Memory γδ T cells enhance angiotensin II-induced blood pressure elevation and sensitize mice to develop hypertension

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Table of Contents

Abstract

Hypertension is the leading risk factor for disease burden and mortality worldwide, and inflammation is a key mediator of hypertension. We recently demonstrated that γδ T cells, which are unconventional lymphocytes that bridge innate and adaptive immunity, play a critical role in hypertension and vascular injury. Absence of γδ T cells blunted the development of hypertension in mice infused with angiotensin (Ang) II. A recent study also showed that an initial exposure to a hypertensive dose of Ang II causes accumulation of memory αβ T cells and sensitizes mice to develop hypertension from a low dose of Ang II. However, whether memory γδ T cells are involved in hypertension is unknown. A subset of γδ T cells referred to as γδT17 cells may also contribute to hypertension. γδT17 cells are prominent producers of interleukin (IL)-17A, a cytokine implicated in the development of hypertension, and the expansion of γδT17 cells is partially dependent upon IL-23 receptor (IL-23R) stimulation. Consequently, we aimed to test two hypotheses regarding the contribution of γδ T cells to hypertension: that memory γδ T cells sensitize mice to develop high blood pressure (BP) from a mild hypertensive challenge, and that Ang II-induced BP elevation and vascular injury would be blunted in mice deficient of IL-23R.

In the first study, we assessed the role of memory γδ T cells in Ang II-induced hypertension in C57BL/6J mice. Two-weeks of pressor dose Ang II infusion sensitized mice to develop hypertension from a subpressor dose of Ang II and resulted in the development of effector memory γδ T cells in mesenteric artery (MA) perivascular adipose tissue (PVAT) and mesenteric lymph nodes (mLNs). Conversely, mice exposed to repeated Ang II infusions had less central memory γδ T cells in the aortic and MA PVAT compared to mice only infused with a single subpressor dose of Ang II. Antibody-mediated depletion of γδ T cells after an initial Ang II hypertensive challenge reduced the pressor response to a subsequent subpressor dose of Ang II, and adoptive transfer of γδ T cells isolated from Ang II-infused mice sensitized normotensive recipient mice to develop hypertension upon infusion with a subpressor dose of Ang II. Consequently, memory γδ T cells develop after an initial hypertensive stimulus and sensitize mice to develop hypertension in response to a mild hypertensive challenge.

In the second study, we investigated the influence of the IL-23R on γδT17 cells and BP in Ang II-induced hypertension. Wild-type (WT) and *Il23r* knock-in (*Il23r*$^{gfp/gfp}$) mice were infused or not with Ang II for 7 or 14 days. *Il-23r*$^{gfp/gfp}$ mice had smaller and stiffer MA and were not

protected from BP elevation or MA dysfunction after 14 days of Ang II infusion. Further, *Il-23r*$^{gfp/gfp}$ mice exhibited more rapid BP elevation at the initiation of hypertension. Seven days of Ang II infusion increased the number of interferon (IFN)-γ-producing γδ T cells in both WT and *Il-23r*$^{gfp/gfp}$ mice, while the number of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells was only increased in *Il-23r*$^{gfp/gfp}$ mice. Irrespective of treatment, *Il-23r*$^{gfp/gfp}$ mice had both a lower γδT17 cell count and IL-$17A^+CD4^+$ T cell frequency, and a higher frequency of IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells compared to WT mice. Blocking IFN-γ reduced the pressor response to Ang II in *Il-23r*$^{gfp/gfp}$ mice. Contrary to our hypothesis, functional IL-23R deficiency exaggerated BP elevation during the initiation of Ang II-induced hypertension via increased IFN-γ.

In conclusion, we demonstrated that memory γδ T cells sensitize mice to develop hypertension to subpressor doses of Ang II, and that paradoxically, IL-23R signaling inhibition exaggerates the initial development of hypertension. The latter occurs via the expansion of IFN-γ-producing T cells. Consequently, selectively targeting memory T cell formation or pathways that augment T cell IFN-γ production may offer new treatment avenues for human hypertension.

Résumé

L'hypertension est le principal facteur de risque de morbidité et de mortalité dans le monde, et l'inflammation un médiateur clé de l'hypertension. Les cellules T γδ, qui sont des lymphocytes non conventionnels et relient l'immunité innée et adaptative, jouent un rôle essentiel dans l'hypertension. L'absence de cellules T γδ a atténué l'élévation de la tension artérielle (TA) chez les souris perfusées avec l'angiotensine II (AngII). Il a été montré qu'une exposition initiale à une dose hypertensive d'AngII provoque une accumulation de cellules T mémoire et sensibilise les souris à élever la TA à une faible dose d'AngII. Cependant, le rôle des cellules T γδ mémoire dans l'hypertension n'a pas été étudié. Un sous-type de cellules T γδ produisant de l'interleukine (IL)-17A, les cellules γδT17, peut également contribuer à l'hypertension. Le développement des cellules γδT17 dépend en partie de la stimulation du récepteur IL-23 (IL-23R). Par conséquent, nous avons décidé de tester deux hypothèses: que les cellules T γδ mémoire sensibilisent les souris à augmenter la TA suite à un défi hypertensif léger, et que l'élévation de la TA et les lésions vasculaires induites par l'AngII soient atténuées chez les souris déficientes en IL-23R.

Dans la première étude, nous avons évalué le rôle des cellules T γδ mémoire dans l'hypertension induite par l'AngII chez des souris C57BL/6J. Deux semaines d'hypertension induite par l'AngII ont entraîné le développement de cellules T γδ mémoire effectrices dans le tissu adipeux périvasculaire (TAPV) des artères mésentériques (AM) et les ganglions lymphatiques mésentériques (GLm) et sensibilisé les souris à élever la TA suite à une faible dose d'AngII. Mais, les souris exposées à des perfusions répétées d'AngII avaient moins de cellules T γδ mémoire centrales dans l'aorte et le TAPV AM. La déplétion des cellules T γδ médié par l'injection d'anticorps a réduit l'élévation de TA à une faible dose d'AngII. Le transfert adoptif de cellules T γδ isolés de souris hypertensives a sensibilisé les souris receveuses à élever la TA à une faible dose d'AngII. Par conséquent, les cellules T mémoire γδ se développent après un stimulus hypertensif initial et sensibilisent les souris à développer une hypertension en réponse à un traitement hypertensif léger.

Dans la deuxième étude, nous avons étudié l'influence de l'IL-23R sur les cellules γδT17 et la TA chez des souris sauvages et *Il23r* knock-in (*Il23r*$^{gfp/gfp}$) infusées ou non avec l'AngII pendant 7 ou 14 jours. Les souris *Il23r*$^{gfp/gfp}$ avaient des AM plus petites et plus rigides et n'étaient pas protégées contre l'élévation de la TA ou le dommage vasculaire après 14 jours d'AngII. En outre, les souris *Il23r*$^{gfp/gfp}$ ont présenté une élévation plus marquée de la TA dans les 7 premier

jours d'AngII. Sept jours de perfusion d'AngII ont accrue le nombre de cellules T γδ productrices d'interféron (IFN)-γ chez tous les souris, tandis que le nombre de cellules T $CD4^+$ et $CD8^+$ productrices d'IFN-γ n'a augmenté que chez les souris *Il23r*$^{gfp/gfp}$. Indépendamment du traitement, les souris *Il23r*$^{gfp/gfp}$ présentaient un nombre de cellules γδT17 et une fréquence des cellules T IL-$17A^+CD4^+$ réduits, ainsi qu'une fréquence accrue de cellules T γδ, $CD4^+$ et $CD8^+$ productrices d'IFN-γ par rapport aux souris sauvages. La neutralisation de l'IFN-γ a réduit la réponse hypertensive à l'AngII chez les souris *Il23r*$^{gfp/gfp}$. Contrairement à notre hypothèse, le déficit en IL-23R a exagéré l'élévation de la TA induite par l'Ang II via une élévation de l'IFN-γ.

En conclusion, nous avons démontré que les cellules T γδ mémoire sensibilisent les souris à élever la TA à une faible dose d'AngII, et que le déficit en IL-23R exagère l'élévation de la TA durant les premiers jours d'AngII via l'expansion de cellules T productrice d'IFN-γ. Par conséquent, le ciblage sélectif de la formation des cellules T mémoire ou des cellules T productrices d'IFN-γ peut offrir de nouvelles avenues de traitement de l'hypertension humaine.

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Finally, I extend thanks to the many lifelong friends I have made over my university studies, both in Montreal and New Brunswick, and to those friends who are no longer with us. While too many to mention here, each has contributed immeasurably to this journey.

Abbreviations

2-HOBA – 2-hydroxybenzilamine

ABPM – Ambulatory blood pressure monitoring

ACE – Angiotensin converting enzyme

Ach – Acetylcholine

ADAM – A disintegrin and metalloproteinases

ADH – Anti-diuretic hormone

Ang II – Angiotensin II

ANOVA – Analysis of variance

AOBP – Automated office blood pressure measurement

APC – Antigen presenting cell

ARB – Angiotensin receptor blocker

AT_1R/AT_2R – Angiotensin receptor

BAT – Brown adipose tissue

BM – Bone marrow

BP – Blood pressure

CANTOS – Canakinumab Anti-inflammatory Thrombosis Outcome Study

CCB – Calcium channel blocker

CCR – Chemokine receptor

CD – Cluster of differentiation

CDR3 – Complementarity determining region 3

cGMP – Cyclic guanosine monophosphate

cLN – Cervical lymph node

CNS – Central nervous system

CO – Cardiac output

DAMP – Damage associated molecular pattern

DBP – Diastolic blood pressure

DC – Dendritic cell

DCT – Distal convoluted tubule

DETC – Dendritic epidermal T cell

DHFR – Dihydrofolate reductase

DOCA – Deoxycorticosterone acetate

EAE – Experimental autoimmune encephalomyelitis

ECM – Extracellular matrix

ENaC – Epithelial sodium channel

eNOS – Endothelial nitric oxide synthase

ER – Endoplasmic reticulum

GFP – Green fluorescent protein

GFR – Glomerular Filtration Rate

GPCR – G-protein coupled receptor

HBPM – Home blood pressure measurement

HLA – Human leukocyte antigen

HMBPP – (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate

HPA – Hypothalamic-pituitary-adrenocortical axis

HSP – Heat-shock protein

HUVEC – Human umbilical vein endothelial cells

IEL – Intraepithelial lymphocytes

IFN-γ – Interferon-γ

IL – Interleukin

IPP – Isopentyl pyrophosphate

JG – Juxtaglomerular

KI – Knock-in

LV – Left ventricle

L-NAME – N^{ω}-nitro-L-arginine methyl ester

MA – Mesenteric artery

MAP – Mean arterial pressure

M-CSF – Macrophage colony-stimulating factor

MHC – Major histocompatibility complex

MICA – MHC class I polypeptide-related sequence A

miR – Micro-RNA

mLN – Mesenteric lymph node

MMF – Mycophenolate mofetil

MMP – Matrix metalloproteinase

MSNA – Muscle sympathetic nerve activity

NADPH – Nicotinamide adenine dinucleotide phosphate

NCC – Na-Cl co-transporter

NF-κB – Nuclear factor kappa B

NK – Natural killer cell

NLRP3 – NOD-like receptor family pyrin domain containing 3

NOS – Nitric oxide synthase

NOX – NADPH oxidase

NR – Nuclear receptor

OVLT – Organum vasculosum of the lamina terminalis

P2X7 – P2X purinoceptor 7

PAMP – Pathogen-associated molecular pattern

PECAM – Platelet endothelial cell adhesion molecule

PNS – Parasympathetic nervous system

PRR – Pattern recognition receptor

PVAT – Perivascular adipose tissue

PWV – Pulse wave velocity

RA – Rheumatoid arthritis

RAAS – Renin-angiotensin-aldosterone system

RAG – Recombinase-activating gene

ROS – Reactive oxygen species

SAM – Sympathetic adrenomedullary system

SBP – Systolic blood pressure

scid – Severe combined immunodeficient

SEM – Standard error of mean

SFO – Subfornical nucleus

sGC – Soluble guanylyl cyclase

SHR – Spontaneously hypertensive rat

siRNA – Small interfering RNA

SLO – Secondary lymphoid organ

SNP – Single nucleotide polymorphism

SNS – Sympathetic nervous system

SOD – Superoxide dismutase

Tc – Cytotoxic T cell

TCR – T cell receptor

T_{CM} – Central memory T cell

T_{EM} – Effector memory T cell

TGF – Tubulo-glomerular Feedback

TGFβ – Transforming growth factor-beta

Th – T helper cell

TIMP – Tissue inhibitor of metalloproteinases

TLR – Toll-like receptor

TNFα – Tumor necrosis factor-alpha

TPR – Total peripheral resistance

Treg – T regulatory cell

T_{RM} – Resident memory T cell

TRP – Transient receptor potential

VEGF – Vascular endothelial growth factor

VSMC – Vascular smooth muscle cell

WAT – White adipose tissue

WT – Wild-type

List of Tables

List of Figures

Chlorophyll-Protein (PerCP)-eFluor™ 710 (PerCP-eF710)-conjugated anti-mouse CD4 (eBioscience, clone RM4-5), Alexa Fluor® 700 (AF700)-conjugated anti-mouse CD8 (Biolegend, clone 53-6.7), Alexa Fluor® 647 (AF647)-conjugated anti-mouse TCRγδ (Biolegend, clone GL3), Phycoerythrin (PE)-Cyanine (Cy)® 7 (PE-Cy7)-conjugated anti-mouse CD44 (BD, clone IM7), Allophycocyanin (APC)/Fire™ 750 (APC/Fire750)-conjugated anti-mouse CD62L (Biolegend, clone MEL-14), PE-CF® 594 (PE-CF594)-conjugated anti-mouse CCR7 (BD, clone 4B12), Alexa Fluor® 488 (AF488)-conjugated anti-mouse CD103 (Biolegend, clone 2E7), and PE-Cy5-conjugated anti-mouse CD69 (Biolegend, clone H1.2F3), and analyzed by flow cytometry. Fluorophores were respectively excited and analyzed with the appropriate laser and band pass filter (BP) (Aqua: 405 nm with 525/50 BP, BV786: 405 nm with 780/60 BP, BV605: 405 nm with 610/20 BP, PerCP-eF710: 488 nm with 695/40 BP, AF700: 640 nm with 730/45 BP, AF647: 640 nm with 670/14 BP, PE-Cy7: 561 nm with 780/60 BP, APC-Fire750: 640 nm with 780/60 BP, PE-CF594: 561 nm with 610/20 BP, AF488: 488 nm with 530/30 BP, PE-Cy5: 561 nm with 670/30 BP). A representative flow cytometry gating strategy of splenocytes of wild-type mice infused with angiotensin II for 14 days is shown. Cells were gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using FSC-A over FSC height (FSC-H) and then again using SSC-A over SSC height (SSC-H). Live cells were gated in the Aqua/FSC-A plot. $CD45^+$ cells were gated in the CD45/FSC-A plot. $CD3^+$ T cells were gated in the CD3/FSC-A plots. γδ T cells were gated in the TCRγδ/CD3 plot. $CD44^+$ γδ T cells were gated in the CD44/FSC-A plot. γδ T_{CM} cells were gated in the CD62L/CCR7 plot. γδ T_{EM} and T_{RM} cell subsets were gated in the CD69/CD103 plot. 92

Figure 3.6: The frequency of $CD4^+$ and $CD8^+$ memory T cells is relatively unchanged following Ang II infusion in comparison to memory γδ T cells. A. Fourteen-day pressor dose Ang II infusion did not alter the frequency of effector memory $CD4^+$ or $CD8^+$ T cells (T_{EM}) in the mesenteric artery (MA) perivascular adipose tissue (PVAT). B. $CD8^+$ T_{EM} were upregulated in the mesenteric lymph nodes (mLN) in response to 14 days of Ang II infusion, but there was no change in $CD4^+$ T_{EM}. C. The frequency of $CD4^+$ and $CD8^+$ central memory T cells (T_{CM}) was unchanged in the aortic PVAT following repeated hypertensive challenges. D. The frequency of $CD4^+$ and $CD8^+$ T_{CM} cells was also unchanged in the MA PVAT following repeated hypertensive challenges. Data are presented as means ± SEM. The data were analyzed using an unpaired t-test. n=6-9; $*P<0.05$ vs. sham… 94

Statement of Originality and Contributions 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 are the first to demonstrate that memory γδ T cells develop in experimental hypertension and that they contribute to the pressor response to Ang II. We are also the first to investigate γδ T cell population dynamics following repeated hypertensive challenges.
- We are the first to show development of effector memory γδ T cells in arteries and lymph nodes following a hypertensive challenge.
- We are the first to demonstrate that adoptive transfer of γδ T cells from mice exposed to a hypertensive challenge can induce a state of pressor hyperresponsiveness to a normally mild hypertensive challenge in recipient animals.
- We are the first to report that functional IL-23R deficiency paradoxically exaggerates initial development of Ang II-induced hypertension, which is mediated in part by IFN-γ.
- We are the first to show that functional IL-23R deficiency is associated with an increase in the number of IFN-γ-producing T cells in the MA PVAT after one week of Ang II infusion.

Contribution of Authors

All chapters of this thesis were written by Kevin Comeau and edited by Dr. Ernesto L. Schiffrin. The body of this thesis was prepared in manuscript format; therefore, the contributions of collaborating authors are detailed below.

Chapter III: Angiotensin II-Induced Memory γδ T Cells Sensitize Mice to a Mild Hypertensive Stimulus

- Kevin Comeau: Design of experiments, technical setup for experiments, troubleshooting experimental techniques, preparation of osmotic minipumps and antibodies for injection, collection of tissues, flow cytometry panel design and optimization, flow cytometry sample preparation and staining, flow cytometry acquisition, γδ T cell isolation by MACS for adoptive transfer, statistical analysis, creation of figures, and writing and editing the manuscript.
- Brandon Shokoples: Collection of tissues (partial), preparation of samples for flow cytometry staining (partial), and technical input regarding data analysis and flow cytometry protocols.
- Antoine Caillon: Technical input regarding flow cytometry panel design and data analysis, experimental design (partial), and technical input on experimental techniques for sample collection and flow cytometry sample preparation.
- Pierre Paradis: Experimental design, supervision of work, evaluation of raw data, and drafting of 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 drafting and revision.

Chapter IV: Angiotensin II induced steeper blood pressure elevation in IL-23 receptor-deficient mice: Role of interferon-γ-producing T cells

- Kevin Comeau: Technical setup for flow cytometry experiments, collection of tissues for flow cytometry experiments, flow cytometry panel design and optimization, in vitro activation of immune cells for determination of cytokine production, intracellular and extracellular flow cytometry staining, flow cytometry acquisition, statistical analysis of flow cytometry data, preparation of anti-IFN-γ and control antibodies for injection, creation of figures, and writing and editing the manuscript.
- Brandon Shokoples: Pressurized myography setup and data collection (partial), preparation of osmotic minipumps and surgical implantation, statistical analysis of blood pressure and myography data, creation of figures, and writing and editing the manuscript.
- Akinori Higaki: Pressurized myography setup and data collection (partial), collection and analysis of blood pressure data by telemetry (partial), flow cytometry phenotyping (partial).
- Nathanne S. Ferreira: Pressurized myography setup and data collection (partial).
- Antoine Caillon: Technical input regarding flow cytometry panel design and data analysis, experimental design (partial), collection of tissues for flow cytometry (partial), and technical input on flow cytometry sample preparation.
- Olga Berillo: Genotyped mice by PCR to monitor the *Il23r*$^{gfp/gfp}$ genotype.
- Mohamed Oukka: Donated *Il23r*$^{gfp/gfp}$ mice.
- Pierre Paradis: Experimental design, supervision of work, evaluation of raw data, and drafting of 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 drafting and revision.

1. Introduction and Literature Review

1.1 Hypertension and its Relevance to Society and Medicine

Elevated blood pressure (BP), or hypertension, is the leading risk factor for cardiovascular disease, disease burden, and mortality worldwide (1). The development of hypertension is a complex and multifactorial process that encompasses a wide range of environmental, lifestyle, and genetic factors that can directly influence BP or the mechanisms underlying BP regulation. Public awareness campaigns for the prevention and control of hypertension have helped increase the rate of treatment among Canadians, with the rate of controlled hypertension increasing from 13.2% in 1992 to 64.6% in 2009 (2). However, the prevalence of a hypertension diagnosis among Canadians aged 20-79 remains quite high, averaging 22.4% for the period of 2016-2019 (3).

Surprisingly, the rate of hypertension among Canadians has remained stable at around 20% since 1992, showing the need for alternative methods for prevention and treatment (2, 3). With such a high prevalence in society, hypertension represents a large burden to healthcare systems. In 2010, the estimated national cost of managing these patients was $13.9 billion, with a projected cost of $20.5 billion in 2021 (4). At the level of the individual, this represents a cost of approximately $2053 per annum directly attributable to hypertension, not including any costs associated with comorbidities brought on by sustained BP elevation. A meta-analysis including data from 195 countries found that having a systolic BP above 140 mmHg in 2015 was associated with an annual mortality rate of 106.3 deaths per 100,000 people, and an estimated loss of 143 million disability-adjusted life years (5). Consequently, proper diagnosis and control of hypertension is essential to maintaining a healthy and productive society while minimizing the burden on healthcare providers.

1.2 Blood Pressure Measurement and Diagnosis of Hypertension

1.2.1 The Need for an Accurate Definition of Hypertension

Hypertension is often described as a “silent killer”, referring to the fact that individuals can suffer from it for years and not show symptoms until significant organ and tissue damage has occurred. Increased public awareness regarding hypertension and its consequences has helped encourage

patients with elevated BP to seek treatment and begin BP monitoring and treatment regimens earlier in the progression of the condition. It is important to treat hypertension and manage elevated BP as early as possible, as hypertension progressively leads to irreversible multiorgan system damage. As a result, properly defining hypertension represents a key opportunity to diagnose and initiate treatment at the right time, thereby reducing preventable comorbidities. Therefore, the defining features to diagnose hypertension are updated on a regular basis to reflect the changing social, economic, and healthcare landscape for a given region.

1.2.2 The Hypertension Canada 2020 Guidelines for the Diagnosis, Risk Assessment, Prevention, and Treatment of Hypertension

BP is the result of the combination of blood exerting a force on the walls of arteries and the reciprocal force that arteries apply to blood in response to this force. The compliance and elasticity of arteries has a broad impact on BP regulation and resting BP. During ventricular systole blood is pumped from the heart into the aorta, where it continues to peripheral arteries and exerts an elevated pressure referred to as systolic BP (SBP). During ventricular diastole blood ceases to flow from the left ventricle into the aorta while continuing to flow to peripheral arteries, and as a result the pressure against the arterial walls drops. This lower limit of BP is referred to as diastolic BP (DBP). SBP, DBP, or a combination of both are used to assess and diagnose hypertension in the clinical setting.

According to the Hypertension Canada 2020 Guidelines for the Diagnosis, Risk Assessment, Prevention, and Treatment of Hypertension, the BP threshold for a diagnosis of hypertension is a mean SBP ≥135 mmHg or mean DBP ≥85 mmHg as determined by automated office BP (AOBP) measurement (6). If AOBP is not available, the non-AOBP threshold for diagnosis is a SBP of ≥140 mmHg and/or DBP ≥90 mmHg. For patients with diabetes, the threshold for diagnosis for both AOBP and non-AOBP measurement is a SBP ≥130 mmHg and/or DBP ≥80 mmHg. If the threshold for diagnosis in a non-diabetic patient is exceeded, follow up out-of-office measurement is recommended to confirm the presence of hypertension and rule out the possibility of the white coat effect interfering with a correct diagnosis. The threshold for diagnosis for ambulatory BP monitoring (ABPM) is a mean 24-hour SBP ≥130 mmHg and/or DBP ≥80 mmHg, or a mean daytime SBP ≥135 mmHg and/or DBP ≥85 mmHg. Finally, a home BP monitoring (HBPM) series (two readings taken each morning and evening for seven days,

discarding the readings of the first day and averaging the last six days) SBP ≥135 mmHg and/or DBP ≥85 mmHg represents another out-of-office threshold for diagnosing hypertension. AOBP is the preferred in-office measurement while ABPM is the preferred out-of-office measurement.

1.3 Pathophysiology of Hypertension

1.3.1 Categories of Hypertension: Essential (or Primary) Versus Secondary Hypertension

BP and its regulatory mechanisms can be influenced by a wide array of physical, cognitive, and genetic factors that encompass lifestyle, environment, and family history. As a result, most cases of hypertension do not have a single identifiable underlying cause and this is termed essential, or primary hypertension (7). Essential hypertension (as it is commonly known, but which would be more appropriately called primary hypertension) can develop and progress as the result of many initiating or contributing factors, including aging, excess dietary sodium, obesity, alcohol consumption, tobacco and/or nicotine consumption, low potassium intake, insulin resistance, and stress (8-10). These factors are additive and impact important physiological systems, including the endocrine, renal, nervous, and cardiovascular system, yielding a complex etiology termed the mosaic theory of hypertension (11). Proposed in 1949, the first definition for the mosaic theory of hypertension postulated that dysregulation of the nervous, cardiovascular, renal, and endocrine systems results in the hypertensive phenotype. Over time this definition has been revisited and expanded to account for advances in research and medicine. As of 2020, the mosaic theory includes further nuances such as inflammation, oxidative stress, vascular function and mechanics, anatomical, neural, and humoral factors, as well as the pillars of heritable traits, genetic background and the environment (Figure 1.1) (12).

In contrast to essential hypertension, secondary hypertension occurs associated to an underlying cause. Typically, secondary hypertension is attributed to renal disease including renal parenchymal and vascular disease, the latter comprising renal artery stenosis which can be due to renal artery fibromuscular dysplasia or to atherosclerosis, and adrenal disease, particularly primary aldosteronism. Some additional examples of underlying causes for secondary hypertension include autoimmune disorders like systemic lupus erythematosus, cardiovascular abnormalities such as coarctation of the aorta, endocrine thyroid disorders such as hyper- and hypothyroidism, congenital neurological defects, as well as disordered breathing during sleep (13). In the case of secondary

hypertension, attention should be directed to treating the underlying cause of the increased BP when this is possible.

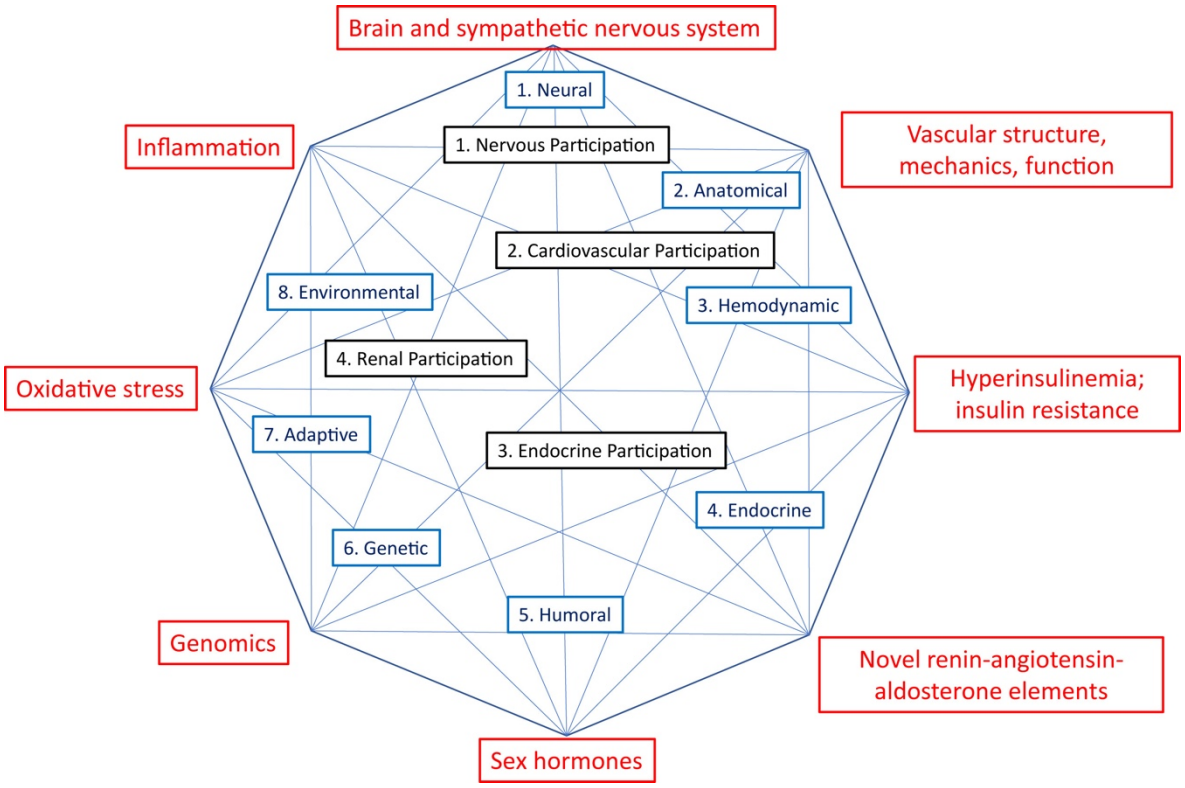

Figure 1.1: The revised 2020 interpretation of the mosaic theory of hypertension. How the original four elements from Irving Page's 1949 mosaic theory (in black text) fit into the 2020 mosaic theory (red text), along with the elements added in 1982 when it was initially revisited (blue text). Adapted with permission from Touyz *et al.* (12).

1.3.2 Pathogenic Mechanisms Underlying Hypertension

BP is considered a function of cardiac output (CO) and total peripheral resistance (TPR). CO is determined simply by multiplying heart rate (HR) by stroke volume (SV), while TPR is a more complex measurement that represents the vasoconstrictive and elastic properties of the peripheral vasculature. Fundamentally, TPR is the resistance to blood flow imparted by the vasculature that must be overcome to push and circulate blood. Mechanistically, TPR is determined by three factors: length of vessels, blood viscosity, and vessel radius. Vessel radius is the most significant

determinant of resistance, and this is reflected in the Hagen-Poiseuille equation. The Hagen-Poiseuille equation describes the relationship between pressure, fluid resistance, and flow rate, considering blood viscosity and the length and radius of the vessel. Therefore, the ability of vessels to vasodilate, vasoconstrict, and maintain a minimum diameter is essential to maintaining healthy BP and preventing end-organ damage. This is especially relevant to the pathogenesis of hypertension, where reduced vessel diameter due to unchecked vasoconstriction, a poor vasodilatory response, and/or arterial stiffening due to remodeling all work to further increase BP. Factors which determine CO and the impact of alterations to CO on BP will be discussed. Consequently, the mechanisms underlying the development of hypertension must involve factors that directly influence CO and/or TPR.

1.3.3 The Kidney: Abnormal Renal Sodium Transport

The kidney plays a pivotal role in the regulation of blood volume, salt transport, and vascular tone, and abnormal renal function has a direct impact on BP. It has been demonstrated that kidney function has an almost linear correlation with the prevalence of hypertension, where patient populations that have low estimates of glomerular filtration rate (GFR) concurrently have the highest rates of hypertension (14). Further, a 2003 autopsy study published in the New England Journal of Medicine found that patients with a history of primary hypertension and/or left ventricular hypertrophy had significantly fewer glomeruli per kidney compared to age, sex, weight, and height matched controls (15). Considering that the number of functional nephrons will impact estimates of GFR, this study provides an important link between renal anatomy, renal function, and primary hypertension. However, it should be noted that abnormal handling of salt in the renal tubules can lead to increases in BP despite a normal GFR. Consuming sodium induces a transient increase in plasma sodium concentration and BP, but for most healthy individuals this is short-lived and inconsequential (16).

Fundamentally, high plasma sodium concentrations result in expanded plasma volume due to salt and water transport being closely related, and this augments arterial BP by increasing cardiac preload and cardiac output (17, 18). High plasma sodium concentrations can also directly affect the structure and function of the vascular wall. A 1952 study demonstrated that the intimal and medial layers of renal arteries collected from hypertensive patients had increased concentrations of both sodium and water (19). These vessels were described as “water-logged” and further studies

revealed that salt and water retention in the vascular wall contributed to arterial wall thickening in hypertension (20, 21). Additionally, high plasma sodium concentrations can increase vascular reactivity to angiotensin (Ang) II (22). Normotensive rats fed a high-salt diet exhibited an increased pressor response to injected Ang II versus rats fed a normal salt diet, and normotensive rats fed a high-salt diet in the absence of Ang II did not exhibit increased BP (23). One central mechanism for reducing BP following an increase in plasma sodium concentration and plasma volume is pressure natriuresis. When arterial BP is increased, this increases renal perfusion pressure and in turn, sodium and water excretion. Following the excretion of excess salt and water, the volume of extracellular fluid is reduced and BP normalized (24).

Pressure-natriuresis is modulated within the kidney through a mechanism known as tubulo-glomerular feedback (TGF). TGF is a negative feedback loop in which the concentration of sodium chloride is sensed downstream in the nephron by macula densa cells in the wall of the thick ascending limb, and the concentration of sodium chloride indicates the GFR of an individual nephron (25). GFR can then be augmented or depressed mainly by vasodilating or vasoconstricting the afferent arteriole, respectively, depending on if macula densa cells detect abnormally low or high sodium chloride delivery. In the case of an abnormally low GFR, reduced sodium chloride delivery to the macula densa induces signaling that culminates in vasodilation of the afferent arteriole and vasoconstriction of the efferent arteriole, thereby increasing glomerular filtration pressure, tubular fluid flow, and GFR. Increasing the fluid flow rate in the thick ascending limb allows for less dilution of the tubular fluid and this increases sodium chloride delivery to the macula densa. Conversely, if the macula densa senses high sodium chloride concentrations associated with an abnormally high GFR, then glomerular flow will be decreased by vasoconstricting the afferent arteriole and allowing for vasodilation of the efferent arteriole.

In individuals with chronic hypertension there is a shift in the pressure natriuresis relationship to maintain sodium balance in the face of increased arterial pressures. Ang II is an important global regulator of pressure natriuresis, and in normotensive individuals modulation of Ang II maintains appropriate sodium balance over a wide range of intakes with minimal effect on BP (26). Mechanistically, when salt intake is high Ang II is suppressed, and when salt intake is low Ang II is increased, and this maintains BP and sodium excretion at normal levels. For example, if salt intake is increased then sodium balance can be maintained without an increase in arterial pressure. This is able to occur as negative regulation of Ang II shifts the set point for pressure

natriuresis to lower BP levels, which allows the kidneys to maintain sodium excretion even at normal arterial pressures. Consequently, dysregulation of Ang II can contribute to dysfunction of the pressure natriuresis relationship. Experimentally, both pharmacologic inhibition of Ang II and infusion of Ang II into dogs decreased the slope of the pressure natriuresis relationship, meaning that these dogs became salt-sensitive and BP must be increased to maintain sodium excretion (27). Therefore, appropriate control of systemic Ang II is necessary to maintain pressure natriuresis and sodium homeostasis with minimal effect on BP, and dysregulation of Ang II can lead to abnormal pressure natriuresis, salt-sensitivity, and subsequent BP elevation in order to effectively excrete sodium. While it is understood that pressure natriuresis is altered in hypertension, controversy exists among research groups as to whether all forms of chronic hypertension are supported by impaired pressure natriuresis (28).

For many patients, the kidney cannot respond appropriately to increased plasma sodium concentrations, resulting in exaggerated BP increases. This is indicative of an abnormal pressure-natriuresis relationship, and these individuals are considered to be salt-sensitive. Salt-sensitivity refers to a BP abnormality reliant on salt intake, and as mentioned above the renal system is not able to efficiently manage excess sodium without alterations to BP (29). Salt-sensitive patients often exhibit fluid retention due to an impaired ability to excrete sodium. Salt sensitivity is associated with several genetic factors, and genes have been discovered in patients with salt sensitivity that contain alleles associated with increased aldosterone activity and increased epithelial sodium channel (ENaC) activity in the distal nephron (30). While the renal system has often been the focus of investigations into salt-sensitive hypertension, the surface of the vascular endothelium, known as the endothelial glycocalyx layer, is negatively charged and may also be able to regulate and store sodium in a non-osmotic manner (31). Dysfunction of the vascular endothelium and/or vascular smooth muscle cells (VSMCs), leading to an impaired ability to respond to salt challenge with the appropriate vasodilatory response, could represent another explanation for salt sensitivity in hypertensive patients. This hypothesis comes from the observation that salt-resistant individuals respond to salt loading with rapid vasodilation, while salt sensitive individuals fail to vasodilate and exhibit elevated BP (32). Therefore, other tissues involved in non-osmotic salt storage (such as skin through macrophage sodium storage and skeletal muscle) may have a role in salt-sensitive hypertension, and salt can influence immune activation and homeostasis, further contributing to BP increases (33). Recent studies have confirmed a role

for salt in activating immune cells in the context of hypertension, and it was shown that the gut microbiota plays an important role in mediating salt-induced immune activation (34). A 2017 study by Wilck *et al.* demonstrated that a salt challenge reduced intestinal survival of *Lactobacillus* spp. and resulted in an upregulation of proinflammatory interleukin (IL)-17A-producing T helper (Th17) cells in both mice and humans (35). To assess the contribution of salt and the gut microbiota to the development of Th17 cells, the authors induced experimental autoimmune encephalomyelitis (EAE) in mice, as the development of EAE is known to be heavily influenced by gut microbiota. Additionally, the authors fed mice either a high salt or normal salt diet and administered a subset of these mice *L. murinus* orally. They found that mice that were fed a high salt diet had increased Th17 cells and worsened EAE compared to EAE mice that were fed a normal salt diet. In contrast, mice that were fed a high salt diet and treated with *L. murinus* had less severe EAE and did not exhibit increased Th17 cells. Taken together, it is conceivable that a high salt diet could also contribute to microbiome abnormalities and inappropriate immune activation in hypertension. However, more studies are needed to gain a clearer understanding of the interactions between the gut microbiome and blood pressure.

1.3.4 Overview of the Renin-Angiotensin Aldosterone System

The primary roles of the renin-angiotensin-aldosterone system (RAAS) include regulation of systemic blood volume, maintenance of perfusion to critical organ systems, and regulation of systemic vascular resistance. Renin, Ang II, and aldosterone all work in concert to elevate and/or maintain BP. Signals which activate RAAS include decreased renal perfusion pressure, reduced salt delivery to the distal convoluted tubule, and sympathetic nervous system (SNS) activation. Mechanistically, RAAS activation is initiated by the macula densa and juxtaglomerular (JG) cells of the nephron (36). When renal blood flow is reduced, the macula densa senses reduced sodium chloride delivery and signals to JG cells to release active renin after its cleavage from prorenin. Renin thus enters the circulation. JG cells can also be stimulated to release renin independent of the macula densa, as JG cells act as baroreceptors in the afferent arteriole that can respond to high or low perfusion pressure by reducing or increasing the production of renin, respectively (37). Further, SNS-mediated activation and stimulation of β-adrenergic receptors on JG cells can induce renin synthesis (38). It is within the blood that renin encounters its target, angiotensinogen, which is inactive and constitutively secreted by the liver. Renin then enzymatically cleaves

angiotensinogen, forming Ang I, which is also physiologically inactive. Although angiotensinogen has been considered a "passive" aspect of the RAAS, research has shown that it can be oxidatively modified (39). The oxidation state of angiotensinogen impacts the ability of renin to cleave it to its active form, and this may affect RAAS-dependant BP regulation.

Following conversion of angiotensinogen to Ang I, Ang I is then converted into either Ang-(1-9), Ang-(1-7), or Ang II via a family of angiotensin converting enzymes (ACE) (40). Formation of Ang II is catalyzed by ACE in the vascular endothelium of the lungs and kidneys, while ACE2 is implicated in the production of Ang-(1-7) from Ang II, and Ang-(1-9) from Ang I (41, 42). ACE is also known to degrade bradykinin to inactive forms in the circulation, and this in turn reduces the serum concentration of vasodilators (43). Both Ang-(1-7) and Ang-(1-9) play a protective role in the heart and vasculature, reducing inflammation and counterbalancing the proinflammatory and prohypertensive actions of Ang II (44, 45). On the other hand, Ang II is arguably the most studied member of the angiotensin family and understanding its physiological activity and mechanism of action has led to large advances in treating hypertensive patients. The biological activity of Ang II is initiated by binding to the angiotensin type I (AT_1R) and type II (AT_2R) G-protein coupled receptors (GPCRs) within various cell subsets and tissues (46). Circulating Ang II is known to have a relatively short half-life (<1 minute) in plasma before being degraded into Ang III and Ang IV by peptidases, whereas the in-vivo half-life of Ang II in heart, kidney, and adrenal gland is much longer at ~15 minutes (47). The known physiological effects of Ang III and IV binding to cognate receptors is still relatively limited, but Ang III is known to interact with AT_1R and AT_2R with similar downstream effects as the binding of Ang II, but with reduced activity (48). However, within the central nervous system (CNS) Ang II can be degraded by aminopeptidase A to Ang III, and Ang III is an important effector neuropeptide that controls ADH release and BP (49). Interestingly, brain Ang III synthesis can be inhibited by the selective aminopeptidase A inhibitor, EC33, which has antihypertensive effects (50). Therefore, aminopeptidase A inhibitors could represent a novel class of antihypertensives. Finally, Ang IV binds AT_4R, and it has been shown to have cardioprotective (51, 52) and cognitive benefits (53).

1.3.5 Angiotensin II, Aldosterone, and Blood Pressure Regulation

Ang II is generally understood to be the most potent vasoconstrictor of the angiotensin peptide hormone family, and because of its high potency it is implicated in the development of

hypertension. There are several important BP regulation mechanisms that involve Ang II, and dysregulation of Ang II and its associated pathways has deleterious consequences to the cardiovascular system. An important downstream effect of Ang II synthesis is the stimulation of aldosterone release from the zona glomerulosa of the adrenal cortex (54). Aldosterone, a nuclear receptor (NR)-binding steroid hormone, increases sodium reabsorption and potassium excretion in the collecting duct and distal tubule of the nephron via stimulation of luminal sodium channels and basolateral sodium-potassium ATPases (55). Aldosterone-mediated increases in salt and water reabsorption lead to increased plasma volume and augmented BP.

In addition to the aldosterone-mediated effects on sodium and water homeostasis, aldosterone directly influences vascular function and physiology by contributing to inflammation, oxidative stress, endothelial dysfunction, and deleterious vascular remodeling (56). Sprague-Dawley rats infused with aldosterone exhibited increased BP, higher systemic oxidative stress, small artery hypertrophic vascular remodeling, and increased vascular collagen and fibronectin deposition (57). Conversely, treatment with spironolactone, a competitive aldosterone receptor antagonist, reduced BP, decreased levels of oxidative stress, and corrected hypertrophic remodeling in mesenteric arteries of aldosterone-infused rats. As Ang II induces the release of aldosterone, the effects of Ang II and aldosterone can be teased apart by treating Ang II-infused rodents with spironolactone. Ang II-infused Sprague-Dawley rats had increased BP, vascular dysfunction, and vascular remodeling indicated by an increased media-to-lumen ratio; however, treatment with spironolactone reduced BP, improved the endothelium-dependent vasodilatory response, and partially corrected the increased media-to-lumen ratio caused by Ang II infusion (58). In addition, spironolactone administration reduced aortic reactive oxygen species (ROS) generation and normalized plasma markers of oxidative stress in Ang II-infused animals. Therefore, some of the oxidative stress attributed to Ang II infusion is mediated through the actions of aldosterone. Aldosterone may also augment vascular Ang II signaling, as it was demonstrated that aldosterone infusion increased the density of Ang II binding sites on mesenteric artery VSMCs in Sprague-Dawley rats (59), and exposure of cultured rat aortic VSMCs to aldosterone enhanced Ang II-induced signal transduction in vitro (60). Regarding inflammation, aldosterone enhances myocardial and vascular inflammation in hypertension, as Ang II-infused rats treated with eplerenone, an aldosterone antagonist similar to spironolactone, have less myocardial and perivascular injury and reduced macrophage infiltration compared to Ang II-treated controls (61).

Aldosterone infusion has been shown to upregulate the expression of cell adhesion molecules associated with leukocyte recruitment and infiltration (62), which could further augment vascular and myocardial inflammation in hypertension. In hypertensive patients, treatment with eplerenone led to BP control and a smaller media collagen-to-elastin ratio, resulting in reduced arterial stiffness in addition to reductions in circulating inflammatory mediators (63). Consequently, aldosterone mediates a significant portion of the downstream vascular and renal consequences of RAAS activation.

While Ang II is a relatively fast-acting peptide hormone that binds GPCRs on the cell surface, binding of aldosterone to NRs and induction of gene expression is a slower process that can take hours to days to reach peak activity (64). However, aldosterone also has rapid non-genomic effects by binding membrane receptors, with one example being the induction of ROS production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXes) via G-protein and MAPK signaling cascades (64). In addition, the rapid effects of aldosterone may act in synergy with AT1R. Genomic effects of aldosterone include net retention of sodium via synthesis of ion channels, leading to increased blood osmolarity, and in turn, increased blood and extracellular fluid volume due to subsequent water retention. In the context of low BP/low perfusion, this increased circulating volume serves to heighten BP and return renal perfusion sensors (JG cells) to steady state. Deoxycorticosterone acetate (DOCA), which is closely related to aldosterone, is an endogenous steroid hormone often used to generate animal models of hypertension due to its ability to alter the regulation of central pressor mechanisms. DOCA administration results in chronic mineralocorticoid overproduction, salt-dependent hypertension, and SNS-mediated immune recruitment (65).

Ang II acts on the brain, where it increases sympathetic neuron activity (66) and further increases BP through several mechanisms. The first is binding within the hypothalamus where it induces feelings of thirst to promote fluid intake (67). The second is stimulating the release of antidiuretic hormone (ADH) from the supraoptic nucleus of the posterior pituitary, which further promotes water retention by causing insertion of aquaporin channels at the collecting duct of the kidney (68). Finally, Ang II reduces the sensitivity of the baroreceptor reflex (discussed in 1.3.7), preventing baroreceptors from sensing and responding to BP increases (69). In concert, these mechanisms serve to increase blood volume and BP by increasing fluid intake and reducing fluid loss, while simultaneously blunting the baroreceptor reflex.

Ang II also has a direct role in regulating vascular tone through AT_1R-dependant vasoconstriction of systemic arterioles, thereby resulting in increased systemic vascular resistance (70). Activation of AT_1R on smooth muscle cells results in a cascade of intracellular signaling that culminates in the release of intracellular calcium, which complexes with calmodulin and activates myosin light chain kinase, which phosphorylates the myosin light chain and induces vasoconstriction of vascular smooth muscle. Therefore, inappropriate regulation of Ang II activity directly impacts the vasculature, inducing vascular dysfunction while promoting the hypertensive state. In hypertension, Ang II augments BP by increasing total body water and salt, increasing vascular resistance, and promoting sympathetic neuron activation. Ang II also has a direct impact on immune cell function via binding angiotensin receptors expressed on T cells (71). As a result of Ang II having such a prominent role in BP homeostasis, many treatments for hypertension have targeted RAAS components.

1.3.6 Neural Regulation of Blood Pressure

Several cardiovascular centres in the medulla oblongata contribute to BP regulation in the long and short term. One such centre is the area postrema, which contributes to BP control and is responsive to circulating Ang II. Ang II induces the area postrema to suppress the baroreceptor reflex (discussed in 1.3.7) and enhance SNS activity, leading to increased BP (72). Surgical ablation of the area postrema in rats infused with Ang II for 5 days resulted in blunted hypertension compared to Ang II-treated sham-lesioned rats (73). Some regions of the brain are also able to sense and respond to changes in blood osmolality. Neurons within the subfornical organ (SFO) of the hypothalamus have salt-sensitive Na(x) channels that detect circulating sodium levels and modify salt-intake behavior (74). Similarly, the organum vasculosum of the lamina terminalis (OVLT) has neurons that can detect sodium and Ang II in the circulation and cerebrospinal fluid and respond by modulating body fluid homeostasis through thirst and SNS activation (75). A recent study of OVLT function in rats found that optogenetic activation of OVLT neurons in conscious Sprague-Dawley rats induced frequency-dependent increases in arterial BP and heart rate concomitant with SNS activation (76). On the other hand, lesion of OVLT attenuated both DOCA-salt-induced hypertension (77) and Ang II + high-salt-induced hypertension (78) in rats. Together, these regions of the brain integrate afferent signals regarding fluid homeostasis and RAAS signaling and respond accordingly to maintain BP and fluid balance.

In addition to the above mechanisms of neural regulation of BP, research groups have described a CNS-mean arterial pressure (MAP) set point (79). In this mechanism, the CNS adjusts sympathetic and parasympathetic activation to maintain adequate coronary and cerebral perfusion. It has been hypothesized that the CNS-MAP set point shifts to a higher BP threshold in hypertension, resulting in enhanced basal SNS activity. Hypertensive patients commonly display enhanced SNS activity, adding support to this hypothesis (80).

1.3.7 The Sympathetic Nervous System: Acute and Long-Term Stress

The sympathetic arm of the autonomic nervous system plays an important role in BP regulation, and in turn, the progression of hypertension (81). SNS activation causes the heart to increase both HR and SV, leading to enhanced CO. However, in the periphery, SNS activation results in vasoconstriction and increased peripheral vascular resistance. In the kidneys, SNS stimulation induces afferent arteriole vasoconstriction, resulting in reduced GFR and increased renal tubular water reabsorption and sodium retention. Interestingly, several clinical trials have investigated the effectiveness of renal denervation as a treatment for resistant hypertension, and results have generally been favourable (82). A 2021 observational study of patient responsiveness to renal denervation found that 48 months after the procedure, patients who were responsive and exhibited reductions to BP had significantly less major adverse cardiovascular events than patients who were not responsive to the procedure (83). Further, a 2022 study published in The Lancet found that renal denervation concomitant with administration of anti-hypertensive medications resulted in lasting BP reductions without any major safety concerns (84).

Apart from the direct actions of the SNS on the heart, vasculature, and kidneys, the SNS can also engage the RAAS by causing renin release from juxtaglomerular cells, promoting downstream prohypertensive responses through Ang II signalling. Both chronic stress and the acute stress response (“fight or flight” reaction) trigger changes in cardiovascular function and BP through SNS activation (85). The acute stress response increases heart rate, heart contractility, systemic vasoconstriction, and upregulates the levels of epinephrine and norepinephrine secreted by the adrenal medulla and sympathetic nerves, respectively. The sympathetic-adrenal-medullary (SAM) and hypothalamic-pituitary-adrenocortical (HPA) axes are the primary mediators of the stress response. Regarding the SAM axis, stress activates preganglionic sympathetic neurons in the thoracolumbar spinal cord that project to vertebral ganglia, and in turn, project to organs

including the adrenal medulla. Within the HPA axis, exposure to stress activates neurons in the paraventricular nucleus of the hypothalamus, causing the release of hormones (such corticotrophin releasing hormone and ADH). The downstream effect of HPA activation is the release of glucocorticoids from the inner adrenal cortex, which can potentiate SNS-mediated cardiovascular activation.

The baroreceptor reflex accounts for transient changes in BP, minimizing variation in systemic arterial BP in the short-term to maintain BP near a normal homeostatic set point (86). The baroreceptor reflex is a negative feedback mechanism in which specialized neurons called baroreceptors within the wall of the aortic arch and carotid sinus feel tension in concert with BP changes and respond extremely rapidly by modulating the autonomic nervous system, which in turn, modifies heart rate and vascular resistance to keep BP at a normal set point (Figure 1.2) (87). If BP is too high, baroreceptors in the wall of the aortic arch and carotid sinus will stretch and fire action potentials through the vagus and carotid sinus nerves, respectively, to the nucleus tractus solitarius of the brainstem. Signal integration results in parasympathetic nervous system (PNS) activation, SNS suppression, and the release of acetylcholine (Ach). Ach induces vasodilation of peripheral vessels and reduces cardiac output, thereby reducing BP. To the contrary, if BP is too low then the baroreceptor reflex will enhance SNS activity and suppress PNS activity, leading to the release of epinephrine and norepinephrine, which induce peripheral vasoconstriction and increases cardiac output to augment BP. Interestingly, targeting the baroreceptor reflex may offer a novel treatment for resistant hypertension. Electrical field stimulation of carotid baroreceptor axons has been shown to acutely reduce SNS activity and BP in patients, and the therapy is being investigated as a heart failure treatment (88, 89).

While the baroreceptor reflex accounts for transient changes in BP, longer term BP regulation by the SNS has been debated. Recent evidence has linked measurements of muscle sympathetic nerve activity (MSNA) to BP. Young men and women do not have a relationship between MSNA and MAP, however, men and women over the age of 40 demonstrate a strong correlation between increased MSNA and increased MAP (90). Additionally, the effect of sex was significant as MSNA correlated with MAP more strongly in older women than older men. As long term psychosocial stress is associated with hypertension, and hypertensive patients have a higher average incidence of psychosocial stress compared to normotensive patients, the long and short term SNS-mediated stress responses represent important pieces of the hypertensive mosaic (91).

In general, the mechanisms underlying long-term BP regulation by the SNS remain poorly understood, and more work needs to be done to address this aspect of SNS-mediated BP regulation. However, recent studies have suggested that stress can stimulate an amygdalar pathway that results in sympathetic stimulation of the bone marrow, leading to inflammatory responses (92). Notably, amygdalar activity was able to independently and robustly predict cardiovascular disease events; therefore, it is conceivable that this stress pathway could also worsen hypertension.

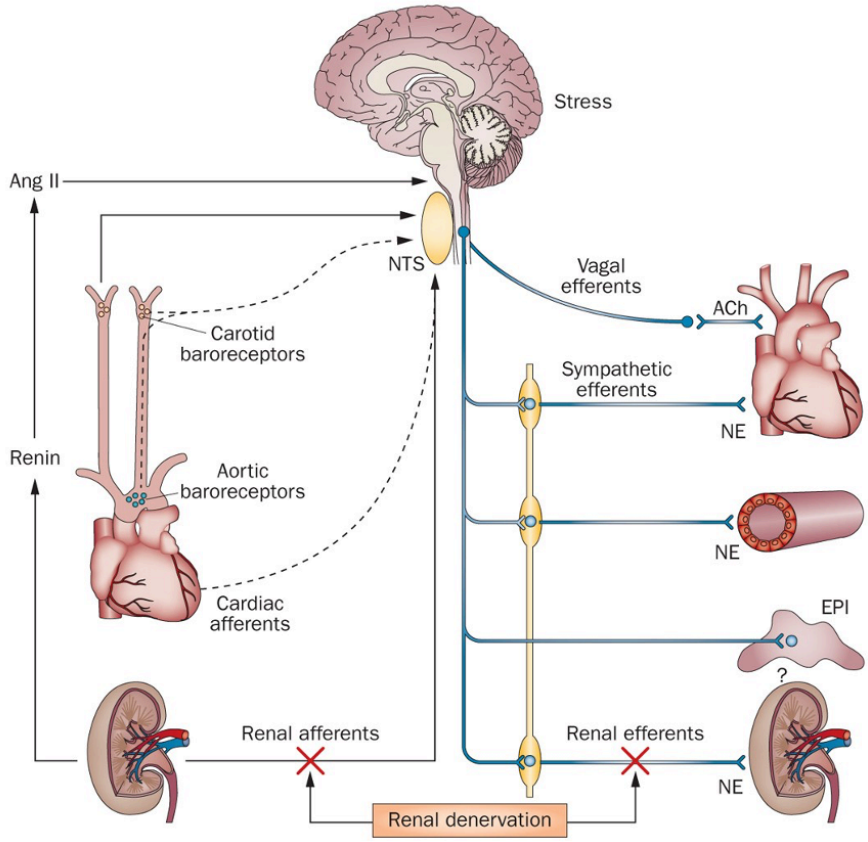

Figure 1.2: The baroreceptor reflex. Aortic and carotid baroreceptors send signals to the nucleus tractus solitarius (NTS), whereupon the signals are integrated, and the sympathetic and parasympathetic arms of the nervous system can be activated or inhibited accordingly. Sympathetic efferents signal through norepinephrine (NE) and epinephrine (EPI), leading to increased blood pressure (BP), while parasympathetic efferents signal through acetylcholine (Ach), reducing BP. Ang II augments BP and contributes to the baroreceptor reflex to low BP. Renal denervation shows favourable effects by reducing BP in resistant hypertension. Solid arrows

represent excitatory pathways while dashed arrows represent inhibitory pathways. Adapted with permission from Victor, R. (89).

1.3.8 The Hypertensive Heart

As BP is a function of CO and TPR, alterations to CO via increasing or decreasing SV and/or HR will influence systemic BP. Increasing HR and/or CO will increase BP, and this is important to maintaining perfusion in the face of changing oxygen requirements during physical activity. However, in the context of hypertension these factors serve to worsen the condition. Acute increases in TPR due to the action of endogenous vasoconstrictors, or chronic increases in TPR due to deleterious vascular remodeling as a result of hypertension, will result in increased load on the left ventricle (LV). This is due to the LV having to work harder to maintain blood circulation through stiff, non-compliant arteries, which would normally possess a high degree of elasticity. Over time, as in the case of hypertension, pressure overload will cause the LV to undergo maladaptive remodeling due to the added load put on cardiomyocytes, leading to LV hypertrophy. Experimentally, it was demonstrated in vivo that cardiac synthesis of myosin heavy chain increased 30% within hours of pressure overload (93). In the short term, LV hypertrophy helps normalize myocardial stress and maintain ejection performance in the face of pressure overload (94). However, in the long term LV hypertrophy is accompanied by cardiomyocyte apoptosis and cardiac fibrosis, which contribute to high rates of heart failure, stroke, coronary events, and overall increased mortality (95). In humans, LV hypertrophy was shown to influence cardiac SNS activity, with the degree of LV hypertrophy having a positive correlation with cardiac norepinephrine release in untreated hypertensive patients (96). Longitudinal clinical studies have shown that aggressive BP control may result in regression of LV hypertrophy (97). However, regression of LV hypertrophy remains infrequent despite BP control, and patients who are female, obese, or diagnosed with diabetes or metabolic syndrome are more likely to not have regression of LV hypertrophy.

1.3.9 Hypertension and Environmental Hazards

There are many known and hypothesized etiological factors in the development of hypertension. Some well-described examples include obesity, insulin resistance, excess alcohol consumption,

high salt intake, and living a sedentary and/or high-stress lifestyle (7). While behavioral modification and lifestyle changes can contribute to reducing BP, some nearly unavoidable aspects of urban life may be risk factors for hypertension. Regular exposure to aircraft (98) and urban (99) noise pollution, as well as short- and long-term exposure to urban air pollution (100) is strongly associated with hypertension. Incidentally, a population-based cohort study of over 1.6 million Toronto residents from 2001 to 2015 found that high levels of road traffic noise pollution was associated with an increased risk for both diabetes and hypertension (101). Surprisingly, individuals within the highest income bracket had the largest risk for diabetes and hypertension in association with daily road traffic noise compared to those in the lowest income bracket, and this could be due to high income earners having high-stress careers.

1.3.10 The Genetics of Hypertension: Complex, Broad, and Pleiotropic

While measures can often be taken to reduce the negative impact of the environment on cardiovascular health, lifestyle accommodations must be made to account for unmodifiable genetic variables. An example of this is reducing salt intake in salt-sensitive individuals, with many aspects of salt-sensitivity being associated with genetic factors. Early experiments with rats gave rise to the idea that genetic factors play an important role in hypertension. A landmark 1962 publication by Dahl *et al.* reported on selective inbreeding experiments that resulted in the separation of two strains of rats, one salt sensitive and the other not (102). Since the advent of genome sequencing, research has uncovered countless single nucleotide polymorphisms (SNPs), micro-RNAs (miRs) (103), DNA modifications (DNA methylation and histone modifications (104)), and other genetic and epigenetic factors associated with hypertension (105). These factors are pleiotropic and encompass a wide range of cells, tissues, and organ systems, both directly related to the cardiovascular system and seemingly unrelated. SNPs may be in coding or non-coding regions, and the persistent pleiotropy of BP SNPs results in limitations in our ability to understand the impact of any single SNP on its own (106).

Recent publications have investigated the presence of differentially expressed miRs in hypertensive patients, and several miRs relevant to inflammation have been detected in hypertensive human cohorts (107). Small interfering RNAs (siRNAs) may provide a new therapeutic avenue to reduce BP, with studies in spontaneously hypertensive rats (SHRs) demonstrating that subcutaneous injection of siRNAs targeting hepatic angiotensinogen can

provide stable and sustained elimination of Ang II, and this could complement traditional pharmaceuticals that target RAAS components (108).

1.3.11 Classes of Common Antihypertensive Medications

Lifestyle modification remains the first line of treatment for hypertension, and dietary changes, physical activity, and weight loss can all help to reduce BP (6). However, lifestyle changes alone are often unable to reduce BP in many patients with established hypertension. As a result, a wide variety of pharmaceuticals have been developed with the aim to reduce BP back to healthy levels. First line monotherapy drugs recommended for treating patients with hypertension without other compelling indications include diuretics (thiazide and thiazide-like), β-blockers, ACE inhibitors, angiotensin receptor blockers (ARBs), or long-acting calcium channel blockers (CCBs) (6). In addition, single-pill combination therapies are often used as a first-line treatment, in which two or more of the above classes of medications are combined in a single pill for ease of delivery and increased effectiveness. Each category of antihypertensive medication exploits a different aspect of the cardiovascular system to reduce BP. For example, renin inhibitors, ACE inhibitors, and ARBs all function by inhibiting different components of the RAAS. Renin inhibitors (such as aliskiren) inhibit the rate-limiting reaction of RAAS activation, the conversion of angiotensinogen to Ang I by renin, the inhibition of which results in absence of Ang II (109). In contrast, ACE inhibitors (such as captopril and enalapril) function by reducing both the production of Ang II and the hydrolysis of the vasodilator bradykinin through inhibition of ACE. Finally, ARBs (such as losartan and valsartan) work by selectively blocking the AT_1R, thereby blocking Ang II-dependent vasoconstriction and sodium retention. Consequently, ARBs are more selective as they have a high affinity for the AT_1R and do not directly influence global Ang II production or target the AT_2R. There is controversy on whether ACE inhibitors or ARBs are preferable for treatment of cardiovascular disease and hypertension, but it seems likely that there are few differences between the final effects of either, and they are used in most cases indistinguishably.

Unlike drugs that target components of the RAAS, β-blockers (such as propranolol) target receptor binding sites for the catecholamines epinephrine and norepinephrine on β-adrenergic receptors of the SNS (110). Blocking β-adrenergic activation reduces SNS-mediated increases in HR, cardiac contractility, and systemic vasoconstriction. This is especially useful in young hypertensive patients, in which SNS overactivation contributes to hypertension. On the contrary,

CCBs function by disrupting the movement of calcium through calcium channels, disrupting several physiological processes. Dihydropyridine CCBs (such as amlodipine) lower BP by reducing arterial vasoconstriction (which is dependent on intracellular calcium flux) and promoting vasodilation of VSMCs (111). Alternatively, non-dihydropyridine CCBs generally lower BP by blocking calcium flux within the myocardium, thereby reducing the force of contraction of the myocardium (reducing cardiac contractility), slowing the conduction of electrical activity within the heart (reducing heart rate), and promoting vasodilation. Some classes of CCBs can also block calcium signaling in the zona glomerulosa of the adrenal cortex, reducing aldosterone production (112). This is especially useful in patients with hypertension related to primary aldosteronism, where there is an overproduction of aldosterone due to adrenal hyperplasia or tumours (113).

In contrast to the other classes of antihypertensive medications, diuretics reduce BP by promoting natriuresis and diuresis and reducing plasma volume, although the mechanism whereby BP is actually reduced remains controversial. This is achieved by altering the reabsorption of sodium and water in the kidney, and different classes of diuretics have unique mechanisms of action. Thiazide diuretics (such as hydrochlorothiazide) function by inhibiting the sodium-chloride symporter within the distal convoluted tubule, resulting in water retention within urine (114). Alternatively, loop diuretics (such as furosemide) function by inhibiting reabsorption of sodium in the ascending loop of the nephron, leading to enhanced water excretion, although they are less effective as antihypertensives except in chronic kidney disease and renal failure. Diuresis also results in excretion of other important ions, including potassium and calcium, and excessive diuresis can induce a state of hypokalemia or hypocalcemia, respectively. As a result, drugs have been developed to spare ions from being excreted in excess. Potassium-sparing diuretics include aldosterone antagonists such as spironolactone, eplerenone, and more recently finerenone and exaserone, as well as epithelial sodium channel blockers like amiloride and triamterene. Both thiazide diuretics and potassium-sparing diuretics are considered to be calcium-sparing, while loop diuretics promote calcium excretion which can affect bone density in aging patients. In fact, thiazide diuretics can result in hypercalcemia and hypocalciuria, with the latter useful in patients with hypercalciuria and renal stones.

1.4 The Arterial Wall

1.4.1 Structure and Composition of the Arterial Wall

Arteries provide the physical infrastructure to circulate oxygenated blood. The three main categories of arteries are large elastic arteries, medium muscular arteries, and small arteries/arterioles. The arterial wall is made up of three distinct layers: the tunica intima, tunica media, and tunica externa or adventitia, which will be discussed below.

1.4.2 The Tunica Externa/Adventitia

The outermost layer of the arterial wall, the adventitia, is composed of collagen and elastin rich extracellular matrix (ECM), fibroblasts, nerve cells (nervi vasorum), and infiltrating immune cells. The adventitia itself is vascularized by small vessels known as vasa vasorum, which carry oxygen and nutrients to the cells of the arterial wall. Expansion and proliferation of vasa vasorum is associated with vascular disease, and vasa vasorum may serve as a conduit for leukocyte entry into the adventitia in inflammatory contexts (115). Finally, the adventitia is also responsible for anchoring the vessel to the surrounding tissue.

1.4.3 The Tunica Media and Vascular Smooth Muscle

The middle layer of the arterial wall, the tunica media, is a highly functional layer composed of VSMCs, elastic membranes, and collagen. The smooth muscle cells of the tunica media are responsible for carrying out vasoconstriction and vasodilation, while collagen provides structural support and strength for the vessel and elastin allows the vessel to recoil and be flexible under load. Loss of flexibility and increased stiffness of the arterial wall is associated with increased deposition of collagen and fibronectin within the tunica media, and a high ratio of collagen to elastin is associated with high arterial stiffness (116). Consequently, the tunica media is an important site of hypertensive vascular remodeling and increases in media thickness are indicative of worsened hypertension. Indeed, an increased ratio of media thickness to internal lumen size was found to be associated with the occurrence of cardiovascular events in patients (117).

1.4.4 The Tunica Intima and Vascular Endothelium

The innermost layer of the arterial wall, the tunica intima, is composed of a highly sensitive monolayer of vascular endothelial cells (simple squamous epithelial cells) anchored to the tunica media via a basement membrane composed of collagen and laminins. The blood-facing luminal surface of endothelial cells is coated with a highly specialized layer of receptors, carbohydrates, lipids, proteins, glycoproteins, proteoglycans, and other macromolecules, termed the glycocalyx, which acts as a selectively permeable barrier and provides a smooth luminal surface for blood and platelets to circulate without aggregating (118). Further, vascular endothelial cells can sense mechanical forces generated by circulating blood and respond by secreting factors into the lumen or underlying VSMCs, fine tuning vascular tone (119). In the context of inflammation, vascular endothelial cells can be activated and express adhesion molecules and chemotactic signals that promote diapedesis, or the movement of leukocytes from the circulation to sites of infection or injury beyond the vasculature (120). Following endothelial activation as a result of injury or infection, surface expression of integrins, selectins, and adhesion molecules induces slow rolling of leukocytes, followed by arrest, adhesion, crawling, scanning, and finally transmigration from the vascular lumen to interstitial tissue following chemokine signals and chemotactic gradients (121). This is especially relevant to hypertension, where circulating immune cells can detect vascular injury via activated endothelial cells, infiltrate the vessel wall, and contribute to local inflammation from within the vascular tissue (122).

1.4.5 Perivascular Adipose Tissue Supports Vessel Structure and Function

Perivascular Adipose Tissue (PVAT) refers to the fat that surrounds blood vessels. PVAT plays a structural role in supporting vessels in addition to its secretory and endocrine capabilities. Mammals possess three distinct types of adipose tissue: white adipose tissue (WAT) that functions primarily to store energy as lipids, brown adipose tissue (BAT) that has thermogenic properties and aids in maintenance of body temperature, and beige adipose tissue that can acquire a BAT-like phenotype upon cold exposure (123). PVAT can resemble WAT or BAT depending on vessel localization. For example, thoracic aortic PVAT is morphologically similar to BAT, and experiments in mice deficient in PVAT revealed that PVAT plays an important role in maintaining intravascular temperature (124). In contrast to thoracic aortic PVAT, PVAT surrounding mesenteric arteries and the abdominal aorta is more WAT-like in appearance, with large lipid

droplets (125). Interestingly, PVAT surrounding the aorta and mesenteric arteries was found to have expression of all renin-angiotensin system components except renin (126). In addition, PVAT is capable of secreting adiponectin, which is known to possess anti-inflammatory and anti-diabetic properties (127). Adiponectin also has potent anticontractile properties via its ability to upregulate nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) (128). Therefore, PVAT-derived adiponectin has the ability to protect against vascular dysfunction in hypertension. In obesity, WAT has been shown to release Ang II (129), and PVAT can release additional factors that influence vascular tone including norepinephrine and nitric oxide (130). In addition, vasa vasorum in the adventitia adjacent to PVAT could act as a conduit for vasoactive compounds or immune cell infiltration in an "outside-in" manner, rather than having to cross the luminal side of the vessel wall (131). In general, PVAT has a demonstrated ability to secrete factors that influence vascular reactivity, vascular inflammation, and vascular tone (132). However, more work needs to be done to determine the signals that induce PVAT to secrete factors that aggravate hypertension and determine if PVAT represents a druggable target.

1.5 Vascular Oxidative Stress and Remodeling

1.5.1 Reactive Oxygen Species: Sources and Implications

ROS are highly reactive molecules derived from molecular oxygen that can interact with and modify important cellular macromolecules. While ROS are important signaling molecules in many physiological contexts, conditions that increase oxidative stress, such as hypertension, result in an overabundance of ROS that can irreversibly modify proteins and lipids (133). In the case of enzymes, lipids, and genetic material, oxidation by ROS can promote dysfunction in these systems or prevent functioning altogether. Generation of ROS is increased in both experimental and clinical hypertension (134, 135), and treatment with antioxidants has been shown to improve vascular function and reduce end-organ damage (136). Primary sources of ROS generation in hypertensive blood vessels include NOX enzymes (137), uncoupled NOS (138), xanthine oxidase, and mitochondrial (139) and endoplasmic reticulum (ER) oxidative stress (140). While vascular dysfunction promotes ROS production, increased levels of ROS potentiate and further develop vascular dysfunction, leading to a positive feedback loop of increasing oxidative stress and worsening vascular function.

1.5.2 Linking Oxidative Stress to Vascular Dysfunction: Nitric Oxide Production

The primary role of NOX enzymes is to produce ROS, and NOX-derived ROS are important to physiological cell processes including proliferation, migration, and differentiation (141). However, NOX-derived ROS can induce further ROS generation by disrupting other biochemical processes, an example being inactivation of tetrahydrobiopterin (BH_4), leading to eNOS uncoupling and further ROS generation (142). Protein levels of NOX enzymes are increased in clinical hypertension, and a recent study found links between aging, hypertension, eNOS uncoupling, and increased expression of NOX5 in hypertensive patients (143). Further, Ang II has been shown to be a powerful activator of NOX, promoting ROS production (144).

Mechanistically, excessive ROS generated by NOXes can interact with eNOS-derived nitric oxide to form peroxynitrite, an unstable and reactive molecule that oxidizes BH_4 into BH_2, lowering the availability of BH_4 required by eNOS to generate nitric oxide (145). Nitric oxide is integral to the regulation of vascular tone, whereby nitric oxide binds soluble guanylyl cyclase (sGC) within VSMCs, leading to generation of the second messenger cyclic guanosine monophosphate (cGMP), which then modulates various cellular components including inactivating myosin light chain kinase and activating myosin light chain phosphatase, leading to vasodilation (146, 147). The main consequence of eNOS uncoupling is generation of ROS instead of nitric oxide, leading to further oxidative stress, reduced bioavailability of nitric oxide for vasodilation, and augmented vascular dysfunction. Evidence suggests that upregulating the activity of dihydrofolate reductase (DHFR), which is the rate limiting salvage enzyme for eNOS cofactor BH_4, may help abrogate eNOS uncoupling and restore eNOS function in hypertension (148).

1.5.3 Vascular Oxidative Stress Promotes Inflammation

Oxidative stress and inflammation are closely linked phenomena that can propagate one another in the context of hypertension. Excessive oxidative stress damages cellular machinery needed to maintain homeostasis, leading to expression of markers of tissue injury that attract immune cells (149). However, an additional consequence of vascular oxidative stress is the potential for formation of neoantigens. Neoantigens are traditionally associated with cancer, where they represent tumor-specific antigens generated by mutations which would not normally be present in

any healthy somatic cells (150). However, highly immunogenic neoantigens could form in hypertension under conditions of excessive oxidative stress. Neoantigens may arise from oxidatively modified proteins and lipids, and in turn, trigger activation of immune cells. Putative neoantigens specific to hypertension have remained elusive, but recent studies have implicated isolevuglandin-modified protein adducts as potential neoantigen candidates (151). Isolevuglandins (or isoketals) are highly reactive oxidation products formed as a result of ROS-mediated lipid peroxidation, and they have the ability to extensively cross-link proteins and alter protein function (152). Immune cells with highly specific T cell receptors that lack a requirement for antigen presentation, namely γδ T cells, may provide more information on neoantigens in hypertension (153). As of yet, very few γδ T cell receptor (TCR) antigens have been discovered, so identifying these antigens may shed light onto new molecules implicated in hypertension as γδ T cells have been demonstrated to play a critical role in hypertension (154).

1.5.4 Hypertensive Vascular Remodeling

It has long been understood that increased peripheral resistance is the principal driving factor behind essential hypertension (155). Deleterious remodeling of resistance arteries is both a consequence of high BP and a driver of further BP increases, and may represent the first manifestation of end-organ damage (156). Gluteal biopsies from patients with essential hypertension show that the remodeling is inward and eutrophic rather than hypertrophic, which results in an increased media-to-lumen ratio and a smaller lumen with no increase in the media cross-sectional area, the latter being the true measure of hypertrophy of the media (Figure 1.3) (157, 158). In animal models, eutrophic remodeling of resistance arteries is commonly observed in hypertension associated with the involvement of the RAAS (159). However, hypertrophic remodeling is primarily observed in large arteries as well as in patients with secondary hypertension, such as renovascular hypertension (160) and in hypertensive diabetics (161), as a result of smooth muscle hypertrophy. Components of the vessel wall are very sensitive to stress and mechanical forces, and alterations in BP manifest as changes in gene expression within the cells of the vascular wall (162). Gene expression programs for eNOS, local production of Ang II, and synthesis of transforming growth factor-β (TGFβ) are influenced by changes to cyclic stretch and stress, and activation of these genes in the context of pressure overload influences ECM synthesis and vascular wall composition (163). It is not fully understood how cells of the vessel

wall sense a changing mechanical environment, but integrins have been found to play a role in sensing intramural stress by fibroblasts and smooth muscle cells (164), and endothelial cells are able to sense shear stress through cadherin complexes (165). A recent review of endothelial cell molecular sensors described a mechanosensory pathway composed of platelet endothelial cell adhesion molecule (PECAM-1) complexed with vascular endothelial growth factor receptor (VEGFR)-2/VEGFR-3 and VE-cadherin (166). This complex is speculated to sense shear stress at endothelial cell junctions and respond by initiating a signaling cascade that activates various transcription factors and gene expression programs related to endothelial function. Other actions initiated by activation of this mechanosensory complex include cytoskeletal remodeling, formation of stress fibers in the direction of flow, and modulation of eNOS. Finally, transient receptor potential (TRP) ion channels on vascular endothelial cells can interact with these mechanosensory pathways and influence several vascular characteristics, such as vascular tone, vascular permeability, oxidative damage, vascular remodeling, and adhesion of immune cells.

To respond to changing mechanical forces, the composition of the vascular wall and ECM is highly dynamic yet regulated. Matrix metalloproteinases (MMPs) carry out modifications to the composition of the vascular wall by hydrolyzing ECM components like elastic fibers and collagens, and MMP activity can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) (167). Consequently, the equilibrium of MMPs and TIMPs determines vascular ECM turnover, and changes to this equilibrium due to stress or inflammation is implicated in hypertension. Other proteases are also involved in vascular remodeling. A role for a disintegrin and metalloproteinases (ADAMs) has been observed in hypertension, with ADAM-17 being involved in shedding of TGFβ and tumor necrosis factor-alpha (TNFα) in hypertensive mouse carotid arteries, and both TGFβ and TNFα contribute to deleterious vascular remodeling in hypertension (168). Further, some serine and cysteine proteases modulate vascular remodeling by activating MMPs or degrading elastin/collagen in the extracellular space, respectively (169, 170). Factors that can influence ECM turnover in blood vessels include circumferential wall stress signals, shear stress, inflammatory cytokines and chemokines, as well as growth factors and oxidative stress (171). The inflammatory pathways involved in promoting vascular remodeling will be discussed in section 1.7.

Stiff, hypertensive arteries are associated with fragmentation or rupture of elastin fibers, the predominant ECM constituent in the tunica media determining vascular mechanical properties

(172), and elastin degradation reduces arterial compliance and elasticity (173). Conversely, hypertensive maladaptive remodeling of the arterial wall is associated with increased collagen deposition and an increased collagen to elastin ratio in hypertensive patients, which leads to further limitations in the distensibility of the vessel (174). Other molecules that can influence arterial stiffness include fibronectin, fibulin, glycosaminoglycans, and proteoglycans (175). Consequently, vascular wall composition is highly dynamic and responsive to mechanical stress. While the initial stages of vascular remodeling seem to provide a protective and adaptive mechanism to avoid tissue injury and account for increased mechanical forces from high BP, inability to return BP back to healthy levels augments the remodeling process to further develop and quickly become maladaptive.

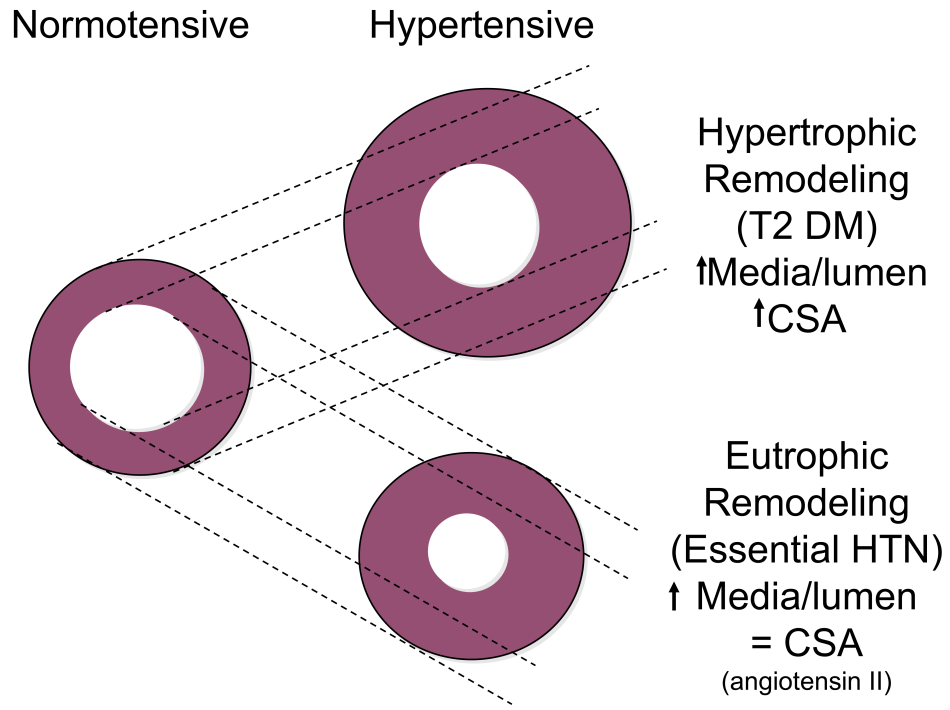

Figure 1.3: Eutrophic versus hypertrophic vascular remodeling. Patients with secondary hypertension, as in the case of type 2 diabetes mellitus (T2 DM) or renovascular hypertension, develop more pronounced hypertrophic remodeling due to smooth muscle hypertrophy. Conversely, individuals with essential hypertension develop more pronounced inward eutrophic remodeling, as do animals made hypertensive by infusion of Ang II. Eutrophic remodeling preserves vessel cross sectional area (CSA) but reduces the lumen diameter, resulting in an increased media to lumen ratio. Hypertrophic remodeling results in vessels developing a larger

CSA, an increased media to lumen ratio, and preserved lumen diameter. Adapted with permission from Schiffrin et al. (159).

1.5.5 Vascular Stiffness and Measurements of Pulse Wave Velocity

Advanced, maladaptive vascular remodeling due to longstanding hypertension and unchecked vascular inflammation leads to dramatically stiffer and thicker blood vessels, and the degree of stiffening is determined mainly by the amount of collagen and elastin. Stiffness can be inferred in the clinic via measurements of pulse wave velocity (PWV), as stiffer vessels demonstrate a proportionally faster PWV. The pressure wave generated by ejection of blood from the heart is partially reflected at arterial junctions and diffusely reflected as it encounters portions of the arterial tree with changing stiffness (116). In turn, these reflections travel back towards the aorta and can be measured in each point of the cardiac cycle. Young, compliant, and healthy arteries exhibit a comparatively slow PWV, and wave reflections arrive later in the cardiac cycle increasing diastolic pressures. Conversely, older, stiffer arteries demonstrate a much higher PWV and reflected waves arrive much sooner, increasing systolic pressures. The augmented systolic pressure generated by rapid pulse wave reflections in stiff vessels can be represented by the augmentation index, which is indicative of the increased afterload and work put onto the LV to overcome pressure generated by reflected waves (176). Augmented pressures represent a further mechanism in which the LV is overworked, leading to LV hypertrophy. However, augmentation index is also influenced strongly by left ventricular myocardial shortening velocity in addition to arterial stiffness and wave reflection, leading to controversy in its use as a proxy for the magnitude of wave reflection and arterial stiffness (177).

1.6 The Immune System in Hypertension

1.6.1 Early Investigations into the Role of the Immune System in Hypertension

If inflammation is considered to be a spectrum with homeostasis at one end, the stress response in the middle, and inflammation at the other end, increases in BP leading to increased forces imparted on vessels will result in a shift from homeostasis, to the stress response, and finally towards inflammation and maladaptive remodeling in an attempt to return to the homeostatic set point

(178). Immunity and inflammation are notably absent from the initial 1949 conceptualization of the mosaic theory of hypertension (11). However, newer iterations of the mosaic theory, including the 1982 revisited and expanded theory (179), and the latest 2020 definition in the Canadian Journal of Cardiology (12), have made note of and subsequently expanded on the role of the immune system in hypertension.

Immunological research techniques evolve quickly, and it is not uncommon for dogmas within the field to be challenged, reimagined, or toppled altogether. Consequently, the scope of pathological conditions attributed to immune cells and their effector functions is constantly expanding. This is also true in the case of hypertension. Most research works investigating the role of the immune system in the pathogenesis of hypertension have been published within the last three decades. A literature search for "immune system AND hypertension" in the National Library of Medicine PubMed database yields an average of less than 200 relevant publications per year until 2006, whereupon the annual number increases substantially, eventually reaching 746 publications in 2021 (numbers as of August 12th, 2022).

Indications that the immune system plays a role in hypertension began appearing in the scientific literature in the latter half of the twentieth century. In 1976, a study investigating DOCA-salt hypertension in mice found that nude mice, which lack both a functional thymus and mature lymphocytes, had blunted hypertension in response to salt-feeding compared to haired-mice (180). The authors also noted "round cell infiltration" in the renal vasculature of the haired mice, and thymus grafting into nude mice recapitulated the hypertensive response. Nearly a quarter-century later, a pioneering study by Muller *et al.* (2000) demonstrated that blocking nuclear factor kappa B (NF-$_{\kappa}$B), an immune transcription factor with broad proinflammatory implications, ameliorates inflammation and protects from end-organ damage in a human renin and angiotensinogen double-transgenic rat model of hypertension (181). The following year in 2001, another important study using Sprague-Dawley rats discovered that treatment with the immunosuppressant mycophenolate mofetil (MMF), which blocks immune cell infiltration, diminishes the pressor response to high salt after Ang II challenge (182). It was later confirmed that treatment with MMF also attenuated DOCA-salt hypertension in Sprague-Dawley rats via reducing T cell kidney infiltration and cytokine production, as well as increasing nitric oxide availability (183). In 2005, De Ciuceis *et al.* were the first to identify a specific cell line involved in the pathogenesis of hypertension (184). Ang II-infused osteopetrotic mice, which are deficient in macrophage colony-stimulating factor

(M-CSF), exhibited blunted hypertension and reduced endothelial dysfunction, vascular remodeling, and oxidative stress, compared to Ang II-infused wild-type (WT) mice. Osteopetrotic mice have a severe deficiency of macrophages due to defective M-CSF production. As a result, vascular macrophage infiltration and accumulation was inhibited in Ang II-infused osteopetrotic mice. Consequently, these experiments demonstrated a role for macrophages in the development of hypertension and vascular dysfunction, opening the door to future studies on immune cells in hypertension. Indeed, two years later, Guzik *et al.* (2007) demonstrated that T cells play a role in both Ang II and DOCA-salt induced hypertension, as genetic deletion of recombinase-activating gene (*Rag*; *Rag-1*$^{-/-}$) blunted the pressor response and prevented vascular dysfunction (122). Conversely, the authors found that adoptive transfer of T cells but not B cells, both of which are diminished in *Rag-1*$^{-/-}$ mice, restored the hypertensive response.

The 2005 study by De Ciuceis *et al.* and the 2007 study by Guzik *et al.* represent important milestones in research on hypertension, as they were the first to clearly demonstrate roles for innate and adaptive immunity, respectively, in the pathogenesis of hypertension (122, 184). Building on these reports, a 2010 publication by Crowley *et al.* demonstrated that severe combined immunodeficient (*scid*) mice, which lack mature lymphocytes, exhibit a blunted pressor response and reduced cardiac and renal injury from Ang II infusion (185). Three years later, Mattson *et al.* (2013) demonstrated that Dahl salt-sensitive rats that had inactivating mutations introduced to the *Rag1* gene exhibited blunted hypertension in response to a high-salt diet (186). Thanks to major advances in the technology used for immunological research, recent investigations into inflammation and hypertension have been able to pinpoint the role of individual components of the immune system, with both protective and deleterious effects being attributed to cells representing innate and adaptive immunity.

1.6.2 Hypertension is a Form of Systemic, Sterile Inflammation

Inflammation represents a rapid, unspecific, and oftentimes local response to invading pathogens, irritants, and injury, in an attempt to contain the offending agent, limit further damage, and promote repair. While this is normally beneficial in the case of eliminating or neutralizing infectious agents, in the case of autoimmunity and disorders that do not involve infection this is oftentimes maladaptive. Hypertension involves many mechanisms that contribute to inflammation, including high levels of oxidative stress (143), increased BP and mechanical stress on cells of the heart and

vessel wall (187), abnormal sodium handling (17), neuroinflammation and neuroimmune interactions (188), and more recently, dysregulation of the gut microbiome (189). On a fundamental level vascular inflammation involves three central factors: the vascular wall, circulating immune cells, and the signals that initiate their interaction. The vessel wall and vascular remodeling were described in sections 1.4 and 1.5, and in subsequent sections the focus will shift to the role of immune cells, cytokines, and what signals promote immune cell activation in hypertension.

1.7 Important Cytokines Involved in the Progression of Hypertension

Cytokines are a broad class of immunomodulatory compounds composed of peptide, protein, and glycoprotein constituents that can promote, reduce, or fine-tune the inflammatory response. Therefore, cytokines represent a potential therapeutic target to reduce end-organ damage associated with hypertension and inflammation. Studies have characterized differential production of various cytokines among hypertensive versus non-hypertensive patients and animals, and further investigations have attempted to block, infuse, or modify the signaling pathways that culminate in cytokine production and release. Additionally, anti-inflammatory cytokines that reduce the immune response may be of utility to researchers attempting to prevent excess inflammation in conditions like hypertension or autoimmunity. Summaries of some prominent proinflammatory and anti-inflammatory cytokines important to the pathogenesis of hypertension are included in the subsequent sections.

1.7.1 Interferon-γ

The proinflammatory cytokine interferon-γ (IFN-γ) contributes to the pathogenesis of hypertension. Seven days of Ang II infusion caused C57BL/6 mice to have elevated IFN-γ mRNA expression and pronounced endothelial dysfunction compared to sham-treated mice, and genetic knockout of IFN-γ significantly improved the endothelium-dependent and independent vasodilatory response (190). In terms of BP, Saleh *et al.* (2015) demonstrated that IFN-$\gamma^{-/-}$ mice were less sensitive to Ang II infusion than WT mice, as IFN-γ knockout animals developed a 20 mmHg lower increase in BP than WT mice after 14 days of Ang II infusion (191). In support of these findings, Markó *et al.* (2012) demonstrated that Ang II-infused IFN-γR knockout mice

exhibited improved GFR and reduced kidney inflammation, cardiac hypertrophy, and cardiac T cell and macrophage infiltration compared to WT Ang II-treated mice (192). However, Guzik *et al.* found that while WT C57BL/6 mice infused with Ang II had increased T cell IFN-γ production after two weeks, administering a neutralizing anti-IFN-γ antibody did not reduce vascular ROS production or the pressor response (122). Consequently, the model of hypertension and dose and length of Ang II infusion may explain some of the variation among experimental outcomes, as these factors will influence the pressor response and level of immune activation.

1.7.2 TNFα

TNFα contributes to the progression of hypertension. Plasma levels of TNFα are increased in hypertensive patients (193), and incubation of human umbilical vein endothelial cells (HUVECs) with plasma from hypertensive patients with or without a TNFα neutralizing antibody (infliximab) resulted in reduced HUVEC apoptosis with TNFα inhibition (194). Animal studies also indicate that TNFα plays a role in the vascular abnormalities associated with hypertension. In a mouse model of metabolic syndrome, New Zealand Obese mice displayed increased visceral fat and BP, as well as hypertrophic remodeling of mesenteric resistance arteries, impaired endothelium-dependent relaxation, high-levels of PVAT ROS production and oxidative stress, and a notable upregulation of TNFα mRNA within the PVAT (195). It was later confirmed that TNFα contributes to vascular oxidative stress, as WT C57BL/6 mice infused with Ang II and treated with a TNFα inhibitor exhibited a reduced pressor response and reduced vascular ROS compared to mice given an IgG control (122). Further, neutralization of TNFα with infliximab in a SHR model of hypertension blunted BP increases and prevented the development of LV hypertrophy (196).

Apart from the vascular abnormalities attributed to TNFα, TNFα is also implicated in glomerular injury and renal dysfunction, and it interacts with RAAS components. In vitro incubation of human podocytes with Ang II upregulates TNFα-related gene expression and cytokine release, and blocking AT_1R or AT_2R with Losartan or PD123319, respectively, dampened TNFα production (197). In addition, neutralizing TNFα with an antibody reduced the pro-proliferative effects of Ang II, demonstrating an interaction between these pathways. In general, TNFα is speculated to contribute to hypertension mainly by its actions on the vasculature.

1.7.3 IL-1β

IL-1β is a proinflammatory cytokine belonging to the IL-1 cytokine superfamily, which induces the expression of genes related to promoting inflammation (198). IL-1 family cytokines are described to be "early-response" cytokines, referring to the fact that they are released in the earliest stage of the immune response. Recognition of IL-1β by the IL-1 receptor stimulates T cell production and release of IL-6 (199) and IL-17A (200). IL-1β also alters the permeability of the vascular wall by inducing changes to endothelial cell-cell junctions, which in turn, may promote immune cell infiltration (201). Plasma levels of IL-1β are higher in hypertensive patients, and IL-1β concentrations are associated with arterial stiffness as determined by PWV (193). The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), which involved over 10,000 participants, demonstrated that the overall risk for major adverse cardiovascular events could be decreased by inhibiting IL-1β with Canakinumab, a monoclonal antibody (202). However, a secondary analysis saw no reduction in BP or rates of incident hypertension in patients receiving Canakinumab, indicating that the mechanisms underlying reduced risk for adverse cardiovascular events is BP-independent (203). In general, more work needs to be done to address the role of IL-1β in hypertension from a mechanistic standpoint.

1.7.4 IL-10

IL-10 is an important anti-inflammatory cytokine produced by a wide range of immune cells, including macrophages, DCs, and various T cell subsets (204). IL-10 functions as an autocrine or paracrine signal to halt proinflammatory cytokine and chemokine production by innate and adaptive immune cells, including macrophages, neutrophils, dendritic cells, and T cells. In addition, IL-10 can inhibit the expression of costimulatory molecules on antigen presenting cells (APCs), the expression of which is required for T cell activation (205). While IL-10 is generally understood to blunt T cell responsiveness via inhibiting APC activity, IL-10 has also been demonstrated to act directly on proinflammatory cluster of differentiation (CD)4^+ helper T cell subsets to induce T cell non-responsiveness or anergy. Additionally, IL-10 promotes the survival of anti-inflammatory $CD4^+$ regulatory T cell (Treg) subsets.

IL-10 has been shown to play a role in animal models of hypertension. IL-10 knockout mice exhibited an exaggerated BP response to Ang II infusion compared to Ang II-treated C57BL/6J WT mice, and subcutaneous infusion of IL-10 into WT mice blunted Ang II-induced

BP elevation (206). In addition, WT mice made hypertensive by Ang II infusion had reduced plasma concentrations of IL-10, showing that IL-10 plasma availability is impacted by Ang II-induced hypertension. A 2020 study by Gillis *et al.* found similar results in male SHRs infused with IL-10 (207). The authors observed that male SHRs exhibit higher BP than female SHRs, and infusion of IL-10 reduced SBP in male but not female SHRs. The observed sex difference regarding BP in relation to IL-10 infusion may be explained by the fact that female SHRs have higher plasma IL-10 concentrations than male SHRs at baseline, which may explain the lower BP observed in female SHRs. In turn, female SHRs may already have sufficient levels of circulating IL-10, and infusion of additional IL-10 did not yield any BP-reducing benefit in female SHRs. Mechanistically, IL-10 is known for having the ability to reduce immune cell activation/activity, but IL-10 could also reduce BP by modulating NOS activity. IL-10 augments nitric oxide production under pro-inflammatory conditions in vitro via increased endothelial expression of NOS in human cell lines (208). Consequently, IL-10 and the cells that produce it could represent novel therapeutic targets to reduce hypertension-induced inflammation.

1.7.5 IL-17A

The IL-17 family of cytokines consists of six structurally similar members, IL-17A to IL-17F. The role of IL-17A (commonly referred to as IL-17) in hypertension has been studied more extensively than the other IL-17 members; therefore, IL-17A will be the focus of this section. The more nuanced roles of IL-17B-F in homeostasis and disease are less well understood, but roles have been found for these cytokines in recruiting immune cells, promoting skin and intestinal barrier maintenance, aiding tumor surveillance, and eliminating pathogens (209). IL-17A acts on a wide range of cell types that influence the progression of hypertension and its associated sterile inflammation. Mechanistically, IL-17A can inhibit endothelial cell nitric oxide production by promoting phosphorylation of eNOS inhibitory site, threonine 496, reducing the availability of endogenous vasodilators (210). IL-17A in combination with TNFα also induces VSMC expression of inflammatory cytokines and chemokines, which promotes immune cell recruitment to the vessel wall (211). In the aorta, IL-17A promotes collagen synthesis by aortic fibroblasts, which contributes to aortic stiffening in hypertension (212). Several studies have provided evidence that genetic deletion or pharmacological blockade of IL-17A lowers BP and reduces end-organ damage. Madhur *et al.* (2010) demonstrated that Ang II-infused WT mice had upregulated T cell

IL-17A production, and that Ang II-infused IL-17$^{-/-}$ mice developed 30 mmHg lower BP than Ang II-infused WT animals after four weeks (211). A more recent study by Saleh *et al.* (2016) confirmed these findings by blocking IL-17A with an anti-IL-17A antibody in Ang II-infused WT mice, leading to reduced BP (213). The authors found that blocking IL-17A or the IL-17A receptor lowered BP and reduced renal and vascular lymphocyte infiltration, but blocking IL-17F had no effect on BP or renal function. In contrast, a study by Markó *et al.* (2012) found that antibody-mediated neutralization of IL-17A did not reduce BP and cardiac hypertrophy in mice infused with Ang II (1.44 mg/kg/day Ang II, 14 days), but this could be the result of the study's combination of high Ang II dose and length of infusion (192).

Clinical data also indicates that IL-17A could play a role in human hypertension, as a positive correlation was found between serum IL-17A concentrations and duration of hypertension in patients with uncontrolled hypertension (214). In contrast to the above findings, Caillon *et al.* (2016) proposed that IL-17A may be protective in the hypertensive cardiovascular system: IL-17A could promote adaptive flow-mediated outward vascular remodeling by augmenting eNOS-dependant relaxation and MMP activity in mesenteric arteries (215). However, the literature generally points to IL-17A having a deleterious role in the pathogenesis of hypertension.

1.7.6 IL-23

In 2003, Aggarwal *et al.* demonstrated that T cell IL-17A production is increased in response to IL-23, a cytokine produced by activated macrophages and dendritic cells that binds the IL-23 receptor (IL-23R) (216). Over the following decades, IL-23 has been found to promote IL-17A production by CD4^{+} Th17 cells and γδT17 cells (217), and IL-23, in combination with IL-1β, can generate extrathymic proinflammatory γδT17 cells (218). As evidence has accumulated for IL-17A having a deleterious role in the development of hypertension, studies have more recently suggested a link between IL-23 signalling and hypertension. In a 2016 study by Schaalan *et al.*, authors observed that serum levels of IL-23 were increased in obese normotensive and hypertensive patients compared to healthy controls, and serum concentrations aligned with the severity of hypertension (219). A more recent 2020 study by Ye *et* al. confirmed these results, as the authors observed that hypertensive patients exhibited higher serum concentrations of IL-23, and IL-23 concentrations were positively correlated with both BP and severity of hypertension (220).

However, genetic and pharmacologic manipulation of IL-23 signaling in animal studies of hypertension has yielded inconclusive results. In 2020, Lee *et al.* reported that injection of recombinant IL-23 into Dahl salt-sensitive but not salt-resistant rats resulted in hypertension via activation of pathogenic Th17 lymphocytes (221). Yet Krebs *et al.* (2014) failed to find a protective effect for ablation of IL-23R signaling, as *Il23r*$^{-/-}$ DOCA-salt and Ang II-treated mice displayed accelerated albuminuria and renal end-organ damage (222). Additionally, Markó *et al.* (2012) demonstrated that neutralizing antibodies against IL-23R did not slow the progression of Ang II-induced hypertension in mice (192). In summary, the IL-23 signalling axis regulates, in part, the production of IL-17A by proinflammatory CD4^{+} Th17 cells and γδT17 cells. However, abrogation of IL-23R signaling by genetic knockout or antibody inhibition has yielded inconclusive results in animal studies of hypertension, leaving more work to be done.

1.8 Innate Immune Cells in Hypertension

The innate immune system is a nonspecific first line of defense against pathogens that encompasses passive anatomical barriers such as the skin and mucous membranes, chemical barriers such as secreted mucous, stomach acid, and digestive enzymes, in addition to active defenses such as the complement system, circulating clotting factors, and innate immune cells that help initiate and sustain the adaptive immune response. Cells of the innate immune system are ubiquitous, and they tend to possess specialized surface receptors, gene expression programs, and effector capabilities when compared across different tissues. Innate immune cells respond very quickly to immune insult by producing cytokines and chemokines in addition to recruiting other immune cells to the site of inflammation.

1.8.1 Common Mechanisms of Immune Surveillance Used by Innate Immune Cells

Innate immune cells are known to possess various pattern recognition receptors (PRRs), including the well-characterized family of toll-like receptors (TLRs) that can sense pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), the latter of which may be generated by hypertension-related tissue injury (Figure 1.4). Indeed, various studies have characterized DAMPs that are increased in hypertension, including heat shock proteins and ECM components, which can activate TLR signaling and increase the inflammatory response

(223). One of the most studied TLRs in hypertension is TLR4, and a review of TLR4 signaling in animal models of hypertension found eight recent studies that demonstrated reduced BP with TLR4 blockade, and three studies that did not (224). From a mechanistic standpoint, blockade of TLR4 reduced oxidative stress and inflammation in hypertension, independent of BP reductions (225). Compared to WT mice, TLR4-deficient mice show diminished Ang II-induced vascular ROS production and aortic NOX activity, and increased expression of extracellular superoxide dismutase (SOD) which scavenges superoxide anion and converts it into oxygen and hydrogen peroxide (226). While TLRs are traditionally associated with the innate immune system, TLR signaling is also important to T cell activation and differentiation (227), which will be discussed in subsequent chapters. Innate immune cells can influence the overall immune response via the production and release of cytokines and chemokines, and via the uptake of antigens through phagocytosis for processing and antigen presentation to adaptive immune cells.

1.8.2 Inflammasome

Inflammasomes are a family of multi-protein complexes belonging to the innate immune system that are assembled by PRRs in response to stimulation by PAMPs or DAMPs. In hypertension, the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is perhaps the most thoroughly investigated of the inflammasomes. The NLRP3 inflammasome mediates the secretion of proinflammatory cytokines IL-1β and IL-18 (228). NLRP3 activation requires an initial priming event, which can be initiated by a wide range of factors such as TLR4 activation or other proinflammatory stimuli that upregulate NF-κB expression. This priming event results in assembly of the NLRP3 inflammasome complex and production of pro-IL-1β and IL-18. Following the priming event, recognition of DAMPs (ex: ATP) triggers K^+ efflux through the P2X purinoceptor 7 (P2X7) receptor, resulting in the activation of caspase-1 and subsequent cleavage and release of the pro-inflammatory cytokines, IL-1β and IL-18, which contribute to hypertension and cardiovascular disease (229). IL-18 is an important costimulatory cytokine necessary for IFN-γ production by antigen-specific T cells, and IL-18 could enhance inflammation in hypertension via promoting IFN-γ production (230).

In humans, increased inflammasome gene expression and circulating IL-1β is strongly associated with increased risk for hypertension and vascular dysfunction, as well as all-cause mortality in individuals over 60 years of age (231). Regarding experimental animal models of

hypertension, *Nlrp3*$^{-/-}$ mice are protected against Ang II-induced gestational hypertension (232), and pharmacological inhibition of NLRP3 reduces BP, renal damage, and dysfunction in C57BL/6J mice made hypertensive by uninephrectomy and administration of DOCA-salt (233). Therefore, modulation of inflammasome activation represents a potential therapeutic avenue to develop new antihypertensives. Targeting NLRP3 in early hypertension could prevent inappropriate production of "early-response" cytokines that work to initiate a larger immune response.

1.8.3 Monocytes and Macrophages

Monocytes/macrophages are key regulators of inflammation and tissue repair, and they contribute to inflammation and end-organ damage in hypertension. Monocytes are generated in the bone marrow before migrating to the periphery and differentiating into various subsets of macrophages or dendritic cells (DCs) (234). Macrophages are characterized as either proinflammatory (M1) or anti-inflammatory (M2), which are known to produce IL-1β, IL-6, IL-23, IFN-γ, and TNFα, or IL-10, respectively (235). IFN-γ is considered to be the main cytokine associated with M1 activation, and macrophage production of IFN-γ contributes to inflammation in hypertension (236). Ablation of lysozyme M-positive macrophages attenuates BP increases, prevents vascular dysfunction, and reduces vascular superoxide production in mice infused with Ang II (237). This was reversed upon adoptive transfer of monocytes, implicating monocytes and macrophages in the pathogenesis of hypertension. Providing further support for macrophages having a deleterious role in the pathogenesis of hypertension, mice deficient in M-CSF are protected against Ang II induced BP elevation, vascular remodeling, endothelial dysfunction, and exhibit reduced vascular macrophage infiltration and ROS (184). In DOCA-salt hypertension, mice devoid of M-CSF are also protected from BP increases, vascular remodeling, oxidative stress, and endothelial dysfunction, echoing the protection from Ang II-induced hypertension (238).

As chemokines mediate immune cell migration and accumulation, antagonizing chemokine receptors is another method to investigate the role of immune cell subsets in hypertension. A 2015 study by Moore *et al.* found that antagonizing chemokine receptor 2 (CCR2), which is highly expressed in both macrophages and aortas of Ang II-treated mice, blunted aortic macrophage accumulation, reduced aortic collagen deposition, and resulted in lower BP in Ang II-treated mice (239). From a translational perspective, isolated human monocytes co-cultured with human aortic

endothelial cells undergoing cyclical 5% or 10% stretch underwent conversion to more activated and differentiated monocyte intermediates, which had markedly stimulated mRNA expression of IL-6, IL-1β, IL-23, and TNFα (240). In response to in vitro stimulation with Ang II, human macrophages upregulate the synthesis and release of the proinflammatory cytokine IL-6, which could promote inflammation in the vascular wall and contribute to hypertension in vivo (241). In summary, proinflammatory macrophages contribute to the pathogenesis of hypertension and represent a potential therapeutic avenue.

1.8.4 Dendritic Cells

DCs bridge innate and adaptive immunity by taking up, processing, and presenting antigens to T cells, which then undergo TCR-mediated activation, proliferation, and differentiation to augment the immune response. Antigen presentation to T cells requires costimulatory interactions, specifically B7 ligands (CD80/86) on APCs with the T cell coreceptor, CD28 (242). Indeed, both DC-mediated antigen presentation and costimulatory signals are important processes in the pathogenesis of hypertension. Ang II infused mice have increased activated ($CD86^+$) DCs in secondary lymphoid organs, and pharmacological blockade of B7-CD28 interactions reduced BP increases in response to Ang II and DOCA-salt (243). Further evidence that APCs play a role in hypertension was demonstrated by Hevia *et al.* (2018); ablation of murine $CD11c^+$ APCs enhanced natriuresis, reduced mediators of inflammation (Nox, IL-1β, TNFα and IL-6), and prevented hypertension induced by Ang II and salt (244). A comprehensive study by Kirabo *et al.* (2014) on the role of DCs in murine hypertension found that: 1) Ang II infusion increased DC superoxide production, 2) isoketal adducts, an immunogenic species of modified proteins induced by oxidative stress, formed as a result of hypertension and accumulated in DCs, 3) following accumulation of isolevuglandins, DCs became activated and expressed high levels of costimulatory molecules and proinflammatory cytokines (IL-6, IL-1β, and IL-23), and 4) these levuglandin-activated DCs promoted T cell survival, proliferation, and cytokine production, augmenting inflammation (151). Additionally, the authors found that isolevuglandins and DCs contribute to DOCA-salt hypertension, and transfer of DCs from Ang II-infused hypertensive mice into normotensive mice sensitized recipient mice to develop hypertension to a subpressor dose of Ang II. Conversely, scavenging isoketals with 2-hydroxybenzilamine (2-HOBA) inhibited BP increases in both the Ang II and DOCA-salt mouse models of hypertension. In summary, induction of oxidative stress

favours isoketal formation which leads to DC and ultimately T cell activation. DC-mediated antigen presentation and costimulatory interactions with T cells are important mechanisms that contribute to the pathogenesis of hypertension and end organ damage.

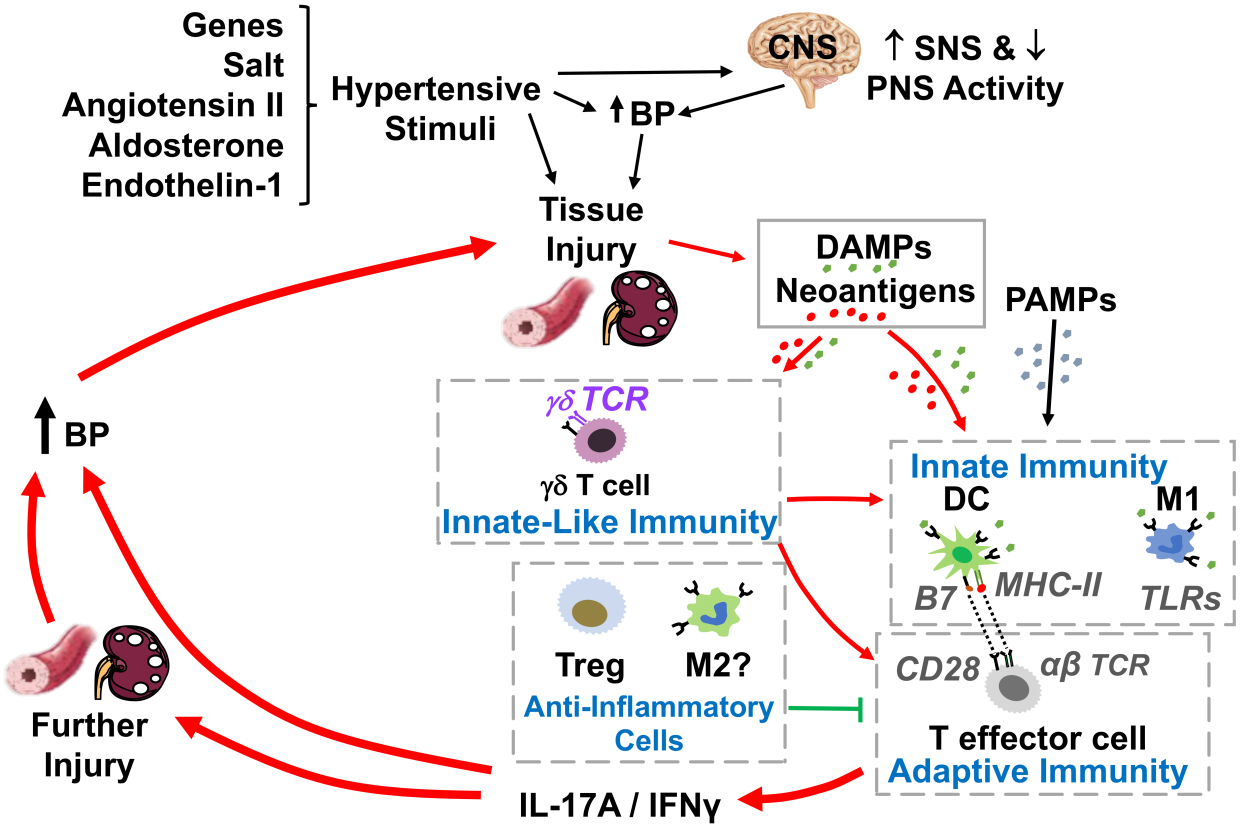

Figure 1.4: Mechanisms behind immune activation in hypertension. Increased blood pressure (BP) leads to damage to the vasculature and kidneys and triggers the release of damage associated molecular patterns (DAMPs) and the formation of neoantigens. DAMPs and neoantigens are recognized by toll-like receptors (TLRs) and T cell receptors (TCRs), on innate immune cells and T cells, respectively. Dendritic cells (DCs) present antigens and costimulatory molecules to αβ T cells to promote full T cell activation. γδ T cells do not require antigen processing or major histocompatibility complex (MHC) presentation by antigen presenting cells (APCs) for TCR-mediated activation. Additionally, γδ T cells are unique in their ability to present antigens to αβ T cells like professional APCs. Anti-inflammatory regulatory T cells (Tregs) can suppress the immune response, while the role of M2 macrophages is unclear. Activated γδ and αβ T cells

produce proinflammatory cytokines, notably IL-17A and IFN-γ, which augment inflammation, promote vascular dysfunction, and contribute to BP increases.

1.9 Adaptive Immunity in Hypertension

1.9.1 T Cells Orchestrate the Adaptive Immune Response

T lymphocytes are a diverse and powerful pool of adaptive immune cells able to initiate and subsequently direct a strong immune response. T lymphocytes are broadly categorized by TCR composition, with $CD4^+$ and $CD8^+$ αβ T cells representing a much larger proportion of circulating and tissue-resident lymphocytes compared to T cells bearing γ and δ TCR chains (245). Surrounding the TCR is the CD3 co-receptor complex, which transduces signals away from the TCR to promote lymphocyte activation. The CD3 complex is composed of four chains, one CD3γ, one CD3δ, and two CD3ε chains, which associate with the TCR and CD3ζ to generate an effective activation signal upon TCR binding of cognate antigen.

T lymphocyte progenitor cells originate in the bone marrow. They then home to the thymus and undergo positive and negative selection to promote alloreactive and prevent autoreactive clones, respectively (246). It is in the thymus where $CD3^+$ lymphocytes become either $CD4^+$ or $CD8^+$ single-positive cells or remain $CD4^-CD8^-$ double negative. T cells subsequently traffic to and gain immune competence in secondary lymphoid organs upon encountering APCs. APCs present antigens to $CD8^+$ and $CD4^+$ T cells via major histocompatibility complex (MHC)-I and MHC-II, respectively. MHC-I and MHC-II molecules are responsible for delivering short antigen peptides to the cell surface and specifically binding TCR co-receptors CD8 and CD4. Antigenic stimulation and exposure to costimulatory interactions and a milieu of cytokines in the local microenvironment shapes the effector fate of T cells. Prominent T cell effector subsets include cytotoxic, helper, and Treg cells, with each type able to express a distinct profile of cytokines and receptors depending on the immune context (247).

1.9.2 T Cells Play a Key Role in the Pathogenesis of Hypertension

The 2007 study by Guzik *et al.* demonstrating that Ang II-infused and DOCA-salt-treated *Rag1* KO mice have blunted hypertension was an important milestone in research into adaptive

immunity in hypertension, and it was the first publication to show a clear role for T cells in hypertension (122). Surprisingly, research groups noticed that protection from hypertension was lost in *Rag1* KO mice purchased from Jackson Laboratory in 2015-2016, and this was attributed to spontaneous genetic drift (248). However, the concept of T cells being a primary driver of hypertension and vascular dysfunction remains uncontested. Since the pivotal 2007 study by Guzik *et al.*, the roles of $CD4^+$, $CD8^+$, and γδ T cells in the pathogenesis of hypertension has been a topic of increasing interest. The following sections will review the roles of various T cell subsets in the progression of hypertension and vascular damage.

1.9.3 $CD4^+$ T-Helper Cells Potentiate Hypertension-Induced Inflammation

Naïve $CD4^+$ T cells primarily develop into Treg or helper (Th) cell subsets. Generally, Treg cells scale down immune responses by producing anti-inflammatory mediators (such as IL-10), while helper T cells positively regulate and fine tune immune responses via the production of proinflammatory mediators (such as IL-17A and IFN-γ) (Figure 1.4) (247). $CD4^+$ Th1 and Th17 cells are known to produce high levels of either IFN-γ or IL-17, respectively, and both IFN-γ and IL-17A promote inflammation (refer to section 1.7 for a more detailed overview on the roles of IL-17A and IFN-γ in hypertension). Studies have shown a deleterious role for $CD4^+$ Th1 and Th17 cells in the development of hypertension and vascular injury: rats made hypertensive by administration of DOCA-salt have increased renal and cardiac-infiltrating Th17 cells compared to animals treated with the mineralocorticoid inhibitor spironolactone (249). A recent study of humans with resistant hypertension found increased numbers of circulating $CD4^+IL\text{-}17^+$ Th17 cells compared to individuals with normal BP (250), and circulating $CD4^+$ T cells isolated from hypertensive patients show a predilection for higher IL-17A and IFN-γ production versus normotensive controls (251). Further, a study by Saleh *et al.* (2016) determined that the main T cell subsets producing IL-17A in the kidney and aorta in response to four weeks of Ang II infusion were γδ T cells and $CD4^+$ Th17 cells (213). Consequently, $CD4^+$ T-helper cells are capable of augmenting hypertension-induced inflammation and mediating hypertensive end-organ damage through the release of proinflammatory cytokines.

1.9.4 $CD4^+$ T Regulatory Cells Dampen the Inflammatory Response

In contrast to helper T cells, Treg cells may reduce hypertension-associated inflammation (Figure 1.4). A 1992 study by Fannon *et al.* revealed that non-helper T cells were reduced in SHRs from two-weeks of age onwards (252). Over the following decades, researchers observed decreased Tregs concomitant with the onset of hypertension, and it is likely that Tregs represent the non-helper T cells that Fannon *et al.* (1992) saw decrease in SHRs. In 2009, Viel *et al.* found that Dahl salt-sensitive rats exhibited reduced Treg activity (253). However, transfer of chromosome 2 which contains genes encoding inflammatory mediators from normotensive Brown Norway rats into Dahl rats reduced BP and upregulated Treg activity. In 2011, Barhoumi *et al.* demonstrated that hypertension induced by 2 weeks of 1000 ng/kg/min Ang II infusion resulted in a reduced frequency of $Foxp3^+$ Tregs in the renal cortex of WT C57BL/6J mice (254). Conversely, adoptive transfer of Tregs attenuated increases in BP in response to Ang II infusion. In general, the balance of proinflammatory versus anti-inflammatory T cell subsets is an important consideration in the development of hypertension. Rats made hypertensive by administration of DOCA-salt have reduced renal and cardiac-infiltrating Tregs compared to animals treated with the mineralocorticoid inhibitor spironolactone (249).

In humans, a study of children with primary hypertension revealed that hypertensive children possess a lower frequency of Tregs than normotensive children, and a reduced level of $CD4^+$ Tregs was found to be associated with increased target organ damage and arterial stiffness (255). Further, Tregs have a role in amplifying sex differences in the development of hypertension. A 2022 literature review by Comeau *et al.* suggests that hypertension in males is associated with increased proinflammatory $CD4^+$ Th17 cells, while protection from hypertension observed in premenopausal women may be attributable to increased $CD4^+$ Tregs and IL-10 production (256). Conversely, the loss of protection from hypertension associated with menopause may be attributable to changes within the Treg compartment.

1.9.5 $CD8^+$ T Cells Contribute to the Development of Hypertension

Upon antigenic stimulation, naïve $CD8^+$ T cells mature into cytotoxic T cells capable of producing IFN-γ and TNFα and augmenting inflammation. Due to these characteristics, $CD8^+$ T cells contribute to the progression of hypertension. In 2014, Trott *et al.* demonstrated that $CD8^+$ T cells are critical to Ang II- and DOCA-salt-induced hypertension in mice (257). After 14 days of Ang

II infusion, WT and $CD4^{-/-}$ mice had significantly higher SBP than $CD8^{-/-}$ mice, and $CD8^{-/-}$ mice exhibited a blunted hypertensive response to both Ang II and DOCA-salt treatment. Conversely, adoptive transfer of $CD8^+$ T cells but not $CD4^+CD25^-$ (non-Treg $CD4^+$ T cells) T cells recapitulated the hypertensive response to Ang II in *Rag-1*$^{-/-}$ mice. Finally, the authors determined that an oligoclonal population of $CD8^+$ T cells had accumulated in the kidneys of hypertensive mice, and WT and $CD4^{-/-}$ mice retained water and sodium in response to a sodium/volume challenge whereas $CD8^{-/-}$ mice did not. Consequently, $CD8^+$ cells may also contribute to salt sensitivity in hypertension. Liu *et al.* (2017) hypothesized that $CD8^+$ T cells contribute to hypertension by upregulating Na-Cl co-transporter (NCC) expression in renal distal convoluted tubule cells (DCT) (258). Adoptive transfer of $CD8^+$ T cells induced remarkably elevated BP in response to a high-salt diet and promoted T cell infiltration and upregulation of NCC in the kidneys of DOCA-salt treated mice. In vitro co-culture of DCT cells with $CD8^+$ T cells confirmed that $CD8^+$ T cells were responsible for the observed upregulation of NCC in DCTs, and that the upregulation occurred in a ROS-dependant manner.

$CD8^+$ T cells may also contribute to the increased incidence of hypertension seen with aging. In a study of human hypertension, newly diagnosed hypertensive patients (treatment naïve) exhibited an increased proportion of IFN-γ-producing senescent $CD8^+$ T cells compared to normotensive controls (259). Aging is associated with accumulation of highly inflammatory senescent T cells with altered immune functionality, and senescent $CD8^+$ T cells have been found to advance animal models of hypertension. Adoptive transfer of senescent $CD3^+$ T cells from aged mice into Ang II-infused *Rag-1*$^{-/-}$ mice resulted in a more dramatic accumulation of IFN-γ-producing $CD8^+$ T cells in the aorta, kidney, and heart than mice populated with T cells from young mice (260). Additionally, Ang II infusion increased BP to a greater extent in mice transferred aged (senescent) cells than mice transferred young cells. In conclusion, $CD8^+$ T cells contribute to the pathogenesis of hypertension, both through promoting inflammation and contributing to abnormal renal salt handling.

1.10 γδ T Cells: Bridging Innate and Adaptive Immunity

Gamma delta (γδ) T cells are unconventional lymphocytes with characteristics of both innate and adaptive immune cells, bridging these two arms of the immune system (261). γδ T cells have

unique abilities when compared to $CD4^+$ and $CD8^+$ αβ T cells, such as non-MHC restricted antigen recognition and rapid induction of potent cytotoxic activity (262). γδ T cells can circulate through blood and lymphoid organs or become resident cells in peripheral tissues and barrier surfaces such as the gut (γδ intraepithelial lymphocytes, IELs) and skin (γδ dendritic epidermal T cells, DETCs) (263). Further, γδ T cells can act as APCs (264), and human γδ T cells are effective at cross presentation of antigens to $CD8^+$ T cells (265). Surprisingly, γδ T cells can be even more effective at cross-presentation than monocyte-derived DCs in vitro. Mouse γδ T cells express MHC II and co-stimulatory molecules after activation in vitro via coculture with anti-CD3 and anti-CD28 antibodies, demonstrating that γδ T cells can express the protein machinery required to influence other T cell subsets (266). Importantly, γδ T cells are capable of producing large amounts of proinflammatory cytokines, namely IL-17A and IFN-γ, which contribute to inflammatory diseases as well as the development of hypertension (267).

1.10.1 γδ T Cell Nomenclature

Human and murine γδ T cells are subdivided by TCR subunit composition, and slight differences exist regarding the nomenclature of each species. Human γδ T cells are divided into two main subsets following TCRδ chain usage, namely $Vδ1^+$ and $Vδ2^+$ γδ T cells, although other TCRδ-chain variants exist at low frequencies. $Vδ1^+$ γδ T cells possess more TCRγ chain diversity (Vγ2/3/4/5/8) than $Vδ2^+$ γδ T cells, which are almost exclusively $Vγ9^+$ (268, 269). Roughly 5% of $CD3^+$ lymphocytes in the peripheral blood of healthy adults are γδ T cells (270), and the majority of these circulating γδ T cells bear a Vγ9Vδ2 TCR (263). The remainder are mostly $Vδ1^+$ with even fewer $Vδ3^+$ and $Vδ5^+$ γδ T cells in the circulation. Human Vγ9Vδ2 T cells are not only numerous, but also clinically relevant, as upon exposure to cells expressing non-peptide antigens, Vγ9Vδ2 γδ T cells respond with a cascade of immune reactions leading to αβ T cell activation, cytokine release, and cytotoxicity (271).

Murine γδ T cells are subdivided based on the same nomenclature system. However, murine isoforms differ from those of humans. Mouse γδ T cells are primarily grouped based on Vγ chain usage, and Vγ chains are described using either the Heilig & Tonegawa or Garman naming systems. In the Garman system, seven Vγ chain subsets (Vγ1.1, Vγ1.2, Vγ1.3, Vγ2, Vγ3,

Vγ4, and Vγ5) are used to subdivide murine γδ T cells (272), and these seven Vγ chain subsets align with the seven Vγ chain subsets in the Heilig & Tonegawa naming system (Vγ1, Vγ2, Vγ3, Vγ4, Vγ5, Vγ6, and Vγ7, respectively) (273). Important murine γδ T cell subsets include $V\gamma1^+$ and $V\gamma7^+$ IELs in the gastrointestinal tract, $V\gamma5V\delta1^+$ DETCs in the epidermis, $V\gamma4^+$ γδ T cells in the dermis, $V\gamma6V\delta1^+$ γδ T cells in female reproductive tissue, tongue, and peritoneal cavity, and $V\gamma1^+$ and $V\gamma4^+$ γδ T cells in secondary lymphoid organs (SLOs) and lung tissue (Heilig and Tonegawa notation) (274). Murine γδ T cells show diversity regarding Vδ chain usage, however, they are normally classified exclusively based on Vγ chain expression (275). Murine γδ T cells encompass approximately 3% of spleen and lymph node $CD3^+$ lymphocytes in rodents (276), and they exhibit a similar ability to produce IL-17A and IFN-γ to human γδ T cells (263). Generally, production of IL-17A is mostly limited to $V\gamma4^+$ and $V\gamma6^+$ cells (277). However, $V\gamma1^+$ cells can also produce IL-17A under some conditions, which was demonstrated in an immunodeficient mouse model of chronic granulomatous disease where mice lack functional NOX (278). Conversely, IFN-γ production is generally associated with $V\gamma1^+$, $V\gamma5^+$, and $V\gamma7^+$ cells (267).

1.10.2 Antigen Recognition by γδ T Cells

While γδ T cells are referred to as "innate-like" lymphocytes, γδ T cells express TCRs that undergo V(D)J recombination similar to αβ T cells, placing them under the umbrella of the adaptive immune system. However, only a small fraction of putative γδ TCR ligands have been discovered (279). γδ T cells perform immune surveillance using a combination of receptors, including the γδ TCR, TLRs, and natural killer receptors, allowing them to recognize self-proteins such as classical and non-classical MHC molecules, including MHC class I polypeptide-related sequence A (MICA) and CD1c, as well as pyrophosphate-containing small molecules (termed phosphoantigens), heat-shock proteins, and lipids (via CD1) (280). Some examples of these small non-peptide phosphoantigens include mevalonate pathway-derived isopentyl pyrophosphate (IPP) associated with infected or transformed cells, as well as (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) produced by parasites and bacteria (281). IPP and HMBPP can directly engage the γδ TCR, leading to TCR-dependent lymphocyte activation and subsequent cytotoxic activity (282). Enhanced diversity of γδ TCR V-J and V-D-J junctional regions can be seen in γδ T cells collected from the intestinal epithelium compared to γδ T cells collected from the thymus,

lymph nodes, and epidermis (283). This finding supports the idea that γδ T cells can recognize non-self-molecules using the γδ TCR as the gut is continually exposed to luminal bacterial antigens, which may lead to increased TCR junctional diversity in this region.

The mechanism and strength of γδ T cell activation plays a role in determining what effector cytokines they can produce. During development in the thymus, antigen exposure yields γδ T cells that are more biased to produce IFN-γ upon TCR-mediated activation, while antigen naïve γδ T cells produce more IL-17A following TCR stimulation (284). Similar to how TCR signal strength dictates thymic development of $CD4^{+,}$ $CD8^{+}$, or Treg subsets from αβ T cell progenitors, TCR signal strength induces thymic γδ T cell progenitors to become γδT1 (high TCR signal) or γδT17 (low TCR signal) cells (285). Outside of the thymus, γδ T cells activated by cytokines appear biased towards IL-17A production, while γδ T cells activated through the TCR have a pronounced ability to co-produce IL-17A and/or IFN-γ (286). Additionally, γδ T cells can be polarized to activate and become IL-17A producers under Th17 conditions (culturing with IL-23) or IFNγ-producing under Th1 conditions (culturing with IL-12). Co-culture of cytokine-producing γδ T cell effectors with $CD4^{+}$ T cells revealed that both IL-17A and IFNγ-producing γδ T cells (γδT17 and γδT1, respectively) polarize $CD4^{+}$ T cells to become more Th17, but neither γδT17 or γδT1 affected the polarization of Th1 cells (286). Consequently, γδ T cells can recognize antigens representing both self (stress signals) and non-self (bacterial, viral, parasitic), and respond to the insult by polarizing into immune-competent effectors.

1.10.3 Memory γδ T Cells: A Double-Edged Sword

As γδ T cells have a less restricted repertoire of putative TCR antigens compared to αβ T cells, and the γδ TCR does not require APC antigen processing for TCR activation, it is not surprising that γδ T cells have roles in infection, immunity, and autoimmunity. Knockout animals and adoptive transfer experiments with γδ T cells have revealed a protective role in animal models of *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and influenza viruses (281). γδ T cells also play a deleterious role in the pathogenesis of autoimmune diseases, where they have been noted to contribute to Type 1 diabetes, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus; however, their exact role is not yet clear in most disease contexts (287). A hallmark of the adaptive immune system is the formation of long-lasting memory T cells, which are antigen

experienced T cells residing in lymphoid organs, the circulation, and peripheral tissues, that possess a lower activation threshold and the capability to respond faster and more effectively upon re-exposure with cognate antigen (288). Following T cell antigenic stimulation and the primary immune response, some effector cells undergo further differentiation into long-lived memory T cells (Figure 1.5). Memory T cells are able to provide different signals compared to naïve and effector T cells, and memory T cells are also able to proliferate faster in response to antigen exposure. These characteristics lead to more effective host protection in the face of infection, however, they may also potentiate autoimmune diseases or worsen states of sterile inflammation, such as in the case of cardiovascular disease. Cytokines and chemokines also play a crucial role in TCR activation and cognate antigen recognition in the memory T cell recall response (289).

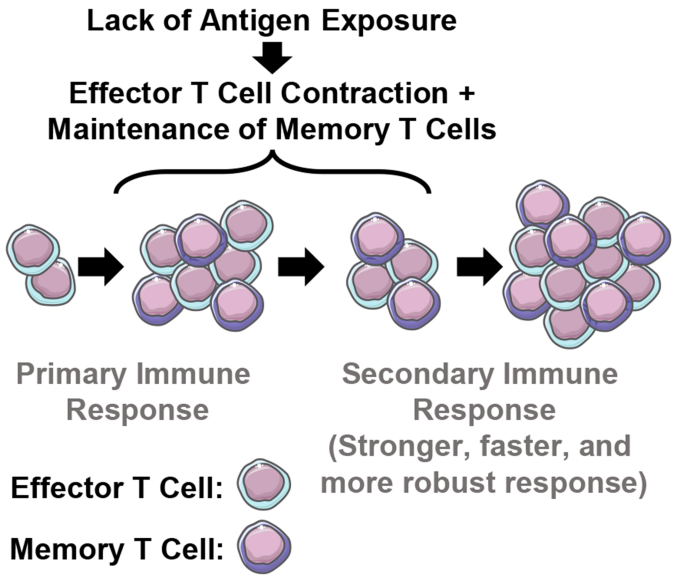

Figure 1.5: Simplified schematic of the memory T cell principle. The primary immune response elicits more effector than memory T cells. After the immune insult has been resolved, effector T cell populations contract due to reduced antigen exposure, and a population of long-lived memory T cells develops. Memory T cells can react more strongly to reencounter with cognate antigen (secondary, tertiary, etc. immune response), leading to quicker and more robust pathogen clearance, or worsened outcomes in the case of autoimmune diseases.

1.10.4 Memory T cell Subsets and Markers

Memory T cells have distinct DNA methylation patterns that contribute to their unique properties, which include increased longevity and rapid activation of effector capabilities (290). Generally, memory T cells can be subdivided into three classes: central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and resident memory T cells (T_{RM}) (291). T_{CM} cells recirculate between SLOs and blood, while T_{EM} cells can recirculate through non-lymphoid tissues, SLOs, and/or blood. Finally, T_{RM} reside permanently in peripheral tissues, SLOs, and local vascular compartments without recirculating.

In mice, T_{CM} cells express lymphoid tissue homing receptors C-C chemokine receptor type 7 (CCR7) and L-selectin (CD62L) (291) (Figure 1.6A). On the other hand, T_{RM} cells express CD69 and integrin alpha E (CD103), which are required for tissue retention and recruitment, respectively (292, 293). In comparison to T_{CM} and T_{RM}, T_{EM} cells do not express any markers associated with lymphoid homing or tissue retention (294). All murine memory T cell subsets express the hyaluronic acid receptor and activation marker, CD44, which is upregulated following cognate antigen recognition and remains upregulated in memory T cells (295). Of important note, naïve murine T cells, while expressing CD62L and CCR7, are distinctly CD44 negative (296).

Human memory T cell populations are subdivided in a similar fashion with some different markers to the mouse (Figure 1.6B). While humans share the expression of CCR7, CD62L, CD69, and CD103 with mice, human memory T cell subsets are further characterized using CD45RA and CD45RO, and are absent of CD44 (297). CD45RA expression is indicative of naïve T cells, while CD45RO expression denotes previous activation in a similar fashion to CD44 in mice (297). Naïve human T cells are $CD45RA^+$ $CD62L^+$ $CCR7^+$, and following TCR-mediated activation the naïve T cells become effector T cells (T_{EFF}) with potent cytotoxic and proinflammatory capabilities (288). T_{EFF} cell populations contract following the primary immune response, however, some T_{EFF} become long-lived $CD45RO^+$ $CD62L^+$ $CCR7^+$ T_{CM} cells (298). T_{CM} cells may then become T_{EM} cells after re-exposure to cognate antigen in the secondary immune response, yielding $CD45RO^+$ $CD62L^-$ $CCR7^-$ effector memory T cells capable of a robust proinflammatory response (299). Alternatively, human T_{RM} cells are $CD45RO^+$ $CD62L^-$ $CCR7^-$ $CD103^+$ $CD69^+$, sharing similar markers to mouse T cells (297). T_{RM} are speculated to develop from T cells that entered tissues during the effector phase of the immune response and remain positioned in the periphery (300). Both murine and human T cells are thought to follow a similar stepwise, progressive shift from

naïve, to T_{EFF}, then T_{CM}, and eventually T_{EM} based on antigen recognition, activation, and the presence of cytokines in the microenvironment (294, 298). However, there is speculation that memory T cell development may not be linear as described above, as several alternate models of memory T cell development have been proposed (301).

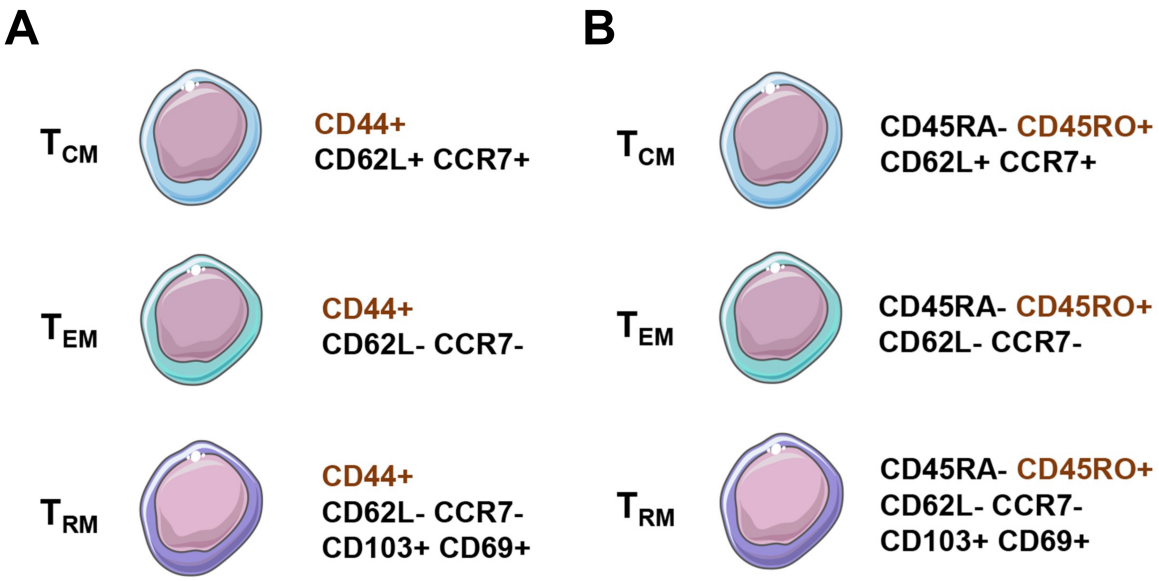

Figure 1.6: Surface markers used for identifying central memory (T_{CM}), effector memory (T_{EM}), and resident memory (T_{RM}) CD4, CD8, and γδ T cells. (A) Surface markers commonly used to identify murine memory T cell subsets. (B) Surface markers commonly used to identify human memory T cell subsets.

1.10.5 γδ T Cells and Immune Memory in Hypertension

In 2017, Caillon *et al.* demonstrated that γδ T cells play a critical role in the development of hypertension and vascular injury (154). Ang II treatment caused an increase in both activation and number of γδ T cells in the spleens of mice after 7 and 14 days of infusion. γδ T cell deficiency due to Tcrδ knockout or injection of γδ T cell-depleting antibodies blunted Ang II-induced BP elevation, small artery endothelial dysfunction, and reduced the activation of $CD4^+$ and $CD8^+$ T cells in the mesenteric artery perivascular adipose tissue (PVAT). In addition, Caillon *et al.* found a correlation between circulating γδ T cells and BP in humans using a multiple linear regression integrating whole blood TCRγ constant region gene expression and age and sex. In 2021, Delaney *et al.* also found a correlation between γδ T cells and long-term BP in humans (302). Cryopreserved

samples from 1195 patients were assayed for immune cells and then correlations were determined with 10-year follow up BP measurements using linear mixed models. The authors found that a one standard deviation increment in the proportion of γδ T cells was associated with a 2.4 mmHg higher average SBP. From a mechanistic standpoint, Li *et al.* (2014) found that cardiac-infiltrating γδ T cells were the main producers of IL-17A in the heart of Ang II-infused mice, and genetic knockdown of γδ T cells abolished IL-17A production and protected against cardiac injury (303). As γδ T cells are a major source of IL-17A and IFN-γ (261), and both IL-17A and IFN-γ contribute to the development of hypertension, we speculate that γδ T cells contribute to hypertension through these cytokines.

One year before the publication by Caillon *et al.*, Itani *et al.* (2016) revealed a role for memory αβ T cells in the development of hypertension (304). The authors found that initial exposure to N^{ω}-nitro-L-arginine methyl ester (L-NAME) or a hypertensive dose of Ang II sensitized mice to develop hypertension in response to a subsequent high-salt diet or infusion of a subpressor dose of Ang II, respectively. The sensitization to mild hypertensive challenges was associated with $CD4^+$ and $CD8^+$ T_{EM} cell accumulation in the kidneys and bone marrow of mice. In addition, inhibition of T_{EM} cell development by destruction of the antigen presenting cell CD70 ligand - T cell CD27 receptor co-stimulatory interaction abrogated the hypertensive response to subsequent mild hypertensive challenges. As long-lived memory γδ T cells have been shown to develop and play a role in models of infection and autoimmunity, and both γδ T cells and memory αβ T cells contribute to the development of hypertension, we speculate that memory γδ T cells develop in hypertension and contribute to the condition. Successive hypertensive insults are predicted to augment the memory response and worsen hypertension-associated inflammation (Figure 1.7).

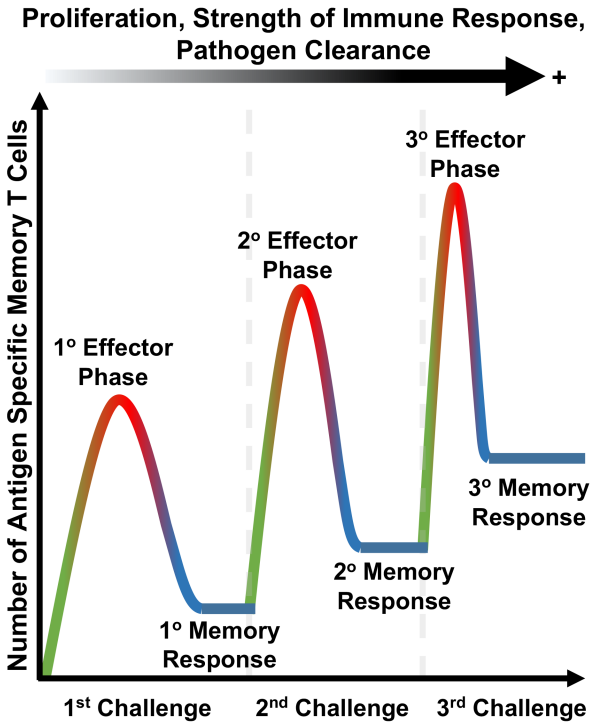

Figure 1.7: A hypertensive challenge induces the formation of memory T cells, which could be reactivated upon subsequent exposure to hypertensive stimuli. Proinflammatory memory T cells that formed as a result of hypertension may be positioned to respond strongly to further immune insults in the form of hypertensive challenges, sensitizing both mice and humans to develop heightened blood pressure.

2. Hypothesis, Objectives, and Experimental Design

2.1 Hypothesis and Objectives

Despite the availability of drugs to treat hypertension, BP remains difficult to control in a large proportion of patients. It is estimated that between 10% and 30% of Canadians diagnosed with hypertension have resistant hypertension, and these individuals have much higher cardiovascular risk (305). Therefore, understanding novel mechanisms in the pathophysiology of hypertension is necessary to generate new treatments for individuals with hypertension that is unresponsive to traditional interventions. Over the last half-century it has been shown that inflammation and innate and adaptive subsets of immune cells are critical to the development of hypertension and vascular dysfunction (12). Recently, our lab was the first to describe a role for γδ T cells in hypertension. Caillon *et al.* (2017) demonstrated that antibody-mediated depletion or genetic ablation of γδ T cells blunted the development of Ang II-induced hypertension in mice, and that TCRγ constant region expression in whole blood was associated with systolic BP in humans (154). γδ T cells are unconventional, "innate-like" T cells that are capable of secreting large volumes of proinflammatory cytokines, including IFN-γ and IL-17A, both of which are involved in sustained hypertension (306). Interestingly, a 2017 study by Muschaweckh *et al.* demonstrated that γδ T cells can be induced in the periphery to produce IL-17A upon stimulation with IL-1β and IL-23 (218), and it is conceivable that these cells could contribute to hypertension. In addition, a 2016 study by Itani *et al.* demonstrated that $CD4^+$ and $CD8^+$ T_{EM} cells develop and play a role in experimental hypertension (304), and γδ T cells can also become memory T cells in contexts of infection and autoimmunity (307). Therefore, we sought to further expand on these concepts by examining two ways in which γδ T cells could contribute to the development of hypertension: through the generation and subsequent reactivation of memory γδ T cells in response to repeat hypertensive challenges, and through cytokine-producing γδ T cell subsets modulated by IL-23R signaling.

We hypothesized that 1. Memory γδ T cells form after an initial exposure to a hypertensive stimulus and that they sensitize mice to develop hypertension to a mild hypertensive challenge, and 2. Ang II-induced BP elevation and vascular injury would be blunted in mice with functional IL-23R deficiency. Therefore, the work presented within this thesis aimed to test these hypotheses.

2.2 Experimental Design

There exist a wide range of animal models that attempt to mimic human hypertension, each with its own benefits and drawbacks. For the studies presented in this thesis we chose to use an Ang II-induced mouse model of hypertension in C57BL/6J mice. This was for several reasons. The RAAS axis is broadly activated in primary hypertension in humans, and it has been understood for many years that elevated levels of circulating Ang II are associated with worsened hypertension in patients (308). In addition, doses of Ang II that are commonly used to induce animal models of hypertension result in BP elevation to a level similar to that of patients with uncontrolled stage 2 hypertension (a SBP of ~140-180 mmHg) (309). The target organ damage seen in Ang II-infused animal models of hypertension is also similar to that of human hypertension, with animals developing varying levels of cardiac hypertrophy, vascular dysfunction, and renal damage, depending on the dose and length of infusion. The pressor dose of Ang II that we chose to use (490 ng/kg/min) is also used extensively among research groups, and the resulting BP phenotype is highly reproducible (122). This allows our study to be easily compared to other studies of immunity in hypertension, and the use of a highly reproducible model that has numerous similarities to human hypertension gives our findings more clinical relevance.

Regarding the mice, we chose to use C57BL/6J mice for our experimental model of hypertension for several reasons. C57BL/6 mice breed rapidly, are easy to maintain, and cost less to house compared to other larger rodents, and they also have a high tolerance for multiple surgical procedures (310). High tolerance for surgery and anesthesia is an especially important consideration in this project, as mice would need to withstand telemetry probe instrumentation and up to three additional procedures to implant and remove osmotic minipumps over the course of nearly two months. Our lab has also had much success using C57BL/6J mice in previous studies that made use of Ang II and telemetry probes. According to the American Heart Association, C57BL/6 mice are more resistant to renal injury than other mouse strains and are not salt sensitive, leading to an ideal model to study RAAS-dependent hypertension (309). Finally, we had to consider using a stain of mice homologous to the knock-in mice used for the study of the IL-23R in hypertension. For the IL-23R experiments, we made use of a strain of IL-23R green fluorescent protein (GFP) knock-in mice, whereupon the IL-23R is deleted and responsiveness to IL-23 inhibited when these mice are bred as homozygotes (311). *Il23r*$^{gfp/gfp}$ mice were generated on a

C57BL/6 background, so it made sense to use strains of mice with similar genetics. Therefore, we chose to use Ang II and C57BL/6J mice for our experimental animal models of hypertension.

The experimental design for the study of memory γδ T cells in hypertension consisted of three main components. In the first set of experiments, we recorded BP and determined memory γδ T cell frequency in response to a single pressor dose Ang II infusion or repeat Ang II infusions (pressor dose followed by subpressor dose of Ang II) in telemetry implanted C57BL/6J mice (Figure 2.1). Memory γδ T cells were profiled in the aortic PVAT, MA PVAT, mLN, BM, and spleen by flow cytometry. In the second part of the study, we exposed telemetry implanted mice to repeat Ang II hypertensive challenges as above and depleted γδ T cells with a monoclonal antibody at the initiation of the subpressor dose Ang II infusion (Figure 2.2). For the final component of the study, we isolated γδ T cells from mice infused with a pressor dose of Ang II and transferred them into recipient, telemetry implanted mice, which were then infused with a subpressor dose of Ang II (Figure 2.3).

In contrast to the experimental design for the study of memory γδ T cells in hypertension, the experimental design for the study of IL-23R in Ang II-induced hypertension consisted of a single 1- or 2-week Ang II infusion. C57BL/6J or *Il23r*$^{gfp/gfp}$ mice were either infused or not with Ang II (490 ng/kg/min) for 7 or 14 days. BP was measured by telemetry and mesenteric artery function and remodeling was evaluated by pressurized myography at 14 days. Cytokine production and surface marker expression was determined by flow cytometry in CD4^{+}, CD8^{+}, and γδ T cells isolated from MA PVAT at 7 days. Finally, BP was recorded in an additional subset of telemetry implanted *Il23r*$^{gfp/gfp}$ mice infused with Ang II for 7 days and injected with anti-IFN-γ or control antibodies.

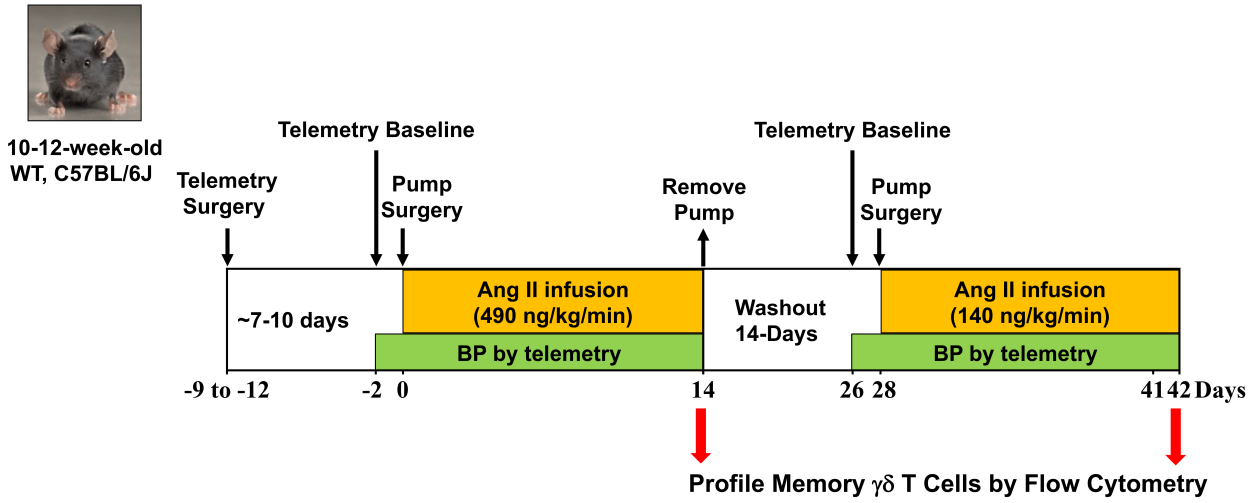

Figure 2.1: Schematic diagram for the study of memory γδ T cell development and BP in response to repeat Ang II hypertensive challenges.

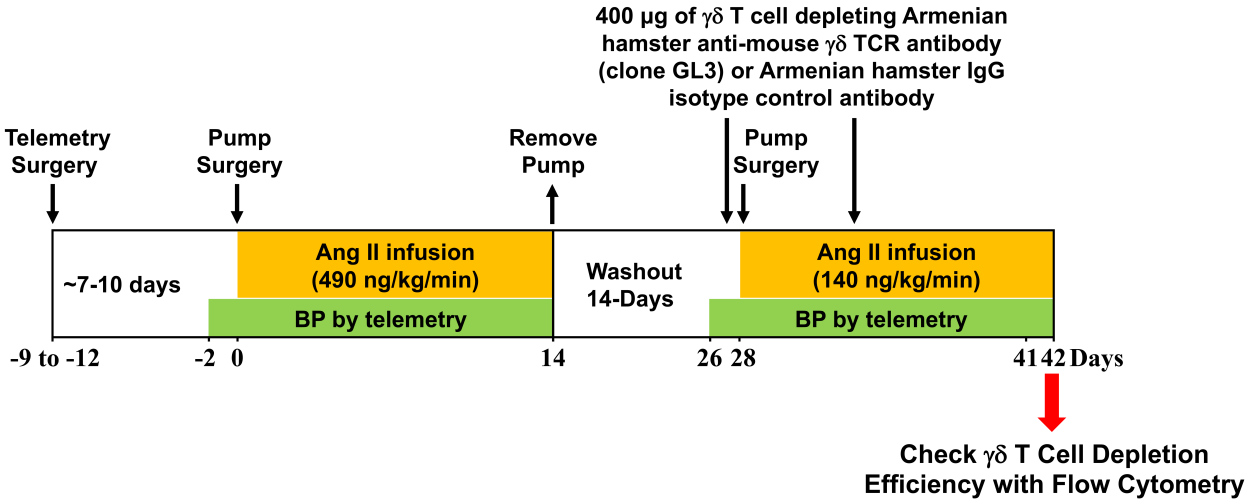

Figure 2.2: Schematic diagram for the study of BP in response to repeat hypertensive challenges in C57BL/6J mice depleted of γδ T cells with an antibody.

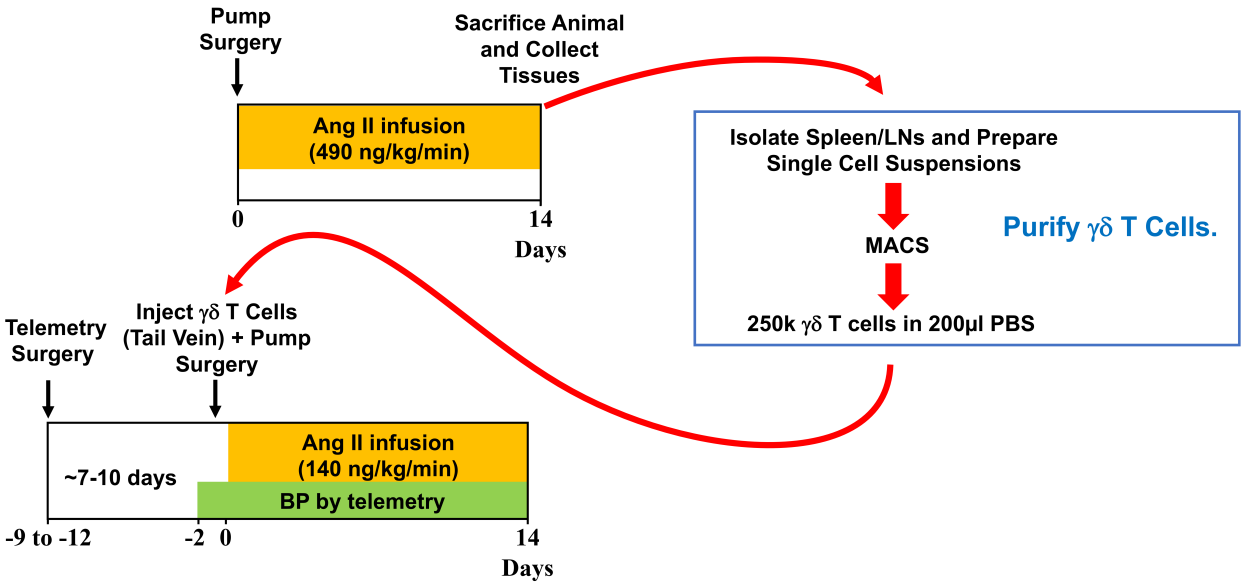

Figure 2.3: Schematic diagram for the study of γδ T cell adoptive transfer and BP elevation in response to a normally subpressor dose of Ang II in C57BL/6J mice.

3. Angiotensin II-Induced Memory γδ T Cells Sensitize Mice to a Mild Hypertensive Stimulus

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Short Title: Memory Gamma Delta T Cells Participate in Hypertension

Number of Figures and Tables: 4

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3.1 Abstract

Background: We recently demonstrated that γδ T cells participate in the pathogenesis of hypertension. Evidence also suggests that memory T cells develop during an initial hypertensive episode, sensitizing mice to develop hypertension to further mild hypertensive challenges. However, whether memory γδ T cells develop and play a role in hypertension remains unknown. We hypothesize that memory γδ T cells develop after a hypertensive challenge and sensitize mice to develop hypertension in response to a subsequent mild hypertensive stimulus.

Methods: Ten-12-week-old C57BL/6J mice were infused or not with a pressor dose of angiotensin (Ang) II (490 ng/kg/min, SC) for two weeks, followed by a two-week washout period, and then infused with a subpressor dose of Ang II (140 ng/kg/min, SC) for two weeks. Blood pressure (BP) was measured by telemetry and memory γδ T cells profiled by flow cytometry. Another group of mice was treated as above and injected IP with 400 μg of an anti-T cell receptor γδ-depleting or isotype control antibody 1 day before and 6 days after the initiation of the subpressor dose Ang II infusion. A final group of mice was injected IV with $2.5x10^5$ live γδ T cells isolated from spleens and lymph nodes of mice infused or not with a pressor dose of Ang II, and following adoptive transfer mice were infused with a subpressor dose of Ang II as above.

Results: Mice exposed to the first hypertensive challenge had a higher systolic BP (SBP) than sham mice at the end of the subpressor hypertensive challenge (149±6 vs. 122±3 mmHg, $P<0.001$). After 14-days of pressor dose Ang II infusion, effector memory γδ T cells increased 5.2-fold in the mesenteric artery (MA) perivascular adipose tissue (PVAT, 1.25±0.37% vs. 0.24±0.12%, $P<0.05$), and 1.8-fold in the mesenteric lymph nodes (mLN, 1.49±0.03% vs. 0.82±0.15%, $P<0.05$) compared to sham treated mice. After a repeat Ang II infusion, central memory γδ T cells decreased by 57% in the aortic PVAT (6.79±1.46% vs. 15.69±2.87%, $P<0.05$) and by 52% in the MA PVAT (0.31±0.08% vs. 0.65±0.13%, $P<0.05$) compared to control mice. Mice depleted of γδ T cells had an average 14 mmHg lower SBP than control mice from day 7 to 14 of the two-week subpressor hypertensive challenge ($P<0.05$). Adoptive transfer of γδ T cells from hypertensive mice induced

an 18 mmHg higher SBP response to a subpressor dose Ang II infusion versus transfer of γδ T cells from sham-treated mice ($P<0.05$).

Conclusion: An initial exposure to a hypertensive stimulus sensitized mice to develop hypertension to a subsequent subpressor hypertensive challenge and resulted in the development of memory γδ T cells. Depleting γδ T cells in mice already exposed to a hypertensive challenge reduced the ability to induce hypertension with a secondary mild hypertensive stimulus. Adoptive transfer of γδ T cells from hypertensive mice sensitized recipient mice to develop hypertension to a mild hypertensive challenge. Consequently, memory γδ T cells develop and contribute to the pathogenesis of hypertension. Understanding the role of memory γδ T cells in hypertension may open new treatment avenues.

3.2 Abbreviations

Ang II – Angiotensin II

ANOVA – Analysis of variance

APC – Antigen-presenting cell

BM – Bone marrow

BP – Blood pressure

CD – Cluster of differentiation

CDR3 – Complementarity determining region 3

cLN – Cervical lymph node

DC – Dendritic cell

HLA – Human leukocyte antigen

IL - Interleukin

IPP – Isopentenyl pyrophosphate

L-NAME – N^{ω}-nitro-L-arginine methyl ester

MA PVAT – Mesenteric artery perivascular adipose tissue

MHC – Major histocompatibility complex

mLN – Mesenteric lymph node

RA – Rheumatoid arthritis

SBP – Systolic blood pressure

SEM – Standard error of mean

TCR – T cell receptor

T_{CM} – Central memory T cell

T_{EM} – Effector memory T cell

T_{RM} – Resident memory T cell

3.3 Introduction

High blood pressure (BP) or hypertension is the leading risk factor for cardiovascular disease, disease burden, and mortality worldwide (1). However, the etiology of hypertension is unclear, and its mechanisms are complex. Both innate (monocyte/macrophages) and adaptive immune cells (T lymphocytes) play an important role in the development of hypertension in mouse models (2). Innate immune cells initiate and subsequently direct the adaptive immune response, and this could occur in hypertension and vascular injury. There is a small subset of T cells expressing γδ T cell receptors (TCRs) instead of conventional αβ TCRs. γδ T cells are unconventional lymphocytes that could play a role in bridging the innate and adaptive immune system (3, 4). These "innate-like" T cells respond rapidly to the pro-inflammatory cytokines interleukin (IL)-1β and IL-23 secreted by innate cells by producing IL-17A, a cytokine involved in sustained hypertension and vascular dysfunction (5). These cells typically migrate directly from the thymus to tissues such as the skin and the intestine (6). Furthermore, γδ T cells present a limited TCR diversity within a specific tissue, which they utilize to engage with only one or a few antigens. γδ T cells recognize small molecules such as phosphate and peptide antigens in a non-major histocompatibility complex (MHC)-restricted manner via their δ TCR and the complementarity determining region 3 (CDR3) (7). Recently, we demonstrated that γδ T cells play a critical role in hypertension and vascular injury (8). Angiotensin (Ang) II caused an increase in the number and activation level of γδ T cells after 7 days of infusion. Deficiency of γδ T cells due to *Tcrδ* knockout or injection of γδ T cell-depleting antibodies prevented Ang II-induced BP elevation, small artery endothelial dysfunction, and spleen and mesenteric artery perivascular adipose tissue (MA PVAT) cluster of differentiation (CD)4^+ and $CD8^+$ T cell activation.

Upon an initial exposure to an antigen, some activated T cells become memory T cells that can be reactivated upon subsequent interaction with the previously encountered antigen (9). There are three main memory T cell subsets: central memory T (T_{CM}) cells that are $CD62L^+CCR7^+$ and are commonly found in lymphoid organs, effector memory T (T_{EM}) cells that are $CD62L^-CCR7^-$ and are able to recirculate between lymphoid tissues, blood, and peripheral organs, and tissue-resident memory T (T_{RM}) cells that are $CD62L^-CCR7^-CD69^+CD103^+$ and reside permanently in peripheral tissues (10). Interestingly, memory T cells may play a role in hypertension. It has been shown that initial exposure to N^{ω}-nitro-L-arginine methyl ester (L-NAME) or a hypertensive dose of Ang II sensitizes mice to develop hypertension to a subsequent high-salt diet or subpressor dose

of Ang II, respectively, which was associated with $CD4^+$ and $CD8^+$ T_{EM} cells accumulating in the kidney and bone marrow (BM) (11). Inhibition of T_{EM} cell development by destruction of the antigen presenting cell CD70 ligand - T cell CD27 receptor interaction abrogated the hypertensive responses to subsequent mild hypertensive challenges. There is evidence that memory γδ T cells play a role in infection and autoimmune disease in murine models (12). However, whether memory γδ T cells are involved in hypertension and vascular injury is still unknown.

We hypothesized that memory γδ T cells develop after an initial exposure to a hypertensive stimulus and sensitize mice to develop hypertension to subsequent subpressor hypertensive challenge. We first profiled memory γδ T cells in the BM, spleen, mesenteric lymph nodes (mLN), MA PVAT, and aortic PVAT of mice infused or not with a pressor dose of Ang II for two weeks. We then performed the same profiling on mice implanted with wireless telemetry probes to measure BP, and these mice were infused or not with a pressor dose of Ang II for two weeks followed by a two-week washout period and a second, subpressor-dose Ang II infusion. After profiling, we investigated the contribution of memory γδ T cells to BP elevation in response to repeated hypertensive challenges. We exposed telemetry-implanted mice to both a pressor followed by subpressor dose Ang II infusion as above, and depleted γδ T cells using an anti-TCRγδ-depleting antibody (clone GL3) upon initiation of the subpressor-dose Ang II infusion. Finally, to test whether memory γδ T cells sensitize mice to develop hypertension, we isolated γδ T cells from Ang II-infused or sham-treated mice and adoptively transferred them into normotensive telemetry-implanted mice, which were then infused with a subpressor dose of Ang II.

3.4 Methods

Experimental Design

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed recommendations of the Canadian Council of Animal Care. C57BL/6J WT mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and the colony was maintained on-site for the duration of the study. To determine the role of memory γδ T cells in hypertension, 10-12-week-old male C57BL/6J mice were used to produce a model of repeated Ang II hypertensive challenges. In this model, mice were initially infused or not with a pressor dose of Ang II (490 ng/kg/min, SC) for 2 weeks, followed by a 2-week washout period, and then infused with a subpressor dose of Ang II (140 ng/kg/min) for 2 weeks. The control group received a sham surgery in place of the initial 2-week pressor dose Ang II infusion to isolate the effect of an initial hypertensive stimulus on pressor sensitivity, BP, and memory γδ T cell generation in response to a mild hypertensive challenge.

To determine whether memory γδ T cells develop after a hypertensive challenge, mice were treated as indicated in the model and BP was measured by telemetry in a subset of mice. Briefly, 9-10-week-old male C57BL/6J mice were surgically instrumented with PA-C10 BP telemetry transmitters as recommended by the manufacturer (Data Sciences International, St. Paul, MN, USA). Mice were allowed to recover for 7 to 10 days, and BP determined from two days prior to and during each Ang II treatment period. Mice with a baseline systolic BP (SBP)>130 mmHg were excluded to ensure that all BP increases were due to treatment. At the end of the studies, mice were anesthetized with 3% isoflurane mixed with O_2 at 1 L/min. The efficiency of anesthesia was checked by rear foot squeezing, and then tissues were collected after either the initial pressor dose Ang II infusion or after repeated Ang II infusions. Memory γδ and αβ T cells were profiled by flow cytometry in BM isolated from the tibia and femur, and in the spleen, mLN, aortic PVAT, and MA PVAT. Extracellular markers and fluorochrome-conjugated monoclonal antibodies used for flow cytometry are presented in Table 3.1, and a representative gating strategy is presented in Figure 3.5.

In order to demonstrate whether memory γδ T cells participate in the pressor response to Ang II, mice were treated as indicated in the model and injected IP with 400 μg of γδ T cell-depleting Armenian hamster anti-mouse TCRγδ antibody (clone GL3) or Armenian hamster IgG isotype control antibody 1 day before and 6 days after the initiation of the subpressor dose Ang II

infusion. BP was measured by telemetry as above and depletion quality verified via flow cytometry in BM, spleen, MA PVAT, and aortic PVAT at the end of the study. A minimum depletion quality of 80% was chosen as a cut-off value for this experiment.

Finally, to find out whether memory γδ T cells sensitize mice to develop hypertension to a mild hypertensive insult, C57BL/6J mice were infused or not with a pressor dose of Ang II for 14 days and γδ T cells were isolated from cervical lymph nodes (cLNs), mLNs, and spleens, and purified via two successive sorts using magnetic beads (MACS, Miltenyi). After isolating γδ T cells and verifying purity and cell count via flow cytometry and automated cell counting, respectively, 2.5 x 10^5 live γδ T cells were injected intravenously (tail vein injection) into normotensive C57BL/6J recipient mice, which were then infused with a subpressor dose of Ang II for 14 days. BP was measured by telemetry in recipient mice. γδ T cell isolation required two donor mice for each recipient mouse. At the end of the study, mice were weighed, anesthetized with isoflurane, and tissues collected.

Data analysis

Results are presented as mean ± standard error of mean (SEM). Normality and homogeneity of variance of the data were verified with a Shapiro-Wilk test and equal variance test, respectively. Comparisons of BP were performed using a two-way analysis of variance (ANOVA) for repeated measures. Other comparisons between two groups were analyzed using an unpaired two-tailed t-test except for verification of γδ T cell depletion quality, where a Mann-Whitney rank sum test was used. The ANOVA tests were followed by a Student-Newman-Keuls post-hoc test. Statistical tests were performed in SigmaPlot version 13 (Systat Sotware, San Jose, CA). $P<0.05$ was considered statistically significant.

3.5 Results

3.5.1 Two-weeks of pressor dose Ang II infusion sensitized mice to develop hypertension to a subpressor dose of Ang II after a two-week washout period

To test whether a strong hypertensive challenge would sensitize mice to develop hypertension to a secondary mild hypertensive challenge, we exposed mice to two sequential 14-day Ang II infusions separated by a 14-day washout, and recorded BP (Figure 3.1A). The first hypertensive challenge, which consisted of the pressor dose of Ang II (490 ng/kg/min), caused an elevation in SBP following pump surgery until the end of the 14-day infusion ($P<0.001$, n = 7-8) (Figure 3.1B). SBP remained unchanged in control mice after sham surgery, and both groups of mice had BP equilibrate to baseline levels at the end of the two-week washout period. The SBP increase in response to the secondary, subpressor dose of Ang II (140 ng/kg/min) was higher in mice pre-exposed to hypertensive Ang II treatment than control animals over the last 3 days of telemetry acquisition ($P<0.001$, n = 7-8). The mice exposed to two hypertensive challenges (pressor and then subpressor dose Ang II infusions) had a SBP of approximately 150 mmHg by the end of the study, while control mice only infused with the subpressor dose of Ang II exhibited a SBP below our threshold for hypertension (130 mmHg) at the end of the study. These results demonstrated that an initial hypertensive challenge using a pressor dose of Ang II sensitized mice to develop hypertension to a subpressor dose of Ang II.

3.5.2 Two-weeks of a pressor dose Ang II infusion increased effector memory γδ T cells in the mesenteric artery perivascular adipose tissue and neighboring mesenteric lymph nodes

In order to determine whether Ang II-induced hypertension results in development of memory γδ T cells, we performed flow cytometry phenotyping of memory γδ T cell subsets in the spleen, BM, mLN, MA PVAT, and aortic PVAT of Ang II- or sham-treated mice. A 14-day pressor dose Ang II infusion increased the frequency of γδ T_{EM} cells ($CD44^+CD62L^-CCR7^-CD69^-CD103^-$) 5.3-fold in the MA PVAT and 1.8-fold in the mLN (expressed as a % of total γδ TCR^+ cells, n = 5-8, $P<0.05$) (Figure 3.2A). The frequency of $CD3^+$ T cells that were identified as γδ T_{EM} cells was also increased the MA PVAT and mLN (n = 5-8, $P<0.05$) (Figure 3.2A). In contrast, we observed no change in the frequency of $CD4^+$ or $CD8^+$ T_{EM} cells in the MA PVAT (Figure 3.6A), or in $CD4^+$ T_{EM} cells in the mLN of Ang II-infused mice (Figure 3.6B). However, we observed an increased frequency of $CD8^+$ T_{EM} cells (as a % of either $CD3^+$ T cells or $CD8^+$ T cells) in the mLN of mice

infused with a pressor dose of Ang II for two weeks (n = 6-9, $P<0.05$) (Figure 3.6B). The frequency of $CD3^+$, $CD4^+$, $CD8^+$, and γδ T cells was unchanged when expressed as a proportion of total $CD45^+$ cells (Figure 3.7A), and the total number of $CD45^+$ cells did not differ significantly between treatments (Figure 3.7B). Overall,14 days of Ang II infusion increased the frequency of effector memory γδ T cells in the resistance arteries of the mesenteric bed (MAs and MA PVAT) and neighboring lymph nodes (mLNs), and also upregulated the frequency of $CD8^+$ T_{EM} cells in the mLN.

3.5.3 Mice exposed to a repeat Ang II hypertensive challenge had less central memory γδ T cells in the aortic and MA PVAT compared to mice only exposed to a single, subpressor dose Ang II infusion

To determine the dynamics of memory γδ T cell populations in response to a repeat hypertensive challenge, we performed flow cytometry phenotyping of memory γδ T cell subsets in the spleen, BM, mLN, MA PVAT, and aortic PVAT of mice exposed to a repeat Ang II hypertensive challenge (Figure 3.1A) versus mice that were given a sham surgery instead of the first pressor dose of Ang II. A repeat subpressor Ang II infusion caused a 57% decrease in the frequency of γδ T_{CM} cells in the aortic PVAT (expressed as a % of total γδ TCR^+ cells) and a 52% decrease in the MA PVAT (% of $CD3^+$ cells) (n = 7-9, $P<0.05$) (Figure 3.2B). γδ T_{CM} cells were also decreased in the aortic PVAT when expressed as a frequency of overall $CD3^+$ T cells (n = 7-9, $P<0.05$) (Figure 3.2B). In contrast to memory γδ T cells, we saw no change in the frequency of $CD4^+$ or $CD8^+$ T_{CM} cells in the aortic PVAT or MA PVAT (Figures 3.6C and 3.6D). The frequency of $CD3^+$, $CD4^+$, $CD8^+$, and γδ T cells was unchanged when expressed as a proportion of total $CD45^+$ cells (Figure 3.8A), and the total number of $CD45^+$ cells did not differ significantly between treatments (Figure 3.8B).Overall, priming mice with the first pressor dose of Ang II resulted in a reduction of γδ T_{CM} cells in the aortic and MA PVAT upon subpressor dose Ang II infusion compared to mice that only received a subpressor dose of Ang II.

3.5.4 Depleting γδ T cells blunted blood pressure increases in response to a subpressor dose of Ang II in mice that had already been infused with a pressor dose of Ang II.

Since γδ T cells have been shown to participate in development of hypertension, we sought to determine whether γδ T cells contribute to the pressor hyperresponsiveness that we observed

following an initial hypertensive insult. Therefore, we depleted mice of γδ T cells at the initiation of the subpressor dose Ang II infusion using an anti-TCRγδ-depleting monoclonal antibody (clone GL3). Both groups of mice responded strongly to the pressor dose of Ang II (490 ng/kg/min), but by day 14 of the two-week washout period SBP had reduced to 133±1.6 mmHg in the IgG control group and 130±1.8 mmHg in the γδ T cell depletion group (Figure 3.3). After the washout period, mice that were intravenously administered 400 μg of anti-TCRγδ-depleting antibody (clone GL3) 1 day before and 6 days after the initiation of the subpressor dose Ang II hypertensive challenge had a SBP of 150±4.5 mmHg on day 13 of the 14-day infusion, while the IgG control group had a SBP of 163±1.9 mmHg (P<0.05, n = 7-8). The effect of treatment was significant (P<0.05, n = 7-8) for the second week of the subpressor dose infusion, which encompassed the day after the last antibody injection to the end of the study. Post-hoc analysis of the second week revealed differences in SBP were significant from days 1-6 of 7 (day 7 was not significant, P<0.05 for days 1-6, n = 7-8), with an average difference of 15 mmHg over those 6 days. Depletion quality was verified in all of the animals included in the data, and γδ T cells were 80-99% depleted in the aortic PVAT, MA PVAT, BM, and spleen of animals given the anti-TCRγδ-depleting GL3 antibody (Figure 3.9). Therefore, depleting γδ T cells with an antibody reduced the pressor responsiveness to a secondary mild hypertensive challenge.

3.5.5 Transfer of γδ T cells from Ang II infused mice into normotensive mice sensitized recipient mice to develop pressor hyperresponsiveness to a normally subpressor dose of Ang II

To test the hypothesis that memory γδ T cells can sensitize mice to develop hypertension, we isolated γδ T cells from pressor dose Ang II-infused or sham-treated donor mice and injected them into normotensive recipient mice, which were then infused with a subpressor dose of Ang II. Mice injected with γδ T cells isolated from donors infused with Ang II had SBP reach 158±3.1 mmHg by the last day of the subpressor dose Ang II infusion, while mice transferred γδ T cells from sham-treated donors had a lower SBP of 140±6.4 mmHg at the same time point (P<0.05, n = 7) (Figure 3.4). Post-hoc testing of the BP curve revealed that the increase in BP reached statistical significance for the final seven days of the subpressor Ang II infusion. Consequently, adoptive transfer of γδ T cells from Ang II-infused donor mice into normotensive recipient mice sensitized

recipient mice to develop an exaggerated pressor response to a normally subpressor dose of Ang II.

3.6 Discussion

This study demonstrated that memory γδ T cells develop and play a role in experimental hypertension. We showed that an initial exposure to a pressor dose of Ang II sensitized mice to develop hypertension in response to a subpressor dose of Ang II. Alongside BP increases, memory γδ T cells developed after pressor-dose Ang II infusion and a secondary subpressor dose Ang II infusion elicited further changes among memory γδ T cell populations. Furthermore, depleting γδ T cells after a two-week pressor dose Ang II infusion blunted the pressor response to subsequent subpressor dose Ang II infusion. Finally, adoptive transfer of γδ T cells from Ang II-infused mice induced pressor hyperresponsiveness to a normally subpressor dose of Ang II versus mice transferred γδ T cells isolated from sham-treated donors.

We previously demonstrated a role for γδ T cells in hypertension and vascular injury (8); however, it remains unclear how γδ T cells respond to repeated hypertensive challenges or if memory γδ T cells develop and contribute to high BP. First, we found that pressor dose Ang II infusion increased the frequency of effector memory γδ T cells in the resistance arteries of the mesenteric bed and nearby mLNs. γδ T_{EM} cells have been shown to produce pro-inflammatory effector cytokines (such as IL-17A and/or IFNγ) in the memory response to infection and in autoimmunity (13). In a 2012 study of rheumatoid arthritis (RA), human Vγ9Vδ2$^+$ γδ T_{EM} cells were observed in high numbers in the peripheral blood and synovial fluid of RA patients (14). The T_{EM} cells collected from RA patients demonstrated strong APC capability through high expression of human leukocyte antigen (HLA)-DR and CD80/86, (two molecules associated with professional APC function), in addition to the ability to simultaneously secrete both IFN-γ and IL-17A upon in vitro stimulation with isopentenyl pyrophosphate (IPP). It is conceivable that an enhanced cytokine-producing ability of γδ T_{EM} cells could augment the hypertensive response, as both IFN-γ and IL-17A contribute to the pathogenesis of hypertension (15, 16). However, this has yet to be demonstrated experimentally

Antigen presentation by γδ T_{EM} cells could also exacerbate hypertension by inducing the activation of CD4$^+$ and CD8$^+$ T cells. Caillon *et al.* (2021) demonstrated this concept by co-culturing αβ T cells with γδ T cells harvested from normotensive mice or mice made hypertensive by infusion of Ang II, and observed that γδ T cells from hypertensive but not normotensive mice caused an increase in the fraction of activated (CD69$^+$) αβ T cells (17). Putative neoantigens could

arise from hypertension-induced tissue injury. One possible source of immunogenic neoantigens could be components of damaged vascular tissue in the resistance arteries of the mesenteric bed, where we also observed increased γδ T_{EM} cells. Kirabo *et al.* (2014) discovered that proteins oxidatively modified by highly reactive isoketals accumulate in dendritic cells (DCs) in hypertension, triggering T cell activation, proliferation, and cytokine production (18). It is possible that γδ T_{EM} cells could present similar neoantigens to other T cell subsets, both locally and in nearby mLNs, and it is conceivable that this could lead to increased immune sensitivity to future hypertensive insults. This seems likely given that γδ T cells are able to act as professional APCs to αβ T cells, and γδ T cells can also effectively cross-present antigens to $CD8^+$ T cells (3, 19). Future experiments will hopefully determine if γδ T cells are able to present antigens in the context of hypertension. With respect to the origin of γδ T_{EM} cells, they may have evolved from γδ T_{CM} and/or T_{RM} cells which have encountered cognate antigen and shifted to an effector memory phenotype (13). In contrast, γδ T_{EM} cells could have also evolved from effector γδ T cells elicited by the Ang II infusion that have acquired a memory phenotype following activation, as the dynamics of memory γδ T cell differentiation are not well understood. In addition to γδ T_{EM} cells, we also observed an increased frequency of $CD8^+$ T_{EM} cells in the mLNs of mice infused with a pressor dose of Ang II. Itani *et al.* (2016) demonstrated an accumulation of $CD8^+$ T_{EM} in the kidneys and BM of hypertensive mice (11). However, we have shown that $CD8^+$ T_{EM} cells are also upregulated in lymph nodes proximal to the mesenteric vascular bed, which contains a high concentration of resistance arteries.

In comparison to a single hypertensive challenge, we observed a decreased frequency of γδ T_{CM} cells upon a repeat hypertensive challenge. It is speculated that γδ T_{CM} cells are antigen-experienced and sourced from effector cells that survived immune contraction following an earlier immune response (20). γδ T_{CM} cells could become proinflammatory effector cells, migrate, and/or proliferate upon cognate TCR antigen recognition, which could lead to the reduced frequency of γδ T_{CM} cells in the tissues that we investigated. Notably, T_{CM} cells have been observed to inherit T_{EM} markers and characteristics, along with the ability migrate to the circulation and other tissues following activation (21). This may explain the reduction of γδ T_{CM} that we observed in the aortic and MA PVAT following the second, mild hypertensive challenge. However, we did not observe any significant changes regarding the frequency of γδ T_{EM} cells in the tissues that we studied after

a repeat hypertensive challenge. Therefore, it is possible that γδ T_{CM} cells could be shifting to a γδ T_{EM} phenotype and/or migrating to other tissues that we did not study. Future studies will hopefully shed more light onto the population dynamics of memory γδ T cells in hypertension.

The frequency of total γδ, $CD3^+$, $CD4^+$, and $CD8^+$ T cells was unchanged between treatments when expressed as a proportion of total $CD45^+$ cells, and the number of $CD45^+$ cells was also unchanged (Figures 3.7 and 3.8). This was observed after a single pressor dose Ang II infusion and after repeat Ang II infusions versus respective control groups. Therefore, we can conclude that γδ T_{EM} and T_{CM} cell populations were indeed increased and decreased, respectively, and that these observations were not simply a result of overall changes in the immune cell or T cell profile. Future investigations would benefit from phenotyping memory γδ T cells in locations such as the circulation and kidneys of hypertensive mice, as Itani *et al.* (2016) observed an accumulation of $CD8^+$ and $CD8^+$ T_{EM} cells in the kidneys of mice made hypertensive by a repeat Ang II infusion or L-NAME/high-salt challenge (11). Memory γδ T cells could also traffic to these locations and contribute to hypertension.

Antibody-mediated depletion of γδ T cells led to a reduced ability to induce BP elevation in mice that had already undergone a moderate hypertensive challenge. Mice depleted of γδ T cells exhibited a reduced pressor response to the subpressor dose Ang II hypertensive challenge, indicating blunted pressor sensitivity to a secondary Ang II infusion compared to mice not depleted of γδ T cells. Depletion of γδ T cells, which represent only a small fraction of overall T cells, led to an average 14 mmHg reduction in SBP over the final week of the two-week subpressor dose Ang II infusion. It is worth noting that mice that were depleted of γδ T cells still had γδ T cells present during the initial pressor dose Ang II infusion, and they also retained $CD4^+$ and $CD8^+$ αβ T cells and innate cells that play a role in the development of hypertension during the subpressor dose Ang II infusion. This further confirms a role for γδ T cells in hypertension and indicates that memory γδ T cells also contribute to the development and maintenance of hypertension. A 14 mmHg drop in BP in hypertensive patients could significantly reduce overall morbidity. Therefore, memory γδ T cells show potential as a therapeutic target to reduce BP in hypertension.

Interestingly, controversy exists regarding the ability of the GL3 monoclonal antibody to deplete γδ T cells. A 2009 publication by Koenecke *et al.* demonstrated that the GL3 antibody does not deplete, but rather generates "invisible" γδ T cells by causing TCR internalization, leaving

the cells still present with other surface receptors (22). If this is true, then our data could indicate that γδ T cells contribute to the pathogenesis of hypertension in a TCR-mediated mechanism. GL3 antibody-mediated γδ TCR internalization could prevent antigen recognition, and block both TCR signaling and TCR-mediated cell activation as a result. This could conceivably be exploited to determine the contribution of other γδ T cell immune receptors to the pathogenesis of hypertension, such as toll-like receptors and natural killer receptors (23).

Adoptive transfer of γδ T cells from Ang II-infused mice increased the pressor sensitivity in recipient mice to a normally subpressor dose Ang II. Given the results of the depletion experiments, this finding was not unexpected. However, the magnitude of the BP increase over the control group was surprising given the fact that both groups received the same number of γδ T cells. As the only difference between the two groups was the donor mouse Ang II infusion status, this experiment demonstrates that γδ T cells possess a form of immune memory in the context of Ang II-induced hypertension. The recipient mice were not treated in any way other than being implanted with telemetry probes and the pump containing a subpressor dose of Ang II, therefore, these mice still possessed their own functional immune system including endogenous γδ T cells. The mice transferred γδ T cells from sham-treated animals also demonstrated increased SBP in response to the subpressor dose of Ang II, albeit to a lesser extent than the mice transferred γδ T cells from hypertensive animals. Unlike the mice that were infused with a subpressor dose of Ang II in the first experiment (Figure 3.1B), the mice that were transferred γδ T cells from sham-treated donors did not have SBP return to below our threshold for hypertension by the end of the subpressor dose Ang II infusion (<130 mmHg). Therefore, simply adding additional γδ T cells on top of the endogenous γδ T cells already present in recipient mice appears to be enough to modestly augment pressor responsiveness. Overall, γδ T cells alone are able to confer increased risk of developing hypertension to a mild hypertensive stimulus. We have shown that memory γδ T cells contribute to the pathogenesis of hypertension, and we are the first to show a role for memory γδ T cells in hypertension.

3.7 Conclusion

We previously demonstrated that γδ T cells play a role in Ang II-induced hypertension and vascular injury (8). Here, we observed the dynamics of memory γδ T cell populations following repeated hypertensive challenges. We demonstrated that γδ T_{EM} cells were upregulated in the MA PVAT and mLN after an initial pressor dose infusion of Ang II, and that γδ T_{CM} cells were downregulated in the aortic PVAT and MA PVAT following a repeat Ang II hypertensive challenge. Mice infused with a pressor dose of Ang II showed heightened pressor responsiveness to subsequent subpressor dose Ang II infusion. Depletion of γδ T cells following a pressor dose Ang II hypertensive challenge revealed that removal of functional γδ T cells blunted the pressor response to a low dose of Ang II. Finally, adoptive transfer of γδ T cells from mice infused with a pressor dose of Ang II resulted in recipient mice exhibiting heightened pressor responsiveness to a subpressor dose Ang II infusion. These results show that memory γδ T cells contribute to the pathogenesis of hypertension, and future studies of adaptive immunity in cardiovascular disease would benefit from considering a potential role for memory γδ T cells. Future studies into cardiovascular disease would also benefit from determining the effector cytokines produced by memory γδ T cells. Targeting memory γδ T cells and finding ways to prevent their formation may offer new therapeutic avenues for the treatment of human hypertension.

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3.10 Disclosures

No potential competing interest, financial or otherwise, are reported by the authors.

Data availability

Data are available on request.

3.11 References

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3.12 Figures

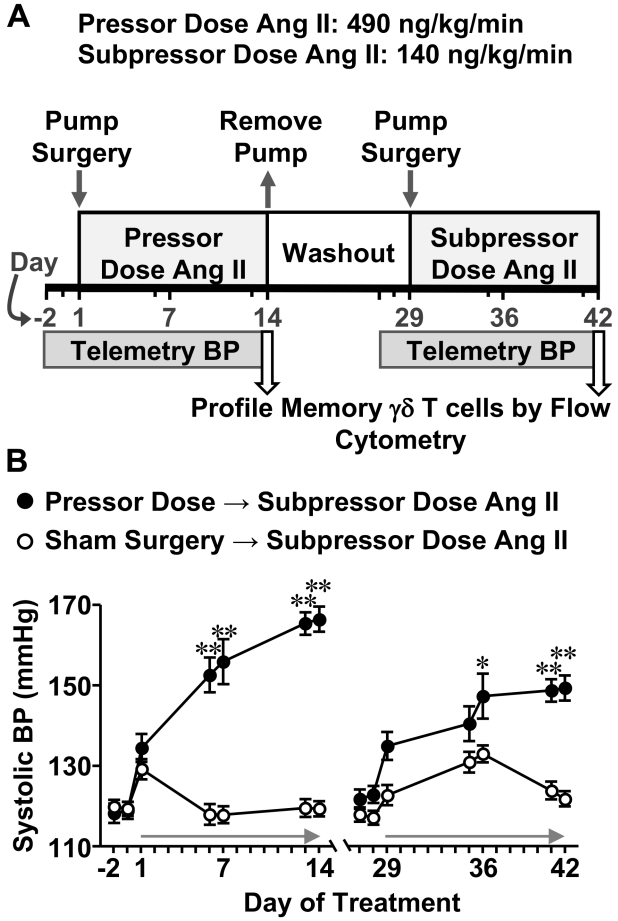


Figure 3.1: Fourteen-day pressor dose angiotensin (Ang) II infusion induced blood pressure (BP) elevation in response to subsequent subpressor Ang II hypertensive challenge. A. Angiotensin II osmotic minipump dosage and experimental design for the mouse model of repeat hypertensive challenge. B. Telemetry results for the model of repeat hypertensive challenge. BP was recorded two days prior to treatment (baseline) and during each hypertensive challenge. Horizontal gray arrows indicate each hypertensive challenge. Data were analyzed using a two-way ANOVA for repeated measures followed by a Student-Newman-Keuls post hoc test. n=7-8; **P*<0.05 and ***P*<0.001 vs. control.

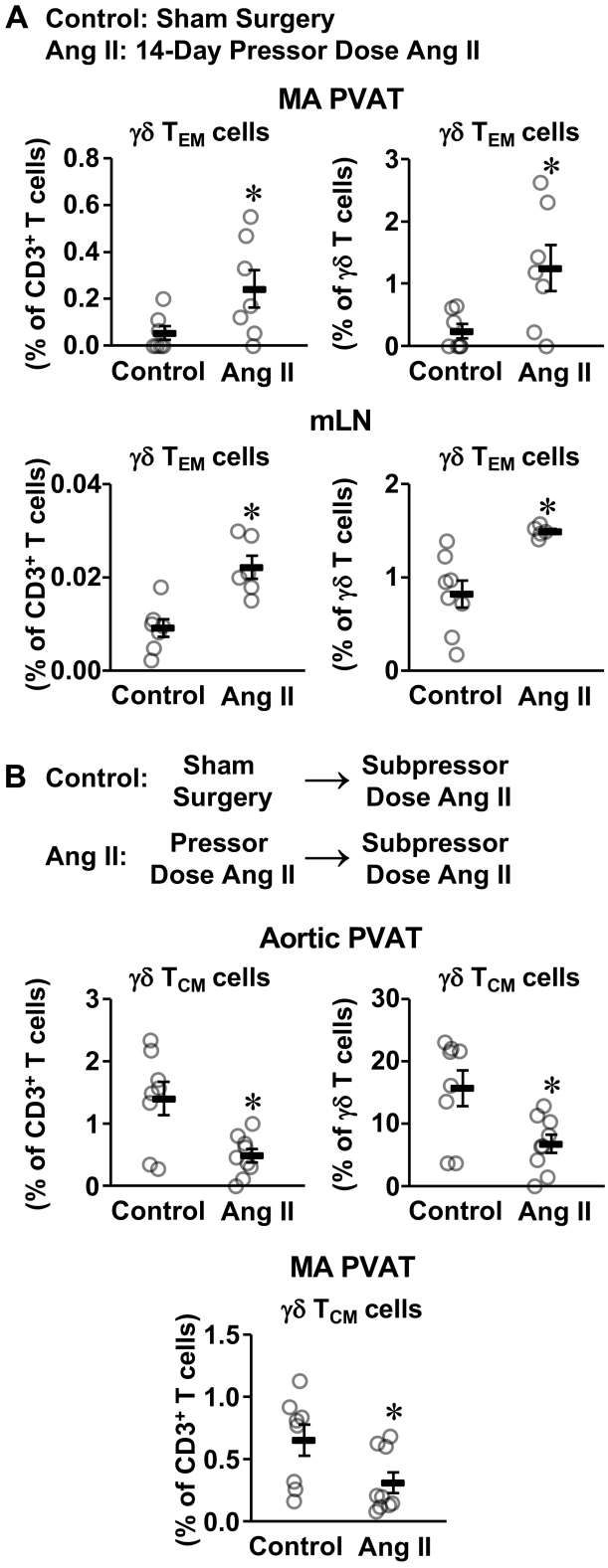


Figure 3.2: A. Fourteen-day pressor dose angiotensin (Ang) II infusion upregulated effector memory γδ T cells in the mesenteric artery (MA) perivascular adipose tissue (PVAT) and mesenteric lymph nodes (mLN). The control group underwent a sham surgery. B. Repeated Ang II hypertensive challenges downregulated central memory γδ T cells in the aortic PVAT and MA PVAT. The control group underwent a sham surgery in place of the initial pressor dose Ang II infusion, but both groups received the subpressor dose Ang II infusion for the final two weeks. Data are presented as means ± SEM. Data were analyzed using an unpaired t-test. n=5-9; **P*<0.05 vs. control.

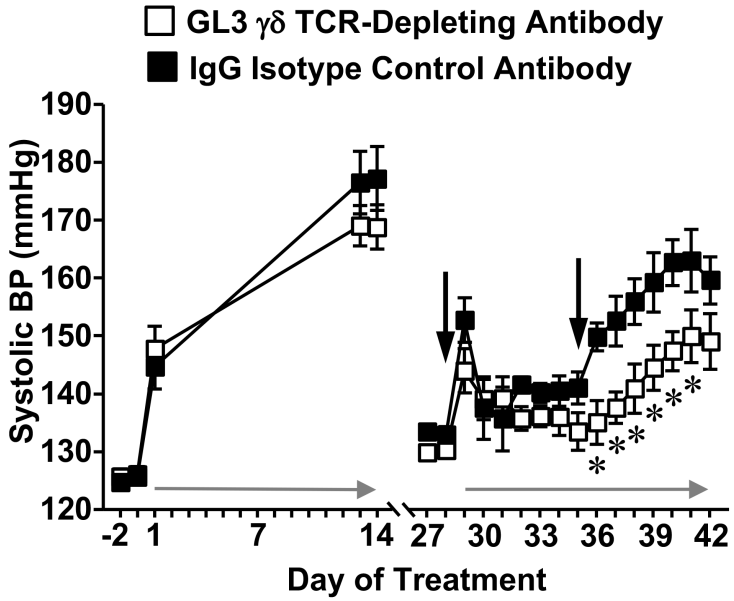


Figure 3.3: Injection of a TCRγδ-depleting antibody at the initiation of and halfway through a two-week subpressor dose angiotensin (Ang) II hypertensive challenge blunted increases in systolic blood pressure (BP) in mice that had already been exposed to a two-week pressor dose Ang II hypertensive challenge. Horizontal gray arrows represent periods of Ang II infusion. Black vertical arrows represent days of control and γδ T cell-depleting antibody injections. Data were analyzed using a two-way ANOVA for repeated measures from day 36 to 42 followed by a Student-Newman-Keuls post hoc test. n=7-8; **P*<0.05 vs. isotype control antibody.

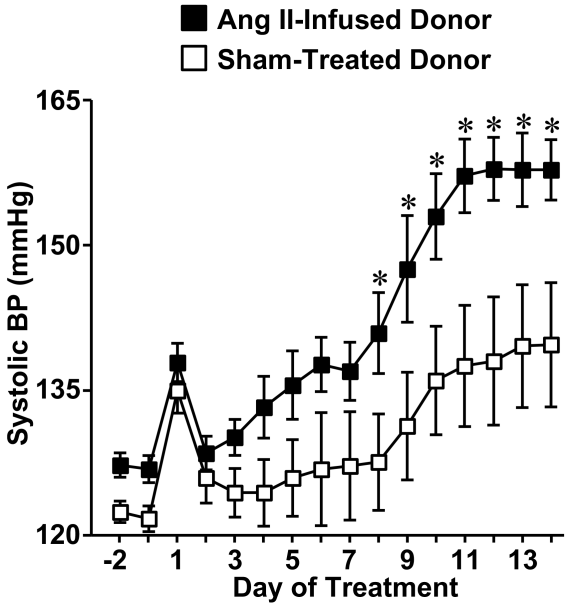


Figure 3.4: Injection of $2.5x10^5$ live γδ T cells harvested from spleens and lymph nodes of mice made hypertensive by two-weeks of angiotensin (Ang) II infusion sensitized recipient mice to develop high blood pressure (BP) in response to a subpressor dose of Ang II. Curves were analyzed using a two-way ANOVA for repeated measures followed by a Student-Newman-Keuls post hoc test. n=7; *P<0.05 vs. sham-treated donor.

3.13 Supplementary Methods

3.13.1 Experimental Design

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed recommendations of the Canadian Council of Animal Care. C57BL/6J WT mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and the colony was maintained on-site for the duration of the study. To determine the role of memory γδ T cells in hypertension, 10-12-week-old male C57BL/6J mice were used to produce a model of repeated Ang II hypertensive challenges. In this model, mice were initially infused with Ang II (490 ng/kg/min, SC) for 2 weeks, followed by a 2-week washout period, and then infused or not with a subpressor dose of Ang II (140 ng/kg/min) for 2 weeks.

To determine if memory γδ T cells develop after a hypertensive challenge, mice were treated as indicated in the model and BP was measured by telemetry in a subset of mice. Briefly, 9-10-week-old male C57BL/6J mice were surgically instrumented with PA-C10 BP telemetry transmitters as recommended by the manufacturer (Data Sciences International, St. Paul, MN, USA). Mice were allowed to recover for 7 to 10 days, and BP determined from two days prior to and during each Ang II treatment period. Mice with a baseline systolic blood pressure (SBP)>130 mmHg were excluded to ensure that all blood pressure increases were due to treatment. Mice were subsequently weighed, anesthetized with isoflurane, and tissues collected after the initial exposure to the hypertensive stimulus, and after the second 2-week mild hypertensive challenge. Memory γδ T cells and activated T cells were profiled by flow cytometry in BM isolated from the tibia and femur, and in the spleen, mLN, aortic PVAT, and MA PVAT.

To see if memory γδ T cells participate in the pressor response to Ang II, mice were treated as indicated in the model and injected IP with 400 μg of γδ T cell depleting Armenian hamster anti-mouse TCRγδ antibody (clone GL3) or Armenian hamster IgG isotype control antibody 1 day before and 6 days after the initiation of the second mild hypertensive challenge. BP was measured by telemetry as above and depletion quality verified via flow cytometry in BM, spleen, MA PVAT, and Ao PVAT. A minimum depletion quality of 80% was chosen as a cut-off value for this experiment.

Finally, to see if memory γδ T cells sensitize mice to develop hypertension to a mild hypertensive insult, C57BL/6J mice were exposed to a hypertensive stimulus or not for 14 days (Ang II, 490 ng/kg/min) and γδ T cells were isolated from cervical lymph nodes (cLNs), mLNs,

and spleen, and purified via two successive sorts using magnetic beads (MACS, Miltenyi). After isolating γδ T cells and verifying purity and cell count via flow cytometry and automated cell counting, respectively, 2.5 x 10^5 live γδ T cells were injected intravenously (tail vein injection) into normotensive C57BL/6J recipient mice, which were then exposed to a mild hypertensive challenge (Ang II, 140 ng/kg/min) for 14 days. BP was measured by telemetry in recipient mice. γδ T cell isolation required two donor mice for each recipient mouse. At the end of the study, mice were weighed, anesthetized with isoflurane, and tissues collected. Profiling of memory γδ T cells and activated T cells was done by flow cytometry in BM, spleen, mLN, aortic PVAT, and MA PVAT.

3.13.2 Flow Cytometry Panel Design

The following anti-mouse fluorochrome-conjugated antibodies were selected to profile γδ T_{CM} cells, γδ T_{EM} cells, and γδ T_{RM} cells, as well as $CD4^+$ and $CD8^+$ memory T cells: Brilliant Violet 786™ (BV786)-conjugated anti-mouse CD45 (BD, clone 30-F11), Brilliant Violet 605™ (BV605)-conjugated anti-mouse CD3 (Biolegend, clone 17A2), Peridinin-Chlorophyll-Protein (PerCP)-eFluor™ 710 (PerCP-eF710)-conjugated anti-mouse CD4 (eBioscience, clone RM4-5), Alexa Fluor® 700 (AF700)-conjugated anti-mouse CD8 (Biolegend, clone 53-6.7), Alexa Fluor® 647 (AF647)-conjugated anti-mouse TCRγδ (Biolegend, clone GL3), Phycoerythrin (PE)-Cyanine (Cy)® 7 (PE-Cy7)-conjugated anti-mouse CD44 (BD, clone IM7), Allophycocyanin (APC)/Fire™ 750 (APC/Fire750)-conjugated anti-mouse CD62L (Biolegend, clone MEL-14), PE-CF® 594 (PE-CF594)-conjugated anti-mouse CCR7 (BD, clone 4B12), Alexa Fluor® 488 (AF488)-conjugated anti-mouse CD103 (Biolegend, clone 2E7), and PE-Cy5-conjugated anti-mouse CD69 (Biolegend, clone H1.2F3). In order to maximize separation of positive and negative populations and minimize background noise, the panel was optimized by titrating the fluorochrome-conjugated monoclonal antibodies using splenocytes and a series of 8 dilutions of the antibody. The lowest dilution with clear positive and negative separation was selected as the best.

3.13.3 Tissue Collection

Mice were anesthetized with isoflurane and depth of anesthesia was confirmed using a toe pinch and observing no pedal withdrawal reflex. An incision was then made through the abdomen of the animal. The spleen was collected first via blunt dissection and placed in 1 mL of phosphate buffered saline (PBS) in the well of a 12-well plate and kept on ice. To harvest the MA PVAT and mLNs, the mesenteric bed was tied off at both ends (near the stomach and rectum) with surgical sutures and excised from the mouse. The mesenteric bed was then kept in a 50 mL tube filled with PBS and placed on ice. The liver, stomach, heart, lungs, and esophagus were then removed from the mouse and discarded to expose the thoracic aorta. The thoracic region of the aorta with attached PVAT was removed from the mouse by cutting inferiorly and superiorly near the celiac trunk and aortic arch respectively. By cutting along the spinal vertebra behind the aorta, the aorta could be removed along with all of its associated PVAT. The aorta and PVAT were then placed in 1 mL of PBS in the well of a 12-well plate and kept on ice. Finally, the right hind limb was removed from the animal by manually dislocating the hip joint and cutting the attached tendons and connective

tissue with small surgical scissors. After removing the limb, the tibia and femur were separated by cutting the knee joint, and each bone was cleaned of any attached tissue. Care was taken to not break the tibia and femur to keep the bone marrow uncontaminated. The tibia and femur were then placed in the well of a 12-well plate and kept on ice. The tissue samples and cell suspensions derived from them were kept on ice throughout this protocol, except during flow cytometry staining.

3.13.4 Flow Cytometry Sample Preparation

Single cell suspensions of splenocytes were prepared by crushing the spleen over the mesh of a 70 μm cell strainer (the mesh was previously cut out from the cell strainer using a blade) in the well of a 12-well plate containing 1 mL of PBS. The back of a 5 mL syringe plunger was used to crush the spleen. Splenocytes were then filtered through a new 70 μm cell strainer into a 50 mL tube, followed by centrifugation. Cells were resuspended in 1 mL of PBS and kept on ice until used. One hunded μL of splenocyte cell suspension was used per test corresponding to $1/10^{th}$ of the spleen.

To isolate the MA PVAT and mLNs, the mesenteric bed was pinned down to a wax dissection plate containing cold PBS, and the cluster of mLNs removed from near the cecum. The mLNs were then gently crushed using the back of a 1 mL syringe plunger over mesh removed from a 70 μm cell strainer in the well of a 12 well plate containing 1 mL of PBS. Cell suspensions were then filtered through a 70 μm cell strainer into a 1.5 mL microtube and washed with PBS. Finally, mLN cells were resuspended in 100 μL of PBS and placed on ice until staining. Sections of MA PVAT were then removed from the mesenteric bed and checked under a microscope for lymph node contamination. The tissue was minced with scissors and then digested in solution containing collagenase A (0.15 U/mL), collagenase type 2 (500 U/mL), elastase (2 U/mL), and trypsin inhibitor (0.5 U/mL) for 30 minutes to one hour at 37°C with gentle agitation. Following digestion, the MA PVAT was filtered through a new 70 μm cell strainer into a 1.5 mL microtube and washed with PBS, then resuspended in 100 μL of PBS and kept on ice until used.

To prepare single cell suspensions of T cells residing within the aortic wall and aortic PVAT, the aorta was first looked at under a microscope to check for any lymph node contamination before being minced with scissors and digested in solution containing collagenase A (0.15 U/mL),

collagenase type 2 (500 U/mL), elastase (2 U/mL), trypsin inhibitor (0.5 U/mL), and hyaluronidase (48 U/mL) for 45 minutes to one hour at 37°C with agitation. The cells were then filtered through a 70 μm cell strainer into a 1.5 mL microtube, washed with PBS, and resuspended in 100 μL of PBS and kept on ice until used.

For BM isolation, the tibia and femur were cleaned with 70% ethanol, cut at each end, and the marrow flushed into a well of a 6-well plate using a 28 g needle and 3-5 mL of RPMI-1640 media. BM cells were then filtered through a 70 μm cell strainer into a 50 mL tube and washed with PBS. Finally, BM cells were resuspended in 200 μL of PBS and kept on ice until used. One hundred μL of BM cell suspension was used per test.

3.13.5 Flow Cytometry Antibody Labeling

Cell suspensions were then transferred into labelled wells of a V-bottom 96-well plate and incubated with LIVE/DEAD™ Aqua (ThermoFisher, cat. no. L34957) for 20 minutes at room temperature (RT) at ¼ of the manufacturer's recommended concentration (1:400), followed by washing with PBS. It was determined via titration experiments carried out by other members of the lab that ¼ of the manufacturer's recommended concentration of LIVE/DEAD™ Aqua was sufficient for staining purposes. Cells were then washed and resuspended in 100 μL of antibody cocktail containing PBS, fluorochrome-conjugated monoclonal antibodies, inactivated fetal bovine serum (6%), and CD16/CD32 Fc block (1%) to limit non-specific binding, and incubated for 30 minutes at 37°C. The amount of each antibody used per 100 μL test was: 0.0625 μg BV786-CD45, 0.125 μg BV605-CD3, 0.05 μg PerCP-eF710-CD4, 0.125 μg AF700-CD8, 0.05 μg AF647-TCRγδ, 0.125 μg PE-Cy7-CD44, 0.1 μg APC-Fire750-CD62L, 0.25 μg AF488-CD103, 0.25 μg PE-CF594-CCR7, and 0.25 μg PE-Cy5-CD69. The high incubation temperature (37°C) was used as recommended by the manufacturer to allow for more favorable binding kinetics for the anti-CCR7 antibody. Fluorescence minus one controls (FMOs) were prepared using leftover splenocytes for proper gate placement, and compensation beads were used for each antibody to compensate for fluorescent spillover between detectors. Cells were then washed with PBS, resuspended in 200 μL PBS per test, and run on the BD LSR Fortessa II flow cytometer immediately following staining to avoid the loss of signal associated with fixation.

3.13.6 Flow Cytometry Gating Strategy

The gating strategy was as follows: cells were first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using FSC-A over FSC height (FSC-H). Singlet cells were gated again using SSC-A over SSC height (SSC-H) to further clean up the data. Live cells were gated in the viability dye/FSC-A plot. Then $CD45^+$ cells were gated in the CD45/FSC-A plot, followed by $CD3^+$ T cells in the CD3/FSC-A plots. Then, $TCR\gamma\delta^+$ cells were gated from the TCRγδ/CD3 plot followed by memory γδ T cells as $CD44^+$ in the CD44/FSC-A plot. Each memory γδ T cell subset was gated from the $TCR\gamma\delta^+CD44^+$ gate. γδ T_{CM} cells were gated as $CD62L^+CCR7^+$ in the CD62L/CCR7 plot. Both γδ T_{EM} and γδ T_{RM} cell subsets were first gated as $CD62L^-CCR7^-$ in the CD62L/CCR7 plot. Then γδ T_{EM} cells were gated as $CD69^-CD103^-$ and γδ T_{RM} cells as $CD69^+CD103^+$ in the CD69/CD103 plot. The gating strategy for memory $CD4^+$ and $CD8^+$ T cells was identical to that of memory γδ T cells, and this gating was carried out following the gating of $CD3^+$ cells.

3.13.7 Isolation and Enrichment of γδ T Cells via Magnetic Bead Sorting

Mice were anesthetized with isoflurane as above. An incision was then made through the abdomen of the animal. The spleen was collected first via blunt dissection and placed in 1 mL of sterile phosphate buffered saline (PBS) in the well of a 12-well plate and kept on ice. Following removal of the spleen, the mesenteric bed was gently pushed to the left side of the animal and cluster of mLNs exposed near the cecum. The mLNs where then cut from the mesenteric bed with care taken to avoid incising the large or small intestines, thereby avoiding bacterial contamination. The mLNs were then placed in 1 mL of sterile phosphate buffered saline (PBS) in the well of a 12-well plate and kept on ice. Finally, an incision was made along the front of the neck and the cLNs exposed by blunt dissecting the skin from underlying muscle tissue. The left and right cLNs were then carefully removed from the neck using small scissors and tweezers and placed in 1 mL of sterile phosphate buffered saline (PBS) in the well of a 12-well plate kept on ice. The animal was then euthanized via removal of the heart.

To maintain as much sterility as possible, cell isolation with magnetic beads was wholly performed inside the confines of a biological safety cabinet. Single cell suspensions of each tissue were prepared by gently crushing spleens and lymph nodes under cut out 70 μm cell strainers in

individual wells of the 12-well plate, as performed above for flow cytometry staining. Suspensions were then filtered once more through a new 40 μm cell strainer into a 50 mL tube. The suspensions were pooled at this point, with two donor suspensions being used per recipient animal injected. Suspensions were then washed with 30-40 mL of cold MACS buffer and then resuspended in 500 μL cold MACS buffer. Suspensions were then incubated with 3 μL of Fc block and 6 μL biotin anti-TCRγδ (BioLegend, clone GL3) for 15 minutes on ice. Cells were subsequently washed twice with 40 mL of MACS buffer each time, and then resuspended in 500 μL of MACS buffer. Two 20 μL samples of each cell suspension were then taken and placed into individual wells of a 96-well plate on ice for unstained and pre-sort flow cytometry control samples. These samples were then quickly washed with PBS and resuspended in 100 μL of fresh, cold PBS to minimize exposure of cells to EDTA in the MACS buffer. Subsequently, 80 μL of Miltenyi UltraPure Anti-Biotin MicroBeads were added to each tube of pooled cell suspension and samples incubated for 20 minutes on ice.

Following labelling with magnetic beads, cells were washed once with 30-40 mL of MACS buffer and resuspended to a final volume of 500 μL. Each suspension was then pipetted through a new 40 μm cell strainer directly into a LS magnetic column (pre-saturated with buffer), and the LS column was subsequently topped up with 5 mL of fresh, cold MACS buffer a total of three times to allow unlabelled cells and debris to pass through. After three washes, the column was carefully removed from the magnet and labelled cells flushed from the column twice, using 5 mL of MACS buffer each time and the included plunger. The enriched suspension of primarily γδ T cells was then added to a new pre-saturated column and the magnetic bead sorting protocol repeated exactly as above.

After the second magnetic beads sort, cells were washed with cold PBS and resuspended in 200 μL of PBS and kept on ice until sample purity and viability had been confirmed. From the final 200 μL sample, one 10 μL aliquot was taken for automated cell counting and one 20 μL aliquot was taken for flow cytometry confirmation of viability and purity. The pre-sort and post-sort flow cytometry samples were then incubated with LIVE/DEAD™ Aqua viability stain in wells of a V-bottom 96-well plate and as described above, and then washed and stained with 2 μL of PE streptavidin for 10 minutes at RT. PE streptavidin binds the biotinylated anti-TCRγδ antibody used to identify γδ T cells for the magnetic bead sorting, and the percentage of PE-positive cells

indicated the purity of γδ T cells in each sample. Samples were then resuspended in cold PBS and quickly brought to the LSR Fortessa II flow cytometer.

Sample viability and γδ T cell purity was determined by gating Aqua-negative and PE-positive populations, respectively. Cell counts were then determined with the 10 μL post-sort aliquot using the brightfield count function on the DeNovix CellDrop™ cell counter. As a quality control to minimize variability among experiments, we set a threshold of a minimum of 75% live γδ T cells per sample in order to proceed with the injection. After considering the viability, purity, and cell count of each sample, it was calculated how much of the remaining 170 μL of γδ T cell suspension would be needed to inject 2.5 x 10^5 viable γδ T cells into recipient mice. These cells were then resuspended in sterile saline and quickly injected via tail vein into recipient mice, which had been implanted with a pump containing a subpressor dose of Ang II the day before.

3.13.8 Data analysis

Results are presented as mean ± standard error of mean (SEM). Comparisons of BP were performed using a two-way analysis of variance (ANOVA) for repeated measures. Other comparisons between two groups were analyzed using a two-tailed t-test or Mann-Whitney rank sum test if assumptions were not met (equality of variance, normality). The ANOVA tests were followed by a Student-Newman-Keuls post-hoc test. Statistical tests were performed in SigmaPlot version 13 (Systat Sotware, San Jose, CA). $P<0.05$ was considered statistically significant.

3.13.9 Supplementary Tables

Table 3.1: Antibodies for flow cytometry profiling of memory γδ T cells.

Antibody	Description	Clone, company
CD45	BV786-conjugated anti-mouse CD45 antibody	30-F11, BD Biosciences
CD3	BV605-conjugated anti-mouse CD3 antibody	17A2, Biolegend
TCRγδ	AF647-conjugated anti-mouse TCRgd antibody	GL3, Biolegend
CD4	PerCP-eF710-conjugated anti-mouse CD4 antibody	RM4-5, eBioscience
CD8	AF700-conjugated anti-mouse CD8 antibody	53-6.7, Biolegend
CD44	PE-Cy7-conjugated anti-mouse CD44 antibody	IM7, BD Biosciences
CCR7	PE-CF594-conjugated anti-mouse CCR7 antibody	4B12, BD Biosciences
CD62L	APC/Fire750-conjugated anti-mouse CD62L antibody	MEL-14, Biolegend
CD103	AF488-conjugated anti-mouse CD103 antibody	2E7, Biolegend
CD69	PE-Cy5-conjugated anti-mouse CD69 antibody	H1.2F3, Biolegend

BV786, Brilliant Violet 786™; BV605, Brilliant Violet 605™; AF647, Alexa Fluor® 647; PerCP-eF710, Peridinin-Chlorophyll-Protein (PerCP)-eFluor™ 710; AF700, Alexa Fluor® 700; PE-Cy7, Phycoerythrin (PE)-Cyanine (Cy)® 7; PE-CF594, Phycoerythrin (PE)-CF® 594; APC/Fire750, Allophycocyanin (APC)/Fire™ 750; AF488, Alexa Fluor® 488; PE-Cy5, Phycoerythrin (PE)-Cyanine (Cy)® 5.

3.13.10 Supplementary Figures and Figure Legends

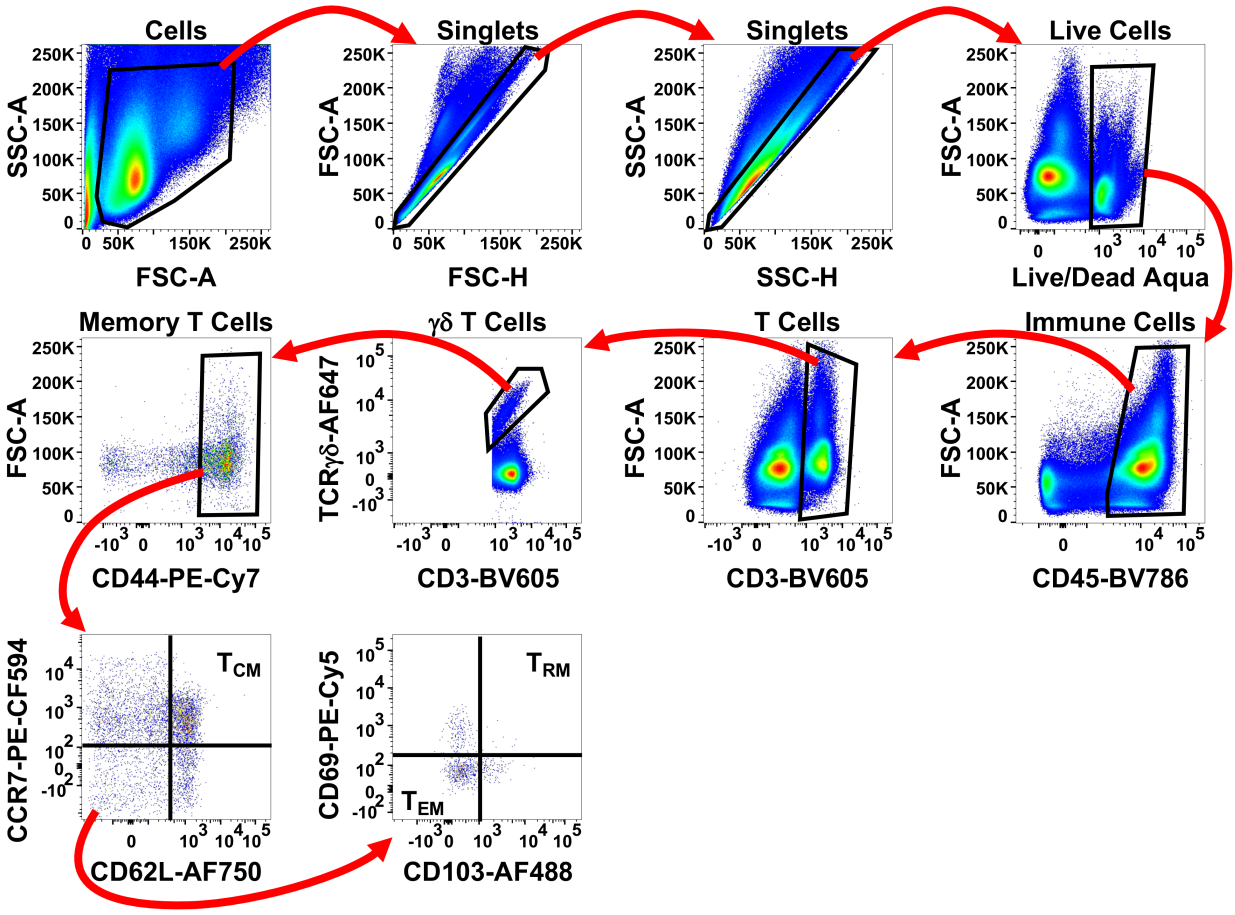


Figure 3.5: Flow cytometry gating strategy for profiling of memory γδ T cells in aortic perivascular adipose tissue (PVAT), mesenteric artery (MA) PVAT, bone marrow, mesenteric lymph nodes (mLN), and spleen. Single cell suspensions were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Brilliant Violet 786™ (BV786)-conjugated anti-mouse CD45 (BD, clone 30-F11), Brilliant Violet 605™ (BV605)-conjugated anti-mouse CD3 (Biolegend, clone 17A2), Peridinin-Chlorophyll-Protein (PerCP)-eFluor™ 710 (PerCP-eF710)-conjugated anti-mouse CD4 (eBioscience, clone RM4-5), Alexa Fluor® 700 (AF700)-conjugated anti-mouse CD8 (Biolegend, clone 53-6.7), Alexa Fluor® 647 (AF647)-conjugated anti-mouse TCRγδ (Biolegend, clone GL3), Phycoerythrin (PE)-Cyanine (Cy)® 7 (PE-Cy7)-conjugated anti-mouse CD44 (BD, clone IM7), Allophycocyanin (APC)/Fire™ 750 (APC/Fire750)-conjugated anti-mouse CD62L (Biolegend, clone MEL-14), PE-CF® 594 (PE-CF594)-conjugated anti-mouse CCR7 (BD, clone 4B12), Alexa Fluor® 488 (AF488)-conjugated anti-mouse CD103 (Biolegend, clone 2E7), and

PE-Cy5-conjugated anti-mouse CD69 (Biolegend, clone H1.2F3), and analyzed by flow cytometry. Fluorophores were respectively excited and analyzed with the appropriate laser and band pass filter (BP) (Aqua: 405 nm with 525/50 BP, BV786: 405 nm with 780/60 BP, BV605: 405 nm with 610/20 BP, PerCP-eF710: 488 nm with 695/40 BP, AF700: 640 nm with 730/45 BP, AF647: 640 nm with 670/14 BP, PE-Cy7: 561 nm with 780/60 BP, APC-Fire750: 640 nm with 780/60 BP, PE-CF594: 561 nm with 610/20 BP, AF488: 488 nm with 530/30 BP, PE-Cy5: 561 nm with 670/30 BP). A representative flow cytometry gating strategy of splenocytes of wild-type mice infused with angiotensin II for 14 days is shown. Cells were gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using FSC-A over FSC height (FSC-H) and then again using SSC-A over SSC height (SSC-H). Live cells were gated in the Aqua/FSC-A plot. $CD45^+$ cells were gated in the CD45/FSC-A plot. $CD3^+$ T cells were gated in the CD3/FSC-A plots. γδ T cells were gated in the TCRγδ/CD3 plot. $CD44^+$ γδ T cells were gated in the CD44/FSC-A plot. γδ T_{CM} cells were gated in the CD62L/CCR7 plot. γδ T_{EM} and T_{RM} cell subsets were gated in the CD69/CD103 plot.

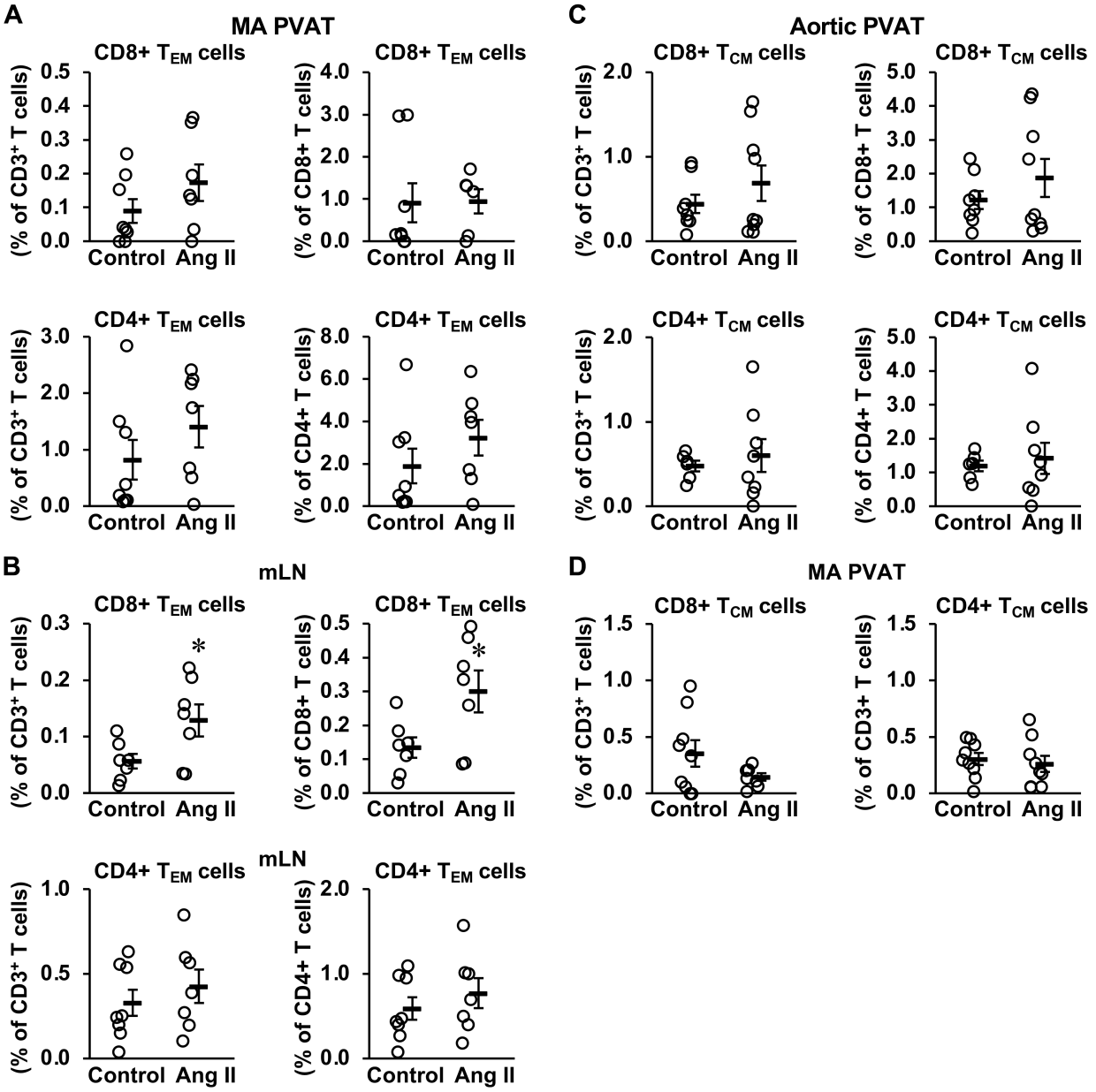
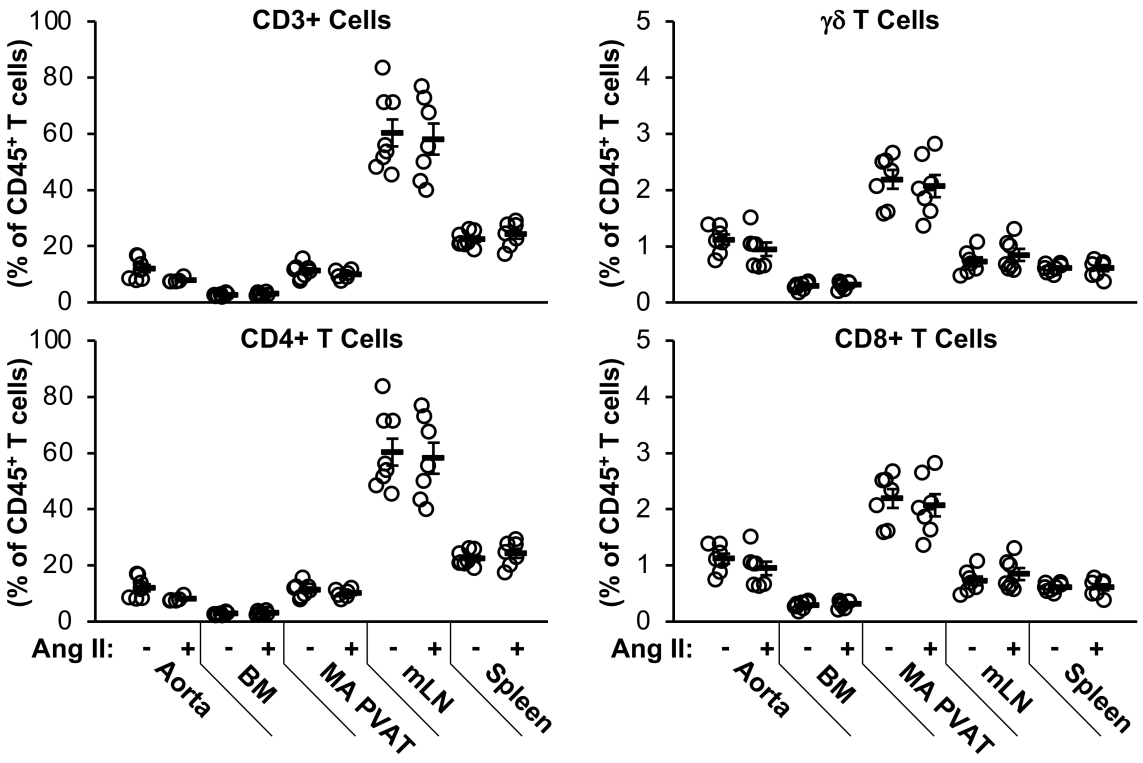


Figure 3.6: The frequency of $CD4^+$ and $CD8^+$ memory T cells is relatively unchanged following Ang II infusion in comparison to memory γδ T cells. A. Fourteen-day pressor dose Ang II infusion did not alter the frequency of effector memory $CD4^+$ or $CD8^+$ T cells (T_{EM}) in the mesenteric artery (MA) perivascular adipose tissue (PVAT). B. $CD8^+$ T_{EM} were upregulated in the mesenteric lymph nodes (mLN) in response to 14 days of Ang II infusion, but there was no change in $CD4^+$ T_{EM}. C. The frequency of $CD4^+$ and $CD8^+$ central memory T cells (T_{CM}) was unchanged in the aortic PVAT following repeated hypertensive challenges. D. The frequency of $CD4^+$ and $CD8^+$ T_{CM} cells was

also unchanged in the MA PVAT following repeated hypertensive challenges. Data are presented as means ± SEM. The data were analyzed using an unpaired t-test. n=6-9; *P<0.05 vs. sham.

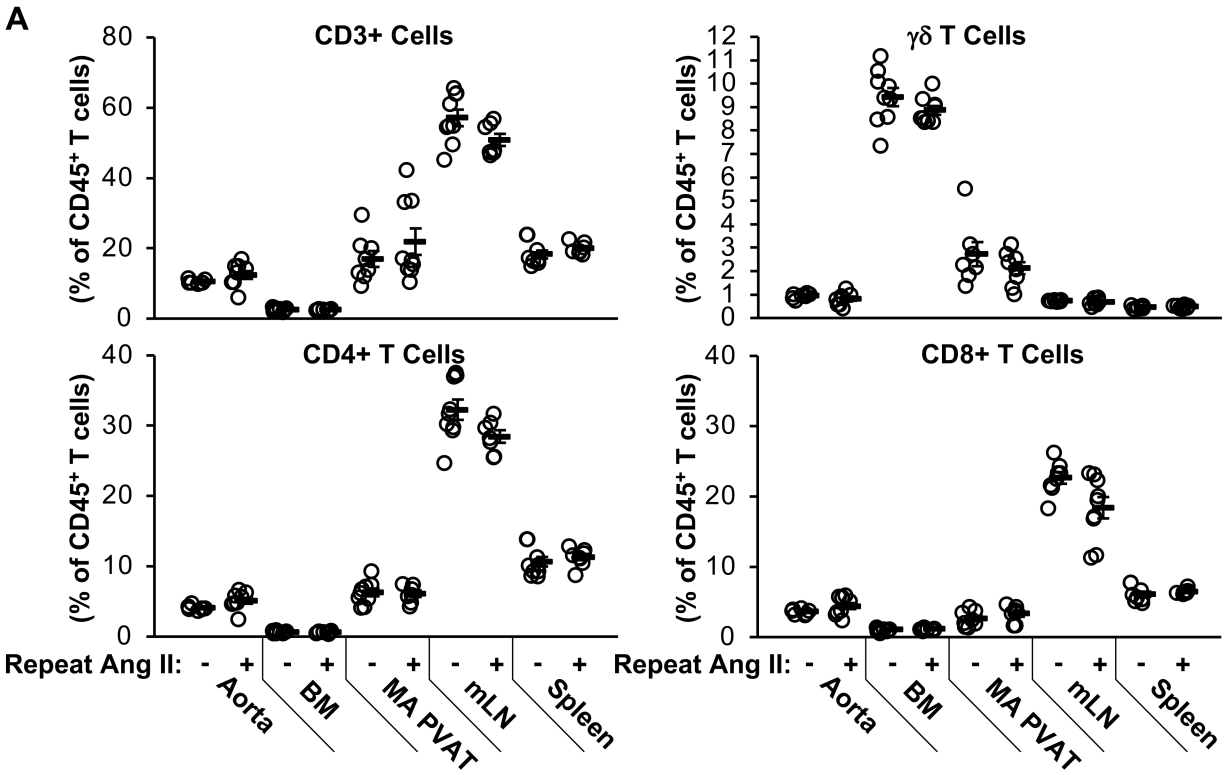
A



B

Tissue	Treatment	# of CD45+ Cells	SEM
Aorta	Sham	5,302	1,156
	Ang II	6,740	1,412
BM	Sham	750,250	38,663
	Ang II	749,145	70,803
MA PVAT	Sham	25,657	7,072
	Ang II	25,722	3,276
mLN	Sham	444,256	146,423
	Ang II	326,215	92,177
Spleen	Sham	875,250	71,643
	Ang II	867,143	158,818

Figure 3.7: The frequency of γδ, $CD3^+$, $CD4^+$, and $CD8^+$ T cells remains unchanged following 14-day pressor dose Ang II infusion. A. Fourteen-day pressor dose Ang II infusion did not significantly alter the frequency of γδ, $CD3^+$, $CD4^+$, or $CD8^+$ T cells expressed as a frequency of total $CD45^+$ immune cells. B. The total number of $CD45^+$ immune cells did not change following two weeks of pressor dose Ang II infusion. Data are presented as means ± SEM. n=5-8.



B

Tissue	Treatment	# of CD45+ Cells	SEM
Aorta	Sham	11,001	1,776
	Ang II	9,854	2,459
BM	Sham	819,444	33,787
	Ang II	827,333	36,408
MA PVAT	Sham	41,380	6,224
	Ang II	49,200	8,493
mLN	Sham	540,313	111,180
	Ang II	351,305	95,380
Spleen	Sham	837,444	42,487
	Ang II	841,333	65,108

Figure 3.8: The frequency of γδ, $CD3^+$, $CD4^+$, and $CD8^+$ T cells remains unchanged following repeat Ang II infusions versus mice that received a sham surgery followed by a subpressor dose Ang II infusion. A. Repeated Ang II infusions did not significantly alter the frequency of γδ, $CD3^+$, $CD4^+$, or $CD8^+$ T cells expressed as a frequency of total $CD45^+$ immune cells. B. The total number of $CD45^+$ immune cells did not change following repeated Ang II infusion. Data are presented as means ± SEM. n=7-9.

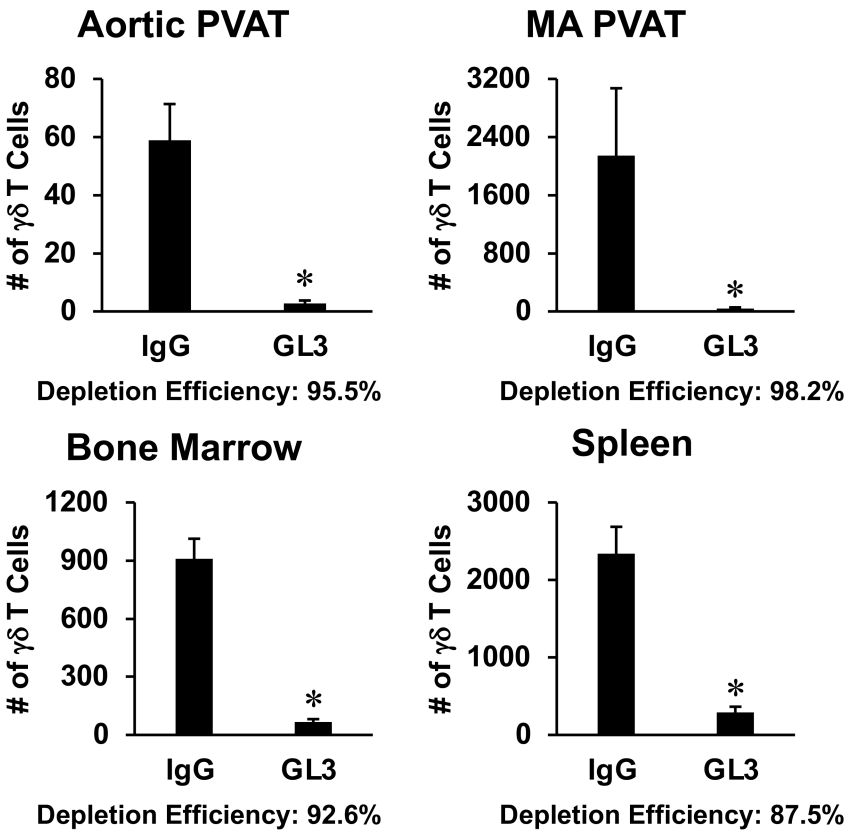


Figure 3.9: Efficiency of antibody-mediated γδ T cell depletion. The number of γδ T cells in the aortic perivascular adipose tissue (PVAT), mesenteric artery (MA) PVAT, bone marrow, and spleen was determined by flow cytometry at the end of the study in wild-type mice injected IP with a γδ T cell-depleting antibody (clone GL3) or IgG isotype control antibody 1 day before and 6 days after the initiation of subpressor dose Ang II infusion. Data are presented as means ± SEM, n = 8-9. Data were analyzed by Mann-Whitney Rank Sum Test. n=8-9; **P*<0.001 vs. IgG.

4. Angiotensin II induced steeper blood pressure elevation in IL-23 receptor-deficient mice: Role of interferon-γ-producing T cells

The previous study demonstrated that memory γδ T cells develop and play a role in Ang II-induced hypertension. However, we were also interested in determining how γδ T cells could contribute to the development of hypertension from a mechanistic standpoint. γδ T cells are known to produce large quantities of IL-17A and/or IFN-γ under inflammatory conditions (261), both of which contribute to the development and maintenance of hypertension (306). Most γδ T cells leave the thymus predetermined to produce either IL-17A or IFN-γ in response to immune insults (320), and these cells are referred to as γδT17 cells and γδT1 cells, respectively. However, some γδ T cells can be induced to become γδT17 cells and produce IL-17A extrathymically in response to stimulation by IL-1β and IL-23 (218). As IL-23 is capable of inducing γδT17 cells in the periphery, the IL-23R represents an interesting target to study in hypertension. IL-23R-mediated extrathymic generation of γδT17 cells could occur in hypertension and lead to increased levels of inflammation. Additionally, the IL-23R is postulated to play a role in stabilizing and maintaining subsets of IL-17A-producing $CD4^+$ (Th17) and $CD8^+$ (Tc17) T cells (321). Therefore, it is conceivable that IL-23R stimulation could augment inflammation in hypertension via promoting extrathymic generation of IL-17A-producing γδ T cells and maintaining populations of IL-17A-producing $CD4^+$ and $CD8^+$ T cells. Conversely, IL-23R inhibition could blunt the development of γδT17 cells and destabilize subsets of Th17 and Tc17 cells, thereby reducing the immune response to a hypertensive insult. We were interested in the latter mechanism, as the IL-23R could represent a novel therapeutic target to treat hypertension. Therefore, these experiments aimed to investigate the consequences of functional IL-23R deficiency with regard to γδT17 cell generation and BP elevation using Ang II-infused *Il23r*$^{gfp/gfp}$ and C57BL/6J mice.

This study demonstrated that functional IL-23R deficiency did not protect against vascular dysfunction and remodeling, and mice lacking a functional IL-23R paradoxically exhibited exaggerated BP increases early in the development of Ang II-induced hypertension. *Il23r*$^{gfp/gfp}$ mice also presented decreased γδT17 cells and a shift toward IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells. Antibody-mediated neutralization of IFN-γ in Ang II-infused *Il23r*$^{gfp/gfp}$ mice blunted BP elevation to 7 days of Ang II infusion, showing that IFN-γ in part mediated the exaggerated hypertensive response seen in *Il23r*$^{gfp/gfp}$ mice. These results highlighted the fact that inhibition of

IL-23R may not be an effective therapeutic approach to reduce hypertension. In addition, we have gained novel mechanistic insight into the dynamics of γδ T cell effector cytokine induction under the control of the IL-23R and demonstrated that IFN-γ-producing T cells are increased in the absence of IL-23R signaling. The article entitled "Angiotensin II induced steeper blood pressure elevation in IL-23 receptor-deficient mice: Role of interferon-γ-producing T cells" was submitted to *Hypertension Research* on August 12th, 2022, as the manuscript was selected to be fast-tracked for publication in this journal.

Angiotensin II-induced a steeper blood pressure elevation in IL-23 receptor-deficient mice: Role of interferon-γ-producing T cells

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Short Title: IL-23 receptor, γδT17 cells and hypertension

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Figures: 4 **Tables:** 0

4.1 Abstract

A subset of IL-17A-producing γδ T cells called γδT17 cells may contribute to the progression of hypertension. γδT17 cell development is in part dependent upon IL-23 receptor (IL-23R) stimulation. We hypothesized that angiotensin (Ang) II-induced blood pressure (BP) elevation and vascular injury would be blunted in *Il23r* knock-in (*Il23r*$^{gfp/gfp}$) mice deficient in functional IL-23R.

To test this, we infused Wild-type (WT) and *Il23r*$^{gfp/gfp}$ mice with Ang II (490ng/kg/min, SC) for 7 or 14 days. We recorded BP by telemetry, assessed vascular function and remodeling using pressurized myography, and profiled T cell populations and cytokine production by flow cytometry. An additional set of *Il23r*$^{gfp/gfp}$ mice was infused with Ang II for 7 days and injected with interferon (IFN)-γ-neutralizing or control antibodies.

Il-23r$^{gfp/gfp}$ mice had smaller and stiffer mesenteric arteries and were not protected against Ang II-induced BP elevation. BP was higher in *Il-23r*$^{gfp/gfp}$ mice than WT mice from day 3 until day 9 of Ang II infusion. *Il-23r*$^{gfp/gfp}$ mice had less γδT17 cells and more IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells than WT mice. Seven days of Ang II infusion led to increased IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells in *Il23r*$^{gfp/gfp}$ mice, whereas only IFN-γ-producing γδ T cells were increased in WT mice. Blocking IFN-γ with a neutralizing antibody reduced the pressor response to 7 days of Ang II infusion in *Il23r*$^{gfp/gfp}$ mice.

Functional IL-23R deficiency increased IFN-γ-producing T cells and exaggerated the initial development of Ang II-induced hypertension, which was in part mediated by IFN-γ.

Keywords: Il23r, IL-17A, hypertension, γδT1, Th1, Tc1

4.2 Introduction

Hypertension is the leading risk factor for cardiovascular disease and mortality worldwide (1-3). Despite the availability of effective antihypertensive drugs for treating hypertension, a substantial proportion of patients are treatment-resistant, in part due to the complex pathophysiology of hypertension (4). Both innate and adaptive immune cells have been shown to play a role in the development of hypertension and vascular injury in mouse models (5). One subset of adaptive immune cells that present a particularly promising target for reducing hypertension are γδ T cells (6).

γδ T cells express γ and δ T cell receptor (TCR) chains instead of the conventional α and β TCR chains observed in $CD4^+$ and $CD8^+$ T cells. γδ T cells are atypical, "innate-like", lymphocytes that can respond rapidly to proinflammatory stimuli without requiring antigen processing or MHC presentation, triggering cytokine production and cytotoxic activity (7). γδ T cells are effective at antigen presentation and cross presentation of antigens to $CD8^+$ T cells (8), supporting the concept that γδ T cells are able to bridge innate and adaptive immunity. Recently, we demonstrated that γδ T cells play a critical role in hypertension and vascular injury (6). Activated γδ T cells increased in the spleens of mice after Ang II-infusion and γδ T cell deficiency due to *Tcrδ* knockout or antibody-mediated depletion blunted Ang II-induced blood pressure (BP) elevation, small artery endothelial dysfunction, and reduced the activation of $CD4^+$ and $CD8^+$ T cells in mesenteric artery (MA) perivascular adipose tissue (PVAT). Interestingly, γδ T cells in adipose tissue are predominantly interleukin (IL)-17A-producing (9), and IL-17A has been shown to be involved in the maintenance of high BP and vascular dysfunction in mice (10,11). Increased levels of IL-17A have also been associated with prehypertension in humans (12). Consequently, we hypothesized that IL-17A-producing γδ T cells contribute to vascular dysfunction and the progression of hypertension.

Two types of γδ T cells are generated during thymic development, γδT1 and γδT17 cells, which produce interferon (IFN)-γ and IL-17A, respectively (13). These cells typically migrate directly from the thymus to tissues such as adipose tissue. Recently, extrathymic generation of γδT17 cells was demonstrated, where IL-1β and IL-23 secreted by innate cells promoted conversion of naïve γδ T cells into γδT17 cells in the periphery (14). Furthermore, IL-23 can induce γδT17 cell expansion. In human hypertension, higher serum concentrations of IL-23 were observed in hypertensive patients and were found to correlate strongly with BP (15,16). In addition, single

nucleotide polymorphisms (SNPs) within the IL23R gene were found to be associated with increased IL-23R mRNA expression, and these SNPs were also found to be more prevalent in patients with hypertension (17). Experimentally, Lee *et al.* (18) demonstrated that injection of recombinant IL-23 into Dahl salt-sensitive rats resulted in the development of hypertension through activation of the Th17 axis and subsequent elevation of circulating IL-17A. Therefore, extrathymic IL-23/IL-23R-induced generation and expansion of γδT17 cells could play a role in the development of hypertension.

We hypothesized that Ang II-induced hypertension is associated with increased numbers of IL-17A-producing γδT17 cells in MA PVAT, and that mice lacking a functional IL-23R will present with less γδT17 cells and blunted vascular injury, T cell activation, and BP elevation in response to Ang II infusion. To test this hypothesis, we utilized *Il23r*$^{gfp/gfp}$ mice that lack a functional IL-23R due to knock-in of enhanced green fluorescent protein into the intracellular domain of the receptor. We infused wild-type (WT) and *Il23r*$^{gfp/gfp}$ mice with Ang II for 14 days, monitoring their BP over the course of the infusion, and then assessed MA function and remodeling post-mortem using pressurized myography. Next, we characterized T cell infiltration and activation in the MA PVAT of WT and *Il23r*$^{gfp/gfp}$ mice infused or not with Ang II for 7 days, followed by assessment of IL-17A and IFN-γ-producing T cell populations. Finally, we utilized an anti-IFN-γ monoclonal antibody to assess the contribution of IFN-γ to Ang II-induced BP elevation in *Il23r*$^{gfp/gfp}$ mice.

4.3 Methods

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 (Protocol #JGH-7258). C57BL/6J WT mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and *Il23r*$^{gfp/gfp}$ mice were generated by Dr. Mohamed Oukka at Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, USA (19). All experimental mice were housed and bred at the Lady Davis Institute for Medical Research. *Il23r*$^{gfp/gfp}$ mice were genotyped by PCR (Figure 4.5 and Table 4.1).

To determine the role of IL-23R on hypertension and vascular injury, a first set of 10 to 12-week-old male C57BL/6J WT and *Il23r*$^{gfp/gfp}$ mice were sham-treated or infused with Ang II

(490 ng/kg/min, SC) for 14 days. In a subset of mice, BP was measured by telemetry 2 days before and during the treatment period. After 14 days of treatment, mesenteric vascular beds attached to the intestine were collected, and endothelial function and vascular mechanical properties were determined in second-order branches of MAs by pressurized myography.

To better understand the role of the IL-23R during the development of hypertension, a second set of mice was sham-treated or infused with Ang II for 7 days, and mesenteric vascular beds attached to the intestine were collected as described above for immune phenotyping by flow cytometry. MAs with attached PVAT (identified here as MA PVAT) were dissected, lymph nodes removed, and a single cell suspension was obtained by enzymatic digestion of the MA PVAT. Immune cells were profiled by flow cytometry using antibodies listed in Table 4.2 and the gating strategy depicted in Figure 4.6. IL-17A- and IFN-γ-producing γδ (γδT17 and γδT1 cells, respectively), $CD4^+$ (Th17 and Th1 cells, respectively), and $CD8^+$ T cells (T cytotoxic 17, [Tc17] and Tc1 cells, respectively) isolated from MA PVAT of mice infused or not with Ang II for 7 days were profiled by flow cytometry, using a combination of extracellular and intracellular marker staining (Table 4.3) and gating strategy depicted in Figure 4.7.

To assess the influence of IFN-γ on the early BP elevation observed in *Il23r*$^{gfp/gfp}$ mice, a new set of *Il23r*$^{gfp/gfp}$ mice were treated with Ang II for 7 days as above and injected with 16.7 mg/kg of anti-IFN-γ-neutralizing antibodies or anti-horseradish peroxidase IgG1 control antibodies (BioXCell, Lebanon, NH, USA) one day prior to and 3 days after the initiation of Ang II-infusion. BP was determined using telemetry as above.

4.3.2 Data analysis

Results are presented as mean ± SEM. Comparisons in BP and concentration-response curve data were performed using a two-way analysis of variance (ANOVA) for repeated measures. Other results were analyzed using a two-way ANOVA or t-test as appropriate. All ANOVA tests were followed by a Student-Newman-Keuls *post-hoc* test. Statistical analysis was performed in SigmaPlot version 12.5 (Systat Sotware, San Jose, CA). $P<0.05$ was considered statistically significant.

4.4 Results

4.4.1 *Il23r*$^{gfp/gfp}$ mice have more pronounced BP elevation during the first 9 days of Ang II infusion and develop vascular dysfunction and remodeling after 14 days

To assess the role of IL-23R in the development of hypertension and vascular injury, we infused mice with Ang II for 14 days. Both WT and *Il23r*$^{gfp/gfp}$ mice had similar increases in systolic BP (SBP; ~45 mmHg) and diastolic BP (DBP; ~35 mmHg) by the end of the 14-day Ang II infusion (Figure 4.1A and 1B). However, *Il23r*$^{gfp/gfp}$ mice exhibited greater SBP and DBP elevation than WT mice early in the development of hypertension, which was from day 3 to 9 of the Ang II infusion (Figure 4.1 A and B). No differences in heart rate (HR) or pulse pressure (PP) were noted between groups (Figure 4.8). In addition, there were no differences in SBP, DBP, or HR between sham-treated WT and *Il23r*$^{gfp/gfp}$ mice (Figure 4.9A-C). However, the PP of *Il23r*$^{gfp/gfp}$ sham-treated mice was significantly higher than WT sham-treated mice (Figure 4.9D).

In both WT and *Il23r*$^{gfp/gfp}$ mice, Ang II infusion led to hypertrophic remodeling with medial thickening (Figure 4.1C), no change in lumen diameter (Figure 4.1D), but enhanced media-to-lumen ratio (Figure 4.1E) and media cross-sectional area (Figure 4.1F). In addition, vascular stiffening as indicated by a leftward shift in the stress-strain curve occurred in both WT and *Il23r*$^{gfp/gfp}$ mice infused with Ang II (Figure 4.1G). However, in line with the enhanced PP seen in *Il23r*$^{gfp/gfp}$ mice, *Il23r*$^{gfp/gfp}$ mice presented with stiffer MAs (Figure 4.1G) and a smaller media-to-lumen ratio and media cross-sectional area compared to WT mice, regardless of treatment (Figure 4.1E, F).

We also assessed MA vascular function as dysfunction of resistance arteries contributes to hypertension and end-organ damage (20). Second order MAs from WT and *Il23r*$^{gfp/gfp}$ mice treated with Ang II had an impaired vasodilatory response to acetylcholine (~25% reduction), which is indicative of endothelial dysfunction (Figure 4.1H). No differences were observed in the endothelium-independent vasodilatory response to the nitric oxide donor sodium nitroprusside (Figure 4.10A) or in the vasoconstrictor response to norepinephrine (Figure 4.10B). These results demonstrated that functional IL-23R deficiency augmented the early pressor response to Ang II and altered resistance artery characteristics.

4.4.2 Fewer γδ T cells in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice

To understand why functional IL-23R deficiency exaggerated initial BP elevation, we investigated immune cell infiltration in the MA PVAT after 7 days of Ang II infusion using flow cytometry. No significant differences in the total number of immune cells ($CD45^+$ cells) or $CD3^+$ T cells were observed between WT and *Il23r*$^{gfp/gfp}$ mice, irrespective of Ang II treatment (Figure 4.11A, B). However, the number and frequency of γδ T cells and activated ($CD69^+$) γδ T cells was lower in *Il23r*$^{gfp/gfp}$ mice compared to WT mice (Figure 4.2), supporting the notion that a functional IL-23R may be important for effector γδ T cell development. Surprisingly, Ang II treatment decreased the frequency of γδ and activated γδ T cells in the MA PVAT of WT mice at day 7 of treatment (Figure 4.2). This contrasts with 14 days of Ang II infusion, where the frequency of activated γδ T cells was observed to increase in the MA PVAT of WT mice (Figure 4.12A).

As γδ T cells were shown to contribute to $CD4^+$ and $CD8^+$ T cell activation in Ang II-induced hypertension (6), we wanted to see if the lower frequency of γδ T cells observed in *Il23r*$^{gfp/gfp}$ mice would influence $CD4^+$ and $CD8^+$ T cell activation. No differences in the number or frequency of activated ($CD69^+$) $CD4^+$ (Figure 4.11C) or $CD8^+$ T cells (Figure 4.11D) were observed between treatments or genotypes. Similarly, no differences were observed in the number or frequency of total $CD8^+$ T cells (Figure 4.11F). However, *Il23r*$^{gfp/gfp}$ mice had a greater frequency of $CD4^+$ T cells regardless of Ang II treatment (Figure 4.11E). In addition to T cells, the number of macrophages and DCs was unchanged following Ang II infusion in WT mice (Figure 4.13). However, Ang II infusion caused an increase in the number of macrophages (2.4-fold) in *Il23r*$^{gfp/gfp}$ mice, and macrophages could modulate the effector functions of T cells. Overall, these results demonstrated that functional IL-23R deficiency resulted in less MA PVAT-infiltrating γδ T cells with a concomitant increase in $CD4^+$ T cells.

4.4.3 Functional IL-23R deficiency decreased MA PVAT-infiltrating γδT17 cells and increased IFN-γ-producing T cells

The differences in initial BP elevation between WT and *Il23r*$^{gfp/gfp}$ mice could not simply be explained by differences in the number of activated γδ, $CD4^+$ or $CD8^+$ T cells. Therefore, we sought to characterize IL-17A-producing T cells as IL-23R signaling is implicated in the expansion of γδT17 cells. We assessed the number and frequency of IL-17A-producing γδT17, Th17, and Tc17 cells. As expected, a large proportion of γδ T cells in WT mice were γδT17 cells (~50-60%),

which were also the most prominent subset of IL-17A-producing T cells (Figure 4.3). Functional IL-23R deficiency caused a ~50% reduction in the number of γδT17 cells in both sham and Ang II treated mice (Figure 4.3A). Furthermore, *Il23r*$^{gfp/gfp}$ mice had a reduced fraction of Th17 cells (Figure 4.3B), but not Tc17 cells (Figure 4.3C) supporting the notion that IL-23R supports the development of γδT17 and Th17 cells. However, Ang II infusion doubled the number of γδT17 cells in *Il23r*$^{gfp/gfp}$ mice (Figure 4.3A) suggesting IL-23R-independent expansion of γδT17 cells occurs in hypertension. Importantly, the number of γδT17 cells in in *Il23r*$^{gfp/gfp}$ mice still did not exceed the number observed in sham-treated WT mice.

As *Il23r*$^{gfp/gfp}$ mice had diminished numbers of IL-17A-producing T cells but higher BP, we investigated another prominent T cell cytokine implicated in hypertension development: IFN-γ. In WT mice, 7 days of Ang II infusion increased the number (2.7-fold) of γδT1 cells but did not alter the number or frequency of Th1 or Tc1 cells (Figure4. 3B and C). In contrast, Ang II infusion augmented the number of IFN-γ-producing γδT1, Th1, and Tc1 cells more than 2-fold in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice after 7 days (Figure 4.3). In fact, the frequency of γδT1, Th1, and Tc1 cells were almost double that of WT mice. Taken together, γδ T cells were the most numerous IL-17A-producing T cell subset in the MA PVAT, and functional IL-23R deficiency favoured a reduction in γδT17 and Th17 cells and an increase in IFN-γ-producing T cells.

4.4.4 Interferon-γ contributes to the accelerated hypertensive response in *Il23r*$^{gfp/gfp}$ mice

To evaluate if IFN-γ contributed to the increased BP observed during the initiation of hypertension in *Il23r*$^{gfp/gfp}$ mice, we assayed whether injection of an anti-IFN-γ neutralizing antibody would blunt Ang II-induced BP elevation in *Il23r*$^{gfp/gfp}$ mice. Neutralization of IFN-γ blunted SBP elevation by 13 mmHg on days 6 and 7 and DBP by 9 mmHg elevation on day 7 of Ang II infusion compared to mice injected with an IgG1 control antibody (Figure 4.4). Mice that received the anti-IFN-γ neutralizing antibody also had a reduction of ~25 beats/min in HR throughout treatment, but no difference in PP (Figure 4.14).

4.5 Discussion

This study showed that *Il23r*$^{gfp/gfp}$ mice are not protected from developing hypertension and vascular injury in response to 2 weeks of Ang II infusion, and contrary to our initial hypothesis, *Il23r*$^{gfp/gfp}$ mice exhibited higher BP after 1 week of Ang II infusion. Furthermore, *Il23r*$^{gfp/gfp}$ mice presented stiffer and smaller MAs than WT mice. T cell profiling by flow cytometry revealed that γδT17 cells were the most numerous subset of IL-17A-producing T cells in WT mice. Further, *Il23r*$^{gfp/gfp}$ mice had a lower number of γδT17 cells, a lower frequency of Th17 cells, and a higher frequency of γδT1, Th1, and Tc1 cells in MA PVAT than WT mice, irrespective of treatment. On the other hand, one week of Ang II infusion resulted in a higher number of γδT1, Th1, and Tc1 cells in *Il23r*$^{gfp/gfp}$ mice, while only γδT1 cells were increased in Ang II-infused WT mice. In general, *Il23r*$^{gfp/gfp}$ mice presented with less γδT17 cells and more IFN-γ-producing T cells, and Ang II infusion resulted in more MA PVAT infiltration of IFN-γ-producing T cells in *Il23r*$^{gfp/gfp}$ mice than in WT mice.

We previously demonstrated a role for γδ T cells in hypertension and vascular injury using mice deficient of γδ T cells due to *Tcrδ* knockout or injection of γδ T cell-depleting antibodies (6). However, it remains unclear which subtype of γδ T cells, γδT17 or γδT1 cells, contributes more to the development of hypertension. γδT17 cells have been shown to be an important source of IL-17A, and the number of γδT17 cells was observed to be increased in the aorta and kidneys of Ang II-infused mice (21). It has also been demonstrated that *Il17a* knockout mice and mice injected with neutralizing antibodies against IL-17A or IL-17 receptor A do not sustain increased BP after 3 or 4 weeks of Ang II infusion (10,21). Further, another study demonstrated that *Il17a* knockout mice exhibited blunted BP elevation in response to 2 weeks of Ang II infusion (22). Consequently, we became interested in determining whether γδT17 cells could be an important inflammatory mediator in hypertension, and if we could reduce BP by blunting their development.

An important receptor implicated in the development of IL-17A-producing γδT17 cells is the IL-23R. IL-23R stimulation by its cognate cytokine, IL-23, can result in effector cytokine commitment and expansion of γδT17 cells extrathymically under inflammatory conditions, and γδ T cells have been shown to be the most prominent IL-23R-expressing immune cell in lymph nodes (14,19). While it is conceivable that inhibiting the IL-23R signaling axis could blunt the development of γδT17 cells and reduce both inflammation and the pressor response, we observed

that *Il23r*$^{gfp/gfp}$ mice were not protected against Ang II-induced BP elevation or vascular injury. Notably, *Il23r*$^{gfp/gfp}$ mice exhibited exaggerated BP elevation after 7 days of Ang II infusion, which implied that functional IL-23R deficiency intensified the initial development of Ang II-induced hypertension. Very few studies have investigated the role of IL-23/IL-23R signaling in T cell effector cytokine commitment in the context of hypertension. However, existing studies have found conflicting results. For example, it was shown that injection of IL-23 increased BP in Dahl salt-sensitive rats, but not in Dahl salt-resistant rats (18). In the same study, Dahl salt-sensitive rats were found to have a greater increase in serum IL-17A and in the fraction of circulating Th17 cells following injection of IL-23 compared to salt-resistant rats. In contrast, BP elevation induced by a very severe model of hypertension combining unilateral nephrectomy, 2 weeks of deoxycorticosterone acetate treatment, salt in the drinking water, and high dose of Ang II (1.2 μg/kg/min, SC), was unaffected by *Il23a* knockout (*Il23a*$^{-/-}$ mice), with *Il23a* encoding one of the two IL-23 subunits, Il-23p19 (23). *Il23a*$^{-/-}$ mice exhibited an increase in the albumin/creatinine ratio at days 3 and 7 but not at day 12 of pro-hypertensive treatment. Furthermore, hypertensive *Il23a*$^{-/-}$ mice presented with more renal damage characterized by increased glomerular injury and proteinaceous casts, and *Il23a*$^{-/-}$ mice also possessed more renal γδ T cells. In another mouse study, injection of anti-IL-23R neutralizing antibodies did not affect BP elevation induced by infusion of a large dose of Ang II (1 μg/kg/min) (24). However, Ang II-induced BP elevation was not maintained and BP dropped by 30 mmHg by the end of the study. While no cardiac hypertrophy developed in either anti-IL-23R or control antibody-injected mice, cardiac infiltration of $CD4^+$ and $CD8^+$ T cells and macrophages was blunted by IL-23R neutralization. These studies suggest that IL-23R inhibition confers no benefit in the form of protection from hypertension in severe models of hypertension. We further demonstrated that *Il23r*$^{gfp/gfp}$ mice are not protected from a milder hypertension induced by a lower dose of Ang II, and that mice deficient of a functional IL-23R actually develop more severe hypertension after one week of Ang II infusion. Therefore, our results are congruent with previous studies that demonstrated no protective role for IL-23R inhibition in hypertension.

Investigation of IL-17A- and IFN-γ-producing T cells after 7 days of Ang II infusion confirmed that γδT17 cells are the more prominent subset of cytokine-producing γδ T cells and the most numerous T cell source of IL-17A in the MA PVAT of WT mice. Neither count nor frequency of MA PVAT-infiltrating γδT17, Th17 or Tc17 cells was affected by 7 days of Ang II infusion in

WT mice. This could imply that γδT17 cells do not play an appreciable role at the initiation of hypertension, however, more studies would need to be carried out to determine if this is in fact the case. A longer Ang II infusion may be necessary to observe an increase in IL-17A-producing T cells in PVAT as reported in the kidney after 28 days of Ang II treatment (21). Interestingly, Ang II augmented γδT1 cells but not Th1 or Tc1 cells in MA PVAT of WT mice, and this may explain why WT mice had lower BP than *Il23r*$^{gfp/gfp}$ mice early in the Ang II infusion. Conversely, *Il23r*$^{gfp/gfp}$ mice developed increased γδT1, Th1, and Tc1 cells in response to 7 days of Ang II infusion. Although γδT1 cells represent a comparatively small population of cells, they could significantly contribute to Ang II-induced inflammation, BP elevation, and vascular injury via production of IFN-γ (6).

As predicted, mice lacking a functional IL-23R had reduced numbers of γδT17 cells in MA PVAT. Still, an appreciable number of γδT17 cells were detected in *Il23r*$^{gfp/gfp}$ mice, suggesting that these γδT17 cells were of IL-23R-independent embryonic thymic origin (13). To the contrary, the number of Th17 and Tc17 cells was unchanged by functional IL-23R deficiency, although the frequency of Th17 cells was reduced. This finding was unsurprising given that the IL-23R is not required for initial polarization of Th17 and Tc17 cells (25). IL-23R signaling is speculated to play a role in stabilizing and expanding the IL-17A-producing phenotype in Th17 and Tc17 cells (26), and this could explain both the reduced frequency of Th17 cells and the shift toward IFN-γ-producing T cells at baseline and in response to Ang II infusion in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice. The increase in IFN-γ-producing T cells may also be explained by the comparatively high phenotypic plasticity of γδT17 and Th17 cells regarding effector cytokine production (25,27), as these cells can shift from IL-17A to IFN-γ production. This concept would support the notion of IL-23R playing a role in stabilizing populations of IL-17A-producing T cells in the context of our results, and it is also conceivable that a similar phenotypic plasticity could exist in Tc17 cells. The enhanced T cell IFN-γ response to Ang II infusion in *Il23r*$^{gfp/gfp}$ mice may also explain why these mice had stiffer and smaller MAs in addition to developing higher BP, as IFN-γ could enhance vascular inflammation via promoting further immune activation. Altogether, this suggests that appropriate balance of T cell IL-17A and IFN-γ production is relevant to both BP and vascular remodeling in response to Ang II-induced hypertension, and that this balance is lost in *Il23r*$^{gfp/gfp}$ mice.

Il23r$^{gfp/gfp}$ mice had a general increase in IFN-γ-producing T cells in the MA PVAT after 7 days of Ang II treatment. However, the number of γδT17 cells was also increased in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice in response to Ang II infusion, which may seem to contradict the hypothesized role for IL-23R in γδT17 development. Nonetheless, it is conceivable that these Ang II-responsive γδT17 cells are of thymic origin and are not dependent on IL-23R signaling. *Il23r*$^{gfp/gfp}$ mice had less γδT17 cells than WT mice irrespective of treatment, and the elevation in number of γδT17 cells in response to Ang II infusion in *Il23r*$^{gfp/gfp}$ mice did not exceed the number of γδT17 cells in sham-treated WT mice. These findings support the concept that *Il23r*$^{gfp/gfp}$ mice lack the ability to generate γδT17 cells in the periphery, and this could explain the reduced number of γδT17 cells in *Il23r*$^{gfp/gfp}$ mice versus WT mice. Further, *Il23r*$^{gfp/gfp}$ mice still possessed γδT17 cells that responded to the Ang II infusion, and these cells are likely of thymic origin.

It is unclear whether the elevation in number of γδT17 cells in *Il23r*$^{gfp/gfp}$ mice contributed to BP elevation at the initiation of hypertension. However, IFN-γ-producing γδT1, Th1, and Tc1 cells were elevated in *Il23r*$^{gfp/gfp}$ mice, and IFN-γ participated in Ang II-induced BP elevation. Antibody-mediated neutralization of IFN-γ blunted BP elevation in response to 7 days of Ang II infusion in *Il23r*$^{gfp/gfp}$ mice, and this confirmed that the elevated BP observed in Ang II-infused *Il23r*$^{gfp/gfp}$ mice was in part mediated by IFN-γ. A role for IFN-γ in the progression of hypertension is already well established. Kamat *et al.* (22) observed blunted BP elevation and preserved renal function assessed by saline challenge in *Ifng*$^{-/-}$ mice infused with Ang II for 14 days. These findings are especially pertinent to our results as we used the same dose of Ang II (490 ng/kg/min) to induce hypertension, and we also found that BP elevation was blunted in Ang II-infused mice injected with an IFN-γ-neutralizing antibody. Consequently, *Il23r*$^{gfp/gfp}$ mice show more severe BP elevation during the first week of Ang II infusion and develop more IFN-γ-producing T cells in the MA PVAT than WT mice. Mechanistically, the elevated pressor response to Ang II infusion seen in *Il23r*$^{gfp/gfp}$ mice was in part dependent on IFN-γ.

In summary, *Il23r*$^{gfp/gfp}$ mice that are deficient in functional IL-23R had reduced γδT17 cells but were not protected from developing hypertension and vascular injury in response to 2 weeks of Ang II infusion. In fact, *Il23r*$^{gfp/gfp}$ mice exhibited exaggerated BP elevation during the initiation of hypertension. We confirmed that γδT17 cells are the most numerous IL-17A-producing T cell subset in the MA PVAT, and *Il23r*$^{gfp/gfp}$ mice presented with less γδT17 cells and

more IFN-γ-producing γδT1, Th1, and Tc1 cells compared to WT mice. One week of Ang II infusion sharply increased the infiltration of IFN-γ-producing γδT1, Th1, and Tc1 cells in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice. Accordingly, the present study demonstrated that functional IL-23R deficiency does not protect against hypertension. Rather, *Il23r*$^{gfp/gfp}$ mice displayed more severe BP elevation early in the development of Ang II-induced hypertension concomitant with an upregulation of IFN-γ-producing T cells. Neutralization of IFN-γ in vivo confirmed that the more severe Ang II-induced hypertension seen in *Il23r*$^{gfp/gfp}$ mice was mediated in part through IFN-γ.

4.6 Disclosures

None.

4.7 Acknowledgements

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4.9 Figures

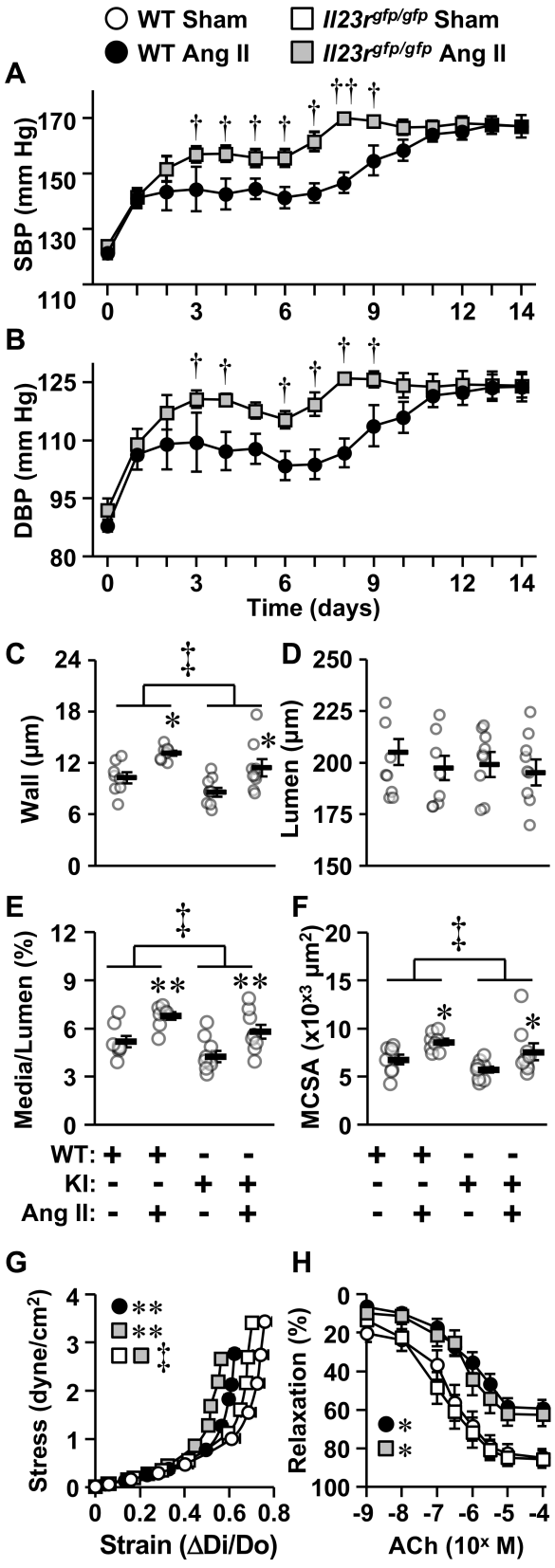


Figure 4.1: IL-23R plays a role in the initiation of hypertension and does not participate in the maintenance of high BP and vascular injury caused by angiotensin (Ang) II infusion. Telemetric systolic (SBP, A) and diastolic blood pressure (DBP, B), and mesenteric artery wall thickness (C), lumen diameter (D), media/lumen (E), media cross-sectional area (MCSA, F) arterial stress-strain relationship (G) and arterial dilatory responses to acetylcholine (H) were determined in wild-type (WT) or *Il23r* knock-in (KI, *Il23r*$^{gfp/gfp}$) mice treated or not with Ang II (490 ng/kg/min) for 14 days. ΔD is the change in lumen diameter for a given intraluminal pressure and Do is the original diameter measured at 3 mmHg. Data are presented as means ± SEM, n= 6 in A and B and 8-9 in C-H. Data was analyzed using a two-way ANOVA with repeated measure in A, B and H, and a two-way ANOVA in C-G), with all ANOVA's followed by a Student-Neuman-Keuls *post hoc* test. In D, the strain values at 140 mmHg (the last point) were used for analysis. **P*<0.05 and ***P*<0.001 versus respective sham, †*P*<0.05 and ††*P*<0.01 versus WT Ang II, and ‡*P*<0.05 versus the WT genotype. gfp, green fluorescence protein.

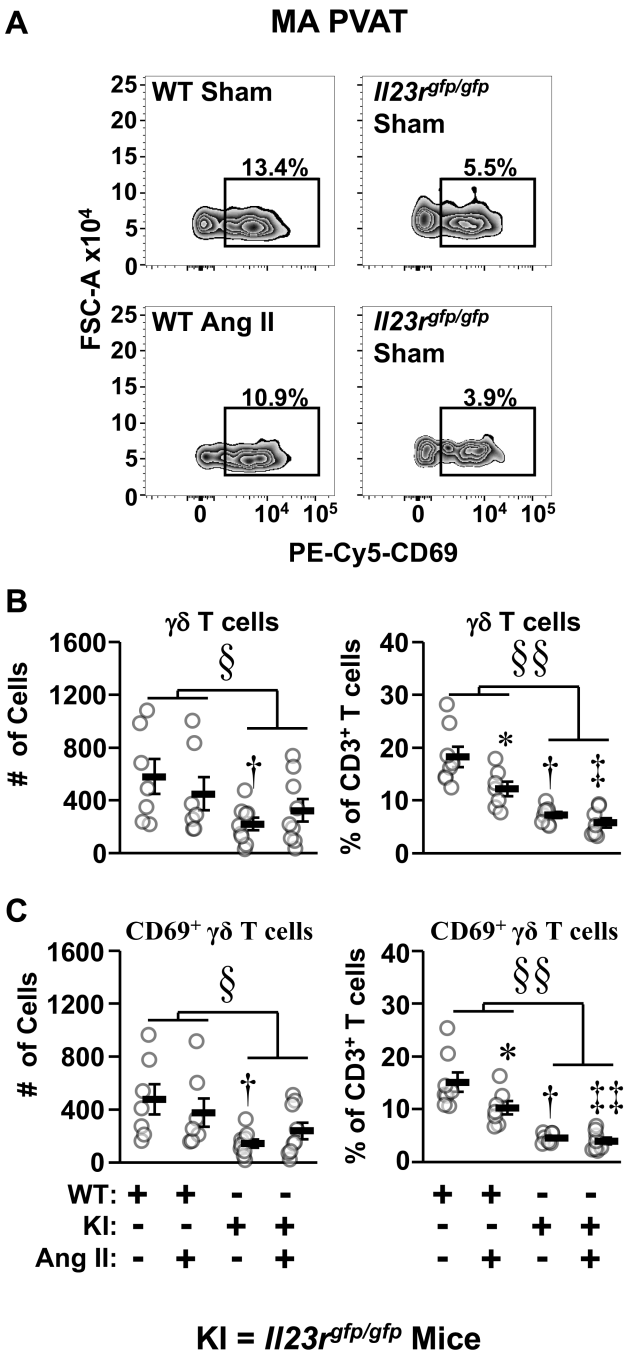


Figure 4.2: *Il23r*$^{gfp/gfp}$ mice presented less γδ T cells in in the mesenteric artery perivascular adipose tissue. Representative final flow cytometry gating for quantifying the number (#) and frequency (% of CD3$^+$ T cells) of activated (CD69$^+$) γδ T cells (A). The number of γδ T cells (B) and activated (CD69$^+$) γδ T cells (C) in the mesenteric artery with adherent perivascular adipose tissue (MA PVAT) of wild-type (WT) or *IL-23r*$^{gfp/gfp}$ mice (knock-in, KI) mice after 7 days of Ang II infusion (490ng/kg/min) or not were determined by flow cytometry. The full gating strategy is presented in Figure 4.6 in the online supplement. Data are presented as means ± SEM, n= 7-9. Data was analyzed using a two-way ANOVA followed by a Student-Neuman-Keuls *post hoc* test. **P*<0.05 versus respective sham, †*P*<0.05 versus WT Sham, ‡*P* <0.05 and ‡‡*P* <0.001 versus WT Ang II, and §*P*<0.05 and §§*P*<0.001 versus the WT genotype. CD, cluster of differentiation, Cy5, cyanine 5; FSC-A, forward scatter-area; gfp, green fluorescence protein; PE, phycoerythrin.

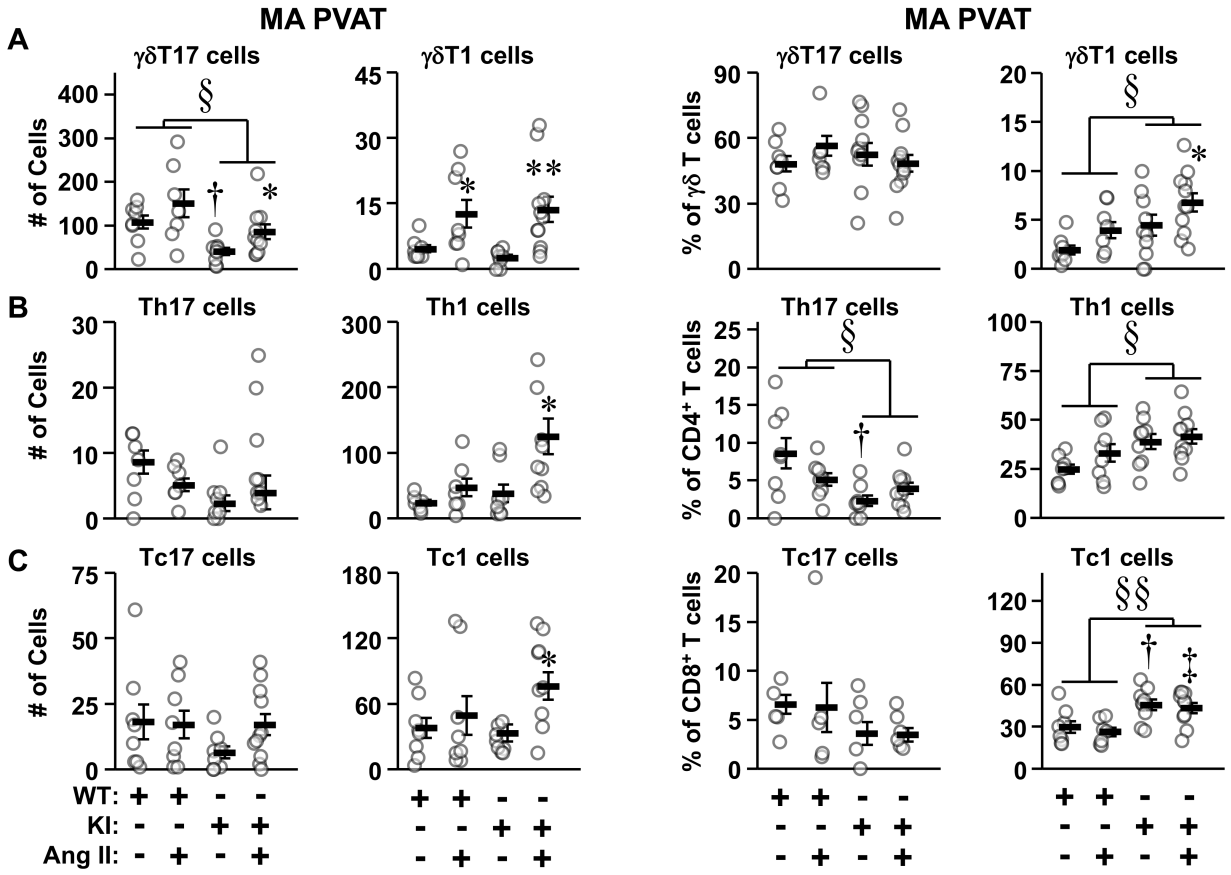


Figure 4.3: Ang II promoted the development of IFN-γ-producing T cells in the mesenteric artery (MA) perivascular adipose tissue (PVAT) of *IL-23r*$^{gfp/gfp}$ mice. The number and frequency of IL-17A-producing γδ (A, γδT17 cells), CD4$^+$ (B, Th17 cells) and CD8$^+$ T cells (C, Tc17 cells) and IFN-γ-producing γδ (A, γδT1 cells), CD4$^+$ (B, Th1 cells) and CD8$^+$ T cells (C, Tc1 cells) in the MA PVAT of wild-type (WT) or *IL-23r*$^{gfp/gfp}$ mice (knock-in, KI) treated or not for 7 days with Ang II (490ng/kg/min) were determined by flow cytometry. Cytokine production was induced by treatment with phorbol 12-myristate 13-acetate and ionomycin prior to flow cytometry analysis. A representative gating strategy is provided in Figure 4.7. Data are presented as means ± SEM, n= 6-11. Data was analyzed using two-way ANOVA followed by a Student-Neuman-Keuls *post hoc* test. *P<0.05 and **P<0.001 versus respective sham; †P <0.05 versus WT sham, and ‡P <0.01 versus WT Ang II, and §P<0.05 and §§P<0.001 versus the WT genotype. gfp, green fluorescence protein.

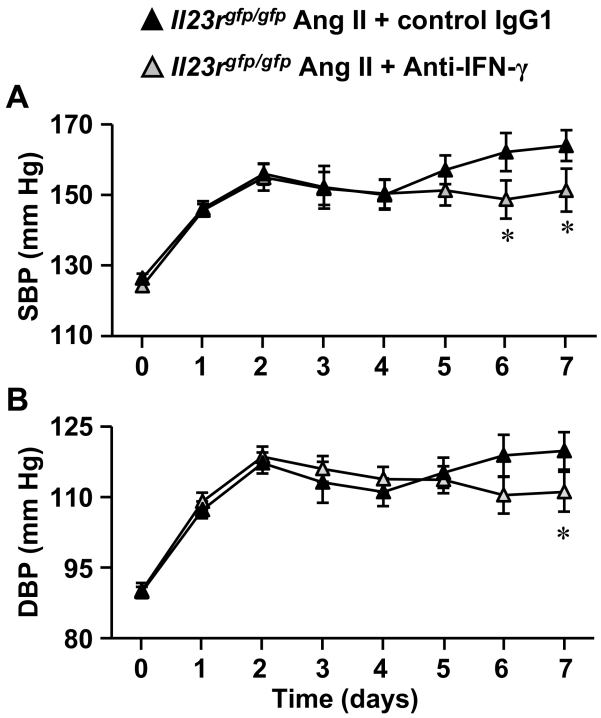
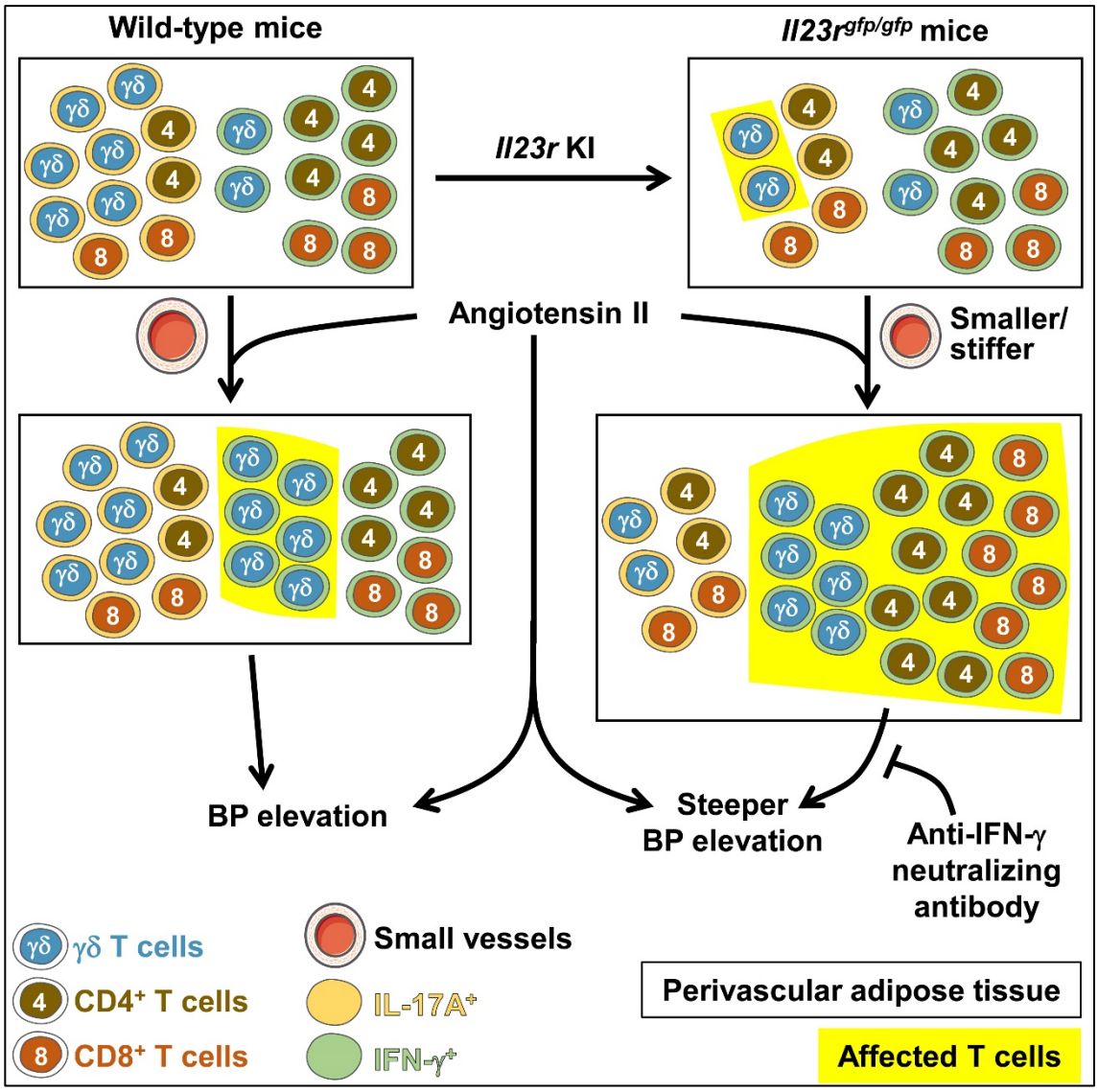


Figure 4.4: Interferon-gamma contributes to the accelerated hypertensive response in *Il23r*$^{gfp/gfp}$ mice. Systolic (SBP, A) and diastolic blood pressure (DBP, B) were determined by telemetry in *Il23r*$^{gfp/gfp}$ mice infused with angiotensin (Ang) II (490 ng/kg/min, SC) for 7 days and injected with 16.7 mg/kg of anti-IFN-γ neutralizing or anti-horseradish peroxidase IgG1 control antibodies one day prior to and 3 days after the initiation of Ang II infusion. Data are presented as means ± SEM, n= 9-10. Data was analyzed using a two-way ANOVA with repeated measure followed by a Student-Neuman-Keuls *post hoc* test. *P<0.05 vs mice receiving the IgG1 control antibodies. gfp, green fluorescence protein.

GRAPHIC ABSTRACT



4.10 Supplementary Figures

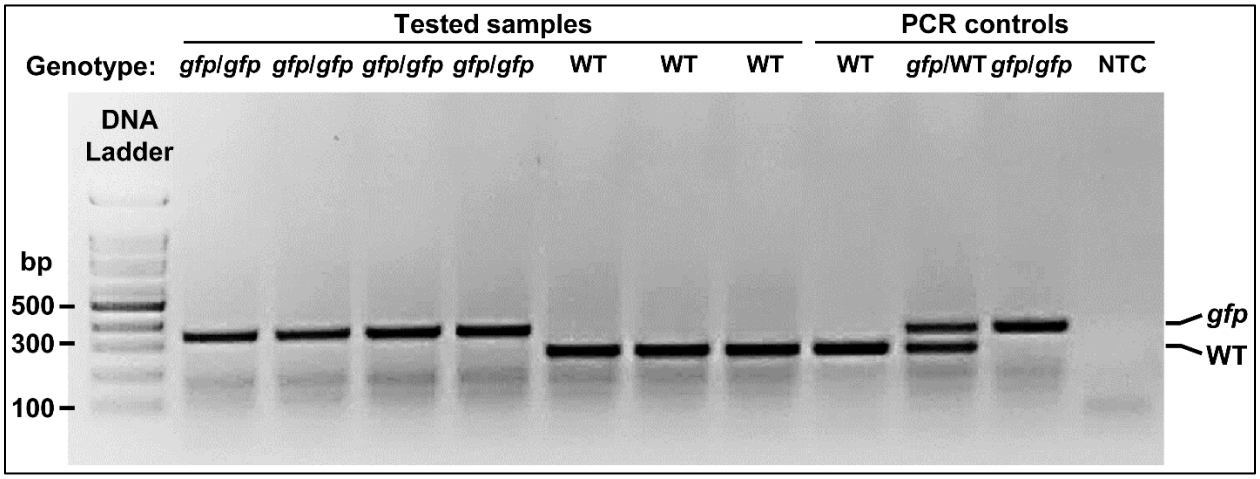


Figure 4.5: Representative PCR genotyping of interleukin 23 receptor (*Il23r*) exon 9 green fluorescent protein knock-in (*gfp*/*gfp*) mice. The PCR was designed to reveal the presence of knock-in (*gfp*) and wild-type (WT) allele by amplifying respectively a 350 base pair (bp) *Il23r-gfp* fragment and a 266 bp *Il23r* fragment. NTC, no template control.

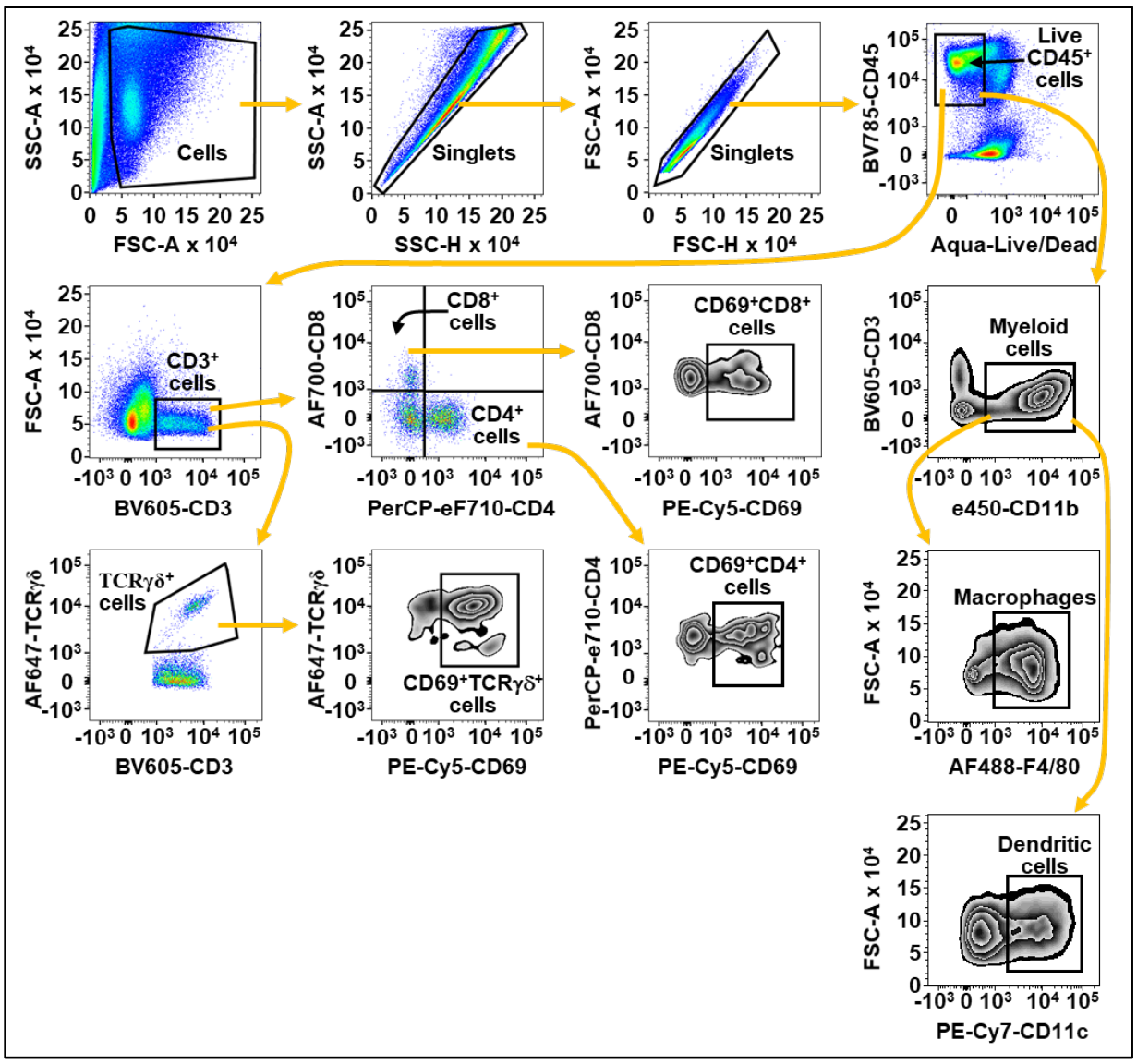


Figure 4.6: Representative immune cell profiling gating strategy using surface markers. Cells are first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using FSC-A over FSC height (FSC-H). Singlet cells were gated again using SSC-A over SSC height (SSC-H) to further clean up the data. Live $CD45^+$ cells were gated in the CD45/Live/Dead plot, followed by $CD3^+$ T cells in the FSC-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, $CD69^+$ $CD4^+$, $CD8^+$ and γδ T cells were gated in CD4/CD69, CD8/CD69 and TCRγδ/CD69 plot, respectively. Myeloid cells were gated from live cells as CD11b+ in the CD3/CD11b plot. Macrophages were subsequently identified as $F4/80^+$ in the FSC-A/F4/80 plot and Dendritic cells as $CD11c^+$ in the FSC-A/CD11c plot. AF488, AF647 and AF700, Alexa-Fluor

647 and 700; BV605 and 785, Brilliant Violet 605 and 785; CD, cluster of differentiation; Cy, Cyanine; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein.

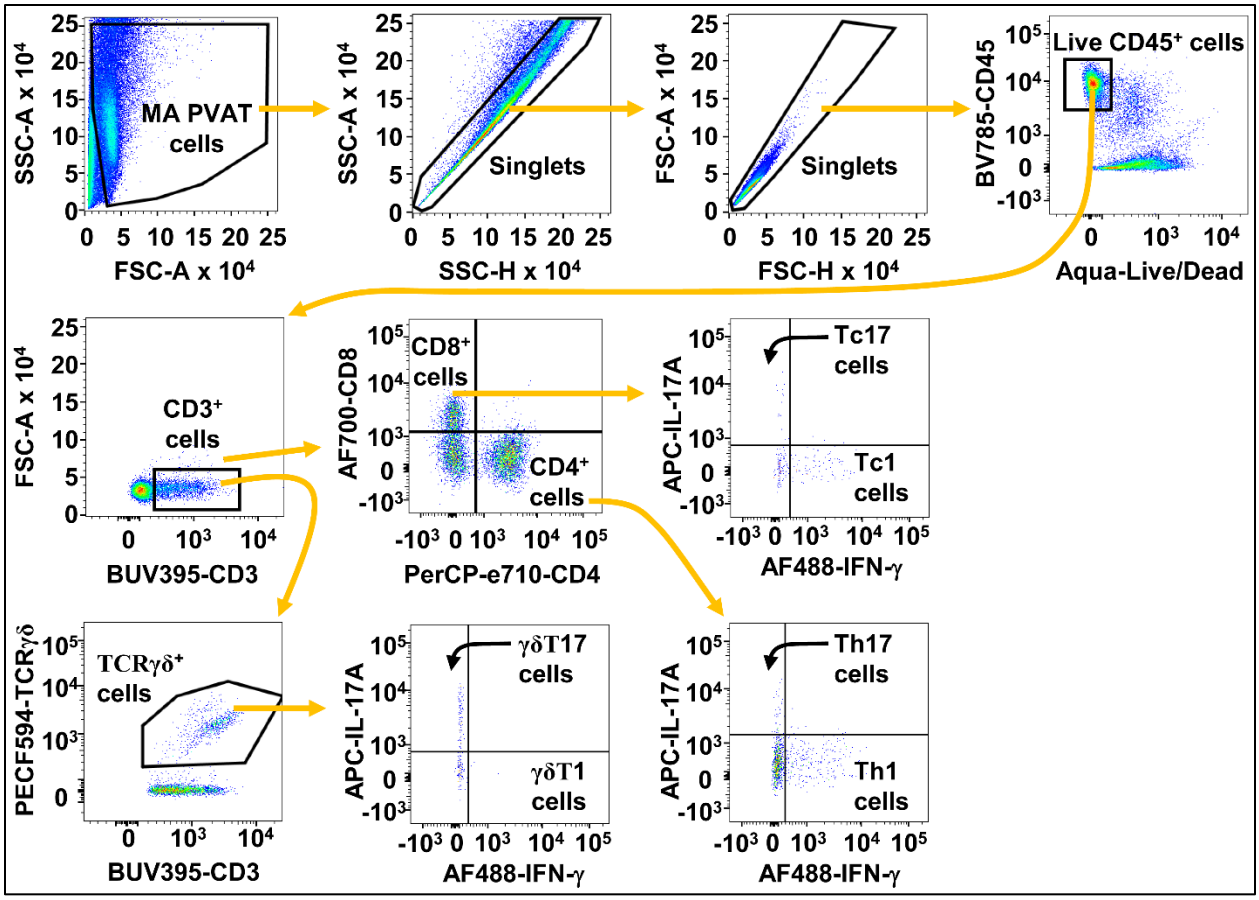


Figure 4.7: Representative gating strategy for interleukin (IL)-17A and interferon-gamma (IFN-γ)-producing T cells. Cells are first gated in the side scatter area (SSC-A)/forward scatter area (FSC-A) plot. Singlet cells were gated using FSC-A over FSC height (FSC-H). Singlet cells were gated again using SSC-A over SSC height (SSC-H) to further clean up the data. Live CD45$^+$ cells were gated in the CD45/Live/Dead plot, followed by CD3$^+$ T cells in the FSC-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 (T cytotoxic 17, Tc17) and IFN-γ-producing γδ (γδT1), CD4$^+$ (Th1) and CD8$^+$ T cells (Tc1) cells were gated in 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.

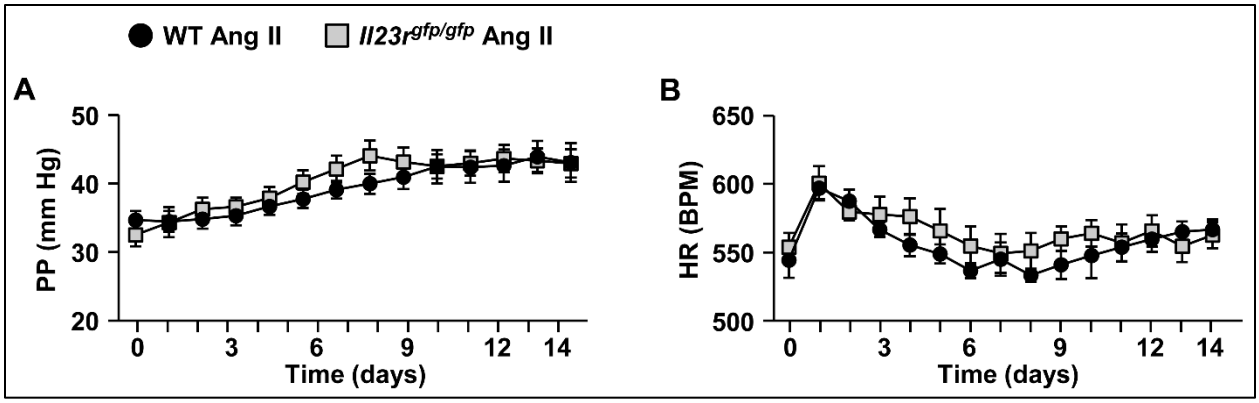


Figure 4.8: Pulse pressure (PP) and heart rate (HR) of wild-type (WT) and *Il23r*$^{gfp/gfp}$ mice infused with angiotensin (Ang) II (490 ng/kg/min, SC) for 14 days. PP and HR were determined by telemetry at baseline and during Ang infusion. Data are presented as means ± SEM, n= 6. BPM, beats per minute; gfp, green fluorescence protein.

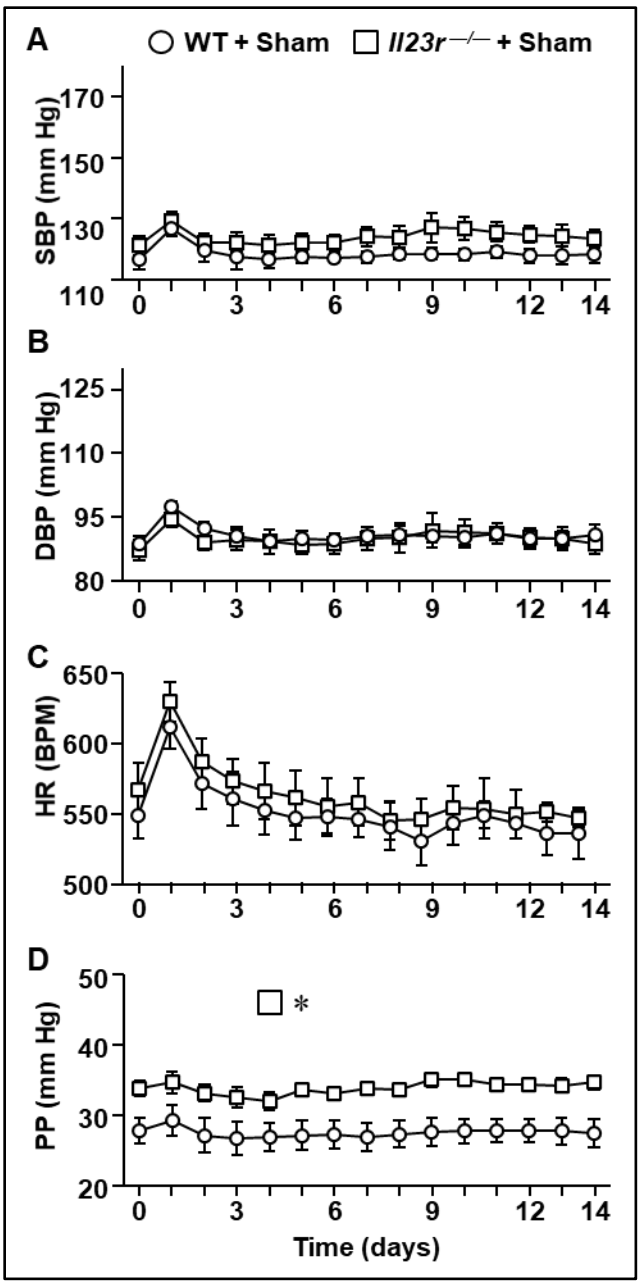


Figure 4.9: Systolic blood pressure (SBP, A), diastolic BP (DBP, B) heart rate (HR, C), and Pulse pressure (PP, D) were determined by telemetry of WT and *Il23r*$^{gfp/gfp}$ sham-treated for 14 days. Data are presented as means ± SEM, n= 5-6. Data for PP was analyzed using a t-test for the area under the curve. **P*<0.05 versus WT Sham BPM, beats per minute; gfp, green fluorescence protein.

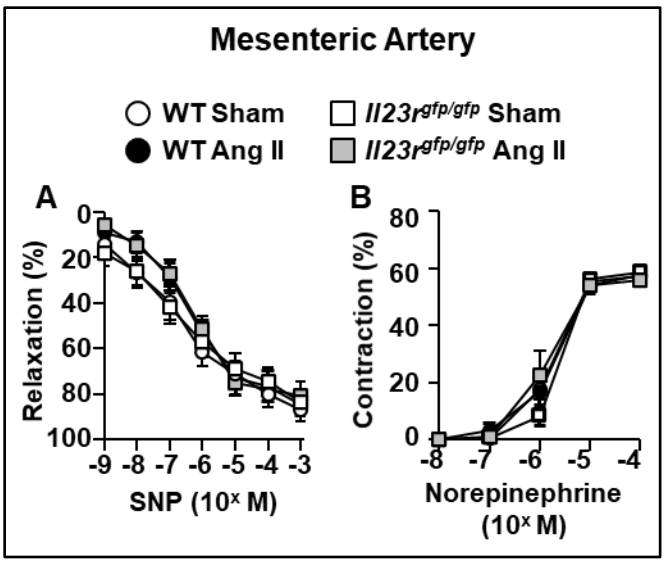


Figure 4.10: Endothelium-independent vasodilation of mesenteric arteries from WT or *Il23r*$^{gfp/gfp}$ mice, pre-constricted with norepinephrine (NE), to increasing doses of sodium nitroprusside (SNP, A) conducted using pressurized myography. Vascular Contraction responses to increasing concentrations of NE (B). Data are presented as means ± SEM, n= 8-9. gfp, green fluorescence protein.

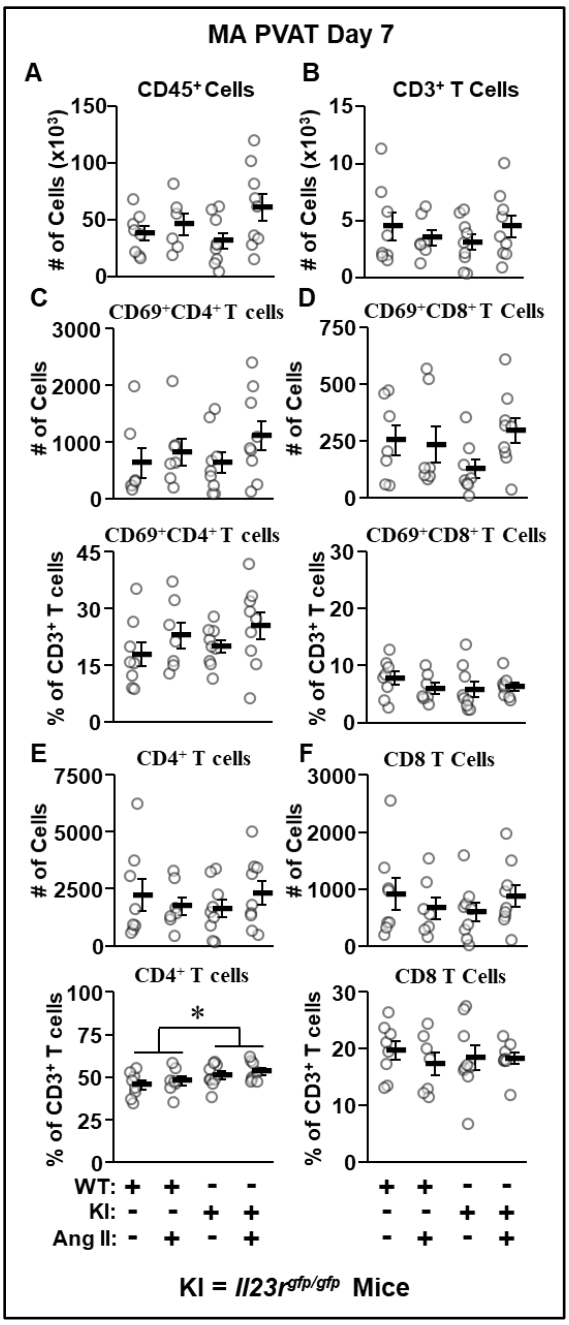


Figure 4.11: Flow cytometry was used to quantify the number of infiltrating leukocytes (A), $CD3^+$ T cells (B), the number and frequency (% of $CD3^+$ T cells) of activated ($CD69^+$) $CD4^+$ T cells (C) and $CD8^+$ T cells (D), and the number and frequency of total $CD4^+$ T cells (E) and $CD8^+$ T cells (F) in the mesenteric artery with adherent perivascular adipose tissue (MA PVAT) of wild-type (WT) or *IL-23r*$^{gfp/gfp}$ mice (knock-in, KI) mice after 7 days of Ang II infusion (490ng/kg/min) or not. The gating strategy is presented in Figure 4.6 in the online supplement. Data are presented as means ± SEM, n= 6-9. Data was analyzed using a two-way ANOVA followed by a Student-

Neuman-Keuls *post hoc* test. **P*<0.05 versus the WT genotype. CD, cluster of differentiation, gfp, green fluorescence protein.

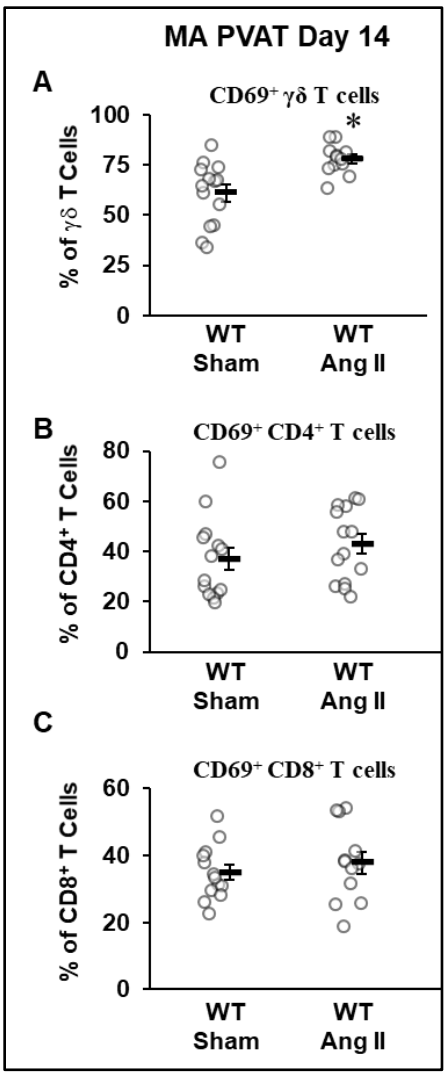


Figure 4.12: Flow cytometry was used to quantify the frequency (% of parent population) of activated ($CD69^+$) γδ T cells (A), $CD4^+$ T cells (B) and $CD8^+$ T cells (C), in the mesenteric artery perivascular adipose tissue (MA PVAT) of wild-type (WT) mice after 14 days of Ang II infusion (490ng/kg/min) or not. The gating strategy is presented in Figure 4.6 in the online supplement. Data are presented as means ± SEM, n= 12-14. Data were analyzed using a t-test. *P<0.01 versus WT sham. CD, cluster of differentiation.

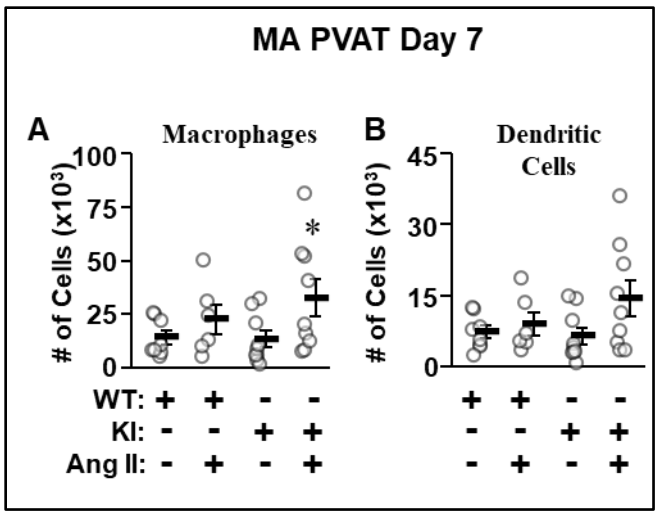


Figure 4.13: Flow cytometry was used to quantify the number of infiltrating macrophages (A), and dendritic cells (B) in the mesenteric artery perivascular adipose tissue (MA PVAT) of wild-type (WT) or *IL-23r*$^{gfp/gfp}$ mice (knock-in, KI) mice after 7 days of Ang II infusion (490ng/kg/min) or not. The gating strategy is presented in Figure 4.6 in the online supplement. Data are presented as means ± SEM, n= 7-9. Data was analyzed using a two-way ANOVA followed by a Student-Neuman-Keuls *post hoc* test. **P*<0.05 versus respective sham. gfp, green fluorescence protein.

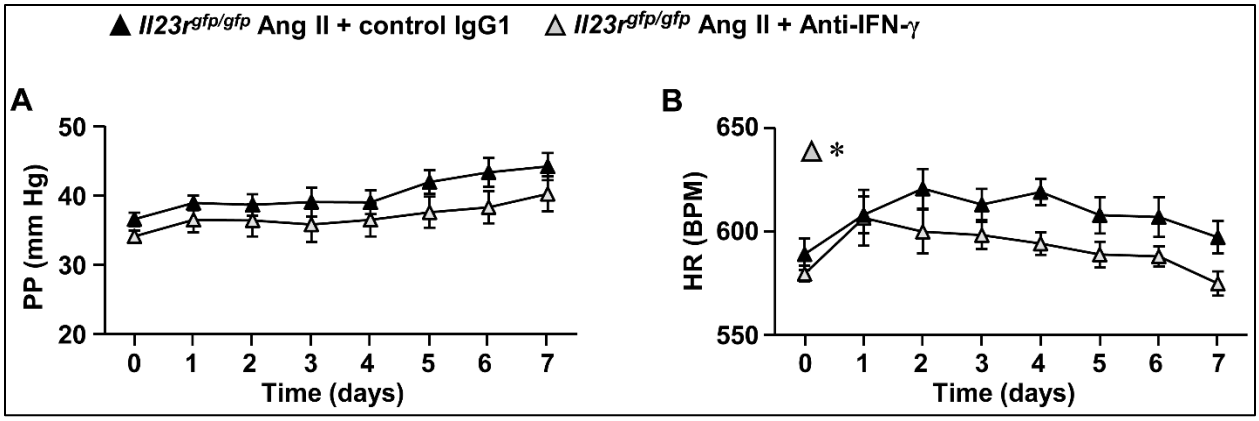


Figure 4.14: Pulse pressure (PP) and heart rate (HR) of *Il23r*$^{gfp/gfp}$ mice infused with angiotensin (Ang) II (490 ng/kg/min, SC) for 7 days and injected with 16.7 mg/kg of anti-IFN-γ neutralizing or anti-horseradish peroxidase IgG1 control antibodies one day prior to and after 3 days initiation of Ang II infusion. PP and HR were determined by telemetry at baseline and during the Ang infusion. Data are presented as means ± SEM, n= 9-10. Data was analyzed using a two-way ANOVA with repeated measure followed by a Student-Neuman-Keuls *post hoc* test. **P*<0.05 versus IgG1. BPM, beats per minute; gfp, green fluorescence protein.

4.11 Supplementary Tables

Table 4.1: Oligonucleotide primers used for PCR.

DNA	Primers	Product size (bp)
Il23r-egfp	F: 5′- TGGTTGCCTGCACCAATTTAAAAG -3′ R: 5′- ACCCCTAGGAATGCTCGTCAAG -3′	350
Il23r	F: 5′- GATCATCTTATGGCTGGTCCTC -3′ R: 5′- GAGTGAGACAGTGTAGCCACAGAT -3′	266

This table displays the forward (F) and reverse (R) oligonucleotide primers used for genotyping and the product sizes of interleukin 23 receptor (*Il23r*)-enhanced green fluorescent protein (*egfp*) fragment and a fragment of the *Il23r* gene. bp, base pairs.

Table 4.2: Flow cytometry monoclonal antibodies for T cell profiling with surface makers

Antigen	Antibodies	Clone, company (catalogue #)
CD45	BV785-conjugated rat anti-mouse CD45 antibody	30-F11, BioLegend (103149)
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)
CD11b	eFlour 450-conjugated rat anti-mouse CD11b antibody	M1/70, eBioscience (48-0112-80)
CD11c	PE-Cy7-conjugated Armenian hamster anti-mouse CD11c antibody	N418, BioLegend (117318)
F4/80	AF488-conjugated rat anti-mouse F4/80 antibody	BM8, BioLegend (123120)

AF488, AF647 and AF700, Alexa-Fluor 488, 647 and 700; BV605 and 785, Brilliant Violet 605 and 785; CD, cluster of differentiation; Cy5, Cyanine 5; Cy7, Cyanine 7; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; TCR, T cell receptor.

Table 4.3: Flow cytometry monoclonal antibodies for interleukin-17A and interferon-gamma-producing T cell profiling

Antigen	Intra/extra-cellular staining	Antibodies	Clone, company (catogue #)
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.

4.12 Supplementary Methods

The study was approved by the Animal Care Committee of the Lady Davis Institute and McGill University and followed the recommendations of the Canadian Council of Animal Care (Protocol #JGH-7258).

4.12.1 Animals

C57BL/6J wild-type (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Interleukin (IL)-23 receptor (IL-23R) knock-in mice having an IRES-EGFP (internal ribosome entry site-enhanced green fluorescent protein) cassette replacing exon 9 of the IL-23R gene (*Il23r*) were generated by Dr. Mohamed Oukka at Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, USA (1). Functional *Il23r* knockout ($IL23R^{gfp/gfp}$) mice were generated by crossing heterozygote *Il23r* knock-in mice. $IL23R^{gfp/gfp}$ mice were identified by genotyping of ear punch DNA as described below. Exon 9 encodes the intracellular domain of the receptor, and $IL23R^{gfp/gfp}$ mice fail to respond to IL-23. All experimental WT and $Il23r^{gfp/gfp}$ mice were generated in our animal facility. They were housed in a conventional facility under sterile conditions with constant temperature and humidity and 12-hour light/12-hour dark cycles. Mice were fed with a normal-salt diet (Teklad Global 18% protein rodent diet with 0.2% of NaCl, Envigo, Lachine, QC, Canada).

4.12.2 Genotyping

DNA was extracted from an ear punch using a quick method (2) with a modification (3). 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 45 min. 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 15 min. At the end of the digestion, the tubes were vortexed and centrifuged. Samples were stored at -20ºC until used.

$Il23r^{gfp/gfp}$ mice were identified using PCR by the amplification of a 350 bp *Il23r-egfp* fragment and the absence of a 266 bp *Il23r* fragment. PCR was performed using 2 µL of supernatant of the ear punch digestion mixture with 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 S1. The PCR conditions were 3 minutes at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s 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) after which an image was taken (Fig. 4.5).

4.12.3 Angiotensin II treatment

Mice were infused with angiotensin (Ang) II as previously described (4). In brief, 9.5-11-week-old male C57BL/6J WT and *Il23r*$^{gfp/gfp}$ mice were anesthetized with 3% isoflurane mixed with O_2 at 1 L/min, the efficiency of anesthesia was checked by the rear foot squeezing, and then surgically implanted SC with ALZET micro-osmotic pumps model 1002 (Durect Corporation, Cupertino, CA) infusing angiotensin II (490 ng/kg/min) for 14 or 7 days, as recommended by the manufacturer. To minimize the post-operation pain, the non-steroidal anti-inflammatory drug carprofen (20 mg/Kg) was administered SC, and 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.

4.12.4 Injection of IFN-γ neutralizing antibodies

Il23r$^{gfp/gfp}$ mice were injected IP with 16.7 mg/kg of anti-IFN-γ neutralizing antibodies or anti-horseradish peroxidase IgG1 control antibodies (BioXCell, Lebanon, NH, USA) one day prior to and 3 days after the initiation of Ang II-infusion (see above).

4.12.5 Blood pressure determination

Blood pressure (BP) was determined by telemetry as previously described (4). Briefly, 8.5-10-week-old male C57BL/6J WT and *Il23r*$^{gfp/gfp}$ mice were anesthetized with isoflurane as above, and were surgically implanted SC as recommended by the manufacturer (Data Sciences International, St. Paul, MN). As above to minimize the post-operation pain, 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 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 continued to be acquired for 14 days.

4.12.6 Collection of tissues

At the end of the protocol, mice were weighed and then anesthetized with isoflurane as above. The mesenteric vascular bed attached to the intestine with the ends of the intestines ligated using surgical sutures was harvested in ice cold 4°C oxygenated (95% air–5% CO_2) Krebs solution (pH 7.4) for endothelial function and mechanic studies or in ice-cold phosphate buffered saline (PBS) for flow cytometry studies. 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. The spleen was collected in ice-cold PBS for preparation of the controls for flow cytometry. Mice were euthanized by cervical dislocation.

4.12.7 Endothelial function and mechanics

The mesenteric artery endothelial function and mechanics were investigated after mounting on a pressure myograph (Living Systems Instrumentation, Burlington, VA, USA) as previously described (5). Briefly, second-order branches of mesenteric arteries were dissected from the mesenteric arterial tree (160 to 240 μm) and mounted on glass pipettes of pressurized myograph chambers. Vessels were equilibrated for 30 minutes at 45 mmHg intraluminal pressure in myograph chambers superfused with 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 exogenous NE (10^{-8}–10^{-4} mol/L). 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 norepinephrine (5 x 10^{-5} mol/L). Vascular mechanical properties (remodeling and stiffening) were evaluated in the absence of vascular tone by incubation in a

calcium (Ca^{2+}) free Krebs solution supplemented with the 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 mmHg and the media cross-sectional area, media/lumen ratio, stress and strain were calculated as previously described (6).

4.12.8 Flow cytometry

One set of mice was used for immune cell profiling with surface markers and another one set for IL-17A and interferon (IFN)-γ-producing T cell profiling. Since mesenteric artery/PVAT samples do not have enough T cells present to prepare unstained and fluorescence minus one (FMO) controls a spleen was used for their preparation.

The spleen was transferred to a well of a 12-well tissue culture plate containing 1 mL of RPMI medium (ThermoFisher Scientific, Waltham, MA). The nylon mesh of a 70-µm cell strainer (Sarstedt, Saint-Léonard, QC, Canada) was cut out using a razor blade and placed over a spleen. The spleen was crushed using the back of a 3 mL syringe plunger underneath a 70-µm nylon mesh until dissociated. The splenocyte suspension was filtered through a 70-µm cell strainer placed on top of a 50-mL centrifuge tube. The cell strainer was washed with 1 mL of RPMI to free cells from the filter. The cell suspension was stored on ice until T cell profiling or *in vitro* T cell activation. The mesenteric vascular bed attached to the intestine was transferred to a dissection dish containing cold PBS placed on a cold ice pack under the direct view of a dissecting microscope at low magnification. The cluster of mesenteric lymph nodes proximal to the cecum was excised with scissors. Small lymph nodes that could reside within the adipose tissue of the mesenteric bed were removed. Thereafter, the mesenteric arteries and PVAT was cut out from the intestine with scissors and transferred to a 100-mm tissue culture dish sitting on an ice pack. The tissue was further checked to remove any remaining lymph nodes. Mesenteric arteries with perivascular adipose tissue (PVAT) were then minced with scissors and digested in 800 µL of enzyme digestion medium at 37ºC for 25 minutes in a 1.5 mL-conical tube with gentle agitation within a hybridization oven (VWR, Mississauga, ON, Canada). The enzyme digestion medium contained Roswell Park Memorial Institute 1640 (RPMI) medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 0.15 U/ml of collagenase A, 500 U/ml of collagenase type 2, 2 U/ml of elastase and 0.5 U/ml of soybean trypsin inhibitor that was filtered with 0.22 µm Millex GP filter unit (EMD Millipore, Billerica, MA, USA). Collagenase A that is a Roche product (Mannheim,

Germany) was obtained from Cedarlane (Burlington, ON, Canada), and all the other enzymes were purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). At the end of the digestion, the cell suspension was filtered through a 70-µm nylon mesh cell strainer (Sarstedt) fitted on top of a new 1.5-mL conical tube to remove debris. The mesh was washed with 200 µL of RPMI medium to free cells from the filter. The cell suspensions was kept on ice until T cell profiling or *in vitro* T cell activation.

For T cell profiling, cell suspensions were centrifuged at 410 x g for 5 minutes at 4°C. Each mesenteric arteries/PVAT cell sample was resuspended in 100 µL of cold PBS and transferred to one well per sample of a V-bottom 96-well plate. Splenocytes were resuspended in 500 µL of PBS and 50 µL of splenocyte suspension (equivalent to 10% of the spleen) per unstained and FMO control was transferred to a respective well of the plate. The cells underwent Live/dead staining. The plate was centrifuged at 410 x g for 5 minutes at 4°C. The cell pellet in the unstained control well was resuspended with 100 µL of PBS and all the other cell pellets with 100 µL of LIVE/DEAD™ Fixable Aqua Dead Cell Stain solution (Cat. # L34957, ThermoFisher Scientific) and incubated 20 minutes at 4°C. Afterwards, mesenteric arteries/PVAT cells were stained for extracellular markers. The plate was centrifuged at 410 x *g* for 5 minutes at 4°C and the cells were washed with PBS. The cell pellet in the unstained control well was resuspended with 100 µL of PBS and all the other cell pellets with 100 µL of PBS containing a cocktail of fluorochrome-conjugated monoclonal antibodies diluted 1/100 (Table 4.2, full panel for the mesenteric arteries/PVAT cells or specific panel lacking one fluorochrome-conjugated monoclonal antibody for each FMO), 1% CD16/CD32 Fc receptor block (clone 2.4G2, BD Biosciences, Mississauga, ON, Canada), and 5% fetal bovine serum (inactivated FBS, Life Technologies, Burlington, ON, Canada). The unstained splenocyte control was resuspended in 100 µL of PBS. Cells were incubated 15 minutes at 4°C. Then, the plate was centrifuged at 410 x *g* for 5 minutes at 4°C, and cells were fixed in 1% paraformaldehyde (PFA) diluted in PBS. The plate was centrifuged at 410 x *g* for 5 minutes at 4°C, cell pellets resuspended in 150 µL PBS for splenocytes and 100 µL for mesenteric arteries/PVAT cells transferred into Axygen® 1.1 mL polypropylene cluster tubes, and placed in the refrigerator until acquisition on the flow cytometer.

For determination of IL-17A- and IFN-γ-producing T cells, cell suspensions were centrifuged at 410 x g for 5 minutes at 4°C and cell pellets 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), and 2 μM Monensin (Biolegend, San Diego, CA, USA) in RPMI medium. Each sample was transferred into a well of a 12-well tissue culture plate 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. Each Mesenteric arteries/PVAT cell pellet was resuspended in 100 μL of cold PBS and transferred to one well per sample of a V-bottom 96-well plate. Splenocyte pellets were resuspended in 500 μL of PBS and 50 μL of splenocyte suspension per unstained and FMO control was transferred to a respective well of the plate. Then, cells but not the unstained control underwent staining as above for Live/dead and extracellular markers using fluorochrome-conjugated monoclonal antibodies listed in Table 4.3. Thereafter, the plate was centrifuged at 410 x *g* for 5 minutes at 4°C, and cells were resuspended in 100 μL PBS containing 10% saponin and both APC-17A and AF488-IFN-γ monoclonal antibodies diluted 1:100 (see Table 4.3 for details) for the mesenteric arteries/PVAT cells, specific panel lacking one fluorochrome-conjugated monoclonal antibody for each FMO or no antibody for the unstained control, and incubated 30 minutes at 4°C. Finally, the plate was centrifuged at 410 x *g* for 5 minutes at 4°C, cells were resuspended in 150 μL PBS for splenocytes and 100 μL for mesenteric arteries/PVAT cells, transferred into Axygen® 1.1 mL polypropylene cluster tubes, and placed in a refrigerator until acquisition on the flow cytometer.

Flow cytometry was performed on a BD LSR Fortessa cell analyzer (BD Biosciences). Unstained control and FMO controls were used to adjust parameters of the cytometer and the gates. Data analysis was performed using FlowJo software (version 10.6, Tree Star Inc., Ashland, OR). Flow cytometry gating strategies are presented in Figures 4.6 and 4.7.

4.12.9 References

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5. Discussion

The studies described in this thesis aimed to understand two distinct γδ T cell-mediated pathophysiological mechanisms in Ang II-induced hypertension. In the first study we sought to determine if memory γδ T cells develop after an initial exposure to an Ang II hypertensive stimulus, and if memory γδ T cells sensitize mice to develop hypertension to a subpressor dose Ang II hypertensive challenge. We demonstrated that effector memory γδ T cells develop after two weeks of pressor dose Ang II infusion in C57BL/6J mice, and that mice that had been exposed to a prior Ang II hypertensive challenge were sensitized to develop hypertension to a mild Ang II hypertensive challenge. We showed that antibody-mediated depletion of γδ T cells in WT mice that had already been exposed to a prior Ang II hypertensive challenge reduced BP increases in response to a subsequent mild Ang II hypertensive challenge. Finally, we demonstrated that adoptive transfer of γδ T cells isolated from Ang II-infused donor mice sensitized recipient mice to develop higher BP to subpressor dose Ang II infusion than mice transferred γδ T cells from sham-treated mice.

In the second study we sought to determine whether the generation of proinflammatory γδT17 cells would be limited in mice lacking a functional IL-23R, and if this would blunt Ang II-induced hypertension. The development and expansion of γδT17 cells is understood to be in-part dependent upon IL-23R stimulation (337), and given that both IL-17A and γδ T cells contribute to hypertension, we hypothesized that Ang II-induced BP elevation and vascular injury would be reduced in *Il23r* knock-in (*Il23r*$^{gfp/gfp}$) mice that are deficient of functional IL-23R. However, we found that *Il23r*$^{gfp/gfp}$ mice do not exhibit blunted hypertension or reductions in vascular injury in response to 14 days of Ang II infusion. Contrary to our initial hypothesis, *Il23r*$^{gfp/gfp}$ mice had smaller and stiffer mesenteric arteries and were not protected from BP elevation or vascular dysfunction after 14 days of Ang II infusion. *Il23r*$^{gfp/gfp}$ mice exhibited more rapid BP elevation at the initiation of hypertension compared to WT mice and had decreased γδT17 cell numbers and an increased frequency of IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells, irrespective of treatment. We investigated the initiation of Ang II-induced hypertension and found that 7 days of Ang II infusion increased the number of IFN-γ-producing γδ T cells in WT mice, while 7 days of Ang II infusion increased the number of IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells in *Il23r*$^{gfp/gfp}$ mice. To investigate the mechanism behind the BP elevation we observed in *Il23r*$^{gfp/gfp}$ mice, we blocked

IFNγ with a neutralizing antibody in vivo. This reduced the pressor response to Ang II in *Il23r*$^{gfp/gfp}$ mice. Therefore, contrary to our hypothesis, functional IL-23R deficiency exaggerated BP elevation during the initiation of Ang II-induced hypertension via an increase in IFN-γ producing T cells.

Each of the above-mentioned studies investigated a unique aspect of γδ T cell-mediated immunity in experimental hypertension. In 2017, our lab was the first to describe a role for γδ T cells in hypertension. Caillon *et al.* demonstrated that antibody-mediated depletion or genetic knockout of TCRδ$^+$ cells blunted Ang II induced hypertension in mice, and that TCRγ constant region expression in whole blood was associated with systolic BP in humans (154). Given the strong evidence put forth by Caillon *et al.*, we sought to further expand on these initial experiments by examining memory T cell formation and effector cytokine production by γδ T cells.

5.1 Memory γδ T Cells and Hypertension

Compelling evidence gathered from animal models and clinical research has demonstrated that γδ T cells can become long-lived memory T cells (307). While the γδ T cell memory response has been explored extensively in models of infection and autoimmunity, the idea of memory γδ T cells playing a role in hypertension is an entirely novel concept. Itani *et al.* (2016) were the first to demonstrate that memory T cells contribute to the pathogenesis of both L-NAME/high-salt and Ang II-induced hypertension in mice (304). The authors observed an accumulation of CD4$^+$ and CD8$^+$ T_{EM} cells in the kidneys and bone marrow of hypertensive mice. Further, they showed that adoptive transfer of 0.5x10^6 bone-marrow αβ T_{EM} cells isolated from mice exposed to an L-NAME/high-salt challenge into recipient mice that were then fed a high salt diet led to robust T_{EM} cell trafficking to the bone marrow and kidneys of recipient mice. To the contrary, no adoptively transferred T_{EM} cells were detected in the kidneys of recipient mice fed a normal salt diet. While these experiments reveal that memory αβ T cells develop in experimental hypertension and display memory characteristics in the form of salt sensitivity, a major limitation of this study is that the authors did not report on BP changes among animals adoptively transferred T_{EM} cells and rechallenged with a high salt diet. The authors also chose to use a limited number of memory markers, selecting only CD44 and CD62L to differentiate memory T cell subsets. In contrast, we have demonstrated that adoptive transfer of 0.25x10^6 γδ T cells from Ang II-infused mice leads to

robust BP elevation in response to a subpressor dose of Ang II in recipient mice. We also used additional markers to differentiate memory T cell subsets, which included CCR7, CD103, and CD69, in addition to CD44 and CD62L. This allowed us to identify memory T cell subsets with higher confidence, as $CD44^+CD62L^-CCR7^-$ T_{EM} and T_{RM} cells cannot be distinguished without the addition of T_{RM} markers CD69 and CD103. Further, we have shown that memory γδ T cells are present in the aorta, mesenteric arteries, and mesenteric lymph nodes, and that they are responsive to Ang II stimulation. This observation represents another novel concept in hypertension research, as memory T cells have not yet been observed in the arterial wall or surrounding PVAT of hypertensive vessels.

In agreement with the study by Itani *et al.* (304), we were also able to show that an initial pressor dose infusion of Ang II sensitizes mice to develop hypertension in response to a subsequent subpressor dose of Ang II. This lends support to the concept of "autopotentiation" in hypertension, which describes a phenomenon where initially subpressor doses of Ang II administered repeatedly or continuously eventually lead to a state of pressor hyperresponsiveness and the development of hypertension (338, 339). As the initial dose of Ang II in our model was considered to be a BP-elevating pressor dose, we demonstrated that a single hypertensive challenge is also able to induce pressor hyperresponsiveness to a further mild hypertensive challenge. Autopotentiation was first observed in Ang II-infused rabbits in 1963 (339), well before the immune system had been linked to the development of hypertension. Although unknown to the authors of these early studies, our data suggests that memory T cells may contribute to the phenomenon of autopotentiation in Ang II-induced hypertension.

5.1.1 Comparing Induction of Memory γδ T Cells and Memory αβ T cells

Unlike TCRs found on $CD4^+$ and $CD8^+$ αβ T cells, the γδ TCR does not require intracellular antigen processing or MHC-mediated antigen presentation (153). Furthermore, no absolute requirement for costimulatory interactions/receptors has been identified thus far for γδ TCR-mediated activation. Therefore, memory γδ T cell formation may be distinct from that of αβ T cells, as costimulatory interactions are necessary for the generation of memory $CD4^+$ and $CD8^+$ T cells (340). However, this does not exclude a role for costimulatory interactions in γδ T cell activation and acquisition of memory characteristics. In some animal models of infection,

costimulatory molecules present on γδ T cells such as CD27 and CD28 play a role in modulating γδ T cell effector functions. For example, CD28-deficient mice had a reduced number of activated γδ T cells upon infection with *Plasmodium berghei* and were unable to expand IFN-γ$^+$ and IL-17A$^+$ γδ T cell subsets (341). In contrast, mice deficient in CD28 exhibited no reductions regarding the expansion or differentiation of IL-17A or IFN-γ-producing γδ T cells in a mouse model of *Listeria monocytogenes* infection (342). While no studies have investigated CD28 or other costimulatory molecules in memory γδ T cell activation or induction as of yet, these costimulatory interactions could play a role. Consequently, blunting the generation of memory γδ T cells by genetic or pharmacologic intervention presents with additional challenges to blunting the formation of memory CD4$^+$ and CD8$^+$ T cells.

The costimulatory interaction between CD27 on T cells and CD70 on APCs is required for formation of memory CD4$^+$ and CD8$^+$ T cells (343, 344), and Itani *et al.* blocked this interaction by knocking out CD70, which prevented the formation of memory αβ T cells in their mouse model of repeated Ang II infusions (304). However, CD27-CD70 interactions can also contribute to γδ T cell effector functions, including promoting the differentiation of IFN-γ-producing γδT1 cells in the thymus and expansion of peripheral γδT1 subsets at early stages of immune responses (345). As IFN-γ contributes to the development of hypertension and γδ T cells are an important source of IFNγ (324), the study by Itani *et al.* (304) fails to account for any potential off target effects of CD70 knockout on γδ T cell cytokine production or expansion. Additionally, the authors observed that CD70 knockout resulted in a blunted increase in double negative T cells in the kidneys of mice infused with Ang II for 14 days. This further supports the notion that some of the observed BP-lowering effects of CD70 knockout could be a result of γδ T cells, as most γδ T cells are double negative for CD4$^+$ and CD8$^+$ (346). Determining whether CD70 knockout affects memory γδ T cell formation would add considerable insight into the mechanisms of memory acquisition by γδ T cells. Therefore, altered γδ T cell function and/or expansion as a result of CD70 knockout could contribute to the reduced pressor sensitivity to low dose Ang II infusion that was attributed to loss of memory αβ T cells. The effect of CD70 knockout on memory γδ T cell formation is unknown and given the significant BP elevation that we observed in our γδ T cell adoptive transfer experiments, this would be an important consideration for future studies.

5.1.2 Neoantigens, the γδ TCR, and Possible Mechanisms Behind Memory γδ T Cell Generation and Activation in Hypertension

In the first study presented in chapter two, we observed an increase in γδ T_{EM} cells in the MA PVAT and mLNs following 14 days of pressor dose Ang II infusion. Itani *et al.* (2016) were the first to demonstrate that $CD4^+$ and $CD8^+$ T_{EM} cells develop in the bone marrow and kidneys in response to L-NAME/high salt treatment or infusion of Ang II (304). However, we are the first to demonstrate that γδ T_{EM} cells also develop in Ang II-induced hypertension, and that they are increased in the resistance arteries of the mesenteric bed and nearby lymph nodes. Ang II is a potent initiator of vascular oxidative stress, and oxidative stress is an important trigger for vascular inflammation (347). While many chemokines, adhesion molecules, and proinflammatory cytokines that both induce and maintain vascular inflammation have been identified in hypertension, no specific TCR antigens have been found as yet. It is generally understood that memory T cell development is dependent on antigen exposure, yielding antigen-specific memory T cell subpopulations (348). While the process of antigen-driven memory T cell generation has been investigated in various models of infection and immunity in the context of $CD4^+$ and $CD8^+$ memory T cells, the process in which memory γδ T cells develop is poorly understood and the literature scarce. This issue is further complicated by the unique ability of γδ T cells to undergo TCR-mediated activation without the need for antigen processing by an APC or MHC presentation of antigen fragments (153). However, studies of human, non-human primate, and murine memory γδ T cells in infection and autoimmunity demonstrate that memory γδ T cells are indeed antigen-specific like memory αβ T cells (307). Therefore, we must consider what molecules could arise and act as neoantigens in the context of hypertension and speculate as to how putative neoantigens could induce memory γδ T cell development in the vasculature of the mesenteric bed and nearby mLNs.

There is potential for the formation of immunogenic molecules, or neoantigens, in hypertensive vessels, and an important study by Kirabo *et al.* (2014) demonstrated that Ang II-induced hypertension resulted in the formation of isoketals in the aortic wall (151). Isoketals are formed as a result of free radical-mediated lipid peroxidation of arachidonic acid (349), a process that could occur in vessels experiencing high levels of oxidative stress. Isoketals are highly reactive and are able to react with lysine residues on proteins, leading to protein cross-linking. Moreover, the authors observed that isoketal protein adducts accumulated in DCs, and adoptive transfer of

DCs from Ang II-infused mice not only promoted T cell proliferation and cytokine production, but also sensitized recipient mice to develop hypertension to a low dose of Ang II upon adoptive transfer (151). It is possible that isoketal protein adducts could activate γδ T cells, but this has yet to be demonstrated experimentally. The γδ TCR has been shown to recognize CD1 glycoproteins on APCs and CD1 functions to present lipid antigens to T cells (350, 351). This lends further clues as to putative neoantigens in hypertension. Peroxidation of arachidonic acid membrane lipids can generate a multitude of lipid oxidation products other than isoketals, and it is possible that species of oxidized lipids could be presented by CD1 molecules to γδ T cells in the context of hypertension (352). Therefore, there exists the possibility that a wide array of putative neoantigens could exist in the form of lipid oxidation products, which in turn could activate γδ T cells and promote memory γδ T cell generation in a CD1-mediated mechanism. Future studies of the role of γδ T cells in hypertension would benefit from investigating this mechanism.

When we depleted γδ T cells with a monoclonal antibody we observed a significant reduction in the pressor response to low-dose Ang II infusion in mice that had already been infused with a previous pressor dose of Ang II. While we have reported that the GL3 antibody depleted γδ T cells, contradictory data exists as to whether γδ T cells are truly depleted in response to treatment with the GL3 clone. A study published in 2009 by Koenecke *et al.* demonstrated that the GL3 and UC7 anti-TCRγδ antibody clones compete for the same epitope, and induce TCR internalization or blocking rather than depletion (318). If this is indeed the case, then it is conceivable that the BP-lowering effects that we observed with GL3 antibody administration could be a result of γδ TCR blocking or signaling inhibition rather than depletion of γδ T cells as a whole. While this is highly speculative, it would support the notion of antigen-driven memory γδ T cell generation and TCR-mediated γδ T cell activation in Ang II-induced hypertension, and suggest that γδ T cells contribute to the pathogenesis of hypertension in a TCR-dependent mechanism.

As γδ T cells are capable of professional antigen presentation (264, 316), it is not surprising that we observed an increase in γδ T_{EM} cells in lymph nodes located proximal to the arteries of the mesenteric bed. It was previously demonstrated that human γδ T_{EM} cells express high levels of APC-related molecules HLA-DR and CD80/86 and are able to present soluble antigens to $CD4^+$ T cells (316). While limited to speculation, γδ T_{EM} cells could be trafficking to lymph nodes to present antigens to other immune cells in our model of Ang II-induced hypertension. The strong

pressor response that we observed to a mild dose of Ang II in mice that were adoptively transferred γδ T cells from Ang II-infused animals could also be a result of antigen presentation by memory γδ T cells to other immune cell subsets. Determining the expression of APC-related surface molecules on γδ T cells following a hypertensive challenge would add mechanistic insight into the role of γδ T cells in hypertension, and this would give clues as to the contribution of antigen presentation by γδ T cells to the development of hypertension.

5.2 IL-23R Signaling Inhibition and Hypertension

We previously showed that γδ T cells play a role in the development of hypertension and vascular injury (154), however, it is unknown which subset of cytokine-producing γδ T cells, γδT17 or γδT1, contributes more to the development of hypertension. It was previously demonstrated that γδT17 cells represent an important source of IL-17A, and γδT17 cells were found to be increased in the aorta and kidneys of Ang II-infused mice (213). Furthermore, *Il17a* knockout mice and mice injected with neutralizing antibodies against IL-17A or the IL-17 receptor A do not sustain increased BP after 3 or 4 weeks of Ang II infusion (211, 213). *Il17a* knockout also reduced BP elevation in mice after 2 weeks of Ang II infusion (353). The IL-23R is required for de novo generation and expansion of γδT17 cells extrathymically under inflammatory conditions, and γδ T cells were shown to be the major immune cell type expressing IL-23R in lymph nodes (311, 354). Accordingly, we speculated that inhibition of IL-23R signaling would reduce the fraction of γδT17 cells, and in turn, blunt the development of hypertension and vascular injury.

In opposition to our hypothesis, we observed that *Il23r*$^{gfp/gfp}$ mice were not protected from Ang II-induced BP elevation or vascular injury, and *Il23r*$^{gfp/gfp}$ mice actually exhibited higher BP at the initiation of hypertension. Other studies of the role of the IL-23R in hypertension have also reported conflicting results. Injection of IL-23 increased BP in Dahl salt-sensitive rats but not in Dahl salt-resistant rats and resulted in a greater increase in serum IL-17A and in the fraction of circulating Th17 cells in the salt-sensitive rats (355). In another study, a very severe model of hypertension which combined unilateral nephrectomy, 2 weeks of treatment with deoxycorticosterone acetate, salt added to the drinking water, and infusion with a high dose of Ang II (1.2 μg/kg/min, SC) was used to induce hypertension in mice (222). The authors observed that BP elevation was unaffected by *Il23a* gene knockout in this model, with *Il23a* encoding one of the

two IL-23 subunits, Il-23p19. Conversely, the urine albumin/creatinine ratio was increased in *Il23a*$^{-/-}$ mice at days 3 and 7 but not at day 12, and hypertensive *Il23a*$^{-/-}$ mice presented an increased fraction of renal γδ T cells. In another mouse study, BP elevation induced by infusion of Ang II (1 μg/kg/min) was not affected by injection of anti-IL-23R neutralizing antibodies compared to mice given IgG control antibodies (192). Given that: 1. IL-23R signaling inhibition led to no protection from BP increases, 2. the primary T cell role attributed to IL-23 signaling is expansion and maintenance of IL-17A-producing T cells, and 3. γδT17 cells were still numerous in *Il23r*$^{gfp/gfp}$ mice in our study, we must consider other mechanisms that result in the development of IL-17A-producing lymphocytes.

5.2.1 The Role of IL-23R Signaling in T Cell Effector Polarization

It may seem contradictory that IL-23R agonism exacerbates hypertension while IL-23R inhibition does not blunt BP increases. However, generation of Tc17, Th17, and γδT17 is not solely dependent on IL-23R signaling. While we did observe reduced numbers of γδT17 cells in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice, a significant number of γδT17 cells remained. This supports the notion that generation of γδT17 cells is IL-23-independent in the context of embryonic thymic origin (313). While αβ T cells generally leave the thymus as naïve T cells and subsequently acquire effector functions in the periphery, most γδ T cells commit to an effector lineage before leaving the thymus (320). This contributes to the ability of γδ T cells to respond rapidly to immune insult in a similar manner to cells of the innate immune system. However, some γδ T cells leave the thymus as naïve cells that can be induced to produce cytokines in the periphery. Stimulation of naïve γδ T cells by IL-1β and IL-23 in the periphery induces extrathymic generation of γδT17 cells (218). Therefore, the reduction in the number of γδT17 cells that we observed in the MA PVAT of *Il23r*$^{gfp/gfp}$ mice could reflect extrathymic γδT17 commitment being inhibited. In contrast, neither Th17 nor Tc17 cell numbers were altered in *Il23r*$^{gfp/gfp}$ mice, which was unsurprising given that IL-23R signaling is not required for the polarization of Th17 or Tc17 cells (356). Initially, IL-23 was thought to be essential for Th17 differentiation, however, it was later discovered that naïve CD4^{+} T cells do not express IL-23R, and IL-23R expression needed to be induced for IL-23 to upregulate Th17 cells (357). Consequently, both Th17 and Tc17 cells can be effectively generated in the presence of cytokines such as IL-6, IL-21, and TGFβ, with IL-23 being speculated to mainly

play a role in stabilizing the phenotype of Th17 and Tc17 cells (321). In addition, inhibition of IL-23 signaling likely has a nominal effect on the expansion and generation of IL-17A-producing lymphocyte populations, whereas administration of exogenous IL-23 could augment BP by expanding and maintaining populations of γδT17, Th17, and Tc17, explaining the contradictory results between exogenous IL-23 administration and inhibition of IL-23R signaling in hypertension.

5.2.2 IFN-γ-Producing T Cells Are Increased in *Il23r*$^{gfp/gfp}$ Mice: Potential Mechanisms and Relevance to Hypertension

We found an increase in the number of IFN-γ-producing γδ, $CD4^+$, and $CD8^+$ T cells in Ang II-infused *Il23r*$^{gfp/gfp}$ mice, but only IFN-γ-producing γδ T cells were increased in number in Ang II-treated WT mice. It is unknown why Ang II increased the number of Th1 and Tc1 cells in *Il23r*$^{gfp/gfp}$ but not in WT mice. One possible explanation could be that if less T cells are committing to IL-17A production, this could leave more T cells able to become IFN-γ producers under inflammatory conditions. For example, we observed a reduced frequency of Th17 cells and an increased frequency of Th1 cells in the *Il23r*$^{gfp/gfp}$ genotype compared to WT. As $CD4^+$ and $CD8^+$ T cells are thought to acquire an effector phenotype in the periphery (320), it is conceivable if less cells are polarizing to IL-17A production then there will be more able to become IFN-γ producers. Additionally, if IL-23 is required to stabilize populations of IL-17A-producing T cells (321), then some Th17 and Tc17 could lose the IL-17A-producing phenotype in the absence of IL-23 stimulation and become Th1-like cells. This hypothesis is further supported by the fact that lineages of IL-17A-producing T cells possess a high degree of late developmental plasticity and are able shift from IL-17A to IFN-γ production (358). Therefore, it is possible that a phenotypic switch from IL-17A to IFN-γ production could be enhanced in the absence of IL-23R signaling, and this could explain the increase in Th1 and Tc1 that we observed in *Il23r*$^{gfp/gfp}$ mice but not WT mice.

In the final experiment of the study, we blocked IFN-γ in vivo with a monoclonal antibody and observed a reduced pressor response to 7 days of Ang II infusion. We concluded that IFN-γ was likely behind the increased BP at the initiation of hypertension, and the increase in γδT1, Th1, and Tc1 cells that we observed using flow cytometry supports this hypothesis. Several studies have shown a role for IFN-γ in the progression of hypertension and cardiovascular injury. Han et al.

(2012) showed that knockout of the gene encoding IFN-γ (*Ifng*) did not affect BP elevation induced by 7 days of a large dose of Ang II (1500 ng/kg/min), but *Ifng* knockout did decrease immune cell infiltration in the heart (359). The lack of protection from BP increases observed in *Ifng* knockout mice could be explained by the authors use of such a high dose of Ang II. On the other hand, a study by Kamat *et al.* (2015) found reduced BP elevation and preserved renal function assessed by a saline challenge in $Ifng^{-/-}$ mice infused with the same dose of Ang II as in this study for two weeks (490 ng/kg/min) (330). Finally, another study found that *Ifng* knockout did not decrease BP elevation but did reduce cardiac hypertrophy and cardiac infiltration of T cells and macrophages in response to a 14-day infusion of a large dose of Ang II (1 μg/kg/min) (192). The lack of a BP lowering effect in this study may also be attributed to the use of a very high dose of Ang II. Consequently, the BP elevation that we observed during the initiation of hypertension in $Il23r^{gfp/gfp}$ mice is in part mediated by IFN-γ, the production of which is heavily reliant on T cells.

Limitations

A major limitation of these studies is that experimental hypertension does not encompass all of the aspects of human hypertension, and animal models typically represent a simplified picture of hypertension as a whole. Another consideration is the short time frame in which mice are made hypertensive by infusion of Ang II. While human hypertension is considered to be a chronic condition that develops slowly over the course of years or decades, mouse models of hypertension are typically induced in the span of days to weeks. Therefore, hypertension-induced target organ damage may not have a chance to fully develop in most animal models of hypertension, including our Ang II mouse model. In addition to the rapid induction of hypertension in animal models, humans generally develop hypertension more with increasing age. This is a caveat in most studies of murine hypertension as a balance must be found between the age of mice, feasibility of maintaining a colony, and the tolerance of mice for anesthesia and multiple surgical procedures. In our model of repeat hypertensive challenges for example, we needed to use mice that would be young and healthy enough to tolerate up to three osmotic minipump or sham surgeries as well as telemetry probe implantation.

The telemetry probe itself could also affect experimental results due to its effect on cerebral blood flow, as the tip of the probe is inserted through the left common carotid artery. As a result, the artery is occluded and blood flow to the brain somewhat reduced in these animals. Telemetry instrumented mice tend to move more slowly and be more docile than littermates not instrumented with telemetry probes. However, the right common carotid artery and circle of Willis are together able to effectively compensate for the occluded left side, and telemetry implanted animals exhibit relatively normal behavior in the context of eating, grooming, and nest building. Therefore, recording BP by telemetry in both Ang II- and sham-treated mice is important to ensure that the results are comparable between groups. While tail cuff BP measurement may be less invasive, using telemetry probes allows us to measure BP parameters uninterrupted and with higher accuracy, and mice do not need to be handled or restrained before, during, or after BP recording. In addition, no animal training is required to acquire BP by telemetry, while mice undergoing tail cuff BP measurement must go through a period of acclimation and training to reduce the effect of stress on BP measurements. However, stress will somewhat impact tail cuff BP measurement regardless of training, and it is impossible to quantify the contribution of animal stress to BP

outcomes. Therefore, BP measurement by telemetry is a more reproducible technique that is less prone to noise and user error than tail cuff.

An additional concern is the validity of genetically altered strains of mice in comparison to pharmacological activation or blockade of a given pathway/receptor/molecule of interest. The issue that arises from using genetically modified strains of mice is that it is difficult if not impossible to evaluate the impact of the specific genetic alteration on the embryonic and early post-natal development of these animals. Pathways that are seemingly unrelated could be significantly changed in knockout and knock-in animals, resulting in off-target effects, which has the potential to mask important results or lead to false conclusions. Therefore, it is difficult to understand the full extent of a given experimental outcome in genetically modified research animals. In contrast, pharmacological interventions allow research animals to develop normally, both embryonically and post-birth, and the intervention can be given at a specific timepoint. For the study of the IL-23R and γδT17 cells in Ang II-induced hypertension we used *Il23r*$^{gfp/gfp}$ mice. These mice have the intracellular portion of the IL-23R replaced by an enhanced green fluorescent protein reporter, and heterozygotes can report cellular IL-23R expression while homozygotes lack a functional IL-23R. However, it is possible that these animals could possess other differences from WT animals due to embryonic and/or post-natal development in the absence of a functional IL-23R, and this represents a limitation of our model. While genetically altered animals can give clues as to how a cell, pathway, or mechanism of interest develops and functions, findings drawn from these studies cannot be applied to humans or considered as a potential therapeutic avenue. Conversely, studies which incorporate a druggable target and pharmacologic intervention can be more readily applied to the clinical setting and are therefore more translational.

There are also limitations regarding some of the experimental techniques used in the studies presented in this thesis, with flow cytometry being the most notable. Flow cytometry is an effective tool to study immune cell marker expression and cytokine production, however, results gathered using flow cytometry naturally underrepresent the true number of immune cells in a given tissue. This is primarily a consequence of sample preparation as some cells will be lost to the walls of tubes or wells or in the supernatant following washing steps. In addition, some cells die during the staining protocol, and this is unavoidable considering the numerous washing steps, the need for mechanical and/or enzymatic dissociation of tissues, and the use of substances that are tough on cells and cell membranes such as paraformaldehyde and saponin. Reporting on cell frequency is

more robust in this regard, as frequencies are less dependent on absolute cell numbers and are more reflective of the immune system as a whole. Frequency data is also less influenced by variables that may affect the number of cells in a given sample, with examples including individual animal size and sample manipulation during flow cytometry staining such as washing steps. However, cell numbers can be reported with high confidence if care is taken in the preparation of cell suspensions for flow cytometry acquisition. For example, the technique used to generate single cell suspensions from MA PVAT samples for flow cytometry phenotyping is highly reproducible if the individual carrying out the work is both rigorous and experienced, as in the case of the IL-23R study. Therefore, while we have taken precautions to minimize the use of experimental techniques that present with serious limitations, our studies have their own inherent limitations that must be considered when interpreting our results.

Conclusion and Perspectives

In conclusion, the studies presented in this thesis provide novel insight into the role of γδ T cells in hypertension. In the first study, we demonstrated that memory γδ T cells develop in response to a hypertensive stimulus, and that they sensitize mice to develop hypertension to a mild hypertensive insult. We are the first to show that memory γδ T cells are present in hypertensive vessels and PVAT, and we are also the first to present data regarding γδ T cell depletion and adoptive transfer in the context of repeat hypertensive challenges. This study challenges the traditional "innate-like" view of γδ T cells, as we have demonstrated that γδ T cells can acquire characteristics of memory T cells following a BP challenge. Functionally, we showed that depleting γδ T cells in mice already exposed to a hypertensive challenge reduced the pressor sensitivity to a subsequent mild hypertensive stimulus. Conversely, mice that retained γδ T cells responded much more strongly to a secondary mild hypertensive stimulus. Most importantly, transfer of γδ T cells from mice that had been exposed to a hypertensive insult induced a state of pressor hyperresponsiveness in recipient mice. Therefore, memory γδ T cells could represent a novel therapeutic target in hypertension. Depleting or limiting the development of memory γδ T cells in hypertensive and prehypertensive individuals may provide protection from BP increases, and future studies will hopefully determine if memory γδ T cells contribute to human hypertension.

In contrast to the study of memory γδ T cells, we have shown that the IL-23R does not likely represent an effective target for reducing BP in hypertensive individuals. We showed that functional IL-23R deficiency led to higher BP early in the development of hypertension and offered no protection against BP increases or vascular dysfunction. Importantly, this study described the consequences of IL-23R inhibition to cytokine-producing T cell subsets in the arteries of the mesenteric bed. We demonstrated that in the absence of IL-23R signaling there is an increased proportion of IFN-γ-producing T cells, and these cells are further increased in response to Ang II infusion. We also showed that the increased BP observed in Ang II-infused *Il23r*$^{gfp/gfp}$ mice is in part mediated through the actions of IFN-γ. Taken together, the work presented in this thesis provides novel insight into the contribution of γδ T cells to experimental hypertension and opens the door to future studies that could lead to new anti-hypertensive treatments.

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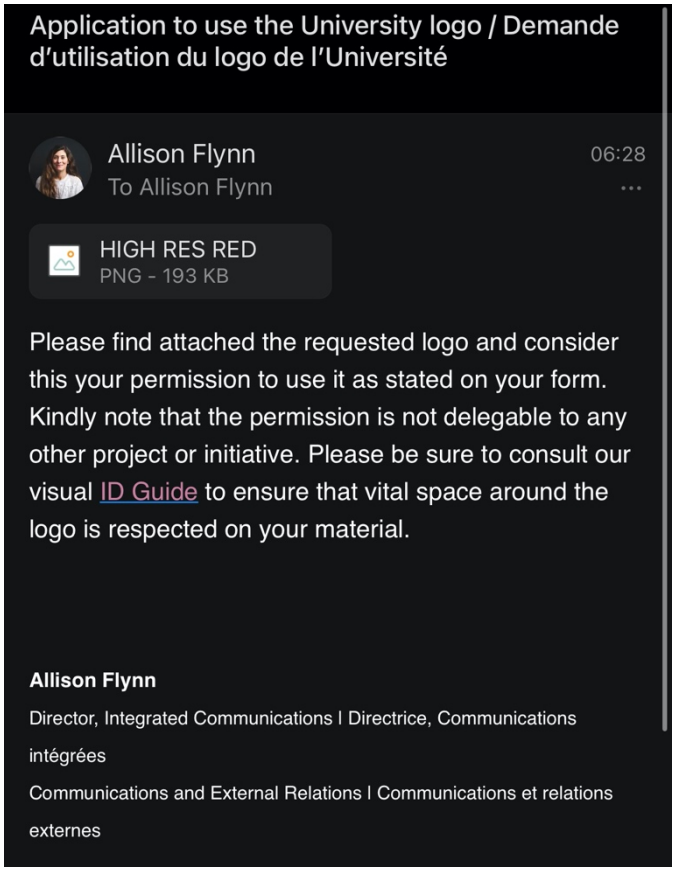

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