MONASH University

Roles for Tissue-Infiltrating T cells during Hypertension

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Abstract

Hypertension remains a major risk factor for death due to heart failure, stroke and vascular disease. Currently, while there are treatments to control high blood pressure, surprisingly 20-30% of patients remain unresponsive and resistant to multidrug therapies, which highlights the need for new approaches to treat cases of uncontrolled hypertension. Current research has implicated an important contribution of the immune system and inflammation to the development of hypertension. There is strong evidence of complex mechanisms involving the immune system and accumulation of specialized immune cells called T cells in blood pressure-controlling organs during hypertension. Although we know that T cells are important to the development of hypertension, the challenge is to identify how T cells promote the progression of hypertension. This will facilitate discovery of novel targeted immunomodulatory therapies, as opposed to broad-spectrum immunosuppressants, to control inflammation associated with hypertension.

In Chapter 3 of this thesis, the aim was to characterise the phenotype of infiltrating T cells of blood pressure-controlling organs to identify potential T cell-derived proinflammatory mediators during hypertension. T cells were isolated from various organs of normotensive and hypertensive mice, and were stimulated to produce cytokines, reactive oxygen species (ROS) and chemokines such as CCL2. Elevated T cell infiltration was observed in aorta, kidney and brain from hypertensive mice compared to normotensive controls. However no differences in cytokine release across various organs (aorta, kidney, brain, blood and spleen) were observed. Strikingly, distinct phenotypes of T cells during hypertension were identified, where T cells from the aorta and kidney of hypertensive mice produced significantly greater ROS and CCL2. These findings highlight a unique T

cell phenotype that is localized to the vasculature and kidneys of hypertensive mice, and T cell-derived CCL2 and ROS may be important mediators that promote local inflammation during hypertension.

In Chapter 4, the effect of the elevated T cell activation on vascular function was assessed. A novel approach of directly stimulating vascular infiltrating T cells in *situ* using monoclonal anti-CD3/CD28 antibodies was employed to examine the effect of T cell activation on vascular function. *In situ* stimulation of T cells in the vasculature exacerbated endothelial dysfunction in intact aorta from angiotensin (Ang) II-induced hypertensive mice. This effect appeared to be ROS-dependent, since co-treatment with the ROS scavenger tempol abolished both ROS production and augmented endothelial dysfunction. This effect was also only observed in aorta obtained from Ang II-induced hypertensive mice, which supports the findings from Chapter 3, where vascular T cells release greater ROS during hypertension. Interestingly, the T cell infiltrate within the perivascular adipose tissue (PVAT) was indeed playing a direct role in modulating endothelium-dependent vasorelaxation, as exacerbation of endothelial dysfunction was abolished upon removal of PVAT.

Finally, in Chapter 5, using a world-first, live-cell tracking technique we identified that T cells in the vasculature appear to form immunological synapses with antigen presenting cells (APCs), which is representative of antigen recognition. This novel imaging approach is new to the field of hypertension and involved incubating fluorescently-labelled T cells from hypertensive (hT cells) or normotensive mice (nT cells) with hypertensive or normotensive CD11c-YFP mouse aorta – APCs that express CD11c also express a yellow fluorescent protein. Following incubation, vessels are imaged using confocal microscopy

and time-lapse recordings are obtained to measure the markers of dynamic interactions including T cell velocity and duration of interaction. A greater number of T cell-APC interactions were observed between hT cells and APCs from hypertensive mouse aorta compared to APCs from normotensive aorta. Moreover these interactions involved slowing of T cell velocity and increased duration of interactions, which are representative of antigen recognition. Therefore, this chapter identified that vascular infiltrating T cells are recruited and recognize cognate antigens within diseased hypertensive vessels, which underscores the existence vascular antigens that are unique to hypertension and the potential stimuli the evokes vascular inflammation

Collectively, this thesis reports the first potential mechanistic evidence of how infiltrating T cells that accumulate in blood pressure-controlling organs during hypertension – particularly the vasculature. Ultimately, the evidence presented has identified that preventing T cell recruitment and activation as a therapeutic targets for the treatment of inflammation associated with hypertension.

Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original papers published in peer reviewed journals 2 unpublished manuscripts. The core theme of the thesis is 'Adaptive immune mechanisms in hypertension'. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Pharmacology under the supervision of Dr Antony Vinh and Prof. Robert Widdop.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

Signature: 

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Date: 02/03/2017

Declarations

In the case of Chapter 3, 4 and 5, the nature and extent of my contribution to the work was the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
3	Differential phenotypes of tissue-infiltrating T cells during angiotensin II-induced hypertension in mice	*Published*	75%; Performed the majority of experiments, conception and design of experiments, data analysis, prepared figures and wrote the manuscript	• Mr Henry Diep Assistance with performing L-012 chemiluminescence; 2%	No
				• Ms Iresha Spizzo Assistance with tissue processing; 2%	No
				• Prof Grant R. Drummond Provided intellectual advice for experimental design and editing of manuscript; 1%	No
				• Dr Antony Vinh • Prof Robert E Widdop Conception and design of experiments, drafting and editing of manuscripts; 20%	No

Declarations

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
4	Direct *in situ* stimulation of vascular infiltrating T cells exacerbates angiotensin II-induced endothelial dysfunction	*Returned for revision*	73% Performed the majority of experiments, conception and design of experiments, data analysis, prepared figures and wrote the manuscript	• Mr Henry Diep Assistance with performing L-012 chemiluminescence; 3%	No
				• Ms Iresha Spizzo Assistance with vascular function experiment; 3%	No
				• Prof Grant R. Drummond Provided intellectual advice for experimental design and editing of manuscript; 1%	No
				• Dr Antony Vinh • Prof Robert E Widdop Conception and design of experiments, drafting and editing of manuscripts; 20%	No

Declarations

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
5	Vascular T cell-Antigen Presenting cell interactions during Hypertension	*Manuscript*	73% Performed the majority of experiments, conception and design of experiments, data analysis, prepared figures and wrote the manuscript	• Mr Ethan Tan Contributed to experiments for the Met-RANTES data; 4%	Yes
				• Ms Anna Wang Assistance with tissue processing; 1%	Yes
				• Prof Grant R. Drummond Provided intellectual advice for experimental design and editing of manuscript; 1%	No
				• Prof Michael J Hickey Provide intellectual advice for the animal model and CD11c YFP+ mice; 1%	No
				• Dr Antony Vinh • Prof Robert E Widdop Conception and design of experiments, drafting and editing of manuscripts; 20%	No

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** 02/03/2017
Main Supervisor's Signature		**Date** 02/03/2017
Supervisor's Signature		**Date** 02/03/2017

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

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Publications from PhD Candidature

Primary Author Original Publications

1. **Wei Z.**, I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh. "Differential Phenotypes of Tissue-Infiltrating T Cells During Angiotensin Ii-Induced Hypertension in Mice." *PloS one* 9, no. 12 (2014): e114895

Primary Author Manuscripts

2. **Wei Z**, Spizzo I, Diep H, Wang A, Drummond GR, Widdop RE, Vinh A."**Vascular T cell-Antigen Presenting Cell interaction during Hypertension**." (Aimed Journal: *Immunity*)
3. **Wei Z**, Spizzo I, Diep H, Drummond GR, Widdop RE, Vinh A. **"Direct stimulation of T cells in aorta induces endothelial dysfunction**." (Revision submitted to *Scientific Reports*)

Co-authored publications

1. Mirabito KM, Hilliard LM, **Wei Z**, Tikellis C, Widdop RE, Vinh A, Denton KM. "Role of Inflammation and the Angiotensin Type 2 Receptor in the Regulation of Arterial Pressure During Pregnancy in Mice." *Hypertension* 64, no. 3 (2014): 626-31.
2. Kim HA, Whittle SC, Lee S, Chu HX, Zhang SR, **Wei Z**, Arumugam TV, Vinh A, Drummond GR, Sobey CG."Brain Immune Cell Composition and Functional Outcome after Cerebral Ischemia: Comparison of Two Mouse Strains." *Frontiers in cellular neuro* 8, (2014): 365.

Conference Abstracts

Oral Presentation

1. **Wei, Z.,** Wang, A, I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh"Vascular T cell-Antigen Presenting Cell interactions during Hypertension." *High Blood Pressure Research Council of Australia, (2015; Melbourne)* **HBPRCA Student Finalist Oral Presentation**
2. **Wei, Z.,** I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh "Dynamic T cell-Antigen Presenting Cell interactions and Direct T cell Activation within the Vascular Wall during Hypertension." *Council on Hypertension 2015 Scientific Meeting, (2015; Washington DC)*
3. **Wei, Z.,** I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh. "Differential Phenotypes of Tissue-Infiltrating T Cells During Angiotensin II-Induced Hypertension in Mice. *High Blood Pressure Research Council of Australia conference (2013; Melbourne*)

Poster Presentation

1. **Wei, Z.,** Wang, A, I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh"Dynamic T cell-Antigen Presenting Cell interactions and Direct T cell Activation within the Vascular Wall during Hypertension." *Conference on Immune Mechanisms of Cardiovascular Disease and Stroke (2015; Krakow*)
2. **Wei, Z.,** I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh. "Visualization of T cells and their dynamic movements within with in aorta from Angiotensin II infused mice." *High Blood Pressure Research Council of Australia conference (2014; Adelaide*
3. **Wei, Z.,** I. Spizzo, H. Diep, G. R. Drummond, R. E. Widdop, and A. Vinh. "Role of Tissue-infiltrating T cells during Angiotensin II-induced hypertension" *The First*

Meeting of Australian & European Consortium on Immune Mechanisms in Vascular Disease & Stroke (2013; Prato)

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Abbreviations

Ach - Acetylcholine

ADRF - Adipocyte-derived relaxing factor

Ang II - Angiotensin II

ANOVA – Analysis of Variance

APC - Antigen Presenting Cell

AV3V - Anteroventral third ventricle

BAFF-$R^{-/-}$ - B-cell-activating factor receptor-deficient

BrdU - Bromodeoxyuridine

CBA - cytometric bead array

CCL - C-C Chemokine Ligand

CCR - C-C Chemokine Receptor

cGMP - Cyclic guanosine 3', 5'-cyclic monophosphate

CNS - Central nervous system

CTLA4-Ig - Cytotoxic T-Lymphocyte Antigen 4 – Antibody

CVD - Cardiovascular Disease

CVO - Circumventricular organs

CXCL - C-X-C Motif Chemokine Ligand

DC - Dendritic cells

DCF - 2',7'-dichlorodihydrofluorescein diacetate

DOCA-salt - Deoxycorticosterone acetate-salt

DN - Double Negative

EAE - Experimental autoimmune encephalomyelitis

FACS - Fluorescence activated cell sorting

FMO - Fluorescence minus one

FOV - Random fields of view

FoxP3 - Forkhead box P3

FSC - Flow Cytometry standard

GATA3 - GATA binding protein 3

GM-CSF - Granulocyte/ Macrophage colony stimulating factor

HAART - Highly active antiretroviral therapy

HIV - Human immunodeficiency virus

HPA - Hypothalamic pituitary axis

hT - Hypertensive T cell

L-NAME - Nω-nitro-L-arginine methyl ester

LPS - Lipopolysaccharide

ICV - Intracerebroventricular

IFN-γ - Interferon-gamma

IL - Interleukin

iNOS - Inducible Nitric Oxide Synthase

MAS - Monash Animal Services

Met-RANTES - Methiolynated-RANTES

MHC - Major Histocompatibility Complexe

MMF - Mycophenolate mofetil

MNC - Mononuclear Cell

M-CSF - Macrophage colony-stimulating factor

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

nT - Normotensive T cell

Nox - NADPH oxidase

NK - Natural Killer Cell

PBS - Phosphate Buffer Solution

PDBu - Phorbol 12, 13-dibutyrate

PMA - Phorbol 12-myristate 13-acetate

PVAT - Perivascular adipose tissue

PVN - Paraventricular nucleus

RANTES - Regulated-on-activation, normal-T-expressed-and-secreted

RAG-1$^{-/-}$ - Recombinase Activating Gene-1 Deficient

RBC - Red Blood Cell

ROS - Reactive Oxygen Species

RUPP - Reduced uterine perfusion pressure

SBP - Systolic Blood Pressure

SCID - Severe Combined Immunodeficiency

S.E.M - Standard Error of the Mean

SHR - Spontaneous hypertensive rat

SLE - Systematic Lupus Erythematosus

SNS - Sympathetic nervous system

SOD - extracellular superoxide dismutase

SSC - Side Scater

TCR - T cell Receptor

TGF-β - Transforming Growth Factor-beta

Th - T helper Cell

TLR - Tool-like receptor

TNF-α - Tumour Necrosis Factor-alpha

Treg - T regulatory cell

VCAM - Vascular cell adhesion molecule

VSMC - Vascular smooth muscle cells

WKY- Wistar-Kyoto rats

YFP - Yellow fluorescent protein

Chapter 1:

General Introduction

1.1 Hypertension

Hypertension, defined by blood pressure ≥140/90 mmHg, is a common risk factor for cardiovascular disease and stroke, which are the leading source of morbidity and mortality in Western societies (W.H.O, 2013).[1] Hypertension is a widespread disease that involves multiple risk factors including obesity, diabetes, excessive alcohol or salt intake, ageing and stress.[5] However, the causes for about 95% of hypertension remain unclear. While there are current treatments that can control high blood pressure, such as angiotensin converting enzyme inhibitors or angiotensin receptor blockers,[6] recent clinical studies have shown that about 15-25% of cases of hypertension are resistant to current anti-hypertensive therapies.[7, 8] Although, the etiology of hypertension remains unclear, recent studies have implicated inflammation, namely the immune system in the development and progression of hypertension.[4] It is now understood that T cells are required for the pathogenesis of hypertension and that hypertension is associated with increased T cell infiltration into the organs that control blood pressure such as the aorta, kidneys and brain.[9, 10] However, the functional contribution of these infiltrating T cells to the development of hypertension remains speculative and understudied.

Animal models for research are valuable tools for understanding the pathophysiology and developing therapeutic treatments for a disease. Various murine models of experimental hypertension have been developed to mimic hypertensive responses observed in humans. The short life span, small size, and relatively low cost of murine models allows a better research of the pathophysiology, genetic factors, development and progression of hypertension. Different pathways are involved in the development of hypertension, thus

to understand the role of T cells and adaptive immunity in the progression of hypertension, various models of experimental hypertension including Angiotensin (Ang) II-induced hypertension, DOCA-salt-induced hypertension, Spontaneously hypertensive rats (SHR) and Dahl-salt sensitive hypertension have been consistently implicated in the field.[4,9,10] Nevertheless, since animal studies do not predict sufficient certainty what will happen in humans, thus clinical trails are vital for extrapolation to humans.

1.2 The Immune Systems

The immune system protects the body against external pathogens such as viruses and bacteria. It consists of innate and adaptive immune systems, which interact to mount an immune response. The innate immune system is the body's first line of defence and protects against pathogens in a non-specific mechanism. It includes physical and chemical barrier defences such as skin and mucousal lining, which prevent pathogen entry and migration.[11] Cells of the innate immune system include phagocytic cells such as macrophages, neutrophils, dendritic cells (DCs) and also natural killer (NK) cells, which either engulf and/or directly destroy the pathogen.[2] Innate immune cells express pattern recognition receptors, which allow them to recognise pathogen associated molecular motifs such as lipopolysaccharide (LPS) and double stranded RNA through toll-like receptors (TLRs). Following recognition of these pathogens, a series of cellular responses including reactive oxygen species (ROS) production and release of cytokine and chemokines ensues.[12]

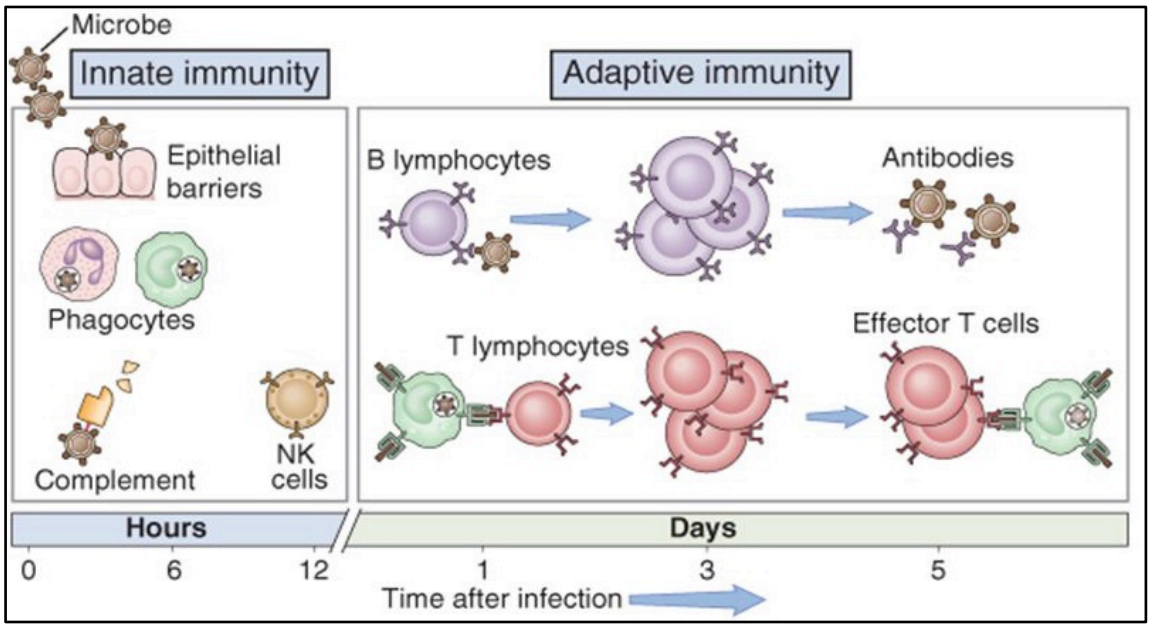

Figure 1.1. Involvement of immune cells during infection. The innate immune response provides the initial defence against infections via barrier defences and innate immune cells including phagocytes (DCs, neutrophils and macrophages) and natural killer cells. The adaptive immune response involves lymphoid cells that include B lymphocytes and T lymphocytes. Activation of the adaptive immune response is most commonly initiated following innate immune activation and is maximal days after activation. (Abbas *et al.*, 2005.[2])

In contrast to the innate immune system, the adaptive immune system is highly specific and has the ability to recognize and retain memory of specific pathogens.[2] Through the activation of specialised cells called lymphocytes, adaptive immunity mounts a response to infection by adapting and neutralizing the pathogen to prevent further infection. One of the hallmarks of adaptive immunity is that upon secondary exposure to the antigen the immune response mounted will be rapid and stronger, and thus it is also called acquired immunity. The adaptive immune system consists of B lymphocytes and T lymphocytes (Figure 1.1).[2] B lymphocytes are responsible for humoral immunity and respond to circulating antigens or pathogens by secreting specific antibodies to neutralize the infection. B cells can also act as antigen presenting cells (APCs) and promote activation and differentiation of T cells.[13] T lymphocytes are responsible for cell-mediated immunity and act to kill the pathogens that reside in or have infected host cells, and these cells will form the focus of this thesis in the setting of hypertenson.[2] Following an initial exposure to an antigen, both B and T lymphocytes undergo clonal expansion, which involves proliferation of effector B and T cells. Mature B cells secrete antibodies against the specific antigen, whereas helper T cells activate innate immune cells including macrophages to kill phagocytosed microbes. Cytotoxic T cells kill infected cells to eliminate reservoirs of infection. After removal of the infection, activated lymphocytes undergo apoptosis and the immune response subsides and returns to homeostasis. However, importantly a small number of lymphocytes will become memory cells that are responsible for the stronger and more efficient response upon second exposure (Figure 1.2). [2, 14]

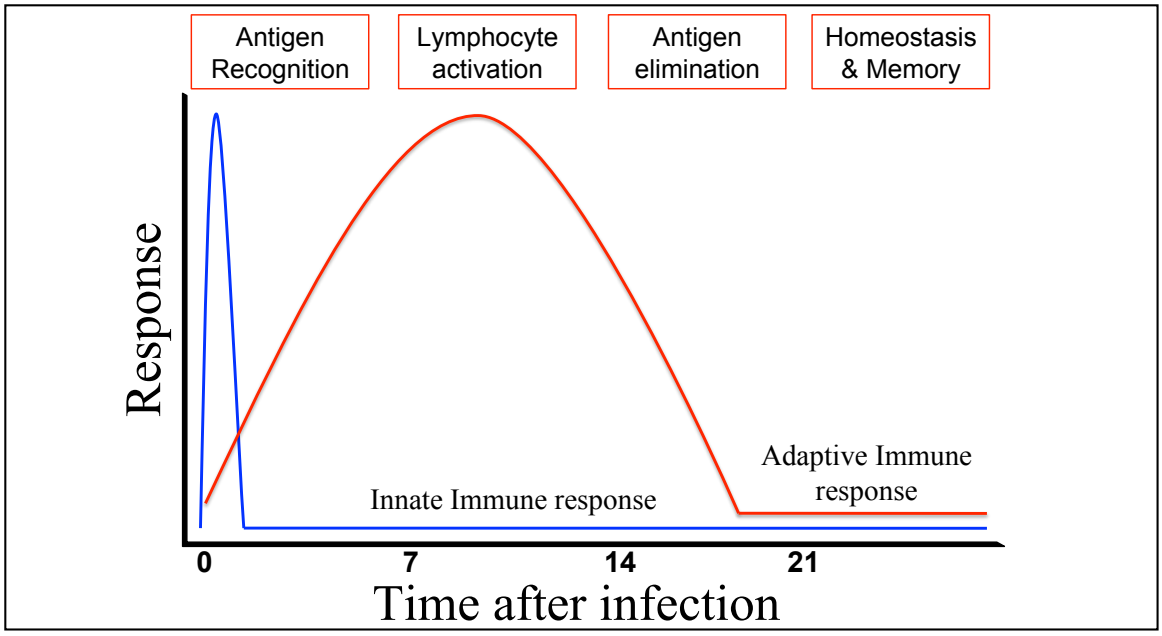

Figure 1.2. Time line of the adaptive immune response. Following exposure to antigens, naive lymphocytes become activated and undergo proliferation to remove the pathogen. After the antigens are eliminated, the majority of the lymphocytes undergo apoptosis and return the body to homeostasis. Only a few memory cells survive and provide lifelong immunity against the pathogen. (Adapted from Abbas *et al.*, 2005.[2])

While the innate and adaptive immune systems are distinct in function, there are significant interactions between the two systems. For example, lymphocytes require innate cells such as dendritic cells to present antigens in order to be activated.[15] Conversely, activated T cells can also promote microbicidal properties of innate cells such as macrophages.[16] Importantly, both innate and adaptive immune responses work in concert to eliminate pathogens and maintain health of the host.[16]

1.3 T Lymphocytes

1.3.1 T Lymphocyte Activation and Subtypes

T lymphocytes can be divided into several subtypes and subsets that all produce various responses to infection and immune homeostasis. The predominant subtypes are T helper (Th) cells ($CD4^+$), cytotoxic T cells ($CD8^+$) and double negative cells (DN; $CD4^-CD8^-$). DN cells lack the expression of the CD4 and CD8 surface proteins and their functional role is not completely understood.[17] T lymphocytes have a restricted specificity for antigens and recognise peptides or antigens that are presented by antigen presenting cells (APCs) such as dendritic cells.[2] This interaction is known as an immunological synapse. Classically, APCs engulfs foreign microbes that are processed into specific epitopes/peptides and presented in the context of MHC molecules (Figure 1.3). Th cells are known to recognise MHC class II molecules, whilst cytotoxic T cells recognise MHC class I molecules. T cells express T cell receptors (TCRs), that recognise antigen/MHC complexes and initiate T cell activation.[18] Immediately following this "antigenic signal", T cells require a second signal known as the "costimulatory signal" to complete activation and maturation into an effector T cell. The best described costimulatory pathway is the B7/CD28-axis which involves binding of B7 ligands, CD80/CD86, on APCs to the CD28 co-receptor on T cells (Figure 1.3).[4] In the absence of a costimulatory signal, T cells will undergo apoptosis or become anergic – a state of inactivity.[2]

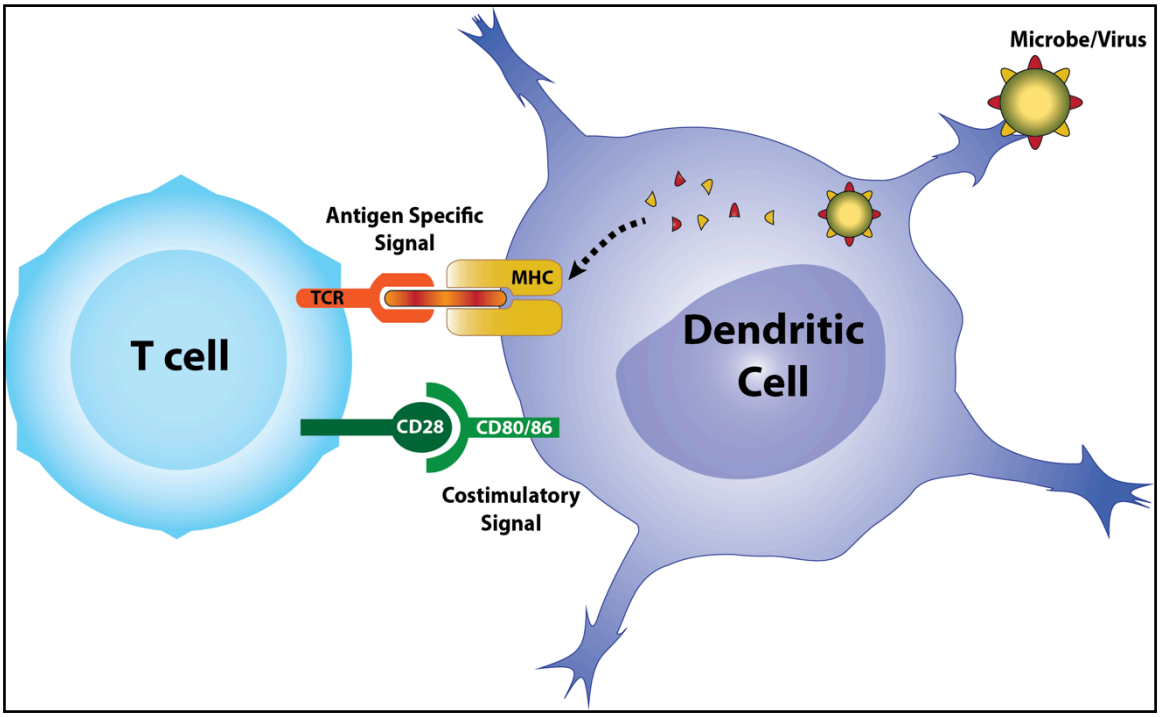

Figure 1.3. Classical T cell activation. APCs such as DCs engulf microbes such as viruses and bacteria. These microbes are digested and processed into peptides that are then presented in the context of a MHC molecule. TCR recognize this complex that together with a costimulatory signal, initiates T cell activation. (Adapted from Abbas *et al.*, 2005;[2] Harrison *et al.*, 2010a[4])

Following T cell activation, cytotoxic T cells release enzymes such as granzymes and perforin to kill the infected cell. In contrast, Th cells can differentiate into various Th subsets to exert effector functions depending on the type of infection/microbe, governed by the cytokines released by APCs during the immunological synapse (Figure 1.4).[19] Upon activation, the predominant function of Th cells is to secrete cytokines to promote inflammation by recruitment of various leukocytes such as monocytes and macrophages, and are the principal mediators of communication in the immune system.[2] Th1 polarisation is favoured by interleukin 12 (IL-12) and is characterised by expression of transcription factors T-bet and signal transducer and activator of transcription 4

(STAT4).[19] Th1 cells are known to secrete interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), which promote inflammation by activating innate cells such as NK cells and macrophages to kill phagocytosed microbes including viruses and bacteria.[20] Th2 polarisation is favoured by release of IL-4 from mast cells and basophils, where the Th2-specific transcription factors STAT6 and GATA binding protein 3 (GATA3) are upregulated.[19] Cytokine secretion from Th2 cells predominantly include IL-4, IL-5, and IL-10 and are known to promote allergic responses and also inflammatory responses to parasites and helminths.[21, 22] Th17 cells are a relatively new T cell subset that produces a pro-inflammatory cytokine known as IL-17, IL-21 and IL-22.[23-25] Th17 cells are regulated by the ROR-γT transcription factor and cytokines including transforming growth factor-β (TGF-β), IL-6 and IL-21 are known to promote Th17 polarisation.[20] To maintain specific T cell polarization to eliminate an antigen, each subset can also inhibit the polarization of naïve T cells to other subsets through the cytokines that they release.[2] For example the Th1 cytokine IFN-γ inhibits Th2 proliferation by directly down regulating GATA3 expression.[26] Another specialised Th cell is the T regulatory (Treg) cell whose predominant function is to regulate the activation of T cells and maintain tolerance to self-antigens.[2] Treg cells are developed under the influence of TGF-β and IL-2 with specific expression of forkhead box P3 (FoxP3) and STAT6. Tregs suppress the immune system by releasing anti-inflammatory cytokines such as TGF-β, IL-10 and IL-35.[19 22]

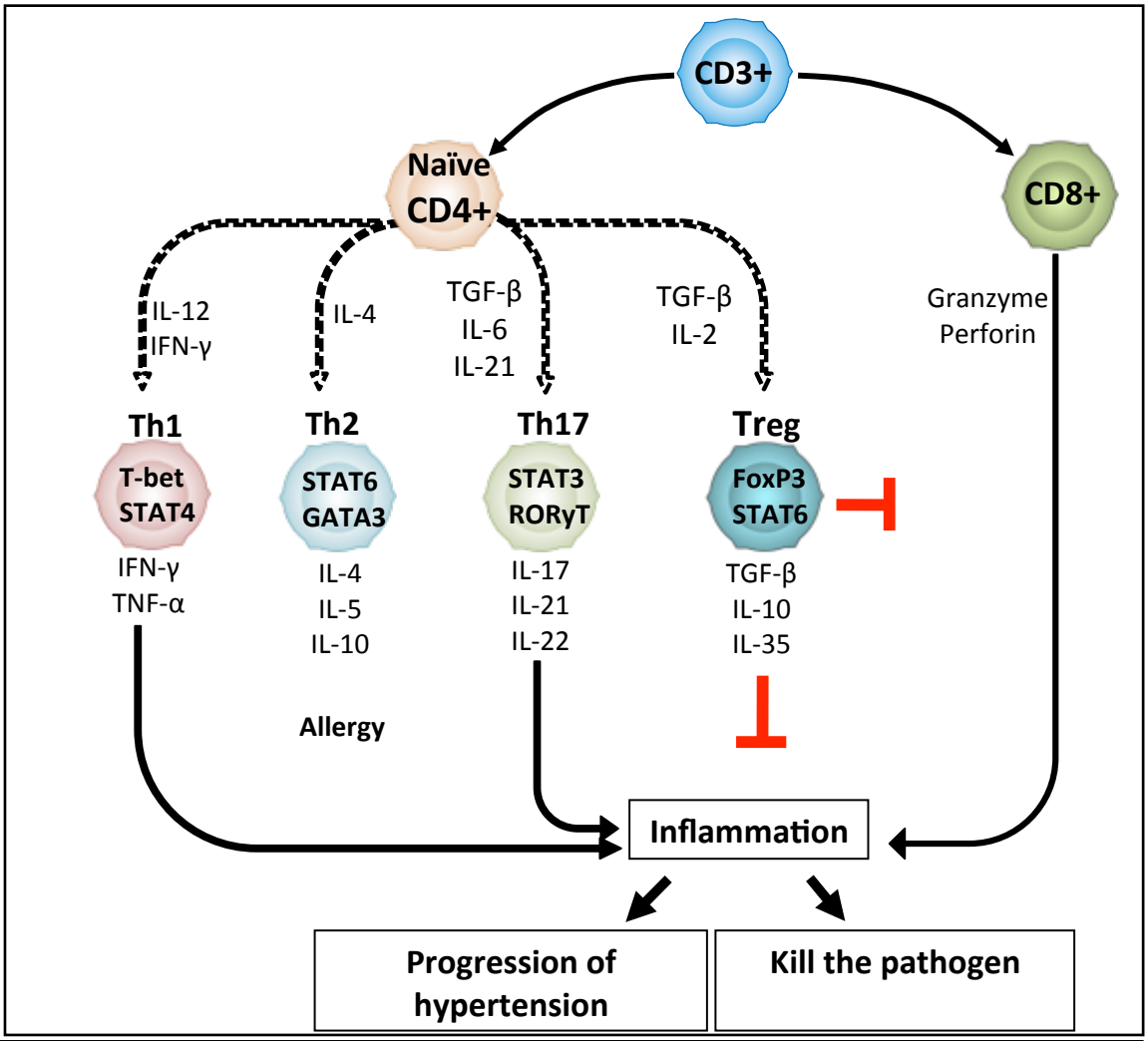

Figure 1.4. T cell subtypes/subsets and their role in the adaptive immune response. Following antigen presentation CD4+ and CD8+ T cells become effector T cells. CD4+ Th cells can polarise to Th1, Th2, Th17 and Treg cells in response to the cytokine environment. These cells secrete pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-17 and IL-4, which cause inflammation and signal innate cells to kill the pathogen. Cytotoxic T cells act to kill the pathogens by releasing cytotoxic enzymes. Treg cells secrete cytokines IL-10 and TGF-β that regulate immune responses. An imbalance in either Th polarisation can promote inflammatory responses associated with diseases including hypertension (Adapted from Abbas *et al.*, 2005[2])

1.3.2 T cell phenotypes and Inflammatory Disorders

The functional role of T cells in inflammatory diseases has been studied for decades, especially in autoimmune diseases such as rheumatoid arthritis[27] and systematic lupus erythematosus (SLE).[28] Rheumatoid arthritis is associated with elevated Th1 cytokines especially TNF-α[29], whereas SLE is more likely a Th2 driven disease but also presents with abnormal expression of Th1 cytokines IFN-γ and TNF-α.[30] Therefore, there is much to be learnt on the role of T cells in hypertension from other related disciplines.

Rheumatoid arthritis is known to be associated with infiltrating Th cells into synovial joints. As discussed above, Th cells are a large source of cytokines that can promote local inflammation as well as recruitment of other immune cells. In rheumatoid arthritis there are elevated pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6, IL-1β, Granulocyte-macrophage colony-stimulating factor (GM-CSF) [29] and chemokines such as Chemokine C-C motif ligand 13 (CCL13),[31] where TNF-α has been documented to be of major importance in the pathogenesis of rheumatoid arthritis. However, there is also upregulation of homeostatic regulatory cytokines, IL-10 and TGF-β, which can suppress the pro-inflammatory response.[27] Importantly, research into T cells in rheumatoid arthritis has led to novel treatments to treat this disease such as the TNF-α inhibitor, etanercept [32] and IL-6 antagonist tocilizumab [33] which inhibit or neutralize TNF-α and IL-6 respectively. Moreover, in the synovial fluid from rheumatoid arthritis patients, an elevated proportion of IFN-γ production from CD4+ and CD8+ T cells has been reported.[34] Studies have shown that T cells in atherosclerosis also have elevated activation,[35, 36] and Th1 cells that release IFN-γ and TNF-α have been suggested to play a role in promoting atherosclerosis.[37] Similarly in SLE, there are elevated production of the

cytokines such as IL-17, IL-4 and IL-10.[32] Since SLE is a multi-systemic autoimmune disease in which many immune cell subsets play a significant role in the disease pathogenesis, by identifying the predominant cytokines produced in SLE, cytokine-based therapies like those mentioned above, have been developed to treat SLE.[32]

With respect to the current study, research on T cells in hypertension has only recently begun to expand and much of the knowledge gaps on the functional roles of T cells during hypertension are yet to be identified. As with many inflammatory diseases, identification of the contribution of T cells to the development of hypertension may also lead to the discovery of targeted therapeutic treatment approaches to treat inflammation associated with hypertension

1.4 Evidence Linking Immunity, T cells, CNS and Hypertension

1.4.1 Innate immunity

The cells of the innate immune system are known to contribute to the development of hypertension. It has been demonstrated that Ang II-induced hypertension is associated with an increase in vascular inflammation with elevated monocyte and macrophage accumulation.[38, 39] Moore et al suggested that Ly6C^{hi} monocyte and macrophage accumulation in the aorta – particularly of the M2 resident macrophage phenotype - plays an important role in Ang II-induced hypertension. Inhibition of macrophage accumulation with a CCR2 antagonist, INCB3344 prevented Ang II-induced vessel fibrosis and blunted hypertensive response.[40] Chan et al also have shown that INCB3344 reduced the aortic expression of CCR2 mRNA and macrophage infiltration in DOCA salt-induced hypertension, and was associated with blunted Ang II-induced pressor responses.[41] It was

suggested that homozygous osteoporotic mice (Op/Op), which are deficient in macrophage colony-stimulating factor (m-CSF) are protected from hypertension and endothelial dysfunction in mesenteric arteries in response to Ang II-infusion. M-CSF functions as a chemotactic factor for monocytes, regulates effector functions of mature monocytes and macrophages, and modulates inflammatory responses by stimulating the production of other cytokines, adhesion molecules, and growth factors.[42] Op/Op mice showed reduced vascular remodeling and vascular cell adhesion molecule (VCAM)-1 expression, which suggested a critical role of macrophages, monocytes and proinflammatory mediators in Ang II-induced hypertenson.[39] Interestingly, adoptive transfer of monocytes into these Op/Op mice restored hypertensive responses to Ang II, thereby implicating that cells of the monocyte lineage play an important role in hypertension.[39] There is also some clinical evidence associating innate immunity and hypertension. Neutrophil to lymphocyte ratios are increased in patients with hypertension, which suggests the involvement of innate immunity to the development of human hypertension.[43, 44]

1.4.2 Adaptive immunity

There are an expanding number of recent studies examining T cells and hypertension. However, research into the role of T cells in hypertension first began in the 1960-70's where mice that received a thymectomy exhibited blunted hypertensive responses.[45] Okuda and colleagues demonstrated that immunosuppression attenuates hypertension in rats with partial renal infarction.[46] Moreover, passive transfer of lymph node cells from rats with renal infarction induces hypertension in healthy rats.[46, 47] Since T cells mature in thymus and can reside in lymph nodes, these studies suggested that T cells might play an

important role in hypertension. Although a considerable amount of studies in the 1960-70s suggested that immune cells contribute in hypertension, there is a lack of knowledge on the mechanisms of immune cells that contributes to the pathogenesis of hypertension. Further research in this field stalled, possibly attributed to lack of understanding of immune system and limited technologies of that era for further investigation.

In 2007, Guzik et al published a seminal paper implicating T cells in hypertension that reignited this field of research. This paper has been cited 840 times since October 2007, which highlights the impact and novelty in hypertension research. Guzik and colleagues showed that recombinase activating gene-1 deficient (RAG-$1^{-/-}$) mice, which lack T and B cells, exhibit a blunted hypertensive response to angiotensin (Ang) II- or DOCA (deoxycorticosterone acetate) salt-induced hypertension.[10] Importantly, Guzik et al demonstrated that upon adoptive transfer of T but not B cells into RAG-$1^{-/-}$ mice, hypertension was restored. Consistent with this finding, Mattson and colleagues demonstrated that RAG-$1^{-/-}$ Dahl salt sensitive rats also exhibited blunted development of hypertension.[48] Furthermore, Crowley et al showed blunted hypertensive responses to Ang II infusion in severe combined immunodeficiency mice (SCID; mice lacking in T and B lymphocytes).[49] Interestingly, in human immunodeficiency virus (HIV)-infected patients that have reduced CD4+ T cells, the incidence of hypertension in reduced.[50-52] A cohort study conducted by Seaberg and colleagues (2005) suggested that highly active antiretroviral therapy (HAART) normalised the prevalence of hypertension in HIV patients to the level of uninfected patients.[53]

Adoptive transfer of T cells in RAG-1$^{-/-}$ mice also lead to impaired endothelium-dependent vasodilation and increased vascular reactive oxygen species (ROS) production that is associated with Ang II infusion, which was absent in RAG-1$^{-/-}$ mice without adoptive transfer.[10] In wild type mice, Ang II-induced hypertension was associated with an increase in the proportion of circulating T cells bearing surface markers CD69, CD44^{high} and CCR5, which represent an activated or effector T cell phenotype.[10] Particularly relevant to this thesis, Ang II-induced hypertension was also shown to increase infiltration of leukocytes, which were predominantly T cells, into perivascular adipose tissue (PVAT) surrounding the aorta. Others have shown that hypertension is associated with increased infiltration of T cells into the kidney.[54, 55] Prevention of T cell infiltration into the kidney has also been reported to lower blood pressure.[56, 57] Using a humanised mouse model where human T cells were injected into immunodeficient recipient mice, human T cell infiltration in aorta and kidney was elevated following Ang II-infusion, with increased circulating IL-17A and IFN-γ producing CD4+ and CD8+ cells.[58] This study highlighted that T cell infiltration associated with hypertension is not limited to mouse T cells, and human T cells also respond in the similar fashion.

Although adoptive transfer of B cells into RAG-1$^{-/-}$ mice did not restore hypertension in the Guzik study,[10] a possible role for B cells was not ruled out. Coventionally, B cells require T cells to be activated and in the Guzik study, adoptive transfer of B cells was performed without any T cells. Therefore, a role for B cells in hypertension may have been masked due to the approach. In fact, B cells may contribute via multiple mechanisms including antibody secretion[59] and the presentation of antigens to T cells.[60] Chan et al showed that Ang II-induced hypertension is associated with increased splenic

B cell activation as well as circulating IgG antibodies, which was not seen in B-cell-activating factor receptor-deficient (BAFF-$R^{-/-}$) mice that lack mature B cells.[59] Furthermore, hypertension was abolished in BAFF-$R^{-/-}$ mice and mice with pharmacological deletion of B cell using anti-CD20 antibody.[59]

1.4.3 Central control of Immune system during hypertension

Hypertension is associated with peripheral inflammation that may directly influence renal and vascular function. Nevertheless, central control of immunity in hypertension also appears to play an important role. The brain and central nervous system (CNS) is essential to processing neurohumoral signals from the periphery in order to maintain blood pressure and fluid homeostasis. The brain and immune system are the two major systems of the body and cross talk between the two systems is important in to maintaining homeostasis of immune system.[61, 62] The sympathetic nervous system (SNS) and hypothalamic pituitary axis (HPA) are two pathways that are important in central regulation of immune response and blood pressure. Primary (thymus and bone marrow) and secondary (spleen and lymph nodes) lymphoid organs are well innervated by the sympathetic nervous system (SNS) and many immune cells including T cells, express receptors for neurohormones such as noradrenaline and Ang II.[63, 64] Blood pressure controlling regions of the brain such as the circumventricular organs (CVO) have recently been shown to regulate peripheral T cell activity. Intracerebroventricular (ICV) administration of Ang II enhanced sympathetic nerve outflow and peripheral cytokine production in spleen.[65] Lob et al (2010) found that deletion of extracellular superoxide dismutase (SOD3) increased ROS production in neurons of the blood pressure controlling regions of the CVO and promoted sympathetic outflow, which exacerbated responses to

non-pressor-doses of Ang II.[66] Importantly, this manipulation of the central nervous system, elevated the proportion of circulating activated T cells as well as vascular T cell infiltration associated with Ang II infusion.[66] Marvar and colleagues identified that electronic lesioning of the anteroventral third ventricle (AV3V) region of the CVO in mice abolished peripheral T cell activation and hypertension in response to Ang II, which suggested that central signals are paramount for T cell activation and vascular inflammation associated with Ang II-induced hypertension.[65, 67] However, the enhanced vascular inflammation and T cell activation during hypertension may also contribute the elevation in blood pressure. Interestingly, Marvar and colleagues suggested that activation of T cells is sensitive to rises in blood pressure alone, as infusion of noradrenaline in AV3V lesioned mice is still able to induce a pressor response and increased peripheral T cell activity, which is reversed in mice co-treated with the anti-hypertensive drug hydralazine.[67] Recently, it was demonstrated that stress-dependent hypertension also triggers T cell activation and promotes T cell-mediated inflammatory responses, which may exacerbate experimental hypertension.[68]

The immune system can also regulate the CNS, where periphery inflammatory cytokine production provides feedback to the brain.[69-71] A study by Winklewski et al suggested a feed forward mechanism induced by peripheral chronic inflammation that leads to brain inflammation, increased SNS activity and ultimately neurogenic hypertension.[72]

1.4.4 Regulation and pharmacological inhibition of immune system during hypertension

There is now evidence that Treg cells play a regulatory role in the development of

hypertension and may represent a novel target to treat high blood pressure. The predominant function of Treg cells is to regulate the activation of T cells and maintain tolerance to self-antigens.[2] These cells suppress T cell mediated immunity by production of IL-10 and transforming growth factor-β (TGF-β).[22] *Scurfy* mice, which lack Treg cells, suffer from multi-organ lymphoproliferative diseases and do not survive past 16-25 days after birth.[73] Barhoumi and colleagues showed that Ang II-infusion caused a 43% reduction in renal expression of the transcription factor for Treg cells, FoxP3.[74] They also showed that Treg cell number was unchanged in the aorta but decreased in the kidney in Ang II-treated hypertensive mice when compared to normotensive controls. Moreover, splenic Treg cells were reduced in Ang II-induced hypertensive mice, which was associated with induction of Treg cell apoptosis.[75] Importantly, adoptive transfer of Treg cells into Ang II-induced hypertensive mice significantly inhibited the development of hypertension, which was associated with a reduction in Ang II-induced elevations in plasma inflammatory cytokine levels and oxidative stress.[74] Evidently, impaired regulation of the immune system by Treg cells and IL-10 correlates with several forms of experimental hypertension, which may account for the heightened adaptive immunity related to the development of high blood pressure.

Immunoregulatory drugs have been suggested to have anti-hypertensive effects and prevent end stage organ failure associated with hypertension. One of the earliest studies investigating the link between immunity and hypertension demonstrated that the immunosuppressant, cyclophosphamide, blunted hypertension in SHRs.[76] Rodríguez-Iturbe et al reported that mycophenolate mofetil (MMF), an immunosuppressant used to prevent transplanted organ rejection, also induced anti-hypertensive effects in SHRs.[57]

Furthermore, in a rat model of neurogenic hypertension, ICV infusion of an anti-inflammatory antibiotic, minocycline also significantly blunted experimental hypertension.[77]

1.4.5 Antigen presentation and T cell activation during hypertension

As mentioned above, T cells are activated in response to presentation of foreign or 'non-self' antigens via dynamic interactions with APCs.[2,78] Vinh and colleagues (2010) reported that T cells were activated classically involving antigen presentation and costimulation during hypertension.[79] This finding suggested that various hypertensive stimuli might prompt the formation of self-antigens or neoantigens (acquired antigen) that may be specific and activate T cells during hypertension, which can then be presented by APCs to evoke T cell activation.[79] Blockade of T cell costimulation with Cytotoxic T-Lymphocyte Antigen 4 – Antibody (CTLA4-Ig), a current therapy used to treat inflammation in rheumatoid arthritis, blunted Ang II- and DOCA salt-induced hypertension.[79] Importantly, an analogue of CTLA4-Ig was recently shown to reduce systolic blood pressure (SBP) in humans following renal transplant, [80] thus supporting the potential translation of immunosuppressive agents to control blood pressure. Past studies have implicated unknown arterial antigens in hypertension that may be presented by APCs to T cells resulting in classical activation of the adaptive immune system,[81] however, the hypertension-specific neoantigen(s) have yet to be identified. [82] Recently, Harrison's group identified isoketal-modified proteins, which may represent some of the neoantigens presented by DCs to activate T cells and promote hypertension. Scavenging of isoketals using 2-hydroxybenzylamine (2-HOBA) abolished experimental hypertension.[83]

1.4.6 T cell recruitment during hypertension

T cells and immune cells accumulate in the kidney and vasculatures during experimental hypertension that likely contribute to the end-organ damage.[3, 10] As chemotactic proteins that direct the migration of various leukocytes from the blood into sites of infection and inflammation,[84, 85] chemokines have been implicated as trafficking signals in recruiting T cells into target organs.[86, 87] T cells and other leukocytes are known to express greater chemokine receptors during hypertension.[10, 41] CCL5, formerly known as regulated-on-activation, normal-T-expressed-and-secreted (RANTES) has been shown to be an important chemokine during hypertension, binding to C-C chemokine receptor type 5 (CCR5). CCR5 that is expressed on T cells, macrophages and DCs binds to CCL3, CCL4 and CCL5,[88, 89] chemotactic proteins that are known to promote leukocyte and T cell recruitment. During Ang II-induced hypertension, increased T cell infiltration correlates with elevated mRNA expression of CCL5, [10] and elevated CCL5 is associated with vascular dysfunction.[90] Guzik and his colleagues have shown elevated expression of CCR5 on T cells during Ang II-induced hypertension,[10] particularly in the aortic PVAT.[90] Recently, it was shown that mice with genetic deletion or pharmacologically inhibition of CCR5 using Methiolynated-RANTES (Met-RANTES) are protected from vascular leukocyte and T cell infiltration, as well as ROS production and endothelial dysfunction associated with Ang II induced hypertension.[90] While antagonism of CCR5 using Met-RANTES abolished infiltration of T cells and monocytes, and subsequently reduced the progression of atherosclerosis in mice,[91] surprisingly CCR5-deficiency or inhibition did not evoke reductions in blood pressure or prevent renal or cardiac end organ damage.[92]

C-C chemokine ligand type 2 (CCL2) is another vital chemokine that promotes leukocyte recruitment, and elevated tissue CCL2 production is associated with enhanced local inflammatory response. T cells are a known source of CCL2,[84, 93] although macrophages and other somatic cells such endothelial cells are also important sources of CCL2.[94] Leukocytes including T cells are known to express C-C chemokine receptor type 2 (CCR2), and elevated CCL2 expression in the local tissues leads to recruitment of CCR2-expressing leukocytes to further promote the inflammatory response. Previous studies have shown that inhibition of CCR2 reduces macrophage infiltration, which can attenuate inflammatory diseases such as crescentic glomerulonephritis.[95] Bush et al reported increased expression of CCL2 in inflammatory diseases including untreated essential hypertension and Ang II-induced hypertension.[96] Vascular macrophage infiltration in Ang II-treated CCR2 knockout mice is also reduced, [96, 97] indicating the importance of CCR2 in recruitment of macrophages during hypertension. More recently, CCR2 inhibition has been shown to reduce blood pressure in Ang II- and DOCA-salt induced hypertensive mice.[46-41] Moreover, leukocyte infiltration was significantly inhibited, which underscores the importance of the CCL2-CCR2 axis in vascular inflammation associated with hypertension.

1.5 Functional Roles of Tissue-Infiltrating T cells during Hypertension

The precise role of the immune system in the development of hypertension remains unclear, however, it is clear that hypertension is associated with accumulation T cells and lymphocytes in blood pressure controlling organs such as the vasculature, kidney and brain.[4, 98] The functional role of these tissue-infiltrating T cells constitutes a major

unanswered question that will be addressed within this thesis. There are several prominent mechanisms that are highly relevant to hypertension and local inflammation, which include pro-inflammatory mediator release such as cytokine and ROS production.

1.5.1 T cell – derived inflammatory cytokines

A likely mechanism proposed in T cell-induced hypertension is through the release of cytokines, which promote inflammation in various blood pressure-controlling organs.[3] Studies from the Harrison group reported that there are more Th1 cells in hypertensive mice, shown by an increased systemic levels of Th1 specific cytokines IFN-γ and TNF-α.[10] Vinh et al (2010) also showed that lymph node T cells isolated from hypertensive mice exhibit increased Th1 cytokine production compared with normotensive mice.[79] Shao and colleagues reported findings in SHRs that were consistent with increased levels of Th1 cytokines.[99] Madhur et al showed that Ang II-infused hypertensive mice have increased IL-17 production, and mice deficient in IL-17 exhibited a blunted hypertensive response.[100] Human data also showed increased circulating IL-17 level in hypertensive type-2 diabetic patients, suggesting the Th17 cells may be important in hypertension.[101] More recently, Saleh et al have demonstrated that IL-17A and IFN-γ played an important role in Ang II-induced hypertension and mice lacking IFN-γ or IL-17A exhibited blunted pressor responses to hypertensive stimuli.[102] However, inhibition of Th1 cytokines using agents such as the TNF-α antagonist, etanercept, to treat experimental hypertension has been equivocal, suggesting that cytokine production by itself may not be the most important and/or a hypertension-specific mechanistic role for T cells during hypertension.[10, 103-105] Collectively, T cell-derived cytokines contribute to hypertension. However, past studies have focused on circulating or splenic T cells in relation to

hypertension,[3, 4] but have not studied the role of tissue-infiltrating T cells that represent local effects of T cells and inflammation, which was examined in this thesis.

1.5.2 PVAT and Hypertension

Perivascular adipose tissue (PVAT) adjacent to adventitia of the aorta is a special compartment that regulates homeostasis function of aorta by releasing adipokines and other mediators.[106-109] It has been shown that PVAT promotes vasodilatation in healthy rat aorta and porcine coronary artery,[107, 108] and inhibits aortic contractions by releasing adipocyte-derived relaxing factor (ADRF).[107] Interestingly, it was suggested that surrounding microenvironment could influence the phenotype of adipocyte.[110] During obesity, release of pro-inflammatory cytokines such as TNF-α and IL-6 is elevated in PVAT, which suggest a unique adipose depot that is prone to inflammation,[111] and heightened inflammation is known to impair ADRF-induced anti-contractile effects on the vasculature.[112]

There is increasing evidence suggesting the crucial role of PVAT inflammation, vessel remodelling and vascular stiffness during disease states including hypertension.[109, 113-115, 116]. The specific mechanism of PVAT in promoting vascular dysfunction and hypertension is not fully understood, but studies have suggested that PVAT are known to be a reservoir for immune cell residence.[48, 117] There is strong evidence showing elevated accumulation of immune cells and CCR5+ T cells in PVAT during hypertension.[3, 10, 48, 117] Therefore, T cells and other immune cell infiltrates are likely an important link between PVAT inflammation and vascular dysfunction during hypertension.

1.5.3 ROS and Hypertension and T cell-derived ROS

Reactive oxygen species (ROS), such as superoxide, play an important role in physiologic and cellular processes. It is known that elevated ROS induces greater oxidative stress, which promotes inflammation and organ dysfunction. ROS formation influences the mechanisms involved in innate immunity, cell and tissue growth, cell signaling and apoptosis.[118] Several studies have showed that elevated oxidative stress and ROS act as contributors to the progression of cardiovascular disease, such as hypertension.[119, 120] Moreover, ROS may play a role in T cell activation function since increased brain and vascular ROS enhanced peripheral T cell activation and subsequently lead to hypertension.[121, 122] As reviewed elsewhere, elevated ROS promote hypertension and impairs vascular function by interference with NO or endothelium-dependent vasodilatation.[123, 124] Furthermore, ROS was suggested to activate the NF-kappaB pathway and promote release of inflammatory cytokines in the adventitia,[125-127] and act as chemotactic signal for greater chemokines release[90, 98] that ultimately promote immune cell accumulation and local inflammation.

T cells express NADPH oxidase 2 (Nox2), which is a major source of T cell-derived ROS production in blood pressure-controlling organs during cardiovascular disease (CVD) progression.[119, 128] There are several different subunits of NADPH oxidase, including Nox1, Nox2 and Nox4. Nox2 is known as the prototypical phagocytic NOX and is expressed in T cells, granulocytes, neutrophils and macrophages, and plays a fundamental role in host defence and innate immunity.[119] Although the ROS produced from T cells is much less than other immune cells such as macrophages and neutrophils. It was suggested that stimulation of T cells could induce rapid generation of ROS, which could contribute

to local oxidative stress, proliferation and differentiation of T cells.[129] T cell-derived ROS has been shown to be important to the development of hypertension.[10] Guzik and his colleagues have shown that adoptive transfer of T cells from mice deficient in p47phox (an important subunit of Nox2 and Nox1 oxidase) into RAG-$1^{-/-}$ mice only partially restored hypertensive responses to Ang II.[10] This observation highlighted the role of T cell-derived ROS in hypertension, and that T cells may be a local source of ROS following T cell infiltration in blood pressure-controlling organs.[10]

In support of this notion, the PVAT is also a large source of ROS that can interfere with nitric oxide (NO)-dependent relaxation in the vasculature.[130] Rey and colleagues reported that ROS directly impairs endothelium-derived NO-dependent vasorelaxation.[131] It was suggested that ROS in the adventitial layer might be dismutated to H_2O_2 that can act as a paracrine signalling agent to induce endothelial dysfunction.[132] [127] Furthermore, adventitial ROS was suggested to activate cyclooxygenase to enhance prostaglandin-induced vasoconstriction as well as stimulate VSMC to contract.[133] While a strong causal link between adventitial ROS and vascular dysfunction exists, T cells may represent the pathophysiological source of ROS that is amplified with abundance of infiltrating T cells in the PVAT during hypertension. The capacity of infiltrating T cells to produce ROS in the setting of hypertension will be interrogated in this thesis.

1.6 Conclusion and aims of this Thesis

Collectively, there is much evidence suggesting that T cells play a crucial role in the development of hypertension. The current immunological basis for hypertension that was postulated by the Harrison group suggests that increases in blood pressure lead to the

development of neoantigens that can be presented to T cells leading to their activation. Activated effector T cells egress sublymphoid organs and are recruited to blood pressure controlling organs via chemokines such as CCL5. Excessive T cell recruitment into the blood pressure controlling organs is then thought to promote local inflammation and end-organ dysfunction (Figure 1.5). However, a major knowledge gap that remains unclear is their specific local contribution to end-organ dysfunction. A functional role that directly promotes inflammation an/or hypertension remains largely unknown.

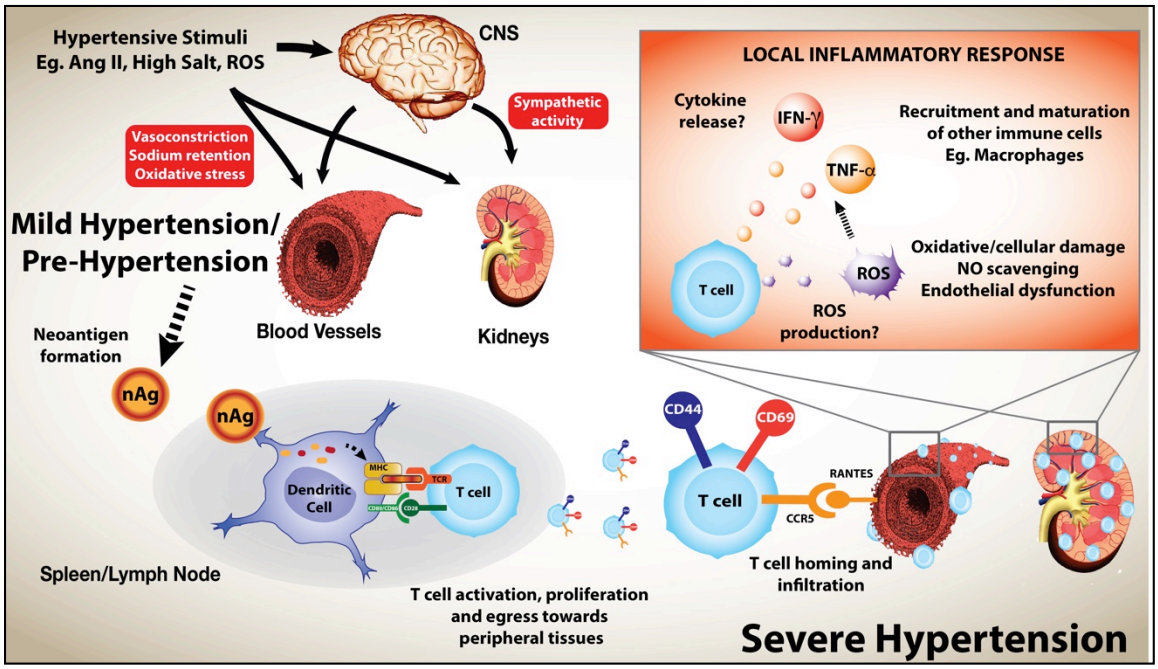

Figure 1.5. Current immunological basis for hypertension. Hypertensive stimuli promotes mild levels of hypertension that can induce formation of neoantigens that are processed by APCs such dendritic cells. Presentation of these unique epitopes to T cells in sublymphoid organs leads to T cell activation and egress. Activated T cells infiltrate in the vasculature and kidney and are hypothesised to release inflammatory cytokines or ROS to promote end-organ dysfunction and subsequently exacerbate hypertension. (Adapted from Harrison et al., 2010[3])

The aims of this thesis are to provide an extensive investigation of the functional roles of tissue-infiltrating T cells during hypertension. This thesis will interrogate whether

infiltrating T cell exhibit differential phenotypes in the setting of hypertension by directly measuring T cell-derived cytokines, ROS and chemokine profiles of infiltrating T cells (Chapter 3). Additionally, the influence of infiltrating T cells on vascular function will be investigated using in situ T cell activation paired with vascular reactivity studies (Chapter 4). Finally, since it was suggested that increases in blood pressure induces formation of neo-antigens that are processed by antigen presenting cells and presented to T cells for activation, Chapter 5 of this thesis specifically examines whether APCs directly present antigens to infiltrating T cells in explanted aorta using live cell imaging - a world first, which may allow us to identify whether or not there are unique hypertension-induced antigens within the vasculature.

Specifically, the aims of my thesis are:

Aim 1

To investigate the differential phenotypes of tissue-infiltrating T cells during Ang II-induced hypertension

Aim 2

To investigate the effect of *in situ* T cell stimulation on the aortic vascular reactivity during Ang II-induced hypertension

Aim 3

a. To develop an ex vivo explanted aorta assay to visualize live T cell and APC interactions in real time
b. To examine the effect of hypertension on T cell – APC interaction in vasculature
c. To examine cross-reactivity of hypertensive T cells within different models of hypertension

Chapter 2:

General Methods

2.1 Ethics Approval

All experiments were approved by Monash University Animal Ethics Committee (Ethics approval numbers: MARP/2011/085, MARP/2012/054, MARP/2013/059) and conducted in accordance with the National Health and Research Council Australia guidelines for the care and use of animals in research.

2.2 Total Animals Studied

A total number of 384 were used, consisting of 141 C57BL6/J mice (male, 8-12 weeks) used in chapter 3, 93 C57BL6/J mice in chapter 4, and 73 C57BL6/J mice and 77 CD11c-YFP+ transgenic mice ($CD11c^+$ cells express yellow fluorescent protein, male, 8-12 weeks) in chapter 5.

2.3 Mouse Models of Hypertension

Mice were obtained from Monash Animal Services (MAS) and housed on a 12-hour light/dark cycle with food and water provided *ad libitum*. For Ang II-induced hypertension, mice were anaesthetised with inhaled-isoflurane. A small incision into the subscapular space was made to insert an osmotic minipump (Alzet model 2002) containing either vehicle (0.15M NaCl, 1% acetic acid) or Ang II (0.7 mg/kg/day; Bachem, Switzerland) for 14 days. Deoxycorticosterone acetate-salt (DOCA-salt) hypertension was induced as previously described.[79] In brief, left kidney uninephrectomy was performed on inhaled-isoflurane anaesthetized mice following ligation of the proximal and distal renal arteries using 6-0 Dysilk sutures. A small incision was also made at the subscapular space and a DOCA (2.4 mg/day; Innovative research of America,

USA) or sham pellet was inserted. Drinking water was also replaced with saline (0.9% NaCl) during the 3-week treatment period in the DOCA-treated mice.

2.4 Blood Pressure Monitoring

Systolic blood pressures (SBP) were measured using a non-invasive tail-cuff apparatus (MC4000 Blood Pressure Analysis System, Hatteras Instruments) prior to treatment and on day 14. For DOCA-salt mice, blood pressures were measured at days 7, 14 and 21. For each time point the mean systolic blood pressures for ≥20 measurements were recorded and analysed.

2.5 Tissue Harvesting

At the end of each treatment period, mice were euthanized by carbon dioxide asphyxiation. This procedure was approved by the Animal Ethics Committee of Monash University. The thoracic cavity was exposed to allow access to the heart and 0.05 ml clexane (400 U/ml) was injected into the left ventricle. Blood was withdrawn via cardiac puncture from the right ventricle using a 1 mL syringe containing 0.05 ml of clexane (400 U/mL). Mice were perfused retrogradely via abdominal aorta using a perfusion pump (MasterFlex L/S Easy-LOAD II, Model 77200-62) with phosphate buffer solution (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4) for five minutes. Relevant organs for each study including the spleen, kidneys, brain and aorta with PVAT-intact were removed and maintained on ice in either PBS or cold Krebs-bicarbonate solution (NaCl 118mM, KCl 4.7mM, KH_2PO_4 1.2mM, $MgSO_4.7H_2O$ 1.2mM, $CaCl_2$ 2.5mM, $NaHCO_3$ 25mM and glucose 11.7mM; pH 7.4).

2.6 Sample Preparation for flow cytometry and cytokine and ROS measurements

2.6.1 Blood Processing

Whole blood was mixed with 10 ml of red blood cell (RBC) lysis buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 6.0mM EDTA) for 5 minutes at room temperature. The sample was then washed with 40 mL PBS and centrifuged at 1200 RPM for 10 minutes at 4^{o}C. Following centrifugation the supernatant was discarded and the lysis step was repeated. The sample then resuspended with 5 ml of PBS and the number of viable cells was counted using a Countess Automated Cell Counter (Invitrogen, model C10281) via trypan blue exclusion 0.4% (Invitrogen).

2.6.2 Spleen Processing

Single cell suspensions of spleens were prepared by mechanical dissociation of the spleen in 1 ml of PBS for 4 minutes and then passed through a 70μm filter. Samples were washed with 25 ml PBS and centrifuged at 1200 RPM for 10 minutes at 4^{o}C. Following centrifugation supernatants were discarded, and samples then resuspended in 30 ml of PBS and counted as described for the blood.

2.6.3 Aorta, Kidney and Brain Processing

Aortic and kidney samples were mechanically dissociated in 1 ml digestion buffer containing collagenase type XI (125 U/mL), hyaluronidase (600 U/mL) and collagenase type I-S (450 U/mL) in $Ca2^{+}/Mg^{2+}$-supplemented PBS (Sigma, St Louis, MO, USA) for 4 minutes. Samples were then transferred to a 24-well plate and incubated at 37^{o}C for 45 minutes and agitated every 10 minutes. Following digestion, samples were passed through a 70μm filter and centrifuged at 1200 RPM for 10 minutes. Aortic samples were resuspended in 90 μL of MACs buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2mM EDTA). Kidney and brain samples were further subjected to percoll density-gradient centrifugation where samples were resuspended in 2 mL of 40% (kidney) or 30% (brain) isotonic percoll solution (GE Healthcare Life Science), and then gently underlaid with 2 mL of 60% (kidney) or 70% (brain) percoll solution respectively (Figure 2.1). Samples were then centrifuged at 2700 RPM for 20 minutes at room temperature with the centrifuge brake off. Mononuclear cells (MNCs) at the interface of the percoll solutions were collected and washed by centrifugation in MACs buffer.

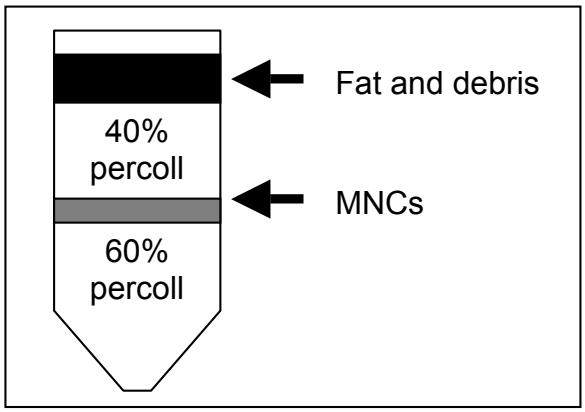

Figure 2.1. Representative figure of kidney sample following centrifugation through a percoll gradient.

2.7 T cell Enrichment

To isolate T cells from single cell suspensions (Figure 2.2), cell pellets were resuspended in 90 µL of MACs buffer mixed with 10 µL of anti-CD90.2 magnetic microbeads (Miltenyi Biotec) per 10^7 total cells (CD90.2 is a general T cell marker). The samples were incubated at 4°C for 15 minutes and then washed with 2 mL of MACs buffer and

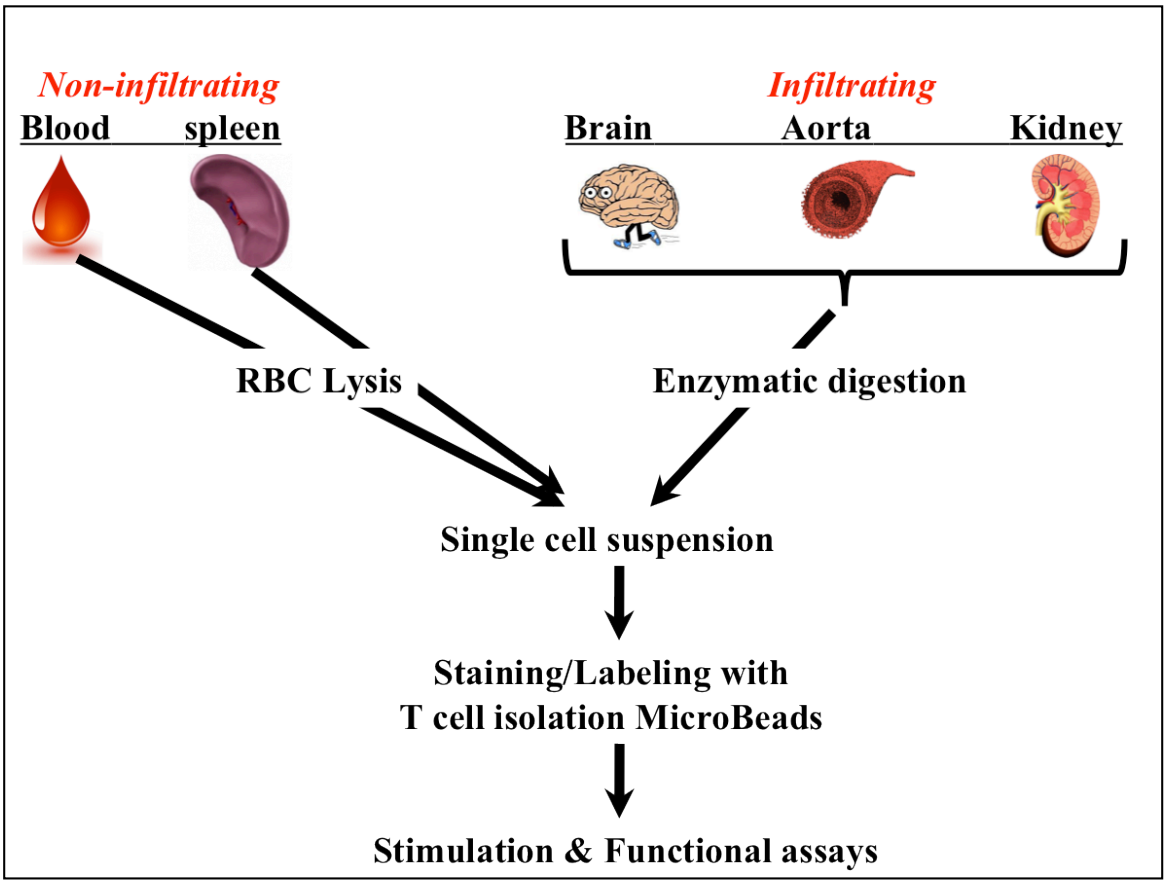

Figure 2.2. Flow chart of sample preparation T cell enrichment and/or functional assays. At the end of treatment, blood, spleen, brain, aorta and kidneys were harvested. Blood and spleen were lysed with RBC lysis buffer, whereas brain, kidneys and aorta were enzymatically digested. All samples were processed in single cell suspensions that were then stained with anti-CD90.2 microBeads to isolate T cells for further stimulation and detection of T cell-derived cytokines, chemokines and ROS production.

centrifuged at 1200 RPM for 10 minutes at 4^{o}C. The cells were then positively isolated using a magnetic column as per manufacture's instructions. The magnetic separation procedure was repeated to increase the purity of $CD3^+$ T cells. Following isolation, the cells were counted, resuspended in culture media and allocated for functional assays. For splenic T cell isolation for imaging experiments detailed in Chapter 5, T cells were enriched using Pan T cell magnetic microbeads negative isolation kit (Miltenyi Biotec) as per manufacture's instructions. For further isolation of splenic $CD4^+$ and $CD8^+$ T cells, a multi-step enrichment procedure was used where Pan T cells were negatively isolated first, followed by positive isolation of $CD4^+$ (L3T4 microbeads) and $CD8^+$ (Ly-2 microbeads) T cells.

2.8 *In situ* T cell stimulation for cytokine and ROS detection

2.8.1 Anti-CD3/CD28 stimulation for quantitative cytokine detection

For quantification of cytokine release, enriched T cells were cultured in complete RPMI 1640 media (10% fetal bovine serum, penicillin 100 U/ml/streptomycin 100 μg/ml and L-glutamine 2mM) in flat-bottomed 96-well plates coated with anti-CD3 (5μg/mL; clone 145-2C11; Biolegend) monoclonal antibodies for 48 hours at 37^{o}C with 5% CO_2 in presence of anti-CD28 (clone 37.51; 5μg/mL; Biolegend) monoclonal antibodies as previously described.[79] To detect the amount of T cell-derived cytokine and chemokine production from cultured media samples and standards, a cytometric bead array (CBA; BD Biosciences) was performed according to manufacturer's instructions and analysed using a LSRII flow cytometer (BD Biosciences). FCAP Array 3.0 (BD Biosciences) was used to analyse the data as outlined in Figure 2.3.

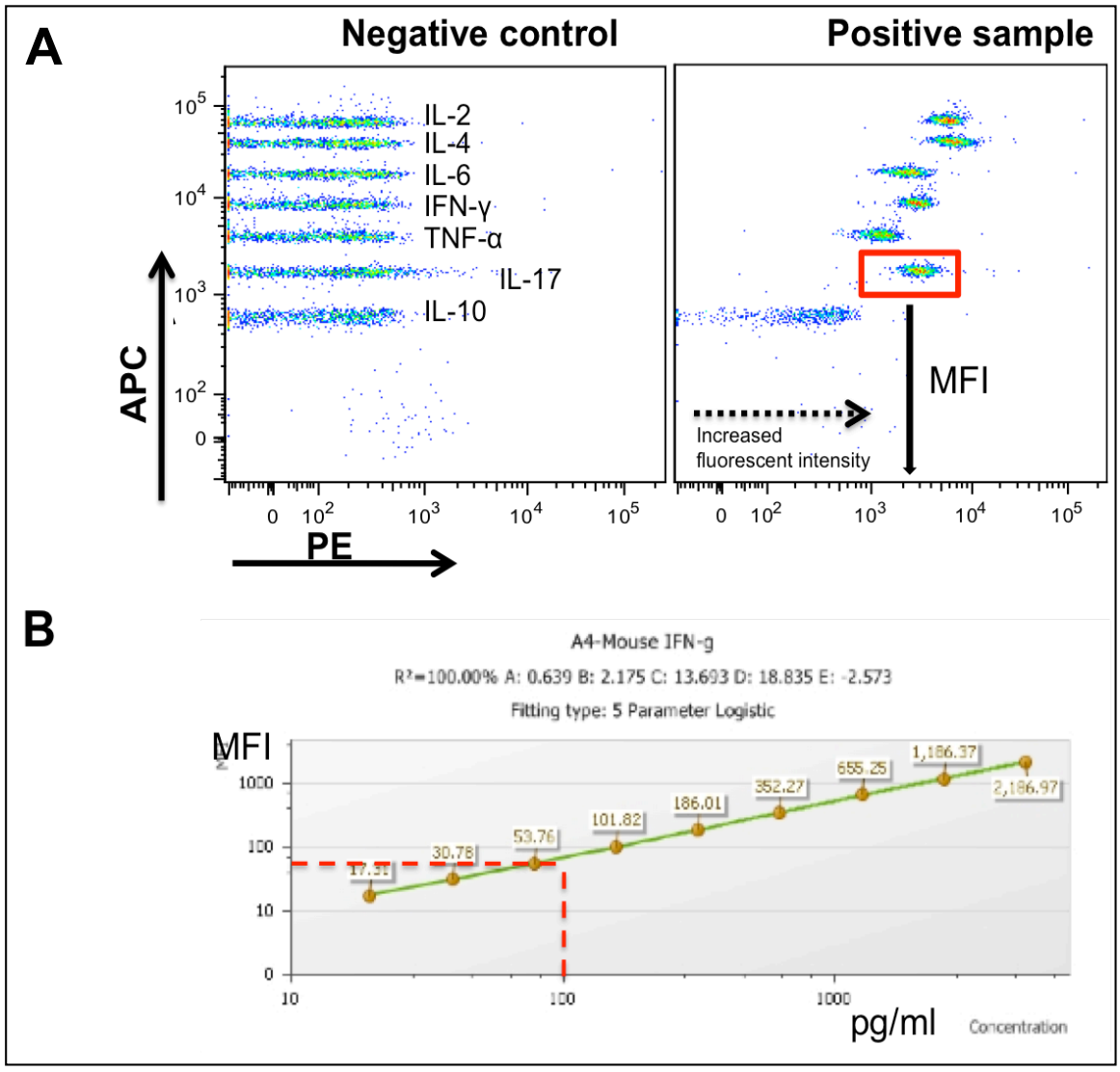

Figure 2.3: Using Flow cytometry and FCAP Array 3.0 to analyse the data. (A) Example plot from the CBA in a negative control (left) and actual sample (right). Bead populations were separated from each other according to the level of APC fluorescence intensity pre-loaded onto each group of beads, which were also labelled with a specific antibody to each individual cytokines/chemokine as shown on the figure. In the presence of cytokines, a greater fluorescent signal on the PE channel is detected and the amount of cytokine in the sample is calculated from the standard curve (B) using FCAP Array 3.0. (NB. PE and APC are fluorochromes linked to the beads to separate relative fluorescent signals)

2.8.2 PMA-Ionomycin stimulation and intracellular staining for flow cytometry

For intracellular cytokine detection, T cells were not enriched since $CD3^+$ T cells could be gated using flow cytometry. To identify the effect of Ang II-induced hypertension on various cytokine-producing T cells, single cell suspension were stimulated in a round-bottomed 96-well plate with phorbol 12-myristate 13-acetate (PMA; 100 μg/ml) and ionomycin (1 μg/ml) for 5 hours at 37°C with 5% CO_2 in the presence of golgi transport inhibitors, Golgi plug™ / Golgi stop™ (1μl/ml; BD Bioscience), which prevent the transport of cytokines out of the cell. In addition, unstimulated and fluorescence minus one (FMO) controls, were also included for subsequent gating for cytokine-producing T cells (NB. FMO controls contained every antibody in the panel except the marker of interest; Figure 2.4 C). Following stimulation, isolated leukocytes were washed by centrifugation in MACs Buffer at 1200 RPM for 10 minutes and resuspended in PBS. Cell pellets were resuspended in 100 μL of the aqua live/dead stain (Life Technologies) for 15 minutes protected from light at 4°C. Cells were then washed with 150 μL of MACs buffer and centrifuged at 1500 RPM for 5 minutes at 4°C. Supernatants were then discarded and cell pellets were stained with the respected antibody master mix for surface markers (Table 2.1), and were incubated for 25 minutes protected form light at 4°C. Following incubation, cells were then washed with 150 μL of MACs buffer and centrifuged and then resuspended in 200 μl Fix/Permeabilisation buffer (eBioscience) for 30 minutes in the dark at 4°C to fix and permeabilise the cells. Permeabilised cells were then washed in Perm Wash buffer (eBioscience) and were stained with an intracellular antibody master mix for 15 minutes in dark at room temperature (Table 2.1). Samples were then washed with perm wash buffer and centrifuged at 1500 RPM for 5 minutes at

4°C. Finally, the samples were resuspended in 200ul of 1% paraformaldehyde in MACs buffer and analysed on the flow cytometer.

Table 2.1: Antibodies employed for cell staining in section 2.8.2 for cell markers, cytokine and chemokines.

Cells/Cytokine/ Chemokine markers	Host/Isotype	Fluorochrome	Clone	Supplier	Dilution
Live/Dead		Aqua		Life Technologies	1:1000
CD3	Mouse BALB/c IgG1, gamma	BV421	SP34-2	BD Bioscience	1:100
CD45	Rat IgG2b, kappa	APC-Cy7	30-F11	Biolegend	1:500
CD4	Mouse IgG2b, kappa	BV605	OKT4	Biolegend	1:200
CD8	Rat IgG2a, kappa	PerCp-Cy5.5	53-6.7	Biolegend	1:100
CD44	Rat IgG2b, kappa	APC-Cy7	IM7	Biolegend	1:200
CD69	Armenian Hamster IgG	PE-Cy7	H1.2F3	Biolegend	1:500
IL-4 (Intracellular)	Rat IgG1, kappa	PE-Cy7	11B11	Biolegend	1:2000
IL-17 (Intracellular)	Rat IgG1, kappa	FITC	TC11-18H10.1	Biolegend	1:100
IFN-γ (Intracellular)	Rat IgG1, kappa	AF700	XMG1.2	Biolegend	1:1000
TNF-α (Intracellular)	Rat IgG1, kappa	PE	TN3-19.12	Biolegend	1:1000
MCP-1 (CCL2) (Intracellular)	Armenian Hamster IgG	PE	2H5	Biolegend	1:200
CMH_2DCFA (Intracellular)		DCF		Thermo Fisher Scientific	1μM

2.8.3 Quantitative and qualitative T cell-derived ROS detection

To quantify amount of ROS produced from T cells, L-012 chemiluminescence was used. ROS formation was detected under basal conditions and following stimulation with the direct activator of protein kinase C, phorbol 12,13-dibutyrate (PDBu). The chemiluminescence probe L-012 and PDB were diluted in Krebs-HEPES buffer (5.78mM NaCl, 0.35mM KCL, 0.30mM $MgSO_47H_2O$, 0.14mM KH_2PO_4, 2.1mM $NaHCO_3$, 2mM D-glucose, 5.21mM Na-Hepes, 2.5mM $CaCl_2$, dH_2O, pH7.4). A 96-well optiplate was used for detection of ROS using L-012 chemiluminescence. In each well 150 μL of the L-012 containing Krebs-HEPES assay solution was added (170 μL in basal wells). Fifty μL of T cell suspension (10^6 cells/ml) were added to each well (duplicates). Just prior to loading the plate, 20 μL of PDBu (100 μM) was added to samples designated for stimulation. ROS formation was detected as an average of the final 20 cycles detected using a Plate Chameleon^{TM}V (Hidex). Signals were averaged from duplicates and were normalized to cell counts, where cells were counted using haemocytometer with trypan blue exclusion before loaded onto the plate at 10^6 cells/ml. ROS formation from the sham- and Ang II-treated group were compared as were comparisons between the basal and PDBu-stimulated signals.

To identify the proportion of ROS positive T cells and T cell subsets, intracellular ROS detection was performed in aortic single cell suspensions prepared as described above. Cell suspensions were stimulated with PDBu (100 μM) for 30 minutes at 37°C. Cells were then stained with a cell permeable ROS-sensitive dye, 5-(and-6)-chloromethyl-29, 79-dichlorodihydrofluorescein diacetate, acetyl ester (CMH_2DCFDA; 1 μM) for a further 30 minutes at 37°C. Following incubation, cells were washed and stained with antibodies for T

cells surface markers for flow cytometry as described above.

2.9 Flow cytometry

2.9.1 Flow cytometry analysis

Samples were analysed on a LSR II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Countbright^{TM} count beads (Life Technologies) were added to each sample (kidney, brain, aorta), in order to calculate the absolute number of cells in the samples. For blood and spleen, 20,000 $CD3^+$ cells were recorded, and for aorta, brain and kidney 20,000 counting bead events were recorded. Countbright^{TM} absolute counting beads were used to determine the total number of cells in each aortic, brain and kidney sample. Absolute cell numbers in the samples were calculated as: number of events x total number of beads / number of beads events added x dilution factor.

2.9.2 Gating Strategies

Figure 2.4 shows the representative gating strategy employed for flow cytometric analyses. Single cells were gated by forward size scatter-height (FSC-H) and forward size scatter-area (FSC-A) to exclude doublets. For blood and spleen, lymphocytes were gated based on size and granularity. Live cells were gated from this lymphocyte population, followed by $CD3^+$ T cell gating from the live population. $CD4^+$ and $CD8^+$ cells were subsequently gated from $CD3^+$ population, from which other cytokine and chemokine markers including IL-4, IL-17, IFN-γ, TNF-α, CCL2 and DCF were then gated. A similar gating strategy was employed for aorta, brain and kidney except the common leukocyte marker, CD45, was gated from the singlets, from which $CD3^+$ cells and subsequent markers were then isolated. To help identify gating boundaries an unstimulated and FMO

controls were used. Specific T cell derived cytokines were represented as percentage of their respective parent population. In a separate subset of samples, CCL2 and DCF unstimulated controls (minus PDBu) were also analysed to establish gates for CCL2- and ROS-producing T cells.

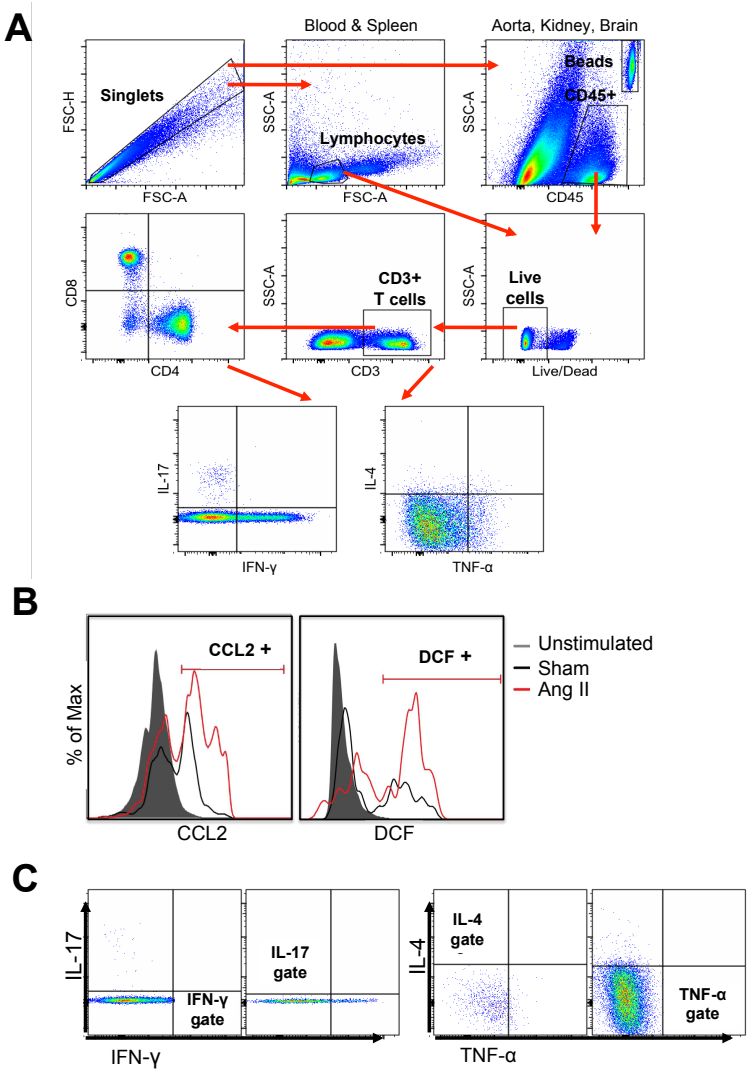

Figure 2.4: Gating of cytokine producing T cells from blood, spleen, aorta, kidneys and brain samples. (A) Singlet cells were gated first, and then $CD45^+$ cells or lymphocytes were gated from the singlet cell population. Live cells were then gated from the CD45+ or lymphocytes, and then $CD3^+$ cells were gated from the live cells. $CD4^+$ and $CD8^+$ cells were gated from CD3+ cells; and other cytokine producing T cells (Th1, Th2 and Th17 cells) were gated based on the cytokine that they release. (B) Representative histograms and gating strategy of CCL2- and ROS- producing T cells from aorta of unstimulated, sham- and Ang II-treated mice. (C) Gating of FMO controls for gating cytokine-producing T cells. The gating was set up so that no cytokine producing T cells were obtained.

2.10 Drugs

The drugs used are listed below.

Name	Company
(Val^5)-Angiotensin II	Bachem, Switzerland
Monoclonal antibody anti-CD3	Biolegend, San Diego
Monoclonal antibody anti-CD28	Biolegend, San Diego
CountBright^{TM} absolute counting beads	Life Technologies, New York
Trypan Blue staining 0.4%	Life Technologies, New York
Far Red	Life Technologies, New York
SNARF-1	Invitrogen
Anti-CD90.2 magnetic MicroBeads	Miltenyi Biotec
CD4 (L3T4) MicroBeads, mouse	Miltenyi Biotec
CD8a (Ly-2) MicroBeads, mouse	Miltenyi Biotec
Pan T cell isolation kit II, mouse	Miltenyi Biotec
Th1/Th2/Th17 Kit Cytometric Bead Array	BD Biosciences
Mouse Inflammation Kit Cytometric Bead Array	BD Biosciences
Fix/Permeabilisation Buffer	BD Biosciences
Isoflurane,	Baxter Healthcare, NSW
Tempol	Sigma-Aldrich, St. Louis
Bovine Serum Albumin (BSA)	Sigma Aldrich, St. Louis
Percoll solution	GE Healthcare Life Science
Acetylcholine	Sigma-Aldrich, St. Louis
Sodium nitroprusside	Sigma-Aldrich, St. Louis
U46619	Jomar Life Sciences
Dihydroethidium	Invitrogen
L-012 sodium salt	WAKO Pure Chemical, Japan
Phorbol 12,13-dibutyrate	Calbiochem

2.11 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Graphpad Prism 7 (GraphadPad Software Inc., San Diego, CA, USA.) For comparisons between two groups, data were compared by Student's unpaired t-test. For multiple comparisons between 3 or more groups data were analysed by one-way ANOVA with Bonferroni post-hoc test. For comparison between two groups with more than one variable data were analysed using two-way ANOVA followed by Bonferroni post hoc test. $P< 0.05$ was considered statically significant.

Chapter 3:

Differential Phenotypes of T Cells During Hypertension in Mice

PLOS ONE

RESEARCH ARTICLE

Differential Phenotypes of Tissue-Infiltrating T Cells during Angiotensin II-Induced Hypertension in Mice

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Abstract

Hypertension remains the leading risk factor for cardiovascular disease (CVD). Experimental hypertension is associated with increased T cell infiltration into blood pressure-controlling organs, such as the aorta and kidney; importantly in absence of T cells of the adaptive immune system, experimental hypertension is significantly blunted. However, the function and phenotype of these T cell infiltrates remains speculative and undefined in the setting of hypertension. The current study compared T cell-derived cytokine and reactive oxygen species (ROS) production from normotensive and hypertensive mice. Splenic, blood, aortic, kidney and brain T cells were isolated from C57BL/6J mice following 14-day vehicle or angiotensin (Ang) II (0.7 mg/kg/day, s.c.) infusion. T cell infiltration was increased in aorta, kidney and brain from hypertensive mice. Cytokine analysis in stimulated T cells indicated an overall Th1 pro-inflammatory phenotype, but a similar proportion (flow cytometry) and quantity (cytometric bead array) of IFN-γ, TNF-α, IL-4 and IL-17 between vehicle- and Ang II- treated groups. Strikingly, elevated T cell-derived production of a chemokine, chemokine C-C motif ligand 2 (CCL2), was observed in aorta ($\sim$6-fold) and kidney in response to Ang II, but not in brain, spleen or blood. Moreover, T cell-derived ROS production in aorta was elevated $\sim$3 -fold in Ang II-treated mice ($n=7$; $P<0.05$). Ang II-induced hypertension does not affect the overall T cell cytokine profile, but enhanced T cell-derived ROS production and/or leukocyte recruitment due to elevated CCL2, and this effect may be further amplified with increased infiltration of T cells. We have identified a potential hypertension-specific T cell phenotype that may represent a functional contribution of T cells to the development of hypertension, and likely several other associated vascular disorders.

Introduction

Hypertension is a common risk factor for cardiovascular disease and stroke, which are the major causes of morbidity and mortality in Western societies (W.H.O, 2013) [1]. While current anti-hypertensive therapies can maintain blood pressure homeostasis in some patients, surprisingly 10–15% of cases of human hypertension remain resistant to these therapies, whether used alone or in combination [2, 3]. Moreover, despite extensive research, the etiology of hypertension still remains unclear and novel approaches need to be developed to treat this condition. Recent studies have implicated inflammation and activation of the immune system in the development of hypertension [4]. It is now well defined that T cells are required for the development of hypertension, which infiltrate organs that control blood pressure such as the aorta and kidneys [5, 6]. However, the functional contribution of these infiltrating T cells to the local inflammatory response during hypertension remains speculative and under-studied.

T lymphocytes can be divided into several subtypes and subsets that all produce various responses to infection and immune homeostasis. The predominant subtypes are T helper (Th) cells (CD4+) and cytotoxic T cells (CD8+), but a population of double negative cells also exist (DN; CD4-CD8-). Approximately 95% of all T cells express a membrane-bound T cell receptor (TCR) comprised of α and β subunits, which is capable of recognizing specific antigens presented in the context of a major histocompatibility complex. A smaller population of T cells (5–10%) express a different TCR comprised of γ and δ subunits that recognize antigens that are usually not presented by MHC molecules. Antigen presenting cells such as dendritic cells and macrophages engulf foreign antigens and can present antigen-specific epitopes to T cells. In the presence of innate cytokines such as IL-12/IFN-γ, IL-4 and IL-23, Th cells (CD4+) polarise to Th1, Th2 and Th17 cells respectively [7]. Polarised Th subsets secrete adaptive immune cytokines that also include IFN-γ, TNF-α (both Th1), IL-4 (Th2) and IL-17 (Th17), which mount an immune response involving reciprocal activation of innate cells such as macrophages and eosinophils, as well as B cells of the adaptive immune system to remove the pathogen [7]. Cytotoxic T cells (CD8+) also act to kill pathogens by releasing cytotoxic enzymes [7]. DN cells lack the expression of both the surface proteins CD4 and CD8 and their functional role is still not completely understood [8]. The role of T cells in inflammatory diseases has been studied for decades, especially in autoimmune diseases such as rheumatoid arthritis [9] and systemic lupus erythematosus (SLE) [10]. Rheumatoid arthritis is known to be associated with infiltrating Th cells into synovial joints. As discussed above, Th cells are a large source of cytokines that can promote local inflammation as well as recruitment of other immune cells. In the setting of rheumatoid arthritis, pro-inflammatory cytokine levels are elevated, which includes IFN-γ, TNF-α, IL-6, IL-1, GM-CSF [11] and chemokines such as chemokine C-C motif ligand 2 (CCL2) [12] and CCL13 [13]. TNF-α has been documented to be of major importance in the pathogenesis of rheumatoid

arthritis. However, there is also upregulation of homeostatic regulatory cytokines, IL-10 and TGF-β, which can suppress the pro-inflammatory response [9]. Importantly, research into T cells in rheumatoid arthritis has led to novel treatments to treat this disease such as the TNF-α inhibitor, etanercept [14] and IL-6 antagonist tocilizumab [15] which inhibit or neutralize TNF-α and IL-6 respectively.

A likely mechanism by which T cells may contribute to hypertension is through the release of cytokines, which promote inflammation in various blood pressure-controlling organs [4]. We have previously reported an elevation in Th1 activity in hypertensive mice shown by increased lymphoid Th1 specific cytokines IFN-γ and TNF-α [6, 16]. Shao and colleagues [17] also suggested that there are more Th1 cells in hypertensive rats. Although past studies have shown T cell-derived cytokines are associated with hypertension, they have focused on circulating or secondary lymphoid organ T cells in the setting of hypertension, [4, 6, 16] and have not studied the role of tissue-infiltrating T cells that represent local effects of T cells and inflammation. Moreover, although chemokines such as CCL2, and ROS production have been implicated in hypertension [6, 18], whether infiltrating T cells are a source of these influential mediators has not been examined in the setting of hypertension.

To our knowledge, we have conducted the first phenotypical analysis of aortic, renal and brain-infiltrating T cells from normotensive and hypertensive mice. After isolating T cells from various blood pressure-controlling organs we measured T cell-derived cytokine and ROS production. We report that organ-specific T cells from hypertensive mice exhibit phenotypically different profiles that may be responsible for local leukocyte recruitment and oxidative stress during hypertension.

Materials and Methods

Animals

Male C57BL6/J mice aged 10–12 weeks (n=66–75) were obtained from Monash Animal Services (MAS) and housed on a 12 hour light/dark cycle with food and water provided *ad libitum*. Isoflurane-anaesthetized mice were randomly allocated to receive either vehicle (0.03M NaCl, 1% acetic acid) or Ang II (0.7 mg/kg/day) via osmotic minipumps (Alzet model 2002) implanted subcutaneously for 14 days. All surgical and treatment procedures were approved by the Monash Animal Research Platform Ethics Committee (Approval number MARP/2011/85).

Blood Pressure Monitoring

Systolic blood pressures (SBP) were measured using a non-invasive tail-cuff apparatus (MC4000 Blood Pressure Analysis System, Hatteras Instruments) prior to treatment and on day 14 to confirm hypertension (SBP > 140 mmHg) was achieved. At each time point over 20 measurements were recorded and averaged.

Tissue Harvesting & Preparation

At the end of each treatment period, mice were euthanized by carbon dioxide asphyxiation and blood and tissue were harvested for further analysis. This procedure was approved by the Monash Animal Research Platform Ethics Committee (Approval number MARP/2011/85). Cardiac puncture was employed to extract blood from the right ventricle. Mice were perfused using a perfusion pump (Model 77200-62 easy-load II, MasterFlex) with phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) for five minutes. Spleen, kidneys, brain and aorta with perivascular fat intact were removed and maintained on ice.

Blood and spleen were prepared as previously described [6]. Aortic, brain and kidney samples were dissociated in 1.5 mL digestion buffer containing collagenase type XI (125 U/mL), hyaluronidase (600 U/mL) and collagenase type I-S (450 U/mL) with gentleMACS Octo Dissociator (Miltenyi Biotec) for 2 minutes and then digested at 37℃ for 45 minutes. Following digestion, samples were pressed through a 70 μm filter and centrifuged at 1200 RPM for 10 minutes. Supernatants were then discarded and aortic samples were ready for further analysis. Kidney and brain samples were further subjected to percoll gradient centrifugation. Samples were resuspended in 2 mL of 40% (kidney) or 30% (brain) isotonic percoll solution (GE Healthcare Life Science) and then gently underlaid with 2 mL of 60% or 70% percoll solution respectively. Samples were then centrifuged at 2700 RPM with the centrifuge brake turned off for 25 minutes at room temperature. Following centrifugation, adipocytes and debris were aspirated off the top layer and mononuclear cells (MNCs) were collected at the interface of both solutions.

T cell Enrichment

To isolate T cells from tissue samples, cell pellets were incubated with anti-CD90.2 labeled magnetic microbeads (Miltenyi Biotec). The cells were then isolated using a magnetic column as per manufactures instructions. Cell purity of enriched cells was confirmed to be $>$90% using flow cytometry. Following isolation, the cells were counted then resuspended in culture media and allocated for T cell-derived cytokine and ROS detection.

Quantitative Cytokine and chemokine detection (Cytometric Bead Array)

Isolated T cells from various organs were seeded in 96-well plates coated with anti-CD3 antibody (BD PharMingen) and cultured for 48 hours in presence of anti-CD28 (BD Pharmingen) as previously described [16]. Following stimulation, conditioned media was used to analyze levels of IFN-γ, TNF-α, IL-4, IL-17 and CCL-2 using a cytometric bead array (CBA, BD Biosciences). To determine phenotypical differences between T cells from hypertensive and normotensive mice, cytokine amount was normalized to T cell number.

Detection of T cell derived superoxide production

To detect the extracellular ROS produced from isolated T cells, L-012 chemiluminescence was employed. ROS formation was detected under basal conditions and following stimulation with the direct activator of protein kinase C, phorbol 12,13-dibutyrate (PDBu; 100 μM). Enriched cells were resuspended in Krebs-HEPES buffer (in mM: NaCl 118; KCl 4.7; KH_2PO_4 1.2; $MgSO_4{\cdot}7H_2O$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 11.7; HEPES 20, pH 7.4). Following a 30 minute incubation period, L-012 (100 mmol/L) was added to each well, which was then loaded into a Hidex Chameleon Luminescence detector. All samples were performed in triplicate and photon emissions were recorded for 30 cycles at 2 min intervals. Photon emissions (relative light units per second) were then averaged over the final 20 cycles.

To enumerate the proportion of ROS positive T cells and T cell subsets, intracellular ROS detection was performed in aortic samples that were harvested and digested, but not subjected to T cell enrichment as described above. Cell suspensions were stimulated with PDBu (100 μM) for 30 minutes at 37℃. Cells were then stained with a cell permeable ROS-sensitive dye, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; 1 μM) for a further 30 minutes at 37℃. Following incubation, cells were washed and stained with live/dead Aqua stain (Life Technologies) for 15 minutes at 4℃. After washing with FACS buffer (PBS with 0.5% bovine serum albumin) cells were stained with fluorochrome-conjugated antibodies for surface markers including CD45 (leukocytes; APC-Cy7; 30-F11), CD3 (T cells; APC; 145-2C11), CD4 (T-helper cells; BV605; RM4-5) and CD8 (cytotoxic T cells; PerCP-Cy5.5; 53–6.7). Cells were then analysed on a LSR II flow cytometer for (BD Biosciences). Unstimulated controls (minus PDBu) were also analysed to establish gates for ROS-producing T cells.

Qualitative Intracellular Cytokine detection and Flow Cytometry

To identify the proportion of T cells and T cell subsets producing each cytokine, intracellular staining and flow cytometry was performed. In a subset of animals, processed tissues samples were not subjected to T cell enrichment and were stimulated with phorbol 12-myristate 13-acetate (PMA; 100 μg/ml)/ionomycin (1 μg/ml) for 4 hours at 37℃ with 5% CO_2 in the presence of protein transport inhibitors (Golgi plug/Golgi stop, BD Bioscience), which prevent the transport of cytokines out of the cell. Cells were washed and stained with live/dead stain (Life Technologies) and fluorochrome-conjugated antibodies for surface markers as described for intracellular ROS detection. Cells were then fixed and permeabilised using a Fix/Permeabilisation Buffer (eBiosciences) for intracellular staining of cytokines including IFN-γ(AlexaFluor700; XMG1.2), TNF-α (PE; TN3-19.12), IL-4 (PE-Cy7; 11B11), IL-17 (FITC; TC11-18H10.1). In a separate subset of samples, CCL2 (FITC; 2H5) was also stained to detect intracellular T cell-specific CCL2 production. Cells were then analysed using an LSR II flow cytometer (BD

Biosciences). Negative unstimulated controls and fluorescence minus one (FMO) controls were analyzed to establish gates for cytokine-producing T cells.

Statistical analyses

All data was expressed as mean ± standard error of mean (SEM). Changes in blood pressure were analyzed using two-way repeated measures ANOVA. Unpaired t-tests were used to compare vehicle and Ang II groups. A p-value of <0.05 was considered significant.

Results

Systolic Blood Pressure (SBP)

Using non-invasive tail-cuff measurements hypertension was confirmed in Ang II-treated mice (Day 14 SBP: vehicle 116±3 vs Ang II 158±2, $P<0.0001$).

T cell Infiltration into Organs

Flow cytometry was used to quantify the number of T cells (CD3+) in various organs. Compared to vehicle-infused mice, Ang II-infusion did not alter the number of circulating or splenic T cells. However, Ang II-infusion did increase T cell numbers in the aorta, kidney and brain (Fig. 1). In the aorta and kidney, Ang II-infusion tended to increase all CD4+, CD8+ and DN T cell subsets compared to vehicle-treated mice (S1 Figure). However, only the aortic CD4+ and double negative T cells and kidney CD4+ T cells were of the effector phenotype ($CD44^{hi}$+$CD62L^{lo}$; S2 Figure). We also observed an increase in FoxP3+ T regulatory cell number in aorta, and a trend towards an increase in kidney from Ang II-infused mice (S3 Figure).

Intracellular Cytokine Analysis

Using intracellular staining and flow cytometric analysis, cytokine profiles of T cells in various tissues and organs was compared (Fig. 2). In T cells isolated from blood and all organs, a greater proportion of T cells produced typical Th1 cytokines (IFN-γ and TNF-α) relative to Th2 (IL-4) or Th17 (IL-17) (Fig. 2B). However, no significant differences in cytokine-producing T cells were observed between vehicle- and Ang II-treated mice.

Although no overall changes in Th1, Th2 and Th17 cytokines were observed in CD3+ cells, further analysis of organ-specific T cell subsets (CD4+, CD8+ and DN cells) revealed differences in cytokine production in certain subsets. Cytokine-producing T cell subsets from various organs of vehicle- or Ang II-treated mice revealed 3 unique subsets (Table 1). A significant increase in the proportion of aortic CD8+ T cells and kidney DN T cells that produced IFN-γ was observed in Ang II-treated mice while a trend towards greater proportion of IFN-γ-producing DN T cells in the blood of Ang II-treated mice was also observed (Table 1).

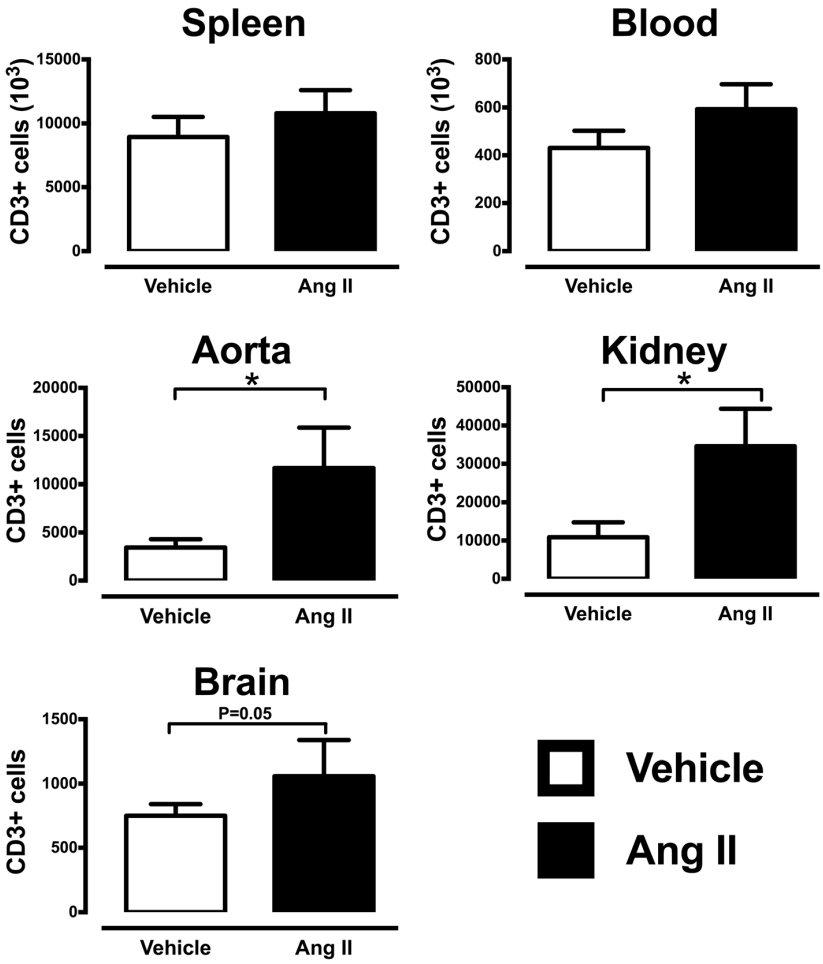

Fig. 1. Total tissue infiltrating T cell (CD3+) infiltration into various organs. Flow cytometry was employed to count the number of T cells in vehicle- and Ang II treated mice in (A) non-infiltrating organs (blood and spleen) and (B) infiltrating organs kidney, brain and aorta. (*P<0.05 Vs vehicle; Unpaired t test; n=12–19). (NB. Cell counts for kidney; brain and aorta were normalized to the cell counting beads).

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We also examined the proportion of T cells that produced the chemoattractant CCL2 in aorta and kidney. Interestingly, a greater proportion of CCL2-producing aortic and kidney T cells was observed in Ang II-infused mice (Figs. 3A and 3B), which appeared to be derived from CD4+ and CD8+ T cell subsets in the aorta, but DN T cells only in the kidney (Fig. 3B).

Cytometric Bead Array Analysis of Cytokines and CCL2

Following T cell isolation using the CD3+ magnetic microbeads, flow cytometry confirmed greater than 90% of the isolated cells were CD3+ T cells in all organs.

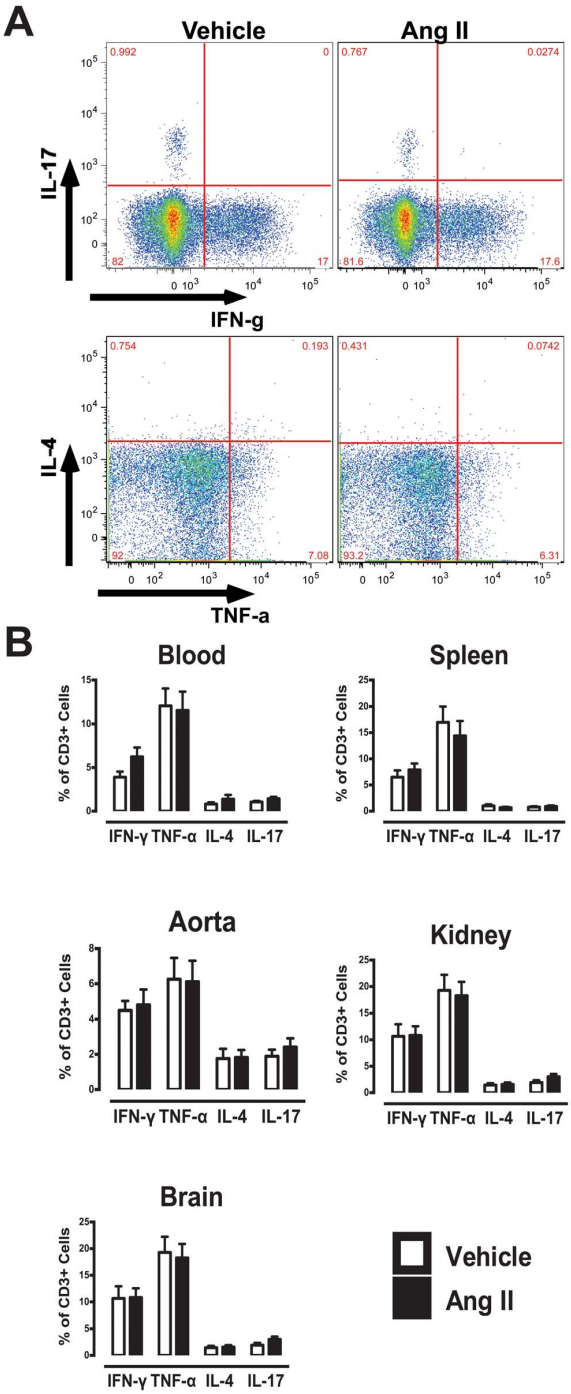

Fig. 2. Intracellular FACS analysis of cytokine producing T cells (CD3+). (A) Representative flow cytometry plots and gating strategy of IFN-γ, TNF-α, IL-4 and IL-17-producing T cells in blood. (B) Mean data of cytokine-producing T cells in vehicle- and Ang II-treated group in various organs (n=11–19).

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Table 1. Proportion of cytokine producing CD4+, CD8+ and DN T cell subsets in various organs from vehicle- and Ang II-infused mice.

		IFN-γ		TNF-α		IL-4		IL-17	
		Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II
Blood	**CD4**	2.0±0.3	1.7±0.2	14.6±2.8	16.5±2.7	0.5±0.1	0.4±0.1	0.7±0.2	1.1±0.2
	CD8	5.5±1.0	7.0 ±12	9.6±2.5	6.8±1.7	0.4±0.1	0.8±0.2	0.4±0.1	0.5±0.1
	DN	7.2 ±1.1	12.3 ±2.5	12.0±3.6	9.5±2.5	2.7±0.8	3.1±0.8	7.3±1.2	9.3±1.1
Spleen	**CD4**	3.2 ±0.6	3.4 ±0.3	20.3±3.7	21.3±3.8	1.6±0.1	0.6±0.2	0.6±0.1	0.7±0.2
	CD8	5.6 ±1.4	8.23±1.5	13.3±3.2	9.5±2.7	1.1±0.3	0.9±0.2	0.5±0.2	0.5±0.2
	DN	13.4 ±2.8	18.1 ±3.0	7.1±1.3	8.8±1.3	3.1±0.9	2.9±0.6	4.1±0.5	3.2±0.4
Aorta	**CD4**	7.4±1.5	6.1±1.4	8.7±2.3	12.9±2.8	0.7±0.2	1.2±0.4	0.8±0.3	1.1±0.3
	CD8	2.8±0.6	5.0±0.7 *	2.9±0.6	5.0±1.1	3.0±0.8	3.8±1.0	1.1±0.4	1.7±0.4
	DN	3.4±0.9	4.1±1.0	5.2±1.2	6.1±1.4	2.5±1.0	4.7±1.7	5.9±1.7	4.2±0.7
Kidney	**CD4**	5.7±2.2	9.9±2.6	3.6±0.7	3.7±1.1	2.0±0.5	1.7±0.5	1.7±0.3	1.5±0.4
	CD8	2.7±1.0	2.6±0.7	5.6±1.5	4.1±1.0	3.4±0.7	2.8±0.7	0.5±0.2	1.5±0.6
	DN	4.5±1.5	8.2±1.8 *	3.2±0.6	6.1±3.5	0.7±0.2	1.7±0.5	4.7±1.2	3.7±0.7
Brain	**CD4**	9.0±2.2	8.9±2.2	21.7±3.3	28.4±4.5	0.9±0.3	0.7±0.3	0.4±0.2	0.7±0.3
	CD8	11.7±3.1	11.4±2.3	16.4±3.7	13.7±2.6	1.2±0.4	2.9±0.8	1.9±0.6	2.3±0.7
	DN	9.5±2.7	14.4±3.2	12.7±3.8	7.4±2.4	2.7±1.5	5.4±1.9	8.7±3.4	8.1±3.7

*P<0.05 Vs Vehicle, Unpaired t –test, n=11–19.

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CD11b positive cells (myeloid cells including macrophages) represented ≤1% of all leukocytes (Fig. 4A). Consistent with intracellular FACS analysis, a cytometric bead array revealed a predominant Th1 cytokine phenotype was observed from T cells of all organs, but no significant differences in T cell-derived cytokine production following anti-CD3/CD28-stimulation was observed in vehicle- or Ang II-treated mice (Fig. 4B). Blood and kidney T cells appeared to produce more cytokines compared to other organs. T cell-derived IL-6 and IL-10 production was also detected from most organs, however, no differences were observed between T cells from normotensive and hypertensive mice (S4 and S5 Figures).

The levels of the chemokine, CCL2, following anti-CD3 stimulation were also measured using a CBA (Fig. 5). Interestingly, while there were no differences in T cell-derived CCL2 production from T cells of blood and spleen of vehicle- or Ang II-treated mice, tissue infiltrating T cells of Ang II-treated mice produced significantly greater CCL2 in aorta (~6 fold) and kidney (~3 fold). No differences in CCL2 production were observed in brain (Fig. 5B).

T cell–Derived ROS production

L-012 chemiluminescence was used to detect the amount of ROS produced by T cells from various organs. There was no difference in the basal level of T cell-derived ROS between vehicle- and Ang II-treated mice (data not shown). The amount of T cell-derived ROS following PDBu stimulation was significantly elevated in tissue-infiltrating T cells from aorta of Ang II-treated mice (Fig. 6A), but this difference was not observed from T cells of any other organs. Flow

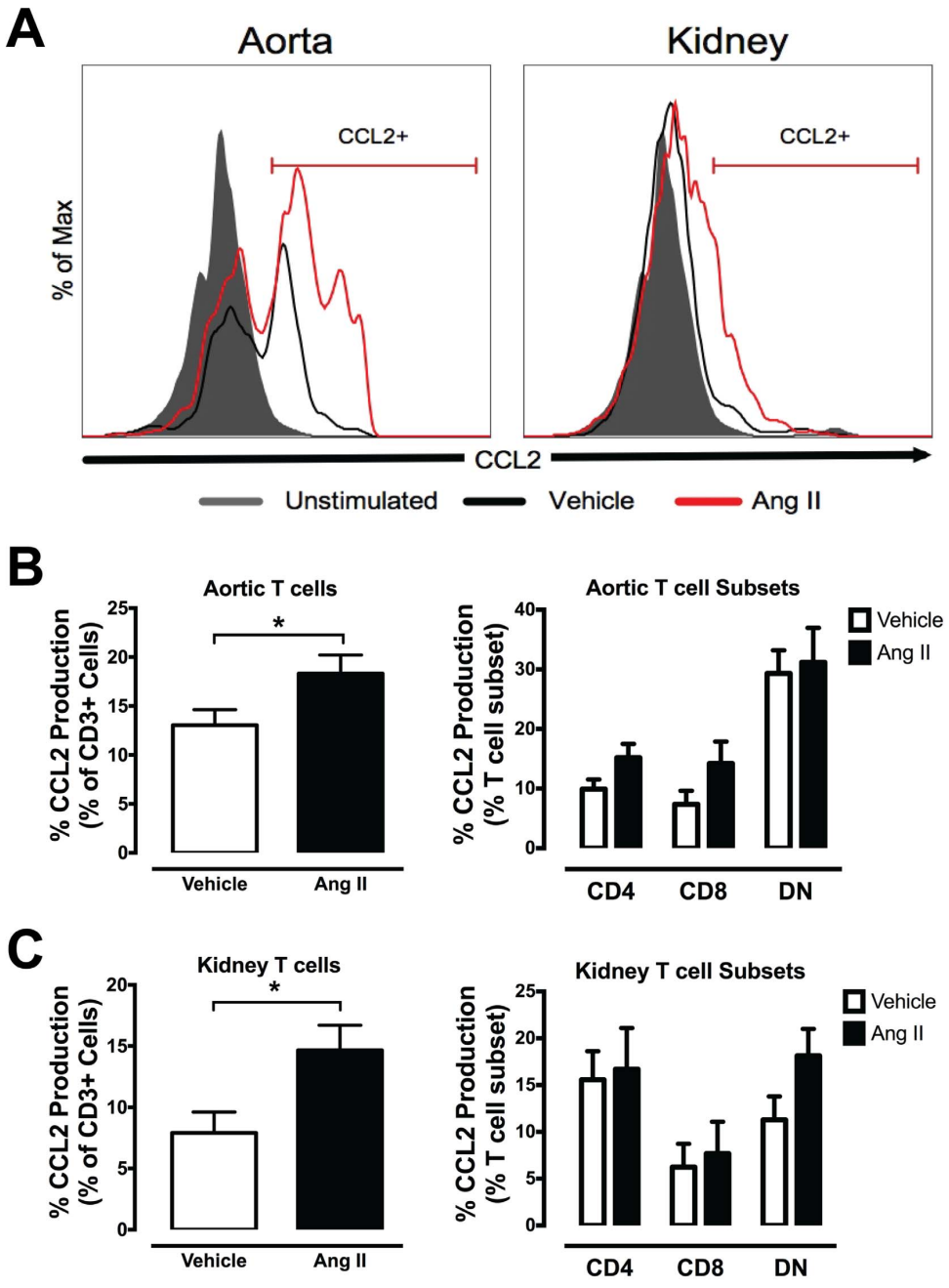

Fig. 3. Intracellular FACS analysis of CCL2-producing T cells from aorta and kidney. (A) Representative histograms and gating strategy of CCL2+ T cells from aorta (left) and kidney (right) of vehicle and Ang II-infused mice. (B) Mean data of aortic and (C) kidney T cells (left) and T cell subsets (right) that produce CCL2 in response to PMA-ionomycin stimulation. (*P<0.05 Vs vehicle; Unpaired t test; n=5–10).

doi:10.1371/journal.pone.0114895.g003

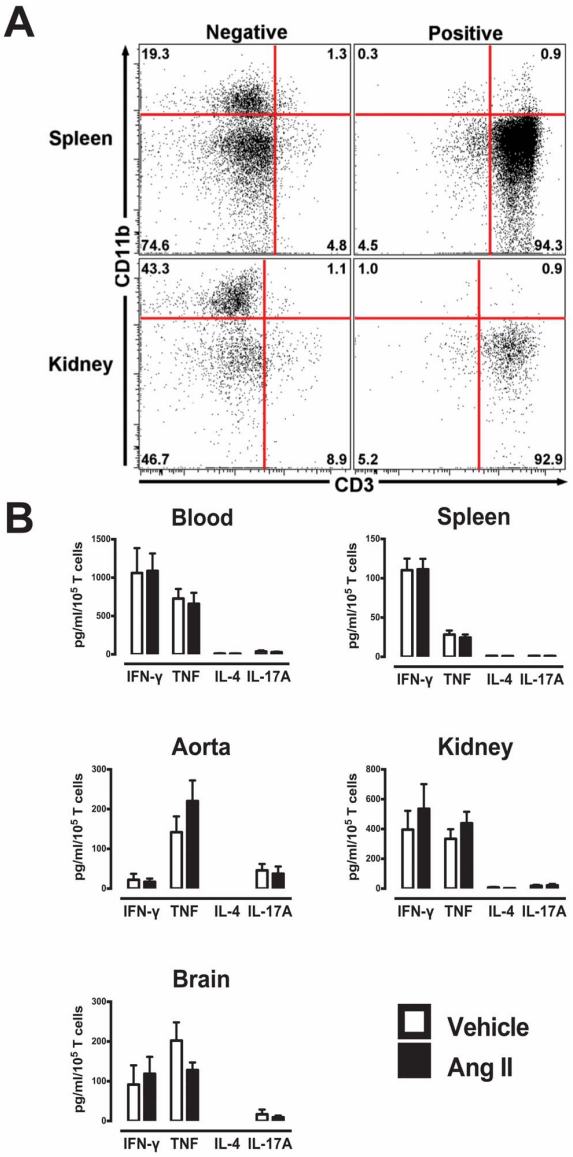

Fig. 4. Quantitative cytokine production from isolated T cells from various organs. (A) Purity of T cell (CD3+) isolation. Representative flow cytometry plots of spleen and kidney samples following magnetic bead T cell isolation. Negative samples represent cells remaining after T cells had been extracted, and the positive samples represent extracted cell samples. (B) Mean data of cytokine production after anti-CD3 stimulation using CBA assay. Data represented as total amount of cytokine produced (A) in pg/ml per 10^5 T cells in blood, spleen, aorta, kidney and brain (n=7–11).

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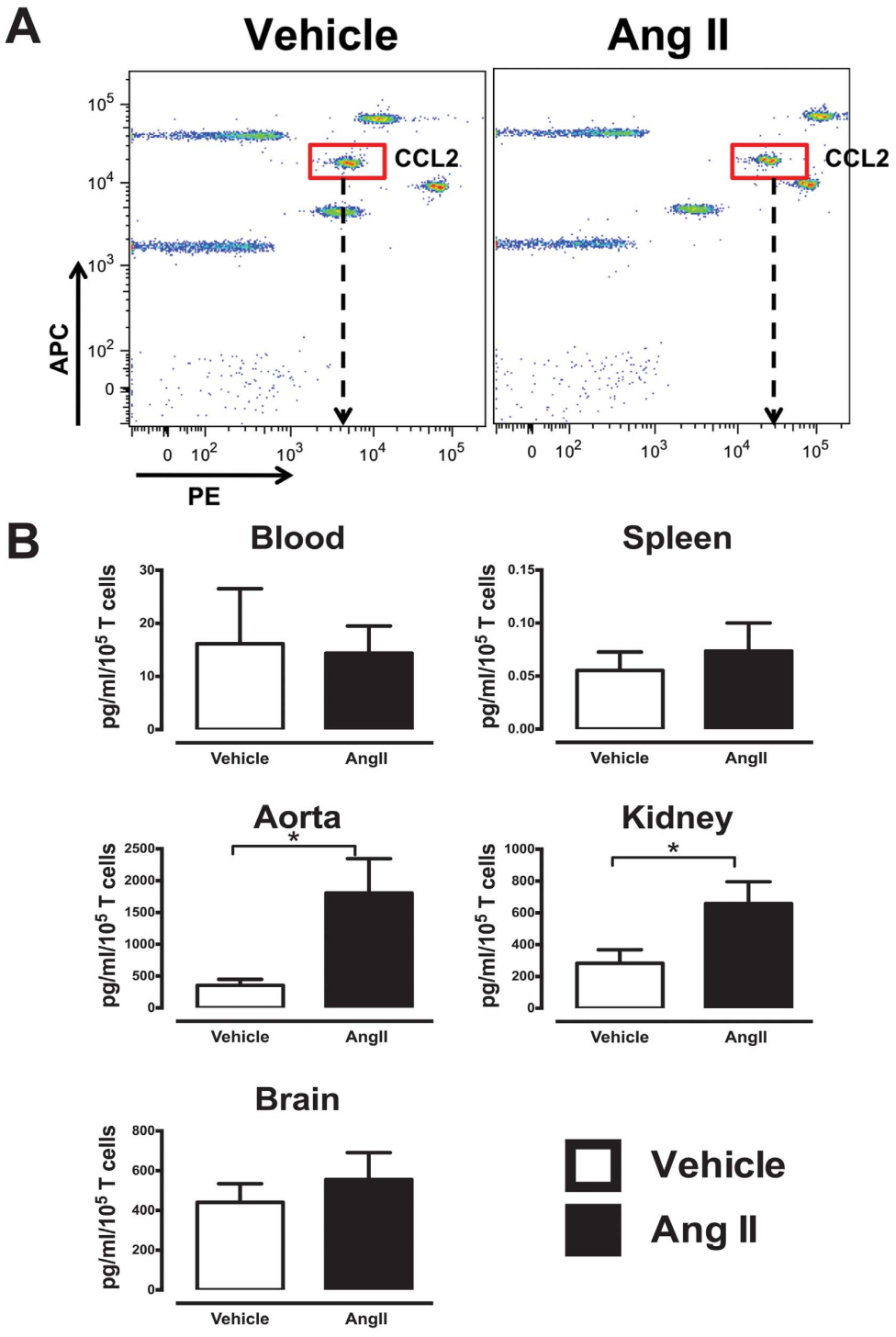
A
Vehicle
Ang II
CCL2
CCL2
APC
PE
B
Blood
Spleen
Aorta
Kidney
Brain
pg/ml/10^5 T cells
Vehicle
AngII
*
Vehicle
Ang II

Fig. 5. Infiltrating T cells from hypertensive mice produce greater CCL2. (A) Representative CBA assays plot and gating of CCL2 in a vehicle and Ang II aortic sample. Arrow denotes the mean fluorescence intensity of each sample. (B) Quantitative analysis of amount of CCL2 produced using CBA assay. Data represented as total amount of cytokine produced in pg/ml per 10^5 T cells in blood, spleen, aorta, kidney and brain (n=11–22). (*P<0.05 Vs vehicle; Unpaired T test; n=7–8).

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cytometric analysis demonstrated a significant increase in PDBu-stimulated intracellular ROS production in aortic T cells from Ang II-infused mice compared to vehicle-treated mice (Fig. 6B and 6C). Interestingly, this increase was only evident in CD4+ and DN T cell subsets within the aorta (Fig. 6C).

Discussion

The major findings of the present study were that the majority of T cells were of the Th1 pro-inflammatory phenotype and produced relatively the same amount of cytokines in normotensive and hypertensive mice, although there was greater T cell infiltration per se into aorta and kidney in Ang II-infused mice. By contrast, there was enhanced aortic CCL2 and ROS production from each T cell-infiltrate during Ang II-induced hypertension, which may represent a hypertension-specific and unique T cell phenotype that is likely to contribute to hypertension and associated vascular diseases.

Hypertension is now considered an inflammatory and immune system disorder. While it is known that there is increased infiltration of T cells into important blood pressure controlling organs during hypertension, the patho-physiological function of these infiltrating T cells remains unclear. Current literature suggests that one of the major contributions of T cells to hypertension is through the release of cytokines from CD4+ T (helper) cell subsets that promote local inflammation, which under normal physiological conditions would facilitate removal of foreign pathogens [6, 17, 19]. While we and others have reported significant elevations of Th1 cytokines, these observations have been limited to T cells isolated from secondary lymphoid organs [16, 17] and thus no studies have directly examined the cytokine profiles of T cells isolated directly from blood pressure-controlling organs. Using well-established immunological tools to define T cell cytokine profiles, we thoroughly examined T cell-derived cytokine production in response to either anti-CD3 or PMA-ionomycin in various tissues. From our studies, it is clear that for the Th cytokines we have analysed, hypertension is not associated with differences in the proportion of cytokine-producing T cells or the amount of cytokine release per T cell from normotensive and Ang II-infused hypertensive mice. We observed no differences in the amount of cytokines produced from non-infiltrating (blood and spleen) compared to infiltrating T cells. However, there was a predominant Th1 phenotype with greater IFN-γ and TNF-α production, which most likely reflects the C57BL6/J mouse strain [20, 21]. C57BL6/J mice are also more prone to hypertension and associated systemic and vascular inflammation compared to Th2 prone BALB/c mice, which

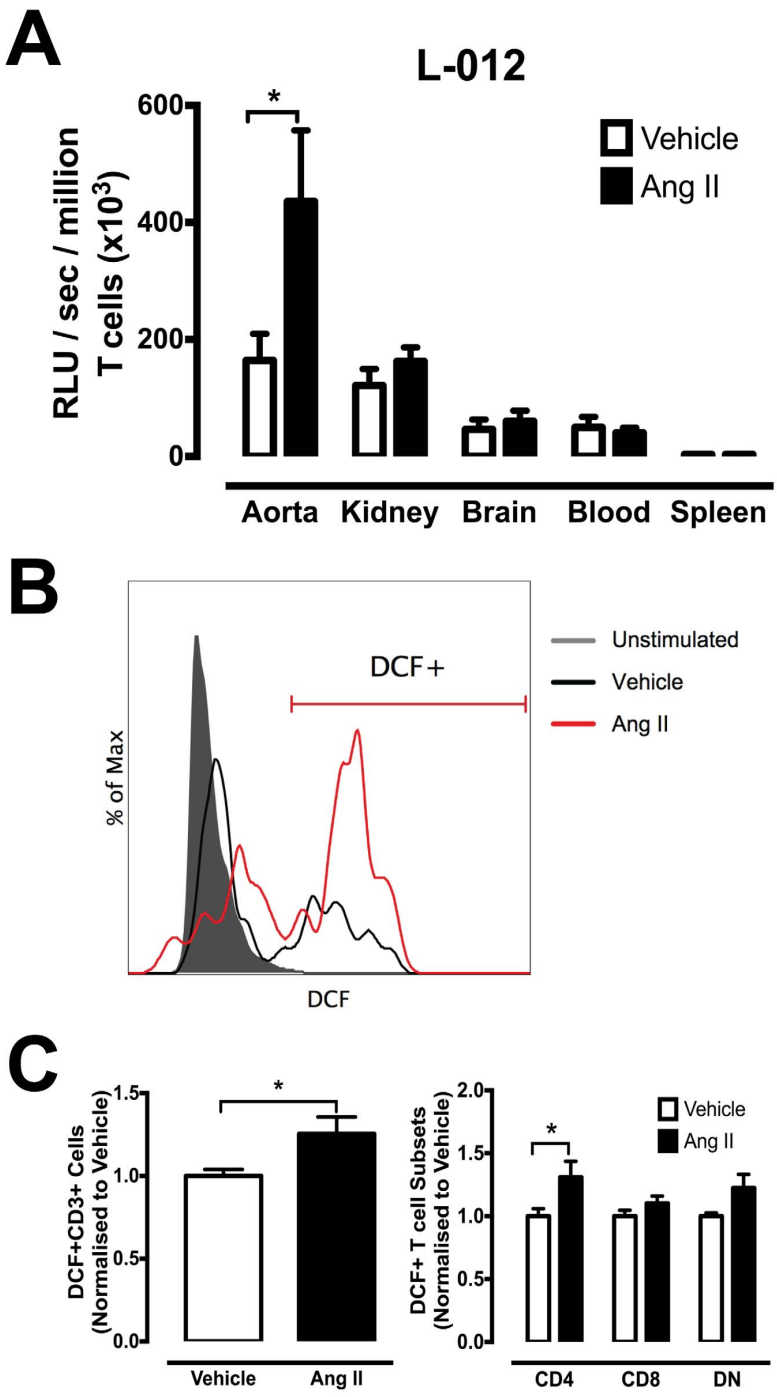

Fig. 6. Effect of Ang II-induced hypertension on T cell-derived ROS production. (A) L-012 enhanced chemiluminescence detection of extracellular ROS from various tissue samples from vehicle and Ang II-infused mice. Data represented as the amount of ROS produced in RLU/sec per million T cells (x10^3) with

PDBu stimulation in kidney, brain blood and spleen (*P<0.05 Vs vehicle; n=7–14). (B) Representative histograms and gating strategy of DCF+T cells from aorta. (C) Mean DCF+T cells normalized to response in corresponding vehicle T cells (left) and T cell subsets (right) (*P<0.05 Vs vehicle; n=5–11).

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would further support an association between hypertension and the Th1 phenotype [22]. However, inhibition of Th1 cytokines using agents such as the TNF-α antagonist, etanercept, to treat experimental hypertension has been equivocal, and consistent with our findings, it suggests that cytokine production may not be the most important or hypertension-specific mechanistic role for T cells during hypertension[6, 23–25]. Our findings however, do not rule out a contribution of cytokines to local inflammation. Consistent with the current literature, we observed greater T cell infiltration into blood pressure-controlling organs such as the aorta, kidney and for the first time we showed in brain that there is increased T cell infiltration during hypertension [6, 26–28]. We also observed greater FoxP3+ T regulatory cell infiltration into the aorta of hypertensive mice, which may suggest non-selective recruitment of T cells during hypertension. Although this differs from a previous study that examined aortic T regulatory cell number in Ang II-infused mice, this may be due to the quantitative approach used. Barhoumi and colleagues [29] used immunofluorescence as opposed to flow cytometry in the current study, which is a fully quantitative technique and examined total FoxP3+ cell across the full length of the aorta. Nonetheless, while infiltrating T cells may not produce more pro-inflammatory cytokines during hypertension, greater accumulation of T cells possibly increases the overall cytokine levels locally, thereby promoting inflammation associated with hypertension. A corollary from our findings may be that prevention of the initial T cell infiltration into blood pressure-controlling organs represents a potential therapeutic strategy to negate inflammation associated with hypertension.

We identified three T cell subsets that were distinct during hypertension. We discovered that there were unique subsets that produced more IFN-γ in specific organs in hypertension, including CD8+ cells in aorta, DN cells in kidney and possibly DN cells in blood (Table 1). While their specific role remains unidentified, these are unique phenotypes that are specifically elevated during hypertension. To our knowledge, no studies have examined specific T cell subsets in hypertension; however, unique subsets have been observed in other immune diseases. In the synovial fluid from patients with rheumatoid arthritis, an elevated proportion of IFN-γ producing CD4+ and CD8+ T cells have been reported [30]. Wahlstrom et al [31] have also shown increased IFN-γ- and TNF-α-producing CD4+ and CD8+ T cell subsets in the bronchoalveolar lavage fluid from sarcoidosis patients; findings that have in fact aided further elucidation of the pathogenic mechanisms in sarcoidosis. Therefore, like other immune disorders, identification of these unique subsets in hypertension represents a step forward in the understanding the local pathology of T cells in the setting of hypertension, and warrants further investigation into the function of these phenotypes.

Importantly, we also identified a distinct infiltrating T cell phenotype during hypertension, where a significantly greater proportion of T cells in aorta and kidney produced CCL2, which also translated to greater amounts of CCL2 detected in conditioned media of stimulated T cells. This increase was absent in circulating or splenic T cells, and since there was increased T cell infiltration into these tissues, CCL2 production may be further amplified during hypertension. CCL2 is a vital chemokine that promotes leukocyte recruitment, and elevated production can result in an enhanced local inflammatory response [12, 32], which may lead to advanced inflammation and organ dysfunction resulting in overt hypertension. T cells are known to produce CCL2 [33, 34], although macrophages are also an important source [35]. Consistent with our findings, previous studies have shown increased CCL2 production from infiltrating T cells in neoplasia/cancer [36, 37] and infiltrating leukocytes in arthritis [12, 38]. Our findings suggest that a hypertension-specific function of infiltrating T cells during hypertension may be to promote further leukocyte recruitment via increased CCL2 production. We speculate that recruited leukocytes, such as macrophages, may then further promote inflammation through cytokine/chemokine release in a feed-forward fashion. While elevated CCL2 in blood pressure-controlling organs has been shown [39], to our knowledge this is the first evidence that T cells are an important local source of CCL2 that may constitute a vital contribution to inflammation associated with hypertension. Leukocytes are known to express CCR2, to which CCL2 and several other chemokines such as CCL8, CCL13 and CCL27 can bind. Mice deficient in CCR2 exhibit decreased macrophage and monocyte infiltration into the arterial wall during Ang II-induced hypertension [39], suggesting that the CCR2:CCL2 axis is vital for leukocyte recruitment during hypertension. More recently we reported that CCR2 inhibition reduces vascular macrophage accumulation and reverses pressor responses in DOCA-salt induced hypertensive mice [18]. Thus the CCR2:CCL2 axis is an important pathway during hypertension and associated vascular disease, and may be initially driven by infiltrating T cells leading to sequalae of pro-inflammatory events.

In addition to elevated CCL2 production, a greater proportion of infiltrating T cells from aorta of hypertensive mice were shown to produce higher levels of intracellular and extracellular ROS. It is well established that elevated ROS results in much greater oxidative stress and promotes inflammation and organ dysfunction during hypertension. [40–42]. Guzik and colleagues [6] demonstrated that adoptive transfer of T cells can lead to impaired endothelium-dependent vasodilatation and increased ROS production following Ang II infusion in RAG-$1^{-/-}$ mice [6]. Importantly, adoptive transfer of T cells deficient in p47phox (an important subunit of Nox2 and Nox1 oxidase) into RAG-$1^{-/-}$ mice failed to fully restore hypertensive responses to Ang II, highlighting the potential role of T cell-derived ROS in hypertension [6]. Interestingly, this phenotypical difference was only localized to aortic T cells (predominantly CD4+ and DN T cell subsets) and was absent in all T cells from other organs, suggesting distinct regional effects of T cells during hypertension. Indeed it has been documented that T cells can behave differently in the presence of different cells [42]. Infiltrating T cells of the kidney

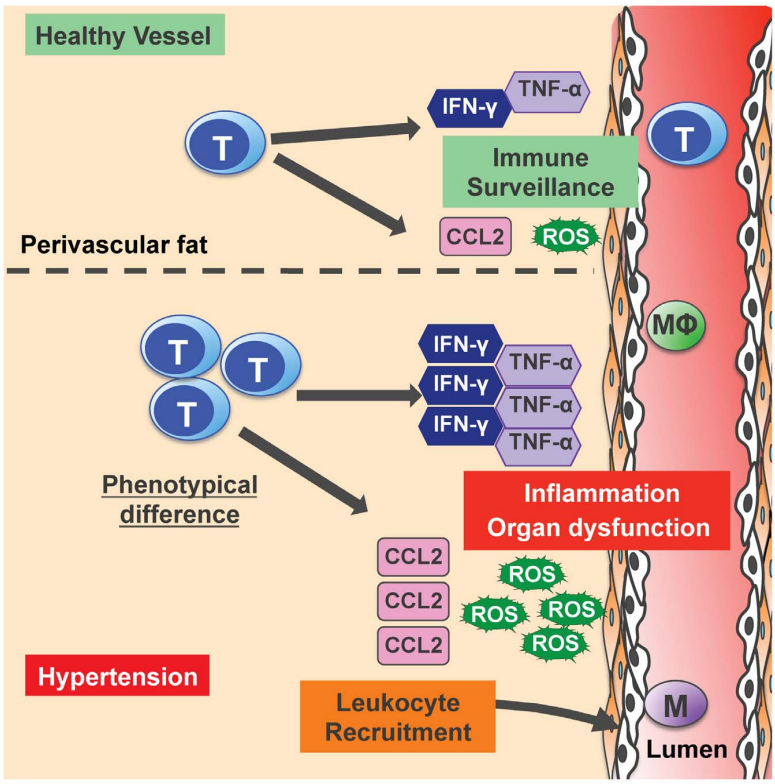

Fig. 7. Potential functions of infiltrating T cells during hypertension. Based on the results to our study and current literature, we speculate that in healthy vessels, there is basal level of T cell infiltration that may be required for immune surveillance. During hypertension, there is greater T cell infiltration localized to the perivascular fat of the aorta. Although each T cell infiltrate produces that same amount of cytokines as in healthy vessels, greater T cell infiltrate during hypertension may result in an overall net increase cytokines produced. However, indicative of a phenotypical difference, T cell infiltrates in vessels of hypertensive mice produce greater CCL2 and ROS, which can result in enhanced leukocyte recruitment such as macrophages (MΦ), oxidative stress and local inflammation that can lead to vascular dysfunction and exacerbate hypertension.

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have been suggested to promote oxidative stress and hypertension in Dahl salt-sensitive rats [43]. We did not observe greater T cell-derived ROS from kidney T cells, however, it is highly likely that other immune cells that can be recruited by T cells, such as macrophages or neutrophils, could act as sources of ROS in the kidney. Importantly, there was no change in ROS production in T cells from non-infiltrating organs (blood and spleen), which is consistent with CCL2 effects where an increase in CCL2 production was limited to infiltrate only. Indeed there is functional interplay between ROS and CCL2. ROS are known to increase CCL2 expression and infiltration of inflammatory cells in pressure-overloaded rat hearts [44]. Chen et al [45] reported that inhibition of NADPH oxidase reduced CCL2 mRNA accumulation in vascular endothelial cells, indicating that ROS plays an important role in the modulation of CCL2 gene expression. It is important to note

that we employed the Ang II-infusion model of hypertension, and Ang II may directly stimulate the effects we have observed in CCL2 and ROS production. However, based on previous studies, the interplay between T cells and the development of hypertension has been observed in several models of hypertension such as DOCA-salt- and noradrenaline-induced hypertension, which suggests that elevated pressure induces activation of the adaptive immune system. Moreover, DOCA-salt-induced hypertension is also associated with elevated aortic CCL2 mRNA expression [18]. Importantly, most human hypertension is associated with greater plasma levels of Ang II, particularly frequent in malignant hypertension [46, 47]. Thus, infiltrating T cells may be responsible for the functional interplay of oxidative stress and leukocyte chemotaxis and may represent a vital function of T cells during hypertension.

Collectively, we have demonstrated that cytokine profiles from infiltrating T cells are not differentially modulated by Ang II-induced hypertension, but rather the local milieu may be influenced by greater accumulation of T cells during hypertension. Moreover, aortic and kidney T cells exhibit greater CCL2 and ROS production (aorta only), and represent a hypertension-specific phenotype that may promote the local inflammatory responses by recruiting leukocytes and causing oxidative stress (Fig. 7). Based on our findings, specifically targeting leukocyte recruitment and homing of T cells to blood pressure controlling organs, may represent a potential therapeutic approach to reduce the burden of inflammation that is associated with hypertension and likely many other related vascular disorders.

Supporting Information

S1 Figure. Total number of (A) aortic and (B) kidney infiltrating T cell subsets in vehicle and Ang II-infused mice.
doi:10.1371/journal.pone.0114895.s001 (TIFF)

S2 Figure. Effector phenotype ($CD44^{hi}$+$CD62L^{lo}$) of (A) aortic and (B) kidney infiltrating T cell subsets in vehicle and Ang II-infused mice. (*P<0.05, ***P<0.001 Vs vehicle; Unpaired t test; n=6–14).
doi:10.1371/journal.pone.0114895.s002 (TIFF)

S3 Figure. FoxP3+ T regulatory cell infiltration. (A) Representative gating strategy for FoxP3+ cells (T regulatory cells) in aorta and kidney. After exclusion on dead cells, total leukocytes (CD45+), T cells (CD3+) and T cell subsets (CD4+, CD8+, DN) were sequentially gated. Finally Foxp3+ cells were gated from CD4+ T cells. (B) Mean data of infiltrating FoxP3+ T cells within aorta (left) and kidney (right) from vehicle and Ang II-infused mice. (*P<0.05 Vs vehicle; Unpaired t test; n=6).
doi:10.1371/journal.pone.0114895.s003 (TIF)

S4 Figure. IL-6 production from blood and organ-isolated T cells. Quantitative analysis of amount of IL-6 produced following anti-CD3/CD28 stimulation using

a CBA. Data represented as total amount of IL-6 produced in pg/ml per 10^5 T cells in blood, spleen, aorta, kidney and brain (n=11–22).
doi:10.1371/journal.pone.0114895.s004 (TIFF)

S5 Figure. IL-10 production from blood and organ-isolated T cells. Quantitative analysis of amount of IL-10 produced following anti-CD3/CD28 stimulation using CBA assay. Data represented as total amount of IL-10 produced in pg/ml per 10^5 T cells in blood, spleen, aorta and kidney (n=11–22).
doi:10.1371/journal.pone.0114895.s005 (TIFF)

Author Contributions

Conceived and designed the experiments: ZW REW AV. Performed the experiments: ZW IS HD AV. Analyzed the data: ZW REW AV. Contributed reagents/materials/analysis tools: IS HD REW AV. Wrote the paper: ZW GRD REW AV.

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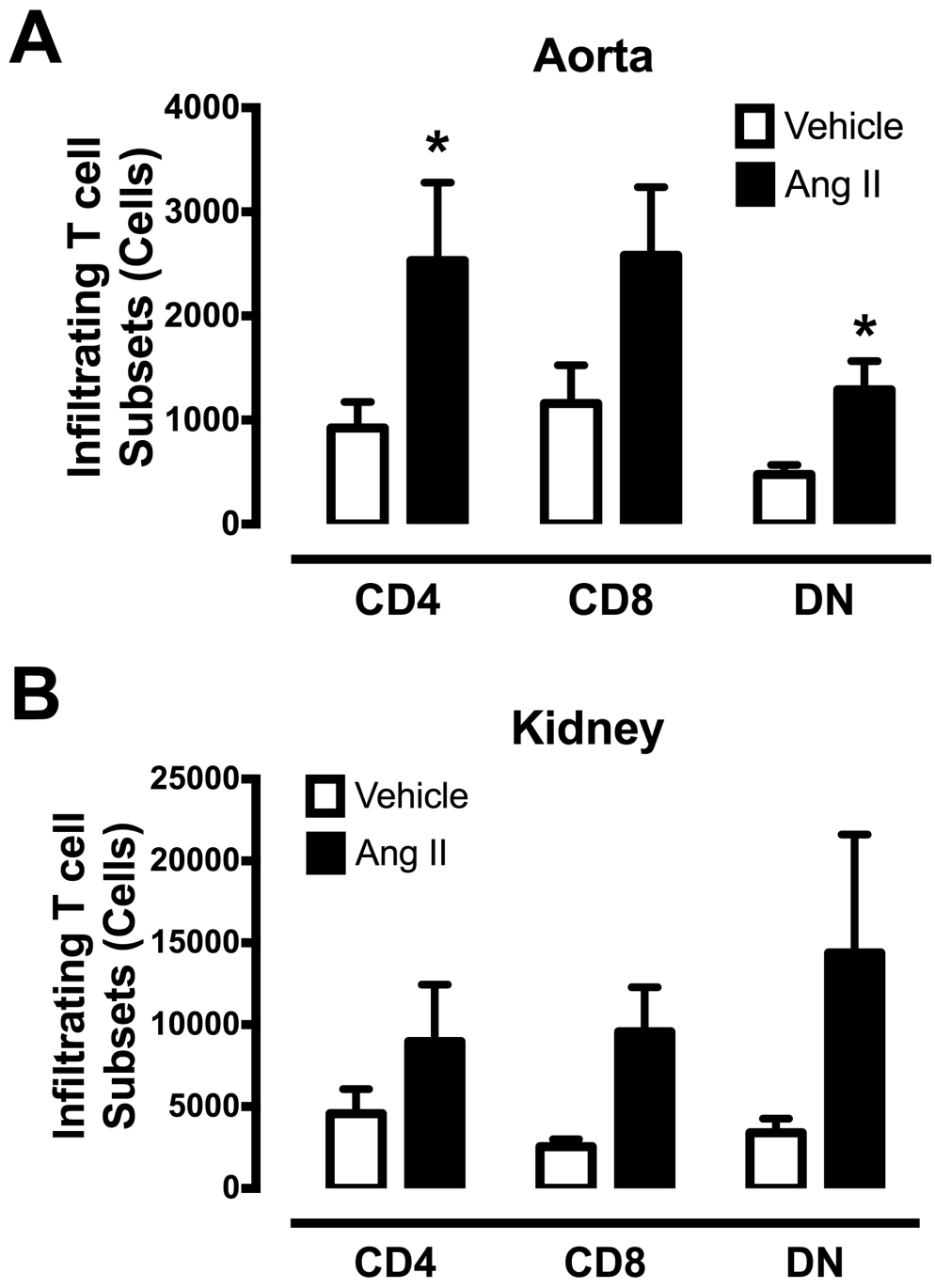
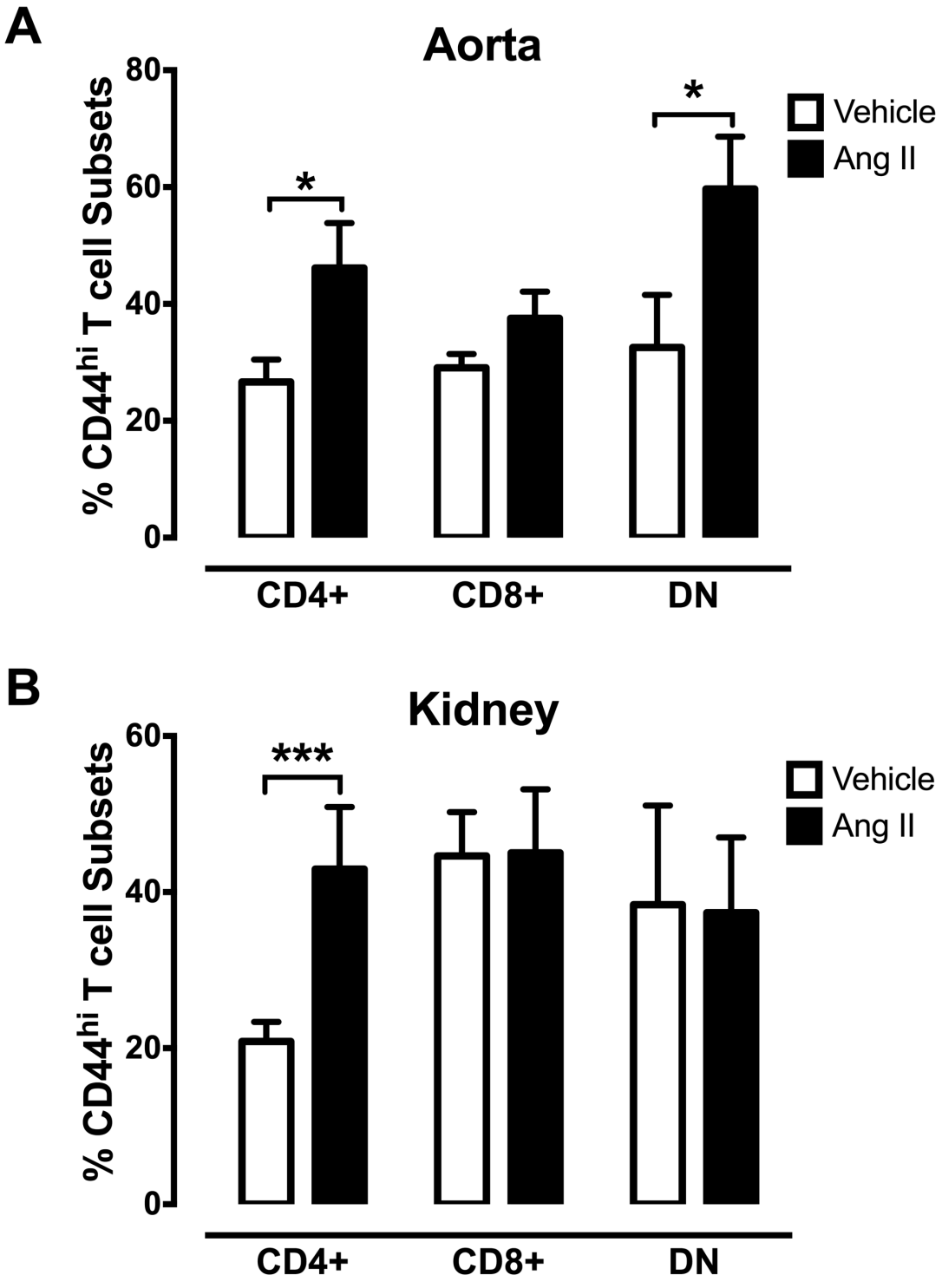
Supplemental Material

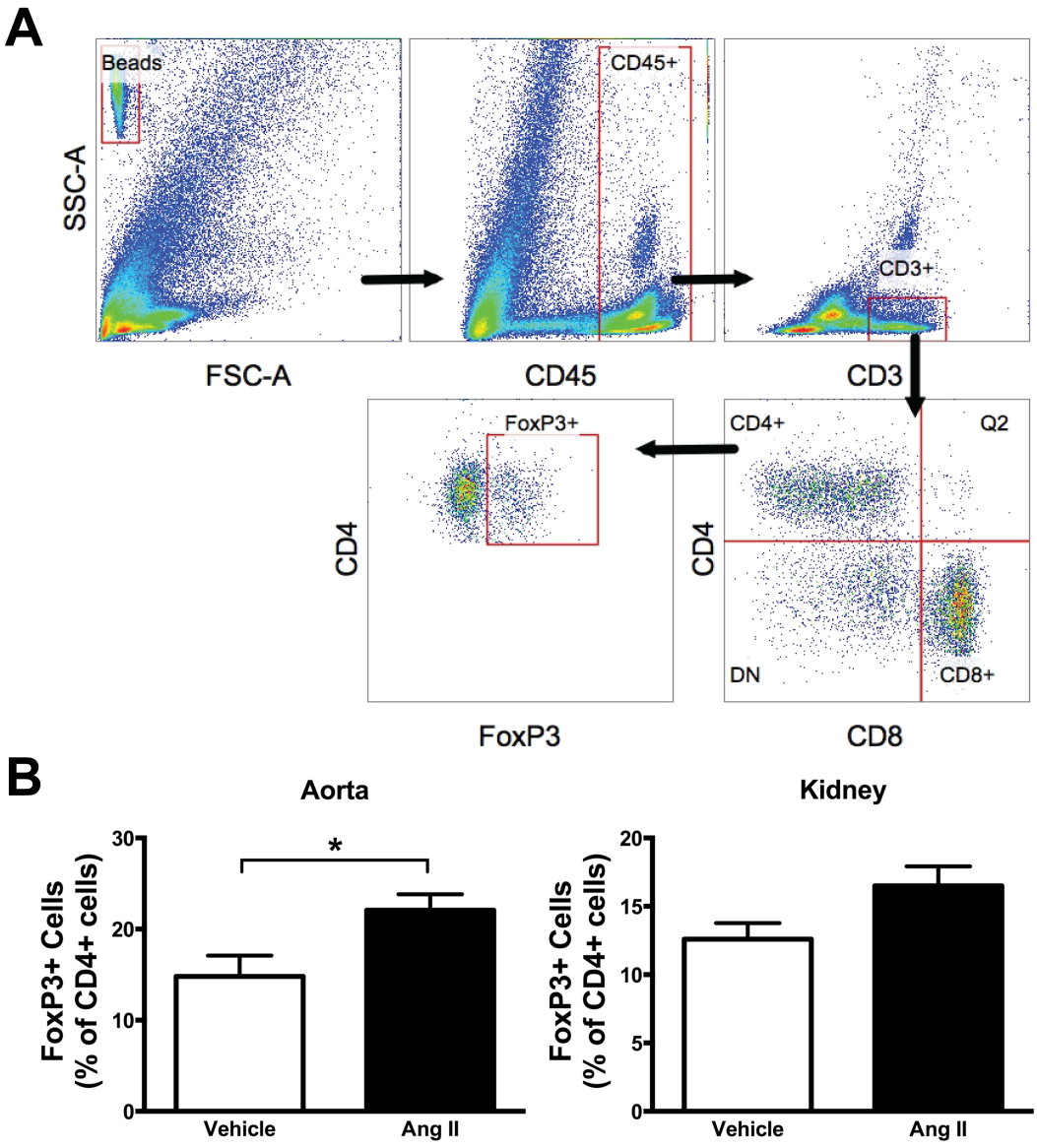


S1 Figure. Total number of (A) aortic and (B) kidney infiltrating T cell subsets in vehicle and Ang II-infused mice.

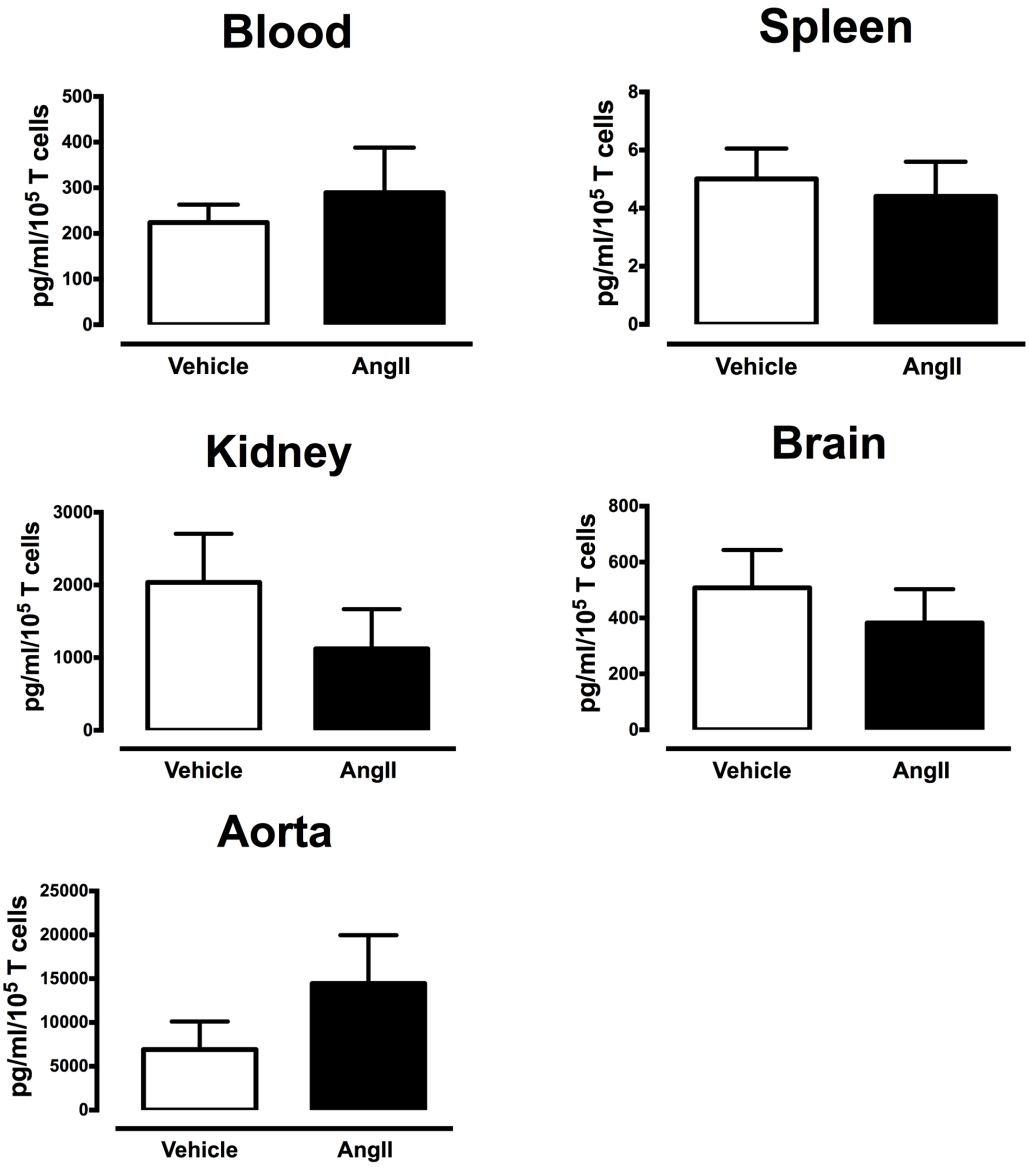


S2 Figure. Effector phenotype (CD44hi+CD62Llo) of (A) aortic and (B) kidney infiltrating T cell subsets in vehicle and Ang II-infused mice. (*P<0.05, ***P<0.001 Vs

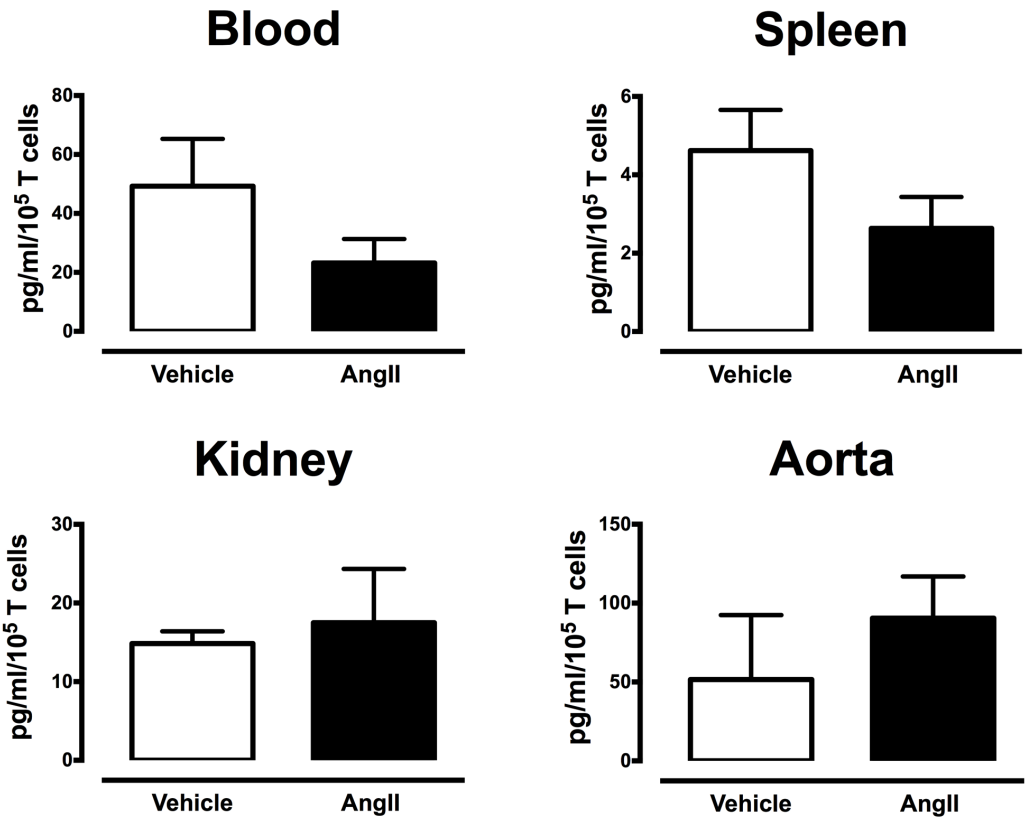
vehicle; Unpaired t test; n=6–14).



S3 Figure. FoxP3+ T regulatory cell infiltration. (A) Representative gating strategy for FoxP3+ cells (T regulatory cells) in aorta and kidney. After exclusion on dead cells, total leukocytes (CD45+), T cells (CD3+) and T cell subsets (CD4+, CD8+, DN) were sequentially gated. Finally Foxp3+ cells were gated from CD4+ T cells. (B) Mean data of infiltrating FoxP3+ T cells within aorta (left) and kidney (right) from vehicle and Ang II-infused mice. (*P<0.05 Vs vehicle; Unpaired t test; n=6).



S4 Figure. IL-6 production from blood and organ-isolated T cells. Quantitative analysis of amount of IL-6 produced following anti-CD3/CD28 stimulation using a CBA. Data represented as total amount of IL-6 produced in pg/ml per 10^5 T cells in blood, spleen, aorta, kidney and brain (n=11–22).



S5 Figure. IL-10 production from blood and organ-isolated T cells. Quantitative analysis of amount of IL-10 produced following anti-CD3/CD28 stimulation using CBA assay. Data represented as total amount of IL-10 produced in pg/ml per 10^5 T cells in blood, spleen, aorta and kidney (n=11–22).

Chapter 4: Effect of Tissue-Infiltrating T cells on vascular dysfunction

DIRECT IN SITU STIMULATION OF VASCULAR INFILTRATING T CELLS EXACERBATES ANGIOTENSIN II-INDUCED ENDOTHELIAL DYSFUNCTION

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Running title: Vascular T cells Exacerbate Endothelial Dysfunction

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Abstract

Experimental hypertension is associated with significant accumulation of T cells into the perivascular adipose tissue (PVAT) surrounding the aorta. However, whether T cells directly influence vascular function during hypertension remains unclear. The current study aimed to determine the effect of *in situ* stimulation of infiltrating T cells on endothelial function during hypertension. C57BL/6J mice received 14-day sham or angiotensin (Ang) II (490 ng/kg/min) infusion via osmotic minipump. Following sham/Ang II infusion, aorta rings with and without PVAT-intact were incubated for 16 hours with monoclonal anti-CD3 and anti-CD28 antibodies to directly stimulate T cells. *In vitro* isolated vessel organ bath experiments revealed that *in situ* activation of local vascular T cells exacerbates Ang II-induced endothelial dysfunction, but had no effect on sham-treated mouse aorta. T cell-mediated exacerbation of endothelial dysfunction was absent in vessels without PVAT or in the presence of reactive oxygen species (ROS) scavenger, tempol. Dihydroethidium staining and L-012-enhanced chemiluminescence showed that ROS production was augmented in vessels following anti-CD3/CD28-stimulation in aorta obtained from Ang II-treated mice, which was attenuated in vessels co-treated with tempol. This is the first evidence that *in situ* activation of perivascular T cell infiltrates exacerbates endothelial dysfunction during hypertension potentially via a ROS-dependent mechanism.

Introduction

Hypertension is a highly prevalent cardiovascular disease that affects one-third of the population in the world. It is a major risk factor for myocardial infarction, stroke, renal failure and atherosclerosis, which are a major source of death in western societies. In last decade, there is increasing evidence that inflammation and immunity plays an important role in the pathogenesis of hypertension.[1] A seminal study implicated a role for T cells in the development of hypertension, and highlighted the accumulation of T cells within the aortic wall of hypertensive mice. While T cell infiltrates were primarily localised to the perivascular adipose tissue (PVAT) surrounding the aorta, the specific pathophysiological role of these T cell infiltrates remains unclear.[2-6]

Adipose tissue is traditionally considered as site for energy storage. However, PVAT and visceral fat have been documented to be sites of accumulation for immune cells.[4,7,8] Increasingly, there are studies suggesting a role for PVAT in vascular function, inflammation and vessel remodelling.[9-12] The homeostatic function of the PVAT appears to be protective since healthy rat aorta and porcine coronary artery with PVAT intact, exhibit blunted vasoconstriction responses.[13,14] However, in disease states such as atherosclerosis, upregulation of inflammatory gene expression together with accumulation of macrophages and T lymphocytes at the border between lamina adventitia and PVAT in human aorta was reported, [15,16] which suggests the potential role of PVAT in promoting vascular inflammation. Guzik et al demonstrated that T cells are crucial in the development of experimental hypertension,[4] where recombinase activating gene-1 deficient (RAG-1$^{-/-}$) mice, which lack T and B cells, are protected from vascular

dysfunction.[4] In the setting of hypertension, infiltration of leukocytes including T cells into PVAT,[4] is associated with endothelial dysfunction since the absence of T cells in recombinase activating gene-1 deficient (RAG-1$^{-/-}$) mice, restores endothelium-dependent vasorelaxation responses. Many recent studies have shown that recruitment of T cells into target organs such as blood vessel and kidney, is associated with in the pathogenesis of hypertension[3,4,17] and end organ damage by promoting local inflammation.[7,18]

In light of these reports, T cells may potentially be an important link between PVAT modulation of vascular function and disease. A likely mechanism proposed in T cell-induced hypertension is through the release of cytokines and ROS, which can promote inflammation and vascular remodelling in various blood pressure-controlling organs.[19] NADPH oxidise-derived ROS production is essential in the maintenance of normal vascular homeostasis.[20,21] However, there is much evidence that elevated oxidative stress and ROS is associated with the progression of cardiovascular disease, such as hypertension.[22,23] While the majority of studies have focused on endothelial- and vascular smooth muscle cell (VSMC)-derived ROS in vascular disease[22], it has become apparent that the adventitia can also be a major source of ROS. Csanyi and colleagues[24] reported that the adventitia is the predominant site of ROS production, and that aortic adventitial-derived ROS promotes endothelial dysfunction. T cells are also a source of ROS which is primarily produced via NADPH oxidase 2 (Nox2).[22,25] Although the quantity of ROS produced from T cells is relatively less than other immune cells such as macrophages and neutrophils, Jackson et al suggested that stimulation of T cells could induce rapid generation of ROS, which could contribute to local oxidative stress.[26] Guzik and his

colleagues suggested that T cell-derived ROS contributes to hypertension, since adoptive transfer of T cells from mice deficient in p47phox (an important subunit of Nox1 and Nox2) into RAG-1$^{-/-}$ mice only partially restored hypertensive responses to Ang II.[4] This observation highlighted the role of T cell-derived ROS in hypertension, and that T cells may be a local source of ROS following T cell infiltration to cause damage in the vasculature.[4] Indeed, we recently identified that isolated aortic T cells showed a significant increase in phorbol 12,13-dibutyrate (PDBu)-stimulated intracellular ROS production in Ang II-infused mice compared to vehicle-treated mice.[5]

While T cells are thought to accumulate in the PVAT and release ROS, the direct effect of T cell-derived ROS on vascular function during hypertension has not been determined. Therefore, the current study investigated the effect of directly activating T cell infiltrates within an intact aorta *in situ*, on vascular function during hypertension. We identified that *in situ* stimulation of vascular T cells augments endothelial dysfunction in diseased hypertensive mouse vessels, which was not observed in aorta obtained from sham-treated mice. This study highlights a potential pathophysiological role for infiltrating T cells in vascular dysfunction associated with hypertension.

Methods

Animals and experimental design

This study was approved by Monash University Animal Ethics Committee (Project MARP/2013/059) and was performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. A total of 61 Male C57BL6/J

mice (8-12 weeks, 25-32g) were obtained from Monash Animal Services (MAS) and housed on 12 hour light/dark cycle with food and water provided *ad libitum*. Inhaled isoflurane-anaesthetized mice were randomly allocated to receive 14-day infusion of either vehicle (0.15M NaCl, 1% acetic acid) or Ang II (490ng/kg/min) via osmotic minipumps (Alzet model 2002) implanted subcutaneously.

Blood pressure monitoring

Systolic blood pressures (SBP) were measured using a non-invasive tail-cuff apparatus (MC4000 Blood Pressure Analysis System, Hatteras Instruments) prior to treatment and on day 14 to confirm elevations in blood pressure. At each time point over 20 measurements were recorded and averaged.

Tissue harvesting and in situ T cell stimulation

Mice were euthanased by CO_2 inhalation (procedure approved by the Animal Ethics Committee of Monash University). Whole aorta was removed and placed in ice-cold Krebs-bicarbonate buffer (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5,$NaHCO_3$ 25, and glucose 11.7. Thoracic and abdominal aorta was cut transversely into 3mm ring segments, and half of abdominal aortic rings were cleaned of PVAT and connective tissue under a dissecting microscope. For acute T cell stimulation, vessels were incubated in the organ bath for 2 hours in the presence of anti-CD3 (clone 145-2C11; 5µg/mL, Biolegend) and anti-CD28 (clone 37.51; 5µg/mL; Biolegend) monoclonal antibodies. For long term T cell activation, vessels were placed in complete RPMI 1640 media containing 10% FBS, penicillin (100 U/ml)/streptomycin

(100 μg/ml) and L-glutamine (2 mM) and incubated overnight (16 hours) with anti-CD3/CD28 monoclonal antibodies. To examine the role of ROS on vascular function, analogous 16 hour incubations were performed in the absence or presence of the ROS scavenger, 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol; 200μM; Sigma-Aldrich). Following incubation, some thoracic segments were frozen in OCT for ROS detection using dihydroethidium (DHE) staining.

Assessment of vascular function

In vitro organ bath experiments were used to examine vascular function from isolated vascular rings with or without PVAT following *in situ* T cell stimulation. Two 100 μm stainless steel wires were threaded through the lumen of each aortic ring (~3 mm long) and the rings were then mounted and suspended in vertical 10mL organ baths containing Krebs-bicarbonate solution, maintained at 37°C and continuously bubbled with carbogen (95% O_2 and 5% CO_2). Isometric tension was continuously measured via a force transducer (Grass FT03) interfaced to a MacLab data acquisition device (ADInstruments, Sydney, Australia). Aortic rings were stretched to their optimum resting tension of 0.5 grams and allowed to equilibrate for 40 minutes, during which Krebs-bicarbonate solution was replaced every 15 minutes. Following equilibration, tissues were maximally contracted with the thromboxane A2 analogue, U46619 (0.6 μM). Tissues were then washed with Krebs-bicarbonate solution at 10 minute intervals and allowed to equilibrate for 1 hour or until tissues returned to baseline. Vessels were pre-contracted with U46619 to 60-70% of the maximum contractile response, from which cumulative concentration-response curves to acetylcholine (ACh; 1nM-10μM) were constructed. To examine

vascular smooth muscle cell (VSMC) integrity, concentration-response curves to sodium nitroprusside (SNP) were performed in U46619-pre-contracted vessels. To test the effect of direct T cell stimulation on nitric oxide (NO) bioavailability, vessels were pre-contracted with U46619 to 30% of the maximum contractile response, from which contractions in response to NOs inhibition with Nω-nitro-L-arginine methyl ester (L-NAME; 10μM) was measured.

ROS Detection - DHE staining

Thirty-micron cryosections of pre-treated (unstimulated, anti-CD3/CD28 stimulated ± tempol) frozen thoracic aorta in OCT with perivascular intact were stained with 2μM DHE for 45 minutes in a dark and humidified incubator at 37°C. DHE fluorescence was imaged using Nikon C1 inverted confocal microscope. Images were acquired using a 20x oil-immersion objective and emitted fluorescence of 543nm was detected using a 590/50nm bandpass filter. A z-stack of 6 optical sections spaced 4-5 μm apart with 16 fields of view were acquired at 512x512 pixels, Fluorescence was quantified as intensity per area (arbitrary units/um^2) in the PVAT of vessels using ImageJ Fiji software as previously described.[27]

ROS Detection L-012 enhanced chemiluminescence

To detect the extracellular ROS produced from isolated aortic rings, L-012 enhanced chemiluminescence was employed. Analogous to the vascular function experiments, aortic rings were stimulated *in situ* with monoclonal anti-CD3/CD28 antibodies overnight and then placed in individual wells of a 96-well plate containing Krebs-HEPES buffer (in

mM: NaCl 118; KCl 4.7; KH_2PO_4 1.2; $MgSO_47H_2O$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 11.7; HEPES 20, pH 7.4). Following a 30-minute incubation at 37^oC, L-012 (100 μM) was added to each well, which was then loaded into a Hidex Chameleon Luminescence detector. ROS formation was detected under basal conditions for 30 cycles before phorbol 12,13-dibutyrate (PDBu; 100μM), direct activator of protein kinase C, was added to detect ROS production following stimulation. All samples were measured in triplicate and photon emissions were recorded for 30 cycles at 2-minute intervals. Photon emissions (relative light units per second) were then averaged over the final 20 cycles.

Statistical Analysis

All data was expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism (Version 7.0). Data were analysed using two-way ANOVA, followed by Bonferroni post hoc test. A p-value of <0.05 was considered statistically significant.

Results

Systolic Blood Pressure (SBP)

Using non-invasive tail-cuff measurements, hypertension was confirmed in Ang II-treated mice at day 14 (SBP: vehicle 118±2 vs Ang II 151±4 mmHg; $P<0.0001$, n=28-33).

Effect of in situ T cell stimulation on aortic vasorelaxation and vasocontraction

Vascular reactivity study was employed to study endothelial function using isolated aorta, whereby the local vascular T cells were activated by pre-incubating intact aortic rings

with monoclonal anti-CD3/CD28 antibodies prior to vascular reactivity experiments. In non-stimulated vessels with PVAT-intact, maximal endothelium-dependent vasorelaxation responses to ACh were impaired in aortic rings from Ang II-treated mice compared to sham-treated mice (Figure 1A). Acute stimulation of T cells for 2 hours using monoclonal anti-CD3/CD28 antibodies had no effect on endothelial vasorelaxation responses (Figure 1A) from sham- and Ang II-treated aorta. In contrast, in vessels that were incubated overnight with anti-CD3/CD28, we observed significant exacerbation of endothelial dysfunction in Ang II-treated mouse aorta, which was not observed in sham mouse aorta (Figure 1B). Interestingly, removal of the PVAT abolished the anti-CD3/CD28-induced exacerbation of endothelial dysfunction (Figure 1C). Elevated ROS can damage VSMCs via oxidation of soluble guanylate cyclase, leading to impaired production of cyclic guanosine 3', 5'-cyclic monophosphate (cGMP) and subsequently reduced vasorelaxation to NO donors. To gain additional insight into whether ROS produced in response to *in situ* T cell stimulation directly causes oxidative stress on VSMCs, concentration-response curves to SNP were performed. SNP evoked-vasorelaxation was similar in aorta obtained from sham- and Ang II-treated mice, following anti-CD3/CD28 stimulation, suggesting no impairment of VSMC function following anti-CD3/CD28 stimulation (Figure 1D). Furthermore, stimulating T cells with monoclonal anti-CD3/CD28 antibodies had no effect on maximum contraction and pre-constriction to U46619 in aortic rings from sham- and Ang II-treated mice following overnight treatment with anti-CD3/CD28 in presence or absence of tempol (Supplementary Figure 1).

ROS production following in situ T cell stimulation

DHE staining was used to localise and quantify ROS in vessels following *in situ* anti-CD3/CD28-mediated T cell activation using the overnight incubation protocol described above. In unstimulated vessels, we observed significantly elevated intensity of DHE staining in the PVAT of aorta from Ang II mice (Figures 2A and 2B). Strikingly, in Ang II-treated aorta, ROS production was further augmented following anti-CD3/CD28-stimulation, which was attenuated in vessels co-treated with ROS scavenger, tempol. Consistent these findings, L-012 enhanced chemiluminescence revealed ~2-fold elevation in PDBu-stimulated ROS production in anti-CD3/CD28-stimulated compared to unstimulated Ang II-treated mouse aorta (Figure 2C). No changes in basal ROS production was observed (Figure 2C). This effect was again abolished in the vessels co-incubated with tempol, but importantly, anti-CD3/28-induced elevations in ROS production were absent in vessels without PVAT.

Effect of scavenging ROS in situ on T cell-mediated exacerbation of endothelial dysfunction

We examined whether ROS as a result of T cell activation was causing endothelial dysfunction. We performed analogous experiments with PVAT intact and pre-incubated aortic rings with monoclonal anti-CD3/CD28 antibodies with and without tempol. Consistent with our previous results anti-CD3/CD28 co-incubation caused further impairment of endothelium-dependent vasorelaxation (Figure 3A). In the presence of tempol, the augmented endothelial dysfunction was abolished. We also examined NO bioavailability in vessels following *in situ* T cell stimulation. L-NAME evoked larger

contractile responses from sham-treated mouse aorta compared to Ang II-treated mouse vessels (Figure 3B). Stimulation of aortic rings with anti-CD3/CD28, further reduced L-NAME-mediated contractions compared to unstimulated vessels. This augmented reduction in NO bioavailability effect was reversed in vessels when stimulated in the presence of tempol.

Discussion

For the past decade, it has been known that T cells contribute to the progression of hypertension. However, how an immune cell can directly influence vascular physiology is not clear. Our previous study demonstrated phenotypical differences of vascular infiltrating T cells compared to circulating or splenic T cells during hypertension, such that they release greater amounts of ROS and CCL2.[5] The main finding of the current study was that direct stimulation of T cells within the vasculature *in situ*, exacerbates Ang II-induced endothelial dysfunction in these vessels obtained from hypertensive mice. *In situ* T cell activation also enhanced vascular ROS production, which most likely contributed to the vascular dysfunction. Indeed, tempol abolished ROS and also reversed the augmented endothelial dysfunction. To our knowledge, this is the first study to demonstrate a pathophysiological effect of direct *in situ* stimulation of infiltrating T cells on vascular dysfunction in the setting of hypertension.

Given the strong association between endothelial dysfunction, hypertension and the localised accumulation of T cells in the PVAT surrounding the aorta, a pathophysiological role for T cells on vascular function may not be considered surprising.

Previous studies including the seminal study from Guzik et al, [4] have been largely associative since RAG-1$^{-/-}$ mice are protected from endothelial dysfunction, which is restored following adoptive transfer of T cells. In the current study, we applied a unique approach by stimulating the infiltrating T cells *in situ* within intact aorta using monoclonal antibodies, which are commonly used to stimulate T cells *in vitro* to assess T cell phenotype and function.[28] This is the first study in the field to examine effect of direct *in vitro* T cell stimulation on vascular function, thus acute and overnight stimulation were employed to identify whether the effect is acute or chronic. Interestingly, it was found that acute stimulation for 2 hours did not influence endothelium-dependent vasorelaxation in Ang II-treated mouse aorta, whereas overnight stimulation of T cells further impaired maximal relaxation responses. This finding indicated that a longer period of stimulation was required for activated T cells to promote endothelium dysfunction, possibly due to release of detrimental mediators including cytokines and ROS. In the current study we did not examine the vessels for longer than 16 hours due to concerns of tissue viability. It is important to note that the novelty of this finding and the distinction from previous studies implicating T cells and vascular dysfunction is the activation of T cells within the vasculature, using classical T cell-activating monoclonal anti-CD3/CD28 antibodies, promoted endothelial dysfunction. This effect was only observed in aorta obtained from Ang II-induced hypertensive mice, but not sham-treated mice, which consolidates previous findings from our laboratory suggesting a unique T cell phenotype that is specific to hypertension.[5]

Since infiltrating T cells reside within the PVAT of the aorta during hypertension,[4] the importance of removing the PVAT on vascular function when stimulating T cells *in situ* was examined. PVAT is connected to the laminar adventitia of most conduit vessels and is thought to communicate with the vessel wall to regulate vascular function, inflammation and maintain vascular homeostasis.[11,13,14,29] Previously, PVAT was considered as a passive structural support for the vessel and was removed in many isolated vessel studies. However, PVAT is known to negatively influence vascular contractile responses to noradrenaline in rat aorta.[14] PVAT was also shown to have inhibitory action on aortic contractions by releasing adipocyte-derived relaxing factor (ADRF).[13] However, during postnatal obesity where inflammation is heightened, ADRF-induced anti-contractile effects on the vasculature were abrogated.[30] The findings from the current study suggest a pathological influence of the PVAT in mediating T cell-dependent effects on vascular function, since removal of the PVAT abolished the exacerbation of endothelial dysfunction and ROS production following *in situ* activation of vascular T cells. These observations suggest that the T cell infiltrate within the PVAT from Ang II-treated mouse aorta is indeed playing a direct role in modulating endothelium-dependent vasorelaxation, which is consistent with adipose tissue being considered as reservoir for immune cell residence.[7,8]

Vascular inflammatory diseases such as atherosclerosis are associated with heightened PVAT inflammation that involves the release of pro-inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6),[31] to cause local accumulation of inflammatory cells. Perivascular adipocytes appear to be a

large source of proinflammatory mediators, and produce them in quantities much greater than other adipocytes[16,31]. Recently, PVAT was reported to contribute to vascular stiffness in obese mice,[32] which suggested the importance of PVAT in vascular remodelling. Collectively, it is clear that the PVAT can be considered an important regulatory vascular structure that recruits and harbours immune cells that can exert direct effects on vascular function.

We have previously shown that the aforementioned unique T cell phenotype has a much greater propensity to produce ROS when isolated from hypertensive mouse aorta.[5] In the current study, it was shown for the first time that elevated T cell-derived ROS accumulates in the PVAT during hypertension. Since Nox2 has been suggested to be a primary source of ROS,[22,25] we believe that T cell-derived ROS may be via Nox2 in our system. In support of Nox2 as the source T cell-derived ROS in response to PDBu-stimulation, vascular PDBu-dependent ROS formation has been shown to require Nox2 at least in the cerebral vasculature.[33] Notably, using DHE staining we observed elevated ROS levels throughout the PVAT in aorta from Ang II-treated mice, which was further augmented with T cell activation. This finding suggests that T cell activation alone, is able to directly increase basal ROS production within the PVAT. Although changes in basal ROS were not detected using L-012 chemiluminescence, this most likely reflects the sensitivity of the L-012 assay. However, the intense DHE staining throughout the PVAT following T cell stimulation is also likely in response to T cell-derived cytokine production and subsequent ROS production from other cells of the PVAT including macrophages and adipocytes. Importantly, potential mechanisms involved with the

augmented endothelial dysfunction may involve enhanced ROS production. This notion was supported by reduced NO bioavailability evident in vessels that received T cell stimulation and that the ROS scavenger tempol reversed both the endothelial dysfunction and L-NAME-induced contraction in aortic ring from Ang II-treated mice. Notably, no changes in overall maximal or pre-contractile responses to U46619 were observed following *in situ* T cell stimulation in the absence or presence of tempol. This finding further supports the selective depletion of NO bioavailability since L-NAME contractions were only affected following T cells stimulation. In support of a direct influence of activating T cells on endothelial cells, the stimulation of T cells *in vitro* in vessels obtained from Ang II-induced hypertensive mice did not affect vascular response to SNP. Classically, oxidation of sGC to the heme-free form impairs responses to $NO^{\cdot}$ donors such as SNP, resulting in a rightward shift of the concentration-response curve.[34,35] However, in current study since vasorelaxation to SNP was not affected in hypertensive mouse vessels, it suggested that the sGC was not oxidized.

The mechanism by which highly reactive short-lived free radicals from the outer PVAT affect the endothelium at the lumen is difficult to reconcile. In the current study, we cannot eliminate the possibility that T cell-derived ROS in PVAT may have diffused to the endothelial cells through the culture media, which would not be conceivable in an *in vivo* setting. However, there is strong evidence suggesting that leukocyte and ROS accumulation initiated in the outer adventitial layer can actually progress inwardly through the media inward towards the intima.[36-38] ROS generated in adventitia can potentially act as a paracrine mediator modifying downstream signalling mechanism in

smooth muscle cells to induce endothelial dysfunction.[39] The "outside-in" mechanism proposed by Pagano's group suggests that vascular inflammation initiates development of microvessels in the vasa vasorum.[24] It was identified that during Ang II-induced hypertension, ROS generated in aortic adventitial was 5.5-fold higher than in intima[40] indicating adventitia as a major source of ROS.[41] In addition, ROS released in the outer adventitial layer may be enzymatically dismutated to H_2O_2 that is relatively stable, cell-permeable,[24] and would be capable causing endothelial dysfunction.[42]

Oxidative stress and inflammation are known to be involved in causing endothelial dysfunction and vascular remodelling, and it is evident that there is functional interplay between the two conditions.[43-45] Elevated ROS production can act as a chemotactic signal for leukocytes to release greater amounts of chemokines,[5,46] and T cells are known to be source of cytokines, chemokines (C-C motif) ligand 2 (CCL2) and RANTES.[47] This could also be a feed-forward propagation process *in vitro* where T cell-derived ROS promote recruitment of more immune cells such as macrophages and T cells, which could further contribute to elevated production of ROS. ROS can activate the NF-kappaB pathway and promote release of inflammatory cytokines in the adventitia such as IL-1β, IL-6 and TNF-α,[24,43,48] therefore promoting expression of endothelial adhesion molecules on the endothelial lining of vasa vasorum and leading to the transmigration of inflammatory cells to the PVAT of the conduit vessel.[24] As CCL2 and RANTES are powerful chemoattractants, their overproduction would further amplify the inflammatory response in vessel wall and thus vascular dysfunction. Fukui and colleagues[47] have suggested that elevated inflammatory mediators can enhance NADPH oxidase-induced

ROS production, which further promote oxidative stress vasculature. Although the current study involves isolated vessels and T cell stimulation *in vitro* and is not likely to include these feed forward mechanisms, we speculate that in an *in vivo* setting, the net effect of vascular dysfunction and inflammation following *in situ* T cell activation could be amplified (Figure 4).

In summary, it is known that during hypertension there is greater T cell infiltration into the vasculature. The current study identified that T cells in PVAT directly contributed to endothelial dysfunction, which most likely involved increased ROS and oxidative stress.

Author Contributions

ZW designed and performed experiments, analysed and interpreted data and wrote the manuscript. IS and HD performed experiments and contributed to data analyses. GRD contributed to the design of experiments and interpretation of data. REW and AV designed and performed experiments, analysed and interpreted data and edited the manuscript.

Additional Information

The authors have no competing financial interests to declare.

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Figures Legends

Figure 1. Direct activation of local vascular T cells exacerbates Ang II-induced endothelial dysfunction. Isolated abdominal aortic rings were employed to examine vascular relaxation responses following (A) acute (n=10) and (B) overnight direct T cell stimulation (anti-CD3/CD28) in aortic rings with PVAT intact (*P<0.05; two-way ANOVA at maximum relaxation; n=7-14). (C) Effect of removing PVAT on % relaxation following overnight direct T cell stimulation (n=6-10). (D) Response to SNP in sham- and Ang II- treated mice, with and without Anti-CD3/CD28 co-stimulation (n = 4-5). Data represented as mean ± SEM.

Figure 2. *In situ* T cell stimulation augments Ang II-induced elevations in ROS production (A) Representative DHE staining for superoxide in sham- and Ang II- treated mouse thoracic aorta pre-incubated with vehicle, anti-CD3/CD28 and tempol. Red staining represents DHE+ fluorescence and green staining is autofluorescence in elastin layers in the medial wall. Small dashed box represents the origin of the magnified inserted image. (B) Mean intensity per area (arbitrary units/um^2) of DHE+ staining in PVAT of thoracic aorta. (#P<0.05 Vs same treatment from sham; *P<0.05 2-way ANOVA; n=3-5). (C) Mean ratio of L-012 chemiluminescence detection of extracellular ROS from Ang II aortic rings with and without PVAT intact. Data represented as relative light units (RLU) per second per mg aorta expressed as a ratio to responses from Ang II-treated mouse vessels without anti-CD3/28 stimulation. The right axis and grey thatched bars represent the basal L-012 responses for each treatment group, expressed as a ratio to the PDBu-stimulated response (*P<0.05 Vs vehicle; 2-way ANOVA; n=4-8).

Figure 3. Scavenging ROS during in situ T cell stimulation reverses endothelial dysfunction. (A) Effect of tempol on vascular relaxation in aortic ring from Ang II-induced hypertensive mice with PVAT intact treated with or without anti-CD3/CD28 stimulation. (*P<0.05; two-way ANOVA at Maximum relaxation; n=6-12). (B) Percentage in change in contractile responses to L-NAME in aorta from sham- and Ang II- treated mice, following overnight pre-incubation with anti-CD3/CD28 and/or tempol. (**P<0.01; *P<0.05 Vs normotensive aorta; 2-way ANOVA; n=8-13, normotensive aorta with anti-CD3/CD28+ tempol n=2). Data represented as mean ± SEM.

Figure 4. Potential pathophysiological role of infiltrating T cells. Hypertension is associated with increased T cell accumulation within the vasculature that is localised to the PVAT. Unique to hypertensive mouse aorta, activation of these T cell infiltrates leads to excessive ROS production from T cells and potentially other cells in the PVAT including macrophages and adipocytes that can influence endothelium-dependent vasorelaxation and/or promote PVAT inflammation that can also feed forward to further impair vascular function. (L: lumen, Int: intima, M: media, PVAT: perivascular adipose tissue)

Figures

Figure 1.

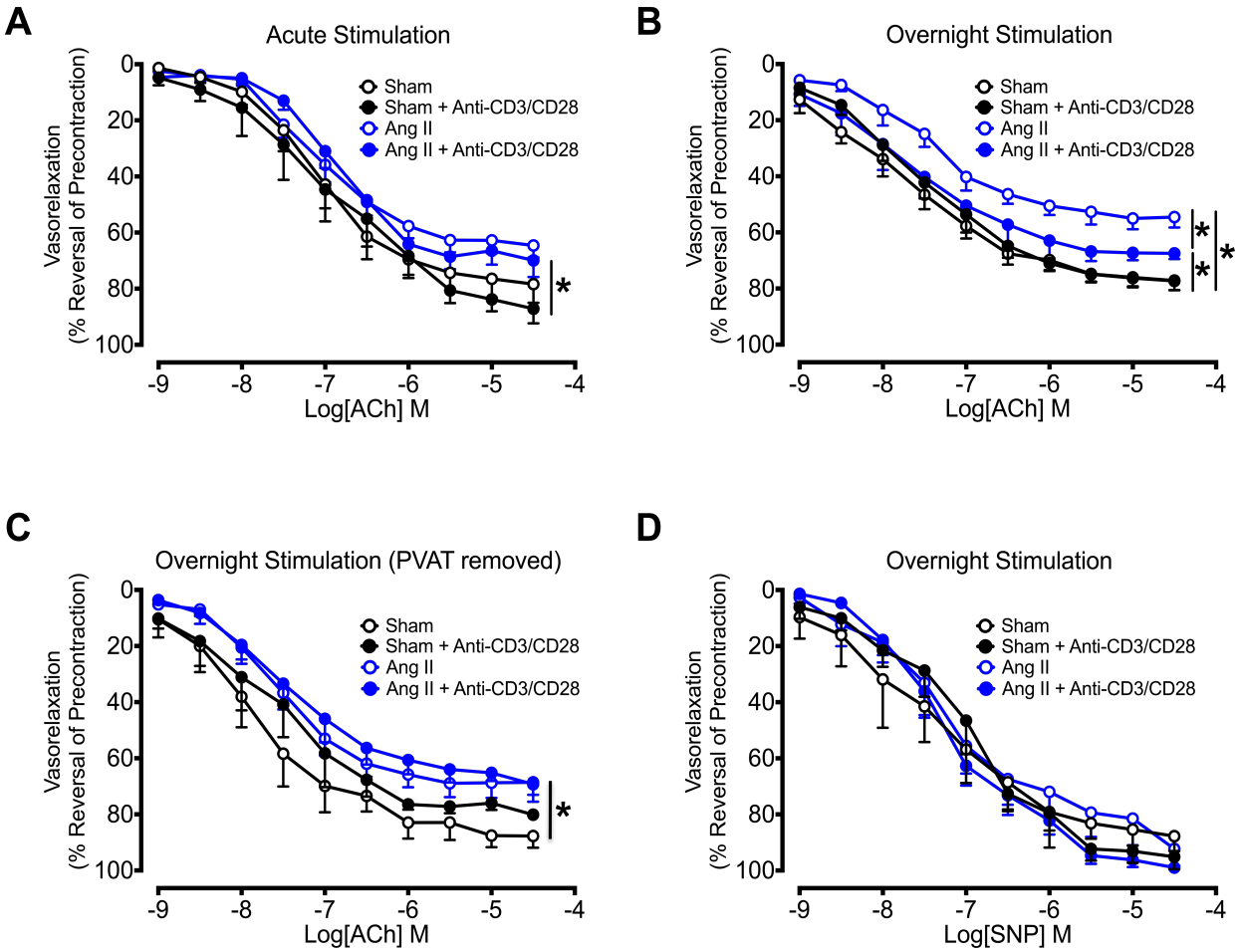


Figure 2.

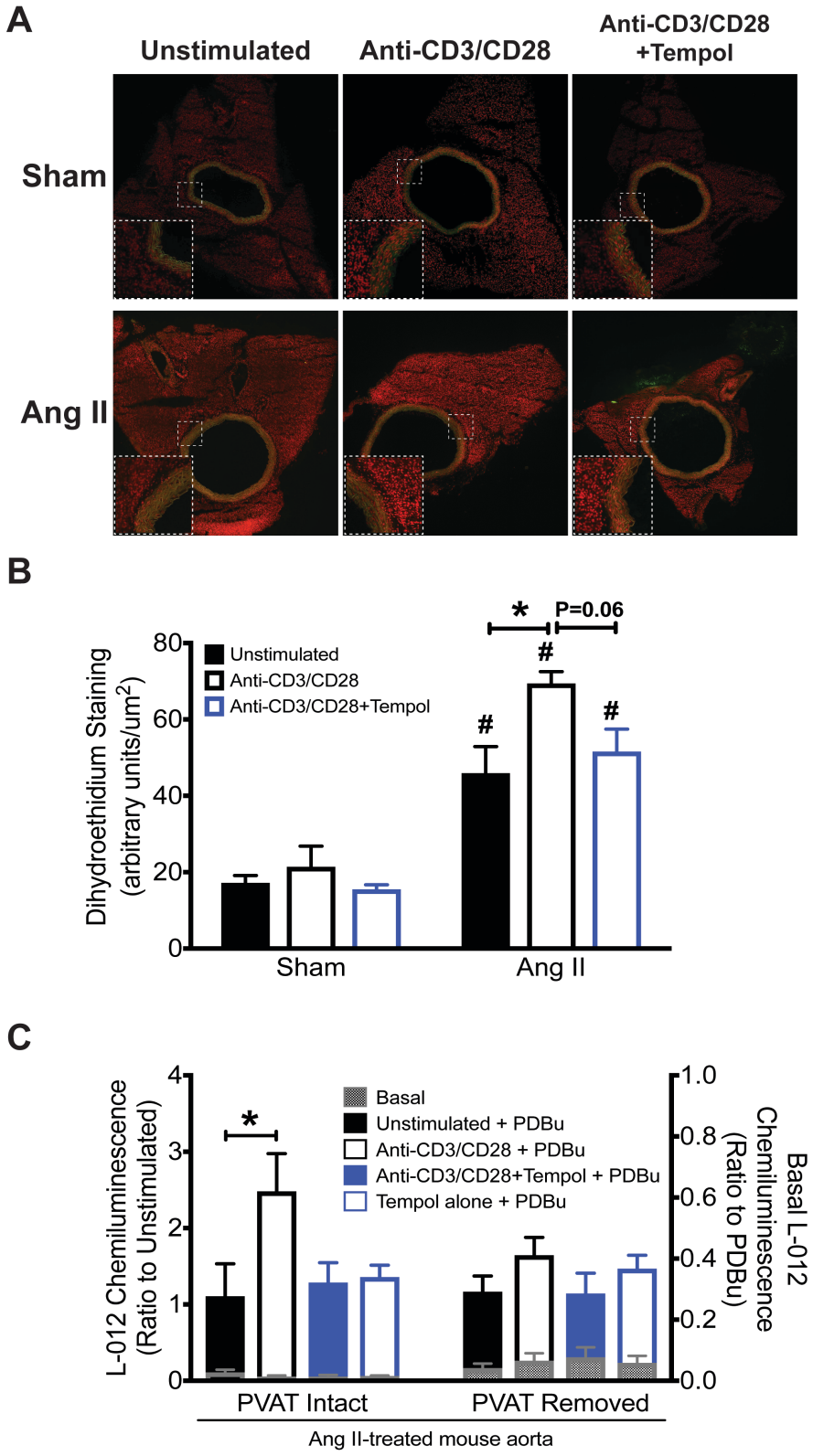

A
Unstimulated
Anti-CD3/CD28
Anti-CD3/CD28 +Tempol
Sham
Ang II
B
Dihydroethidium Staining (arbitrary units/um2)
Unstimulated
Anti-CD3/CD28
Anti-CD3/CD28+Tempol
P=0.06
Sham
Ang II
C
L-012 Chemiluminescence (Ratio to Unstimulated)
Basal L-012 Chemiluminescence (Ratio to PDBu)
Basal
Unstimulated + PDBu
Anti-CD3/CD28 + PDBu
Anti-CD3/CD28+Tempol + PDBu
Tempol alone + PDBu
PVAT Intact
PVAT Removed
Ang II-treated mouse aorta

Figure 3.

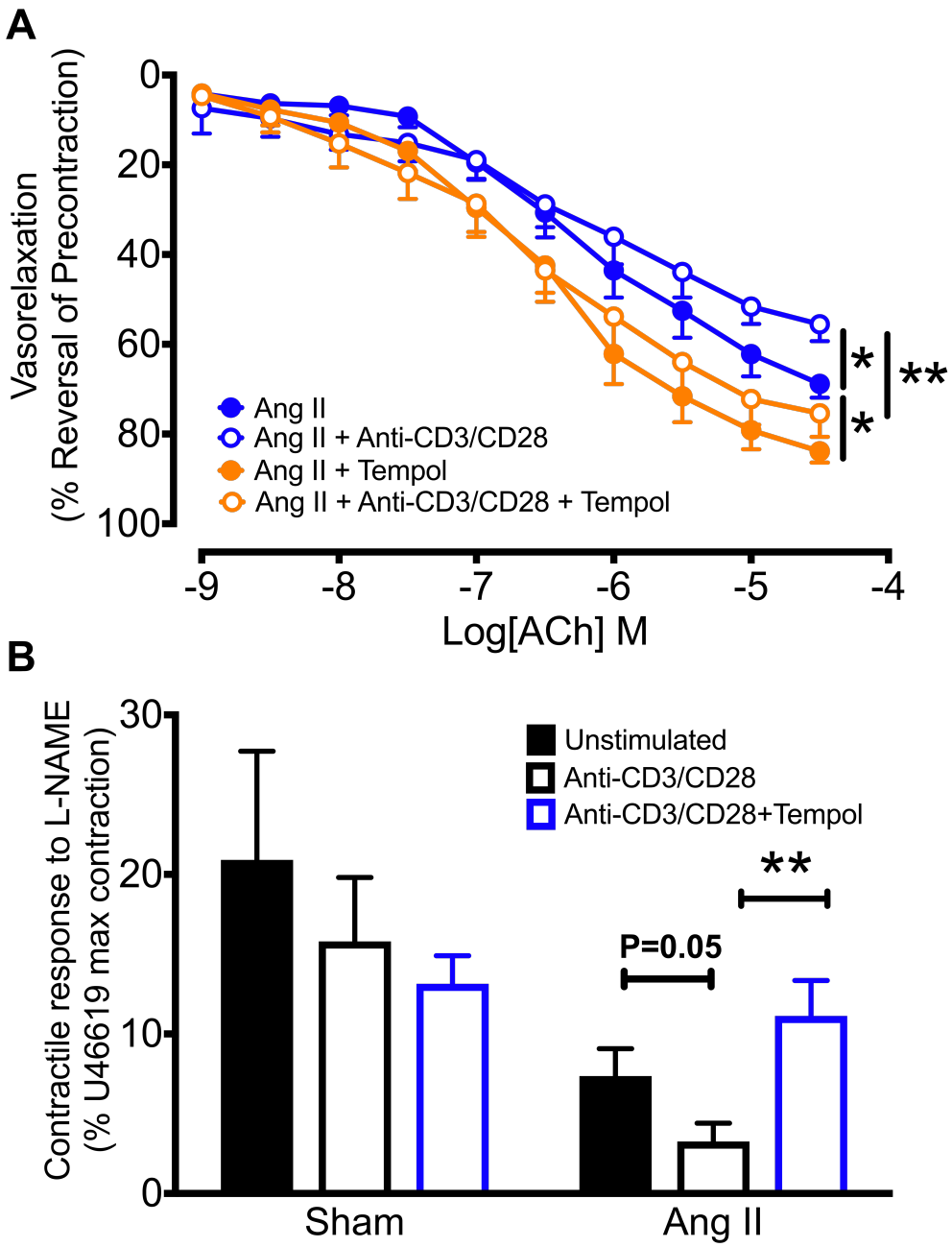
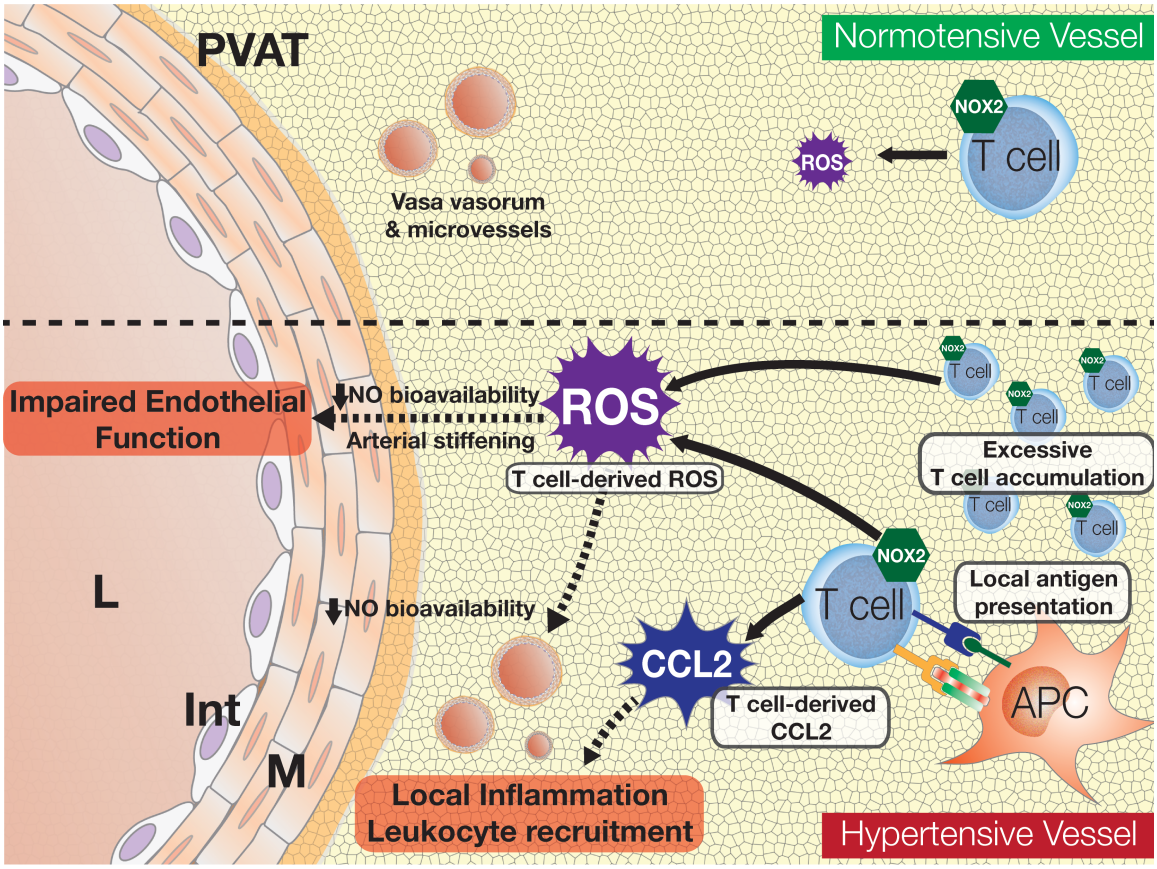

A
Vasorelaxation
(% Reversal of Precontraction)
0
20
40
60
80
100
-9
-8
-7
-6
-5
-4
Log[ACh] M
Ang II
Ang II + Anti-CD3/CD28
Ang II + Tempol
Ang II + Anti-CD3/CD28 + Tempol
*
**
*
B
Contractile response to L-NAME
(% U46619 max contraction)
30
20
10
0
Unstimulated
Anti-CD3/CD28
Anti-CD3/CD28+Tempol
**
P=0.05
Sham
Ang II

Figure 4.


PVAT
Normotensive Vessel
NOX2
T cell
ROS
Vasa vasorum
& microvessels
Impaired Endothelial Function
NO bioavailability
Arterial stiffening
ROS
T cell-derived ROS
Excessive
T cell accumulation
L
NO bioavailability
Local antigen
presentation
CCL2
T cell-derived
CCL2
APC
Int
M
Local Inflammation
Leukocyte recruitment
Hypertensive Vessel

Supplemental Material

DIRECT IN SITU STIMULATION OF VASCULAR INFILTRATING T CELLS EXACERBATES ANGIOTENSIN II-INDUCED ENDOTHELIAL DYSFUNCTION

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Running title: Vascular T cells Exacerbate Endothelial Dysfunction

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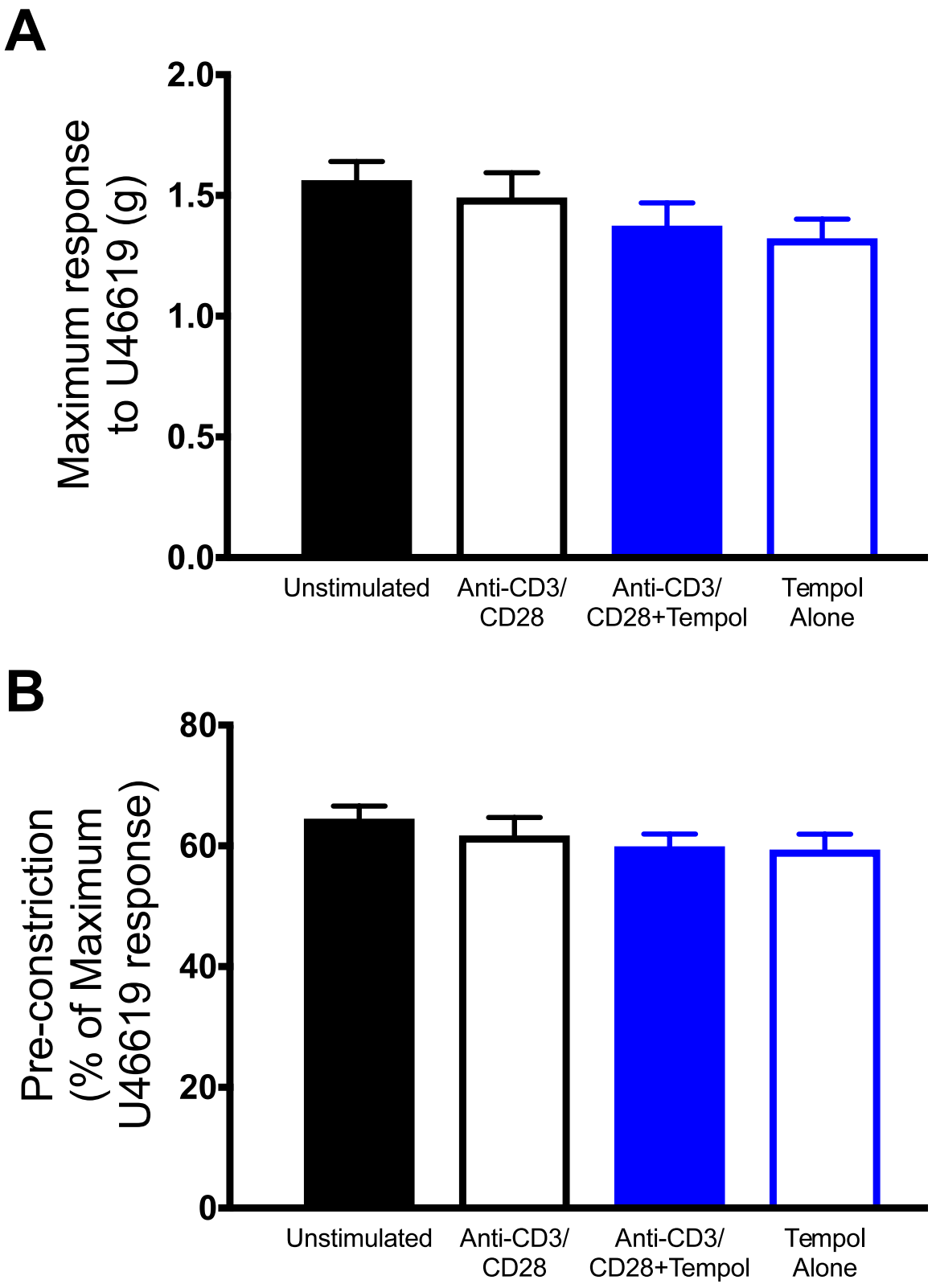
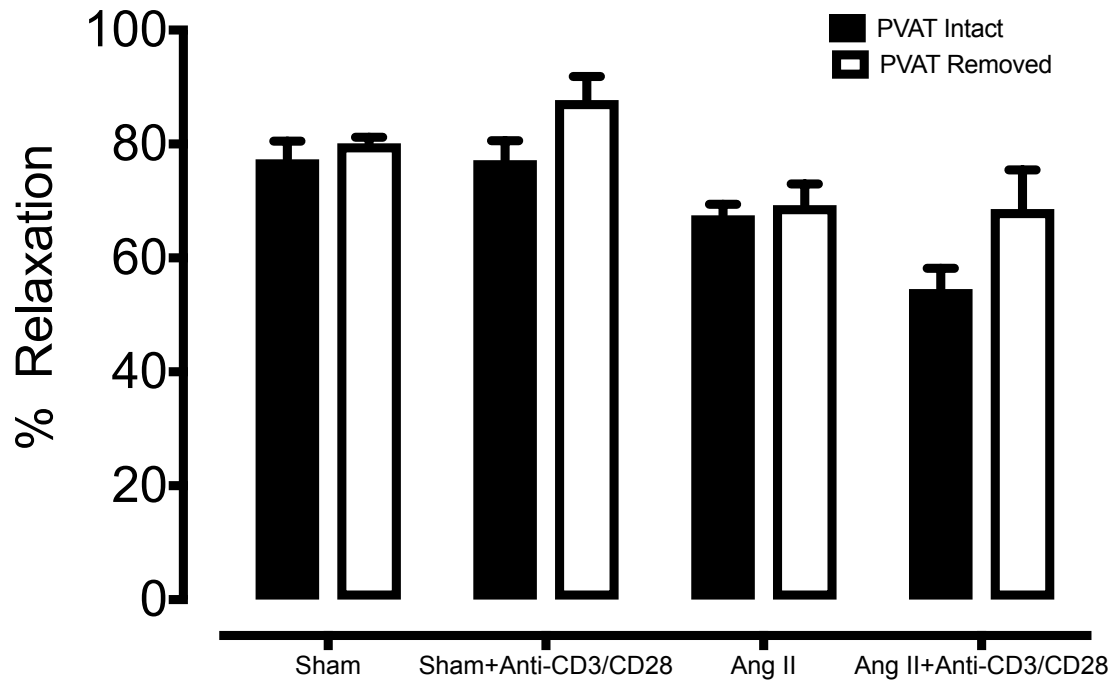

A
Maximum response to U46619 (g)
2.0
1.5
1.0
0.5
0.0
Unstimulated
Anti-CD3/ CD28
Anti-CD3/ CD28+Tempol
Tempol Alone
B
Pre-constriction (% of Maximum U46619 response)
80
60
40
20
0
Unstimulated
Anti-CD3/ CD28
Anti-CD3/ CD28+Tempol
Tempol Alone

Supplementary Figure 1. (A) Maximal contractile and (B)pre-constriction responses to U46619 in isolated aortic rings following 16 hour incubations with anti-CD3/CD28 and/or tempol.



Supplementary Figure 2.% relaxation responses to 1 μM ACH in isolated aortic rings with PVAT intact and removed.

Chapter 5:

Dynamic T Cell Interaction During Hypertension

VASCULAR T CELL-ANTIGEN PRESENTING CELL INTERACTIONS DURING HYPERTENSION

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Abstract

Objectives: T cells contribute to the development of experimental hypertension. While a hypertension-specific neoantigen has been implicated in T cell activation, it is not known whether vascular-infiltrating T cells recognize and are locally activated by an antigen within the vessel wall. The current study identified whether cognate antigens are presented to T cells within the vessel wall of hypertensive mice.

Methods: Splenic T cells were isolated from sham-treated (nT cells) and angiotensin II (Ang II)-infused (0.7mg/kg/day; 14 days; hT cells) C57BL6/J mice. Following anti-CD3/CD28 stimulation (48 hours), cells were fluorescently labelled and co-incubated simultaneously with explanted aorta obtained from sham or Ang II-treated CD11c-YFP$^+$ mice.

Results: In aorta obtained from CD11c-YFP$^+$ mice alone, we detected a ~2-fold increase in CCR5 ligand (CCL3, CCL4 and CCL5) secretion from Ang II-treated mouse aorta compared to sham-treated mouse aorta. In consistent, using confocal microscopy we observed an increase in overall T cell infiltration in explanted aorta obtained from Ang II-treated mice compared to sham, with a greater number (~2-fold) of hT cells infiltration compared to nT cells. Time-lapse recordings of Ang II mouse aorta revealed a greater proportion of T cell-CD11c-YFP$^+$ APC interaction, evidenced by significantly slower velocity and longer duration of interaction and isoketal modified protein scavenger 2-HOBA inhibited this dynamic interaction. Interestingly, CD8$^+$ T cells appeared to have a greater proportion of T cell – APC interactions compared to CD4$^+$ T cells.

Conclusion: These data are the first evidence suggesting that vascular-infiltrating T cells are presented with cognate antigens by APCs within the vessel wall during hypertension, which may contribute to the associated inflammatory response in hypertension.

Introduction

Hypertension is a complex and long-term medical condition and a major risk factor for death due to heart failure, stroke and coronary artery disease.[1] Current literature implicates immune-related mechanisms in the development of experimental hypertension, particularly the role of adaptive immune system and T cells.[2] Guzik and colleagues showed that recombinase activating gene-1 deficient (RAG-$1^{-/-}$) mice, which lack T and B cells, exhibit blunted hypertensive responses to angiotensin (Ang) II- or DOCA (deoxycorticosterone acetate) salt-induced hypertension.[3] However, adoptive transfer of T but not B cells into RAG-$1^{-/-}$ mice restored hypertension. This seminal study highlighted the importance of T cells in the progression of hypertension.

It is well known that hypertension is associated with significant accumulation of T cells into blood pressure-controlling organs, such as the kidney and vasculature where they predominantly accumulate in perivascular adipose tissue (PVAT).[3, 4] In the aorta, this appears to be dependent on regulated-on-activation, normal-T-expressed-and-secreted (RANTES) activity at the CCR5 chemokine receptor,[3] and it was shown that RANTES played an important role in promoting vascular dysfunction.[5] However, the mechanism of how T cells infiltrate and whether they recognise cognate antigens locally in the vessel wall remains unknown. Classically, naïve T cells are primarily activated in the spleen or sub-lymphoid organs where antigen presenting cells (APCs) engulf foreign antigens and present them to T cells in the context of a major histocompatibility complex (MHC), and together with second co-stimulatory signal T cells are fully activated.[2, 6] Interaction of T cells with APCs in lymphoid organs leads to T cell activation, clonal proliferation and release of cytokines.[6] This interaction can be microscopically visualised via time-lapse

recordings of T cells forming immunological synapses with dendritic cells (DC) within lymph nodes.[7, 8] However, it is now clear that antigen-experienced T cells can be reactivated upon peripheral exposure to antigens. Intravital microscopy studies in models of dermal inflammation have shown that antigen-specific $CD4^+$ T cells can be directly reactivated in the skin through secondary antigen presentation.[9] In the setting of vascular disease, time-lapse recordings coupled with 2-photon microscopy revealed augmented T cell-APC interactions between $ApoE^{-/-}$ mouse $CD4^+$ T cells and $CD11c^+$ APCs within the aorta from $ApoE^{-/-}$/CD11 yellow fluorescent protein (YFP) mice.[10] These *dynamic* T cell-APC interactions were defined by slowing of T cell velocity and also increased duration of interaction, which are collectively important indicators of an immunological synapse and presentation of antigens.[10] This finding demonstrated the existence of atherosclerosis-specific antigens that can be presented locally by YFP $CD11c^+$ APCs to $CD4^+$ T cells leading to arterial wall-induced T cell activation, and subsequent proliferation and release of inflammatory cytokines.[10]

Antigen presentation also appears to be vital to the development of hypertension since inhibition of B7/CD28 T cell co-stimulation using CTLA4-Ig, not only prevented but also reversed both Ang II- and DOCA salt-induced hypertension.[11] This finding suggested that classical T cell activation was required in the development of hypertension and it implicated the existence of hypertension-specific 'neoantigens' that could be recognised by T cells. The Harrison group has suggested that highly reactive gamma-isoketoaldehyde modified proteins (isoketals) may represent the hypertension-specific antigens that activate T cells during hypertension,[12] since treating mice chronically with isoketal scavenger 2-hydroxybenzylamine (2-HOBA) was shown to blunt experimental

hypertension.[12] They also demonstrated that these interactions correlated with increased deposition of these isoketals within aorta that were localised to the PVAT, suggesting presence of local antigens within the aorta.[12]

Using a novel imaging approach adapted from Klaus Ley's model[10], this study aimed to investigate whether local vascular antigen presentation also occurs in the setting of hypertension. We demonstrated that indeed disease-specific T cells interacted with DC within the hypertensive mouse aorta and perhaps suggest the presence of cognate antigens that can be presented to T cells in the periphery.

Methods:

Animals

Male C57BL6/J mice (Monash Animal Services, n = 73) and CD11c-YFP^{+} transgenic mice (n = 77, obtained as gift from Professor Michael Hickey; Center for Inflammatory Diseases) aged 8–12 weeks were housed on a 12 hour light/dark cycle with food and water provided *ad libitum*. Isoflurane-anaesthetized mice were randomly allocated to receive either sham (0.15M NaCl, 1% acetic acid) or Ang II (0.7 mg/kg/day) via osmotic minipumps (Alzet model 2002) implanted subcutaneously for 14 days. Deoxycorticosterone acetate-salt (DOCA-salt) hypertension was produced as previously described.[11] To determine the effect of scavenging isoketals on T cell-APC interactions, a separate cohort of Ang II-treated mice received either vehicle, 2-hydroxybenzylamine (0.3 mg/ml, 2-HOBA) or 4- hydroxybenzylamine (0.3 mg/ml, an analogue that exhibits low reactivity with isoketals; 4-HOBA) via drinking water. All surgical and treatment procedures were approved by the Monash Animal Research Platform Ethics Committee (Approval number MARP/2013/059).

Blood pressure monitoring

Systolic blood pressures (SBP) were measured using a non-invasive tail-cuff apparatus (MC4000 Blood Pressure Analysis System, Hatteras Instruments) prior to treatment and on day 14 to confirm hypertension (SBP ≥140 mmHg) was achieved. At each time point over 20 measurements were recorded and averaged.

Preparation of explanted aorta model

T cell enrichment and labelling

At the end of treatment, mice were euthanized by CO_2 asphyxiation and tissues were harvested for further analysis. Spleens were harvested from sham-, Ang II- or DOCA-salt induced hypertensive C57BL6/J mice and were processed into single cell suspensions as previously described.[4] In brief, T cells were enriched via negative isolation using a Pan T cell magnetic microbeads isolation kit (Miltenyi Biotec) as per manufactures instructions. Cell purity of enriched cells was confirmed to be >90% using flow cytometry. For further isolation of splenic CD4+ and CD8+ T cells, a multi-step enrichment procedure was used where Pan T cells were first negatively isolated, followed by positive isolation of CD4+ (L3T4 microbeads) and CD8+ (Ly-2 microbeads) T cells. Isolated cells were seeded in a 96-well plate coated with anti-CD3 (5 μg/mL; clone 145-2C11; Biolegend) monoclonal antibodies and cultured in complete RPMI 1640 medium (10% FBS, penicillin 100 U/ml/streptomycin 100 μg/ml and L-glutamine 2mM) for 48 hours in presence of anti-CD28 (clone 37.51; 5μg/mL; Biolegend) monoclonal antibodies as previously described.[11] Following anti-CD3/CD28 stimulation, cells were harvested and centrifuged at 1200 RPM for 10 minutes. Supernatants were then discarded and T cells were resuspended at 10^6 cells/ml in phosphate buffered solution (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4) with 1% bovine serum albumin (BSA; Sigma-Aldrich). T cells obtained from Ang II-treated mice (hT) were then labelled with 8μM SNARF-1 carboxylic acid, acetate, succinimidyl ester (SNARF-1, Life Technologies), while T cells obtained from sham-treated mice (nT) were labelled with 3μM CellTrace Far Red dye (Life Technologies), each for 15 minutes at 37°C in water bath. Following

staining, cells were then washed and resuspended at a 10^6 cell/ml in complete RPMI 1640 for incubation with explanted aorta.

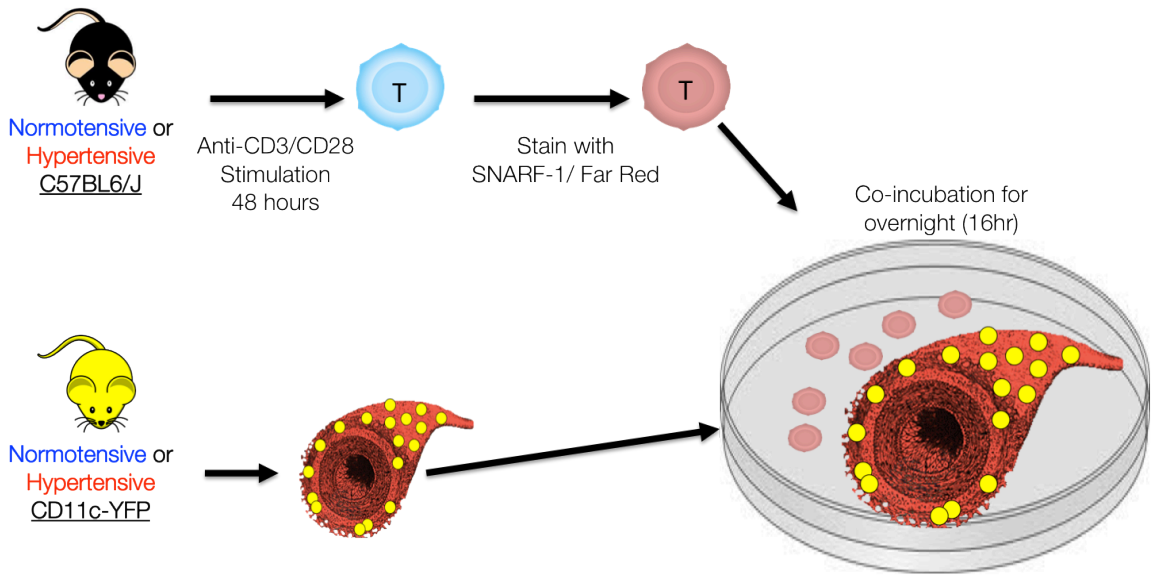


Figure 1. Experimental procedure. On day 1, spleens were extracted from sham or Ang II-treated mice where T cells were isolated using magnetic microbead isolation and stimulated with anti-CD3/CD28 for 48 hours. On day 3, T cells were labelled with SNARF-1(hT) or Far Red(nT) . Aortic sections with PVAT intact were extracted from AngII-infused hypertensive mice and co-incubated with T cells overnight.

Explanted aorta and confocal microscopy

Following T cell staining, CD11c-YFP$^+$ mouse aorta from sham and Ang II-treated mice was harvested. Fluorescently-labelled T cells from sham- (nT) and Ang II- (hT) treated mice (10^6 cells/treatment/ml) were co-incubated simultaneously with explanted aorta for 16 hours at 37°C with 5% CO_2 in complete RPMI 1640 medium Figure 1. Following co-incubation explanted aorta were washed in RPMI 1640 medium and the ends of aorta

were glued to a FluoroDish Cell culture dish with Histoacryl glue (TissueSeal) and was maintained at 37°C with 5% CO_2 in RPMI 1640 medium without phenol red. Time-lapse confocal imaging was performed using a Nikon C1 inverted confocal microscope. A 4x plain and 20x oil-immersion objective was used for image acquisition. Twenty optical slice Z-stacks spaced 4-6 μm apart were acquired at 512 x 512 pixels every minute over a period of 1 hour recording time. Emitted fluorescence was split with 3 dichoric mirrors (488 nm, 543 nm and 633 nm) and passed through filters at 515/30 nm (YFP), 590/50 nm (SNARF-1) and 650LP (CellTrace Far Red).

Image processing and cell tracking

Each video was analysed in the x, y and z plane and Imaris (Version 8.2) was employed for the analysis of live cell images. Fluorescent cells (SNARF-1, Far red, YFP) were tracked to measure total T cell infiltration, number of dynamic interactions, duration of T cell-APC interaction (seconds) and cell velocities (μm/min). Imaris software was used to automatically process 3D video data by detecting cells in each fluorescent channel, and created tracks by linking the detected cells over time. T cells interacting with APCs for more than 5 minutes during the recording time were defined as interacting T cells.

T cell infiltration assay

To test the effect of CCR5 antagonism on T cell infiltration in explanted aorta, nT cells were pre-treated with various concentrations of methiolynated-RANTES (Met-RANTES, 1-100nM) for 30 minutes prior to co-incubation with Ang II-treated aortic rings, and were compared to untreated T cells. As a negative control, non-stimulated T cells were also

concurrently co-incubated with an aortic ring. Following pre-treatment T cells were then co-incubated with Ang II-treated aorta in the continued presence of Met-RANTES or no-treatment for 16 hours at 37°C with 5% CO_2. Aortic rings were then washed in PBS, and fixed in 10% formalin overnight. Vessels were then placed on slides and coverslipped using anti-fade mounting medium with DAPI (Vectashield). A 20x oil-immersion objective was used for image acquisition where 12 optical slice z-stacks spaced 2 μm apart were acquired at 1024 x 1024 pixels. Four random fields of view (FOV) of the PVAT from each aortic ring were captured. Emitted fluorescence was split with dichroic mirrors (408nm and 561nm) and passed through filter at 450/35 nm (DAPI) and 590/50 nm (SNARF-1). Z-stacks from each FOV were maximally projected and labelled infiltrating T cells were then counted and averaged between all FOVs per aortic ring using ImageJ software. All analyses were performed blinded to the treatment groups.

Proinflammatory Chemokine Array

Isolated aortic rings from sham- and Ang II- treated mice with PVAT intact were incubated for overnight (16 hrs at 37°C with 5% CO_2) in complete RPMI 1640 medium. Conditioned media was collected and used to analyse the amount of chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL9, CXCL10, CXCL13) secreted using Biolegend Multi-Analyte Flow Assay Kit (Mouse Proinflammatory Chemokine Panel 13-plex). Samples were analysed on a LSR II flow cytometer (BD Biosciences) and Flow cytometry standard (FSC) files were analysed using BioLegend's LEGENDplex™ Data Analysis Software.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Graphpad Prism 7 (GraphadPad Software Inc., San Diego, CA, USA.) For comparison between two groups, data were compared by Student's unpaired t-test. Velocity of T cells were analysed by one-way ANOVA followed by Bonferroni post-hoc test. For comparison between two groups with more than one variable, data were analysed using two-way ANOVA followed by Bonferroni post-hoc test. $P< 0.05$ was considered statically significant.

Results

Systolic Blood Pressure (SBP)

Ang II-induced hypertension was confirmed in C57BL6/J and CD11c-YFP$^+$ mice, compared to sham-treated mice. (Day 14 SBP: sham 109 ± 4 mmHg Vs Ang II 149 ± 5 mmHg; $P<0.001$, n=63-87).

T cells readily infiltrate Ang II-treated mouse aorta via the CCR5-axis

Initial experiments were employed to localize infiltrating T cells and CD11c-YFP$^+$ APCs that reside in the explanted aorta. SNARF-1-labeled hT cells and CD11c-YFP$^+$ APCs were localized to the PVAT of explanted aorta (Figure 1A). Three dimentional constructions of the Z-stacks revealed that SNARF-1 labelled T cells infiltrated deeply into the PVAT (Figure 1B), with strongly increased T cell infiltration of hT and nT cells in aorta from Ang II-treated mice (Figure 1C).

Since greater T cell recruitment was observed into the PVAT of aorta, a chemokine array was employed to examine the differences in chemokine production from explanted aortas from sham- and Ang II-treated mice prior to T cell co-incubation. Aorta from Ang II-treated mice released greater amount of CCR5 ligands CCL3, CCL4 and CCL5, which are important chemoattractants in recruiting T cells, as well as CCL22 and CXCL13 (Figure 1D). Since there were increased CCR5 ligands, we examined the effect of CCR5 inhibition on T cells infiltration into explanted aorta, where CCR5 axis is important for *ex vivo* T cells infiltration. Interestingly, stimulated hT and nT cells readily infiltrated explanted aorta, which was not observed in naïve or non-stimulated T cells (Figure 2A and B). To determine the importance of the CCR5-axis T cell infiltration in the presence

of Met-RANTES was examined. Pre-treatment and co-incubation with Met-RANTES showed a dose-dependent inhibition of T cell infiltration into PVAT (Figure 2A and B).

Greater Dynamic Interactions between infiltrating hT cells and CD11c-YFP$^+$ APCs during hypertension

Time-lapse recording were captured to track the various dynamic movements three dimensionally (Figure 3A). We observed that cells moved at varying velocities with different displacement throughout the x, y and z plane. Interactions between T cells and APCs were frequent and observed throughout the entire time lapse, however, we observed 2 distinct behaviors: firstly T cells approach an APC at a constant velocity, interacting and moving off without any slowing observed. Secondly, we also observed T cells approaching with slowing of velocity and showing long lasting interactions with CD11c-YFP$^+$ APCs, which typified a dynamic interaction. In some instances, T cell-APC interactions were observed throughout the entire time lapse. (Figure 3B and C; Supplemental Video 1A and B).

To directly compare the movement and T cell-APC interactions of nT and hT cells as noted in figure 3, T cells were co-incubated (labeled with different fluorescent markers) with CD11c-YFP$^+$ mouse aorta from sham- and Ang II-treated mice. Consistent with our earlier findings (Figure 1), this model of explanted aorta also showed that in sham-treated mice, a limited number of nT or hT cells infiltrated the PVAT of aorta (Figure 4A and B). Significant nT and hT cell infiltration was observed in explanted aorta from Ang II-treated mice. Importantly, a greater percentage of T cell-CD11c-YFP$^+$ APC interactions were only observed with hT cells (Figure 4C, Supplemental Video 2A and B). A portion

of hT cells participated in long-lasting interactions with CD11c-YFP$^+$ APCs, which ultimately decreased the dynamic velocity of interacting hT cells in aorta from Ang II treated mice (Supplemental Video 2A and B). Average velocities of total hT cells were also significantly reduced when compared to the total nT cells with interacting hT cells showed a further reduction in velocity in aorta from Ang II-induced hypertensive mice (Figure 4D). Furthermore, interacting hT cells also exhibited an extended durations of interaction compared to nT cells in same aorta (Figure 4E).

We then aimed to address whether the observed enhanced T cell-CD11c-YFP$^+$ APC interactions in Ang II-induced hypertension, involve a hypertensive-specific antigen conserved across different models of hypertension.

The experiment was repeated using T cells isolated from DOCA-Salt induced hypertensive mouse, and co-incubated with aorta from Ang II-treated CD11c-YFP$^+$ mice. Consistent with Ang II-induced hypertension, there appeared to be greater hT cell (DOCA-salt) infiltration into aorta obtained from Ang II-treated CD11c-YFP$^+$ mice and appeared to be greater interactions with CD11c-YFP$^+$ APCs compared to T cells from sham-treated mouse aorta (Figure 5; Supplemental Video 3A and B).

T cell Subset Analysis

With greater infiltration and interaction of hT cell during hypertension, we tested whether there were any preferences for dynamic interactions between CD4$^+$ and CD8$^+$ T cells subtypes in an aorta from Ang II-induced hypertensive CD11c-YFP$^+$ mice (Figure 6A; Supplemental Video 4). Interestingly, there was no difference between CD4$^+$ and CD8$^+$ T infiltration into PVAT (Figure 6B). However, CD8$^+$ T cells from hT cells appeard to have

a significantly greater percentage of interaction with CD11c-YFP$^+$ APCs compare to CD4+ T cells (Figure 6C).

Scavenging of isoketals inhibited T cell-APC interactions in Ang II-induced hypertension

Having shown antigen-specific hT cell interactions with CD11c-YFP$^+$ APCs in aorta from Ang II- treated mice, we then tested whether scavenging isoketal-modified proteins can inhibit T cell infiltration and interactions with CD11c-YFP$^+$ APCs. Co-treating with isoketal scavenger 2-HOBA in drinking water significantly reduced Ang II-induced pressor responses although a similar reduction in blood pressure in mice co-treated with control 4-HOBA was observed (Figure 7A). Compared to Ang II-treated mouse aorta alone, 2-HOBA and 4-HOBA co-treated aorta exhibited similar reductions in T cell infiltration (Figure 7B).

While hT cells interacted more frequently with APCs compared to nT cells in Ang II-treated aorta alone, co-treatment with isoketal scavenger 2-HOBA abolished T cell-APC interactions which was not seen in aorta from 4-HOBA co-treated mice (Figure 7C). Consistent with previous velocity results, in aorta obtained from Ang II-treated mice, hT cells exhibited a significantly slower velocity compared to nT cells. However, hT cells in aorta that were co-treated with 2-HOBA had no difference in velocity compare to nT cells (Figure 7D).

Discussion

Since classical immunological mechanisms are involved in the pathogenesis of hypertension,[13] it is not surprising that ablation of individual cells of the immune system starting with T [3, 14, 15] and B [16] cells, to myeloid-derived cells such macrophages[17] and neutrophils[18], results in blunting of experimental and potentially clinical hypertension.[19] This study aimed to identify why T cells infiltrate the PVAT of aorta in mouse models of hypertension and whether they are activated locally. In this study, we have reported the first evidence that vascular-infiltrating T cells are presented with cognate antigens by APCs within the vessel wall during hypertension, which may potentially induce secondary activation of these T cell infiltrates and promote the progression of hypertension.

This study used a novel explanted aorta model to observe T cell infiltration and the nature of their interactions *ex vivo* – a world first in the field of hypertension. The first important observation was that labelled T cells directly infiltrate the aorta and are localised to the PVAT surrounding the aorta. This is consistent with current knowledge that T cells accumulate in the PVAT in various *in vivo* models of hypertension.[3] We confirmed the elevated chemokine gradient released from Ang II-treated mouse aorta detected using a proinflammatory chemokine bead array. Interestingly, of the multiple chemokines measured, we detected a ~2-fold increase in all CCR5 ligands (CCL3, CCL4 and CCL5) secreted from Ang II-treated mouse aorta compared to sham. This is consistent with the original findings from Guzik and colleagues[3] where regulated-on-activation, normal-T-expressed-and-secreted (RANTES) mRNA expression was significantly elevated in Ang II-infused mouse aorta, and is 3-4 fold higher compared to T cells from peripheral blood

and spleen. This suggested that aorta from Ang II-induced hypertensive mice may be releasing greater amounts of chemokines that recruit T cells and other leukocytes that are known to express chemokine receptors such as CCR5 and CCR2.[3, 20] Indeed, RANTES has been implicated in the infiltration of T cells associated with hypertension. Ang II-induced hypertension was shown to increase infiltration of leukocytes, which were predominantly T cells and correlated with increase in RANTES mRNA expression and circulating T cells bearing C-C chemokine receptor type 5 (CCR5).[3] RANTES is a ligand for the chemokine receptor CCR5, which facilitates homing of T cells to target sites. It was shown that mice deficient in RANTES have reduced vascular leukocyte and T cell infiltration, ROS production and endothelial dysfunction associated with Ang II induced hypertension.[5]

Activated T cells are know to express greater chemokine receptors, therefore, it was vital to validate whether our model of T cell-CD11c-YFP^{+} APC interactions in hypertension required T cells to be activated to increase expression of chemokine receptors for recruitment. We observed significantly increased T cell infiltration into aorta from Ang II-treated mice compared to aorta from sham-treated mice. Using our model, we also confirmed that direct T cell infiltration is indeed dependent on the CCR5-axis since Met-RANTES dose-dependently reduced T cell infiltration into a hypertensive mouse aorta. Even though these results were obtained using an *ex vivo* approach, the CCR5-axis for direct T cell recruitment into the aorta mimics results shown *in vivo*, since inhibition of CCR5-axis prevents accumulation of T cells in PVAT. This further supports the validity of our model to examine aortic T cells infiltration and function.

Although the CCR5-axis appears to be the main chemotactic pathway involved it is also worth noting that we also detected increased release of chemokine CCL22 and CXCL13 in conditioned media of aorta from Ang II-treated mice. These chemokines are also important in lymphocyte recruitment and inflammatory response. CCL22 is predominatly released by DCs and macrophages that target cells expressing CCR4,[21] and displays chemotactic activity for monocytes, DCs, natural killer cells and for chronically activated T lymphocytes.[21] CXCL13 is generally known as chemoattractant for B cells, however expression of CXCL13 in T cells is considered to be associated with follicular T cells.[22] A recent study suggested that CXCL13 acts as a new biomarker for systemic lupus erythematosus,[23] which is an autoimmune disease associated with elevated T cell cytokines.[24]

Therefore, in addition to the CCR5 ligands, it is likely that other chemokines mediate recruitment of other immune cells, as well as T cells, into the PVAT during hypertension. Surprisingly, there was no significant increased in CCL2 released from intact aorta of Ang II-treated mice. Our previous study showed infiltrating T cells in blood pressure controlling organs release greater amount of CCL2 during Ang II-induced hypertension.[4] This discrepancy could be due to the fact that, in the present study, CCL2 release was detected from intact explanted aorta, compared to CCL2 detection from isolated T cells alone (Chapter 3),[4] which may reflect the fact that the aortic tissue is not a homogeneous source of T cells.

Aortic T cells bearing CCR5 are elevated in Ang II-treated mice,[5] and Itani et al found that there is increased memory T cells (CD3+/CD45RO+) in aorta and lymph nodes

during hypertension.[25] Interestingly, the current study showed that stimulated T cells more readily infiltrate into the PVAT compared to non-stimulated or naïve T cells. The result showed elevated T cell infiltration into the aorta during hypertension and we observed a greater number (~2-fold) of hT cells compared to nT cells within aorta from Ang II-treated mice. This may suggest that there were more CCR5 expressing T cells in the spleens from Ang II-induced hypertension. Furthermore, we showed that blocking CCR5 using Met-RANTES prevent the infiltration of T cells into explanted aorta. Interestingly, a recent study showed that CCR5 deficiency had no significant effects on lowering blood pressure and the authors suggested that RANTES-independent pathways could be involved in hypertension.[5] The absence of blood pressure changes could also be explained by the short Ang II-infusion of 2 weeks, where the vascular functional benefits of RANTES knockout or Met-RANTES treatment may need longer treatment to be fully seen.

Since T cells readily infiltrate hypertensive mouse vessels in our model, we had established a platform to observe whether dynamic T cell-APC interactions occur during hypertension in the vessel wall. Coined the "*second touch hypothesis,*" [26] Klaus Ley's group used an $ApoE^{-/-}$ $CD11c\text{-}YFP^{+}$ mouse model of atherosclerosis to observe $CD11c\text{-}YFP^{+}$ APCs interacting with T cells from either wildtype or $ApoE^{-/-}$ mice.[10] They reported that in $ApoE^{-/-}$ mouse isolated T cells exhibit increased T cell-$CD11c\text{-}YFP^{+}$ APC interactions, together with slowing of T cell velocity and also increased duration of interactions compared to wildtype mouse T cells. These findings highlighted that T cells harvested from atherosclerotic mice were able to recognise disease-specific antigens within an atherosclerotic plaque. Given previous work implicating a hypertension-specific

neoantigen,[11] we also similarly asked whether these antigens were presented within the site of T cell accumulation during hypertension. Using analogous indicators we adapted the Ley model to examine vessels from Ang II-induced hypertensive mice. Indeed, aortic CD11c-YFP$^+$ APCs engaged in dynamic interactions with T cells – this was only observed with hT cells. Despite elevated infiltration of nT cells into hypertensive mouse aorta, we observed no change in the percentage of T cell-CD11c-YFP$^+$ APC interactions between hT cells and APCs, which underscores the potential recognition of a hypertension-specific cognate antigen by nT cells. A reverse experiment was also performed, where aorta from sham-treated mice were incubated with nT and hT cells. We theorised that if an identical response was observed in aorta from sham-treated mice where hT cells still interacted more frequently than nT cells, it may have indicated that hT cells are potentially responding to self antigens. However, this was not observed suggesting that hypertensive stimuli might prompt the formation of neoantigens and T cells harvested from Ang II-treated mice readily recognised these neoantigens (Figure 8). In addition, occasionally T cells stopped in areas with no YFP$^+$ cells, which suggests that cells interact with APCs that do not express YFP in CD11c YFP$^+$ mice such as macrophages and B cells. Nevertheless, greater T cell infiltration into the PVAT is observed during hypertneion,[3] and this is mimicked in our explanted aorta model. We speculate a similar effect that hT cells in aorta from Ang II-treated mice exhibit greater interaction *in vivo*.

Previous studies have associated T cell infiltration into the aorta from various models of hypertension.[3, 11, 15] To determine whether antigen-specific interactions between T cells and CD11c-YFP$^+$ APCs is a selective Ang II-induced response, cross reactivity

experiments were performed, where T cells were isolated from spleen of DOCA-salt-induced hypertensive mice and incubated with aorta form Ang II-treated CD11c-YFP$^+$ mice. Strikingly, consistent with the results in Ang II-induced hypertension mouse model, antigen-specific interactions with CD11c-YFP$^+$ APCs was observed in DOCA-salt but not sham T cells, which suggested hypertension specific antigens may potentially be conserved from across different models of hypertension. This is not surprising, since T cells are essential for the development across multiple models of hypertension,[3, 11, 15] and a study from Marvar et al indicated that elevations in blood pressure was the main driving force of T cell activation that promoted inflammation during hypertension.[27] This elevation in blood pressure is thought to induce oxidative modification through rearrangement of H2-isoprostane intermediates in the F2-isoprostane pathway of free radical–mediated lipid peroxidation to vascular proteins leading to the formation of gamma-ketoaldehydes (isoketals) modified proteins.[12]

Harrison's group has suggested possible antigens during hypertension, including isoketal-modified proteins that accumulate in DCs during hypertension, which is associated with secretion of IL-6, IL-1b, IL-23 and increased costimulatory proteins CD80 and CD86.[12] In addition, they also showed that isoketal-loaded DCs activated T cells, particularly CD8+ T cells to induce proinflammatory response and hypertension,[12] and co-treating with isoketal scavengers prevented the hypertensive response. The current study showed that the isoketal scavenger 2-HOBA significantly blunted blood pressure with reduced T cell infiltration. Importantly, scavenging isoketalated proteins using 2-HOBA reversed the Ang II-induced T cell-APCs interaction. Co-treating with 4-HOBA that exhibit low reactivity with isoketals (analogue of 2-HOBA) blunted hypertensive response to Ang II

and reduced T cells infiltration. While this effect of 4-HOBA could be due to reduction in blood pressure, importantly 4-HOBA did not inhibit the T cell-APC interactions, suggesting that isoketal-modified proteins are crucial in antigen presentation to T cells. Therefore, consistent with current literature, the current study demonstrated that hypertension-specific antigens might be isoketal-modified protein, since scavenging with 2-HOBA abolished all local/vascular T cells-APC interaction.

T cells are well known to be essential to the development of hypertension,[3, 4] however the functional role of T cells subsets in hypertension remains unconfirmed. It was shown that both CD4+ and CD8+ T cell infiltration are increased during hypertension,[28] but whether one specific subset is solely responsible for promoting inflammation associated with hypertension is unclear. Harrison's group suggested that CD8+ but not CD4+ T cell accumulation in kidney contributes to hypertension,[28] indicating that T cells subtypes could exert different mechanisms in promoting hypertension. Experiments examining interactions of CD11c-YFP$^+$ APCs with CD4+ and CD8+ T cell subtypes revealed the same degree of infiltration of both subtypes into hypertensive aorta. Interestingly, CD8+ T cells showed a greater percentage of interaction with CD11c-YFP$^+$ APCs compared to CD4+ T cells from same mice. T cells have a restricted specificity for antigens and can only recognise antigens that are presented on major histocompatibility complexes (MHC) expressed on the surface of APCs.[6] CD4+ T cells are known to recognise the MHC class II molecules, whilst CD8+ T cells recognise MHC class I molecules,[6] thus the difference in response to CD11c-YFP+ APCs may highlight the presence or formation of different neoantigens during hypertension.

In addition, although both CD4+ and CD8+ T cells act to remove the pathogens and induce inflammatory responses that may be involved in the pathogenesis of hypertension, their mechanisms are different. T helper CD4 cells can polarise to different phenotypes and secret adaptive immune cytokines such as IFN-γ, TNF-α and IL-17, whereas CD8+ T cells are known as cytotoxic T cells that act to kill pathogens by releasing cytotoxic enzymes.[6] Our previous study showed a greater proportion of IFN-γ producing aortic CD8+ T cells in Ang II-induced hypertension (chapter 3)[4] and consistent with current literature that CD8+ T cells accumulates in kidney and contribute to hypertension.[28] Moreover, CD8-deficient mice exhibit blunted hypertensive responses, and adoptive transfer of CD8+ but not CD4+ T cells into $RAG^{-/-}$ mice reversed blood pressure increase during chronic Ang II infusion.[28, 29] These data implicated the importance of CD8+ T cells in the pathogenesis of hypertension, however CD4+ T cells could still contribute to progression of hypertension through other proinflammatory activities.

In the current study, direct functional outcomes as result of T cell-APC interactions, such as local inflammatory responses remains undefined. However, in other inflammatory disease, such as atherosclerosis, the presentation of antigens to T cells induced T cell proliferation and production of cytokine IFN-γ and TNF-α locally.[10] Thus, it is plausible that presentation of antigens to T cells in PVAT during hypertension promotes greater release of proinflammatory cytokines in target organs, resulting in exacerbation of leukocyte recruitment, inflammatory response and oxidative stress, ultimately leading to endothelial dysfunction (chapter 4). Although these results were obtained using an *ex vivo* approach, the antigen-specific interaction between hT cells and CD11c-YFP APCs in

aorta exists and is a likely mechanism *in vivo* by which the T cells are activated locally contribute to pathogenesis of hypertension.

We have presented a world first model to visualise dynamic T cell-APC interaction in aorta during hypertension, however as an explanted *in situ* setting that mimics *in vivo*, there are limitations. T cells were artificially stimulated *in vitro* using anti-CD3/CD28 antibodies in order to infiltrate into explanted aorta, therefore using T cells that already express greater activation markers, for example from the blood, could be another approach. Preliminary experiments using T cells from blood and lymph node were performed, however due to low yield in number of T cells, infiltrations into aorta were insufficient. For future study, pooling together T cells derived from blood or lymph node from a number of mice with same treatment could be an approach for better yield of T cells.

The functional outcomes from this study were not examined. However, in future experiments, flow cytometry may be used to examine proliferation marker such as Ki67 and BrdU or CBA for cytokine production in conditioned media from co-incubation of T cells and APCs .In addition, the exact hypertension-induced isoketal-modified protein or antigens remains undefined in the field of hypertension. Therefore, using an immunopeptidome approach to screening the antigens presented by APCs in the APC MHC molcule would be valuable to perform to try and identify the specific antigens.

In summary, we conclude that CCR5 chemokine axis is important in T cell recruitment during hypertension, and aorta from Ang II-induced hypertensive mice released greater

CCR5 ligands associated with greater T cells recruitment. The greater dynamic interactions between T cells and CD11c-YFP^{+} APCs may suggest presence of hypertension-specific neoantigens. On the basis of previous literature, presenting antigens to T cells may potentially lead to T cells activation, proliferation and secretion of inflammatory cytokines, and could be the plausible mechanism by which T cell and adaptive immune response promotes hypertension.

Figures

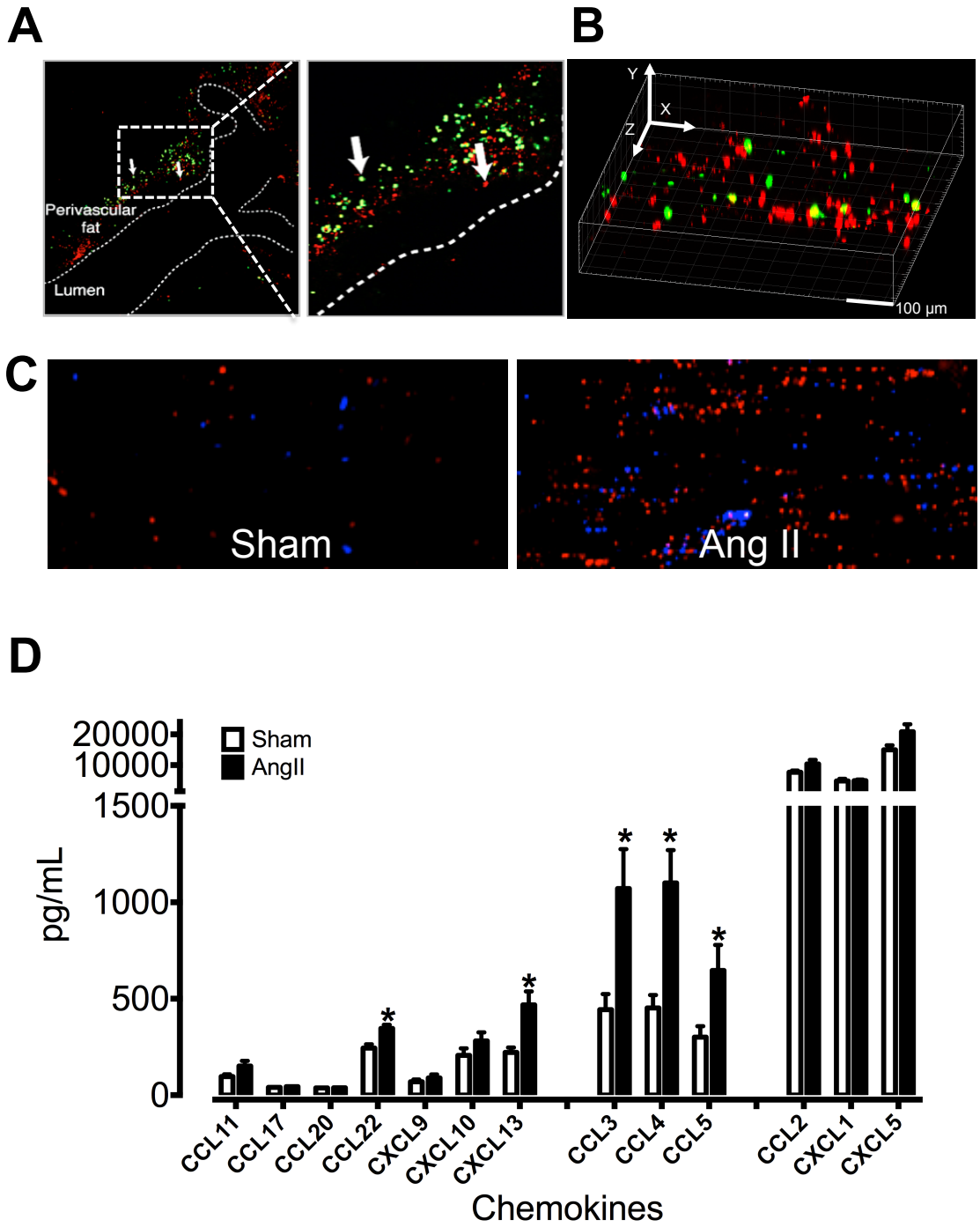
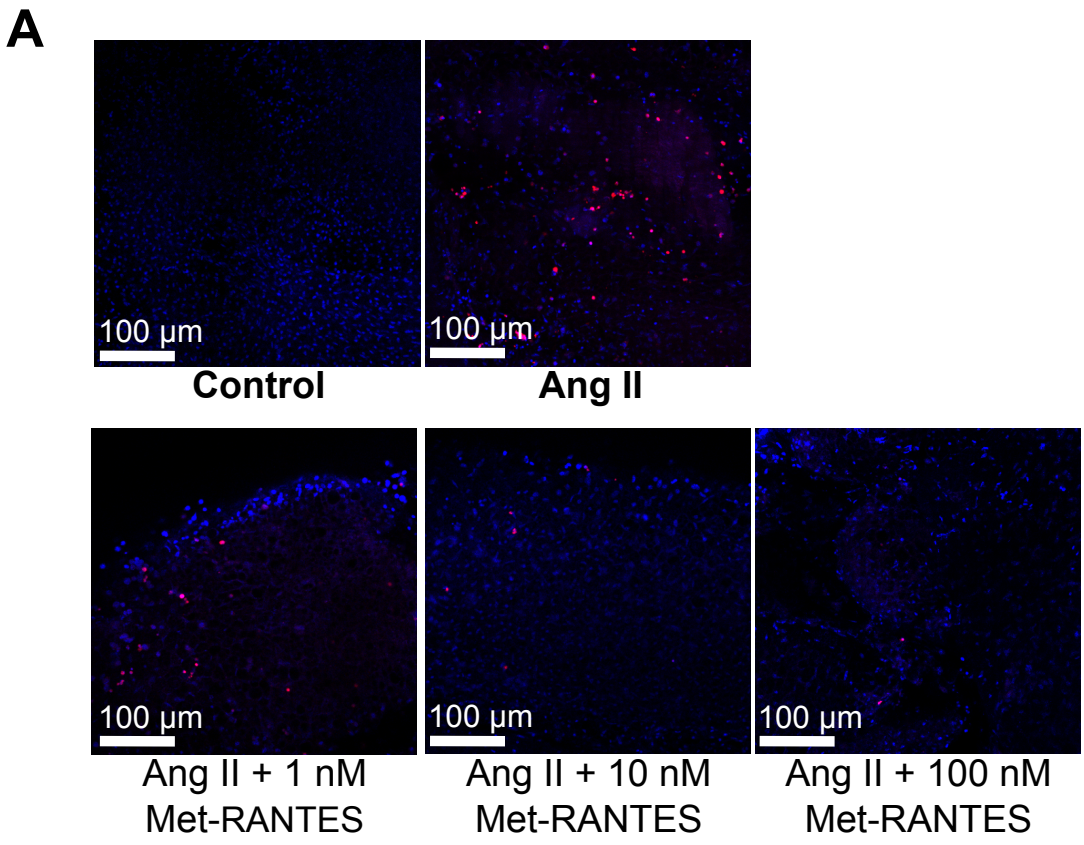

A
B
Perivascular fat
Lumen
Y
X
Z
100 µm
C
Sham
Ang II
D
20000
10000
1500
1000
500
0
pg/mL
Sham
AngII
CCL11
CCL17
CCL20
CCL22
CXCL9
CXCL10
CXCL13
CCL3
CCL4
CCL5
CCL2
CXCL1
CXCL5
Chemokines

Figure 1. Infiltration of T cells in PVAT in model of explanted intact aorta. (A) Localization of APCs (green) and T cells (red) in abdominal aorta. (B) 3D projection along the y-axis (side view) through image stacks [x,y,z]. View of CD11c-YFP^{+} (green), SNARF-labelled hT cells (red) and nT cell (blue) in middle of PVAT of explanted mouse aorta. (C) Infiltrated hT and nT cells in PVAT of aorta from sham- Vs Ang II- treated mice. (D) Aortic chemokine secretion as detected using a CBA assay following overnight incubaton of intact aorta from Ang II- and sham-treated mice. (*$P<0.05$ Vs sham; two-way ANOVA; n=7-14).


A
100 µm
100 µm
Control
Ang II
100 µm
100 µm
100 µm
Ang II + 1 nM Met-RANTES
Ang II + 10 nM Met-RANTES
Ang II + 100 nM Met-RANTES

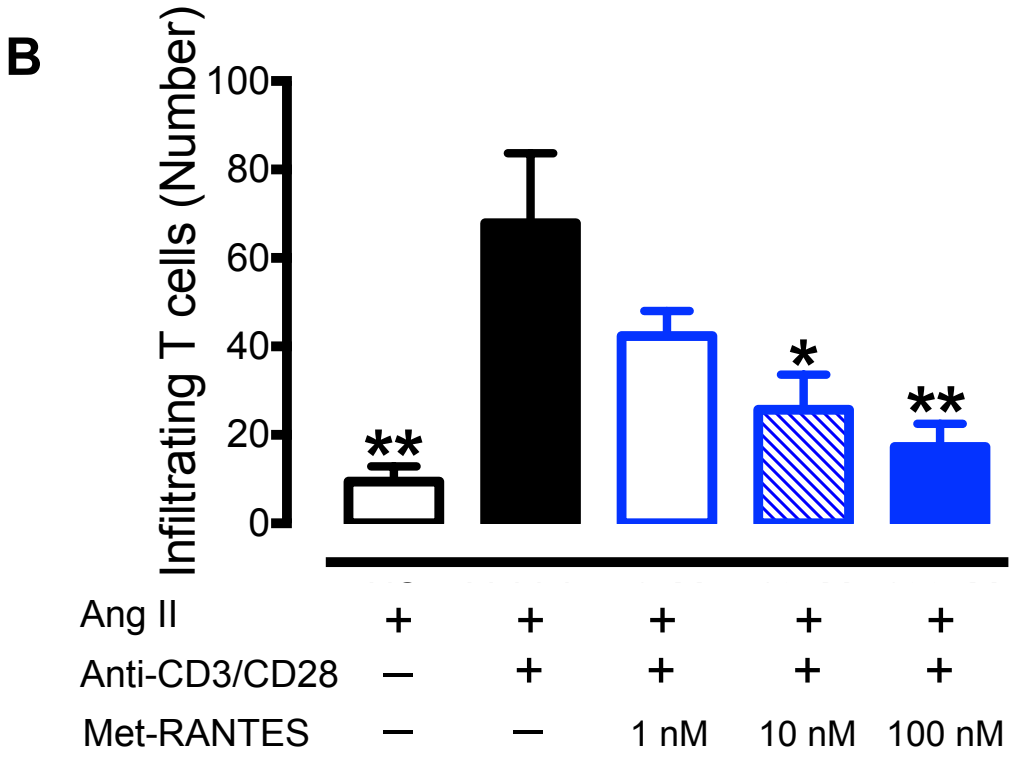

B
Infiltrating T cells (Number)
100
80
60
40
20
0
**
*
**
Ang II + + + + +
Anti-CD3/CD28 – + + + +
Met-RANTES – – 1 nM 10 nM 100 nM

Figure 2. Confocal imaging showing infiltration of nT cells in PVAT of aorta for Ang II - treated mice following pre-treatment with vehicle and Met-RANTES. (A) Representative images of maximum intensity projection along z-axis of infiltrated T cells (red) with DAPI (blue) in PVAT from each representative treatment (control, vehicle, 1 nM, 10 nM and 100 nM of met-RANTES). (B) Mean number of infiltrating T cells into PVAT from each treatment with four fields of view at 20x were analysed per aorta. (*$P<0.05$ Vs Ang II alone; two-way ANOVA; n=6).

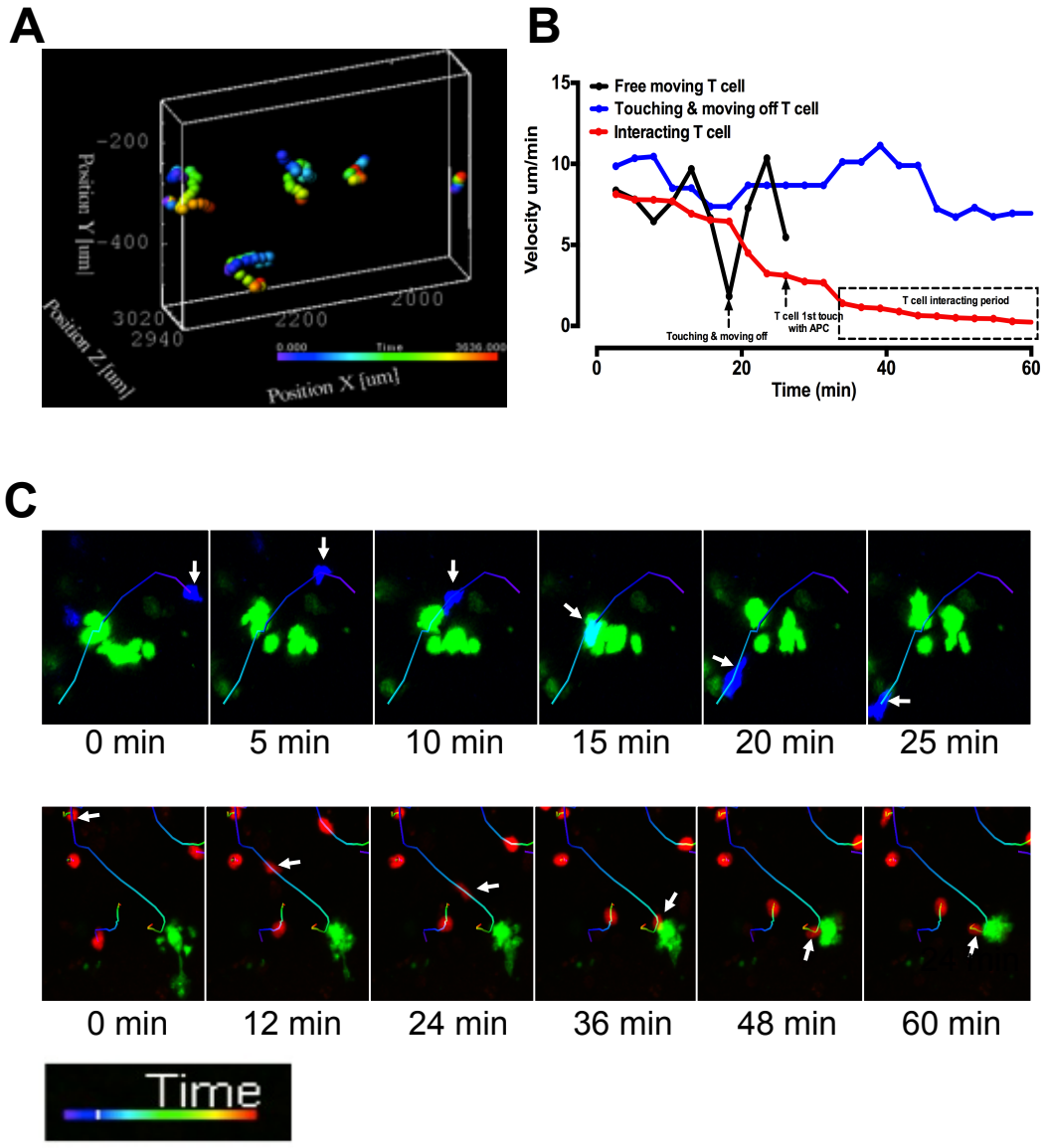

A
B
C
Position Y [um]
Position Z [um]
Position X [um]
-200
-400
3020
2940
2200
2000
Time
Free moving T cell
Touching & moving off T cell
Interacting T cell
Velocity um/min
15
10
5
0
Touching & moving off
T cell 1st touch with APC
T cell interacting period
0
20
40
60
Time (min)
0 min
5 min
10 min
15 min
20 min
25 min
0 min
12 min
24 min
36 min
48 min
60 min
Time

Figure 3. Representative tracking of live T cell movement. (A) Displacement tracks plots in positions of individual T cells over time in 3D projection. (B) Velocities of three individual free moving and interacting T cells. (C) Time-lapse images of interacting and free moving CD11c-YFP^{+}APCs in aortic PVAT. View of CD11c-YFP^{+} (green), SNARF-labelled hT cells (red) and nT cell (blue) in PVAT of explanted mouse aorta. (Arrows denote dynamic movements of T cell)

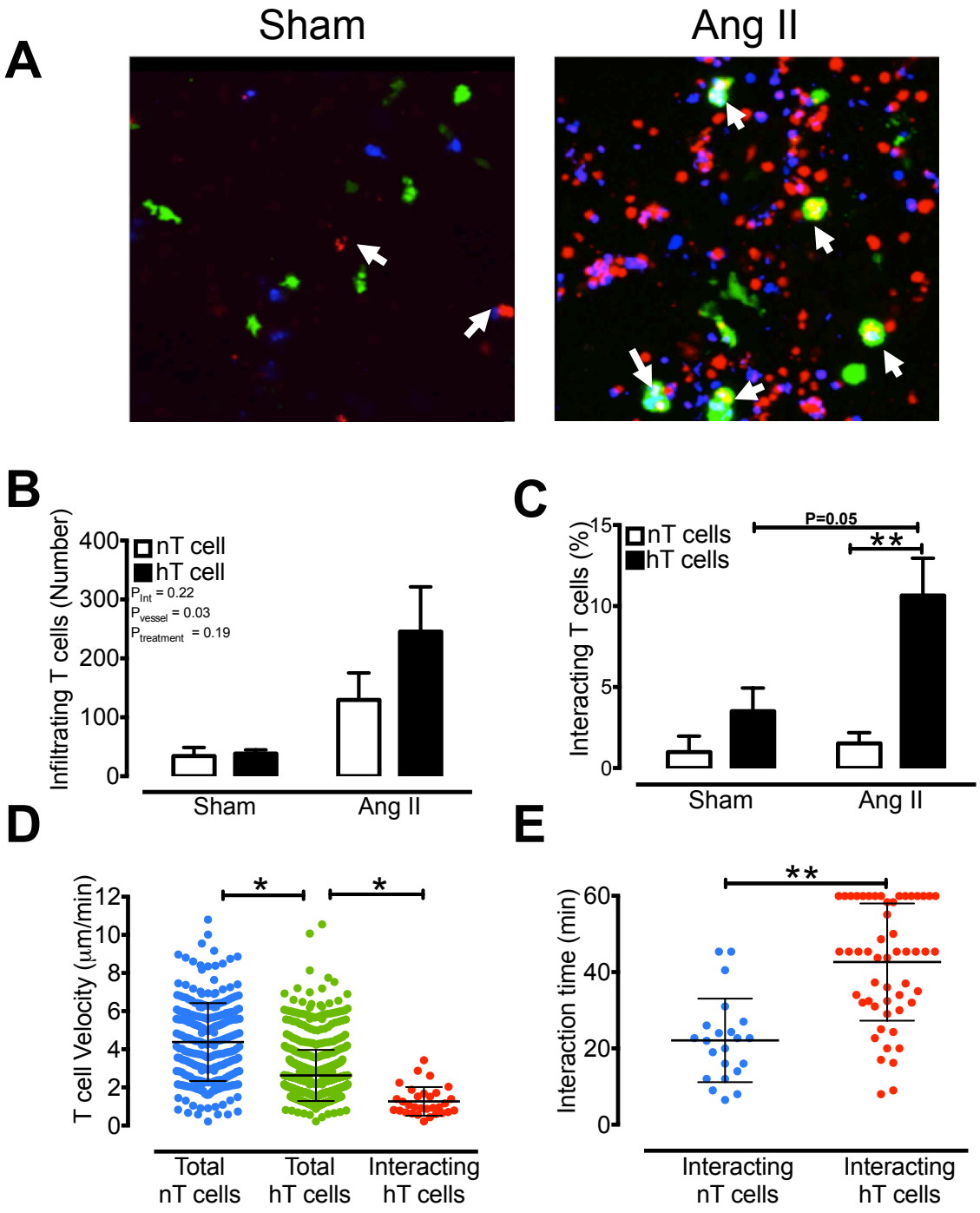

A
Sham
Ang II
B
Infiltrating T cells (Number)
400
300
200
100
0
nT cell
hT cell
P_Int = 0.22
P_vessel = 0.03
P_treatment = 0.19
Sham
Ang II
C
Interacting T cells (%)
15
10
5
0
nT cells
hT cells
P=0.05
**
Sham
Ang II
D
T cell Velocity (μm/min)
12
10
8
6
4
2
0
*
*
Total nT cells
Total hT cells
Interacting hT cells
E
Interaction time (min)
60
40
20
0
**
Interacting nT cells
Interacting hT cells

Figure 4. Confocal imaging of antigen-specific T cells - APC interaction in aortic PVAT in sham- and Ang II - treated mice. (A) Representative images of maximally projected z-stacks showing interaction between CD11c-YFP^{+} APCs (green) with hT (red) and nT (blue) in PVAT. (Arrows denote interactions between T cell and YFP^{+} APCs) (B) Mean infiltrating number and (C) percentage of hT cells and nT cells interacting with APCs in sham and Ang II. (**P<0.01; two-way ANOVA; sham n=5; Ang II n=8-11). (One field of view at 20x was analysed per aorta) (D) Mean velocities of individual infiltrating nT and hT cells in Ang II (E) Mean interaction time between nT and hT cells with APCs in Ang II (*P<0.05; Unpaired T test; n=8-11).

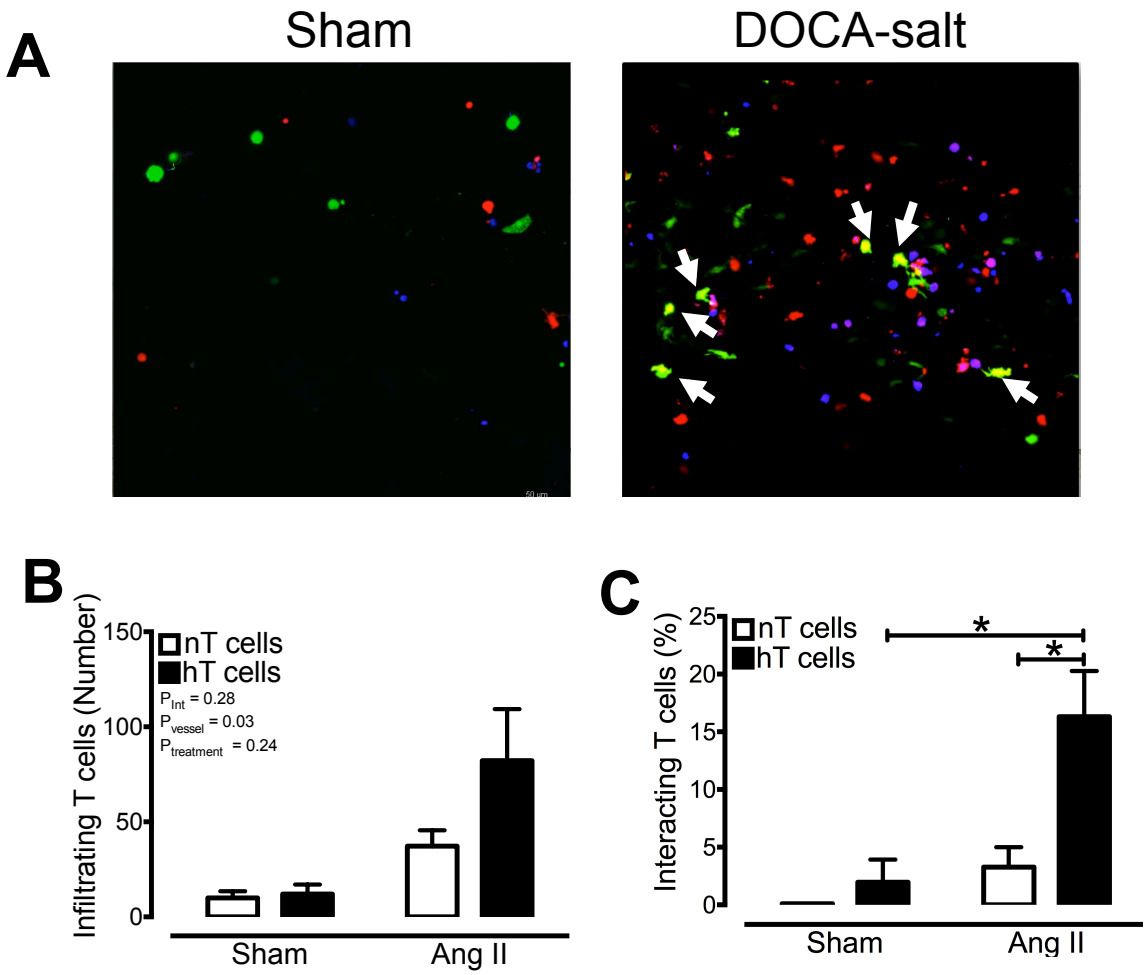


Figure 5. Confocal imaging of antigen-specific T cells - APC interaction in aortic PVAT in sham- and DOCA-salt - treated mice. (A) Representative images of maximally projected z-stacks showing interaction between CD11c-YFP^{+} APCs (green) with DOCA hT (red) and nT (blue) in PVAT. (Arrows denote interactions between T cell and YFP^{+} APCs) (B) Mean infiltrating number and (C) percentage of interacting infiltrated nT cells and hT cell in aorta from sham and DOCA-salt. Data represented as mean ± SEM. (*P<0.05; two-way ANOVA; sham n=3; Ang II n=6) (One field of view at 20x was analysed per aorta)

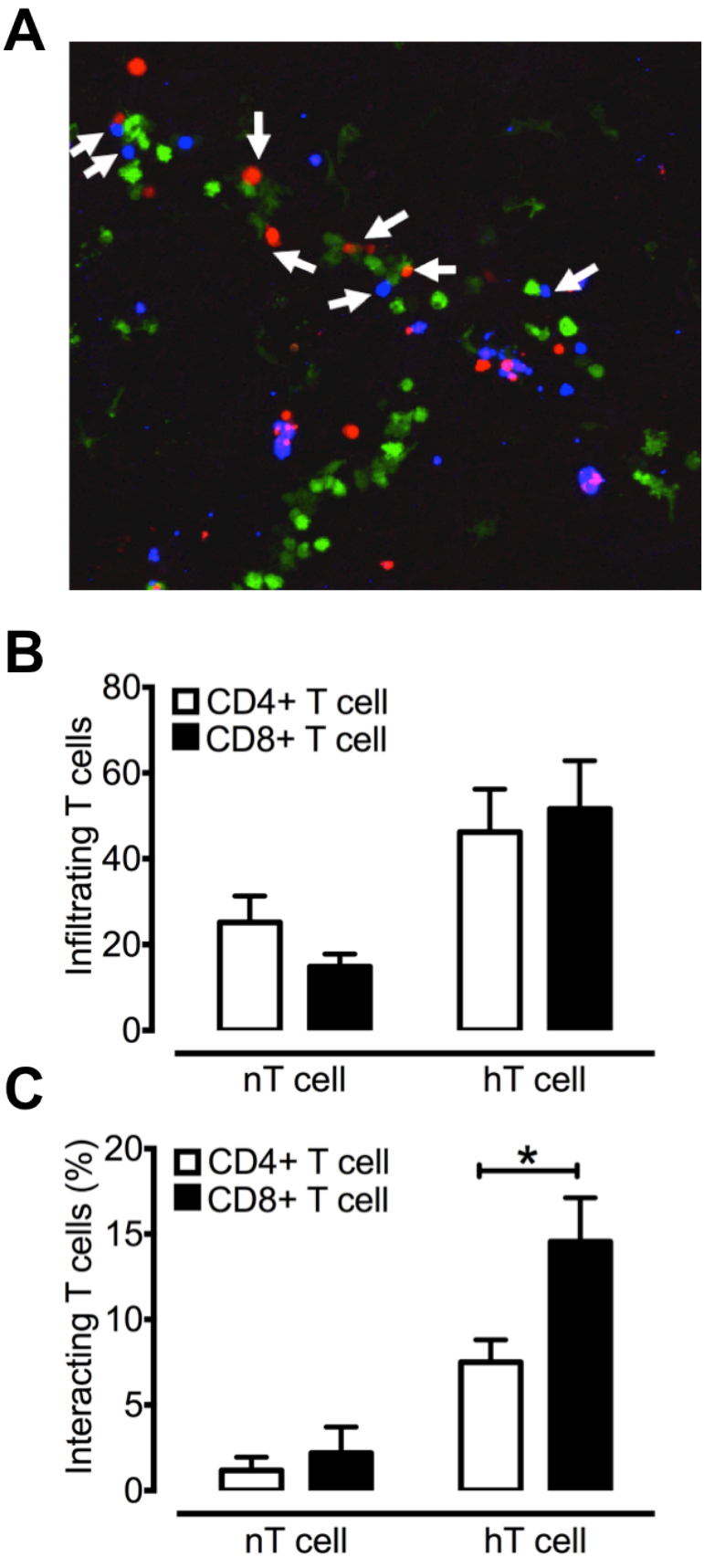

A
B
80
60
40
20
0
Infiltrating T cells
CD4+ T cell
CD8+ T cell
nT cell
hT cell
C
20
15
10
5
0
Interacting T cells (%)
CD4+ T cell
CD8+ T cell
*
nT cell
hT cell

Figure 6. Confocal imaging of antigen-specific T cells – APC (CD4+, CD8+ subset) interaction in aortic PVAT of Ang II - treated mice. (A) Representative images of maximally projected z-stacks showing interaction between CD11c-YFP^+ APCs (green) with CD4+ (red) and CD8+ (blue) T cells in PVAT. (Arrows denote interactions between T cell and YFP^+ APCs) (B) Mean infiltrating number and (C) percentage of interacting infiltrated CD4+ and CD8+ T cells from nT and hT cells during Ang II-induced hypertension (*$P<0.05$; Unpaired T test; n=6-10). (One field of view at 20x was analysed per aorta)

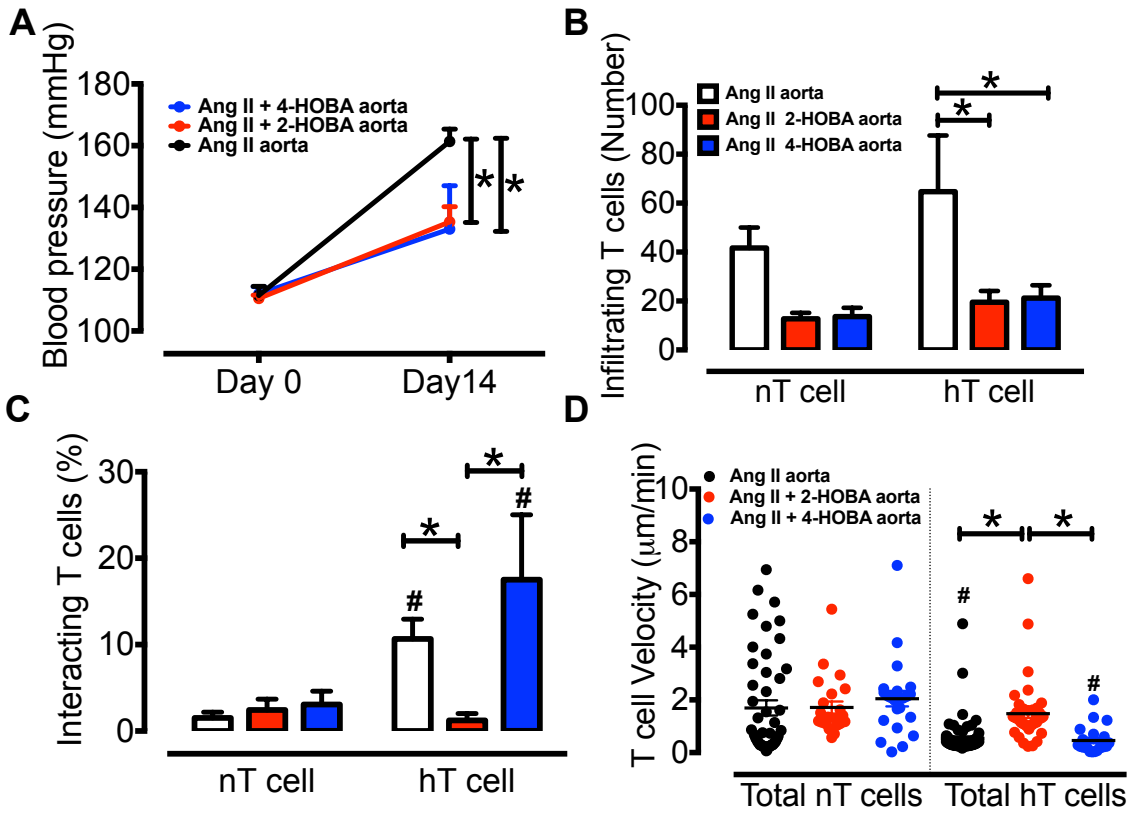


Figure 7. Isoketal-modified proteins contribute to Ang II induced hypertension and elevated antigen-specific interaction between hT cells and APCs. (A) Effect of isoketal scavenger 2-HOBA and control compound 4-HOBA on systolic blood pressure of Ang II - induced hypertension (*P<0.05 two-way ANOVA Vs Ang II aorta; n=3-8). Effect of 2-HOBA on (B) number of infiltrated CD3+ T cell and (C) percentage of T cell-CD11c-YFP^{+} APC interactions with APCs in Ang II - treated mouse aorta (#P<0.05 Vs same treatment from nT cell; *P<0.05 two-way ANOVA; nT cell n=3-4; hT cell n= 8-10;). Data represented as mean ± SEM and n represented as per animal. (D) Velocity of hT and nT cells in PVAT with Ang II, Ang II + 2-HOBA and Ang II + 4-HOBA treated mice (#P<0.05 Vs same treatment from nT cell; *P<0.05; two-way ANOVA; Ang II aorta n=2; Ang II+2-HOBA aorta n=7; Ang II+4HOBA aorta n=4).

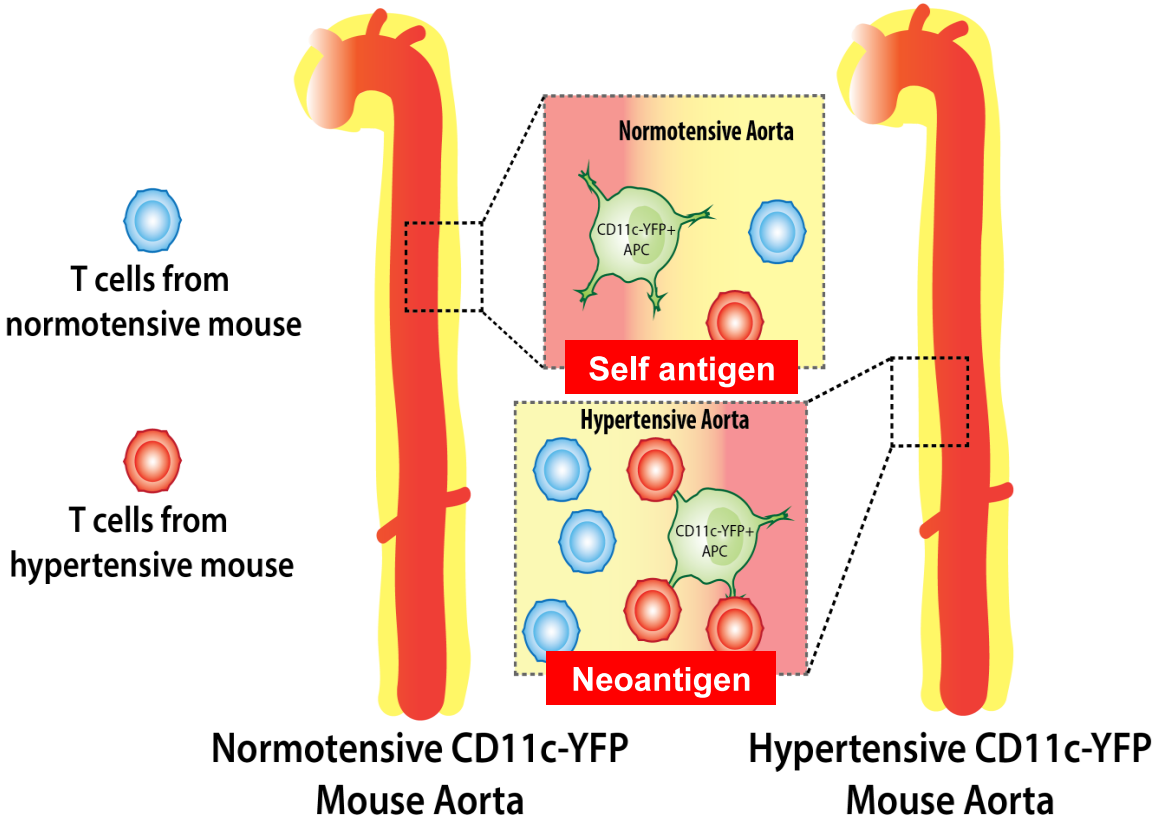


Figure 8. Existence of hypertension specific antigens. In explanted aorta from sham - treated mice there is limited level of T cell infiltration, as the T cells may only respond to the self - antigens. However in the explanted aorta from Ang - II treated mice, elevated amount of CCR5 ligands were released, which attracts greater T cells to infiltrate aorta. Importantly, a greater percentage of dynamic interaction with CD11c-YFP^{+} APCs was only seen in hT cells, which indicate the presence of hypertension specific antigens.

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vascular macrophage accumulation and hypertension by a CCR2 antagonist in deoxycorticosterone/salt-treated mice. *Hypertension*. 2012;60:1207-1212

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Chapter 6:

General Discussion

6.1 Summary of Main Findings

Overall, data presented in this thesis provide novel findings on the functional roles of T cell during hypertension. It addressed significant knowledge gaps of the specific function and local activation of infiltrating T cells that may promote experimental hypertension. Namely, the thesis identified phenotypical differences of tissue-infiltrating T cells during experimental hypertension, which release greater ROS and CCL2. Direct *in situ* activation of local infiltrating T cells also exacerbates endothelial dysfunction that was absent in aorta obtained from sham-treated mice. Notably, we have showed elevated dynamic T cell-APC interactions during hypertension, which highlights the existence of hypertension-specific unique antigens. These findings have advanced our understanding of the role that T cells play in the pathogenesis of hypertension.

6.2 Potential mechanisms underlying tissue-infiltrating T cells in the pathogenesis of hypertension

6.2.1 T cell-dependent inflammation hypertension

Using definitive characterization through cytokine and chemokine inflammatory assays we demonstrated an important role for T cells in the pathogenesis of hypertension. This thesis adds significant weight to the emerging concept of the involvement of adaptive immune system and different phenotypes of T cells during hypertension.

A likely proposed mechanism involved in T cell-induced hypertension is through the local release of cytokines following vascular and renal infiltration.[3] Elevated cytokine

release can promote inflammation by influencing the recruitment and maturation of other immune cells.[3] In chapter 3, we applied classical immunological techniques to phenotype infiltrating T cells. Notably, we demonstrated that the amount cytokines (IL-4, IL-17, IFN-γ and TNF-α) released per isolated T cell and proportion of cytokine producing T cells in Ang II-treated mice were identical to sham-treated mice. This finding suggested that there may not be a unique cytokine-producing T cell phenotype that is specific to hypertension. However, since there is enhanced T cell infiltration into blood pressure controlling organs (aorta, kidney, brain) this may ultimately result in an elevation in the overall local cytokine production due to an increase in the number of cytokine-producing cells, rather than a unique T cell phenotype. Recent studies have shown elevated Th1 (IFN-γ, TNF-α) and Th17 (IL-17) cytokines in the circulation or from splenic T cells in Ang II-induced hypertension.[10, 100, 134,135] Moreover, mice lacking IFN-γ or IL-17A have been shown to exhibit a blunted pressor response to various hypertensive stimuli,[102] which suggested an important role of IL-17A and IFN-γ in the pathogenesis of hypertension. However, there have been recent reports of increases in the number of cytokine-producing T cells that infiltrate various blood pressure-controlling organs.[102] Interestingly, these studies did not express their data as the proportion of cytokine producing T cells as we have in Chapter 3. Since these studies have also shown significant accumulation of T cells in the vasculature and kidney of hypertensive mice compared to sham-treated mice, it is highly probable that if expressed as a percentage of the total T cell infiltrate, they may have observed no differences between T cells isolated from hypertensive mice compared to normotensive mice - much similar to the findings of

Chapter 3. This would support the notion that it is the overall accumulation of cytokine-producing T cells in in blood pressure-controlling organs associated with hypertension that may promote local inflammation and organ dysfunction, as opposed to a unique cytokine producing T cell phenotype. In this context, identifying targets to prevent the initial T cell infiltration may represent a more effective approach to preventing detrimental T cell-dependent effects on hypertension, particularly since cytokine interventions such as etanercept exert equivocal effects on experimental hypertension.[10, 104, 105]

Chemokine and chemokine receptors play important roles in homeostastic regulation and migration of immune cells including T cells.[86, 87] While we did not observe a unique cytokine-producing phenotype in Chapter 3, we discovered a unique T cell chemokine phenotype that released significantly greater amounts of CCL2 that resided in isolated aorta from Ang II-treated mice (Chapter 3). Elevated CCL2 could attract CCR2 expressing monocytes and T cells, and plays an important role in immune cell recruitment in disease.[136, 137] Ang II-induced macrophage infiltration in the vasculature is reduced in CCR2 knockout mice,[96, 97] indicating the importance of CCL2-CCR2 chemokine axis in recruitment of macrophages. Cytometric bead array analysis on conditioned media from infiltrating vascular and renal T cells showed enhanced CCL2 production, which was absent in conditioned media of T cells isolated from the blood or spleen. Interestingly and a potential limitation in Chapter 3 was that only CCL2 was examined as part of a general inflammatory mediator detection kit. In Chapter 5 we used a proinflammatory chemokine

array to examine 12 different chemokines simultaneously (CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL9, CXCL10, CXCL13). We postulate that if samples obtained from Chapter 3 were examined using the proinflammatory chemokine array, we may have also observed elevations in other chemokines that could be released from T cells. In fact, from conditioned media of aorta obtained from Ang II-treated mice we observed enhanced release of CCL3, CCL4, CCL5, CCL22, CXCL13. Admittedly, elevations in these chemokines may not be purely T cell-derived, however, our data certainly suggests that during hypertension in an environment with increased T cell infiltration, there is a greater inflammatory chemoattractive environment in the vessel.

Targeting chemokine receptors to prevent leukocyte infiltration into the blood pressure controlling organs during hypertension may also be a feasible approach to address inflammation associated with hypertension. Antagonism of CCR2 reduces vascular inflammation and dysfunction in Ang II- and DOCA-salt-induced hypertension.[40, 41] Blockade of CXCR4 also prevents T cell accumulation and fibrosis in kidney in DOCA-salt-treated mice.[138] A recent study also showed that inhibition of CCR5 prevents vascular inflammation and endothelial dysfunction during hypertension.[90] Consistent with these findings, in Chapter 5 we identified that T cell infiltration is highly-dependent on the CCR5-chemokine axis. We demonstrate elevated CCR5 chemokine (CCL3, CCL4, CCL5) production in conditioned media of aorta of Ang II-treated mice. Furthermore, using a novel *ex vivo* model of T cell infiltration where stimulated T cells were co-

incubated with Ang II-treated mouse aorta, we showed that blocking CCR5 using Met-RANTES dose-dependently prevented infiltration of T cells into explanted aorta. This is the first study demonstrating that pharmacological targeting of the CCR5-axis reduces acute T cell infiltration in an *ex vivo* explanted aorta model, which further supports the importance of CCR5-axis in T cell infiltration.

6.2.2 Effect of T cell-derived ROS on vascular function during hypertension

This thesis has addressed the direct role of T cell-derived cytokines during hypertension; another pathophysiologically relevant pathway particularly in the context of vascular dysfunction is via T cell-derived ROS. It is well documented that oxidative stress and ROS contribute to the progression of cardiovascular diseases such as atherosclerosis and hypertension via direct effects on endothelium-dependent vasodilatation and other factors such as VSMC proliferation.[119] ROS may also play a role in T cell activation itself since increased brain and vascular ROS has been shown to enhance peripheral T cell activation and subsequently exacerbate to Ang II-induced pressor responses.[10, 121, 122] In Chapter 3, using L-012 chemiluminescence and DCF staining in conjunction with flow cytometry, we identified elevated ROS production that was only observed in T cells isolated from aorta of Ang II-induced hypertensive mouse.[98] This was also supported by data presented in Chapter 4, where we showed increased ROS production following *in situ* T cell activation in whole aorta obtained from Ang II-treated mice using L-012 chemiluminescence and DHE staining. Therefore, using 3 different measurements of ROS between the Chapters 3 and 4, we have consistently shown that activation of

vascular T cells, whether isolated or *in situ*, results in increased ROS production during Ang II-induced hypertension. Importantly, in Chapter 4 we confirmed that these elevations in ROS translate into functional deficits in isolated aorta, which is the first evidence of a direct pathophysiological role for T cells in hypertension. To support a ROS dependent mechanism, co-treatment with ROS scavenger tempol abolished both T cell-derived ROS production and exacerbations in endothelial dysfunction.

Curiously, when identifying ROS-producing areas in DHE stained aortic cross-sections, the areas with the most intense staining were localised to the PVAT. There is indeed evidence that perivascular ROS can actually induce endothelial dysfunction via an "outside-in" mechanism as described by the Pagano group.[131, 139] Pagano's group suggested that ROS produced in the adventitia may also be enzymatically dismutated to H_2O_2 that is cell-permeable, which can diffuse through the vessel wall,[127] and act as paracrine mediator activating downstream signalling to induce endothelial dysfunction.[132, 140] Strikingly, in Chapter 4 exacerbation of endothelial dysfunction in response to T cell activating stimuli was abolished upon removal of PVAT, suggesting that the T cell infiltrates within the PVAT from Ang II-induced hypertensive mouse aorta is indeed playing a direct role in modulating endothelium-dependent vasorelaxation. T cells infiltrate greatly into PVAT during hypertension,[10] where T cells are source of cytokines[3] and release greater ROS during hypertension.[119, 120] Therefore, the PVAT may act as a reservoir for ROS and inflammatory cytokines, which ultimately promotes vascular dysfunction and remodelling. In SHRs, the PVAT has been shown to behave

differentially compared to normotensive Wistar-Kyoto (WKY) rats.[141] Isolated aortic rings obtained from WKY rats with PVAT intact exhibited reduced phenylephrine-induced contractions compared to SHRs aortic with PVAT intact. Interestingly, in a bioassay that transferred the solution incubated with PVAT-intact aorta to aorta with PVAT removed, enhanced relaxation responses in aorta from WKY, but not SHR.[141]

Recently, it is revealed that PVAT also plays a role in controlling vascular function and release ADRF which inhibit aortic contraction and in healthy mice, however during elevated inflammatory conditions such as postnatal obesity, these ADRF-induced relaxation effects on the vasculature were diminished.[107, 108] Studies have indicated that ADRF released from PVAT inhibit vasoconstriction to a number of agonists including phenylephrine, serotonin and Ang II in rat aorta and mesenteric arteries, as well as in human internal thoracic arteries.[142,143,144,145] Evidence also suggests that a diminished effect of NO may also be involved in the PVAT-induced modulation of vascular tone.[146, 147] Heagerty's group showed that incubation with superoxide dismutase and catalase restores PVAT vasorelaxant function in rats with obesity.[146] Furthermore, it was identified that adipose tissue also releases adiponectin, an adipokine with anti-inflammatory activity that is down-regulated in obese male mice.[117] It was demonstrated that, in mouse adipose tissue, adiponectine is inversely correlated with RANTES mRNA, which suggests a potential role of adiponectin in regulating RANTES expression in adipose tissue.[117] Collectively, with relevance to humans, PVAT in individuals with inflammatory diseases such as obesity or hypertension may release greater ROS and

inflammatory cytokines but reduced anti-contractile mediators.

6.3 Hypertension-induced neoantigens and enhanced dynamic T cell-APC interactions as targets for treatment for hypertension

6.3.1 Visualising dynamic vascular T cells behavior and interaction

While studies in chapter 3 and 4 identified the vital roles of T cell-derived chemokine and ROS during hypertension, a fundamental question is how are these infiltrating T cells actually activated in the setting of hypertension? Classical T cell activation is known to be involved in experimental hypertension, where it was shown that chronic treatment with an inhibitor of T cell co-stimulation, CTLA4-Ig, not only prevented, but also reversed both Ang II- and DOCA salt-induced hypertension.[135] From a mechanistic perspective, this study suggests that rather than nonspecific accumulation of previously activated T cells, infiltrated T cells are activated via classical mechanisms and antigen recognition by T cells. Presentation of antigens to naïve T cells typically occurs in sublymphoid organs, and effector antigen-experienced T cells then egress and are recruited to high chemokine expressing inflammatory sites where they may encounter cognate antigens to exert T cell-mediated immunity [2]. A major aspect of this thesis was to develop a novel imaging model to investigate T cell infiltration and dynamic movements throughout the aorta during hypertension. This represented a significant knowledge gap in the field, as T cells are known to infiltrate vessels during hypertension, however their behavior and interaction within the vessel wall remain unidentified. Chapter 5 described the first live real time imaging model to capture T cell behaviors in vessels obtained from Ang II-treated mice

compared to vessels obtained from sham-treated mice. We adapted an approach similar to Klaus Ley's model where they have imaged T cell-APC interaction in mouse models of atherosclerosis.[36] While it was shown that T cells co-incubated with atherosclerotic vessels readily infiltrate the plaque, to validate our model in hypertension, the infiltration of T cells into aorta was firstly examined. We identified that a hypertensive mouse aorta is a prerequisite for T cells to infiltrate into the PVAT. Stimulation of enriched T cells from the spleen was also required, as the effector T cells are known to express greater chemokine receptors.[10] It is known that T cells bearing CCR5 are elevated during Ang II-induced hypertension, at least in the circulation, aorta and kidneys.[10, 90] We showed CCR5-dependent migration of T cells into the PVAT, where inhibition of CCR5 using Met-RANTES was able to dose-dependently inhibit chemotaxis. Interestingly, T cells obtained from sham-treated mice (nT cells) can also infiltrate hypertensive mouse aorta, however, the number was reduced compared to T cells obtained from Ang II-treated mice (hT cells), which may suggest that there are apparent differences between T cells from spleen of normotensive and hypertensive mice. Observations of elevated infiltration of various T cell phenotypes (e.g. Th1, Th2, Th17) including $FoxP3^+$ T regulatory cell infiltration into the aorta during hypertension (Chapter 3), suggests that recruitment of T cells during hypertension may be non-selective, as effector T cells are recruited towards a high chemokine gradient. Nonetheless, given the dependency on the CCR5-axis, T cell infiltration in our *ex vivo* model resembles T cell recruitment in *vivo.* and may be used as a potential new migration assay to evaluate agents that prevent T cell migration in hypertension. Compared to classic T cell migration assays, such as Boyden chamber, the

live cell-imaging model incorporating a fully intact vessel may be more physiologically relevant in diseased vascular tissue.

6.3.2 T cell-APC interaction

Since we had validated our explanted aorta model in the setting of hypertension, the major use of this technique was to examine whether there is dynamic T cell-APC interactions in aorta obtained from Ang II-induced hypertensive mice. Live cell imaging models have been applied to examine dynamic T cell-APC and activation in other diseases, such as skin inflammation,[148] encephalomyelitis lesions[143] and liver granulomas.[144] During *leishmania* major infection, activated $CD4^+$ T cells were shown to enter inflamed tissue irrespective of their antigen specificity, but dynamic interactions with infected phagocytes was only observed in antigen presentation by $CD4^+$ T cells.[148] In the setting of hypertension, we demonstrated that in aorta obtained from Ang II-treated mice, greater hT cell-APC interactions were observed compared to nT cells, likely due to antigen presentation to T cell. Harrison's group have suggested that isoketal-modified proteins may be the neoantigens during hypertension since there is significant deposition of these modified proteins in the aortic PVAT of hypertensive mice and scavenging isoketals using 2-HOBA blunted pressor responses to Ang II and DOCA-salt.[83] Consistent with these findings, we have demonstrated that chronic treatment with 2-HOBA in Ang II-infused mice also significantly inhibited the interaction between YFP^+ APCs and hT cells, in addition to blunting pressor responses. Cross reactivity experiments where T cells from a DOCA-salt-induced hypertension were co-incubated with vessels

from Ang II-treated CD11c-YFP mice suggested the possibility that hypertension specific antigens may be conserved from across different models of hypertension. Notably, although both $CD4^+$ and $CD8^+$ T cell interactions with YFP^+APCs were elevated in vessels from Ang II-treated mice, $CD8^+$ T cells appeared to have a greater proportion of interactions with CD11c-YFP^+ APCs compared to $CD4^+$ T cells. This may suggest that predominant role for $CD8^+$ T cells in promoting vascular inflammation during hypertension, which has been suggested in the kidney of Ang II-treated mice.[151] However, since $CD8^+$ and $CD4^+$ recognise antigens in the contexts of different MHC molecules (MHC Class I and II, respectively), elevations in T cell-APC interactions in both subsets, may also suggest the presence of multiple distinct neoantigens during hypertension.

An important discovery, from our imaging studies in Chapter 5, was the nature of the hypertension-specific interaction. It was anticipated that if we observed similar elevations in interactions between hT cells and APCs in aorta from Ang II- or sham-treated mice compared to nT cells, we could speculate that the antigen was indeed a self-antigen. As sham-treated mouse aorta would not have been exposed to the potential stressors related to hypertension, which are postulated to induce neoantigen formation, any recognition of antigens by hT cells in sham-treated mouse aorta could only be classed as self-antigens. This would imply that hypertension was indeed an autoimmune disorder. However, we have determined that hT cells only recognize antigens within vessels of hypertensive mice, which clearly indicates that existence of neoantigens that are unique to

hypertension. In addition, similar arguments have been used in studies of other T cell dependent cardiovascular diseases such as atherosclerosis, although identifying the potential neoantigens in that context has proven challenging but some promising candidates have been identified.[146]

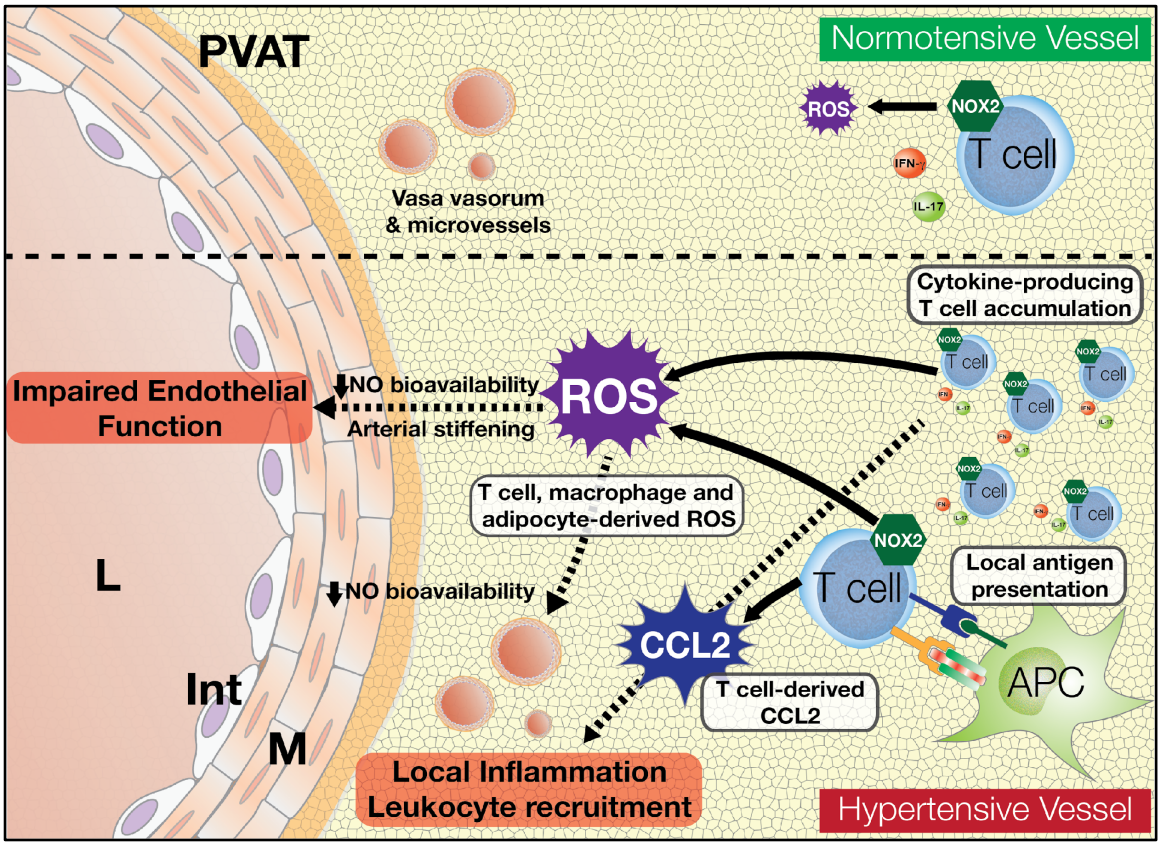

Figure 6.1. Schematic highlighting the role vascular infiltrating T cells during hypertension. This thesis has identified that T cells that infiltrate the vasculature during hypertension release the same amount of cytokines such as IFN-γ and IL-17. However, in hypertensive vessels the excessive accumulation of T cells can result in an overall net increase in aortic cytokine levels. Infiltrating T cells in hypertensive vessels also release greater ROS and CCL2, suggestive of a differential phenotype that is unique to the vasculature during hypertension. The excessive T cell-derived ROS and/or ROS produced from other PVAT cells such as macrophages and adipocytes, can then reduce NO bioavailability and promote endothelial dysfunction. Importantly, we have also identified that infiltrating T cells in the aorta of hypertensive mice also recognise cognate antigens that are only present in diseased hypertensive mouse vessels, but not healthy normotensive mouse vessels.

6.4 Limitations and Future approaches

In Chapters 3 and 5 it was found that CCL2 and CCR5 ligands (CCL3, CCL4, CCL5) appears to play an important role in T cells recruitment, however, contribution of other chemokines such as CXCL13 cannot be ignored. In future studies, using cytometric bead arrays to screen other chemokines and antagonising these chemokine pathways would provide valuable information on the mechanisms of T cell recruitment. Remarkably, in Chapter 5 we revealed the potential of multiple hypertension specific antigens due to difference in presentation of antigen to $CD4^+$ and $CD8^+$ T cells, and blocking of anti-major histocompatibility complex class I or II molecules may help to confirm the nature of hypertension-specific antigens. It has been shown that, in experimental autoimmune encephalomyelitis (EAE), blocking MHC II molecules decreased the number of interacting pathogenic T cells within the CNS.[149] Another important question yet to be answered relates to which type of T cell is primarily responsible for inflammation in the setting of hypertension and how it promotes severe hypertension. A future approach to discover this would be using immunopeptidome assays to examine the antigens presented by APCs, by isolating APCs and process the antigens that are in the MHC molecule to sequence the specific antigens. Guzik's landmark study has reignited research into adaptive immunity and hypertension. It appears that T cells are possibly not the initial cause of hypertension but perhaps more likely a consequence, that then promotes the progression to more severe hypertension. Based on Dr Harrison's hypothesis, neoantigens that may be specific for hypertension might be formed after a period of mild hypertension. This would suggest that preventing immune activation via intervention at

this early stage would be of potential benefit to preventing overt hypertension. Moreover, if this progressive process of immune activation in hypertension was true, it also further reinforces the need to monitor blood pressures routinely to identify high-risk states such as pre-hypertension. It would be of great interest to identify when T cells become activated in the development of hypertension and whether this timing correlates with the severity of hypertension. Furthermore, the current study only examined the infiltrated T cells in aorta but not other small resistance arteries that also play an important role in regulating blood pressure and hypertension. Interestingly, preliminary study showed some phenotypical difference in terms of T cell infiltration into mesenteric arteries, therefore further investigations would be beneficial in future studies.

6.5 Clinical implications of Main Findings

Our findings have furthered our understanding on the mechanisms by which T cells promote hypertension and support the rationale of potentially targeting inflammation as a treatment for severe hypertension. The ideal approach would be to target inflammation and immunity without the burden of global immunosuppression by identifying and using selective anti-inflammatory agents.

Based on our findings in Chapter 3, where we found no specific cytokine-producing T cell phenotype infiltrating blood pressure-controlling organs, targeting T cell-derived cytokines may not be the most efficacious approach to controlling T cell-mediated

inflammation. In contrast, preventing T cell entry in the vasculature and other organs may represent a superior approach since we have shown that when activated, T cell infiltrates at least in the vasculature, are capable of releasing excessive ROS that can directly impair endothelial function. Moreover, restricting the entry of T cells will also reduce excess chemokine CCL2 production, as well as prevent the overall increase in cytokine levels that results from T cell accumulation. During the course of this PhD, there have been some studies that have addressed targeting vascular immune cell chemotaxis during hypertension. Chan et al, demonstrated that blocking CCR2 using antagonist INCB3344 reversed the DOCA salt-induced macrophages infiltration and importantly blunted the elevated blood pressure in DOCA salt-treated mice.[41] Highly relevant to this thesis, the Guzik group examined the effect of chronic MET-RANTES treatment on the development of Ang II-induced hypertension.[90] Interestingly, although MET-RANTES treatment did not prevent the Ang II-mediated pressor responses, vascular infiltration of T cells was reduced and endothelial function was indeed improved by CCR5 inhibition. This is consistent with our evidence showing the importance of the CCR5-axis in vascular T cell recruitment. In regards to the development of hypertension, we speculate that perhaps longer treatment periods with MET-RANTES could potentially result in reversal of hypertension as a result of reduced inflammation.

One important finding that may translate to clinical applications in the long-term, is the confirmation that infiltrating T cells recognise cognate antigens in the aorta during hypertension. With this knowledge at hand, future studies directed at isolating and

characterising antigens from the aorta using immunopeptidome approaches could pave the way to developing immunizations or tolerisation vaccines. It is known that blocking T-cell co-stimulation using CTLA4-Ig analogue prevents experimental hypertension.[79] Clinical evidence also proved that inhibiting T cells activation using a CTLA4-Ig analogue reduced rebound hypertension in patients associated with kidney transplantation.[80] However, while CTLA4-Ig is well tolerated and readily prescribed clinically, rather than inhibiting T cell activation globally and risking off-target effects, selective immunisation against hypertension-specific antigens could circumvent the development of inflammation associated with hypertension. In fact, in SHRs, immunisation against heat shock protein 70 (HSP70) has been shown to reduce renal inflammation and blood pressure in salt-sensitive hypertension.[153,154] However, this is indeed an expanding area of basic research and much research is still required to translate from bench to bedside.

Based on the collective evidence obtained thus far from studies on experimental hypertension, it has been postulated that involvement of the immune system and inflammation stems from initial changes in blood pressure.[155] It is thought that mild increases in blood pressure through well known hypertensive stimuli such as Ang II, high salt and ROS, leads to damage and modifications to proteins that may then serve as neoantigens that are specific for hypertension.[83] Importantly, initial presentation of neoantigens to T cells, leads to activation and expansion of effector T cells that will target the hypertension-specific antigen(s).[79,83] Indeed, this thesis has implicated a vascular

antigen(s) that is presented and recognised by effector T cells from hypertensive mice. Effector T cells are then recruited via a chemokine gradient into blood pressure controlling organs, where they recognise cognate antigens and mount a local inflammatory response. Inflammation of the vessels and kidney leads to end-organ damage and dysfunction that ultimately further promotes hypertension and the risk of an end-stage cardiovascular event.[155] Currently, there is no evidence to support this hypothesis in clinical hypertension. However, since the initial rise in blood pressure appears to be vital to activation of the immune system during experimental hypertension, it could be postulated that targeting patients at a state of pre-hypertension (130-139 mmHg) would be far more beneficial than later stages of clinical hypertension. This would circumvent the potential activation of the immune/inflammatory axis. However, since many patients are likely to have established hypertension when diagnosed, re-purposing immunomodulatory agents that are currently used to treat other inflammatory disorders may potentially be an immediate therapeutic strategy to control hypertension.

6.6 Conclusion

In summary, studies in this thesis have expanded our current understanding of the role of infiltrating T cells of blood pressure controlling organs – namely the vasculature. Collectively, the findings of this thesis have addressed specific knowledge gaps in the field regarding T cell-mediated inflammation, effects on vascular function and mechanisms of T cell activation. Novel tools and approaches to studying vascular T cell chemotaxis and behaviour have also been discovered, refined and described in this thesis.

Excitingly, our findings raise the possibility that therapeutic targeting of infiltrating T cells and their local activation could be used as a tool for preventing severe hypertension and associated end organ damage.

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Appendix

Role of Inflammation and the Angiotensin Type 2 Receptor in the Regulation of Arterial Pressure During Pregnancy in Mice

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Abstract—During normal pregnancy the renin–angiotensin system is activated, yet pregnant women are resistant to the pressor effects of angiotensin II. Our aim was to determine the role of the angiotensin type 2 receptor (AT_2R) in the regulation of arterial pressure, natriuresis, and immune cell infiltration during pregnancy. Mean arterial pressure was measured via telemetry, and flow cytometry was used to enumerate immune cell infiltration in 14-week-old wild-type and AT_2R knockout mice during gestation. In wild-type mice, mean arterial pressure decreased during gestation, reaching a nadir at gestational day 9 (–6±2 mm Hg) and returned to near preconception levels during late gestation. In AT_2R-deficient mice, the midgestational decrease in mean arterial pressure was absent. Furthermore, mean arterial pressure was significantly increased during late gestation compared with wild-type mice (≈10 mm Hg). As expected, circulating immune cell activation was suppressed during pregnancy. However, this response was absent in AT_2R-deficient mice. While renal immune cell infiltration was similar between the genotypes, there was a significant T cell phenotypic switch toward a proinflammatory T-helper 1 phenotype in AT_2R-deficient mice. These data indicate that the AT_2R plays an important role in arterial pressure regulation and may modulate T cell activation and renal cytokine production during pregnancy. Therefore, deficits in AT_2R expression may contribute to pregnancy-induced hypertension and thus represents a potential therapeutic target. **(*Hypertension*. 2014;64:626-631.)** • **Online Data Supplement**

Key Words: arterial pressure ■ inflammation ■ pregnancy ■ renal

During a normotensive pregnancy circulating angiotensinogen, renin activity, and angiotensin II (AngII) are increased, yet pregnant women are resistant to the pressor effects of AngII.[1,2] Recently, we have gained strong evidence that the angiotensin type 2 receptor (AT_2R) blunts pressor responsiveness, sodium retention, tubuloglomerular feedback, and renal vasoconstrictor responses to AngII in female rodents.[3–5] The renal responses to AngII are also different in healthy men and women,[6] and it has been suggested that the explanation for these disparities might also lie with differences in AT_2R function.[7] Given that estrogen enhances AT_2R expression,[8,9] the AT_2R may contribute to the cardiovascular and renal adaptations that occur during pregnancy.

While the immune system is suppressed during normotensive pregnancy, the pathophysiology of preeclampsia is characterized by immune system activation.[10] It has been demonstrated that T cells, part of the adaptive immune system, are essential for the development of AngII-induced hypertension.[11] Within the kidney, the angiotensin type 1 receptor (AT_1R) increases production of the proinflammatory T-helper (Th) 1 cytokine, interferon-γ (IFN-γ), and decreases production of the anti-inflammatory Th2 cytokine interleukin-4.[12] Skewing of Th cells toward a Th1 phenotype is well established in several inflammatory disorders including hypertension and preeclampsia.[12,13] Recent studies indicate that AT_2R agonism with compound 21 elicits anti-inflammatory actions, skewing the Th1:Th2 balance toward the Th2 phenotype.[14,15] Thus, the AT_2R may contribute to the increase in Th2 cytokines and subsequent suppression of Th1-mediated immune responses during normotensive pregnancies, whereas AT_2R deficiency may promote an increase in the Th1:Th2 ratio and pregnancy-induced hypertension.

We hypothesized that during pregnancy the AT_2R modulates natriuresis and contributes to the suppression of the immune system, which facilitates the decrease in arterial pressure during pregnancy. Our aims were to examine mean arterial pressure (MAP) throughout pregnancy and the renal and placental immune responses on gestational day (Gd) 8 and Gd16 and renal excretory function at Gd18 in wild-type (WT) and AT_2R-knockout (AT_2R-KO) mice.

Methods

Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. MAP was measured via radiotelemetry at baseline, during gestation and 2 weeks postpartum in WT (FVB/N)

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and AT$_2$R-KO mice. In additional cohorts, immune system activation, infiltration, and the proportion of ex vivo–stimulated T cells producing Th1 (IFN-γ, tumor necrosis factor α, interleukin-17) and Th2 (interleukin-4) cytokines were determined using flow cytometry, renal excretory function was measured via 24-hour urine collection, and renal and placental gene expression of renin angiotensin system (RAS) components (AT$_{1a}$R, AT$_{1b}$R, AT$_2$R, angiotensin-converting enzyme 2 [ACE2], and mas receptor [MasR]) were determined using real-time reverse transcription-polymerase chain reaction. See the online-only Data Supplement for full methods.

Results

Arterial Pressure

Preconception MAP was similar in WT and AT$_2$R-KO mice (94±1 versus 92±1 mm Hg, respectively, P=0.2). During pregnancy, MAP decreased in WT mice. This gestational decrease in MAP reached a nadir of –6±2 mm Hg at Gd9 (P<0.01 as compared with baseline; Figure 1). Compared with WT, the midgestational decrease in MAP was abolished in AT$_2$R-KO mice (P_g=0.04; Figure 1). Near term, MAP returned to baseline levels in WT mice; however, during late gestation MAP was augmented in AT$_2$R-KO mice (2±3 versus 13±7 mm Hg at Gd20, P<0.05). In mice with radiotelemetry probes that continued to record MAP for the full 2-week postpartum period, it was observed that MAP returned to preconception levels in both WT (88±2 mm Hg; n=6) and AT$_2$R-KO (93±3 mm Hg; n=4) mice.

Renal Excretory Function

Basal body weight was similar between the genotypes and increased during pregnancy (P_t<0.001; Table S1 in the online-only Data Supplement). Water intake and urine flow were increased at Gd18 (both P_t<0.05), but the increases were not different between the genotypes (Table S1). Sodium intake was increased at Gd18 as compared with baseline (P_t=0.004; Figure 2A). In contrast, there was no significant difference in sodium excretion between the genotypes or during pregnancy (Figure 2B). Determination of sodium balance (sodium intake minus excretion) demonstrated that during pregnancy there was net sodium retention (P_t=0.008). Moreover, this effect was enhanced in AT$_2$R-KO mice (P_{g*t}=0.05; Figure 2C). Basal albuminuria was not significantly different between the genotypes. During pregnancy albuminuria decreased 38±6% in the WT and increased 57±35% in the AT$_2$R-KO mice (P_{g*t}<0.001; Table S1). There were no significant differences in fetal number, fetal weight, placental weight, or placental efficiency between the genotypes (Table S2).

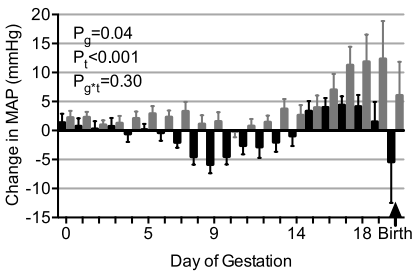

Figure 1. Change in 24-hour mean arterial pressure (MAP) during pregnancy compared with baseline in wild-type (■; n=7) and angiotensin type 2 receptor knockout (■; n=8) mice. Data are presented as mean±SEM. Data were analyzed using repeated-measures ANOVA with the factors genotype (P_g), time (P_t), and their interaction, followed by Tukey post hoc tests. P<0.05 was considered statistically significant.

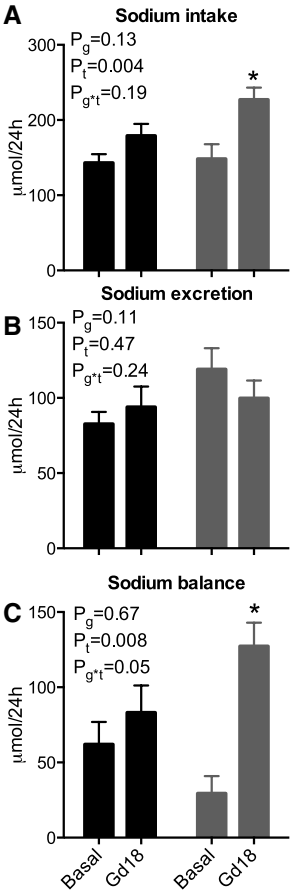

Figure 2. Sodium balance studies in wild-type (WT) and angiotensin type 2 receptor knockout (AT$_2$R-KO) mice at baseline and on gestational day (Gd) 18. Sodium intake (**A**), sodium excretion (**B**), and sodium balance (**C**; sodium intake minus sodium excretion) of WT (■; n=8) and AT$_2$R-KO (■; n=6) mice. Data are presented as mean±SEM. Data were analyzed using repeated-measures ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. *P<0.05 vs basal AT$_2$R-KO.

Immune System Activation

Circulating

There was no significant difference in the proportion of circulating T cells (CD3+) between the genotypes at baseline or during gestation (P_{g*t}=0.4; Figure S4A). The proportion of the T cell subpopulations characterized by CD4+ (Th cells), FoxP3+ (T regulatory cells), and CD8+ (cytotoxic T cells) expression was similar between the genotypes at baseline and unchanged during pregnancy (Figure S4B–S4D). Analysis of circulating Th cells demonstrated that at baseline Th activation was significantly lower in AT$_2$R-KO as compared with WT mice (Figure 3A and 3B). Circulating Th activation was suppressed during pregnancy in WT but unaffected in AT$_2$R-KO mice (Figure 3A and 3B). Cytotoxic T cell activation was also suppressed during pregnancy; however, this was not significantly different between the genotypes (data not shown).

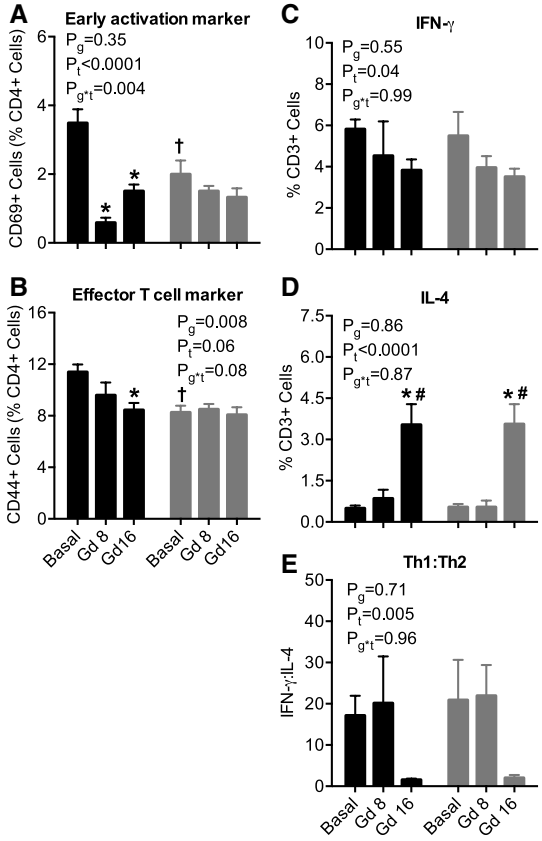

Figure 3. Circulating T cell activation in wild-type (WT; ■; n=4–12 per group) and angiotensin type 2 receptor knockout (■; n=4–12 per group) mice at baseline, gestation day (Gd) 8, and Gd16. CD4+ (**A**) early and (**B**) effector T cell activation, the proportion of ex vivo-stimulated T cells producing (**C**) interferon-γ (IFN-γ) and (**D**) interleukin (IL)-4, and the (**E**) Th1:Th2 ratio. Data are presented as mean±SEM. Data were analyzed using an ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. †P<0.05 vs respective WT group. *P<0.05 vs respective basal group. #P<0.05 vs respective Gd8 group.

The proportion of IFN-γ- and interleukin-4-producing T cells (Figure 3C and 3D) and the Th1:Th2 ratio were similar between the genotypes at baseline (Figure 3E). During gestation, the proportion of T cells producing the IFN-γ at Gd8 and Gd16 decreased to a similar extent in WT and AT_2R-KO mice (P_t=0.04; P_{g*t}=0.99; Figure 3C). Conversely, the proportion of T cells producing interleukin-4 was significantly increased at Gd16 in both genotypes (P_t<0.0001; P_{g*t}=0.87; Figure 3D). Consequently, at Gd16 the circulating Th1:Th2 ratio was similarly decreased from basal between the genotypes (P_t=0.005; P_{g*t}=0.96; Figure 3E). There were no significant differences in circulating tumor necrosis factor-α- and interleukin-17-producing T cells between the genotypes at baseline or during gestation (Figure S5A and S5D).

Renal

Basal renal leukocyte and T cell infiltration were similar in WT and AT_2R-KO females, and there were no significant differences in renal cytokine expression or the Th1:Th2 ratio (Figure 4). During pregnancy, renal T cell infiltration was similar between the genotypes (P_g=0.84, P_{g*t}=0.87; Figure 4B).

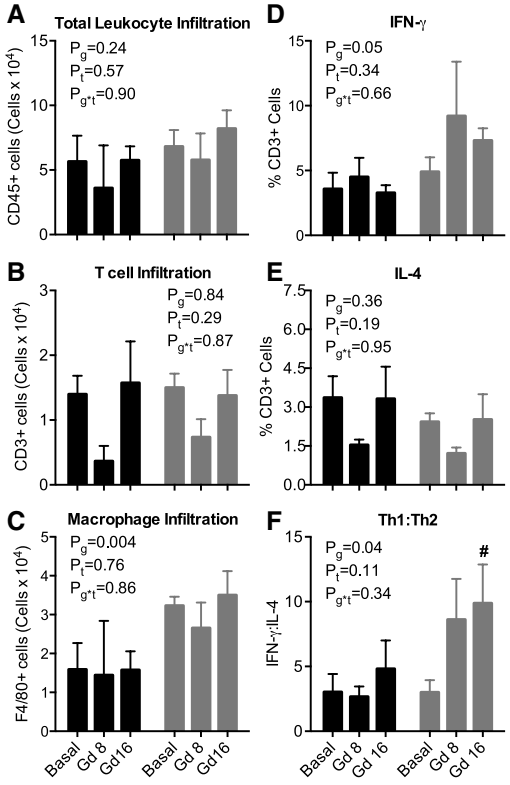

Figure 4. Renal immune cell infiltration and cytokine production in wild-type (WT; ■; n=4–12 per group) and angiotensin type 2 receptor knockout (■; n=4–12 per group) mice at baseline, gestation day (Gd) 8, and Gd16. Immune cell (**A**), T cell (**B**), and macrophage infiltration (**C**), the proportion of ex vivo-stimulated T cells producing interferon-γ (IFN-γ; **D**) and interleukin (IL)-4 (**E**), and the Th1:Th2 ratio (**F**). Data are presented as mean±SEM. Data were analyzed using an ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. †P<0.05 vs respective WT group.

However, compared with WT counterparts, the proportion of T cells producing IFN-γ was increased in AT_2R-KO females during pregnancy, representing a phenotypic change in the T cells infiltrating the kidney at Gd8 (4.5±1.5 and 9.2±4.2% CD3+ cells, respectively) and Gd16 (4.9±1.7 and 7.3±0.9% CD3+ cells, respectively; P_g=0.05, P_{g*t}=0.66; Figure 4D). There were no significant differences in the proportion of T cells producing tumor necrosis factor-α and interleukin-17 between the genotypes at baseline or during gestation (Figure S5B and S5E). Comparison of the renal Th1:Th2 ratios demonstrated a trend at Gd8, with AT_2R-KO mice having a higher Th1:Th2 ratio than WT mice (P=0.06; Figure 4F). At Gd16, the renal Th1:Th2 ratio was significantly increased in AT_2R-KO mice compared with baseline (P=0.04; Figure 4F). The number of macrophages infiltrating the kidney was also greater in AT_2R-KO than WT mice at baseline, and this difference was maintained during gestation (P_g=0.004, P_{g*t}=0.6; Figure 4C).

Placenta

The expected decrease in placental Th1:Th2 ratio was observed at Gd16 as compared with Gd8 in both genotypes (Figure S6F). There were no significant differences in T cell infiltration or cytokine production between the genotypes (Figures S5C, S5F, S6B, S6D, and S6E).

Renal and Placental Gene Expression

Compared with WT mice, AT$_2$R-KO mice had lower basal renal AT$_{1a}$R and AT$_{1b}$R gene expression (Figure S7A and S7B, both $P<0.05$). Basal renal ACE2 and MasR gene expression were not significantly different between the genotypes. During pregnancy there was no change in AT$_{1a}$R, AT$_2$R, or MasR gene expression (Figure S7). In WT mice, renal AT$_{1b}$R gene expression was reduced at Gd18 as compared with basal ($P=0.02$). In pregnant AT$_2$R-KO mice, renal ACE2 expression was significantly higher at Gd8 versus Gd16 ($P=0.04$). Pregnancy altered the expression of components of the RAS in the placenta with marked increases in AT$_{1a}$R, ACE2, and MasR. However, there was no significant impact of AT$_2$R deficiency (see Results in the online-only Data Supplement and Figure S8).

Discussion

There were 3 major findings in the present study. Firstly, the AT$_2$R mediates the normal midgestational decrease in arterial pressure and contributes to arterial pressure regulation during late gestation. These findings extend previous observations of the arterial pressure-lowering effects of the AT$_2$R during pregnancy.[16–18] Secondly, in AT$_2$R-KO mice there was a phenotypic switch in the T cells infiltrating the kidney toward a proinflammatory phenotype, resulting in an increase in the renal Th1:Th2 ratio at Gd16. Moreover, the increase in the Th1:Th2 ratio was observed only in the kidneys of pregnant AT$_2$R-KO mice, which markedly contrasted the reduced Th1:Th2 ratios in the circulation and placentae as per regular pregnancies. Finally, as expected, pregnant mice were in positive sodium balance. However, sodium retention was enhanced in pregnant AT$_2$R-KO mice, and this may have contributed to the increase in arterial pressure in late gestation. Therefore, the AT$_2$R plays an important role in arterial pressure regulation and may modulate renal immune cell phenotype as well as pressure-natriuresis during pregnancy. Our studies demonstrate that the AT$_2$R contributes to the normal cardiovascular and renal adaptations that facilitate the gestational decrease in arterial pressure. A corollary of this is that deficits in AT$_2$R expression may contribute to pregnancy-induced hypertension.

Pregnancy in mammals, including humans[19] and mice,[20] is characterized by a midgestational fall in arterial pressure, with MAP returning to preconception levels during the peripartum period. In the present study, we have documented for the first time the full impact of the AT$_2$R on arterial pressure during pregnancy in mice. It was demonstrated that during gestation, the AT$_2$R mediates the normal midgestational decrease in arterial pressure, because the fall was absent during pregnancy in AT$_2$R-KO mice. Furthermore, AT$_2$R deficiency was associated with an elevation in arterial pressure during late gestation. Previously, Takeda-Matsubara et al[18] observed a significant increase in systolic arterial pressure during late gestation as compared with prepregnancy levels in AT$_2$R-KO mice. However, this study did not observe the normal midgestational decrease in arterial pressure in WT (C57BL/6) mice and consequently did not report an effect of AT$_2$R deletion on arterial pressure during midgestation. Conversely, Chen et al[16] and Carey et al[17] have demonstrated that pharmacological and genetic AT$_2$R deficiency abolishes the normal midgestational decrease in arterial pressure. However, these studies observed no effect of AT$_2$R deficiency on arterial pressure during late gestation. The discord between these findings and those of the current study most likely reflects methodological differences. In our study, MAP was measured using radiotelemetry, which is the gold standard method to measure arterial pressure. The earlier studies used tail-cuff plethysmography to measure arterial pressure, which may mask subtle differences in arterial pressure during pregnancy because of the inherent stress involved with restraint and frequent handling.[21] Thus, our data unequivocally demonstrate that the normal midgestational decrease observed in the arterial pressure profile of pregnant WT mice is absent in AT$_2$R-KO mice and, importantly, that AT$_2$R deficiency leads to higher arterial pressure during late gestation.

To protect the feto-maternal unit during pregnancy, the immune system is normally suppressed.[10] Previous studies investigating Th1:Th2 ratio during pregnancy have measured IFN-γ and interleukin-4 levels and shown a distinct shift toward the Th2 phenotype, both systemically and within placentae.[22–24] IFN-γ and interleukin-4 are the principal cytokines that promote Th1 and Th2 differentiation, respectively.[25,26] In the present study we have shown a similar pattern, with a decrease in the Th1:Th2 ratio observed in the circulation and placentae of pregnant WT mice. Surprisingly, AT$_2$R deficiency in mice did not alter the Th1:Th2 ratio within the circulation or placentae as hypothesized. Thus, the findings of the present study suggest that AT$_2$R deficiency does not affect the systemic immune response. Furthermore, because placental AT$_2$R expression decreased during gestation in WT mice and there was no change in the placental Th1:Th2 ratio or number of live fetuses between the genotypes, it is not likely that AT$_2$R deficiency is associated with poorer fetal outcomes. This is consistent with a previous report suggesting that the AT$_2$R does not play a pivotal role in the placenta.[27] Conversely, placental ACE2 and MasR gene expression was increased during late gestation in both genotypes. This finding is consistent with previous studies which have demonstrated that uteroplacental levels of Ang(1–7) and ACE2 are elevated during pregnancy.[28,29] Thus the ACE2/Ang(1–7)/MasR pathway may play an important role in uteroplacental blood flow and fetal growth.

The most remarkable finding of the present study was that although there was no difference in the number of T cells infiltrating the kidney between pregnant WT and AT$_2$R-KO mice, AT$_2$R deficiency promoted a phenotypic shift of renal T cells and an increase in the renal Th1:Th2 balance in pregnant AT$_2$R-KO mice. A recent study using the AT$_2$R agonist compound 21 showed that the AT$_2$R reduces stimulation of a proinflammatory cytokine, interleukin-6, by inhibiting the transcription factor, nuclear factor-κB.[14] Nuclear factor-κB controls the expression of proinflammatory cytokines, chemokines, adhesion molecules, and growth factors which all play a critical role in mediating the proinflammatory actions of the Th1 phenotype.[10] The importance of this finding has been demonstrated in pregnant spontaneously hypertensive rats. Hypertension in the spontaneously hypertensive rat is ameliorated during pregnancy, an effect that is associated with a reduction in the renal AT$_1$R:AT$_2$R ratio and decreased inflammation as indicated by downregulation of

nuclear factor-κB in the kidney.[30] Collectively these findings suggest that the AT_2R suppresses AT_1R-mediated immune system activation within the kidney during pregnancy. Because the increase in the renal Th1:Th2 ratio preceded the rise in arterial pressure during late gestation in AT_2R-KO mice, this suggests that renal inflammation may contribute to pregnancy-induced hypertension.

During pregnancy there was an increase in sodium intake; however, sodium excretion did not change. Thus, pregnant mice were in a state of positive sodium balance. Significantly, the sodium retention was greater in pregnant AT_2R-KO than WT mice. Renal adaptations to pregnancy promote sodium retention, which facilitates the marked increase in plasma volume. Moreover, sodium retention during pregnancy occurs while both natriuretic and antinatriuretic signals are activated.[31] However, previous studies in pregnant rats have demonstrated that the pressure-natriuresis relationship is blunted during late gestation.[31,32] In the present study, arterial pressure was significantly elevated at Gd18 above prepregnancy levels in AT_2R-KO mice. This finding coupled with the similar natriuretic response at Gd18 between WT and AT_2R-KO mice indicates a rightward shift of the pressure-natriuresis relationship in AT_2R-KO mice during late gestation. These results suggest that the blunted pressure-natriuresis relationship observed in normotensive pregnancy is, at least in part, AT_2R mediated. This is consistent with previous findings, which have demonstrated that the AT_2R modulates pressure-natriuresis in nonpregnant rodents.[3,4] Consequently, deficits in AT_2R expression seem to alter the normal renal adaptations to pregnancy. Indeed, albuminuria was increased in pregnant AT_2R-KO mice compared with WT mice.

Contrary to our hypothesis, renal AT_2R expression during pregnancy did not increase in WT females, nor did other components (ACE2 and MasR) of the protective arm of the RAS. However, we, and others, have previously demonstrated that AT_2R, ACE2, and MasR gene expression is greater in female than in male rodents.[5,9,33] Furthermore, here we demonstrate that these high levels of expression persist during pregnancy. In contrast, AT_2R expression was significantly increased in the aorta, renal artery, and kidneys of pregnant normotensive rats at Gd14 as compared with nulliparous females, and this time point correlated with the peak increase in renal function.[34] In another study in pregnant ewes, AT_2R expression was increased in the uterine artery and unchanged in the aorta.[35] In these studies, AT_1R expression was unchanged between virgin and pregnant females. Therefore, females have high expression of the protective components of the RAS, which are maintained or increased in a regional and temporal manner during pregnancy, in various animals. Furthermore, as AngII binds with ≈15-fold greater affinity to the AT_2R as compared with the AT_1R,[36] this may underlie the reduced pressor responsiveness to AngII during pregnancy. Alternatively, the facts that plasma levels of Ang(1–7) increase ≈16-fold during normotensive pregnancy in women and Ang(1–7) is able to elicit its actions via the AT_2R, as well as its own receptor, MasR, suggest that Ang(1–7) may play a pivotal role in countering the pressor actions of AngII during normal pregnancy.[37] Future studies are needed to further characterize the role of the depressor RAS pathways in the normal regulation of arterial pressure during pregnancy.

Perspectives

Given that women with a history of preeclampsia are at an increased risk for developing cardiovascular disease in later life, understanding the mechanisms regulating arterial pressure during pregnancy are of importance. Our study demonstrates a key role for the AT_2R in the normal regulation of arterial pressure during pregnancy. Therefore, the enhanced AT_2R:AT_1R ratio observed in females may underlie reduced pressor responsiveness to AngII during normal pregnancy. Conversely, increased sensitivity to AngII during preeclampsia is associated with a decrease in the AT_2R:AT_1R ratio.[38] Thus, deficits in the depressor RAS pathways may be a predisposing factor to the development of pregnancy-induced hypertension and future cardiovascular risk.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first study to unequivocally demonstrate that the angiotensin type 2 receptor (AT$_2$R) mediates the normal midgestational decrease in arterial pressure and that the AT$_2$R contributes to the regulation of arterial pressure during late gestation in mice.
- This modulates immunity during gestation in mice.

What Is Relevant?

- AT$_2$R deficiency abolished the midgestation decrease in arterial pressure that was observed in wild-type mice and contributed to higher arterial pressure during late gestation in AT$_2$R knockout mice.
- AT$_2$R deficiency increased the renal Th1:Th2 balance during late gestation.
- AT$_2$R knockout mice had a higher arterial pressure during pregnancy as compared with wild-type mice, yet they excreted the same amount of sodium, suggesting a rightward shift in the pressure-natriuresis relationship.

Summary

The AT$_2$R plays a protective role in arterial pressure regulation during pregnancy in mice. A consequence of this is that deficits in AT$_2$R expression may contribute to the development of pregnancy-induced hypertension. Therefore, AT$_2$R agonist therapy represents a novel approach to treating pregnancy-induced hypertension.

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Brain immune cell composition and functional outcome after cerebral ischemia: comparison of two mouse strains

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Inflammatory cells may contribute to secondary brain injury following cerebral ischemia. The C57Bl/6 mouse strain is known to exhibit a T helper 1-prone, pro-inflammatory type response to injury, whereas the FVB strain is relatively T helper 2-prone, or anti-inflammatory, in its immune response. We tested whether stroke outcome is more severe in C57Bl/6 than FVB mice. Male mice of each strain underwent sham surgery or 1 h occlusion of the middle cerebral artery followed by 23 h of reperfusion. Despite no difference in infarct size, C57Bl/6 mice displayed markedly greater functional deficits than FVB mice after stroke, as assessed by neurological scoring and hanging wire test. Total numbers of $CD45^+$ leukocytes tended to be larger in the brains of C57Bl/6 than FVB mice after stroke, but there were marked differences in leukocyte composition between the two mouse strains. The inflammatory response in C57Bl/6 mice primarily involved T and B lymphocytes, whereas neutrophils, monocytes and macrophages were more prominent in FVB mice. Our data are consistent with the concept that functional outcome after stroke is dependent on the immune cell composition which develops following ischemic brain injury.

Keywords: cerebral ischemia-reperfusion, immune cell infiltration, inflammation, middle cerebral artery occlusion, stroke, Th1/Th2 balance

INTRODUCTION

Stroke is the 4th leading cause of death after heart disease, cancer and chronic lower respiratory disease, and over a third of survivors are left with major neurological injury (Go et al., 2013). Approximately 85% of stroke cases are of the ischemic type (Go et al., 2013), in which an embolus or local thrombus causes occlusion of a major cerebral artery and results in disruption of brain blood flow. Whilst thrombolysis by intravenous recombinant tissue plasminogen activator (rt-PA) may be effective in improving outcome by promoting reperfusion, it has a number of limitations, including a short therapeutic window of 3–4.5 h (<10% of stroke patients receive rt-PA) (Gravanis and Tsirka, 2008). For further advances in the clinical treatment of ischemic stroke, the complex mechanisms of cellular injury following cerebral ischemia must be elucidated to provide novel targets for future therapies.

It is now established that the initial insult in ischemic stroke is followed by induction of cytokines and chemokines, which attract numerous inflammatory cell types to the damaged brain region, which ultimately contribute to secondary brain injury (Gelderblom et al., 2009; Chu et al., 2014). Growing evidence indicates the importance of T lymphocytes in cerebral ischemic damage, whereby they become activated and infiltrate the brain within 24 h (Yilmaz et al., 2006; Hurn et al., 2007; Urra et al., 2009a), although the mechanism(s) underlying their actions are not fully clear. Recombination activating gene 1-deficient mice, which lack T and B lymphocytes, have less severe brain injury following cerebral ischemia, and this protection is lost upon reconstitution with T but not B lymphocytes (Yilmaz et al., 2006; Kleinschnitz et al., 2010).

T lymphocytes ($CD3^+$ cells) are mostly comprised of $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T (Tc) cell subpopulations, both of which are thought to play detrimental roles in ischemic stroke (Yilmaz et al., 2006). There is also evidence that regulatory T lymphocytes (Tregs; $CD4^+CD25^+FoxP3^+$) may modulate the severity of stroke outcome (Liesz et al., 2009), despite exerting acutely detrimental effects by promoting intravascular coagulation during reperfusion (Kleinschnitz et al., 2013). Major distinct Th cell types include Th1 and Th2, and are defined according to the cytokines they release (Abbas et al., 1996). In general terms, Th1 cells promote an inflammatory response through secretion of pro-inflammatory cytokines [e.g., interleukin(IL)-2, IL-12, interferon(IFN)-γ, and tumor necrosis factor(TNF)-α], whereas Th2 cells promote a humoral or allergic response by

secretion of anti-inflammatory cytokines (e.g., IL-4, IL-10, and IL-13) (Arumugam et al., 2005; Jin et al., 2010).

Clarification of the importance of Th1 and Th2 immunity in acute stroke is needed to define the complex evolution of cerebral ischemic injury and potentially identify therapeutic strategies to limit stroke injury. Here, we have studied representative mouse strains commonly accepted as Th1-dominant (C57Bl/6) and Th2-dominant (FVB) (Whitehead et al., 2003) to investigate stroke outcome in a prototypical Th1- or Th2-prone immune environment, respectively.

MATERIALS AND METHODS

ANIMALS

This study fully adheres to the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010). All animal experiments were conducted in accordance with National Health and Medical Research Council of Australia guidelines for the care and use of animals in research and approved by the Monash University Animal Ethics Committee (Projects SOBSB/2010/10 and SOBSB/2011/112). A total of 201 male mice (C57Bl/6: $n = 89$, 19–33 g; FVB: $n = 112$, 25–39 g) aged 8–15 weeks were studied. The mice had free access to water and food pellets before and after surgery. Thirty-seven mice were excluded from the study because they: (1) died during surgical procedure (C57Bl/6: $n = 12$; FVB: $n = 24$) or (2) were euthanized prior to 24 h as per institutional ethics requirements due to severe functional impairment (C57Bl/6: $n = 1$).

TRANSIENT FOCAL CEREBRAL ISCHEMIA

Focal cerebral ischemia was induced by transient intraluminal filament occlusion of the right middle cerebral artery (MCA) as described previously (Jackman et al., 2009; Brait et al., 2010). Mice were anesthetized with ketamine-xylazine (80 and 10 mg/kg, respectively; intraperitoneal). Rectal temperature was monitored and maintained at 37.5 ± 0.5°C throughout the procedure and until animals regained consciousness using an electronic temperature controller (Testronics, Kinglake, Victoria, Australia) linked to a heat lamp. The right proximal common carotid artery was clamped, and a 6–0 nylon monofilament with silicone-coated tip (Doccol Co., Redlands, CA, USA) was inserted and gently advanced into the distal internal carotid artery, 11–12 mm distal to the carotid bifurcation, occluding the MCA at the junction of the Circle of Willis. Severe (~80%) reduction in regional cerebral blood flow (rCBF) was confirmed using transcranial laser-Doppler flowmetry (Perimed, Järfälla, Sweden) in the area of cerebral cortex supplied by the MCA. The filament was then tied in place and the clamp was removed. After 1 h of cerebral ischemia, the monofilament was retracted to allow reperfusion for 23 h. Reperfusion was confirmed by an immediate increase in rCBF, which reached the pre-ischemic level within 5 min. The wound was then closed and the animal was allowed to recover. Regional CBF was recorded for 30 min after the induction of reperfusion. Sham-operated mice were anesthetized and the right carotid bifurcation was exposed, dissected free from surrounding connective tissue but no filament was inserted. All animals were administered 1 mL of sterile saline via a subcutaneous injection for rehydration after surgery.

NEUROLOGICAL ASSESSMENT

At the end of the experiment (24 h after induction of stroke/sham surgery), neurological assessment was performed using a modified six-point scoring system (Jackman et al., 2009; Brait et al., 2010): 0, normal motor function; 1, flexion of torso and contralateral forelimb when mouse is lifted by the tail; 2, circling when mouse held by the tail on a flat surface; 3, leaning to the one side at rest; 4, no spontaneous motor activity; 5, death within 24 h. A hanging wire test was also performed in which mice were suspended from a wire 30 cm high for up to 180 s, and the average time of 3 trials with 5-min rest periods in between was recorded. Neurological assessment was evaluated by an observer blinded to experimental groups.

CEREBRAL INFARCT AND EDEMA VOLUMES

Mice were killed at 24 h by inhalation of isoflurane, followed by decapitation. The brains were immediately removed and snap frozen with liquid nitrogen. Coronal sections (30 μm) separated by ~420 μm were obtained and stained with thionin (0.1%) to delineate the infarct. Images of the sections were captured with a CCD camera mounted above a light box. Infarct volume was quantified as described previously (Jackman et al., 2009; Kim et al., 2012) using image analysis software (ImageJ, NIH, Bethesda, MD, USA), and corrected for brain edema, estimated using the following formula: corrected infarct volume = [left hemisphere area − (right hemisphere area − right hemisphere infarct area) × (thickness of section + distance between sections)] (Tsuchiya et al., 2003; Xia et al., 2006). Edema-corrected infarct volumes of individual brain sections were then added giving a three-dimensional approximation of the total infarct volume. Total, cortical and subcortical infarct volumes were quantified individually.

GROSS CEREBROVASCULAR ANATOMY

For gross comparison of cerebrovascular anatomy, some naïve animals ($n = 3$ of each strain, without any surgical procedures) were deeply anesthetized by inhalation of isoflurane, the thorax was opened and intracardial perfusion was performed with PBS, followed by 4% paraformaldehyde and finally 4% Evans blue solution in 20% gelatin.

FLOW CYTOMETRY

On each occasion when flow cytometry was utilized, we studied at least one post-stroke mouse together with a time-matched sham-operated control mouse of each strain. Animals were euthanized at 24 h by inhalation of isoflurane, followed by blood removal by cardiac puncture and the whole mouse was then intracardially perfused with phosphate-buffered saline (PBS) and brain, blood, and spleen were collected. Leukocytes were purified from blood using red blood cell lysis buffer (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 3 mmol/L EDTA). Spleens were mechanically dissociated and passed through 70 μm nylon cell strainers (BD Falcon, Bedford, MA, USA) to obtain a single-cell suspension. Cells were then lysed with red blood cell lysis buffer and washed with PBS containing 1% bovine serum albumin. The brain was removed from the skull and after removing the cerebellum and olfactory bulb, was separated into left (contralateral) and

right (ischemic) hemispheres. Each hemisphere was dissociated mechanically in digestion buffer containing collagenase type XI (125 U/mL), hyaluronidase (60 U/mL), and collagenase type I-S (450 U/mL) in $Ca2^{+}/Mg2^{+}$-supplemented PBS (Sigma, St Louis, MO, USA), and incubated at 37°C for 30 min with gentle agitation. The mixture was then passed through 70 μm nylon cell strainers to obtain a single-cell suspension. After washing with PBS (1200 rpm, 10 min), the cell pellet was resuspended in 3 mL 30% percoll (GE Healthcare, Uppsala, Sweden), underlaid with 70% percoll, and centrifuged for 20 min at 2400 rpm at room temperature without the use of a brake. The cells at the interphase of two density gradients were collected and washed with PBS containing 1% bovine serum albumin (1200 rpm, 10 min) for staining. All cells were incubated with appropriate antibodies listed in **Table 1** at 4°C in darkness for 20 min. After staining, cells were analyzed by LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (Tree Star Inc., Ashland, OR, USA). Countbright counting beads (Invitrogen, Carlsbad, CA, USA) were included to define the absolute number of cells in the samples.

GATING STRATEGY

Single cells were identified by forward scatter, and dead cells (7-amino actinomycin D^{+}) were excluded. Cells were gated for $CD45^{+high}$ and $CD45^{+med}$ populations as described previously (Chu et al., 2014). $CD45^{+high}$ populations were then divided into lymphoid cells, which include: B cells ($B220^{+}$), T cells ($CD49b^{+}CD90^{-}NK1.1^{-}$), thymocytes ($CD49b^{-}CD90^{+}NK1.1^{-}$), NK cells ($CD49b^{+}CD90^{+}NK1.1^{+}$), and NKT cells ($CD49b^{+}CD90^{-}NK1.1^{+}$); and myeloid cells ($CD11b^{+}$). $CD45^{+med}CD11b^{+}F4/80^{+}$ cells were considered microglia. Two panels of antibodies were used, one of which was employed with each animal. Panel 1 enabled the counting of microglia and myeloid-derived leukocytes (i.e., $CD11b^{+}$ cells), which include: neutrophils ($Ly6G^{+}$), monocytes ($Ly6C^{+}$), macrophages ($F4/80^{+}$), and dendritic cells ($CD11c^{+}$); whereas Panel 2 divided lymphocytes into: B cells ($CD19^{+}$) and T cells ($CD3^{+}$). T cells were then further subdivided into $CD4^{+}$ T cells, $CD8^{+}$ T cells, $CD4^{-}CD8^{-}$ T cells, and $CD4^{+}CD25^{+}$ T cells. Fluorescence-minus-one were included as negative controls to define positive populations for F4/80, CD11c, Ly6C, CD19, CD3, and CD25.

Table 1 | Summary of antibodies used for flow cytometry.

Antigen	Host/Isotype	Supplier
CD8a-APC	Rat IgG2a, kappa	BD Phamingen
CD19-PE	Rat IgG2a, kappa	BioLegend
CD11c-Brilliant Violet 570	ArHam IgG	BioLegend
CD25-PE-Cy7	Rat IgG1, lambda	BioLegend
CD4-FITC	Rat IgG2b, kappa	BioLegend
CD45-APC-Cy7	Rat IgG2b, kappa	BioLegend
CD49b-PE	ArHam IgG	BioLegend
CD90.2-PE	Rat IgG2b, kappa	BioLegend
Ly6C-FITC	Rat IgG2c, kappa	BioLegend
Ly6G-PE-Cy7	Rat IgG2a, kappa	BioLegend
NK1.1-PE	Mouse IgG2a, kappa	BioLegend
CD11b-eFluro450	Rat IgG2b, kappa	eBioscience
CD3-eFluro450	Rat IgG2b, kappa	eBioscience
F4/80-APC	Rat IgG2a, kappa	eBioscience
7-Amino-actinomycin D (7AAD)		Invitrogen

CYTOKINE MEASUREMENT

Single-cell suspensions of brain, blood and spleen were obtained as described for flow cytometry. All cells were resuspended in complete RPMI 1640 media supplemented with heat inactivated fetal bovine serum (10% w/v), streptomycin and penicillin (100 U/mL), L-glutamine (1%) and 2-mercaptoethanol (50 mM). Blood and spleen cells were seeded at 200,000 cells/well in a 96-well plate coated with anti-CD3. All cells in the brain hemisphere were seeded. Blood, spleen, and contralateral brain hemisphere of sham-operated mice was seeded as unstimulated controls. Recombinant mouse IL-2 (20 ng/mL) was added and cells were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After stimulation, cells were spun down (15,000 rpm, 5 min) and supernatant was collected. Samples were analyzed for 7 key inflammatory cytokines (IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α) using Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences, Franklin Lakes, NJ, USA). Samples and standards were prepared according to manufacturer's protocol. After adding capture beads, cells were analyzed by LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FCAP Array software (BD Biosciences, Franklin Lakes, NJ, USA).

STATISTICAL ANALYSIS

Values are presented as mean ± standard error. Results of the hanging wire test, infarct volume and flow cytometry, comparing C57Bl/6 and FVB sham- or stroke-operated mice, were analyzed using one-way analysis of variance with Bonferroni *post-hoc* test with selected multiple comparisons or a Student's unpaired *t*-test, as appropriate. The neurological deficit score was expressed as the median result per group and was analyzed using a Kruskal-Wallis test with Dunn's *post-hoc* test. A P value <0.05 was considered statistically significant. Statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS

CEREBRAL BLOOD FLOW PROFILE AND MORTALITY

CBF was similarly reduced by ∼80% in both C57Bl/6 and FVB mice following insertion of the monofilament (**Figure 1A**). No significant differences in CBF profiles were observed between C57Bl/6 and FVB mice. Mortality rates at 24 h after cerebral ischemia were 8.8% (3/34) and 2.9% (1/34) in C57Bl/6 and FVB mice, respectively (**Figure 1B**). Mice of both strains had no significant differences in the cerebrovascular anatomy; in particular, the posterior communicating arteries were present in both strains (Supplementary Figure 1).

NEUROLOGICAL FUNCTION

Mice of both strains typically had no neurological deficit (score of 0) at 24 h after sham surgery (**Figure 1B**). Following cerebral ischemia, both C57Bl/6 and FVB mice had significant

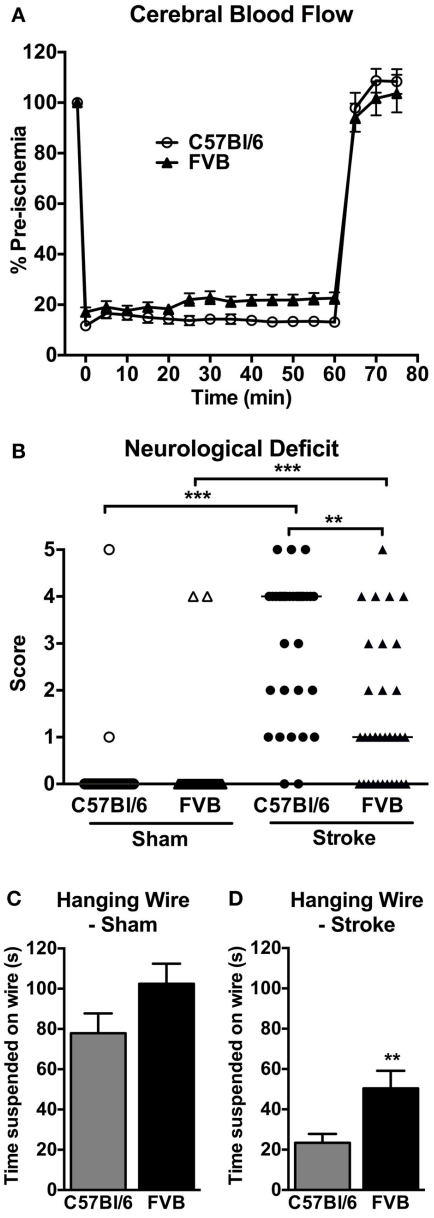

FIGURE 1 | (A) Regional cerebral blood flow during 1 h middle cerebral artery occlusion ($n = 30$–31); (B) neurological deficit ($n = 29$–34; $^{**}P < 0.01$, $^{***}P < 0.001$; Kruskal-Wallis with Dunn's post-tests) and (C,D) hanging wire performance ($n = 28$–33; $^{**}P < 0.01$; Student's unpaired *t*-test) at 24 h after cerebral ischemia. There was less functional impairment in FVB mice compared to C57Bl/6 mice. Data are mean ± s.e.m. in **(A,C,D)**, and are median scores in **(B)**.

neurological deficit compared to sham-operated mice of the same strain, although C57Bl/6 mice had greater deficit than FVB mice. Similarly, in the hanging wire test sham-operated C57Bl/6 and FVB mice achieved comparable hanging times (**Figure 1C**), whereas FVB mice achieved ∼2-fold longer hanging times than C57Bl/6 mice at 24 h following cerebral ischemia (**Figure 1D**).

INFARCT AND EDEMA VOLUMES

Representative coronal sections of C57Bl/6 and FVB brains at 24 h after MCA occlusion are shown in **Figures 2A,B**, respectively. C57Bl/6 and FVB mice had similar total infarct (**Figure 2C**), cortical infarct (**Figure 2E**), subcortical infarct (**Figure 2F**) and edema (**Figure 2D**) volumes.

LEUKOCYTE INFILTRATION IN THE BRAIN

There was a ∼4-fold increase in the total number of leukocytes in the ischemic hemisphere of C57Bl/6 mice compared to sham-operated mice ($P < 0.01$, **Figure 3A**). There also tended to be an increase in total leukocytes following ischemia in FVB mice, but there were ∼40% fewer leukocytes than in C57Bl/6 mice (**Figure 3A**). Myeloid cells ($CD11b^+$), comprising neutrophils ($Ly6G^+$), dendritic cells ($CD11b^+CD11c^+$), macrophages ($F4/80^+$) and monocytes ($Ly6C^+$), were increased by a ∼15-fold in the ischemic hemisphere of FVB mice to levels that were twice those in C57Bl/6 mice ($P < 0.01$, **Figure 3B**). By contrast, lymphoid cells were increased by 2–3-fold following ischemia in C57Bl/6 mice, whereas there was no change in lymphoid cell numbers in the brains of FVB mice following stroke (**Figure 3C**). No significant effect of stroke was observed in the number of microglia ($CD45^{+med}CD11b^+F4/80^+$) in either strain (**Figure 3D**).

Among myeloid cells, neutrophils were most prevalent, with these cells being ∼4-fold more numerous in ischemic hemispheres of FVB than in C57Bl6 mice (**Figure 4A**). There was a similar profile of both macrophage and $Ly6C^{low}$ monocyte numbers in the ischemic brain following stroke, with also ∼4-fold more of these cells in FVB than C57Bl/6 mice (**Figures 4B,C**). $Ly6C^{high}$ monocytes and total monocytes were present in similar numbers in the ischemic brains of the two strains (**Figures 4D,E**). In contrast, the number of dendritic cells in the ischemic hemisphere was ∼3-fold higher in C57Bl/6 than FVB mice (**Figure 4F**).

Among lymphoid cells, there were marked increases in numbers of both B cells and T cells in the post-ischemic brain of C57Bl/6 mice but not FVB mice (**Figures 5A,B**). Further analysis of T cell subpopulations indicated that the increase in C57Bl/6 mice was mostly due to infiltration of $CD4^+CD25^-$ ("T helper") cells and not to $CD8^+$ ("cytotoxic") nor $CD4^+CD25^+$ T cells, which includes Tregs (**Figures 5C–E**).

Overall, despite similar compositions of immune cells in the brains of C57Bl/6 and FVB mice following sham surgery, there were marked differences between strains after stroke with lymphoid:myeloid cells representing ∼80:20 in total (**Figure 6**). While a similar ratio persisted in C57Bl/6 mice after ischemia, there was a markedly different leukocyte composition in FVB mice after stroke, with a reversal of the lymphoid:myeloid ratio to ∼20:80 (**Figure 6**). There were few differences in blood composition of leukocytes between strains or after stroke (**Figure 7**).

SPLENIC LEUKOCYTE NUMBERS

At 24 h after stroke there was a tendency for a reduction in the total number of splenic leukocytes in both mouse strains (**Figure 8A**). There were fewer splenic myeloid cells in FVB vs. C57Bl/6 mice, due to lower numbers of neutrophils and $Ly6C^{high}$ monocytes (**Figures 8B,D,G**), whereas there were higher

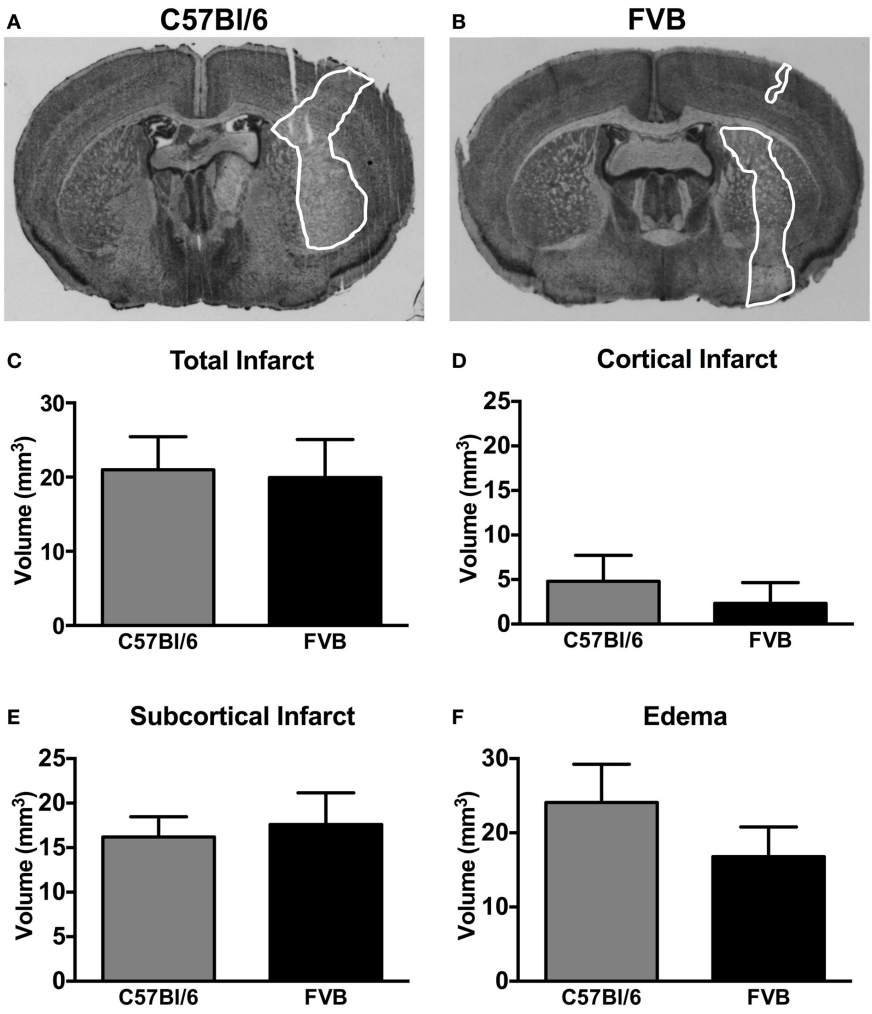

FIGURE 2 | Representative coronal brain sections from a (A) C57Bl/6 mouse and a (B) FVB mouse 24 h after 1 h middle cerebral artery occlusion (infarct areas are outlined in white). Infarct [**(C)**, total; **(D)**, cortical; **(E)**, subcortical] and **(F)** edema volumes are also shown ($n = 11$ per group; Student's unpaired *t*-test). There was no difference in the infarct volume between two strains of mice. Data are mean ± s.e.m.

numbers of $CD4^+$, $CD8^+$, and total T cells in that strain (**Figures 8J–L**). There was no significant reduction in any cell population (**Figures 8C,E,F,H,I**) except for 40–50% fewer T cells in FVB mice (**Figures 8J–L**). Consistent with these data, spleen weight was slightly reduced at 24 h after stroke in both strains, with the difference reaching statistical significance in FVB mice only (Supplementary Figure 2).

CYTOKINE LEVELS

Brain cytokine analysis at 24 h indicated that stroke resulted in substantially higher mean levels of IL-4, IL-6, TNF-α, IFN-γ, IL-10, and IL-17A in both C57Bl/6 and FVB mice (Supplementary Figure 3). There was a tendency for higher levels of IL-4, IL-6, TNF-α, and IFN-γ in the ischemic hemisphere of FVB than of C57Bl/6 (Supplementary Figure 3). There were no clear trends in cytokine levels in blood and spleen were generally similar in sham-operated mice of each strain, and there were no marked effects of stroke after 24 h in either strain (Supplementary Figures 4, 5).

DISCUSSION

There is growing evidence that T lymphocytes may influence the development of ischemic injury and functional deficit following experimental stroke (Iadecola and Anrather, 2011; Brait et al., 2012). For example, mice lacking T cells are reported to have smaller infarcts and improved functional outcome after focal ischemia compared to wild-type mice (Yilmaz et al., 2006; Hurn et al., 2007; Urra et al., 2009a; Kleinschnitz et al., 2013). Furthermore, there may be differential effects of $CD4^+$ T cell subsets on stroke outcome, such as exacerbation by Th1 cells and amelioration by Th2 cells of brain infarct development and functional deficit (Xiong et al., 2011; Gu et al., 2012). There is also clinical evidence that single nucleotide polymorphisms in the genes of Th1 and Th2 cytokines, and molecules that regulate

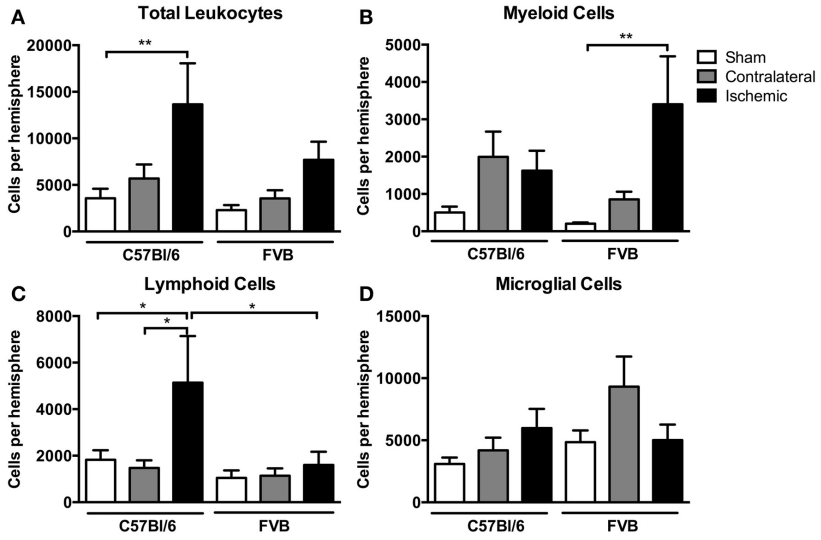

FIGURE 3 | Flow cytometric quantification of (A) total leukocytes and (B–D) leukocyte subsets in the brain at 24 h after 1 h middle cerebral artery occlusion. Data are shown for the contralateral and ischemic hemispheres, and compared with sham control mice of the same strain (n values: total leukocytes $n = 13$–16, subsets $n = 6$–9; $^{*}P < 0.05$, $^{**}P < 0.01$; One-Way ANOVA with Bonferroni post-tests). There was a greater number of leukocytes, predominantly lymphoid cells, infiltrating the brain of C57Bl/6 mice compared to FVB mice. Data are mean ± s.e.m.

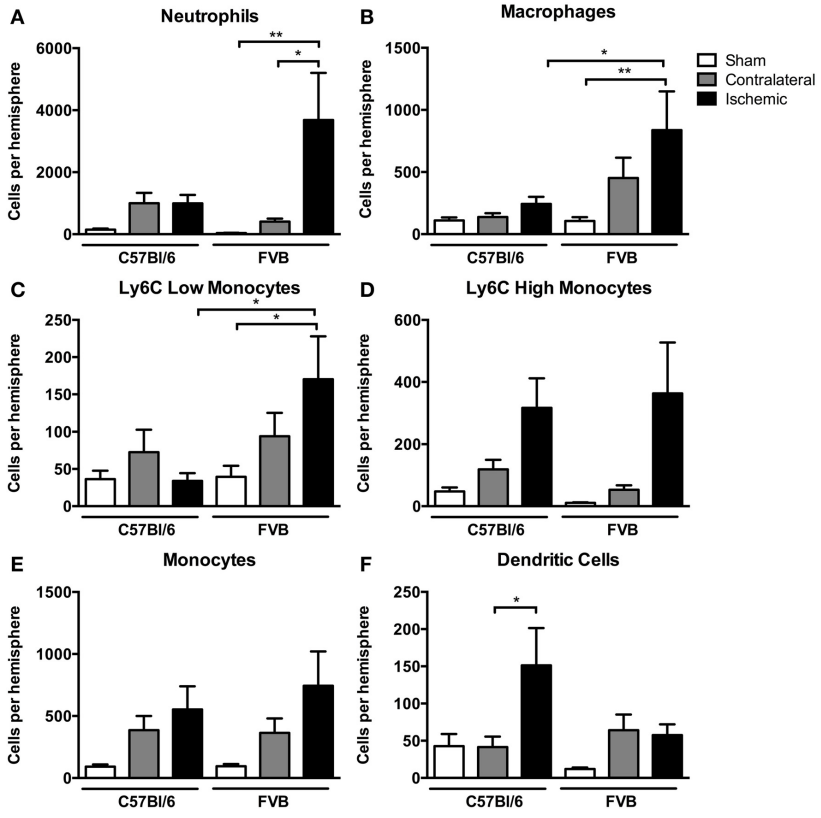

FIGURE 4 | Quantification of myeloid cell subpopulations [(A), neutrophils; (B), macrophages; (C), Ly6C low monocytes; (D), Ly6C high monocytes; (E), monocytes; (F), dendritic cells] in the brain 24 h after 1 h middle cerebral artery occlusion. Data are shown for the contralateral and ischemic hemispheres, and compared with sham control animals of the same strain ($n = 6$–9; $^{*}P < 0.05$, $^{**}P < 0.01$; One-Way ANOVA with Bonferroni post-tests). Data are mean ± s.e.m.

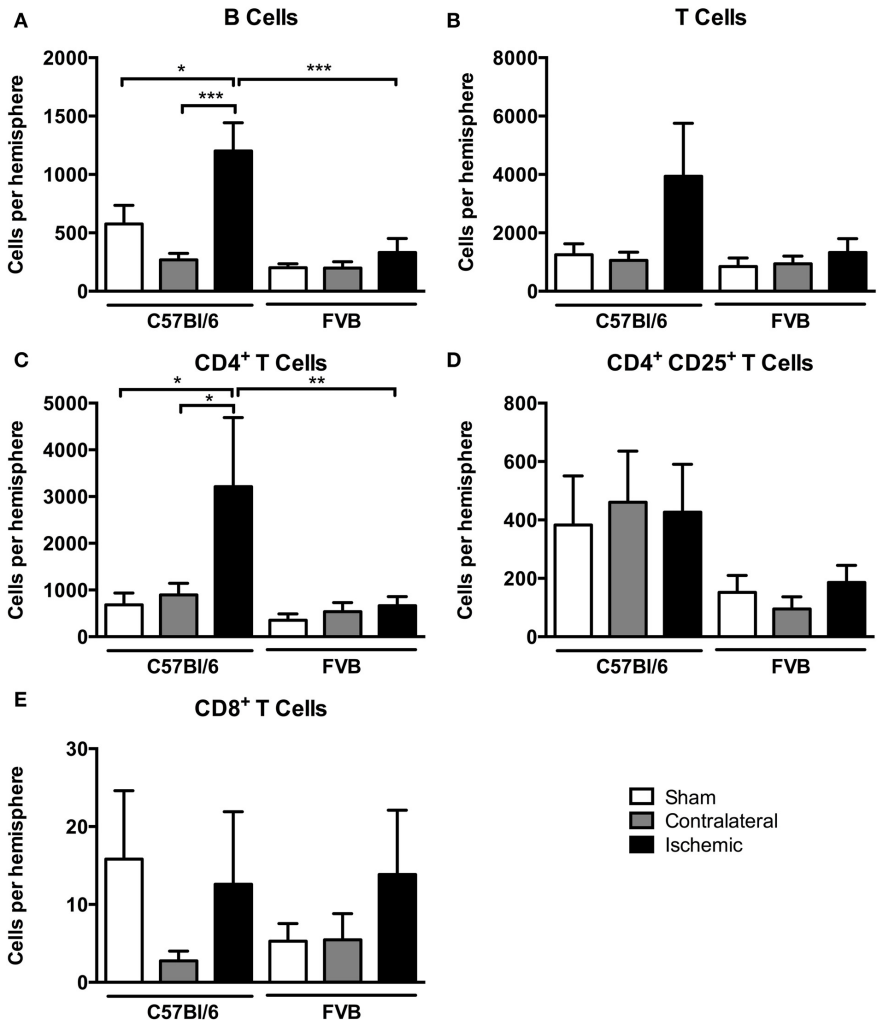

FIGURE 5 | Quantification of lymphocyte subpopulations [(A), B cells; (B), T cells; (C), CD4$^+$ T cells; (D), CD4$^+$ CD25$^+$ T cells; (E), CD8$^+$ T cells] in the brain 24 h after 1 h middle cerebral artery occlusion. Data are shown for the contralateral and ischemic hemispheres, and compared with sham control animals of the same strain ($n = 4$–8; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; One-Way ANOVA with Bonferroni post-tests). Data are mean ± s.e.m.

their transcription rate or their functionality, may predispose to immune responses of differing strength and thus contribute to the risk of stroke (Marousi et al., 2008).

Our study has examined representative mouse strains commonly accepted as Th1-dominant (C57Bl/6) and Th2-dominant (FVB) (Whitehead et al., 2003) to investigate stroke outcome at 24 h in prototypical Th1- vs. Th2-prone immune environments, respectively. Our data generally support the concept that Th1-prone immunity in C57Bl/6 results in a more severe functional outcome after stroke compared to Th2-prone FVB mice. For example, spleen levels of IL-4 and IL-10 were 3–4-fold higher in control FVB vs. C57Bl/6 mice. Yet, with no significant differences in cerebrovascular anatomy, degree of ischemic insult caused by MCA occlusion, and ultimately in the developed infarct size between the two mouse strains, there was a markedly different profile of immune cell infiltration in the ischemic hemispheres of C57Bl/6 and FVB mice.

Despite a similar immune cell composition in the brains of sham-operated C57Bl/6 and FVB mice, which comprised myeloid and lymphoid cells in a ∼20:80 ratio, after ischemia there was an overall increase in infiltrating cell numbers in both strains whereby this ratio was preserved in C57Bl/6 but was converted to ∼80:20 in FVB mice. The magnitude of total leukocyte infiltration into the brain of C57Bl/6 was approximately twice that observed in FVB, and these strain differences occurred in the absence of any notable stroke-related systemic differences such as cell numbers, cell composition or cytokine profile in either blood or spleen. Striking increases were noted to occur particularly in the number of innate immune cells such as neutrophils, macrophages and LyC6^{+low} monocytes infiltrating the ischemic FVB brain, whereas the most prominent increases in C57Bl/6 mice occurred in the numbers of infiltrating T and B lymphocytes and dendritic cells—key cells for adaptive immunity. These responses were associated with some strain differences in the

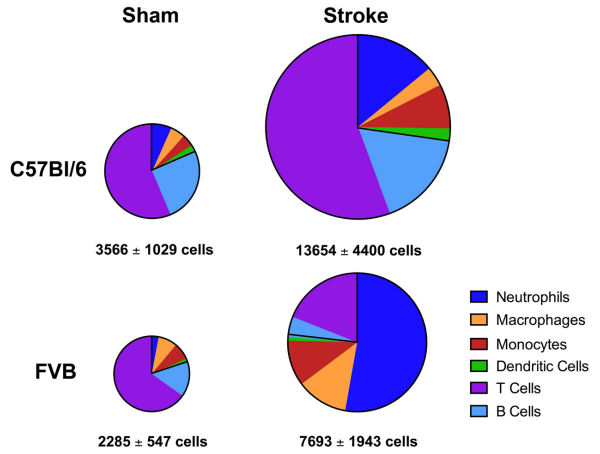

FIGURE 6 | Pie charts summarizing number and composition of immune cells in ischemic hemispheres of C57Bl/6 and FVB mice at 24 h after 1 h middle cerebral artery occlusion. The area of each pie is proportional to the number of leukocytes per hemisphere (also shown as mean ± s.e.m.; total leukocytes $n = 13$–16, myeloid cells $n = 6$–9, lymphocytes $n = 4$–8). There was a greater number of leukocytes, predominantly lymphoid cells, infiltrating the brain of C57Bl/6 mice compared to FVB mice.

mean levels of certain cytokines in the ischemic hemisphere, such as a tendency for larger amounts of both Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-6) cytokines to be present in FVB than C57Bl/6 mice. However, it is not possible from the present data to discern whether these differences in brain cytokine levels may have been a cause or effect of the different post-ischemic immune cell profiles.

We found marked differences in neutrophil content of ischemic brains between FVB and C57Bl/6 mice. Clinical and experimental data suggest that neutrophils are the most abundant cell type in the brain after ischemia (Akopov et al., 1996; Gelderblom et al., 2009; Chu et al., 2014) and their accumulation is correlated with the severity of brain infarct and neurological deficit (Akopov et al., 1996). Activated neutrophils have been shown to promote the release of free radicals and cytokines, which further recruit leukocytes to the damaged area (Harris et al., 2005). However, it is controversial whether neutrophils contribute directly to secondary brain damage or have a mild neuroprotective role in cerebral ischemia. Animal studies have shown that increased infarct size is associated with neutrophil elimination (Takizawa et al., 2002) whilst others have shown that neutrophils may not contribute directly to infarct size (Beray-Berthat et al., 2003; Harris et al., 2005; Brait et al., 2011). Our data in FVB mice suggest that neutrophil number is not directly associated with infarct size in that a ∼100-fold increase in brain infiltration after ischemia did not result in a bigger infarct size than in C57Bl/6 mice where the increase was markedly less. In previous study of parasite infection, neutrophils were found to play an early role in the induction of the Th2 response that develops in Th2-prone Balb/C mice but not in C57Bl/6 mice (Tacchini-Cottier et al., 2000). Thus, it is possible that the infiltration of neutrophils into the ischemic brains of FVB mice was associated with the induction of a less severe Th2-type immune response.

There were also markedly greater numbers of macrophages and Ly6C^{low} monocytes (the latter are considered to be anti-inflammatory) present in the FVB brains after cerebral ischemia. Macrophages and monocytes produce inflammatory cytokines and upregulate adhesion molecules in endothelial cells, thereby promoting neutrophil accumulation and migration (Chiba and Umegaki, 2013). Analogous to Th cells, macrophages are highly plastic cells and can polarize into two distinct activated macrophage subsets depending on the microenvironment (Kigerl et al., 2009). The classic or M1 activated cells are characterized by their capacity to present antigen, high production of nitric oxide and reactive oxygen species and of pro-inflammatory cytokines. In contrast, alternative or M2 activated cells are involved in scavenging of debris, angiogenesis, tissue remodeling and repair (Kigerl et al., 2009). Macrophages from Th1 strains (e.g., C57Bl/6, B10D2) are known to be more readily activated (e.g., to produce nitric oxide) than macrophages from Th2 strains (e.g., Balb/C, DBA/2) (Mills et al., 2000). Ly6C^{+low} monocytes are known to exhibit M2 characteristics (Geissmann et al., 2010). At the early stages following ischemic stroke, resident microglia and newly recruited macrophages appear to have a M2 phenotype that gradually transforms into an M1 phenotype in peri-infarct regions (Hu et al., 2012). It is possible that the greater number of macrophages and Ly6C^{+low} monocytes in FVB mice at 24 h after stroke represents more numerous M2-like cells contributing to a milder inflammatory environment in that strain. Further insight into the polarity of macrophages in FVB mice is needed.

We observed an increase in dendritic cells in the ischemic brain of C57Bl/6 mice at 24 h. Dendritic cells are involved in antigen presentation during immune cell activation and in the maintenance of peripheral tolerance through modulation of the immune response (Thompson and Thomas, 2002), but their role in outcome after cerebral ischemia is currently unclear.

There was a marked infiltration of lymphoid cells, particularly B and CD4$^+$ T cells (i.e., Th cells), into the ischemic hemisphere of C57Bl/6 mice. T lymphocytes enter the brain by 24 h after ischemic stroke (Gelderblom et al., 2009; Kleinschnitz et al., 2013; Chu et al., 2014), and both CD4$^+$ and CD8$^+$ T cells contribute to post-ischemic injury in mice (Arumugam et al., 2005; Kleinschnitz et al., 2013). It is conceivable that the infiltration of CD4$^+$ cells, which occurred selectively in C57Bl/6 mice following stroke, contributed to a more severe level of brain inflammation than in FVB mice despite a similar infarct volume. Tregs are a subset of CD4$^+$ T cells that are reported to play a protective, immunomodulatory role in the brain over several days after stroke (Liesz et al., 2009), but a detrimental role during more acute conditions (Kleinschnitz et al., 2013). We found no significant changes after stroke in either CD8$^+$ cells or CD4$^+$CD25$^+$ T cells, which will largely consist of Tregs.

Effects of B cells on stroke outcome are poorly understood. B cells can function as antigen-presenting cells to activate cytotoxic CD8$^+$ T cells, but there is also data to suggest that an IL-10-producing subpopulation of regulatory B cells may limit injury in experimental stroke (Ren et al., 2011; Bodhankar

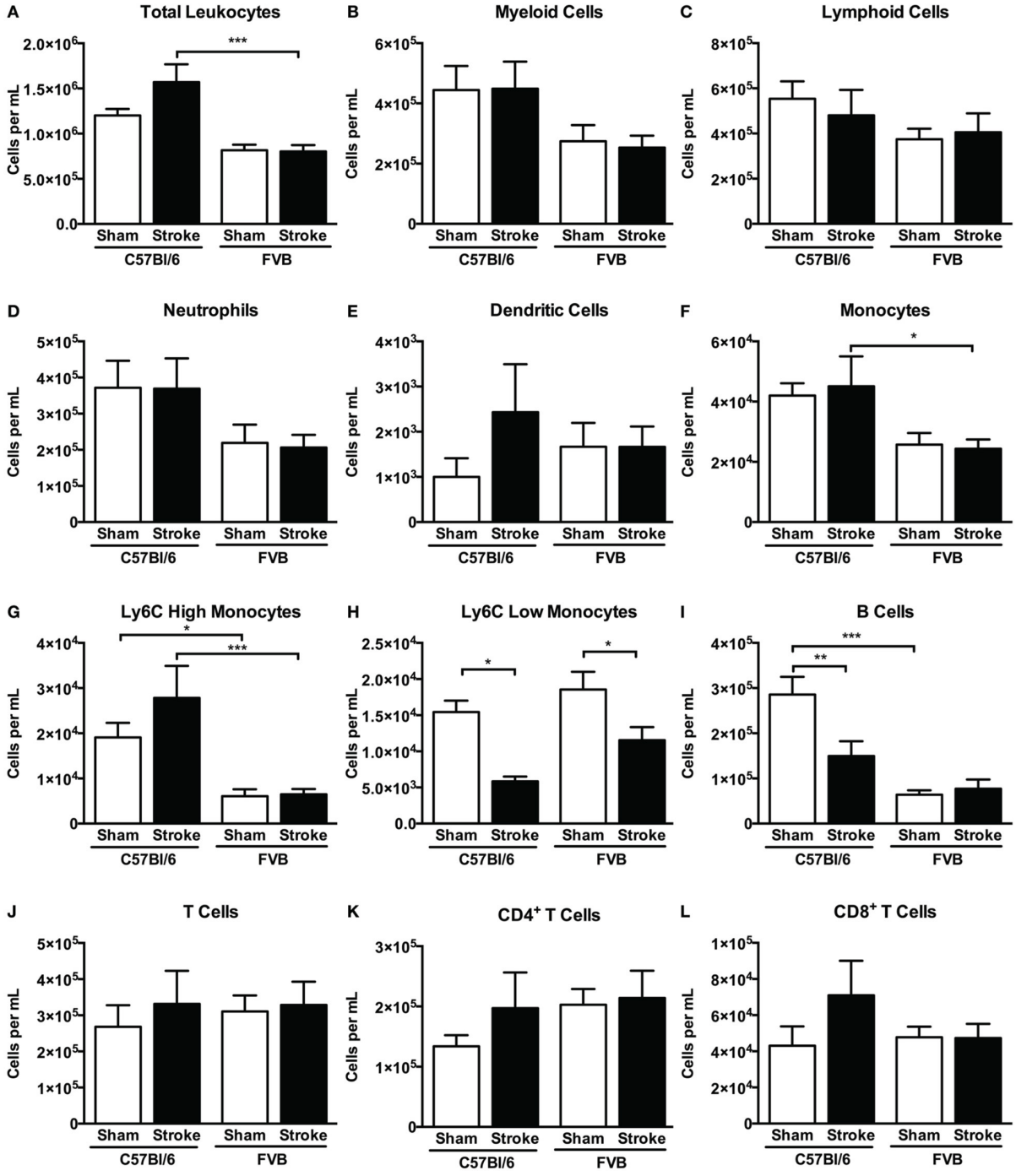

FIGURE 7 | Quantification of (A) total leukocytes and (B–L) leukocyte subsets in blood 24 h after 1 h middle cerebral artery occlusion, compared with sham control animals of the same strain (total leukocytes $n = 16–28$, myeloid cells $n = 9–17$, lymphocytes $n = 7–12$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; One-Way ANOVA with Bonferroni post-tests). Data are mean ± s.e.m.

et al., 2013). In addition, poor outcome in stroke patients is associated with reduced levels of circulating B cells (Urra et al., 2009b). Interestingly, we found that B cell infiltration into the brain following ischemia occurred selectively in C57Bl/6 mice (**Figure 5A**). Furthermore, whereas the number of circulating B cells was ∼5-fold higher in control C57Bl/6 vs. FVB mice, stroke selectively reduced the number of B cells in the blood of the former strain (**Figure 7I**). Further study is necessary to clarify

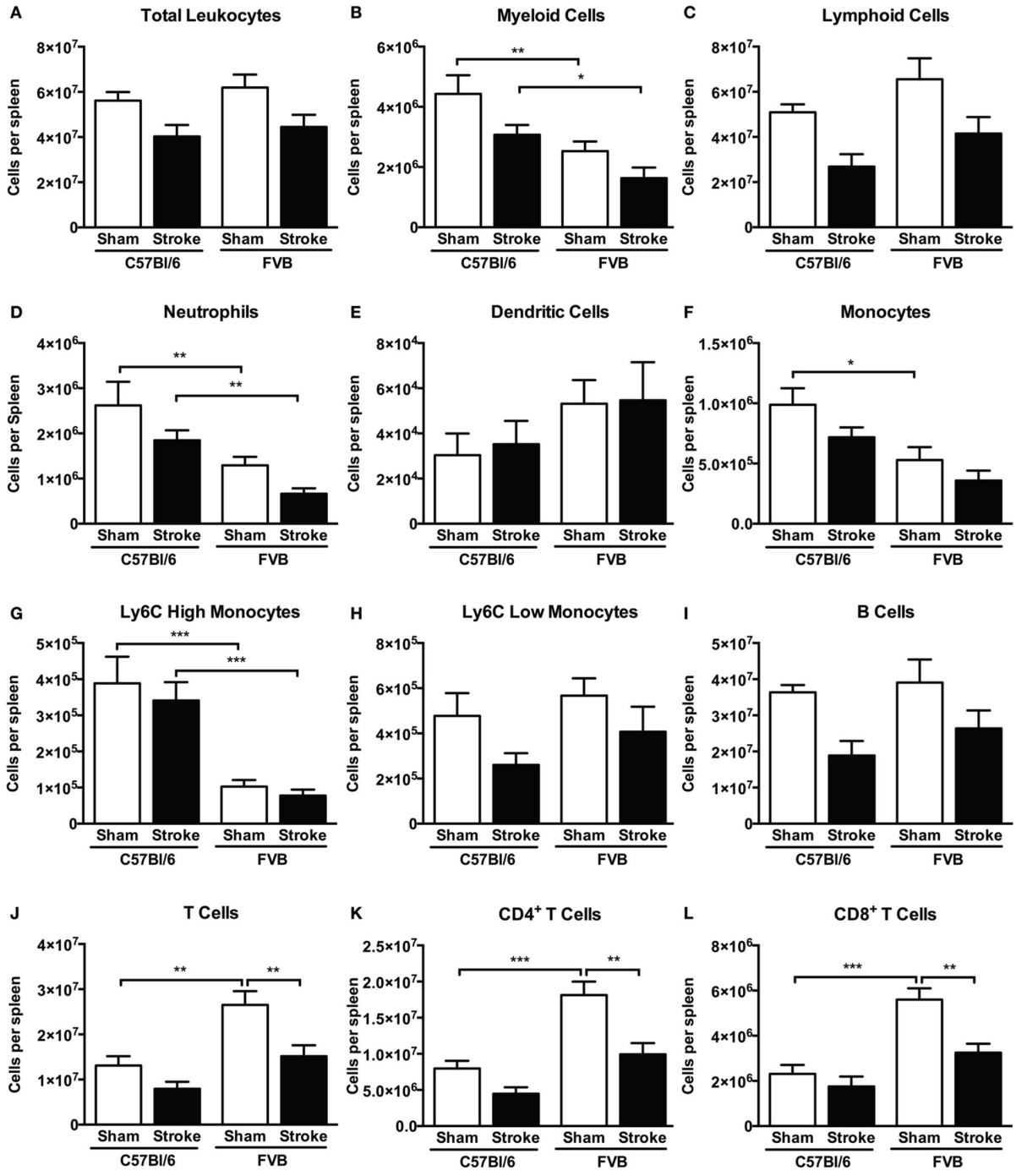

FIGURE 8 | Quantification of (A) total leukocytes and (B–L) leukocyte subsets in spleen 24 h after 1 h middle cerebral artery occlusion, compared with sham control animals of the same strain (total leukocytes $n = 17–29$, myeloid cells $n = 9–17$, lymphocytes $n = 6–12$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; One-Way ANOVA with Bonferroni post-tests). Data are mean ± s.e.m.

the importance of these strain differences in B cell number and distribution for stroke outcome.

In summary, we observed a profound difference in post-stroke functional outcome which was associated with a markedly contrasting number and composition of cells infiltrating the injured brain, despite similar systemic immune cell and cytokine profiles between the two mouse strains. The data therefore suggest that the nature of the inflammatory response to brain ischemia

can vary considerably, and it may consequently impact the functional outcome independently of the volume of injured tissue. It would appear that the early infiltration into the ischemic brain tissue of certain innate/myeloid cell types, such as neutrophils, macrophages and Ly6C^{+low} monocytes, rather than cells of the adaptive immune system, such as B and T lymphocytes, may assist in achieving a milder level of functional deficit.

We acknowledge that while differences in biological responses between mouse strains that possess varying genetic and immunological profiles may provide a useful tool to gain some mechanistic insight into immune cell-mediated ischemic brain injury, studies of mice of the same genetic background are needed to provide definitive conclusions regarding these complex mechanisms. Moreover, if such findings are relevant for developing effective therapies for stroke patients, it will be interesting to determine whether individuals predisposed to Th2-prone immunity, including conditions such as asthma and allergy, might experience milder brain inflammation and functional deficit after stroke.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00365/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Role of Inflammation and the Angiotensin Type 2 Receptor in the Regulation of Arterial Pressure During Pregnancy in Mice

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Abstract—During normal pregnancy the renin–angiotensin system is activated, yet pregnant women are resistant to the pressor effects of angiotensin II. Our aim was to determine the role of the angiotensin type 2 receptor (AT_2R) in the regulation of arterial pressure, natriuresis, and immune cell infiltration during pregnancy. Mean arterial pressure was measured via telemetry, and flow cytometry was used to enumerate immune cell infiltration in 14-week-old wild-type and AT_2R knockout mice during gestation. In wild-type mice, mean arterial pressure decreased during gestation, reaching a nadir at gestational day 9 (–6±2 mm Hg) and returned to near preconception levels during late gestation. In AT_2R-deficient mice, the midgestational decrease in mean arterial pressure was absent. Furthermore, mean arterial pressure was significantly increased during late gestation compared with wild-type mice (≈10 mm Hg). As expected, circulating immune cell activation was suppressed during pregnancy. However, this response was absent in AT_2R-deficient mice. While renal immune cell infiltration was similar between the genotypes, there was a significant T cell phenotypic switch toward a proinflammatory T-helper 1 phenotype in AT_2R-deficient mice. These data indicate that the AT_2R plays an important role in arterial pressure regulation and may modulate T cell activation and renal cytokine production during pregnancy. Therefore, deficits in AT_2R expression may contribute to pregnancy-induced hypertension and thus represents a potential therapeutic target. **(*Hypertension*. 2014;64:626-631.)** • **Online Data Supplement**

Key Words: arterial pressure ■ inflammation ■ pregnancy ■ renal

During a normotensive pregnancy circulating angiotensinogen, renin activity, and angiotensin II (AngII) are increased, yet pregnant women are resistant to the pressor effects of AngII.[1,2] Recently, we have gained strong evidence that the angiotensin type 2 receptor (AT_2R) blunts pressor responsiveness, sodium retention, tubuloglomerular feedback, and renal vasoconstrictor responses to AngII in female rodents.[3–5] The renal responses to AngII are also different in healthy men and women,[6] and it has been suggested that the explanation for these disparities might also lie with differences in AT_2R function.[7] Given that estrogen enhances AT_2R expression,[8,9] the AT_2R may contribute to the cardiovascular and renal adaptations that occur during pregnancy.

While the immune system is suppressed during normotensive pregnancy, the pathophysiology of preeclampsia is characterized by immune system activation.[10] It has been demonstrated that T cells, part of the adaptive immune system, are essential for the development of AngII-induced hypertension.[11] Within the kidney, the angiotensin type 1 receptor (AT_1R) increases production of the proinflammatory T-helper (Th) 1 cytokine, interferon-γ (IFN-γ), and decreases production of the anti-inflammatory Th2 cytokine interleukin-4.[12] Skewing of Th cells toward a Th1 phenotype is well established in several inflammatory disorders including hypertension and preeclampsia.[12,13] Recent studies indicate that AT_2R agonism with compound 21 elicits anti-inflammatory actions, skewing the Th1:Th2 balance toward the Th2 phenotype.[14,15] Thus, the AT_2R may contribute to the increase in Th2 cytokines and subsequent suppression of Th1-mediated immune responses during normotensive pregnancies, whereas AT_2R deficiency may promote an increase in the Th1:Th2 ratio and pregnancy-induced hypertension.

We hypothesized that during pregnancy the AT_2R modulates natriuresis and contributes to the suppression of the immune system, which facilitates the decrease in arterial pressure during pregnancy. Our aims were to examine mean arterial pressure (MAP) throughout pregnancy and the renal and placental immune responses on gestational day (Gd) 8 and Gd16 and renal excretory function at Gd18 in wild-type (WT) and AT_2R-knockout (AT_2R-KO) mice.

Methods

Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. MAP was measured via radiotelemetry at baseline, during gestation and 2 weeks postpartum in WT (FVB/N)

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and AT$_2$R-KO mice. In additional cohorts, immune system activation, infiltration, and the proportion of ex vivo–stimulated T cells producing Th1 (IFN-γ, tumor necrosis factor α, interleukin-17) and Th2 (interleukin-4) cytokines were determined using flow cytometry, renal excretory function was measured via 24-hour urine collection, and renal and placental gene expression of renin angiotensin system (RAS) components (AT$_{1a}$R, AT$_{1b}$R, AT$_2$R, angiotensin-converting enzyme 2 [ACE2], and mas receptor [MasR]) were determined using real-time reverse transcription-polymerase chain reaction. See the online-only Data Supplement for full methods.

Results

Arterial Pressure

Preconception MAP was similar in WT and AT$_2$R-KO mice (94±1 versus 92±1 mm Hg, respectively, P=0.2). During pregnancy, MAP decreased in WT mice. This gestational decrease in MAP reached a nadir of –6±2 mm Hg at Gd9 (P<0.01 as compared with baseline; Figure 1). Compared with WT, the midgestational decrease in MAP was abolished in AT$_2$R-KO mice (P_g=0.04; Figure 1). Near term, MAP returned to baseline levels in WT mice; however, during late gestation MAP was augmented in AT$_2$R-KO mice (2±3 versus 13±7 mm Hg at Gd20, P<0.05). In mice with radiotelemetry probes that continued to record MAP for the full 2-week postpartum period, it was observed that MAP returned to preconception levels in both WT (88±2 mm Hg; n=6) and AT$_2$R-KO (93±3 mm Hg; n=4) mice.

Renal Excretory Function

Basal body weight was similar between the genotypes and increased during pregnancy (P_t<0.001; Table S1 in the online-only Data Supplement). Water intake and urine flow were increased at Gd18 (both P_t<0.05), but the increases were not different between the genotypes (Table S1). Sodium intake was increased at Gd18 as compared with baseline (P_t=0.004; Figure 2A). In contrast, there was no significant difference in sodium excretion between the genotypes or during pregnancy (Figure 2B). Determination of sodium balance (sodium intake minus excretion) demonstrated that during pregnancy there was net sodium retention (P_t=0.008). Moreover, this effect was enhanced in AT$_2$R-KO mice (P_{g*t}=0.05; Figure 2C). Basal albuminuria was not significantly different between the genotypes. During pregnancy albuminuria decreased 38±6% in the WT and increased 57±35% in the AT$_2$R-KO mice (P_{g*t}<0.001; Table S1). There were no significant differences in fetal number, fetal weight, placental weight, or placental efficiency between the genotypes (Table S2).

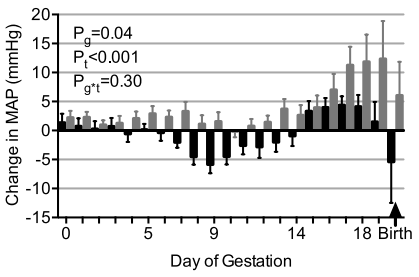

Figure 1. Change in 24-hour mean arterial pressure (MAP) during pregnancy compared with baseline in wild-type (■; n=7) and angiotensin type 2 receptor knockout (■; n=8) mice. Data are presented as mean±SEM. Data were analyzed using repeated-measures ANOVA with the factors genotype (P_g), time (P_t), and their interaction, followed by Tukey post hoc tests. P<0.05 was considered statistically significant.

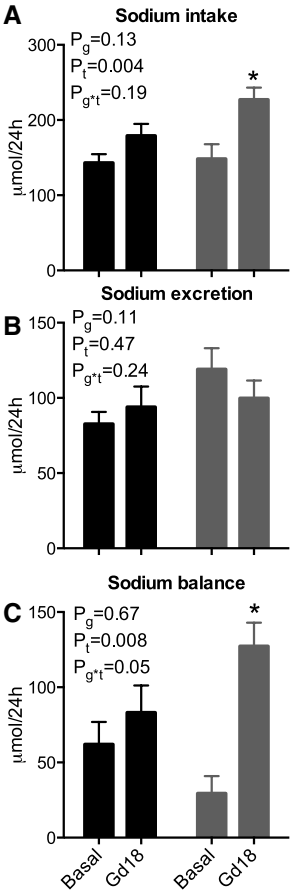

Figure 2. Sodium balance studies in wild-type (WT) and angiotensin type 2 receptor knockout (AT$_2$R-KO) mice at baseline and on gestational day (Gd) 18. Sodium intake (**A**), sodium excretion (**B**), and sodium balance (**C**; sodium intake minus sodium excretion) of WT (■; n=8) and AT$_2$R-KO (■; n=6) mice. Data are presented as mean±SEM. Data were analyzed using repeated-measures ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. *P<0.05 vs basal AT$_2$R-KO.

Immune System Activation

Circulating

There was no significant difference in the proportion of circulating T cells (CD3+) between the genotypes at baseline or during gestation (P_{g*t}=0.4; Figure S4A). The proportion of the T cell subpopulations characterized by CD4+ (Th cells), FoxP3+ (T regulatory cells), and CD8+ (cytotoxic T cells) expression was similar between the genotypes at baseline and unchanged during pregnancy (Figure S4B–S4D). Analysis of circulating Th cells demonstrated that at baseline Th activation was significantly lower in AT$_2$R-KO as compared with WT mice (Figure 3A and 3B). Circulating Th activation was suppressed during pregnancy in WT but unaffected in AT$_2$R-KO mice (Figure 3A and 3B). Cytotoxic T cell activation was also suppressed during pregnancy; however, this was not significantly different between the genotypes (data not shown).

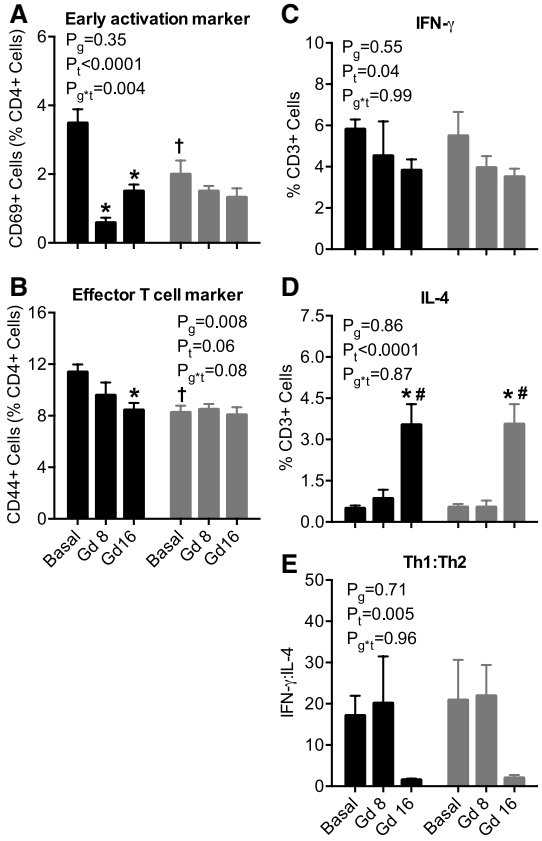

Figure 3. Circulating T cell activation in wild-type (WT; ■; n=4–12 per group) and angiotensin type 2 receptor knockout (■; n=4–12 per group) mice at baseline, gestation day (Gd) 8, and Gd16. CD4+ (**A**) early and (**B**) effector T cell activation, the proportion of ex vivo-stimulated T cells producing (**C**) interferon-γ (IFN-γ) and (**D**) interleukin (IL)-4, and the (**E**) Th1:Th2 ratio. Data are presented as mean±SEM. Data were analyzed using an ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. †P<0.05 vs respective WT group. *P<0.05 vs respective basal group. #P<0.05 vs respective Gd8 group.

The proportion of IFN-γ- and interleukin-4-producing T cells (Figure 3C and 3D) and the Th1:Th2 ratio were similar between the genotypes at baseline (Figure 3E). During gestation, the proportion of T cells producing the IFN-γ at Gd8 and Gd16 decreased to a similar extent in WT and AT_2R-KO mice (P_t=0.04; P_{g*t}=0.99; Figure 3C). Conversely, the proportion of T cells producing interleukin-4 was significantly increased at Gd16 in both genotypes (P_t<0.0001; P_{g*t}=0.87; Figure 3D). Consequently, at Gd16 the circulating Th1:Th2 ratio was similarly decreased from basal between the genotypes (P_t=0.005; P_{g*t}=0.96; Figure 3E). There were no significant differences in circulating tumor necrosis factor-α- and interleukin-17-producing T cells between the genotypes at baseline or during gestation (Figure S5A and S5D).

Renal

Basal renal leukocyte and T cell infiltration were similar in WT and AT_2R-KO females, and there were no significant differences in renal cytokine expression or the Th1:Th2 ratio (Figure 4). During pregnancy, renal T cell infiltration was similar between the genotypes (P_g=0.84, P_{g*t}=0.87; Figure 4B).

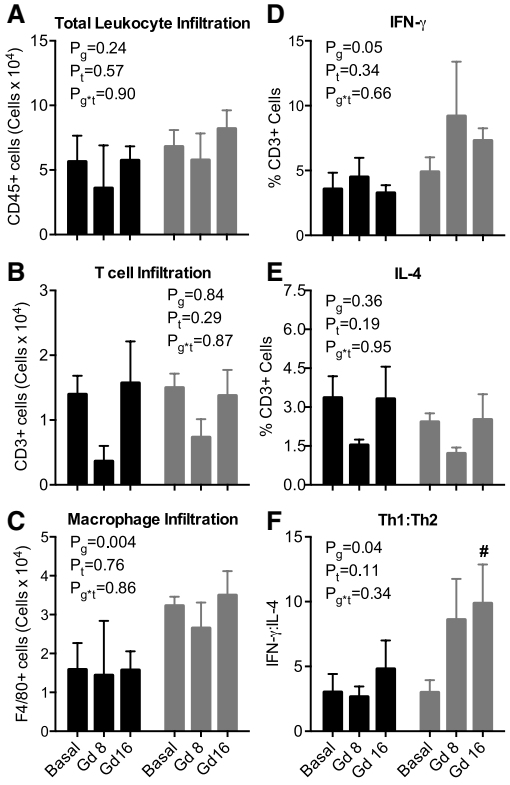

Figure 4. Renal immune cell infiltration and cytokine production in wild-type (WT; ■; n=4–12 per group) and angiotensin type 2 receptor knockout (■; n=4–12 per group) mice at baseline, gestation day (Gd) 8, and Gd16. Immune cell (**A**), T cell (**B**), and macrophage infiltration (**C**), the proportion of ex vivo-stimulated T cells producing interferon-γ (IFN-γ; **D**) and interleukin (IL)-4 (**E**), and the Th1:Th2 ratio (**F**). Data are presented as mean±SEM. Data were analyzed using an ANOVA with the factors genotype (P_g), time (P_t), and their interaction. Tukey post hoc tests were performed where appropriate. †P<0.05 vs respective WT group.

However, compared with WT counterparts, the proportion of T cells producing IFN-γ was increased in AT_2R-KO females during pregnancy, representing a phenotypic change in the T cells infiltrating the kidney at Gd8 (4.5±1.5 and 9.2±4.2% CD3+ cells, respectively) and Gd16 (4.9±1.7 and 7.3±0.9% CD3+ cells, respectively; P_g=0.05, P_{g*t}=0.66; Figure 4D). There were no significant differences in the proportion of T cells producing tumor necrosis factor-α and interleukin-17 between the genotypes at baseline or during gestation (Figure S5B and S5E). Comparison of the renal Th1:Th2 ratios demonstrated a trend at Gd8, with AT_2R-KO mice having a higher Th1:Th2 ratio than WT mice (P=0.06; Figure 4F). At Gd16, the renal Th1:Th2 ratio was significantly increased in AT_2R-KO mice compared with baseline (P=0.04; Figure 4F). The number of macrophages infiltrating the kidney was also greater in AT_2R-KO than WT mice at baseline, and this difference was maintained during gestation (P_g=0.004, P_{g*t}=0.6; Figure 4C).

Placenta

The expected decrease in placental Th1:Th2 ratio was observed at Gd16 as compared with Gd8 in both genotypes (Figure S6F). There were no significant differences in T cell infiltration or cytokine production between the genotypes (Figures S5C, S5F, S6B, S6D, and S6E).

Renal and Placental Gene Expression

Compared with WT mice, AT$_2$R-KO mice had lower basal renal AT$_{1a}$R and AT$_{1b}$R gene expression (Figure S7A and S7B, both $P<0.05$). Basal renal ACE2 and MasR gene expression were not significantly different between the genotypes. During pregnancy there was no change in AT$_{1a}$R, AT$_2$R, or MasR gene expression (Figure S7). In WT mice, renal AT$_{1b}$R gene expression was reduced at Gd18 as compared with basal ($P=0.02$). In pregnant AT$_2$R-KO mice, renal ACE2 expression was significantly higher at Gd8 versus Gd16 ($P=0.04$). Pregnancy altered the expression of components of the RAS in the placenta with marked increases in AT$_{1a}$R, ACE2, and MasR. However, there was no significant impact of AT$_2$R deficiency (see Results in the online-only Data Supplement and Figure S8).

Discussion

There were 3 major findings in the present study. Firstly, the AT$_2$R mediates the normal midgestational decrease in arterial pressure and contributes to arterial pressure regulation during late gestation. These findings extend previous observations of the arterial pressure-lowering effects of the AT$_2$R during pregnancy.[16–18] Secondly, in AT$_2$R-KO mice there was a phenotypic switch in the T cells infiltrating the kidney toward a proinflammatory phenotype, resulting in an increase in the renal Th1:Th2 ratio at Gd16. Moreover, the increase in the Th1:Th2 ratio was observed only in the kidneys of pregnant AT$_2$R-KO mice, which markedly contrasted the reduced Th1:Th2 ratios in the circulation and placentae as per regular pregnancies. Finally, as expected, pregnant mice were in positive sodium balance. However, sodium retention was enhanced in pregnant AT$_2$R-KO mice, and this may have contributed to the increase in arterial pressure in late gestation. Therefore, the AT$_2$R plays an important role in arterial pressure regulation and may modulate renal immune cell phenotype as well as pressure-natriuresis during pregnancy. Our studies demonstrate that the AT$_2$R contributes to the normal cardiovascular and renal adaptations that facilitate the gestational decrease in arterial pressure. A corollary of this is that deficits in AT$_2$R expression may contribute to pregnancy-induced hypertension.

Pregnancy in mammals, including humans[19] and mice,[20] is characterized by a midgestational fall in arterial pressure, with MAP returning to preconception levels during the peripartum period. In the present study, we have documented for the first time the full impact of the AT$_2$R on arterial pressure during pregnancy in mice. It was demonstrated that during gestation, the AT$_2$R mediates the normal midgestational decrease in arterial pressure, because the fall was absent during pregnancy in AT$_2$R-KO mice. Furthermore, AT$_2$R deficiency was associated with an elevation in arterial pressure during late gestation. Previously, Takeda-Matsubara et al[18] observed a significant increase in systolic arterial pressure during late gestation as compared with prepregnancy levels in AT$_2$R-KO mice. However, this study did not observe the normal midgestational decrease in arterial pressure in WT (C57BL/6) mice and consequently did not report an effect of AT$_2$R deletion on arterial pressure during midgestation. Conversely, Chen et al[16] and Carey et al[17] have demonstrated that pharmacological and genetic AT$_2$R deficiency abolishes the normal midgestational decrease in arterial pressure. However, these studies observed no effect of AT$_2$R deficiency on arterial pressure during late gestation. The discord between these findings and those of the current study most likely reflects methodological differences. In our study, MAP was measured using radiotelemetry, which is the gold standard method to measure arterial pressure. The earlier studies used tail-cuff plethysmography to measure arterial pressure, which may mask subtle differences in arterial pressure during pregnancy because of the inherent stress involved with restraint and frequent handling.[21] Thus, our data unequivocally demonstrate that the normal midgestational decrease observed in the arterial pressure profile of pregnant WT mice is absent in AT$_2$R-KO mice and, importantly, that AT$_2$R deficiency leads to higher arterial pressure during late gestation.

To protect the feto-maternal unit during pregnancy, the immune system is normally suppressed.[10] Previous studies investigating Th1:Th2 ratio during pregnancy have measured IFN-γ and interleukin-4 levels and shown a distinct shift toward the Th2 phenotype, both systemically and within placentae.[22–24] IFN-γ and interleukin-4 are the principal cytokines that promote Th1 and Th2 differentiation, respectively.[25,26] In the present study we have shown a similar pattern, with a decrease in the Th1:Th2 ratio observed in the circulation and placentae of pregnant WT mice. Surprisingly, AT$_2$R deficiency in mice did not alter the Th1:Th2 ratio within the circulation or placentae as hypothesized. Thus, the findings of the present study suggest that AT$_2$R deficiency does not affect the systemic immune response. Furthermore, because placental AT$_2$R expression decreased during gestation in WT mice and there was no change in the placental Th1:Th2 ratio or number of live fetuses between the genotypes, it is not likely that AT$_2$R deficiency is associated with poorer fetal outcomes. This is consistent with a previous report suggesting that the AT$_2$R does not play a pivotal role in the placenta.[27] Conversely, placental ACE2 and MasR gene expression was increased during late gestation in both genotypes. This finding is consistent with previous studies which have demonstrated that uteroplacental levels of Ang(1–7) and ACE2 are elevated during pregnancy.[28,29] Thus the ACE2/Ang(1–7)/MasR pathway may play an important role in uteroplacental blood flow and fetal growth.

The most remarkable finding of the present study was that although there was no difference in the number of T cells infiltrating the kidney between pregnant WT and AT$_2$R-KO mice, AT$_2$R deficiency promoted a phenotypic shift of renal T cells and an increase in the renal Th1:Th2 balance in pregnant AT$_2$R-KO mice. A recent study using the AT$_2$R agonist compound 21 showed that the AT$_2$R reduces stimulation of a proinflammatory cytokine, interleukin-6, by inhibiting the transcription factor, nuclear factor-κB.[14] Nuclear factor-κB controls the expression of proinflammatory cytokines, chemokines, adhesion molecules, and growth factors which all play a critical role in mediating the proinflammatory actions of the Th1 phenotype.[10] The importance of this finding has been demonstrated in pregnant spontaneously hypertensive rats. Hypertension in the spontaneously hypertensive rat is ameliorated during pregnancy, an effect that is associated with a reduction in the renal AT$_1$R:AT$_2$R ratio and decreased inflammation as indicated by downregulation of

nuclear factor-κB in the kidney.[30] Collectively these findings suggest that the AT_2R suppresses AT_1R-mediated immune system activation within the kidney during pregnancy. Because the increase in the renal Th1:Th2 ratio preceded the rise in arterial pressure during late gestation in AT_2R-KO mice, this suggests that renal inflammation may contribute to pregnancy-induced hypertension.

During pregnancy there was an increase in sodium intake; however, sodium excretion did not change. Thus, pregnant mice were in a state of positive sodium balance. Significantly, the sodium retention was greater in pregnant AT_2R-KO than WT mice. Renal adaptations to pregnancy promote sodium retention, which facilitates the marked increase in plasma volume. Moreover, sodium retention during pregnancy occurs while both natriuretic and antinatriuretic signals are activated.[31] However, previous studies in pregnant rats have demonstrated that the pressure-natriuresis relationship is blunted during late gestation.[31,32] In the present study, arterial pressure was significantly elevated at Gd18 above prepregnancy levels in AT_2R-KO mice. This finding coupled with the similar natriuretic response at Gd18 between WT and AT_2R-KO mice indicates a rightward shift of the pressure-natriuresis relationship in AT_2R-KO mice during late gestation. These results suggest that the blunted pressure-natriuresis relationship observed in normotensive pregnancy is, at least in part, AT_2R mediated. This is consistent with previous findings, which have demonstrated that the AT_2R modulates pressure-natriuresis in nonpregnant rodents.[3,4] Consequently, deficits in AT_2R expression seem to alter the normal renal adaptations to pregnancy. Indeed, albuminuria was increased in pregnant AT_2R-KO mice compared with WT mice.

Contrary to our hypothesis, renal AT_2R expression during pregnancy did not increase in WT females, nor did other components (ACE2 and MasR) of the protective arm of the RAS. However, we, and others, have previously demonstrated that AT_2R, ACE2, and MasR gene expression is greater in female than in male rodents.[5,9,33] Furthermore, here we demonstrate that these high levels of expression persist during pregnancy. In contrast, AT_2R expression was significantly increased in the aorta, renal artery, and kidneys of pregnant normotensive rats at Gd14 as compared with nulliparous females, and this time point correlated with the peak increase in renal function.[34] In another study in pregnant ewes, AT_2R expression was increased in the uterine artery and unchanged in the aorta.[35] In these studies, AT_1R expression was unchanged between virgin and pregnant females. Therefore, females have high expression of the protective components of the RAS, which are maintained or increased in a regional and temporal manner during pregnancy, in various animals. Furthermore, as AngII binds with ≈15-fold greater affinity to the AT_2R as compared with the AT_1R,[36] this may underlie the reduced pressor responsiveness to AngII during pregnancy. Alternatively, the facts that plasma levels of Ang(1–7) increase ≈16-fold during normotensive pregnancy in women and Ang(1–7) is able to elicit its actions via the AT_2R, as well as its own receptor, MasR, suggest that Ang(1–7) may play a pivotal role in countering the pressor actions of AngII during normal pregnancy.[37] Future studies are needed to further characterize the role of the depressor RAS pathways in the normal regulation of arterial pressure during pregnancy.

Perspectives

Given that women with a history of preeclampsia are at an increased risk for developing cardiovascular disease in later life, understanding the mechanisms regulating arterial pressure during pregnancy are of importance. Our study demonstrates a key role for the AT_2R in the normal regulation of arterial pressure during pregnancy. Therefore, the enhanced AT_2R:AT_1R ratio observed in females may underlie reduced pressor responsiveness to AngII during normal pregnancy. Conversely, increased sensitivity to AngII during preeclampsia is associated with a decrease in the AT_2R:AT_1R ratio.[38] Thus, deficits in the depressor RAS pathways may be a predisposing factor to the development of pregnancy-induced hypertension and future cardiovascular risk.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first study to unequivocally demonstrate that the angiotensin type 2 receptor (AT$_2$R) mediates the normal midgestational decrease in arterial pressure and that the AT$_2$R contributes to the regulation of arterial pressure during late gestation in mice.
- This modulates immunity during gestation in mice.

What Is Relevant?

- AT$_2$R deficiency abolished the midgestation decrease in arterial pressure that was observed in wild-type mice and contributed to higher arterial pressure during late gestation in AT$_2$R knockout mice.
- AT$_2$R deficiency increased the renal Th1:Th2 balance during late gestation.
- AT$_2$R knockout mice had a higher arterial pressure during pregnancy as compared with wild-type mice, yet they excreted the same amount of sodium, suggesting a rightward shift in the pressure-natriuresis relationship.

Summary

The AT$_2$R plays a protective role in arterial pressure regulation during pregnancy in mice. A consequence of this is that deficits in AT$_2$R expression may contribute to the development of pregnancy-induced hypertension. Therefore, AT$_2$R agonist therapy represents a novel approach to treating pregnancy-induced hypertension.