SEX-DIFFERENCES IN THE IMMUNE AXIS OF PERIVASCULAR ADIPOSE TISSUES: IN HEALTH AND ADIPOSITY-INDUCED HYPERTENSION

By

Ramya Kalyana Kumar

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology – Doctor of Philosophy

2020

ABSTRACT

SEX-DIFFERENCES IN THE IMMUNE AXIS OF PERIVASCULAR ADIPOSE TISSUES: IN HEALTH AND ADIPOSITY-INDUCED HYPERTENSION

By

Ramya Kalyana Kumar

Perivascular adipose tissue (PVAT) is protective and reduces the contraction of blood vessels in health. Increased immune cell infiltration and loss of the anti-contractile function of PVAT occurs with obesity and hypertension. Thus, our overall hypothesis was that **T cells and macrophages in mesenteric PVAT promote release of IL-10 that causes vasorelaxation in health.** In addition, we hypothesized that **immunological PVAT dysfunction precedes adiposity-induced hypertension (alike in males and females).** The research detailed in this dissertation provides evidence to prove or disprove our hypotheses: (1) A community of naïve, regulatory, activated and memory-type immune cells including T cells and macrophages exist in mesenteric (MRPVAT, white PVAT around mesenteric resistance arteries) and aortic (APVAT, brown PVAT around thoracic aorta) PVATs in health; (2) Mesenteric PVAT-derived IL-10 neither causes direct vasorelaxation nor reduces vasoconstriction directly or indirectly, thereby not contributing to the anti-contractile nature of PVAT in healthy mice and rats; and (3) At 10 (pre-hypertensive stage) and 17 (onset of hypertension) weeks of diet, there were greater adiposity-induced immune cell changes (CD4+ memory T cells and M2- like macrophages) in MRPVAT and APVAT from females *vs* males. At 24 weeks (with

progression of hypertension), immune changes in PVATs began to occur in males and diminish in females.

Taken together, these findings are important to know because assessing immunological PVAT function at an early stage has the potential to prevent the development of hypertension. Future studies building on this work should investigate the role of immune cells in PVATs in health and probe into the sex-differences in the immune-mechanisms contributing to vascular tone regulation in adiposity-induced hypertension.

To my mentors, past and present.
To the supramental force in the universe that guides me.

ACKNOWLEDGEMENTS

Realizing and thanking the amazing people that have bolstered me through this beautiful journey makes this dissertation more meaningful. This work was possible only with the support of wonderful mentors, colleagues, friends and family. My BIG THANKS to each of you for guiding, inspiring and supporting me through a fulfilling journey towards my doctoral degree. I would like to use this space to specifically thank some of them who deserve a special mention.

To my mentor, Dr. Stephanie Watts: You have been inspirational, guiding and pushing me in the right direction. I truly admire how you put your mentee's progress first. Your work ethic, passion for science and kindness are some of the many qualities about you that have motivated me in the last four years. Your love and dedication to pharmacology has encouraged me to pursue a career in this field. More than being a teacher, a mentor and a role model, you have truly been my science mother.

Choosing my committee members has been one of the best choices I have made in my PhD life. I sincerely thank each one of you for always cheering but also challenging me to think deeper into my science. Dr. Cheryl Rockwell: Thank you for giving me the space and freedom to work in your laboratory. I have genuinely felt adopted by the Rockwell lab at many times. A dissertation with an immunology focus would have been just a dream without your generous inspiration and guidance at all times. Dr. Gregory

Fink: You have always given me the time with a big smile when I have walked into your door with new data. Thank you for being my statistics guru! You are a key reason for bringing out my love for big picture thinking in science and life in general. Dr. Andres Contreras: You have been very kind, motivating and supporting me throughout my PhD. Thank you for pushing me towards focusing on particular areas for my thesis when I have wanted to happily get distracted in all the ways my project could go. Dr. James Galligan: I loved our spontaneous hallway conversations about my science and progress. Thank you for never telling me a 'no' when I came up to try a new set of experiments. Each of you have been very generous, kind and flexible with me, guiding me to see the light at the end of the tunnel.

Choosing the Watts lab has been one of the most rewarding decisions in my career. Mrs. Emma Flood: You are my first American friend and so dear will you be forever. I have never before had a more cheerful, colorful, optimistic person to work with in a lab. You bring so much sunshine and joy when you are around me- thank you! Mrs. Janice Thompson: You have literally been a lab mother to me. Anything that has made me smile or cry, you knew you were going to hear all about it- Thank you for giving me this space and for your warmth. Ms. Alexis: You always reminded me of a 'little' mature female version of my brother. I love your 'it'll be fine' attitude. Thank you for all the laughter and good times we have had! Dr. David Ferland: You have been a friendly, wonderful colleague, a computer-geek and my go-to senior graduate student. I would like to thank

the all the members of the Watts lab, especially Dr. Nadia Ayala-Lopez, Maleeha Ahmad, Dr. Carolina Restini, Dr. Bridget Seitz for their support throughout.

Dr. Anne Dorrance: You have been a terrific graduate program coordinator. Thank you for being such an empathetic and caring mentor to me. Dr. Rick Neubig: Thank you for prioritizing the career of graduate students and their well-being as a measure of our department's success. Administrative staff (Ms/ Mr Jake Wier, Erica Lange, Heather deFeijter-Rupp, Bradley Robinson, Beverly Dickinson, Darlene Whitman, Stephen Stofflet, Patricia Gregory, Wendy Rohen): Big cheers to you for your ever-smiling willingness to help in my years working in our department. The Rockwell lab (Dr. Yining Jin, Dr. Robert Freeborn, Dr. Alex Turley, Mr. Luca Kaiser, Ms. Alison): I have literally lived in your lab for so many months together. I wouldn't have learned so many things in my world of science, if not for you all. Drs. James Luyendyk, Adam Lauver, Sudin Bhattacharya, Yongliang Yang, Nathan Tycocki and DiCarlo: Thank you for opening your doors for your support and the nurturing collaborations.

Mrs. Nancy Cuddeback: I am so fortunate to have you in my life! Thank you for truly being my Michigan mother. I am amazed beyond words at your generosity when you opened your door for me, your positive view of the world and your 'kindness to all' policy. Mr. Balto: I wish you could read this. You are my first other-species love. Thank you for being so loving and providing me a fresh perspective of life. My thanks to the entire Padden and Cuddeback families for adopting me, you have been a blessing to me away

from home. Mrs. Beth Hayward: My special thanks to you and the Hayward crew for also being my extended family in Lansing. I have felt energized and rejuvenated every time after we met.

Mr. Kalyana Kumar and Mrs. Ramalakshmi: Thank you for trusting me, showing me the power of believing in oneself and chasing one's dreams. You have been the kind of parents anyone would yearn for! Mr. Ashwin: Thank you for being a pillar of support. I cannot ask for a better brother. Mr. Anand: I am blessed to have married you. Thank you for all the driving (and of course more!) you have done to make this long-distance relationship seem easy through my doctoral years. Mr. Sundarram and Mrs. Prabha: How many parents-in-law take pride in their daughter-in-law's career, like the two of you always have. Thank you for being my new parents. Mr. Vignesh and Mrs. Ajita: You both have been my tremendous cheerleaders. Thank you Ajita, for being a 'sister-from-another-mother' to me. My friends Ms. Saranya, Ms, Muthulakshmi, Mr. Sivakumar, Ms. Sreesubha and Ms. Sowmya for their encouragement and positivity through these years as before.

There have been several others whose names have not been mentioned here but have played a role in seeing my PhD through. My heartfelt thanks to them. I immensely thank the universe and the superior power for being my constant source of energy and surrounding me with really amazing people.

TABLE OF CONTENTS

LIST OF TABLES xi

LIST OF FIGURES xii

KEY TO SYMBOLS AND ABBREVIATIONS xiv

CHAPTER 1 Overall Introduction 1
- The Discovery of Perivascular Adipose Tissue's Hidden Potential 2
- PVAT is more than just Adipocytes 5
- PVAT is Anatomically and Functionally Different from Other Adipose Tissues 5
- All PVATs don't Function Alike! 6
- Why Focus on Immune Cells? 9
- Immune Cells in PVATs and non-PVAT Fats in Homeostasis 10
- Role of Immune Cells and Cytokines in Vascular Tone Regulation in Health 12
- Obesity Meets Hypertension: The Crisis 16
- Obesity and Hypertension: Wars Led by Immune Cells 17
- Rationale for the Use of the Dahl S HFD-Induced Hypertension Model 19
- Do Males and Females Use Different Weapons to Fight Obesity and Hypertension? 20
- Immunological PVAT Dysfunction *vs* Hypertension Development 21
- Overall Hypotheses 22
- REFERENCES 23

CHAPTER 2 Naïve, Regulatory, Activated and Memory Immune Cells Co-Exist in PVATs that are Comparable in Density to Non-PVAT Fats in Health 35
- Introduction 37
- Materials and Methods 39
- Results 46
- Discussion 62
- REFERENCES 68

CHAPTER 3 Interleukin-10 Does Not Contribute to The Anti-Contractile Nature of PVAT in Health 73
- Introduction 75
- Materials and Methods 76
- Results 85
- Discussion 102
- REFERENCES 108

CHAPTER 4 Sex-Differences in T Cells and Macrophages in Perivascular Adipose Tissues Precede High-Fat Diet-Induced Hypertension 112
Introduction 114
Materials and Methods 116
Results 119
Discussion 136
REFERENCES 141

CHAPTER 5 Overall Discussion 145
Summary of New and Novel Findings 146
Discussion 152
Possible Reasons for the Unexpected Sex-Differences 157
Limitations 159
Big Questions to Consider when Moving Forward 160
Future Directions 161
Current Challenges with Immune Modulators for the Treatment of Obesity and Hypertension 164
Conclusion and Broad Implications of our Findings 167
REFERENCES 169

LIST OF TABLES

Table 2.1 Immunophenotyping antibodies for flow cytometry 40

Table 2.2 Antibody panel design for flow cytometry 40

Table 2.3 Definitions of specific immune sub-populations 44

Table 2.4 Overall immune cell densities in spleen, PVATs and non-PVAT fats in male and female SD rats 50

Table 2.5 Overall immune cell percentages in spleen, PVATs and non-PVAT fats in male and female SD rats 61

Table 3.1 Exogenous IL-10 was not anti-contractile in the SD rat: pharmacological parameters 93

Table 3.2 PVAT remained anti-contractile with short term genetic depletion of endogenous IL-10: pharmacological parameters 98

Table 3.3 PVAT remained anti-contractile with prolonged genetic depletion of endogenous IL-10: pharmacological parameters 101

LIST OF FIGURES

Figure 1.1 Histological images of MRPVAT and APVAT 4

Figure 1.2 Anatomical locations of PVAT and non-PVAT fats investigated 8

Figure 1.3 Graphical representation of overall hypotheses 22

Figure 2.1 Basic gating strategy for flow cytometry experiments 47

Figure 2.2 PVATs contained a denser immune cell community *vs* their respective non-PVAT fats in female rats only 49

Figure 2.3 MRPVAT in females had a higher naïve CD4 T cell population *vs* APVAT (females) and MRPVAT (males) 52

Figure 2.4 PVATs were mostly similar in numbers of CD8 T cell subtypes *vs* their respective non-PVAT fats 54

Figure 2.5 PVATs and non-PVAT fats had similar numbers of CD25 expressing B cells 56

Figure 2.6 MRPVAT had a greater density of CD68+ macrophage subpopulation *vs* APVAT and RP fat in females 58

Figure 2.7 Summary of findings, as pie charts 60

Figure 3.1 IL-10 mRNA was present in male wildtype rats (MRPVAT) and mice (SMPVAT) 86

Figure 3.2 IL-10 receptor was present in male rat mesenteric and male mouse superior mesenteric arteries 88

Figure 3.3 Acute exposure to exogenous IL-10 did not cause direct vasorelaxation in mesenteric arteries from both wildtype male rats and mice 90

Figure 3.4 Acute exposure to exogenous IL-10 was not anti-contractile in rat mesenteric resistance arteries 92

Figure 3.5 Validation of the IL-10 knockout mouse 95

Figure 3.6 PVAT remained anti-contractile with short-term genetic depletion of endogenous IL-10 97

Figure 3.7 PVAT was anti-contractile even with prolonged genetic depletion of endogenous IL-10 100

Figure 3.8 Summary (Graphical abstract) 107

Figure 4.1 Development and progression of hypertension with high-fat feeding 120

Figure 4.2 Bulk RNA-sequencing of MRPVAT from both sexes after 24 weeks on diet 122

Figure 4.3 Overall snapshot of adaptive and innate immune cells in PVATs along the time-course of development and progression of hypertension 125

Figure 4.4 Sex-differences in T cell and macrophage subtypes in PVATs before the development of hypertension 127

Figure 4.5 Sex-differences in T cell and macrophage subtypes in PVATs peak at 17 weeks on diet, with the onset of hypertension 129

Figure 4.6 Sex-differences in immune cells diminish with the progression of hypertension after 24 weeks on diet 131

Figure 4.7 Magnitude of expression of markers of M2-like macrophages and T regulatory cells over the time course of hypertension development and progression 133

Figure 4.8 Summary (Graphical abstract) 135

Figure 5.1 Graphical representation of findings in Chapters 1 and 2 148

Figure 5.2 Graphical representation of findings in Chapter 3 (MRPVAT) 150

Figure 5.3 Graphical representation of findings in Chapter 3 (APVAT) 151

KEY TO SYMBOLS AND ABBREVIATIONS

ACh	acetylcholine
ADRF	adipose-derived relaxing factor
Ang	angiotensin
ANOVA	analysis of variance
APVAT	PVAT around thoracic aorta
ATM	adipose tissue macrophages
°C	celcius (degrees)
CaCl2	calcium chloride
CD	control-diet
CD#	cluster of differentiation
CDC	center for disease control and prevention
cDMEM	complete Dulbecco's Modified Eagle Medium
COX	cyclo oxygenase
C_t	cycle threshold
CTLA	cytotoxic T lymphocyte associate protein
CVD	cardiovascular disease
Dahl S	Dahl salt-sensitive
DEG	differentially expressed genes
DNA	deoxy ribonucleic acid
EC_{50}	half-maximal effective concentration

ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
Emax	maximal effect
eNOS	endothelial notric oxide synthase
ET	endothelin
F	female
FDA	food drug administration
Foxp3	forkhead box p3
FSC	forward scatter
HFD	high-fat diet
ICOSL	inducible T cell costmulatory ligand
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL10 RA	receptor for IL-10
ILC	innate lymphoid cells
iNKT	invariant natural killer cells
Io	ionomycin
JAK	janus kinases
K2PO4	potassium phosphate
KCl	potassium chloride
KO	knockout

LPS	lipo polysaccharide
M	male
MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MCP	macrophage chemoattractant protein
MFI	mean fluorescence intensity
$MgSO_4.H_2O$	magnesium sulfate monohydrate
MHC	major histocompatibility complex
mr	mouse recombinant
MRA	mesenteric resistance artery
mRNA	messenger ribonucleic acid
MRPVAT	PVAT around mesenteric resistance vessels
N	number of animals
nAb	neutralizing antibody
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NK	natural killer
NO	nitric oxide
OVX	overiectomy
PBS	phosphate buffered saline
PCA	principal component analysis
PE	phenylephrine

PMA	phorbol myristate acetate
PPARγ	peroxisome proliferator-activated receptor- gamma
PSS	physiological salt solution
PVAT	perivascular adipose tissue
qPCR	semi-quantitative polymerase chain reaction
rcf	relative centrifugal force
RP	retroperitoneal
RPL13a	ribosomal protein L13a
rr	rat recombinant
SAT	subcutaneous adipose tissue
SD	Sprague Dawley
SEM	standard error of mean
SHR	spontaneously hypertensive rats
SMA	superior mesenteric artery
SMC	smooth muscle cell
SMPVAT	PVAT around superior mesenteric vessels
SS	subscapular
SSC	side scatter
STAT	signal transducer and activator of transcription proteins
SVF	stromal vascular fraction
TLR	toll-like receptor
TNF	tumor necrosis factor

Treg	regulatory T cells
UCP	uncoupling protein
VAT	visceral adipose tissue
VSMC	vascular smooth muscle cell
WAT	white adipose tissue
WT	wildtype

CHAPTER 1

Overall Introduction

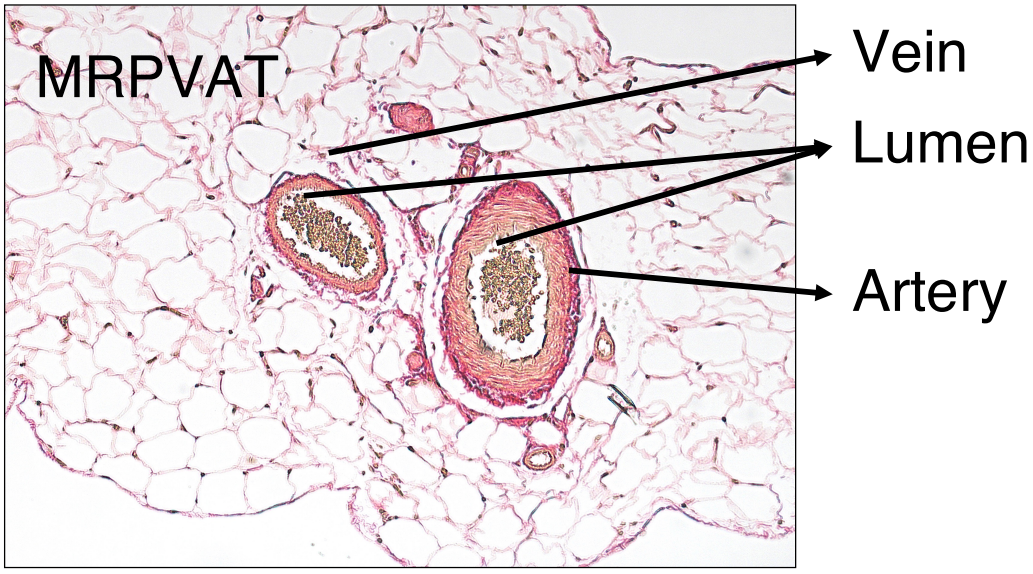
The Discovery of Perivascular Adipose Tissue's Hidden Potential

Perivascular adipose tissue (PVAT) is the fat that surrounds almost all the peripheral blood vessels greater than 100 μm. PVAT was routinely removed from vascular (patho)physiology studies in the lab and during coronary artery surgeries in the clinic. Seminal findings by Soltis and Cassis in 1991 incited the importance of what is now called the 'tunica adiposa' or the unofficial fourth layer of a blood vessel: PVAT [Soltis EE 1991]. They discovered that rat aorta with PVAT constricted lesser to norepinephrine, a vasoconstrictor agonist compared to a blood vessel without PVAT [Soltis EE 1991]. This is called the classic 'anti-contractile' function of PVAT and is experimentally observed as a rightward (lower potency of the agonist as measured by EC_{50}) and/ or downward shift (reduced maximal contraction measured by E_{max}) of an agonist response curve.

This anti-contractile function of PVAT occurs with various agonists such as angiotensin (Ang) II, serotonin, endothelin (ET)-1 and phenylephrine (PE); in multiple vascular beds including the mesenteric circulation, coronary arteries and limb vessels, observed in the mouse, rat, pig, dog and human [T Szaz 2013]. So, is this anti-contractile effect because PVAT is a bulky obstructive layer impeding the agonist to get to the blood vessel to exert its function? Beginning with Gollasch *et al*, solution-transfer studies [Gollasch M 2012] have demonstrated that factors released from PVAT are responsible, at least in part, for its anti-contractile function and the term ADRF (adipose-derived relaxing factor) was thus coined by Lohn *et al* in 2002 [Lohn M 2002]. A few individual molecules have been implicated in mediating the relaxant or anti-contractile effects of

PVAT such as adiponectin, nitric oxide (NO), hydrogen sulfide, angiotensin (1-7) and methyl palmitate [Fesus G 2007, Dubrovska G 2004, Beltowski J 2013, Verlohren S 2004, Victorio JA 2016, Lee YC 2011]. This anti-contractile nature is in part endothelium-dependent, mediated by NO and calcium-activated potassium channel activation and in part endothelium-independent, mediated by hydrogen peroxide and soluble guanylate cyclase activation [Gao YJ 2007, Dubrovska G 2004]. Thus, by local regulation of vascular tone, PVAT helps control (lower) blood pressure in healthy subjects.

A



B

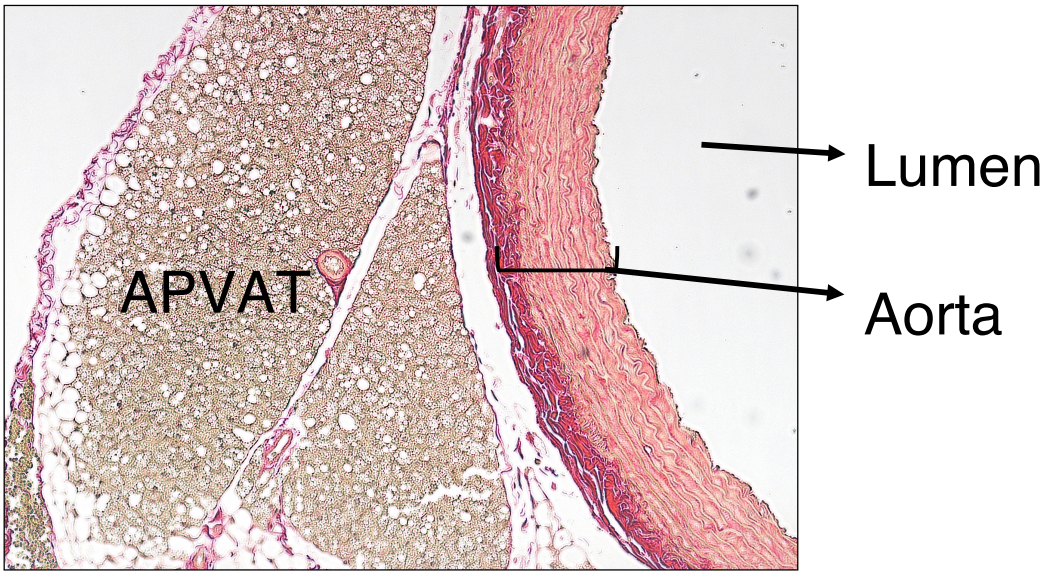


Figure 1.1 Histological images of MRPVAT and APVAT
Histological image (20X) of PVAT around the mesenteric resistance vessels (MRPVAT; white PVAT) **(A)** and thoracic aorta (APVAT; brown PVAT) **(B)** from a Dahl salt-sensitive rat. Formalin-fixed paraffin embedded section, stained with picrosirius red.

PVAT is more than just Adipocytes

Adipocytes occupy a significant volume within PVAT and are typically considered the major source of the vasorelaxant or anti-contractile factors secreted from PVAT. But, PVAT is anatomically a far more complex tissue. It is composed of not only adipocytes, but also pre-adipocytes, fibroblasts, mesenchymal stem cells, endothelial cells, immune cells and nerves [Szasz T 2013]. ***Is there a community of immune cells in PVAT that could, by their secretions (cytokines) contribute to the anti-contractile nature of PVAT in health? Chapters 2 and 3 of this thesis work were dedicated to answer just this.***

PVAT is Anatomically and Functionally Different from Other Adipose Tissues

PVAT gets the privilege of being in such close proximity to the blood vessels, that the other non-PVAT adipose tissues lack. By knocking out peroxisome proliferator-activated receptor (PPAR)γ in smooth muscle cells (SM22α-Cre) Chang *et al* created a mouse that was devoid of thoracic aortic PVAT (APVAT) and mesenteric PVAT while the other non-PVAT fats [subscapular (SS) brown adipose tissue fat, gonadal, inguinal, subcutaneous] remained intact [Chang L 2012]. They proved that mice lacking both non-PVAT SS fat and PVAT had a significantly lower intravascular temperature than mice lacking SS fat only. This suggests that PVAT's control over vascular health regulation is superior to other fats. Creation of this PPARγ knockout (KO) mouse also indicated that PVATs and other non-PVAT fats have different origins and developmental properties.

PVAT's secretory profile is different from other adipose tissues. Human coronary artery PVAT expressed lower levels of adipocyte differentiation and maturation markers such as lipoprotein lipase, glycerol phosphate 3 dehydrogenase and perilipin *vs* SAT and VAT [Chatterjee TK 2009]. APVAT expresses lesser levels of adipokines including adiponectin, leptin, resistin and lipid-oxidation genes *vs* subcutaneous (SAT) and visceral (VAT) adipose tissues [Fitzgibbons TP 2011, Chatterjee TK 2009]. Adipocytes isolated and cultured from SAT, VAT and radial artery PVAT demonstrated that the adipocytes from PVAT produced a greater concentration of angiogenic factors and cytokines such as hepatocyte growth factor, vascular endothelial growth factor, macrophage chemoattractant protein (MCP)-1 and thrombospondin 1 *vs* SAT and VAT adipocytes [Rittig K 2012, Fitzgibbons TP 2011, Chatterjee TK 2009]. Thus, findings from other adipose tissues such as gonaldal (epididymal, periovarian), retroperitoneal (RP), perirenal and SS fats may not be directly applicable to PVAT. PVAT deserves to be considered and studied on its own merits.

All PVATs don't Function Alike!

The commonly studied PVATs in rodents are around thoracic aorta (APVAT), superior mesenteric vessels (SMPVAT) and mesenteric resistance vessels (MRPVAT). But one would quickly notice in the literature that MRPVAT and SMPVAT are not well clarified in many studies, where it is simply called 'mesenteric PVAT'. So, why might this be of concern?

Let's start here: Are PVATs different from each other? APVAT is morphologically similar to the brown fat, SS fat, with multilocular (multiple small lipid droplets) adipocytes and expression of uncoupling protein-1 (UCP-1) which imparts the brown color to the fat [Fitzgibbons 2011] **(Figure 1.1A)**. SS fat is important for generating heat *via* non-shivering thermogenesis in newborns. MRPVAT adipocytes resemble that of white adipose tissue (WAT), with unilocular (single large lipid droplet) adipocytes lacking UCP-1 **(Figure 1.1B)**. WAT is well known for storing energy in the form of triglycerides, which can be mobilized *via* lipolysis when needed [Fitzgibbons 2011]. SMPVAT is considered a mix of WAT and SS fat [Staffan Hildebrand 2018]. Thus, APVAT, MRPVAT and SMPVAT are anatomically located along different locations of the vascular tree **(Figure 1.2)**, contain adipocytes that are morphologically different from each other and are involved in different homeostatic functions. APVAT surrounds the aorta, a conduit artery that stiffens in hypertension, while MRPVAT and SMPVAT (part of the visceral fat that positively correlates with obesity) surround the mesenteric resistance vessels and superior mesenteric vessels respectively that contribute to total peripheral resistance. This is why it is absolutely essential to specify clearly which PVAT is being studied and no studies (to our knowledge) directly comparing the different types PVATs have been reported. ***Comparing APVAT and MRPVAT, along with their respective non-PVAT fats at the immune cell level, is one of the goals of Chapter 2 of this work.*** Such a comparison would allow us to understand if PVAT-specific experimental/ pharmacological intervention could be possible in the future.

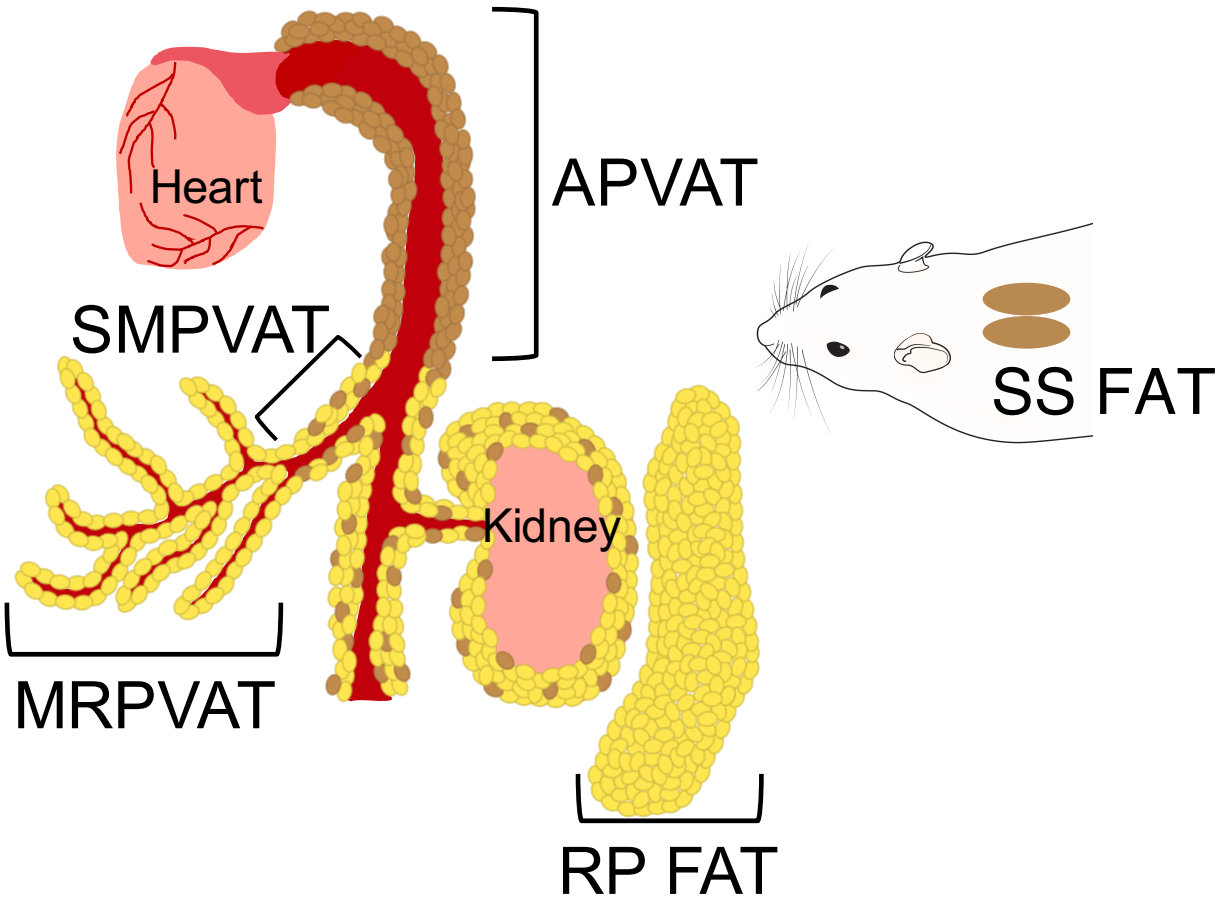


Figure 1.2 Anatomical locations of PVAT and non-PVAT fats investigated
PVATs and non-PVAT fat comparators focused in this work [Modified from Nicholas Brown 2014]. PVAT around thoracic aorta (APVAT; brown PVAT), superior mesenteric vessels (SMPVAT; mix of white and brown PVATs) and mesenteric resistance vessels (MRPVAT, white PVAT); non-PVAT fats: retroperitoneal (RP) white fat, subscapular (SS) brown fat. For aesthetics, white fat has been colored yellow here.

Why Focus on Immune Cells?

Immune aggregates in human adipose tissue were first discovered in 1874 and called 'milky spots' [Ravier 1874]. Through the next century, surgeons realized that cells in omentum (the fat that spreads over stomach, liver and intestines) had powers such as wound healing, ability to defend against peritonitis and to localize inflammation [Platell C 2000]. What are the different immune cell types in adipose tissues? Could different types of adipose tissues contain a different community of immune cells? Why do adipose tissues need immune cells? How do immune cells interact with their neighboring cells within the adipose tissue? These complex questions have only now started to be answered in the last few decades.

The real adipose-immune story began in 1993, when Gokhan Hotamisligil and Bruce Spiegelman discovered that the pro-inflammatory cytokine tumor necrosis-alpha (TNF-α) was associated with obesity [Hotamisligil G 1993]. Then, a decade later, Ferrante *et al* reported that macrophages accumulate in VAT that were the major source of TNF-α in obese mice [Weisberg SP 2003]. This way, the immune cells in adipose tissues began to be recognized in connection with obesity. Since then, many studies have focused on immune cells and cytokines in non-PVAT fats in the context of cardiovascular diseases (CVD) such as obesity, hypertension and atherosclerosis, compared to a relatively fewer studies in PVATs [Nishimura S 2009, Feuerer M 2009, Lynch L 2009, 2012, Weisberg SP 2003]. But, we need to first understand the composition and homeostatic functions of immune cells in adipose tissues, including PVATs, in the context

of health before we turn to pathophysiological conditions. This approach will nurture opportunities to harness the inherent functions of immune cells in specific PVATs for future explorations towards selective pharmacologic interventions to prevent/ reverse immune dysfunction in chronic metabolic diseases.

Immune Cells in PVATs and non-PVAT Fats in Homeostasis

PVAT, like most of the other organs, is likely subject to immune surveillance. Most of the knowledge about the adipose-immune axis comes from studies on WAT (VAT or SAT). VAT contains a dynamic pool of resident immune cells, including CD4 [Winer S 2009], CD8 T cells [Nishimura S 2009], T regulatory (Treg) cells [Feuerer M 2009], natural killer NK cells [Boulenourar S 2017], invariant(i) NKT cells [Lynch L 2009, 2012], $\gamma\delta$ T cells, B cells [Withers SB 2011], macrophages [Weisberg SP 2003, Lumeng CN 2007, Wentworth JM 2010], mast cells [Liu J 2009] and eosinophils [Wu D 2011] in health. In situ carboxyfluorescein succinimidyl ester labeling of specific immune cells indicated that many of these immune cells are tissue (lung) resident with little recirculation between the lung airway and peripheral immune niches [Takamura S 2018, Ely KH 2006]. Similar to the lung, could immune cells also reside in adipose tissues in health? One reason for immune cells to be adipose-resident may be that a rapid response can be generated to diverse antigen types including but not limited to endogenous lipid antigens in adipose tissue, and microbial and metabolite-derived antigens from the gut. Resident innate lymphoid cells (ILC2) in VAT are alert and responsive to parasite infections [Molofsky AB 2013, Everaere I 2018]. Memory T cells in WAT promote rapid effector memory

responses to parasitic infections [Han SJ 2017]. However, lineage tracing studies are needed to understand if such a 'resident' niche of immune cells exists in PVATs.

Adipose homeostasis is a dynamic and frequent process requiring changes in lipid handling including lipid storage, lipid oxidation and lipolysis adapting to daily challenges such as feeding, fasting and cold exposure. Macrophages in lean AT (ATMs) are usually M2-like anti-inflammatory cells expressing CD301, CD206, arginase 1, IL-10 and TGF-β [Russo L 2018]. ATMs are typically resident cells that perform housekeeping functions such as replenishing injured and apoptotic cells, scavenging debris, promoting insulin sensitivity, regulating angiogenesis and remodeling the extracellular matrix [Russo L 2018, Fitzgibbons TP 2016, Caspar-Bauguil S 2015, DY Oh 2014]. ILC2 activated by IL-33 produce methionine enkephalin and drive thermogenesis [JR Brestoff 2015]. $\gamma\delta$T cells in adipose tissue are the major producers of IL-17A after cold exposure and IL-17 regulates synthesis of IL-33, a cytokine important for thermogenesis [Villarroya F 2018, Mahlakoiv T 2019]. IL-33 in turn also directs Treg expansion and improves glucose handing [Cipolletta D 2012]. CD22+CD19+CD45R+ regulatory B cells exist in murine and human SAT and they constitutively produce IL-10 [Nishimura S 2013]. These cells, similar to Tregs, are important regulators of glucose tolerance and insulin sensitivity in lean adipose tissue. Certain immune cells within lean adipose tissue contribute to local homeostasis by modulating/ regulating other immune subtypes in adipose tissue. For example, ILC1 and NK cells are inversely associated with macrophage survival in lean adipose tissue in mice [Boulenouar S 2017]. On the other hand, ILC2 activation by either

IL-33 or IL-25 triggers accumulation of eosinophils and ATMs in mice [Kane H 2019]. Immune cells can also interact with other immune cells *via* co-stimulatory molecules instead of secretions/cytokines. For instance, ILC2s express ICOSL (inducible T cell costimulatory ligand) and OX40L that interact with ICOS+/OX40+ T regs and Th2 cells, thus granting them license to expand WAT-resident iNKT cells in mice that are important producers of IL-10 in health [Molofsky AB 2015, Halim TYF 2018]. Thus, immune cells in adipose tissues are important for maintaining this local homeostasis and insulin sensitivity, thus ultimately contributing to systemic metabolic homeostasis.

PVAT has been left behind in terms of understanding whether a diverse community of innate and adaptive immune cells co-exist in PVATs in health. And if so, how different are they from other non-PVAT fats? This is exactly the new knowledge we add through Chapter 2 of this thesis work to the field.

Role of Immune Cells and Cytokines in Vascular Tone Regulation in Health

Only two studies have investigated the vascular tone regulation by immune cells in PVATs. First, the anti-contractile function of MRPVAT was intact in small mesenteric arteries from healthy CD11b-DTR (macrophage-deficient) mice [Withers SB 2011]. But when the arteries with fat from wildtype and CD11b-DTR mice were exposed to aldosterone (that triggers inflammation), the anti-contractile nature of PVAT was lost only in the wildtype mice. This suggested that macrophages activated in an inflammation setting were responsible for the loss of anti-contractile effect of PVAT [Withers SB 2011].

However, the second study using delta ΔdblGATA-1 mice (eosinophil deficient) demonstrated reduced anti-contractile function of MRPVAT in small mesenteric resistance arteries that was restored by intravenous injection of purified eosinophils *vs* WT. In the same study, eosinophils, when directly added to the norepinephrine-constricted mesenteric arteries, caused a rapid (within 30 seconds) direct vasorelaxation. This relaxation was greater in the presence of PVAT, was concentration-dependent (100 to 3000 cells) and mediated *via* mediators such as NO and adiponectin rather than direct cell to cell contact [Withers SB 2017]. *These studies were important in kindling our interests towards probing into PVAT-derived immune cells and their primary secretions (cytokines) as important contributors to the anti-contractile nature of PVAT in health.*

The vascular effects of cytokines have been evaluated in the context of sepsis, inflammation, endothelial dysfunction and other vascular disorders [Elisabet Vila 2005]. One reason may be that inflammation and thrombosis (the initial response to an insult in any CVD; Nagareddy P 2013) are inevitably associated with a 'cytokine storm' that ultimately leads to vascular dysfunction. Hence, most of the vascular studies have been performed using pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6.

<u>Short-term exposure (*in vitro*: 2 minutes to 1 hour; *in vivo*: 20 minutes to 2 hours):</u> *The following studies have been done in the absence of PVAT around the blood vessels.* Lower concentrations of TNF-α (0.01 to 100 ng/ mL) did not demonstrate/ promote any vascular effect (vasoconstriction, vasorelaxation, internal diameter changes) in first order

cremaster arterioles of rat [TM Glembot 1996], rat aorta [Vicaut E 1985] and human resistance arteries [Pickkers P 2002]. However, higher concentrations of TNF-α (1.25 to 10 μg/mL) caused constriction of bronchial artery of sheep [Wagner EM 2000], promoted norepinephrine (NE)-induced contraction of human internal mammary artery, femoral artery and abdominal artery [Iversen PO 1999] and inhibited endothelium-dependent relaxation to acetylcholine in bovine intralobular pulmonary arteries [Greenberg S 1993]. IL-1 (20- 60 ng/ mL) promoted Ang II-induced vasoconstriction in the rat aorta [Vicaut E 1985], while no change was observed in PE-induced contraction in the rat first order cremaster arterioles [Minghini A 1998]. IL-6 (0.8-10 mM) promoted NE-induced contraction of human internal mammary artery, femoral artery and abdominal artery [Iversen PO 1999]. However, another study reported no changes occurring with IL-6 (500-1000 units/ mL) with regards to PE-induced contraction or ACh-induced vasorelaxation in rat first order cremaster arterioles [Minghini A 1998]. A lower concentration of IL-10 (20 ng/mL) did not alter the internal diameter of rat first order cremaster arterioles [Tan BB 2001]. However, very high concentrations (0.02-0.1 mM) of IL-10 caused norepinephrine-induced contraction in human internal mammary artery, femoral artery and abdominal artery [Iverson PO 1999]. Those cytokines (TNF-α and IL-6) that exerted rapid vasoconstrictive effects with short-term exposure, vasoconstriction was mediated by endothelin-1 or cyclo-oxygenase COX-2 [Vila E 2005]. One recent study reported the ability of recombinant IL-33 (3 ng/ mL) to cause an immediate rapidly decaying relaxation of mouse mesenteric resistance arteries (without PVAT), mediated by NO [Saxton SN 2020]. These taken together suggest that the vascular effect of a cytokine is based on

species, anatomical location of the vascular bed, the exposure time, concentration of the cytokine and the presence of co-stimulatory molecules. Hence moving forward, it is critical to evaluate the direct vascular effects of a cytokine taking all this into consideration. *This is exactly how* ***Chapter 3*** *was born.*

Long-term exposure (*in vitro*: 5 hours to 48 hours; *in vivo*: 5 hours to 3 days): Studies have included human temporal artery, isolated rat mesenteric vascular bed and rat middle cerebral arteries for long-term cytokine exposures. In all these studies, the cytokines used (TNF-α, IL-1β and IL-6) have caused (*in vivo* administration) or enhanced (*ex vivo* exposure) agonist-induced vasoconstriction that appear to be COX-2 mediated. Regardless, all cytokine-mediated vascular effects seem to be endothelium-dependent [Vila E 2005]. And, a point to note is that ALL these *in vitro* vascular studies have been done in the absence of PVAT. As we will learn in Chapter 2, PVATs contain a plethora of immune cell subtypes in healthy subjects. These immune cells are the major producers of the vasoactive cytokines we have discussed above. So, have we missed something in these studies by cleaning PVAT off of blood vessels? ***What cytokines could PVAT make in health? Could PVAT-derived endogenous cytokines cause vasorelaxation or contribute to the anti-contractile function of PVAT that would reduce blood pressure in healthy subjects?*** These possibilities remain unexplored yet. ***Chapter 3 of this thesis is aimed at bridging this knowledge gap.***

Obesity Meets Hypertension: The Crisis

With the newly adopted guidelines, more than 45% of adults have hypertension (systolic pressure >130 mmHg or a diastolic pressure > 80 mmHg), a significant portion of which is uncontrolled [Whelton PK 2018, Clement DL 2017]. Up to three-quarters of those with hypertension are obese (body mass index ≥ 30 kg/ m^2) and obese patients are 3.5 times more likely to develop hypertension [Saxton SN 2019]. Most of the deaths linked with obesity associated hypertension are caused by hypertension [Clement DL 2017, Hall JE 2015, Nguyen T 2012]. Hypertension is definitely one of the most preventable causes of death. Although non-compliance to medication owing to undesirable side effects and genetic predisposition are important players in determining whether hypertension is controllable or not, the market space to accommodate new novel therapeutic targets is not saturated yet.

PVAT may be one of the factors that links adiposity to hypertension. PVAT mass throughout the vascular tree increases in obesity in both the rodent and the human (Greenstein AS 2009, Marchesi C 2009, Ketonen J 2010, Lehman SJ 2010). Given that PVAT is anti-contractile in health, one would expect that its anti-contractile effect would be amplified with more PVAT mass in obesity. However, multiple independent investigators have reported increased immune cell infiltration in adipose tissues including PVATs and a loss of PVAT's anti-contractile nature with obesity and hypertension in humans and rodents [Zaborska KE 2017, Costa RM 2018, Fernandez-Alfonso MS 2017, Fernandez-Alfonso MS 2013, Schinzari F 2017, Szasz T 2013, Withers SB 2014, Xia N

2016, Xia N 2017]. Vasculature is undoubtedly an important player in blood pressure regulation. Arterial blood pressure is regulated by total peripheral resistance and cardiac output. And, the peripheral resistance is primarily determined by small mesenteric resistance vessels and arterioles [Christensen KL 2001]. Hence, we focused on mouse superior mesenteric (~300-350 μm) and rat third order small mesenteric resistance arteries (~150-200 μm) and the PVAT around these vessels for the vascular function studies in **Chapter 3**.

Obesity and Hypertension: Wars Led by Immune Cells

Systemic inflammation is a common underlying factor in both obesity and hypertension. Treatment with immunosuppressive agents such as the lymphocyte inhibitor mycophenolate mofetil or genetic deletion of recombinase activating gene (Rag1; lacks functional T and B cells) attenuated hypertension in male Dahl salt-sensitive (Dahl S) rats [Mattson DL 2006, Mattson DL 2013]. In addition, genetic deletion of CD247, one of the genes required for T cell activation and proliferation, lowered blood pressure in male Dahl S rats [Rudemiller N 2014]. In humans, increased circulating levels of IL-6 and high-sensitivity C reactive protein (markers of inflammation) correlate with obesity [Rexrode KM 2003]. TNF-α mRNA and protein levels in human VAT also correlate with BMI and decrease following dietary weight loss [Kern PA 1995]. Targeted deletion of MCP-1 or its receptor CCR2 in mice adipose tissues offered protection against inflammation and insulin resistance, suggesting a destructive role for macrophages [Kanda H 2006]. On the other hand, Rag null mice on a HFD exhibited greater weight

gain, adipocyte hypertrophy and impaired glucose tolerance *vs* their wildtype, suggesting a protective role for lymphocytes [Winer S 2009].

In both hypertension and obesity, CD8+ T cells [major producers of TNF and interferon (IFN)γ], T_H17 cells (IFNγ, IL-17, IL-22), $\gamma\delta$T cells (TNF-α, IFNγ, CCL5, IL-17), B cells (immunoglobulin IgG), monocytes and macrophages (reactive oxygen species, TNF-α) infiltrate into non-PVAT WAT, ultimately exacerbating hypertension or weight gain, glucose intolerance and insulin resistance, thus worsening systemic metabolomic parameters in obesity in experimental male rodents [Drummond GR 2019, McLaughlin T 2017]. Treg (major producers of IL-10), iNKT (IL-10) and myeloid-derived suppressor (IL-33) cells help prevent hypertension development, kidney damage and help preserve insulin sensitivity [Drummond GR 2019, McLaughlin T 2017].

T cells (Th17, CD4, CD8) have been the most studied cell type in APVAT in association with AngII-induced hypertension [Guzik TJ 2007, Mikolajczyk TP 2016, Itani HA 2017]. Other immune cell types such as B cells, NK cells, macrophages and dendritic cells have been only associated with inflammation in PVATs with hypertension in mice [Nosalski R 2017]. Virtually every immune cell type (proinflammatory macrophages being the most studied) in WAT has been implicated in rodent models of diet-induced obesity [McLaughlin T 2017]. As detailed so far, there are multiple studies investigating the immune system primarily in non-PVAT adipose tissues in the experimental models of obesity and hypertension separately. ***Hence as a field, we need to investigate the***

composition and activation status of the immune community of PVATs in the context of adiposity-associated hypertension. Discovering this is one of the goals of Chapter 4.

Rationale for the Use of the Dahl S HFD-Induced Hypertension Model

Most rodent models do not exhibit robust reproducible hypertension with a HFD [Fernandez R 2018, Nizar JM 2016, Mark AL 1999]. The Dahl S rat, as the name suggests, is sensitive to a high (4%) salt intake in developing hypertension [Taylor LE 2016]. Like most other rodent models of experimental hypertension, females develop lesser magnitude of hypertension and end organ (kidney) damage *vs* males in this Dahl S-high salt model. In our experiments, the Dahl S rats were fed a HFD (60% kCal from fat and normal salt; specific details in **Chapter 3**) or control diet (10% kCal), from weaning for 24 weeks. We call this a model of adiposity-induced hypertension as criteria to define rodent obesity does not exist, unlike the human. Both males and females of Dahl S rats developed increased visceral adiposity along with hypertension [Fernandes R 2018]. However, it is important to note that the body weights of HFD-fed rats were not statistically greater than their controls, thus achieving a metabolically unhealthy lean condition. ***Thus, this served as a novel reliable model to question whether the immune cell changes at the PVAT level preceded the development of hypertension and was different in females vs males, given that both the sexes reached the same magnitude of high blood pressure within the same time course (Chapter 3)***.

Do Males and Females Use Different Weapons to Fight Obesity and Hypertension?

Greater increase in TNF-α (mRNA) in the liver was reported in high fat diet (HFD)-fed male *vs* female mice [Ganz M 2014]. Another study reported greater circulating IL-6 in HFD-fed male *vs* female mice. Additionally, adipose-specific deletion of IL-6 resulted in attenuated weight gain only in the females [Navia B 2014]. Premenopausal women are generally protected from hypertension and CVDs *vs* age-matched men. However, this protection is lost with the onset of menopause [Taylor LE 2016]. Male Dahl S rats had a higher increase in blood pressure *vs* females in response to 4% salt intake. In the same study, ovariectomy (OVX) resulted in equal magnitude of blood pressure raise in both the sexes [Brinson KN 2014]. OVX also worsened HFD-induced increase in blood pressure in the female mice, which could be attenuated with 17-β estradiol supplementation [Gupte M 2012, Wang Y 2015]. Multiple such studies have revealed sex-differences in PVATs in hypertension (in association with adipokines) and obesity separately (in association with immune cells). For example, MRPVAT from male SHRSP rats had greater leptin expression *vs* respective males. Additionally, exogenous incubation of MRA+PVAT from female SHRSP with resistin (40 ng/ mL for 4 hours) impaired K_{ATP} mediated vasorelaxation [Small HY 2019]. Decreased number of crown-like structures were observed in non-PVAT WAT from HFD-fed female *vs* male mice [Nickelson KJ 2012]. *Studies evaluating sex-differences in the immune axis in PVATs with obesity, hypertension and especially in adiposity-induced hypertension are limited.*

Immunological PVAT Dysfunction *vs* Hypertension Development

This is the chicken or egg paradox. Much of our knowledge about time course of immune cell changes in PVATs comes from studies in atherosclerosis in mice (a CVD). Macrophage and T cell recruitment into APVAT preceded the development of endothelial dysfunction and oxidative stress [Durpes MC 2015, Skiba DS 2017]. Macrophage and B cell content further elevated in the later stages of atherosclerosis [Galkina E 2006, Moos MPW 2005]. Proinflammatory molecules such as IL-8, RANTES and MCP-1 were also increased with progression of atherosclerosis [Henrichot E 2005]. But we do not know whether the immune changes in PVATs precede adiposity-induced hypertension, another CVD. This is critical to know because we can then explore possibilities to block/prevent the immunological dysfunction of PVAT, such that the development of hypertension can be prevented. ***Hence, a rigorous time-course study to understand the dynamic changes in the immune compartment of PVATs was performed as detailed in Chapter 4***.

Overall Hypotheses

T cells and macrophages in mesenteric PVAT promote release of IL-10 that causes vasorelaxation in health; immunological PVAT dysfunction precedes adiposity-induced hypertension (alike in males and females).

Specific Aims Overview

Aim 1: To test that PVAT has an active immune population in health **(Chapter 2)**.

Aim 2: To test that PVAT-derived interleukin (IL)-10 causes vasorelaxation, contributing to the anti-contractile function of PVAT in health **(Chapter 3)**.

Aim 3: To test that immune cell infiltration in PVATs precedes the development of adiposity-induced hypertension (similar in males and females) **(Chapter 4)**

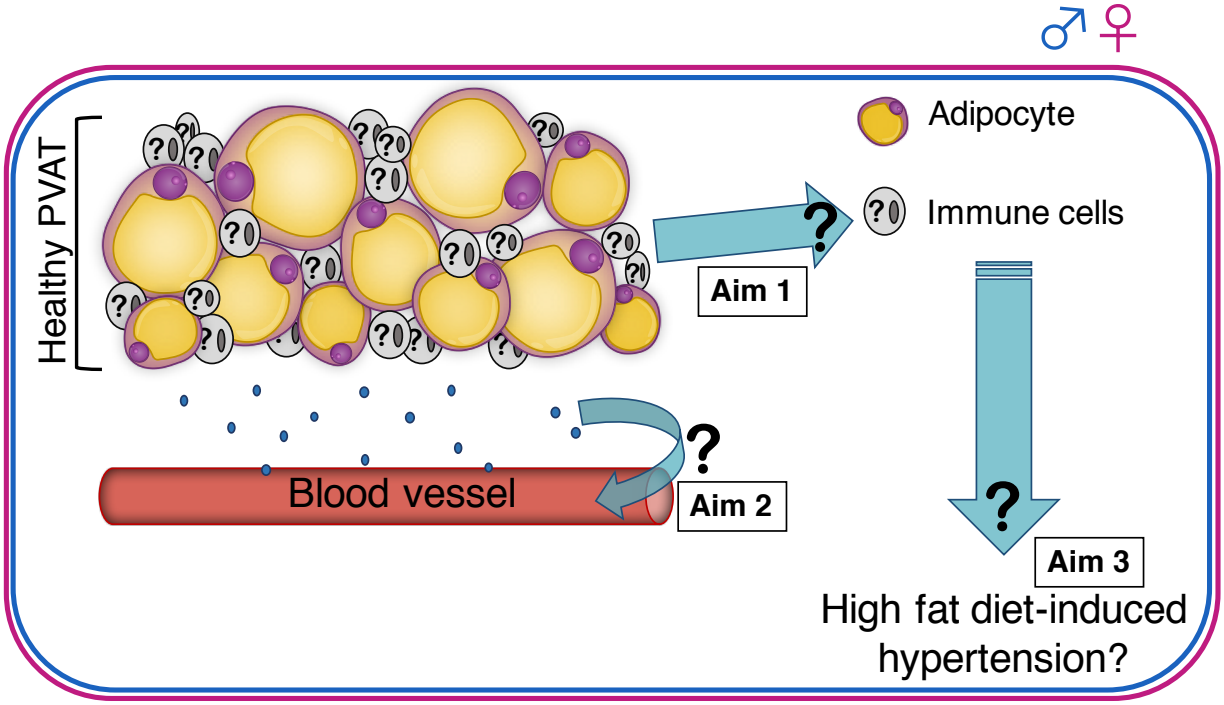


Figure 1.3 Graphical representation of overall hypotheses

REFERENCES

REFERENCES

Bełtowski J. (2013). Endogenous hydrogen sulfide in perivascular adipose tissue: role in the regulation of vascular tone in physiology and pathology. *Can. J. Physiol. Pharmacol.* 91, 889–898. doi:10.1139/cjpp-2013-0001.

Boulenouar S, Michelet X, Duquette D, Alvarez D, Hogan AE, Dold C, et. al. (2017). Adipose Type One Innate Lymphoid Cells Regulate Macrophage Homeostasis through Targeted Cytotoxicity. *Immunity* 46, 273–286. doi:10.1016/j.immuni.2017.01.008.

Brestoff JR and Artis D. (2015). Immune regulation of metabolic homeostasis in health and disease. *Cell* 161, 146–160. doi:10.1016/j.cell.2015.02.022.

Brinson KN, Rafikova O and Sullivan JC. (2014). Female sex hormones protect against salt-sensitive hypertension but not essential hypertension. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R149-57. doi:10.1152/ajpregu.00061.2014.

Brown, N. K., Zhou, Z., Zhang, J., Zeng, R., Wu, J., Eitzman, D. T., Chen, Y. E., and Chang, L. (2014). Perivascular adipose tissue in vascular function and disease: a review of current research and animal models. *Arterioscler. Thromb. Vasc. Biol.* 34, 1621–1630. doi:10.1161/ATVBAHA.114.303029.

Caspar-Bauguil S, Kolditz C-I, Lefort C, Vila I, Mouisel E, Beuzelin D, et. al. (2015). Fatty acids from fat cell lipolysis do not activate an inflammatory response but are stored as triacylglycerols in adipose tissue macrophages. *Diabetologia* 58, 2627–2636. doi:10.1007/s00125-015-3719-0.

Chang L, Villacorta L, Li R, Hamblin M, Xu W, Dou C, Zhang J, Wu J, Zeng R and Chen YE. (2012). Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-γ deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. *Circulation* 126, 1067–1078. doi:10.1161/CIRCULATIONAHA.112.104489.

Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G, et. al. (2009). Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. *Circ. Res.* 104, 541–549. doi:10.1161/CIRCRESAHA.108.182998.

Christensen KL and Mulvany MJ. (2001). Location of resistance arteries. *J. Vasc. Res.* 38, 1–12. doi:10.1159/000051024.

Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, Benoist, C and Mathis D. (2012). PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* 486, 549–553. doi:10.1038/nature11132.
Clement, D. L. (2017). Poor blood pressure control: what can we do? *J. Hypertens.* 35, 1368–1370. doi:10.1097/HJH.0000000000001347.

Costa RM, Neves KB, Tostes RC and Lobato NS. (2018). Perivascular adipose tissue as a relevant fat depot for cardiovascular risk in obesity. *Front. Physiol.* 9, 253. doi:10.3389/fphys.2018.00253.

Drummond, GR, Vinh A, Guzik TJ and Sobey CG. (2019). Immune mechanisms of hypertension. *Nat. Rev. Immunol.* 19, 517–532. doi:10.1038/s41577-019-0160-5.

Dubrovska G, Verlohren S, Luft FC and Gollasch M. (2004). Mechanisms of ADRF release from rat aortic adventitial adipose tissue. *Am. J. Physiol. Heart Circ. Physiol.* 286, H1107-13. doi:10.1152/ajpheart.00656.2003.

Durpès M-C, Morin C, Paquin-Veillet J, Beland R, Paré M, Guimond M-O, Rekhter M, King GL and Geraldes P. (2015). PKC-β activation inhibits IL-18-binding protein causing endothelial dysfunction and diabetic atherosclerosis. *Cardiovasc. Res.* 106, 303–313. doi:10.1093/cvr/cvv107.

Ely KH, Cookenham T, Roberts AD and Woodland DL. (2006). Memory T cell populations in the lung airways are maintained by continual recruitment. *J. Immunol.* 176, 537–543. doi:10.4049/jimmunol.176.1.537.

Everaere L, Ait Yahia S, Bouté M, Audousset C, Chenivesse C and Tsicopoulos A. (2018). Innate lymphoid cells at the interface between obesity and asthma. *Immunology* 153, 21–30. doi:10.1111/imm.12832.

Fernandes R, Garver H, Harkema JR, Galligan JJ, Fink GD and Xu H. (2018). Sex Differences in Renal Inflammation and Injury in High-Fat Diet-Fed Dahl Salt-Sensitive Rats. *Hypertension* 72, e43–e52. doi:10.1161/HYPERTENSIONAHA.118.11485.

Fernández-Alfonso MS, Gil-Ortega M, García-Prieto CF, Aranguez I, Ruiz-Gayo M and Somoza B. (2013). Mechanisms of perivascular adipose tissue dysfunction in obesity. *Int. J. Endocrinol.* 2013, 402053. doi:10.1155/2013/402053.

Fernández-Alfonso MS, Somoza B, Tsvetkov D, Kuczmanski A, Dashwood M and Gil-Ortega M. (2017). Role of perivascular adipose tissue in health and disease. *Compr. Physiol.* 8, 23–59. doi:10.1002/cphy.c170004.

Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft FC and Gollasch M. (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc. Res.* 75, 719–727. doi:10.1016/j.cardiores.2007.05.025.

Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et. al. (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 15, 930–939. doi:10.1038/nm.2002.
Fitzgibbons TP and Czech MP. (2016). Emerging evidence for beneficial macrophage functions in atherosclerosis and obesity-induced insulin resistance. *J. Mol. Med.* 94, 267–275. doi:10.1007/s00109-016-1385-4.

Fitzgibbons TP, KoganS, Aouadi M, Hendricks GM, Straubhaar J and Czech MP. (2011). Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1425-37. doi:10.1152/ajpheart.00376.2011.

Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ and Ley K. (2006). Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J. Exp. Med.* 203, 1273–1282. doi:10.1084/jem.20052205.

Ganz M, Csak T and Szabo G. (2014). High fat diet feeding results in gender specific steatohepatitis and inflammasome activation. *World J. Gastroenterol.* 20, 8525–8534. doi:10.3748/wjg.v20.i26.8525.

Gao YJ, Lu C, Su LY, Sharma AM and Lee RMKW. (2007). Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br. J. Pharmacol.* 151, 323–331. doi:10.1038/sj.bjp.0707228.

Glembot TM, Britt LD and Hill MA. (1996). Endotoxin interacts with tumor necrosis factor-alpha to induce vasodilation of isolated rat skeletal muscle arterioles. *Shock* 5, 251–257. doi:10.1097/00024382-199604000-00004.

Gollasch M. (2012). Vasodilator signals from perivascular adipose tissue. *Br. J. Pharmacol.* 165, 633–642. doi:10.1111/j.1476-5381.2011.01430.x.

Greenberg S, Xie J, Wang Y, Cai B, Kolls J, Nelson S, Hyman A, Summer WR and Lippton H. (1993). Tumor necrosis factor-alpha inhibits endothelium-dependent relaxation. *J. Appl. Physiol.* 74, 2394–2403. doi:10.1152/jappl.1993.74.5.2394.

Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, et. al. (2009). Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* 119, 1661–1670. doi:10.1161/CIRCULATIONAHA.108.821181.

Gupte M, Thatcher SE, Boustany-Kari CM, Shoemaker R, Yiannikouris F, Zhang X, Karounos M and Cassis LA. (2012). Angiotensin converting enzyme 2 contributes to sex differences in the development of obesity hypertension in C57BL/6 mice. *Arterioscler. Thromb. Vasc. Biol.* 32, 1392–1399. doi:10.1161/ATVBAHA.112.248559.

Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C and Harrison DG. (2007). Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J. Exp. Med.* 204, 2449–2460. doi:10.1084/jem.20070657.

Halim TYF, Rana BMJ, Walker JA, Kerscher B, Knolle MD, Jolin HE, et. al. (2018). Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells. *Immunity* 48, 1195-1207.e6. doi:10.1016/j.immuni.2018.05.003.

Hall JE, do Carmo JM, da Silva AA, Wang Z and Hall ME. (2015). Obesity-induced hypertension: interaction of neurohumoral and renal mechanisms. *Circ. Res.* 116, 991–1006. doi:10.1161/CIRCRESAHA.116.305697.

Han S-J, Glatman Zaretsky A, Andrade-Oliveira V, Collins N, Dzutsev A, Shaik J, et. al. (2017). White adipose tissue is a reservoir for memory T cells and promotes protective memory responses to infection. *Immunity* 47, 1154-1168.e6. doi:10.1016/j.immuni.2017.11.009.

Henrichot E, Juge-Aubry CE, Pernin A, Pache J-C, Velebit V, Dayer J-M, Meda P, Chizzolini C and Meier CA. (2005). Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler. Thromb. Vasc. Biol.* 25, 2594–2599. doi:10.1161/01.ATV.0000188508.40052.35.

Hildebrand S, Stümer J and Pfeifer A. (2018). PVAT and its relation to brown, beige, and white adipose tissue in development and function. *Front. Physiol.* 9, 70. doi:10.3389/fphys.2018.00070.

Hotamisligil GS, Shargill NS and Spiegelman BM. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259, 87–91. doi:10.1126/science.7678183.

Itani HA, McMaster WG, Saleh MA, Nazarewicz RR, Mikolajczyk TP, Kaszuba AM, et. al. (2016). Activation of Human T Cells in Hypertension: Studies of Humanized Mice and Hypertensive Humans. *Hypertension* 68, 123–132. doi:10.1161/HYPERTENSIONAHA.116.07237.

Iversen PO, Nicolaysen A, Kvernebo K, Benestad HB and Nicolaysen G. (1999). Human cytokines modulate arterial vascular tone via endothelial receptors. *Pflugers Arch.* 439, 93–100. doi:10.1007/s004249900149.

Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et. al. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* 116, 1494–1505. doi:10.1172/JCI26498.

Kane H and Lynch L. (2019). Innate immune control of adipose tissue homeostasis. *Trends Immunol.* 40, 857–872. doi:10.1016/j.it.2019.07.006.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R and Simsolo RB. (1995). The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J. Clin. Invest.* 95, 2111–2119. doi:10.1172/JCI117899.

Ketonen J, Shi J, Martonen E and Mervaala E. (2010). Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. *Circ. J.* 74, 1479–1487.

Lee Y-C, Chang H-H, Chiang C-L, Liu C-H, Yeh J-I, Chen M-F, Chen P-Y, Kuo J-S and Lee TJF. (2011). Role of perivascular adipose tissue-derived methyl palmitate in vascular tone regulation and pathogenesis of hypertension. *Circulation* 124, 1160–1171. doi:10.1161/CIRCULATIONAHA.111.027375.

Lehman SJ, Massaro JM, Schlett CL, O'Donnell CJ, Hoffmann U and Fox CS. (2010). Peri-aortic fat, cardiovascular disease risk factors, and aortic calcification: the Framingham Heart Study. *Atherosclerosis* 210, 656–661. doi:10.1016/j.atherosclerosis.2010.01.007.

Liu J, Divoux A, Sun J, Zhang J, Clément K, Glickman J N, et. al. (2009). Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat. Med.* 15, 940–945. doi:10.1038/nm.1994.

Löhn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M and Sharma AM. (2002). Periadventitial fat releases a vascular relaxing factor. *FASEB J.* 16, 1057–1063. doi:10.1096/fj.02-0024.

Lumeng CN, Bodzin JL and Saltiel AR. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* 117, 175–184. doi:10.1172/JCI29881.

Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, Balk SP, O'Shea D, O'Farrelly C and Exley MA. (2012). Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* 37, 574–587. doi:10.1016/j.immuni.2012.06.016.

Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG and O'Farrelly C. (2009). Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur. J. Immunol.* 39, 1893–1901. doi:10.1002/eji.200939349.

Mahlakõiv T, Flamar AL, Johnston LK, Moriyama S, Putzel GG, Bryce PJ and Artis D. (2019). Stromal cells maintain immune cell homeostasis in adipose tissue via production of interleukin-33. *Sci. Immunol.* 4. doi:10.1126/sciimmunol.aax0416.

Marchesi C, Ebrahimian T, Angulo O, Paradis P and Schiffrin EL. (2009). Endothelial nitric oxide synthase uncoupling and perivascular adipose oxidative stress and inflammation contribute to vascular dysfunction in a rodent model of metabolic syndrome. *Hypertension* 54, 1384–1392. doi:10.1161/HYPERTENSIONAHA.109.138305.

Mark AL, Correia M, Morgan DA, Shaffer RA and Haynes WG. (1999). State-of-the-art-lecture: Obesity-induced hypertension: new concepts from the emerging biology of obesity. *Hypertension* 33, 537–541. doi:10.1161/01.hyp.33.1.537.

Mattson DL, James L, Berdan EA and Meister CJ. (2006). Immune suppression attenuates hypertension and renal disease in the Dahl salt-sensitive rat. *Hypertension* 48, 149–156. doi:10.1161/01.HYP.0000228320.23697.29.

Mattson DL, Lund H, Guo C, Rudemiller N, Geurts AM and Jacob H. (2013). Genetic mutation of recombination activating gene 1 in Dahl salt-sensitive rats attenuates hypertension and renal damage. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304, R407-14. doi:10.1152/ajpregu.00304.2012.

McLaughlin T, Ackerman SE, Shen L and Engleman E. (2017). Role of innate and adaptive immunity in obesity-associated metabolic disease. *J. Clin. Invest.* 127, 5–13. doi:10.1172/JCI88876.

Minghini A, Britt LD and Hill MA. (1998). Interleukin-1 and interleukin-6 mediated skeletal muscle arteriolar vasodilation: in vitro versus in vivo studies. *Shock* 9, 210–215. doi:10.1097/00024382-199803000-00009.

Molofsky AB, Nussbaum JC, Liang H-E, Van Dyken SJ, Cheng LE, Mohapatra A, Chawla A and Locksley RM. (2013). Innate lymphoid type 2 cells sustain visceral

adipose tissue eosinophils and alternatively activated macrophages. *J. Exp. Med.* 210, 535–549. doi:10.1084/jem.20121964.

Molofsky AB, Savage AK and Locksley RM. (2015). Interleukin-33 in Tissue Homeostasis, Injury, and Inflammation. *Immunity* 42, 1005–1019. doi:10.1016/j.immuni.2015.06.006.

Moos MPW, John N, Gräbner R, Nossmann S, Günther B, Vollandt R, Funk CD, Kaiser B and Habenicht AJR. (2005). The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 25, 2386–2391. doi:10.1161/01.ATV.0000187470.31662.fe.

Nagareddy P and Smyth SS. (2013). Inflammation and thrombosis in cardiovascular disease. *Current opinion in hematology*, *20*(5), 457–463. doi.org/10.1097/MOH.0b013e328364219d.

Navia B, Ferrer B, Giralt M, Comes G, Carrasco J, Molinero A, et. al. (2014). Interleukin-6 deletion in mice driven by aP2-Cre-ERT2 prevents against high-fat diet-induced gain weight and adiposity in female mice. *Acta Physiol (Oxf)* 211, 585–596. doi:10.1111/apha.12328.

Nguyen T and Lau DCW. (2012). The obesity epidemic and its impact on hypertension. *Can. J. Cardiol.* 28, 326–333. doi:10.1016/j.cjca.2012.01.001.

Nickelson KJ, Stromsdorfer KL, Pickering RT, Liu T-W, Ortinau LC, Keating AF and Perfield JW. (2012). A comparison of inflammatory and oxidative stress markers in adipose tissue from weight-matched obese male and female mice. *Exp. Diabetes Res.* 2012, 859395. doi:10.1155/2012/859395.

Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et. al. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 15, 914–920. doi:10.1038/nm.1964.

Nishimura S, Manabe I, Takaki S, Nagasaki M, Otsu M, Yamashita H, et. al. (2013). Adipose natural regulatory B cells negatively control adipose tissue inflammation. *Cell Metab.* 18, 759–766. doi:10.1016/j.cmet.2013.09.017.

Nizar JM, Dong W, McClellan RB, Labarca M, Zhou Y, Wong J, et. al. (2016). Na+-sensitive elevation in blood pressure is ENaC independent in diet-induced obesity and insulin resistance. *Am. J. Physiol. Renal Physiol.* 310, F812-20. doi:10.1152/ajprenal.00265.2015.

Nosalski R and Guzik TJ. (2017). Perivascular adipose tissue inflammation in vascular disease. *Br. J. Pharmacol.* 174, 3496–3513. doi:10.1111/bph.13705.

Oh DY and Walenta E. (2014). The role of omega-3 fatty acid receptor GPR120 in insulin resistance. *Int. J. Obes. Suppl.* 4, S14-6. doi:10.1038/ijosup.2014.5.

Pickkers P, Netea MG, van der Meer JWM and Smits P. (2002). TNFalpha and IL-1beta exert no direct vasoactivity in human isolated resistance arteries. *Cytokine* 20, 244–246. doi:10.1006/cyto.2002.2004.

Platell C, Cooper D, Papadimitriou JM and Hall JC. (2000). The omentum. *World J. Gastroenterol.* 6, 169–176.

Rexrode KM, Pradhan A, Manson JE, Buring JE and Ridker PM. (2003). Relationship of total and abdominal adiposity with CRP and IL-6 in women. *Ann. Epidemiol.* 13, 674–682. doi:10.1016/s1047-2797(03)00053-x.

Rittig K, Dolderer JH, Balletshofer B, Machann J, Schick, F, Meile T, et. al. (2012). The secretion pattern of perivascular fat cells is different from that of subcutaneous and visceral fat cells. *Diabetologia* 55, 1514–1525. doi:10.1007/s00125-012-2481-9.

Rudemiller N, Lund H, Jacob HJ, Geurts AM and Mattson DL. (2014). PhysGen Knockout Program. CD247 modulates blood pressure by altering T-lymphocyte infiltration in the kidney. *Hypertension* 63, 559–564. doi:10.1161/HYPERTENSIONAHA.113.02191.

Russo L and Lumeng CN. (2018). Properties and functions of adipose tissue macrophages in obesity. *Immunology* 155, 407–417. doi:10.1111/imm.13002.

Saxton SN, Clark BJ, Withers SB, Eringa EC and Heagerty AM. (2019). Mechanistic links between obesity, diabetes, and blood pressure: role of perivascular adipose tissue. *Physiol. Rev.* 99, 1701–1763. doi:10.1152/physrev.00034.2018.

Saxton SN, Whitley AS, Potter RJ, Withers SB, Grencis R and Heagerty AM. (2020). Interleukin-33 rescues perivascular adipose tissue anti-contractile function in obesity. *Am J Physiol Heart Circ Physiol.* doi: 10.1152/ajpheart.00491.2020.

Schinzari F, Tesauro M and Cardillo C. (2017). Endothelial and Perivascular Adipose Tissue Abnormalities in Obesity-Related Vascular Dysfunction: Novel Targets for Treatment. *J. Cardiovasc. Pharmacol.* 69, 360–368. doi:10.1097/FJC.0000000000000469.

Skiba DS, Nosalski R, Mikolajczyk TP, Siedlinski M, Rios FJ, Montezano AC, et. al. (2017). Anti-atherosclerotic effect of the angiotensin 1-7 mimetic AVE0991 is mediated by inhibition of perivascular and plaque inflammation in early atherosclerosis. *Br. J. Pharmacol.* 174, 4055–4069. doi:10.1111/bph.13685.

Small HY, McNeilly S, Mary S, Sheikh AM and Delles C. (2019). Resistin Mediates Sex-Dependent Effects of Perivascular Adipose Tissue on Vascular Function in the Shrsp. *Sci. Rep.* 9, 6897. doi:10.1038/s41598-019-43326-z.

Soltis EE and Cassis LA. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin. Exp. Hypertens. A* 13, 277–296. doi:10.3109/10641969109042063.

Szasz T, Bomfim GF and Webb RC. (2013). The influence of perivascular adipose tissue on vascular homeostasis. *Vasc. Health Risk Manag.* 9, 105–116. doi:10.2147/VHRM.S33760.

Takamura S. (2018). Niches for the Long-Term Maintenance of Tissue-Resident Memory T Cells. *Front. Immunol.* 9, 1214. doi:10.3389/fimmu.2018.01214.

Tan BB, Prewitt RL and Britt LD. (2001). Interleukin-10 prevents loss of tone of rat skeletal muscle arterioles exposed to endotoxin. *J. Surg. Res.* 100, 110–115. doi:10.1006/jsre.2001.6105.

Taylor LE and Sullivan JC. (2016). Sex differences in obesity-induced hypertension and vascular dysfunction: a protective role for estrogen in adipose tissue inflammation? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 311, R714–R720. doi:10.1152/ajpregu.00202.2016.

Verlohren S, Dubrovska G, Tsang S-Y, Essin K, Luft FC, Huang Y and Gollasch M. (2004). Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. *Hypertension* 44, 271–276. doi:10.1161/01.HYP.0000140058.28994.ec.

Vicaut E, Baudry N and Hou X. (1994). Nitric oxide-independent response to acetylcholine by terminal arterioles in rat cremaster muscle. *J. Appl. Physiol.* 77, 526–533. doi:10.1152/jappl.1994.77.2.526.

Victorio JA, Fontes MT, Rossoni LV and Davel AP. (2016). Different Anti-Contractile Function and Nitric Oxide Production of Thoracic and Abdominal Perivascular Adipose Tissues. *Front. Physiol.* 7, 295. doi:10.3389/fphys.2016.00295.

Vila, E., and Salaices, M. (2005). Cytokines and vascular reactivity in resistance arteries. *Am. J. Physiol. Heart Circ. Physiol.* 288, H1016-21. doi:10.1152/ajpheart.00779.2004.

Villarroya F, Cereijo R, Villarroya J, Gavaldà-Navarro A and Giralt M. (2018). Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab.* 27, 954–961. doi:10.1016/j.cmet.2018.04.006.

Wagner EM. (2000). TNF-alpha induced bronchial vasoconstriction. *Am. J. Physiol. Heart Circ. Physiol.* 279, H946-51. doi:10.1152/ajpheart.2000.279.3.H946.

Wang Y, Shoemaker R, Thatcher SE, Batifoulier-Yiannikouris F, English VL and Cassis LA. (2015). Administration of 17β-estradiol to ovariectomized obese female mice reverses obesity-hypertension through an ACE2-dependent mechanism. *Am. J. Physiol. Endocrinol. Metab.* 308, E1066-75. doi:10.1152/ajpendo.00030.2015.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112, 1796–1808. doi:10.1172/JCI19246.

Wentworth JM, Naselli G, Brown WA, Doyle L, Phipson B, Smyth GK, Wabitsch M, O'Brien PE and Harrison LC. (2010). Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes* 59, 1648–1656. doi:10.2337/db09-0287.

Whelton PK, Carey RM, Aronow WS, Casey DE, Collins KJ, Dennison Himmelfarb, C, et. al. (2018). 2017 acc/aha/aapa/abc/acpm/ags/apha/ash/aspc/nma/pcna guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: executive summary: A report of the american college of cardiology/american heart association task force on clinical practice guidelines. *Hypertension* 71, 1269–1324. doi:10.1161/HYP.0000000000000066.

Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, et. al. (2009). Normalization of obesity-associated insulin resistance through immunotherapy. *Nat. Med.* 15, 921–929. doi:10.1038/nm.2001.

Withers SB, Agabiti-Rosei C, Livingstone DM, Little MC, Aslam R, Malik RA and Heagerty AM. (2011). Macrophage activation is responsible for loss of anticontractile function in inflamed perivascular fat. *Arterioscler. Thromb. Vasc. Biol.* 31, 908–913. doi:10.1161/ATVBAHA.110.221705.

Withers SB, Forman R, Meza-Perez S, Sorobetea D, Sitnik K, Hopwood T, et. al. (2017). Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. *Sci. Rep.* 7, 44571. doi:10.1038/srep44571.

Wu D, Molofsky AB, Liang H-E, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, Chawla A and Locksley RM. (2011). Eosinophils sustain adipose alternatively activated

macrophages associated with glucose homeostasis. *Science* 332, 243–247. doi:10.1126/science.1201475.

Xia N, Horke S, Habermeier A, Closs EI, Reifenberg G, Gericke A, et. al. (2016). Uncoupling of Endothelial Nitric Oxide Synthase in Perivascular Adipose Tissue of Diet-Induced Obese Mice. *Arterioscler. Thromb. Vasc. Biol.* 36, 78–85. doi:10.1161/ATVBAHA.115.306263.

Xia N and Li H. (2017). The role of perivascular adipose tissue in obesity-induced vascular dysfunction. *Br. J. Pharmacol.* 174, 3425–3442. doi:10.1111/bph.13650.

Zaborska, K. E., Wareing, M., Edwards, G., and Austin, C. (2017). Loss of anti-contractile effect of perivascular adipose tissue in offspring of obese rats. *Int J Obes (Lond)* 41, 997. doi:10.1038/ijo.2017.17.

CHAPTER 2

Naïve, Regulatory, Activated, and Memory Immune Cells Co-exist in PVATs that are Comparable in Density to Non-PVAT Fats in Health

This Chapter was adapted from

Kumar RK, Jin Y, Watts SW, Rockwell CE. (2020). Naïve, regulatory, activated and memory immune cells co-exist in PVATs that are comparable in density to non-PVAT fats in health. *Front Physiol*. 11:58. doi: 10.3389/fphys.2020.00058.

Abstract

Perivascular adipose tissue (PVAT), the fat surrounding peripheral blood vessels, is protective and reduces the contraction of blood vessels in health. PVAT is composed of adipocytes, stromal cells and immune cells. Recent work supports eosinophils as one of the cell types key to the anti-contractile nature of PVAT in health. Hence, we hypothesized that there exists a basally activated immune cell community in healthy PVAT that is distinctly different from non-PVAT fats. PVATs were from around mesenteric resistance vessels (MRPVAT- white fat) and thoracic aorta (APVAT- brown fat). Non-PVATs included retroperitoneal (RP fat- white fat) and subscapular (SS fat- brown fat) while the spleen was a positive control. Tissues were harvested from adult male and female Sprague Dawley rats. Six primary immune cell types were identified in PVATs. T cells (CD4 and CD8), B cells, natural killer (NK) cells, macrophages, mast cells and neutrophils in the stromal vascular fraction of each fat were identified using 9-color flow cytometry. PVATs contained a higher number of total immune cells *vs* their respective non-PVAT fats in females. Females had a higher number of T cells in MRPVAT *vs* males. Females also had a greater number of T cells and total immune cells in APVAT *vs* males. Further, activation, differentiation and/or polarization of various immune cell types were similarly determined by flow cytometry. PVATs were similar to their respective non-PVAT fats in density of recently activated B cells (B220+ CD25+). However, MRPVAT in females had a higher number of naïve CD4 T cells *vs* MRPVAT in males and APVAT in females. MRPVAT also had denser naïve CD8 T cells *vs* APVAT in females. Overall, this research for the first time has identified a community of discrete populations of immune cells

(naive/recently activated/regulatory/memory) in healthy PVATs. Contrary to our hypothesis, they are more similar than different in density to their respective non-PVAT fats.

Introduction

Perivascular adipose tissue (PVAT) contains heterogeneous cell populations including adipocytes, pre-adipocytes, fibroblasts, immune cells, endothelial cells and nerves [Szaz T 2012, Meijer RI 2011]. Like many other organs, adipose tissues including PVATs are likely subject to immune surveillance. Type-2 immune cells [regulatory T cells (Tregs), invariant natural killer cells (iNKT), M2-like macrophages and eosinophils] and their associated cytokines [interleukin (IL)-4, IL-5, IL-13, IL-10, transforming growth factor-ß] have been identified in healthy non-PVAT white and brown adipose tissues [Chawla A 2011, Wu D, Schipper HS 2012a, Lynch L 2016, Nguyen DCA 2011, Feuerer M 2009]. In health, the immune cells in non-PVAT fats interact with each other and also with other cell types in the adipose tissue, contributing towards maintaining the anti-inflammatory status of the tissue and preserving insulin sensitivity [Schipper HS 2012b]. They also contribute to brown fat activation, thermogenesis, white fat browning and clearing cellular debris in healthy PVATs [Schipper HS 2012b]. B cells secrete immunoglobin M antibodies that promote phagocytosis of apoptotic cells in healthy white adipose tissue (WAT) [Baumgarth N 2011]. Although eosinophils, Tregs and macrophages have been identified in healthy PVATs, only eosinophils in PVATs from healthy mice have been recognized as a key cell type to the anti-contractile nature of PVAT [Withers SB 2017]. Could there be

a community of immune cells in PVAT that are responsible for the anti-contractile nature of PVAT in health? This question can't be answered until we know the relative immune composition and activation status of immune cells in PVATs in steady-state health. *Hence, our current study was focused on discovering the primary immune cell types and their basal activation status in PVATs, relative to the respective non-PVAT fats in both males and females in health.*

PVATs are in close proximity to the blood vessels and can directly influence vascular tone, differing from non-PVAT fats [Gollasch M 2012]. As such, we tested the hypothesis that PVAT contains a basally activated immune cell community in health, distinct from their respective non-PVAT fats. We postulate such an immune cell community to be protective in health. The current study focused on flow cytometric analyses of two PVATs - MRPVAT (white adipose tissue-like PVAT, located around small mesenteric resistance vessels) and APVAT (brown adipose tissue-like PVAT, located around thoracic aorta). Two non-PVAT adipose tissues- retroperitoneal fat (RP fat, non-PVAT white fat, found behind the kidneys) and subscapular fat (SS fat, non-PVAT brown fat, situated at the back of the neck region) were used as their respective non-PVAT fat-type comparators. Use of these four tissues from the same rats helped us answer two questions. First, are MRPVAT and APVAT different in immune composition? Second, are the PVATs different from non-PVATs? The spleen served as a positive control, given that it is a well-characterized secondary lymphoid organ. This also added confidence to the immunophenotyping data obtained in PVATs and other fats. This was especially

important given the scarcity of flow cytometry work with rat adipose tissues, unlike mouse adipose tissues. We discovered a steady-state immune population in PVATs in health, a portion of which are basally activated, differentiated and/or polarized. But in contrast to our hypothesis, the immune subpopulations of PVATs are more similar in density than different to their respective non-PVAT fats.

Materials and Methods

Animals

Animal maintenance and experimental protocols were approved by the Michigan State University Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for Animal Care and Use of Laboratory Animals (2011). Male and female Sprague Dawley rats (350 g males and 250 g females, between 12-14 weeks of age, Charles River, Indianapolis, IN USA; RRID: RGD_10395233) were used. Animals were maintained on a 12/12 light/dark cycle at 22-25 °C. They were fed *ad-libitum* (#8940 irradiated Teklad 22/5 rodent diet). Prior to all dissections, the rats were anesthetized with sodium pentobarbital (60-80 mg/kg, i.p.) and death was assured by creating a bilateral pneumothorax. Tissue dissection/processing proceeded as described below in section 2.3.

Antibodies used

Table 2.1 and **Table 2.2** in the supplementary list the antibodies used for immunophenotyping studies.

Antibody	Fluorochrome	Supplier	Clone	Dilution
Live/dead	Zombie aqua	Biolegend		1:1000
CD45	APC/Cy7	BD Bioscience	OX-1	1:200
CD3	PE	BD Bioscience	1F4	1:100
CD4	BV605	BD Bioscience	OX-35	1:50
CD8a	PerCPeFluor710	eBioscience	OX-8	1:100
B220	PE/Cy7	BD Bioscience	OX-39	1:50
CD68	AF647	Biorad	ED1	1:10
CD161	APC	Biolegend	3.2.3	1:100
HIS48	FITC	BD Bioscience	HIS-48	1:100
FcεRI	FITC	EMD Millipore	γ-subunit	1:25

Table 2.1 Immunophenotyping antibodies for flow cytometry

Antibody	Fluorochrome	Supplier	Clone	Dilution
Baseline				
Live/dead	Zombie aqua	Biolegend		1:1000
CD45	APC/Cy7	BD Bioscience	OX-1	1:200
CD3	FITC	BD Bioscience	1F4	1:100
T cell panel				
CD4	BV605	BD Bioscience	OX-35	1:50
CD8	PerCPeFluor710	eBioscience	OX-8	1:100
CD25	PE	BD Bioscience	OX-39	1:100
CD134	BV711	BD Bioscience	OX-40	1:50
CD45RC	AF680	Santa Cruz	OX-22	1:50
Foxp3	AF647	Biolegend	150D	1:20
B cell panel				
B220	PE/Cy7	eBioscience	HIS24	1:50
CD25	BV605	BD Bioscience	OX-39	1:50
Macrophage panel				
CD68	AF700	Biorad	ED1	1:10
CD86	AF647	Biolegend	24F	1:50
CD163	PE	Thermofisher	ED2	1:50
MHCII	BV711	BD Bioscience	OX-6	1:50

Table 2.2 Antibody panel design for flow cytometry

Immune cell isolation and flow cytometry

Using flow cytometry, innate immune cells (macrophages, neutrophils, mast cells), adaptive immune cells (T cells, B cells) and NK cells were identified and quantified. Live cells were either determined by using propidium iodide staining separately or Zombie aqua stain added to each cell preparation. Consistently, approximately 85-90% viable cells were obtained in every sample preparation. All the tissues were harvested from the same animals. Immune cells are reported as number of cells normalized to tissue weight in milligrams. The flow cytometric data were analyzed using Attune NxT software (v 2.6). An unstained control sample for each tissue-type was used to: (i) adjust forward and side scatter so that the cell populations of interest are on scale; and (ii) adjust the photomultiplier tube gain for each fluorochrome detector so that the peak mean fluorescence intensity of each channel was within 10^3 and 10^4 on a log scale. Spectral overlap was auto-compensated using single color compensation controls using compensation beads (Cat #01-2222-42) and the same compensation values were applied to all the tissues/rats.

Splenocyte isolation and processing

Spleens were mechanically disrupted by a syringe plunger and filtered through a 40 μm filter. The single cell suspension obtained was then washed with Dulbecco's modified eagle medium. Red blood cell lysis was performed by adding ammonium-chloride-potassium lysis buffer and incubating for 2 minutes on ice. The splenocytes were washed twice with PBS containing 1% fetal bovine serum (FACS/ flow buffer) and labeled

with fluorescent antibodies after FcR blocking (CD32, Cat #550271). Viability was assessed with propidium iodide (1:30 in flow buffer, Cat #421301) immediately before analysis. In flow cytometry studies assessing steady state status of immune cells, viability was measured using Zombie-aqua dye (1: 1000 in dPBS, 77143). All flow cytometry assays were performed using Attune NxT acoustic focusing cytometer from Life Technologies.

Stromal vascular fraction (SVF) isolation from adipose tissues

APVAT, MRPVAT, RP fat and SS fat were all dissected from the same rats. All the immune cell populations were quantified from the same fat samples, while another set of experiments were performed to phenotype the activation, differentiation and/or polarization of various immune populations. The fats were removed from the blood vessels where appropriate, blotted dry and weighed. The adipose tissues were minced with scissors, collagenase (1 mg/ ml; type-I, Cat # LS004196) digested at 37 ºC for about 1 hour. The cell suspensions were sequentially filtered through 100 μm and 40 μm filters. The flow through contained cells lesser than 40 μm, so adipocytes were eliminated. Upon washing with flow buffer and centrifugation at 300 rcf for 5 minutes, a cell pellet which is called the SVF, was obtained.

Surface labeling of immune cells

Ammonium-chloride-potassium red blood cell lysis buffer (400 μL; Cat #10-548E) was added to the SVF pellet, gently pipette-mixed and incubated on ice for 2 minutes to

destroy red blood cells. The red blood cell-lysed SVF was washed twice with flow buffer and labeled with fluorescent antibodies (30 minutes incubation) after blocking Fc receptors with purified anti-CD32 antibody (10 minutes).

Intracellular labeling to identify T regulatory cells

After the surface labeling was complete (described above), intracellular labeling was performed to identify cells containing Foxp3 as Tregs, using the Foxp3 transcription factor staining buffer set (Cat #5523). The cells were incubated with a fixation/ permeabilization buffer for 1 hour at room temperature in the dark and centrifuged at 700 rcf for 5 minutes. The supernatants were carefully discarded (pipetted out to minimize loss of cells) and the cells were washed with permeabilization buffer. The supernatants were carefully discarded again after centrifugation at 700 rcf. The intracellular label (Foxp3 for Treg cells) prepared in permeabilization buffer was incubated with the cells at room temperature for 30 minutes in the dark. The cells were then washed twice with permeabilization buffer and resuspended finally in flow buffer for flow cytometry analysis.

Defining immune cell subtypes

CD4 T and CD8 T cells were further classified either as *naïve cells*, *recently activated cells* expressing early/late activation marker*, regulatory or memory cells*. B cells expressing CD25 were defined as *recently activated cells.* Macrophages were classified as either *M1-like or M2-like* **(Table 2.3)**. **Tables 2.2** and **2.1** list the panel design details and specific definitions, respectively, for each of the identified subpopulations.

Surface markers	Definitions	Reference
T cell subtypes		
CD4+Foxp3-CD25+ or CD8+Foxp3-CD25+	Recently activated T cells (early marker)	[Stephens LA 2004]
CD4+OX40+ or CD8+OX40+	Recently activated T cells (late marker)	[Stephens LA 2004]
CD4+Foxp3+ or CD8+Foxp3+	Regulatory T cells	[Abe Y 2009]
CD4+CD25-CD45RC- or CD8+CD25-CD45RC-	Memory T cells	[Han SD 2017, Luettig B 2001]
CD4+CD25-CD45RC+ or CD8+CD25-CD45RC+	Naïve T cells	[Luettig B 2001]
B cell subtype		
B220+CD25+	Recently activated B cells	[Amu S 2006]
Macrophage subtypes		
CD68+CD86+MHCII+	Classically activated M1-like macrophages	[Wang M 2017]
CD68+CD163+	Alternatively activated M2-like macrophages	[Yu E 2016]

Table 2.3 Definitions of specific immune sub-populations

Data presentation and statistics

Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla CA; RRID: SCR_002798). Gating for immune cells were done by 3 people (2 blinded and 1 unblinded), analyses were done twice by one person to avoid any bias and ensure rigor and reproducibility. All gating and analyses yielded very similar results. Hence, data presented in this paper are from one person's gating and analyses. Two-way ANOVA with Tukey's multiple comparison test was used to determine statistical significance in all the flow cytometry experiments. Thus, two types of comparisons were possible: between the fats within each sex and between the sexes within each fat. A $P<0.05$ was considered to be statistically significant.

Statistical power was calculated *a priori*. However, because it is very hard to define a specific effect size for biologically relevant differences in immune cell populations between adipose tissues, we had to use a 'best guess approach' based on our preliminary data. Standard deviations for power analyses were obtained by averaging data (cell counts and percentages) on CD68+ macrophages and CD3+ T cells from a mix of males and females from preliminary studies done in the lab. The average standard deviation observed was 34% of the mean values. We chose a standardized effect size (Cohen's d) of 0.8, as that is a standard criterion used to define a "large" effect size (i.e. biologically meaningful). The "n" value per group suggested for a Cohen's d of 0.8, a power of 0.8 and 5% type-I error rate was 26. Hence, we acknowledge that our studies (n=3-6), like most others in the field, are underpowered. However, since fractions of immune cells in

adipose tissue have been reported to increase dramatically (e.g. ~5% macrophages to ~50% macrophages) during weight gain [13], we are confident that our analyses would be adequate to detect such large, biologically relevant changes as statistically significant.

Results

Gating strategy to identify and characterize immune cells in PVATs

Figure 2.1 depicts our general strategy for identifying CD3+ T cells, B220+ B cells, and CD68+ macrophages along with their subpopulations, CD161+ NK cells, HIS48+ neutrophils and FcεRI+ mast cells by flow cytometry. Cell clumps and cellular debris were first excluded by analysis of cell size on a forward-side scatter plot **(Figure 2.1A)**. This step appreciably reduced autofluorescence in SVF preparations. Then, the doublets **(Figure 2.1B)** and Zombie aqua+ dead cells **(Figure 2.1C)** were excluded. Next, CD45+ leukocytes were selected **(Figure 2.1D)**, followed by CD3+ T cell selection. CD3+ T cells were further classified into CD4+ and CD8+ T cells and their subpopulations **(Figure 2.1E)**. From the CD3- cells, B220+ B cells (and CD25+ B cells) were selected **(Figure 2.1F)**. Then, from the CD3-B220- population, individual innate immune cell niches that included macrophages (and their subtypes), NK cells, neutrophils and mast cells were selected **(Figure 2.1F)** as shown by the markers in **Tables 2.1 and 2.2**.

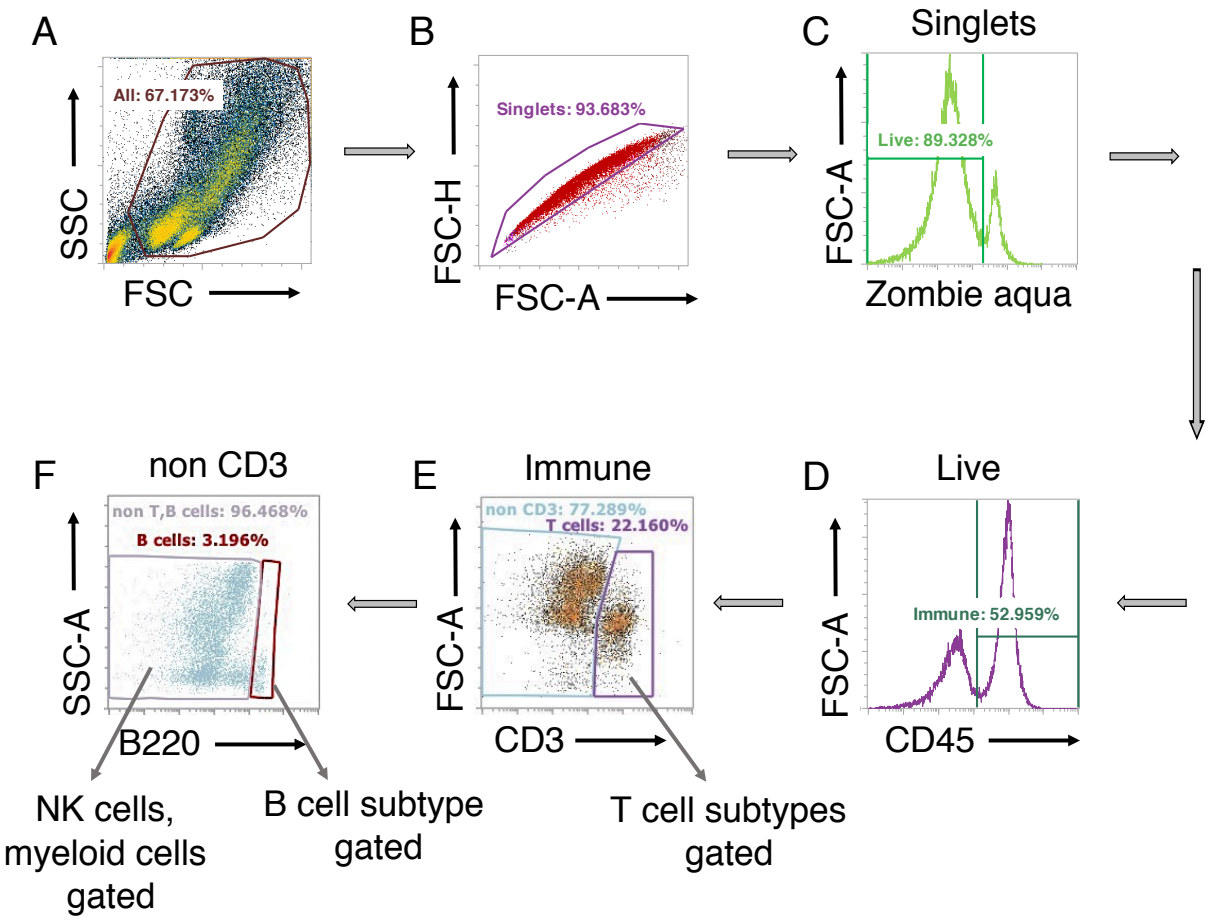


Figure 2.1 Basic gating strategy for flow cytometry experiments
A side-scatter (SSC) *vs* forward scatter (FSC) plot with all the events recorded. Cells were selected 'All' after size-based exclusion of cellular debris **(A)**. Forward scatter- height *vs* area plot allowed for selection of 'singlets', avoiding cells sticking to each other as doublets **(B)**. 'Viable/live' cells (zombie aqua negative) were then positively gated from the singlets **(C)**. From this live cell pool, $CD45^+$ 'immune' cells were gated **(D)**. From the immune cells, $CD3^+$ 'T cells' were selected. From this CD3+ cells, CD4/CD8 T cells and their subpopulations were further gated **(E)**. Then from the $CD3^-$ cells, B220 expressing cells were gated as B cells. $B220^+CD25^+$ cells were further gated from B cells. $CD3^-B220^-$ cells were further gated into NK cells or myeloid cells and their subtypes **(F)**.

PVATs contained a higher number of immune cells *vs* their respective non-PVAT fats, in female rats only

Six primary immune cell types: T cells, B cells, macrophages, NK cells, mast cells and neutrophils were quantified by flow cytometry in PVATs. Spleen served as the positive control. APVAT had a higher number of T cells (both sexes) per mg tissue *vs* MRPVAT and, the spleen contained 10 times greater number of immune cells *vs* both the PVATs (both sexes) **(Figure 2.2A)**. Females had a greater number of T cells *vs* males in APVAT **(Figure 2.2A)**. MRPVAT had a higher number of macrophages (both sexes), T cells (females) and total immune cells (females) *vs* RP fat **(Figure 2.2B)**. Females had a higher number of T cells and total immune cells in MRPVAT *vs* males **(Figure 2.2B)**. APVAT had a higher number of T cells (females) and total immune cells (females) *vs* SS fat. Females had a greater number of T cells in APVAT *vs* males **(Figure 2.2C)**. Raw data values with average number of each immune cell type (per milligram tissue) in PVATs, non-PVAT fats and spleen are presented in **Table 2.4**.

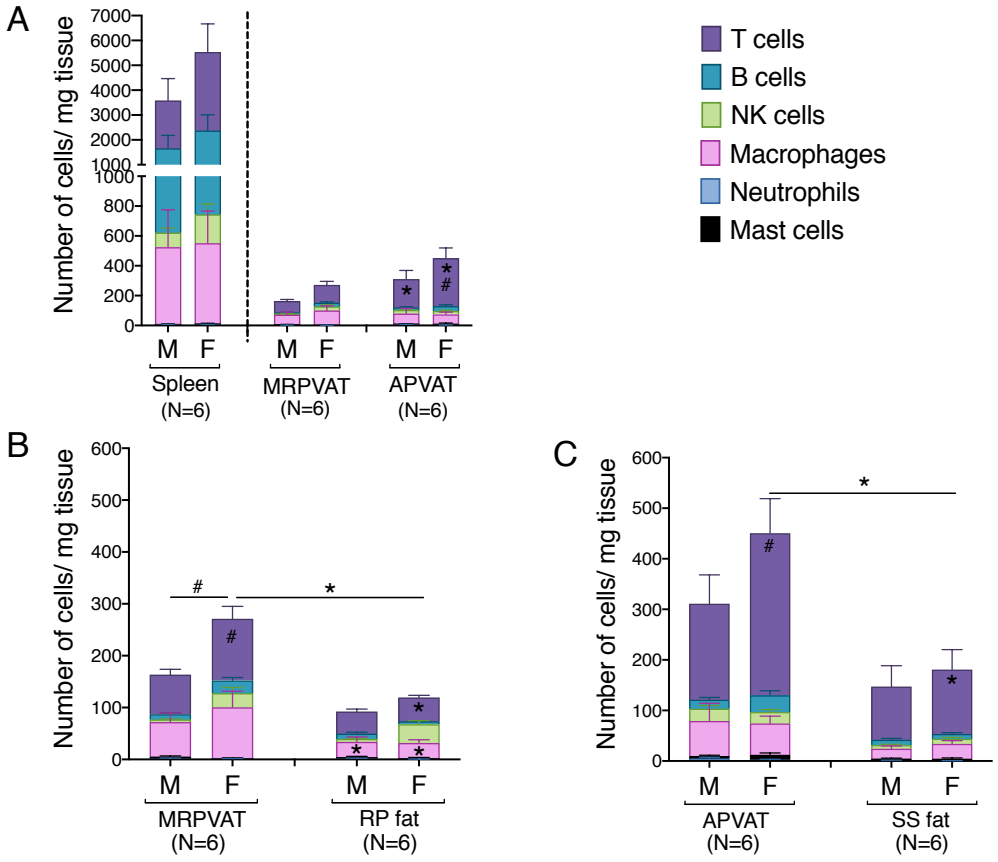


Figure 2.2 PVATs contained a denser immune cell community *vs* their respective non-PVAT fats in female rats only

Immunophenotyping of mesenteric resistance (MR)PVAT, thoracic aortic (A)PVAT, retroperitoneal (RP) fat, subscapular (SS) fat and spleen, in healthy male and female SD rats. T cells, B cells, natural killer (NK) cells, macrophages, neutrophils and mast cells were quantified by flow cytometry. Each immune cell type is presented as absolute cell counts normalized to tissue weight (in milligram). Comparisons between **(A)** MRPVAT and APVAT along with spleen. APVAT had a higher number of T cells (both sexes) per mg tissue *vs* MRPVAT. Females had a greater number of T cells *vs* males in APVAT **(B)** MRPVAT and non-PVAT white RP fat. MRPVAT had a higher number of macrophages (both sexes), T cells (females) and total immune cells (females) *vs* RP fat. Females had a higher number of T cells and total immune cells in MRPVAT *vs* males **(C)** APVAT and non-PVAT brown SS fat. APVAT had a higher number of T cells (females) and total immune cells (females) *vs* SS fat. Females had a greater number of T cells in APVAT *vs* males. Bars represent means±SEM for the number of animals indicated by N. A $P<0.05$ by 2-way ANOVA was considered statistically significant. * and # inside the stacks in the graph represent a significant difference in the specific stack of immune cells between the two fats within each sex or between the sexes within each fat, respectively. * and # outside the stacks in the graph represent a significant difference in the sum total of immune cells between the two fats within each sex or between the sexes within each fat, respectively.

	Spleen	MRPVAT	APVAT	RP fat	SS fat
T cells (male)	1926.5±877.5	76.2±10.2	190±57.1	42.7±4.6	105.4±41.1
(female)	3162.3±1130.3	118.8±23.8	320.7±68.3	45.8±4.2	127.2±39.5
B cells	1038±516.4	10.4±2.5	17.6±4.5	10.1±2.7	10.4±2.7
	1628.4±634.4	24.7±5.6	33.1±8.9	5.8±0.7	9.7±2.4
NK cells	97.2±30.5	4.9±0.96	24.3±6.2	5.7±3.4	7.4±1.5
	193.1±70.3	27.4±10.9	22.6±4.9	36.1±6.9	10±2.8
Macrophages	515.1±250.4	66.6±17.5	69.3±34.9	29.5±9.5	19.5±5.7
	538.7±215.2	98±31.1	62.1±14.4	29.1±6.2	29.3±6.3
Neutrophils	4.5±3	1.9±0.6	5.3±2.1	1.8±0.5	1.3±0.4
	4.8±1.9	1.1±0.4	2.4±1.1	0.5±0.1	0.5±0.2
Mast cells	4.9±1.6	3.5±1.4	4.6±1.5	2.6±1.5	3.4±1
	7.2±2.3	1.3±0.7	9.8±3.7	2.2±1.1	4.1±1.5

Table 2.4 Overall immune cell densities in spleen, PVATs and non-PVAT fats in male and female SD rats

Immune subpopulations (for data graphed in ***Figure 2.2***) in Spleen, MRPVAT, APVAT, RP fat and SS fat in male and female SD rats. Data presented in the table are average numbers of each immune cell type per milligram tissue±SEM. Statistics are presented in ***Figure 2.2***.

MRPVAT in females had a higher naïve CD4 T cell population *vs* APVAT (females) and MRPVAT (males)

CD4 T cells were classified as recently activated (expressing early marker CD25 or late marker OX40), regulatory (Foxp3+), memory (CD45RC-) or naïve (CD45RC+) in PVATs and their respective non-PVATs. MRPVAT in females had a greater number of naïve CD4 T cells *vs* APVAT in females and MRPVAT in males **(Figure 2.3A)**. The spleen contained ~7 times greater number of total CD4 T cell subtypes *vs* both the PVATs (both sexes) **(Figure 2.3A)**. MRPVAT in females had a higher number of naïve CD4 T cells *vs* males **(Figure 2.3B)**. MRPVAT and APVAT had similar density of the CD4 T cell subpopulations that were analyzed *vs* RP fat and SS fat respectively **(Figure 2.3B, 2.3C)**.

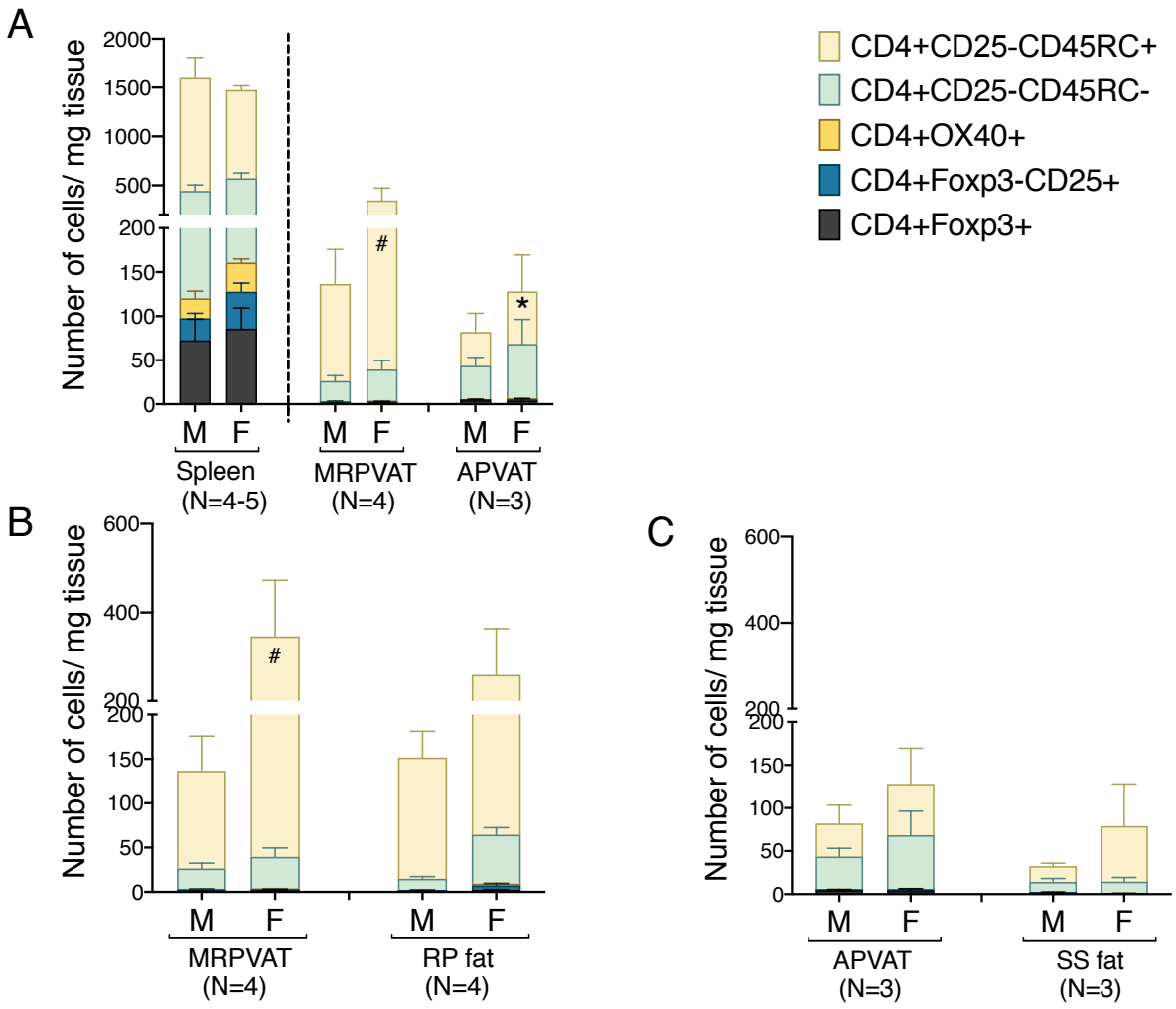


Figure 2.3 MRPVAT in females had a higher naïve CD4 T cell population *vs* APVAT (females) and MRPVAT (males)

Immunophenotyping of mesenteric resistance (MR)PVAT, thoracic aortic (A)PVAT, retroperitoneal (RP) fat, subscapular (SS) fat and spleen, in healthy male and female SD rats. CD4 T cell subtypes (CD4+CD25+, CD4+OX40+, CD4+Foxp3+, CD4+CD45RC- and CD4+CD45RC+ cells) were quantified by flow cytometry. Each immune cell type is presented as absolute cell counts normalized to tissue weight (in milligram). Comparisons between **(A)** MRPVAT and APVAT along with spleen. MRPVAT in females had a greater number of naïve CD4 T cells *vs* APVAT in females and MRPVAT in males **(B)** MRPVAT and non-PVAT white RP fat. Females had a greater number of naïve CD4 T cells *vs* males in MRPVAT **(C)** APVAT and non-PVAT brown SS fat. Bars represent means±SEM for the number of animals indicated by N. A $P<0.05$ by 2-way ANOVA was considered statistically significant. * and # inside the stacks in the graph represent a significant difference in the specific stack of immune cells between the two fats within each sex or between the sexes within each fat, respectively.

MRPVAT had a greater density of naïve CD8 T cells *vs* APVAT in female rats only

CD8 T cell subpopulations were classified into naïve, recently activated, regulatory and memory phenotypes, similar to that of CD4 T cells. MRPVAT had a greater density of naive CD8 T cells *vs* APVAT in females only **(Figure 2.4A)**. The spleen consisted of ~7 times greater number of classified CD8 T cells *vs* both PVATs **(Figure 2.4A)**. MRPVAT and APVAT had similar density of the CD8 T cell subtypes that were analyzed *vs* RP fat and SS fat respectively **(Figure 2.4B, 2.4C)**.

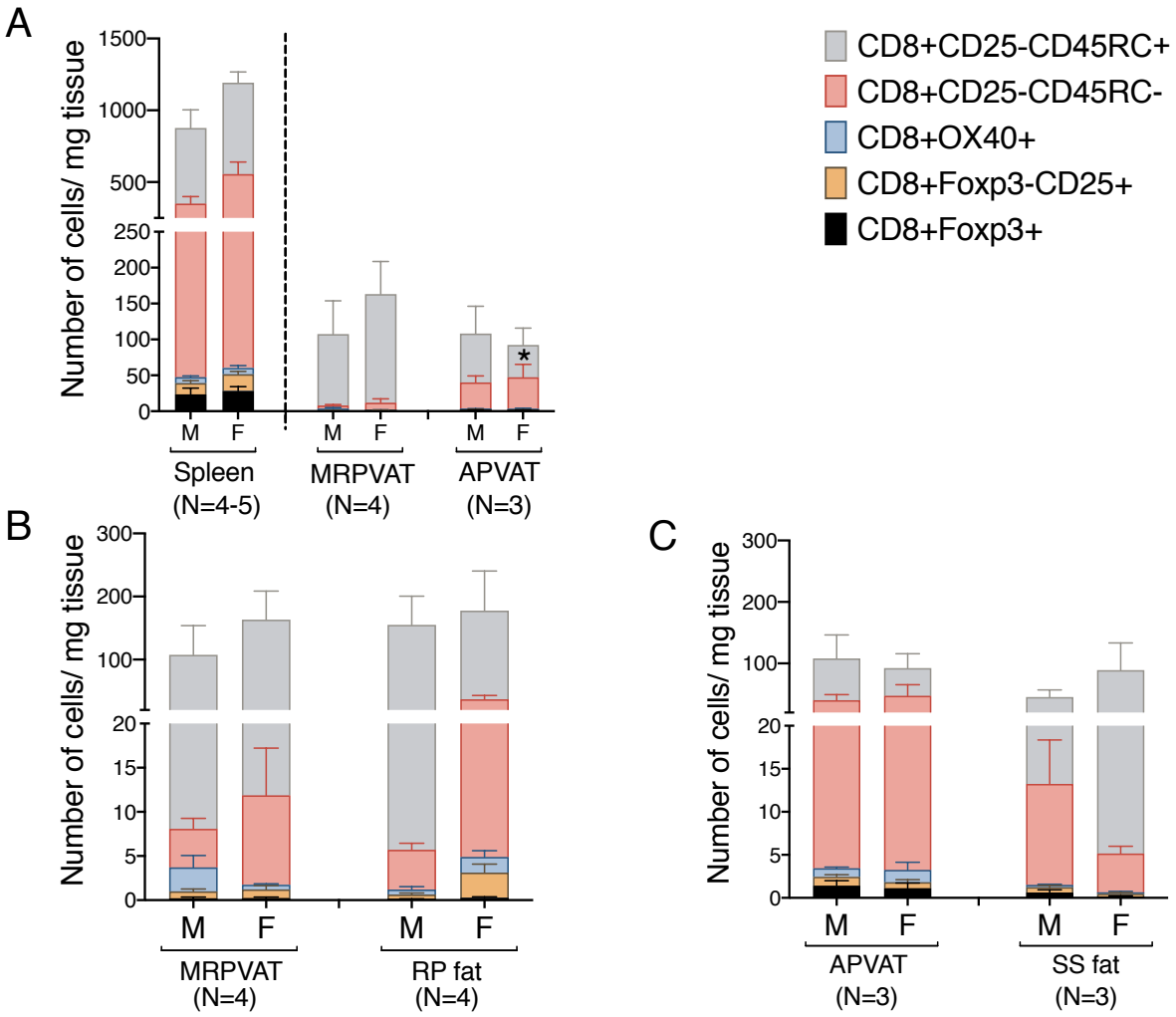


Figure 2.4 PVATs were mostly similar in numbers of CD8 T cell subtypes *vs* their respective non-PVAT fats

Immunophenotyping of mesenteric resistance (MR)PVAT, thoracic aortic (A)PVAT, retroperitoneal (RP) fat, subscapular (SS) fat and spleen, in healthy male and female SD rats. CD8 T cell subtypes (CD8+CD25+, CD8+OX40+, CD8+Foxp3+, CD8+CD45RC- and CD8+CD45RC+ cells) were quantified by flow cytometry. Each immune cell type is presented as absolute cell counts normalized to tissue weight (in milligram). Comparisons between **(A)** MRPVAT and APVAT along with spleen. MRPVAT had a greater density of naive CD8 T cells *vs* APVAT in females **(B)** MRPVAT and non-PVAT white RP fat **(C)** APVAT and non-PVAT brown SS fat. Bars represent means±SEM for the number of animals indicated by N. A $P<0.05$ by 2-way ANOVA was considered statistically significant. * inside the stack in the graph represents a significant difference in the specific stack of immune cells between the two fats within each sex.

PVATs were similar to non-PVAT fats in density of CD25 expressing B cells

B cells expressing early marker CD25 were classified as recently activated. Both PVATs had a similar density of CD25+ B cells **(Figure 2.5A)**. Spleen was composed of ~10 times greater density of CD25 expressing B cells *vs* both PVATs **(Figure 2.5A)**. MRPVAT and APVAT had a similar CD25+ B cell density *vs* RP fat and SS fat respectively **(Fig 2.5B, 2.5C)**.

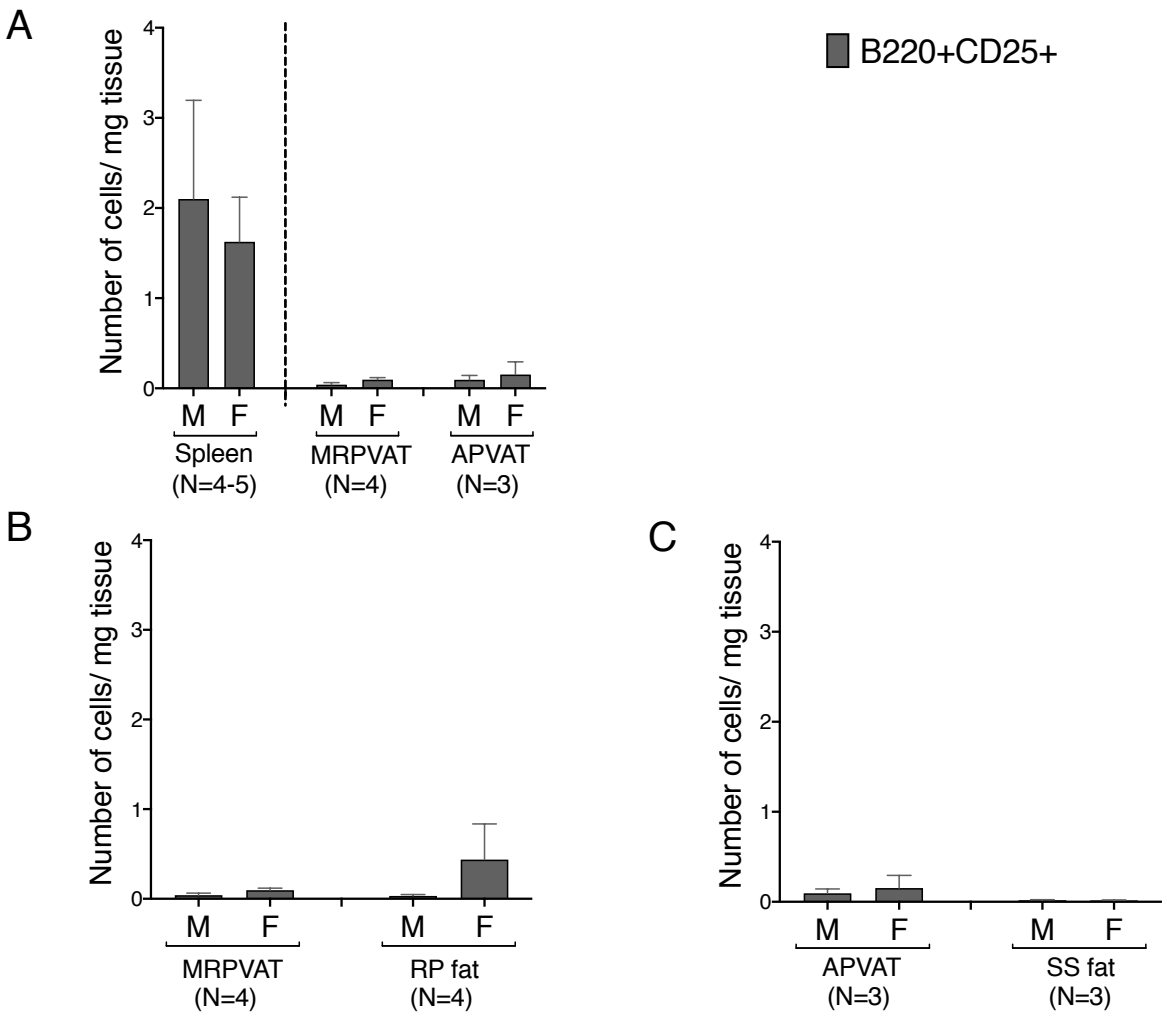


Figure 2.5 PVATs and non-PVAT fats had similar numbers of CD25 expressing B cells

Immunophenotyping of mesenteric resistance (MR)PVAT, thoracic aortic (A)PVAT, retroperitoneal (RP) fat, subscapular (SS) fat and spleen, in healthy male and female SD rats. B220CD25 cells were quantified by flow cytometry and is presented as absolute cell counts normalized to tissue weight (in milligram). Comparisons between **(A)** MRPVAT and APVAT along with spleen **(B)** MRPVAT and non-PVAT white RP fat **(C)** APVAT and non-PVAT brown SS fat. Bars represent means±SEM for the number of animals indicated by N.

MRPVAT contained an increased number of CD68+ macrophage subpopulations *vs* APVAT and RP fat, in female rats only

The density of CD68+ macrophages in PVATs was comparable to that of spleen, save for MRPVAT in females that consist of ~4 times greater number of CD68+ macrophage subpopulations **(Figure 2.6A)**. In females, MRPVAT had a greater number of CD68+CD86+MHCII+ macrophages and total CD68+ macrophage subpopulations *vs* APVAT and male MRPVAT **(Figure 2.6A)** and *vs* RP fat **(Figure 2.6B)**. APVAT and SS fat had comparable numbers of CD68+ macrophage subtypes **(Figure 2.6C)**.

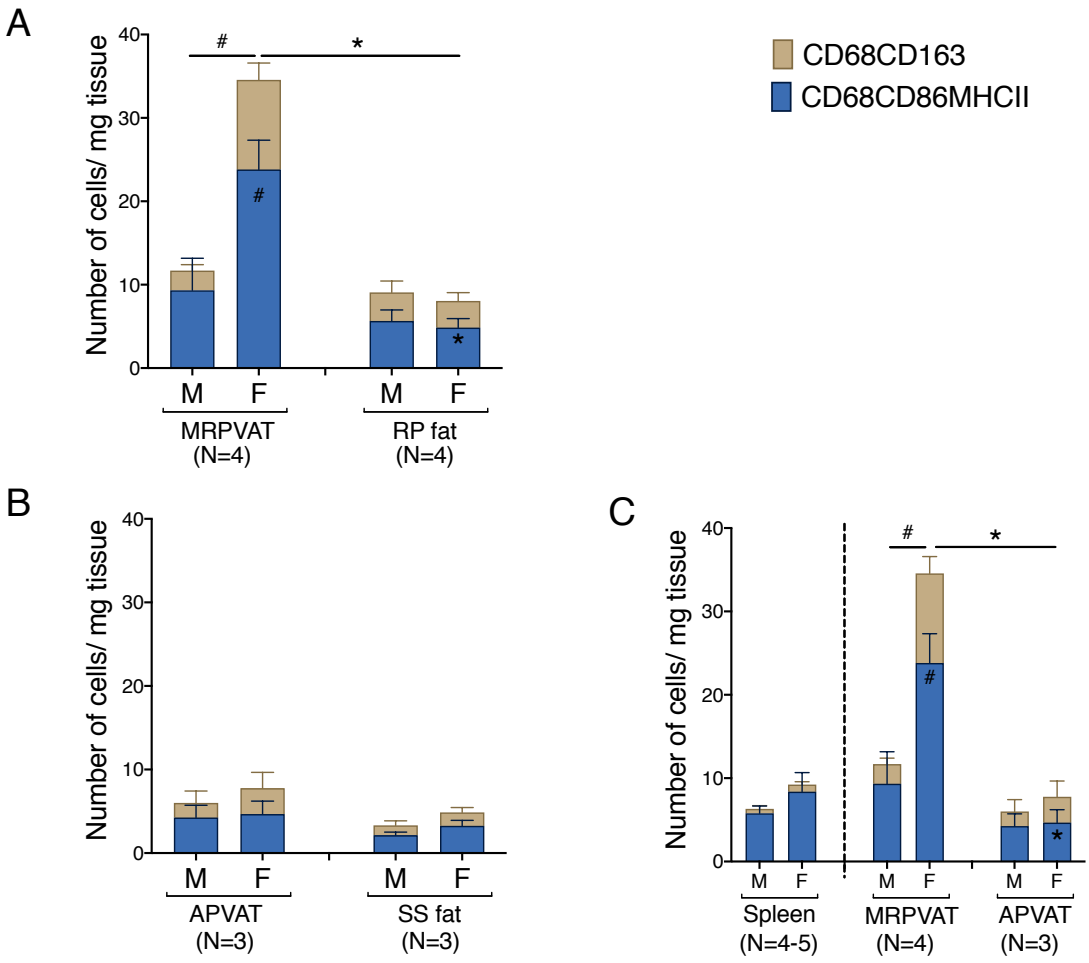


Figure 2.6 MRPVAT had a greater density of CD68+ macrophage subpopulation *vs* APVAT and RP fat in females

Immunophenotyping of mesenteric resistance (MR)PVAT, thoracic aortic (A)PVAT, retroperitoneal (RP) fat, subscapular (SS) fat and spleen, in healthy male and female SD rats. Mature CD68+ macrophages (CD68+CD163+ and CD68+CD86+MHCII+ cells) were quantified by flow cytometry. Each immune cell type is presented as absolute cell counts normalized to tissue weight (in milligram). Comparisons between **(A)** MRPVAT and APVAT along with spleen. In females, MRPVAT had a greater number of CD68+CD86+MHCII+ macrophages and total CD68+ macrophage subpopulations *vs* APVAT (females) and MRPVAT (males). **(B)** MRPVAT and non-PVAT white RP fat. In females, MRPVAT had a greater number of CD68+CD86+MHCII+ macrophages and total CD68+ macrophage subpopulations *vs* RP fat **(C)** APVAT and non-PVAT brown SS fat. Bars represent means±SEM for the number of animals indicated by N. A $P<0.05$ by 2-way ANOVA was considered statistically significant. * and # inside the stacks in the graph represent a significant difference in the specific stack of immune cells between the two fats within each sex or between the sexes within each fat, respectively. * and # outside the stacks in the graph represent a significant difference in the sum total of immune cells between the two fats within each sex or between the sexes within each fat, respectively.

Immune cell subpopulations in healthy PVATs were more similar than different *vs* non-PVAT fats

Six primary immune cell types (both innate and adaptive) co-exist in PVATs and these can be subdivided based on activation, differentiation and/or polarization status. Macrophages, T cells, B cells and NK cells constituted about 80% of the total immune population in adipose tissues including PVATs **(Figure 2.7)**. The numbers outside each wedge represent the percentages of the respective cell subpopulations that were classified [percentages of immune cell subpopulations listed in **Table 2.3** (excluding naïve T cells), as percentage of each primary immune cell type in the respective pie sector]. This figure gives us an overview of the community of immune cells that are contained in PVATs. ***PVAT vs non-PVAT fat:*** PVATs contained a similar percentage of each immune cell type *vs* their respective non-PVAT fats. ***White vs brown fat:*** MRPVAT and RP fat (white) were rich in CD68+ macrophages and CD68+ macrophage subtypes, while APVAT and SS fat (brown) were T-cell rich. ***Males vs Females:*** Females **(Figure 2.7)** consisted of a greater fraction of NK cells (MRPVAT, RP fat and SS fat), T cells (APVAT) and a lesser percentage of 'other' cells (all fats) defined as unidentifiable population of immune cells *vs* males **(Figure 2.7)**. Raw data values with average percentages of each immune cell type in PVATs, non-PVATs and spleen are presented in **Table 2.5**.

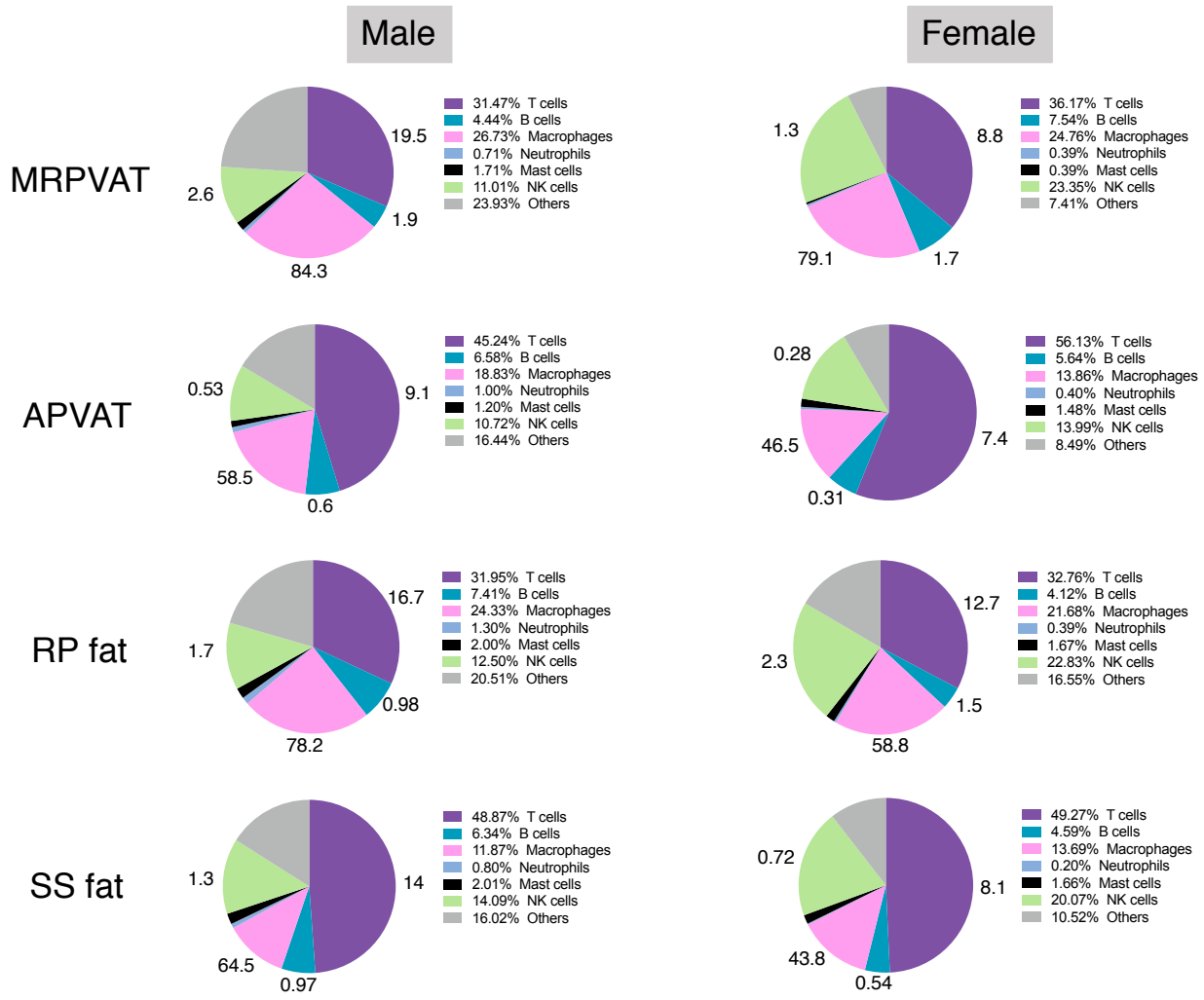


Figure 2.7 Summary of findings, as pie charts
Pie chart representation of each immune cell type as a percentage of total immune composition in MRPVAT, APVAT, RP fat and SS fat in males **(left)** and females **(right)**. The numbers outside represent the percentages of immune cell subpopulations listed in **Table 2.3** (excluding naïve T cells) as percentage of each primary immune cell type in the respective pie sector.

	Spleen	MRPVAT	APVAT	RP fat	SS fat
T cells (male)	39.2±4	31.2±3.3	45.4±6.8*	31.9±4.1	48.6±5.4
(female)	40.9±2.7	37.4±4.8	56.7±2.4*, #	33.4±2.1	50.4±4.5
B cells	20±2.8	4.4±1	6.6±2.4	7.4±1.9	6.3±1.1
	20.5±2.1	7.8±1.3	5.7±1.2	4.2±0.5	4.7±1
NK cells	8.6±0.6	10.9±2.5	10.8±1	12.5±2.5	14.1±2.8
	11.9±2.3	24.1±3.7	14.1±3.1	23.3±3	20.5±2.7
Macrophages	10.5±1.5	26.5±7.5	18.9±6.5	24.3±7.2	11.8±2.9
	7.6±1	25.6±6.8	14±3.7	22.1±4.8	14±3.4
Neutrophils	0.1±0.1	0.7±0.2	1±0.3	1.3±0.4	0.8±0.2
	0.1±0	0.4±0.1	0.4±0.1	0.4±0.1	0.2±0.1
Mast cells	0.1±0	1.7±0.9	1.2±0.2	2±1.2	2±0.4
	0.1±0	0.4±0.1	1.5±0.3	1.7±0.9	1.7±0.3

Table 2.5 Overall immune cell percentages in spleen, PVATs and non-PVAT fats in male and female SD rats

Immune subpopulations (for data graphed as pie charts in ***Figure 2.7***) in Spleen, MRPVAT, APVAT, RP fat and SS fat in male and female SD rats. Data presented in the table are average percentages of each immune cell type ±SEM. A $P<0.05$ by 2-way ANOVA was considered statistically significant. * represents a significant difference in T cell percentage between MRPVAT and APVAT (both sexes). # represents a significant difference in T cell percentage between males and females in APVAT.

Discussion

The current study tested the hypothesis that an activated immune population exists in PVATs of healthy rats and that would be different *vs* their respective non-PVAT fats. Flow cytometry was primarily employed. This work is important because; (1) the study describes a reliable method to isolate immune cells from multiple adipose tissues in rats and quantify them by flow cytometry; (2) it is the first time a study has been designed to compare the immune composition of two types of PVATs with their respective non-PVAT fat controls in both sexes in health; and (3) discrete immune subpopulations (naïve, recently activated, regulatory and memory type) have been reported in PVATs in healthy rats for the first time.

Immune cell percentages/numbers identified in the spleen in the current study are consistent with the previous studies in Sprague Dawley rats [Morris DL 1997]. The current study has identified both innate and adaptive immune cells homing in healthy PVATs. Approximately 15-30% of SVF were macrophages in adipose tissues including PVATs. This is similar to other reports where close to 15% of visceral adipose tissue is macrophages in lean mice, and are predominantly found in the interstitial spaces between adipocytes [Weisberg SP 2003, Lumeng CN 2008]. In our present study, T, B and NK cells accounted for nearly 40-50% of immune cells in healthy PVATs and non-PVATs. This is consistent with a recent report where lymphocytes (T and B cells) were ~38% of live SVF cells in non-obese human abdominal subcutaneous WAT [Acosta JR 2016]. We observe a minor (~2-3% of immune cells) mast cell and neutrophil population in PVATs

and non-PVATs, which is also consistent with the existing literature in mice WAT [Ferrante AW 2013]. A distinct and substantial pool of memory T and B subsets have been identified in PVATs and non-PVATs here. This finding is also consistent with earlier mice studies that have revealed the presence of different subpopulations of memory T cells in non-PVAT WATs [Masopust D 2001, Han SJ 2017]. By contrast, our study has identified ~80-95% macrophages that are MHCII+ but other studies in mice that have reported 55% of MHCII+ macrophages in epididymal WAT in mice [Morris DL 2013]. This disparity in observations may be due to species differences in MHCII+ macrophages or due to different antibodies used for identifying macrophages (F4/80 in mice *vs* CD68 in rats).

In health, is the immune system of PVATs functionally similar to non-PVAT fats?

The current study has identified a *similar* immune cell subtypes between PVATs and their respective non-PVAT fats in health. Our study has also revealed that MRPVAT and RP fat are rich in macrophages and T cells while APVAT and SS fat are T cell rich. Gene and protein expression studies from other labs reveal striking similarities between thoracic aortic PVAT and classical interscapular brown adipose tissue in mice [Fitzgibbons TP 2011, Hildebrand S 2018] that are significantly different from WAT. This aligns well with our current findings and suggests that MRPVAT may be functionally similar to its respective non-PVAT RP fat, but different from APVAT. However, on the contrary, some previous studies also suggest that PVATs may be functionally different from their respective non-PVAT fats. Smooth muscle-specific peroxisome proliferator

activated receptor gamma knockout in mice resulted in selective loss of mesenteric and aortic PVAT, leaving the other fats (interscapular brown adipose tissue, gonadal WAT and subcutaneous WAT) intact [Chang L 2012]. This strongly supports that PVATs have different developmental properties than non-PVAT fats. Other studies have reported that visceral, gonadal and subcutaneous fats hold distinct memory T cell gene signatures *vs* spleen in mice [Han SJ 2017]. This suggests that anatomically different adipose tissues may possess functionally distinct immune sub-populations. The close proximity of PVAT to the blood vessels (unlike other non-PVAT fats), the differences in the local tissue microenvironment and interaction with other immune/ non-immune cells [Mahlakoiv T 2019] within the tissue are all likely to determine the fate and physiological functions of these immune cells in PVATs.

Potential roles of the mature immune reservoir in PVATs

Finding what appears to be a recently activated, memory and/or polarized subtype of T cells, B cells and macrophages in the PVATs of healthy rats raises a number of questions. *First*, why and how do immune cells in healthy PVATs become activated or acquire memory? *Second*, what functions do these different immune subtypes at steady state perform in healthy PVATs?

In our current study, the majority of T cells in adipose tissues including PVATs were either of naïve or memory phenotype. Non-PVAT WAT has an immune compartment that nurtures long-term maintenance in health and rapid re-activation of

memory T cells in disease [Han SJ 2017]. Over 80% of CD68+ macrophages are MHCII+ in PVATs in health. MHCII-mediated antigen presentation is critical for the development and maintenance of visceral adipose tissue Treg cells in healthy mice [Zeng Q 2018]. PVATs may serve similar functions and may participate in classical immunological host defense against pathogens. The endogenous activators of immune cells in PVATs in health have not yet been discovered. However, activated immune cells in PVATs may also be essential to produce regulatory molecules and other mediators to promote specific functions in other immune niches. WAT-resident anti-inflammatory macrophages, Tregs and eosinophils directly/promote release of anti-inflammatory cytokines such as IL-10, transforming growth factor-β [Gong D 2012, Grant RW 2015, Oh SA 2013]. These steady state immune cells and the cytokines released promote tissue repair and extracellular matrix remodeling, clear cell debris, aid in lipolysis, adipogenesis, angiogenesis and overall help maintain insulin sensitivity [Schipper HS 2012b]. The paracrine action of PVAT on the vasculature may be much stronger than other adipose depots due to PVATs location relative to vascular tunica media and adventitia [Gollasch M 2012, Noblet JN 2015, Goodpaster BH 2005]. Immune cells may either directly release factors that are anti-contractile or promote other cells in PVAT (e.g. adipocytes) to release relaxants. For example, IL-10 and IL-1β produced by macrophages and Tregs inhibit vascular smooth muscle cell contraction [Marceau F 2010, Zemse SM 2010]. PVAT-resident eosinophils directly release catecholamines that stimulate adipocytes to produce adiponectin and nitric oxide, *via* β–3 adrenoreceptors, which cause vasorelaxation [Withers SB 2017].

Third, what provokes the protective PVAT-resident immune population to decline/maladapt in pathologies such as obesity, hypertension and atherosclerosis? In disease, increased hypoxia/oxidative stress, excess free fatty acids, increased metabolic damage associated molecular patterns and pattern associated molecular patterns are some known triggers that activate the innate immune cells which in-turn present the antigens to the adaptive immune cells in non-PVAT adipose tissues [Guzik TJ 2017]. Further studies are warranted to identify the initiators and mechanisms of maladaptation of immune cells in PVAT in disease.

Limitations

We recognize several limitations of our current work. First, the percentage of immune cells as determined by our gating strategy in each tissue does not total up to a hundred. Several reasons could explain this: (i) eosinophils and B cell subtypes could not be detected by flow cytometry due to lack of reliable surface markers/ flow cytometry antibodies that can detect eosinophils and other B cell subtypes in rats; (ii) the adipose tissues could contain other immune cell types such as dendritic cells, type 2 innate lymphoid cells (ILC 2) and other immune cell types not expressing the flow markers that have been used in this study; (iii) PVATs and other fats of healthy rats contain unidentified/ poorly defined immune cell populations. Second, future work to determine the functional capabilities of these identified immune subpopulations would be helpful for a more precise definition and understanding of immune cells in PVAT. Third, the estrous staging of the female rats was not determined in this study. Fourth, lineage tracking

studies would be necessary to clarify the origin of the immune cells identified in PVATs. Finally, collagenase digestion is the gold standard method for immune cell isolation from adipose tissues. Although the effect of collagenase on immune cell activation is unknown, interpretations should be made with caution as surface expression of markers could have been altered with enzymatic treatment.

Conclusion

In summary, this study for the first time has identified discrete subpopulations of T cells, B cells, NK cells and macrophages in healthy PVATs of both male and female Sprague Dawley rats, that are not distinctly different from non-PVAT fats. The current study not only highlights the similarities in the immune composition of PVATs *vs* non-PVAT fats, males *vs* females, but also the local heterogeneity of different PVATs in health. This leads to the question of what the different immune cell subtypes in healthy subtypes do and if we can exploit these similarities to develop broad-spectrum immunotherapeutic targets to white or brown fats including PVATs.

REFERENCES

REFERENCES

Abe Y, Urakami H, Ostanin D, Zibari G, Hayashida T, Kitagawa Y and Grisham MB. (2009). Induction of Foxp3-expressing regulatory T-cells by donor blood transfusion is required for tolerance to rat liver allografts. *PLoS ONE* 4, e7840. doi:10.1371/journal.pone.0007840.

Acosta JR, Douagi I, Andersson DP, Bäckdahl J, Rydén M, Arner P and Laurencikiene J. (2016). Increased fat cell size: a major phenotype of subcutaneous white adipose tissue in non-obese individuals with type 2 diabetes. *Diabetologia* 59, 560–570. doi:10.1007/s00125-015-3810-6.

Amu S, Gjertsson I, Tarkowski A and Brisslert M. (2006). B-cell CD25 expression in murine primary and secondary lymphoid tissue. *Scand. J. Immunol.* 64, 482–492. doi:10.1111/j.1365-3083.2006.01832.x.

Baumgarth N. (2011). The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat. Rev. Immunol.* 11, 34–46. doi:10.1038/nri2901.

Chang L, Villacorta L, Li R, Hamblin M, Xu W, Dou C, Zhang J, Wu J, Zeng R and Chen YE. (2012). Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-γ deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. *Circulation* 126, 1067–1078. doi:10.1161/CIRCULATIONAHA.112.104489.

Chawla A, Nguyen KD and Goh YPS. (2011). Macrophage-mediated inflammation in metabolic disease. *Nat. Rev. Immunol.* 11, 738–749. doi:10.1038/nri3071.

Ferrante AW. (2013). The immune cells in adipose tissue. *Diabetes Obes. Metab.* 15 Suppl 3, 34–38. doi:10.1111/dom.12154.

Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, et al. (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 15, 930–939. doi:10.1038/nm.2002.

Fitzgibbons TP, Kogan S, Aouadi M, Hendricks GM, Straubhaar J and Czech MP. (2011). Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1425-37. doi:10.1152/ajpheart.00376.2011.

Gollasch M. (2012). Vasodilator signals from perivascular adipose tissue. *Br. J. Pharmacol.* 165, 633–642. doi:10.1111/j.1476-5381.2011.01430.x.

Gong D, Shi W, Yi S, Chen H, Groffen J and Heisterkamp N. (2012). TGFβ signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol.* 13, 31. doi:10.1186/1471-2172-13-31.

Goodpaster BH, Krishnaswami S, Harris TB, Katsiaras A, Kritchevsky SB, Simonsick EM, Nevitt M, Holvoet P and Newman AB. (2005). Obesity, regional body fat distribution, and the metabolic syndrome in older men and women. *Arch. Intern. Med.* 165, 777–783. doi:10.1001/archinte.165.7.777.

Grant RW and Dixit VD. (2015). Adipose tissue as an immunological organ. *Obesity (Silver Spring)* 23, 512–518. doi:10.1002/oby.21003.

Guzik TJ, Skiba DS, Touyz RM and Harrison DG. (2017). The role of infiltrating immune cells in dysfunctional adipose tissue. *Cardiovasc. Res.* 113, 1009–1023. doi:10.1093/cvr/cvx108.

Han S-J, Glatman Zaretsky A, Andrade-Oliveira V, Collins N, Dzutsev A, Shaik J, Morais da Fonseca D, Harrison OJ, Tamoutounour S, Byrd AL, et al. (2017). White adipose tissue is a reservoir for memory T cells and promotes protective memory responses to infection. *Immunity* 47, 1154-1168.e6. doi:10.1016/j.immuni.2017.11.009.

Hildebrand S, Stümer J and Pfeifer A. (2018). PVAT and its relation to brown, beige, and white adipose tissue in development and function. *Front. Physiol.* 9, 70. doi:10.3389/fphys.2018.00070.

Luettig B, Kaiser M, Bode U, Bell EB, Sparshott SM, Bette M and Westermann J. (2001). Naive and memory T cells migrate in comparable numbers through the normal rat lung: only effector T cells accumulate and proliferate in the lamina propria of the bronchi. *Am. J. Respir. Cell Mol. Biol.* 25, 69–77. doi:10.1165/ajrcmb.25.1.4414.

Lumeng CN, DelProposto JB, Westcott DJ and Saltiel AR. (2008). Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* 57, 3239–3246. doi:10.2337/db08-0872.

Lynch L, Hogan AE, Duquette D, Lester C, Banks A, LeClair K, Cohen DE, Ghosh A, Lu B, Corrigan M, et al. (2016). iNKT Cells Induce FGF21 for Thermogenesis and Are Required for Maximal Weight Loss in GLP1 Therapy. *Cell Metab.* 24, 510–519. doi:10.1016/j.cmet.2016.08.003.

Mahlakõiv T, Flamar AL, Johnston LK, Moriyama S, Putzel GG, Bryce PJ and Artis D. (2019). Stromal cells maintain immune cell homeostasis in adipose tissue via production of interleukin-33. *Sci. Immunol.* 4. doi:10.1126/sciimmunol.aax0416.

Marceau F, deBlois D, Petitclerc E, Levesque L, Drapeau G, Audet R, Godin D, Larrivée J-F, Houle S, Sabourin T, et al. (2010). Vascular smooth muscle contractility assays for inflammatory and immunological mediators. *Int. Immunopharmacol.* 10, 1344–1353. doi:10.1016/j.intimp.2010.08.016.

Masopust D, Vezys V, Marzo AL and Lefrançois L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291, 2413–2417. doi:10.1126/science.1058867.

Meijer RI, Serne EH, Smulders YM, van Hinsberg VWM, Yudkin JS, Eringa EC (2011). Perivascular adipose tissue and its role in type 2 diabetes and cardiovascular disease. *Curr Diab Rep.* 11(3):211-217. doi: 10.1007/s11892-011-0186-y.

Morris DL and Komocsar WJ. (1997). Immunophenotyping analysis of peripheral blood, splenic, and thymic lymphocytes in male and female rats. *J. Pharmacol. Toxicol. Methods* 37, 37–46.

Morris DL, Cho KW, Delproposto JL, Oatmen KE, Geletka LM, Martinez-Santibanez G, Singer K and Lumeng CN. (2013). Adipose tissue macrophages function as antigen-presenting cells and regulate adipose tissue CD4+ T cells in mice. *Diabetes* 62, 2762–2772. doi:10.2337/db12-1404.

Nguyen Dinh Cat A, Briones AM, Callera GE, Yogi A, He Y, Montezano AC and Touyz RM. (2011). Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors. *Hypertension* 58, 479–488. doi:10.1161/HYPERTENSIONAHA.110.168872.

Noblet JN, Owen MK, Goodwill AG, Sassoon DJ and Tune JD. (2015). Lean and obese coronary perivascular adipose tissue impairs vasodilation via differential inhibition of vascular smooth muscle K+ channels. *Arterioscler. Thromb. Vasc. Biol.* 35, 1393–1400. doi:10.1161/ATVBAHA.115.305500.

Oh SA and Li MO. (2013). TGF-β: guardian of T cell function. *J. Immunol.* 191, 3973–3979. doi:10.4049/jimmunol.1301843.

Schipper HS, Prakken B, Kalkhoven E and Boes M. (2012a). Adipose tissue-resident immune cells: key players in immunometabolism. *Trends Endocrinol. Metab.* 23, 407–415. doi:10.1016/j.tem.2012.05.011.

Schipper HS, Rakhshandehroo M, van de Graaf SFJ, Venken K, Koppen A, Stienstra R, Prop S, Meerding J, Hamers N, Besra G, et al. (2012b). Natural killer T cells in adipose tissue prevent insulin resistance. *J. Clin. Invest.* 122, 3343–3354. doi:10.1172/JCI62739.

Stephens LA, Barclay AN and Mason D. (2004). Phenotypic characterization of regulatory CD4+CD25+ T cells in rats. *Int. Immunol.* 16, 365–375. doi:10.1093/intimm/dxh033.

Szaz T and Webb RC (2012). Perivascular adipose tissue: more than just structural support. *Clin Sci (Lond).* 122(1):1-12. doi: 10.1042/CS20110151.

Wang M, Fijak M, Hossain H, Markmann M, Nüsing RM, Lochnit G, Hartmann MF, Wudy SA, Zhang L, Gu H, et al. (2017). Characterization of the Micro-Environment of the Testis that Shapes the Phenotype and Function of Testicular Macrophages. *J. Immunol.* 198, 4327–4340. doi:10.4049/jimmunol.1700162.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112, 1796–1808. doi:10.1172/JCI19246.

Withers SB, Forman R, Meza-Perez S, Sorobetea D, Sitnik K, Hopwood T, Lawrence CB, Agace WW, Else KJ, Heagerty AM, et al. (2017). Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. *Sci. Rep.* 7, 44571. doi:10.1038/srep44571.

Wu D, Molofsky AB, Liang H-E, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, Chawla A and Locksley RM. (2011). Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* 332, 243–247. doi:10.1126/science.1201475.

Yu E, Goto M, Ueta H, Kitazawa Y, Sawanobori Y, Kariya T, Sasaki M and Matsuno K. (2016). Expression of area-specific M2-macrophage phenotype by recruited rat monocytes in duct-ligation pancreatitis. *Histochem. Cell Biol.* 145, 659–673. doi:10.1007/s00418-016-1406-y.

Zemse SM, Chiao CW, Hilgers RHP and Webb RC. (2010). Interleukin-10 inhibits the in vivo and in vitro adverse effects of TNF-alpha on the endothelium of murine aorta. *Am. J. Physiol. Heart Circ. Physiol.* 299, H1160-7. doi:10.1152/ajpheart.00763.2009.

Zeng Q, Sun X, Xiao L, Xie Z, Bettini M and Deng T. (2018). A Unique Population: Adipose-Resident Regulatory T Cells. *Front. Immunol.* 9, 2075. doi:10.3389/fimmu.2018.02075.

CHAPTER 3

Interleukin-10 Does Not Contribute to the Anti-Contractile Nature of PVAT in Health

Abstract

Perivascular adipose tissue (PVAT) is protective and reduces contraction of blood vessels in health. PVAT is composed of adipocytes, multiple types of immune cells and stromal cells. Interleukin (IL)-10, an anti-inflammatory cytokine usually produced by T cells, B cells and macrophages, was identified as one of the highly expressed (mRNA) cytokines in the mesenteric PVAT of healthy rats. One report suggested that exogenous IL-10 causes relaxation of mouse mesenteric arteries, also suggesting that IL-10 maybe a potential anti-contractile factor. Hence, we hypothesized that **PVAT-derived IL-10 causes vasorelaxation and/or reduces vasoconstriction, thus contributing to the anti-contractile nature of PVAT in health.** Mesenteric arteries from rats and mice expressed the receptor for IL-10 (in tunica intima and media) as determined by immunohistochemistry. Mesenteric resistance arteries for rats and superior mesenteric artery for mice were used for isometric contractility studies. Increasing concentrations [0.4- 100 ng/mL] of recombinant rat/ mouse (rr/ mr) IL-10 or vehicle was directly added to half-maximally constricted (phenylephrine, PE) vessels (without PVAT, with endothelium). IL-10 did not cause a direct vasorelaxation. Further, the ability of rrIL-10 to cause a rightward or downward shift of a vasoconstriction-response curve was tested in the rat. The vessels were incubated with rrIL-10 [100 ng/mL or 10 ng/mL] or vehicle for 1.5 hours in the tissue bath followed by a cumulative PE [10^{-8}-10^{-4} M] or U46619 [10^{-10}-10^{-5} M] response curve. The maximal contractions and EC_{50} values were similar in IL-10 incubated vessels *vs* vehicle. Thus, acute exposure of exogenous IL-10 did not reduce local vasoconstriction. To further test if endogenous IL-10 from PVAT was anti-contractile, superior mesenteric arteries from IL-10 WT and KO mice, with and without PVAT, were subjected to increasing concentrations of PE. The anti-contractile nature of PVAT was preserved with both short-term and prolonged depletion (using younger and older mice, respectively) of endogenous IL-10 in males and females. Contrary

to our hypothesis, PVAT-derived IL-10 neither caused vasorelaxation nor reduced local vasoconstriction directly/ indirectly. Therefore, IL-10 does not contribute to the anti-contractile nature of PVAT in healthy rodents.

Introduction

In health, the presence of PVAT inhibits agonist-induced vasoconstriction in vessels from humans and rodents [Soltis EE 1991, Lohn M 2002]. This is called the classic 'anti-contractile' nature of PVAT, experimentally observed as a rightward or downward shift in an agonist contractile response curve. PVAT also directly promotes vasorelaxation [Lohn M 2002, Verlohren S 2004], which would reduce blood pressure in healthy subjects.

The molecules responsible for the anti-contractile/ vasorelaxing functions of PVAT in health may include adiponectin, nitric oxide, and angiotensin (1-7). These factors have been thought of as primarily derived from PVAT adipocytes [Szasz T 2013]. But in addition to adipocytes and stromal cells, we discovered that a community of innate and adaptive immune cells co-exist in PVATs [Kumar RK 2020]. These immune cells may either directly release factors that are anti-contractile or promote other cells in PVAT (*e.g.* adipocytes) to release vasorelaxants. Recent work supports eosinophils as critical to the anti-contractile actions of PVAT in health [Withers SB 2017]. Unlike the considerable literature which supports a pathological function of immune cells in cardiovascular disease [Virdis A 2015], this paper raised the provocative idea that under healthy conditions, the immune cells in PVAT may actively contribute to the anti-contractile nature of PVAT.

Macrophages and T cells are the dominant immune cells in PVATs in health [Kumar RK 2020]. They are the major producers of the anti-inflammatory master regulatory cytokine interleukin (IL)-10 [Saraiva M 2010]. IL-10 was reported to be a direct vasorelaxant in the presence of endothelium in mesenteric arteries of wildtype (WT) male mice [Kassan M 2011]. This led us to **hypothesize that PVAT-derived IL-10 causes vasorelaxation and/ or reduces local vasoconstriction, thus contributing to the anti-contractile nature of PVAT in healthy rodents**. The term 'healthy' was used as no purposeful disease induction was invoked. Experiments were designed to test: (1) the contribution of IL-10 to the anti-contractile nature of PVAT in two species: rats and mice; (2) exogenous *vs* endogenous (or direct *vs* indirect effects) IL-10 to cause vasorelaxation/ reduce vasoconstriction and (3) if the anti-contractile nature of PVAT was lost with short-term or prolonged depletion of IL-10 in males and females. Younger and older (or short-term and prolonged) IL-10 KO/ WT mice were used because prolonged (not short-term) genetic depletion of IL-10 leads to activation of systemic inflammation in KO mice [Sikka G 2013] and the vascular effects of depleting IL-10 might take longer to appear without disease induction. Contrary to our hypothesis, we discovered that PVAT-derived IL-10 neither caused vasorelaxation nor reduced vasoconstriction in healthy rodents. These studies support that IL-10 is not involved in the anti-contractile nature of PVAT in health.

Materials and Methods

Animals

Animal maintenance and experimental protocols were approved by the MSU Institutional Animal Care and Use Committee, and compiled with the National Institutes

of Health Guide for Animal Care and Use of Laboratory Animals (2011). All the animals were maintained on a 12/12 light/dark cycle at 22-25 °C, fed on *ad-libitum.*

Rats: Male Sprague Dawley rats (SD; 8-12 weeks of age, Charles River, IN, USA; RRID: RGD_10395233) were used. Prior to all dissections, the rats were anesthetized with sodium pentobarbital (60-80 mg/kg, i.p.) and death was assured by creating a bilateral pneumothorax.

Mice: Male and female IL-10 knockout KO mice on C57BL/6 background (13 weeks of age and 26 weeks of age; RRID: IMSR_JAX:002251) and age-matched male and female C57BL/6 wildtype WT mice (~13 weeks old and ~26 weeks old; RRID: IMSR_JAX:000664) were obtained from Jackson Laboratories, ME, USA. Mice were euthanized in compliance with American Veterinary Medical Association recommendations. IL-10 KO mice were validated as described in section in the methods **(Figure 3.5)**.

Tissue handling and dissection

Third order mesenteric arteries from rats and superior mesenteric artery from mice were isolated and, where appropriate, cleaned of PVAT in a silastic-impregnated dish filled with physiological salt solution [PSS; in mM; 130 NaCl; 4.7 KCl; 1.17 $MgSO_4 \cdot 7H_2O$; 1.18 K_2HPO_4; 14.8 $NaHCO_3$; 5.5 dextrose; 0.03 $CaNa_2$ ethylenediametetraacetic acid;

1.6 $CaCl_2$ (pH 7.2)]. In other instances, PVAT was dissected from vessels and retroperitoneal (RP) fat was cleaned off of small blood vessels and used for qPCR.

RNA isolation and RT-qPCR

MRPVAT (PVAT around the mesenteric resistance vessels) and RP (retroperitoneal) fat from male WT SD rats, and SMPVAT (PVAT around the superior mesenteric vessels) and RP fat from male WT mice were homogenized in 2 mL tubes (Omni, Kennesaw, GA) with 1.4 mm ceramic bead media (cat #19-645, Omni) using the Omni Bead Ruptor.

Rat: RNA was isolated with the Zymo Quick-RNA Mini Prep Kit (Cat # R1054, Zymo Research, CA, USA), then quantified on a NanoDrop 2000c (Thermo Scientific). cDNA was reverse transcribed using the RT^2 First Strand Kit (cat# 330404, Qiagen, MD, USA). RT-qPCR was performed on a QuantStudio 7 Flex Real-Time PCR system using RT^2 SYBR Green ROX Master Mix (Cat #330521, Qiagen) with the following parameters: 95 ºC for 20 s; 95 ºC for 1 s and 60 ºC for 20 s for 40 cycles, followed by a melt curve to determine the presence of a single PCR product. RT2 profiler PCR array kit (#PARN-053ZE-4, Qiagen) was used to measure IL-10 and housekeeping gene beta-2-microglobulin, according to manufacturer's protocol.

Mouse: RNA was isolated with the Zymo Quick-RNA Mini Prep Kit (Cat # R1054, Zymo Research, Irvine, CA), then quantified on a NanoDrop 2000c (Thermo Scientific).

cDNA was reverse transcribed using the High Capacity cDNA kit (cat# 4368814, Thermo Fisher Scientific). RT-qPCR was performed on a QuantStudio 7 Flex Real-Time PCR system using Fast SYBR Green Master Mix (Cat # 4385612, Thermo Fisher Scientific) with the following parameters: 95 ºC for 20 s; 95 ºC for 1 s and 60 ºC for 20 s for 40 cycles, followed by a melt curve to determine the presence of a single PCR product. All primer sequences were retrieved from Primer Depot (http://primerdepot.nci.nih.gov/) and synthesized by Integrated DNA Technologies. The primer sequences are: IL-10 forward primer: 5'-ACCAGCTGGACAACATACTGC-3'; IL-10 reverse primer: 3'-ATTTCTGGGCCATGCTTCTCT-5' and RPL13A (ribosomal housekeeping gene) forward primer: 5'-GTTGATGCCTTCACAGCGTA-3', RPL13A reverse primer: 5'-AGATGGCGGAGGTGCAG-3'.

Relative IL-10 expression for the rat/ mouse was normalized to the respective housekeeping gene and the data were analyzed with the 2^{-dC_t} method in which C_t is the threshold cycle.

Immunohistochemistry

All tissue sections were formalin-fixed and paraffin embedded. Mesenteric vessels with MRPVAT from male WT SD rats, superior mesenteric vessels with SMPVAT from male WT mouse and male WT mouse spleen were embedded and sectioned (8 μm thick) by Michigan State University's Investigative Histopathology Lab. WT male SD rat spleen sections (5 μm thick) were purchased from Zyagen (San Diego, CA, USA). The rat/ mouse

spleens were used as biological positive controls for IL-10RA. Using standard immunohistochemistry protocols, slides were de-waxed and antigens unmasked. All the slides were then blocked with a species-specific blocking serum (1.5% goat serum in PBS; Vector Laboratories, CA, USA) for 60 minutes at room temperature. IL-10 RA primary antibody (1:100, RRID:AB_2847938, Abcam) was used in both the rat and the mouse (it has proven reactivity to both). After overnight incubation at 4^{o}C with primary antibody in blocking buffer or blocking buffer alone (for negative control), all the sections were exposed to anti-rabbit secondary antibody (1:1000, #PK6101; Vector Laboratories, CA, USA) for 45 minutes. ImmPACT NovaRED HRP substrate kit (#SK-4805) for rat slides and DAB with HRP substrate kit (#SK-4100) for mouse sllides from Vector Laboratories (Burlingame, CA) were used. Hematoxylin (#H2404, Vector Laboratories, CA, USA) was used as a nuclear stain. Nikon Eclipse TE2000 inverted microscope (Nikon, Otowara, Japan) was used to capture bright field images using the 40X objective. Images were captured using a Nikon Digital Sight DS-Qil camera and Nikon NIS Elements BR 3.0 software. Adjustments in brightness and contrast for all the images were applied uniformly across each image. Images representative of five animals were selected for the figures.

Isometric contraction

Third order mesenteric arteries from rats and superior mesenteric artery from mice, cleaned of fat (-PVAT) or with fat intact (+PVAT) and endothelium intact, were mounted into a multi wire myograph system 620 (Danish Myo Technology, Denmark). Data were

acquired using a PowerLab Data Acquisitions unit (ADInstruments, CO, USA). Baths contained warmed, oxygenated PSS. Rings were pulled to optimum resting tension (13.3 kPa) and equilibrated for 30 minutes with washes every 10 min. The vessels were exposed to an initial concentration of 10 μM phenylephrine (PE), an α-1 adrenergic agonist, to test viability and to elicit maximum contraction. Tissues were washed and returned to baseline. Presence of the endothelium was verified with a tissue relaxation caused with addition of 1 μM acetylcholine (ACh) after inducing a half-maximal PE-induced contraction. The tissues were washed and returned to baseline. It was ensured that all the vessels (from both rats and mice) that were exposed to exogenous IL-10 had demonstrated at least 80% relaxation with 1μM ACh. All the cumulative response curves were performed with sufficient time necessary for a response to plateau prior to addition of the next concentration. However, if no change in response was recorded within 4 minutes, the next concentration was applied. Tissues were taken through one of the protocols below.

Protocol I: Direct relaxation caused by IL-10

Male SD rat mesenteric resistance arteries without PVAT and 13 week-old male WT mouse superior mesenteric artery without PVAT were used. The arteries were half-maximally constricted with PE and then mouse (mr)/ rat (rr) recombinant IL-10 (0.4 ng/ mL to 100 ng/ mL; mr: #575802 Biolegend, CA, USA; rr: #522-RLB R&D Systems, MN, USA) or their respective vehicle controls (mouse: 20 mM Tris and 150 mM NaCl pH 7.5

in PBS; rat: PBS+0.2% BSA) were applied in a cumulative fashion. Same volume of vehicle as mr/ rrIL-10 was added to the vessels from the respective species.

Protocol II: Ability of IL-10 to shift a contractile curve

Rat mesenteric resistance arteries with and without PVAT were used. Either rrIL-10 (10 ng/ mL or 100 ng/ mL) or vehicle (PBS+ 0.2% BSA; same volume as rrIL-10) was added for 90 minutes without washing, following which a cumulative PE (10^{-8} to 10^{-4} M) or U46619 a thromboxane A_2 agonist (10^{-10} to 10^{-5} M; #16450 Cayman Chemical Company, MI, USA) response curve was performed. PE and U46619 curves were performed on different rats.

Protocol III: Vascular effects with genetic loss of IL-10

Two ages of IL-10 KO and WT mice were used: 13 week and 26 week-old males and females. The superior mesenteric artery with or without PVAT were subjected to a cumulative PE (10^{-8} to 10^{-4} M) concentration response curve.

Cytokine assays (IFN-γ and IL-10) and ELISA

Splenocyte preparation

The spleens isolated from rats/ mice were mechanically disrupted by a syringe plunger and filtered through a 40 μm filter in complete Dulbecco's Modified Eagle Medium (cDMEM) supplemented with 10% fetal bovine serum (Biowest LLC, MO, USA), 25 mM HEPES, 50 µM 2-mercaptoethanol, nonessential amino acids (1X final concentration from

100X stock solution), 100 U/ml penicillin, and 100 μg/ml streptomycin (cDMEM). The single cell suspension obtained was then washed with cDMEM and then, red blood cell lysis was performed by adding ammonium-chloride-potassium lysis buffer and incubating for 2 minutes on ice. The splenocytes were further washed twice with cDMEM and counted using a hemocytometer.

IFN-γ ELISA in rat splenocytes

Wells of a 96 well flat-bottom plate were pre-coated with anti-CD3 antibody in PBS (1: 200; #14003085, eBiosciences, CA, USA) or PBS alone and incubated at 4 ºC overnight. The wells were emptied and 1 million splenocytes per well were added, with and without 100 ng/mL rat recombinant IL-10. Simultaneously, the wells that received anti-CD3 antibody also received anti-CD28 antibody (1:100; #200902, Biolegend, CA, USA). After 72 hours, the supernatants were collected and rat IFN-γ ELISA (#BMS621, Thermo Fisher Scientific, CA, USA) was performed according to the manufacturer's protocol. This is the standard assay to evaluate the activity of IL-10 [David F Fiorentino 1989].

IFNγ ELISA in mouse splenocytes

Wells of a 96 well flat-bottom plate received 1 million splenocytes per well. The cells received either purified hamster anti-mouse CD3 (1.5 μg/ mL; #14003386 eBiosciences, CA, USA), purified hamster anti-mouse CD28 (1.5 μg/ mL; #14028186, eBiosciences, CA, USA) and an $F(ab')_2$ fragment specific for anti-Syrian hamster IgG

crosslinker (#107006142, Jackson ImmunoResearch Laboratories, PA, USA) or nothing, with and without mouse recombinant IL-10 (100 ng/ mL). After 72 hours, the supernatants were collected and mouse IFNγ ELISA (#430801; Biolegend, CA, USA) was performed as per manufacturer's protocol.

IL-10 ELISA in mouse splenocytes

One million murine splenocytes at a concentration of $2x10^6$ cells/mL were seeded out in a 48 well flat bottom plate. The cells were activated using cell activation cocktail phorbol myristate acetate (PMA; 40 nM)/ ionomycin (Io; 0.5 μM) (#423301; Biolegend, CA, USA) or vehicle (Dimethyl Sulfoxide, #D2650; Sigma Aldrich, MO, USA) for 48 hours. It was necessary to activate the splenocytes with PMA/Io because basal level of IL-10 protein is challenging to be detected with the commercially available ELISA kits. Subsequently, IL-10 concentration in the supernatants was assessed using ELISA (#431411; Biolegend, CA, USA).

Data Presentation and Statistics

Data are reported as mean ± SEM for the number of animals N. Statistical analysis was performed with GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla CA; RRID: SCR_002798). mRNA measures were reported as 2^{-dCt} and unpaired t-test was performed. For all ELISAs, one-way ANOVA with Tukey's multiple comparison's test was performed. Contraction was reported as a percentage of the initial contraction to 10 μM

PE and relaxation was reported as a percentage of half-maximal PE-induced contraction. Two-way ANOVA with posthoc Tukey test was performed for isometric contractility studies. For all the data presented, a $P < 0.05$ was considered statistically significant. Potencies (-log EC_{50}) and maximal effect were calculated using GraphPad Prism 8.0. When a response didn't plateau, (-log EC_{50}) and maximal effect values automatically estimated by GraphPad Prism was used.

Results

IL-10 mRNA was present in PVATs of male SD rats (MRPVAT) and wildtype mice (SMPVAT)

IL-10 mRNA was present in MRPVAT in male SD rats, in 10 times greater quantity *vs* RP fat, a non-PVAT white fat **(Fig 3.1A)**. IL-10 mRNA was also present in SMPVAT and RP fat in similar quantities in male WT mice **(Fig 3.1B)**.

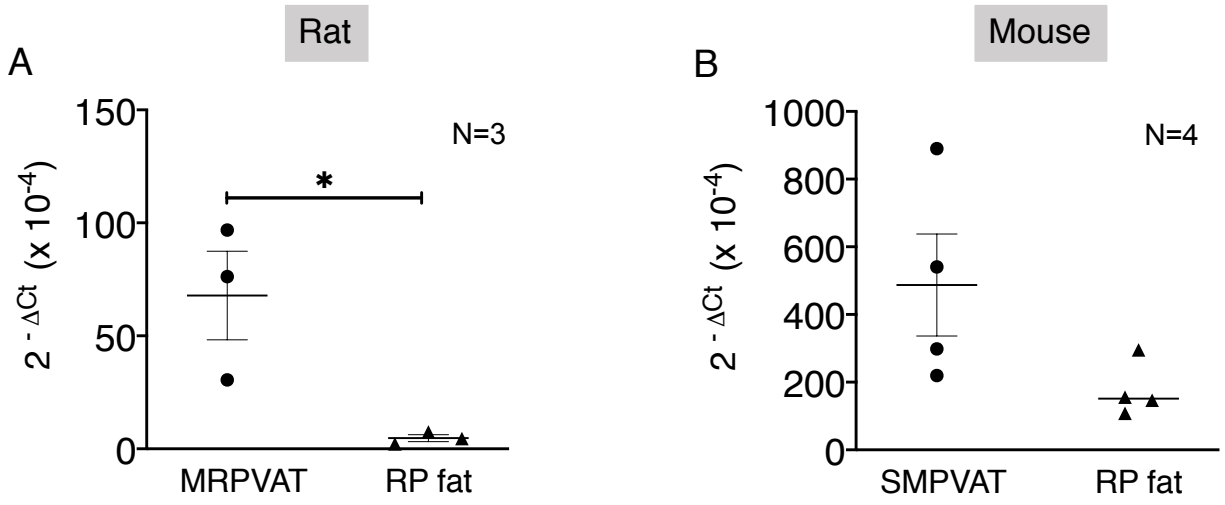


Figure 3.1 IL-10 mRNA was present in male wildtype rats (MRPVAT) and mice (SMPVAT)

IL-10 mRNA quantified by real time-qPCR in MRPVAT and RP fat from male SD rats **(A)** and in SMPVAT and RP fat from male WT mice **(B)**. Reference gene: beta-2-microglobulin for the rat and RPL13a for the mouse. Data were normalized to the respective reference genes. Each dot represents an animal and means±SEM is plotted in the graphs. * represents $p< 0.05$ by an unpaired student t-test for the number of animals indicated by N. Mean C_t±SEM for reference genes: rat MRRPVAT= 17.6±0.2 ; rat RP fat= 16.8±0.3 ; mouse SMPVAT= 32.4±0.4; mouse RP fat= 24±0.4.

The receptor for IL-10 was present in mesenteric resistance arteries (male rats) and superior mesenteric artery (male wildtype mice)

For IL-10 to be potentially vasoactive, the receptor for IL-10, IL-10RA, needs to be present in the mesenteric arteries. IL-10 RA (pointed by yellow arrows) was expressed in tunica intima and tunica media of mesenteric resistance arteries and MRPVAT in male rats **(Figure 3.2C, D)** and in the tunica intima and tunica media of superior mesenteric artery and SMPVAT in male WT mice **(Figure 3.2G, H)**. Spleen from rats **(Fig 3.2A, B)** and mice **(Fig 3.2E, F)** served as biological positive controls.

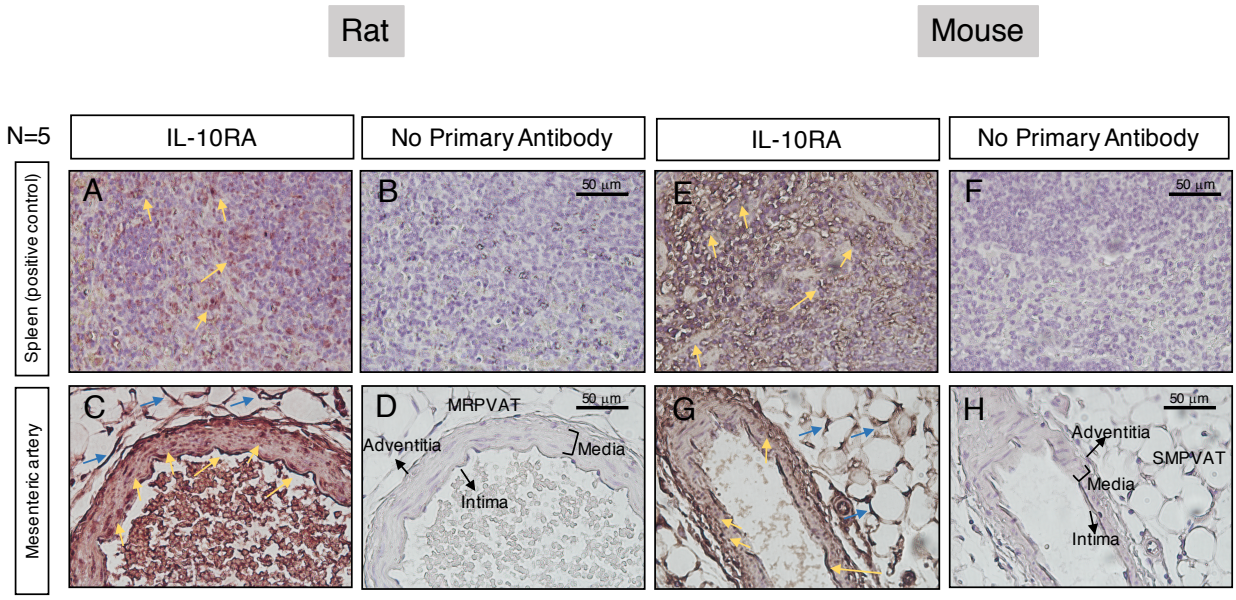


Figure 3.2 IL-10 receptor was present in male rat mesenteric and male mouse superior mesenteric arteries

Brightfield images (40X objective) using a primary antibody against IL-10RA (pointed with yellow arrows) on the spleen **(A, B)** and mesenteric arteries (yellow arrows) + MRPVAT (blue arrows) **(C, D)** from male WT SD rats; on the spleen (yellow arrows) **(E, F)** and superior mesenteric artery (yellow arrows) + SMPVAT (blue arrows) from male WT mice **(G, H)**. Images are representative of 5 animals (indicated by N). Spleen: biological positive control; No primary antibody: technical negative control; Tunica intima, media, adventitia and MRPVAT **(D)** or SMPVAT **(H)** are marked in tissues without primary antibody for reference.

Acute exogenous IL-10 did not cause direct vasorelaxation in both wildtype rats and mice

Cytokines have been used in the range of 10 to 100 ng/ mL concentrations in vascular contractility studies with rat aorta (IL-8, IL-2) and in *ex vivo* studies with adipose tissue (IL-10) [Ohkawa F 1995, Rajbhandari P 2017]. Hence, 10 ng/ mL and 100 ng/ mL of rr/ mr IL-10 or 0.4 to 100 ng/ mL for IL-10 cumulative concentration response curves were chosen for the current study.

Both rrIL-10 and mrIL-10 (used in respective species) reduced IFN-γ secreted by cultured splenocytes activated for 72 hours by anti-CD3/anti-CD28; this reduction was statistically significant in the mouse but not in the rat **(Figure 3.3A, D)**. Hence, the rr and mr IL-10 proteins used in the current study were biologically active molecules at the concentrations used in contractility experiments. Vehicle controls or rr/mr IL-10 was added to half-maximally constricted arteries (constricted with PE) in a cumulative concentration-dependent manner to the respective species. Representative tracings demonstrated the inability of rr/ mr IL-10 to relax PE-induced half-maximal contraction **(Figure 3.3B, E)**. Tracings from multiple experiments were then quantified and are shown in **Figure 3.3C and F**.

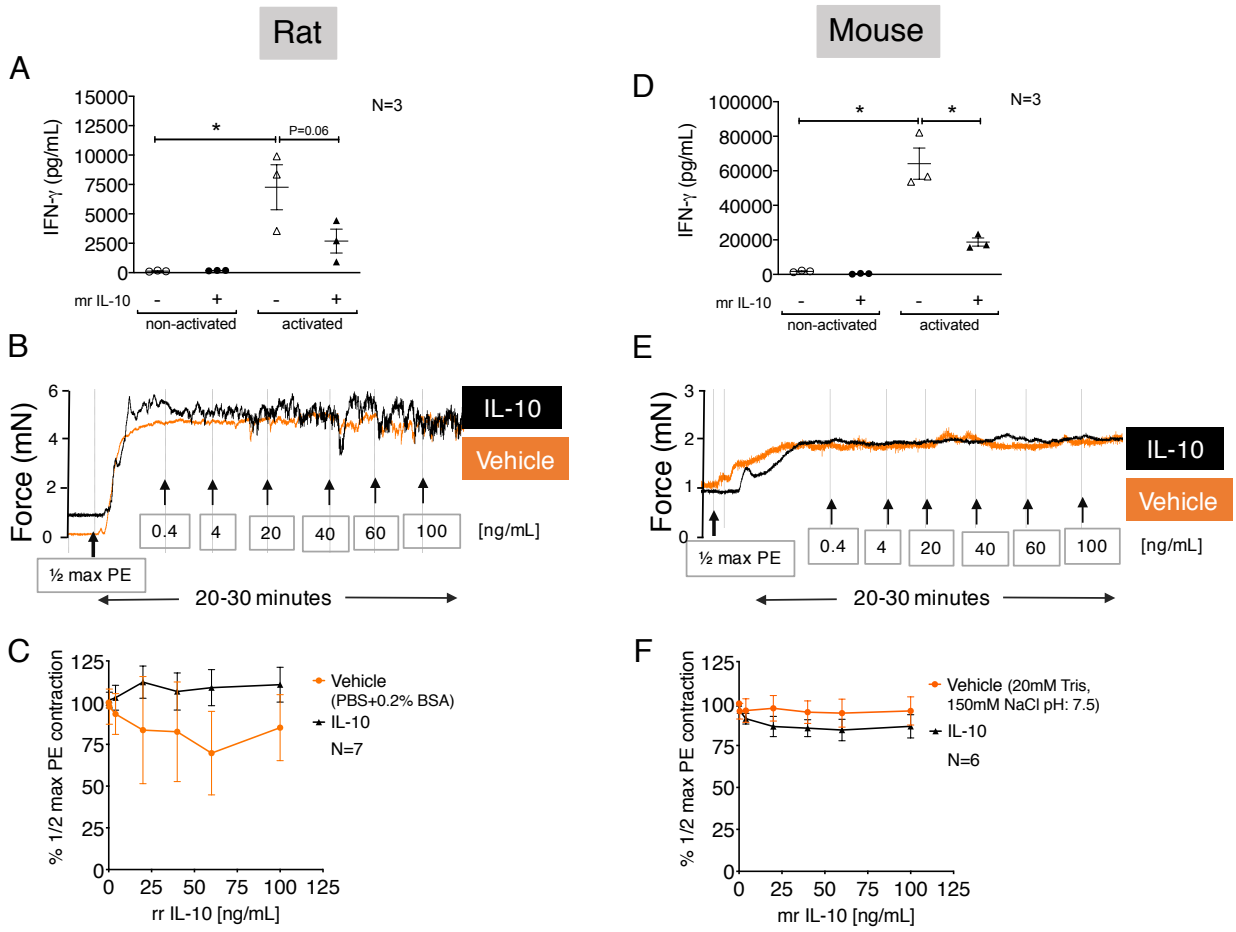


Figure 3.3 Acute exposure to exogenous IL-10 did not cause direct vasorelaxation in mesenteric arteries from both wildtype male rats and mice

IFN-γ measures by ELISA with/ without activation, with/ without mr/ rr IL-10 (100 ng/ mL; added to respective species) using splenocytes isolated from male SD rats **(A)** and male WT mice **(D)**. Representative tracings of half maximally constricted (with PE) rat third-order mesenteric resistance arteries (no PVAT, with endothelium; **B**) and mouse superior mesenteric artery (no PVAT, with endothelium; **E**) subject to a cumulative rr/ mr IL-10 (0.4 ng/ mL to 100 ng/ mL; added to respective species) concentration response curve and their quantification **(C, F)**. * represents $p<0.05$ by one-way ANOVA with Tukey's multiple comparisons test. Points represent means±SEM and the response to IL-10 is normalized to the respective half-maximal PE-induced contraction. Half-maximal PE-induced contraction (mN): Vehicle (rat)= 5.8±0.7; rrIL-10= 5.1±0.5; Vehicle (mouse)= 1±0.08; mrIL-10= 1.8±0.3. N= number of animals.

Acute exogenous IL-10 did not directly reduce vasoconstriction in mesenteric resistance arteries of wildtype rats

Third order mesenteric resistance arteries were incubated with rrIL-10 (10 ng/mL or 100 ng/mL) or vehicle to test if rrIL-10 caused a rightward shift of an agonist response curve. But neither the PE concentration response curve **(Figure 3.4A, B)** nor the U46619 concentration response curve **(Figure 3.4C, D)** shifted rightward in the presence of either low (10 ng/mL) or high (100 ng/mL) concentrations of IL-10. The potencies (-log EC_{50} [M]) and the maximal contractions of the curves were similar in the presence of IL-10 and vehicle **(Table 3.1).**

Together, exogenous acute exposure of IL-10 neither caused a direct vasorelaxation nor was anti-contractile. We further tested if endogenous IL-10 derived from PVAT was anti-contractile. IL-10 KO mice were used to assess if the anti-contractile nature of PVAT was lost with short-term or prolonged depletion (using younger and older mice, respectively) of endogenous IL-10.

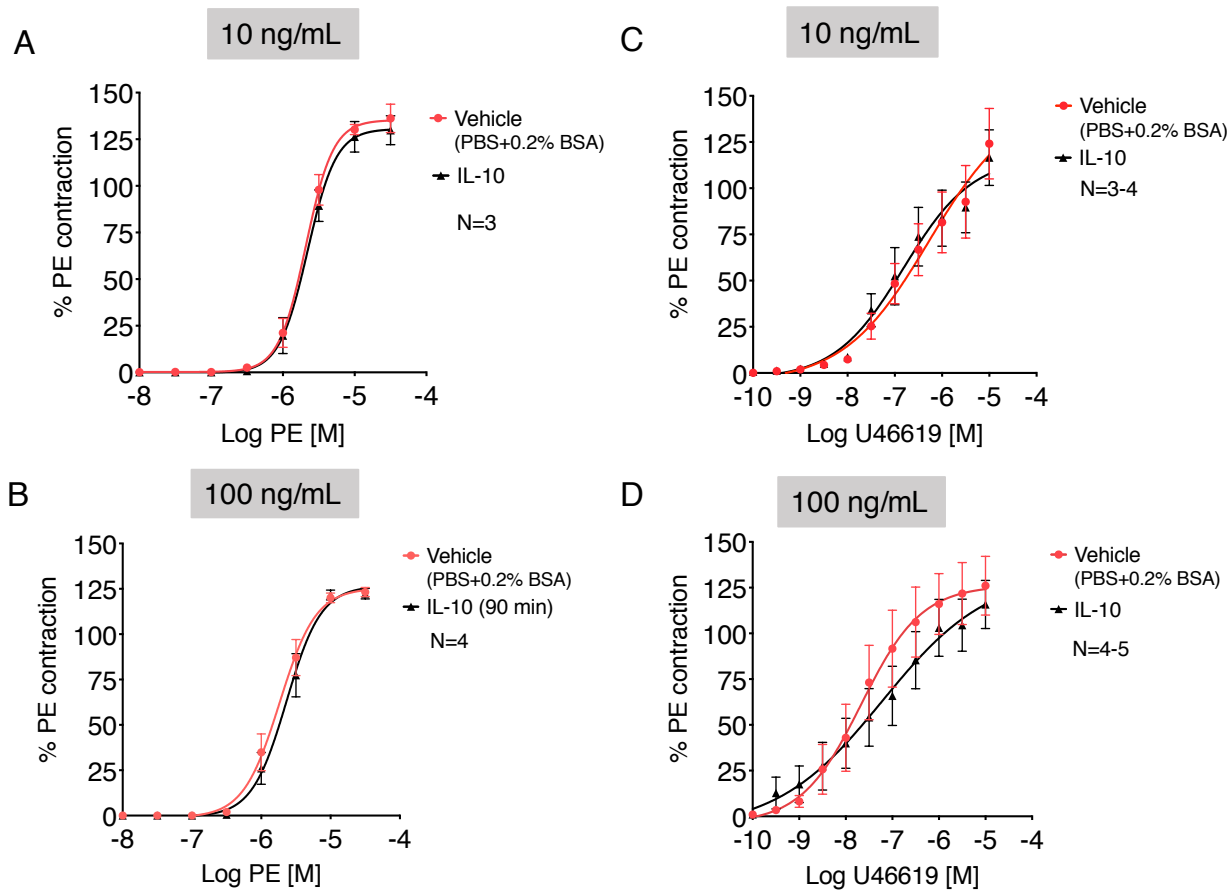


Figure 3.4 Acute exposure to exogenous IL-10 was not anti-contractile in rat mesenteric resistance arteries

PE-induced cumulative contraction (10^{-8} to 10^{-4} M) of isolated rat third order mesenteric resistance artery without PVAT (with endothelium) after incubation with vehicle (PBS+0.2% BSA) or rrIL-10 (10 ng/mL: **A**; 100 ng/mL: **B**) for 90 minutes in the tissue bath. U46619-induced contraction (10^{-10} to 10^{-5} M) of the rat third order mesenteric resistance artery without PVAT (with endothelium) after incubation with vehicle (PBS+0.2% BSA) or rrIL-10 (10 ng/mL: **C**; 100 ng/mL: **D**) for 90 minutes in the tissue bath. Points represent means±SEM. N represents the number of animals (and 2 vessels per animal).

Concen -tration of rrIL10 (ng/ mL)	Agonist	Exposure condition	-logEC_{50} [M]	Maximum contraction (%10 μM PE)	Initial PE contraction (mN)
10	PE	Vehicle	5.7±0.03	135.3±3.8	8.4±1.90
		IL-10	5.7±0.04	130.5±4.8	9.2±1.20
	U46619	Vehicle	7.1±0.12	100.0±6.1	8.2±1.20
		IL-10	7.2±0.30	112.0±7.0	8.0±0.80
100	PE	Vehicle	5.7±0.05	125.4±4.9	8.8±1.50
		IL-10	5.6±0.05	126.6±5.3	9.8±1.70
	U46619	Vehicle	7.7±0.28	126.3±13.2	5.3±0.70
		IL-10	7.2±0.60	133.5±41.0	7.3±1.60

Table 3.1 Exogenous IL-10 was not anti-contractile in the SD rat: pharmacological parameters
List of pharmacological parameters of data presented in ***Figure 3.4***.

Validation of the IL-10 knockout mouse

Stimulation of cultured splenocytes with a pan-immune activator such as PMA/Io for 48 hours increased IL-10 secretion in both male and female WT mice but not in the IL-10 KO mice. Thus, IL-10 KO mice were validated using an IL-10 ELISA. **(Figure 3.5)**.

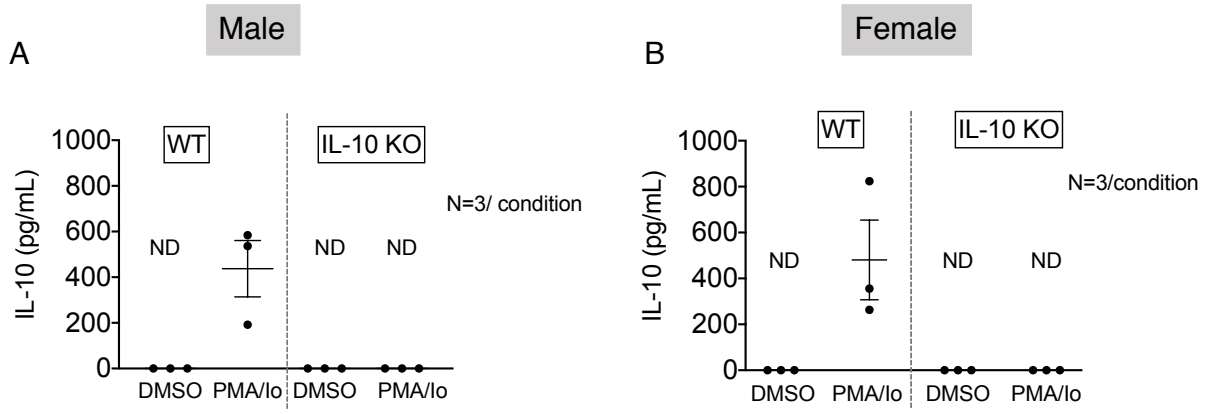


Figure 3.5 Validation of the IL-10 knockout mouse
IL-10 measured by ELISA (as pg/ mL) on splenocytes isolated from 13 week-old male **(A)** and female **(B)** IL-10 KO and age-matched WT, incubated with phorbol myristate acetate/ ionomycin (PMA/Io) or vehicle (DMSO) for 48 hours. Each dot represents an animal N and means±SEM are quantified. ND=not detectable.

PVAT still remained anti-contractile with short-term genetic depletion of endogenous IL-10 in mice

In isolated mouse superior mesenteric artery, the anti-contractile nature of PVAT was present in both WT and IL-10 KO mice at 13 weeks of age (younger mice) in males **(Figure 3.6A)** and females **(Figure 3.6B)**. The rightward/ downward shift in the contractile response curve in arteries with PVAT was not lost even with IL-10 depletion **(Table 3.2)**.

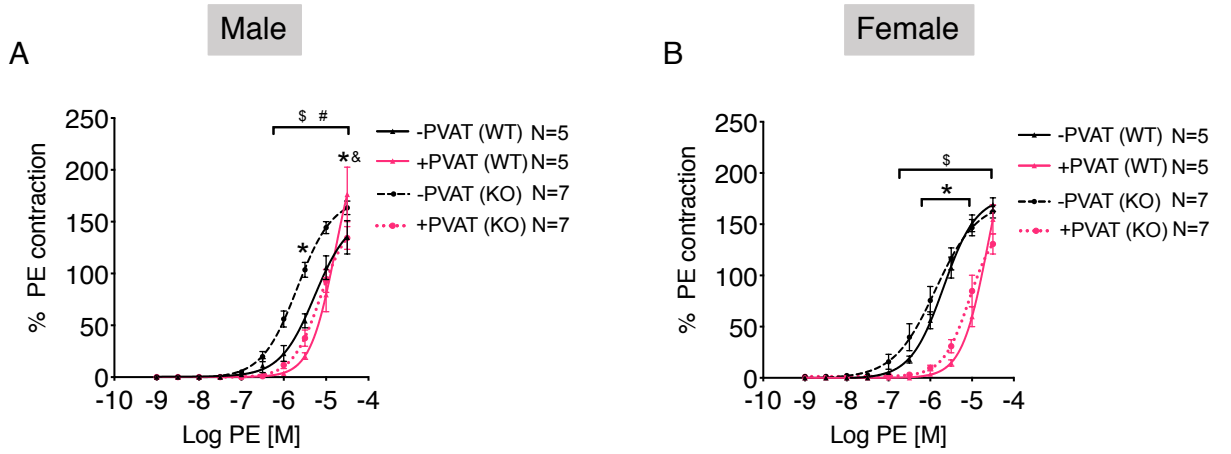


Figure 3.6 PVAT remained anti-contractile with short-term genetic depletion of endogenous IL-10

Cumulative PE-concentration response curves (10^{-8} to 10^{-4} M) in superior mesenteric arteries with/ without PVAT from 13 week-old male **(A)** and female **(B)** IL-10 KO and respective age-matched WT mice. Points represent means±SEM for the number of animals N. Two-way ANOVA with posthoc Tukey test was used and a $P<0.05$ was considered statistically significant. Symbols denote significant differences in contraction between: with and without PVAT in WT (*); with and without PVAT in KO ($); WT and KO in tissues without PVAT (#); WT and KO in tissues with PVAT (&).

Mouse strain	Tissue type	-logEC_{50} [M]	Maximum contraction (as %10 μM PE)	Initial PE contraction (mN)
WT (13 wk)	-PVAT	M: 5.3±0.14 F: 5.7±0.06	156.2±20.2 177.6±7.7	1.9±0.50 2±0.20
	+PVAT	M: 4.8±0.26 F: 4.6±0.30*	245.2±87 259.3±116.7	1.8±0.40 2.8±0.08
KO (13 wk)	-PVAT	M: 5.7±0.06 F: 5.9±0.10	172.8±7.2 172.4±12.7	2.6±0.30 2.9±0.40
	+PVAT	M: 5.1±0.09 F: 5.1±0.10$^{\$}$	153.8±15.4 152.5±19.0	2.8±0.50 3.5±0.70

Table 3.2 PVAT remained anti-contractile with short term genetic depletion of endogenous IL-10: pharmacological parameters

Pharmacological parameters of data presented in ***Figure 3.6***. A P< 0.05 by one-way ANOVA with Tukey's posthoc test was considered statistically significant. * and $^{\$}$ represent significant differences in –logEC_{50} values between –PVAT and +PVAT in WT females and KO females respectively; M=male; F=female.

The anti-contractile nature of PVAT was still intact with prolonged genetic depletion of endogenous IL-10 in mice

The anti-contractile function of PVAT was intact **(Table 3.3)** in response to PE-induced vasoconstriction in both sexes of WT mice at 26 weeks of age (older mice), as expected. However, surprisingly, the anti-contractile nature of PVAT was also still evident in both males **(Figure 3.7A)** and females **(Figure 3.7B)** of IL-10 KO mice at 26 weeks of age. However, there was a leftward shift in the KO *vs* WT PE-response curves in females only, in the absence of PVAT **(indicated by # in Table 3.3)**.

There were no differences in EC_{50} values of PE-induced contraction and maximal PE contractions between 13 and 26 weeks-old mice (-PVAT/ +PVAT, male/ female, WT/ KO; comparing **Tables 3.2 and 3.3**).

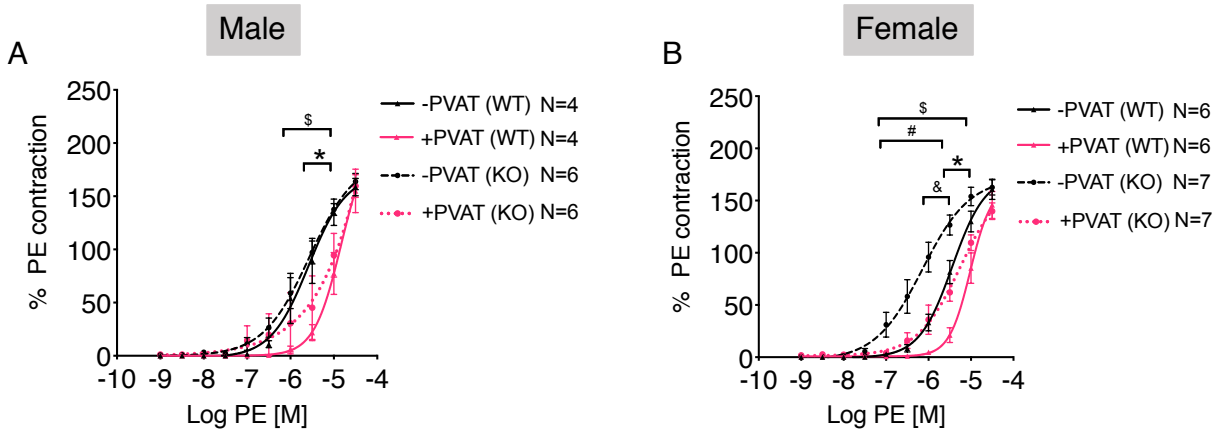


Figure 3.7 PVAT was anti-contractile even with prolonged genetic depletion of endogenous IL-10

Cumulative PE-concentration response curves (10^{-8} to 10^{-4} M) in superior mesenteric arteries with/ without PVAT from 26 week-old male **(A)** and female **(B)** IL-10 KO and respective age-matched WT mice. Points represent means±SEM for the number of animals N. Two-way ANOVA with posthoc Tukey test was used and a $P<0.05$ was considered statistically significant. Symbols denote significant differences in contraction between: with and without PVAT in WT (*); with and without PVAT in KO ($); WT and KO in tissues without PVAT (#); WT and KO in tissues with PVAT (&).

Mouse strain	Tissue type	-logEC_{50} [M]	Maximum contraction (as %10 μM PE)	Initial PE wake up (mN)
WT (26 wk)	-PVAT	M: 5.6±0.15 F: 5.4±0.08	171.2±20.2 174.6±12.3	2.6±0.50 2.3±0.60
	+PVAT	M: 4.8±0.25 F: 5.0±0.08*	209.7±66.7 169.7±16.5	1.8±0.30 3.4±0.60
KO (26 wk)	-PVAT	M: 5.6±0.14 F: 6.1±0.13#	185.7±18.6 171.7±13.1	3.1±0.70 3.3±0.60
	+PVAT	M: NC F: 5.2±0.20$	NC 172.6±26.8	3.5±1.00 5.7±1.20

Table 3.3 PVAT remained anti-contractile with prolonged genetic depletion of endogenous IL-10: pharmacological parameters

Pharmacological parameters of data presented in ***Figure 3.7***. A $P < 0.05$ by one-way ANOVA with Tukey's posthoc test was considered statistically significant. * and $ represent significant differences in –logEC_{50} values between –PVAT and +PVAT in WT females and KO females respectively; # denotes significant difference in –logEC_{50} values between WT and KO in –PVAT tissues in females. M=male; F=female; NC= not calculable (curve not plateaued and value could also not be estimated by GraphPad Prism 8.1)

Discussion

The current study tested the hypothesis that PVAT-derived IL-10 causes a direct vasorelaxation and/ or reduces vasoconstriction, thus contributing to the anti-contractile function of PVAT in health. **New and Novel:** This is the first study to report testing of the idea that PVAT-derived IL-10 may contribute to the anti-contractile nature of PVAT in health. This study has evaluated: (1) direct *vs* indirect effects of IL-10 on vascular tone regulation; (2) the anti-contractile nature of PVAT with short-term *vs* prolonged depletion of endogenous IL-10 in both sexes; and (3) vasoactive potential of IL-10 in two rodent species.

IL-10 as a direct vasorelaxant in health: a conundrum

Our results revealed that IL-10 neither caused direct vasorelaxation nor reduced vasoconstriction in arteries from healthy rats and mice **(Figures 3.3-3.7)**. This is in agreement with the finding that preincubation with 20 ng/mL of IL-10 did not affect the ability of rat cremaster artery to constrict to PE [Tan BB 2001]. But our finding is in disagreement with another published study [Kassan M 2011] that reported IL-10 to cause a direct vasorelaxation in mesenteric arteries (-PVAT, with endothelium) of 8 week-old male WT mice. These suggested the possibility of a species difference in the vasoactive potential of IL-10. Thus, we performed experiments using both the rat and the mouse in the current study. However, one reason for the controversy could be that while Kassan *et. al.* showed that 60 ng of IL-10 could directly vasorelax, the exact concentration used was not reported, thus making a direct comparison difficult. We used no higher than 100

ng/ mL because physiological range of endogenous IL-10 protein in serum of healthy human adults has been reported to be only 13 pg/mL [Kleiner G 2013]. In addition, the source and activity of the recombinant IL-10 protein were not reported in the Kassan 2011 paper.

The pleiotropic effects of IL-10 in health

IL-10 KO mice exhibit chronic systemic inflammation, colitis and frailty, all of which are associated with increased sensitivity to adrenergic agonists [Westbrook RM 2017]. Studies suggest that IL-10 KO mice have enhanced sensitivity to β-adrenergic signaling that is responsible for enhanced thermogenic gene induction *vs* WT [Rajbhandari P 2018]. In the current study, there was a leftward shift in the PE curve in tissue without PVAT from 26 week females (as determined by EC_{50} values in **Table 3.3**) *vs* respective WT, suggesting that the arteries of IL-10 KO mice in females only became more sensitive to the α1-adrenergic agonist, PE, with age. IL-10 KO females exhibited stronger immune responses elicited to infections *vs* IL-10 KO males [Guilbault C 2002]. Hence, although these sex-differences in the current study were unexpected, other sex differences in IL-10 KO mice are well known [Guilbault C 2002].

Previous findings have identified IL-10RA in mature adipocytes of inguinal white adipose tissue in mice [Rajbhandari P 2018]. This aligns with our finding that IL-10RA was present not just in the mesenteric arteries but also in adipocytes in PVAT in both mice and rats **(Figure 3.2).** This also suggests that IL-10 is likely not indirectly triggering

release of a substance from PVAT that could cause vasorelaxation. Bone marrow derived IL-10 acts on adipocytes *via* IL-10RA to limit thermogenesis and confer resistance to obesity [Rajbhandari P 2018]. IL-10 may also be involved in maintaining PVAT-immune homeostasis and insulin sensitivity in health. Future studies are needed to better understand the biological purpose of IL-10 in PVATs in health.

Could IL-10 be vasoprotective in disease?

The current study supports that the anti-contractile nature of PVAT does not depend on short-term or prolonged absence of endogenous IL-10 **(Figure 3.6, 3.7)**. One possibility may be that physiological compensation occurred when IL-10 was knocked out, such that the anti-contractile nature of PVAT was maintained even when systemic inflammation occurred in IL-10 KO mice. However, the use of exogenous IL-10 in the current study helped rule out physiological compensation and supported that IL-10 is not a vasorelaxant or an anticontractile factor. However, future *in vivo* IL-10 infusion studies in the IL-10 KO mice would be helpful.

The only known receptor for IL-10 is IL-10R. IL-10 homodimer binds to IL-10 RA, activates JAK/STAT and Akt cascades to exhibit immunoregulatory functions [Verma R 2016]. In mice, IL-10 limits multiple pathologies such as angiotensin-II induced hypertension, diabetes, endothelin-1-induced vascular injury. In these pathologies, IL-10 either rescues or aids endothelium-dependent vasorelaxation. It does so by increasing eNOS expression/ activity or inhibiting NADPH oxidase activity by p38 MAPK-dependent

mechanism [Lima VV 2016, Gunnett CA 2002, Giachini FR 2008, Kassan M 2011]. Hence, care was taken to preserve the endothelium in vessels that were exposed exogenous IL-10 in the present study. Additionally, treatment with the anti-inflammatory cytokine IL-10 impaired production of pro-inflammatory cytokines such as TNF-α, IL1-β and IL-6 [Kessler B 2017]. These together suggest that IL-10 may act like brakes to the immune system such that the vascular functions of IL-10 are visible and better appreciable when the immune system is actively responding to an insult *vs* in a healthy state.

Limitations

We acknowledge several limitations in the current study. First, the protein level of endogenous IL-10 in PVATs in health was not measured as its levels are below the detection limits of any reliable commercially available kit. However, studies have reported obese human white adipose tissue a source of IL-10 protein [Juge-Aubry CE 2005]. Thus, adipose tissue is capable of synthesizing IL-10 protein. Second, *in vivo* infusion of exogenous IL-10 or disease induction was not done, because the intent of this study was to understand if the biologically available IL-10 in PVATs was vasoactive (anti-contractile) in health. Third, the vasoactive potential of endogenous IL-10 in rat could not be evaluated due to lack of availability of an IL-10 KO rat. Fourth, different arteries for mice (superior mesenteric) and rats (mesenteric resistance) were used. This was purposeful and ensured confidence that IL-10 was not vasoactive, using two different types of blood vessels (superior mesenteric artery=surrounded by a mix of white and brown PVATs;

mesenteric resistance artery=surrounded by white PVAT) across two species. Finally, IL-10 receptor antagonists could not be used in the rats due to lack of availability of reliable antagonists.

Conclusion

IL-10 (mRNA) was present in the mesenteric PVAT and its receptor IL-10RA was present in the mesenteric arteries of both mice and rats. However, in both species, IL-10 did not cause a direct vasorelaxation and IL-10 in PVAT also did not cause a reduction in vasoconstriction directly or indirectly. Hence, IL-10 does not contribute to the anti-contractile nature of PVAT in health.

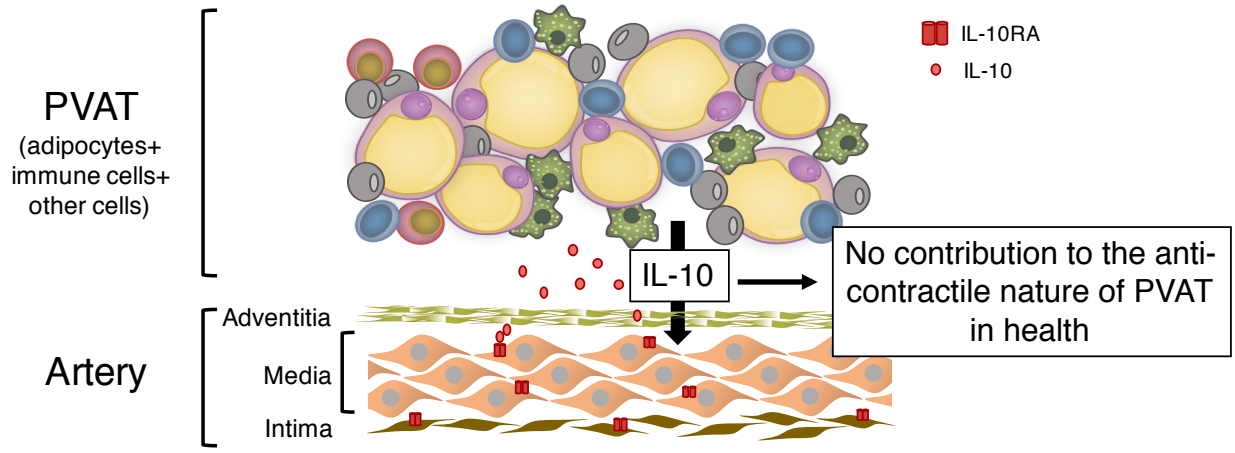


Figure 3.8 Summary (Graphical abstract)
PVAT-derived IL-10 is neither a direct vasorelaxant nor an anticontractile factor in healthy rodents.

REFERENCES

REFERENCES

Giachini FR, Zemse SM, Carneiro FS, Lima VV, Carneiro ZN, Callera GE, Ergul A, Webb RC and Tostes RC. (2008). Interleukin-10 attenuates vascular responses to endothelin-1 via effects on ERK1/2-dependent pathway. *Am J Physiol Heart Circ Physiol.* 296(2):H489-96. doi: 10.1152/ajpheart.00251.2008. Epub 2008 Dec 12.

Guilbault C, Stotland P, Lachance C, Tam M, Keller A, Thompson-Snipes L, Cowley E, Hamilton TA, Eidelman DH, Stevenson MM and Radzioch D (2002) Influence of gender and interleukin-10 deficiency on the inflammatory response during lung infection with Pseudomonas aeruginosa in mice. Immunology.;107(3):297-305. doi: 10.1046/j.1365-2567.2002.01508.x.

Gunnett CA, Heistad DD and Faraci FM. (2002). Interleukin-10 protects nitric oxide-dependent relaxation during diabetes: role of superoxide. *Diabetes.* 51(6):1931-7. doi: 10.2337/diabetes.51.6.1931.

Juge-Aubry CE, Somm E, Pernin A, Alizadeh N, Giusti V, Dayer JM and Meier CA. (2005) Adipose tissue is a regulated source of interleukin-10. *Cytokine*. 21;29(6):270-4. doi: 10.1016/j.cyto.2004.10.017.

Kassan M, Galan M, Partyka M, Trebak M and Matrougui K. (2011) Interleukin-10 released by CD4(+)CD25(+) natural regulatory T cells improves microvascular endothelial function through inhibition of NADPH oxidase activity in hypertensive mice. *Arterioscler Thromb Vasc Biol.* 31(11):2534-42. doi: 10.1161/ATVBAHA.111.233262.

Kessler B, Rinchai D, Kewcharoenwong C, Nithichanon A, Biggart R, Hawrylowicz CM, Bancroft GJ and Lertmemongkolchai G. (2017) Interleukin 10 inhibits pro-inflammatory cytokine responses and killing of Burkholderia pseudomallei. *Sci Rep.* 20;7:42791. doi: 10.1038/srep42791.

Kleiner G, Marcuzzi A, Zanin V, Monasta L and Zauli G. (2013). Cytokine levels in the serum of healthy subjects. *Mediators Inflamm.* 2013:434010. doi: 10.1155/2013/434010.

Kumar RK, Jin Y, Watts SW and Rockwell CE. (2020). Naïve, Regulatory, Activated, and Memory Immune Cells Co-exist in PVATs That Are Comparable in Density to Non-PVAT Fats in Health. *Front Physiol.* 11;11:58. doi: 10.3389/fphys.2020.00058.

Lima VV, Zemse SM, Chiao CW, Bomfim GF, Tostes RC, Webb RC and Giachini FR. (2016). Interleukin-10 limits increased blood pressure and vascular RhoA/Rho-kinase signaling in angiotensin II-infused mice. *Life Sci.* 15;145:137-43. doi: 10.1016/j.lfs.2015.12.009.

Löhn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M and Sharma AM. (2002). Periadventitial fat releases a vascular relaxing factor. *FASEB J.* 16(9):1057-63. doi: 10.1096/fj.02-0024com.

Ohkawa F, Ikeda U, Kanbe T, Kawasaki K and Shimada K. (1995). Effects of inflammatory cytokines on vascular tone. *Cardiovasc Res.* 30(5):711-5.

Rajbhandari P, Thomas BJ, Feng AC, Hong C, Wang J, Vergnes L, Sallam T, Wang B, Sandhu J, Seldin MM, Lusis AJ, Fong LG, Katz M, Lee R, Young SG, Reue K, Smale ST and Tontonoz P. (2018). IL-10 Signaling Remodels Adipose Chromatin Architecture to Limit Thermogenesis and Energy Expenditure. *Cell.* 172(1-2):218-233.e17. doi: 10.1016/j.cell.2017.11.019.

Saraiva M and O'Garra A. (2010). The regulation of IL-10 production by immune cells. *Nat Rev Immunol.* 10(3):170-81. doi: 10.1038/nri2711.

Sikka G, Miller KL, Steppan J, Pandey D, Jung SM, Fraser CD 3rd, Ellis C, Ross D, Vandegaer K, Bedja D, Gabrielson K, Walston JD, Berkowitz DE and Barouch LA. (2013) Interleukin 10 knockout frail mice develop cardiac and vascular dysfunction with increased age. *Exp Gerontol.* 48(2):128-35. doi: 10.1016/j.exger.2012.11.001.

Soltis EE and Cassis LA. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. Clin Exp Hypertens A. 13(2):277-96. doi:10.3109/10641969109042063.

Szasz T, Bomfim GF and Webb RC. (2013). The influence of perivascular adipose tissue on vascular homeostasis. *Vasc Health Risk Manag.* 9:105-16. doi: 10.2147/VHRM.S33760.

Tan BB, Prewitt RL and Britt LD. (2001). Interleukin-10 prevents loss of tone of rat skeletal muscle arterioles exposed to endotoxin. *J Surg Res.* 100(1):110-5. doi: 10.1006/jsre.2001.6105.

Verlohren S, Dubrovska G, Tsang SY, Essin K, Luft FC, Huang Y and Gollasch M. (2004). Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. *Hypertension.* 44(3):271-6. doi: 10.1161/01.HYP.0000140058.28994.ec.

Verma R, Balakrishnan L, Sharma K, Khan AA, Advani J, Gowda H, Tripathy SP, Suar M, Pandey A, Gandotra S, Prasad TS and Shankar S. (2016). A network map of Interleukin-10 signaling pathway. *J Cell Commun Signal.* 10(1):61-7. doi: 10.1007/s12079-015-0302-x.

Virdis A, Duranti E, Rossi C, Dell'Agnello U, Santini E, Anselmino M, Chiarugi M, Taddei S and Solini A. (2015). Tumour necrosis factor-alpha participates on the endothelin-

1/nitric oxide imbalance in small arteries from obese patients: role of perivascular adipose tissue. *Eur Heart J.* 36(13):784-94. doi: 10.1093/eurheartj/ehu072.

Westbrook RM, Yang HL, Langdon JM, Roy CN, Kim JA, Choudhury PP, Xue QL, di Francesco A, de Cabo R and Walston J. (2017). Aged interleukin-10tm1Cgn chronically inflamed mice have substantially reduced fat mass, metabolic rate, and adipokines. *PLoS One.* 12(12):e0186811. doi: 10.1371/journal.pone.0186811.

Withers SB, Forman R, Meza-Perez S, Sorobetea D, Sitnik K, Hopwood T, Lawrence CB, Agace WW, Else KJ, Heagerty AM, Svensson-Frej M and Cruickshank SM. (2017). Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. *Sci Rep.* 7:44571. doi: 10.1038/srep44571.

CHAPTER 4

Sex-Differences in T cells and Macrophages in Perivascular Adipose Tissues Precede High-Fat Diet-induced Hypertension

Abstract

Perivascular adipose tissue (PVAT) may connect adiposity to hypertension because of its vasoactive functions and proximity to blood vessels. Both sexes of Dahl S rat become equally hypertensive when fed a high fat diet (HFD). We hypothesized that immune cell changes in PVATs precede the development of HFD-induced hypertension. Further, both sexes would have similar immune cell composition in PVATs with the development and progression of hypertension. Male and female Dahl S rats were fed a regular (10% calories from fat; CD) diet or a HFD (60%) from weaning. PVATs from around the thoracic aorta (APVAT) and small mesenteric vessels (MRPVAT) were harvested at 10 weeks (pre-hypertensive), 17 weeks (onset) or 24 (hypertensive) weeks on diet. RNA-sequencing in MRPVAT at 24 weeks indicated sex-differences with HFD (> CD) and diet-differences in males (> females). The top (2 out of 7) immune processes with the maximum number of differentially expressed genes were associated with immune effector processes and leukocyte activation. Macrophages and T cells (and their activation status), neutrophils, mast, B and NK cells were measured by flow cytometry. Sex-specific changes in the number of CD4 memory T cells (males > females) and M2-like macrophages (females > males) in PVATs occur with a HFD before hypertension develops. Sex-differences became more prominent with the development and progression of hypertension, driven by the diet (HFD > CD). These findings suggested that though the magnitudes of increased blood pressure were equivalent in both sexes, how they reached that magnitude was immunologically different in the PVATs.

Introduction

Over 45% of all adults in the U.S. have hypertension and up to 75% of those with hypertension are obese [Whelton PK 2017, Hall JE 2015]. Both in obesity and hypertension, perivascular adipose tissue (PVAT), the fat around the peripheral blood vessels, loses its protective anti-contractile function partly due to local inflammation and oxidative stress [Szasz T 2013]. Due to PVAT's proximity to blood vessels and its functional capabilities, PVAT may be one of the links connecting obesity to hypertension [Noblet JN 2015, Gollasch M 2012].

PVAT contains multiple types of innate and adaptive immune cells in both sexes of healthy Sprague Dawley rats [Kumar RK 2020]. T cells may play a causal role in the development of hypertension in high salt-induced hypertension in male Dahl salt-sensitive (Dahl S) rats [Mattson DL 2013, De Miguel C 2010]. APVAT (PVAT around the thoracic aorta) inflammation and infiltration of macrophages and T cells precede atherosclerotic plaque development (another cardiovascular disease) [Skiba DS 2017]. This led us to **hypothesize that immune cell infiltration in PVATs precedes the development of high fat diet (HFD)-induced hypertension.** These immune cells in PVATs may directly (*via* cytokines) or indirectly (*via* other cells in PVATs/ adipokines) alter vascular tone, thus leading to hypertension [Nosalski R 2017, Almabrouk TAM 2018, Small HY 2019].

Sex-differences in PVAT functions in obesity-induced hypertension (in association with resistin and estrogen) have been previously reported [Small HY 2019, Taylor LE

2016]. Although T cell and macrophage counts increase in APVAT with AngII-hypertension in male mice, the occurrence of sex-differences in the immune system in PVATs with the development and progression of hypertension is little known [Nosalski R 2017, Mikolajczyk TP 2016]. Both males and females of the Dahl S rat strain demonstrated increased adiposity and became equally hypertensive when fed a HFD [Fernandez R 2018]. Hence, we also **hypothesized that both sexes of Dahl S rats would have similar immune cell composition and activation status in PVATs prior to and with the development and progression of HFD-induced hypertension.** Two types of PVATs in each sex were studied- MRPVAT (white adipose tissue-type PVAT around small mesenteric resistance vessels) and APVAT (brown adipose tissue-type around the thoracic aorta). Bulk RNA sequencing (MRPVAT) and flow cytometry (both PVATs) were employed to test the hypotheses. In support of our first hypothesis, we discovered that changes in specific subsets of T cells and macrophages in PVATs precede the development of HFD-induced hypertension. But contrary to our second hypothesis, clear sex-differences (females > males) in T cell and macrophage subsets were observed in both PVATs (MRPVAT > APVAT) prior to, and with the development and progression of hypertension. Although it is beyond the hypothesis and scope of this paper, the vascular implications of these immune changes in PVATs are important next steps.

Materials and Methods

Animals

Animal maintenance and experimental protocols were approved by the MSU Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for Animal Care and Use of Laboratory Animals (2011). Male and female Dahl salt sensitive rats were purchased from Charles River Laboratory at 3 weeks of age and were randomly fed a normal salt (0.3% NaCl) CD (D12450J, kcal from saturated fat 10%+ carbohydrate, 70%+ protein 20%) or a high fat diet (D12492, kcal from saturated fat 60%+ carbohydrate 20%+protein 20%; Research Diets, Inc). Animals were maintained on a 12/12 light/dark cycle at 22-25 °C, fed on *ad-libitum f*or 10, 17 or 24 weeks. Blood pressures were measured by tail cuff on multiple groups of rats that were used in the current study **(Figure 4.1)**. These measures correlate with blood pressures measured by radiotelemetry, as previously published by our group [Fernandez R 2018].

Bulk mRNA sequencing: sample processing of MRPVAT

Males and females fed a CD or HFD for 24 weeks (5 per group) were used. RNA was extracted from MRPVAT using the Quick RNA MiniPrep kit (Cat# R1054) that includes a DNase step to remove genomic DNA. Purity, concentration, and integrity of mRNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE USA) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA USA). All samples had a 260:280 nm ratio between 1.9 and 2.1 and RNA integrity number ≥ 7.5. Novogene Corporation Inc (Sacramento, CA, USA) performed

Quality Control for the RNA isolation that occurred at Michigan State University. Comparative libraries were constructed, library quality control was certified and sequencing was conducted.

Flow cytometry

Prior to all dissections, the overnight fasted rats were anesthetized with sodium pentobarbital (60-80 mg/kg, i.p.) and death was assured by creating a bilateral pneumothorax. MRPVAT and APVAT were dissected from blood vessels and weighed at 10, 17 or 24 weeks of diet, from each rat. All the immune cell populations were quantified from the same fat samples to phenotype the activation, differentiation and/or polarization of various immune populations.

Stromal vascular fraction (SVF) isolation from PVAT

The PVATs were minced with scissors and collagenase (1 mg/ ml; type-I, Cat # LS004196) digested at 37 ºC for about 1 hour. The cell suspensions were sequentially filtered through 100 μm and 40 μm filters. The flow through contained cells less than 40 μm, thus eliminating the adipocytes. Upon washing with flow buffer and centrifugation at 300 rcf for 5 minutes, a cell pellet containing the SVF, was obtained.

Surface labeling of immune cells

Ammonium-chloride-potassium red blood cell lysis buffer (400 μL; Cat # 10-548E) was added to the SVF pellet, gently pipette-mixed and incubated on ice for 2 minutes to

destroy red blood cells. The red blood cell-lysed SVF was washed twice with flow buffer (PBS+ 10% FBS) and labeled with fluorescent antibodies (30 minutes incubation) after blocking Fc receptors with purified anti-CD32 antibody (10 minutes). Intracellular staining to identify Tregs was performed as previously described [Kumar RK 2020]. Viability was measured using Zombie-aqua dye (1: 1000 in dPBS, Cat# 77143). All flow cytometry assays were performed using an Attune NxT acoustic focusing cytometer from Life Technologies. Antibody and panel design details are in **Tables 2.1 and 2.2**.

Data presentation and statistics

Bulk mRNA sequencing: mRNA isolated (at Michigan State University) from MRPVAT of both sexes fed a CD or a HFD were transcriptomic sequenced *via* Illumina platform by Novogene (Sacramento, CA, USA). After quality control, the paired-end clean reads were mapped onto reference genome using STAR (v2.5). HTSeq (v 0.6.1) was used to count the read numbers of each gene. The gene read counts matrix was fed into DESeq2 (v 1.24.0) to identify the differentially expressed genes (DEGs). To identify the outliers in the samples, principal component analysis was done with 10 principal components and the rotation matrix was used to calculate the mahalanobis distance among the samples. To improve the stability of data, median (instead of mean) and mean absolute deviation around the median were used. For data analysis in R, tidyverse (v 1.3.0) and Rattus.norvegicus (v 1.3.1) libraries were used. For transcriptome analyses, the p-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate. Genes with an adjusted P-value <0.05 and absolute fold

change >1, identified by DESeq2 were assigned as DEGs. To identify which immune system processes were most affected by a HFD or sex-differences, seven interpretable immune-system related Gene Ontology terms were chosen and the number of DEGs in each term was reported.

Flow cytometry studies: Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software Inc., LaJolla CA). Two-way ANOVA with Tukey's multiple comparison test was used to determine statistical significance. A $P<0.05$ was considered statistically significant. Thus, two types of comparisons along the time course study were possible: between the sexes within each diet (with male as base) and between the diets within each sex (with CD as base).

Results

The magnitude and timeline of hypertension development with a high fat diet is similar in male and female Dahl S rat

Both male and female Dahl S rats fed a HFD, progressively developed hypertension. At 10 weeks of diet, the mean arterial pressures (MAP) was similar between CD and HFD-fed animals, independent of sex. At 17 weeks of diet, HFD-fed rats had significantly increased MAP *vs* CD in the respective sexes. At 24 weeks, MAP in HFD-rats were significantly greater than their respective CD-fed rats in both sexes. However, the magnitude and timecourse of hypertension development was similar between the male and the female Dahl S rats **(Figure 4.1)**.

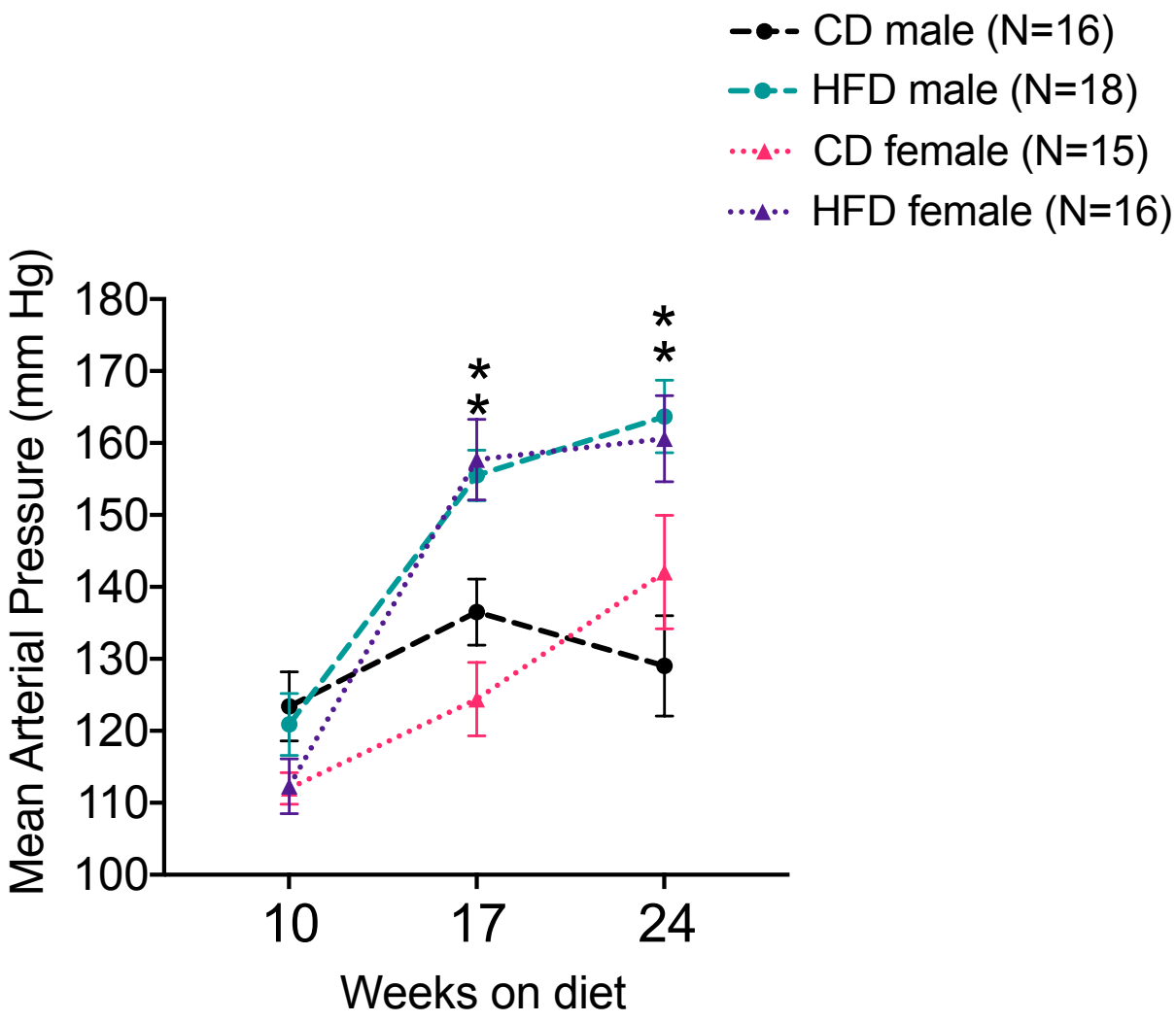


Figure 4.1 Development and progression of hypertension with high-fat feeding Comparison of mean arterial pressure (MAP) measured by tail cuff at 10, 17 and 24 weeks of control (CD) or high-fat (HFD) diet, in both males and females. Data are presented as mean+SEM. * denotes significant differences between CD and HFD within a sex at a given time point.

Bulk RNA-sequencing reveals sex-differences with HFD (> CD) and diet-differences in males (> females), in MRPVAT after 24 weeks of diet

RNA sequencing was performed on MRPVAT from male and female Dahl S rats on a CD or HFD for 24 weeks, a time point at which both sexes on a HFD were equally hypertensive *vs* their respective CD rats **(Figure 4.1)**. Principal components analysis (PCA) revealed the clustering patterns of the samples. G75FHF5 and G73FHF3 were statistical outliers. Hence, the remaining analyses excluded these animals. A clear separation of males from females along the PC2 axis suggested sex-differences in the transcriptomics **(Figure 4.2A)**. As this study focuses on the immune cells in PVAT, only the immune system related DEGs were chosen. HFD induced more sex-differences *vs* CD (155 *vs* 29 unique DEGs). There were 51 shared DEGs between the diets, representing diet/ blood pressure-independent (age-related) sex-differences in gene expression **(Figure 4.2B)**. The top two (with the most number of DEGs) immune processes associated with the sex-differences influenced solely by the HFD-induced hypertension ('HFD unique') were immune effector processes and leukocyte activation **(Figure 4.2C)**. There were a greater number of diet-induced differences in males (115 DEGs) *vs* females (6 DEGs). Also, there were no shared DEGs between the sexes, suggesting that the DEGs are strongly sex-dependent **(Figure 4.2D)**. The top two unique immune processes associated with the diet-differences in males ('male unique') were also immune effector processes and leukocyte activation. A similar conclusion could not be drawn for the females given the low number of unique DEGs **(Figure 4.2E)**.

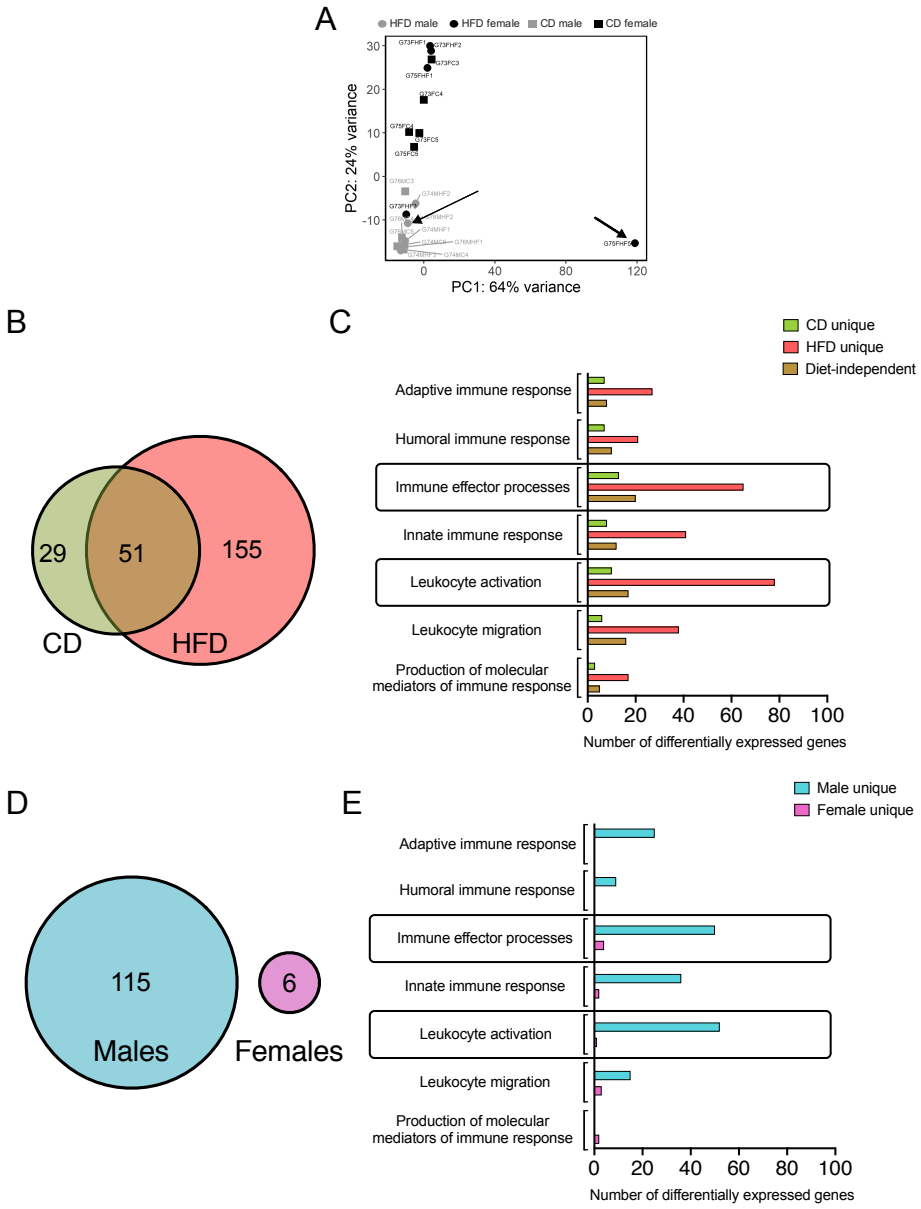


Figure 4.2 Bulk RNA-sequencing of MRPVAT from both sexes after 24 weeks on diet

Bulk RNA sequencing was performed on MRPVAT at 24 weeks on diet, from both sexes. Principal Component Analysis contained all the 20 samples (5 sample/ diet/ sex) **(A)** Venn diagram with the number of immune-related differentially expressed genes (DEGs) with respect to sex differences with HFD *vs* CD **(B)** and the specific immune system processes affected **(C)**. Venn diagram with the number of immune-related DEGs with respect to diet differences in males *vs* females **(D)** and the specific immune system processes affected **(E)**. G#: group number; M: male, F: female; C- CD; HF- HFD.

Overall changes in innate and adaptive immune profiles in PVATs with progression of hypertension in males *vs* females

Six primary immune cell types: T cells (CD4, CD8), B cells, macrophages, NK cells, mast cells and neutrophils were quantified in MRPVAT **(Figure 4.3; panels A-C)** and APVAT **(Figure 4.3; panels D-F),** in both sexes after being fed a control diet (CD) or high fat diet (HFD) for **10, 17 or 24 weeks**. Corresponding blood pressure measures in **Figure 4.1**. In the following figures, comparisons between the sexes within each diet (with male as base) and between the diets within each sex (with CD as base) were made.

MRPVAT: At 10 weeks, females on a CD had a greater number of CD4 T cells and sum of total immune cell counts *vs* CD males and HFD females **(Figure 4.3A)**. At 17 weeks, females on a HFD had a greater sum of total immune cell counts *vs* CD females **(Figure 4.3B)**. At 24 weeks, females on a HFD had a lesser number of CD4 T cells vs CD females **(Figure 4.3C)**. Although no diet-differences were observed in males, this finding is partially consistent with RNA seq data that diet-differences occur in females.

APVAT: There were no differences in the primary immune cell types between the sexes or diets **(Figure 4.3D)**. At 17 weeks, females had reduced numbers of CD4, CD8 and sum of total immune cell counts *vs* males, independent of the diet **(Figure 4.3E)**. At 24 weeks, females on a CD continued to have a lesser number of CD4 T cells *vs* CD males **(Figure 4.3F)**. Males had no overall immune cell changes in PVATs at all time

points of study. Also, APVAT in general has 3-7 times greater density of immune cells *vs* MRPVAT.

T cells (dominant adaptive immune cell type in PVATs) and macrophage (dominant innate immune cell type in PVATs) became the focus to sub-characterize them as active/ naïve/ memory/ effector phenotypes.

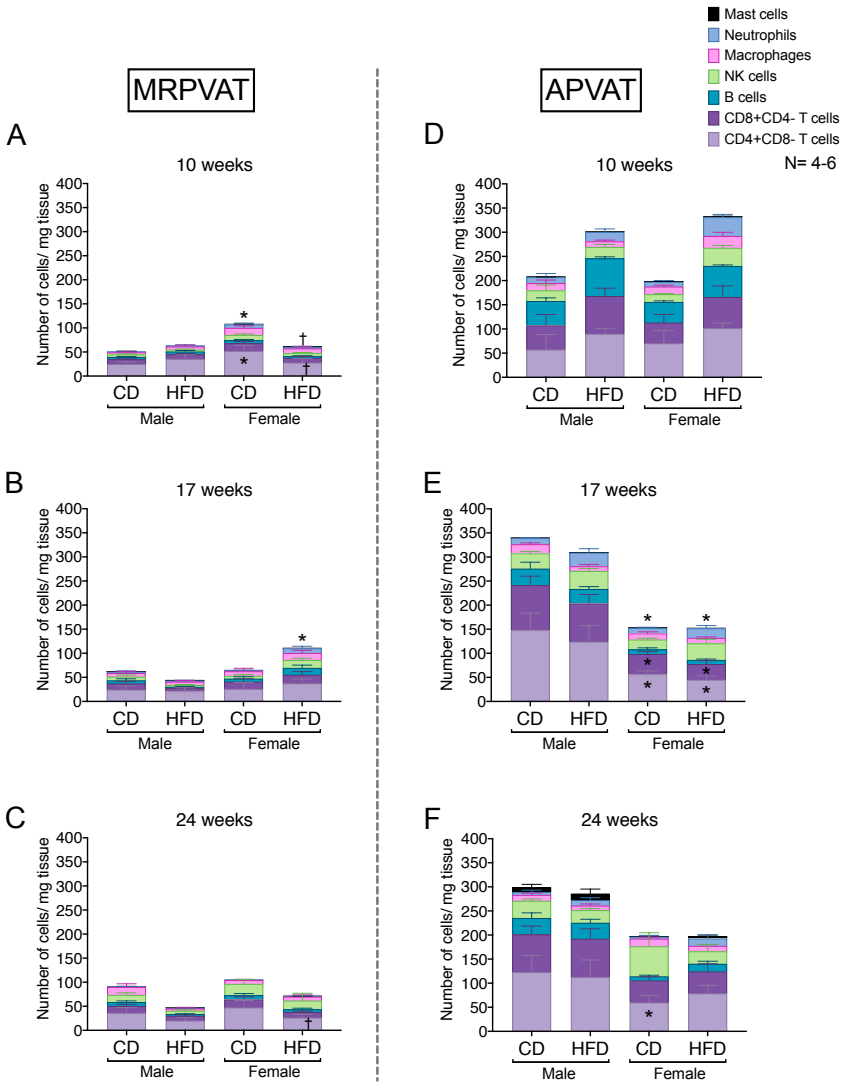


Figure 4.3 Overall snapshot of adaptive and innate immune cells in PVATs along the time-course of development and progression of hypertension

Immunophenotyping of mesenteric resistance PVAT (MRPVAT) and thoracic aortic PVAT (APVAT) in male and female Dahl S rats fed a control diet (CD) or a high fat diet (HFD) for 10 **(A,D)**, 17 **(B,E)** or 24 **(C,F)** weeks. Bars represent means±SEM for the absolute cell counts of each immune cell type, normalized to tissue weight (in milligram). Number of animals is indicated by N. A $P<0.05$ by 2-way ANOVA with Tukey's test for multiple comparison, was considered statistically significant. * and † inside the stacks in the graph represent a significant difference in the specific stack of immune cells between the sexes within each diet and between the diets within each sex respectively. * and † outside the stacks in the graph represent a significant difference in the sum total of immune cells between the sexes within each diet and between the diets within each sex respectively.

At 10 weeks, CD4+ memory T cell counts decrease in HFD females (*vs* males) in MRPVAT while M1-like macrophage numbers increase in HFD females (*vs* males) in APVAT before hypertension

Subtypes of CD4 T cells, CD8 T cells and macrophages were quantified in MRPVAT **(Figure 4.4; panels A-C)** and APVAT **(Figure 4.4; panels D-F)**, after **10 weeks** on a CD or HFD in both sexes by flow cytometry. CD4 and CD8 T cells were classified as recently activated (expressing early marker CD25 or late marker OX-40), regulatory (Foxp3+), antigen-experienced/ memory (CD45RC-) or naïve (CD45RC+). CD68+ macrophages were classified as M1-like (expressing CD86 and MHCII) or M2-like (CD163+).

MRPVAT: Females on a CD had a greater number of CD4 memory T cells *vs* males on a CD, while females on a HFD contained a reduced number of CD4 memory T cells *vs* HFD males and CD females **(Figure 4.4A)**. Females on a HFD had reduced CD8 T cell counts *vs* females on a CD **(Figure 4.4B)**. Females on a CD had a greater number of M1-like macrophages *vs* males on a CD, while CD females had a greater number of both M1-like and M2-like macrophages *vs* HFD females **(Figure 4.4C)**.

APVAT: There were no differences in CD4 and CD8 T cell subtypes between the sexes or diets **(Figure 4.4D, 4.4E)**. Females on a HFD had greater number of M1-like macrophages *vs* males on a HFD **(Figure 4.4F)**.

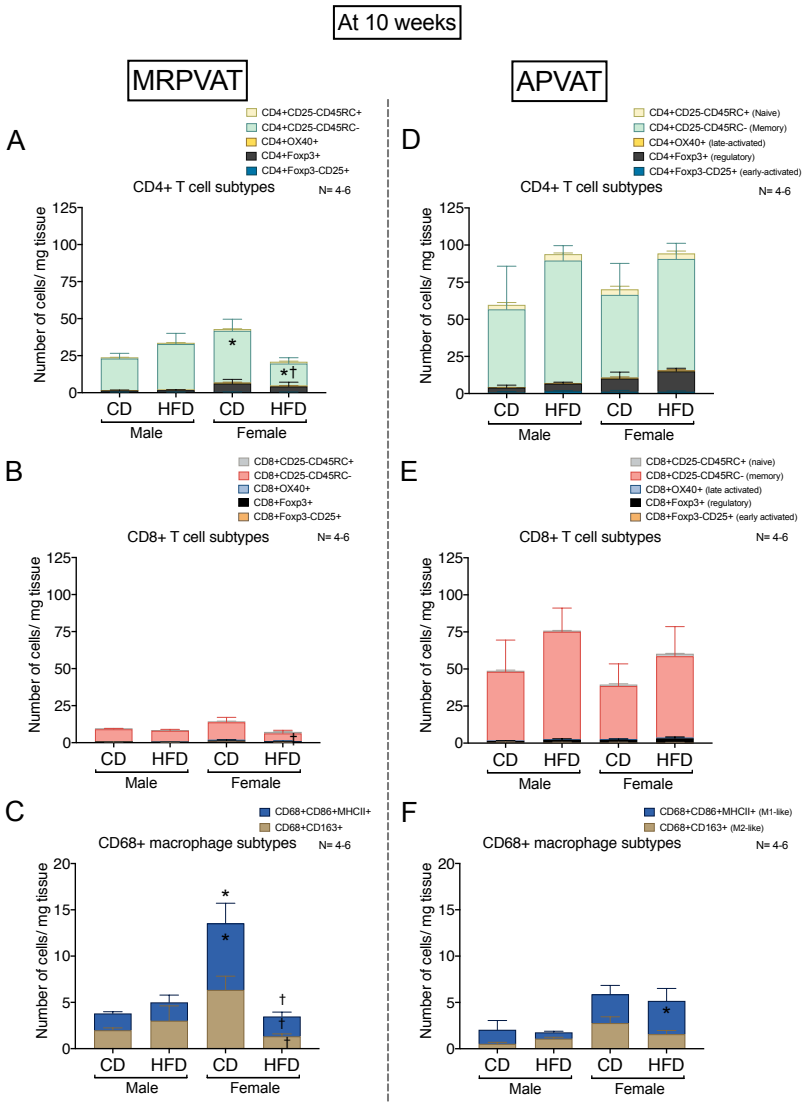


Figure 4.4 Sex-differences in T cell and macrophage subtypes in PVATs before the development of hypertension

Subtypes of CD4+, CD8+ T cells and CD68+ macrophages in MRPVAT and APVAT of both sexes at **10 weeks on diet (before hypertension)** were quantified by flow cytometry. Subtypes of CD4+: CD4+CD25+, CD4+OX40+, CD4+Foxp3+, CD4+CD45RC- and CD4+CD45RC+ cells **(A,D)**, CD8+ T: CD8+CD25+, CD8+OX40+, CD8+Foxp3+, CD8+CD45RC- and CD8+CD45RC+ cells **(B,E)** and CD68+ CD68+CD163+ and CD68+CD86+MHCII+ macrophages **(C,F)** were measured. Bars represent means±SEM for the absolute cell counts of each immune cell type, normalized to tissue weight (in milligram). Number of animals is indicated by N. A $P<0.05$ by 2-way ANOVA with Tukey's test for multiple comparison, was considered statistically significant. * and † inside/ outside the stacks in the graph represent a significant difference in the specific stack of immune cells/ sum total of immune cells between the sexes within each diet and between the diets within each sex respectively.

At 17 weeks, immune composition changes occur with the onset of hypertension that is mostly diet-dependent in MRPVAT (females> males) and diet-independent in APVAT

Subtypes of CD4 T cells, CD8 T cells and macrophages were quantified in MRPVAT **(Figure 4.5; panels A-C)** and APVAT **(Figure 4.5; panels D-F)**, after **17 weeks** on a CD or HFD in both sexes by flow cytometry.

MRPVAT: Females on a HFD had a greater number of CD4 memory T cells *vs* HFD males and CD females **(Figure 4.5A)**. Females on a HFD had a greater number of CD8 memory T cells *vs* HFD males **(Figure 4.5B)**. Females on a HFD had a greater number of M2 macrophages *vs* HFD males **(Figure 4.5C)**.

APVAT: Females had a lesser number of CD4 and CD8 memory T cells *vs* males independent of the diet **(Figure 4.5D, 4.5E)**. Females on a HFD had a greater number of M2-like macrophages *vs* HFD males. Males on a HFD had a reduced M1 macrophage count *vs* CD males **(Figure 4.5F)**.

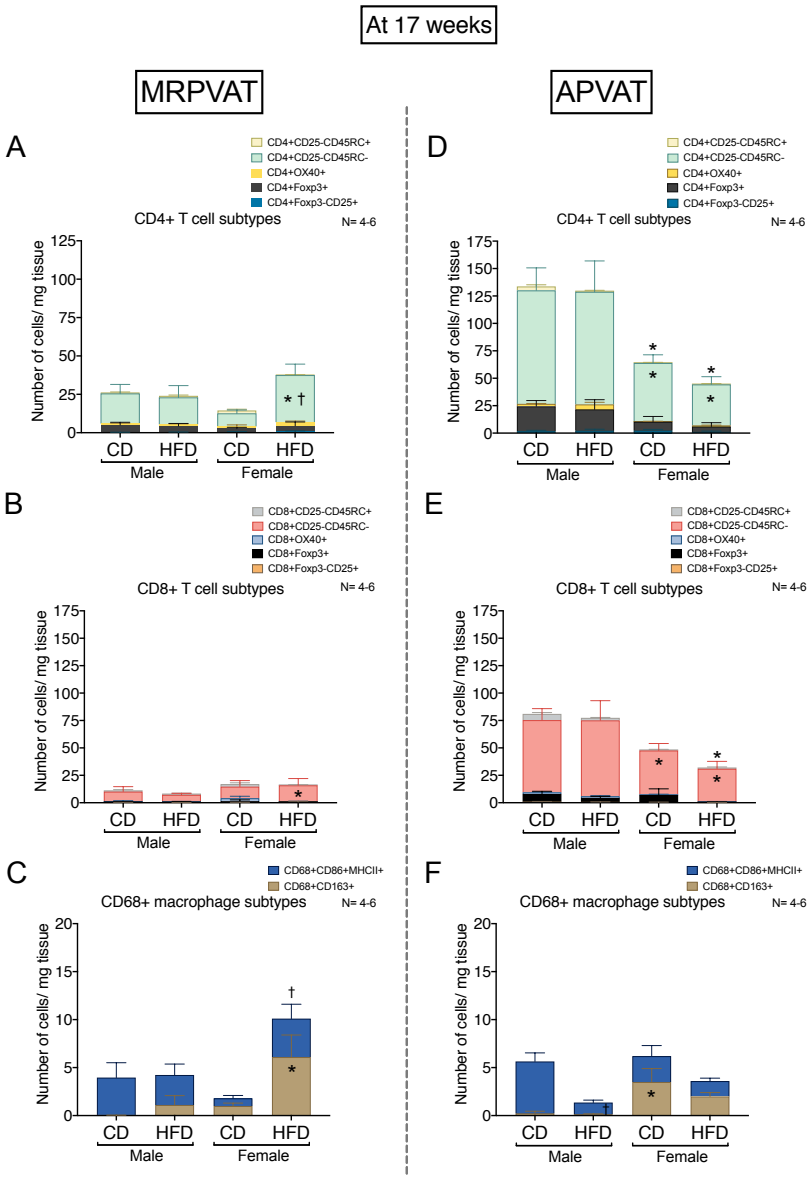


Figure 4.5 Sex-differences in T cell and macrophage subtypes in PVATs peak at 17 weeks on diet, with the onset of hypertension

Subtypes of CD4+, CD8+ T cells and CD68+ macrophages in MRPVAT and APVAT of both sexes at **17 weeks on diet (onset of hypertension with HFD)** were quantified by flow cytometry. Subtypes of CD4+ T cells **(A,D)**, CD8+ T cells: cells **(B,E)** and CD68+ macrophages **(C,F)** were measured. Bars represent means±SEM for the absolute cell counts of each immune cell type, normalized to tissue weight (in milligram). Number of animals is indicated by N. A $P<0.05$ by 2-way ANOVA with Tukey's test for multiple comparison, was considered statistically significant. * and † inside/ outside the stacks in the graph represent a significant difference in the specific stack of immune cells/ sum total of immune cells between the sexes within each diet and between the diets within each sex respectively.

At 24 weeks, diet-dependent immune composition changes with hypertension in PVATs begin to occur in males but diminish in females

CD4 T cell, CD8 T cell and macrophage subtypes were quantified in MRPVAT **(Figure 4.6; panels A-C)** and APVAT **(Figure 4.6; panels D-F)**, after **24 weeks** on a CD or HFD in both males and females by flow cytometry.

MRPVAT: HFD fed rats had a lesser number of CD4 memory T cells *vs* CD fed rats independent of sex **(Figure 4.6A)**. There were no differences in CD8 T cell subtypes between the sexes or diets **(Figure 4.6B)**. Males on a HFD had a lesser number of M1-like macrophages *vs* CD males **(Figure 4.6C)**. This is consistent with the RNA seq results that diet-differences were prominent in males *vs* females.

APVAT: Females on a CD had a reduced number of CD4 memory T cells *vs* males on a CD **(Figure 4.6D)**. Females had a reduced number of CD8 memory T cells *vs* males, independent of diet **(Figure 4.6E)**. There were no differences in macrophage subtypes between the sexes or diets **(Figure 4.6F)**.

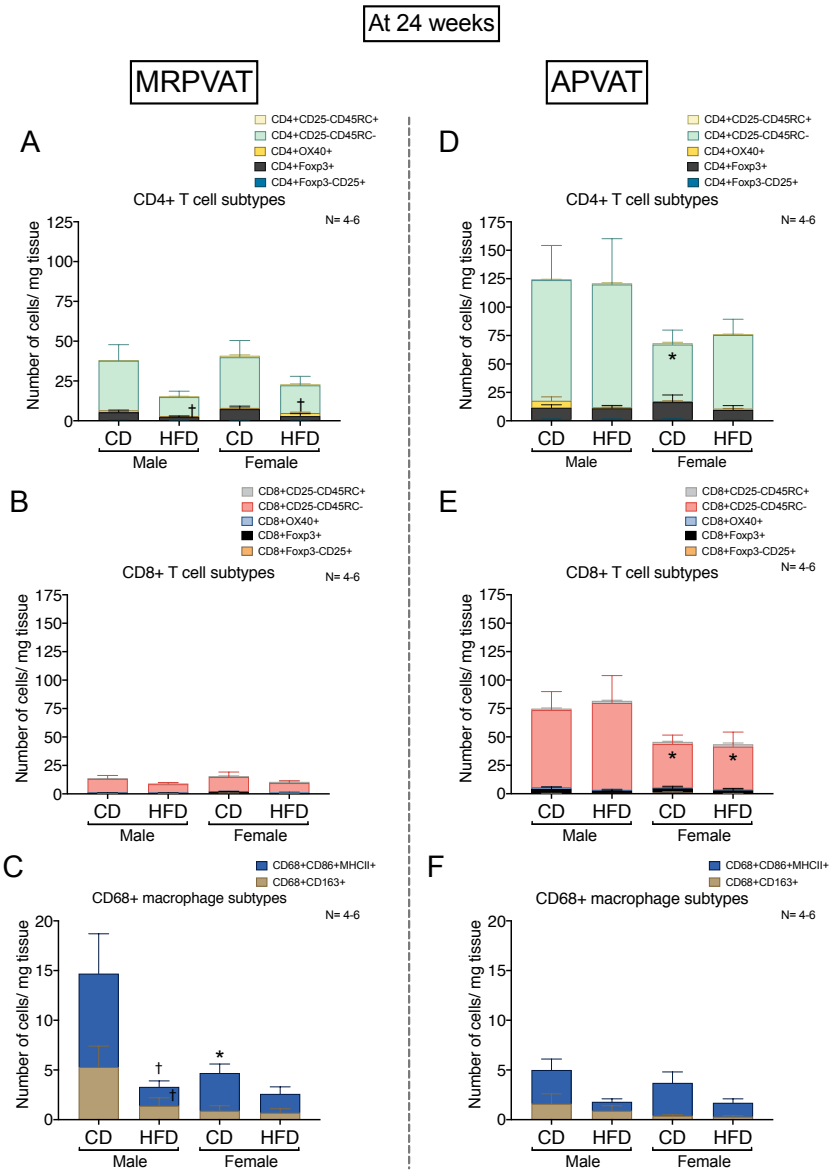


Figure 4.6 Sex-differences in immune cells diminish with the progression of hypertension after 24 weeks on diet

Subtypes of CD4+, CD8+ T cells and CD68+ macrophages in MRPVAT and APVAT of both sexes at **24 weeks on diet (hypertensive with HFD)** were quantified by flow cytometry. Subtypes of CD4+ T cells **(A,D)**, CD8+ T cells: cells **(B,E)** and CD68+ macrophages **(C,F)** were measured. Bars represent means±SEM for the absolute cell counts of each immune cell type, normalized to tissue weight (in milligram). Number of animals is indicated by N. A $P<0.05$ by 2-way ANOVA with Tukey's test for multiple comparison, was considered statistically significant. * and † inside/ outside the stacks in the graph represent a significant difference in the specific stack of immune cells/ sum total of immune cells between the sexes within each diet and between the diets within each sex respectively.

Females on a HFD have higher expression of M2-like macrophage (at 10 weeks, pre-hypertensive) and regulatory T cell markers (at 17 weeks, onset of hypertension) in PVATs *vs* HFD males

Mean fluorescence intensity (MFI) was used to quantify the magnitude of expression (different from absolute cell counts) of markers of specific immune cells over the time course of hypertension development and progression. MFI of all the markers used in the study were quantified. Only those that indicated sex or diet differences were included here. CD163 expression on CD163+ macrophages, Foxp3 expression on CD4+Foxp3+ and CD8+Foxp3+ T cells were quantified in MRPVAT **(Figure 4.7; panels A-C)** and APVAT **(Figure 4.7; panels D-F)**, after **10, 17 or 24 weeks** on a CD or HFD in both sexes by flow cytometry.

MRPVAT: Females on a HFD had greater expression of CD163 on CD163+ macrophages at 10 and 17 weeks *vs* HFD males **(Figure 4.7A)**. Females on a HFD had greater expression of Foxp3 on CD4+Foxp3+ and CD8+Foxp3+ T cells at 17 weeks *vs* HFD males **(Figure 4.7B, 4.7C)**.

APVAT: Females on a HFD had greater expression of CD163 on CD163+ macrophages at 10 weeks *vs* HFD males **(Figure 4.7D)**. Females on a HFD had greater expression of Foxp3 on CD4+Foxp3+ and CD8+Foxp3+ T cells at 17 weeks *vs* HFD males **(Figure 4.7E, 4.7F)**. Sex-differences with a HFD was prominent in terms of MFI, aligning with the RNA-seq data.

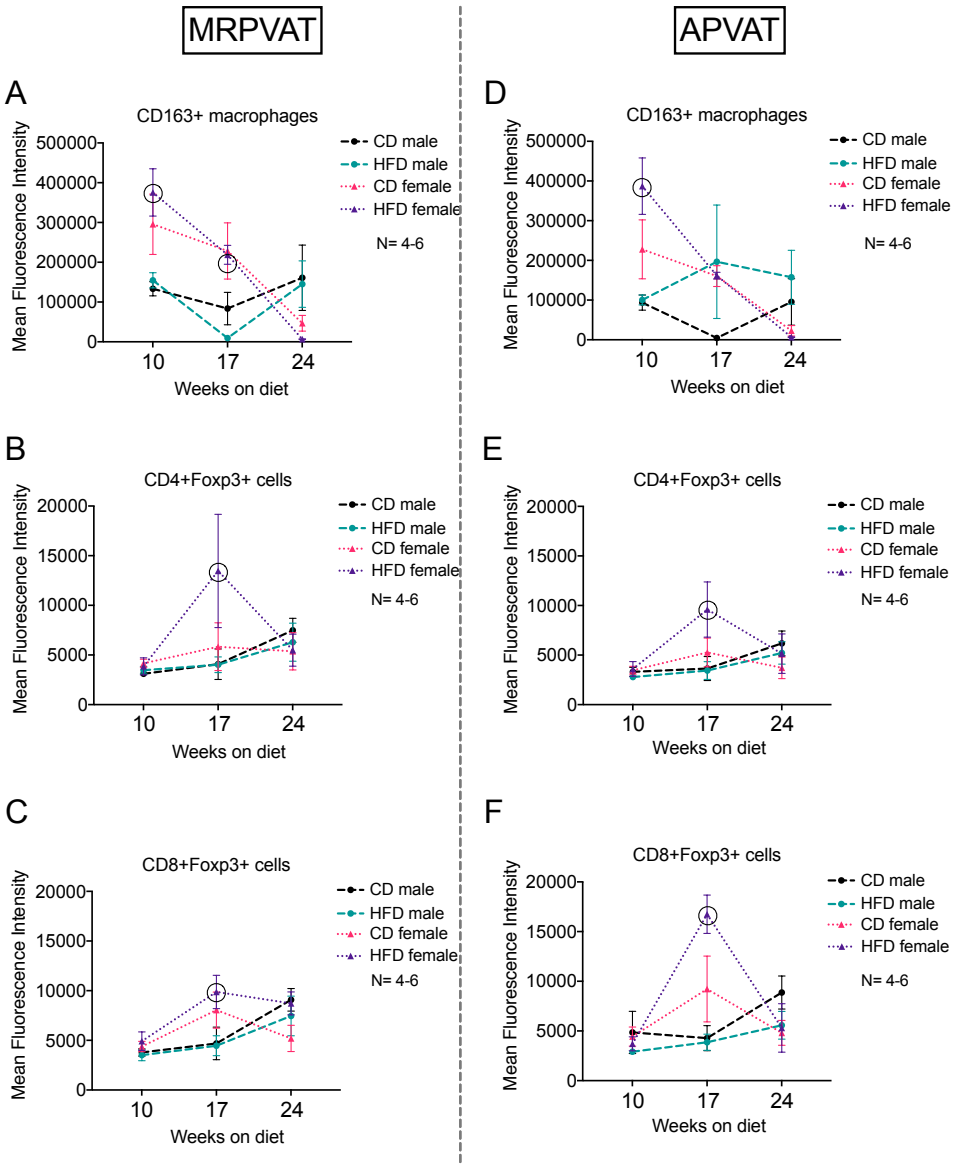


Figure 4.7 Magnitude of expression of markers of M2-like macrophages and T regulatory cells over the time course of hypertension development and progression

Mean fluorescence intensities (MFI) of CD163 on CD163+ M2-like macrophages **(A,D)**, Foxp3 on CD4+ **(B,E)** and CD8+ **(C,F)** T regulatory cells in MRPVAT and APVAT of both sexes over the time course on diet were quantified by flow cytometry. Points represent means±SEM of the magnitude of expression (measured by MFI) of the specified marker. Number of animals is indicated by N. A $P<0.05$ by 2-way ANOVA with Tukey's test for multiple comparison, was considered statistically significant. Circles in the graph represent a significant difference in MFI between the sexes within a diet at a given time point.

Summary

Basal sex-differences in T cell and macrophage subtypes exist in PVATs in the Dahl S strain (at 10 weeks on a CD). After 10 weeks on HFD (before hypertension develops): sex-differences in T cell and macrophage subtypes were observed. Diet-dependent changes occurred in both males and females in a PVAT-specific manner. After 17 weeks on HFD (onset of hypertension): HFD-dependent changes were the most prominent in MRPVAT and diet-independent changes in APVAT. After 24 weeks on HFD (hypertensive): HFD-induced immune changes started to occur in males and diminish in females (also reflected in sequencing studies) in the PVATs **(Figure 4.8)**.

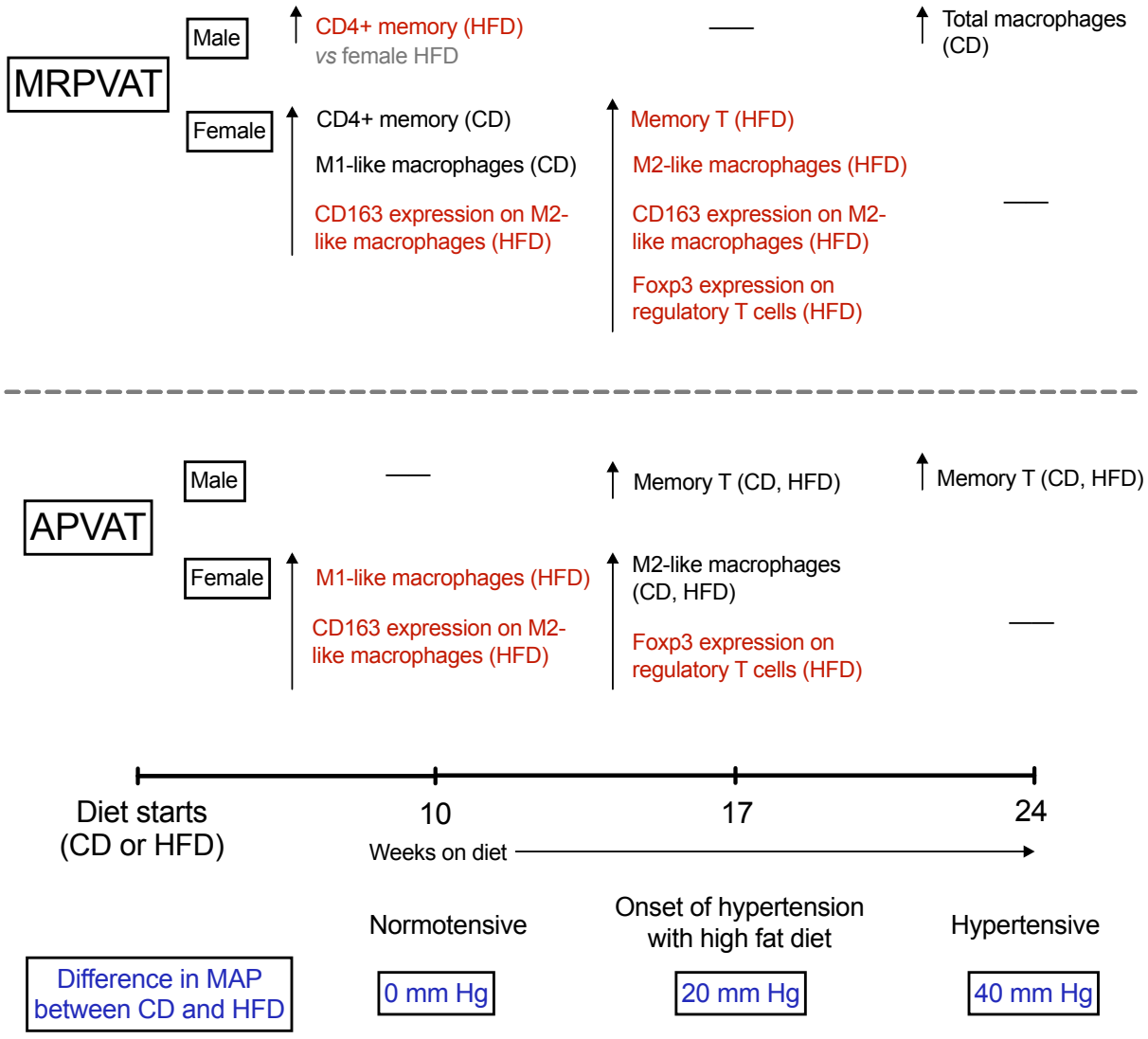


Figure 4.8 Summary (Graphical abstract)
Sex-differences within a diet associated with the dynamic immune cell changes in MRPVAT and APVAT prior to, with the development and with the progression of HFD-induced hypertension.

Discussion

Treatment with the lymphocyte inhibitor mycophenolate mofetil lowers the increases in blood pressure in male Dahl S rats, suggesting a role for T and B cells in high salt diet-induced hypertension [Spradley FT 2013]. Mice lacking either functional macrophages or T and B cells resisted increases in BP with Ang-II treatment [Saely CH 2012]. Hence, immune cells are important causative links to hypertension development. The reason the current study focused on whether specific immune cell population changes in PVATs precede hypertension development is that such a finding would support immune changes as a cause rather than just an effect of hypertension.

APVAT had 3-7 times higher density of immune cells *vs* MRPVAT in Dahl S rats (current study) and SD rats in both sexes [Kumar RK 2020]. This difference may be due to the differences in PVAT functions or the differences in the functions of blood vessels they surround. Only MRPVAT was chosen for the RNA-seq study because MRPVAT is a part of the visceral adipose tissue that increases risk for CVDs and MRPVAT surrounds blood vessels that control peripheral resistance [Saely CH 2012]. The RNA-seq data at 24 weeks in MRPVAT support two main ideas. First, diet-differences in immune cell changes occurred in males (males> females)- this aligns with the flow cytometry data **(Figure 4.2 and Figure 4.6)**. Second, sex-differences in immune changes in MRPVAT are more prominent with a HFD than a CD (particularly **Figure 4.7**). This is, however, in contradiction to the flow cytometry data that sex-differences diminish at 24 weeks. Flow cytometry panels are by necessity more limited in scope compared to the sequencing data. Thus, most of the gene

expression changes that were revealed were not able to be assessed by flow cytometry. Also, cell numbers of a given type as measured by flow cytometry could diminish even if those cells expressed more of a specific set of genes as measured by bulk RNA-seq, making a direct comparison difficult.

HFD-induced hypertension vs physiological aging

Physiological aging is associated with increased visceral adipose tissue mass, systemic low grade inflammation and increased cardiovascular disease risk [Mau T 2019, Zemancikova A 2019]. All these phenotypes overlap with that of our experimental model. Hence, effort was taken to tease out the gene expression differences (from the sequencing study) that were solely associated with a HFD vs those that were 'diet-independent or CD-dependent' or in fact 'age-related'. Although sex-differences were not originally anticipated in this model, we confirmed findings from the sequencing data with the flow cytometry studies.

Sex as a biological factor for immunological changes in PVAT: causal for development of hypertension?

Sex-differences in the immune system functions, in PVAT functions and in the development and progression of hypertension are well-recognized in both humans and rodents [Small HY 2019, Spradley FT 2013, Tipton AJ 2014]. In the current study, there were sex-differences in T cells and macrophage sub-populations in PVATs even at baseline. Females exhibit greater changes in immune populations (especially T cell and

macrophage subtypes) with HFD-induced hypertension. This aligns with a previous finding that T cell profile in abdominal adipose tissue of females changes more with aging in mice vs males [Spradley FT 2013]. T cells, by contributing to macrophage recruitment, may thus precede macrophage infiltration in obesity [Nishimura S 2009]. Also, we observed an increase in memory T cells in MRPVAT (HFD-dependent and time point-dependent sex differences) and in APVAT (in males only and diet independent). Similarly, memory T cells increased in blood, vasculature and kidneys of hypertensive mice [Itani HA 2015]. In our study, although there were no differences in the number of Tregs with hypertension in both sexes, females on a HFD with moderate increase in blood pressure *vs* age-matched HFD males had increased expression of Foxp3 on Tregs, consistent with females maintaining more Tregs *vs* males in Ang II hypertension and SHR [Pollow DP 2014, Tipton AJ 2014]. In our study, the findings that blood pressure rises equally in both sexes with a HFD and that the basal immunological differences in PVATs are exacerbated with HFD-induced hypertension together suggest that the immune mechanisms in PVATs driving hypertension are different in males *vs* females.

Are all the immune changes in PVATs detrimental?

Macrophages and T cells were the top two immune cell types identified to play a role in the sex-differences in the development and progression of HFD-induced hypertension in the current study. HFD-fed mice had increased M1-like macrophage infiltration in APVAT [Almabrouk TAM 2018]. Ang II increased proinflammatory monocyte/macrophage infiltration in PVAT in Treg-deficient mice [Mian MO 2016].

Presently, a surge in the M1-like macrophage levels was observed at 10 weeks (CD females in MRPVAT and HFD females in APVAT), suggesting that immunological PVAT dysfunction precedes the development of hypertension. Although M2-like macrophages are in general protective, several studies point to the role of M2-like macrophages in dysfunctional adipose tissue, insulin-resistance [Wentworth JM 2010] or vascular remodeling and fibrosis [Moore JP 2015]. Thus, deeper phenotypic characterization of the macrophage and other immune cell subtypes identified in the current study would be an excellent next step to understand their function.

Limitations

Dendritic cells and eosinophils [Withers SB 2017] could not be included due to the lack of availability of reliable flow cytometry markers in the rat. The origin of the specific immune cell subtypes identified in PVATs with HFD over the time course is not interpretable from the current study. Factors known to be linked to sex-differences in hypertension- gonadal hormones, cytokines and adipokines were not the focus of this study and were not measured. Also, estrous staging females was not done. RNA-sequencing at 10- and 17-week time points were not performed because the blood pressures were the most elevated at 24 weeks with a HFD. Female CD *vs* HFD demonstrated a few DEGs which was unexpected given the blood pressure differences observed. However, the quality of the input RNA and sequencing studies eliminates this being an experimental error. Finally, the functional roles or consequences of the dynamic

immune cell changes in PVAT warrants further studies. This is of real interest to the future studies in our group.

Conclusion

This rigorous time course study identified that immune cells (especially T cell and macrophage subtypes) in PVATs respond to a HFD before hypertension occurs in both sexes of Dahl S rats. Additionally, sex-differences in PVATs exist at baseline and become more prominent, driven by a HFD with the development and progression of hypertension.

Immunological changes in PVAT precede the development of CVDs such as HFD-induced hypertension (current study) and atherosclerosis. This is important to know because assessing PVAT function at an early stage has the potential to prevent the development of hypertension/ other CVDs. Clinically, women are more prone to adverse drug reactions in all anti-hypertensive drug groups [Rydberg DM 2018]. Thus, gender-based and tissue (PVAT)-specific pharmacological treatments may reduce the dosage and limit drug toxicity and may also lead to better blood pressure control.

REFERENCES

REFERENCES

Almabrouk TAM, White AD, Ugusman AB, Skiba DS, Katwan OJ, Alganga H, et. al. (2018). High Fat Diet Attenuates the Anticontractile Activity of Aortic PVAT via a Mechanism Involving AMPK and Reduced Adiponectin Secretion. *Front Physiol*. 9:51. doi: 10.3389/fphys.2018.00051.

De Miguel C, Das S, Lund H and Mattson DL. (2010). T lymphocytes mediate hypertension and kidney damage in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol.* 298(4):R1136-42. doi: 10.1152/ajpregu.00298.2009.

Fernandes R, Garver H, Harkema JR, Galligan JJ, Fink GD and Xu H. (2018). Sex Differences in Renal Inflammation and Injury in High-Fat Diet-Fed Dahl Salt-Sensitive Rats. *Hypertension*. 72(5):e43-e52. doi: 10.1161/HYPERTENSIONAHA.118.11485.

Gollasch M. (2012). Vasodilator signals from perivascular adipose tissue. *Br J Pharmacol.* 165(3):633-42. doi: 10.1111/j.1476-5381.2011.01430.x.

Hall JE, do Carmo JM, da Silva AA, Wang Z and Hall ME. (2015). Obesity-induced hypertension: interaction of neurohumoral and renal mechanisms. *Circ Res*. 116(6):991-1006. doi: 10.1161/CIRCRESAHA.116.305697.

Itani HA and Harrison DG. (2015). Memories that last in hypertension. *Am J Physiol Renal Physiol*. 308(11):F1197-9. doi: 10.1152/ajprenal.00633.2014.

Kumar RK, Jin Y, Watts SW and Rockwell CE. (2020). Naïve, regulatory, activated and memory immune cells co-exist in PVATs that are comparable in density to non-PVAT fats in health. *Front Physiol*. 11:58. doi: 10.3389/fphys.2020.00058. eCollection 2020.

Mattson DL, Lund H, Guo C, Rudemiller N, Geurts AM and Jacob H. (2013). Genetic mutation of recombination activating gene 1 in Dahl salt-sensitive rats attenuates hypertension and renal damage. *Am J Physiol Regul Integr Comp Physiol.* 304(6):R407-14. doi: 10.1152/ajpregu.00304.2012.

Mau T and Yung R. (2019). Adipose tissue inflammation in aging. *Exp Gerontol*. 2018;105:27-31. doi: 10.1016/j.exger.2017.10.014.

Mian MO, Barhoumi T, Briet M, Paradis P and Schiffrin EL. (2016). Deficiency of T-regulatory cells exaggerates angiotensin II-induced microvascular injury by enhancing immune responses. *J Hypertens*. 34(1):97-108. doi: 10.1097/HJH.0000000000000761.

Mikolajczyk TP, Nosalski R, Szczepaniak P, Budzyn K, Osmenda G, Skiba D, et. al. (2016) Role of chemokine RANTES in the regulation of perivascular inflammation, T-cell

accumulation, and vascular dysfunction in hypertension. *FASEB J.* 30(5):1987-99. doi: 10.1096/fj.201500088R.

Moore JP, Vinh A, Tuck KL, Sakkal S, Krishnan SM, Chan CT, et. al. (2015). M2 macrophage accumulation in the aortic wall during angiotensin II infusion in mice is associated with fibrosis, elastin loss, and elevated blood pressure. *Am J Physiol Heart Circ Physiol*. 309(5):H906-17. doi:10.1152/ajpheart.00821.2014.

Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et.al. (2009) CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med*. 15(8):914-20. doi: 10.1038/nm.1964.

Noblet JN, Owen MK, Goodwill AG, Sassoon DJ and Tune JD. (2015). Lean and Obese Coronary Perivascular Adipose Tissue Impairs Vasodilation via Differential inhibition of Vascular Smooth Muscle K+ Channels. *Arterioscler Thromb Vasc Biol.* 35(6):1393-400. doi: 10.1161/ATVBAHA.115.305500.

Nosalski R and Guzik TJ. (2017). Perivascular adipose tissue inflammation in vascular disease. *Br J Pharmacol*. 174(20):3496-3513. doi: 10.1111/bph.13705.

Pollow DP, Uhrlaub J, Romero-Aleshire M, Sandberg K, Nikolich-Zugich J, Brooks HL and Hay M. (2014). Sex differences in T-lymphocyte tissue infiltration and development of angiotensin II hypertension. *Hypertension*. 64(2):384-390. doi: 10.1161/HYPERTENSIONAHA.114.03581.

Rydberg DM, Mejyr S, Loikas D, Schenck-Gustafsson K, von Euler M and Malmström RE. (2018). Sex differences in spontaneous reports on adverse drug events for common antihypertensive drugs. *Eur J Clin Pharmacol.* 74(9):1165-1173. doi:10.1007/s00228-018-2480-y.

Saely CH, Geiger K and Drexel H. (2012). Brown versus white adipose tissue: a mini-review. *Gerontology*. 58(1):15-23. doi: 10.1159/000321319.

Skiba DS, Nosalski R, Mikolajczyk TP, Siedlinski M, Rios FJ, Montezano AC, et.al. (2017). Anti-atherosclerotic effect of the angiotensin 1-7 mimetic AVE0991 is mediated by inhibition of perivascular and plaque inflammation in early atherosclerosis. *Br J Pharmacol.* 174(22):4055-4069. doi: 10.1111/bph.13685.

Small HY, McNeilly S, Mary S, Sheikh AM and Delles C. (2019). Resistin Mediates Sex-Dependent Effects of Perivascular Adipose Tissue on Vascular Function in the Shrsp. *Sci Rep*. 9(1):689. doi: 10.1038/s41598-019-43326-z.

Spradley FT, De Miguel C, Hobbs J, Pollock DM and Pollock JS. (2013). Mycophenolate mofetil prevents high-fat diet-induced hypertension and renal glomerular injury in Dahl SS rats. *Physiol Rep*. 1(6):e00137. doi: 10.1002/phy2.137.

Szasz T, Bomfim GF and Webb RC. (2013). The influence of perivascular adipose tissue on vascular homeostasis. *Vasc Health Risk Manag.* 9:105-16. doi: 10.2147/VHRM.S33760.

Taylor LE and Sullivan JC. (2016). Sex differences in obesity-induced hypertension and vascular dysfunction: a protective role for estrogen in adipose tissue inflammation*? Am J Physiol Regul Integr Comp Physiol*. 311(4):R714-R720. doi: 10.1152/ajpregu.00202.2016.

Tipton AJ and Sullivan JC. (2014). Sex differences in blood pressure control: are T lymphocytes the missing link? *Hypertension*. 64(2):237-9. doi: 10.1161/HYPERTENSIONAHA.114.03688.

Wentworth JM, Naselli G, Brown WA, Doyle L, Phipson B, Smyth GK, Wabitsch M, O'Brien PE and Harrison LC. (2010). Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes*. 59(7):1648-56. doi: 10.2337/db09-0287.

Whelton PK, Carey RM, Aronow WS, Casey DE Jr, Collins KJ, Dennison Himmelfarb C, et. al. (2018). 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Hypertension. 71(6):1269-1324. doi: 10.1161/HYP.0000000000000066. Epub 2017 Nov 13. *Erratum in: Hypertension*. 2018;71(6):e136-e139.

Withers SB, Forman R, Meza-Perez S, Sorobetea D, Sitnik K and Hopwood T. (2017). Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. *Sci Rep*. 17;7:44571. doi: 10.1038/srep44571.

Zemančíková A and Török J. (2019). Influence of Age on Anticontractile Effect of Perivascular Adipose Tissue in Normotensive and Hypertensive Rats. *Oxid Med Cell Longev*. 9314260. doi: 10.1155/2019/9314260.

CHAPTER 5

Overall Discussion

Summary of New and Novel Findings

Chapter 1: Naïve, regulatory, activated and memory immune cells co-exist in PVATs that are comparable in density to non-PVAT fats in health **(Figure 5.1)**.

1. MRPVAT and APVAT contained naïve, activated, regulatory and memory- type innate and adaptive immune cells including T cells, B cells, NK cells, macrophages, mast cells and neutrophils in the healthy SD rat (alike in males and females).
2. MRPVAT and APVAT were similar in their immune composition, densities and activation status *vs* RP fat and SS fat (their respective non-PVAT fat comparators) respectively.
3. APVAT had a higher density of immune cells *vs* MRPVAT in both the male and female SD rat.

Chapter 2: IL-10 did not contribute to the anti-contractile nature of PVAT in health **(Figure 5.1)**.

4. IL-10 (mRNA) was one of the top three cytokines expressed in MRPVAT of SD rats in health.
5. The only known receptor for IL-10 was present in the tunica media, intima and PVATs of mesenteric resistance arteries in the male SD rat and superior mesenteric artery in wildtype male C57BL/6 mouse.
6. In both the rat mesenteric resistance arteries and the mouse superior mesenteric artery (without PVAT), exogenous IL-10 did **not** cause a direct vasorelaxation.

7. Exogenous IL-10 (low and high concentrations) also did not rightward shift a vasoconstrictant agonist response curve in the rat mesenteric resistance artery.
8. Short-term or prolonged depletion of endogenous IL-10 (use of 13 and 26 week-old IL-10 KO mice) still did **not** lead to loss of the anti-contractile nature of PVAT in the mouse superior mesenteric arteries.

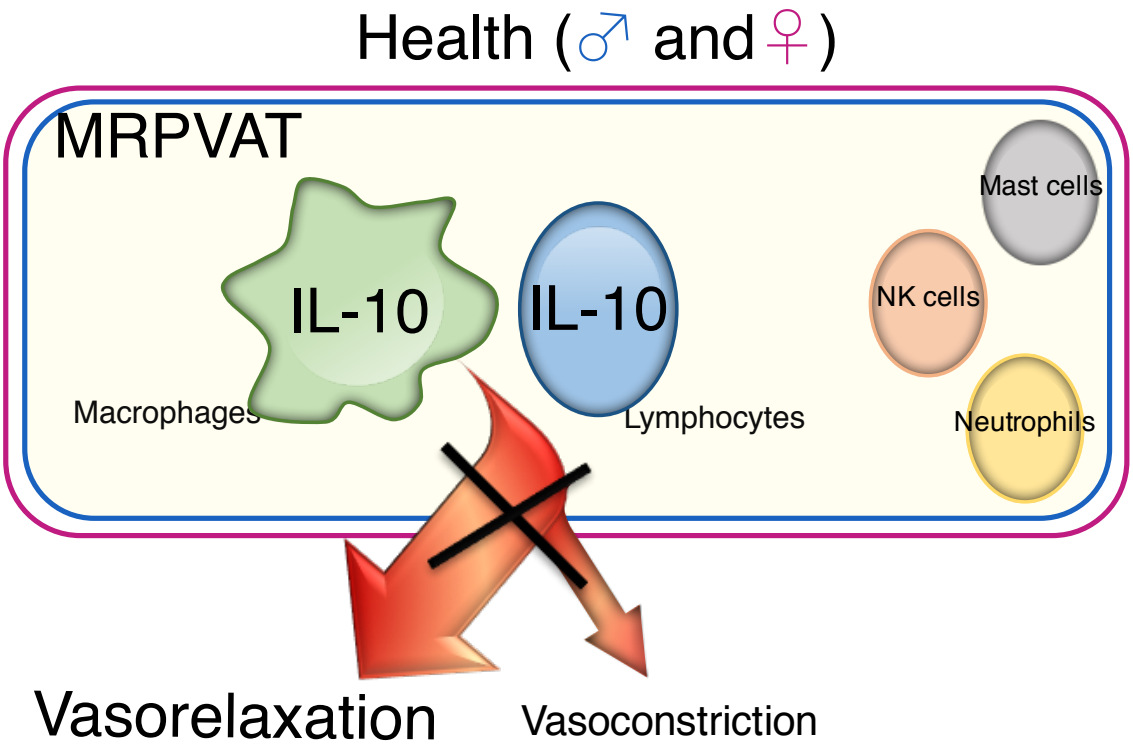


Figure 5.1 Graphical representation of findings in Chapters 1 and 2

Chapter 3: Sex-differences in T cells and macrophages in PVAT precede high-fat diet-induced hypertension **(Figures 5.2 and 5.3)**.

9. Basal sex-differences in T cell and macrophage subtypes existed in PVATs of Dahl S rats.
10. Sex-differences in memory T cell and M2-like macrophage densities in MRPVAT and APVAT preceded the development of HFD-induced hypertension.
11. With the onset of hypertension (17 weeks of diet), sex-differences in T cell and macrophage subtypes were HFD-dependent in MRPVAT and diet-independent in APVAT.
12. At 10 (pre-hypertensive stage) and 17 (onset of hypertension) weeks, there were greater HFD-induced immune cell (especially T cell and macrophage subtypes) changes in MRPVAT and APVAT from females *vs* males.
13. At 24 weeks (full progression of hypertension), HFD-induced immune changes (memory T cells and macrophages) began to appear in males and diminish in females.

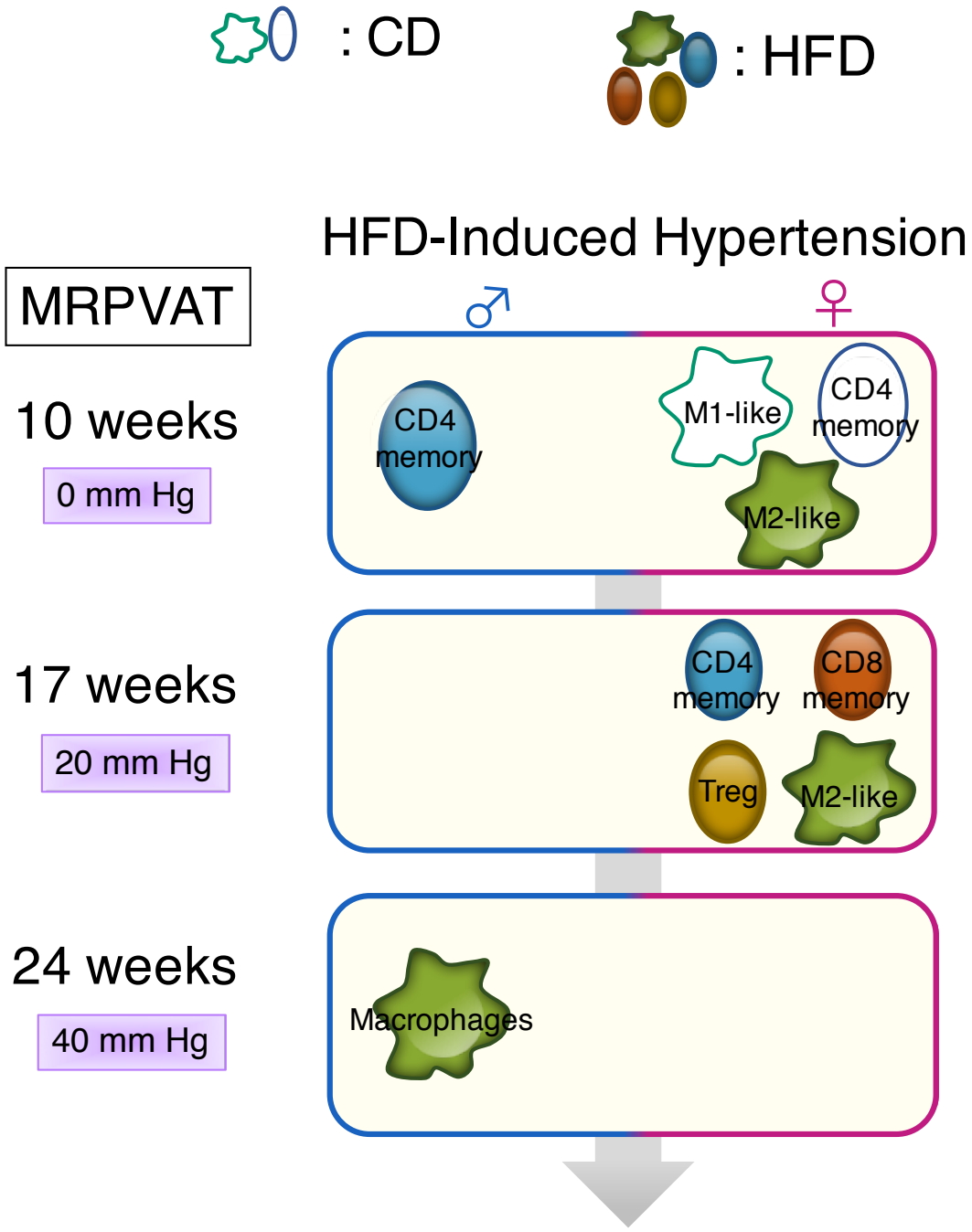


Figure 5.2 Graphical representation of findings in Chapter 3 (MRPVAT)
Sex-differences in immune cell activation status (numbers/ mean fluorescence intensity) in MRPVAT. (Key: eg, at 10 weeks, higher density/ MFI of M1-like macrophages occur in CD females *vs* CD males). Differences in mean arterial pressure between HFD and respective CD-fed rats are presented as mm Hg (measured by radiotelemetry [Fernandez R 2018]) with 10, 17 and 24 weeks on diet.

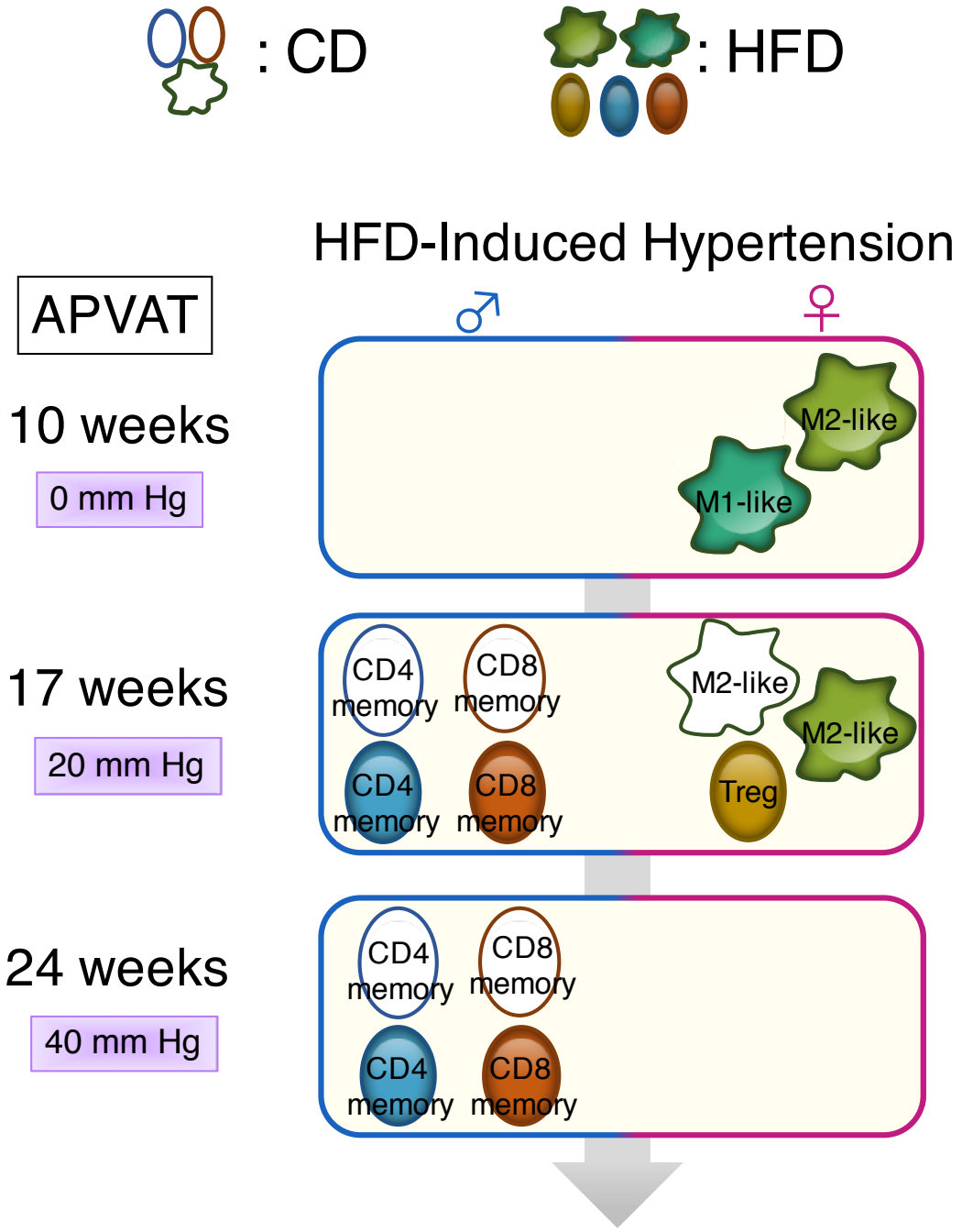


Figure 5.3 Graphical representation of findings in Chapter 3 (APVAT)
Sex-differences in immune cell activation status (numbers/ mean fluorescence intensity) in APVAT. (Key: eg, at 10 weeks, higher density/ MFI of M1-like macrophages occur in HFD females *vs* HFD males). Differences in mean arterial pressure between HFD and respective CD-fed rats are presented as mm Hg (measured by radiotelemetry [Fernandez R 2018]) with 10, 17 and 24 weeks on diet.

Discussion

This dissertation tested the overall hypothesis that **T cells and macrophages in PVAT promote release of IL-10 that causes vasorelaxation in health; immunological PVAT dysfunction precedes adiposity-induced hypertension (alike in males and females)**. Many studies have identified multiple types of innate and adaptive immune cells in non-PVAT adipose tissues (especially WAT) in the context of hypertension, obesity and atherosclerosis [McLaughlin T 2017, Skiba DS 2017]. Thus, we first needed to understand the diversity of the immune cell community and their activation status in PVATs in health (homeostasis). We purposefully designed experiments to compare MRPVAT (white PVAT around mesenteric resistance vessels, the vessels that are important in regulating total peripheral resistance) and APVAT (brown PVAT around thoracic aorta, the conduit vessel that stiffens in hypertension). This was important given the fact that MRPVAT and APVAT are morphologically different and have diverse biological functions to achieve, as reverberated throughout this thesis. Another intentional comparison was performed between PVATs and their respective non-PVAT fat comparators. This served as an important first step towards understanding whether PVAT was special and if PVAT-specific experimental interventions might be possible in the future.

We first discovered that PVAT has a rich community of naive, activated, regulatory and memory type innate and adaptive immune cells in health **(Figure 2.1)**. One may ask if the immune cells in PVATs are infiltrating from the circulation, owing to PVAT's close

proximity to the blood vessels or if these immune cells are truly PVAT-residing. It is possible that the identified immune cells are brought to the PVAT from primary immune organs (spleen and thymus), secondary immune organs (local lymph nodes) or from the circulation. Lineage tracing studies using genetically engineered rodents will help unravel if the immune cells identified in PVATs originated in PVATs. This will also be a foundational step to understand whether the homeostatic anti-inflammatory immune cells in PVATs switch to a proinflammatory phenotype in pathologies such as obesity and hypertension or if PVATs recruit them from other immune organs/ blood when needed.

Why do adipose tissues including PVATs contain immune cells? One reason could be that the immune cells in PVATs serve roles similar to non-PVAT adipose tissues towards maintaining tissue homeostasis (clearing debris, maintaining insulin sensitivity, assist in ECM remodeling etc) [Fitzgibbons TP 2016, Russo L 2018]. DNA (by 16S metagenomics sequencing) of Firmicutes and Bacteroidetes were predominantly present in mesenteric adipose tissues (similar to fecal and ileum samples) of wildtype C57BL/6 mice [Lluch J 2015]. In addition, the same study reported that Deferribacters phylum was present only in the mesenteric adipose tissue (and not in ileum, liver, skeletal muscle, heart or brain). This raises the hypothesis that MRPVAT has a microbiome on its own that is different from that of APVAT (a brown PVAT, from a different anatomical location) or RP fat (a white non-PVAT fat). This could also another possible reason why immune cells live in adipose tissues including PVATs. Although immune cell densities in PVATs were similar to their like non-PVAT fats **(Chapter 2)**, their biological purpose and functions

in PVATs may differ from their non-PVAT fats, because PVATs surround the blood vessels unlike other non-PVAT fats. These exact thoughts provoked us to investigate if the cytokines made by these immune cells in PVATs could contribute to vascular tone regulation in health **(Chapter 3)**.

We identified IL-10 mRNA as one of the top three cytokines (out of 84 chemokines and cytokines) present in MRPVAT of wildtype male rats and was also expressed in the SMPVAT in wildtype male mice **(Figure 3.1)**. IL-10 is a classic anti-inflammatory master regulatory cytokine. For IL-10 to exert vasoactive properties, its only known receptor IL-10RA needs to be present in the rat mesenteric resistance arteries (MRA) and mouse superior mesenteric arteries (SMA). We identified IL-10 RA protein in the tunica media (containing vascular smooth muscle cells VSMCs) and tunica intima (composed of endothelial cells) of MRA (in rats) and SMA (in mice). The effect of cytokines on SMCs from other organs (such as uterus, urinary bladder, lungs and intestine) have been documented, where the cytokines may regulate SMC function by directly acting on the SMCs (by binding to their respective receptors) or indirectly by releasing mediators from other immune cells/ other cell types that can further act on SMCs. For example, IL-1β and IFN-γ, by directly binding to their surface receptors on rat ileal SMCs, reduced LPS-induced SMC contractility [Lodato RF 1999]. IgE, by binding to its receptors on mast cells, triggered the release of mast cell mediators including serotonin, histamine and leukotrienes, and indirectly augmented uterine SMC contractility during labor in pregnant

guinea pigs [Bytautiene E 2008]. These ideas geared us to test the direct *vs* indirect vascular effects of IL-10.

As stated earlier in this work, PVAT is anti-contractile in health. So, could the IL-10 made by the PVAT be one of the factors directly or indirectly responsible for this anti-contractile nature of PVAT? Contrary to our hypothesis, IL-10 (exogenous) was neither a direct vasorelaxant **(Figure 3.3)** nor did it directly decrease vasoconstriction **(Figure 3.4)** in mesenteric arteries from healthy rodents. To confirm the biological capability of the recombinant proteins (mouse and rat), we used the classic assay of measuring IFNγ in the supernatants of cultured activated splenocytes with and without recombinant IL-10 added to respective species. As expected, the activated splenocytes secreted lesser IFNγ in the presence of IL-10 *vs* in the absence of IL-10 **(Figure 3.3)**. This was an important experiment to assure that the recombinant proteins we used were biologically active, but just not vasoactive. However, maybe the endogenous PVAT-derived IL-10 could indirectly contribute to the anti-contractile nature of PVAT. Thus we turned to the use of arteries with and without PVAT from IL-10 KO and age-matched WT mice. The anti-contractile property of PVAT was intact even when IL-10 was knocked out (short-term KO, 13 week old mice) **(Figure 3.5)**.

However, it is important to note that IL-10 KO mouse is commonly used as a model of systemic chronic inflammation [Sikka G 2013]. So, as inflammation worsens with age in IL-10 KO mice [Sikka G 2013], we did not want to miss the opportunity to observe

vascular changes that might kick in following the inflammation phenotype. This was the reason why we examined older (26 week age) IL-10 KO and WT mice. However, at 26 weeks of age, the anti-contractile function of PVAT was maintained in the mesenteric arteries from IL-10 KO mouse **(Figure 3.6)**. These studies thoroughly demonstrated that PVAT-derived IL-10 did not contribute to the anti-contractile nature of PVAT, either directly or indirectly. This finding provokes a critical question: What else does IL-10 do in the PVAT? Could it be involved in PVAT homeostasis? We have speculated the possibilities in connection with the existing literature, in detail in **Chapters 1 and 3**. The crux is that IL-10 probably functions like brakes to the immune system overdrive in pathological conditions. The importance of brakes (IL-10) in a car (immune system in PVAT) is realized only when the car is in motion (immune-overdrive in disease) and not stationary (homeostasis). So, could IL-10 offer vasoprotection in disease? To answer this, we first need to probe into how these immune cells in PVATs change in composition/ densities, activation status and secretions in a disease setting.

To understand the dynamic interplay of PVAT (a fat) with the vasculature (blood vessel), we proceeded to take advantage of the HFD-fed Dahl S rat model, developed by Dr. Gregory Fink in our cardiovascular group at MSU [Fernandez R 2018]. This rat model exhibited hypertension when on a HFD. Both males and females developed similar magnitude of hypertension in the same time period. This finding along with the findings from **Chapters 2 and 3** that we did not observe sex-differences in immune composition/ vasoactive functions of IL-10 in health, led us to hypothesize that the changes in immune

cell activation status in PVATs in the Dahl S model would be similar in both males and females, with the development and progression of adiposity-induced hypertension.

Studies in atherosclerosis reported that T cell and macrophage infiltration into APVAT preceded the plaque development in mice [Skiba DS 2017]. Hence, we tested the hypothesis that immunological PVAT dysfunction precedes the development of hypertension in HFD-fed Dahl S rats. We considered only APVAT and MRPVAT (and not their non-PVAT fat comparators) for this study. This was based on the fact that the immune cell densities and activation status were similar between PVATs and their respective non-PVAT fats in health **(Chapter 1)**. Overall, with the development and progression of hypertension, diet-dependent immune changes occurred in MRPVAT while diet-independent changes in APVAT **(Chapter 4 and Figure 5.2)**. But contrary to our hypothesis, females fed a HFD had a greater density of T cell and macrophage subtypes in PVATs before (10 weeks) and with the onset (17 weeks) of hypertension *vs* males **(Figures 4.2 and 4.3)**. However, with progression of hypertension (24 weeks), immune changes in PVATs began to appear in HFD-fed males and diminish in HFD-fed females. **(Figure 4.4)**.

Possible Reasons for the Unexpected Sex-Differences

Several reasons could explain the sex-differences we observed. (1) ***Sex-hormones and genes:*** Females before menopause are more protected from cardiovascular and metabolic complications *vs* age-matched men. Estrogen is

considered a key player responsible for this safety bubble in women [Taylor LE 2018]. Mattson *et al* reported that substitution of chromosomes 1,5,7,13,16 and 18 from Brown Norway rats onto the Dahl S rats attenuated the development of salt-induced hypertension in males [Mattson DL 2008]. On the other hand, the same study also demonstrated that substitution of chromosomes 1 and 5 (and not 7,13,16 and 18) decreased the development of hypertension in females [Mattson DL 2008]. These suggest the direct positive association (maybe even causation) between genetics and the development of hypertension; (2) **Cytokines:** Kidneys from female SHR had more IL-10+ cells while males contained more IL-6+ and IL-17+ cells [Tipton AJ 2014]. Sex-differences in the circulating/ tissue-specific levels of proinflammatory cytokines such as TNF-α, IL-6, IL-1β and TLRs that have been positively correlated with obesity and hypertension have been reported [Henstridge DC 2019] (3) **Adipokines:** Women have increased circulating leptin *vs* males, while it is the opposite in rodents in experimental hypertension/ obesity. However, circulating leptin levels are positively associated with obesity and hypertension [Considine RV 1996, Landt M 1998] (4) **Microbiota:** This is a relatively recent concept. Germ-free (no gut microbiota) mice did not develop hypertension and vascular dysfunction with AngII [Karbach SH 2016]. Gut microbiome differed between genders in a BMI-specific manner, with higher *Firmicutes* to *Bacteriodetes* ratio in obese women *vs* men, correlating with increased inflammation and activation of the immune system, thus worsening metabolic syndrome (including hypertension and obesity) [Beale AL 2019]. From these studies, an important question arises: Do systemic changes in sex-hormones, cytokines, adipokines or microbiota lead to immune cell changes in PVAT or

vice versa? It is, however, a challenging task to establish a causal (with sex hormones being the top suspects) link to sex-differences in obesity and hypertension, because of the number of confounding variables involved (including diet, environment, socio-economic status, genetic variability and microbiome), that need to be separated out to point to the specific root cause of sex-differences.

Limitations

We recognize several limitations in our studies. Many of these have been detailed at the end of **Chapters 2, 3 and 4**.

First, while we have broadly and deeply characterized the community of immune cells in PVATs in health and adiposity-induced hypertension, the functional roles of these immune cells is a key question that still remains to be answered. Second, IL-10 protein levels were not measured in our study **(Chapter 3)**. This was because IL-10 protein levels at baseline (without stimulation in health) were below the detection limit of any commercially available assays. Moreover, IL-10 can be subject to post transcriptional modifications that may affect its biological activity (such as vasoactive potential) [Mark J Powell 2000, Zhang H 2019]. Hence, with the development of more sensitive assays in the future, this is an important measure to be made. Third, although sex-differences in our studies were unexpected, factors associated with sex-differences (such as the steroid hormones, cytokines and adipokines) have not been measured as it was beyond the scope of this work. Finally, another inherent limitation was that we used a rat model to

study the convergence of immune system in health and hypertension. Rat is undeniably a great model to study adiposity and hypertension as it allows us to integrate findings from other hypertension/ adiposity rat models. However, the genetic (various genetically modified mice) and pharmacological tools to investigate the immune system is far better available in the mouse than in the rat. This limitation can be overcome as more readily-available reproducible tools become available to dive into the immune mechanisms in the rat.

Big Questions to Consider when Moving Forward

1. How much of a change in immune cell density or function in PVATs or other organs should we be worried or concerned about with respect to the development and progression of obesity/ hypertension? Statistical significance (mostly a $P< 0.05$) only would indicate with 95% confidence that a difference observed between groups, does not occur by chance. For instance, if a 7% statistically significant increase in macrophage numbers occurs in MRPVAT in HFD *vs* CD in males- does this really matter to the development or progression of hypertension?

2. Will the immune level changes in PVAT observed in the Dahl S model of HFD-induced hypertension used in this work translate to other forms of diet-induced hypertension or other experimental models of hypertension?

3. We have observed differences in MRPVAT *vs* APVAT in terms of sex-differences in dynamic changes in composition and activation status of immune cells

(Figures 5.2 and 5.3). Does one tissue/ PVAT matter the most to drive hypertension in our model- such as APVAT or MRPVAT?

4. What changes (immune/ RAAS/ sympathetic/ adipose/ ECM-level) in which organs (vasculature/ kidneys/ heart/ brain) are key considerations to drive the development and progression of HFD-induced hypertension?

Future Directions

Although there are multiple interesting alternate or sequential hypothesis relative to the findings of the present work, a few specific pressing questions of key interest have been identified as below.

We did not observe differences in PVATs *vs* their respective non-PVAT fat comparators in immune composition/ activation status in PVATs from healthy rats **(Chapter 2)**. Thus, an alternate hypothesis that emerges is that the immune cells identified perform different functions in PVATs *vs* their like non-PVAT fats. In addition, PVAT-derived IL-10 did not contribute to the anti-contractile function of PVAT in health **(Chapter 3)**. T cells and macrophages are the most abundant and non-naïve (being either activated or regulatory or memory type) immune cells in both MRPVAT and APVAT **(Chapter 2)**. So, could PVAT-derived T cells and/ or macrophages (such as eosinophils; [Withers SB 2017]) or other cytokines directly or indirectly contribute to the anti-contractile nature of PVAT in health? This question can be addressed by isometric contractility studies using arteries with and without PVAT from genetically modified rats such as Rag

null rats that lack functional lymphocytes; by surgically depleting T cells by thymectomy; and/ or by pharmacologically depleting macrophages by clodronate liposomes.

We observed sex-differences in T cell and macrophage subtypes before, during and after HFD-induced hypertension in Dahl S rats **(Chapter 4)**. Moreover, females demonstrated greater changes in T cell and macrophage subtypes over the course of hypertension development *vs* males. In addition, another study from our group has demonstrated greater renal injury in males *vs* females in the same animal model [Fernandez R 2018]. These together provoke the hypothesis that males would have more proinflammatory cytokines and adipokines in PVATs *vs* females. One quick way to test this would be to measure the protein levels of proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-6 by a cytokine array or ELISA and adipokines such as chemerin, leptin, resistin by an adipokine array in PVATs in the HFD-Dahl S model (both sexes). The cytokines and adipokines mentioned here should be of prime interest as they are each individually positively associated with hypertension and obesity [Ma D 2009, Tanase DM 2019, Zhang L 2010, Gu P 2015, Izquierdo AG 2019, Ernst MC 2010, Steppan CM 2001, Caer C 2017]

Given that we observed immune changes in PVATs with hypertension, can we prevent the development or reverse the progression of HFD-induced hypertension by treating the Dahl S rats with immunosuppressants (such as mycophenolate mofetil; [De Miguel C 2010]). Does estrogen play a causal role in the sex-differences observed in

PVAT-derived immune cells in **Chapter 4**? Treatment with 17-β-estradiol or ovariectomizing the Dahl S rats before/ after a high fat diet feeding prevent hypertension development/ reverse the progression of hypertension respectively can help address this question. SD rats on the same HFD do not develop hypertension [Fernandez R 2018] as the Dahl S rats do in our study. How is the SD rat immunologically different from the Dahl S rat? Is the Dahl S rat a possible autoimmune model? Testing for presence of circulating auto-antibodies and auto-reactive T cells in the Dahl S rats would be one way to understand if the Dahl S model demonstrates autoimmunity. Measuring the levels of proinflammatory cytokines (such as TNF-α, IFN-γ, IL-17, IL-1β, IL-6) in the supernatants of immune cells cultured (with and without activation) from the spleens/ PVATs/ non-PVAT fats from both sexes of healthy SD *vs* Dahl S rats would serve as an important next step to answer many of our questions (functional role of immune cells in the context of SD *vs* Dahl S, male *vs* female Dahl S, PVAT *vs* non-PVAT fats). One recent study reported that intraperitoneal administration of IL-33 caused eosinophilia and restored the anti-contractile of MRPVAT of HFD-fed hypertensive mice [Saxton SN 2020]. Thus, finally, a key question emerges: In the current work, how do these immune cell changes in PVAT contribute to vascular tone regulation in adiposity-induced hypertension? Surgical or pharmacological depletion of T cells and macrophages (as described above) at specific times along the time course (10, 17 and 24 weeks) in our HFD-fed Dahl S rat model and investigating the anti-contractile nature of PVATs in mesenteric arteries using a wire myograph will be one of the many directions one could go.

Current Challenges with Immune Modulators for the Treatment of Obesity and Hypertension

Previously, obesity and hypertension were considered behavioral disorders and as a result, most treatment options have relied on lifestyle modifications. However, over the decades, several studies have unraveled the complexity (such as genetics and environment) in the pathogenesis of hypertension and obesity. Pharmacological interventions along with exercise, diet and behavioral changes would help in better control of these pathologies [Apovian CM 2015].

Hypertension: At present, common treatment options for hypertension in humans are adrenoreceptor antagonists (such as prazosin), angiotensin converting enzyme inhibitors (perindopril), angiotensin receptor blockers (irbesartan), mineralocorticoid antagonists (spironolactone), diuretics (thiazides) or vasodilators (Ca channel blockers) [Whelton PK 2017]. These treatment strategies have certainly reduced fatal and non-fatal side effect outcomes and associated end-organ damage [Ettehad D 2016]. More than 45% of adults in the US are hypertensive, 20% are resistant to conventional anti-hypertensive treatment protocols/ combination therapies. Differences in ethnicity [Brewster LM 2016], genetic variability [Seidel E 2017, Shahin MH 2016, Heidari F 2017, Ware JS 2017] and phenotypic differences contribute to the challenges posed to the current anti-hypertensive treatments. Hence there is a clear need to discover new and novel anti-hypertensive pharmacological interventions.

Beginning in the 1980's, several studies have pointed towards using pan-immunosuppressants (cyclophosphamide, azathioprine) [Khraibi AA 1984, Tinsley JH 2009] or targeted immune modulators (neutralizing antibodies, nAb, for cytokines such as IL-6, TNF-α, IL-1β, IL-17, IFN-γ or depleting specific immune cell types such as anti-TCR$\gamma\delta$ or hindering cell-cell interactions with drugs like cytotoxic T-lymphocyte associated protein CTLA4-Ig) to lower blood pressure in rodents [Hashmat S 2016, Filho AG 2013, Rodriguez-Iturbe B 2017, Caillon A 2017]. The non-specific pan-immune modulators demonstrate severe limitations from clinical perspective due to an unfavorably high risk-to-benefit ratio. Cytokine release syndrome (infliximab, muromonab; [Lichtenstein L 2015, Bugelski PJ 2012]), cardiac conduction disorders and cardiomyopathy (chloroquine, cyclophosphamide; [Al-Bari MAA 2015, Curigliano G 2016]), increased thrombotic events (anti-CD40L; [Sidiropoulos PI 2004]), risk of infections and multi organ toxicities (CTLA4-Ig, anti-CD20, IFN-γ nAb; [Seibert FS 2014, Hansel TT 2010, Kelchtermans H 2008]) are the risks associated with the use of these pharmaceuticals for the treatment of hypertension. The specific details regarding dosing, efficacy and toxic side effects are well-documented/ tabulated elsewhere [Bomfim GF 2019]. However, several other candidates including anti-TLR-4, tocilizumab (IL-6 nAb) and canakinumab (anti-IL-1β) have shown promise for the treatment of hypertension so far [Monnet E 2017, Kim SC 2017, Shah SR 2018]. Moreover, novel modalities such as albumin-bound sirolimus (mTOR inhibitor) nanoparticles has better pharmacokinetics, biodistributuion and shows better promise for the treatment of pulmonary arterial hypertension *vs* oral sirolimus [Simon M 2019].

Obesity: Obesity has been recognized as an epidemic and is being tracked by the CDC since the 1960s. Obesity is accompanied by several co-morbidities including hypertension, type 2 diabetes, dyslipidemia, obstructive sleep apnea, coronary artery disease osteoarthritis and even certain forms of cancers [Guo F 2016]. Many of these pathologies can be prevented or reduced in intensity if obesity is treated/ prevented. Phentermine (a sympathomimetic amine that suppresses appetite) was the first Food Drug Administration (FDA)-approved anti-obesity drug [Sehnert KW 1963, Rider JA 1963]. Since then, many other monodrugs such as serotonin-releasing agents (fintepla, redux), lipase inhibitors (orlistat), amine reuptake inhibitors (sibutramine) and combination drugs such as amine activity stimulants (empatic), antihyperglycemics (pramlintide) have been FDA-approved for obesity treatment [Powell AG 2011, Aronne LJ 2011]. But several of the initially FDA-approved anti-obesity drugs have been withdrawn due to long-term safety concerns associated with monotherapies including but not limited to psychiatric/ cognitive/ cardiovascular adverse events and teratogenicity [Astrup A 2011, Adan RA 2013]. Combinatorial treatment regimens such as pramlintide (leptin + amylin analogs) are more efficacious and demonstrate a comparatively better safety profile *vs* monotherapy [Powell AG 2012]. Obesity is undeniably a chronic inflammatory condition which requires continuous long-term treatment. So, how could immunosuppressants act as anti-obese drugs? Unfortunately, pan-immunosuppressants such as glucocorticoids, tacrolimus (calcineurin inhibitor) and evrolimus, sirolimus (mTOR inhibitors) lead to weight gain [Leslie WS 2007]. Specific targeted immune modulators have not been used (to the best of our knowledge) as anti-obesity medications thus far.

A Brighter Future? Immune modulators/ immunosuppressants are not currently used to treat primary hypertension or obesity due to efficacy and safety concerns. With the second generation of targeted immune modulators (such as those targeting JAK-STAT pathway), there is promise in terms of reduced systemic toxicity/ side effects [Schwartz DM 2017]; and improved safety profile and efficacy. As we are inching towards personalized treatment possibilities, better understanding the immune pathophysiology of obesity, hypertension and obesity-induced hypertension along with continuous strife for betterment of targeted/ selective immune modulators offer promise for better treatment options. Development of adipose-tissue specific or in fact, PVAT-specific targeted immunotherapies would be beneficial to reduce the downside of systemic immunosuppressants for the treatment of obesity and hypertension. However, the biggest challenge is that there are currently no known PVAT-selective genes/ proteins/ cells/ cellular components that have been identified yet. Thus, PVAT-specific therapy is currently just a wishful dream.

Conclusion and Broad Implications of our Findings

Our venture through the immune cell community within PVATs in health and adiposity-induced hypertension has opened several opportunities of research. Understanding the intricate details regarding the composition and activation status of immune cells at the level of PVAT (a fat tissue that surrounds peripheral blood vessels) is critical to further our understanding of the vasoactive capabilities of these cells/ their secretions in pathological states. This research comes at a time when obesity-associated

hypertension is a world-wide epidemic and novel pharmacological targets are absolutely needed. Our unexpected discovery of sex-differences in the immune axis of PVATs with adiposity-associated hypertension also has opened Pandora's box. This research highlights the importance of considering both sexes in pre-clinical studies, gears towards understanding whether the use of targeted (tissue/ gender) immunosuppressant drugs to treat obesity/ hypertension might prove beneficial. Finally, this work provides a strong foundation to focus on PVAT as a novel possibility for selective immune-cell intervention to prevent or treat cardiovascular diseases involving PVAT dysfunction.

REFERENCES

REFERENCES

Adan RA. (2013). Mechanisms underlying current and future anti-obesity drugs. *Trends Neurosci.* 36(2):133-40. doi: 10.1016/j.tins.2012.12.001.

Al-Bari MA. (2015). Chloroquine analogues in drug discovery: new directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. *J Antimicrob Chemother.* 70(6):1608-21. doi: 10.1093/jac/dkv018.

Apovian CM, Aronne LJ, Bessesen DH, McDonnell ME, Murad MH, Pagotto U, Ryan DH and Still CD. (2015). Endocrine Society. Pharmacological management of obesity: an endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 100(2):342-62. doi: 10.1210/jc.2014-3415.

Aronne LJ, Powell AG and Apovian CM. (2011). Emerging pharmacotherapy for obesity. *Expert Opin Emerg Drugs.* 16(3):587-96. doi: 10.1517/14728214.2011.609168.

Astrup A, Dyerberg J, Elwood P, Hermansen K, Hu FB, Jakobsen MU, et. al. (2011). The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010? *Am J Clin Nutr*. 93(4):684-8. doi: 10.3945/ajcn.110.004622.

Beale AL, Kaye DM and Marques FZ. (2019). The role of the gut microbiome in sex differences in arterial pressure. *Biol Sex Differ.* 10(1):22. doi: 10.1186/s13293-019-0236-8.

Bomfim GF, Cau SBA, Bruno AS, Fedoce AG and Carneiro FS. (2019). Hypertension: a new treatment for an old disease? Targeting the immune system. *Br J Pharmacol.* 176(12):2028-2048. doi: 10.1111/bph.14436.

Brewster LM, van Montfrans GA, Oehlers GP and Seedat YK. (2016). Systematic review: antihypertensive drug therapy in patients of African and South Asian ethnicity. *Intern Emerg Med.* 11(3):355-74. doi: 10.1007/s11739-016-1422-x.

Bugelski PJ and Martin PL. (2012). Concordance of preclinical and clinical pharmacology and toxicology of therapeutic monoclonal antibodies and fusion proteins: cell surface targets. *Br J Pharmacol.* 166(3):823-46. doi: 10.1111/j.1476-5381.2011.01811.x.

Bytautiene E, Vedernikov YP, Saade GR, Romero R and Garfield RE. (2008). IgE-independent mast cell activation augments contractility of nonpregnant and pregnant

guinea pig myometrium. *Int Arch Allergy Immunol.* 147(2):140-6. doi: 10.1159/000135701.

Caër C, Rouault C, Le Roy T, Poitou C, Aron-Wisnewsky J, Torcivia A, Bichet JC, Clément K, Guerre-Millo M and André S. (2017). Immune cell-derived cytokines contribute to obesity related inflammation, fibrogenesis and metabolic deregulation in human adipose tissue. *Sci Rep.* 7(1):3000. doi: 10.1038/s41598-017-02660-w.

Caillon A, Mian MOR, Fraulob-Aquino JC, Huo KG, Barhoumi T, Ouerd S, Sinnaeve PR, Paradis P and Schiffrin EL. (2017). γδ T Cells Mediate Angiotensin II-Induced Hypertension and Vascular Injury. *Circulation.* 135(22):2155-2162. doi: 10.1161/CIRCULATIONAHA.116.027058.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et. al. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med.* 334(5):292-5. doi: 10.1056/NEJM199602013340503.

Curigliano G, Cardinale D, Dent S, Criscitiello C, Aseyev O, Lenihan D and Cipolla CM. (2016). Cardiotoxicity of anticancer treatments: Epidemiology, detection, and management. *CA Cancer J Clin.* 66(4):309-25. doi: 10.3322/caac.21341.

De Miguel C, Das S, Lund H and Mattson DL. (2010). T lymphocytes mediate hypertension and kidney damage in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol.* 298(4):R1136-42. doi: 10.1152/ajpregu.00298.2009.

Ernst MC and Sinal CJ. Chemerin: at the crossroads of inflammation and obesity. (2010). *Trends Endocrinol Metab.* 21(11):660-7. doi: 10.1016/j.tem.2010.08.001.

Ettehad D, Emdin CA, Kiran A, Anderson SG, Callender T, Emberson J, Chalmers J, Rodgers A and Rahimi K. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. *Lancet.*;387(10022):957-967. doi: 10.1016/S0140-6736(15)01225-8.

Fernandes R, Garver H, Harkema JR, Galligan JJ, Fink GD and Xu H. (2018). Sex Differences in Renal Inflammation and Injury in High-Fat Diet-Fed Dahl Salt- Sensitive Rats. *Hypertension.* 72(5):e43-e52. doi: 10.1161/HYPERTENSIONAHA.118.11485.

Filho AG, Kinote A, Pereira DJ, Rennó A, dos Santos RC, Ferreira-Melo SE, Velloso LA, Bordin S, Anhê GF and Moreno Junior H. (2013). Infliximab prevents increased systolic blood pressure and upregulates the AKT/eNOS pathway in the aorta of spontaneously hypertensive rats. *Eur J Pharmacol.* 700(1-3):201-9. doi: 10.1016/j.ejphar.2012.11.059.

Fitzgibbons TP and Czech MP. (2016). Emerging evidence for beneficial macrophage functions in atherosclerosis and obesity-induced insulin resistance*. J Mol Med (Berl).* 94(3):267-75. doi: 10.1007/s00109-016-1385-4.

Gu P, Cheng M, Hui X, Lu B, Jiang W and Shi Z. (2015). Elevating circulation chemerin level is associated with endothelial dysfunction and early atherosclerotic changes in essential hypertensive patients. *J Hypertens.* 33(8):1624-32. doi: 10.1097/HJH.0000000000000588.

Guo F and Garvey WT. (2016). Cardiometabolic disease risk in metabolically healthy and unhealthy obesity: Stability of metabolic health status in adults. *Obesity (Silver Spring).* 24(2):516-25. doi: 10.1002/oby.21344.

Hansel TT, Kropshofer H, Singer T, Mitchell JA and George AJ. (2010). The safety and side effects of monoclonal antibodies*. Nat Rev Drug Discov.* 9(4):325-38. doi: 10.1038/nrd3003.

Hashmat S, Rudemiller N, Lund H, Abais-Battad JM, Van Why S and Mattson DL. (2016). Interleukin-6 inhibition attenuates hypertension and associated renal damage in Dahl salt-sensitive rats. *Am J Physiol Renal Physiol.* 311(3):F555-61. doi: 10.1152/ajprenal.00594.2015.

Heidari F, Vasudevan R, Mohd Ali SZ, Ismail P and Arkani M. (2017). RAS Genetic Variants in Interaction with ACE Inhibitors Drugs Influences Essential Hypertension Control. *Arch Med Res.* 48(1):88-95. doi: 10.1016/j.arcmed.2017.03.003.

Henstridge DC, Abildgaard J, Lindegaard B and Febbraio MA. (2019). Metabolic control and sex: A focus on inflammatory-linked mediators. *Br J Pharmacol.* 176(21):4193-4207. doi: 10.1111/bph.14642.

Izquierdo AG, Crujeiras AB, Casanueva FF and Carreira MC. (2019). Leptin, Obesity, and Leptin Resistance: Where Are We 25 Years Later? *Nutrients*. 11(11):2704. doi: 10.3390/nu11112704.

Karbach SH, Schönfelder T, Brandão I, Wilms E, Hörmann N, Jäckel S, et. al. (2016) Gut Microbiota Promote Angiotensin II-Induced Arterial Hypertension and Vascular Dysfunction. *J Am Heart Assoc.* 5(9):e003698. doi: 10.1161/JAHA.116.003698.

Kelchtermans H, Billiau A and Matthys P. (2008). How interferon-gamma keeps autoimmune diseases in check. *Trends Immunol.* 29(10):479-86. doi: 10.1016/j.it.2008.07.002.

Khraibi AA, Norman RA Jr and Dzielak DJ. (1984). Chronic immunosuppression attenuates hypertension in Okamoto spontaneously hypertensive rats. *Am J Physiol.* 247(5 Pt 2):H722-6. doi: 10.1152/ajpheart.1984.247.5.H722.

Kim SC, Solomon DH, Rogers JR, Gale S, Klearman M, Sarsour K and Schneeweiss S. (2017). Cardiovascular Safety of Tocilizumab Versus Tumor Necrosis Factor Inhibitors in Patients With Rheumatoid Arthritis: A Multi-Database Cohort Study. *Arthritis Rheumatol.* (6):1154-1164. doi: 10.1002/art.40084.

Landt M, Gingerich RL, Havel PJ, Mueller WM, Schoner B, Hale JE and Heiman ML. (1998). Radioimmunoassay of rat leptin: sexual dimorphism reversed from humans. *Clin Chem.* 44(3):565-70. PMID: 9510863.

Leslie WS, Hankey CR and Lean ME. (2007). Weight gain as an adverse effect of some commonly prescribed drugs: a systematic review. *QJM.* 100(7):395-404. doi: 10.1093/qjmed/hcm044.

Lichtenstein L, Ron Y, Kivity S, Ben-Horin S, Israeli E, Fraser GM, Dotan I, Chowers Y, Confino-Cohen R and Weiss B. (2015). Infliximab-Related Infusion Reactions: Systematic Review. *J Crohns Colitis.* 9(9):806-15. doi: 10.1093/ecco- jcc/jjv096.

Lluch J, Servant F, Païssé S, Valle C, Valière S, Kuchly C, et. al. (2015). The Characterization of Novel Tissue Microbiota Using an Optimized 16S Metagenomic Sequencing Pipeline. *PLoS One.* 10(11):e0142334. doi: 10.1371/journal.pone.0142334.

Lodato RF, Khan AR, Zembowicz MJ, Weisbrodt NW, Pressley TA, Li YF, Lodato JA, Zembowicz A and Moody FG. (1999) Roles of IL-1 and TNF in the decreased ileal muscle contractility induced by lipopolysaccharide. *Am J Physiol.* 276(6):G1356-62. doi: 10.1152/ajpgi.1999.276.6.G1356.

Ma D, Feitosa MF, Wilk JB, Laramie JM, Yu K, Leiendecker-Foster C, Myers RH, Province MA and Borecki IB. (2009). Leptin is associated with blood pressure and hypertension in women from the National Heart, Lung, and Blood Institute Family Heart Study. *Hypertension.* 53(3):473-9. doi: 10.1161/HYPERTENSIONAHA.108.118133.

Mattson DL, Dwinell MR, Greene AS, Kwitek AE, Roman RJ, Jacob HJ and Cowley AW Jr. (2008). Chromosome substitution reveals the genetic basis of Dahl salt-sensitive hypertension and renal disease. *Am J Physiol Renal Physiol.* 295(3):F837-42. doi: 10.1152/ajprenal.90341.2008.

McLaughlin T, Ackerman SE, Shen L and Engleman E. (2017). Role of innate and adaptive immunity in obesity-associated metabolic disease. *J Clin Invest.* 127(1):5-13. doi: 10.1172/JCI88876.

Monnet E, Lapeyre G, Poelgeest EV, Jacqmin P, Graaf K, Reijers J, Moerland M, Burggraaf J and Min C. (2017). Evidence of NI-0101 pharmacological activity, an anti-TLR4 antibody, in a randomized phase I dose escalation study in healthy volunteers receiving LPS. *Clin Pharmacol Ther.* 101(2):200-208. doi: 10.1002/cpt.522.

Powell AG, Apovian CM and Aronne LJ. (2011). New drug targets for the treatment of obesity. *Clin Pharmacol Ther.* 90(1):40-51. doi: 10.1038/clpt.2011.82.

Powell AG, Apovian CM and Aronne LJ. (2012). The combination of phentermine and topiramate is an effective adjunct to diet and lifestyle modification for weight loss and measures of comorbidity in overweight or obese adults with additional metabolic risk factors. *Evid Based Med.* 17(1):14-5. doi: 10.1136/ebm.2011.100103.

Powell MJ, Thompson SA, Tone Y, Waldmann H and Tone M. (2000). Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. *J Immunol.* 165(1):292-6. doi: 10.4049/jimmunol.165.1.292.

Rider JA and Moeller HC. (1963). Double-blind evaluation of the use of phentermine in treating obesity. *Appl Ther.* 5:523-4. PMID: 13982231.

Rodriguez-Iturbe B, Pons H and Johnson RJ. (2017). Role of the Immune System in Hypertension. *Physiol Rev.* 97(3):1127-1164. doi: 10.1152/physrev.00031.2016.

Russo L and Lumeng CN. (2018). Properties and functions of adipose tissue macrophages in obesity. *Immunology.* 155(4):407-417. doi: 10.1111/imm.13002.

Saxton SN, Whitley AS, Potter RJ, Withers SB, Grencis RK and Heagerty AM. (2020). Interleukin-33 rescues perivascular adipose tissue anti-contractile function in obesity. Am *J Physiol Heart Circ Physiol.* doi: 10.1152/ajpheart.00491.2020.

Simon M, Gomberg-Maitland M, Oudiz RJ, Machado R, Rischard F, Elinoff JM et. al. (2019). Severe pulmonary arterial hypertension treated with ABI-009, nab-sirolimus, an mTOR inhibitor. J Heart Lung Transplant. 28(4) S487. doi: 10.1016/j.healun.2019.01.1238.

Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M and O'Shea JJ. (2017). JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nat Rev Drug Discov*. 17(1):78. doi: 10.1038/nrd.2017.267.

Sehnert KW. (1963). Development of phentermine, an appetite-control drug. *Clin Med (Northfield).* 70:400-3. PMID: 15445969.

Seibert FS, Steltzer J, Melilli E, Grannas G, Pagonas N, Bauer F, Zidek W, Grinyó J and Westhoff TH. (2014). Differential impact of belatacept and cyclosporine A on central

aortic blood pressure and arterial stiffness after renal transplantation. *Clin Transplant.* 28(9):1004-9. doi: 10.1111/ctr.12413.

Seidel E and Scholl UI. (2017). Genetic mechanisms of human hypertension and their implications for blood pressure physiology. *Physiol Genomics.* 49(11):630-652. doi: 10.1152/physiolgenomics.00032.2017.

Shah SR, Abbasi Z, Fatima M, Ochani RK, Shahnawaz W, Asim Khan M and Shah SA. (2018). Canakinumab and cardiovascular outcomes: results of the CANTOS trial. *J Community Hosp Intern Med Perspect.* 8(1):21-22. doi: 10.1080/20009666.2018.1428023.

Shahin MH and Johnson JA. (2016). Mechanisms and pharmacogenetic signals underlying thiazide diuretics blood pressure response. *Curr Opin Pharmacol.* 27:31-7. doi: 10.1016/j.coph.2016.01.005.

Sidiropoulos PI and Boumpas DT. (2004). Lessons learned from anti-CD40L treatment in systemic lupus erythematosus patients. *Lupus.* 13(5):391-7. doi: 10.1191/0961203304lu1032oa.

Sikka G, Miller KL, Steppan J, Pandey D, Jung SM, Fraser CD, et. al. (2013) Interleukin 10 knockout frail mice develop cardiac and vascular dysfunction with increased age. *Exp Gerontol.* 48(2):128-35. doi: 10.1016/j.exger.2012.11.001.

Skiba DS, Nosalski R, Mikolajczyk TP, Siedlinski M, Rios FJ, Montezano AC, et. al. (2017). Anti-atherosclerotic effect of the angiotensin 1-7 mimetic AVE0991 is mediated by inhibition of perivascular and plaque inflammation in early atherosclerosis. *Br J Pharmacol.* 174(22):4055-4069. doi: 10.1111/bph.13685.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS and Lazar MA. (2001). The hormone resistin links obesity to diabetes. *Nature.* 409(6818):307-12. doi: 10.1038/35053000.

Tanase DM, Gosav EM, Radu S, Ouatu A, Rezus C, Ciocoiu M, Costea CF and Floria M. (2019). Arterial Hypertension and Interleukins: Potential Therapeutic Target or Future Diagnostic Marker? *Int J Hypertens.* 2019:3159283. doi: 10.1155/2019/3159283.

Taylor LE and Sullivan JC. (2016). Sex differences in obesity-induced hypertension and vascular dysfunction: a protective role for estrogen in adipose tissue inflammation? *Am J Physiol Regul Integr Comp Physiol.* 311(4):R714-R720. doi: 10.1152/ajpregu.00202.2016.

Tinsley JH, Chiasson VL, South S, Mahajan A and Mitchell BM. (2009). Immunosuppression improves blood pressure and endothelial function in a rat model of

pregnancy- induced hypertension. *Am J Hypertens.* 22(10):1107-14. doi: 10.1038/ajh.2009.125.

Tipton AJ and Sullivan JC. (2014). Sex differences in blood pressure control: are T lymphocytes the missing link? *Hypertension.* 64(2):237-9. doi: 10.1161/HYPERTENSIONAHA.114.03688.

Ware JS, Wain LV, Channavajjhala SK, Jackson VE, Edwards E, Lu R, et. al. (2017). Phenotypic and pharmacogenetics evaluation of patients with thiazide-induced hyponatremia. *J Clin Invest.* 127(9):3367-3374. doi: 10.1172/JCI89812.

Whelton PK, Carey RM, Aronow WS, Casey DE Jr, Collins KJ, Dennison Himmelfarb C, et. al. (2018) ACC/AHA/AAPA/ ABC/ACPM/AGS/ APhA/ASH/ ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Hypertension.* 71(6):1269-1324. doi: 10.1161/HYP.0000000000000066.

Withers SB, Forman R, Meza-Perez S, Sorobetea D, Sitnik K, Hopwood T, et. al. (2017). Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. *Sci Rep.* 7:44571. doi: 10.1038/srep44571.

Zhang H and Kuchroo V. (2019). Epigenetic and transcriptional mechanisms for the regulation of IL-10. *Semin Immunol.* 4:101324. doi: 10.1016/j.smim.2019.101324.
Zhang L, Curhan GC and Forman JP. Plasma resistin levels associate with risk for hypertension among nondiabetic women. (2010). *J Am Soc Nephrol.* 21(7):1185-91. doi: 10.1681/ASN.2009101053.