transient focal cerebral ischemia models resulted in decreased infarct size, and neuronal death and improved survival.(147, 148) Interestingly, a protective effect in P2X7 KO mice has not been demonstrated. Le Feuvre and colleagues saw no improvement in infarct volume 24 hours after inducing temporary cerebral ischemia in P2X7 KO mice, but did see an improvement using an IL-1 receptor antagonist.(149) In an acute ischemic stroke model in mice, P2X7 KO led to larger edema size within the first 24 hours reperfusion, but not after 72 hours.(150) It is possible that P2X7 activation on microglia by low concentrations of ATP after cerebral I/R provides neuroprotection,(150-152) while prolonged stimulation of P2X7 on glial and neural cells results in cellular death and inflammation.(147, 148, 153) Therefore, P2X7 appears to be a double edged sword in cerebral I/R injury, with P2X7 activation initially providing a neuroprotective benefit, but

with prolonged activation shifting to become a pro-inflammatory mediator exaggerating cerebral ischemic injury.

2.9 Therapeutic Potential of P2X7 Intervention

Downstream targets of P2X7 activation, mainly IL-1β, have been investigated in several clinical studies for efficacy in managing cardiovascular disease and have yielded promising results. The CANTOS trial using the IL-1β antagonist canakinumab, was one of the first trials to demonstrate that the risk for recurrent cardiovascular disease could be decreased by lowering inflammation without lowering systemic lipid levels.(44) However, patients on canakinumab had a significantly increased risk of fatal infection, although there was no difference in all-cause mortality between groups (median patient follow up of ~3.7 years). Targeting P2X7 rather than IL-1β could have several advantages. First, P2X7 activation is a major mediator of IL-1β production, but not the only one, and whether prolonged use of a P2X7 antagonist would also increase the risk of fatal infection is unclear at this time, although clinical trials conducted thus far with P2X7 antagonists have had limited to no serious adverse advents reported for up to 6 months of treatment. Additionally, P2X7 antagonism has the added benefit of blocking other downstream effects of P2X7 activation that can be deleterious to health, such as cellular death. Finally, P2X7 antagonism may be especially beneficial in patients with cardiovascular disease and metabolic disorders such as hyperlipidemia or hyperglycemia. In pre-clinical models, P2X7 antagonism was able to diminish inflammasome activation by non-nucleotide agonists such as oxidized low density lipoproteins, glucose and palmitate, highlighting an additional benefit when treating disorders such as atherosclerosis.(93, 101)

Although animal models targeting P2X7 in cardiovascular disease have shown favourable results, to date, there have been no clinical trials investigating P2X7 antagonism in cardiovascular disease. Over 70 patents for P2X7 antagonists have been filed, with several P2X7 antagonists having undergone clinical investigation for various inflammatory conditions with mixed results (Table 1).(154) AstraZeneca's P2X7 antagonist (AZD9056) had no effect on reduction of inflammatory biomarkers or disease index in patients with chronic obstructive pulmonary disease or rheumatoid arthritis, but modestly improved the disease index in Crohn's disease (specifically decreased nociception) despite no reduction in inflammatory biomarkers.(155-157) Similarly, Pfizer's P2X7 antagonist (CE-224,535) was inefficacious in lowering disease activity or

inflammatory biomarkers in rheumatoid arthritis patients inadequately controlled by methotrexate.(158) Ex-vivo analysis had demonstrated that AZD9056 was able to inhibit IL-1β ex-vivo in human monocytes, and therefore it was postulated that in these pathologies inhibiting the P2X7-IL-1β and IL-18 inflammatory axis was insufficient to control disease progression and that other inflammatory cytokines could potentially be major contributors.(156) Due to the failure of these drugs to adequately suppress systemic inflammation, both companies abandoned their clinical trials after completion of phase II.(155-158) Recently, a phase II clinical trial by Evotec and Second Genome investigating P2X7 antagonism in nonalcoholic steatohepatitis was also terminated due to an unfavorable risk-benefit profile with their P2X7 inhibitor.(159)

Despite underwhelming results from early clinical trials, the recent crystallization of P2X7 has further facilitated the development of more targeted P2X7 antagonist therapeutic strategies that could further enhance clinical efficacy.(160) Janssen has designed new P2X7 agents for diagnosis and treatment of mood disorders that can penetrate the blood-brain barrier and have shown encouraging results in phase I clinical trials.(161-163) Specific interest has begun to emerge at targeting P2X7 variants in disease settings. Biosceptre has developed a monoclonal antibody to an epitope termed E200, which is associated with nonfunctional variants of P2X7 and has demonstrated efficacy in a phase I clinical trial for the treatment of basal cell carcinoma.(164) As P2X7 variants may also contribute to the pathogenesis of cardiovascular disease, such as I/R injury, it is an interesting avenue of research that merits more attention. Recently, a P2X7 specific nanobody, $1/10^{th}$ the size of an antibody, was developed that was able to block P2X7 mediated IL-1β release with 1000x greater potency than Janssen's or AstraZeneca's small molecule inhibitors JNJ47965567 and AZ10606120.(165, 166) The enhanced specificity of P2X7 antagonists opens the door for potentially targeting other P2X7 variants in disease settings, and will be an interesting avenue of research to follow over the coming years.

Despite the lack of efficacy for disease management of early P2X7 antagonists in human clinical trials, they provide evidence for the relative tolerability of P2X7 antagonists, as limited to no serious adverse advents were reported in the majority of clinical trials conducted so far. Therefore, since animal models have demonstrated a potential benefit for P2X7 antagonism in the context of hypertension, atherosclerosis and heart disease, and clinical trials have provided a precedent for safety of P2X7 directed inhibitors, P2X7 antagonists may represent a viable therapeutic option in the management of cardiovascular disease.

2.10 Conclusion

P2X7 is a key player in promoting inflammatory responses to tissue injury. In cardiovascular disease, P2X7 activation promotes endothelial dysfunction and inflammation that drives kidney, and cardiac dysfunction, atherosclerosis, hypertension development and the progression of heart failure. Pre-clinical models investigating P2X7 receptor KO or antagonism in cardiovascular disease have shown promising results in attenuating disease. Current clinical trials of P2X7 antagonists have shown P2X7 antagonists to be mostly well tolerated and therefore, P2X7 inhibition may represent an untapped resource for the management of cardiovascular disease.

2.11 Acknowledgements

None.

2.12 Source of Funding

The work of the authors was supported by the CIHR First Pilot Foundation Grant 143348, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program, and by the Canada Fund for Innovation, to ELS, and by the Fonds de recherche Santé Quebec (FRQS) bourse 289184, Lady Davis Institute/TD Bank Studentship award and Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship to BGS.

2.13 Disclosures

None.

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*Table 2.1: Current and past clinical trials investigating the efficacy of P2X7 antagonism for disease management. *Estimated patient recruitment number..*

Company	Compound Name	Indication	Phase	Study Centres	Year Completed	Number of patients enrolled (completed)	Results	Ref.
Astrazeneca	AZD9056	Chronic Obstructive Pulmonary Disease	Phase II	28 centres across Bulgaria, Germany, Hungary, Sweden and United Kingdom	2006	271(120)	Safe, tolerable, no effect on lung function	(155)
		Crohn's Disease	Phase II	10 centres across Belgium, France, Germany, Austria and Hungary	2007	34(30)	Safe, tolerable, improvement in Crohn's Disease Activity Index, no decrease in inflammatory markers (CRP)	(157)
		Rheumatoid Arthritis	Phase II	51 centres across Argentina, Australia, Belgium, Canada, Czech Republic, France, Mexico, Poland, Romania, Russian Federation, Slovakia and the United States	2009	385(316)	Safe, tolerable, no-improvement in disease	(156)
Biosceptre	nfP2X7 antibody	Basal Cell Carcinoma	Phase I	3 sites across the United States	2014	21(20)	Safe, tolerable, reduction in lesion size	(164)
Evotec/Second Genome	SGM 1019	Nonalcoholic steatohepatitis	Phase II	10 sites across the United States	2019	9	Phase II terminated due to unfavorable risk-benefit profile	(159)
GlaxoSmithKline	GSK1482160	Inflammatory Pain	Phase I	One centre in the United Kingdom	2009	10(10)	Not possible to achieve level of pharmacology (>90%IL-1b inhibtion) within an adequate safety margin	(167)
Pfizer	CE-224,535	Rheumatoid Arthritis	Phase II	24 centres across Chile, Czech Republic, Mexico, Poland, Republic of Korea, Spain and the United States	2009	100(86)	Safe, tolerable, no improvement in disease condition	(158)
Janssen	JNJ-54175446	Mood Disorders	Phase II	5 centres across the United Kingdom	Underway	142*	Recruitment suspended due to Covid-19 pandemic	(161, 163)

Chapter 3: Hypothesis, Objectives and Experimental Design

3.1 Hypothesis and Objectives

Despite intensive efforts, and although BP control in Canada is among the highest in the world, there remains a large proportion of patients with resistant hypertension. Both the innate and adaptive immune systems play an essential role in the development of hypertension and cardiovascular injury, making the immune axis a compelling target for a new class of anti-hypertensives.(135)

P2RX7 is expressed throughout most of the body but is abundantly expressed in both innate and adaptive immune cells and therefore impacts both arms of the immune system.(383) Innate immune cells play a role in the initiation and subsequent direction of the adaptive immune response, a mechanism that may be misdirected in hypertension to cause cardiovascular injury. For instance, Itani *et al*. (121) demonstrated that CD70 on innate immune cells was necessary for generating T_{EM} cells during an initial hypertensive episode. These T_{EM} cells sensitize mice to develop hypertension to a second mild hypertensive challenge, leading to end-organ damage. Evidence has emerged that P2RX7 contributes to T_{EM} formation and retention in other disease settings and may similarly contribute to T_{EM} formation in hypertension.(247, 248) In hypertension, turbulent blood flow, endothelial stretching or cardiovascular injury may cause the release of ATP that could contribute to the activation of P2RX7. Indeed, extracellular ATP is elevated in murine models of hypertension and hypertensive humans, providing a potential mechanism for P2RX7 activation.(103) Therefore, stimulation of P2RX7 could play a critical role in the activation and persistence of an activated immune system in hypertension.

We hypothesized that P2RX7 KO or antagonism would attenuate Ang II-induced blood pressure elevation and cardiovascular injury by blunting the activation of innate and adaptive immune cells.

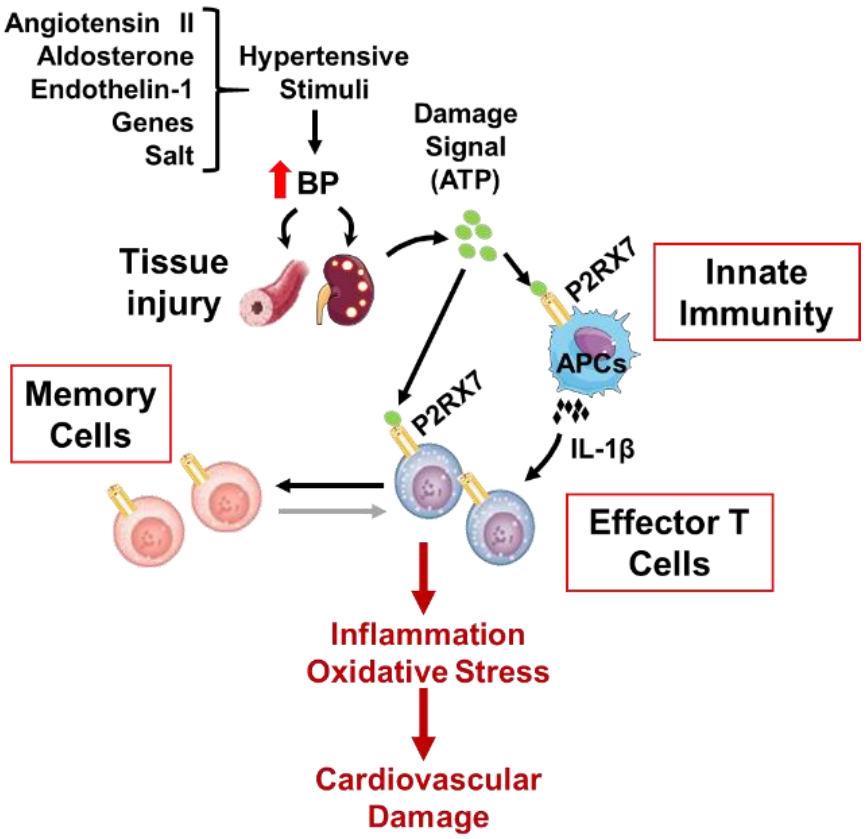

Figure 3.1: Proposed role of P2RX7 in hypertension-associated immune activation and cardiovascular damage. Increased BP induces transient tissue injury, causing ATP release, which stimulates IL-1β production and release from innate immune cells, and T_{EM} formation through P2RX7 activation. Together this increases inflammation and oxidative stress leading to cardiovascular damage.

The work of this thesis aimed to **1)** determine whether Ang II-induced hypertension, cardiovascular injury and activation of innate and adaptive immune cells would be blunted in *P2rx7*$^{-/-}$ mice or mice receiving a P2RX7 antagonist; **2)** determine the relative contribution of P2RX7 versus NLRP3 signalling in the development of hypertension, vascular injury and immune activation.

3.2 Experimental Design

Several commonly employed animal models of hypertension have been designed to mirror as closely as possible human hypertension. For our project, we chose the Ang II-induced mouse model of hypertension in C57BL/6J mice for several reasons. Elements of the RAAS axis are broadly activated in human primary hypertension, and BP elevation from Ang II-infusion closely

resembles BP in stage 2 hypertensives (SBP of ~140-180 mmHg).(384) The model is also reproducible across species (384), and as of 2013, ~48% of NIH-funded research in hypertension used an Ang II model of hypertension. Therefore using an Ang II model allows our results to be more comparable to most studies published in the field.(385) Ang II also engages numerous systems involved in hypertension (discussed in Chapter 1.2.1), such as the CNS, immune system, kidney and cardiac system. Within two weeks of Ang II infusion of doses >400ng/kg/min, vascular dysfunction appears characterized by endothelial dysfunction, inward remodelling, infiltration of immune cells and enhanced ROS production. Similarly, cardiac remodelling and dysfunction are apparent within two weeks of Ang II treatment, especially with larger doses of Ang II. Thus, we chose two weeks of Ang II infusion as our target duration for the experiments outlined in this thesis

Mice, rather than other rodents or large animals, were chosen due to their ease of breeding, lower cost, amenability to developing hypertension, and similarity in the presentation of hypertension to humans. Specifically, the C57BL/6J mouse strain was used as they had *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice commercially available on the C57BL/6J background. Furthermore, C57BL/6 mice are recommended by the American Heart Association for studying RAAS-dependent hypertension.(384) In addition, C57BL/6 mice tolerate multiple operations well, unlike other strains, (386) making them suitable for our purposes where the mice undergo surgery for telemetry probe implantation and then a separate surgery for osmotic mini-pump insertion.

The experimental design for aim 1) and aim 2) of this thesis are available below in Figure 3.2 and Figure 3.3, respectively. In aim 1) we chose a dose of 1000ng/kg/min of Ang II to provoke cardiac injury, whereas in aim 2) we used a lower dose of 490ng/kg/min to more closely mimic the slower progression of hypertension in humans and to discern more minor differences in Ang II-mediated vascular dysfunction. In previous studies, the P2RX7 antagonist, AZ10606120 dihydrochloride (AZ106) was administered by a daily I.P. injection.(387) To limit the handling of the mice and potential interference with BP recordings, we tested whether the compound was stable for 2-weeks at body temperature in a slightly acidic environment, mimicking the conditions that would be present if the drug was co-administered with Ang II in a mini-osmotic pump. After two weeks in the above conditions, we confirmed the compound was stable by demonstrating it was capable of inhibiting IL-1β production from splenocytes to a comparable degree of fresh AZ106 (Figure 3.4). The dose chosen for AZ106 was the maximum soluble dose in water (694 ng/kg/min) that could be infused with Ang II in a mini-osmotic pump. Before proceeding with

Aim 1), we tested to ensure the dose of AZ106 chosen was non-lethal and non-hepatotoxic by comparing liver weights to vehicle-treated animals in a pilot group of 6 mice (Figure 3.5). Once confirmed, we proceeded with the study as outlined in Aim 1 and Figure 3.2.

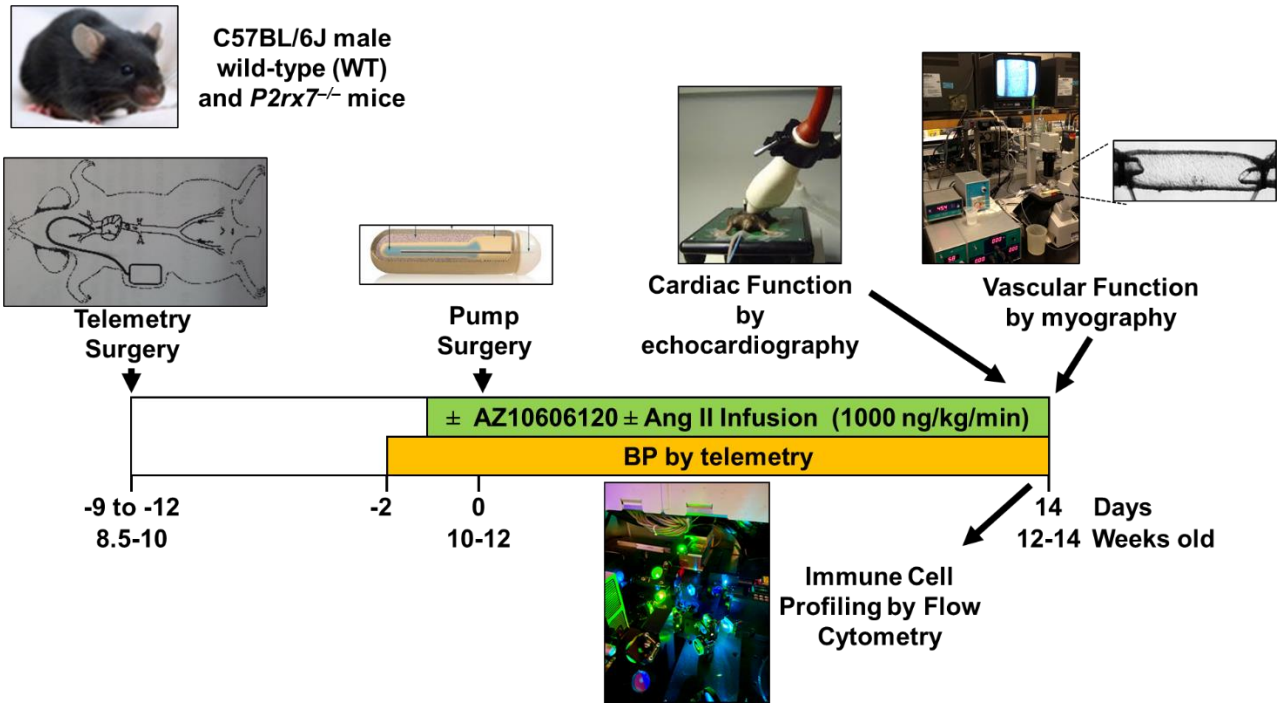

Figure 3.2: Schematic diagram for the endpoints of Aim 1.

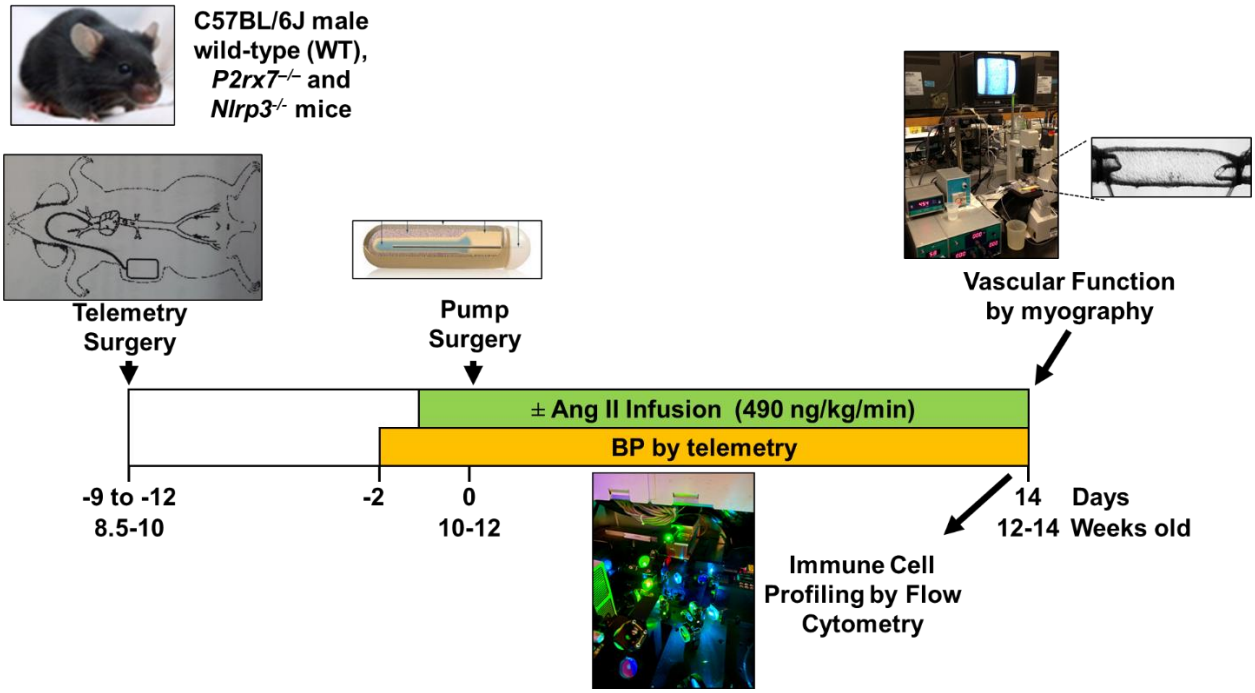

Figure 3.3: Schematic diagram for the endpoints of Aim 2.

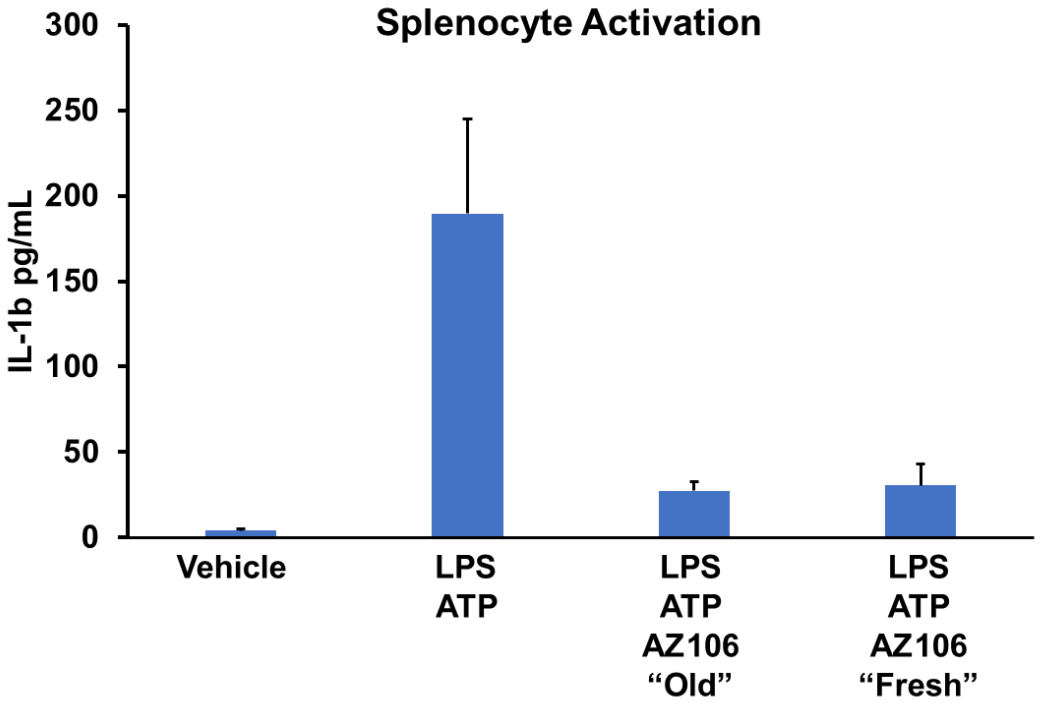

Figure 3.4: AZ106 stability test. Splenocytes were isolated and then primed with lipopolysaccharides (LPS; 1µg/mL) for two hours without or with "Old" AZ106 (1µM) or "Fresh" AZ106 (1µM). After which, the cells were activated for one hour with 1mM ATP. IL1β concentration was quantified from the supernatant using an ELISA. "Old" AZ106 constituted AZ106 that was dissolved in 5 x 10-6 M acetic acid (pH ~5.12) and left at body temperature for two weeks to mimic conditions within the mini-osmotic pump. "Fresh" AZ106 represented AZ106 taken directly from the preserved stock solution. N=3.

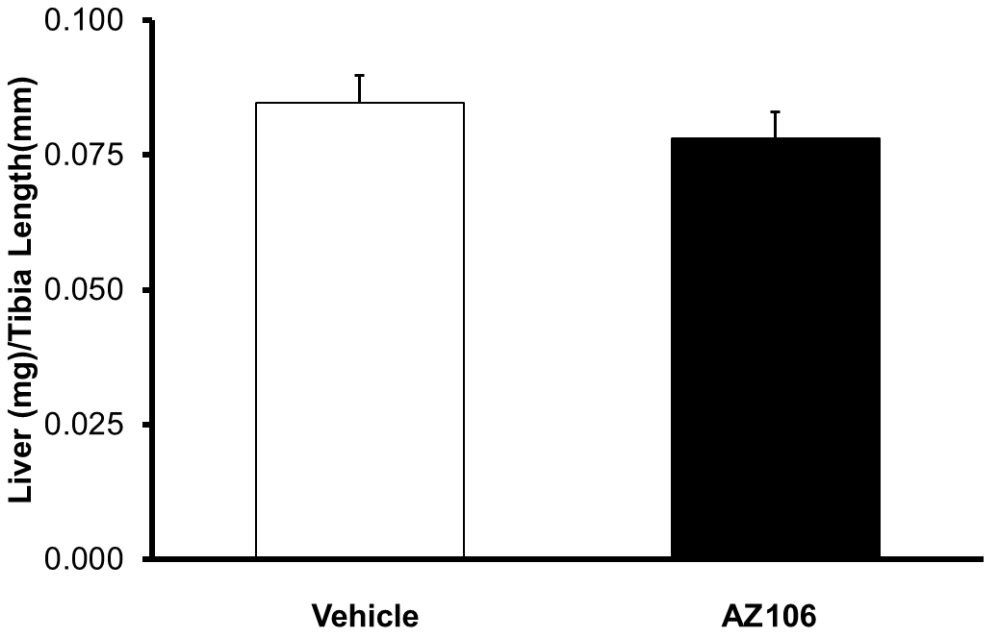

Figure 3.5: Liver weights of vehicle-treated mice or mice receiving the P2RX7 antagonist (694ng/kg/min) for 14 days, corrected to tibia length. N=6.

Chapter 4: P2RX7 Contributes to Angiotensin II-induced Hypertension, Vascular Injury, and T cell Activation, But Its Involvement in Cardiac Dysfunction Is Unclear

Previous reports have suggested that P2RX7 may contribute to the development of hypertension by regulating kidney function; however, no studies have addressed whether P2RX7 affects vascular or cardiac function during hypertension. This chapter aimed to establish whether or not P2RX7 contributes to Ang II-induced immune activation, hypertension, and cardiovascular injury. Particularly we were interested in assessing the accumulation and activation of immune cells in the PVAT, a prominent site for inflammation in hypertension. Our goal was to determine whether innate immune cell activation and subsequent adaptive immune cell activation would be attenuated in the PVAT of mice with P2RX7 signalling inhibited. We had a particular interest in looking for the development of T_{EM} cells in the PVAT, as they are a recently characterized immune population in hypertension, which had not previously been documented in the PVAT. As inflammatory cells in the PVAT contribute to vascular remodelling and dysfunction, our goal was also to assess the extent of vascular damage. Finally, hypertension contributes to LV hypertrophy and cardiac dysfunction, and we wished to determine whether P2RX7 could represent a target for attenuating this dysfunction.

This study confirmed that *P2rx7* KO or antagonism decreased BP and attenuated immune activation and vascular injury. Interestingly, we discovered that *P2rx7* KO mice developed intensified cardiac dysfunction and hypertrophy, whereas this was not the case with P2RX7 antagonism. In addition, using the UK Biobank, we identified 3 SNPs for *P2RX7* that correlated with an increased odds of hypertension, suggesting P2RX7 is also relevant in human hypertension. This study highlighted that P2RX7 antagonism may present a viable target for treating hypertension-associated inflammation, lowering BP and attenuating vascular but not cardiac damage.

The article entitled “P2RX7 antagonism blunts angiotensin II-induced hypertension, vascular injury and T cell activation without the cardiac dysfunction induced by *P2rx7* KO” is in preparation to be submitted for publication. The main manuscript is followed by the Online Supplement, which includes the expanded materials and methods section as well as supplemental figures.

P2RX7 antagonism blunts angiotensin II-induced hypertension, vascular injury and T cell activation without exacerbation of cardiac dysfunction induced by *P2rx7* knockout

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Keywords: blood pressure, cardiac dysfunction, endothelial dysfunction, inflammation, memory T cells

4.1 Abstract

Background: It is now well established that inflammation contributes to the pathogenesis of hypertension, however the initial trigger for inflammation and immune activation is not clear. Damage-associated molecular patterns such as extracellular ATP are elevated in murine models of hypertension and hypertensive humans, suggesting that purinergic receptors may be involved in hypertension. Further, a hypomorphic single nucleotide polymorphism for the purinergic receptor P2X7 is associated with a decreased risk of essential hypertension. Therefore, we sought to identify whether P2RX7 contributes to angiotensin II-induced hypertension and cardiovascular injury.

Methods: We took wild-type and *P2rx7*$^{-/-}$ mice and infused them with Ang II (1000ng/kg/min) for 14 days, monitoring their blood pressure using radiotelemetry. At the end of the protocol, we assessed their cardiac function using echocardiography, the function and mechanical properties of resistance arteries using pressurized myography and assessed immune activation using flow cytometry. In a second group of mice, we utilized a P2RX7 antagonist (AZ10606120 dihydrochloride) to assess similar endpoints as above.

Results: We observed that *P2rx7*$^{-/-}$ mice had attenuated systolic blood pressure but not diastolic BP elevation, resulting in attenuated pulse pressure compared to WT mice. *P2rx7*$^{-/-}$ mice were also protected against Ang II-induced aortic stiffening, and endothelial dysfunction and hypertrophic remodelling of resistance arteries. These changes were associated with decreased activation of $CD4^+$ and $CD8^+$ T cells in the perivascular adipose tissue and reduced propagation of effector memory T cells. However, *P2rx7*$^{-/-}$ mice had exacerbated cardiac dysfunction, with a greater left ventricle mass, a dilated LV chamber, and a more significant fractional shortening impairment than WT mice. Mice receiving a P2RX7 antagonist had lower SBP and DBP, preserved endothelial function in resistance arteries and attenuated T cell activation. Most importantly, mice receiving the P2RX7 antagonist had comparable cardiac function to WT mice.

Conclusion: P2RX7 may present a viable target for attenuating BP, and associated vascular damage, while lowering inflammation and immune activation.

4.2 Introduction

Hypertension remains the leading risk for cardiovascular disease and mortality worldwide.(1) It is now widely accepted that the immune system contributes to the pathogenesis of hypertension and cardiovascular injury; however, the initial trigger for immune activation is unclear.(2) Damage-associated molecular patterns (DAMPs) are endogenous danger signals that play a key role in engaging an immune response.(3) They are released during tissue injury or infection and consist of molecules such as heat shock proteins, uric acid, cholesterol crystals or extracellular adenosine triphosphate (ATP). Recently it was demonstrated that plasma ATP levels are elevated in hypertensive patients in comparison to normotensive controls or patients with well-controlled hypertension, suggesting ATP could act as a DAMP in hypertension.(4)

Extracellular ATP activates a class of receptors referred to as purinergic receptors. There are two broad classes of purinergic receptors, P1 receptors and P2 receptors, of which P2 receptors preferentially respond to ATP. P2 receptors are subdivided into 2 categories, metabotropic G-protein-coupled P2Y receptors and ligand-gated ionotropic P2X receptors. P2Y receptors have varied biological functions depending on the subtype expressed and the tissue they are expressed in.(5) P2X receptors (P2X1-7) act as functional trimers, and when stimulated by ATP, they serve as non-specific cation ion channels, particularly for K^+, Na^+, and Ca^{2+}. Of particular interest is the purinergic receptor P2X7 (P2RX7) which has been implicated in numerous cardiovascular diseases, including hypertension.(6)

P2RX7 is unique from the other P2X channels in that it typically requires high concentrations of extracellular ATP (100-1000x physiologic) for activation.(7) Once activated, a cation flux ensues, regulating numerous cell signalling pathways, including inflammatory signalling, cellular death, and T lymphocyte development and function.(8) A role for P2RX7 has been established in numerous elements pertinent to the pathogenesis of hypertension. P2RX7 was demonstrated to contribute to the vascular tone of the kidney helping regulate glomerular perfusion pressure and natriuresis, with P2RX7 antagonism enhancing sodium natriuresis in rats.(9, 10) Furthermore, P2RX7 antagonism or knockout (KO) protected against vascular injury in models of atherosclerosis, and P2RX7 has been implicated in contributing to cardiac dysfunction during ischemic injuries.(6) Single nucleotide polymorphisms (SNP) in P2RX7 have been identified that correlate strongly with systolic and diastolic blood pressure (rs598174) in a Caucasian population, and a hypomorphic SNP (rs3751143) was correlated with a decreased risk of essential

hypertension in a population of Chinese postmenopausal women.(11, 12) Therefore, P2RX7 seems to be posed at the crossroads for regulating target organ damage in the context of hypertension, particularly vascular and cardiac dysfunction.

We hypothesized that *P2rx7* deficient mice or mice administered with the P2RX7 antagonist (AZ10606120) would experience attenuated vascular and cardiac dysfunction and remodelling due to decreased immune cell activation. We infused wild-type (WT) and P2rx7 deficient mice with angiotensin (Ang) II and monitored their blood pressure via radiotelemetry to test this hypothesis. Next, we assessed the development of cardiac hypertrophy and dysfunction using echocardiography and assessed resistance artery dysfunction using pressurized myography. To investigate whether *P2rx7*$^{-/-}$ diminished immune activation, we profiled immune cell populations in the spleen, bone marrow and aortic perivascular adipose tissue (PVAT) using flow cytometry. Finally, we assessed the utility of a pharmacological P2RX7 antagonist for diminishing Ang II-induced blood pressure elevation, vascular and cardiac dysfunction and remodelling, and immune cell activation.

4.3 Methods

A detailed methods section can be found in the online data supplement.

4.3.1 Experimental Design

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed the Canadian Council of Animal Care recommendations. C57BL/6J WT and *P2rx7*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and a colony was maintained on-site for the duration of the study.

To understand the role of P2RX7 in Ang II-induced hypertension and cardiovascular injury, 10-12-week-old male C57BL/6J WT and *P2rx7*$^{-/-}$ mice were infused or not with Ang II (1000 ng/kg/min, SC) for 14 days. A sub-group of mice were implemented with radio telemetric probes, and their blood pressure and heart rate were continuously measured by telemetry 1 day before and during the treatment period. At the end of the treatment, prior to euthanasia, cardiac function was assessed via echocardiography.(13) Afterward, the mice were euthanized, blood was collected via cardiac puncture in a tube of EDTA, the mesenteric vascular beds attached to the intestine were collected, and endothelial function and vascular mechanical properties were determined in second-order branches of MA by pressurized myography, the ventricle of the heart was dissected and snap-frozen in liquid nitrogen for RT-qPCR analysis, and the aorta with

perivascular adipose tissue (PVAT) was collected and preserved in 4% PFA or Tissue-Tek OCT. In a second group of mice, the spleen, femurs, tibias and aorta with PVAT were collected in ice-cold phosphate-buffered saline and used for immune phenotyping by flow cytometry.

To determine whether P2RX7 antagonism attenuated Ang II-induced hypertension without exacerbating cardiac dysfunction, a group of Ang II treated WT mice were infused with the P2RX7 antagonist AZ10606120 dihydrochloride (AZ106) (694 ng/kg/min) or vehicle for 14 days. BP was determined by telemetry, mesenteric artery function using pressurized myography, cardiac function by ultrasound and infiltration of activated immune T cells in the spleen, bone marrow and aortic PVAT by flow cytometry.

In up to 384,653 unrelated White British UK Biobank participants, associations with measured systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and hypertension were modelled using linear or logistic regression, as appropriate, for 1 324 variants within 50 kb of *P2RX7* transcription. For variants which remained significant after Bonferroni correction for the number of variants tested, associations reported in the GWAS Catalog (37) were extracted. For additional details, see the supplemental methods.

4.3.2 Data analysis

Results are presented as means ± standard error of the mean (SEM). Comparisons in BP and concentration-response curve data were carried out using a two-way analysis of variance (ANOVA) for repeated measures. Other comparisons between more than two groups were made using a two-way ANOVA. A Student-Newman-Keuls post-hoc test followed all ANOVA tests. Comparisons of two groups were conducted using an unpaired t-test. ANOVA and *t*-tests were performed in SigmaPlot version 13 (Systat Software, San Jose, CA). $P<0.05$ was considered statistically significant.

4.4 Results

4.4.1 P2rx7 Knockout Reduces Angiotensin II-induced Systolic BP and Pulse Pressure Elevation

To determine the role of P2RX7 in hypertension, we treated WT and *P2rx7*$^{-/-}$ mice with Ang II (1000ng/kg/min) for 14 days and continuously monitored their BP using radiotelemetry. WT and *P2rx7*$^{-/-}$ mice had similar baseline SBP (122±1 vs 124±1 mmHg), and PP (34±2 vs 29±2 mmHg) (Figure 4.1A and C). After 14 days of treatment, *P2rx7*$^{-/-}$ mice displayed a significantly attenuated

increase in SBP (~12 mmHg), along with reduced PP (~16 mmHg) compared to WT mice, but no significant differences in DBP (Figure 4.1B). Both WT and *P2rx7*$^{-/-}$ mice had an increase in heart rate with Ang II-infusion, however starting at day 9 of infusion *P2rx7*$^{-/-}$ mice had a greater heart rate (~40 beats per minute greater) than WT mice (Figure 4.1D).

4.4.2 Ang II-Induced Vascular Remodelling and Endothelial Dysfunction Are Attenuated By P2rx7 Knockout

Increased PP is associated with aortic stiffness and may drive endothelial dysfunction.(14) So, we sought to identify whether stiffening of the aorta in WT or *P2rx7*$^{-/-}$ mice had occurred with Ang II infusion. A classical way to non-invasively assess arterial function of the aorta is through pulse wave velocity (PWV) analysis using ultrasound, with faster PWVs representing stiffer arteries.(15) We found that Ang II treatment induced aortic stiffening in WT mice but not in *P2rx7*$^{-/-}$ mice (Figure 4.1E). To further quantify aortic stiffening, we assessed the collagen content in the media and adventitia using picrosirius red staining and fibronectin content using immunofluorescence. We saw no differences in collagen (Figure 4.10A, B) or fibronectin density (Figure 4.10D, E) in the media of the vasculature in the sham or Ang II treated groups. In the adventitia, we observed increased fibronectin density only in the WT mice treated with Ang II (Figure 4.1F) and saw no differences in relative collagen content in the adventitia (Figure 4.10C). Therefore, increased adventitial fibronectin content in the WT mice treated with Ang II most likely accounts for the aortic stiffening observed in these mice.

In addition, endothelial dysfunction and remodelling in the microvasculature can contribute to hypertension development.(16) In WT mice, acetylcholine-induced mesenteric artery endothelial dilatation was impaired in Ang II treated WT mice by ~22%, but not in *P2rx7*$^{-/-}$ mice (Figure 4.1G), implying that Ang II-induced endothelial dysfunction was blunted by *P2rx7* KO. To assess the contribution of nitric oxide synthase to endothelial function, we used a NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Mesenteric arteries from both WT and *P2rx7*$^{-/-}$ mice had diminished vasodilatory response to Ach after pre-incubation with L-NAME with ~30% and ~40% maximal relaxation responses respectively (Figure 4.1G). Ang II-treated WT mice had further impairment of ~16% compared to WT sham, indicating at least part of the endothelial dysfunction observed in Ang II-treated WT mice is NOS-independent. Ang II-treated *P2rx7*$^{-/-}$ mice had no impairment in NOS-independent vasodilation. No differences were observed between groups in the

vasodilation response to the nitric oxide donor sodium nitroprusside (SNP) (Figure 4.11A) or the constrictor response to norepinephrine (NE) (Figure 4.11B).

Vascular remodelling can be classified as either eutrophic (an increased media to lumen ratio [M/L] without an increase in media cross-sectional area [MCSA]) or hypertrophic (increased M/L and MCSA), with the latter being associated with increased peripheral vascular resistance and more severe hypertension.(16) Ang II infusion promoted hypertrophic remodelling in WT mice treated with Ang II, with an increased M/L (1.25-fold) and increased MCSA (1.33-fold). In contrast, Ang II promoted eutrophic remodelling in *P2rx7*$^{-/-}$ mice with an increased M/L (1.23-fold) but not MCSA (Figure 4.1H). Both WT and *P2rx7*$^{-/-}$ mice infused with Ang II underwent small arterial stiffening, as indicated by a similar left-ward displacement in the stress-strain curve (Figure 4.11C).

4.4.3 Plasma ATP Concentrations and Immune Cell Expression of P2RX7 Increases in WT Mice Treated With Ang II

As was previously reported in hypertensive conditions, (4) we observed a 2.4-fold increase in plasma ATP concentrations in WT mice treated with Ang II (Figure 4.2A). In addition, we observed that macrophages, dendritic cells (DCs), $CD4^+$ and $CD8^+$ T cells in the aortic PVAT had increased expression of P2RX7 after Ang II treatment (Figure 4.2B). This ATP concentration (~5μM) was shown to enhance antigen-presenting capabilities of DCs in a P2RX7-dependent manner, allowing for activation of $CD8^+$ T cells through upregulation of the co-stimulatory molecule CD86.(4) Therefore, we investigated the infiltration of $CD86^+$ macrophages and DCs into the aortic PVAT, anticipating to see less in the *P2rx7*$^{-/-}$ mice. However, unlike previous reports, we did not see a decreased number of $CD86^+$ macrophages or DCs in *P2rx7*$^{-/-}$ mice treated with Ang II. In both WT mice and *P2rx7*$^{-/-}$ mice, Ang II induced a similar accumulation of activated DCs ($CD11b^+CD11c^+CD86^+$) and activated macrophages ($CD11b^+F4/80^+CD86^+$) into the aortic PVAT (Figure 4.2C). Despite the lack of differences in the accumulation of innate cells in the aortic PVAT, the P2X7 receptor may influence the ability of innate cells to activate T cells through modulating IL-1β release. We then tested the ability of bone-marrow-derived macrophages (BMDMs) and DCs (BMDCs) from WT and *P2rx7*$^{-/-}$ mice to produce IL-1β. BMDMs and BMDCs from WT mice primed with lipopolysaccharide and stimulated with ATP released IL-1β, but *P2rx7*$^{-/-}$ BMDMs and BMDCs were unable to significantly produce IL-1β,

confirming that ATP mediated IL-1β release from BMDMs and BMDCs is primarily mediated through P2RX7 (Figure 4.2D).

4.4.4 Angiotensin II Infusion Promotes CD8$^+$ T Cell Activation, IFN-γ Production and Effector Memory Formation, Which is Attenuated by P2rx7 Knockout

IL-1β is an important cytokine for promoting T cell activation(17), and abhorrent signalling may promote altered T cell profiles in *P2rx7*$^{-/-}$ mice. Therefore, we analyzed the immune populations of WT and *P2rx7*$^{-/-}$ mice treated or not with Ang II using flow cytometry. Ang II-infusion resulted in a 3.8-fold increased infiltration of activated CD8$^+$CD69$^+$ T cells in the aortic PVAT, which was blunted in *P2rx7*$^{-/-}$ mice (Figure 4.2E). We also observed an ~3.4-fold and 2.5-fold increase in the number of activated CD4$^+$CD69$^+$ and γδ$^+$CD69$^+$ T cells in the aortic PVAT of WT mice treated with Ang II (Figure 4.12A), with a similar trend observed in the *P2rx7*$^{-/-}$ mice treated with Ang II with a 1.8-fold (*P=.07)* and 2.1-fold (*P=.06*) increase in activated CD4$^+$ and γδ T cells respectively.

IFN-γ has been identified as a critical contributor to the pathogenesis of hypertension (18), so we sought to investigate whether IFN-γ signalling was diminished in *P2rx7*$^{-/-}$ mice. The frequency of IFN-γ producing CD8$^+$ T cells in the spleen increased in Ang II-treated WT mice compared to their sham-treated counterparts, whereas this increase in IFN-γ production did not occur in the *P2rx7*$^{-/-}$ mice (Figure 4.2F). Besides CD8$^+$ T cells, IFN-γ can also be produced from Th1 CD4$^+$T cells and γδT1 cells. The frequency of IFN-γ producing γδT1 increased 2.9-fold in WT mice treated with Ang II, whereas there was no significant increase in the *P2rx7*$^{-/-}$ mice (Figure 4.12B). We observed no difference in the frequency of IFN-γ producing Th1 cells with Ang II-treatment or between genotypes (Figure 4.12B). IL-17 has also been identified as an important cytokine that mediates the development of hypertension.(19) We observed a 1.8-fold increase in the frequency of IL-17 producing Th17 cells in the spleen of WT mice treated with Ang II, which was attenuated in the *P2rx7*$^{-/-}$ mice (Figure 4.12C). No significant differences were observed in the frequencies of IL-17 producing CD8$^+$ or γδ T cells (Figure 4.12C). These results suggest that *P2rx7*$^{-/-}$ may hinder the development of both IFN-γ and IL-17 producing T cells, potentially accounting for the attenuated hypertensive responses observed in *P2rx7*$^{-/-}$ mice.

4.4.5 Ang II-induced Generation of T Effector Memory Cells is Attenuated in P2rx7$^{-/-}$ *Mice*

Effector memory T cells (T_{EM}), characterized by the expression of CD44$^+$ and a lack of expression of L-selectin (CD62L), have been shown to develop after exposure to hypertensive stimuli,

sensitizing mice to develop hypertension to otherwise mild hypertensive stimuli.(20) Recently, it was documented that *P2rx7*$^{-/-}$ mice have an impaired ability to form and retain T_{EM} cells after infection with acute lymphocytic choriomeningitis virus.(21) Therefore, we wanted to see if *P2rx7*$^{-/-}$ mice in our model had a decreased frequency of T_{EM} after Ang II exposure. As previously reported (20, 22), we saw an increase in the frequency of $CD8^+$ T_{EM} in the bone marrow of WT mice (1.5-fold) after 2 weeks of Ang II infusion (Figure 4.3A). Furthermore, in the spleen, a reservoir for memory T cells, we observed an increased frequency of $CD4^+$ T_{EM} (1.5-fold) and $\gamma\delta^+T_{EM}$ (1.5-fold; Figure 4.3B). In addition, we also observed an increase in $CD4^+$ T_{EM} (1.5-fold), $\gamma\delta^+T_{EM}$ (1.3-fold) and a strong trend to increase in $CD8^+$ T_{EM} cells (1.4-fold, P=0.05) in the aortic PVAT (Figure 4.3C). Ang II did not significantly increase the frequency of $CD4^+$, $CD8^+$, or γδ T_{EM} in any of these tissues in the *P2rx7*$^{-/-}$ mice. However, it is noteworthy that *P2rx7*$^{-/-}$ sham mice had a greater frequency of $CD8^+$ T_{EM} cells than WT sham mice in the bone marrow (1.3-fold) and more $\gamma\delta^+T_{EM}$ in the spleen (1.2-fold), but these differences were lost after Ang II treatment. Therefore, impaired generation of T_{EM} in *P2rx7*$^{-/-}$ mice may also contribute to the anti-hypertensive response we observed.

4.4.6 Ang II-induced Cardiac Dysfunction is Exacerbated in P2rx7$^{-/-}$ *Mice*

Left ventricle (LV) hypertrophy is a characteristic feature of hypertension(23), and hypertension accounts for a significant proportion of congestive heart failure cases.(24) Therefore, we sought to determine whether *P2rx7*$^{-/-}$ mice would have attenuated cardiac hypertrophy and dysfunction in response to Ang II. Both WT and *P2rx7*$^{-/-}$ mice treated with Ang II underwent cardiac hypertrophy as demonstrated by increased heart weight measured post euthanasia, corrected to body weight or tibia length (Table 4.1). Using echocardiography, we assessed cardiac function prior to euthanasia. The estimated LV mass corrected to body weight increased in both WT (1.5-fold) and *P2rx7*$^{-/-}$ mice (1.7-fold) but was significantly larger in *P2rx7*$^{-/-}$ compared to WT mice (1.2-fold). WT mice treated with Ang II underwent concentric remodeling (an increase in relative wall thickness), with an increase in posterior wall thickness, but preserved LV internal diameter. Conversely, *P2rx7*$^{-/-}$ mice treated with Ang II had a diminished posterior wall thickness compared to treated WT mice, and developed a dilated LV (indicative of eccentric remodeling). Fractional shortening, a measurement of LV function, decreased in both WT and *P2rx7*$^{-/-}$ mice, but was considerably worse in *P2rx7*$^{-/-}$ mice indicating exacerbated cardiac dysfunction. These results suggest that despite

attenuated BP and inflammation *P2rx7*$^{-/-}$ mice treated with Ang II experience exacerbated cardiac dysfunction and hypertrophy.

4.4.7 The P2RX7 Antagonist AZ10606120 Attenuates Ang II-Induced Hypertension

As *P2rx7*$^{-/-}$ mice displayed attenuated hypertensive responses to Ang II but exacerbated cardiac dysfunction, we wanted to investigate whether a P2RX7 antagonist would have a similar effect. We took WT mice and treated them with Ang II as above, with or without the continuous infusion of the P2RX7 antagonist AZ106 and assessed similar endpoints. Starting around day 6 of Ang II treatment SBP, DBP and HR were lower (~26 mmHg, ~22 mmHg and ~87 BPM respectively) in mice treated with the P2RX7 antagonist and stayed lower, except for HR, until the end of Ang II-infusion (Figure 4.4A, B and D). Pulse pressure was significantly elevated in control mice from days 4-14, but only on days 7-9 in the mice treated with the P2RX7 antagonist; however, there were no significant differences in pulse pressure between control and P2RX7 antagonist treated mice (Figure 4.4C).

4.4.8 Endothelial Dysfunction, but Not Arterial Remodelling or Stiffening, Was Attenuated With P2RX7 Antagonism

In line with the lack of differences observed in pulse pressure in mice receiving the P2RX7 antagonist, there were no differences observed in aortic pulse wave velocity (aortic stiffening) (Figure 4.4E). Similarly, no difference was observed in the stress-strain curves, M/L or MCSA of mesenteric arteries from control mice or mice receiving the P2RX7 antagonist (Figure 4.13A-C). As with the *P2rx7*$^{-/-}$ mice, P2RX7 antagonism prevented Ang II-induced endothelial dysfunction (Figure 4.4F). No differences were observed in NE-mediated vasoconstriction (Figure 4.13D), vasodilation to Ach after pre-treatment with L-NAME (Figure 4.13E), or vasodilatory response to SNP (Figure 4.13F).

4.4.9 Infiltration of Activated T Cells Into the Aortic PVAT is Attenuated with P2RX7 Antagonism

The number of activated $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells were decreased by approximately half in the aortic PVAT of mice treated with the P2RX7 antagonist (Figure 4.5A), with a similar trend observed for activated $\gamma\delta^+CD69^+$ T cells (Figure 4.14A). In addition, we saw a decreased number of $CD8^+$ T_{EM} and a strong trend for decreased $\gamma\delta^+$ T_{EM} in the aortic PVAT (Figure 4.5B), and a reduced frequency of $CD4^+$ T_{EM} in the spleen (Figure 4.5C). We did not see a decreased

frequency of $CD8^+$ T_{EM} in the bone marrow (Figure 4.14D), $\gamma\delta^+$ T_{EM} in the spleen (Figure 4.14E) or $CD4^+$, $CD8^+$ or $\gamma\delta^+$ T_{EM} in the aortic PVAT, despite decreased cell count (Figure 4.14F). We also profiled the IFN-γ and IL-17 producing capacity of splenocytes, however unlike with *P2rx7*$^{-/-}$ mice, we did not observe a decrease in frequency of IFN-γ or IL-17 producing $CD4^+$, $CD8^+$ or $\gamma\delta^+$ T cells in mice treated with a P2RX7 antagonist (Figure 4.14B and C). These results highlight differences in the mechanism of T cell activation between KO of P2RX7 and acute P2RX7 antagonism

4.4.10 P2RX7 Antagonism Did Not Exacerbate Ang II-induced Cardiac Dysfunction

We also assessed cardiac function in mice receiving the P2RX7 antagonist to determine whether they are protected from or experience exacerbated cardiac dysfunction. There were no differences in heart weight, LV mass, wall thickness or chamber diameter in mice receiving the P2RX7 antagonist (Table 4.2). Furthermore, fractional shortening was comparable to Ang II-treated WT mice not receiving the P2RX7 antagonist. Interestingly, there were differences in stroke volume and cardiac output, which were ~1.5-fold and ~1.4-fold greater, respectively, in the mice receiving the P2RX7 antagonist compared to the mice receiving the vehicle. Accordingly, the aortic root was also larger (1.1-fold) in mice receiving the P2RX7 antagonist.

To further understand what was driving the differences observed in cardiac function, we used RT-qPCR to examine the expression of hypertrophy and fibrosis marker genes in cardiac ventricles. Ang II-induced ~3-20-fold increased expression of the hypertrophy markers atrial natriuretic peptide (ANP; *Nppa*), α-skeletal actin 1 (*Acta1*), and β-myosin heavy chain 7 (*Myh7b*) regardless of genotype or of receiving the P2RX7 antagonist (Figure 4.15A). P2RX7 antagonism did trend to decrease the expression of α-skeletal actin 1 (P =0.05) compared to *P2rx7*$^{-/-}$ mice treated with Ang II, bringing its expression to a comparable level with the Ang II-treated WT mice. Transforming growth factor β (*Tgfb1*), a pro-fibrotic cytokine, was significantly increased in the ventricles of *P2rx7*$^{-/-}$ mice treated with Ang II, but no other group (Figure 4.15B). Interestingly, expression of collagen type Ia (*Col1a*) and collagen type IIIa (*Col3a*) were elevated in *P2rx7*$^{-/-}$ sham mice compared to WT sham, with Ang II having no significant effect. In contrast, treatment with the P2RX7 antagonist resulted in a significant reduction in both type I and type III collagen compared to Ang II-treated WT and *P2rx7*$^{-/-}$ mice. Taken together, it appears that *P2rx7*$^{-/-}$ mice treated with Ang II have a tendency to increased expression of hypertrophy and fibrosis genes,

which is modestly attenuated with P2RX7 antagonism and may partially account for the exacerbated cardiac dysfunction observed in *P2rx7*$^{-/-}$ mice.

4.4.11 Single Nucleotide Polymorphisms in P2rx7 Are Associated with Hypertension in the UK Biobank.

Previous studies have reported significant associations between SNPs in *P2RX7* and human BP.(11, 12) Among up to 386 653 White British UK Biobank participants, three variants within 50 kb of *P2RX7* transcription were associated with at least one blood pressure trait after Bonferroni correction (Table 4.3). These three variants, representing one independent signal ($r^2 \geq 0.86$), included the *P2RX7* 3' UTR variant rs28969479. Each copy of rs28969479-A was associated with a 0.55 mmHg increase in DBP (95% CI, 0.31 to 0.79 mmHg; $p = 6.9 \times 10^{-6}$) and an 8% increase in the odds of hypertension (odds ratio per risk allele, 1.08; 95% CI, 1.03 to 1.12; $p = 1.1 \times 10^{-3}$). The only association reported for these three variants in the GWAS Catalog was between rs139429176 and birth weight (38): the allele associated with increased diastolic blood pressure was also associated with decreased birth weight (-0.07 Z-score; 95% CI, -0.10 to -0.05; $p = 1.3 \times 10^{-8}$).

4.5 Discussion

This study adds to the growing evidence that targeting the immune system and inflammation presents a viable target for new anti-hypertensive therapies. It has previously been demonstrated that P2RX7 regulates kidney function and inflammation during hypertensive challenges (9, 10, 25, 26). However, our study is the first to investigate the contribution of P2RX7 signalling in regulating vascular and cardiac dysfunction in hypertension. We demonstrated that genetic KO of *P2rx7* or P2RX7 antagonism attenuates Ang II-induced hypertension, vascular injury, and immune system activation. However, we were unable to identify a protective role for antagonizing P2RX7 signalling in hypertension-associated cardiac hypertrophy or dysfunction. P2RX7 antagonism did not affect cardiac function and remodelling, whereas *P2rx7* KO aggravated cardiac dysfunction and hypertrophy, unveiling an intrinsic defect with the *P2rx7*$^{-/-}$ mice. Furthermore, we identified 3 SNPs in P2RX7 correlated with an increased odds of hypertension using the UK Biobank, suggesting that P2RX7 may be a relevant target in human hypertension.

In a previous study, Zhao *et al*.(4) revealed that P2RX7 contributes to the priming of the immune system during hypertensive stimuli by enhancing the cross-talk between innate immune cells (DCs and macrophages) and adaptive immune cells (T-cells) in mouse models of

hypertension. Our findings support that P2RX7 contributes to T cell activation in the context of hypertension. We validated previous results that circulating plasma ATP concentrations are increased in a hypertensive setting(4) and further demonstrated that P2RX7 expression on innate and adaptive immune cells increased after Ang II treatment. We also demonstrated that either *P2rx7* KO or antagonism attenuated the accumulation of activated T cells in the PVAT. These results lend credence to the hypothesis that ATP can act as a trigger in hypertension, facilitating immune activation through P2RX7 signalling. However, unlike Zhao *et al*.(4), we did not find differences in DC or macrophage CD86 expression between WT and *P2rx7*$^{-/-}$ mice. The expression of CD86 was elevated in both genotypes treated with Ang II. Instead, P2RX7 mediated production of IL-1β was abrogated in BMDMs, and BMDCs from *P2rx7*$^{-/-}$ mice, and we suggest that diminished IL-1β signalling accounts for the observed suppression of T cell activation.

A novel finding of this study was that P2RX7 antagonism or KO attenuated the development of T_{EM} cells in the context of hypertension. Previous reports have shown that $CD4^+$ and $CD8^+$ T_{EM} develop in response to hypertensive insults and predispose mice to develop exacerbated hypertension and end-organ damage in response to further, mild hypertensive challenges.(20, 22) Expanding upon their research, we demonstrated for the first time the accumulation of T_{EM} cells in the aortic PVAT. These cells are uniquely placed to respond to changes in BP and could contribute to the adverse vascular remodelling and endothelial dysfunction that occurs with long periods of hypertension in human populations. The blunted development of $CD4^+$ and $CD8^+$ T_{EM} observed in the mice lacking functional P2RX7 may also mean these mice would be protected against enhanced pressor responses and inflammation to mild hypertensive stimuli, but this remains to be confirmed.

Herein, we identified for the first time that γδ T_{EM} cells also accumulate in the spleen and aortic PVAT after an Ang II challenge, which could be blunted by *P2rx7* KO or antagonism. The role of γδ T_{EM} cells in hypertension is currently unknown. γδ T cells are a relatively rare subset of T cells representing ~1-10% of circulating T cells, and are significant producers of IL-17 and IFN-γ.(2) Recently, it was demonstrated that a deficiency in γδ T cells due to *Tcrδ* KO or injection of γδ T cell-depleting antibodies, prevented Ang II-induced BP elevation, small artery endothelial dysfunction and activation of $CD4^+$ and $CD8^+$ T cells.(27) Therefore, it is likely that γδ T_{EM} cells contribute to hypertension-associated vascular damage and immune activation, but this remains to be confirmed. We also observed a general trend of decreased activation of γδ T cells in the aortic

PVAT of *P2rx7*$^{-/-}$ mice or mice receiving the P2RX7 antagonist. P2RX7 activation has been demonstrated to be important for γδ T cell maturation and lineage choice (28), and therefore attenuated P2RX7 signalling in γδ T cells may account for the decreased number of activated and T_{EM} γδ T cells observed.

The attenuated vascular dysfunction in our study could result from several factors. First, T cells are known to contribute to vascular dysfunction through the production of inflammatory cytokines and ROS generation. Since we observed a reduced accumulation of activated T cells in the PVAT, this could account for the attenuated vascular dysfunction. Another likely explanation is that P2RX7 antagonism led to abrogated NLRP3-mediated IL-1β production. IL-1β has previously been demonstrated to induce endothelial dysfunction (29, 30) and contribute to vascular remodelling.(31) IL-1 receptor KO (31) or treatment with anakinra (29), an IL-1 receptor antagonist, can prevent endothelial dysfunction and attenuate vascular remodelling. Since we reported abrogated IL-1β production from BMDMs and BDMCs without functional P2RX7, this could account for the attenuated vascular dysfunction. Finally, as preventing γδ T cell activation in hypertension was demonstrated to decrease activation of $CD4^+$ and $CD8^+$ T cells, as well as vascular damage (27), the attenuated vascular damage, and $CD4^+$ and $CD8^+$ T cell activation in *P2rx7*$^{-/-}$ mice or mice receiving the P2RX7 antagonist may in part be mediated through suppressed γδ T cell activation.

P2RX7 has been shown to contribute to cardiac fibrosis, remodelling and dysfunction in other pathologies including myocardial ischemia.(6) Therefore, we anticipated that genetic KO of *P2rx7* or P2RX7 antagonism would attenuate Ang-II induced cardiac dysfunction and hypertrophy. Contrary to our initial hypothesis, *P2rx7*$^{-/-}$ mice were not protected from Ang II-induced cardiac dysfunction. In fact, the *P2rx7*$^{-/-}$ mice treated with Ang II had a significantly greater LV mass index and a greater LVIDd than their WT counterparts. In addition, *P2rx7*$^{-/-}$ mice displayed enhanced cardiac dysfunction as defined by decreased fractional shortening. Mice receiving the P2RX7 antagonist did not share these findings and instead experienced comparable hypertrophy and cardiac dysfunction to vehicle-treated WT mice. The results observed in our *P2rx7*$^{-/-}$ mice treated with Ang II are similar to previous reports of *P2rx7*$^{-/-}$ mice in other disease settings. In an autoimmune model of cardiomyopathy, *P2rx7*$^{-/-}$ mice were more prone to develop dilated cardiomyopathy than WT mice.(32) In a separate study, *P2rx7*$^{-/-}$ mice fed a high-fat diet for 16 weeks developed cardiac dysfunction and underwent LV hypertrophy, whereas WT mice

did not.(33) In addition, they noted that *Col1a* expression was also elevated in *P2rx7*$^{-/-}$ mice fed a high-fat diet for 16 weeks, whereas *Col3a* expression had an apparent trend to being higher at baseline. Similarly, we observed elevated expression of both *Col1a* and *Col3a* gene expression in *P2rx7*$^{-/-}$ sham mice compared to WT mice. Furthermore, in our study *P2rx7*$^{-/-}$ mice treated with Ang II had increased expression of TGF-β, whereas no other group did. These results seem to suggest that *P2rx7*$^{-/-}$ mice are prone to develop cardiac fibrosis. Interestingly, mice treated with the P2RX7 antagonist had decreased expression of both *Col1a* and *Col3a,* suggesting that unlike *P2rx7* KO, P2RX7 antagonism may protect against Ang II-induced cardiac fibrosis. Furthermore, hypertrophy markers were also elevated in *P2rx7*$^{-/-}$ mice. At baseline *P2rx7*$^{-/-}$ mice had a greater expression of the hypertrophy marker *Acta1* than WT mice, and after Ang II-infusion there was a trend to increased expression of both *Acta1* and *Nppa* compared to WT mice. In contrast, mice receiving the P2RX7 antagonist had comparable expression of hypertrophy markers in the ventricle compared to WT mice.

Pressure overload, such as in hypertension, induces concentric remodelling, increasing LV wall thickness while preserving LV internal diameter. WT mice in our study treated with Ang II underwent concentric remodelling, whereas *P2rx7*$^{-/-}$ mice did not. It has been documented that concentric remodelling is ERK-dependent (34). Since P2RX7 activation is a known regulator of ERK signalling (35), this possibly accounts for the absence of concentric remodelling in *P2rx7*$^{-/-}$ mice. In fact, *ERK*$^{-/-}$ mice treated with Ang II share similar characteristics with *P2rx7*$^{-/-}$ mice, such as an enlarged left ventricle, impaired fractional shortening, and a dilated left ventricular internal diameter in diastole.(34) Another likely explanation for the decline in cardiac function in *P2rx7*$^{-/-}$ mice is the recently described role for P2RX7 in mitochondrial fitness. In the absence of P2RX7, mitochondrial function in cardiac tissue was severely impaired.(36) In fact, the authors found that *P2rx7*$^{-/-}$ mice displayed exercise intolerance with significantly impaired cardiac function, larger hearts, declined stroke volume, fractional shortening, ejection fraction and cardiac output than their WT (C57BL/6J) counterparts. Therefore *P2rx7*$^{-/-}$ mice may not be able to adequately adapt to increased energy demands in the heart leading to the functional impairment and adverse remodeling we observed. Importantly in our study WT mice receiving the P2RX7 antagonist did not display exacerbated cardiac dysfunction and hypertrophy as was seen in the *P2rx7*$^{-/-}$ mice, suggesting that P2RX7 antagonism may not have these same cardiotoxic effects. Supporting this, more than 8 clinical trials investigating P2RX7 antagonists in various conditions have been

conducted so far with none reporting adverse cardiac events with up to 6 months of treatment.(6) It will be important however, to conduct long-term studies to ensure that P2RX7 antagonism is not cardiotoxic.

Using the UK Biobank, we identified three novel SNPs for P2RX7 associated with an increased odds for hypertension, rs28969479, rs139429176, and rs116953937. Furthermore, rs139429176 has also been associated with decreased birth weight, suggesting that this SNP has physiologic manifestations.(38) These SNPs add to the growing list of SNPs for P2RX7 linked to hypertension or cardiovascular disease. In a study of familial hypertension, including 248 Caucasian families (1425 individuals), the SNP rs598174 for P2X7 was strongly associated with systolic and diastolic ambulatory BP.(11) In a Chinese population of postmenopausal women, a hypomorphic single nucleotide polymorphism (rs3751143) for P2X7 was associated with a decreased risk of primary hypertension.(12) The fact that we did not find an association for these SNPs may simply reflect the different populations investigated (i.e. white British Europeans compared to Chinese women) or the differences in our analysis methods. For example, the Palomino-Doza study specifically investigated familial hypertension, whereas we did not.

Collectively our data support the notion that extracellular ATP may serve as a DAMP in the context of hypertension, triggering activation of the immune system through P2RX7 activation. We observed that P2RX7 deficiency or antagonism attenuated activation and infiltration of T cells into the PVAT and prevented the development of T_{EM} cells which have been shown to increase susceptibility to hypertension and end-organ damage. We have identified three novel SNPs using the UK Biobank correlated with human hypertension, reinforcing the hypothesis that P2RX7 plays a role in human hypertension. Furthermore, we have demonstrated that pharmaceutical antagonism of P2RX7 attenuated Ang II-induced immune activation, BP elevation and vascular injury. As several P2RX7 antagonists have already completed Phase I clinical testing, investigation into the effects of P2RX7 antagonists for treatment of human hypertension, vascular damage and immune activation should be able to be pursued without delay.

4.6 Sources of Funding

The work of the authors was supported by the CIHR First Pilot Foundation Grant 143348, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program, and by the Canada Fund for Innovation,and a Distinguished James McGill Professor Award to ELS, by the Fonds de recherche Santé Quebec (FRQS) bourse 289184, Lady

Davis Institute/TD Bank Studentship award and Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship to BGS, by the McGill Department of Medicine Gordon Phillips Fellowship to KC, George Thanassoulis is a Chercheur Boursier Clinicien – Senior from the Fonds de Recherche Santé – Québec, George Thanassoulis and James C. Engert hold an operating grant from the Canadian Institutes for Health Research, and George Thanassoulis holds operating grants from the Heart and Stroke Foundation of Canada and the National Institutes of Health (R01 HL128550).

4.7 Acknowledgements

Analyses conducted using the UK Biobank Resource were performed under Application Number 41025. The authors are grateful to Veronique Michaud for her excellent technical support.

4.8 Disclosures

George Thanassoulis has received consulting fees from Ionis Pharmaceuticals and has participated in advisory boards for Amgen and Sanofi.

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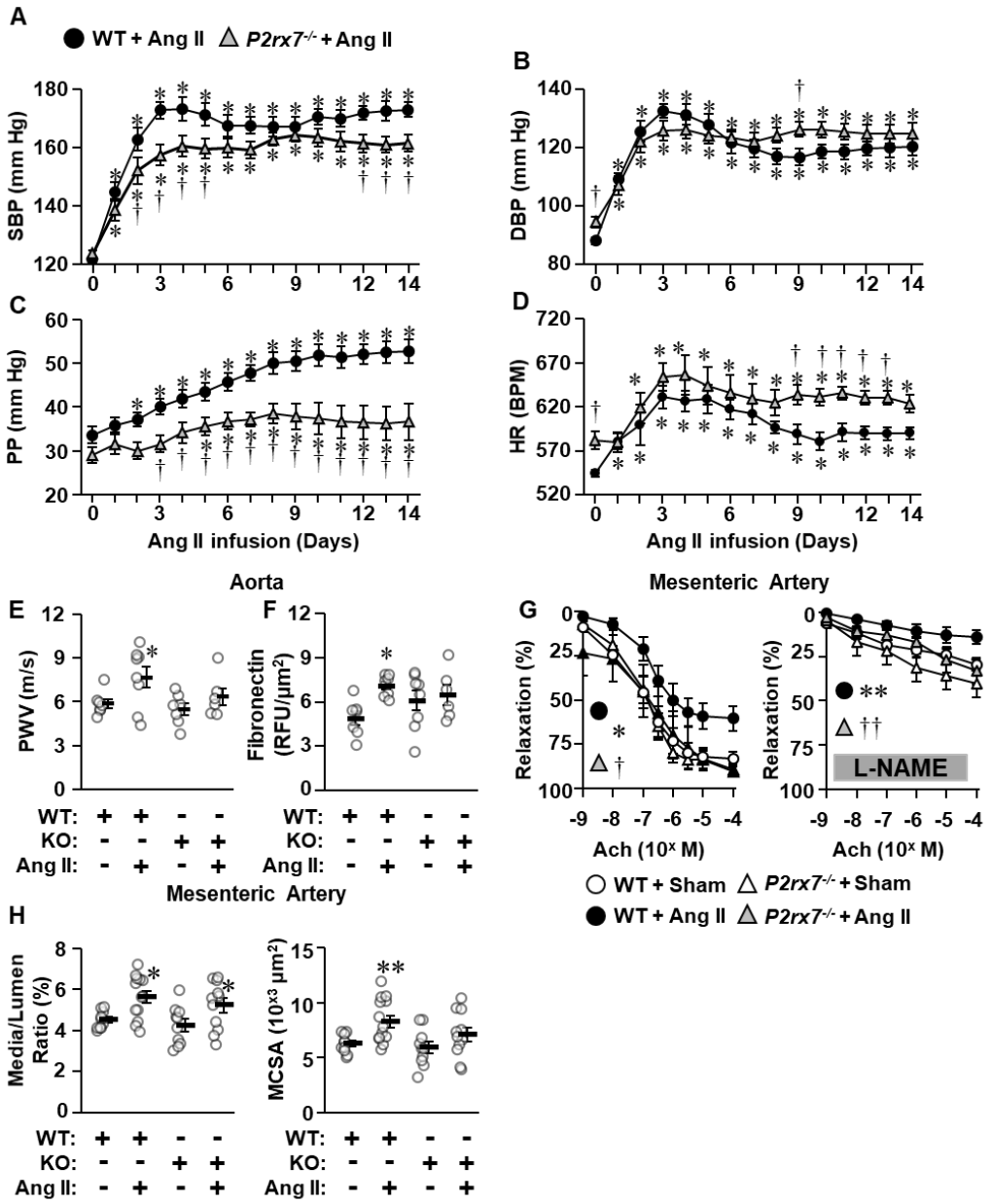

*Figure 4.1: P2rx7$^{-/-}$ attenuates Ang II-induced hypertension and vascular dysfunction.*Systolic blood pressure (SBP, A), diastolic BP (DBP, B), pulse pressure (PP, C), and heart rate (HR, D) were determined via telemetry in WT and *P2rx7*$^{-/-}$ mice treated for 14 days with Ang II (1000ng/kg/min). Aortic pulse wave velocity (PWV) was estimated using ultrasound (E). Adventitial fibronectin content was evaluated using immunofluorescence and expressed as relative fluorescence units (RFU) per μm2 (F). Endothelial-dependent relaxation responses of mesenteric arteries from WT or *P2rx7*$^{-/-}$ mice, pre-constricted with norepinephrine (NE), to increasing doses

of acetylcholine without or with the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (G). Media/lumen (H) and media cross-sectional area (MCSA) of mesenteric arteries at 45 mmHg intraluminal pressure. Data are presented as means ± SEM, n= 7 for A-D, n= 6-9 for E-G and n=10-14 for H. Data were analyzed using a two-way ANOVA for repeated measures for A-D and a two-way ANOVA for E-H. A Student-Neuman-Keuls post hoc test followed all ANOVA tests. The area under the curve was used for analysis in G and H. *P<0.05 and **P<0.01 versus respective baseline (day 0) or sham, †P<0.05 and ††P<0.01 versus WT Ang II.

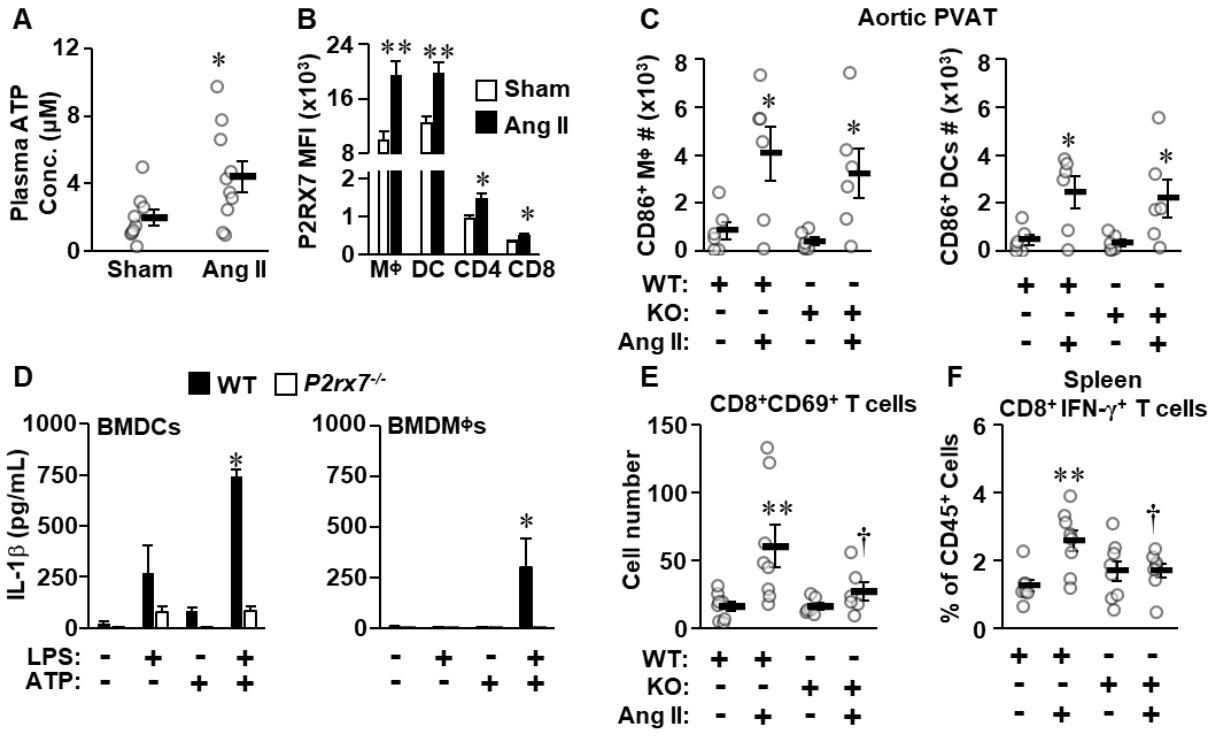

Figure 4.2: Deficiency of P2RX7 leads to decreased CD8⁺ T cell activation and cytokine production. Plasma ATP concentrations were quantified using a luminometer (A). Flow cytometry was used to measure P2RX7 surface expression (mean fluorescence intensity [MFI]) on immune cells (B), quantify macrophage (Mϕ) and dendritic cell (DC) activation (C) and $CD8^+$ T cell activation (E) in the aortic perivascular adipose tissue (PVAT). IL-1β production from bone marrow-derived dendritic cells and macrophages (BMDMs and BMDCs respectively) primed or not with lipopolysaccharides (LPS) and stimulated or not with ATP, quantified via ELISA (D). Flow cytometry was used to quantify the number of activated ($CD69^+$) $CD8^+$ (E).The frequency of IFN-γ producing $CD8^+$ T cells was determined by flow cytometry after activation with phorbol 12-myristate 13-acetate and ionomycin (F). Gating strategies for panel***s*** (C), (E), and (F) are provided in Figure 4.7, 4.8, and 4.9 respectively. Data are presented as means ± SEM, n= 8-10 for A, n= 6-8 for B, C and E and n=4-7 for D. Data was analyzed using an unpaired t-test for A and B, and a two-way ANOVA for C and E. All t-tests and ANOVA tests were followed by a Student-Neuman-Keuls post hoc test. For D a Kruskal-Wallis One Way Analysis of Variance on Ranks was used. *P<0.05 and **P<0.01 versus respective sham, †P<0.05 versus WT Ang II.

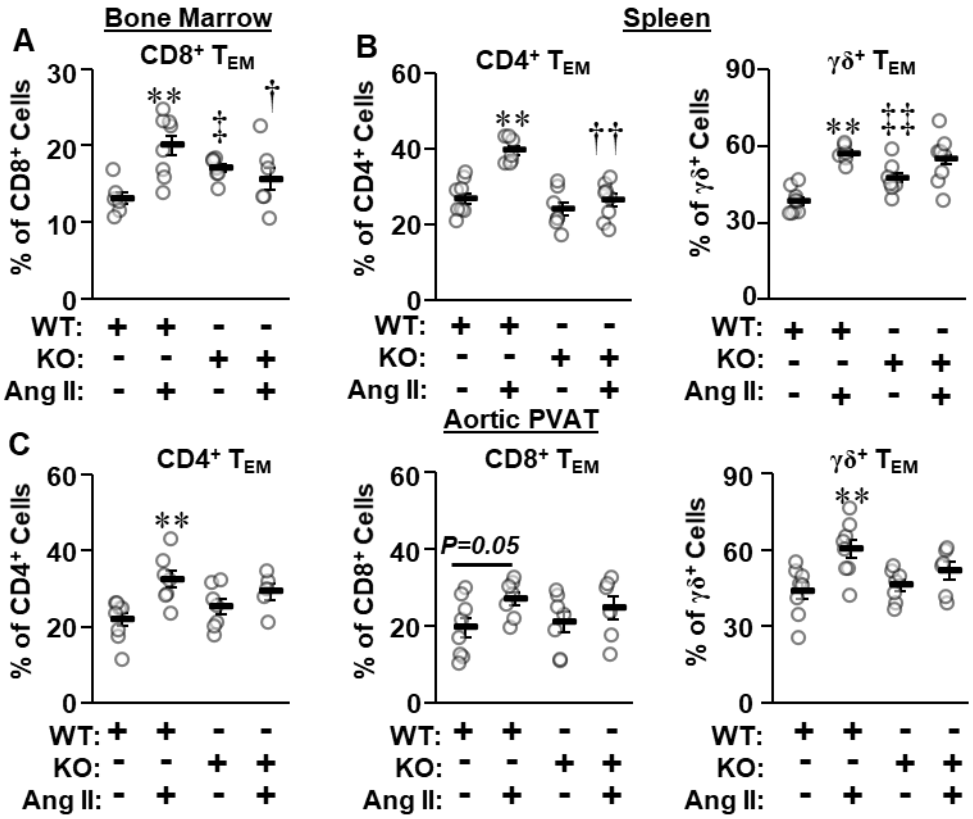

Figure 4.3: The generation of T_{EM} cells after Ang II-infusion are attenuated in P2rx7$^{-/-}$ mice. Flow cytometry was used to quantify the frequency of $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T effector memory (T_{EM}; $CD44^+CD62L^-$) cells in the bone marrow (A) aortic perivascular adipose tissue (PVAT) (B), and spleen (C). A representative gating strategy is presented in supplemental Figure 4.8. Data are presented as means ± SEM, n= 5-8, and were analyzed using a two-way ANOVA followed by a Student-Neuman-Keuls post hoc test. **P<0.01 versus respective sham, †P<0.05 and ††P<0.01 versus WT Ang II, ‡P< 0.05 and ‡‡ P< 0.01 vs WT Sham.

Table 4.1: P2rx7$^{-/-}$ mice treated with Ang II have exacerbated cardiac hypertrophy and dysfunction

Parameter	WT		*P2rx7*$^{-/-}$	
	Sham	Ang II	Sham	Ang II
Body Weight (g)	28.3±0.6	25.6±0.7*	30.6±1.0	28.0±1.1*†
Tibia Length (mm)	18.0±0.1	17.6±0.1	18.0±0.2	18.0±0.2
Heart Weight/Body Weight (mg/g)	4.5±0.1	5.9±0.2**	4.7±0.1	6.3±0.5**
Heart Weight/Tibia Length (mg/mm)	7.2±0.2	8.6±0.3**	8.0±0.3	9.6±0.5**
Lung Weight/Body Weight (mg/g)	5.2±0.	6.4±0.6	5.3±0.4	6.1±0.4
Lung Weight/Tibia Length (mg/mm)	8.1±0.2	9.8±0.9	8.9±0.4	9.7±0.4
Echocardiographic Parameters				
LV Mass/Body Weight (mg/g)	4.2±0.2	6.3±0.2**	4.2±0.2	7.3±0.5**†
LVIDd (mm)	3.5±0.1	3.5±0.1	3.8±0.1	4.3±0.2*††
LVPWd (mm)	0.76±0.03	1.09±0.05**	0.76±0.03	0.94±0.05*†
LV RWT	0.52±0.04	0.66±0.04*	0.45±0.02	0.46±0.01††
LV Systolic Function				
FS (%)	43.8±2.4	32.5±3.1*	37.3±2.9	20.2±3.1**†
SV (μL)	62.8±4.6	95.2±9.8*	73.9±3.0	92±14.5
CO (mL/min)	33.3±2.5	49.4±5.0*	39.4±1.8	48.8±8.1
HR (BPM)	530±7	519±10	533±4	528±8
Aortic Root (mm)	1.36±0.02	1.59±0.05**	1.44±0.04	1.60±0.04*

Body weight, tibia length and heart mass corrected to tibia length or body weight of WT or *P2rx7*$^{-/-}$ mice treated or not with Ang II. Echocardiograph parameters taken in m-mode included estimated left ventricle (LV) mass corrected to body weight, LV internal diameter in diastole (LVIDd), LV posterior wall thickness in diastole (LVPWd), relative wall thickness (RWT), fractional shortening (FS) and heart rate (HR). The aortic root (diameter in mm) was calculated from an m-mode image of the aorta. Stroke volume (SV) was estimated from the pulse wave doppler of the aorta. Data are presented as means ± SEM, n= 8-11 for body weight, tibia length and heart mass corrected to tibia length or body weight. n=5-8 for echocardiography parameters. Data were analyzed using a 2-way ANOVA followed by a Student-Neuman-Keuls post hoc test. * P <0.05 and ** P <0.001 versus respective sham and † P <0.05 and †† P <0.01 versus WT + Ang II.

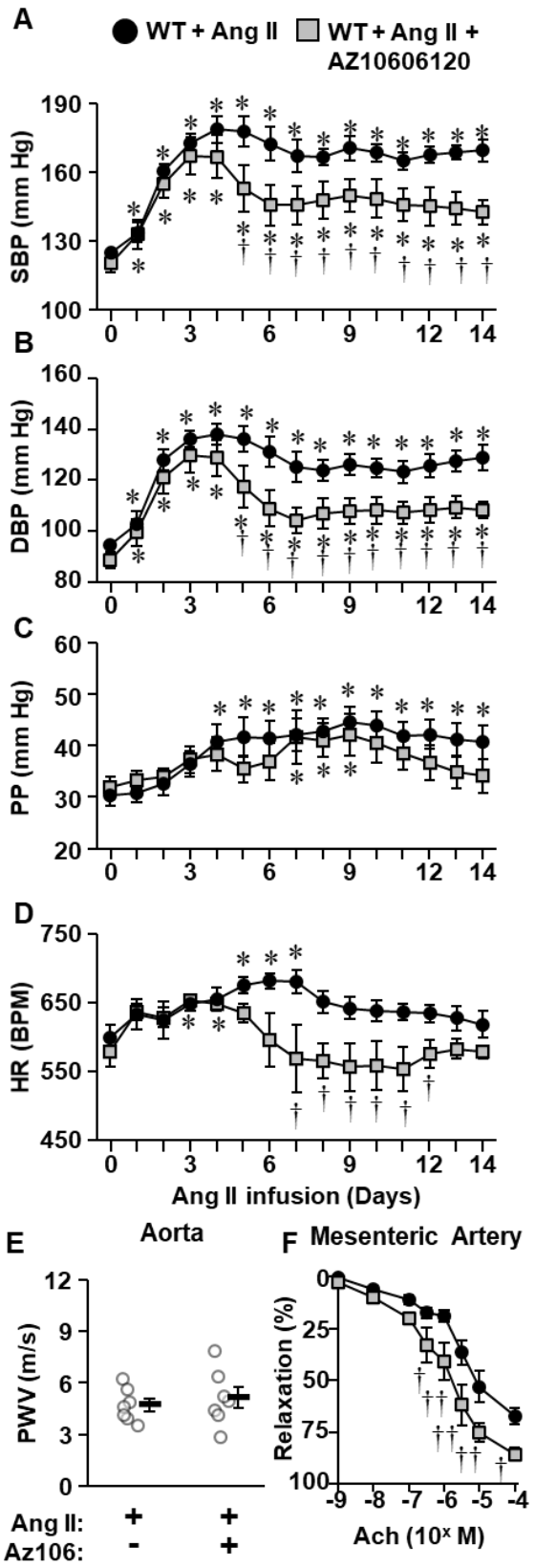

Figure 4.4: P2RX7 antagonism attenuates Ang II-induced hypertension and resistance artery endothelial dysfunction. Systolic blood pressure (SBP, A), diastolic BP (DBP, B), pulse pressure (PP, C), and heart rate (HR, D) were determined via telemetry in WT mice treated for 14 days with Ang II (1000ng/kg/min), with or without the P2RX7 antagonist AZ10606120 (694 ng/kg/min). Aortic pulse wave velocity (PWV) was estimated using ultrasound measured in meters/second (m/s) (E). Endothelium-dependent relaxation responses of mesenteric arteries pre-constricted with norepinephrine (NE), to increasing doses of acetylcholine (F). Data are presented as means ± SEM, n=5-7. Data was analyzed using a two-way ANOVA with repeated measure for A-D, and F, and an unpaired t-test for E. All tests were followed by a Student-Neuman-Keuls post hoc test. *P<0.05 versus respective baseline (day 0), †P<0.05 and ††P<0.01 versus WT Ang II.

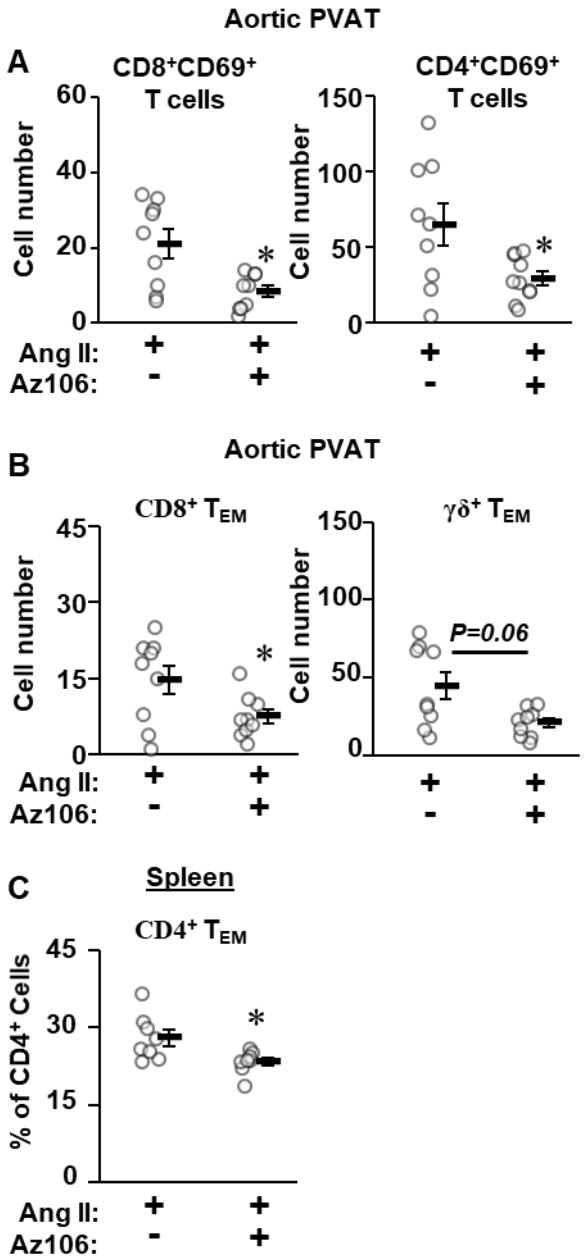

Figure 4.5: Infiltration of activated T cells into the aortic PVAT is attenuated with P2RX7 antagonism. Flow cytometry was used to quantify the number of activated ($CD69^+$) $CD4^+$, and $CD8^+$ T cells (A) the number of $CD8^+$ and $\gamma\delta^+$ T effector memory (T_{EM}; $CD44^+CD62L^-$) cells in the aortic perivascular adipose tissue (PVAT) (B) and the frequency of $CD4^+$ T_{EM} in the spleen (C). A representative gating strategy is presented in Figure 4.8. Data are presented as means ± SEM, n=8-10. The data were analyzed using an unpaired t-test followed by a Student-Neuman-Keuls post hoc test. *P<0.05 versus WT Ang II.

Table 4.2: P2RX7 antagonism does not exacerbate cardiac hypertrophy and dysfunction in mice treated with Ang II.

	WT	
Parameter	Ang II	Ang II + AZ10606120
Body Weight (g)	23.8±0.6	25.0±0.4
Tibia Length (mm)	17.4±0.2	17.5±0.2
Heart Weight/Body Weight (mg/g)	7.2±0.2	6.8±0.2
Heart Weight/Tibia Length (mg/mm)	9.7±0.2	9.7±0.3
Lung Weight/Body Weight (mg/g)	8.0±0.7	8.6±0.7
Lung Weight/Tibia Length (mg/mm)	11.0±0.9	12.2±0.9
Echocardiographic Parameters		
LV Mass/Body Weight (mg/g)	6.1±0.2	6.2±0.4
LVIDd (mm)	3.5±0.1	3.8±0.1
LVPWd (mm)	1.05±0.04	1.06±0.05
LV RWT	0.62±0.04	0.54±0.02
LV Systolic Function		
FS (%)	29.9±3.8	28.9±3.3
SV (μL)	99.0±12.6	144.1±5.3*
CO (mL/min)	51.7±6.5	74.7±2.9**
HR (BPM)	523±3	518±5
Aortic Root (mm)	1.59±0.03	1.69±0.04*

Body weight, tibia length and heart mass corrected to tibia length or body weight of WT mice treated with Ang II or WT mice treated with Ang II and the P2RX7 antagonist AZ10606120. Echocardiograph parameters taken in m-mode included estimated left ventricle (LV) mass corrected to body weight, LV internal diameter in diastole (LVIDd), LV posterior wall thickness in diastole (LVPWd), relative wall thickness (RWT), fractional shortening (FS) and heart rate (HR). The aortic root (diameter in mm) was calculated from an m-mode image of the aorta. Stroke volume (SV) was estimated from the pulse wave doppler of the aorta. Data are presented as means ± SEM, n= 8-10 for body weight, tibia length and heart mass corrected to tibia length or body weight. n=7 for echocardiography parameters. Data were analyzed using a T-test. * P <0.01 WT + Ang II group.

Table 4.3: Associations of variants with Bonferroni $p \leq 0.05$ for at least one blood pressure trait.

Variant	EA	OA	Systolic Blood Pressure			Diastolic Blood Pressure			Pulse Pressure			Hypertension		
			mmHg per Allele (95% CI)	p	p Bonf	mmHg per Allele (95% CI)	p	p Bonf	mmHg per Allele (95% CI)	p	p Bonf	Odds Ratio per Allele (95% CI)	p	p Bonf
rs28969479	A	G	0.37 (-0.05, 0.78)	0.085	1	0.55 (0.31, 0.79)	6.9×10^{-6}	9.1×10^{-3}	-0.18 (-0.47, 0.11)	0.22	1	1.08 (1.03, 1.13)	9.3×10^{-4}	1
rs139429176	T	C	0.35 (-0.06, 0.76)	0.098	1	0.55 (0.31, 0.78)	6.5×10^{-6}	8.7×10^{-3}	-0.20 (-0.48, 0.09)	0.18	1	1.08 (1.03, 1.12)	1.1×10^{-3}	1
rs116953937	G	C	0.34 (-0.07, 0.76)	0.11	1	0.54 (0.30, 0.78)	9.1×10^{-6}	0.012	-0.20 (-0.49, 0.09)	0.18	1	1.08 (1.03, 1.12)	1.2×10^{-3}	1

Abbreviations: Bonf, Bonferroni; EA, effect allele; OA, other allele.

Online Supplement

4.10 Detailed Methods

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed the Canadian Council of Animal Care recommendations.

4.10.1 Animals

C57BL/6J wild-type (WT) and *P2rx7*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and a colony was maintained on-site for the duration of the study. The animals were housed in a conventional facility under sterile conditions with constant temperature and humidity and a 12-hour light/12-hour dark cycle. They were fed a regular salt diet (Teklad Global 18% protein rodent diet with 0.2% of NaCl, Envigo, Lachine, QC, Canada). *P2rx7*$^{-/-}$ mice were confirmed by genotyping DNA obtained from an ear punch as described below.

4.10.2 Genotyping

DNA was extracted from an ear punch using a modified quick method.(1, 2) Ninety µL of basic digestion buffer (25 mM NaOH and 0.2 mM EDTA, pH 12) were added to the tubes containing the piece of ear punch, and the mixture was heated at 95ºC for 40 minutes. The tubes were then vortexed to ensure tissue disruption and maximal DNA release. The remaining material and lysis solution was quickly centrifuged at 12,000 x *g*, and then incubated at 95°C for an additional 30 minutes. At the end of the digestion, the tubes were vortexed and centrifuged. Samples were stored at -20ºC until use.

Genetically deficient *B6.129P2-P2rx7*$^{tm1Gab/J}$ (Purinergic receptor P2X7 [*P2rx7*]$^{-/-}$) mice (strain #005576) were identified using PCR by the amplification of a 200 bp *P2rx7*^{tm1Gab} fragment and the absence of a 246 bp *P2rx7* fragment. PCR was performed using 2 µL of supernatant of the ear punch digestion mixture with a TopTaq DNA Polymerase kit (Qiagen, Foster City, CA, USA). The PCR reaction contained 1.25 units/reaction of TopTaq DNA Polymerase, 200 µM of each dNTP, 0.5 µM of each oligonucleotide primer, and 1x CoralLoad. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and are presented in Table 4.4. The PCR conditions were 3 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C.

PCR products were run on a 2% agarose gel containing 1x TAE (40 mM tris-acetate, 1 mM EDTA) buffer and 1 μg/mL RedSafe™ nucleic acid stain (FroggaBio, Toronto, ON, Canada). Images were taken using ChemiDoc XRS+ Gel Imaging System (Bio-Rad) (Figure 4.6).

4.10.3 Angiotensin II Treatment

Ten to 12-week-old male C57BL/6J wild-type (WT) and *P2rx7*$^{-/-}$ mice were sham-treated or infused with Ang II (1000 ng/kg/min, SC) for 14 days. Mice were anesthetized with 3% isoflurane mixed with O_2 at 2 L/min. The depth of anesthesia was confirmed by rear foot squeezing. The non-steroidal anti-inflammatory drug carprofen (20 mg/Kg) was injected SC to minimize post-operational pain. The mice were then surgically implanted SC with ALZET mini osmotic pumps (Model 1002, Durect Corporation, Cupertino, CA), infusing Ang II (1000 ng/kg/min) for 14 days, as recommended by the manufacturer. A drop of an analgesic mixture, lidocaine (20 mg/mL)/bupivacaine (5 mg/mL), was applied to the surgical site before closure. Control mice underwent sham surgery. An additional group of 10-12-week-old C57LBl/6J WT mice were implanted with ALZET mini osmotic pumps (Model 2002, Durect Corporation, Cupertino, CA), infusing 1000 ng/kg/day of Ang II with or without the P2RX7 antagonist AZ10606120 dihydrochloride (694 ng/kg/min; Alomone Labs, Israel) for 14 days and treated as described above.

4.10.4 Blood Pressure Determination

Blood pressure (BP) was determined by telemetry as previously described.(3) 8.5-10-week-old male WT and *P2rx7*$^{-/-}$ mice were anesthetized with isoflurane and surgically implanted with the telemetry probes as recommended by the manufacturer (Data Sciences International, St. Paul, MN). Carprofen (20 mg/Kg) was injected SC, and a drop of lidocaine (20 mg/mL)/bupivacaine (5 mg/mL) mixture was applied to the surgical site before closure. The mice recovered for 7-10 days with carprofen administered once a day for the first two recovery days. BP was determined every 5 minutes for 10 seconds for two days, after which mice underwent sham surgery or were implanted with micro-osmotic pumps as above, and BP was recorded for 14 days.

4.10.5 Pulse Wave Velocity

Aortic stiffness was assessed in mice by determining the aortic pulse wave velocity (PWV) using ultrasound with the Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, ON, Canada) as previously described in Sprague–Dawley rats.(4) Mice were anesthetized with isoflurane, and the heart rate was maintained between 500 and 550 beats/min. A 40 MHz frequency probe was

used to acquire the pulse wave doppler signal of the blood flow at the root of the aorta and in the abdominal aorta at the level of the femoral aortic bifurcation while simultaneously recording the electrocardiogram tracings. PWV was calculated by the quotient of the aortic length (L) by the transit time (ΔT) of the PW to travel from the aortic valve (T1) to the aortic bifurcation (T2). L was measured at necropsy with a silk and a ruler. T1 and T2 were obtained by calculating the difference in the elapsed time from the peak of the R wave of the QRS complex to the onset of the flow wave at the level of the ascending aorta and the aortic bifurcation, respectively.

4.10.6 Determination of Cardiac Function

Echocardiography was performed prior to euthanasia, after 14 days of Ang II-infusion, using the Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, ON, Canada) and a 40-MHz imaging transducer to examine cardiac function as previously described.(5) Images were analyzed using Vevo Lab version 3.2.6. The mice were anesthetized with isoflurane, and the heart rate was maintained between 500 and 550 beats per minute. The transducer was positioned on the left anterior side of the chest. The heart was first imaged in the 2-dimensional mode of the long-axis view of the left ventricle. Next, B-mode imaging was used to measure the diameter of the pulmonary outflow tract (POT) and then pulse wave doppler measurements were acquired for the pulmonary artery after aligning the transducer in the direction of the blood flow. Measurements of the pulmonary artery velocity-time integral (PaVTI) and peak velocity were taken. The right ventricular stroke volume (RV SV) (1000*PaVTI in cm*π* ((POT in mm/20)²)), cardiac output (RV SV/1000*HR) and max pressure gradient (4 x ((PV Peak Velocity in mm/s /1000)²)) were estimated. Left ventricle (LV) outflow tract pulse wave doppler flow spectra were obtained from the apical view, where the LV outflow tract (LVOT) was measured. Aortic VTI and max pressure gradient were also calculated as described for the pulmonary artery. A 2-D short-axis view of the heart was then used. The M-mode cursor was positioned perpendicular to the anterior and posterior wall to measure the LV internal dimension during diastole and end-systole (LVIDd and LVIDs, respectively). LV fractional shortening (FS) was calculated as ((LVIDd-LVIDs)/LVIDd))x100. Relative wall thickness was calculated as ((LV anterior wall thickness in diastole + LV posterior wall in diastole)/LVIDd).

4.10.7 Collection of Tissues

At the end of the protocol, mice were weighed and then anesthetized with isoflurane. Organs were collected in ice-cold phosphate-buffered saline (PBS), dried, weighed and then snap frozen or preserved in 4% PFA or Tissue-Tek OCT. The mesenteric vascular bed attached to the intestine with the ends of the intestines ligated using surgical sutures was harvested in ice-cold Krebs solution (pH 7.4) for endothelial function and mechanic studies. The Krebs solution was oxygenated (95% air– 5% CO2) and contained 120 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 4.7 mmol/l KCl, 1.18 mmol/l KH_2PO_4, 1.18 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 0.026 mmol/l EDTA and 5.5 mmol/l glucose. The spleen and aorta with perivascular adipose tissue (PVAT) were collected in ice-cold PBS for flow cytometry, histological or immunofluorescence studies. Mice were euthanized by cervical dislocation.

4.10.8 Endothelial Function and Mechanics

Mesenteric artery endothelial function and mechanics were investigated after mounting on a pressure myograph system (Living Systems Instrumentation, Burlington, VA, USA) as previously described.(6) Second-order branches of the mesenteric arterial tree were dissected (160 to 240 μm) and mounted on glass pipettes of pressurized myograph chambers. Vessels were maintained at an intraluminal pressure of 45 mmHg in Kreb's solution bubbled continuously with 95% air and 5% CO_2. Media and lumen diameters were measured by a computer-based video imaging system (Living Systems Instrumentation). Vessels were considered viable when they constricted to >60% of their resting lumen diameter in response to extraluminal application of 125 mmol/L potassium chloride plus 10^{-5} mol/L norepinephrine (NE). Vascular contractile properties were assessed by extraluminal perfusion with exogenous NE. Endothelium-dependent (10^{-9}-10^{-4} mol/L acetylcholine) and independent (10^{-9}-10^{-3} mol/L sodium nitroprusside) relaxations were assessed in vessels precontracted with NE (5 x 10^{-5} mol/L). Nitric oxide availability was evaluated by an acetylcholine concentration-response curve repeated after 20 minutes incubation with the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} mol/L). Vascular mechanical properties (remodeling and stiffening) were evaluated in the absence of vascular tone by incubation in a Ca^{2+} free Krebs solution supplemented with a Ca^{2+} chelating agent (EGTA, 10 mmol/L). Media thickness and lumen diameter were measured in response to incremental augmentations of intraluminal pressure from 3 to 140 mm Hg and the media cross-sectional area, media/lumen ratio, stress and strain were calculated as previously described.(7)

4.10.9 Immunofluorescence Detection of Fibronectin

The aortic arch was embedded in Tissue-Tek OCT and stored at -80ºC until use. Cryosections 5µm thick were used to assess the expression levels of fibronectin by immunofluorescence microscopy. Tissue cryosections were fixed in an acetone:methanol (1:1) mix for 10 minutes at room temperature (RT) and washed with PBS containing 0.1 % Tween-20 (PBST) twice for 10 minutes. Sections were blocked for 1 hour at RT with PBST containing 10% normal goat serum. Sections were then incubated overnight at 4°C with rabbit anti-fibronectin (1:1000, Millipore/Chemicon) antibody in a blocking solution overnight at 4°C. The sections were washed 3 times with PBST before being blocked again for 1 hour at RT. The sections were then incubated with Alexa Fluor 594 goat anti-rabbit (1:400, Invitrogen) antibody in the blocking solution for 1 hour at RT and then washed 3 times with PBST and stained in 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Vector Laboratories, Burlingame, CA, USA) diluted in PBS for 5 minutes. The slides were washed with PBS 1 time before being mounted with mounting medium Fluoromount (Sigma-Aldrich). Fluorescent images were captured using an upright microscope Leica DM2000 with a Cy3, Texas Red, FITC or DAPI filters, as appropriate, and analyzed with ImageJ software. The expression levels of fibronectin were determined by quantifying the relative fluorescence per μm^2 of aortic wall media or adventitia and reported as fold change relative to their respective control group. The elastin autofluorescence and DAPI-stained nuclei were used to locate the wall media and PVAT area.

4.10.10 Histological Studies

The descending thoracic aorta was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 8µm. The sections were washed in PBS for 5 min and stained with 0.1% Sirius red solution (Sigma-Aldrich, St. Louis, MO, USA) prepared in saturated picric acid for 60 min. Excess stain was removed by rinsing in two baths of 87 mM acetic acid for 1 and 3 minutes. Sections were then dehydrated in successive 2-minute baths of ethanol (95%, 100% and 100%) and immersed in two xylene baths for 3 minutes each. Sections were then mounted using Eukitt Mounting Medium (Electron Microscopy Sciences, Hatfield, PA, USA). White and polarized light images were captured using an Olympus BX60F5 polarizing microscope (Olympus Canada, Richmond Hill, ON, Canada). Images were analyzed using ImageJ software

4.10.11 Measurement of Plasma ATP

Plasma ATP concentration was determined using Molecular Probes' ATP Determination Kit (ThermoFisher Scientific, Waltham, MA) as described by the manufacturer. Blood was collected via cardiac puncture, and 200µL was transferred to a tube of EDTA and placed on ice. Samples were then centrifuged at 1000 x g for 15 minutes at 4 ºC. Afterwards, the supernatant was transferred to a fresh Eppendorf and centrifuged at 10 000 x g for 10 minutes at 4ºC. The supernatant was then transferred to a new Eppendorf and snap-frozen in liquid nitrogen before being stored at -80 ºC until use. Ten uL from samples with no indication of hemolysis were pipetted into an OptiPlate-96 well plate (ThermoFisher Scientific, Waltham, MA), and then transferred to a Berthold Detection Systems Orion II microplate Luminometer (Berthold Technologies GmbH & Co. KG, Germany) with an auto-injector. 90uLof the standard reaction solution was injected into each well, followed by a 100-second delay before measuring the luminosity for 10 seconds. ATP concentration was extrapolated from an ATP standard curve. Each sample was done in duplicate, and the values were averaged.

4.10.12 Flow Cytometry

Immune cell profiles in the spleen and aortic PVAT were determined by flow cytometry. Single-cell suspensions of splenocytes were prepared by crushing the spleen with the back of a 1 mL syringe plunger over a 70 µm cell strainer in the well of a 12-well plate containing 1 mL RPMI-1640 media. Splenocytes were then centrifuged and resuspended in 1 mL of PBS. One hundred µl of splenocyte cell suspension was used per test, corresponding to 1/10 of the spleen. Bone marrow was harvested from the tibia and femur of one leg, and flushed with ice cold Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12) and filtered through a 70 µm cell strainer. Bone marrow cells were then centrifuged, resuspended in 400µL and 200µL (half the sample) was used per test. The thoracic region of the aorta with PVAT was removed from the mouse by cutting inferiorly and superiorly near the celiac trunk and aortic arch respectively. Cutting along the spinal vertebra behind the aorta removed all associated PVAT with the aorta. The aorta was then viewed under a microscope to check for any lymph node contamination before being minced with scissors and digested in a solution containing collagenase A (1 mg/mL), collagenase type 2 (500 U/mL), elastase (2 U/mL), trypsin inhibitor (0.25 mg/mL), and hyaluronidase (0.5 mg/mL) for 45 minutes to one hour at 37^{o}C with agitation. The cells were then filtered (70 µm), washed with PBS, and resuspended in 100 µl of PBS (1 test) before staining. Isolated cell suspensions were stained with

a Live/Dead fixable Aqua dead cell stain (ThermoFisher Scientific, Waltham, MA) or Zombie UV fixable viability kit (Biolegend, San Diego, CA, USA) and thereafter incubated with a cocktail containing rat anti-mouse CD16/CD32 Fc receptor block and specific antibodies against immune cell markers in phosphate-buffered saline supplemented with 5% Fetal bovine serum. Descriptions of antibodies used are available in Table 4.5 and Table 4.6.

For determination of IL-17A and IFN-γ producing T cells, %50 of the splenocyte suspension described above was centrifuged at 410 x g for 5 minutes at 4°C and cell pellets were resuspended in 1 mL of activation cocktail per tube. The activation cocktail consisted of 0.05 μg/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Oakville, ON, Canada), 1 μg/mL ionomycin (Sigma-Aldrich, Oakville, ON, Canada), and 2 μM Monensin (Biolegend, San Diego, CA, USA) in RPMI medium. Each sample was transferred into a 12-well tissue culture plate well and placed in a tissue culture incubator set at 37°C and 5% CO_2 for 4 hours. Thereafter, cells were transferred into 1.5 mL conical tubes and centrifuged at 410 x g for 5 minutes at 4°C. Cells were resuspended in 100 μL of Zombie UV fixable viability kit (Biolegend, San Diego, CA, USA), transferred to one well per sample of a V-bottom 96-well plate and incubated for 20 minutes at room temperature. Samples were then washed with 100uL of PBS and then centrifuged at 410 x g for 5 minutes at 4°C. The samples were then incubated with a cocktail containing rat anti-mouse CD16/CD32 Fc receptor block and specific antibodies against immune cell markers (Table 4.7). Cells were fixed with 1% PFA and then stained intracellularly for IL-17A and IFN-γ using PBS containing 10% saponin.

Flow cytometry was performed on the BD LSR Fortessa cell analyzer. Fluorescence minus one was used to adjust the gates, and *P2rx7*$^{-/-}$ mice were used as a negative control for P2RX7 expression. Data analysis was performed using FlowJo software version 10 (version 10.6, Tree Star Inc., Ashland, OR), and P2RX7 expression was presented as mean fluorescence intensity. Flow cytometry gating strategies are available in Figure 4.7, 4.8 and 4.9.

4.10.13 Inflammasome Activation of Bone Marrow-Derived Macrophages and Dendritic Cells

Bone marrow was harvested from the tibia and femurs of 10-14-week-old WT and *P2rx7*$^{-/-}$ mice and used to generate bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). Briefly, bone marrow was flushed with ice-cold Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12) and filtered through a 70 μm cell strainer. Live cells were counted in

0.2% Trypan blue stain using a Neubauer cell counting hemocytometer. For BMDMs, 1.5 million cells were seeded per well in a six-well plate in DMEM/F12 media supplemented with non-essential amino acids, penicillin-streptomycin, 10% fetal bovine serum (FBS) and 10% L-929 conditioned media. The cells were allowed to differentiate into BMDMs for 8-10 days. Differentiation into BMDMs was confirmed via flow cytometry as $CD11b^{+}F4/80^{+}$ double-positive cells. After differentiation, cells were primed or not with lipopolysaccharides (LPS, 1 µg/mL) for 2 hours, followed by activation with ATP (3 mM) for 2 hours. After incubation, the supernatant was collected, and IL-1β concentration was determined by ELISA. For BMDCs, 15 million cells were seeded in a $100cm^2$ petri dish in RPMI media supplemented with non-essential amino acids, penicillin-streptomycin, 10% FBS and 20ng/mL of granulocyte-macrophage colony-stimulating factor (Sino Biological). After 7-10 days, differentiation into BMDCs was confirmed as $CD11b^{+}F4/80^{-}CD11C^{+}$ cells. After differentiation, BMDCs were transferred to a 96 well u-bottomed plate (200 000 cells/well) and were primed or not with LPS (100 ng/mL) for 24 hours, followed by activation with ATP (3 mM) for 3 hours. After incubation, the supernatant was collected, and IL-1β concentration was determined by ELISA.

4.10.14 RNA Extraction and Reverse Transcription-Quantitative PCR

Total RNA was isolated from the heart's ventricles using the *mir*Vana miRNA isolation kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocols. Frozen tissues (~80-100 mg) were homogenized in 1 mL of mirVana miRNA lysis/binding buffer (ThermoFisher Scientific) for one minute at maximum speed with a Polytron PT 1600 E homogenizer equipped with a dispersing aggregate PT-DA 1607/2EC (Brinkmann Instruments, Mississauga, ON, Canada). The homogenate was then centrifuged at 1,500 x *g* for 5 minutes at 4°C to remove any foam generated during homogenization. Afterwhich, the samples were processed for total RNA extraction using the mirVana miRNA isolation kit according to the manufacturer's protocols. RNA concentration was determined with a Nanodrop spectrophotometer ND-100 V3.1.2 (Thermo Fisher Scientific). RNA quality was evaluated by comparison with the ribosomal RNA (rRNA) 28S and 18S bands and by checking for mRNA smear by electrophoresis of 0.8 µg of RNA with a ribonuclease free 1% agarose gel containing 1X TAE electrophoresis buffer (40 mM M Tris-acetate and 1 mM ethylenediaminetetraacetic acid [EDTA]). RNA was stored at -80°C until used for RT-qPCR.

The mRNA expression levels of interest and ribosomal protein S16 (*Rps16*) were determined by reverse transcription-quantitative PCR. In brief, 1 µg of RNA was reverse-transcribed with the Quantitect RT kit (Qiagen, Foster City, CA, USA). qPCR was carried out with SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada) and the Applied Biosystems 7500 Real-Time PCR System (ThermoFisher Scientific). Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to have a melting temperature (Tm) of 60°C, 1 guanine/cytosine clamp, and amplify a product of 250-350 nucleotides. Primers containing CCCC or GGGG or those that could form hairpins with Tm >50°C determined with the OligoAnalyzer HAIRPIN tool of Integrate DNA Technologies (https://www.idtdna.com/calc/analyzer) were excluded. Primers were designed to avoid genomic DNA (gDNA) amplification. UCSC In-Silico PCR was used to verify whether primers amplified gDNA (http://genome.ucsc.edu/cgi-bin/hgPcr). If primers were amplifying gDNA, they were excluded if their PCR product was <1000 nucleotides. Larger gDNA products would not be expected to be amplified with a 1-minute elongation step (see below). To eliminate any possible contaminating gDNA, RT was performed using QuantiTect Reverse Transcription Kit, including a gDNA elimination step preceding the RT step (Qiagen). The primer pair specificities were verified by *in silico* PCR using the Primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the non-redundant nucleotide collection (nr) to confirm that only the correct targets were amplified.

Primer pairs were validated using a cDNA dilution curve with a qPCR efficiency between 99% and 106%, deemed acceptable. We also ensured that there was only a single amplicon with a melting temperature >80°C in the qPCR dissociation curve. PCR product length was confirmed by electrophoresis with a 2% agarose gel containing 1X TAE electrophoresis buffer and 1 µg/mL RedSafe™ nucleic acid stain (FroggaBio, Toronto, ON, Canada) with a Ready-to-Use 100 bp DNA ladder (FroggaBio). The qPCR conditions were: 30 seconds at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (8) with *Rps16* as a reference gene. The oligonucleotide primers are listed in Table 4.8.

4.10.15 Analysis of the UK Biobank

Hypertension was defined as the presence of a diagnostic code for hypertension (*International Classification of Diseases [ICD]*, 9th Revision 401-405 and 10th Revision I10-I13 and I15) in hospital inpatient records, SBP ≥ 140 mmHg, DBP ≥ 90 mmHg, or self-reported use of blood pressure medication (Table 4.9). Variants were included in the analysis if the minor allele

frequency was ≥ 0.001 and the imputation quality score was ≥ 0.3. Models were adjusted for age, sex, genotype batch, and 20 principal components.

4.10.16 Data Analysis

Results are presented as means ± standard error of the mean (SEM). Comparisons in BP and concentration-response curve data were carried out using a two-way analysis of variance (ANOVA) for repeated measures. Other comparisons between more than two groups were made using a two-way ANOVA. A Student-Newman-Keuls post-hoc test followed all ANOVA tests. Comparisons of two groups were conducted using an unpaired t-test. ANOVA and *t*-tests were performed in SigmaPlot version 13 (Systat Software, San Jose, CA). $P<0.05$ was considered statistically significant.

4.11 Supplemental References

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4.12 Additional Tables

Table 4.4: Oligonucleotide primers used for PCR.

DNA	Primers	Product size (bp)
P2rx7^{tm1Gab}	F: 5'-GCCAGAGGCCACTTGTGTAG-3' R: 5'-TATACTGCCCCTCGGTCTTG-3'	200
P2rx7	F: 5'-TCACCACCTCCAAGCTCTTC-3' R: 5'-TATACTGCCCCTCGGTCTTG-3'	246

This table displays the forward (F) and reverse (R) oligonucleotide primers used for genotyping and the product sizes of mutated purinergic receptor P2X ligand-gated ion channel 7 (*P2rx7*, targeted mutation 1, Christopher A Gabel [*tm1Gab*]) fragment and a fragment of the *P2rx7* gene. bp, base pairs.

Table 4.5: Flow cytometry monoclonal antibodies for innate immune cell profiling with surface makers.

Antigen	Antibodies	Clone, company (catalogue #)
CD11b	EF450-conjugated rat anti-mouse CD11b antibody	M1/70, eBioscience (17-0112-81)
CD11c	PE-Cy7-conjugated Armenian hamster anti-mouse CD11c antibody	N418, Biolegend (117318)
CD39a	AF647-conjugated rat anti-mouse CD39 antibody	Duha59, Biolegend (143808)
CD45	BV785-conjugated rat anti-mouse CD45 antibody	30-F11, Biolegend (103149)
CD86	BV605- conjugated rat anti-mouse CD86 antibody	GL-1, Biolegend (105037)
F4/80	AF488-conjugated rat anti-mouse F4/80 antibody	BM8, Biolegend (123120)
P2RX7	PE-conjugated rat anti-mouse P2RX7 antibody	1F11, Biolegend (148704)

AF488 and AF647, Alexa-Fluor 488 and 647; BV605 and BV785, Brilliant Violet 605 and 785; CD, cluster of differentiation; Cy7, Cyanine 7; EF450, eflour 450; PE, phycoerythrin.

Table 4.6: Flow cytometry monoclonal antibodies for T cell profiling with surface makers.

Antigen	**Antibodies**	**Clone, company (catalogue #)**
CD45	BV786-conjugated rat anti-mouse CD45 antibody	30-F11, BD (564225)
CD3	BV605-conjugated rat anti-mouse CD3 antibody	17A2, Biolegend (100237)
CD4	PerCP-eFluor710-conjugated rat anti-mouse CD4 antibody	RM4-5, eBioscience (46-0042-82)
CD8a	AF700-conjugated rat anti-mouse CD8a antibody	53-6.7, Biolegend (100730)
TCR γδ	AF647-conjugated Armenian hamster anti-mouse TCR δ antibody	GL3, Biolegend (118134)
CD69	PE-Cy5-conjugated Armenian hamster anti-mouse CD69 antibody	H1.2F3, Biolegend (104510)
P2RX7	PE-conjugated rat anti-mouse P2RX7 antibody	1F11, Biolegend (148704)
CD103	AF488-conjugated Armenian hamster anti-mouse CD103 antibody	2E7, Biolegend (121408)
CD197	PE-CF594-conjugated rat anti-mouse CD197 antibody	4B12, BD Bioscience (536596)
CD44	PE-Cy7-conjugated rat anti-mouse CD44 antibody	IM7, BD Bioscience (560569)
CD62L	APC-Fire 750-conjugated rant anti-mouse CD62L antibody	MEL-14, Biolegend (104450)
CD103	AF488-conjugated Armenian hamster anti-mouse CD103 antibody	2E7, Biolegend (121408)

AF488, AF647 and AF700, Alexa-Fluor 488, 647 and 700; APC, allophycocyanin; BD, Becton Dickinson; BV605 and BV786, Brilliant Violet 605 and 786; CD, cluster of differentiation; CF594, cyanine-based fluorescent dye 594; Cy5, Cyanine 5; EF450, eflour 450; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; TCR, T cell receptor.

Table 4.7: Flow cytometry monoclonal antibodies for interleukin-17A and interferon-gamma producing T cell profiling.:

Antigen	**Intra/extra-cellular staining**	**Antibodies**	**Clone, company (catalogue #)**
CD45	extracellular	BV785-conjugated rat anti-mouse CD45 antibody	30-F11, Biolegend (103149)
CD3	extracellular	BUV395-conjugated rat anti-mouse CD3 antibody	17A2, BD Biosciences (740268)
CD4	extracellular	PerCP-eFluor710-conjugated rat anti-mouse CD4 antibody	RM4-5, eBioscience (46-0042-82)
CD8a	extracellular	AF700-conjugated rat anti-mouse CD8a antibody	53-6.7, BioLegend (100730)
TCR γδ	extracellular	PE-CF594-conjugated Armenian hamster anti-mouse TCR δ antibody	GL3, BD Biosciences (563532)
IL-17A	Intracellular	APC-conjugated rat anti-mouse IL-17A	eBio17B7, eBioscience (17-7177-81)
IFN-γ	Intracellular	AF488-conjugated rat anti- IFN-γ antibody	XMG1.2, eBioscience (53-7311-82)

AF488 and AF700, Alexa-Fluor 488 and 700; APC, allophycocyanin; BD, Becton Dickinson; BV785, Brilliant Violet 785; BUV395, Brilliant Ultraviolet 395; CD, cluster of differentiation; CF594, cyanine-based fluorescent dye 594; IFN-γ, interferon-gamma; Il, interleukin; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; TCR, T cell receptor.

Table 4.8: Oligonucleotide primers for quantitative PCR.

Name	Sequence	Product size (bp)
Acta1	F:5'-CTTCCTTTATCGGTATGGAGTCTG-3'	286
	F:5'-TTGGTGATCCACATCTGCTG-3'	
Col1a1	F:5'-TTGGAGGAAACTTTGCTTCC-3'	339
	F:5'-CTCGGTGTCCCTTCATTCC-3'	
Col3a1	F:5'-ATAAGCCCTGATGGTTCTCG -3'	317
	F:5'-AGCTGCACATCAACGACATC -3'	
Myh7	F:5'-GAAGGACTTTGAGTTAAATGCACTC-3'	281
	F:5'-GCCGCATCTTCTGGAACTC-3'	
Nppa	F:5'-TGAGCAGACTGAGGAAGCAG-3'	235
	F:5'-ACTCTGGGCTCCAATCCTG-3'	
Nppb	F:5'-GAGAAAAGTCGGAGGAAATGG-3'	222
	F:5'-CCTACAACAACTTCAGTGCGTTAC-3'	
P2rx7	F:5'-TTGCTTTGGTGAGCGATAAG-3'	219
	F:5'-GCACTTGGCCTTCTGACTTG-3'	
Rps16	F:5'-ATCTCAAAGGCCCTGGTAGC-3'	211
	F:5'-ACAAAGGTAAACCCCGATCC-3'	
Tgfb1	F:5'-GTGGAAATCAACGGGATCAG-3'	277
	F:5'-AGGGTCCCAGACAGAAGTTG-3'	

The forward (F) and reverse (R) oligonucleotide primers used for quantitative PCR of mouse cardiac ventricles. Actin alpha 1 skeletal muscle (*Acta1*), collagen type I alpha 1 chain (*Col1a1*), collagen type III alpha 1 chain (*Col3a1*), myosin heavy chain 7 cardiac muscle beta (*Myh7*), natriuretic peptide type A (*Nppa*), natriuretic peptide type B (*Nppb*), purinergic receptor P2X 7 (*P2rx7*), ribosomal protein S16 (*Rps16*), and transforming growth factor-beta 1 (*Tgfb1*), bp, base pair.

Table 4.9: Overview of the number of hypertensive individuals in the UK Biobank

Total Number of Participants	Number of Hypertensives	Number of Normotensives
384 653	209 734	174 919

Hypertension was defined as a diagnostic code for hypertension in hospital inpatient records, systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or self-reported blood pressure medication.

4.13 Additional Figures

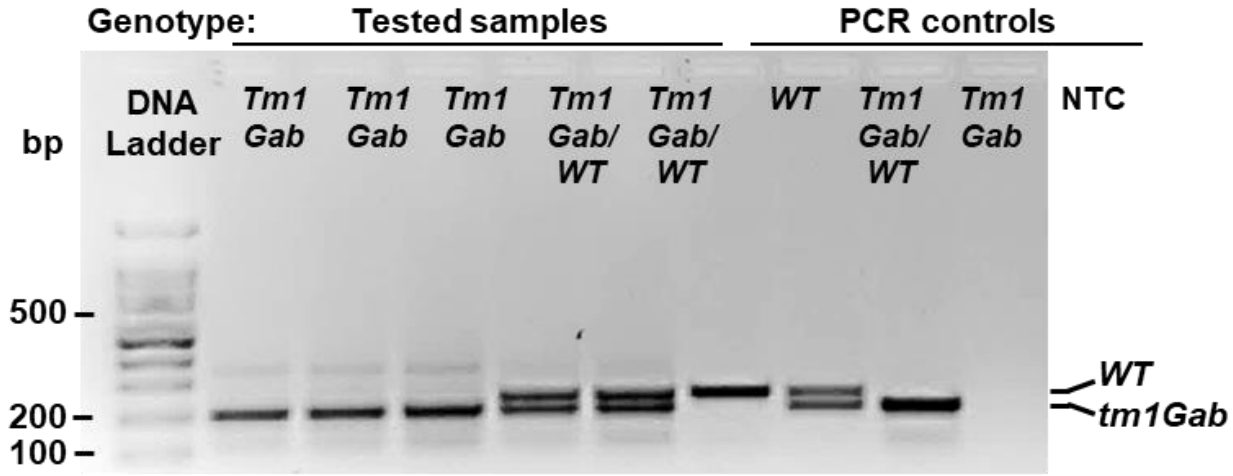

Figure 4.6: P2RX7 KO genotyping. P2rx7$^{-/-}$ samples were identified by visualization of a unique 200 bp fragment using electrophoresis with a 2% agarose gel containing 1X TAE electrophoresis buffer and RedSafe nucleic acid stain with a 100 base pairs (bp) DNA ladder (L). Tm1Gab/WT, P2RX7 heterozygote; WT, C57BL/6J WT; NTC, negative control master mix and water.

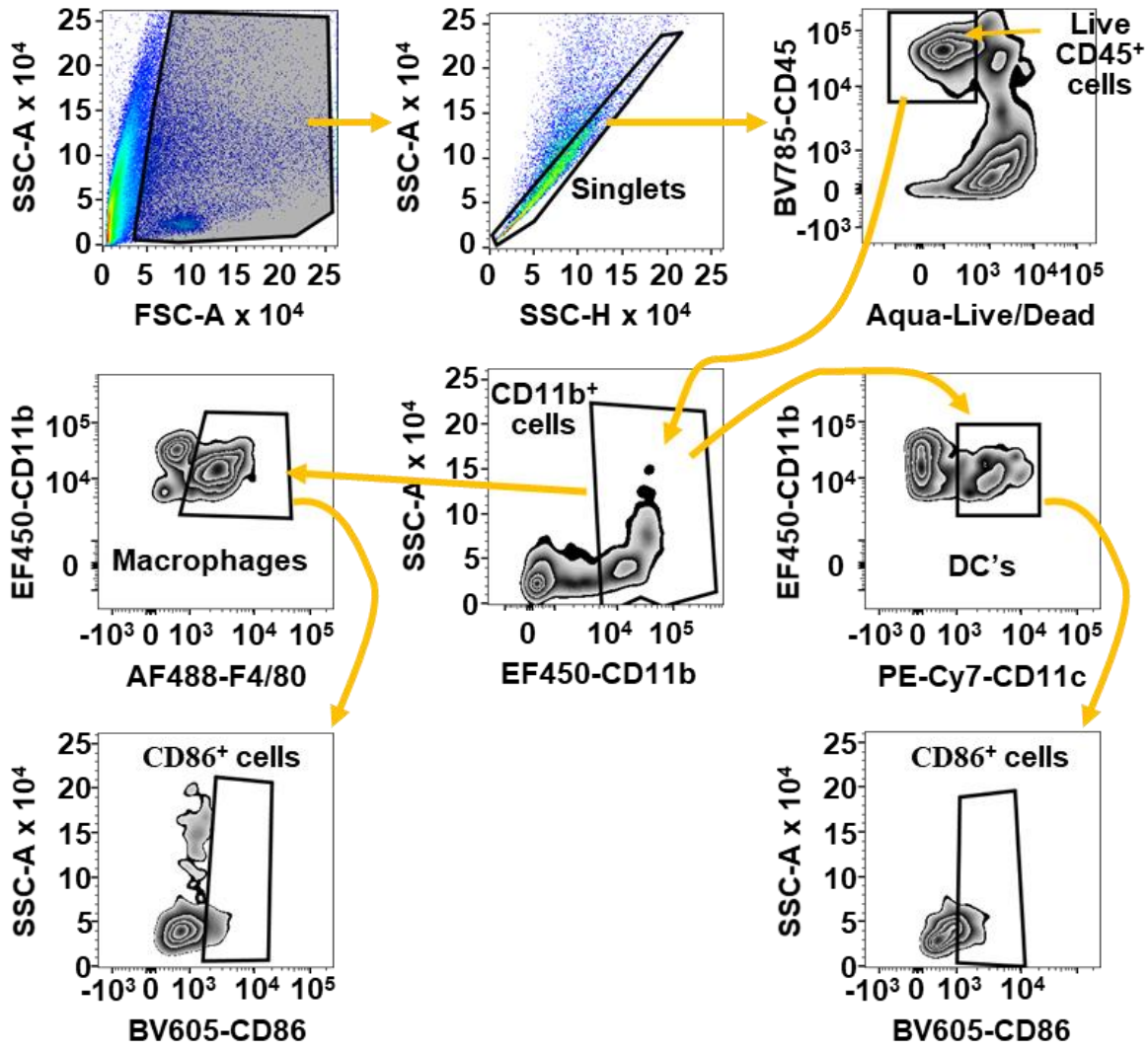

Figure 4.7: Flow cytometry gating strategy of innate immune cells in the aortic perivascular adipose tissue.: Cells are first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using SSC-A over SSC height (SSC-H). Live $CD45^+$ cells were gated in the CD45/Live/Dead plot, followed by $CD11b^+$ myeloid cells in the SSC-A/CD11b plot. Then, macrophages and dendritic cells (DCs) were gated in the CD11b/F4/80 plot and CD11b/CD11c plot, respectively. CD86 positive cells were then gated within macrophage and DC populations using a CD86 flow minus one in a SSC-A/CD86 plot. AF488, Alexa-Fluor 488; BV605 and BV785, Brilliant Violet 605 and 786; CD, cluster of differentiation; Cy7, Cyanine 7; EF450, eflour 450; PE, phycoerythrin.

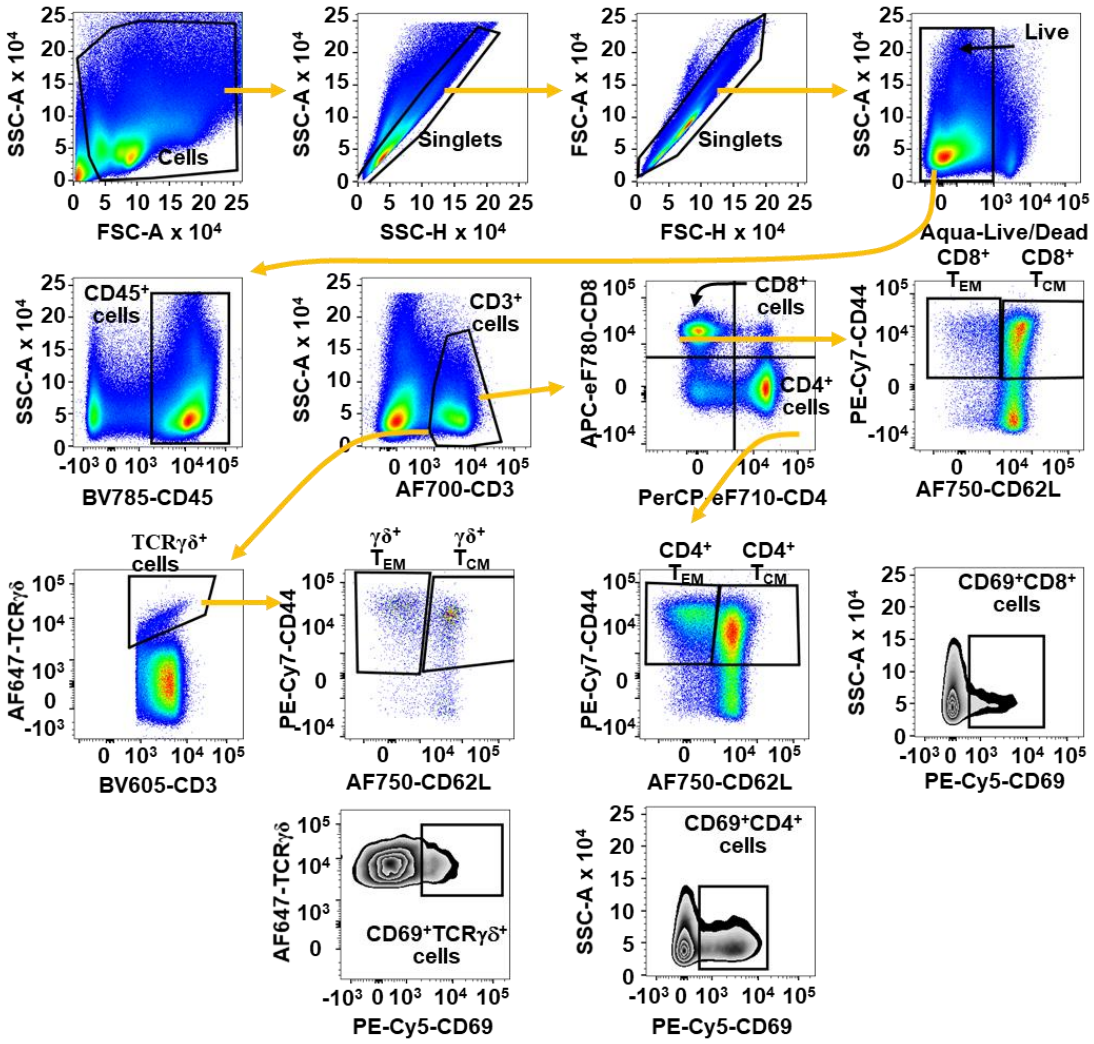

Figure 4.8: Representative flow cytometry gating strategy of T cells. The gating strategy above depicts cells from the spleen, but the same gating strategy was used for T cells in the aortic perivascular adipose tissue, bone marrow and mesenteric lymph nodes**.** Cells were first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using SSC-A over SSC height (SSC-H)). Singlet cells were gated again to clean up the data further using FSC-A over FSC-H. Live lymphocytes were gated using SSC-A/viability dye. $CD45^+$ lymphocytes were gated using SSC-A/CD45, followed by $CD3^+$ T cells in the SSC-A/CD3 plot. Then, $CD4^+$ and $CD8^+$ cells were gated in the CD8/CD4 plot and γδ T cells in the T cell receptor (TCR) γδ/CD3 plot. $CD69^+$ $CD4^+$, $CD8^+$ and γδ T cells were gated in CD4/CD69, CD8/CD69 and TCRγδ/CD69 plot, respectively. T effector memory (T_{EM}) cells were gated within their respective parent populations ($CD4^+$, CD8+, or $\gamma\delta^+$) as $CD44^+$, $CD62L^-$, and T central memory (T_{cm}) as $CD44^+$, $CD62L^+$. AF488, AF647 and AF700, Alexa-Fluor 488, 647 and 700; APC, allophycocyanin; BD, Becton Dickinson; BV605 and BV786, Brilliant Violet 605 and 786; CD, cluster of differentiation; CF594, cyanine-based fluorescent dye 594; Cy5, Cyanine 5; EF450, eflour 450; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; TCR, T cell receptor.

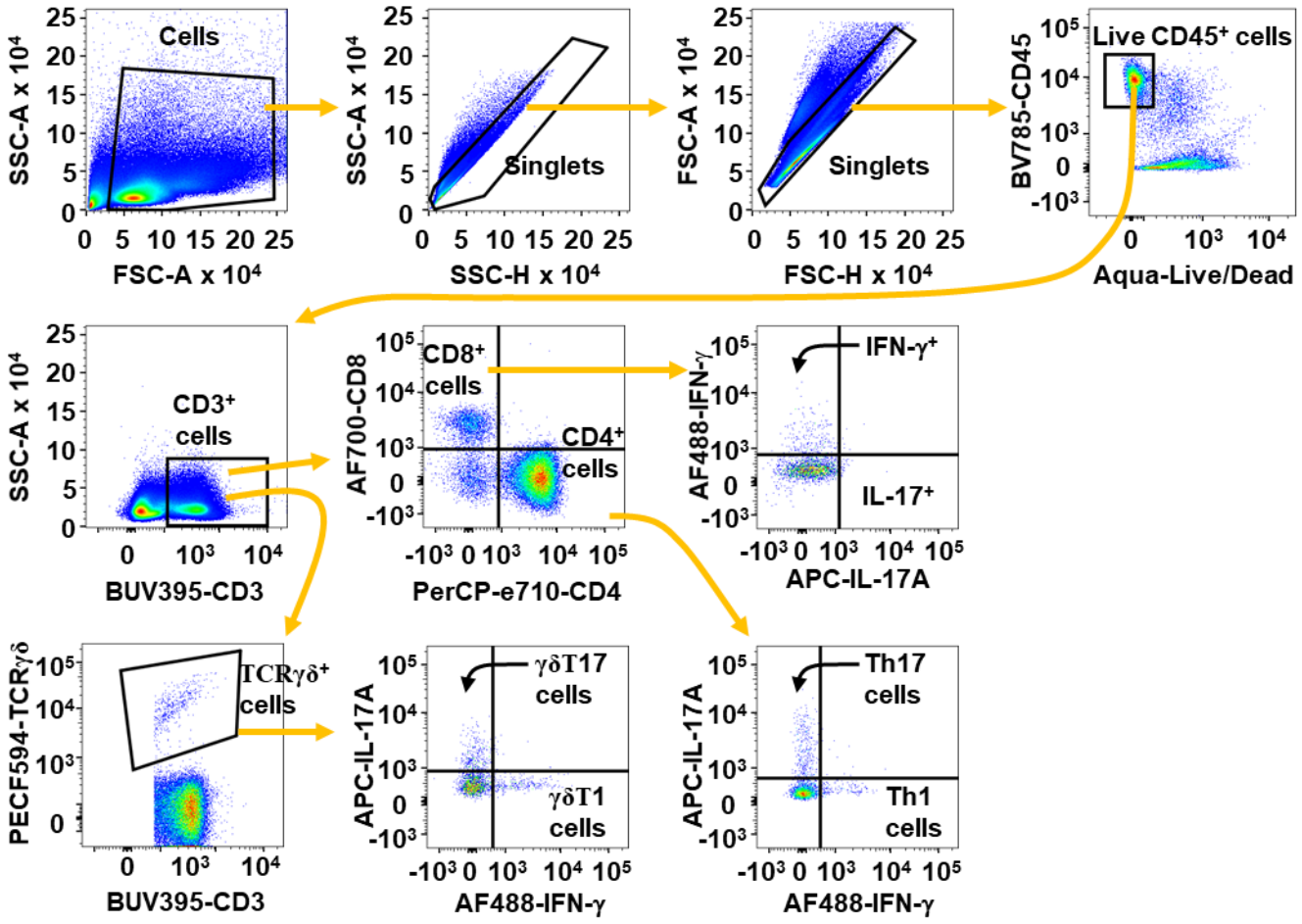

Figure 4.9: T cell activation gating strategy. Representative gating strategy for interleukin (IL)-17A and interferon-gamma (IFN-γ) producing T cells. Cells are first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using SSC-A over SSC height (SSC-H)). Singlet cells were gated again to clean up the data further using FSC-A over FSC-H. Live $CD45^+$ cells were gated in the CD45/Live/Dead plot, followed by $CD3^+$ T cells in the SSC-A/CD3 plot. Then, $CD4^+$ and $CD8^+$ cells were gated in the CD8/CD4 plot and γδ T cells in the T cell receptor (TCR) γδ/CD3 plot. Finally, IL-17A producing γδ (γδT17), $CD4^+$ (T helper 17, Th17) and $CD8^+$ T cells and IFN-γ producing γδ (γδT1), $CD4^+$ (Th1) and $CD8^+$ T cells were gated in the respective IL-17A/IFN-γ plot. AF488 and AF700, Alexa-Fluor 488 and 700; APC, allophycocyanin; BD, Becton Dickinson; BV785, Brilliant Violet 785; BUV395, Brilliant Ultraviolet 395; CD, cluster of differentiation; CF594, cyanine-based fluorescent dye 594; e710, eFluor 710; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein

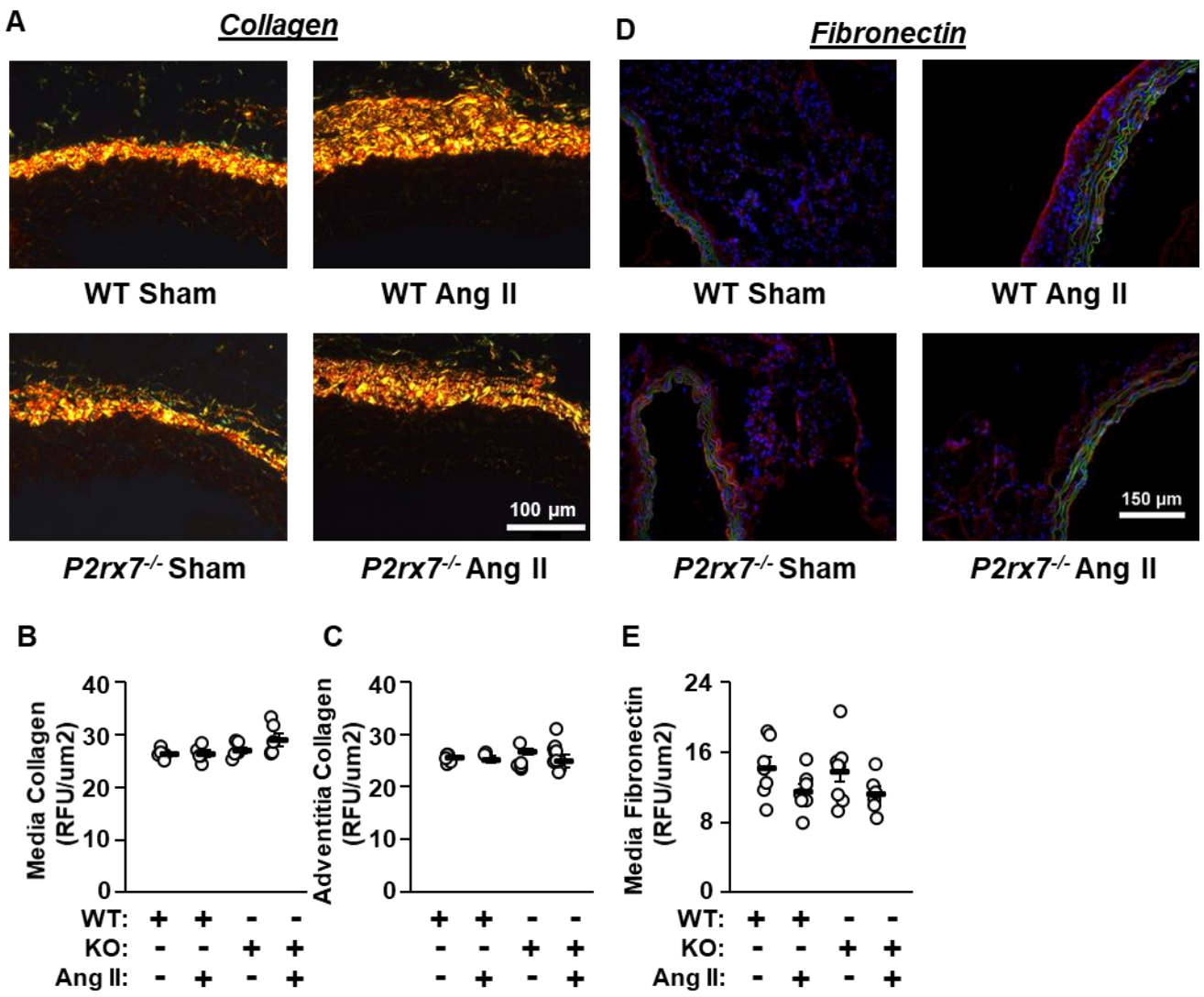

Figure 4.10: Collagen and fibronectin content of the aorta. Representative polarized light images of Sirius red (A) stained sections of the aorta with perivascular adipose tissue from wild-type (WT) or *P2rx7*$^{-/-}$ mice treated or not with angiotensin (Ang) II. Media (B) and adventitial (C) collagen content were assessed from the polarized images and expressed as relative fluorescent units (RFU)/µm^2. Representative images of aortic sections stained with fibronectin (red) are shown. Blue and green represent 4',6-diamidino-2phenylindole (DAPI) fluorescence and elastin autofluorescence, respectively (D). Media fibronectin content is expressed as RFU/µM^2 (E). Values are means± SEM, n=5-7.

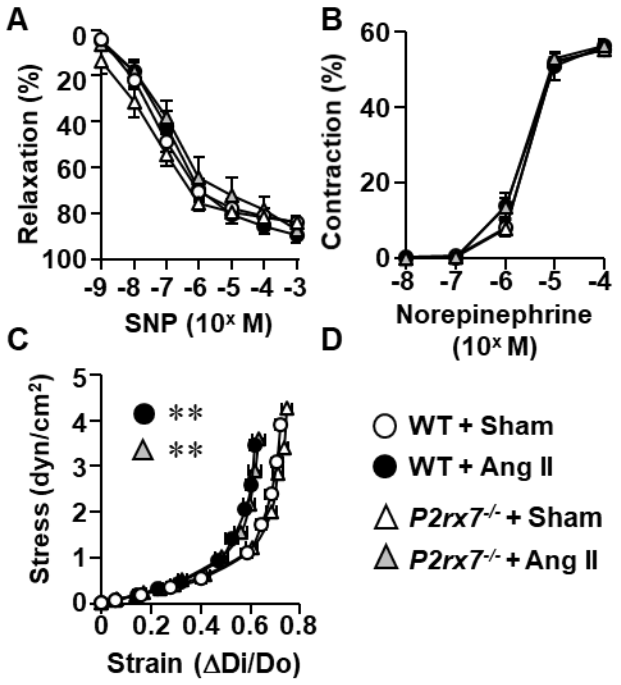

Figure 4.11: Vascular properties of mesenteric arteries. Endothelium-independent vasodilation of mesenteric arteries from WT or *P2rx7*$^{-/-}$ mice, pre-constricted with norepinephrine (NE), to increasing doses of sodium nitroprusside (SNP, A) conducted using pressurized myography. Contraction responses to increasing concentrations of NE (B). Stress-strain curve of mesenteric arteries exposed to incremental increases in intraluminal pressure (C). ΔDi is the change in lumen diameter for a given intraluminal pressure and Do is the original diameter measured at 3 mmHg. Values are means± SEM, n=6-9 for A, B and 10-14 for C. Data were analyzed using a two-way ANOVA with repeated measure for A, B and two-way ANOVA for C, followed by a Student-Neuman-Keuls post hoc test. The strain values at 140 mmHg (the last points) were used for analysis in D. **P<0.01 versus respective sham.

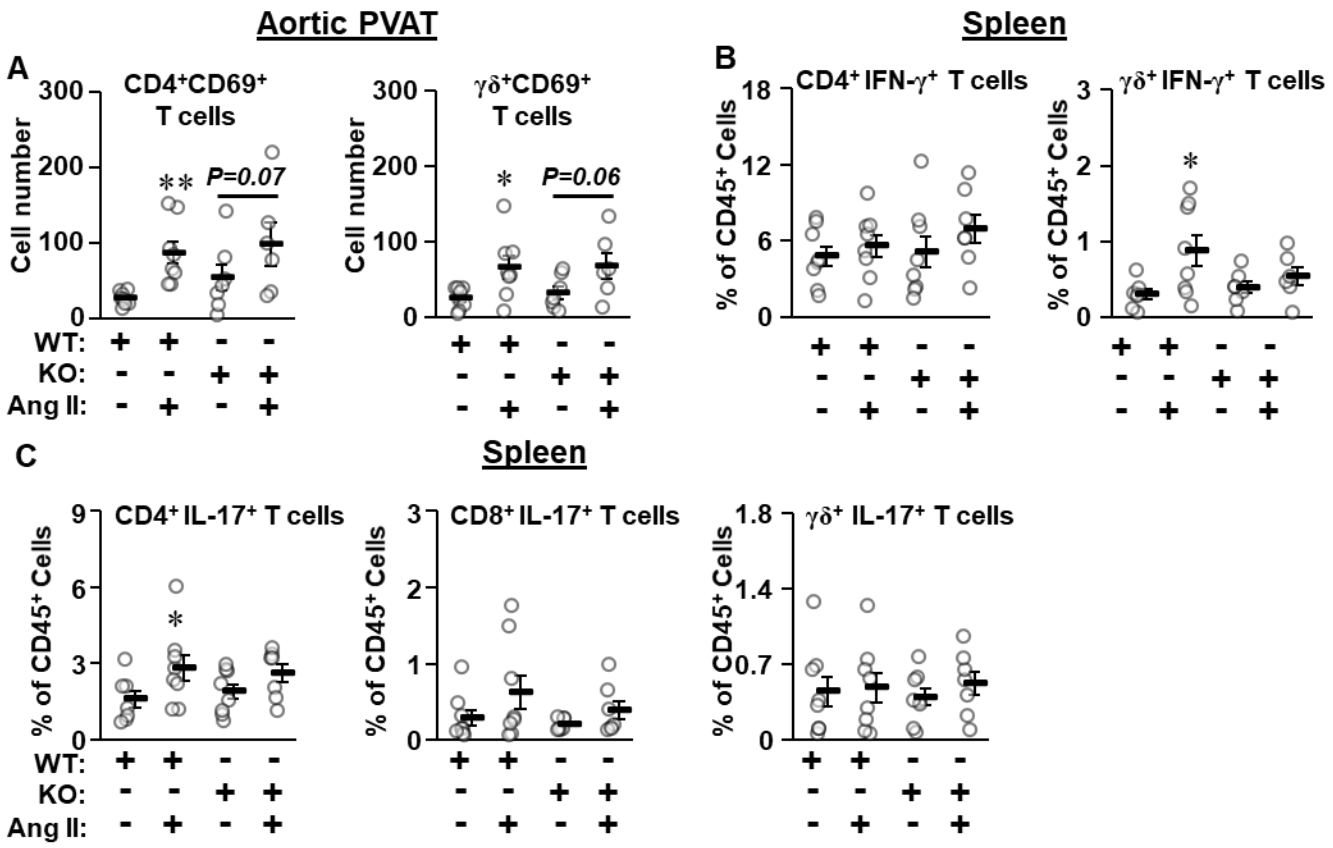

Figure 4.12: T cell activation and cytokine production. Flow cytometry was used to quantify the number of activated ($CD69^+$) $CD4^+$ and $\gamma\delta^+$ T cells (A) in the aortic perivascular adipose tissue (PVAT) in WT and *P2rx7*$^{-/-}$ mice treated or not for 14 days with angiotensin (Ang II). The frequency of IFN-γ (B) and IL-17 (C) producing T cells in the spleen was determined by flow cytometry after activation with phorbol 12-myristate 13-acetate and ionomycin. Gating strategies for panels (A) and (B, C) are provided in supplemental Figure 4.8 and Figure 4.9 respectively. Data are presented as means ± SEM, n= 6-8. Data were analyzed using a two-way ANOVA followed by a Student-Neuman-Keuls post hoc test. **P*<0.05 and ***P*<0.01 versus respective sham.

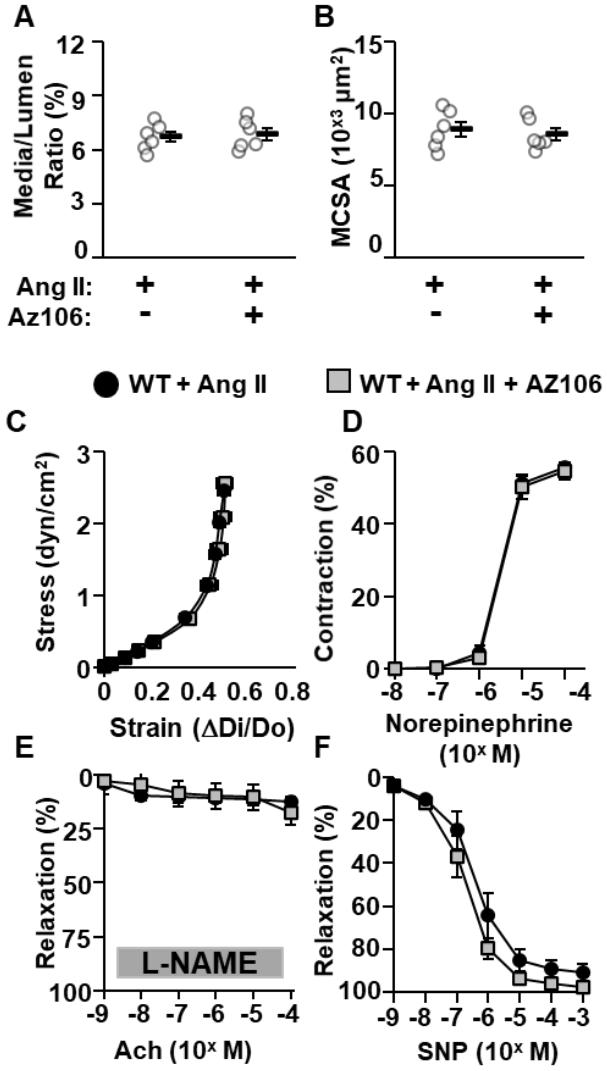

Figure 4.13: Vascular mechanics and function of Ang II-infused mice treated or not with the P2RX7 antagonist AZ10606120. Media/lumen (A) and media cross-sectional area (MCSA) (B) of mesenteric arteries at 45 mmHg intraluminal pressure were recorded using a pressure myography system. The stress-strain curve of mesenteric arteries exposed to incremental increases in intraluminal pressure (C). ΔDi is the change in lumen diameter for a given intraluminal pressure, and Do is the original diameter measured at 3 mmHg. Contraction responses to increasing concentrations of NE (D). Endothelium-dependent relaxation responses of mesenteric arteries, pre-constricted with norepinephrine (NE), to increasing doses of acetylcholine in the presence of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (E). Endothelium-independent vasodilation of mesenteric arteries pre-constricted with norepinephrine (NE), to increasing doses of sodium nitroprusside (SNP) (F). Values are means± SEM, n=6.

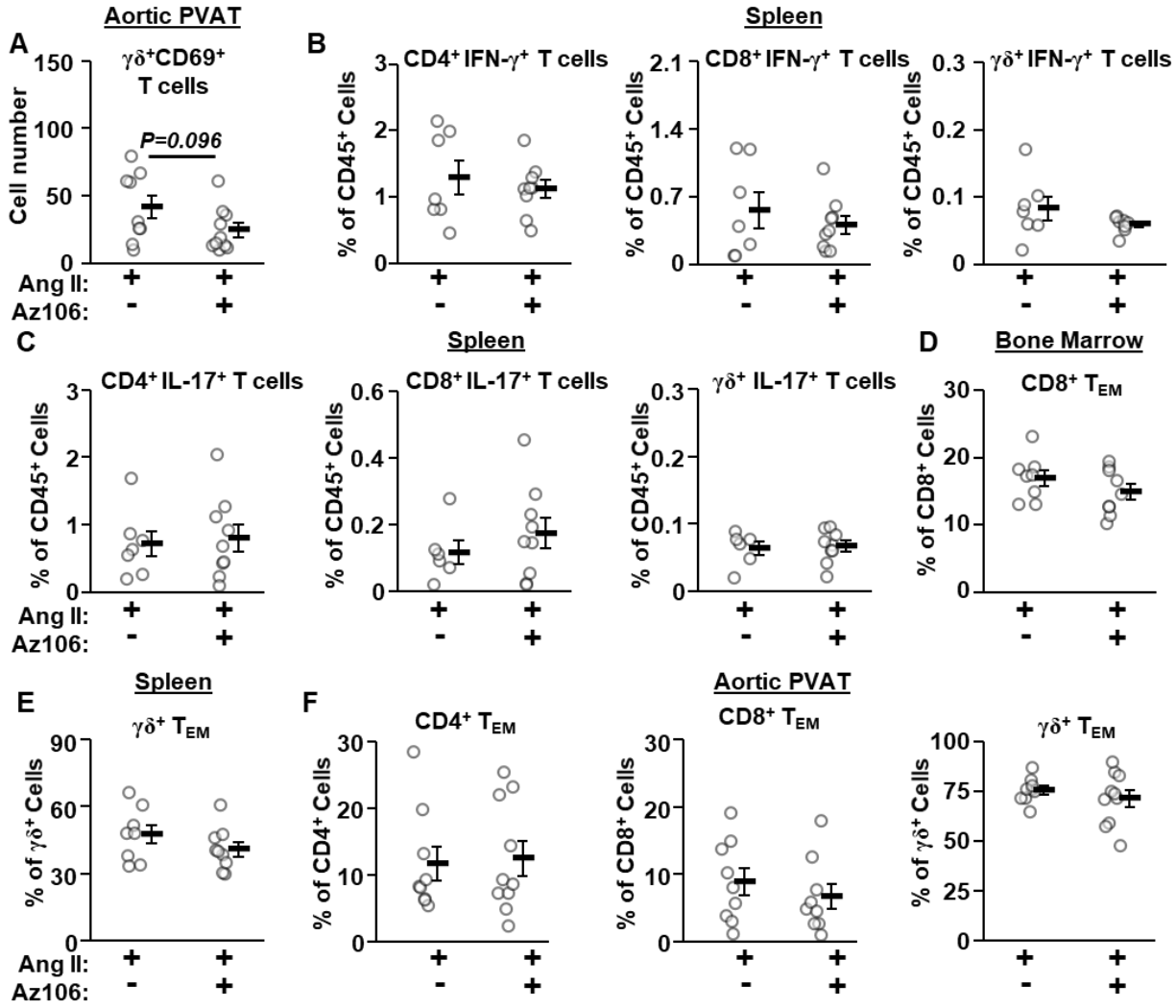

Figure 4.14: T cell activation, cytokine production and memory profile of WT mice after two weeks of Ang II-infusion with or without the P2RX7 antagonist AZ10606120. Flow cytometry was used to quantify the number of activated ($CD69^+$) $\gamma\delta^+$ T cells (A) in the aortic perivascular adipose tissue (PVAT). The frequency of IFN-γ (B) and IL-17 (C) producing T cells in the spleen was determined by flow cytometry after activation with phorbol 12-myristate 13-acetate and ionomycin. Flow cytometry was used to quantify the frequency of $CD8^+$ T effector memory (T_{EM}; $CD44^+CD62L^-$) cells in the bone marrow (D), $\gamma\delta^+$ T_{EM} in the spleen (E) and $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T_{EM} in the aortic PVAT (F). Gating strategies for panels (A, D-F), and (B, C) are provided in Figure 4.8 and Figure 4.9. Values are means ± SEM, n= 7-10. Data were analyzed using an unpaired t-test followed by a Student-Neuman-Keuls post hoc test.

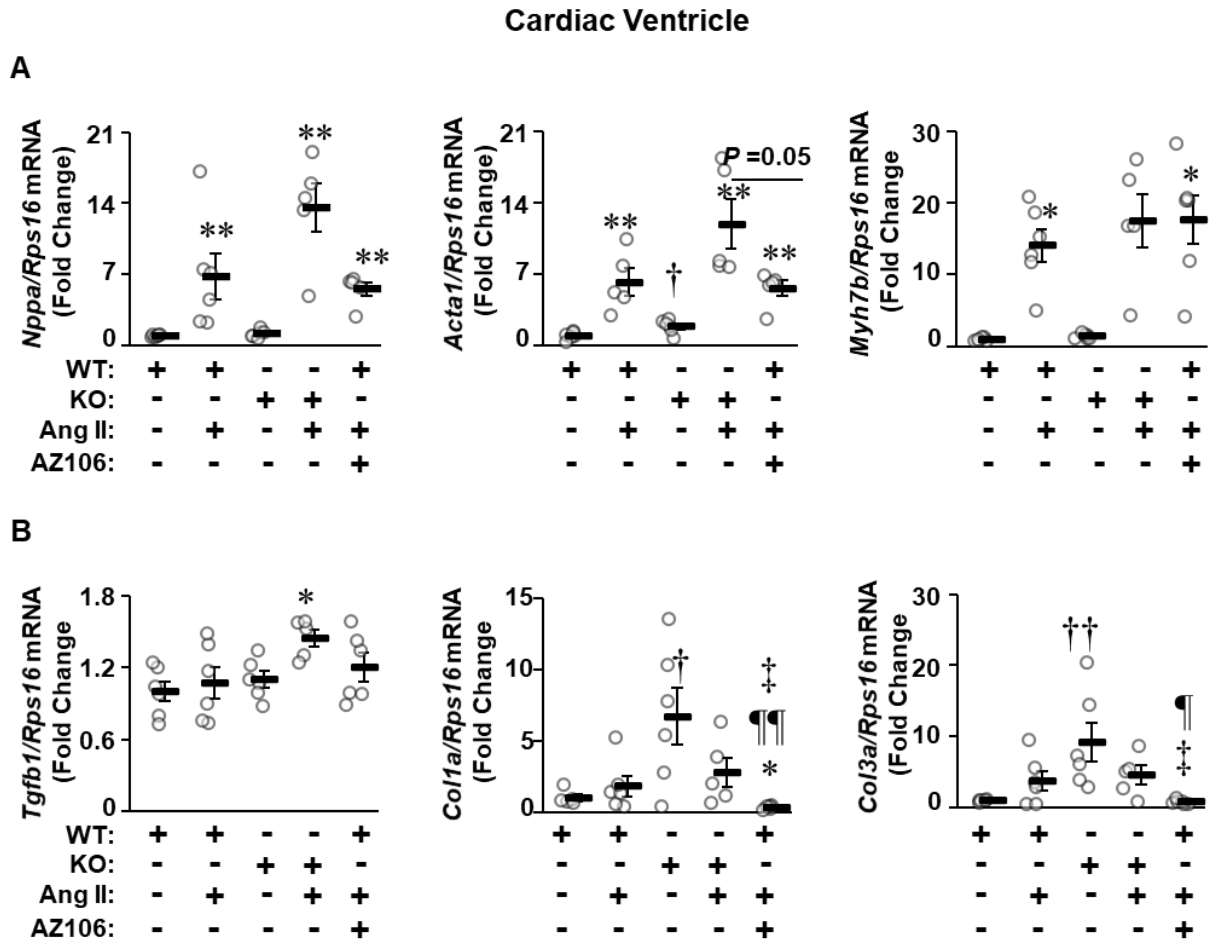

Figure 4.15: Expression of hypertrophy and fibrosis genes are elevated in P2rx7$^{-/-}$ mice. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to assess the expression of hypertrophy genes (A) and fibrosis genes (B) in cardiac ventricles from WT and *P2rx7*$^{-/-}$ mice treated or not with Ang II and Ang II-treated WT mice receiving the P2RX7 antagonist AZ10606120 (AZ106). The mRNA expression was normalized by ribosomal protein S16 (*Rps16*) mRNA levels and expressed as fold change over control. Data are presented as means ± SEM, n=5-6. All RT-qPCR data, except for β-myosin heavy chain 7 (*Myh7b*), were analyzed using 1-way ANOVA followed by a Student-Newman-Keuls post hoc test. *Myh7b* was analyzed using a Kruskal-Wallis 1-way ANOVA on ranks followed by a Dunn's multiple comparison post hoc test. **P*<0.05 and ***P* <0.001 vs respective sham; †*P* <0.05 versus WT Sham, ‡*P*<0.05 versus WT Ang II, ¶¶ *P*<0.01 vs *P2rx7*$^{-/-}$ Ang II. Alpha skeletal actin-1 (*Acta1*), collagen type I alpha 1 chain (*Col1a1*), collagen type III alpha 1 chain (*Col3a1*), atrial natriuretic peptide (*Nppa*), and transforming growth factor-beta 1 (*Tgfb1*).

Chapter 5: The Role of P2RX7 and the Inflammasome in Angiotensin II-Induced Hypertension

This study aimed to delineate the role of P2RX7 from the NLRP3 inflammasome in mediating Ang II-induced hypertension, vascular damage, and immune activation. The previous study demonstrated that the genetic KO of *P2rx7* is protective in the context of Ang II-induced hypertension. Furthermore, other groups have shown a protective role for NLRP3 antagonism or KO in murine models of hypertension. However, it has not been elucidated whether the protective effect of P2RX7 KO or antagonism is mediated through diminished NLRP3 inflammasome activation. This study demonstrated that the decreased immune activation and vascular damage in *P2rx7* KO mice are most likely mediated through diminished NLRP3 activation. In contrast, the decreased BP is mainly independent of the NLRP3 inflammasome. These results align with the large CANTOS trial, which demonstrated that inhibition of IL-1β, a major downstream effector of NLRP3 activation, did not lower BP but decreased adverse cardiovascular events. Therefore, this study proposes that P2RX7 would present a better target than the NLRP3 inflammasome for attenuating hypertension and associated vascular dysfunction and immune cell activation.

The article entitled "P2RX7-NLRP3 inflammasome signalling is critical for Ang II-induced endothelial dysfunction and T cell activation, but primarily it is P2RX7 that contributes to Ang II-induced blood pressure elevation" is in preparation to be submitted for publication upon publication of the manuscript presented in Chapter 4. The main manuscript is followed by the Online Supplement, which includes the expanded materials and methods section and supplemental figures.

P2RX7-NLRP3 inflammasome signalling is critical for Ang II-induced endothelial dysfunction and T cell activation, but primarily it is P2RX7 that contributes to Ang II-induced blood pressure elevation

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Short Title: P2RX7 knockout attenuates hypertension independently from NLRP3

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Keywords: Endothelial dysfunction, blood pressure, inflammation, IL-1β, NLRP3, P2RX7.

5.1 Abstract

Hypertension is the leading risk factor for cardiovascular disease and mortality worldwide. It is now appreciated that chronic inflammation contributes to the pathogenesis of hypertension, and strategies to target this inflammation are of great interest. The purinergic receptor P2X7 plays an important role in facilitating inflammation, in large part through activation of the NLRP3 inflammasome and subsequent release of the pro-inflammatory cytokine interleukin-1β. Previous reports have suggested that both P2RX7 and NLRP3 antagonism could be protective against hypertension and target organ damage, but no studies to date have shown if P2RX7 contributes to hypertension through NLRP3 signalling. C57BL/6 wild-type (WT), *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were infused with angiotensin (Ang) II. Blood pressure was recorded via telemetry, vascular function and remodelling studied using pressurized myography and immune activation using flow cytometry. *P2rx7*$^{-/-}$, but not *Nlrp3*$^{-/-}$ mice, had attenuated systolic blood pressure elevation (~162, 173, and 176 mmHg, *P2rx7*$^{-/-}$, *Nlrp3*$^{-/-}$and WT respectively) in response to Ang II treatment. Both *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated with Ang II had preserved endothelial function and attenuated vascular remodelling. Furthermore, both *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice had attenuated $CD4^+$ and $CD8^+$ T cell activation in the spleen compared to WT mice treated with Ang II. In summary, despite reduced T cell activation, diminished vascular remodelling and preserved endothelial dysfunction, *Nlrp3*$^{-/-}$ mice are not protected from Ang II-induced increases in systolic blood pressure, whereas *P2rx7*$^{-/-}$ mice are. This would suggest the anti-hypertensive effect of P2RX7 antagonism is mediated at least partially through a pathway other than NLRP3 signalling.

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5.2 Introduction

Hypertension is the leading risk factor for mortality worldwide (1), and its prevalence is expected to increase as the global population continues to age. Chronic low-grade inflammation is now appreciated to contribute to the pathogenesis of hypertension.(2) Increased inflammasome gene expression and circulating interleukin (IL)-1β in subjects over the age of 60 years is strongly associated with increased risk for hypertension and vascular dysfunction, as well as all-cause mortality.(3) Thus, targeting the source of inflammation in hypertension is an interesting target for developing new therapeutic agents.

The purinergic receptor P2X7 (P2RX7), is an ATP-gated cation channel that is highly expressed on immune cells. When activated P2RX7 triggers K^+ efflux, leading to NLRP3 activation, caspase-1 cleavage and subsequent activation and release of IL-1β.(4) IL-1β is a pro-inflammatory cytokine that promotes immune activation and is a known driver of endothelial dysfunction, which in itself is a key component for the progression of hypertension and end-organ damage.(5-7) P2RX7, NLRP3 and IL-1β have all been implicated in the progression of cardiovascular disease or hypertension and thus, targeting this axis provides a promising candidate to reduce the inflammation associated with hypertension.

Elevated plasma ATP concentrations have been observed in hypertensive patients compared to normotensive patients or patients with well-controlled hypertension.(8) Similarly, elevated extracellular ATP concentrations have been observed in several animal models of hypertension, providing a potential mechanism for P2RX7 activation.(8-11) We have previously demonstrated that *P2rx7*$^{-/-}$ or pharmaceutical antagonism of P2RX7 attenuated Ang II-induced hypertension, vascular dysfunction, and immune activation, but we did not define if the protective effect was due to diminished NLRP3 signalling.(9) In animal models, *Nlrp3*$^{-/-}$ mice are protected from angiotensin (Ang) II-induced gestational hypertension,(12) whereas NLRP3 antagonism with MCC950 in mice decreased blood pressure (BP), renal dysfunction, and inflammation in a one-kidney/DOCA-salt model of hypertension.(13) Similarly, *Nlrp3*$^{-/-}$ mice are protected against aldosterone-induced hypertension and small arterial hypercontractility, endothelial dysfunction, and vascular remodelling.(7) Together, these studies would suggest that the detrimental effect of P2RX7 in hypertension is mediated through NLRP3 signalling. However, besides activating the NLRP3 inflammasome, P2RX7 has numerous other cellular roles, including promoting cellular death, T lymphocyte maturation and activation, and regulating kidney hemodynamics, which could

contribute to hypertension.(14) Therefore, the goal of this study was to determine if the protective effect of P2RX7 was mediated through NLRP3 inflammasome activation. To test this, we took wild-type C57BL/6J (WT), *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice, treated them for two weeks with Ang II, and monitored their blood pressure via telemetry. Next, we assessed their vascular function and remodelling using pressurized myography. Finally, we assessed global inflammation by analyzing $CD4^+$and $CD8^+$ T cell activation in the spleen using flow cytometry.

5.3 Methods

A detailed methods section can be found in the online data supplement.

5.3.1 Experimental Design

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed the Canadian Council of Animal Care recommendations. WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and a colony was maintained on-site for the duration of the study.

Ten to 12-week-old male WT, *P2rx7*$^{-/-}$, *Nlrp3*$^{-/-}$ mice were infused or not with Ang II (490 ng/kg/min, SC) for 14 days. A sub-group of mice were implemented with radio telemetric probes and their blood pressure and heart rate were continuously measured by telemetry 2 days before and during the treatment period as previously described.(15) At the end of the treatment, the mice were euthanized, the mesenteric vascular beds attached to the intestine were collected, and endothelial function and vascular mechanical properties were determined in second-order branches of mesenteric arteries by pressurized myography. The spleen was collected in ice-cold phosphate-buffered saline and used for immune phenotyping by flow cytometry to assess immune activation.

5.3.2 Data analysis

Results are presented as means ± standard error of the mean (SEM). Comparisons in BP data were carried out using a two-way analysis of variance (ANOVA) for repeated measures. Comparison in heart rate were conducted using a one-way ANOVA on the area under the curve. Other comparisons between more than two groups were made using a two-way ANOVA. All ANOVA tests were followed by a Student-Newman-Keuls *post-hoc* test. ANOVA tests were performed in SigmaPlot version 13 (Systat Sotware, San Jose, CA). $P<0.05$ was considered statistically significant.

5.4 Results

5.4.1 P2rx7$^{-/-}$ Attenuates Ang II-Induced Systolic and Diastolic Blood Pressure Elevation

We first wanted to determine whether the attenuated pressor response we previously observed in *P2rx7*$^{-/-}$ mice was due to diminished NLRP3 inflammasome activation.(9) We took WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated them with Ang II (490ng/kg/min) for 14 days and continuously recorded blood pressure levels by radiotelemetry. Confirming our previous findings, *P2rx7*$^{-/-}$ mice had a decreased pressor response to Ang II, with a 17-mmHg reduction in systolic blood pressure (SBP) and a 15-mmHg reduction in diastolic BP (DBP) compared to treated WT mice (Figure 5.1A and B). Interestingly, *Nlrp3*$^{-/-}$ mice initially had a reduced increase in SBP and DBP in response to Ang II infusion, but by day 14 only DBP remained lower (~10 mmHg), whereas their SBP was similar to Ang II-treated WT mice (~173 mm Hg). Pulse pressure rose similarly in Ang II-treated WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice however there was a trend to higher pulse pressure in the Ang II treated *Nlrp3*$^{-/-}$ mice (Figure 5.1C). Starting by day 7 of treatment *Nlrp3*$^{-/-}$ mice had a lower heart rate (~70 beats per minute lower) than WT or *P2rx7*$^{-/-}$ mice, with the lower heart rate persisting until the end of treatment at day 14 (Figure 5.1D).

5.4.2 Small Artery Endothelial Dysfunction and Hypertrophic Remodelling are Attenuated by P2rx7$^{-/-}$

As endothelial dysfunction and vascular remodelling are key components for the progression of hypertension and end-organ damage, we utilized pressurized myography to investigate whether NLRP3 or P2RX7 dependent signalling contributed to Ang II-induced endothelial dysfunction and vascular remodelling. WT mice treated with Ang II had a ~30% reduction in vasodilatory response to acetylcholine, indicating endothelial dysfunction. Whereas neither *P2rx7*$^{-/-}$ nor *Nlrp3*$^{-/-}$ mice displayed endothelial dysfunction (Figure 5.2A). Sodium nitroprusside (SNP) is a nitric oxide donor and acts directly on vascular smooth muscle cells (VSMC) to induce vasodilation in an endothelium-independent manner. Both *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated with Ang II had a leftward shift in the SNP dose-response curve (Figure 5.2B) compared to treated WT mice, suggesting enhanced sensitivity to NO-mediated vasodilation. As VSMC responses to SNP were different in *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice compared to WT mice, we wanted to investigate whether VSMC mediated contractile responses to norepinephrine would also be altered. However, we found no differences in the contractile responses to norepinephrine between any of the genotypes or treatments (Figure 5.2C). Surprisingly, *Nlrp3*$^{-/-}$ mice presented with stiffer mesenteric arteries

at baseline (Figure 5.2D), whereas baseline arterial stiffness was comparable between WT and *P2rx7*$^{-/-}$ mice. Both WT and *P2rx7*$^{-/-}$ mice underwent similar arterial stiffening after Ang II-infusion, as indicated by a left-ward displacement in the stress-strain curve, leaving arterial stiffness comparable between all genotypes after Ang II-infusion (Figure 5.2D). Resistance arteries from WT mice treated with Ang II underwent hypertrophic remodelling with an increased mesenteric artery media to lumen ratio (M/L) (1.23-fold)), and media-cross-sectional area (MCSA) (1.28-fold) (Figure 5.2E and F). Whereas *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated with Ang II underwent eutrophic remodelling with an increased M/L (1.23-fold) but not MCSA. Hypertrophic remodelling is associated with more severe hypertension (16), and thus the eutrophic remodelling in *P2rx7*$^{-/-}$ mice treated with Ang II might reflect the attenuated BP increases. However, as *Nlrp3*$^{-/-}$ mice did not have attenuated SBP, despite reduced vascular remodelling, it suggests that NLRP3 mediated inflammation may account for the decreased remodelling in both *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice. Of note, the M/L and MCSA of *P2rx7*$^{-/-}$ mice after Ang II treatment was significantly smaller than that of treated *Nlrp3*$^{-/-}$ mice suggesting a combination of attenuated BP and NLRP3 mediated inflammation or other factors may account for the observed differences in *P2rx7*$^{-/-}$ mice.

5.4.3 The NLRP3 Inflammasome Contributes to T Cell Activation in Ang II-Induced Hypertension

Numerous studies have illustrated a critical role for T cell activation in the pathogenesis of hypertension.(2) Since the inflammasome plays a role in activating the adaptive immune system, we used flow cytometry to assess the activation of T cells in the spleen. We observed an increase in the frequency of CD69$^+$ expressing CD4$^+$ (1.7-fold) and CD8$^+$ (1.8-fold) T cells in the WT mice treated with Ang II, indicating T cell activation (Figure 5.3). An increased frequency of activated CD4$^+$ T cells was also observed in the *P2rx7*$^{-/-}$ mice treated with Ang II (1.4-fold), although the frequency was significantly lower than in treated WT mice (1.2-fold) (Figure 5.3B). No significant increase in the frequency of activated CD4$^+$ T cells was observed in *Nlrp3*$^{-/-}$mice. In fact, the frequency of CD69$^+$CD4$^+$T cells was significantly lower than both WT (1.5-fold) and *P2rx7*$^{-/-}$ (1.2-fold) mice treated with Ang II. Neither *Nlrp3*$^{-/-}$ nor *P2rx7*$^{-/-}$ mice had a significant increase in activated CD8$^+$ T cells. The frequencies of global CD4$^+$ and CD8$^+$ populations in the spleen (represented as a frequency of CD45$^+$ cells) were comparable between genotypes (Figure 5.5), indicating only differences in activated T cell populations existed across genotypes.

5.5 Discussion

This study adds to the growing body of evidence that P2RX7 contributes to the pathogenesis of hypertension. Here, we report that *P2rx7*$^{-/-}$ mice have attenuated SBP and DBP, eutrophic rather than hypertrophic small artery remodelling, preserved endothelial function and decreased T cell activation in response to Ang II-infusion. Furthermore, we report that *Nlrp3*$^{-/-}$mice do not share this smaller pressor response to Ang II, despite preserved endothelial function and attenuated vascular remodelling and T cell activation.

Our results demonstrate that the P2RX7-NLRP3 signalling pathway is important in regulating vascular dysfunction and T cell activation in Ang II-induced hypertension, but the BP attenuating effects of P2RX7 knockout appear to be at least partially independent of NLRP3 activation, at least up to 14 days of Ang II-infusion. These results are in contrast to other findings reporting a prominent role of NLRP3 in regulating blood pressure in hypertensive models. In a model of preeclampsia, *Nlrp3*$^{-/-}$ mice were protected against Ang II-induced gestational hypertension with an attenuated rise in SBP.(12) It was recently demonstrated that components of the NLRP3 inflammasome also contribute to aldosterone-induced hypertension in mice. *Nlrp3*$^{-/-}$, *Il-1r*$^{-/-}$ and *caspase-1*$^{-/-}$ mice infused with aldosterone for 14 days were protected against SBP elevation, and vascular dysfunction and remodelling.(7) Furthermore, the protective effect of *Nlrp3*$^{-/-}$ was confirmed to be immune-mediated since the adoptive transfer of *Nlrp3*$^{-/-}$ bone marrow cells into WT mice treated with aldosterone recapitulated the protective effect of *Nlrp3*$^{-/-}$. NLRP3 antagonism has also been shown to be effective at reducing BP, with MCC950 administration in mice decreasing BP, renal dysfunction, renal infiltration of $CD4^+$ T cells and macrophages, and attenuating the production of inflammatory cytokines (IL-1β, IL-18 and IL-17) in a one-kidney/DOCA-salt model of hypertension.(13, 17) Of note, both studies using the NLRP3 antagonist did not present a drop in blood pressure until after 14 days of treatment. In a model of Ang II-induced hypertension, NLRP3 antagonism did not affect BP elevation up to 7 days of treatment, despite attenuated cardiac fibrosis, although the study used a much larger dose of Ang II (1500ng/kg/min) and used tail-cuff to assess BP.(18) Similarly, NLRP3 antagonism did not affect BP in aged (23-31 month-old) mice treated with Ang II (194ng/kg/min) for 10 days.(19) These studies raise the possibility that we could have observed a reduction in BP in the *Nlrp3*$^{-/-}$ mice if we had continued the infusion of Ang II past 14 days. However, Wen *et al.*(20) did not report differences in systolic BP elevation in *Nlrp3*$^{-/-}$ mice compared to WT mice treated with Ang

II (1000ng/kg/min) for 24 days. These disparate results regarding the potential role of NLPR3 in hypertension further support the hypothesis of an independent role of P2RX7 in regulating BP.

The role of P2RX7 in hypertension has also been investigated in several models of hypertension. Pharmaceutical antagonism of P2RX7 reduced blood pressure (BP) in Dahl-salt sensitive rats fed a high-salt diet, while P2X7 gene (*P2rx7*) knockout prevented deoxycorticosterone acetate salt-induced BP elevation and renal damage in mice.(21, 22) Our lab previously demonstrated that genetic knockout of P2RX7 or P2RX7 antagonism attenuated Ang II-induced blood pressure elevation, vascular dysfunction and T cell activation.(9) The diminished T cell activation in these mice was thought to be mediated through impaired IL-1β signalling rather than through B7 ligands (CD80 and CD86) as previously suggested,(8) as we did not observe a difference in CD86 expression in innate immune cells between WT and *P2rx7*$^{-/-}$ mice treated with Ang II. The current study supports the notion that *P2rx7*$^{-/-}$ decreases T cell activation through suppressing NLRP3 inflammasome signalling, as *Nlrp3*$^{-/-}$mice had similarly attenuated T cell activation after Ang II-infusion. Furthermore, these results suggest that the NLRP3 inflammasome is important for T cell activation in Ang II-induced hypertension, independent of reductions in BP.

Inflammation is a known driver of endothelial dysfunction and end-organ damage. The preserved endothelial function in *P2rx7*$^{-/-}$ mice is most likely a reflection of diminished NLRP3 mediated IL-1β production as *Nlrp3*$^{-/-}$mice also had preserved endothelial function despite elevated SBP. In a model of diabetic vascular injury, NLRP3 antagonism resulted in decreased plasma IL-1β and concomitantly preserved endothelial function.(23)This fits with previous reports showing that IL-1β induces endothelial dysfunction (5-7) and that treatment with anakinra, an IL-1receptor antagonist, can prevent endothelial dysfunction in a model of diabetes.(5) However, in the context of human hypertension, targeting IL-1β appears to be ineffective in reducing blood pressure. In the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial, patients treated with Canakinumab, a monoclonal antibody target at IL-1β, saw no reduction in blood pressure or incident hypertension, although they were protected against adverse cardiac events.(24) These results align with the results of the current study in that decreased NLRP3 signalling and thus decreased IL-1β protected against end-organ damage (vascular dysfunction) but did not lower systolic blood pressure in the *Nlrp3*$^{-/-}$mice treated with Ang II for 14 days. This further strengthens the notion that the anti-hypertensive effect of P2RX7 knockout or antagonism is independent of NLRP3 or IL-1β signalling.

Besides NLRP3 signalling, the anti-hypertensive effect of P2RX7 antagonism may be mediated through regulation of renal function. Silencing P2RX7 in a model of diabetes mellitus decreased renin and angiotensin-converting enzyme expression in the renal cortex, increased NO bioavailability and attenuated renal dysfunction.(25) Further P2RX7 antagonism in Ang II-infused rats reduced afferent and efferent arteriole resistance, restoring glomerular plasma flow and filtration rate.(26) Menzies et al. (27) reported a six-fold increase in sodium excretion of Ang II treated rats when given a P2RX7 antagonist coinciding with a decrease in mean arterial pressure. Interestingly, P2RX7 antagonism has no or very modest effects on renal arteriole resistance or renal perfusion pressure in normotensive rats.(26, 28) This corresponds with previous reports that P2RX7 expression is low or absent in the kidneys of normotensive rats but is upregulated in hypertensive states.(26, 29) Therefore, P2RX7 may not have a prominent role in kidney regulation in normotensive or non-disease states but in hypertension or conditions such as diabetes, P2RX7 expression increases and thus can have a prominent role in regulating kidney function and BP.

As P2RX7 antagonism not only inhibits NLRP3-mediated signalling, but can also promote natriuresis, it may present a better therapeutic target than NLRP3 alone. So far no clinical trials have investigated the efficacy of targeting P2RX7 in hypertension or cardiovascular disease. Several studies, including our own, have raised concerns that targeting P2RX7 may have unintended cardiotoxic effects. *P2rx7*$^{-/-}$ mice had altered electrocardiographic patterns, exercise intolerance and developed dilated cardiomyopathy in response to induced auto-immunity against muscarinic M_2 receptors.(30) Similarly, Sarti *et al.(31)* reported that *P2rx7*$^{-/-}$ mice had reduced exercise capacity, with impaired stroke volume, ejection fraction, fractional shortening, and cardiac output, compared to WT mice. We also reported that *P2rx7*$^{-/-}$ mice treated with Ang II had exacerbated cardiac hypertrophy and dysfunction with a greater left ventricle mass and diminished fractional shortening.(9) Importantly, we demonstrated that impaired cardiac function was limited to *P2rx7*$^{-/-}$ mice, as mice treated with the P2RX7 antagonist AZ10606120 did not have exacerbated cardiac hypertrophy or dysfunction in response to Ang II. In addition, P2RX7 antagonists have undergone at least 8 separate clinical trials, with evidence of the relative tolerability of P2RX7 antagonists, as limited to no serious adverse advents were reported in the majority of clinical trials conducted so far.(32) In comparison, earlier trials with the NLRP3 antagonist MCC950 were discontinued due to hepatotoxicity.(33) Therefore, as P2RX7 knockout provided a greater anti-hypertensive effect than NLRP3, and P2RX7 antagonists have

demonstrated clinical safety, this report suggests that P2RX7 presents a better potential therapeutic target than the NLRP3 inflammasome.

5.6 Conclusions and Perspectives

It has been previously reported that both P2RX7 and the NLRP3 inflammasome contribute to the pathogenesis of hypertension. However, no studies to date have assessed whether P2RX7's pro-hypertensive action is mediated through regulating NLRP3 signalling. Here we have reported that both *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice have preserved endothelial function, attenuated vascular remodelling and diminished activation of T cells after Ang II-infusion compared to WT mice suggesting NLRP3 signalling is responsible for these effects. Interestingly, *Nlrp3*$^{-/-}$ mice did not have a reduced pressor response after Ang II infusion, whereas *P2rx7*$^{-/-}$ mice did. These results suggest that the attenuated BP elevation observed in *P2rx7*$^{-/-}$ mice is mediated through a pathway other than the NLRP3 inflammasome. This study indicates that P2RX7 presents a better therapeutic target than the NLRP3 inflammasome for attenuating hypertension and end-organ damage.

5.7 Acknowledgements

The authors are grateful to Veronique Michaud for her excellent technical support.

5.8 Sources of Funding

The work of the authors was supported by the CIHR First Pilot Foundation Grant 143348, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program, and by the Canada Fund for Innovation, and a Distinguished James McGill Professor Award to ELS, by the Fonds de recherche Santé Quebec (FRQS) bourse 289184, Lady Davis Institute/TD Bank Studentship award and Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship to BGS and by the McGill Department of Medicine Gordon Phillips Fellowship to KC.

5.9 Disclosures

None declared.

5.10 References

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Figures

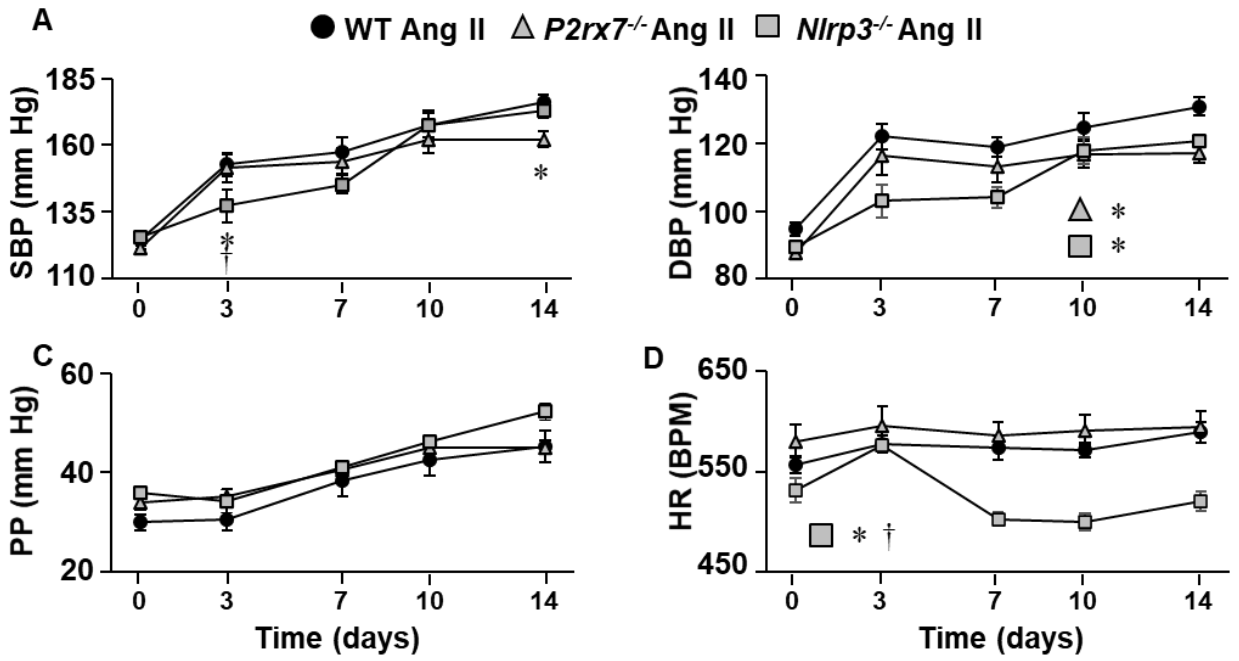

Figure 5.1: P2rx7$^{-/-}$ and Nlrp3$^{-/-}$ mice have different pressor responses to 14 days of angiotensin (Ang) II infusion. Systolic blood pressure (SBP, **A),** diastolic (DBP, **B**), pulse pressure (PP, **C**), and heart rate (HR, **D**) were determined by telemetry in WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated for 14 days with Ang II (490ng/kg/min). Data are presented as means ± SEM, n= 5-8. For **A-C,** *d*ata *were* analyzed using a two-way ANOVA with repeated measure, whereas the area under the curve was used in a 1 way ANOVA for **D.** All tests were followed by a Student-Neuman-Keuls post hoc test. *P <0.05 versus WT Ang II, †P<0.05 versus P2rx7$^{-/-}$ Ang II.

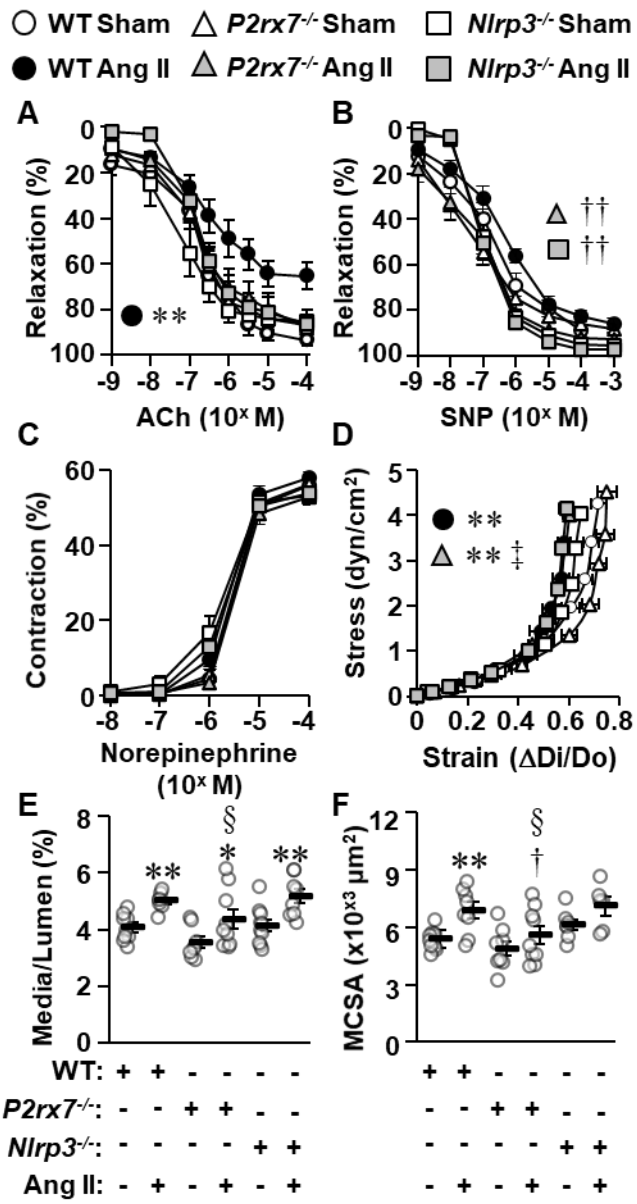

Figure 5.2: Small artery endothelial dysfunction and hypertrophic remodelling are attenuated in P2rx7$^{-/-}$ and Nlrp3$^{-/-}$ mice. Endothelium-dependent (**A**) and -independent (**B**) relaxation responses of mesenteric arteries from WT, *P2rx7$^{-/-}$ Nlrp3$^{-/-}$*mice treated or not for 14 days with Ang II (490ng/kg/min), pre-constricted with norepinephrine (NE, 10^{-5} M), to increasing doses of acetylcholine (Ach) (**A**) or sodium nitroprusside (SNP) (**B**) conducted using pressurized myography. Contraction responses to increasing concentrations of NE (**C**). Stress-strain curve of mesenteric arteries exposed to incremental increases in intraluminal pressure (**D)**. ΔDi is the change in lumen diameter for a given intraluminal pressure and Do is the original diameter measured at 3 mmHg. Media/lumen (**E)** and media cross-sectional area (MCSA) (**F)** of mesenteric arteries at 45 mmHg intraluminal pressure. Data are presented as means ± SEM, n= 6-12 and was analyzed using a two-way ANOVA. All ANOVA tests were followed by a Student-Neuman-Keuls

post hoc test. In **A-C**, the area under the curve was used for analysis, whereas in **D,** the strain values at 140 mmHg (the last points) were used for analysis. **P*<0.05 and ***P*<0.01 versus respective sham, †*P*<0.05 and †*P*<0.01 versus WT Ang II, ‡ *P*<0.05 versus *Nlrp3*$^{-/-}$ sham, § *P*<0.05 versus *Nlrp3*$^{-/-}$ Ang II.

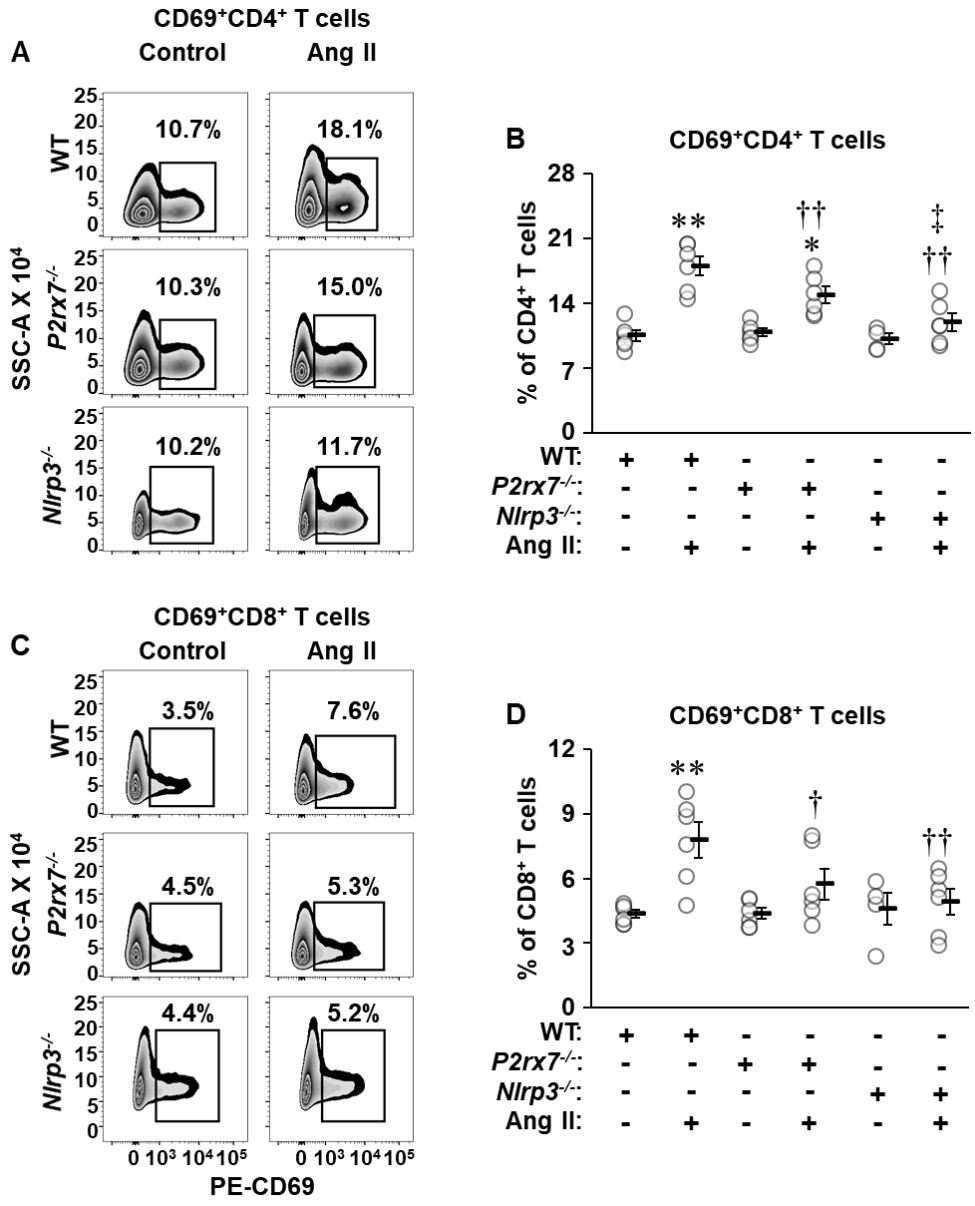

Figure 5.3: The NLRP3 inflammasome contributes to T cell activation in Ang II-induced hypertension. The frequency (%) of activated ($CD69^+$) $CD4^+$ (**A, B)** and $CD8^+$ (**C**-**D**) T cells in the spleens of WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated or not with Ang II for 14 days was determined via flow cytometry. The gating strategy is presented in supplemental figure 1. Representative gating of $CD69^+CD4^+$ (**A**) and $CD69^+CD8^+$ (**B**) in the side scatter area (SSC-A)/phycoerythrin-conjugated (PE) hamster anti-mouse CD69 antibody zebra plot is shown. Data are presented as means±SEM, n=4-6. The data w*ere* analyzed using 2-way ANOVA followed by a Student–Newman–Keuls post hoc test. *P<0.05 and **P<0.01 versus respective sham, †P<0.05 and †† P<0.01 versus WT Ang II, ‡ P<0.05 versus Nlrp3$^{-/-}$Ang II.

Online Supplement

5.11 Detailed Methods

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed the Canadian Council of Animal Care recommendations.

5.11.1 Animals

C57BL/6J wild-type (WT), *P2rx7*$^{-/-}$ (*B6.129P2-P2rx7*$^{tm1Gab/J}$, strain #005576), and *Nlrp3*$^{-/-}$ mice (B6.129S6-*Nlrp3*$^{tm1Bhk/J}$, stock #021302) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and a colony was maintained on-site for the duration of the study. The animals were housed in a conventional facility under sterile conditions with constant temperature and humidity and a 12-hour light/12-hour dark cycle. They were fed a normal salt diet (Teklad Global 18% protein rodent diet with 0.2% of NaCl, Envigo, Lachine, QC, Canada).

5.11.2 Angiotensin II Treatment

Ten to 12-week-old male WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were sham-treated or infused with Ang II (490 ng/kg/min, SC) for 14 days. Mice were anesthetized with 3% isoflurane mixed with O_2 at 2 L/min. The depth of anesthesia was confirmed by rear foot squeezing. The non-steroidal anti-inflammatory drug carprofen (20 mg/Kg) was injected SC to minimize post-operational pain. The mice were then surgically implanted SC with ALZET mini osmotic pumps (Model 1002, Durect Corporation, Cupertino, CA) infusing Ang II (490 ng/kg/min) for 14 days, as recommended by the manufacturer. A drop of analgesic mixture, lidocaine (20 mg/mL)/bupivacaine (5 mg/mL), was applied to the surgical site before closure. Control mice underwent sham surgery.

5.11.3 Blood Pressure Determination

Blood pressure (BP) was determined by telemetry as previously described.[1] 8.5-10-week-old male WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were anesthetized with isoflurane as above and were surgically implanted subcutaneously as recommended by the manufacturer (Data Sciences International, St. Paul, MN). Carprofen (20 mg/Kg) was injected SC, and a drop of lidocaine (20 mg/mL)/bupivacaine (5 mg/mL) mixture was applied to the surgical site before closure. Mice were allowed to recover for 7 to 10 days and carprofen was administered as above once a day for the first two recovery days. BP was determined every 5 minutes for 10 sec for two days, after which

mice underwent sham surgery or were implanted with micro-osmotic pumps as above, and BP was recorded for 14 days.

5.11.4 Endothelial Function and Mechanics

At the end of treatment, the mice were euthanized by cervical dislocation and the mesenteric bed was harvested in ice-cold Krebs solution (pH 7.4). The Krebs solution contained 120 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 4.7 mmol/l KCl, 1.18 mmol/l KH_2PO_4, 1.18 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 0.026 mmol/l EDTA and 5.5 mmol/l glucose. Mesenteric artery endothelial function and mechanics were investigated after mounting on a pressure myograph (Living Systems Instrumentation, Burlington, VA, USA) as previously described.[2] Second-order branches of the mesenteric arterial tree were dissected from the mesenteric arterial tree (160 to 240 μm) and mounted on glass pipettes of pressurized myograph chambers. Vessels were maintained at 45 mmHg intraluminal pressure in Krebs solution bubbled continuously with 95% air and 5% CO_2. Media and lumen diameters were measured by a computer-based video imaging system (Living Systems Instrumentation). Vessels were considered viable when they constricted to >60% of their resting lumen diameter in response to extraluminal application of 125 mmol/L KCl plus 10^{-5} mol/L norepinephrine. Vascular contractile properties were assessed by extraluminal perfusion with potassium chloride (KCl) or exogenous norepinephrine (NE). Endothelium-dependent (10^{-9}-10^{-4} mol/L acetylcholine) and -independent (10^{-8}-10^{-3} mol/L sodium nitroprusside) relaxations were assessed in vessels precontracted with NE (5 x 10^{-5} mol/L). Vascular mechanical properties (remodeling and stiffening) were evaluated in the absence of vascular tone by incubation in a Ca^{2+} free Krebs solution supplemented with a Ca^{2+} chelating agent (EGTA, 10 mmol/L). Media thickness and lumen diameter were measured in response to incremental augmentations of intraluminal pressure from 3 to 140 mm Hg and the media cross-sectional area, media/lumen ratio, stress, and strain were calculated as previously described.[3]

5.11.5 Flow Cytometry

Immune cell profiles in the spleen were determined by flow cytometry. At the end of treatment, the spleen was harvested in ice-cold phosphate-buffered saline (PBS). Single-cell suspensions of splenocytes were prepared by crushing the spleen with the back of a 1 mL syringe plunger over a 70 μm cell strainer in the well of a 12-well plate containing 1 mL RPMI-1640 media. Splenocytes were then centrifuged and resuspended in 1 mL of PBS. One hundred μl of splenocyte cell

suspension was used per test corresponding to 1/10 of the spleen. Isolated cell suspensions were stained with a Live/Dead fixable Aqua dead cell stain (ThermoFisher Scientific, Waltham, MA), and thereafter incubated with a cocktail containing rat anti-mouse CD16/CD32 Fc receptor block and specific antibodies against immune cell markers in phosphate-buffered saline supplemented with 5% Fetal bovine serum. Descriptions of the antibodies used are available in **Error! Reference s ource not found.**.

Flow cytometry was performed on the BD LSR Fortessa cell analyzer. Data analysis was performed using FlowJo software version 10 (version 10.6, Tree Star Inc., Ashland, OR). The flow cytometry gating strategy is available in Figure 5.4.

5.11.6 Data Analysis

Results are presented as means ± standard error of mean (SEM). Comparisons in BP data were carried out using a two-way analysis of variance (ANOVA) for repeated measures. Comparisons in heart rate were conducted using a one-way ANOVA on the area under the curve. Other comparisons between more than two groups were done using a two-way ANOVA. All ANOVA tests were followed by a Student-Newman-Keuls *post-hoc* test. ANOVA tests were performed in SigmaPlot version 13 (Systat Software, San Jose, CA). $P<0.05$ was considered statistically significant.

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5.12 Supplemental References

1. Caillon A, Mian MOR, Fraulob-Aquino JC, Huo KG, Barhoumi T, Ouerd S, Sinnaeve PR, Paradis P, Schiffrin EL. gammadelta T Cells Mediate Angiotensin II-Induced Hypertension and Vascular Injury. *Circulation*. 2017;135:2155-2162.
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Supplementary Figures and Tables

Table 5.1: Antibodies for flow cytometry profiling of T cells.:

Antibodies	Description	Clone, company
CD3	AF700-conjugated rat anti-mouse CD3 antibody	17A2, eBioscience
CD4	PerCP-eF710-conjugated rat anti-mouse CD4 antibody	RM4-5, eBioscience
CD45	BV785-conjugated rat anti-mouse CD45 antibody	30-F11, Biolegend (103149)
CD8a	APC-eF780-conjugated rat anti-mouse-CD8a antibody	53-6.7, eBioscience
CD69	PE-conjugated hamster anti-mouse CD69 antibody	H1.2F3, BD Biosciences

AF700, Alexa Fluor 700, APC, allophycocyanin, APC-eF780, APC-eFluor 780, BV785, Brilliant Violet 785, PE, phycoerythrin, PerCP, peridinin chlorophyll protein, PerCP-eF710, PerCP-eFluor 710.

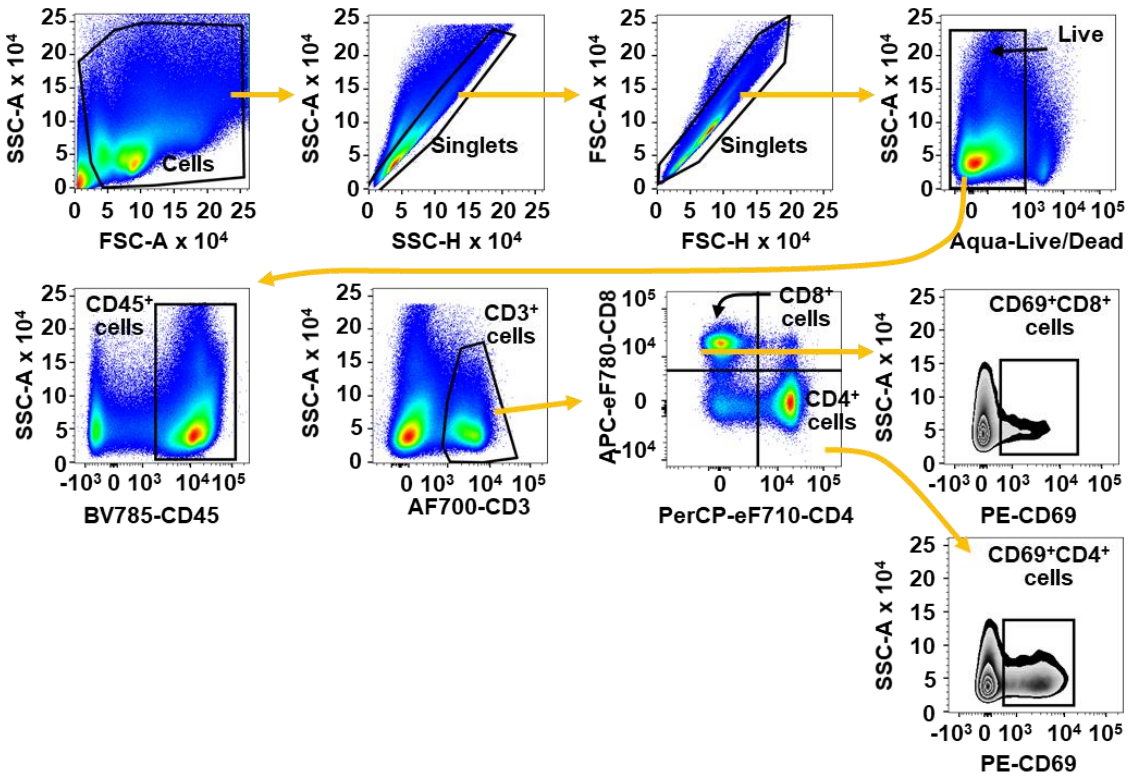

Figure 5.4: Representative Flow cytometry gating strategy of splenic T cells. The profile of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+CD69^+$, and $CD8^+CD69^+$ T cells were determined by flow cytometry in the spleen of WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated as described in the text. Splenocytes were stained with fixable viability dye Aqua, brilliant violet 785 (BV785)-conjugated rat anti-mouse CD45 antibody, Alexa Fluor 700 (AF 700)-conjugated anti-mouse CD3, peridinin chlorophyll protein (PerCP)-eF710-conjugated anti-mouse CD4, allophycocyanin (APC)-eF780-conjugates anti-mouse CD8a, and phycoerythrin (PE)-conjugated anti-mouse CD69 antibodies, followed by flow cytometry analysis. Cells were first gated in the side scatter area (SSC-A) over forward scatter area (FSC-A). Singlet lymphocytes were gated using SSC-A/SSC-height (SSC-H). Singlets were gated again using FSC-A over FSC-H. Live lymphocytes were gated using SSC-A/viability dye. $CD45^+$ lymphocytes were gated using SSC-A/CD45. $CD3^+$ were gated using SSC-A/CD3. $CD4^+$ and $CD8^+$ T cells were gated from $CD3^+$ population. $CD4^+$ and $CD8^+$ T cells were then examined for CD69 expression.

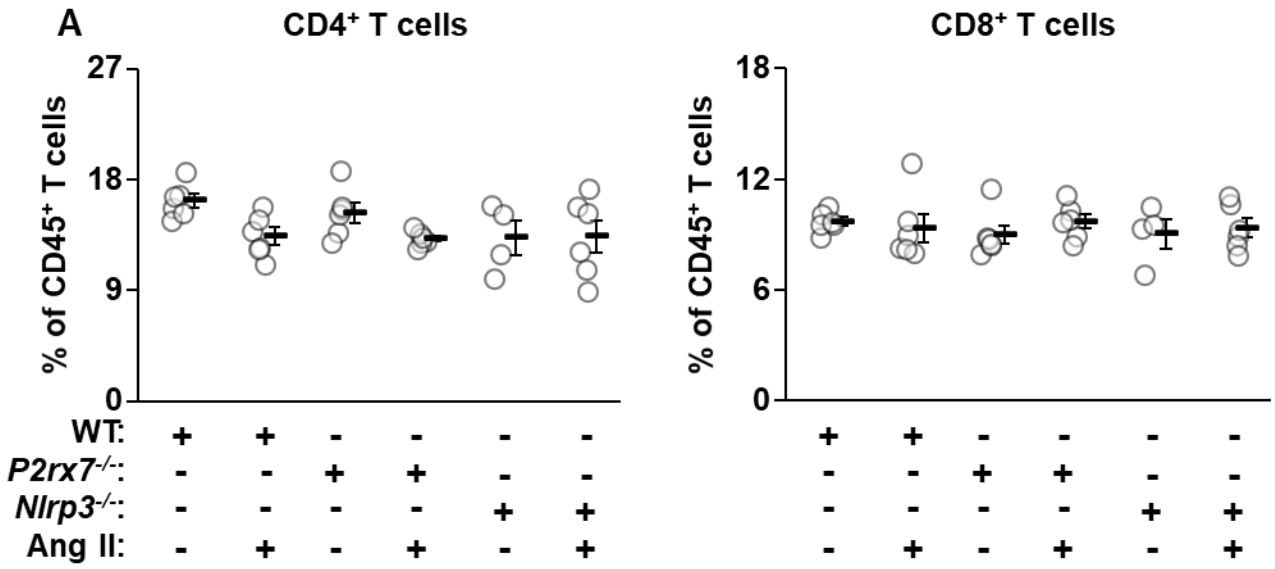

Figure 5.5: The frequency of CD4+and CD8+ T cells in the spleen are not affected by P2RX7 or NLRP3 knockout. The frequency (%) of $CD4^+$ and $CD8^+$ T cells in the spleens represented as a frequency of total immune cells ($CD45^+$ cells) of WT, *P2rx7*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice treated or not with Ang II for 14 days was determined via flow cytometry. The gating strategy is presented in Figure 5.4. Data are presented as means±SEM, n=4-6. The data were analyzed using 2-way ANOVA followed by a Student–Newman–Keuls post hoc test.

Chapter 6: Resident Memory T Cells: An Avenue of Interest for Future Hypertension Research

This chapter represents a brief report identifying an immune cell subset, not identified before in hypertension, with the potential to contribute to the pathological manifestations of hypertension. Here we demonstrate that a pro-inflammatory immune subset, T resident memory cells, develops in response to Ang II-infusion, and *P2rx7* KO or antagonism can attenuate their development.

The letter entitled "Resident Memory T cells: An avenue of interest for future hypertension research" is in preparation to be submitted for publication upon publication of the manuscript presented in Chapter 4.

Resident Memory T Cells: An Avenue of Interest For Future Hypertension Research

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Hypertension remains one of the most prevalent diseases and is the biggest risk factor for mortality worldwide. A growing body of evidence has emerged suggesting memory T lymphocytes play a critical role in the pathogenesis of hypertension. Previous reports have identified that effector and central memory T cells develop in response to hypertensive insults, sensitizing mice to develop hypertension and end-organ damage to otherwise mild or subpressor hypertensive stimuli.(1) However, no studies have investigated the role of resident memory T (T_{RM}) cells in hypertension.

T_{RM} cells are non-circulating immune cells that act as first responders to immunogenic insult.(2) They are positioned at places of previous tissue injury or insult to respond rapidly. When stimulated with their cognate antigen or inflammatory cytokines, these cells can rapidly proliferate, recruit circulating cells and secrete further inflammatory mediators. As hypertension has been shown to generate effector and central memory T cells, we hypothesized that T_{RM} cells would accumulate in the aortic perivascular adipose tissue (PVAT) after a hypertensive insult. We took 10-12-week-old male C57BL/6J wild-type (WT) mice and sham-treated or infused them with angiotensin (Ang II) (1000 ng/kg/min, SC) for 14 days. After 14 days we digested the aorta with perivascular adipose tissue (PVAT) in a solution containing collagenase A (1 mg/mL), collagenase type 2 (500 U/mL), elastase (2 U/mL), trypsin inhibitor (0.25 mg/mL), and hyaluronidase (0.5 mg/mL) and assessed T_{RM} accumulation using flow cytometry. Memory T cells are characterized by the expression of the hyaluronic acid receptor and activation marker CD44, which is upregulated upon antigen encounter and stays expressed in memory T cells. Distinct from other memory T cell subsets, T_{RM} cells do not express the lymphoid homing receptors C-C chemokine receptor 7 (CCR7) or L-selectin (CD62L) but do express tissue retention markers CD69 and integrin alpha E (CD103) (Figure 6.1A). A representative final gating step for $CD4^+$ T_{RM} is provided in Figure 6.1B.

Ang II treatment did not affect the total number of $CD4^+$ or $CD8^+$ T cells in the aortic PVAT, but there was a trend of increased innate-like γδ T cells with Ang II-infusion (Figure 6.1C). In the absence of Ang II treatment, there were almost no T_{RM} cells detectable in the aortic PVAT; however, after two weeks of Ang II-infusion, there was an accumulation of the number and frequency of $CD4^+$, $CD8^+$ and γδ T_{RM} cells (Figure 6.1D and E). Next, we wanted to assess whether we could attenuate the development of these T_{RM} cells in Ang II-induced hypertension. The purinergic receptor P2RX7 is highly expressed on T_{RM} cells, plays a key role in modulating inflammatory responses and in controlling T cell polarization.(3) Based on our previous findings

that Ang II treated *P2rx7*$^{-/-}$ mice or mice treated with the P2RX7 antagonist AZ10606120 had diminished accumulation of activated T cells and effector memory T cells in the aortic PVAT, we hypothesized that we would find decreased T_{RM} in these mice as well.(4)

To test this hypothesis, we studied 10-12-week-old *P2rx7*$^{-/-}$ mice and sham-treated or infused them with Ang II (1000 ng/kg/min, SC) for 14 days. An additional group of WT mice were similarly implanted with osmotic pumps infusing 1000 ng/kg/day of Ang II with the P2RX7 antagonist AZ10606120 dihydrochloride (AZ106) (694 ng/kg/min) for 14 days. P2RX7 knockout or antagonism did not significantly affect the total number of $CD4^+$, $CD8^+$ or γδ T cells in the aortic PVAT, although there was a trend to fewer γδ T cells in AZ106 treated mice (Figure 6.1C). The accumulation of T_{RM} cells in the aortic PVAT noted in the WT mice was attenuated in *P2rx7*$^{-/-}$ mice and WT mice receiving the P2RX7 antagonist AZ106 with one exception. The frequency but not number of γδ T_{RM} in the AZ106 treated mice increased to a similar extent as Ang II-treated WT mice.

This study is the first to show that T_{RM} cells accumulate in the aortic PVAT after a hypertensive insult. As these cells have potent pro-inflammatory potential, it will be interesting to explore if they contribute significantly to the vascular injury associated with hypertension in future studies. As hypertension in humans is a chronic condition, it would also be interesting to assess whether longer or repeated exposures to hypertensive insults would result in further accumulations of T_{RM} cells. It is also important to question whether such a limited number of cells could elicit noticeable pathophysiologic effects. However, it is noteworthy that the techniques used to isolate cells from PVAT may result in T_{RM} cell death through a P2RX7 mediated pathway and thus distort the estimates of T_{RM} cells.(5) Therefore, the number of T_{RM} cells quantified by flow cytometry may be underestimated. Further, as P2RX7 has been shown to mediate T_{RM} cell death during isolation, the differences in T_{RM} populations between WT and *P2rx7*$^{-/-}$or P2RX7 antagonized mice may be much greater than we report here. Taken together this study shows that a highly pro-inflammatory lymphocyte population accumulates in the aortic PVAT after hypertensive insults which can be attenuated by P2RX7 antagonism. The accumulation of T_{RM} cells in response to hypertensive insults has clinical implications as hypertension is a chronic condition, and the reactivation of these memory cells may contribute to hypertension-associated end-organ damage. Thus, the role of T_{RM} in hypertension provides an intriguing avenue for future research.

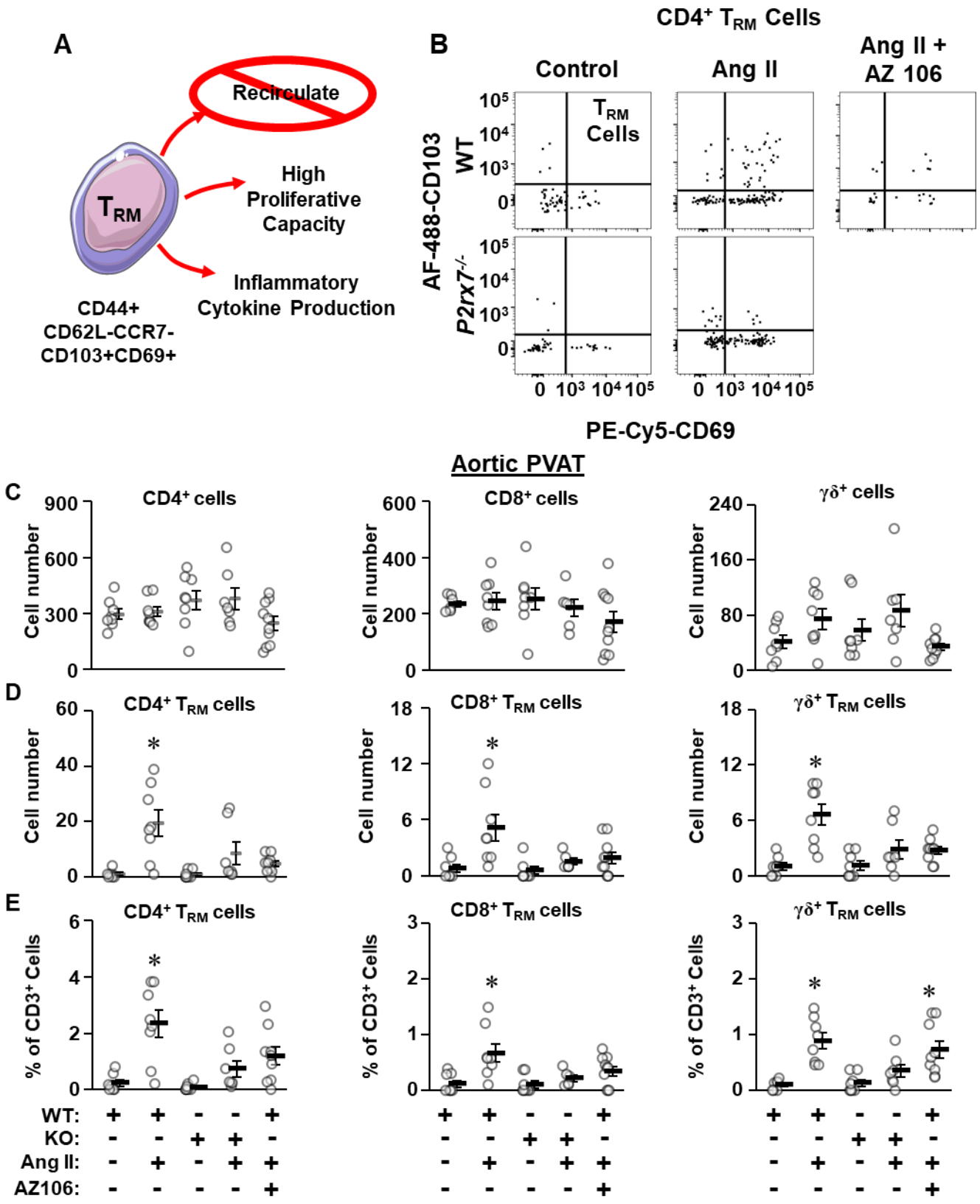
A
Recirculate
T_{RM}
High Proliferative Capacity
Inflammatory Cytokine Production
CD44+ CD62L-CCR7- CD103+CD69+
B
$CD4^+$ T_{RM} Cells
Control
Ang II
Ang II + AZ 106
T_{RM} Cells
WT
$P2rx7^{-/-}$
AF-488-CD103
PE-Cy5-CD69
Aortic PVAT
C
$CD4^+$ cells
$CD8^+$ cells
$\gamma\delta^+$ cells
Cell number
D
$CD4^+$ T_{RM} cells
$CD8^+$ T_{RM} cells
$\gamma\delta^+$ T_{RM} cells
E
% of $CD3^+$ Cells
WT:
KO:
Ang II:
AZ106:

Figure 6.1: Ang II-induced hypertension results in the generation of TRM cells in the aortic PVAT which can be attenuated through P2RX7 antagonism. T resident memory (T_{RM}) cells do not recirculate and instead reside permanently in their tissue of origin, where they can respond rapidly to insult by inducing proliferation and inflammatory cytokine production. They are characterized by the expression of CD44, CD69 and integrin alpha E (CD103), and the absence of C-C chemokine receptor 7 (CCR7) and L-selectin (CD62L) (**A**). Representative final gating step of $CD4^+$ T_{RM} cells in the aortic perivascular adipose tissue in wild-type (WT) mice treated or not for 14 days with angiotensin (Ang) II (1000ng/kg/min) and the P2RX7 antagonist AZ10606120 (AZ106) (694ng/kg/min), and in *P2rx7*$^{-/-}$ mice treated or not with Ang II. Dot plots are shown with T_{RM} cells representing the double-positive Alexa-Flour (AF)-488-$CD103^+$and phycoerythrin (PE)-Cyanine 5 (Cy5)-$CD69^+$ cells (**B**). The number of $CD4^+$, $CD8^+$ and γδ T cells in the aortic PVAT in WT and *P2rx7*$^{-/-}$ (KO) cells treated or not with Ang II and AZ106 was determined by flow cytometry (**C**). The number (**D**) and frequency of $CD3^+$ (**E**) $CD4^+$, $CD8^+$ and γδ T_{RM} cells in the aortic PVAT. Data are presented as means ± SEM, n=6-10. The data were analyzed using a Kruskal-Wallis 1-way ANOVA on ranks followed by a Dunn's multiple comparison post hoc test. **P*<0.05 vs respective sham.

6.1 Acknowledgements

The authors are grateful to Veronique Michaud for her excellent technical support.

6.2 Sources of Funding

The work of the authors was supported by the CIHR First Pilot Foundation Grant 143348, a Canada Research Chair (CRC) on Hypertension and Vascular Research by the CRC Government of Canada/CIHR Program, and by the Canada Fund for Innovation, and a Distinguished James McGill Professor Award to ELS, by the Fonds de recherche Santé Quebec (FRQS) bourse 289184, Lady Davis Institute/TD Bank Studentship award and Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship to BGS and by the McGill Department of Medicine Gordon Phillips Fellowship to KC.

6.3 Disclosures

None declared.

6.4 References

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Chapter 7: Discussion

The work of this thesis sought to determine if suppressing inflammation by targeting P2RX7 would attenuate hypertension and cardiovascular injury. In the first study, we demonstrated that *P2rx7*$^{-/-}$ mice have attenuated hypertension, vascular injury and immune cell activation. However, we noted that *P2rx7*$^{-/-}$ mice developed worse cardiac dysfunction in response to Ang II. In the second part of the study, we utilized a P2RX7 antagonist. We demonstrated that P2RX7 antagonism attenuated hypertension, vascular injury and immune cell activation without exacerbating cardiac dysfunction. In the second study of this thesis, we aimed to delineate the relative contribution of P2RX7 mediated NLRP3 inflammasome activation from other functional roles of P2RX7 signalling in the context of hypertension. We demonstrated that the BP attenuating effect of P2RX7 antagonism appears to be largely independent of the NLRP3 inflammasome. On the other hand, the preservation of endothelial function and attenuated immune activation depends on the NLRP3 inflammasome. Finally, we demonstrated that P2RX7 contributed to the development of T_{EM} cells in hypertension and identified for the first time that T_{RM} cells accumulate in the PVAT of hypertensive animals suggesting that T_{RM} cells could be an avenue for future research in the field of hypertension and immunology.

Much effort has been made to try and identify the initial trigger for immune activation in hypertension. Past results by Kirabo *et al.*(111) posited that isoketal adducts, formed as the result of uncontrolled ROS accumulation, could serve as neoantigens triggering innate and subsequent adaptive immune activation in hypertension. This study provided sound evidence for a likely initiating factor in hypertension. However, recently it was identified that in both hypertensive humans and animals, there are elevated circulating levels of extracellular ATP.(103, 289) This suggested to us that purinergic signalling may also act as an initiating stimulus for immune activation in hypertension. In Chapter 4, we demonstrated that not only is ATP elevated in hypertensive animals, but so is the surface expression of P2RX7 in key immune subsets pertinent to the development of hypertension (i.e. macrophages, DCs and $CD4^+$, and $CD8^+$ T cells). These results reinforced the likelihood that P2RX7 contributed to immune activation during hypertension. The following sections of the thesis will do a deep dive into the suggested role for P2RX7 in immune activation in the context of hypertension, followed by a discussion on the role of P2RX7 in vascular and cardiac dysfunction, and ending with a summary of clinical implications.

7.1 P2RX7 and Immune Activation

T cells have a well-established role in hypertension (115), and thus suppressing T cell activation is an intriguing target for novel anti-hypertensive therapies. T cell activation occurs through three signals: **1**) antigen recognition (TCR/MHC interactions), **2**) co-stimulation (CD28/B7 [CD80/CD86] interactions), and **3**) cytokine mediators, of which innate immune cells are primary facilitators. Our initial hypothesis was that P2RX7's contribution to immune activation in hypertension would be primarily through modulation of the innate immune system. This was based on the fact that innate immune cells express ~10-20x the amount of P2RX7 compared to T cells (Figure 4.2B). We believed that P2RX7 signalling in innate immune cells would contribute to T cell activation through modulation of signal 2) co-stimulation and signal 3) cytokine mediators.

Antigen-presenting cells, including monocyte/macrophages and DCs, are key facilitators for the T cell activation described above. DCs from Ang II treated mice support survival and proliferation of $CD8^+$ T cells.(111) In addition, $CD8^+$ T cells cultured with DCs from Ang II treated mice secrete more IFN-γ, IL-17 and TNF-α than $CD8^+$ T cells cultured with DCs from sham-treated mice.(111) It is believed these stimulatory effects are primarily mediated through CD28/CD86 interactions, as CD86 inhibition blunts Ang II-induced T cell activation, hypertension and vascular injury.(102) Studies have demonstrated that ATP stimulation of P2RX7 promotes the expression of CD86 in both human monocytes(231) and murine DCs.(103) Therefore, we anticipated decreased expression of CD86 in macrophages and DCs from Ang II treated *P2rx7*$^{-/-}$ mice compared to WT mice. However, we did not observe any differences in the expression of CD86 or in the number of CD86 expressing macrophages and DCs between *P2rx7*$^{-/-}$ and WT mice. In both groups, CD86 expression was found to increase after Ang II infusion. This decreased the likelihood that CD28/CD86 interactions were behind the reduced T cell activation we observed in *P2rx7*$^{-/-}$ mice. However, as we never utilized co-culture experiments with macrophages, DCs and T cells, we cannot definitively exclude this option.

The other mechanism through which P2RX7 could promote T cell activation is through signal 3) cytokine mediators. P2RX7 is a well-described activator of the NLRP3 inflammasome, promoting the release of the cytokines IL-1β and IL-18. IL-1β is known to induce the activation of both $CD4^+$ and $CD8^+$ T cells.(407) In Chapter 4, we demonstrated that BMDMs and BMDCs from *P2rx7*$^{-/-}$ mice had blunted IL-1β production. Furthermore, Chapter 5 demonstrated that both

P2rx7$^{-/-}$ and *Nlrp3*$^{-/-}$ mice had attenuated T cell activation after Ang II treatment compared to WT mice. These results strongly suggest that the decreased T cell activation in our hypertensive models was due to diminished IL-1β signalling. However, as we did not quantify IL-18 in our studies, we cannot exclude a role for IL-18 in T cell activation. IL-18, also known as IFN-γ inducing factor, may have contributed to the diminished T cell activation and IFN-γ production in *P2rx7*$^{-/-}$ mice, but this was not determined. Taken together, our results from Chapters 4 and 5 suggest that P2RX7 contributes to T cell activation in hypertension through NLRP3 inflammasome signalling.

The work of this thesis cannot exclude a more direct role for P2RX7 in T cell activation. A study by Yip *et al.* (198) found that ATP is released upon TCR stimulation, triggering P2RX7 activation and subsequent activation of the NFAT signalling pathway. It is possible that the absence of P2RX7 in T cells directly inhibits their activation rather than it being the result of diminished innate-adaptive cross-talk. In support of this theory, we reported an increased expression of P2RX7 in naïve $CD4^+$ and $CD8^+$ T cells in the aortic PVAT of Ang II treated WT mice, suggesting that T cell P2RX7 may be involved in Ang II-induced hypertension. However, caution must be taken with this interpretation. It has been demonstrated that it is not the level of P2RX7 expression on the T cell per se but the differentiation and activation state of the T cell that regulates the effect of P2RX7 signalling in T cells.(419) For example, recently activated ($CD69^+$) cells are less sensitive to P2X7 mediated CD62L cleavage but are more sensitive to P2X7 mediated PS exposure and cell death. The reduced ability to shed CD62L after activation is thought to promote the retention of T cells in lymph nodes to allow for complete differentiation into effector cells. Therefore, extracellular ATP may engage P2RX7 expressed directly on T cells to promote their activation and maturation into effector T cells. However, our study using *Nlrp3*$^{-/-}$ mice would suggest that antagonism of NLRP3 inflammasome signalling, rather than direct action on T cells, is the reason behind diminished T cell activation in hypertension. We cannot exclude that antagonism of both the NLRP3 inflammasome and P2RX7 T cell responses contributed to the diminished T cell activation we report in this thesis.

An interesting observation we had when comparing immune activation between genetic KO of P2RX7 and P2RX7 antagonism was that not all of the attenuated immune responses in *P2rx7*$^{-/-}$ mice were preserved with P2RX7 antagonism. For instance, we did not observe diminished IL-17 or IFN-γ signalling with P2RX7 antagonism. Similar differences in T cell populations between mice receiving a P2RX7 antagonist and *P2rx7*$^{-/-}$ mice were reported in a

paper published in *Oncogene*.(420) *P2rx7*$^{-/-}$ mice had less infiltrating CD8$^+$ T cells and more infiltrating Tregs in the tumour microenvironment. In contrast, mice who received the P2RX7 antagonist (A74003) had increased CD4$^+$ effector T cell infiltration and IFN-γ levels. Furthermore, tumour infiltrating cells in *P2rx7*$^{-/-}$ mice had increased expression of CD39 and CD73, whereas with P2RX7 antagonism, CD39 expression was decreased, resulting in differences in extracellular ATP and adenosine concentrations. In our study we also noticed characteristic differences at baseline between the *P2rx7*$^{-/-}$ mice and WT mice. For instance, *P2rx7*$^{-/-}$ mice had a greater frequency of CD8$^+$ T_{EM} in the bone marrow, γδ T_{EM} in the spleen, and had a greater quantity of T cells in the MA PVAT (data not shown) when compared to WT mice. Our data, along with the study by De Marchi *et al*.(420) suggests there are intrinsic differences in immune cell development in *P2rx7*$^{-/-}$ mice and caution is needed when trying to extrapolate findings from *P2rx7*$^{-/-}$ mice.

7.2 P2RX7 and the Generation of Memory T Cells in Hypertension

A relatively novel concept in hypertension research is the development of immunological memory after exposure to hypertensive insults. Itani *et al*.(143) were the first to demonstrate that T_{EM} cells form after exposure to L-NAME/high salt or Ang II treatment. These T cells rapidly expand upon reexposure to otherwise mild hypertensive challenges resulting in elevated blood pressure and end-organ damage. Our study recapitulated findings from Itani *et al*.(143) and Xiao *et al*.(144), demonstrating the accumulation of T_{EM} cells in the spleen and bone marrow of WT mice treated with Ang II. Expanding upon their research, we showed the accumulation of T_{EM} cells in the aortic PVAT in hypertension for the first time. These cells are uniquely placed to respond to changes in BP and could contribute to the adverse vascular remodelling and endothelial dysfunction that occurs with long periods of hypertension in human populations. Furthermore, we were the first to demonstrate the appearance of γδ T_{EM} cells after hypertensive insults. Our lab has previously shown a role for γδ T cells in hypertension, demonstrating that depletion of γδ T cells attenuates Ang II-induced hypertension and vascular injury.(138) However, the role of γδ T_{EM} cells in hypertension is currently unknown and is being investigated in our laboratory.

In addition to showing that T_{EM} cells develop after Ang II treatment, we also demonstrated that we could inhibit their formation with genetic KO or pharmaceutical antagonism of P2RX7. Genetic deficiency of P2RX7 and P2RX7 antagonism could prevent the accumulation of CD4$^+$, CD8$^+$ and γδ T_{EM} cells to varying degrees in the spleen, bone marrow and aortic PVAT. In a model of infection with acute lymphocytic choriomeningitis virus, P2RX7 was demonstrated to

contribute to the development and maintenance of T_{EM} cells.(248) They reported that memory cells lacking P2RX7 expression displayed mitochondrial dysfunction and were unable to subsist. Whether the attenuated development of T_{EM} cells was a reflection of attenuated hypertension or direct impairment of mitochondrial energetics (247) was not identified in this thesis.

In the final study presented in Chapter 6, we reported that T_{RM} cells developed in the aortic PVAT after Ang II treatment, and their development could be attenuated by inhibiting P2RX7. The role of T_{RM} cells in hypertension is currently unknown. T_{RM} cells are non-circulating cells with potent effector functions that can respond rapidly to pathogenic or tissue insult.(142) Upon activation, T_{RM} cells recruit and activate DCs and $CD8^+$ T cells and produce IFN-γ and granzyme B, processes that can be attenuated by P2RX7 antagonism.(249) It is tempting to speculate that these same effector functions could be engaged in response to hypertension-associated DAMPs and PAMPs, contributing to the pathological vascular changes observed in human hypertension. However, this remains to be addressed but surely will be an exciting topic for future research.

Therefore, P2RX7 appears to present not only a viable target for general immune cell activation but also for preventing the generation of long-lived memory T cells in hypertension.

7.3 P2RX7, Vascular Function and Remodelling

A characteristic feature of progressive hypertension is the development of endothelial dysfunction and remodelling of blood vessels.(61) Endothelial dysfunction is characterized by enhanced contractility, impaired vasodilation and a shift towards a pro-inflammatory state. As pressure is inversely proportional to the fourth power of the vessel radius, any changes to vascular dynamics that result in a diminished radius can drastically affect BP. Therefore, strategies that prevent these maladaptive changes are desirable as they can contribute to significant attenuations in BP. In our model, we demonstrated that *P2rx7*$^{-/-}$ mice were protected against Ang II-induced endothelial dysfunction and hypertrophic remodelling of resistance arteries. This protective effect could be mediated through several potential mechanisms discussed below.

As was mentioned, a characteristic feature of endothelial dysfunction is inflammation. Ang II is a potent inducer of vascular inflammatory responses. It stimulates the expression of cellular adhesion molecules and ROS production that favours immune cell recruitment and activation. Furthermore, immune cells themselves are a source of inflammatory cytokines and ROS that contribute to vascular damage. In this thesis, we demonstrated that either genetic KO or pharmaceutical antagonism of P2RX7 could oppose this effect, reducing the accumulation of

activated T cells in the aortic PVAT. Interestingly, P2RX7 may play a direct role in favouring immune cell recruitment to the PVAT. Abacavir, a drug used to treat HIV, enhanced leukocyte-endothelial interactions by promoting P2RX7 activation.(421) P2RX7 activation decreased leukocyte rolling velocity and increased leukocyte Mac-1 expression, favouring leukocyte endothelial transmigration. The results from this study are supported by similar observations in atherosclerosis models, where P2RX7 was shown to favour immune cell recruitment into atherosclerotic plaques through up-regulation of cellular adhesion molecules on endothelial cells.(356, 361, 364) Therefore, P2RX7 antagonism or genetic KO potentially reduced the accumulation of activated T cells in the PVAT by inhibiting endothelial-leukocyte interactions and subsequent transmigration of leukocytes. The attenuated accumulation of leukocytes in the PVAT could account for the preserved endothelial function and attenuated vascular remodelling we observed.

It is also possible that the preserved endothelial function and reduced remodelling in *P2rx7*$^{-/-}$ mice reflect the lower BP in these mice. However, our results presented in Chapter 5 support the idea that diminished vascular inflammation rather than BP accounts for these effects. In this study, we demonstrated that *Nlrp3*$^{-/-}$ mice also have preserved endothelial function and decreased remodelling despite no significant reductions in SBP after 14 days of treatment. As antagonizing P2RX7 and NLRP3 diminishes IL-1β production, the preserved endothelial function we observed is most likely a result of decreased IL-1β activation, as Il-1β is a known driver of vascular dysfunction and remodelling.(396) However, as both P2RX7 and NLRP3 activation can also induce ROS formation, we cannot exclude this as a possibility to explain the preserved endothelial function and remodelling. As well, inhibition of NLRP3 has been shown to decrease VSMC proliferation (422), and this could account for the decreased MCSA reported in *Nlrp3*$^{-/-}$ and *P2rx7*$^{-/-}$ mice. Taken together, our results suggest that P2RX7 antagonism attenuates endothelial dysfunction through suppressing NLPR3-IL1β signalling, however we can not exclude a role for ROS and attenuated VSMC proliferation.

In addition to attenuated remodelling of resistance arteries, *P2rx7*$^{-/-}$ mice also were protected from aortic stiffening as demonstrated by a decreased PWV compared to Ang II treated WT mice. We observed that WT mice treated with Ang II had an accumulation of fibronectin in the adventitia, whereas the *P2rx7*$^{-/-}$ mice did not. This decreased fibronectin could account for the decreased aortic vascular stiffness observed in *P2rx7* KO mice.(423, 424) It is often speculated

that stiffening of the large elastic arteries precedes small arterial stiffening.(425) Interestingly, the resistance arteries of *P2rx7*$^{-/-}$ mice treated with Ang II underwent vascular stiffening despite no indications of aortic stiffening. The fact that we saw mesenteric artery stiffening but not aortic stiffening challenges the concept that large arterial remodelling precedes resistance artery remodelling. Our results support previous assertations that small artery remodelling may be the earliest manifestation of target-organ damage in hypertension.(426)

Interestingly, P2RX7 antagonism did not result in attenuated vascular remodelling. As the BP was lower in mice receiving the P2RX7 antagonist, it is possible given time, differences in vascular remodelling would appear as the higher BP in WT mice would drive further arterial wall stress and remodelling.(425) However, evidence, summarized by Boutouyrie *et al.*(425), suggests that vascular stiffness precedes BP elevation. Therefore, it is possible that the stiffened arteries in the mice receiving the P2RX7 antagonist may lead to increased BP in these mice given time. Long-term studies investigating P2RX7 antagonism in hypertension would shed light on this issue.

The fact that we did not observe reduced hypertensive responses in NLRP3 mice does not preclude a role for NLRP3 in pathophysiology and potentially in blood pressure management per se. Studies conducted by other groups did not report a blood pressure-lowering effect until after 14 days of NLRP3 antagonism.(345, 377, 422) It may be that prolonged exposure of endothelial cells and target organs to IL-1β leads to progressive dysfunction and maladaptive remodelling, leading to a loss of compliance of the resistance vascular bed and a progressive increase in BP. This would align with a suggested role for chronic inflammation in the progressive development of hypertension. It would also fit with the study that showed IL-1β and inflammasome genes only correlated with worse outcomes in patients over 60, suggesting that chronic NLRP3 signalling is needed for the onset of apparent symptoms.(81) However, the CANTOS trial seems to omit a role for IL-1β in human blood pressure management in the context of hypertension.(12) Therefore, due to the inconsistent findings regarding NLRP3's effect on BP, more studies are needed to clarify the matter.

7.4 P2RX7, NLRP3 and Cardiac Function

A surprising revelation from Chapter 4 was that *P2rx7*$^{-/-}$ mice treated with Ang II developed exacerbated cardiac hypertrophy and dysfunction and displayed characteristics reminiscent of dilated cardiomyopathy. As *P2rx7*$^{-/-}$ mice had lower BP and attenuated inflammation, we anticipated a cardioprotective effect. However, a recently published study helps to explain our

findings. In 2021, Sarti *et al*.(226) demonstrated that P2RX7 is expressed on the mitochondrial membrane, and its expression is increased in instances of oxidative stress. Furthermore, they demonstrated that P2RX7 deficient cells had impaired energy metabolism with lower mitochondrial matric Ca^{2+} content, reduced mitochondrial potential, lower complex 1 expression and thus reduced respiration. These results corroborate previous studies that suggested P2RX7 influences mitochondrial fitness in HEK cells (224) and $CD8^+$ T cells (247, 248), although mitochondrial localization of P2RX7 had not been reported before. P2RX7 has been suggested to help cells metabolically adapt to changes in available energy sources, such as low glucose.(225) Reinforcing this concept, *P2rx7*$^{-/-}$ mice fed a high-fat diet for 16-weeks had a greater increase in plasma glucose, triglycerides, cholesterol and body weight than WT mice.(403) In addition, *P2rx7*$^{-/-}$ mice experienced LV hypertrophy, diastolic dysfunction and surprisingly increased markers of inflammation (IL1β and IL-6), whereas the WT mice did not. Therefore, it appears that P2RX7 expression is critical in cardiac mitochondria to allow cardiomyocytes to adapt to stress, and without mitochondrial P2RX7 expression, mice are prone to developing cardiomyopathy.

Importantly, our study found that two weeks of P2RX7 antagonism did not exacerbate any of our recorded indicators of cardiac dysfunction or hypertrophy. As it would appear that P2RX7 expression on the mitochondria is essential for modulating cellular energetics, the targeting of cell surface P2RX7 expression should not interfere with the mitochondrial role of P2RX7 signalling. In addition, a recent study using cardiomyocytes demonstrated that P2RX7 antagonism could be cardioprotective.(427) The authors demonstrated that apelin-13 administration led to dysregulated reticulophagy. The addition of a P2RX7 antagonist could attenuate this, thereby suppressing cardiomyocyte hypertrophy. In a Transverse aortic constriction pressure overload model, P2RX7 antagonism reduced cardiac fibrosis and dysfunction after 4 weeks by suppressing TGF-β and NLRP3-IL-1β signalling.(428) However, longer-term studies investigating the effects of P2RX7 antagonists on cardiac function are needed to validate that P2RX7 antagonists are indeed not cardiotoxic.

Interestingly *P2rx7*$^{-/-}$ mice had increased HR prior to and after Ang II treatment, whereas *Nlrp3* KO and mice receiving AZ106 had lower HR after Ang II treatment compared to WT mice. The increased HR of *P2rx7*$^{-/-}$ mice seems to be due to a defect in electrical conductance within the heart. Martinez *et al.*(401) reported that *P2rx7*$^{-/-}$ mice have a greater baseline HR (shorter R-R interval) than WT C57BL/6 mice. In addition, they found that *P2rx7*$^{-/-}$ mice have a longer QTc

interval (indicating defects in ventricular depolarization) and had an ST depression (401), which in humans is often associated with hypokalemia and is indicative of cardiac ischemia.(429) As P2RX7 antagonism decreased rather than increased HR, it suggests P2RX7 antagonism does not induce similar defects, although we did not validate this with electrocardiogram tracings in our study. The decreased HR observed in mice receiving the P2RX7 antagonist is most likely a result of suppressed cardiac SNS activation, through diminished NLRP3 signalling, as *Nlrp3*$^{-/-}$ mice in our study also had a lower HR. A trend to lower HR in rats receiving shRNA against NLRP3 has also been reported.(422) The lower HR may result from decreased IL-1β signalling. Injection of an IL-1β inhibitor to the PVN resulted in a decreased HR through suppression of the SNS.(430) Similarly, injection of an NLRP3 antagonist into the PVN was also found to decrease SNS activity (NE release) and BP in a model of salt-sensitive hypertension.(431) Unfortunately, HR was not reported in this study. Further supporting that SNS activity is suppressed, a series of experiments from the same research group demonstrated that post MI, P2RX7 antagonism diminished SNS signaling to the heart resulting in a decreased HR.(323, 324, 329) Therefore, the decreased HR observed in mice receiving a P2RX7 antagonist or in *Nlrp3*$^{-/-}$ mice most likely is the result of decreased SNS activation of the heart.(430)

When conducting echocardiography in mice, the mice are anesthetized, and the heart rate is maintained between 500-550 BPM. Interestingly, when HR was normalized in this manner, we noticed that the SV and CO of mice receiving the P2RX7 antagonist were significantly greater than vehicle-treated WT mice (1.5-fold). Three factors typically affect SV, which includes contractility, preload and afterload.(432) As preload is reflected in diastolic pressure, and diastolic pressure decreased in these mice, we can most likely exclude preload as a causal factor. Afterload is the force opposing the ejection of blood from the heart, which if simplified, is a reflection of arterial BP and systemic vascular stiffness and peripheral resistance. As BP decreased after an initial increase in mice receiving AZ106, it is possible the increased SV is partially due to a decreased afterload. Contractility is the heart's ability to eject blood at a given afterload and preload. It is a combination of force (inotropy) and velocity, regulated by the length of sarcomeres, number and type of actin/myosin filaments, and speed of cross-bridge cycling. In healthy cardiac hypertrophy, such as in trained athletes, cardiac contractility increases in parallel with the degree of cardiac hypertrophy.(433) However, in maladaptive cardiac hypertrophy, cardiac fibrosis, particularly an accumulation of collagen fibres, can decrease contractility.(434) Looking at the LV

mass and LVPWd, in the Ang II treated mice in our study, we can see that the mice underwent cardiac hypertrophy. However, as shown in Figure 4.15, mice receiving the P2RX7 antagonist had decreased fibrosis as demonstrated by decreased mRNA collagen levels. Therefore, the combination of cardiac hypertrophy with decreased collagen could increase cardiac contractility. In culmination with the decreased afterload, increased cardiac contractility could account for the elevated SV and CO observed in the mice receiving the P2RX7 antagonist.

7.5 Clinical Implications

As was introduced at the start of this thesis, no clinical trials specifically designed to investigate the effects of targeting the immune system in treating hypertension have been conducted so far. However, we do have indications that strategies targeting the immune system may be effective in human hypertension. Recent developments have led to the generation of a humanized mouse model in which the murine immune system is replaced by the human immune system. In 2016, using this humanized mouse model, Itani *et al.* demonstrated increased activation of human T cells in hypertension. After Ang II infusion, $CD45^+$ leukocytes, $CD4^+$ T cells and human memory T cells ($CD3^+CD45RO^+$) accumulated in thoracic lymph nodes, aorta and kidney.(121) Preventing hypertension by treatment with hydralazine and hydrochlorothiazide prevented these increases in activated T cells. Analyzing T cells from human patients, they also reported an increase in circulating Th17 cells and IFN-γ producing $CD4^+$ and $CD8^+$ T cells in hypertensive compared to normotensive humans. Therefore it would appear T cell activation contributes to the pathogenesis of human hypertension as well as in rodents. In support of this hypothesis, immune suppression with MMF reduced BP by ~16 mmHg in patients with psoriasis treated for 3 months(9) and 9mmHg in SLE patients treated between 7 and 90 days.(10) These studies provide strong evidence that immune activation similarly contributes to the pathogenesis of human hypertension.

As has been repeated throughout this thesis, the CANTOS trial demonstrated that the risk for recurrent cardiovascular disease could be decreased by lowering inflammation without lowering systemic lipid levels.(11) However, targeting IL-1β alone appears inefficacious in controlling BP.(12) Therefore, targeting P2RX7 may present a better target for the management of human BP as it can suppress other pro-hypertensive elements besides IL-1β signalling. There is evidence that targeting P2RX7 may be advantageous in human hypertension and cardiovascular disease. We identified 3 novel SNPs utilizing the UK Biobank: rs28969479, rs139429176, and

rs116953937, that correlated with an increased odds of having hypertension. Several previous studies had also reported an association between SNPs in P2RX7 and BP. In a study of familial hypertension in Caucasians, a SNP (rs598174) for P2RX7 was strongly associated with systolic and diastolic ambulatory BP.(11) Furthermore, a hypomorphic SNP (rs3751143) was found to be associated with a decreased risk of hypertension in a Chinese population of postmenopausal women.(12) Similarly, a relation for NLRP3 and human BP has been observed. A mutation in the cold-induced autoinflammatory syndrome 1 (*CIAS1)* gene, which encodes for NLRP3, that leads to increased NLRP3 activation, was higher expressed in hypertensive compared to normotensive patients.(435) Male homozygotes of this *CIAS1* gene mutation had 6.4mmHg higher SBP than non-carriers, suggesting NLRP3 activation contributes to human hypertension in male populations. Together, these association studies would suggest P2RX7 has a role in human hypertension.

Besides hypertension, SNPs for P2RX7 have been correlated to other cardiovascular diseases. A rare loss of function SNP (rs28360451) was associated with familial hypertrophic cardiomyopathy in an Indian population, supporting what we found with the *P2rx7*$^{-/-}$ mice.(436) All affected family members had the SNP, whereas none of the unaffected family members possessed it and it was also not found in 100 control patients. In contrast, the loss of function P2X7 variant, rs3751143, was significantly associated with a decreased risk of ischemic heart disease and stroke, especially in individuals with hypertension.(309) Similarly, circulating P2X7 mRNA was found to be predictive of prognosis in acute MI, with elevated P2X7 expression correlating with worse patient outcomes.(382) As several P2RX7 antagonists are advancing through clinical trials, it will be important to watch whether long-term administration of P2RX7 antagonists causes detrimental cardiac effects. If they prove safe to use, targeting P2RX7 may not only present a viable target for managing hypertension, but cardiovascular disease in general as well.

Limitations

A limitation of the studies within this thesis was that our interventions, genetic or pharmaceutical, were initiated prior to the onset of hypertension. In humans, hypertension can often go undiagnosed for years, and anti-hypertensive therapies are not typically initiated until a period of time that can be long after the onset of hypertension. As our hypothesis for this thesis was that P2RX7 acts as an initial trigger for immune activation in the context of hypertension, instituting inflammation and end-organ damage, it is unclear if P2RX7 antagonism in already established hypertension would be effective. That being said, as hypertension is a condition of chronic inflammation, it is likely that DAMPs are continuously released, and the immune system is continually engaged. Therefore P2RX7 antagonism could likely be beneficial in human hypertension. Future studies should be conducted to validate these claims

Another limitation in our study design was the choice of C57BL/6J mice for the basis of our model. C57BL/6J mice possess several mutations that decrease their suitability for comparison to P2RX7 antagonism in our models. C57BL/6J mice have a mutation in the NLRP12 inflammasome gene which results in impaired macrophage-mediated neutrophil recruitment to sites of inflammation.(437) Therefore, recruitment of leukocytes to inflammatory sites such as the PVAT after Ang II treatment may have been slightly inhibited in these mice. However, as we still saw recruitment of inflammatory leukocytes to target tissues in C57BL/6J mice, which was decreased by antagonizing P2RX7, this mutation is unlikely to be a major limitation to our results. More significantly C57BL/6 mice present a natural P451L allelic mutation that impairs the functionality of P2RX7.(270) It was demonstrated that C57BL/6 mice, compared to BALB/c mice, have impaired P2RX7 mediated pore-forming ability and are less sensitive to P2RX7 mediated cellular death. The expression of P2RX7 in T cells from C57BL/6 mice is also considerably lower than in BALB/c mice and correlated with the decreased sensitivity to ATP mediated cellular death observed in T cells from C57BL/6 mice.(438) Furthermore, P2RX7-mediated lymphocyte activation (measured by Ca^{2+} mediated NFAT activation and IL-2 release) is considerably impaired in C57BL/6 mice compared to BALB/c mice, which express fully functional P2X7 receptors.(244) However, in our study, BALB/c mice would not present a viable alternative to C57BL/6 mice, as BALB/c mice are more resistant to developing hypertension and vascular dysfunction and therefore would not be suitable for studying Ang II-induced hypertension and

vascular damage.(439) It was also vital for us to have a WT mouse strain in the same background as the mice used to generate the *P2rx7*$^{-/-}$ mice purchased from Jackson Laboratories to compare responses to Ang II-infusion adequately.

Another limitation of this thesis was the use of a global KO for P2RX7 rather than an inducible or tissue-specific KO model. With a global KO, it is more difficult to tease out the specifics of receptor function, especially with a target such as P2RX7, which is almost ubiquitously expressed and has numerous roles across the body. As well, using a KO model comes with intrinsic issues. These issues include embryonic development issues or physiologic redundancies, where an alternate pathway or receptor could take over the function of P2RX7 in its absence. In addition, the Pfizer KO mice used in this study are not null for P2RX7.(179) Instead, they express a truncated C-terminal variant that hinders but does not completely block trafficking to the cell surface. These receptors are still partially functional as they permit a whole-cell current when stimulated, albeit at ~1/10 current of WT P2RX7 channels. Of note, these receptors were incapable of pore formation, suggesting they may not be able to mediate cellular death responses. As we validated that BMDMs and BMDCs from *P2rx7*$^{-/-}$ mice are incapable of P2RX7 mediated (LPS+ ATP stimulated) IL-1β production, we can confirm some loss of functionality. Still, we cannot definitively say that some function is not preserved in other tissues that may contribute to hypertension. Despite problems with the Pfizer-generated *P2rx7*$^{-/-}$ mice, they are still preferential to the alternative available at the start of the study developed by GlaxoSmithKline (GSK). The GSK generated *P2rx7*$^{-/-}$ mice express functional P2X7K variants, and therefore P2RX7 functionality in T cells is preserved.(186, 440) As a large part of this thesis was investigating T cell activation in the context of hypertension, the GSK mice would not be suitable for our study. In order to circumvent the issues with using a *P2rx7* KO while also making the model more translational, we repeated our experiments using a specific P2RX7 antagonist and recapitulated our results. Despite the limitations described with using C57BL/6 WT and Pfizer generated *P2rx7*$^{-/-}$ mice, our findings presented in this thesis are actually strengthened by their use. As C57BL/6 WT mice have attenuated P2RX7 function, and the *P2rx7*$^{-/-}$ mice have some retained P2RX7 functionality, we were less likely to observe differences. Therefore, any differences we present here in this thesis are most likely underrepresented, and the anti-hypertensive effect of P2RX7 antagonism might be even more pronounced than we observed.

Conclusion and Perspectives

In conclusion, we have established a role of P2RX7 in the pathogenesis of hypertension. For the first time, we have shown that P2RX7 activation contributes to the development of hypertension-associated endothelial dysfunction and vascular remodelling. Further, we demonstrate that by genetic KO of P2RX7 or through pharmaceutical antagonism, we can diminish Ang II-induced activation of the immune system and subsequently decrease BP. Notably, we have demonstrated that P2RX7 contributes to the development of T_{EM} and T_{RM} cells in the context of hypertension and thus may play a role in prolonged hypertension, such as in humans. Interestingly we demonstrated a divergent path between P2RX7 antagonism and *P2rx7* KO regarding cardiac function, with the latter leading to exacerbated cardiac dysfunction in response to Ang II. We have also demonstrated for the first time that the BP-attenuating effect of P2RX7 KO is primarily independent of antagonizing NLRP3 activation; however, diminished vascular damage and attenuated immune activation appear to be dependent on NLRP3 signalling. Taken altogether, the work of this thesis provides evidence that P2RX7 presents a viable target for the treatment of hypertension and associated inflammation and vascular damage.

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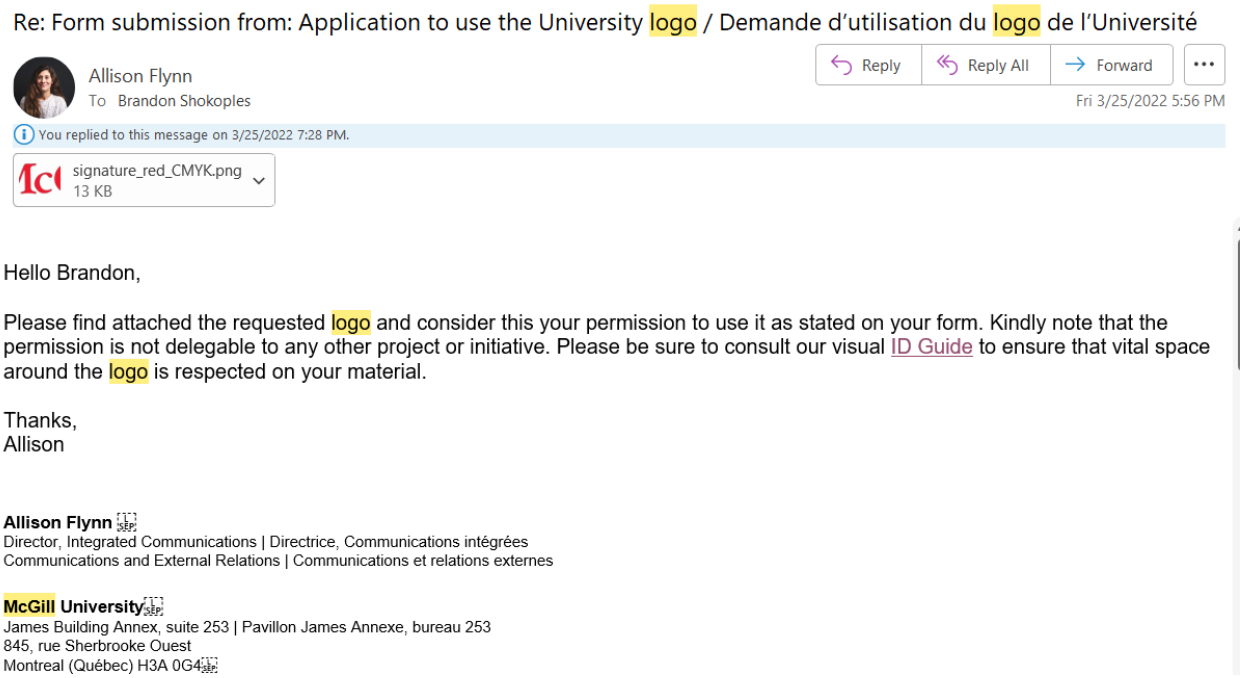

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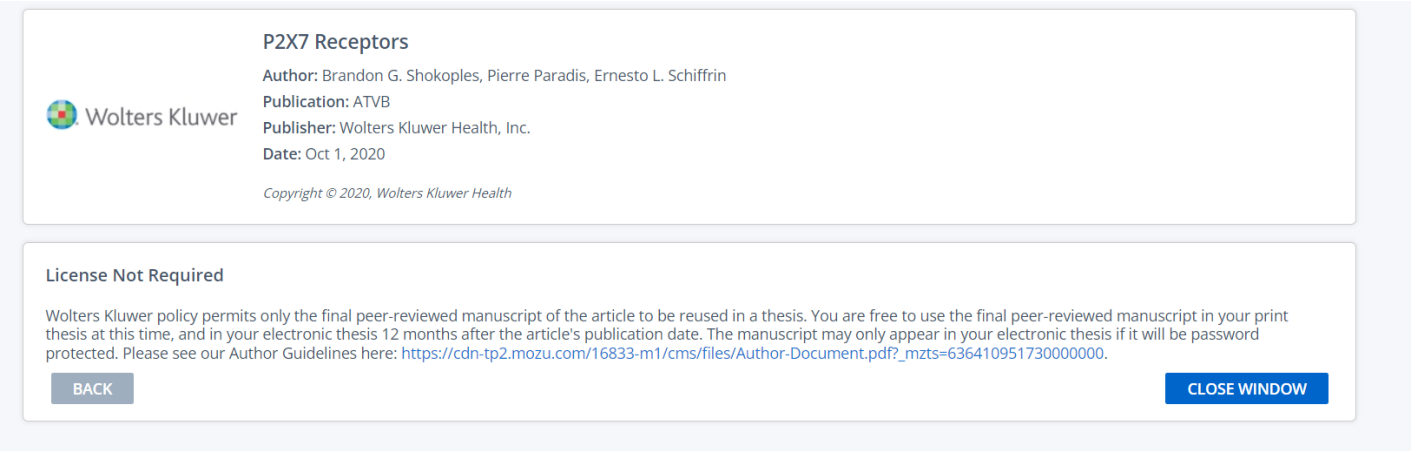

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