SGK1, influenced by salt and IL-17A, promotes hypertension and end-organ damage

By

Allison Elizabeth Norlander

Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in

Molecular Physiology and Biophysics
May, 2017
Nashville, Tennessee

Approved:

Alyssa H. Hasty, Ph.D.

Raymond C. Harris, Jr., M.D.

Jacek J. Hawiger, M.D., Ph.D.

Katherine T. Murray, M.D.

Jens M. Titze, M.D.

To my parents, Lynn and Barry, and my brother Eric, always and infinitely supportive, I couldn't have completed this degree without you.

ACKNOWLEDGEMENTS

I would like to eternally thank both of my mentors, Drs. Meena Madhur and David Harrison. They have taught me an incredible amount over the past 4 years and I am indebted to them for their endless support and guidance. With their help I have learned to become a focused, precise, and dedicated scientist. I am also grateful to my Dissertation Committee Members: Drs. Alyssa Hasty, Ray Harris, Jens Titze, Jacek Hawiger, and Kathy Murray, for their direction, criticism and support that allowed me to complete these studies successfully. I would also like to thank the Department of Molecular Physiology and Biophysics for providing an environment that allowed me to grow as a scientist.

I am also incredibly grateful to all the past and present members of the Madhur and Harrison laboratories, especially all who helped with my studies. In particular, I would like to thank the following individuals. I am grateful to Dr. Dan Trott, for taking me as a rotation student, becoming a friend, and helping me immensely during my first couple years in the laboratory. I would like to wholeheartedly thank Dr. Mohamed Saleh, who without I wouldn't have learned so many techniques, for friendship and for always being so willing to help. I would also like to thank Dr. Hana Itani for her continuous advice and friendship, as well as experimental help, during my graduate career. Furthermore, I would like to thank my fellow graduate students Bethany Dale and Roxana Loperena for their friendship and continuous support.

Finally I would like to thank all of my friends and family (including my cats and my dog!) who have given me so much support and love during this journey. I couldn't have finished without each one of you.

Acknowledgements of Support

This work would have not been possible without the salary support of the Immunobiology of Blood and Vascular Systems Training Program (NIH T32 HL069765-11A) and the F31 Predoctoral Individual National Research Service Award (NIH: NHLBI 1F31HL127986-01); the experiments would not have been completed without the American Heart Association's Strategically Focused Research Network grant (14SFRN20420046) awarded to Drs. Madhur and Harrison.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Introduction

Hypertension, defined as blood pressure ≥ 140/90mmHg, is a worldwide health concern that affects approximately 30% of adults and 70% of the elderly in the United States. Hypertension contributes to cardiovascular disease morbidity and mortality [1]. In 2010 it was ranked as the top single risk factor for global burden of disease and affects individuals in both economically developed and developing nations alike [2]. End-organ damage of the kidneys and vasculature are important manifestations of the disease. As such, those who suffer from hypertension are more likely to develop atherosclerosis, stroke, myocardial infarction, heart failure, chronic kidney disease and dementia [1, 3]. While occasional cases of hypertension are due to identifiable causes, such as renal artery stenosis, pheochromocytoma, excessive adrenal aldosterone production, or monogenetic causes, more than 90% of cases do not have an identifiable etiology and are classified as “essential”. Essential hypertension often co-exists with obesity, disorders of lipid metabolism, aging and insulin resistance, and thus is often considered as part of a complex metabolic phenotype that has myriad manifestations [4].

Classic Concepts of Hypertension

Perturbations of the vasculature, central nervous system and the kidneys have all been implicated in essential hypertension, and likely all contribute to elevations of blood pressure. Yet, the precise manner in which these systems are recruited and interact remains undefined. Blood pressure is the product of cardiac output and systemic vascular resistance. Thus, either cardiac output or systemic vascular resistance must be elevated in chronic hypertension. Interestingly, it appears these play different roles depending on age and duration of hypertension. Fagard and

Stassen measured cardiac output at rest and during exercise in 110 hypertensive individuals ranging from 16 to 64 years of age, and found that cardiac output is elevated in younger individuals (<age 25) with hypertension but was within the normal range in older patients [5]. While this might reflect differences in the etiology of hypertension in younger vs older individuals, this pattern is compatible with the concept that blood volume, and thus cardiac output is elevated early in hypertension, and that there are vascular adaptations that occur later in the disease, associated with a reduction in blood volume, and an increase in systemic vascular resistance.

Why would blood volume be increased in hypertension? There is substantial support for the concept that renal retention of volume and sodium must occur to sustain hypertension; simply stated, in the setting of normal kidneys, an increase in blood volume and elevation of blood pressure leads to a brisk diuresis and ultimately the normalization of blood pressure. The relationship between blood pressure with sodium and water excretion can be described by the 'pressure-natriuresis' relationship defined by Guyton and co-workers. For hypertension to be sustained there must be a rightward shift in this relationship. Any insult or change to the kidney that alters this ability to excrete sodium and water will result in a 'natriuretic handicap;' wherein the mean arterial pressure over which salt and water is excreted will increase to account for a decrease in the capacity of kidney function [6]. This is often not reflected by overt changes in renal function, but by enhanced reabsorption of sodium and water along the nephron regulated through differential activity of the various proximal and distal transporters. Sustained hypertension is associated with vascular rarefaction within the kidney, fibrotic replacement of renal parenchymal tissue, and defects in glomerular function and proteinuria [7, 8]. These defects

in renal function underlie the importance of sodium restriction and use of diuretics in the treatment of hypertension, which act in part to normalize the pressure natriuresis curve.

If blood volume is increased in early hypertension, or in younger age groups, why does it return to normal values with time and why is there a concomitant increase in systemic vascular resistance? Guyton proposed that the increase in cardiac output would lead to “systemic autoregulation”, and that this further raises blood pressure, leading to a pressure natriuresis and return of blood volume (and cardiac output) to near normal levels. It is now clear that hypertension is associated with other events that increase systemic vascular resistance. These events evolve slowly [9, 10]. These include enhanced vasoconstriction due to factors including loss of endothelium-derived nitric oxide, local production of potent vasoconstrictors such as endothelin-1, prostaglandin $H_{2,}$ and angiotensin II (Ang II), and increased production of reactive oxygen species (ROS), such as superoxide. There is also remodeling of vessels in hypertension, initially described by Folkow, in which the vascular lumen is narrowed as the media thickens [11]. Finally, there is frank loss of capillaries and resistance vessels, known as vascular rarefaction, in hypertension. All of these events combine to reduce the cross sectional area of the vasculature and predispose individuals to increased systemic vascular resistance and hypertension [12, 13].

How does the central nervous system contribute? Activation of the sympathetic nervous system results in release of norepinephrine from postganglionic neurons which act through adrenergic receptors on target organs. Increased sympathetic outflow and adrenergic agonists contribute to left-ventricular hypertrophy, vascular remodeling and vascular smooth muscle cell proliferation [14]. Furthermore, the sympathetic nervous system innervates renal tubules, renal vessels and the juxtaglomerular granular (JG) cells of the kidney. Not only does enhanced renal sympathetic

nerve activity result in increased sodium and water reabsorption in the nephron and constriction of the renal vasculature, increased renal sympathetic nerve activity stimulates renin release from JG cells creating a feed-forward mechanism that activates the renin-angiotensin (RAS) system. These changes magnify the activity of renal transporters further increasing sodium and water reabsorption and promoting hypertension [15].

Emerging evidence over the past decade suggests that these alterations stem from, at least in part, an underlying inflammatory condition during which innate and adaptive immune cells become activated, invade target tissues and promote end-organ damage through production of various cytokines and chemokines.

Rodent Models of Hypertension

Several different rodent models are commonly used to experimentally induce and study hypertension. One of these methods is the infusion of Ang II into mice or rats. As described above, Ang II is a hormone responsible for stimulating many effects that lead to hypertension. Ang II is infused into rodents via osmotic mini pumps at pressor doses of either 490 or 1000 ng/kg/min over the course of, commonly, 7,14 or 28 days [16, 17]. A second method, the deoxycorticosterone acetate (DOCA) /salt method, results in induction of a salt sensitive form of hypertension [18, 19]. Salt sensitivity is defined as >5-10% change in blood pressure with salt intake [20]. In this case, investigators remove one kidney, implant a DOCA pellet, and feed the mice or rats 1% salt water over the course of 3 weeks. DOCA is an aldosterone analog and thus results in increased sodium and water reabsorption in the kidney. Another salt-sensitive model is the L-NG-Nitroarginine Methyl Ester (L-NAME) and high salt method for inducing hypertension. In this case animals are initially given L-NAME in the drinking water for two weeks; they then go through a two week washout period and subsequently receive a 4%NaCl

high salt diet for the next three weeks [21]. Genetic adaptations that predispose rats to hypertension are also commonly studied. One such rat is the spontaneously hypertensive rat (SHR). These rats were generated by Okamoto and colleagues who bred a male Wistar Kyoto rat with noticeably elevated blood pressure to a female Wistar Kyoto rat with slightly elevated blood pressure in the 1960s [22]. The development of hypertension in the SHR begins at about 5-6 weeks of age. Another rat strain genetically prone to develop hypertension is the Dahl Salt-Sensitive rat. In the 1960s as well, Lewis K. Dahl began breeding rats together that were susceptible to the effects of a high salt (8% salt) diet, i.e. they developed hypertension and its accompanying comorbidities [23]. Today this strain is still used to investigate mechanisms of salt-sensitive hypertension.

Brief History of the Immune System in Hypertension

The idea that the immune system plays a role in hypertension was first proposed in the 1960s. Grollman et al. showed that the transfer of lymphocytes from rats that had induced unilateral renal infarction to previously normotensive rats caused hypertension in the recipients [24]. Additionally, Grollman et al. demonstrated that immunosuppression lowers blood pressure in rats that had partial renal infarction [25]. Olsen et al. described an inflammatory reaction occurring inside the vasculature of humans with different causes of hypertension; specifically he noted a periadventitial accumulation of T cells and monocytic cells [26].

In the 1970s Svendsen discovered that mice which were thymectomized or athymic nude mice do not maintain hypertension post renal infarction [27]. Ba et al., in the 1980s, discovered that transplant of a thymus from a Wistar-Kyoto (WKY) reduced blood pressure in a recipient spontaneously hypertensive rat (SHR). They also noted that transplanting a compatible thymus into neonatal SHRs resulted in significant suppression of blood pressure if full immunologic

restoration was achieved [28]. These experiments set the stage for recent advances regarding the role of the immune system in hypertension.

Innate Immune System and Hypertension

The innate immune system is responsible for the non-specific early response to antigen that occurs in the body and also for priming the adaptive immune system to said antigen which it subsequently attacks with a specific response. One of the first cell types of the innate immune system formally associated with the development of hypertension was the monocyte. Monocytes are antigen presenting cells (APCs). APCs are specialized cells which present antigen to and thus activate the adaptive immune system. APCs are also composed of macrophages and dendritic cells (DCs). One of the first studies to clarify the importance of monocytes to the development of Ang II-induced hypertension was performed by Wenzel et al. The authors selectively depleted monocytes in mice by treating animals that had inducible expression of the diphtheria toxin receptor on myeloid cells ($LysM^{iDTR}$) with a low dose regimen of the toxin. Depletion of monocytes blunted hypertension and attenuated endothelial dysfunction, a condition resulting in imbalanced levels of vasodilating and vasoconstricting substances in the vessels leading to increased vasoconstriction and systemic resistance during hypertension, relative to controls. The hypertensive response was fully restored by adoptive transfer of wild type monocytes into depleted mice [29].

Our laboratory and others have shown that immune cells infiltrate the kidney and perivascular fat of vessels and release cytokines that alter organ function. Infiltrating APCs, including monocytes and DCs, contain multiple important sensors of the innate immune system allowing them to respond appropriately to pathogens; one of these sensors is the inflammasome. Inflammasomes are complexes activated by different pathogen- and danger- associated molecular patterns

(PAMPs and DAMPs) which lead to the cleavage of IL-β and IL-18 inside the cell. These cytokines are subsequently released to activate pro-inflammatory signaling pathways. Of the 4 inflammasome complexes that exist, the NLRP3 inflammasome reacts to multiple stimuli implicated in the pathogenesis of hypertension, including ROS and other molecules associated with stress and danger responses. Three recent studies have demonstrated the importance of the inflammasome to hypertension. Wang et al. showed that hypertension is blunted in inflammasome-deficient $ASC^{-/-}$ and $NLRP3^{-/-}$ mice using a renin-dependent model of hypertension [30]. ASC is an adaptor protein crucial to formation of an active inflammasome complex. Krishnan et al demonstrated that $ASC^{-/-}$ are protected from both the DOCA/salt and Ang II-induced models of hypertension. These authors also demonstrate that a specific NLRP3 inhibitor reverses DOCA/salt induced blood pressure increases, indicating a causal role for the inflammasome and its key cytokines, IL-1β and IL-18, in the development of hypertension [19]. While Bruder-Nascimento et al. report that long-term aldosterone infusion in mice results in increased plasma levels of IL-1β and increased vascular contractility coupled with decreased endothelial-dependent vascular relaxation in mesenteric arterioles. Mice that lacked either the IL-1 receptor (IL-1R1) or NLRP3 and caspase 1, another important inflammasome component, were protected from those vascular abnormalities [31].

The demonstrated importance of IL-β to the development of both human and rodent hypertension led Zhang et al. to investigate the effect of this cytokine on the kidney. The authors recently showed that genetic deficiency or blockade of IL-1R1 limited the extent to which blood pressure could rise in response to Ang II infusion, adding to previous evidence implicating IL-1 signaling in the pathogenesis of hypertension. Furthermore, they went on to demonstrate that signaling of the IL-1R1 in the kidney prevents intra-renal myeloid cells from maturing into Ly6C+Ly6G+

macrophages which produce nitric oxide (NO). NO is a known suppressor of Na-K-Cl cotransporter 2 (NKCC2). Thus, IL-1R1 signaling effectively activates NKCC2 in the kidney, promoting sodium reabsorption and blood pressure elevation [32].

Additionally, innate immune cells such as monocytes, macrophages and neutrophils contain functional NADPH oxidases which produce ROS to help eliminate invading pathogens. We have consistently found increased levels of ROS in the vasculature in models of hypertension [17, 33, 34]. Therefore, activated innate immune cells in hypertension also contribute to vasoconstriction and vascular remodeling through production of ROS which lowers bioavailability of NO.

The complement system is another important component of the innate immune system and functions to boost the abilities of antibodies and phagocytic cells to clear pathogens. Activation of this system leads to cleavage of the complement proteins, which are constantly in circulation, to their active state. The role of one such protein, anaphylatoxin C5a, in hypertension was recently examined by Weiss et al. The authors found that a majority of C5aR1 positive cells in the kidney were DCs and that renal injury, but not cardiac injury, after unilateral nephrectomy and Ang II infusion was ameliorated in mice lacking C5aR1 [35]. Thus the authors described a previously undefined role for C5a in the development of renal injury during hypertension.

Shah et al. identified a population of myeloid-derived suppressor cells (MDSCs), which are immature myeloid cells capable of regulating and suppressing the immune system, that are increased in the circulation, the kidneys, and the spleen of hypertensive mice. These cells are capable of suppressing T cell proliferation and accumulate in the spleen where they interact with T cells in attempts to counter inflammation and blood pressure increase. The authors demonstrate that depletion of these cells results in an enhanced pro-inflammatory phenotype of T cells in both the spleen and kidney [36]. MDSCs were initially identified in cancer models and their

accumulation during chronic hypertension increases the evidence indicating that inflammatory and immune responses are dysregulated in this disease.

Furthermore, our laboratory has recently identified the key cells that produce collagen and contribute to vascular stiffening in hypertension. The Harrison lab found that cells expressing stem cell antigen 1 (Sca-1) produce large amounts of collagen and take on a fibroblast like phenotype in hypertension. Additionally they found that the majority of cells producing collagen in the aorta during hypertension are bone-marrow derived [37]. This study thus identifies the bulk of collagen producing cells in the aorta as part of the innate immune system.

T cells and Hypertension

In 2007, our group showed that mice deficient in T and B cells ($Rag1^{-/-}$ mice) develop blunted hypertension and have preserved vascular endothelial function when infused with Ang II or DOCA/salt. Interestingly, adoptive transfer of T, but not B, cells restored the hypertensive response. Following Ang II infusion, T cells were shown to have become activated and to have infiltrated the perivascular adipose tissue of mice [38]. This paper was the first to directly link T cells to hypertension.

Two important studies went on to confirm and expand upon the initial findings observed by our group regarding the importance of T cells to the genesis of hypertension. Mattson et al. generated $Rag1^{-/-}$ rats from the Dahl salt sensitive (SS) strain and found that blood pressure increase was blunted in $Rag1^{-/-}$ rats compared to wild type Dahl SS rats after high salt feeding. $Rag1^{-/-}$ rats had less immune cells accumulate in their kidneys after high salt feeding and also had attenuated glomerular and tubular damage compared to Dahl SS rats [39]. Crowley et al. described how *scid*

mice, which lack lymphocyte activity, develop blunted hypertension and cardiac hypertrophy when infused with Ang II [40].

Furthermore, Rudemiller et al. specifically investigated the role of the CD3 zeta chain (CD247), a gene important for T cell signaling, in hypertension. The authors deleted CD247 in Dahl SS rats using zinc-finger nucleases. This deletion resulted in decreased infiltration of T cells into the kidney, lower blood pressure and preserved renal integrity in response to salt [41]. In general, T cell survival is dependent on functionality of Axl, a receptor tyrosine kinase. Axl has been linked to hypertension by several recent GWAS and genomic studies [42]. Batchu et al. demonstrate that $Rag1^{-/-}$ mice that received $Axl^{-/-}$ CD4+ T cells through adoptive transfer developed a blunted blood pressure increase and decreased perivascular adventitial T cell infiltration in response to DOCA/salt induction compared to those animals who received $Axl^{+/+}$ CD4+ T cells. The authors report that the protective phenotype of $Axl^{-/-}$ CD4+ T cells is due to their inability to expand and survive after transfer, a function of Axl [43]. Together, these studies suggest that fully functional T cells are important for the development of hypertension and add more evidence demonstrating the need for these cells for disease progression.

T cell Activation in Hypertension

With verification of T cell involvement in hypertensive development, investigators became curious as to how these cells were being activated during disease progression. One possibility was with involvement of the sympathetic nervous system. To determine if sympathetic outflow and the activation of the sympathetic nervous system could be involved in immune activation, Marvar et al. looked at a specific region in the circumventricular organs (CVO) implicated in blood pressure control, the anteroventral third ventricle (AV3V) region. Previous research has shown that electrolytic lesions which disrupt the AV3V region completely eliminate the central

actions of Ang II. When the authors investigated T cell activation and vascular inflammation in mice which have AV3V lesions, they found that the blood pressure increase and activation and infiltration of T cells were virtually abolished in these mice in response to Ang II [44]. In relation, Xiao et al. recently investigated the importance of sympathetically innervated renal nerves, which transmit sympathetic outflow to the kidneys, to the development of hypertension using renal denervation. The authors found that bilateral renal denervation prevents dendritic cell (DC) activation. This resulted in less T cell activation and infiltration, a blunted blood pressure increase, and preserved renal integrity in response to Ang II [45]. Furthermore, Carnevale et al. recently showed that hypertensive stimuli trigger splenic sympathetic nerve discharge, a response that is necessary for T cell activation and their subsequent egression from the spleen during hypertension [46]. Together, these studies implicate the central nervous system and sympathetic outflow as important for T cell activation and hypertension.

The CVO has also been shown to produce superoxide in response to Ang II. ROS have been linked to hypertension; therefore, Lob et al. investigated the effects of superoxide production in the CVO to T cell activation in hypertension in two studies. In the first, the authors deleted extracellular superoxide dismutase (SOD3) in the CVO of mice using an adenovirus encoding cre-recombinase/loxP based system. They found that deletion of SOD3 in the CVO results in enhanced T cell activation and vascular infiltration in response to Ang II. Moreover, they also found that the sympathetic modulation of the heart was increased. These studies suggest that superoxide production in the CVO may activate T cells through modulation of sympathetic outflow [47]. In the second set of studies, our laboratory implicated ROS production by the NADPH oxidases of the subfornical organ of the CVO as a crucial determinant in blood pressure regulation. Lob et al., again using an adenovirus encoding cre-recombinase/loxP system, deleted

the $p22^{phox}$ subunit of the NADPH oxidase in the SFO. This deletion prevented blood pressure increase and eliminated vascular inflammation and T cell infiltration in these mice in response to Ang II, adding further evidence to the importance of ROS signaling in the CVO to T cell activation in hypertension [48].

Traditionally, monocytes transverse the endothelium and differentiate into macrophages or DCs that function during disease. These activated APCs then migrate to secondary lymphoid organs where they come in contact with and activate T cells. In 2014, our laboratory described the importance of DCs to the activation of T cells during hypertension. Kirabo et al. showed that DCs in mice infused with Ang II become activated and produce increased amounts of ROS, ultimately leading to the production of isoketal-adducted proteins in these cells. These isoketalated proteins are then presented to T cells and promote their proliferation and differentiation [34]. Furthermore, overexpression of the NADPH subunit $p22^{phox}$ in vascular smooth muscle cells in mice resulted in their development of age-related hypertension, inflammation, and aortic stiffening due to increased production of ROS throughout their lifetime. Aortic stiffening is characteristic of aging and is a condition hallmarked by increased collagen deposition and a reduction in aortic compliance. At younger ages and among those not afflicted by cardiovascular disease, the elastic properties of the aorta allow it to act as both a supplier and reservoir of peripheral blood. Half of the blood is ejected during systole and as the aorta recoils during diastole the remaining blood is pushed into circulation to keep the body adequately perfused between heart beats, this is termed the Windkessel effect. Thus, accumulation of collagen or degradation of elastin results in a decrease in flexibility of the aorta and requires higher pressures to force blood into circulation [49, 50]. Wu et al. ultimately found that DCs isolated from these aged mice contain isoketalated proteins and that treatment with the

scavenger, 2-hydroxybenzylamine (2-HOBA), ameliorates age-dependent hypertension, inflammation and end-organ damage [51]. Together, these studies demonstrate the importance of ROS to isoketalated protein production, T cell activation and hypertension.

Recently, studies have also linked gut microbiota to immune activation in hypertension. The human microbiome is dominated primarily by the species *Firmicutes* and *Bacteroidetes*, although, both the *Actinobacteria* and *Proteobacteria* are also present [52]. The gut microbiota can regulate up to 10% of the host's transcriptome, including genes related to immunity [53, 54]. Cotillard et al. have shown that a decrease in microbial gene richness can result in low-grade inflammation [55]. Yang et al investigated the composition of the microbiota from fecal samples taken from the SHR, Ang II-induced hypertensive rats, and from humans with essential hypertension. The authors observed gut dysbiosis, or decrease in microbial richness, and an increased *Firmicutes/Bacteroidetes* ratio in all hypertensive models investigated. Oral administration of minocycline into Ang II infused rats normalized the *Firmicutes/Bacteroidetes* ratio and also attenuated blood pressure, thus connecting gut dysbiosis to activation of the immune system and to hypertension [56]. Furthermore, investigators have observed differences in the gut microbiota between the Dahl Salt-Sensitive and Dahl Salt-Resistant rats, thus adding further evidence implicating differences in microbiota to immune activation, inflammation and hypertension, although no direct mechanism has yet been identified [57]. Moreover, Santisteban et al. show that both SHR and Ang II-infused rats develop changes in their gut pathology; such as increases in their intestinal permeability. These alterations were associated with changes in gut microbial communities relevant to control of blood pressure in these animals. These authors also report an overall increase in sympathetic drive to the gut in these hypertensive animals, suggesting that abnormal sympathetic stimulation of the gut could drive changes in gut

pathology and gut microbial communities contributing to inflammation resulting in immune cell activation and hypertension [58].

T cell Subtypes in Hypertension

α/β T cells are produced in the bone marrow and migrate to the thymus where they undergo negative and positive selection to determine which subtype, either CD4+ or CD8+, they will become. We investigated the role of both of these subtypes of T cells in hypertension and observed oligoclonal expansion of $CD8^+$, but not $CD4^+$, T cells in the kidney. Additionally, $CD8^{-/-}$ mice developed blunted hypertension and preserved kidney and vascular function compared to $CD4^{-/-}$ mice [59]. These data demonstrate that an oligoclonal population of $CD8^+$ cells in the kidney serve to drive the development of hypertension and are most important to the disease. However, $CD4^+$ T cells produce cytokines, such as IL-17A and others, which are also important to disease pathogenesis.

$CD4^+$ T cells are able to differentiate into different subtypes relative to type of infection present or type of response needed (**Figure 1-1**, [60]). Characterization of the importance of each individual T cell subset in hypertension could shed light on potential therapeutic opportunities. Th1 cells are characterized by their expression of the transcription factor Tbet and by their production of large amounts of the cytokine, interferon-γ (IFNγ). Our laboratory found an increase in both $CD4^+$ T cells and $CD8^+$ T cells producing IFNγ in Ang II-induced hypertension, showing the importance of this T cell subset and of IFNγ to the development of the disease [17].

The heart itself is also an important target of activated immune cells during hypertension. Marko et al. have shown that cardiac damage, measured by hypertrophy and fibrosis, and also cardiac inflammation were reduced in IFN-γ receptor (IFN-γR) knockout mice in response to Ang II.

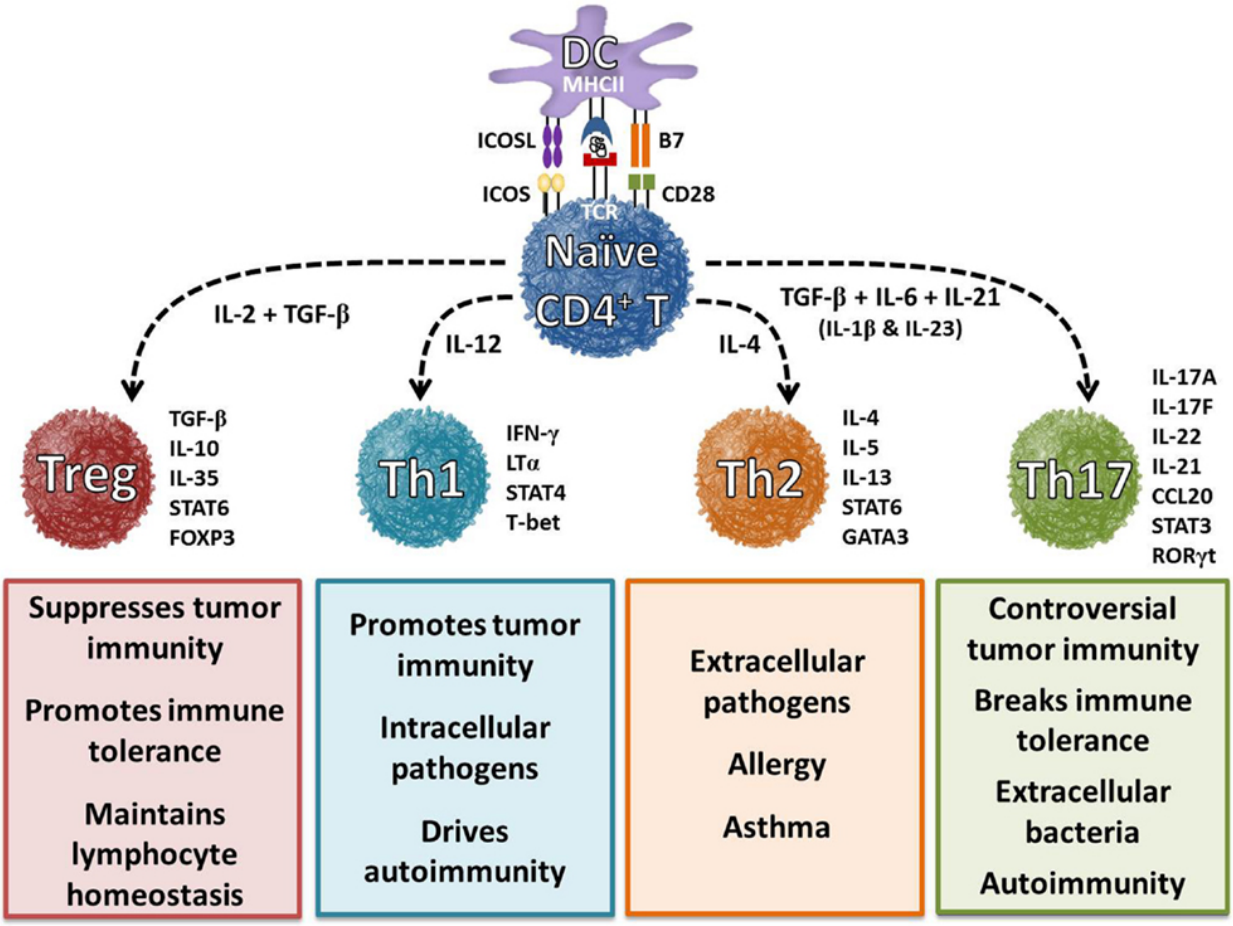


Figure 1-1. T helper subsets and their cytokines. T cells can differentiate into unique subsets specific to the insult that is occurring (Bailey et al.) [60].

However, blood pressure was not changed in these mice. Furthermore, the authors reported that in relation to kidney function in IFN-γR knockout mice, their glomerular function was preserved, while general podocyte survival and functionality was impaired when the animals were exposed to a high dose of Ang II [61]. These results suggest that IFNγ signaling may be important for the function of podocytes. However, Zhang et al investigated IFNγ knockout mice but saw no change in blood pressure or albumin excretion [62].

In keeping with the above, we recently found that the adapter protein LNK (SH3B3) plays an important role in suppressing IFN-γ production and hypertension. T cells of mice lacking this protein were found to produce much greater levels of IFNγ than those of WT mice following infusion of Ang II. Notably, these animals exhibited a marked increase in inflammation during Ang II infusion, and this phenotype was transferable to WT mice by bone marrow transplant or adaptive transfer of LNK-deficient hematopoietic cells. These results indicate that LNK functions as a 'brake' to proliferation and cytokine signaling in hematopoietic cells in which it is expressed in the context of hypertension [17].

Another important cytokine produced by Th1 cells is TNF-α. Zhang et al. show that TNF-$\alpha^{-/-}$ mice develop blunted hypertension and end-organ damage in response to Ang II. The authors also demonstrate that TNF-$\alpha^{-/-}$ mice had enhanced endothelial nitric oxide synthase (eNOS) expression and greater nitric oxide bioavailabilty than controls. These results suggest that TNFα signaling during hypertension limits eNOS expression and renal nitric oxide production. Zhang et al. also studied $Tbet^{-/-}$ mice and found that although blood pressure remained unchanged, nephrin elevation was blunted demonstrating preserved glomerular function [62]. Moreover, Chen et al investigated the effects of Angi II infusion on TNF-R1 (TNF-α receptor) deficient mice. Interestingly, TNF-$R1^{-/-}$ mice displayed increased blood pressure and albuminuria

compared to WT mice after 7 days of Ang II infusion. Additionally, the authors report that TNF-R1$^{-/-}$ mice also have increased urinary TNF-α compared to WT mice after Ang II infusion. Thus, the authors proposed that TNF-α may normally modulate kidney responses to Ang II through TNF-R1 and therefore its deletion results in an exacerbated hypertensive phenotype [63]. Venegas-Pont et al. investigated the contribution of TNF-α to the development of hypertension that arises concomitantly in a genetic mouse model of systemic lupus erythematosus (SLE), a chronic inflammatory disease. The authors infused female SLE (NZBWF1) mice with etanercept, a TNF-α antagonist, or vehicle for 4 weeks. Excitingly, they observed that mean arterial pressure, albuminuria, monocyte/macrophage infiltration and renal cortex NADPH activity were reduced in SLE mice treated with etanercept compared to those given vehicle, thus confirming the importance of TNF-α in development of hypertension in the setting of a chronic inflammatory disease [64]. Taken together, these studies display a contribution of Th1 cells to hypertensive end-organ damage.

In addition, the kidney has its own RAS system. Activation of the intrarenal RAS in the kidney contributes to the development of hypertension and also to kidney end-organ damage. A recent study by O'Leary et al. described how inflammatory cytokines contribute to the activation of intrarenal RAS. The authors found that IL-6, which is produced by monocytes and macrophages, through STAT3 activation, was responsible for elevation of angiotensinogen expression in proximal tubule cells [65]. Moreover, Satou et al had previously demonstrated how IFN-γ regulates angiotensinogen production by renal proximal tubule cells. Their studies showed that treatment of renal proximal tubular cells with IFN-γ for 6 or 12 hours results in a decrease of angiotensinogen production, however, 24 or 48 hours of exposure results in an increase in angiotensinogen production. They went on to discover, through RNA interference studies, that

this biphasic regulation of angiotensinogen is controlled in these cells by STAT1-SOCS1 axis modulation of STAT3 activity [66]. These studies highlight a potential mechanism by which IFN-γ modulates blood pressure.

Hashmat et al. investigated the importance of IL-6 to the development of salt sensitive hypertension. They found that an IL-6 neutralizing antibody administered during the course of high salt feeding ameliorated blood pressure in Dahl salt-sensitive rats. The IL-6 antibody also reduced their urinary albumin excretion, glomerular damage and tubular damage. These investigators further showed that infiltration of monocytes and macrophages was significantly reduced by IL-6 blockade, implicating this cytokine in the pathogenesis of salt sensitive hypertension [67].

Th17 cells are characterized by their expression of the transcription factor retinoic acid receptor-related orphan receptor gamma (RORγt) and of the cytokine IL-17A. Many studies have implicated Th17 cells in different autoimmune diseases. Madhur et al. observed that IL-$17^{-/-}$ mice do not sustain hypertension and are protected from T cell infiltration and vascular dysfunction caused by 4 weeks of Ang II compared to WT mice [33]. These studies were the first to show that signaling from IL-17 is necessary for the maintenance of hypertension. In a later set of studies, our laboratory confirmed the importance of IL-17A to hypertensive development through the use of antibody inhibition. Infusion of monoclonal antibodies to IL-17A or for its receptor, IL-17RA, during the second half of a 4 week Ang II infusion protocol in mice resulted in a reduction in blood pressure, a reduction in renal and vascular inflammation and blunted the development of albuminuria when compared to mice infused with control antibodies. These data suggest that IL-17A antibodies could be a useful treatment for hypertension [68].

Amador et al. found activation of Th17 cells in both the heart and kidney of rats through measurements of IL-17 and RORγt after induction of DOCA/salt hypertension. Moreover, the authors also found that treating DOCA/salt rats with an anti-IL-17 antibody reduced blood pressure and reduced expression of many pro-inflammatory and pro-fibrotic mediators in both the heart and kidney [18]. These studies confirm the importance of IL-17 signaling to the development of hypertension.

Our laboratory also investigated the importance of IL-17A to aortic stiffening in hypertension. We found that IL-17A$^{-/-}$ mice were protected against aortic stiffening [69]. Interestingly, Jiang et al. recently demonstrated the importance of IL-17A and Th17 cells to vascular remodeling during hypertension. The authors show that infusing mice with both Ang II and IFN-y results in decreased collagen 1 and III expression compared to mice only infused with Ang II. They also report a decrease in osteopontin expression in the spleen that coincides with reduced Th17 cell numbers in the spleen in the group that received both IFN-y and Ang II infusion compared to those just receiving Ang II [70]. Thus the authors conclude that IFN-y infusion may inhibit differentiation of CD4+ T cells to Th17 cells and also vascular remodeling during hypertension through modulation of osteopontin expression in the spleen.

Our laboratory provided the first evidence specifically linking the pro-inflammatory cytokines, IFN-γ and IL-17A to activity of kidney transporters in 2015. Specifically, Kamat et al. observed preserved renal function measured by urinary sodium excretion in response to a saline challenge in IFN-γ$^{-/-}$ mice but not WT mice after 2 weeks of Ang II infusion. This may, in part, be explained by a demonstrated decrease in expression of the distal transporters NKCC2 and Na-Cl cotransporter (NCC) coupled with maintenance of pressure natriuresis along the proximal tubule, measured by an expected decrease in sodium and hydrogen exchanger 3 (NHE3) expression, in

IFN-$\gamma^{-/-}$ mice but not in WT mice after 2 weeks of Ang II infusion. Furthermore, IL-17A$^{-/-}$ mice also display preserved renal function measured by urinary sodium excretion in response to a sodium challenge along with maintenance of the pressure natriuretic response, thus reduction in NHE3 expression, after 2 weeks of Ang II infusion compared to WT mice. However, distal tubular transporter expression is not changed compared to WT after 2 weeks of Ang II infusion [71]. These studies demonstrate the importance of both IFN-y and IL-17A to hypertension through their effects resulting in the reabsorption of sodium and water and thus elevated blood pressure.

However, one may question the potential molecular mechanism through which IL-17A and IFN-y act in the kidney cells themselves to cause these effects. To understand the mechanism through which IL-17A acts in the kidney we examined the profile of sodium transporters in the kidneys of WT and IL-17A$^{-/-}$ mice following 4 weeks of Ang II infusion. In WT mice, Ang II infusion caused an almost 2-fold increase in expression of both NCC and the epithelial sodium channel (ENaC), relative to sham. In striking contrast, there was no Ang II-induced increase of NCC or ENaC in IL-17A deficient mice. Additionally, we found that serum and glucocorticoid-regulated kinase 1 (SGK1) mRNA expression increases in the kidneys of WT mice after both 2 and 4 weeks of Ang II infusion, and that this increase was absent in the IL-17A deficient animals. *In vitro* studies revealed an SGK1 dependent increase in NCC activity in mouse distal convoluted tubule cells (mDCT15) and also an SGK1 dependent increase in NHE3 protein expression in human proximal tubule kidney epithelial cells (HK-2). Furthermore, IL-17A$^{-/-}$ mice were protected from renal injury that results from angiotensin II infusion, measured through albuminuria and angiotensinogen abundance in the urine [16]. These studies provide insight into how one inflammatory cytokine, IL-17A, can regulate sodium and water balance in the kidney.

Large amounts of evidence have demonstrated that T cells in hypertension predominantly infiltrate the perivascular adipose tissue and adventitia of vessels to exert their effects. A recent study performed by Mikolajczyk et al. demonstrates that this is effect is, in part, due to the chemokine RANTES (CCL5). Many of the cells, importantly T cells, which infiltrate the perivascular adipose tissue, bear the RANTES receptor CCR5. The authors show that vascular accumulation of IFN-y producing T cells and macrophages and also endothelial dysfunction are blunted in RANTES$^{-/-}$ mice infused with Ang II [72]. However, blood pressure is not decreased in these mice. This may be because deletion of RANTES does not affect kidney inflammation or dysfunction and because of the primary effects of Ang II itself. A later study performed by Rudemiller et al. detailed the protective effects of RANTES in the kidney. The authors show that RANTES deficient mice develop worse kidney damage and increased levels of macrophage infiltration into the kidney compared to controls in response to normotensive unilateral ureteral obstruction, a model of endogenous RAS activation. They go on to show that RANTES unexpectedly limits the pro-inflammatory actions of CCL2 in the injured kidney thereby restraining macrophage accumulation [73].

Anti-inflammatory T regulatory cells (T regs), characterized by their expression of the transcription factor forkhead box P3 (Foxp3) and of the cytokine IL-10, are capable of suppressing the immune response. These cells directly contribute to the maintenance of immune self-tolerance. Kvakan et al. show that adoptive transfer of T regs into Ang II infused mice results in less cardiac hypertrophy and fibrosis of the heart, despite no change in blood pressure. However, the authors do show reduced T cell infiltration into the hearts of animals that received T regs, displaying a critical role for T regs in suppression of the inflammatory response that results from Ang II infusion [74].

Katsuki et al. found that lower numbers of T regs present is crucial for the development of hypertension in the stroke-prone spontaneously hypertensive rat (SHRSP). The authors also observed that a reduction in sympathetic outflow to the spleen via splenic denervation in pre-hypertensive SHRSP rats increases numbers of T regs and delays the onset of hypertension in the rats, further implicating CNS control over T cell activation, differentiation and apoptosis in hypertension [75]. Viel et al found dysfunctional T regs in the Dahl SS rat. When T regs from the Dahl SS rat were compared to Brown Norway rats, the suppressive capacity of T regs was impaired [76].

Barhoumi et al. found that adoptive transfer of T regs into WT mice prevented Ang II- induced blood pressure increases and decreased Ang II-induced renal injury and immune cell infiltration. Additionally, the authors described an Ang II-induced decrease in Foxp3+ T reg cells in the kidney, which would thereby support an inflammatory phenotype in the kidneys in hypertension [77]. Several other groups have shown this as well. Matrougui et al. similarly saw a reduction in T regs in Ang II-infused mice. These authors also noted improved coronary arteriolar endothelial function in mice that received adoptive transfer of T regs [78]. Mian et al. demonstrate that microvascular injury, measured by examining microvascular remodeling and stiffness, was exaggerated in $Rag1^{-/-}$ given T cells from Scurfy mice compared to mice given WT T cells in response to Ang II. Scurfy mice are deficient in T regs because of their mutated Foxp3 gene [79]. Taken together, these data from mice and rats demonstrate that the presence of functional T regs are crucial for controlling the hypertensive response.

A fundamental feature of adaptive immunity and of all T cell subtypes is their ability to form memory cells post antigenic exposure. Memory ensures a swift reactivation of the adaptive immune system in the event that the host sees the antigen at a later date. Memory T cells either

live in secondary lymphoid organs as central memory cells or in the periphery as effector memory cells. Our laboratory found that exposure to hypertensive stimuli results in the production of effector memory T cells that localize to the kidneys and to the bone marrow. These cells appear to be the major sources of IFN-y and IL-17A in the kidneys of hypertensive mice. It is appreciated that memory T cells are able to reside in the bone marrow long term in a quiescent state. We were able to demonstrate that repeated exposure to hypertensive stimuli after initial priming results in reactivation and migration of bone marrow residing memory T cells back to the kidney [21]. These data illustrate a previously undescribed role of memory T cells in hypertension. Interestingly, Olofsson et al. have shown that memory T cells ($CD4^{+}CD44^{hi}CD62L^{lo}$) expressing choline acetyltransferase (ChAT) stimulate activation of eNOS and increase levels of nitrates and nitrites in endothelial cells. Also, T cell ChAT deficient mice have elevated arterial blood pressure. These data suggest a potential therapeutic strategy for hypertension, through manipulation of T cell mediated vasorelaxation [80].

In summary, the data discussed above implicate the innate and adaptive immune responses as critical to the development of hypertension. Each individual cell type or product discussed could potentially be used as a target of future therapeutics for hypertension, which would be aimed at decreasing the inflammatory response in humans in order to complement the various drugs already shown to decrease blood pressure. Moving forward, it is necessary to continue to investigate these processes and to aspire to fully understand the immune contributions to hypertension, a disease that is continually looking more and more autoimmune in nature.

Goals of Thesis

Although our understanding of the role of the adaptive immune system in hypertension is continuously evolving, signaling pathways inside the T cell enabling their activity during

hypertension and also the direct effects their cytokines have on end-organs during disease progression remain poorly understood. Moreover, no studies have yet investigated the role of salt, a substance of which numerous epidemiological studies have linked to hypertension and of which has been demonstrated to cause pathogenic production of IL-17A, a key cytokine in hypertension, when given in excess to differentiating Th17 cells *in vitro*, on its effects on the functionality of immune cells in hypertension [81, 82].

This dissertation aims to address several outstanding questions surrounding the effect of the pro-inflammatory cytokine IL-17A, produced by Th17 cells, on the kidneys themselves, as well as the effect of salt on T cells in hypertension. The salt sensing kinase SGK1, serves as the investigative link between the two studies. SGK1 has not only been described as the intracellular mediator of the Th17 cell's pathogenic production of IL-17A *in vitro*, but is also a canonically important mediator of hypertension within kidney cells, where it functions to facilitate salt and water reabsorption through modulation of distal tubule sodium transporters, thus, promoting the disease. In Chapter 3 we test the hypothesis that SGK1 mediates IL-17A induced salt and water retention during hypertension and in Chapter 4 we test the hypothesis that T cell SGK1 is an important mediator of hypertension. The findings from the studies performed in this thesis enhance our understanding of the role Th17 cells and its cytokines play in hypertension, and also identify therapeutic targets that could be used in conjunction with traditional therapies to help mitigate the disease.

CHAPTER 2

Materials and Methods

Mice

All animal procedures were approved by Vanderbilt University Institutional Animal Care and Use Committee (IACUC), and mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals.

Mice used in Chapter 3**:** Wild-Type (C57Bl/6J) mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were obtained as a generous gift from Dr. Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan) and were generated as previously described [83, 84]. Male mice approximately 10-12 weeks of age were used.

Mice used in Chapter 4: Wild-Type (C57Bl/6J) mice and tg^{CD4cre} mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. SGK1$^{fl/fl}$ mice were obtained as a generous gift from Dr. Aniko Naray-Fejes-Toth (Dartmouth College, Hanover, NH, USA) and were generated as previously described [85]. Male mice approximately 10-12 weeks of age were used.

Surgical Procedures and Blood Pressure Measurements

Angiotensin II infusion: Mice were anesthetized with ketamine/xylazine (90-120 mg/kg + 10mg/kg) (1:1 volume ratio) and 2 or 4 week osmotic mini-pumps (Alzet, DURECT Corporation, model 2002 or 2004) containing angiotensin II (490 ng/kg/min, Sigma) or vehicle (0.08 M sodium chloride/1% acetic acid solution) were inserted subcutaneously.

DOCA-salt Treatment: Mice were anesthetized with ketamine/xylazine (90-120 mg/kg + 10mg/kg; 1:1 volume ratio). The left kidney was surgically removed, a DOCA pellet (100mg, Innovative Research of America) was inserted subcutaneously, and the drinking water was

supplemented with 1% NaCl for 21 days. Control (Sham) mice underwent a similar surgical incision but without nephrectomy and received a placebo pellet. Sham mice received regular drinking water with no NaCl supplementation.

Tail Cuff Blood Pressure Measurements: These experiments measure blood pressure non-invasively. Before the experiment, the person measuring the blood pressure is blinded to the animal's genotype and treatment. 4 animals at a time were placed on an MC4000 Blood Pressure Analysis System (Hatteras Instruments) which was pre-warmed to allow for vasodilation through footpads. The mice were covered with a magnetic black cover to keep them in place. Tails of the mice were threaded through a small blood pressure cuff and gently taped down. The machine measures blood pressure in cycles of 10 and generates an average systolic blood pressure. The first two rounds of 10 are eliminated from analysis, giving the mice time to acclimate, rounds 3 and 4 are averaged together to generate a final systolic blood pressure value for the day. The animals' blood pressure is measured two days per week and averaged to produce a final weekly value [33].

Carotid Radiotelemetry Blood Pressure Measurements: These experiments invasively measure blood pressure. Mice were anesthetized with ketamine/xylazine (90-120 mg/kg + 10mg/kg; 1:1 volume ratio). The left common carotid artery was isolated and the catheter connected to the transducer was inserted into the carotid and advanced until the tip was just inside the thoracic aorta. The transmitter was positioned under the skin on the right flank close to the hindlimb [86]. Following telemetry implantation, mice were allowed to recover for 10 days prior to implantation of osmotic minipumps (described above). Measurements of blood pressure were obtained daily for 4 weeks.

Sacrifice of mice: At the end of each experiment, mice were sacrificed using CO_2 inhalation. Mice were perfused with saline until all blood was cleared from the circulation and then kidneys, aortae, and spleen were extracted for preservation or further analysis.

Transporter profiling (Chapter 3)

Immunoblotting for NHE3, NCC-total, phosphorylated NCC, and gamma ENaC were performed as previously described (Performed by Alicia McDonough's Laboratory at the University of Southern California) [71, 87, 88]

Urine analysis

Chapter 3: Mice were placed in metabolic cages for 24 hour urine collection. Albumin was measured in the urine using an ELISA kit (Albuwell, Exocell). Immunoblotting for albumin and angiotensinogen was performed on urine samples as previously described and according to manufacturer's instructions [87, 88].

Chapter 4: Albumin and creatinine concentrations were measured from a spot urine sample [89] (obtained after 4 weeks of angiotensin II infusion) using ELISA kits from Exocell. Albumin concentration was divided by creatinine concentration in each sample to determine the albumin:creatinine ratio.

Immortalized Cell culture (Chapter 3)

Cytokines IL-17A, IL-17F, and TNFα were obtained from R&D systems. The SGK1 inhibitor, GSK 650394, was obtained from TOCRIS Bioscience. Mouse distal convoluted tubule (mDCT15) cells were cultured as previously described [90]. NEDD4-2 deficient mDCT15 cells were generated as previously described [91]. HK-2 cells were purchased from ATCC. HK-2 cells

or mDCT15 cells were plated in 6 well dishes at a density of 100,000 cells per well and allowed to grow to confluency over the ensuing days before experiments were performed. HK-2 cells were cultured in Keratinocyte SFM (Invitrogen) media in which the Epidermal Growth Factor and Bovine Pituitary Extract were added. Additionally 1% Penicillin/Streptomycin was added to the media. mDCT15 cells were grown in DMEM/F-12 50/50 media in which 5% Fetal Bovine Serum and 1% Penicillin/Streptomycin were added. mDCT15 cells were serum starved overnight before experiments were performed. Cells were treated with experiment specific doses of IL-17A, IL-17F, TNFα and/or GSK 650394.

Immunoblotting (Chapter 3)

For p78SGK1 or NHE3: HK-2 cells or mDCT15 cells plated in 6 well dishes were scraped in cold sorbitol buffer (5% sorbitol, 5mM histidine/imidazole, 0.5mM EDTA with protease and phosphates inhibitors) to isolate protein. Protein was quantified using a Bradford Assay Kit. Samples (30µg of protein) were separated by SDS-PAGE. Samples were then transferred to a nitrocelluse membrane and blocked with either 5% BSA or 5% milk. Membranes were probed using either NHE3 (Millipore) or p78SGK1 (Cell Signaling Technologies). Membranes were then labelled using a BioRad HRP Conjugate and detected with BioRad ECL Chemiluminescence solution. Membranes were imaged with a BioRad ChemiDoc Imager. Blots were then stripped for 15 minutes and re-probed using an antibody to GAPDH (Santa Cruz Biotech). Densitometry was performed using BioRad Image Lab software, and bands of interest were normalized to GAPDH.

For Nedd4-2: Proteins were transferred electrophoretically to PVDF membranes. After blocking with 3% BSA, the membranes were probed with corresponding primary antibodies (anti Nedd4-2

antibody (Abcam, Cambridge, UK, 1:500), anti-actin (Sigma, 1:1000)) and incubated overnight. The blots were washed in TBST. Signal detection for Nedd4-2 and actin was done using IRDye800 rabbit anti-goat IgG antibody, (Rockland immunochemicals, dilution 1:10000) and subsequent scanning of the membrane by the Odyssey Infrared Imager. Intensity of the protein bands was analyzed by using Odyssey Infrared Imaging Software (Li-Cor Biosciences).

Quantitative RT-PCR

Chapter 3: Whole kidneys were homogenized in Trizol using a Bead Beater, and lysates were then subjected to phenol-chloroform extraction. RNA was isolated using an RNeasy Mini Kit (Qiagen). HK-2 cells or mDCT15 cells plated on a 6 well dish were washed and then lysed in RLT Buffer and RNA was extracted using an RNeasy Mini Kit (Qiagen). RNA quantity and purity was measured using a spectrophotometer. cDNA was made using a High Capacity cDNA reverse transcriptase kit (Applied Biosystems). Samples were evaluated for mouse or human SGK1 (Taqman), mouse or human IL-17A (SABiosciences), mouse or human GAPDH (Taqman), and a primer designed against 18s (SIGMA) that works in both species due to sequence conservation. Samples were normalized to GAPDH and then normalized again to one of the control samples. The Relative Quantification (RQ) values are plotted.

Chapter 4: RNA was isolated from T cells using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was then quantified using a DS-11 Spectrophotometer (DeNovix). cDNA was made using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Taqman assay system and probes (Thermo Fisher) were used to detect SGK1, NKCC1, alpha ENaC, beta ENaC, gamma ENaC, NCC, NCX1, NCX2, NHE1, NHE6, and SCN5A. A Taqman assay system and probe for GAPDH (Thermofisher Scientific) was used as an endogenous control. IL-17A mRNA was

quantified using the SYBR Green assay system (RT^2 Primer Assay, Qiagen). Primers designed against 18s (SIGMA) were used as endogenous control for the SYBR Green assay. Gene expression values were calculated based on the comparative Ct method normalized to GAPDH or 18s mRNA and displayed as either relative quantification (with one of the control samples set to '1') for some experiments or fold induction with every control sample set to '1' for other experiments.

NCC activity assay and lentiviral transduction (Chapter 3)

mDCT15 cells were incubated at 37°C with vehicle, 100ng/mL IL-17A, 100ng/mL IL-17F, 100nM GSK 650394, or a combination of the agents. Thirty minutes before uptake, 0.1 mM metolazone (an inhibitor of NCC) or vehicle (DMSO) was added to the media. The media was then changed to a $^{22}Na^{+}$ containing medium with or without 0.1mM metolazone and incubated for 20 minutes. Cells were lysed and radioactivity was measured by liquid scintillation. Protein concentrations were determined by a BCA assay. Uptakes were normalized to nmol/mg protein. NCC activity was defined as the thiazide-sensitive uptake which is the difference in Na uptake with or without metolazone [92]. Inhibition of Nedd4-2 in the mDCT15 cells was performed using lentiviral transduction as described in Arroyo et al [91]. (Experiments were performed by Dr. Benjamin Ko's laboratory at the University of Chicago).

Flow cytometry of renal and aortic leukocytes (Chapter 4)

Single cell suspensions of one kidney were prepared by placing the kidney in digestion media containing RPMI 1640, 5% FBS, 2mg/mL Collagenase D (Roche), and 0.1mg/mL DNase I (SIGMA). Kidneys were homogenized using AutoMACs Dissociator (Miltenyi Biotech) and incubated, shaking, at 37ºC for 20 minutes. Single cell suspensions of thoracic aorta with

surrounding perivascular fat were prepared by placing the thoracic aorta in digestion media containing RPMI 1640, 5% FBS, 1mg/mL Collagenase A (Roche), 1mg/mL Collagenase B (Roche), and 0.1mg/mL DNase I (SIGMA). Aortae were homogenized by hand in 6 well plates using curved scissors and incubated, shaking, at 37ºC for 40 minutes (4). Tissue homogenates were filtered through a 40µM cell strainer after digestion. Single cell suspensions were stained for flow cytometry using the following antibodies/stain: Pacific LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), brilliant violet-510-conjugated (BV510) anti-CD45 antibody (BioLegend, clone 30-F11), peridinin chlorophyll protein-cyanin-5.5-conjugated (PerCP-Cy5.5) anti-CD3 antibody (BioLegend, clone 17A2), phycoerythrin-cyanin-7-conjugated (PE-Cy7) anti-CD8 antibody (BioLegend, clone 53-6.7), allophycocyanin-Hilite-7-conjugated (APC-H7) anti-CD4 antibody (BD Biosciences, clone GK1.5), and Alexa Fluor 488-conjugated anti-F4/80 antibody (BioLegend, clone BM8). A known quantity of calibration (counting) beads (123count eBeads, eBioscience) was added to each sample prior to analysis. Samples were run on a BD FACSCanto II system and analyzed using BD FACSDiva software (BD Biosciences). Gates were set using fluorescence minus one (FMO) controls. Results were normalized using the bead count and expressed as number of cells per kidney or per thoracic aorta.

Intracellular staining for cytokine producing lymphocytes from spleen (Chapter 4)

Spleens were ground and filtered through a 40µM cell strainer and then subjected to red blood cell (RBC) lysis using RBC lysis buffer (eBioscience). Approximately 3 x 10^6 cells were resuspended in RPMI medium containing 5% FBS and subsequently stimulated with 4 µL of Cell Activation Cocktail containing PMA, ionomycin, and the Golgi inhibitor brefeldin A (BioLegend) at 37°C for 3 hours. Cells were then washed and stained first with LIVE/DEAD Fixable Violet dead cell stain (Life Technologies). The following surface antibodies were then

added to the cells for 30 minutes: PerCP-Cy5.5-conjugated anti-CD3 antibody (BioLegend, clone 17A2), BV510-conjugated anti-CD8 antibody (BioLegend, clone 53-6.7) and APC-H7-conjugated anti-CD4 antibody (BD Biosciences, clone GK1.5). Intracellular staining was then performed using the BD Transcription Factor Buffer set according to the manufacturer's instructions (BD Biosciences). Antibodies used for intracellular staining were Alexa-Fluor 488-conjugated anti-IFN-γ antibody (BD Biosciences, clone XMG1.2) and phycoerythrin (PE)-conjugated anti-IL17A antibody (Biolegend, clone TC11-18H10.1). Results are expressed as percentage of cytokine positive cells relative to total $CD4^+$ or $CD8^+$ cells.

Measurements of Vascular Reactivity (Chapter 4)

Isometric tension studies of mesenteric arterioles were performed using 2 mm segments of second order mouse mesenteric arterioles dissected free of perivascular fat. Studies were performed in a small vessel horizontal wire myograph (Danish Myo Technology, models 610M and 620M) containing a physiological salt solution composed of 130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 5 mM glucose and 1.6 mM $CaCl_2$. The isometric tone was recorded for each vessel using LabChart Pro v7.3.7 (AD Instruments). The vessels were equilibrated over a 20 minute period at 37°C. A passive circumference-tension curve was generated for each vessel to determine optimum passive tension to simulate an in vivo transmural pressure of 100 mmHg according to the manufacturer's protocol as previously described with modifications (37). After normalization, vessels were contracted with 60 mM KCl to assess the integrity of the vessel, and then endothelial-dependent and endothelial-independent vascular relaxations were tested using increasing concentrations of acetylcholine or sodium nitroprusside, respectively, after preconstriction with norepinephrine (10μM).

T cell isolation and culture (Chapter 4)

Spleens were isolated from mice and single cell suspensions were prepared as described above. $CD4^+$ and $CD8^+$ naïve T cells, γδ T cells, or $CD3^+$ pan T cells were isolated using Miltenyi cell separation kits according to the manufacturer's instructions and an AutoMACS magnetic cell sorter (Miltenyi Biotec). Cells were plated in RPMI 1640 media containing 10% FBS, 1% Penicillin/Streptomycin, and 50μM β-mercaptoethanol at a density of 200,000 cells/well in a 96 well plate that was pre-coated with no azide/low endotoxin anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) (BD Biosciences). To induce Th17 polarization, cells were treated with 20 ng/mL IL-6, 5 ng/mL TGF-β1, 10 ng/mL IL-1β, and 10 ng/mL of IL-23 for 72 hours at 37°C. These cytokines were purchased from R&D Systems. To determine the effect of sodium chloride (NaCl) on IL-17A and SGK1 expression, some samples received an additional 40 mM NaCl in addition to the Th17 polarizing cytokines described above. For the pharmacological inhibitor studies, cells were pretreated for 30 minutes with 100uM hydrochlorothiazide, 5uM spironolactone, 100uM furosemide, 100uM bumetanide, or equal volume of the corresponding vehicle (ethanol or DMSO). These drugs were purchased from SIGMA. After 30 minutes, cells were treated with Th17 polarizing cytokines with or without an extra 40 mM NaCl as indicated. In these studies, cells were re-treated with the appropriate inhibitors or vehicle after 48 hours and then harvested after an additional 24 hours for a total of 72 hrs incubation at 37°C.

Statistics

Data are expressed as mean ± SEM. Telemetry blood pressure data were analyzed between the two groups of mice using regression with robust standard errors and consideration of clusters of mice. Time was modeled using restricted cubic spline non-linearly in the model. In addition to

the time and group identity variable, the interaction between them was included in the model. The *P*-value for the interaction is reported. All other analyses were performed in GraphPad Prism using repeated measures ANOVA, two-way ANOVA, one-way ANOVA, ratio paired t-test, Student's t-test, and Holm-Sidak's post-hoc test or Bonferroni correction as specified in the figure legends. *P* values ≤ 0.05 were considered significant.

CHAPTER 3

Interleukin 17A regulates renal sodium transporters and renal injury in angiotensin II-induced hypertension

Allison E. Norlander[1], Mohamed A. Saleh[2,3], Nikhil V. Kamat[4], Benjamin Ko[5], Juan Gnecco[6], Linjue Zhu[2], Bethany Dale[1], Yoichiro Iwakura[7], Robert S. Hoover[8], Alicia A. McDonough[4], Meena S. Madhur[1,2]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville TN, USA. [2]Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA. [3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. [4]Department of Cell and Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA. [5]Department of Medicine, Chicago University School of Medicine, Chicago, IL, USA. [6]Department of Microbiology, Immunology and Pathology, Vanderbilt University, Nashville, TN, USA. [7]Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan, [8]Division of Renal Medicine, Department of Medicine, Emory University School of Medicine, Atlanta GA, USA

The contents of this chapter have been published in Hypertension vol. 68 (1), pp. 167-74; July 2016.

Introduction

Hypertension is a leading cause of cardiovascular disease morbidity and mortality [93]. Yet, the pathogenesis of hypertension is still poorly understood, and despite conventional treatment, blood pressure remains uncontrolled in nearly half of the hypertensive population [94]. Emerging evidence from our group and others implicates innate and adaptive immune cells and the cytokines that they produce as pathogenic mediators of this disease and its attendant end-organ damage [95, 96].

Interleukin 17A (IL-17A), a pro-inflammatory cytokine produced predominantly by $CD4^+$ T helper 17 (Th17) cells as well as gamma delta T cells, plays an important role in numerous autoimmune diseases [97]. We have shown that in response to angiotensin II (Ang II) infusion, mice deficient in IL-17A develop an initial increase in blood pressure that is similar to wild type (WT) mice but are unable to sustain these elevated pressures. Blood pressure starts to decline after 2 weeks and is approximately 30 mmHg lower than Ang II infused WT mice by 4 weeks. Moreover, IL-$17A^{-/-}$ mice exhibit reduced vascular inflammation and preserved vascular function in response to Ang II infusion compared to WT mice [33]. In keeping with this, Amador et al. reported a marked increase in Th17 cells in DOCA-salt treated rats and observed that treating DOCA-salt rats with an antibody against IL-17A reduced blood pressure and collagen deposition in the heart and kidneys [18]. We recently showed that Ang II treated IL-$17A^{-/-}$ mice have preserved diuresis and natriuresis in response to an acute saline challenge unlike Ang II treated WT mice which retain salt and water [71]. Consistent with this finding, proximal tubule NHE3 protein abundance was reduced by 40% in IL-$17A^{-/-}$ mice but not WT mice after 2 weeks of Ang II infusion, suggesting a mechanism for enhanced pressure natriuresis in the IL-$17A^{-/-}$ mice.

There are 6 isoforms of IL-17: A through F. IL-17A shares 50% sequence similarity with IL-17F, and both can bind as homo- or heterodimers to the same receptor complex composed of IL-17RA and IL-17RC subunits [98]. The role of IL-17F in hypertension is previously unknown. The goal of the present study was to determine the effect of IL-17A and IL-17F on renal sodium transporters after a prolonged (4 week) period of Ang II infusion, a time when the blood pressure blunting in IL-17A$^{-/-}$ mice is most prominent. In addition, we investigated whether the effects of IL-17A or F are direct effects of these cytokines acting on renal sodium transporters in renal epithelial cells.

Our results demonstrate that IL-17A (but not IL-17F) mediates Ang II induced renal injury and regulates renal sodium transporters, namely NHE3 and NCC, through a serum and glucocorticoid regulated kinase 1 (SGK1) dependent pathway. Moreover, we found that cultured renal proximal tubule and distal convoluted tubule cells produce endogenous IL-17A. This study provides mechanistic insight into how inflammatory cytokines can regulate sodium and water balance and therefore blood pressure.

Results

IL-17A$^{-/-}$ mice fail to sustain the upregulation of distal sodium transporters in response to chronic (4 weeks) angiotensin II infusion: Following Ang II infusion, IL-17A$^{-/-}$ mice exhibit an initial rise in BP similar to WT mice but are unable to sustain these pressures. BP starts to decline around 2 weeks and reaches pressures 30 mmHg lower than Ang II infused WT mice by 4 weeks (Madhur et al. [95] and **Figure 3-1**). Therefore, we performed renal transporter profiling in whole kidneys of WT and IL-17A$^{-/-}$ mice after 4 weeks of Ang II or vehicle (Sham) infusion.

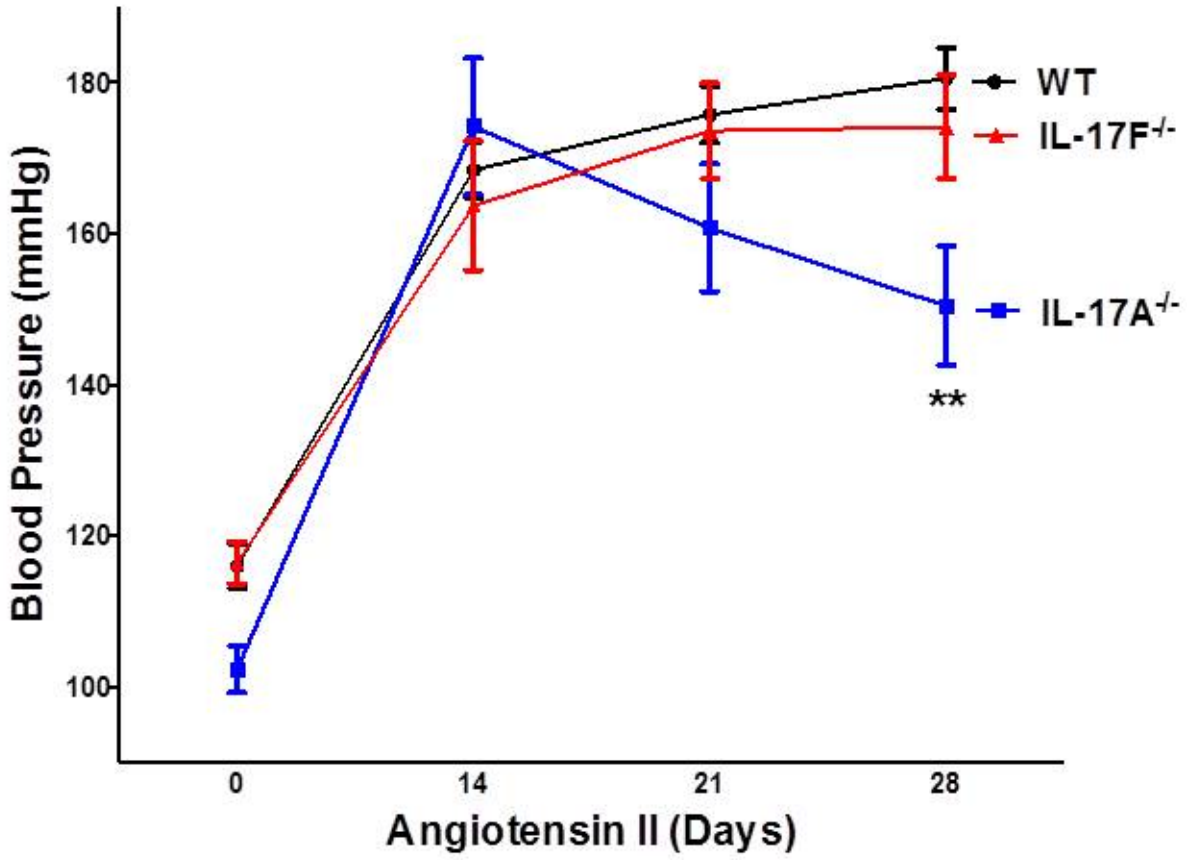


Figure 3-1: Blood pressure is blunted in IL-17A$^{-/-}$, but not IL-17F$^{-/-}$, mice following Ang II infusion. Tail cuff blood pressures at baseline and during 28 days of Ang II infusion (490 ng/kg/min) in WT, IL-17A$^{-/-}$, and IL-17F$^{-/-}$ mice. Two-way ANOVA followed by the Holm-Sidak post-hoc test was performed. ***P*<0.01 vs WT (n=5-13 per group).

Interestingly, at this timepoint, there was a marked difference in the activation of distal sodium transporters between WT and IL-17A$^{-/-}$ mice. As expected, Ang II increased abundance and phosphorylation of NCC and cleavage (activation) of the gamma subunit of ENaC in WT mice, but this was abolished in IL-17A$^{-/-}$ mice (**Figure 3-2**). We previously showed that after 2 weeks of Ang II infusion, distal transporters are activated in IL-17A$^{-/-}$ mice but to a lesser degree than in WT mice [71]. Taken together, this data shows that IL-17A$^{-/-}$ mice fail to sustain the Ang II induced increases in distal convoluted tubule and collecting duct sodium channels, coincident with their decline in blood pressure.

To determine if this effect was specific to the IL-17A isoform, we investigated the effect of IL-17F on blood pressure and renal sodium transporters using IL-17F deficient mice. Blood pressure in IL-17F$^{-/-}$ mice during chronic Ang II infusion was not blunted (**Figure 3-1**) and distal sodium transporter abundance was similar to that observed in WT mice with the exception of a reduction in cleaved γENaC (**Figure 3-2**). Thus, the reductions in blood pressure and distal renal sodium transporter abundance are specific to the IL-17A isoform.

IL-17A regulates renal SGK1 expression: Serum and glucocorticoid regulated kinase 1 (SGK1) is an important mediator of salt and water retention in the kidney through inhibition of Nedd4-2 mediated ubiquitination and degradation of NCC and ENaC in the distal convoluted tubule, thereby enhancing the expression of these transporters on the cell surface [99, 100]. We hypothesized that SGK1 mediates the effects of IL-17A on renal sodium transporters. To test this hypothesis, we performed quantitative RT-PCR on whole kidney lysates from WT and IL-17A$^{-/-}$ mice infused with 2 or 4 weeks of Ang II or vehicle (Sham). SGK1 expression was upregulated 2-fold in kidneys from WT mice infused for 2 or 4 weeks with Ang II (**Figure 3-3**).

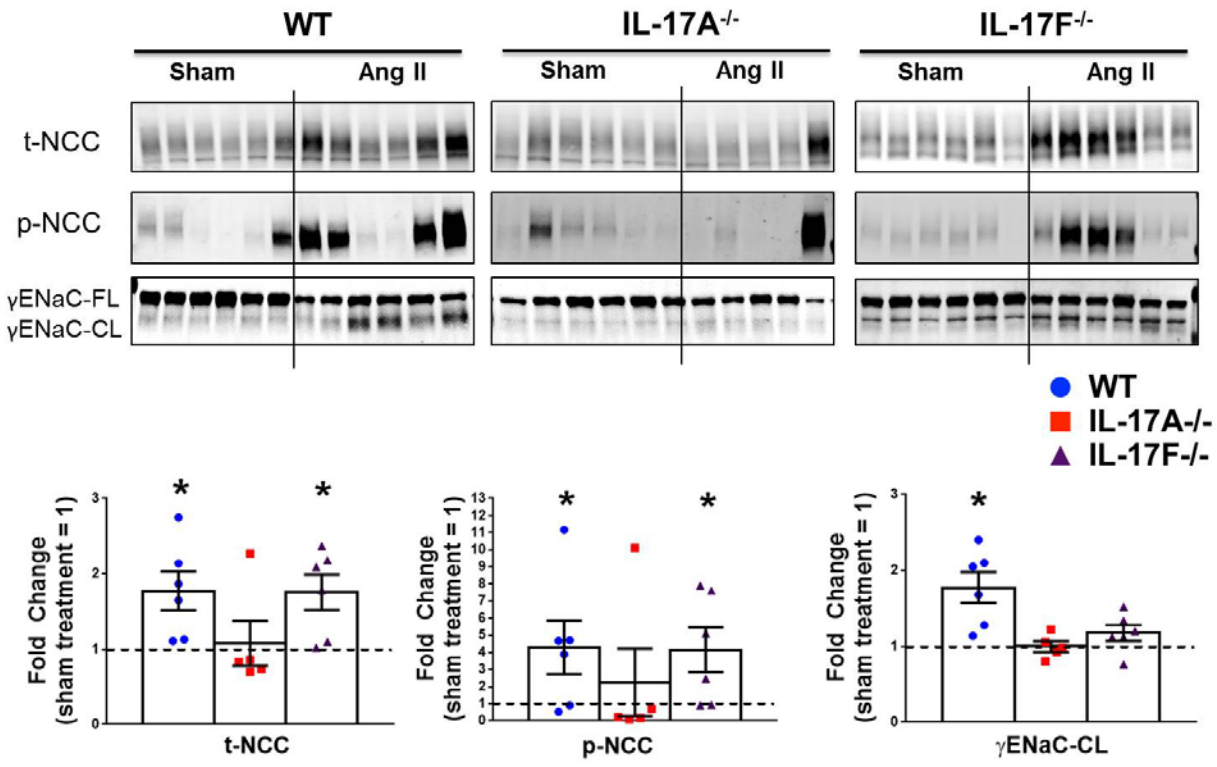


Figure 3-2: IL-17A deficiency blunts the Ang II induced increase in distal sodium transporters. Abundance of total and phosphorylated Na-Cl cotransporter (t-NCC and p-NCC) as well as full length (FL) and cleaved (CL) gamma ENaC were analyzed in whole renal tissue homogenates from wild-type (WT), IL-17A$^{-/-}$, and IL-17F$^{-/-}$ mice after 4 weeks of vehicle (Sham) or angiotensin II (Ang II) infusion. Fold change in transporter abundance in Ang II infused mice compared to vehicle (Sham) infused mice is shown below. Values are plotted as mean±SEM. Student's t-Test was used to compare transporter abundance in Ang II infused animals to the corresponding vehicle infused animals for each genotype. *$P \leq 0.05$ (n=5-6 per group).

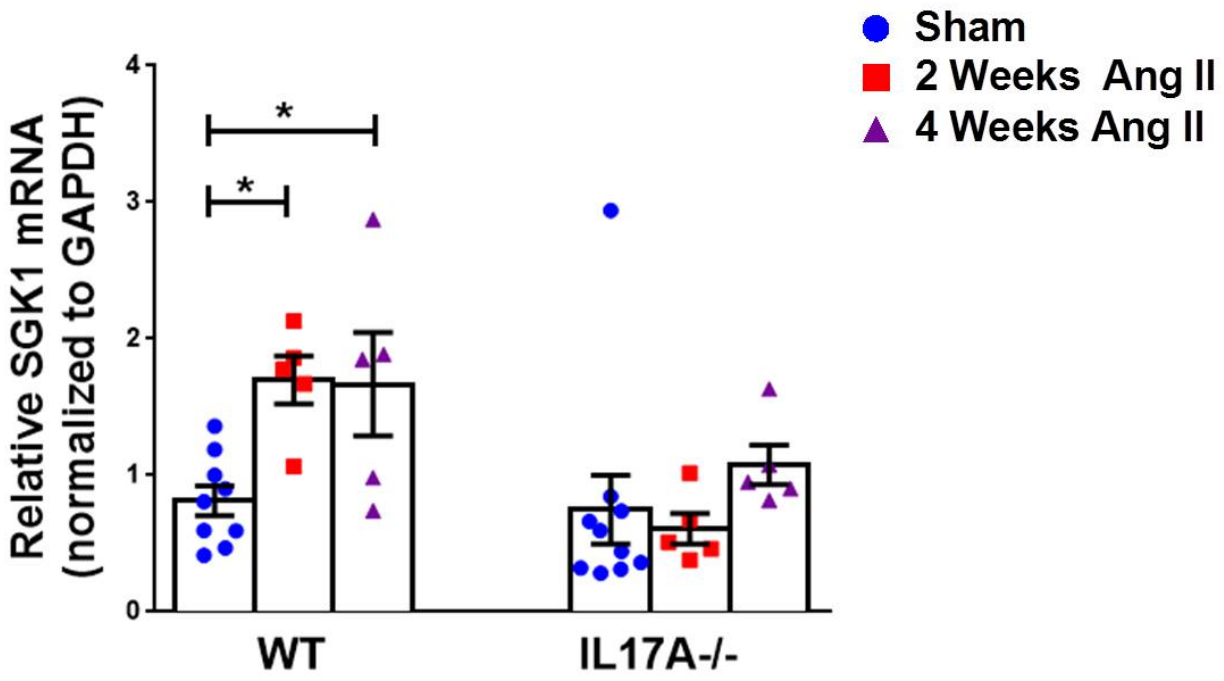


Figure 3-3: IL-17A regulates renal SGK1 expression. WT and IL-17A$^{-/-}$ mice were infused with vehicle (Sham) or Ang II for two or four weeks as indicated. Quantitative real time PCR was performed on RNA extracted from whole renal tissue homogenates and normalized to GAPDH. Relative quantification values are plotted as mean±SEM. Two-way ANOVA followed by Bonferroni post-hoc test was used. *P<0.05 (n=5-10 per group).

In contrast, kidneys from IL-17A$^{-/-}$ mice had no increase in SGK1 expression after either 2 or 4 weeks of Ang II infusion (**Figure 3-3**). This suggests that SGK1 is a potential mediator of the effects of IL-17A in the kidney.

IL-17A, but not IL-17F, directly upregulates NCC activity in cultured mouse distal convoluted tubule (mDCT15) cells via an SGK1/Nedd4-2 dependent pathway: To determine if the effect of IL-17A on distal tubule sodium transporters was a direct and specific effect, we measured NCC activity in a cultured mouse distal convoluted tubule cell line (mDCT15) that has been previously characterized and shown to recapitulate many features of in vivo distal convoluted tubule cells [90]. NCC activity was defined as the metalozone inhibited increase in radioactive sodium uptake. IL-17A, but not IL-17F, significantly increased NCC activity in these cells (**Figure 3-4A**).

We did not detect an increase in SGK1 mRNA with IL-17A treatment of mDCT15 cells (data not shown), but phosphorylation of SGK1 at Serine 78 was increased 1.5 fold after 15 minutes of IL-17A treatment (**Figure 3-4B**). Importantly, the IL-17A induced increased in NCC activity was abrogated by co-treatment with the SGK1 inhibitor, GSK 650394 (**Figure 3-4C**). To test the hypothesis that the effect of IL-17A and SGK1 on NCC activity is mediated via Nedd4-2, we used mDCT15 cells in which Nedd4-2 was silenced by lentiviral transduction of a short hairpin RNA (shRNA) targeting Nedd4-2 or a non-targeting shRNA as control. As shown in **Figure 3-5**, the Nedd4-2 shRNA transducted cells expressed approximately 60% less Nedd4-2 protein by immunoblotting. In these cells, the basal level of NCC activity was increased, and there was virtually no additional effect of IL-17A on NCC activity (**Figure 3-4D**). Taken together, these results suggest that IL-17A induces an increase in NCC activity through phosphorylation of SGK1 and inhibition of Nedd4-2 mediated ubiquitination and degradation of NCC.

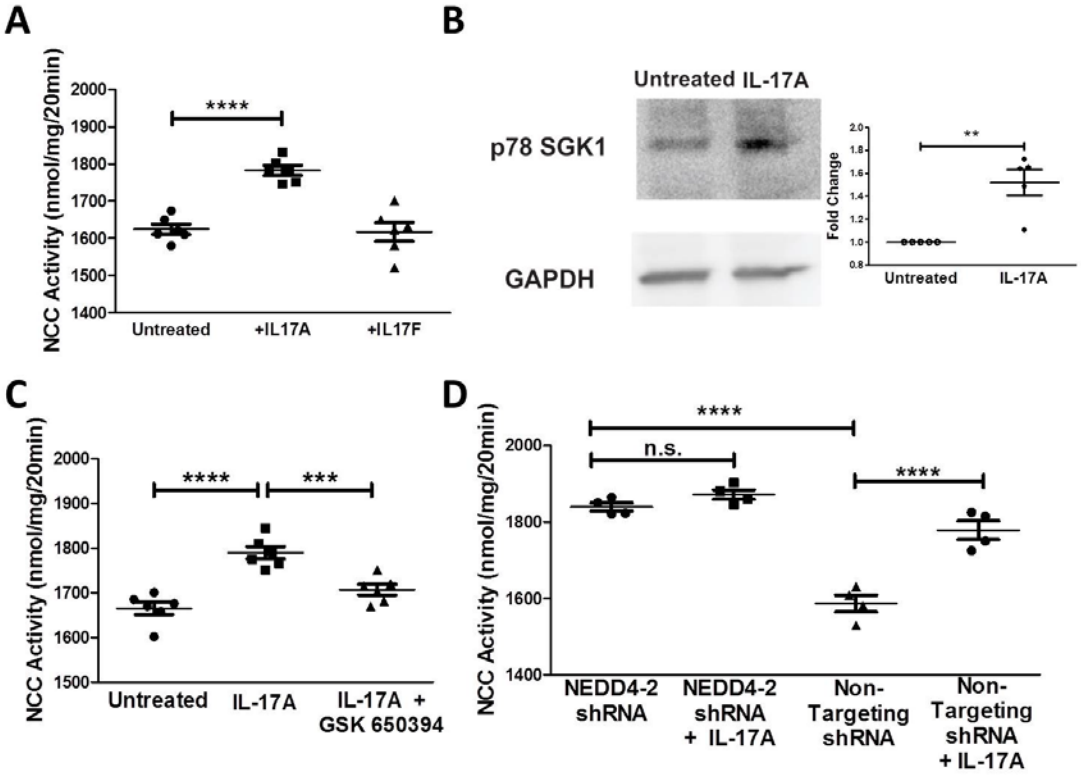


Figure 3-4: IL-17A increases NCC activity in cultured mouse distal convoluted tubule (mDCT15) cells via an SGK1/Nedd4-2 dependent pathway. A) NCC activity in mDCT15 cells with or without treatment with 100ng/mL of IL-17A or IL-17F (n=6 per group). **B)** Representative immunoblot for phosphorylated SGK1 on serine 78 in mDCT15 cells with or without 15 min of treatment with recombinant IL-17A (100ng/ml). Quantification of all blots relative to GAPDH and normalized to untreated is shown on the right (n=5 per group). **C)** NCC activity in mDCT15 cells after treatment with IL-17A or IL-17A plus 100nM of the SGK1 inhibitor, GSK 650394 (n=6 per group). **D)** NCC activity with or without IL-17A treatment in mDCT15 cells lentivirally transducted with a short hairpin RNA (shRNA) to silence NEDD4-2 or a non-targeting shRNA (n=4 per group). All data are plotted as mean±SEM. One-way ANOVA followed by Newman-Keuls post-hoc test was used for panels A, C, and D, and a paired Student's t-Test was used for panel B. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

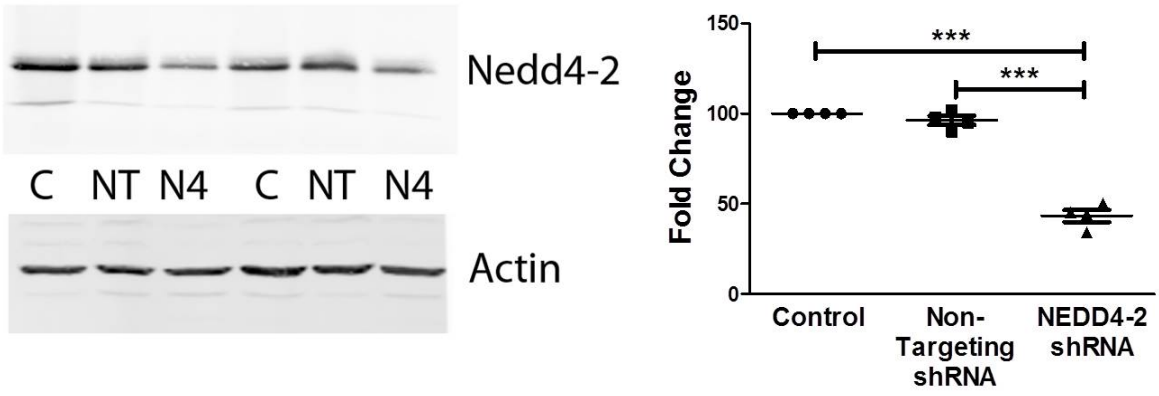


Figure 3-5. NEDD4-2 shRNA decreases NEDD4-2 expression in mDCT15 cells: Representative immunoblot showing amount of NEDD4-2 protein in mDCT15 control cells (C), mDCT15 cells transducted with a non-targeting shRNA (NT), and mDCT15 cells transducted with an shRNA targeting NEDD4-2 (N4). Actin was used as a loading control. Quantification of all blots relative to actin and normalized to control is shown on the right. Repeated measures one-way ANOVA followed by the Newman-Keuls post-hoc test was used. ***$P<0.001$ (n=4).

IL-17A upregulates NHE3 in cultured human proximal tubule (HK-2) cells via an SGK1 dependent pathway: Downregulation of NHE3 is an important mechanism for pressure natriuresis. Our previous study [71] demonstrated that IL-17A$^{-/-}$ mice exhibit suppressed NHE3 abundance at an earlier timepoint (2 weeks) after Ang II infusion compared to WT mice. In the current study, we found that IL-17A$^{-/-}$ mice maintained this decrease in NHE3 abundance, and that WT mice exhibited a similar decrease in NHE3 abundance by 4 weeks of Ang II infusion (**Figure 3-6**). Thus, while pressure natriuresis eventually occurs in both WT and IL-17A$^{-/-}$ mice, it is accelerated in the absence of IL-17A. Thus, we hypothesized that IL-17A has a direct effect on NHE3 regulation in proximal tubule cells. To test this hypothesis, we quantified NHE3 abundance by immunoblotting in cultured human proximal tubule (HK-2) cells treated with or without recombinant IL-17A for 72 hours. Using this in vitro system, we were able to remove the confounding effects of blood pressure and angiotensin II. Interestingly, IL-17A directly upregulated NHE3 protein expression by greater than 2-fold in these cultured cells (**Figure 3-7A**).

To determine whether IL-17A regulates SGK1 in the proximal tubule, we treated HK-2 cells with increasing doses of recombinant IL-17A, IL-17F, or tumor necrosis factor alpha (TNFα) for 24 hours. Interestingly, only IL-17A caused a dose-dependent increase in SGK1 expression by quantitative RT-PCR (**Figure 3-7B**). We then investigated whether IL-17A treatment also increased phosphorylation of SGK1. We found that phosphorylation of SGK1 at Serine-78 increased 2-fold in HK-2 cells after 15 minutes of treatment with IL-17A (**Figure 3-7C**). To determine whether the IL-17A dependent increase in NHE3 was dependent on SGK1, we treated HK-2 cells with increasing doses of the SGK1 inhibitor, GSK 650394, starting 30 minutes prior to IL-17A treatment.

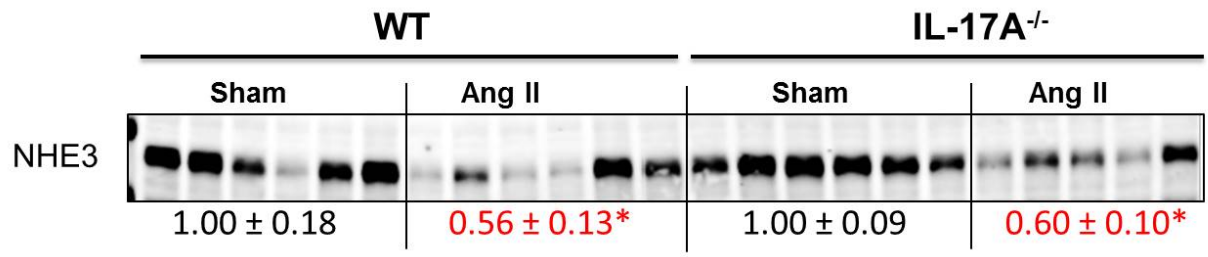


Figure 3-6: NHE3 expression is blunted in WT and IL-17A$^{-/-}$ mice after 4 weeks of Ang II infusion. Abundance of NHE3 in WT and IL-17A$^{-/-}$ mice after 4 weeks of vehicle (Sham) or Ang II infusion was analyzed by immunoblotting in whole renal tissue homogenates. Quantification data are reported under the blots as mean±SEM. Student's t-Test was used to compare transporter abundance in Ang II infused animals to the corresponding vehicle infused animals for each genotype. *P<0.05 (n=5-6 per group).

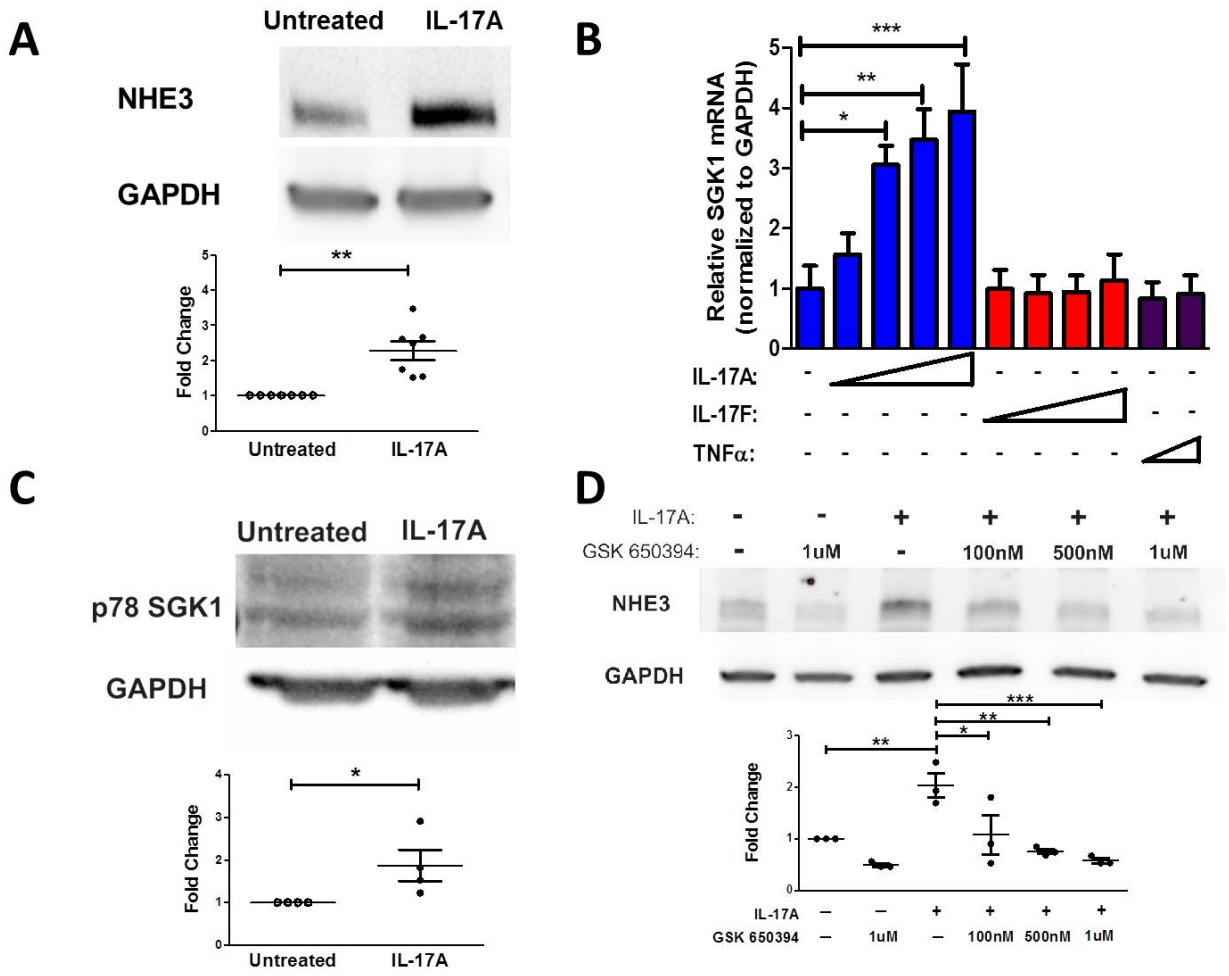


Figure 3-7: IL-17A upregulates NHE3 in cultured human proximal tubule (HK-2) cells via an SGK1 dependent pathway. A) HK-2 cells were incubated with or without 100 ng/mL of recombinant IL-17A for 72 hours. Representative immunoblot for NHE3 and GAPDH is shown with quantification of all blots relative to GAPDH and normalized to untreated shown below (n=7 per group). **B)** HK-2 cells were treated with IL-17A or IL-17F (increasing doses from 1 ng/ml to 100 ng/ml) or TNFα (1ng/ml or 10ng/mL) for 24 hours. Quantitative real time PCR for SGK1 was performed on the cell lysates. Relative quantification normalized to GAPDH is shown (n=4 per group). **C)** Representative immunoblot for phosphorylated SGK1 on serine 78 in HK-2 cells with or without 15 min of treatment with recombinant IL-17A (100ng/ml). Quantification of all blots relative to GAPDH and normalized to untreated is shown below (n=4 per group). **D)** HK-2 cells were pretreated for 30 min with vehicle or the SGK1 inhibitor, GSK 650394, at the indicated concentrations and subsequently treated with or without 100ng/mL of IL-17A for 72 hours. A representative immunoblot for NHE3 and GAPDH is shown. Quantification of all blots relative to GAPDH and normalized to untreated is shown below (n=3 per group). All data are plotted as mean±SEM. One-way ANOVA followed by Bonferroni post-hoc test was used for panels B and D, and a paired Student's t-Test was used for panels A and C. **P*<0.05, ***P*<0.01, ****P*<0.001.

The SGK1 inhibitor reduced the basal expression of NHE3 and caused a dose-dependent decrease in the IL-17A induced NHE3 expression (**Figure 3-7D**) indicating that the effect of IL-17A on NHE3 in proximal tubule cells is mediated by SGK1. Taken together, these data demonstrate that IL-17A increases expression and phosphorylation of SGK1 in the proximal tubule leading to increased abundance of NHE3.

IL-17A$^{-/-}$ mice are protected from glomerular and tubular injury in response to angiotensin II infusion: Albuminuria is a marker of glomerular injury and is known to increase in response to Ang II induced hypertension. To determine the effect of IL-17A and IL-17F on glomerular injury, we measured urinary albumin by ELISA on 24-hr urine samples from WT, IL-17A$^{-/-}$, and IL-17F$^{-/-}$ mice after 4 weeks of vehicle (Sham) or Ang II infusion. WT and IL-17F$^{-/-}$ mice exhibited a marked increase in albuminuria while IL-17A$^{-/-}$ mice were completely protected from glomerular injury during Ang II infusion (**Figure 3-8A**). We then performed immunoblotting to confirm this finding and to quantify urinary angiotensinogen (a marker of tubular injury) in these 24 hr urine samples from WT and IL-17A$^{-/-}$ mice. Interestingly, Ang II infusion induced a marked increase in urinary angiotensinogen in WT mice and this was absent in IL-17A$^{-/-}$ mice (**Figure 3-8B**). Thus IL-17A, but not IL-17F, contributes to the renal end-organ damage that occurs as a result of Ang II induced hypertension.

Renal proximal and distal convoluted tubule cells produce endogenous IL-17A: We showed that IL-17A plays an important role in the regulation of renal sodium transporters both in vivo and in vitro. However, a key question is what is the source of IL-17A in the kidney? We and others have observed an accumulation of immune cells, particularly T lymphocytes, in the kidneys of animals and humans with hypertension [95, 96]. However, there is also evidence that renal epithelial cells may produce their own cytokines such as IL-6, IL-8, and TNF-α [101-103].

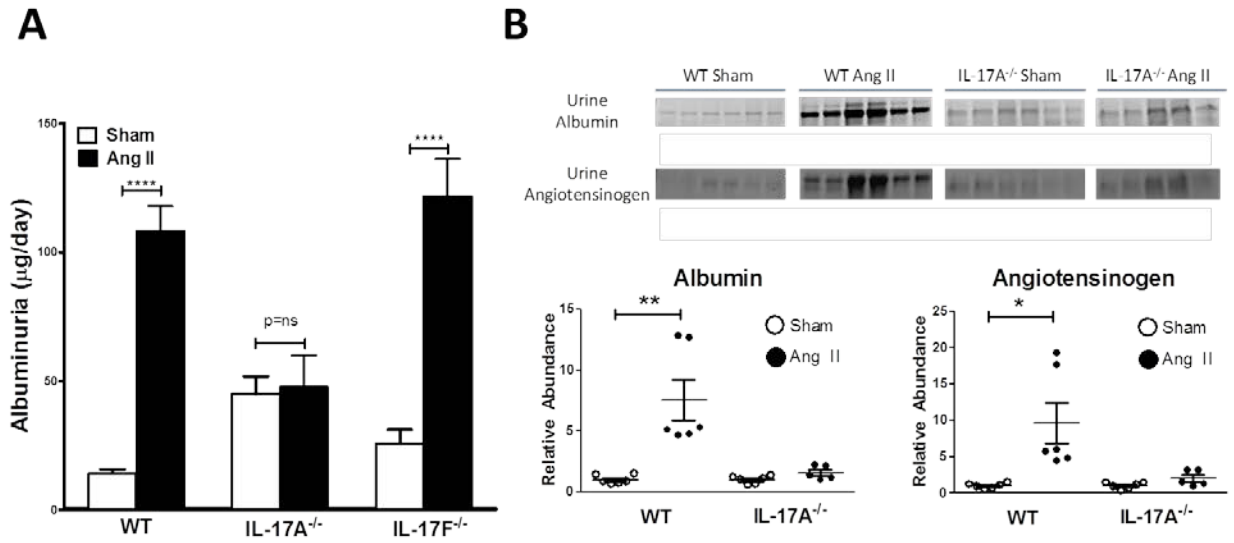


Figure 3-8: IL-17A$^{-/-}$ mice are protected from glomerular and tubular injury in response to angiotensin II infusion. WT, IL-17A$^{-/-}$, or IL-17F$^{-/-}$ mice were infused with vehicle (Sham) or Ang II for 4 weeks and then placed in metabolic cages for 24 hours for urine collection. **A)** Albuminuria was measured via ELISA and corrected for urine volume. **B)** Urinary albumin and angiotensinogen were measured by immunoblot and relative abundance for each group is plotted below. Data are expressed as mean±SEM. Two-way ANOVA followed by Newman-Keuls post-hoc test was used in panel A. Student's t-Test was used in panel B to compare protein levels in Ang II infused animals to the corresponding vehicle infused animals for each genotype. **P*<0.05, ***P*<0.01, *****P*<0.0001 (n=5-6 per group).

By RT-PCR, we confirmed the previously reported observation [104] that HK-2 cells produce IL-17A. Interestingly, we also found that mDCT15 cells express IL-17A (**Figure 3-9**). This was not observed in mouse pulmonary artery smooth muscle cells or human aortic endothelial cells. RNA from mouse CD4+ T cells polarized towards the Th17 lineage served as a positive control. Thus, IL-17A produced by renal tubular cells themselves or from infiltrated T cells can regulate sodium absorption, renal injury, and blood pressure.

Discussion

A major cause and complication of hypertension is renal dysfunction leading to increased sodium and water retention which promotes a rise in blood pressure and compensatory pressure natriuresis. Here we show that the pro-inflammatory cytokine, IL-17A, produced by immune cells as well as renal epithelial cells, can induce the expression and activity of both proximal and distal sodium transporters through an SGK1 dependent pathway, thus counteracting pressure natriuresis and contributing to sodium retention (**Figure 3-10**). Moreover, loss of IL-17A protects against glomerular and tubular injury in response to Ang II induced hypertension as evidenced by attenuation of albuminuria and urinary angiotensinogen levels in the IL-17A$^{-/-}$ mice. In contrast, deficiency of the related cytokine, IL-17F, has little or no effect on blood pressure and renal transporter expression. Interestingly, the effect of IL-17A on renal transporters is dependent on the duration of Ang II infusion. We previously reported that after 2 weeks of Ang II infusion, IL-17A$^{-/-}$ mice down-regulated NHE3, while NHE3 expression was unchanged in WT mice. In both groups, distal sodium transporters such as NCC and ENaC were activated (although to a lesser extent in the IL-17A$^{-/-}$ mice) [71]. Blood pressure starts to normalize in the IL-17A$^{-/-}$ mice after 2 weeks of Ang II infusion, reaching pressures 30 mmHg lower than Ang II infused WT mice by 4 weeks (Madhur et al.[95] and **Figure 3-1**).

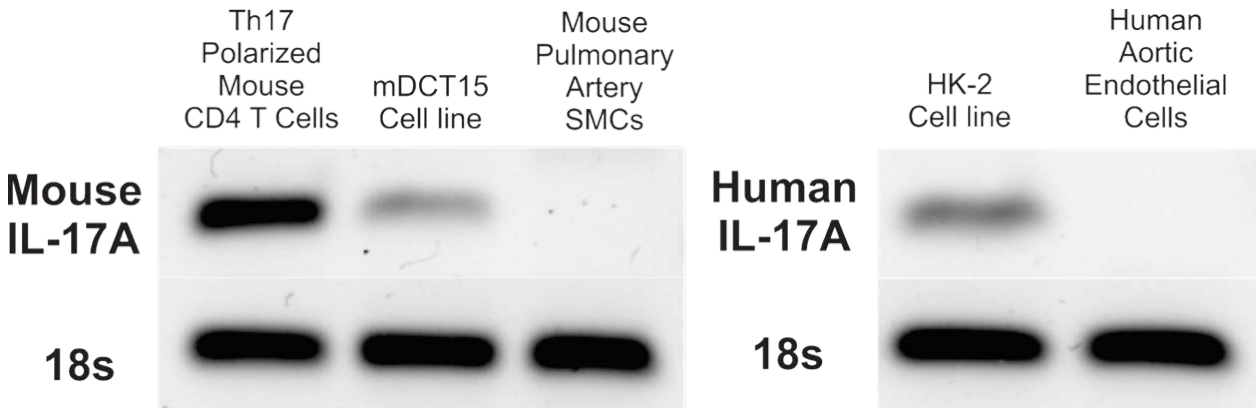


Figure 3-9: IL-17A is produced endogenously by both the mDCT15 and HK-2 kidney cell lines: Representative RT-PCR gel for IL-17A in mDCT15 and HK-2 cells. Mouse Th17 polarized CD4 T cells were used a positive control. IL-17A mRNA was not detected in 2 other cell lines, mouse pulmonary artery smooth muscle cells (SMCs) and human aortic endothelial cells. 18s was used as a control for RNA integrity.

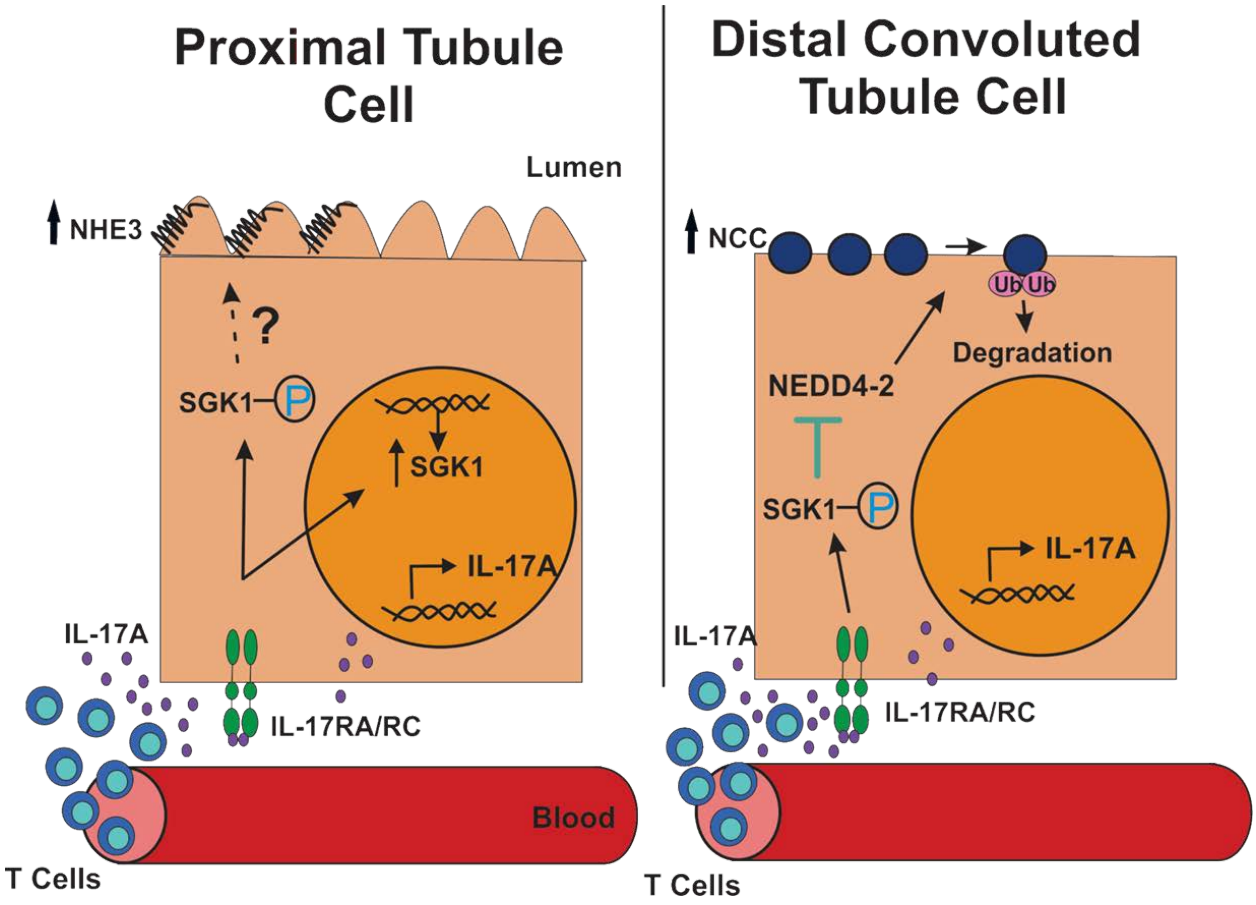


Figure 3-10: Schematic illustrating the effects of IL-17A (endogenous and T cell derived) on both proximal and distal tubule cells in the kidney.

At this 4 week timepoint, the major differences between WT and IL-17A$^{-/-}$ mice are seen in the distal tubule. IL-17A$^{-/-}$ (but not WT) mice have significant blunting of the activation of distal sodium transporters. These are key transporters in the regulation of blood pressure as evidenced by the fact that thiazide diuretics, pharmacological inhibitors of NCC, are still one of the most effective drugs in the treatment of hypertension [105].

Moreover, many of the Mendelian forms of hypertension are due to mutations that influence the activity of NCC and/or the amiloride-sensitive ENaC. Thus, the blood pressure reduction observed after 4 weeks of Ang II infusion in IL-17A$^{-/-}$ mice is likely due to decreased activity of distal sodium transporters. In fact, our data suggests that IL-17A inhibition may be as or more effective than the use of thiazide diuretics or amiloride in the treatment of hypertension and the resultant glomerular and tubular injury.

Urinary angiotensinogen is a biomarker of tubular injury as well as an indicator of the intrarenal renin-angiotensin system (RAS) activity. It is interesting to note that Ang II infusion failed to induce an increase in urinary angiotensinogen in the IL-17A$^{-/-}$ mice. In response to Ang II, proximal tubule cells produce greater levels of angiotensinogen which leads to increased Ang I release into the tubule lumen and subsequently conversion to Ang II by local angiotensin converting enzyme. Ang II then acts on the distal tubule and collecting duct to increase expression of NCC and ENaC [106]. Satou et al. showed that prolonged treatment of renal proximal tubule cells with interferon-γ results in increased angiotensinogen production in these cells. Interferon-γ may thus indirectly activate distal sodium transporters via stimulation of the intrarenal RAS system [107]. It is conceivable that IL-17A may be functioning in a similar manner. However, we did not see an elevation of angiotensinogen by qRT-PCR after IL-17A treatment of HK-2 cells (data not shown). Moreover, our in vitro studies show that IL-17A can

stimulate NCC activity in cultured cells. Nevertheless, we cannot rule out a potential effect of IL-17A on the intrarenal RAS in vivo.

Of note are the differential effects seen between IL-17A versus IL-17F. Although they share 50% sequence similarity and bind the same receptor complex, our results indicate that IL-17A (but not IL-17F) regulates NCC activity, renal injury, and hypertension [97].

Limitations of the present study include the use of one specific mouse strain and one model of hypertension, namely angiotensin II infusion. In addition, we have only tested the effects of IL-17 isoforms on one proximal tubule and one distal tubule cell line. Thus, additional studies using other animal strains, cell lines, and different models of hypertension are necessary to generalize these findings.

Monoclonal antibodies to IL-17A or the IL-17RA receptor have recently been developed for human use. In 2015, the FDA approved the first IL-17A antagonist Secukinumab (a human monoclonal antibody that selectively binds to IL-17A) for the treatment of moderate to severe plaque psoriasis. Also in development are drugs such as Brodalumab, a monoclonal antibody that targets the IL-17RA receptor subunit. This drug is awaiting FDA approval for the treatment of moderate to severe psoriasis. Our data suggests that targeting IL-17A or the IL-17RA/RC receptor complex may be a novel therapeutic strategy for the treatment of hypertension and the associated renal dysfunction.

CHAPTER 4

A salt sensing kinase in T lymphocytes, SGK1, drives hypertension and hypertensive end-organ damage

Allison E. Norlander[1], Mohamed A. Saleh[2,3], Arvind K. Pandey[2], Hana A. Itani[2], Jing Wu[1†], Liang Xiao[2], Jooeun Kang[1], Bethany L. Dale[1], Slavina B. Goleva[1], Liping Du[4], David G. Harrison[1,2], Meena S. Madhur*[1,2]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232, USA.

[2]Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA.

[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

[4]Vanderbilt Center for Quantitative Sciences, Vanderbilt University School of Medicine, Nashville, TN 37232, USA.

[†]Current address: Department of Pharmacology, University of Iowa Carver College of Medicine, Iowa City, IA 52242, USA.

Introduction

Hypertension is a leading cause of death and disability from cardiovascular and renal diseases including myocardial infarction, heart failure, stroke, and chronic kidney disease. The rising prevalence of hypertension, due in part to changes in diet and food processing, makes it a major global health concern. In the U.S. alone, hypertension afflicts approximately one-third of all adults over 18 years of age, and this rises to two-thirds by the age of 60 [108]. In 2010, high blood pressure was ranked as the leading single risk factor for global burden of disease [2].

Evidence from our group and others has demonstrated that hypertension is an inflammatory disease characterized by T cell activation, infiltration of target organs such as the kidney and vasculature, and production of cytokines that lead to salt and water retention, vascular dysfunction, and renal injury [17, 71, 109, 110]. Most notably, we previously demonstrated that mice deficient in the pro-inflammatory cytokine interleukin 17A (IL-17A) exhibit blunted hypertension, attenuated vascular inflammation, and preservation of vascular function in response to angiotensin II induced hypertension [33]. Recently, we showed that IL-17A regulates proximal and distal renal sodium transporters and contributes to angiotensin II induced renal injury (**Chapter 3**) [110]. IL-17A is produced by a subset of CD4+ T helper cells (Th17 cells), a subset of CD8+ cytotoxic T cells (Tc17 cells), and a distinct subset of innate-like gamma delta T cells (γδT17 cells). We found that Th17 cells and γδT17 cells are the major sources of IL-17A in the kidney and vasculature following angiotensin II induced hypertension [111].

Of note, numerous epidemiological and experimental studies have demonstrated a link between dietary salt intake and hypertension [112]. Interestingly, there is now emerging evidence that salt can directly influence immune cell function. Two recent studies demonstrated that elevated

extracellular sodium chloride concentrations of 190 mM, presumably similar to levels found in the interstitium of animals fed a high-salt diet [113, 114], promoted the polarization of naïve T cells into Th17 cells through a serum and glucocorticoid regulated kinase 1 (SGK1) dependent pathway [81, 82]. Using a mouse model of multiple sclerosis known as experimental autoimmune encephalomyelitis (EAE), Wu et al. [81] showed that high salt feeding resulted in increased numbers of Th17 cells in the central nervous system and mesenteric lymph nodes and led to a more rapid and severe form of EAE, an IL-17A mediated disease, in control animals. Interestingly, mice with T cell deficiency of SGK1 exhibited a significantly reduced EAE incidence and severity that was not further exacerbated by high salt feeding. Thus, in addition to regulating salt-responsiveness of T cells, SGK1 appears to mediate salt-independent T cell, and particularly Th17 cell, pathogenicity in autoimmune disease.

Given the importance of IL-17A in hypertension, we therefore hypothesized that T cell SGK1 plays a key role in salt dependent and independent hypertension and hypertensive end-organ damage. To test this hypothesis, we generated mice with T cell specific deletion of SGK1 and induced hypertension using angiotensin II infusion or uninephrectomy followed by deoxycorticosterone acetate – salt (DOCA-salt) treatment. We found that loss of SGK1 in T cells blunts hypertension and completely abrogates renal/vascular inflammation and protects against hypertensive renal and vascular injury. Furthermore, we detected the expression of multiple sodium channels and sodium transporters on T lymphocytes and provide evidence that the sodium-potassium-2 chloride cotransporter 1 (NKCC1) is upregulated in Th17 cells and mediates the salt-induced increase in SGK1. Taken together, these results demonstrate that T cell SGK1 and NKCC1 may serve as novel therapeutic targets for the treatment of hypertension.

Results

T cell SGK1 deficiency attenuates angiotensin II-induced hypertension

To determine the role of T cell SGK1 in hypertension, we generated mice with T cell specific deletion of SGK1 by crossing $SGK1^{fl/fl}$ mice with transgenic mice expressing cre recombinase under the control of the CD4 promoter (tg^{CD4cre}). T cells that express CD4 at any stage during development should undergo cre-recombination and deletion of SGK1. We first investigated the role of T cell SGK1 in angiotensin II (Ang II) induced hypertension. We verified knockdown of SGK1 in CD4+ and CD8+ T cells, SGK1 is knocked down in both cell types because they go through a double positive stage during development in the thymus, and demonstrate that SGK1 is knocked down in some γδ T cells in our T cell culture model (**Figure 4-1A-C**) [115]. Blood pressure (BP) in $SGK1^{fl/fl}tg^{CD4cre}$ mice and $SGK1^{fl/fl}$ controls was measured both noninvasively using the tail-cuff method and invasively using carotid radiotelemetry in freely moving mice. BPs in vehicle-infused (Sham) mice were similar between $SGK1^{fl/fl}tg^{CD4cre}$ mice and $SGK1^{fl/fl}$ controls, demonstrating that T cell deletion of SGK1 does not affect baseline BP (**Figure 4-2A**). However, following infusion of a pressor dose of Ang II (490/ng/kg/min) for 28 days, systolic BP was 25 mmHg lower in the $SGK1^{fl/fl}tg^{CD4cre}$ mice compared to the $SGK1^{fl/fl}$ controls as measured by both tail cuff and telemetry (**Figure 4-2A-B**). Diastolic BPs and mean arterial BPs were similarly reduced in the $SGK1^{fl/fl}tg^{CD4cre}$ mice compared to controls while heart rates were similar between both groups (**Figure 4-2C-E**). As a secondary control, to confirm that cre recombinase expression in T cells is not responsible for the blunted hypertensive phenotype, we measured BP in tg^{CD4cre} mice at baseline and weekly (2 days per week) using carotid radiotelemetry during 28 days of Ang II infusion.

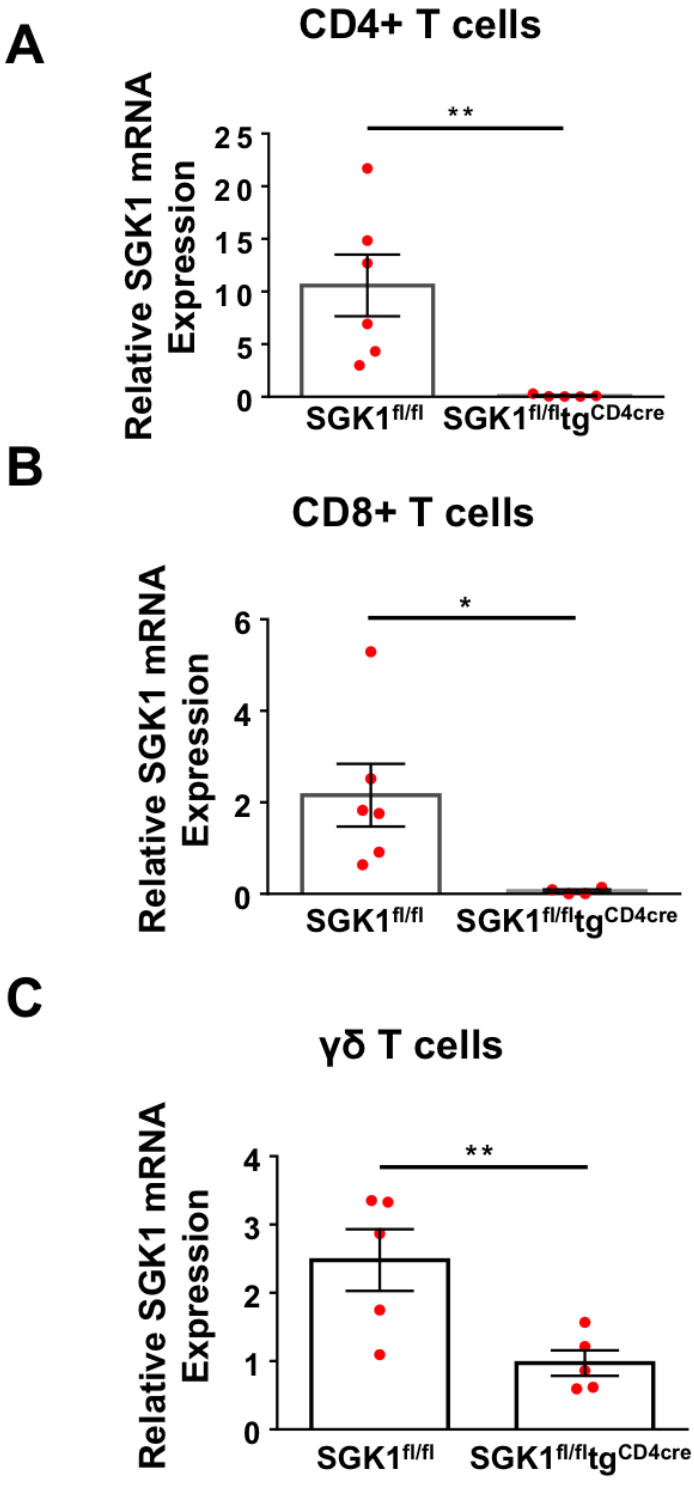


Figure 4-1. SGK1 expression is abolished in CD4⁺ and CD8⁺ T cells and some γδ T cells in SGK1$^{fl/fl}$tg^{CD4cre} mice. Naïve CD4⁺ T cells **(A)**, naive CD8⁺ T cells **(B)**, or γδ T cells **(C)** were isolated from spleens of SGK1$^{fl/fl}$ control and SGK1$^{fl/fl}$tg^{CD4cre} mice and cultured for 3 days on anti-CD3/anti-CD28 coated plates in the presence of Th17 polarizing cytokines plus an excess 40mM NaCl to maximize SGK1 expression which was then quantified by qRT-PCR (**P*<0.05, ***P*<0.01; Student's t-test; n = 4-6 per group). All data are expressed as mean ± SEM.

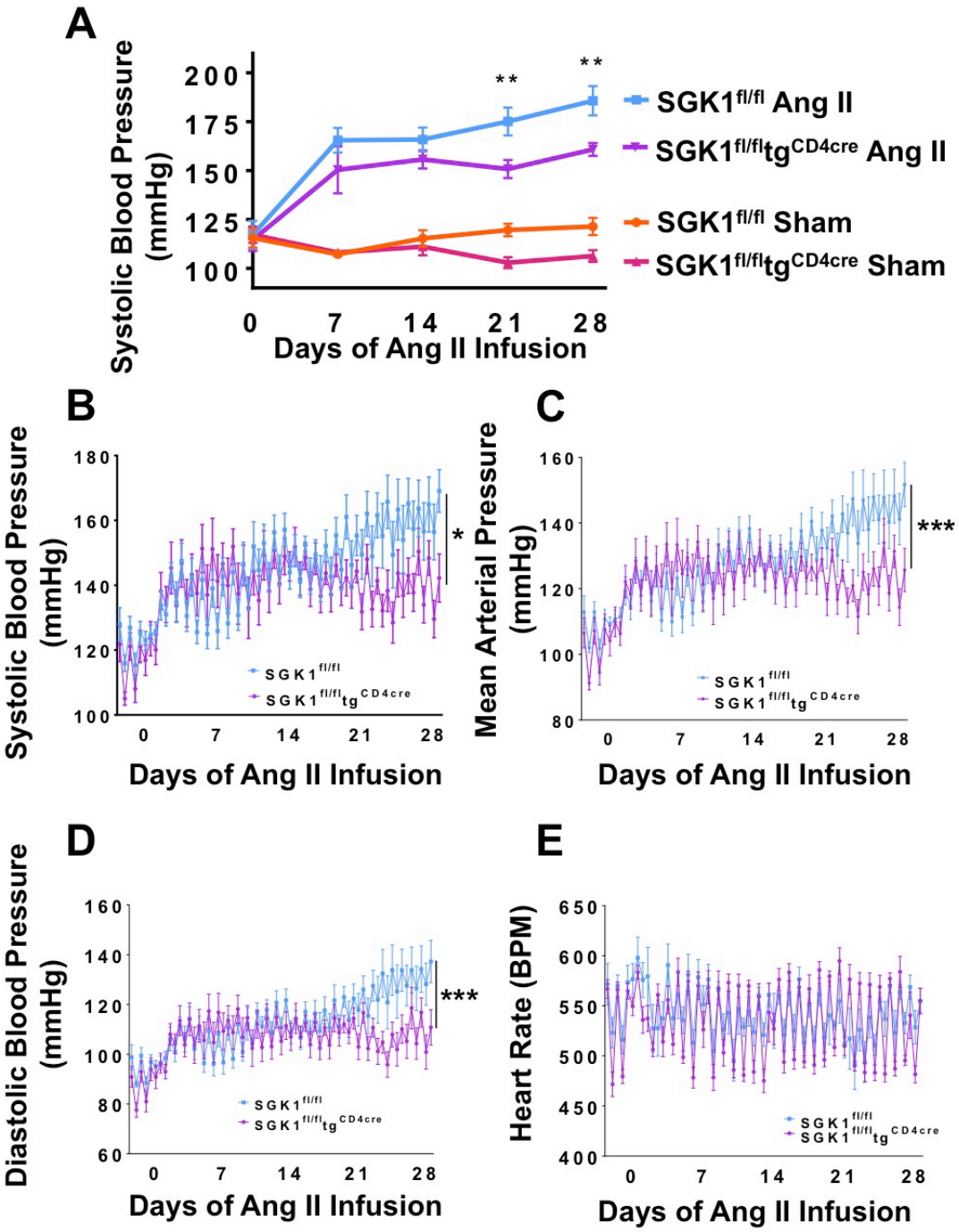


Figure 4-2. T cell SGK1 deficiency attenuates angiotensin II (Ang II)-induced hypertension. **(A)** Systolic blood pressures measured noninvasively using the tail-cuff method over 28 days of Ang II (490ng/kg/min) or vehicle (Sham) infusion in $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice (**P<0.01 between both Ang II infused groups; repeated measures ANOVA/Holm-Sidak's post hoc test; n = 6-7 per group). **(B)** Systolic blood pressures, **(C)** Mean arterial pressures, **(D)** Diastolic blood pressures, and **(E)** Heart rates measured using invasively using carotid radiotelemetry over 28 days of Ang II infusion in $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice (*P<0.05, ***P<0.001; regression analysis; n = 6-7 per group). All data are expressed as mean ± SEM.

The systolic, diastolic and mean arterial pressures of the tg^{CD4cre} mice increased similarly as the $SGK1^{fl/fl}$ controls (**Figure 4-3A-C**). Heart rate was similar among all 3 groups of mice (**Figure 4-2E and Figure 4-3D**).

T cell SGK1 deficiency prevents Ang II induced vascular and renal inflammation

We and others have shown that Ang II increases total leukocyte and T cell infiltration into the perivascular fat and kidneys, and that this is likely an important causal factor for the progression of hypertension and the development of end-organ damage [17, 38, 72].

To determine the role of T cell SGK1 on vascular and renal inflammation, we performed flow cytometry on single cell suspensions of the thoracic aorta and kidney following 28 days of Ang II infusion in $SGK1^{fl/fl}tg^{CD4cre}$ mice and $SGK1^{fl/fl}$ controls. Representative flow cytometry dot plots and gating strategy to detect total leukocytes ($CD45^+$ cells), total T lymphocytes ($CD45^+CD3^+$ cells), and T cell subsets ($CD4^+$, $CD8^+$ and double negative (DN) cells) in the thoracic aorta are shown in **Figure 4-4A**. Baseline levels of total leukocytes and T cells were similar in both groups as shown after vehicle infusion (Sham) in $SGK1^{fl/fl}$ controls and $SGK1^{fl/fl}tg^{CD4cre}$ mice (**Figure 4-4B-F**). Ang II infusion markedly increased total leukocytes, total T cells, and T cell subsets in $SGK1^{fl/fl}$ control mice while this response was completely absent in the $SGK1^{fl/fl}tg^{CD4cre}$ mice (**Figure 4-4B-F**). Representative flow cytometry dot plots and gating strategy to detect renal inflammation are shown in **Figure 4-5A**. Quantification of renal total leukocytes and T cells were similar in both groups after vehicle infusion (Sham) (**Figure 4-5B-F**).

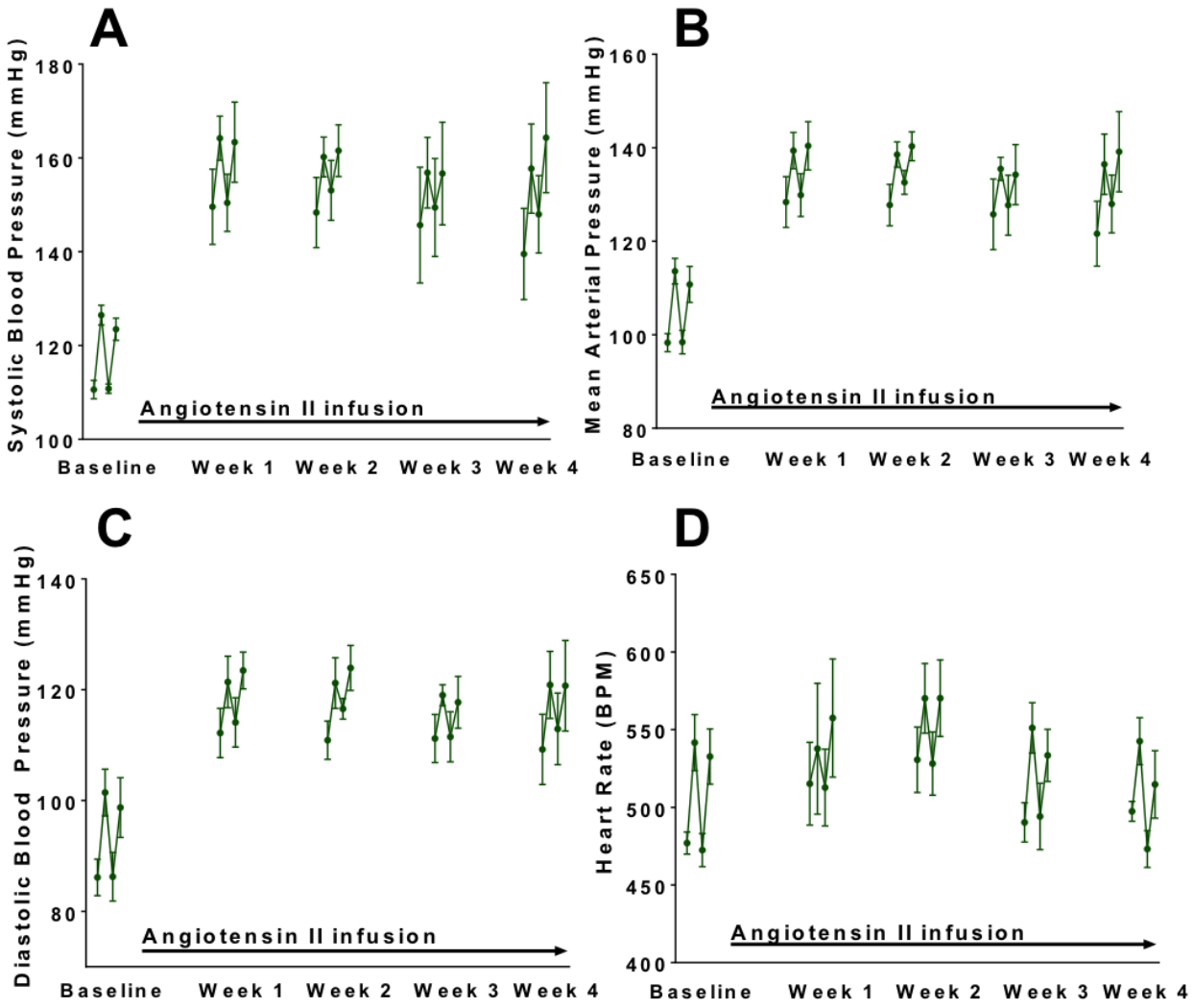


Figure 4-3. Cre recombinase expression in T cells does not affect Ang II-induced blood pressure response. Telemetry recordings of **(A)** systolic blood pressures, **(B)** mean arterial pressures, **(C)** diastolic blood pressures, and **(D)** heart rates in tg^{CD4cre} mice infused with Ang II (490ng/kg/min) for 28 days. Data were recorded for 2 days at baseline and weekly thereafter during Ang II infusion. All data are expressed as mean ± SEM (n = 5).

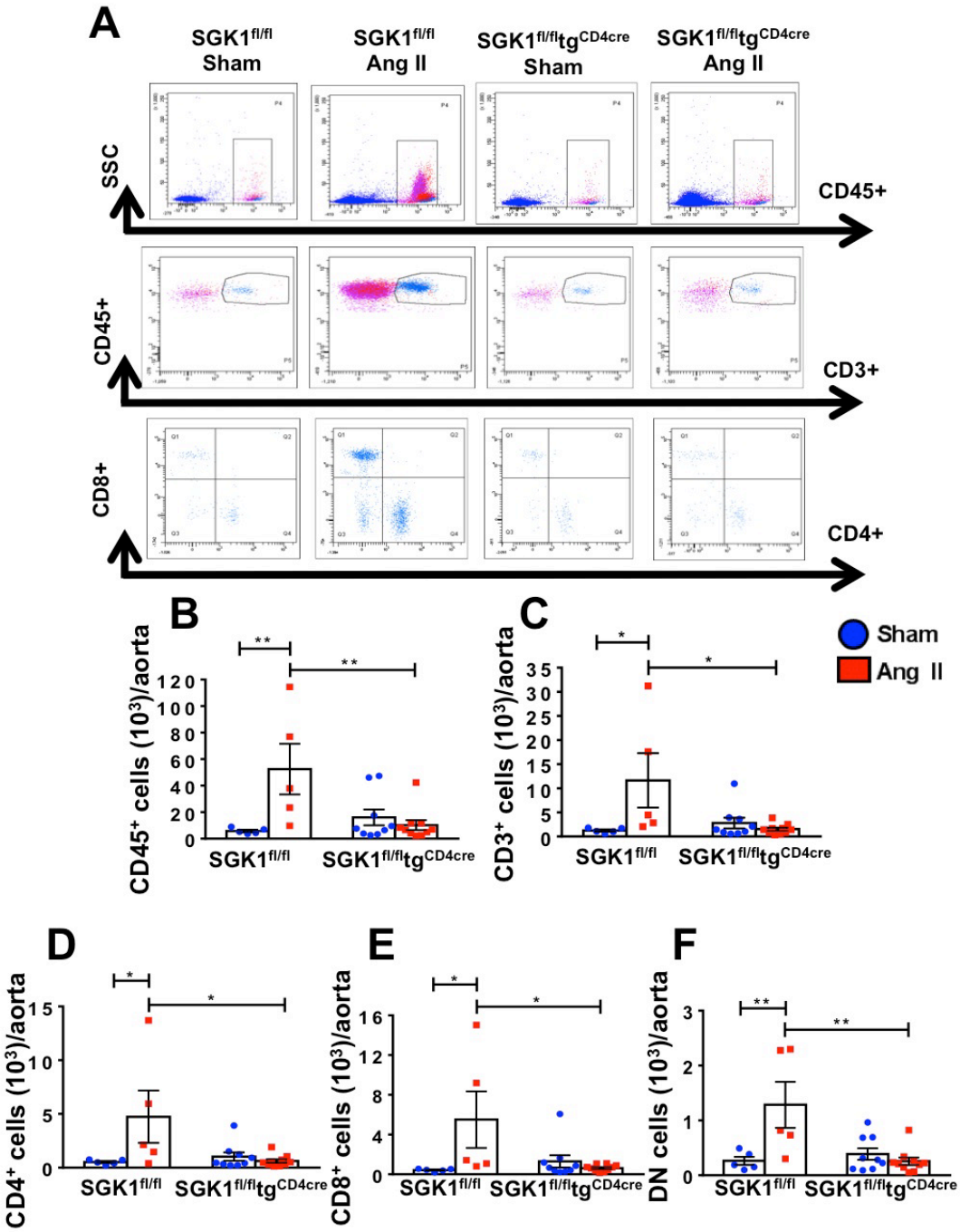


Figure 4-4. T cell SGK1 deficiency prevents Ang II-induced vascular inflammation. (A) Representative flow cytometry dot plots showing gating strategy for total leukocytes ($CD45^+$ cells), total T lymphocytes ($CD45^+CD3^+$ cells), and T cell subsets ($CD4^+$, $CD8^+$, and $CD3^+CD4^-CD8^-$ double negative (DN) cells) in single cell suspensions from the thoracic aorta of $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice infused with Ang II or vehicle (Sham) for 28 days. **(B-F)** Summary data of absolute numbers of indicated cell types per thoracic aorta (**P*<0.05, ***P*<0.01; two-way ANOVA/Holm-Sidak's post hoc test; n = 5-10 per group). All data are expressed as mean ± SEM.

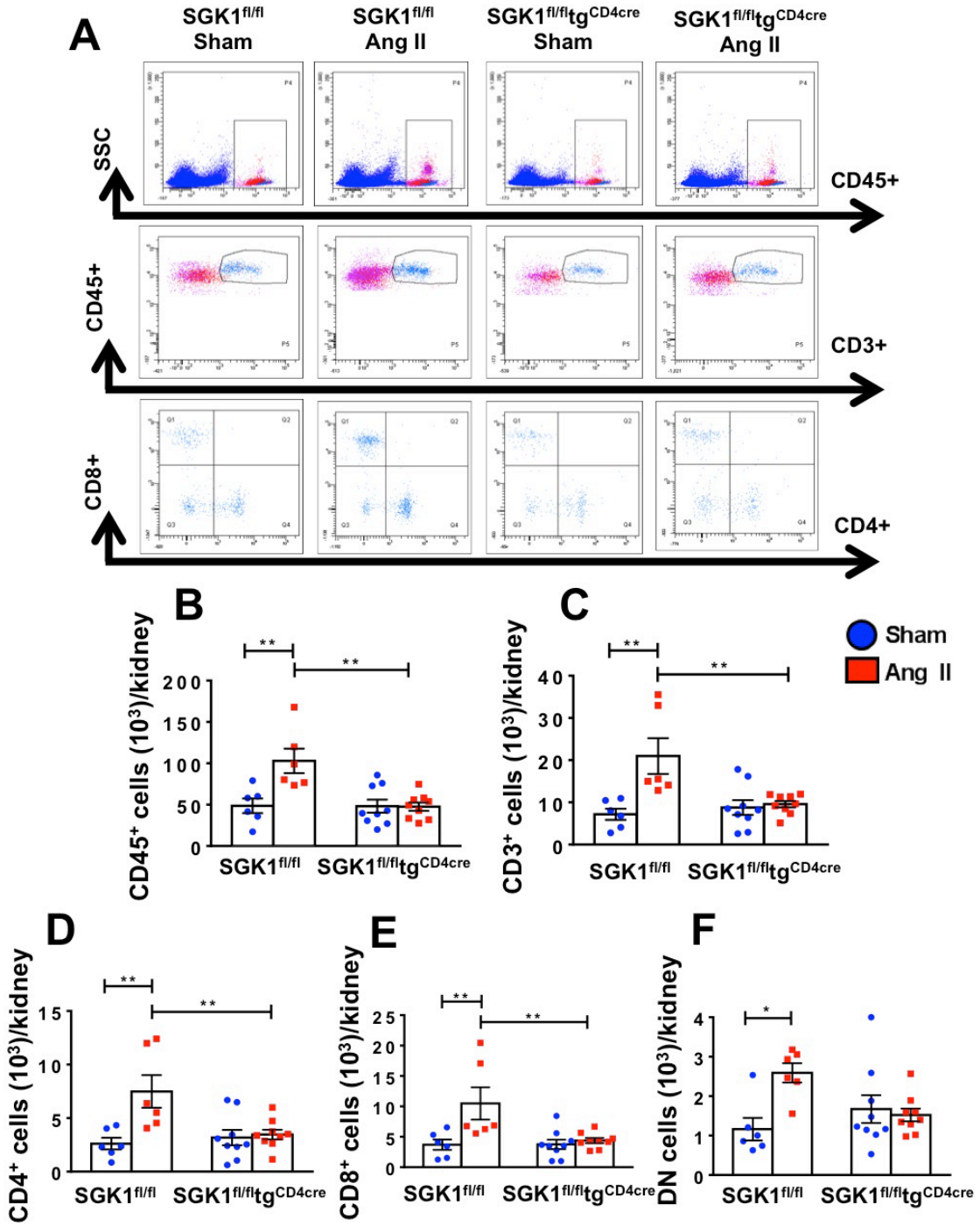


Figure 4-5. T cell SGK1 deficiency prevents Ang II-induced renal inflammation. (A) Representative flow cytometry dot plots showing gating strategy for total leukocytes ($CD45^+$ cells), total T lymphocytes ($CD45^+CD3^+$ cells), and T cell subsets ($CD4^+$, $CD8^+$, and $CD3^+CD4^-CD8^-$ double negative (DN) cells) in single cell suspensions from one kidney of $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice infused with Ang II or vehicle (Sham) for 28 days. **(B-F)** Summary data of absolute numbers of indicated cell types per kidney (**P*<0.05 ***P*<0.01; two-way ANOVA/Holm-Sidak's post hoc test; n = 6-9 per group). All data are expressed as mean ± SEM.

As seen in the aorta, Ang II infusion increased renal total leukocytes and T cell subsets only in $SGK1^{fl/fl}$ controls while $SGK1^{fl/fl}tg^{CD4cre}$ mice were completely protected from Ang II induced renal inflammation. Taken together, these results suggest that T cell SGK1 is required for the development of hypertensive renal and vascular inflammation.

T cell SGK1 deficiency protects against Ang II induced vascular and renal injury

Hypertension is characterized by endothelial dysfunction and glomerular injury [17, 33]. We assessed vascular function of resistance vessels by measuring endothelium-dependent and –independent relaxation of second order mesenteric arterioles from $SGK1^{fl/fl}$ controls and $SGK1^{fl/fl}tg^{CD4cre}$ mice. Mesenteric arterioles from $SGK1^{fl/fl}$ control mice exposed to 4 weeks of Ang II infusion displayed impaired relaxation to acetylcholine compared to vessels isolated from vehicle (Sham) infused mice (**Figure 4-6A**).

Importantly, mesenteric arterioles from $SGK1^{fl/fl}tg^{CD4cre}$ mice displayed no impairment in endothelium-dependent relaxation following Ang II infusion (**Figure 4-6B**). Endothelium-independent relaxation to sodium nitroprusside was not affected by Ang II infusion in both groups of mice (**Figure 4-6C-D**).

We then examined renal injury in these mice by measuring the albumin:creatinine ratio in spot urine following 4 weeks of vehicle (Sham) or Ang II infusion. Albuminuria is a well-known marker of glomerular injury and has been shown to increase following Ang II infusion in WT mice [17, 110]. For these experiments, we used tg^{CD4cre} mice as a secondary control. Ang II infusion resulted in a marked increase in albuminuria in both control groups, $SGK1^{fl/fl}$ and tg^{CD4cre} mice, while the increase in albuminuria in $SGK1^{fl/fl}tg^{CD4cre}$ mice was blunted and non-significant (**Figure 4-6E**).

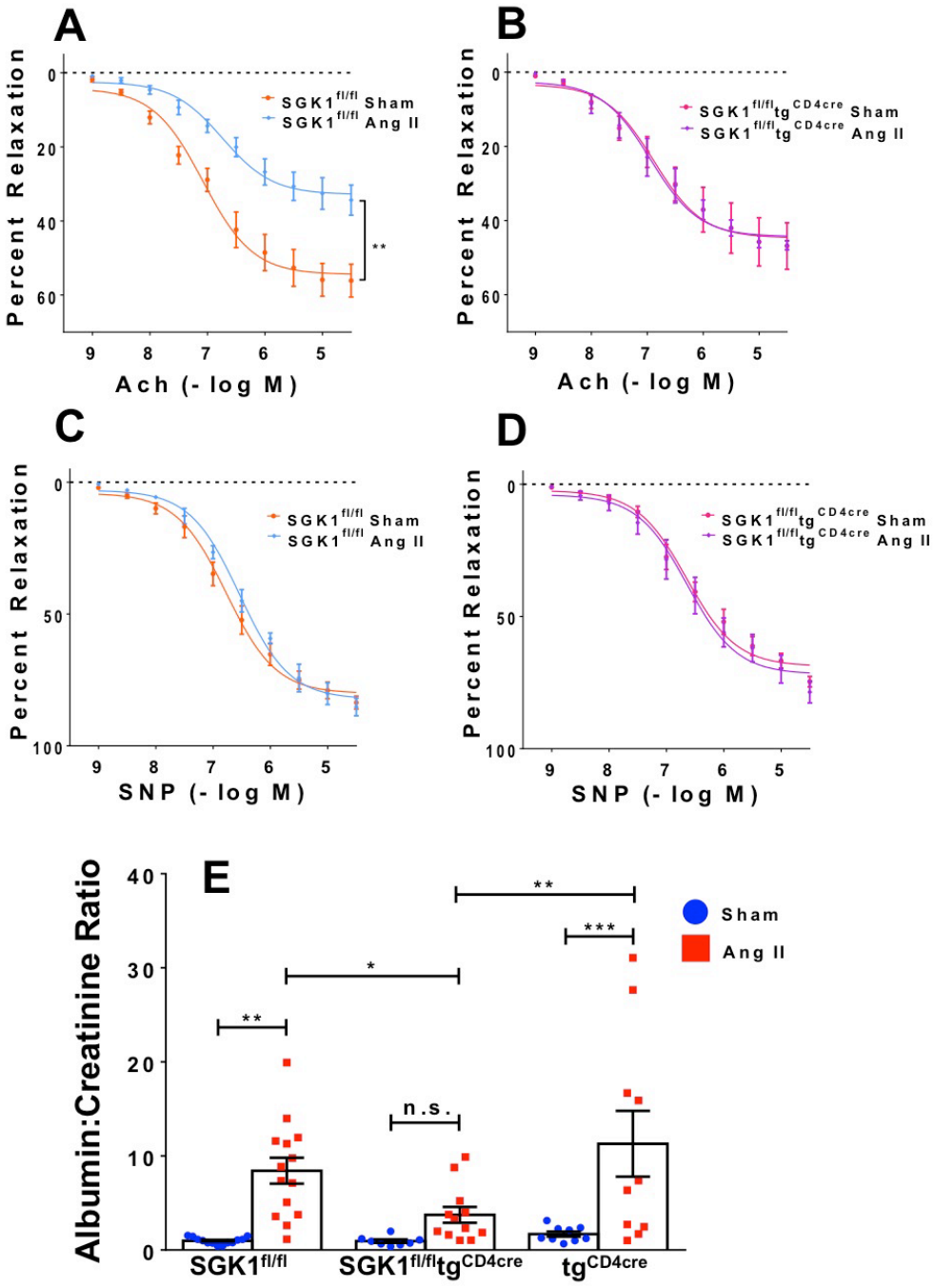


Figure 4-6. T cell SGK1 deficiency protects against Ang II-induced vascular and renal injury. SGK1$^{fl/fl}$ controls, tg^{CD4cre} controls, and SGK1$^{fl/fl}$tg^{CD4cre} mice were infused with Ang II or vehicle (Sham) for 28 days. Endothelium-dependent relaxation to increasing doses of acetylcholine (Ach) was measured in **(A)** SGK1$^{fl/fl}$ mice and **(B)** SGK1$^{fl/fl}$tg^{CD4cre} mice, and endothelium-independent relaxation to increasing doses of sodium nitroprusside (SNP) was measured in **(C)** SGK1$^{fl/fl}$ mice and **(D)** SGK1$^{fl/fl}$tg^{CD4cre} mice (**P<0.05; repeated measures ANOVA/Holm-Sidak's post-hoc test; n=4-6 per group). **(E)** Urinary albumin to creatinine ratio in the indicated groups (*P<0.05, **P<0.01, ***P<0.001; one-way ANOVA/Holm-Sidak's post-hoc test; n = 8-14 per group). All data are expressed as mean ± SEM.

Thus, the absence of renal and vascular inflammation following Ang II infusion in T cell SGK1 deficient mice correlates with protection from Ang II induced renal and vascular injury.

T cell SGK1 deficiency abrogates Ang II induced increase in splenic Th17 cells

We previously showed that Ang II infusion was associated with an increase in IL-17A production from circulating and splenic CD4+ T cells [17, 33]. To determine the effect of SGK1 on Th17 cell numbers in vivo in response to Ang II infusion, we performed intracellular staining and flow cytometry on splenic single cell suspensions from vehicle (Sham) and Ang II infused $SGK1^{fl/fl}$ controls and $SGK1^{fl/fl}tg^{CD4cre}$ mice. Representative flow cytometry images of IL-17A producing CD4+ T cells (Th17 cells) and CD8+ T cells (Tc17 cells) are shown in **Figure 4-7A**. Ang II infusion resulted in a 2-fold increase in splenic Th17 cells in $SGK1^{fl/fl}$ control mice, and this was completely abrogated in $SGK1^{fl/fl}tg^{CD4cre}$ mice (**Figure 4-7B**).

Splenic Tc17 cells were not affected by Ang II infusion or loss of T cell SGK1 (**Figure 4-7C**). Interferon gamma (IFNγ) is produced by CD4+ Th1 and CD8+ Tc1 cells and has been shown to play an important role in hypertension [17]. By intracellular staining and flow cytometry, we did not detect any differences in splenic Th1 or Tc1 percentages in response to Ang II infusion or loss of T cell SGK1 (**Figure 4-8A-C**).

T cell SGK1 deficiency attenuates hypertension and vascular inflammation in a DOCA-salt model of hypertension

Since SGK1 has been shown to mediate salt induced Th17 polarization and maintenance [81, 82], we investigated the role of T cell SGK1 in a deoxycorticosterone acetate (DOCA)-salt model of hypertension in which uninephrectomy is accompanied by implantation of a DOCA pellet and supplementation of the drinking water with 1% NaCl.

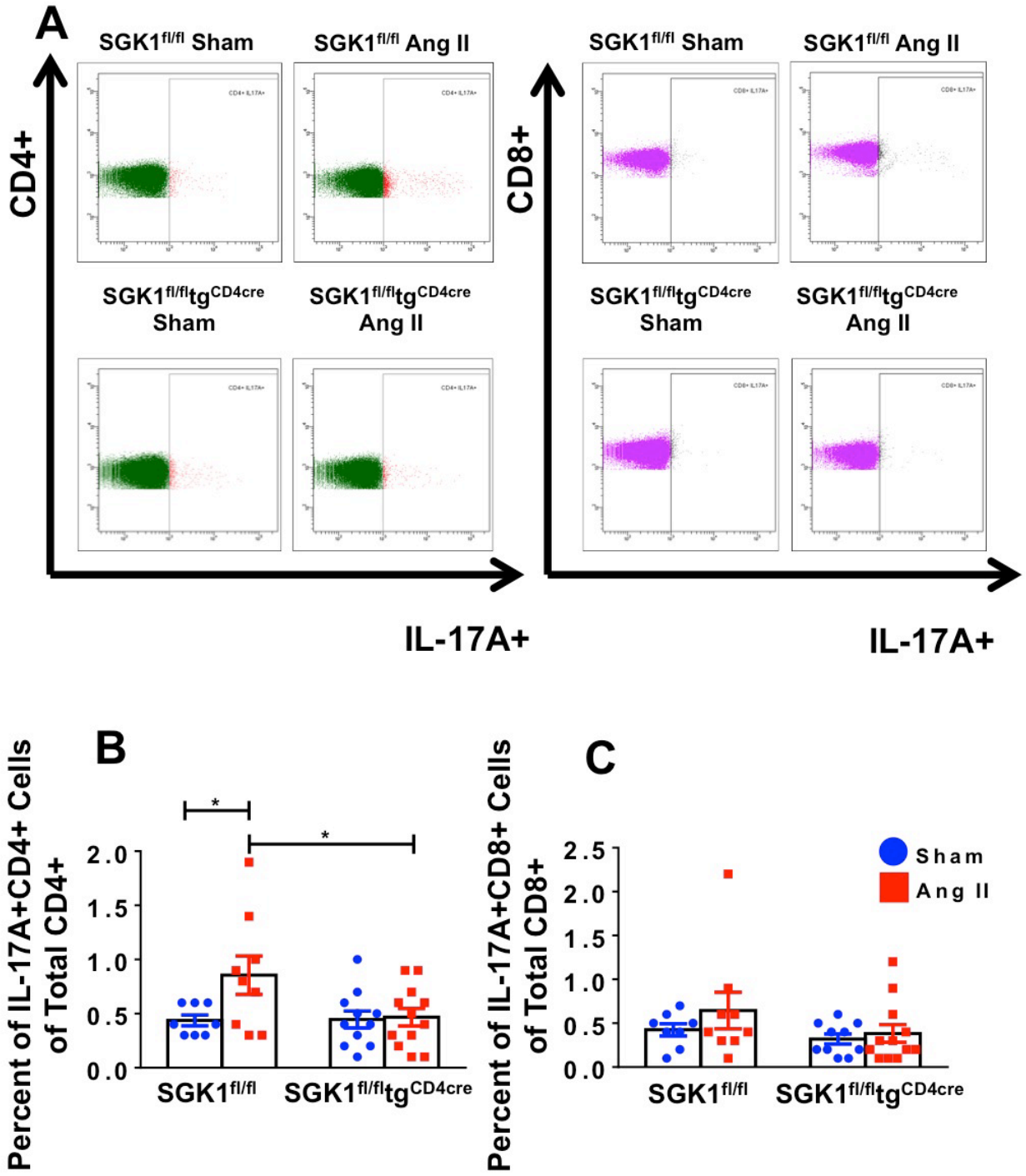


Figure 4-7. T cell SGK1 deficiency abrogates Ang II-induced increase in splenic Th17 cells. **(A)** Representative flow cytometry dot plots for $CD4^+IL\text{-}17A^+$ Th17 cells and $CD8^+IL\text{-}17A^+$ Tc17 cells in splenic single cell suspensions from $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice infused with Ang II or vehicle (Sham) for 28 days. **(B-C)** Summary data of percentages of $CD4^+IL\text{-}17A^+$ Th17 cells out of total $CD4^+$ cells and $CD8^+IL\text{-}17A^+$ Tc17 out of total $CD8^+$ cells in the indicated groups (*P<0.05; one-way ANOVA/Holm-Sidak's post-hoc test; n=8-12 per group). All data are expressed as mean ± SEM.

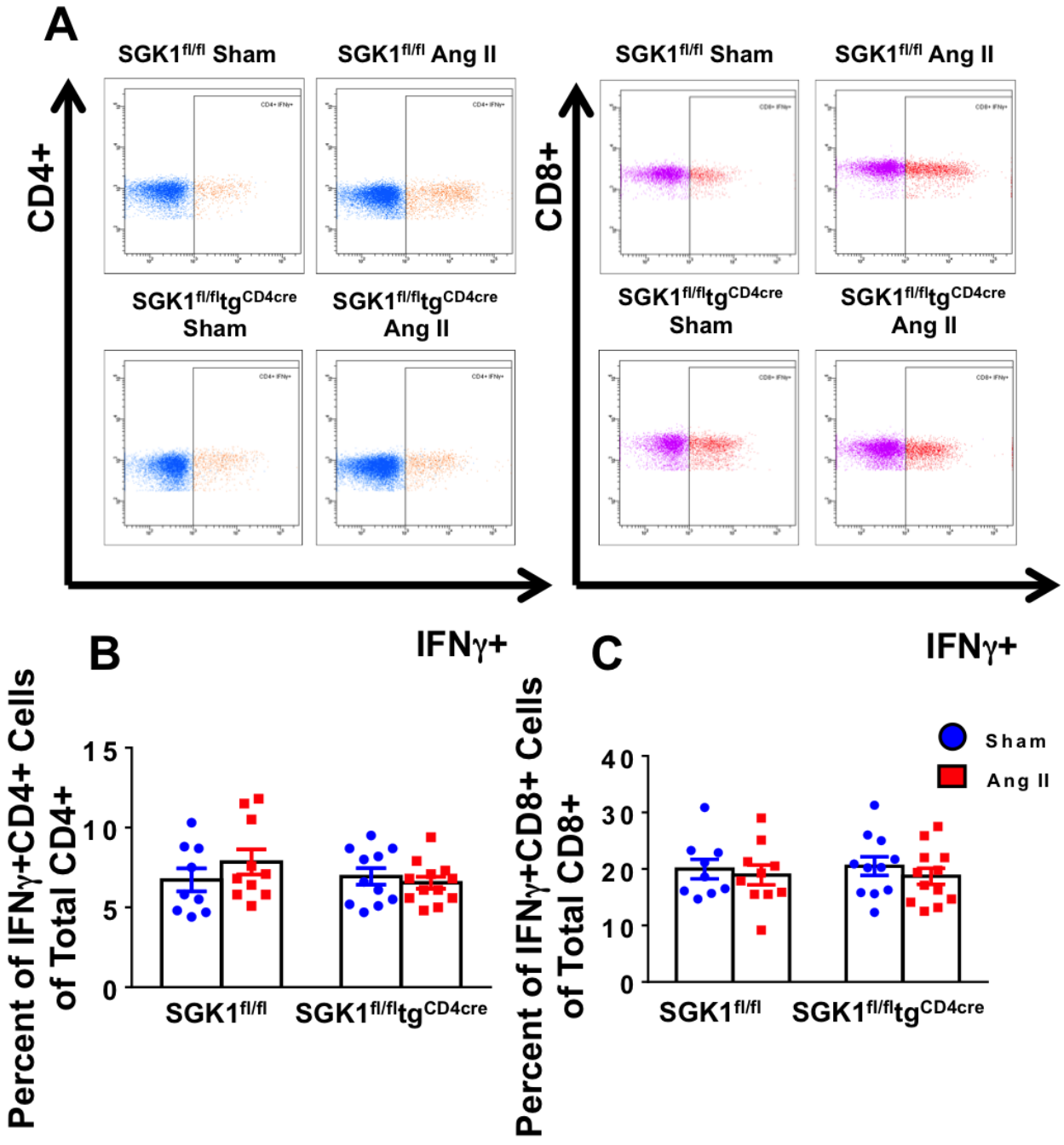


Figure 4-8. Frequency of splenic Th1/Tc1 cells is not altered by Ang II infusion or loss of T cell SGK1. (A) Representative flow cytometry dot plots for $CD4^+IFN\gamma^+$ Th1 cells and $CD8^+IFN\gamma^+$ Tc1 cells in splenic single cell suspensions from $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice infused with Ang II or vehicle (Sham) for 28 days. **(B-C)** Summary data of percentages of $CD4^+IFN\gamma^+$ Th1 cells out of total $CD4^+$ cells and $CD8^+IFN\gamma^+$ Tc1 cells out of total $CD8^+$ cells in the indicated groups. All data are expressed as mean ± SEM (n=9-12 per group).

The development of hypertension was blunted in the $SGK1^{fl/fl}tg^{CD4cre}$ mice with systolic BPs reaching 15 mmHg lower than $SGK1^{fl/fl}$ controls at days 14 and 21 of DOCA-salt treatment (**Figure 4-9A**). DOCA-salt treatment was associated with an increase in total CD45+ leukocytes and CD3+ T cells in the thoracic aorta in $SGK1^{fl/fl}$ control mice, and this was abrogated in $SGK1^{fl/fl}tg^{CD4cre}$ mice (**Figure 4-9B-F**). Thus, T cell SGK1 appears to play an important role in both salt-dependent and –independent hypertension.

NKCC1 is upregulated in Th17 cells and mediates the salt-induced increase in SGK1

To understand how T cells sense extracellular NaCl, we first isolated and cultured naïve $CD4^+$ or $CD8^+$ splenic T cells under Th17 polarizing conditions with or without an excess 40 mM NaCl for 3 days. Expression of IL-17A and SGK1 was measured in these cells by qRT-PCR.

As previously shown in $CD4^+$ T cells [81, 82], we found that IL-17A and SGK1 are upregulated in both $CD4^+$ and $CD8^+$ T cells upon exposure to Th17 polarizing cytokines and further increased by high salt treatment (**Figure 4-10A-D**). We then isolated pan T cells from spleens of WT mice and performed RT-PCR for a panel of sodium channels and transporters. We were able to detect expression of all the channels and transporters listed in **Table 4-1**. Of note, NKCC2, a renal specific isoform of NKCC, was not detected in T cells.

To determine which of these channels/transporters might mediate the salt effect on T cells, we tested various pharmacological inhibitors of sodium transporters as well as an antagonist of the mineralocorticoid receptor (which has been previously shown to regulate the Th17 response to DOCA-salt treatment in rats [18]).

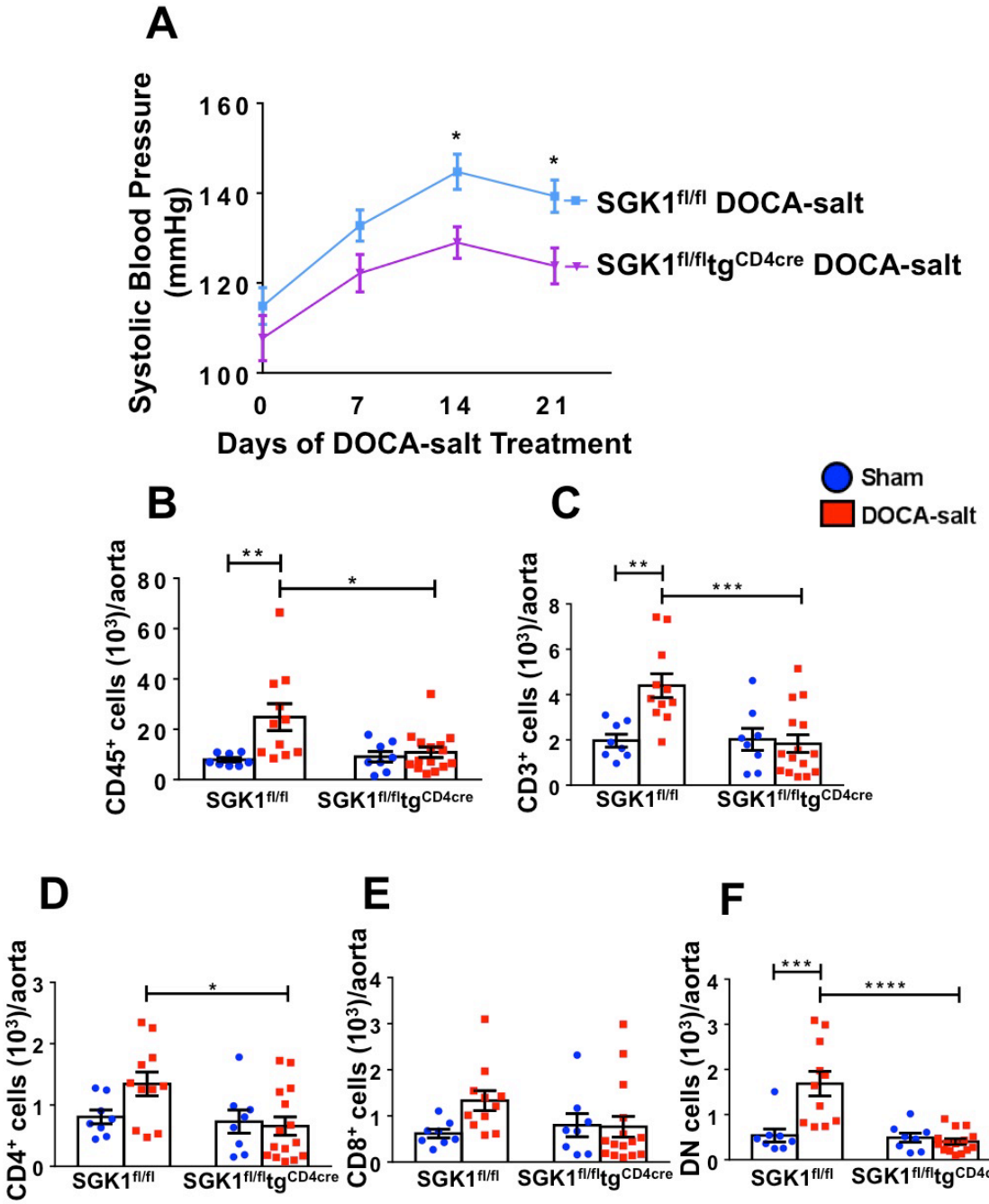


Figure 4-9. T cell SGK1 deficiency attenuates hypertension and vascular inflammation in a DOCA-salt model of hypertension. (A) Systolic blood pressures measured noninvasively using the tail-cuff method in $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice in response to DOCA-salt treatment over 21 days (**P*<0.05; repeated measures ANOVA/Holm-Sidak's post hoc test; n = 8-10 per group). **(B-F)** Summary flow cytometry data of absolute numbers of total leukocytes ($CD45^+$ cells), total T lymphocytes ($CD45^+CD3^+$ cells), and T cell subsets ($CD4^+$, $CD8^+$, and $CD3^+CD4^-CD8^-$ double negative (DN) cells) per thoracic aorta in $SGK1^{fl/fl}$ and $SGK1^{fl/fl}tg^{CD4cre}$ mice after 21 days of DOCA-salt or vehicle (Sham) treatment (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, two-way ANOVA/Holm-Sidak's post hoc test; n = 8-15 per group). All data are expressed as mean ± SEM.

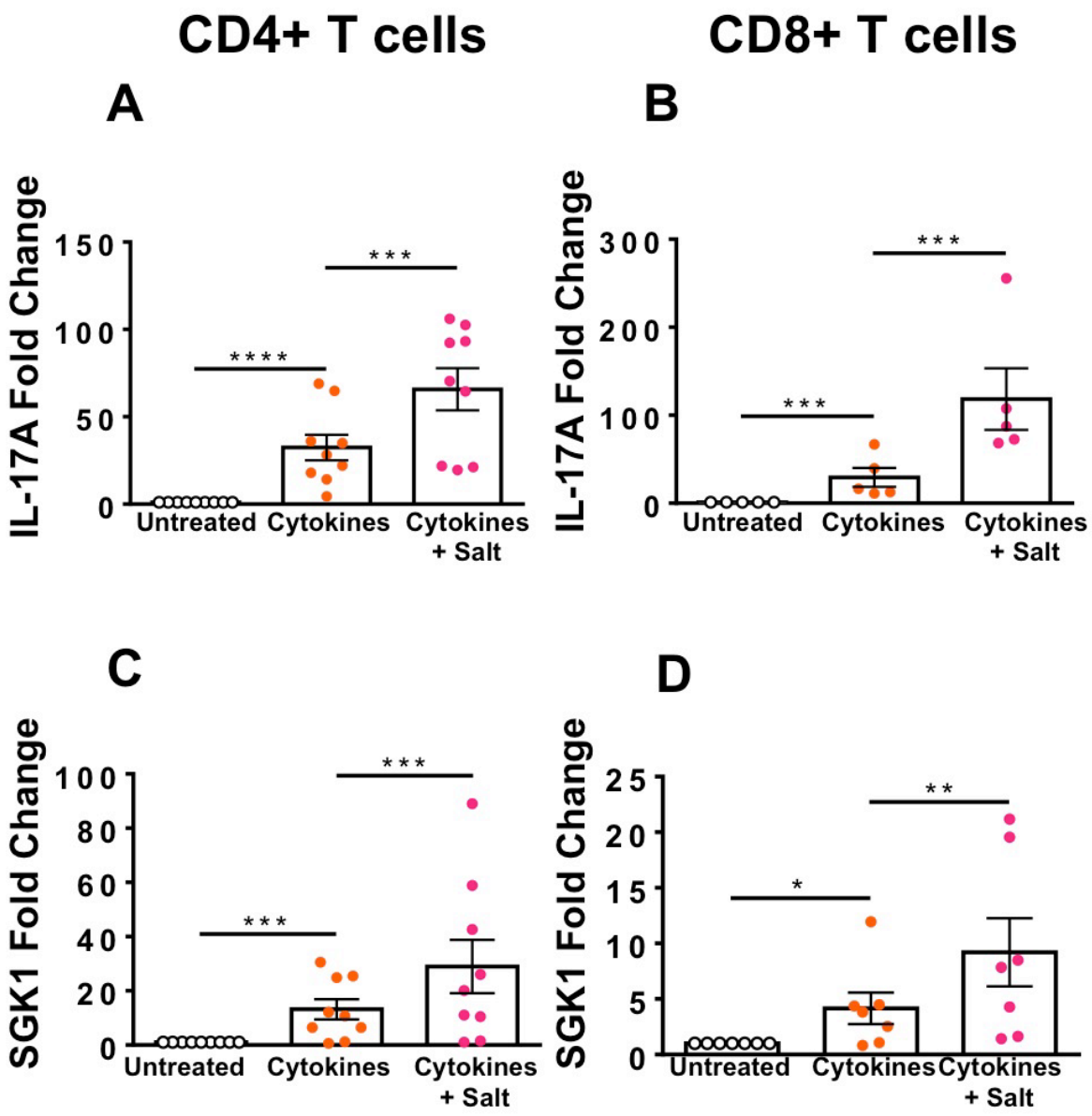


Figure 4-10. Salt enhances SGK1 and IL-17A expression in CD4$^+$ and CD8$^+$ T cells exposed to Th17 polarizing cytokines. Naïve splenic CD4$^+$ T cells **(A and C)** and CD8$^+$ T cells **(B and D)** were isolated from C57Bl/6J wild type (WT) mice and cultured for 72 hours on anti-CD3/anti-CD28 coated plates in the presence or absence of Th17 polarizing cytokines (IL-1β, IL-6, IL-23, TGF-β) (Cytokines) with or without an excess 40 mM NaCl (Salt) as indicated. IL-17A expression **(A and B)** and SGK1 expression **(C and D)** were quantified by qRT-PCR and expressed as fold change relative to untreated cells (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ratio-paired t-test between the indicated groups; n = 5-9 per group). All data are expressed as mean ± SEM.

Sodium Channels and Transporters Expressed by T cells
Epithelial Sodium Channel α,β,γ subunits (ENaC)
Sodium/Chloride Cotransporter (NCC)
Sodium/Calcium Exchanger 1 (NCX1)
Sodium/Calcium Exchanger 2 (NCX2)
Sodium/Hydrogen Exchanger 1 (NHE1)
Sodium/Hydrogen Exchanger 6 (NHE6)
Sodium/Potassium/2 Chloride Cotransporter 1 (NKCC1)
Voltage-gated Sodium Channel 5A (SCN5A)

Table 4-1. Sodium channels and transporters expressed by T cells. Pan $CD3^+$ T cells were isolated from spleens of C57Bl/6J wild type mice and RT-PCR was performed for the indicated sodium channels/transporters. Expression was not changed with infusion of Ang II.

Naïve $CD4^+$ T cells were cultured as above with Th17 polarizing cytokines or Th17 polarizing cytokines plus salt along with hydrochlorothiazide (inhibits NCC), spironolactone (inhibits the mineralocorticoid receptor), furosemide (inhibits NKCC1), or bumetanide (inhibits NKCC1). SGK1 expression was measured by qRT-PCR.

Interestingly, treatment with either furosemide or bumetanide abrogated the salt induced increase in T cell SGK1 expression, suggesting that NKCC1 plays a critical role in the ability of T cells to sense salt, while the other agents had no effect (**Figure 4-11A**). We then quantified NKCC1 expression by qRT-PCR in naïve $CD4^+$ T cells that were untreated or treated with Th17 polarizing cytokines ± salt. NKCC1 was significantly upregulated in $CD4^+$ T cells cultured under Th17 polarizing conditions with a modest further increase when salt was added to the culture media (**Figure 4-11B**). Taken together, NKCC1 appears to be induced in Th17 cells and mediate the salt induced increase in T cell SGK1 expression.

Discussion

Hypertension is a major health concern worldwide, and its prevalence has been compounded by the high salt content of modern processed foods. Among patients with hypertension who report taking prescribed medications, only two-thirds have their blood pressure under control [108]. Moreover, even in patients with reasonable blood pressure control, hypertension is associated with an elevated risk of cardiovascular events [116, 117] presumably due to an unaddressed inflammatory process. But how does salt and inflammation converge to cause hypertension and end-organ damage? Here we show that the salt-sensing kinase, SGK1, in T lymphocytes is critical to the development of hypertension in response to two different hypertensive stimuli – angiotensin II infusion and DOCA-salt treatment.

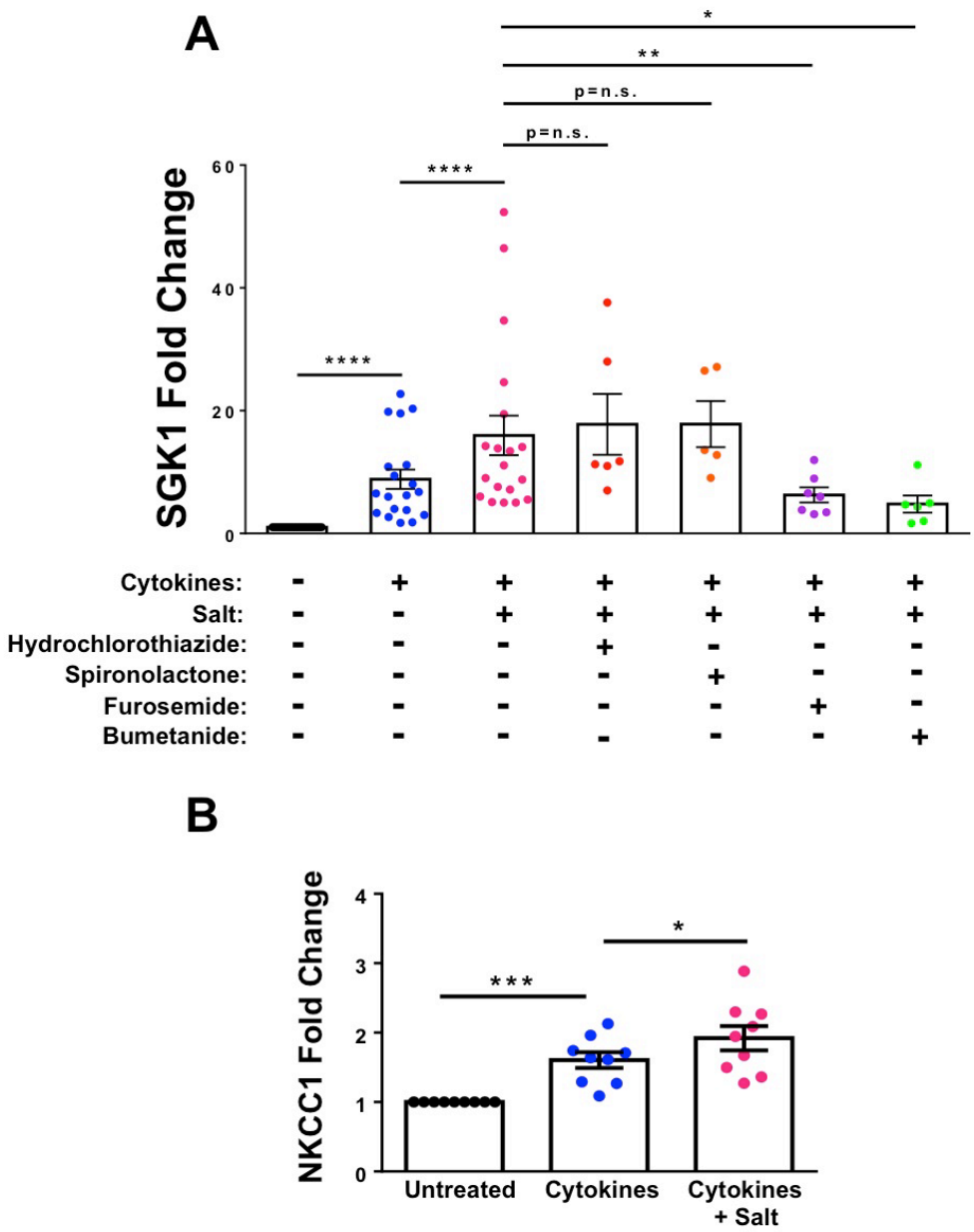


Figure 4-11. NKCC1 is upregulated in Th17 cells and mediates the salt-induced increase in SGK1. (A) Naïve splenic $CD4^+$ T cells were isolated from C57Bl/6J wild type (WT) mice and cultured for 72 hours on anti-CD3/anti-CD28 coated plates in the presence or absence of Th17 polarizing cytokines (Cytokines), an excess 40 mM NaCl (Salt), and co-treatment with DMSO or ethanol (vehicle) or hydrochlorothiazide, spironolactone, furosemide or bumetanide as indicated. SGK1 expression was quantified by qRT-PCR and expressed as fold change relative to untreated cells (*P<0.05, **P<0.01, ****P<0.0001; ratio-paired t-test between the indicated groups with Bonferroni correction for the drug treatment groups; n = 19 in the first three groups and n = 5-7 in each drug treatment group). **(B)** Naïve splenic $CD4^+$ T cells from WT mice were cultured as described above and sodium-potassium-2 chloride co-transporter 1 (NKCC1) expression was quantified by qRT-PCR and expressed as fold change relative to untreated cells (*P<0.05, ***P<0.001; ratio-paired t-test between the indicated groups; n = 9 per group). Bars represent mean ± SEM.

Furthermore, we show that NKCC1 is upregulated in Th17 cells and mediates the T cell response to salt. While loss of T cell SGK1 resulted in a significant reduction in the blood pressure response to both stimuli, it should be noted that blood pressure was still elevated above normal in both models. However, despite this persistent mild elevation in blood pressure, it is remarkable that vascular and renal inflammation and vascular and renal injury were virtually completely abolished by the loss of SGK1 in T cells. This highlights the need to possibly redefine treatment goals in hypertension to focus on reducing the inflammatory substrate that leads to end-organ damage rather than just focusing on the blood pressure itself.

SGK1 is well known for its role in modulating sodium transport in renal epithelial cells of the distal convoluted tubule through upregulation of ENaC and NCC [118]. Recently, Wu et. al and Kleinewietfeld et. al described a novel role for SGK1 in the stability and maintenance of Th17 cells by phosphorylation and inactivation of FoxO1, a direct repressor of the interleukin 23 (IL-23) receptor. IL-23 signaling in Th17 cells maintains the Th17 cell phenotype [81, 82]. Hernandez et al. and Safa et al. subsequently showed that SGK1 inhibits T regulatory (Treg) cells at least in part by phosphorylating and inhibiting both FoxO1 and FoxO3, which are important in regulating Foxp3 expression. Foxp3 is the signature transcription factor required for Treg function [119, 120]. Treg cells have been shown to suppress Ang II induced hypertension and vascular injury [77]. Thus, the mechanism by which loss of T cell SGK1 protects against hypertension and end-organ damage is likely through a shift in the Th17/Treg balance. Indeed, we observed a reduction in percentage of Th17 cells in the spleen following Ang II infusion in our T cell SGK1 deficient mice compared to $SGK1^{fl/fl}$ controls (**Figure 4-7B**).

Salt has also recently been shown to influence antigen-presenting cells such as macrophages. Salt boosts the activation of classical, lipopolysaccharide-induced M1 macrophages and blunts the

activation of alternative M2 macrophages [121, 122]. Zhang et al. described a specific activation state of macrophages induced by high salt which they termed M (Na). This state is characterized by enhanced expression of pro-inflammatory genes and suppressed expression of anti-inflammatory genes [123]. Kirabo et al. demonstrated that dendritic cells activate T cells in hypertension through presentation of isoketal-modified peptides [34]. Of note, the cytokines released by these activated dendritic cells are Th17 polarizing cytokines (IL-6, IL-1β, and IL-23). Thus, in addition to a direct effect of salt on T cells, salt induced activation of T cells in vivo could be potentiated through enhanced activation of antigen presenting cells. Nevertheless, our results demonstrate that deleting SGK1 in T cells alone is sufficient to protect against hypertension and more importantly, against the renal and vascular damage that accompanies hypertension.

An important result of our study is that loss of T cell SGK1 was protective in a salt-independent Ang II model of hypertension. There are at least two possible explanations for this. First, Ang II would be predicted to increase aldosterone, which would then lead to sodium and water retention in the kidney. Indeed, we have shown that animals retain sodium and water after Ang II infusion [59]. Recent work by Titze and colleagues has challenged conventional paradigms of salt homeostasis by demonstrating that animals and humans can store sodium in their skin, muscle, and lymphatics at concentrations exceeding that of plasma without a commensurate increase in water [124]. Thus, Ang II infusion may be associated with increased tissue levels of sodium. Alternatively, Ang II may directly or indirectly increase T cell SGK1 expression or function. We did not detect an increase in T cell SGK1 expression when we added Ang II to our T cell culture media (data not shown). However, this does not exclude an effect of Ang II on T cell SGK1 expression or function in vivo. Of note, Ang II has been shown to increase SGK1 expression and

phosphorylation in other cell types [125, 126]. Moreover, other signals associated with hypertension such as endothelin, glucocorticoids, oxidative stress, and certain cytokines have been shown to increase SGK1 transcription in various contexts [127]. Thus, T cell SGK1 may be a final common pathway by which multiple stimuli cause T cell activation in hypertension.

Interestingly, T cell SGK1 deficiency was associated with a dramatic absence of T cell infiltration into the kidney and vasculature in response to hypertension. One potential possibility for this is that SGK1 affects chemokine receptors on T cells. However, we performed qRT-PCR screening for a number of different chemokine receptors on T cells from $SGK1^{fl/fl}$ controls and $SGK1^{fl/fl}tg^{CD4cre}$ mice following Ang II infusion and did not detect any difference in chemokine receptor expression (data not shown). We and others have shown that IL-17A can induce chemokine expression in mouse mesangial cells and human aortic smooth muscle cells [33, 128]. These chemokines are involved in the recruitment of T cells and monocytes. Thus, through an overall reduction in IL-17A production, loss of T cell SGK1 may be associated with reduced chemokine expression and thus attenuated inflammatory cell recruitment into these tissues. Another intriguing possibility is that SGK1 is involved in T cell migration. Using furosemide and bumetanide, we showed that ion transport through NKCC1 mediates SGK1 upregulation in high salt conditions. Kochl et al recently demonstrated that With No Lysine 1 (WNK1) kinase regulates T cell migration through an NKCC1 dependent pathway [129]. The authors showed that NKCC1 deficiency or treatment with bumetanide reduced CCL21 induced migration of $CD4^+$ T cells. Further studies are needed to determine whether NKCC1 mediated T cell migration may involve signaling through SGK1.

The finding that furosemide and bumetanide inhibit salt-induced T cell SGK1 expression suggests that these commonly used diuretics may actually have a previously unrecognized anti-

inflammatory effect. It is interesting to speculate that these drugs not only help to excrete sodium, but that they may also block the effect of dietary salt on T cells, shifting the balance from a Th17 to a Treg phenotype. Future studies are needed to determine the role of T cell NKCC1 in blood pressure regulation and whether blocking NKCC1 specifically in T lymphocytes would have a beneficial effect on hypertension and hypertensive end-organ damage.

One limitation of this study is that we deleted SGK1 primarily in $CD4^+$ and $CD8^+$ T cells and only a subset of γδ T cells. We previously showed that γδ T cells are a major source of IL-17A in the kidney and vasculature in Ang II induced hypertension [111]. The role of SGK1 and/or salt on γδT cell function is unknown. Nevertheless, we did see a major effect on hypertension and renal/vascular inflammation in our model, suggesting that loss of SGK1 in primarily $CD4^+$ and $CD8^+$ T cells is sufficient to confer protection from hypertensive stimuli. Another limitation is that it is unclear how the excess salt concentrations that we and others have used in vitro correlates with tissue sodium concentrations in vivo in hypertensive animals and humans. It is difficult to quantify tissue sodium in animals, although one study by Wiig et al using energy dispersive X-ray spectrometry/scanning electron microscopy (EDX-SEM) demonstrated that sodium concentrations in the lymph capillaries of DOCA-salt treated rats reached levels comparable to what we used in cell culture [114]. In humans, ^{23}Na magnetic resonance imaging has demonstrated an increase in skin and muscle sodium with aging and hypertension, but due to the methodology used to quantify tissue sodium, a direct correlation with cell culture sodium concentrations is not possible [114].

In conclusion, we have demonstrated that the salt-sensing kinase SGK1 in T lymphocytes plays a critical role in regulating blood pressure and more importantly, renal and vascular inflammation and injury in experimental models of hypertension. Moreover, NKCC1 in T cells may play an important role in how T cells sense and respond to elevated sodium chloride in the extracellular environment. Future studies into how much and where we store sodium and how other signals in addition to sodium activate T cell SGK1 will lead to a deeper understanding of the interplay between salt, inflammation, and hypertension and lead to novel therapeutic targets for this widespread disease.

CHAPTER 5

Conclusions and Future Directions

Synopsis

Hypertension has become a worldwide health concern, and is an important contributor to cardiovascular morbidity and mortality [4, 130]. Specifically, renal and vascular dysfunction, which are both causes and consequences of hypertension, can lead to the development of complications that include myocardial infarction and chronic kidney disease, among others [131]. Throughout the past decade, researchers have intensely focused on uncovering the role inflammation and the immune system play in the development and maintenance of hypertension. Our laboratory and others have determined that both T cells and dendritic cells are important mediators of hypertension, as animals develop a blunted hypertensive response when deficient in either of these cell types [29, 34, 38]. Our laboratory previously demonstrated that deficiency of IL-17A, produced by CD4+ Th17 cells as well as gamma delta (γδ) T cells, results in blunted hypertension and preserved vascular function [33]. However, little was known regarding the actions of IL-17A in the kidney. Epidemiological studies have also associated excess dietary salt intake with the development of hypertension but the precise mechanism by which salt promotes hypertension was poorly understood. Interestingly, recent studies have linked excess salt to pathogenic IL-17A production by Th17 cells [81, 82] via a serum and glucocorticoid-regulated kinase 1 (SGK1) dependent pathway. Moreover, SGK1, a salt-sensing kinase, has also been shown to regulate salt and water retention in the kidney through increased surface expression of distal sodium transporters such as the sodium chloride co-transporter (NCC) and the epithelial sodium channel (ENaC) [81, 82, 132]. Furthermore, a recent study evaluated polymorphisms located in SGK1 in a cohort of hypertensive humans who had completed a dietary salt protocol.

Rao et al. found that two different single nucleotide polymorphisms (SNPs) in SGK1 were associated with elevated blood pressure in humans on a high salt diet; suggesting genotypic analysis of SGK1 in humans could identify individuals prone to salt-sensitive hypertension, connecting SGK1 to human hypertension [133]. Therefore, the overall goal of this dissertation was to determine the role of the SGK1-IL-17A axis in T lymphocytes and the kidney in regulating sodium transport and hypertension. I made the following key observations:

1. IL-17A, produced by Th17 cells and locally by tubule cells, acts through SGK1 on proximal tubule cells to enhance sodium/hydrogen exchanger 3 (NHE3) expression and acts through SGK1 on distal tubule cells to enhance sodium/chloride cotransporter (NCC) activity resulting in enhanced sodium and water retention (**Chapter 3**).
2. T cell SGK1 is an important mediator of salt dependent and independent hypertension. Deficiency of T cell SGK1 results in blunted blood pressure, abrogated vascular/renal inflammation, and preserved vascular/renal function. Sodium/Potassium/2 Chloride Cotransporter 1 (NKCC1) mediates the excess salt induced activation of SGK1 in Th17 cells (**Chapter 4**).

Synthesis of Inflammatory Pathways in Hypertension

As a whole, this thesis adds to the ever increasing body of evidence linking the immune system to the development of hypertension by discovering mechanisms through which salt and IL-17A act to promote hypertension and by identifying biomarkers in human monocytes (**Appendix A**) that confirm the importance of inflammasome activation in hypertensive human subjects. We now believe that inflammation begins as a result of disruption of the Renin-Angiotensin-Aldosterone System (RAAS). Traditionally, renin, a proteolytic enzyme, is released into

circulation by the juxtaglomerular cells of the kidney in response to a decrease in renal perfusion pressure. Renin acts on angiotensinogen, a hormone present in circulation, converting it to angiotensin I. Subsequently, angiotensin converting enzyme (ACE), present in the lungs and kidney, converts angiotensin I to angiotensin II. Angiotensin II has many direct effects that result in increased blood pressure; these include increasing sympathetic activity, promoting vasoconstriction, promoting tubular salt and water reabsorption, and increasing levels of reactive oxygen species (ROS), among others [134, 135]. Importantly, angiotensin II also promotes secretion of aldosterone from adrenal glands. Aldosterone directly acts to enhance salt and water reabsorption in the kidney. Enhanced activation of this system thus results in hypertension by increasing systemic vascular resistance and promoting salt and water reabsorption [136, 137]. We hypothesize that excess sodium is stored in tissues such as the skin and skeletal muscle, and increased salt leads to the activation of NADPH oxidase in dendritic cells. Dendritic cells (DCs) then produce even more ROS. This leads to the formation of isoketals which adduct to lysine residues on proteins in the DCs themselves. DCs are then able to present these isoketal-adducted proteins to T cells resulting in their activation [34, 51]. Importantly, ROS also activates the NLRP3 inflammasome in DCs [138]. Activation of the inflammasome leads to production of the cytokines IL-18 and IL-1β. IL-1β, in combination with IL-6 and TGF-β also secreted by DCs, promotes differentiation of T cells into IL-17A producing Th17 cells [19, 139, 140]. Th17 cells differentiated in areas with elevated interstitial sodium produce increased amounts of IL-17A [81, 82, 141]. IL-17A then further promotes vascular dysfunction by causing impaired vasodilation, vascular stiffness and increased inflammation [109]. IL-17A also causes renal dysfunction and injury by promoting enhanced expression/activity of renal sodium transporters

and by inducing glomerular damage (**Chapter 3**). Thus, IL-17A through induction of vascular and renal dysfunction, contributes to worsening of hypertension (**Figure 5-1**).

Outstanding Questions/Future Directions

Why do inflammatory cytokines alter renal transporter expression and/or activity?

The results described in **Chapter 3** contribute to a newer body of evidence demonstrating the ability of pro-inflammatory cytokines to alter the abundance and activity of renal sodium transporters. We found that IL-17A acts on distal tubule cells to enhance NCC and epithelial sodium channel (ENaC) abundance after chronic (4 weeks) angiotensin II infusion. In a previous paper, we showed that IL-17A acts on proximal tubule cells to enhance NHE3 expression after 2 weeks of angiotensin II infusion [71]. We then verified the actions of IL-17A on both distal tubule NCC and proximal tubule NHE3 using cultured cell lines. Together, these papers suggest that IL-17A biphasically regulates renal sodium channel expression during hypertension, impacting proximal tubule channels first and later enhancing expression of distal channels. However, IL-17A is not the only pro-inflammatory cytokine able to alter renal sodium channel function. In our previous paper, we were also able to demonstrate that IFN-γ regulates the abundance of NHE3, NCC and NKCC2 [71]. Additionally, Zhang et al identified a pathway through which IL-1β signaling in renal myeloid cells decreases nitric oxide production within the kidney resulting in enhanced sodium reabsorption through NKCC2 [32]. Moreover, IL-6 has been shown to regulate ENaC in cultured cortical collecting duct cells and also angiotensinogen, which is converted to angiotensin II by the intrarenal renin-angiotensin system and can then act to regulate local sodium channels, in cultured proximal tubule cells [66, 142]. All of these studies beg the same important questions; specifically, how and why are these cytokines altering renal

transporter expression and functionality? Our findings have shed some light on the mechanisms behind the effects of IL-17A on renal sodium transporter expression. However, further studies are needed to identify the precise mechanisms by which IL-17A acts to activate SGK1 in the proximal tubule, and also if SGK1's actions resulting in enhanced NHE3 expression after IL-17A treatment are mediated by any of the sodium/hydrogen exchanger regulatory cofactor (NHERF) scaffolding proteins. Future studies can also investigate the importance of locally produced IL-17A by the renal tubule cells themselves on basal control of channel expression. Broadly, understanding the mechanisms behind the actions of other cytokines (e.g. IL-1β, IFNγ, IL-6) on renal sodium transporter abundance and activity as well as identifying other cytokines that could contribute to regulation, such as the anti-inflammatory cytokine IL-10, is essential to dissecting the pathophysiology of hypertension and also for the identification of therapeutic targets. In regards to why pro-inflammatory cytokines have these functions, it is possible that these mechanisms evolved to help the body fight off infections. Studies have demonstrated that increased sodium helps to promote pathogenic functions of innate and adaptive immune cells [81, 82, 120, 123]. Thus, the immune system may have evolved a way, through pro-inflammatory cytokine activation of renal transporters, to boost its own functions during times of need, in a feed-forward fashion, to help ward off pathogens. However, activation of the immune system in response to oxidative stress or autoantigens that results in hypertension and other autoimmune diseases may lead to inadvertent and chronic enhanced sodium reabsorption that contributes to exacerbation of disease pathology.

Where do T cells see elevated sodium concentrations and how do they sense it?

Increased dietary salt intake and also activation of renal sodium transporters by immune cell produced cytokines are two methods by which our bodies can, in theory, increase their sodium

stores, and importantly, both methods have been linked to hypertension [71, 143]. If worsening of hypertension is indeed due to activation and proliferation of cells of the immune system in locations where sodium concentrations reach levels of 190mmol/L, the amount determined by Wu et al. and Kleineweitfeld et al. that induces the most IL-17A production by Th17 cells, where are T cells chronically seeing such high levels of sodium [81, 82]? The answer may lie in recent research by Dr. Jens Titze and colleagues whose group has demonstrated that both animals and humans can store elevated concentrations of sodium in their skin, muscle and lymphatics. Interestingly, storage of sodium in these areas occurred without a commensurate increase in water [124]. The prior dogma was that water and sodium must be stored together in such a way as to maintain equilibrium between plasma and extracellular tissue sodium concentration. While ^{23}Na MRI is currently being used in select sites to quantify tissue sodium in humans, it has proven more difficult to quantify tissue sodium in animals. One method, however, energy dispersive X-ray spectrometry/scanning electron microscopy (EDX-SEM), is promising and was performed by Wiig et al in a study involving DOCA-salt treated rats; additionally, Titze and colleagues commonly ash mice to determine organ sodium content [114]. Currently, our laboratory is attempting to optimize ^{23}Na MRI on anesthetized mice in order to visualize locations of elevated sodium content. Understanding where and how much sodium T cells encounter during hypertension is important to designing specialized therapeutics to combat the inflammatory aspect of this disease.

In addition to understanding *where* T cells see excess sodium, it is necessary to understand *how* T cells sense excess sodium when exposed to it. Our findings demonstrate that Th17 cells, in particular, sense and respond to excess sodium through the sodium channel NKCC1. These data have significant implications as the commonly prescribed diuretics, bumetanide and furosemide,

which both inhibit NKCC1 and NKCC2, may be exerting an anti-inflammatory effect. Our data also revealed that T cells isolated from spleens of mice express at least 8 different sodium channels/subunits or transporters at baseline. Thus, it will be essential in future studies to determine which T cell subsets express which channels/transporters; and whether salt, via influx through these or other channels, alters T cell function in hypertension. Additionally, it is crucial to extrapolate this concept to other immune cell types. For example, do dendritic cells, macrophages, monocytes, B cells, etc. express sodium channels? Are they functional? Are we also inhibiting the actions of these cell types by administering diuretics to patients? All of these questions are important to answer if we want to truly understand the pathogenesis of hypertension and how salt and inflammation converge to worsen the disease.

Significance of IL-17A inhibition as a potential anti-hypertensive treatment: an alternative or addition to diuretics?

In **Chapter 3** we put forth evidence demonstrating that global deletion of IL-17A blunts the development of albuminuria, a marker of renal injury, in response to 4 weeks of angiotensin II infusion in comparison to WT mice. As stated in the synopsis, global deletion of IL-17A was also linked previously by our laboratory to preservation of vascular function and blunted blood pressure responses to 4 weeks of angiotensin II infusion [33]. Together, these papers suggest that treatment of sustained hypertension with monoclonal antibodies to either IL-17A or its receptor, IL-17RA, would result in the lowering of blood pressure and preservation of the current state of functionality of the kidneys and the vasculature. This hypothesis was supported by data in a paper we recently published. We demonstrated that administration of IL-17A or IL-17RA monoclonal antibodies to mice during weeks 3 and 4 of a 4 week administration of angiotensin II resulted in a reduction of blood pressure and levels of renal/vascular inflammation and renal

albuminuria compared to mice administered control IgG monoclonal antibody [68]. Thus, it is possible that IL-17A monoclonal antibodies as a treatment for hypertension could replace the use of diuretics, as we suggest in our published discussion from **Chapter 3**, as they combat inflammation in addition to preserving renal function. However, this theory was called into question due to evidence discovered later which was described in **Chapter 4** and discussed in relation to salt in the previous section. As stated, furosemide and bumetanide actually act to block the excess salt induced pathogenic phenotype of Th17 cells and thereby inhibit an aspect of the underlying inflammatory condition in hypertension. Thus, future studies in this area would explore the actions of monoclonal antibodies and diuretics in combination to battle established hypertension. It is possible that treating mice for the second two weeks of a 4 week angiotensin II experiment with both diuretics and anti-IL-17A or anti-IL-17RA monoclonal antibodies would result in an additive effect and lower blood pressure even more. Or, they would act in conjunction to provide long term and stable preservation of kidney function lowering the risk of development of chronic kidney disease. Or, we would discover that one works better than the other after directly comparing animals treated with diuretics versus animals treated with anti-IL-17A or anti-IL-17RA antibodies during the same experiment. Thus, it is important to understand how these drugs would work together and if there is benefit for prescribing both or one versus the other, as many patients with uncontrolled hypertension take more than 3 different drugs to combat their blood pressure and the fewer they need the better. With that said, currently, two drugs Secukinumab (which binds IL-17A selectively and is FDA approved) and Brodulamab (which selectively binds the IL-17A receptor and is currently recommended for approval by an FDA advisory panel) act on the IL-17A pathway and are used to treat psoriasis patients. Future studies can employ the treatment of hypertensive patients with these drugs in addition to or in

replacement of their current drug regimen to determine if the effects seen in mice extend to humans, if they do, that would imply that treatment with anti-IL-17A or anti-IL-17RA monoclonal antibodies is a therapeutic option for the general population battling the disease.

Contribution of SGK1 in other cell types to hypertensive development

SGK1 is a salt sensing kinase, however, it also responds to numerous other stimuli including mineralocorticoids, oxidative stress and cytokines [127]. Oxidative stress, cytokines, and aldosterone, a mineralocorticoid, have all been linked to hypertension and stimulate or activate multiple types of immune cells. Our model, in which we deleted SGK1 in T cells by crossing SGK1 floxed mice with mice expressing cre recombinase under the control of the CD4 promotor, deleted SGK1 in most CD4+ and CD8+ T cells and a subset of γδ T cells. We found that this deletion blunts blood pressure, abrogates renal/vascular inflammation, and preserves renal/vascular function. If deleting SGK1 in just one component of immune cells results in such a drastic attenuation of disease, it stands to reason that a molecule that is activated by so many things could contribute to worsening of disease through its activation in other immune cell types, and therefore is worth investigating. First, as stated in **Chapter 4** in detail, it is necessary to explore deletion of SGK1 in all γδ T cells, as they are such an avid producer of IL-17A during hypertension. Additionally, some dendritic cells express CD4 on their cell surfaces [144, 145]. As such, we have effectively deleted SGK1 in a subset of dendritic cells. To date we have not classified the importance of this subset of dendritic cells to the development of hypertension in our model. Still, we may have impacted the functionality of a subset of dendritic cells, potentially impacting their ability to activate the adaptive immune response in this disease. In relation, salt boosts the activation of M1 macrophages while suppressing the activation of M2

macrophages [121, 122]. Thus, it stands to reason that salt may affect the functionality of dendritic cells as well since macrophages and dendritic cells can both differentiate from a common progenitor, the monocyte. Moreover, Kirabo et al. described the excess production of reactive oxygen species by dendritic cells isolated from mice infused with angiotensin II [34]. As SGK1 is stimulated by both of these substances, active dendritic cells may need functional SGK1 in order to uptake or present antigen, or to migrate appropriately to activate T cells, without these cells possessing functional SGK1, the disease may be attenuated. Thus, the combination of altering T cell and some dendritic cell function may explain why the reduction in inflammation in our model in response to angiotensin II and DOCA-salt was so dramatic. Ongoing and future studies will therefore explore the functionality of $CD4^+$ SGK1 deficient dendritic cells in hypertension and their responsiveness to salt and oxidative stress.

Conclusion

Taken together, the studies described in this thesis have advanced our knowledge of the impact Th17 cells, their cytokine IL-17A, and their interaction with salt have on the pathophysiology of hypertension (**Figure 5-1**). It has become clear that the effects of IL-17A on hypertensive end-organ damage is multi-factorial, and adds further evidence to support the idea that hypertension itself is an autoimmune disease. Our findings also helped to decipher why salt, in excess, is detrimental to people with hypertension, as it is able to promote the response of pathogenic Th17 cells. Ultimately, these studies further back the importance of evaluating anti-IL-17A monoclonal antibodies as therapy for hypertension and also have identified SGK1 in T cells as a novel target for hypertensive therapeutics.

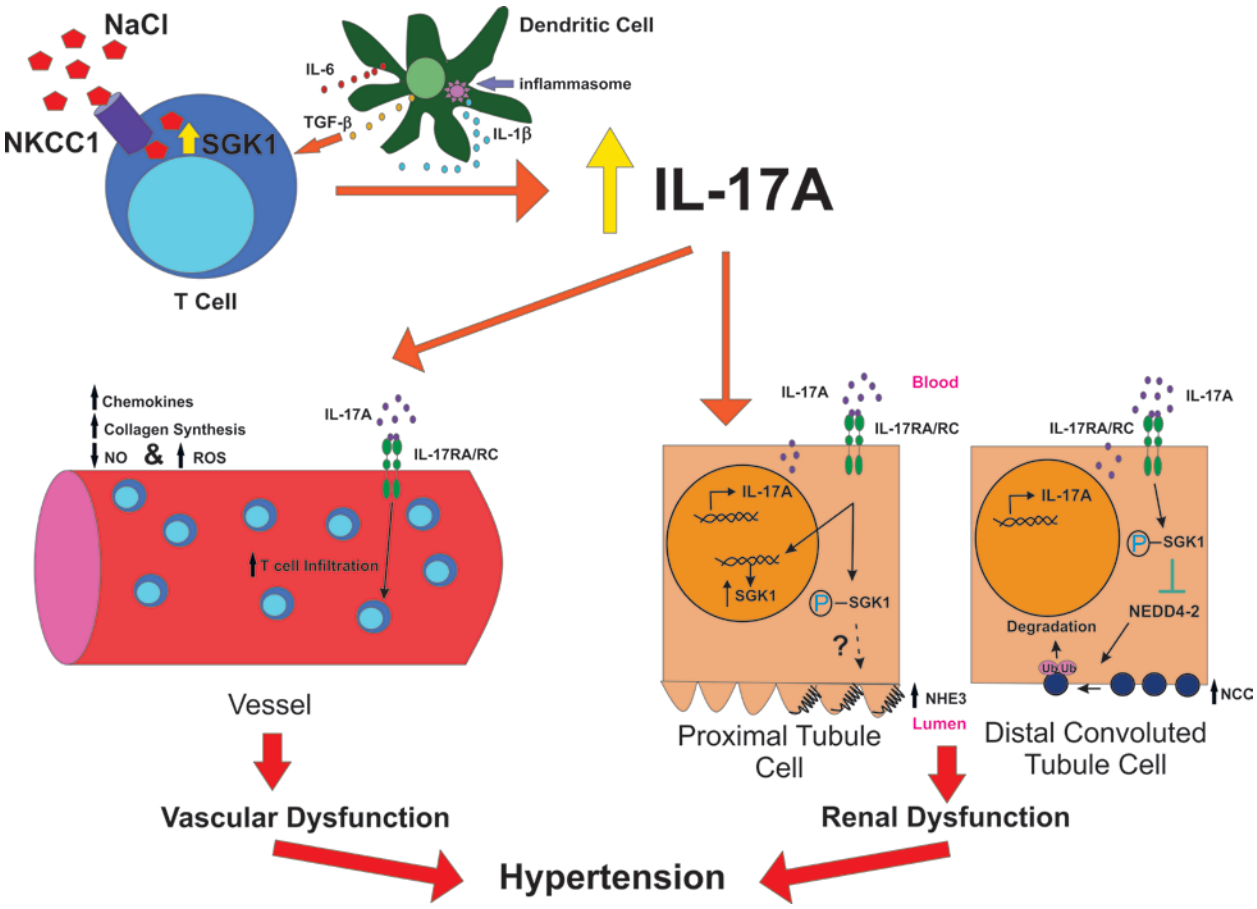


Figure 5-1. Thesis Summary Schematic. Salt flows into T cells resulting in upregulation of SGK1 and increased production of IL-17A. IL-17A acts on the kidneys and vasculature resulting in end-organ damage/dysfunction and hypertension.

Appendix A

Human monocyte transcriptional profiling identifies an inflammatory gene signature in hypertension

Allison E. Norlander[1]*, Cristi L. Galindo[2]*, Fernando Elijovich[3], Cheryl L. Laffer[3], Juan S. Gnecco[4], and Meena S. Madhur[1,3]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, USA. [2]Division of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, TN, USA. [3]Division of Clinical Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA. [4]Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, TN, USA.

*These authors contributed equally to this work

Introduction

Hypertension is a leading cause of death from myocardial infarction, stroke, heart failure, and chronic kidney disease. Emerging evidence from our group and others indicates that hypertension is an inflammatory disease in which antigen presenting cells, particularly monocyte-derived dendritic cells, present neo-antigens to T lymphocytes which then leads to T cell activation, infiltration of target organs, and the production of pro-inflammatory cytokines that cause sodium and water retention, vascular dysfunction, and renal injury [29, 34, 109, 110, 146].

Circulating monocytes are pluripotent cells capable of differentiating into dendritic cells, macrophages, and microglial cells based on environmental cues. Monocytes can also present antigen without changing phenotype. Monocytes from patients with hypertension secrete more pro-inflammatory cytokines (including interleukin 1β, IL-1β)[147, 148], demonstrate enhanced migration [149], exhibit increased adherence to endothelial cells[150], and produce more reactive oxygen species [150]. However, these prior studies used monocytes from patients that were either untreated or temporarily stopped treatment. Therefore, an important question is whether monocytes from treated hypertensive individuals are still dysfunctional and whether correcting this dysfunction would lead to improved blood pressure and cardiovascular risk reduction. Interestingly, Kirabo et al. [34] recently demonstrated that monocytes from treated hypertensive patients exhibit higher levels of isoketals, which are oxidatively modified fatty acids that can adduct to proteins, potentially leading to the formation of neo-antigens.

To understand more thoroughly the genes and pathways that are altered in hypertensive monocytes, we performed RNA sequencing of the entire monocyte transcriptome from 5

normotensive and 7 treated hypertensive individuals. We identified genes that were differentially expressed between the two groups and validated several of them in an independent cohort of 6 normotensive and 9 hypertensive subjects. The identified transcriptomic signature of hypertensive monocytes suggests enhanced inflammasome activity in hypertension. Importantly, the identified transcripts could potentially serve as biomarkers for disease severity and as novel therapeutic targets.

Methods

Patients: Whole blood (40 ml) was obtained from 5 normotensive and 7 hypertensive subjects recruited at Vanderbilt for the original cohort. One normotensive subject was sampled on 2 separate days to obtain a technical replicate that could be used to test the internal consistency of the RNAseq assay. An additional 6 normotensive and 9 hypertensive subjects were recruited for the validation cohort. Hypertension was defined as a systolic blood pressure greater than 140 mmHg, a diastolic blood pressure greater than 90 mmHg, or treatment with antihypertensive agents, regardless of blood pressure. Exclusion criteria were confirmed or suspected secondary causes of hypertension, diabetes mellitus type I or II, concomitant illness requiring corticosteroids or immunosuppressants, recent (within 3 months) vaccination against any infectious agent, active ongoing malignancy, severe psychiatric disorders, or the presence of HIV/AIDS. Informed consent was obtained prior to the blood draw. The protocol was approved by the Vanderbilt Institutional Review Board.

Monocyte Isolation and RNA extraction: Whole blood was mixed 1:1 with cold PBS + EDTA. The mixture was layered on top of Ficoll (GE life sceinces) and centrifuged for 30 minutes. The ‘buffy coat’ layer was isolated and washed 3 times with cold PBS + EDTA. The isolated

peripheral blood mononuclear cells (PBMCs) were stained using Monocyte Isolation kit II, human (Miltenyi) and the monocytes were negatively selected using an AutoMACs cell separator. Monocytes were spun down and RNA was extracted using an RNeasy kit (Qiagen), which included a DNaseI treatment step. The purity and quantity of RNA samples were measured using a Denovix DS-11 Spectrophotometer or a NanoDrop ND-1000 Spectrophotometer.

RNA Sequencing: cDNA library construction and RNA sequencing was performed by VANTAGE (Vanderbilt Technologies for Advanced Genomics). cDNA Library preparation was accomplished using the Illumina TruSeq stranded mRNA Sample Preparation Kit, and Rev. D of the protocol. Samples were sequenced on the Illumina HiSeq 2500 using v3 SBS chemistry. Libraries were sequenced on a Single Read 50bp run at 30 million passing filter reads/sample. Details of RNA Sequencing protocols are available on: http://vantage.vanderbilt.edu/resources/grant-text/.

RNA Sequencing Analysis: A total of 13 samples were obtained from 12 patients (their characteristics are in **Table A-1**). Based on post-alignment quality assurance and control (QA/QC), all samples were of high fidelity; i.e., Phred quality score = 37.00 ± 0.31, indicating a high probability that the bases were accurately identified. The Phred score = $-10*\log_{10}$ (Probability of Error). For all 13 samples, the base call accuracy was estimated to be > 99.9%. For statistical analyses, samples were separated into two groups: hypertensive (n = 7) and normotensive (n = 5). Two samples were collected for one of the 5 normotensive subjects (technical replicates), obtained on separate days, allowing us to perform two separate sets of analyses to increase the robustness of the data and reduce false positives due to RNASeq technical variation.

	Normotensive Controls (n=5)	Hypertensive Patients (n=7)	p value
Age (years)	47.9±6.8	50.4±2.7	
Sex (F/M)	4/1	7/0	
Race (W/B)	5/0	6/1	
Duration of HTN (years)	NA	8.9±4.8	
Systolic BP (mmHg)	112.6±7.2	136.7±5.7	<0.02
Diastolic BP (mmHg)	64.8±4.8	78.5±5.7	<0.06
MAP (mmHg)	80.7±5.0	97.9±5.0	<0.03
BMI (kg/m^2)	26.9±3.9	33.4±3.7	
Antihypertensive Drugs:			
ACEi		3	
ARB		2	
ACEi + TZ + CCB		1	
CCB		1	

Table A-1: Demographics table for original cohort: n = number of subjects in each group, HTN = hypertension, BP = blood pressure, MAP = mean arterial pressure, BMI = body mass index. ACEi=angiotensin converting enzyme inhibitors; ARB=angiotensin AT1 receptor blockers; TZ=thiazide diuretics; CCB=dihydropyridine calcium channel blockers. Significant and borderline results for the unpaired t-test and chi-square comparisons between groups are in the rightmost column. Lack of a value indicates lack of statistical significance.

Analysis 1 included all hypertensive and normotensive subjects (true biological replicates), with the normotensive replicated subject represented by the first sample. Analysis 2 was identical to Analysis 1, except the second sample from that subject was used. While we were interested in obtaining a global view of transcriptional differences between hypertensive and normotensive subjects, we especially wanted to identify transcripts with the highest likelihood of being true and reproducible findings. For this purpose, we employed 12 separate methodologies (6 each for Analysis 1 and 2), using various combinations of alignment, quantification, statistical, and annotation strategies. As shown in **Table A-2**, this strict analysis yielded a total of 60 transcripts that were identified as significant (multiple hypothesis-corrected p value < 0.05; fold difference > 1.5).

Quantitative RT-PCR: Monocyte RNA was converted to cDNA using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). The cDNA was then probed for the following genes; all probes were purchased from Taqman: Arg1, CCL4, MMP8, VSIG4, PGLYRP1 and IL18RAP. Results were normalized to GAPDH and subsequently normalized to 1 of the normotensive patients. The data are plotted as Relative Quantification values.

Statistics: Statistical analysis for RNAseq was performed using Partek Flow, with the following parameters: Alignment: STAR and TopHat2/Bowtie2; Quantification: Cufflinks and P/E (Partek's optimization of the expectation-maximization algorithm [151]); Statistics: Cuffdiff, GSA, and ANOVA; Annotations: RefSeq and Aceview. Stepwise multivariate regression analyses were carried out to identify genes, the expression of which ($\log_{10}$ of normalized RPKM; reads per kilobase per million mapped reads) could be a determinant of blood pressure. Univariate correlation analysis was used for hypothesis generation.

Alignment	Quant	Stats	Annot	Analysis Set 1	Analysis Set 2	Total (1 OR 2)	Common (1 AND 2)
				Number RNA entities with p<0.05, FD > 1.5			
STAR	Partek E/M	GSA	RefSeq	624	651	810	465
			Aceview	1,790	1,670	2,280	1,180
		ANOVA	RefSeq	525	549	698	376
			Aceview	1,643	1,535	2,104	1,074
Bowtie2 + TopHat2	Cufflinks	CuffDiff	RefSeq	1,155	1,115	1,659	611
			Aceview	962	944	1,316	590
All genes identified using STAR				2,051	1,956	2,620	1,387
All genes identified using TopHat2				1,763	1,717	2,569	911
All differential genes				3,457	3,332	4,757	2,032
Genes commonly altered *in every* STAR analysis				289	290	376	**204**
Genes commonly altered *in every* TopHat2 analysis				333	342	381	**294**
Final Stringent Gene List				88	80	108	**60**

Table A-2: Overview of Statistical Results: Analysis Sets 1 and 2 are defined in more detail in the Supplementary methods section. Quant = Quantification, Stats = Statistics, Annot = Annotation

If the expression of a gene correlated with MAP within one group of subjects (either controls or hypertensive patients) or within both, the gene was considered a candidate regressor and included in the multivariate analysis. In contrast, if the expression of a gene only correlated with MAP in all subjects analyzed together, it was not included as a hypothetical regressor because it was assumed that such correlations reflect dichotomization of subjects by both blood pressure status and level of gene expression. RT-PCR data are represented as mean±SEM. Comparisons of characteristics between the normotensive and hypertensive groups were done with one-tailed Student's t-Tests if data were normally distributed, with one-tailed Mann-Whitney tests if data were not normally distributed, or with chi-squares for categorical variables. A p-value <0.05 was considered significant.

Results

RNASeq analysis: The demographics of the 5 normotensive and 7 hypertensive subjects in the original cohort are given in **Table A-1.** The hypertensive patients had higher blood pressures despite treatment and slightly higher (albeit not statistically significant) body mass indices (BMI). The strict combinatorial analytical approach described in Methods (see online supplement) yielded a total of 60 transcripts in the monocytes isolated from these subjects that were identified as significantly differentially expressed after multiple-hypothesis correction (p value < 0.05; fold difference > 1.5) between normotensive and hypertensive groups (**Table A-2**). These 60 transcripts along with their fold-difference and p-values are shown in **Table A-3**. A positive fold-difference indicates the gene is upregulated in hypertensive individuals, and a negative fold-difference indicates the gene is downregulated in hypertensive individuals.

Symbol	Gene Name	p value	FD
ABCA13	ATP-binding cassette, sub-family A (ABC1), member 13	7.0×10^{-03}	48.2
SLPI	secretory leukocyte peptidase inhibitor	1.0×10^{-02}	18.1
TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	7.2×10^{-03}	12.7
DEFA4	defensin, alpha 4, corticostatin	1.7×10^{-02}	11.3
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	8.8×10^{-03}	11.2
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	1.2×10^{-02}	10.9
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	1.2×10^{-02}	10.5
LCN2	lipocalin 2	8.2×10^{-03}	10.1
MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	4.1×10^{-03}	10.0
CCL4	chemokine (C-C motif) ligand 4	8.4×10^{-03}	9.6
ATP2C2	ATPase, Ca++ transporting, type 2C, member 2	1.2×10^{-02}	9.3
MKI67	marker of proliferation Ki-67	1.2×10^{-02}	9.0
ARG1	arginase 1	8.6×10^{-03}	8.7
LTF	lactotransferrin	5.0×10^{-03}	8.6
HJURP	Holliday junction recognition protein	1.1×10^{-02}	8.4
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	1.4×10^{-02}	8.3
PGLYRP1	peptidoglycan recognition protein 1	6.1×10^{-03}	8.2
RPL9	ribosomal protein L9	1.3×10^{-03}	8.2
CAMP	cathelicidin antimicrobial peptide	3.7×10^{-03}	7.5
RORA	RAR-related orphan receptor A	1.1×10^{-02}	7.5
CRISP3	cysteine-rich secretory protein 3	7.4×10^{-03}	6.6
FAM20A	family with sequence similarity 20, member A	2.7×10^{-03}	6.3
PDZD4	PDZ domain containing 4	1.9×10^{-03}	6.0
SAMD3	sterile alpha motif domain containing 3	2.4×10^{-03}	5.9
IL18RAP	interleukin 18 receptor accessory protein	6.5×10^{-03}	5.7
SH2D1A	SH2 domain containing 1A	8.4×10^{-03}	5.5
PTGDR	prostaglandin D2 receptor (DP)	3.4×10^{-03}	5.4
CD247	CD247 molecule	1.4×10^{-02}	5.4
GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX)	3.7×10^{-02}	5.3
PTGES	prostaglandin E synthase	1.3×10^{-02}	4.8
AFAP1	actin filament associated protein 1	1.6×10^{-03}	4.6
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	1.7×10^{-02}	4.4
HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	7.9×10^{-03}	4.3
NKG7	natural killer cell granule protein 7	1.6×10^{-02}	4.1
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa	6.8×10^{-03}	4.1

	gelatinase, 92kDa type IV collagenase)		
CCL3	chemokine (C-C motif) ligand 3	8.8×10^{-03}	4.0
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	5.5×10^{-03}	3.8
TNF	tumor necrosis factor	3.2×10^{-03}	3.1
ANKRD33B	ankyrin repeat domain 33B	1.5×10^{-03}	2.4
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	6.0×10^{-03}	2.3
MMRN1	multimerin 1	1.6×10^{-02}	2.3
CXCR1	chemokine (C-X-C motif) receptor 1	1.9×10^{-02}	2.3
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.7×10^{-03}	2.2
CPNE5	copine V	1.6×10^{-02}	2.0
CETP	cholesteryl ester transfer protein, plasma	2.5×10^{-02}	2.0
FCHO2	FCH domain only 2	1.2×10^{-04}	1.9
MS4A4A	membrane-spanning 4-domains, subfamily A, member 4A	2.5×10^{-03}	1.8
MCTP2	multiple C2 domains, transmembrane 2	1.0×10^{-03}	1.8
JAK3	Janus kinase 3	1.0×10^{-03}	1.7
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	9.8×10^{-03}	1.6
GPRIN3	GPRIN family member 3	9.0×10^{-03}	1.6
FHL1	four and a half LIM domains 1	5.5×10^{-03}	1.6
CR1	complement component (3b/4b) receptor 1 (Knops blood group)	2.2×10^{-02}	1.5
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	1.3×10^{-02}	-1.6
PID1	phosphotyrosine interaction domain containing 1	9.9×10^{-03}	-1.6
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	3.0×10^{-03}	-1.6
FLT3	fms-related tyrosine kinase 3	1.6×10^{-02}	-1.7
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	2.0×10^{-02}	-1.8
VSIG4	V-set and immunoglobulin domain containing 4	5.4×10^{-03}	-1.9
ACCS	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)	4.0×10^{-03}	-2.1

Table A-3: List of the 60 Significant Genes and their fold-change: P value = average p value across 12 analyses, including Cuffdiff with multiple hypothesis correction. FD = average fold-difference across all 12 analyses, after conversion of Cuffdiff log2 values to fold-difference values. A positive fold-difference indicates the gene is upregulated in hypertensive individuals and a negative fold-difference indicates the gene is downregulated in hypertensive individuals.

Hierarchical clustering (**Figure A-1A**) and Principal Components Analysis (PCA, **Figure A-1B**) of the 60 most robust genes demonstrated that the combined expression profile was sufficient to differentiate hypertensive from normotensive subjects.To identify known and predicted protein-protein interactions among the 60 significantly altered genes, we employed the online network analysis tool, STRING v10 [152, 153], which yielded a large and multi-connected network of differentially expressed genes and inferred transcriptional regulators (**Figure A-1C**).

Upstream regulatory analysis using Ingenuity Pathway Analysis (IPA) inferred activation of NF-κB, IL-1β, and the adaptor protein myeloid differentiation primary response 88 (MYD88). These three molecules were predicted to be activated and are all part of the IL-1β signaling pathway that culminates in regulation of 21 of the 60 differentially expressed genes identified by RNASeq (F**igure A-2**). We also employed IPA to identify non-biased and significantly enriched functions in these 60 genes. The most statistically enriched disorder was "Inflammatory Response", with a *p* value range of 5.4×10^{-4} to 4.6×10^{-15} (**Table A-4**), which essentially indicates a very low probability that this functional category would be represented by a significant portion of 60 randomly selected genes. Based on causal prediction analysis, which uses individual relationships curated from the literature to infer functional directionality from transcriptional directionality (i.e., up- or down-regulation of each functionally related gene), the functions associated with inflammatory responses were activated (Z score= 2.595 to 2.943). Enriched molecular and cellular functions include immune cell migration and signaling, phagocytosis, proliferation of vascular smooth muscle cells, and T cell development (**Table A-4**).

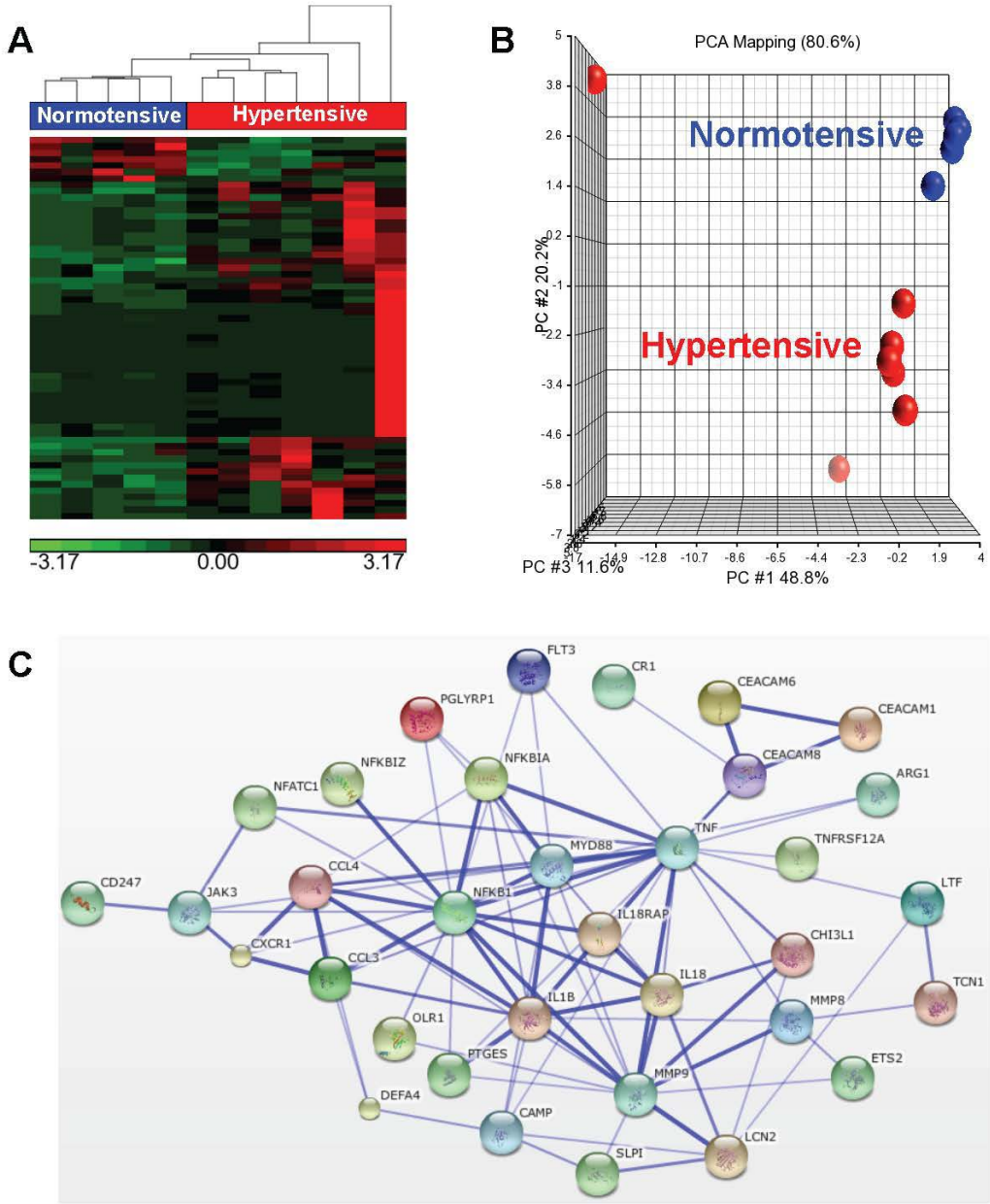


Figure A-1: Genes from normotensive and hypertensive monocytes are different by RNAseq Analysis: (**A)** Hierarchical clustering of normalized RPKM (reads per kilobase per million mapped reads) for those genes that were identified as significantly differentially expressed using RNASeq**.** Intensity of red or green is used to show fold increase or decrease, respectively. Columns represent individual samples, and each row represents one gene. **(B)** Principal components analysis (PCA) of the 60 significant genes. Three components were sufficient to describe 80.6% of the variability among samples (48.8%, 20.2%, and 11.6% on the X, Y, and Z axes, respectively). **(C)** Protein network produced using the online analysis tool STRING v10. Analysis included the 60 significantly altered transcripts and most significant predicted regulators. Unconnected entities were removed, resulting in the network shown. Thickness of lines represent level of confidence (thicker = higher confidence).

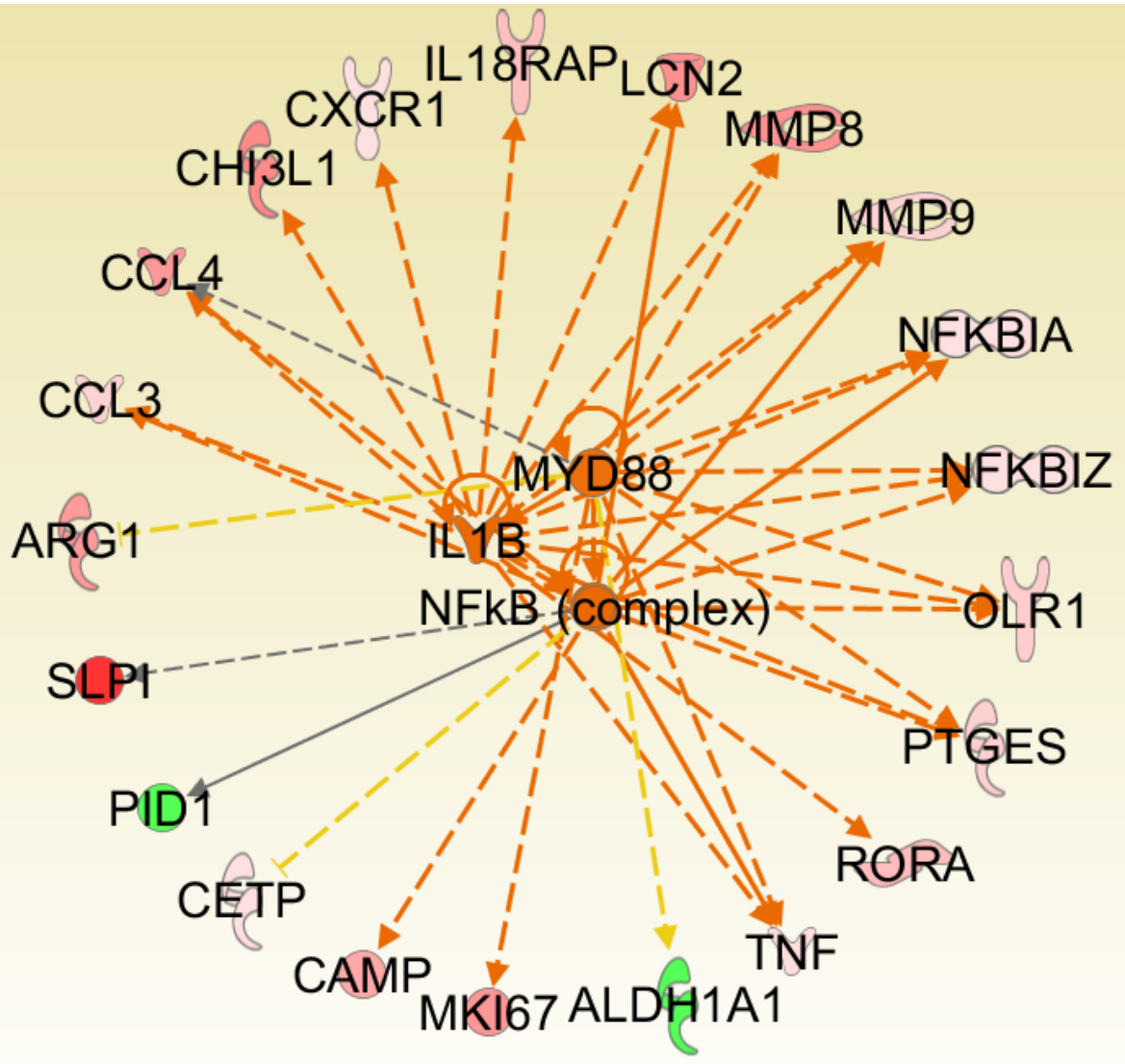


Figure A-2. Functional Protein Network: Functional network generated from upstream regulator analysis using Ingenuity Pathway Analysis (IPA) software. The three central proteins were predicted to be activate using IPA (p values = 1.5 X 10^{-11} to 1.5 X 10^{-9}, Z scores = 2.9 to 3.9). Peripheral genes are overlaid with RNASeq expression values. Red or green color represents up- or down-regulation, respectively. Intensity of the color represents the degree of change. Orange lines with arrows indicate that directionality of the downstream gene is consistent with predicted activation of the up-stream regulator. Yellow lines indicate inconsistent results between gene expression and IPA's knowledge base for that particular interaction. Gray lines indicate that directionality cannot be resolved.

Top Diseases and Bio Functions	Enrichment		Predicted Activation	
	p value	No.	*Z score	Direction
DISEASES AND DISORDERS				
Inflammatory Response	$5.4 \times 10^{-4} – 4.6 \times 10^{-15}$	33	NA	
Activation of leukocytes	4.6×10^{-15}	22	2.943	Activated
Inflammatory response	4.2×10^{-13}	21	2.670	Activated
Activation of myeloid cells	7.5×10^{-13}	15	2.685	Activated
Activation of phagocytes	2.5×10^{-12}	15	2.569	Activated
Activation of macrophages	2.0×10^{-11}	12	2.595	Activated
MOLECULAR AND CELLULAR FUNCTIONS				
Cellular Movement	$5.8 \times 10^{-4} – 4.4 \times 10^{-16}$	32	NA	
Cell movement of mononuclear leukocytes	6.5×10^{-12}	16	2.975	Activated
Cell movement of lymphocytes	9.7×10^{-11}	14	2.802	Activated
Migration of cells	1.0×10^{-10}	28	2.668	Activated
Cell movement of monocytes	5.9×10^{-8}	8	2.740	Activated
Migration of mononuclear leukocytes	7.0×10^{-8}	11	2.823	Activated
Cell-cell Signaling and Interaction	$5.5 \times 10^{-4} – 4.6 \times 10^{-16}$	30	NA	
Cellular Function and Maintenance	$5.5 \times 10^{-4} – 4.0 \times 10^{-10}$	28	NA	
Engulfment of cells	1.7×10^{-6}	10	2.649	Activated
Phagocytosis of cells	3.7×10^{-5}	7	2.050	Activated
Cellular Development	$5.7 \times 10^{-4} – 1.5 \times 10^{-9}$	29		
Proliferation of smooth muscle cells	5.7×10^{-5}	7	2.006	Activated
Proliferation of vascular smooth muscle cells	1.4×10^{-4}	5	2.173	Activated
Development of lymphocytes	2.2×10^{-4}	9	2.264	Activated
T cell development	5.5×10^{-4}	8	2.609	Activated
PHYSIOLOGICAL SYSTEM DEVELOPMENT AND FUNCTION				
Immune Cell Trafficking	$5.6 \times 10^{-4} – 4.4 \times 10^{-16}$	30	NA	
Hematological Dev/Function	$5.8 \times 10^{-4} – 2.7 \times 10^{-15}$	31	NA	
Tissue Morphology	$5.8 \times 10^{-4} – 8.4 \times 10^{-10}$	27	NA	
Quantity of CD4+ T-lymphocytes	1.2×10^{-5}	6	2.200	Activated

Table A-4: Functional Analysis of Significant Genes: Main functional categories shown in bold, Sub-categories are italicized. *Positive and negative Z scores of >2.0 are generally considered as significant for activation and inhibition, respectively.

Regression Analysis of Gene Determinants of Blood Pressure: The expression (log_{10} normalized RPKM; reads per kilobase per million mapped reads) of 15 genes (ABCA13, ARG1, cAMP, CEACAM1, CEACAM8, CHI3L1, CRISP3, LCN2, LTF, MMP8, OLR1, PGLYRP1, PTGES, SLPI, and TCN1) correlated significantly with mean arterial pressure (MAP) within the group of normotensive controls (r=0.91 to 0.99) and among all patients together (r=0.65 to 0.79), whereas that of three additional genes (FAM20A, MMP9 and FCER1A) correlated significantly within the controls only (r=0.89 to 0.97). By multivariate regression, only LTF and PGLYRP1 remained significant predictors of MAP variability in normotensive controls.

The expression of 4 genes (CD247, GZMH, PDZD4 and PTGDR) correlated significantly with MAP within the hypertensive subjects (r=0.77 to 0.88) and among all patients together (r=0.70 to 0.80). By multivariate regression, the only significant predictor of MAP variability in hypertensive subjects was GZMH.

The expression of IL18RAP correlated significantly with MAP of controls (r=0.93), hypertensive patients (r=0.78) and of all subjects analyzed together (0.89). By multivariate regression, the expression of this gene was the only significant determinant of MAP in all subjects.

The univariate correlations for the four significant genes in multivariate analyses are shown in **Figure A-3**. The corresponding models for MAP based on expression of these genes are shown in **Table A-5.**

Quantitative RT-PCR in the validation cohort: The demographics of these 6 normotensive and 9 hypertensive subjects are given in (**Table A-6**). Blood pressures and BMI were slightly but not significantly higher in hypertensives compared to normotensives.

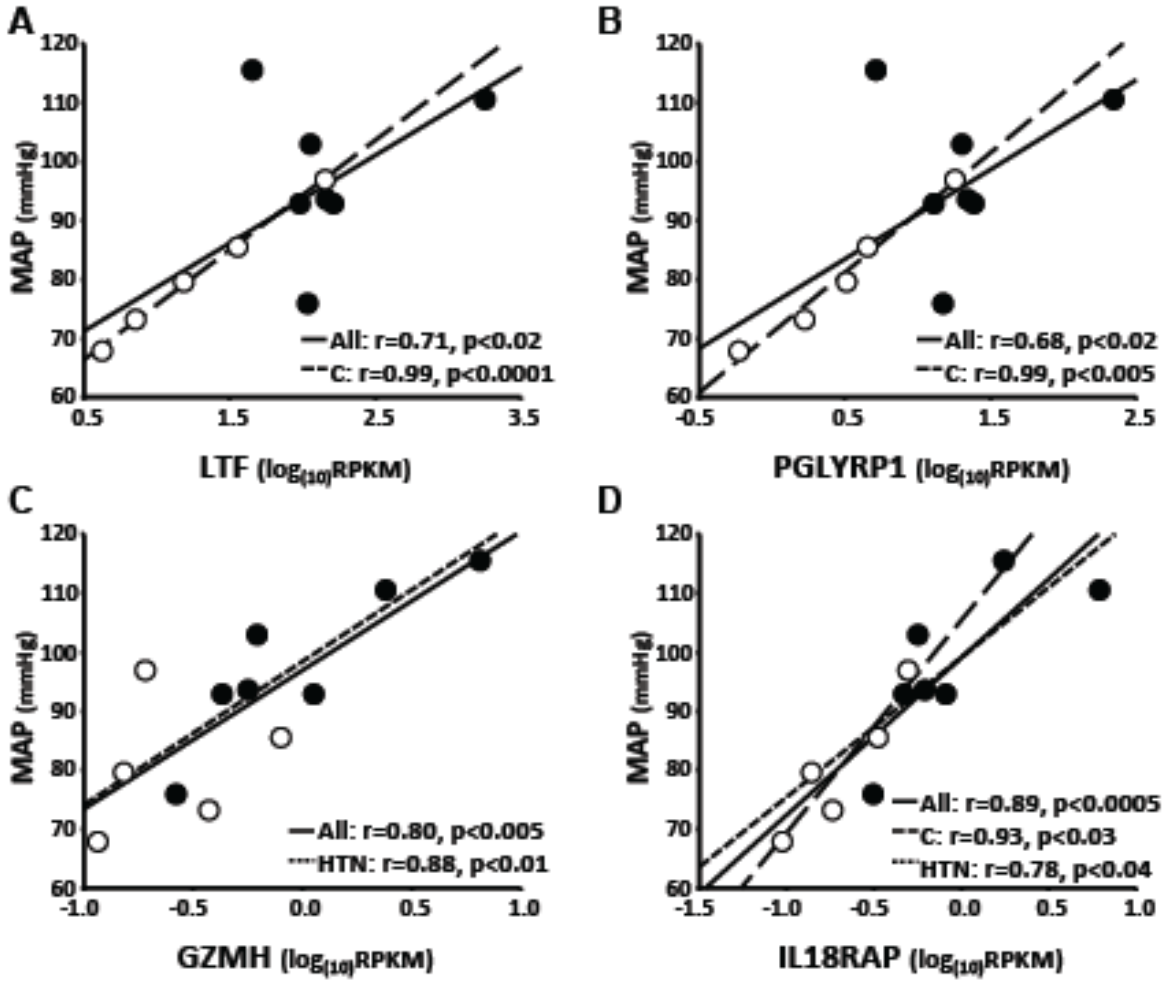


Figure A-3: Regression Analyses of MAP from Genes that were Significant Predictors: Univariate regressions of mean arterial pressure (MAP) on the expression ($log_{(10)}$RPKMs) of those genes that were significant predictors of MAP in multivariate regression analyses: **(A)** lactotransferrin, LTF, **(B)** peptidoglycan recognition protein 1, PGLYRP1, **(C)** granzyme H, GZMH and **(D)** interleukin 18 receptor accessory protein, IL18RAP. Control normotensive subjects are represented by open dots, and hypertensive patients are shown by closed dots. Pearson correlation coefficients and their significance are given for controls (C, n=5), hypertensive subjects (HTN, n=7) or all subjects analyzed together (All, n=12). $P<0.05$ was considered significant.

Gene	Group	Model	R^2	p-value
LTF and PGLYRP1	C	MAP = 60.01 (±0.41) + 14.60 (±0.53)*$\log_{10}$LTF + 4.54 (±0.59)*$\log_{10}$PGLYRP1	0.99	p<0.0001
GZMH	H	MAP = 98.45 (±2.60) + 24.28 (±5.86)*$\log_{10}$GZMH	0.77	p<0.009
IL18RAP	All	MAP = 99.15 (±2.50) + 26.75 (±4.42)*$\log_{10}$IL18RAP	0.79	p<0.0002

Table A-5: Multivariate Regression Models for MAP using expression levels of LTF, PGLYRP1, GZMH, and/or IL18RAP: C = normotensive controls, H = hypertensive patients, All = all subjects analyzed together

	Normotensive Controls (n=6)	Hypertensive Patients (n=9)	p value
Age (years)	42.2±6.3	50.4±3.2	
Sex (F/M)	3/3	7/2	
Race (W/B/A)	4/1/1	7/2/0	
Duration of HTN (years)	NA	15.0±3.8	
Systolic BP (mmHg)	128.0±5.5	140.4±8.9	
Diastolic BP (mmHg)	72.7±3.0	78.3±2.5	
MAP (mmHg)	91.1±3.1	99.0±4.3	
BMI (kg/m^2)	25.7±2.6	30.2±2.4	
Antihypertensive Drugs:			
None		1	
TZ + TMT		2	
CCB + α1B		1	
CCB + SP		1	
ACEi		2	
ACEi + TZ		1	
ARB + TZ + CCB		1	

Table A-6: Demographics Table for Validation Cohort: n = number of subjects in each group, HTN = hypertension, BP = blood pressure, MAP = mean arterial pressure, BMI = body mass index. TZ=thiazide diuretics; TMT=triamterene; CCB=dihydropyridine calcium channel blockers; α1B=peripheral alpha-1 adrenergic receptor blockers; SP=spironolactone; ACEi=angiotensin converting enzyme inhibitors; ARB=angiotensin AT1 receptor blockers. Unpaired t-test and chi-square comparisons were performed between groups. Lack of a p value in the rightmost column indicates lack of statistical significance.

Frequency and type of antihypertensive treatment was slightly different from the original cohort. We examined 8 of the 60 genes by quantitative RT-PCR (qPCR): 4 were the ones that we found to predict MAP in multivariate models (**Figure A-3**) and the other 4 were chosen due to known or predicted effects on inflammation (ARG1, CCL4, MMP8, and VSIG4). Six out of these 8 genes were significantly altered in the predicted direction (with GZMH showing a trend but not reaching statistical significance), demonstrating that our findings are robust and reproducible (**Figure A-4 and Figure A-5**).

Discussion

Despite conventional treatment, hypertension remains uncontrolled in nearly 50% of patients with the disease [108]. Even when blood pressure is relatively controlled, hypertensive patients are still at an elevated risk of cardiovascular events [116, 117]. This suggests that the risk of the hypertensive process may be due to factors other than blood pressure, which are insufficiently addressed by current pharmacological therapy. Our study suggests that altered monocyte function, reflected by altered gene expression, may be at least partly responsible for the residual cardiovascular risk in patients with hypertension. Using RNAseq, we identified 60 RNA transcripts that are differentially expressed between monocytes from normotensive versus treated hypertensive subjects. We chose 8 of these genes to validate by qPCR in a separate cohort of normotensive and hypertensive subjects. Remarkably, we were able to validate the predicted changes in 6 of the 8 genes tested despite the fact that the two cohorts differed in their anti-hypertensive medication profile. Multivariate regression analyses revealed that 2 genes (LTF and PGLYRP1) significantly correlated with MAP in control subjects and thus may be involved in blood pressure homeostasis. One gene, GZMH, significantly correlated with MAP only in hypertensive subjects and thus may be a marker of injury in hypertension.

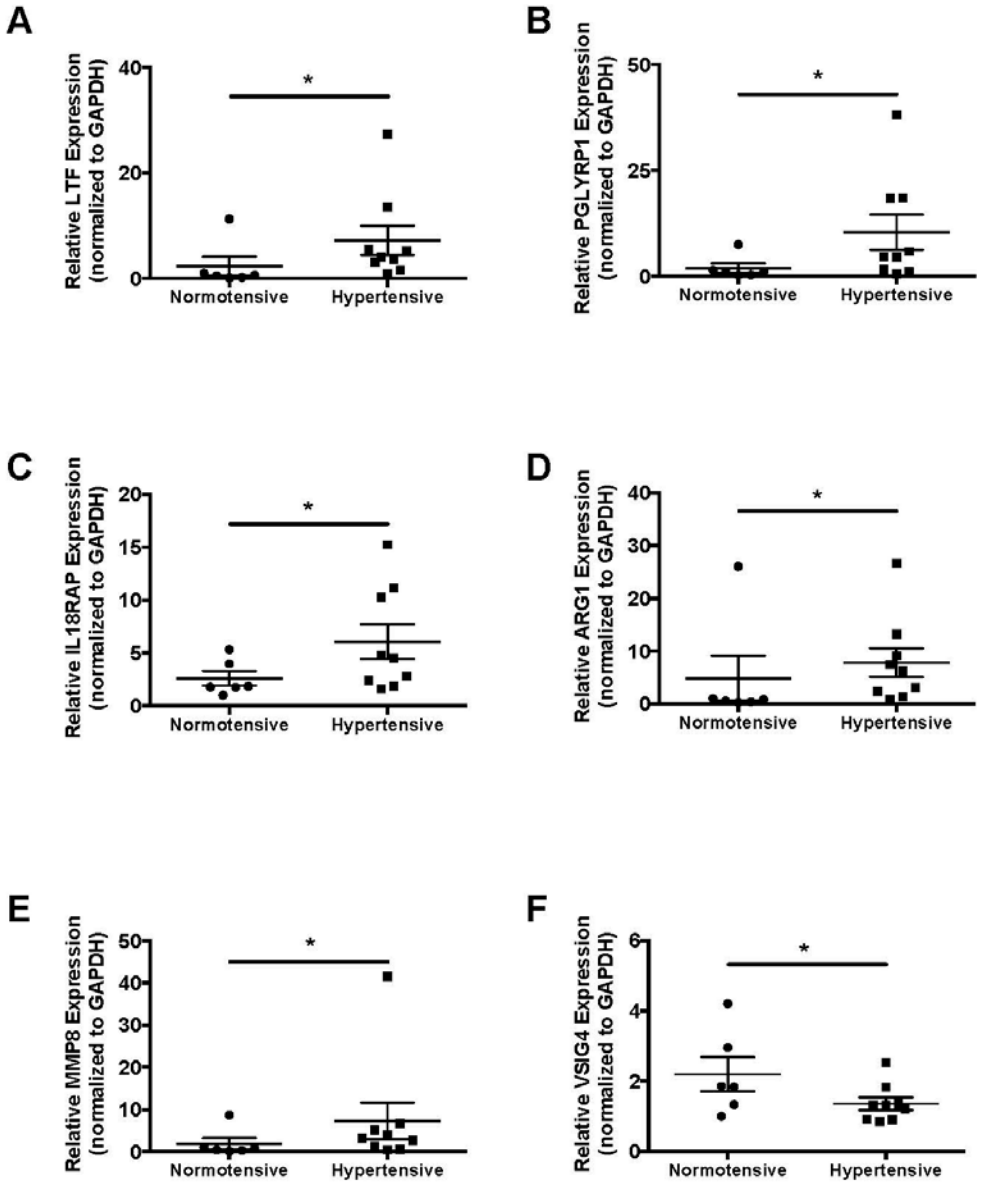


Figure A-4: RT-PCR for selected genes in the validation cohort: Relative PCR quantification (normalized to GAPDH) of **(A)** lactotransferrin, LTF **(B)** peptidoglycan recognition protein 1, PGLYRP1 **(C)** interleukin 18 receptor accessory protein, IL18RAP **(D)** arginase 1, ARG1 **(E)** matrix metalloproteinase 8, MMP8 and **(F)** V-set and Ig domain-containing 4, VSIG4 in a new cohort of normotensive (n=6) and hypertensive (n=9) patients. Data are plotted as mean±SEM. **P*<0.05.

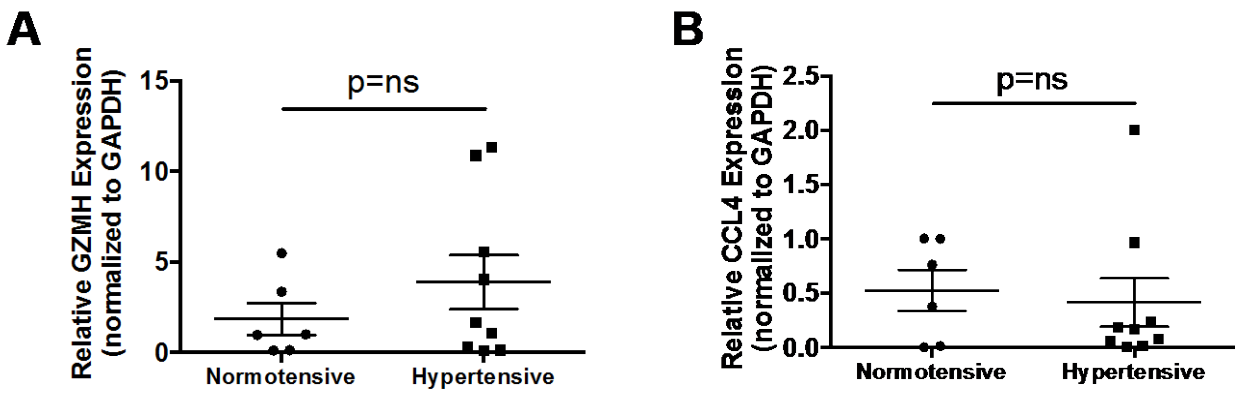
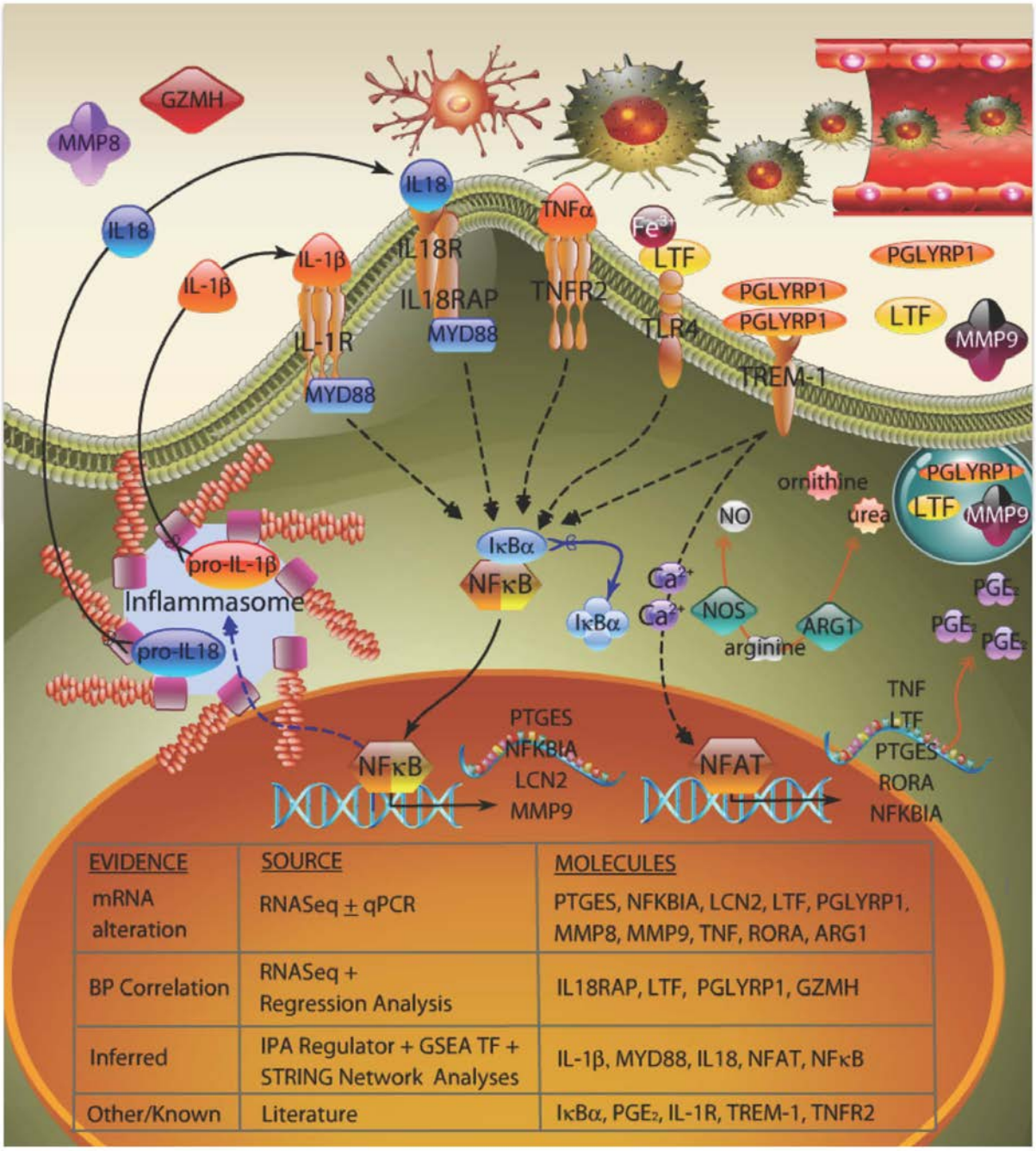


Figure A-5: Genes non-significant by RT-PCR in the validation cohort: Relative PCR quantification (normalized to GAPDH) of **(A)** granzyme H, GZMH and **(B)** chemokine (C-C motif) ligand 4, CCL4 in a new cohort of normotensive (n=6) and hypertensive (n=9) patients. Data are plotted as mean±SEM. **P*<0.05.

Of note, IL18RAP significantly correlated with MAP within normotensive and hypertensive subjects and in all subjects combined. We were able to develop a putative model for monocyte activation and signaling in hypertension using several of the molecules identified through RNAseq, regression analyses, qPCR validation, and inferred through systems biology approaches and the literature (**Figure A-6**).

Interestingly, 21 of the 60 genes identified are downstream of the IL-1β pathway. IL18RAP (also known as IL18Rα) binds to the IL18 receptor and enhances IL18 signaling [154]. Both IL-1β and IL18 are products of the inflammasome, a multimeric protein complex important in innate immunity (**Figure A-6)**. There is emerging evidence that the inflammasome and its products also play a critical role in hypertension [154, 155]. The inflammasome is activated by damage associated molecular pattern receptors (DAMPs) and pathogen associated molecular pattern receptors (PAMPs) that bind to toll-like receptors and signal through NFκB. The exact DAMP or PAMP that activates the inflammasome in hypertension is unknown. While one would speculate that a DAMP might contribute to immune activation in hypertension, our results suggest that the PAMP, peptidoglycan recognition protein 1 (PGLYRP1), might play an important role. PGLYRP1 binds to triggering receptor expressed on myeloid cells (TREM)-1 either as a complex with bacterial peptidoglycan or in the absence of bacterial peptidoglycan when it is multimerized [156]. Signaling through TREM-1 results in activation of NFκB and NFAT mediated transcription (**Figure 4**). Interestingly, PGLYRP1 has been implicated in reactive oxygen species (ROS) formation in neutrophils [157]. Since hypertensive monocytes express more isoketals, a by-product of oxidative injury [34], it is attractive to hypothesize that PGLYRP1 is upstream of ROS production in monocytes.



EVIDENCE	SOURCE	MOLECULES
mRNA alteration	RNASeq ± qPCR	PTGES, NFKBIA, LCN2, LTF, PGLYRP1, MMP8, MMP9, TNF, RORA, ARG1
BP Correlation	RNASeq + Regression Analysis	IL18RAP, LTF, PGLYRP1, GZMH
Inferred	IPA Regulator + GSEA TF + STRING Network Analyses	IL-1β, MYD88, IL18, NFAT, NFκB
Other/Known	Literature	IκBα, PGE_2, IL-1R, TREM-1, TNFR2

Figure A-6: Schematic of monocyte signaling pathway identified: Putative monocyte signaling pathway produced using ePath3D (Protein Lounge) based on study analysis results and review of the literature. Type of evidence and source for each molecule are listed in tabular form at the bottom of the figure.

Notably, Rohatgi et al [158] showed that increased PGLYRP1 levels are associated with all major cardiovascular risk factors (including hypertension), with inflammatory markers such as C-reactive protein and interleukin 18, and with coronary artery calcium, aortic wall thickness, and abdominal aortic plaque burden.

Other genes that we validated by qPCR or that we found to correlate with MAP in regression analyses that are particularly interesting include lactotransferrin (LTF), arginase 1 (ARG1) and V-set and immunoglobulin domain containing 4 (VSIG4). Recombinant human LTF induces maturation of human monocyte-derived dendritic cells, leading to up-regulation of HLA class II, CD83, CD80 and CD86 costimulatory molecules, and CXCR4 and CCR7 chemokine receptors [159]. ARG1 is involved in the metabolism of arginine to urea and ornithine which is involved in wound healing. Increased ARG1 activity theoretically depletes arginine and thus could reduce nitric oxide synthesis by the nitric oxide synthases (**Figure A-6**). While ARG1 is a marker of alternatively activated M2 macrophages in mice, this is not necessarily true in humans [160]. The role of ARG1 in hypertensive monocytes requires further study. VSIG4 is one of the few genes that was down-regulated in hypertensive monocytes. VSIG4 is a B7 family-related protein that has been shown to function as a negative regulator of T cell activation [161]. Thus, the downregulation of VSIG4 could therefore promote T cell activation and cytokine production in hypertension.

REFERENCES

1. Lionakis, N., et al., *Hypertension in the elderly.* World J Cardiol, 2012. **4**(5): p. 135-47.
2. Bromfield, S. and P. Muntner, *High blood pressure: the leading global burden of disease risk factor and the need for worldwide prevention programs.* Curr Hypertens Rep, 2013. **15**(3): p. 134-6.
3. WHO *A global brief on hypertension*. 2013.
4. Carretero, O.A. and S. Oparil, *Essential hypertension. Part I: definition and etiology.* Circulation, 2000. **101**(3): p. 329-35.
5. Fagard, R. and J. Staessen, *Relation of cardiac output at rest and during exercise to age in essential hypertension.* Am J Cardiol, 1991. **67**(7): p. 585-9.
6. Landsberg, L., *On Rounds: 1000 Internal Medicine Pearls, Clinical Aphorisms and Related Pathophysiology.* 2015.
7. Chade, A.R., *Renal vascular structure and rarefaction.* Compr Physiol, 2013. **3**(2): p. 817-31.
8. Klahr, S., et al., *The effects of dietary protein restriction and blood-pressure control on the progression of chronic renal disease. Modification of Diet in Renal Disease Study Group.* N Engl J Med, 1994. **330**(13): p. 877-84.
9. Guyton, A.C., *The relationship of cardiac output and arterial pressure control.* Circulation, 1981. **64**(6): p. 1079-88.
10. Guyton, A.C., *Abnormal renal function and autoregulation in essential hypertension.* Hypertension, 1991. **18**(5 Suppl): p. III49-53.
11. Folkow, B., *Physiological aspects of primary hypertension.* Physiol Rev, 1982. **62**(2): p. 347-504.
12. Greene, A.S., et al., *Microvascular rarefaction and tissue vascular resistance in hypertension.* Am J Physiol, 1989. **256**(1 Pt 2): p. H126-31.
13. Prewitt, R.L., Chen, II, and R. Dowell, *Development of microvascular rarefaction in the spontaneously hypertensive rat.* Am J Physiol, 1982. **243**(2): p. H243-51.
14. Mancia, G., et al., *Sympathetic activation in the pathogenesis of hypertension and progression of organ damage.* Hypertension, 1999. **34**(4 Pt 2): p. 724-8.
15. DiBona, G.F., *Nervous kidney. Interaction between renal sympathetic nerves and the renin-angiotensin system in the control of renal function.* Hypertension, 2000. **36**(6): p. 1083-8.
16. Norlander, A.E., Saleh, M.A., Kamat, N.V., Ko, B., Gnecco, J., Zhu, L., Dale, B., Iwakura, Y., Hoover, R.S., McDonough, A., Madhur, M.S., *Interleukin 17A promotes hypertension through induction of proximal and distal renal sodium transporters via an SGK1 dependent pathway.* Hypertension, 2016.
17. Saleh, M.A., et al., *Lymphocyte adaptor protein LNK deficiency exacerbates hypertension and end-organ inflammation.* J Clin Invest, 2015. **125**(3): p. 1189-202.
18. Amador, C.A., et al., *Spironolactone decreases DOCA-salt-induced organ damage by blocking the activation of T helper 17 and the downregulation of regulatory T lymphocytes.* Hypertension, 2014. **63**(4): p. 797-803.
19. Krishnan, S.M., et al., *Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice.* Br J Pharmacol, 2016. **173**(4): p. 752-65.

20. Sullivan, J.M., *Salt sensitivity. Definition, conception, methodology, and long-term issues.* Hypertension, 1991. **17**(1 Suppl): p. I61-8.
21. Itani, H.A., et al., *CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli.* Circ Res, 2016. **118**(8): p. 1233-43.
22. Okamoto, K. and K. Aoki, *Development of a strain of spontaneously hypertensive rats.* Jpn Circ J, 1963. **27**: p. 282-93.
23. Rapp, J.P., *Dahl salt-susceptible and salt-resistant rats. A review.* Hypertension, 1982. **4**(6): p. 753-63.
24. Okuda, T. and A. Grollman, *Passive transfer of autoimmune induced hypertension in the rat by lymph node cells.* Tex Rep Biol Med, 1967. **25**(2): p. 257-64.
25. White, F.N. and A. Grollman, *Autoimmune Factors Associated with Infarction of the Kidney.* Nephron, 1964. **1**: p. 93-102.
26. Olsen, F., *Inflammatory cellular reaction in hypertensive vascular disease in man.* Acta Pathol Microbiol Scand A, 1972. **80**(2): p. 253-6.
27. Svendsen, U.G., *The role of thymus for the development and prognosis of hypertension and hypertensive vascular disease in mice following renal infarction.* Acta Pathol Microbiol Scand A, 1976. **84**(3): p. 235-43.
28. Ba, D., et al., *Restoration of T cell depression and suppression of blood pressure in spontaneously hypertensive rats (SHR) by thymus grafts or thymus extracts.* J Immunol, 1982. **128**(3): p. 1211-6.
29. Wenzel, P., et al., *Lysozyme M-positive monocytes mediate angiotensin II-induced arterial hypertension and vascular dysfunction.* Circulation, 2011. **124**(12): p. 1370-81.
30. Wang AQ, S.A., Nussberger J, Ives A, Bagnoud N, Shaefer S, Tschopp J, Burnier M., *Renin-Dependent Hypertension in Mice Requires the NLRP3- Inflammasome.* Journal of Hypertension, 2014. **3**(6).
31. Bruder-Nascimento, T., et al., *NLRP3 Inflammasome Mediates Aldosterone-Induced Vascular Damage.* Circulation, 2016. **134**(23): p. 1866-1880.
32. Zhang, J., et al., *Interleukin-1 Receptor Activation Potentiates Salt Reabsorption in Angiotensin II-Induced Hypertension via the NKCC2 Co-transporter in the Nephron.* Cell Metab, 2016. **23**(2): p. 360-8.
33. Madhur, M.S., et al., *Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction.* Hypertension, 2010. **55**(2): p. 500-7.
34. Kirabo, A., et al., *DC isoketal-modified proteins activate T cells and promote hypertension.* J Clin Invest, 2014. **124**(10): p. 4642-56.
35. Weiss, S., et al., *The complement receptor C5aR1 contributes to renal damage but protects the heart in angiotensin II-induced hypertension.* Am J Physiol Renal Physiol, 2016: p. ajprenal 00040 2016.
36. Shah, K.H., et al., *Myeloid Suppressor Cells Accumulate and Regulate Blood Pressure in Hypertension.* Circ Res, 2015. **117**(10): p. 858-69.
37. Wu, J., et al., *Origin of Matrix-Producing Cells That Contribute to Aortic Fibrosis in Hypertension.* Hypertension, 2016. **67**(2): p. 461-8.
38. Guzik, T.J., et al., *Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction.* J Exp Med, 2007. **204**(10): p. 2449-60.
39. Mattson, D.L., et al., *Genetic mutation of recombination activating gene 1 in Dahl salt-sensitive rats attenuates hypertension and renal damage.* Am J Physiol Regul Integr Comp Physiol, 2013. **304**(6): p. R407-14.

40. Crowley, S.D., et al., *Lymphocyte responses exacerbate angiotensin II-dependent hypertension.* Am J Physiol Regul Integr Comp Physiol, 2010. **298**(4): p. R1089-97.
41. Rudemiller, N., et al., *CD247 modulates blood pressure by altering T-lymphocyte infiltration in the kidney.* Hypertension, 2014. **63**(3): p. 559-64.
42. Huan, T., et al., *Integrative network analysis reveals molecular mechanisms of blood pressure regulation.* Mol Syst Biol, 2015. **11**(4): p. 799.
43. Batchu, S.N., et al., *Role of Axl in T-Lymphocyte Survival in Salt-Dependent Hypertension.* Arterioscler Thromb Vasc Biol, 2016. **36**(8): p. 1638-46.
44. Marvar, P.J., et al., *Central and peripheral mechanisms of T-lymphocyte activation and vascular inflammation produced by angiotensin II-induced hypertension.* Circ Res, 2010. **107**(2): p. 263-70.
45. Xiao, L., et al., *Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II-Induced Hypertension.* Circ Res, 2015. **117**(6): p. 547-57.
46. Carnevale, D., et al., *A cholinergic-sympathetic pathway primes immunity in hypertension and mediates brain-to-spleen communication.* Nat Commun, 2016. **7**: p. 13035.
47. Lob, H.E., et al., *Induction of hypertension and peripheral inflammation by reduction of extracellular superoxide dismutase in the central nervous system.* Hypertension, 2010. **55**(2): p. 277-83, 6p following 283.
48. Lob, H.E., et al., *Role of the NADPH oxidases in the subfornical organ in angiotensin II-induced hypertension.* Hypertension, 2013. **61**(2): p. 382-7.
49. Cavalcante, J.L., et al., *Aortic stiffness: current understanding and future directions.* J Am Coll Cardiol, 2011. **57**(14): p. 1511-22.
50. Sethi, S., et al., *Aortic stiffness: pathophysiology, clinical implications, and approach to treatment.* Integr Blood Press Control, 2014. **7**: p. 29-34.
51. Wu, J., et al., *Immune activation caused by vascular oxidation promotes fibrosis and hypertension.* J Clin Invest, 2016. **126**(1): p. 50-67.
52. Qin, J., et al., *A human gut microbial gene catalogue established by metagenomic sequencing.* Nature, 2010. **464**(7285): p. 59-65.
53. Sommer, F., et al., *Site-specific programming of the host epithelial transcriptome by the gut microbiota.* Genome Biol, 2015. **16**: p. 62.
54. Min, Y.W. and P.L. Rhee, *The Role of Microbiota on the Gut Immunology.* Clin Ther, 2015. **37**(5): p. 968-75.
55. Cotillard, A., et al., *Dietary intervention impact on gut microbial gene richness.* Nature, 2013. **500**(7464): p. 585-8.
56. Yang, T., et al., *Gut dysbiosis is linked to hypertension.* Hypertension, 2015. **65**(6): p. 1331-40.
57. Mell, B., et al., *Evidence for a link between gut microbiota and hypertension in the Dahl rat.* Physiol Genomics, 2015. **47**(6): p. 187-97.
58. Santisteban, M.M., et al., *Hypertension-Linked Pathophysiological Alterations in the Gut.* Circ Res, 2016.
59. Trott, D.W., et al., *Oligoclonal CD8+ T cells play a critical role in the development of hypertension.* Hypertension, 2014. **64**(5): p. 1108-15.
60. Bailey, S.R., et al., *Th17 cells in cancer: the ultimate identity crisis.* Front Immunol, 2014. **5**: p. 276.

61. Marko, L., et al., *Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage.* Hypertension, 2012. **60**(6): p. 1430-6.
62. Zhang, J., et al., *Tumor necrosis factor-alpha produced in the kidney contributes to angiotensin II-dependent hypertension.* Hypertension, 2014. **64**(6): p. 1275-81.
63. Chen, C.C., et al., *TNFR1-deficient mice display altered blood pressure and renal responses to ANG II infusion.* Am J Physiol Renal Physiol, 2010. **299**(5): p. F1141-50.
64. Venegas-Pont, M., et al., *Tumor necrosis factor-alpha antagonist etanercept decreases blood pressure and protects the kidney in a mouse model of systemic lupus erythematosus.* Hypertension, 2010. **56**(4): p. 643-9.
65. O'Leary, R., et al., *Macrophage-derived interleukin 6 contributes to angiotensin II mediated angiotensinogen stimulation in renal proximal tubular cells.* Am J Physiol Renal Physiol, 2016: p. ajprenal 00482 2015.
66. Satou, R., et al., *Interferon-gamma biphasically regulates angiotensinogen expression via a JAK-STAT pathway and suppressor of cytokine signaling 1 (SOCS1) in renal proximal tubular cells.* FASEB J, 2012. **26**(5): p. 1821-30.
67. Hashmat, S., et al., *Interleukin-6 Inhibition Attenuates Hypertension and Associated Renal Damage in Dahl Salt-Sensitive Rats.* Am J Physiol Renal Physiol, 2016: p. ajprenal 00594 2015.
68. Saleh M. A., N.A.E., Madhur M.S., *Inhibition of Interleukin-17A, but not Interleukin-17F, signaling lowers blood pressure, and reduces end-organ inflammation in angiotensin II-induced hypertension.* J Am Coll Cardiol Basic Trans Science, 2016. **1**(7): p. 606-16.
69. Wu, J., et al., *Inflammation and mechanical stretch promote aortic stiffening in hypertension through activation of p38 mitogen-activated protein kinase.* Circ Res, 2014. **114**(4): p. 616-25.
70. Jiang, L., et al., *Mechanism of IFN-gamma in regulating OPN/Th17 pathway during vascular collagen remodeling of hypertension induced by ANG II.* Int J Clin Exp Pathol, 2015. **8**(11): p. 14433-40.
71. Kamat, N.V., et al., *Renal transporter activation during angiotensin-II hypertension is blunted in interferon-gamma-/- and interleukin-17A-/- mice.* Hypertension, 2015. **65**(3): p. 569-76.
72. Mikolajczyk, T.P., et al., *Role of chemokine RANTES in the regulation of perivascular inflammation, T-cell accumulation, and vascular dysfunction in hypertension.* FASEB J, 2016.
73. Rudemiller, N.P., et al., *C-C Motif Chemokine 5 Attenuates Angiotensin II-Dependent Kidney Injury by Limiting Renal Macrophage Infiltration.* Am J Pathol, 2016. **186**(11): p. 2846-2856.
74. Kvakan, H., et al., *Regulatory T cells ameliorate angiotensin II-induced cardiac damage.* Circulation, 2009. **119**(22): p. 2904-12.
75. Katsuki, M., et al., *Decreased proportion of Foxp3+ CD4+ regulatory T cells contributes to the development of hypertension in genetically hypertensive rats.* J Hypertens, 2015. **33**(4): p. 773-83; discussion 783.
76. Viel, E.C., et al., *Immune regulation and vascular inflammation in genetic hypertension.* Am J Physiol Heart Circ Physiol, 2010. **298**(3): p. H938-44.
77. Barhoumi, T., et al., *T regulatory lymphocytes prevent angiotensin II-induced hypertension and vascular injury.* Hypertension, 2011. **57**(3): p. 469-76.

78. Matrougui, K., et al., *Natural regulatory T cells control coronary arteriolar endothelial dysfunction in hypertensive mice.* Am J Pathol, 2011. **178**(1): p. 434-41.
79. Mian, M.O., et al., *Deficiency of T-regulatory cells exaggerates angiotensin II-induced microvascular injury by enhancing immune responses.* J Hypertens, 2016. **34**(1): p. 97-108.
80. Olofsson, P.S., et al., *Blood pressure regulation by CD4+ lymphocytes expressing choline acetyltransferase.* Nat Biotechnol, 2016. **34**(10): p. 1066-1071.
81. Wu, C., et al., *Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1.* Nature, 2013. **496**(7446): p. 513-7.
82. Kleinewietfeld, M., et al., *Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells.* Nature, 2013. **496**(7446): p. 518-22.
83. Nakae, S., et al., *Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses.* Immunity, 2002. **17**(3): p. 375-87.
84. Ishigame, H., et al., *Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses.* Immunity, 2009. **30**(1): p. 108-19.
85. Fejes-Toth, G., et al., *Epithelial Na+ channel activation and processing in mice lacking SGK1.* Am J Physiol Renal Physiol, 2008. **294**(6): p. F1298-305.
86. Davis, M.E., et al., *Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training.* Am J Physiol Heart Circ Physiol, 2003. **284**(4): p. H1449-53.
87. Gonzalez-Villalobos, R.A., et al., *The absence of intrarenal ACE protects against hypertension.* The Journal of clinical investigation, 2013. **123**(5): p. 2011-23.
88. Nguyen, M.T., et al., *Differential regulation of Na+ transporters along nephron during ANG II-dependent hypertension: distal stimulation counteracted by proximal inhibition.* American journal of physiology. Renal physiology, 2013. **305**(4): p. F510-9.
89. Kurien, B.T. and R.H. Scofield, *Mouse urine collection using clear plastic wrap.* Lab Anim, 1999. **33**(1): p. 83-6.
90. Ko, B., et al., *A new model of the distal convoluted tubule.* American journal of physiology. Renal physiology, 2012. **303**(5): p. F700-10.
91. Arroyo, J.P., et al., *Nedd4-2 modulates renal Na+-Cl- cotransporter via the aldosterone-SGK1-Nedd4-2 pathway.* J Am Soc Nephrol, 2011. **22**(9): p. 1707-19.
92. Ko, B., et al., *Phorbol ester stimulation of RasGRP1 regulates the sodium-chloride cotransporter by a PKC-independent pathway.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(50): p. 20120-5.
93. Nwankwo T, Y.S., Burt V, Gu Q., *Hypertension among adults in the united states: National Health and Examination survey, 2011-2012.* NCHS data brief, 2013. **133**.
94. Wang, T.J. and R.S. Vasan, *Epidemiology of uncontrolled hypertension in the United States.* Circulation, 2005. **112**(11): p. 1651-62.
95. Harrison, D.G., et al., *Inflammation, immunity, and hypertension.* Hypertension, 2011. **57**(2): p. 132-40.
96. Guzik, T.J., et al., *Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction.* The Journal of experimental medicine, 2007. **204**(10): p. 2449-60.

97. Gu, C., L. Wu, and X. Li, *IL-17 family: cytokines, receptors and signaling.* Cytokine, 2013. **64**(2): p. 477-85.
98. Gaffen, S.L., *Structure and signalling in the IL-17 receptor family.* Nature reviews. Immunology, 2009. **9**(8): p. 556-67.
99. Lang, F., C. Stournaras, and I. Alesutan, *Regulation of transport across cell membranes by the serum- and glucocorticoid-inducible kinase SGK1.* Molecular membrane biology, 2014. **31**(1): p. 29-36.
100. Boase, N.A. and S. Kumar, *NEDD4: The founding member of a family of ubiquitin-protein ligases.* Gene, 2015. **557**(2): p. 113-22.
101. Woltman, A.M., et al., *Interleukin-17 and CD40-ligand synergistically enhance cytokine and chemokine production by renal epithelial cells.* Journal of the American Society of Nephrology : JASN, 2000. **11**(11): p. 2044-55.
102. O'Donnell, M.P., et al., *Renal cell cytokine production stimulates HIV-1 expression in chronically HIV-1-infected monocytes.* Kidney international, 1998. **53**(3): p. 593-7.
103. Macica, C.M., et al., *TNF production by the medullary thick ascending limb of Henle's loop.* Kidney international, 1994. **46**(1): p. 113-21.
104. Loverre, A., et al., *IL-17 expression by tubular epithelial cells in renal transplant recipients with acute antibody-mediated rejection.* American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 2011. **11**(6): p. 1248-59.
105. Verouti, S.N., et al., *Regulation of blood pressure and renal function by NCC and ENaC: lessons from genetically engineered mice.* Current opinion in pharmacology, 2015. **21**: p. 60-72.
106. McMaster, W.G., et al., *Inflammation, immunity, and hypertensive end-organ damage.* Circulation research, 2015. **116**(6): p. 1022-33.
107. Satou, R., et al., *Interferon-gamma biphasically regulates angiotensinogen expression via a JAK-STAT pathway and suppressor of cytokine signaling 1 (SOCS1) in renal proximal tubular cells.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2012. **26**(5): p. 1821-30.
108. Nwankwo, T., et al. *Hypertension Among Adults in the United States: National Health and Nutrition Examination Survey, 2011-2012*. 2013; Available from: http://www.cdc.gov/nchs/data/databriefs/db133.pdf.
109. McMaster, W.G., et al., *Inflammation, immunity, and hypertensive end-organ damage.* Circ Res, 2015. **116**(6): p. 1022-33.
110. Norlander, A.E., et al., *Interleukin-17A Regulates Renal Sodium Transporters and Renal Injury in Angiotensin II-Induced Hypertension.* Hypertension, 2016. **68**(1): p. 167-74.
111. Saleh, M.A., A.E. Norlander, and M.S. Madhur, *Inhibition of Interleukin-17A, But Not Interleukin-17F, Signaling Lowers Blood Pressure, and Reduces End-Organ Inflammation in Angiotensin II–Induced Hypertension.* JACC: Basic to Translational Science, 2016. **1**(7): p. 606-616.
112. Lev-Ran, A. and M. Porta, *Salt and hypertension: a phylogenetic perspective.* Diabetes Metab Res Rev, 2005. **21**(2): p. 118-31.
113. Machnik, A., et al., *Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism.* Nat Med, 2009. **15**(5): p. 545-52.

114. Wiig, H., et al., *Immune cells control skin lymphatic electrolyte homeostasis and blood pressure.* J Clin Invest, 2013. **123**(7): p. 2803-15.
115. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.
116. Blacher, J., et al., *Residual cardiovascular risk in treated hypertension and hyperlipidaemia: the PRIME Study.* J Hum Hypertens, 2010. **24**(1): p. 19-26.
117. Struthers, A.D., *A new approach to residual risk in treated hypertension--3P screening.* Hypertension, 2013. **62**(2): p. 236-9.
118. Ellison, D.H., *Ubiquitylation and the pathogenesis of hypertension.* J Clin Invest, 2013. **123**(2): p. 546-8.
119. Safa, K., et al., *Salt Accelerates Allograft Rejection through Serum- and Glucocorticoid-Regulated Kinase-1-Dependent Inhibition of Regulatory T Cells.* J Am Soc Nephrol, 2015. **26**(10): p. 2341-7.
120. Hernandez, A.L., et al., *Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells.* J Clin Invest, 2015. **125**(11): p. 4212-22.
121. Jantsch, J., et al., *Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense.* Cell Metab, 2015. **21**(3): p. 493-501.
122. Binger, K.J., et al., *High salt reduces the activation of IL-4- and IL-13-stimulated macrophages.* J Clin Invest, 2015. **125**(11): p. 4223-38.
123. Zhang, W.C., et al., *High salt primes a specific activation state of macrophages, M(Na).* Cell Res, 2015. **25**(8): p. 893-910.
124. Titze, J., *Sodium balance is not just a renal affair.* Curr Opin Nephrol Hypertens, 2014. **23**(2): p. 101-5.
125. Stevens, V.A., et al., *The role of SGK-1 in angiotensin II-mediated sodium reabsorption in human proximal tubular cells.* Nephrol Dial Transplant, 2008. **23**(6): p. 1834-43.
126. Baskin, R. and P.P. Sayeski, *Angiotensin II mediates cell survival through upregulation and activation of the serum and glucocorticoid inducible kinase 1.* Cell Signal, 2012. **24**(2): p. 435-42.
127. Lang, F., F. Artunc, and V. Vallon, *The physiological impact of the serum and glucocorticoid-inducible kinase SGK1.* Curr Opin Nephrol Hypertens, 2009. **18**(5): p. 439-48.
128. Paust, H.J., et al., *The IL-23/Th17 axis contributes to renal injury in experimental glomerulonephritis.* J Am Soc Nephrol, 2009. **20**(5): p. 969-79.
129. Kochl, R., et al., *WNK1 kinase balances T cell adhesion versus migration in vivo.* Nat Immunol, 2016. **17**(9): p. 1075-83.
130. Chockalingam, A., N.R. Campbell, and J.G. Fodor, *Worldwide epidemic of hypertension.* Can J Cardiol, 2006. **22**(7): p. 553-5.
131. Nwankwo, T., et al., *Hypertension among adults in the United States: National Health and Nutrition Examination Survey, 2011-2012.* NCHS Data Brief, 2013(133): p. 1-8.
132. McCormick, J.A., et al., *SGK1: a rapid aldosterone-induced regulator of renal sodium reabsorption.* Physiology (Bethesda), 2005. **20**: p. 134-9.
133. Rao, A.D., et al., *Polymorphisms in the serum- and glucocorticoid-inducible kinase 1 gene are associated with blood pressure and renin response to dietary salt intake.* J Hum Hypertens, 2013. **27**(3): p. 176-80.

134. Dikalov, S.I. and R.R. Nazarewicz, *Angiotensin II-induced production of mitochondrial reactive oxygen species: potential mechanisms and relevance for cardiovascular disease.* Antioxid Redox Signal, 2013. **19**(10): p. 1085-94.
135. Sachse, A. and G. Wolf, *Angiotensin II-induced reactive oxygen species and the kidney.* J Am Soc Nephrol, 2007. **18**(9): p. 2439-46.
136. Mascolo, A., et al., *New and old roles of the peripheral and brain renin-angiotensin-aldosterone system (RAAS): Focus on cardiovascular and neurological diseases.* Int J Cardiol, 2017. **227**: p. 734-742.
137. Atlas, S.A., *The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition.* J Manag Care Pharm, 2007. **13**(8 Suppl B): p. 9-20.
138. Abais, J.M., et al., *Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector?* Antioxid Redox Signal, 2015. **22**(13): p. 1111-29.
139. Blanco, P., et al., *Dendritic cells and cytokines in human inflammatory and autoimmune diseases.* Cytokine Growth Factor Rev, 2008. **19**(1): p. 41-52.
140. Kushwah, R. and J. Hu, *Role of dendritic cells in the induction of regulatory T cells.* Cell Biosci, 2011. **1**(1): p. 20.
141. Kopp, C., et al., *23Na magnetic resonance imaging-determined tissue sodium in healthy subjects and hypertensive patients.* Hypertension, 2013. **61**(3): p. 635-40.
142. Li, K., et al., *Interleukin-6 stimulates epithelial sodium channels in mouse cortical collecting duct cells.* Am J Physiol Regul Integr Comp Physiol, 2010. **299**(2): p. R590-5.
143. Ha, S.K., *Dietary salt intake and hypertension.* Electrolyte Blood Press, 2014. **12**(1): p. 7-18.
144. Vremec, D., et al., *CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen.* J Immunol, 2000. **164**(6): p. 2978-86.
145. Shortman, K., *Burnet oration: dendritic cells: multiple subtypes, multiple origins, multiple functions.* Immunol Cell Biol, 2000. **78**(2): p. 161-5.
146. Saleh, M.A., et al., *Lymphocyte adaptor protein LNK deficiency exacerbates hypertension and end-organ inflammation.* J Clin Invest, 2015.
147. Dorffel, Y., et al., *Preactivated peripheral blood monocytes in patients with essential hypertension.* Hypertension, 1999. **34**(1): p. 113-7.
148. Wirtz, P.H., et al., *Glucocorticoid sensitivity of circulating monocytes in essential hypertension.* Am J Hypertens, 2004. **17**(6): p. 489-94.
149. Zhao, Z., et al., *Increased migration of monocytes in essential hypertension is associated with increased transient receptor potential channel canonical type 3 channels.* PLoS One, 2012. **7**(3): p. e32628.
150. Dorffel, Y., et al., *Preactivated monocytes from hypertensive patients as a factor for atherosclerosis?* Atherosclerosis, 2001. **157**(1): p. 151-60.
151. Li, B., et al., *RNA-Seq gene expression estimation with read mapping uncertainty.* Bioinformatics, 2010. **26**(4): p. 493-500.
152. Snel, B., et al., *STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene.* Nucleic Acids Res, 2000. **28**(18): p. 3442-4.
153. Szklarczyk, D., et al., *STRING v10: protein-protein interaction networks, integrated over the tree of life.* Nucleic Acids Res, 2015. **43**(Database issue): p. D447-52.
154. Krishnan, S.M., et al., *IL-1beta and IL-18: inflammatory markers or mediators of hypertension?* Br J Pharmacol, 2014. **171**(24): p. 5589-602.

155. Krishnan, S.M., et al., *Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice.* Br J Pharmacol, 2015.
156. Read, C.B., et al., *Cutting Edge: identification of neutrophil PGLYRP1 as a ligand for TREM-1.* J Immunol, 2015. **194**(4): p. 1417-21.
157. Dziarski, R., et al., *Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice.* Blood, 2003. **102**(2): p. 689-97.
158. Rohatgi, A., et al., *The association between peptidoglycan recognition protein-1 and coronary and peripheral atherosclerosis: Observations from the Dallas Heart Study.* Atherosclerosis, 2009. **203**(2): p. 569-75.
159. Spadaro, M., et al., *Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells.* FASEB J, 2008. **22**(8): p. 2747-57.
160. Thomas, A.C. and J.T. Mattila, *"Of mice and men": arginine metabolism in macrophages.* Front Immunol, 2014. **5**: p. 479.
161. Vogt, L., et al., *VSIG4, a B7 family-related protein, is a negative regulator of T cell activation.* J Clin Invest, 2006. **116**(10): p. 2817-26.