

**ROLE OF THE ADAPTIVE IMMUNE SYSTEM IN ANGIOTENSIN II INDUCED  
VASCULAR REMODELING AND STIFFENING**

by

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**DEDICATION**

*To my beloved family*

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## ABSTRACT

Elevation of blood pressure leads to structural and functional alterations in vasculature, resulting in increased arterial stiffness, which in turn is a predictor of future hypertension and cardiovascular risks. Angiotensin II (Ang II) plays a crucial role in blood pressure regulation. In addition to its hemodynamic effects, Ang II activates both innate and adaptive immunity. The objective of this study is to define the roles of CD4<sup>+</sup> T lymphocyte subsets in the progression of vascular remodeling and stiffening induced by Ang II. A mouse model of Ang II infusion was used to induce hypertension and vascular diseases. In the WT mice, Ang II infusion led to an increased aortic stiffness within 7 days of the treatment as well as an increase in aortic remodeling within 14 days of the treatment. Interestingly, RAG1<sup>-/-</sup> mice, lacking functional T and B lymphocytes were prevented from the vascular stiffening and remodeling caused by Ang II. Characterization of T cell subsets in the perivascular aortic infiltrates showed that there was a sequential activation of peri-aortic Th1 and Th17 during the time course of Ang II treatment, which was associated with the initial increased aortic stiffness and the subsequent remodeling, respectively. To extend the concept, roles of suppressive regulatory T cells (Tregs) were further examined. Proliferation of Tregs was successfully induced *in vivo* using a cytokine complex of IL-2 and anti-IL-2 mAb clone JES6-1. Ang II-infused mice that received the IL-2/anti-IL-2 complex exhibited a reduced vascular remodeling and stiffening caused by Ang II. Stimulation of Tregs with the IL-2/anti-IL-2 complex also suppressed the Th1 and Th17 responses and reduced immune cells infiltrates in the aortas. Since hypertension is closely related to the kidney and renal homeostasis is also

tightly regulated by Ang II, the kidney function was determined in this Ang II-hypertensive model. In the wild type mice, two weeks infusion of Ang II resulted in an increased glomerular filtration rate (GFR) whereas immunodeficient RAG1<sup>-/-</sup> mice exhibited a marked decrease in GFR. Subsequent experiments showed that Th17 was crucial in renal hemodynamic response to Ang II, partly by regulating secretion of vasodilatory prostaglandin E<sub>2</sub>.

## CHAPTER 1: INTRODUCTION

### Statement of the problem

Hypertension is a common clinical pathology worldwide. The *World Health Statistics 2012* reports that one in three adults has high blood pressure, which contributes to 50 percent of all deaths from stroke and heart disease. In the United States, about 78 million individuals have high blood pressure and only half of them have it controlled. Despite the availability of effective antihypertensive drugs, that prevalence of hypertension in the U.S. population is projected to increase 7.2% by 2030 (Go et al., 2013). Hypertension is generally accepted as a major risk factor for cardiovascular and renal complications. Chronic hypertension, listed as a primary cause of death, leads to vascular pathology, cerebral incidents, heart attack, renal failure, and dementia (Messerli, Williams, & Ritz, 2007; Staessen, Wang, Bianchi, & Birkenhager, 2003). Elevation of blood pressure leads to structural alterations in the vasculature, resulting in an increased arterial stiffness, which may promote target organ damage (Benetos, Laurent, Asmar, & Lacolley, 1997; Chirinos, 2012). On the contrary, a recent Framingham cohort study indicated that aortic stiffness is rather a cause of hypertension than a result. Assessed by carotid-femoral pulse wave velocity, this study showed that aortic stiffness is jointly associated with future systolic hypertension but initial blood pressure is not associated with aortic stiffness (Kaess et al., 2012). Although, the existing evidence confirms the association between hypertension and vascular stiffening, how they are related is not well understood. In the other words, is arterial stiffness the result or the cause of hypertension?

More importantly, despite intensive research efforts, the etiology of hypertension remains ambiguous and the molecular mechanism of its pathogenesis have not been elucidated. Current therapeutics may be effective in reducing blood pressure but do not reverse the underlying mechanism and the pathological changes remain progressive.

There is a general agreement that blood pressure is regulated by sodium and water balance and vasomotor tone. Hypertension, therefore, is a consequence of changes in these factors, including perturbations of the renin-angiotensin system and sodium regulation, increased sympathetic tone and peripheral arterial resistance, and vascular dysfunction and hypertrophy. In the present study, we propose to demonstrate the novel concept that the adaptive immune system may affect this neuroendocrine system and play a role in aortic remodeling and stiffening during the development of hypertension. Our hypothesis is supported by the established role of immune cells in the pathogenesis of atherosclerosis, where the disease is also associated with increased arterial wall thickening and arterial rigidity (Song, Leung, & Schindler, 2001; van Popele et al., 2001).

To study hypertension and vascular pathology, we used Angiotensin II (Ang II) infusion, a well-established model for primary hypertension, postmenopausal hypertension, preeclampsia, vascular remodeling, and vascular aging (Qin, 2008). Ang II is a primary effector hormone that regulates blood pressure and contributes in large measure to the cardiovascular pathology in humans. Beside its hemodynamic effect, Ang II acts as a vasoactive hormone, regulating expression of growth factor and promoting cell proliferation and fibrosis (Castoldi et al., 2003; Che et al., 2008; Ruiz-Ortega et al.,

2001). Recently, the effect of Ang II has been extended to acting as a proinflammatory mediator which activates the adaptive immune response, specifically lymphocyte activation (Nataraj et al., 1999) and alters the balance of helper T lymphocyte subsets (Shao et al., 2003). Another significant study also indicates the pivotal role of the renin-angiotensin system in autoimmune disease, multiple sclerosis (Platten et al., 2009). In this study, blocking Ang II signaling suppresses auto-reactive Th1 and Th17 responses and promotes suppressive regulatory T cells (Platten et al., 2009). It follows that these immune cells may be responding in the same fashion in the vasculature under the action of Ang II. Existing evidence in humans also supports the association between CD4<sup>+</sup> helper T cells and hypertension (Dong, He, Wang, Xie, & Wang, 2005; Herrera, Ferrebuz, MacGregor, & Rodriguez-Iturbe, 2006; Saito, Umekage et al., 1999; Saito, Sakai et al., 1999; Seaberg et al., 2005). Also, in rodents, immunodeficient mice have a reduced blood pressure response to Ang II (Guzik et al., 2007) and adoptive transfer of suppressive regulatory T cells reduces Ang II induced vascular injury and hypertension (Barhoumi et al., 2011).

Collectively, a potential role of the adaptive immune system, particularly CD4<sup>+</sup> T lymphocytes in the progression of hypertension and vascular remodeling has been suggested; however, the defined roles of each of the T lymphocytes subsets in this process have not been elucidated. Therefore, in this study we propose to examine, first, the temporal relationship between stiffness of large arteries and the progression of hypertension, and second, the role of CD4<sup>+</sup> T cell subsets, including Th1, Th2, Th17, and regulatory T cells, in vascular structural remodeling and stiffness.

## Literature review

The increase in arterial stiffness is an age-related process and a consequence of various diseases such as diabetes, atherosclerosis, and hypertension (Franklin, 2005; Zieman, Melenovsky, & Kass, 2005). As reviewed by Zieman et al. (Zieman et al., 2005), arterial stiffening is an independent marker for increased cardiovascular risks, including stroke, heart failure, coronary artery disease, mortality and morbidity in hypertension, and renal disease. A recent clinical study has shown that aortic stiffness is positively and significantly associated with hypertension, diabetes, chronic renal disease, and dyslipidemia, whereas peripheral arterial stiffness has only a significant correlation with hypertension (Wohlfahrt et al., 2013). This finding emphasizes the significance of large artery stiffness in vascular pathology. Increased large artery stiffness with aging is mainly due to vascular structural alterations that include collagen deposition, fragmentation and reduction of elastin, and an increase in cross-linking by advanced glycation end-products (AGEs) (Fleenor, 2012). Sustained elevation of blood pressure leads to vascular hypertrophy and collagen accumulation which can be attributed to increased mechanical wall stress and/or local humoral factors (Benetos et al., 1997). Understanding the mechanisms underlying vascular remodeling and stiffening will provide potential targets for future therapeutic development. In this review, basic arterial mechanics and vascular remodeling will be addressed. Also, the novel role of adaptive immune components in the development of vascular pathology, particularly the role of T lymphocytes, will be discussed.

### *Arterial mechanics*

Distensible elastic arteries are important for a proper cardiac function in a closed circulatory system. The upstream large arterial tree acts as an elastic reservoir to expand and store a portion of stroke volume with each systole and then recoil elastically and discharge this volume to the periphery vessels during each diastole. This phenomenon is known as the Windkessel effect, which allows a steady blood flow throughout the cardiac cycle and helps to decrease work load to the heart (Shadwick, 1999; Wagenseil & Mecham, 2009).

The components of the arterial wall that account for its elastic properties are collagen and elastin deposited by smooth muscle cells in the medial layer (Wagenseil & Mecham, 2009). These two extracellular matrix proteins (ECM) have distinct elastic properties. At physiological pressure, less than 10% of collagen fibers are engaged and the artery wall represents the elasticity of elastin. At higher pressure, more collagen fibers are recruited and the vessel becomes progressively stiffer in order to restrict aortic distension and prevent aortic aneurysm or rupture (Greenwald, Moore, Rachev, Kane, & Meister, 1997; Roach & Burton, 1957; Shadwick, 1999; Wagenseil & Mecham, 2009).

Hemodynamic forces as well as extrinsic factors such as hormones, and salt and glucose regulation exert influence on vascular structure and stiffening (Zieman et al., 2005). Under normal conditions, there are three mechanical stresses that apply to the arterial wall: (1) circumferential stress induced by blood pressure, (2) wall shear stress induced by blood flow, and (3) axial stress induced by elongation of surrounding tissues

(Hayashi & Naiki, 2009; Humphrey, Eberth, Dye, & Gleason, 2009; Wagenseil & Mecham, 2009). Alterations in these stresses, for example, by sustained elevation of blood pressure, result in changing dimensions, geometry, and microstructure of the blood vessels. The process is termed growth and remodeling (Humphrey et al., 2009).

It has been postulated that the remodeling process could actually be an adaptive response of the arterial wall to maintain a homeostatic mechanical state (Humphrey, 2008a). As long as the mechanical stress is present, remodeling will continue; however, once the remodeling has proceeded to achieve the ideal stress-strain values the remodeling processes will cease (Grinnell, 1994). Proposed mechanisms that are involved in the arterial remodeling process include fibrosis, hyperplasia of the arterial intima and media, changes in vascular collagen and elastin, endothelial dysfunction, and arterial calcification (van Vark et al., 2012). Although the molecular mechanism that controls vascular remodeling is not well established, the three cell types located in the vasculature—the vascular endothelial cells, smooth muscle cells (VSMCs), and adventitial fibroblasts—have been shown to mediate the process (Humphrey et al., 2009) by modulating the extracellular matrix proteins (ECM), which provide structural integrity and elasticity of the vessel wall.

#### *Arterial wall structure*

Arterial walls consist of three layers: the tunica intima, tunica media, and tunica adventitia. The intima is the innermost layer on the luminal surface of the arteries, consisting of a single layer of endothelial cells and a subendothelial area containing a thin

layer of connective tissues and SMCs (Waller et al., 1992). Endothelial cells lining the intima are capable of producing elastin and recruiting SMCs to the vessel walls. The intima is important in atherosclerosis and restenosis but seems to play little role in the mechanical properties of the vessel (Wagenseil & Mecham, 2009). The medial layer comprises most of the thickness of the arterial wall (Safar, Blacher, Mourad, & London, 2000) and contributes to its mechanical properties. The media consists of SMCs and connective tissues, including elastic fibers, collagen, and proteoglycans. The elastin is arranged in lamellae and functions to produce an elastic reservoir to distribute stress evenly throughout the wall. SMCs and collagen fibers reside in between and connect the elastic lamellae. The mechanical properties of the vessel wall are mostly contributed by the elastin and collagen components since it has been shown that elimination of SMCs does not significantly alter the static elastic properties of rat aortas (Berry, Greenwald, & Rivett, 1975). The outermost layer is the adventitia, which is surrounded by perivascular tissues and composed of mainly collagen fibers produced by fibroblasts. These stiff collagen fibers help maintain the vascular structure especially at very high pressure. Studies show that the adventitia contains residential progenitor cells capable of differentiating to SMCs that migrate to the medial and intimal layers (Wagenseil & Mecham, 2009).

#### *Vascular remodeling in hypertension*

Hypertension is known to be directly associated with arterial stiffening and remodeling. Elevated BP over time can lead to vascular remodeling, hypertrophy, and

hyperplasia—structural changes that produce intrinsic arterial stiffening. Untreated hypertension may accelerate the rate of large artery stiffness and thus perpetuate a vicious cycle of sustained hypertension, which further increases large artery stiffness (Franklin, 2005). A clinical study by Benetos et al in 2002 indicates high blood pressure is a major determinant of progressive aortic stiffness (Benetos et al., 2002). In the other way around, aortic stiffness is also an independent predictor and is associated with a greater risk of future hypertension in non-hypertensive individuals (Dernellis & Panaretou, 2005; Liao et al., 1999; Kaess et al., 2012). These studies suggest that the bi-directional association between vascular stiffness and hypertension.

In animal models, hypertension leads to reduced elastin fraction and increased ratios of the amount of collagen to that of elastin, which correspond to wall stiffness. Arterial wall thickening is also observed in hypertension. VSMC hypertrophy but not hyperplasia may contribute to this increase in wall thickness (Olivetti, Anversa, Melissari, & Loud, 1980; Owens, Rabinovitch, & Schwartz, 1981), probably by promoting extracellular matrix (ECM) synthesis.

Balance of the ECM, including collagen and elastin, is mostly regulated by the matrix metalloprotease (MMP) family. MMPs degrade the ECM, resulting in fragmented and discontinuous elastin fibers as shown by the observation that increased MMP-2 expression in aged rats is localized near the break sites of the elastic lamellae (Li, Froehlich, Galis, & Lakatta, 1999). In addition to elastin degradation, MMPs play a role in vascular remodeling by producing uncoiled, stiffer collagen fibers (Zieman et al.,

2005). An animal study showed that MMP-2 expression and activity are significantly increased in DOCA-salt hypertensive rats (Watts et al., 2007). In addition there is a decrease in concentration of MMP-1 and an increase in that of TIMP-1, an inhibitor of MMP, in serum of hypertensive patients with left ventricular hypertrophy, suggesting a reduction of collagen degradation in the course of hypertension (Laviades et al 1998).

The stiffness of collagen and elastin can also be increased by an additional formation of crosslinking, via enzymatic and non-enzymatic pathways. By enzymatic pathways, elastin crosslinks can be initiated by lysyl oxidase, which deaminates the lysine side chain of tropoelastin molecules to form covalent crosslinks, desmosine and isodesmosine (Kagan & Sullivan, 1982). Lysyl oxidase also mediates crosslinking of collagen by deaminating the lysine and hydroxylysine residues, resulting in collagen crosslinks that are less susceptible to degradation (van der Slot et al., 2004; van der Slot et al., 2005). Previous studies have shown a tendency of increasing the amount of mature crosslinks with hypertension (Hayashi & Naiki, 2009) and upregulation of lysyl oxidase in the vessels of hypertensive rats. A study of heart failure in humans also indicates that increased expression of lysyl oxidase correlates with increased collagen-crosslinking and probably collagen-mediated left ventricular stiffness (Lopez et al., 2009).

#### *Role of the renin-angiotensin system in vascular remodeling*

The renin-angiotensin system is known to play a critical role in VSMC growth and regulation of ECM proteins in the vessel wall. Ang II stimulates synthesis of ECM

components, mainly collagen, by VSMCs, partly through its effect on cytokines, growth factors, and endothelin-1 (ET-1) (Briones 2010, Jose Tunon 2000).

Ang II, acting through AT1 receptors, stimulates proliferation of medial VSMCs and adventitial fibroblasts in coronary arterioles of Wistar rats (McEwan et al 1998). In arterial SMCs, Ang II enhances production of TGF- $\beta$  correlated with an increased in collagen synthesis (Ford et al 1999). This finding was also found in renal mesangial cells (Ruiz-otega M et al 1995). Previous studies showed that TGF- $\beta$  signaling dictates collagen type I release from VSMCs. The molecular mechanisms involve Smad2 phosphorylation and  $\alpha\beta$ -integrin activation. More importantly, treatment of hypertensive mice with neutralizing TGF- $\beta$ 1 antibody reduces artery stiffness (Belmadani, Zerfaoui, Boulares, Palen, & Matrougui, 2008). In addition to TGF- $\beta$ , Ang II also increases collagen synthesis by adventitial fibroblasts via induction of connective tissue growth factor (CTGF) (Che et al., 2008). It has been suggested that Ang II-induced collagen synthesis is partly mediated by ET, since an ET antagonist has been shown to prevent Ang II-induced collagen type 1 gene activation and ECM deposition (Chatziantoniou, Boffa, Ardaillou, & Dussaule, 1998; Gomez-Garre et al., 1996).

In addition to its effect on growth factors, the action of Ang II on vascular remodeling may be partly due to increased production of reactive oxygen species (ROS). Ang II promotes ROS generation by stimulating NADH and NADPH oxidase activity in cultures VSMCs (Griendling, Minieri, Ollerenshaw, & Alexander, 1994). A number of studies have shown the role of ROS in blood pressure regulation. Ang II-induced

hypertension was delayed in mice lacking a catalytic subunit of NADPH oxidase (Haque & Majid, 2011). Also, treatment with antioxidants such as vitamin C and E, free radical scavengers, or tetrahydrobiopterin attenuates the development of hypertension (Montezano & Touyz, 2012).

Recently, hypertension has been widely considered a chronic low-grade inflammatory disease (Briones 2010), associated with immune and inflammatory cells (macrophages, lymphocytes, and other leukocytes) present in the vasculature (Schiffrin 2011). Ang II is known to be a proinflammatory mediator, modulating immune and inflammatory cells (Ruiz-Ortega et al., 2001). By acting through AT1 receptors on immune cells, Ang II promotes splenic lymphocyte proliferation via activation of calcineurin phosphatase (Nataraj et al., 1999). Besides, Ang II induced upregulation of proinflammatory cytokine IL-6 is mediated by NF- $\kappa$ B pathway (Han Y et al 1999). In the arterial vasculature, Ang II stimulation of aortic fibroblasts induces fibroblast proliferation and proinflammatory factors, particularly MCP-1 and IL-6 (Tieu et al., 2011). The adventitial derived MCP-1 in turn recruits monocytes, which may support infiltration of lymphocytes into vasculatures. Lymphocytes, a main component of adaptive immune system, appear to participate in vascular diseases.

#### *Immune mechanisms in hypertension*

Adaptive immunity, defined as a response of the immune system to a specific antigen that typically generates immunological memory, is composed of B and T lymphocytes (Alberts, 2002). B lymphocytes are capable of producing antibodies,

thereby mediating humoral immunity. T lymphocytes consist of two main distinct populations, which are T helper cells (Th; expressing CD4 receptors) and cytotoxic T cells (CTLs; expressing CD8 molecules). Upon antigenic stimulation, helper T cells secrete cytokines to activate other T cells as well as other immune cells including B cells and macrophages. In contrast, CTLs work by killing cells that are infected with pathogens (Abbas, Abul K., Lichtman, Andrew H., Pillai, Shiv., 2007).

There is existing evidence supporting the role of the adaptive immune system in hypertension. In humans, treatment with mycophenolate mofetil, a lymphocyte specific immunosuppressant, significantly reduces systolic and diastolic blood pressure in psoriasis and rheumatoid arthritis patients (Herrera et al., 2006). Additionally, several studies indicate immune activation and alteration of Th1/Th2 balance in women with preeclampsia (Dong et al., 2005; Saito et al., 1999; Saito et al., 1999). More importantly, HIV patients with low CD4 T lymphocyte counts have low incidence of hypertension, which is increased when patients are being treated with highly aggressive antiretroviral therapy (HAART) (Seaberg et al., 2005). The concept is supported by studies in rodents. Suppression of immune cells with mycophenolate mofetil reduces Ang II-producing cells in the renal tubulointerstitium and prevents the development of salt-sensitive hypertension in Sprague-Dawley rats (Rodriguez-Iturbe et al., 2001). Another study in genetically immunodeficient mice revealed a crucial role of T lymphocytes in the development of hypertension. In this study, the authors show that mice lacking T and B lymphocytes (RAG1<sup>-/-</sup> mice) have a reduced hypertensive response and are protected from vascular dysfunction induced by either Ang II infusion or desoxycorticosterone

acetate (DOCA) salt. Interestingly, adoptive transfer of T but not B cells restores the hypertensive response to Ang II in these immunodeficient RAG1<sup>-/-</sup> mice. Also, Ang II was demonstrated to increase T cell activation markers and T cell infiltrates in the perivascular adipose tissue and the adventitia in this study (Guzik et al., 2007). Finally, infusion of Ang II results in an imbalance of CD4 T cell subsets with Th1 domination (Shao et al., 2003). Collectively, these studies suggest a strong contribution of T lymphocytes, possibly CD4 helper T cells, to hypertension and vascular remodeling.

#### *CD4 T lymphocyte subsets*

Naïve CD4 helper T cells can be activated and differentiated into distinct effector subtypes, which secrete various cytokines that activate or modulate other immune cells such as macrophages, B lymphocytes, and cytotoxic T cells. The initial step of T cell activation is the interaction of T cell receptor (TCR) and co-receptor CD4 with antigen-MHC II complex presented by professional antigen presenting cells (APCs). TCR is coupled with CD3 molecules which mediate a biochemical signaling cascade upon antigen recognition by TCR. Among the APCs, dendritic cells (DCs) play the most important role in CD4 T cell differentiation and proliferation by providing costimulatory signals in addition to the antigenic signal. Costimulation involves binding of CD28, expressed on all naïve CD4 T cells, to CD80/86 on activated DCs, among other signals. Several distinct effector CD4 subsets have been identified. These include T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), regulatory T cells (Tregs), T-helper 9 (Th9), and follicular helper T cells (Tfh) (Luckheeram, Zhou, Verma, & Xia, 2012). The fate of

T cell differentiation is dependent on the local cytokine milieu, as well as the costimulatory signal, the strength of the TCR signal (Tao, Constant, Jorritsma, & Bottomly, 1997), and the dendritic cells subsets (Kohu et al., 2009).

Th1 cells are important to immune responses against intracellular pathogens and are responsible for some immune-mediated diseases such as Crohn's disease. Their main cytokine production includes IFN- $\gamma$ , lymphotoxin, and IL-2 (Zhu, Yamane, & Paul, 2010). IFN- $\gamma$  activates macrophages and enhances phagocytic activity while IL-2 is necessary for proliferation of CD8 cytotoxic T cells and regulatory T cells.

Differentiation of Th1 cells requires the presence of IFN- $\gamma$  and IL-2. APCs are a major source of IL-2, which in turn stimulates natural killer (NK) cells to secrete IFN- $\gamma$  (Luckheeram et al., 2012). The master transcription factor for Th1 differentiation is the T-box transcription factor (Tbet), which induces IFN- $\gamma$  production and upregulation of IL-12R $\beta$ 2 (Rengarajan, Szabo, & Glimcher, 2000).

Th2 cells mediate host defense against extracellular parasites, including helminths and play a crucial role in the induction of allergic diseases. Th2 cells produce various cytokines involved in allergic reactions and hypersensitivity, such as IL-4, IL-5, IL-9, IL-10, IL-13, IL-35, and IL-25. Differentiation of Th2 depends on IL-4 and IL-2. IL-4-mediated STAT6 activation induces expression of GATA3, a master regulator of Th2. GATA3 has been shown to 1) induce expression of Th2 cytokines including IL-5 and IL-13 (Rengarajan, Szabo, & Glimcher, 2000), 2) induce selective proliferation of Th2 through upregulating a transcriptional repressor Gfi-1, and 3) inhibit Th1 differentiation

by interacting with Tbet (Zhu, Yamane, Cote-Sierra, Guo, & Paul, 2006). However, GATA3 alone is not sufficient for IL-4 production, which also requires IL-2-mediated STAT5 activation for inducing and maintaining accessibility of the *il4* locus (Zhu & Paul, 2008).

Th17 cells are implicated in host defense against extracellular bacteria and fungi and have a crucial role in the induction of many autoimmune diseases. Th17 cells produce IL-17a, IL-17f, IL-21, and IL-22. IL-17 induces proinflammatory cytokines, including IL-6, IL-1, TNF- $\alpha$  as well as chemokines such as CXCL8, resulting in chemotaxis of inflammatory cells to sites of infection. During immune responses against extracellular bacteria and fungi, IL-17 promotes migration and activation of neutrophils. IL-21 provides an amplifying signal to further promote differentiation and proliferation of Th17 whereas IL-22 participates in mucosal host defense against pathogens. The initial step of Th17 differentiation requires TGF- $\beta$  and IL-6. IL-21 and IL-23 then play a crucial role in the proliferation and maintenance of the differentiated cells (Harrington et al., 2005; Zhu & Paul, 2008). The orphan nuclear receptor ROR $\gamma$ t was shown to be the key transcription factor for Th17 differentiation by inducing transcription of genes encoding IL-17 and IL-17f (Ivanov et al., 2006)

Regulatory T cells (Tregs), expressing CD4 and CD25 surface molecules, naturally arise from thymus and play a crucial role in maintaining self-tolerance as well as immune homeostasis after clearance of pathogens (Fontenot & Rudensky, 2004). Tregs suppress activation and proliferation of multiple immunocompetent cells using

various molecular and cellular mechanisms as reviewed by Shevach, 2009. Tregs secrete suppressive cytokines such as IL-10, IL-35, and TGF- $\beta$  that directly inhibit effector T cells, and may also function as cytotoxic T cells to kill these responder cells. Because of their high expression of CD25, the IL-2 receptor  $\alpha$  chain, Tregs are able to “steal” IL-2 from the responder T cells leading to cytokine-deprivation of these effector cells. Via these mechanisms, Tregs also inhibit the function of APCs and other innate immune cells (Shevach, 2009). CD4<sup>+</sup>CD25<sup>+</sup> Tregs can also be induced in the periphery from naïve CD4 T cells and are designated inducible Tregs (iTregs). TGF- $\beta$  is a key cytokine for iTreg differentiation in the periphery and also the development of Tregs in the thymus. Downstream TGF- $\beta$  signaling activates Smad3, which induces expression of Forkhead transcription factor Foxp3, a master regulator of Tregs (Tone et al., 2008). Foxp3 is specifically expressed in CD4<sup>+</sup>CD25<sup>+</sup> Tregs and is required for their development and function (Fontenot, Gavin, & Rudensky, 2003). The Scurfy mouse strain, which has a defective *Foxp3* gene exhibits hyperactivation of CD4 T cells and overproduction of proinflammatory cytokines. The mutant is lethal in hemizygous males within a month after birth. In humans, mutation of the *Foxp3* gene results in the fatal autoimmune disease, IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome), which is considered the human counterpart of Scurfy (Sakaguchi et al., 2006; Sakaguchi, Yamaguchi, Nomura, & Ono, 2008)

Th9 cells were initially characterized as a subset of Th2 producing IL-9 (Luckheeram et al., 2012). However, recent studies have shown that IL-9 is produced exclusively by a distinct population of Th cells, designated Th9. In vitro, Th9 can be

generated by polarizing naïve CD4 T cells with TGF- $\beta$  and IL-4, during antigenic stimulation or with anti-CD3/CD28 antibodies (Tan & Gery, 2012). It has been suggested that Th9 may contribute to allergic diseases. IL-9 has been demonstrated to increase after allergen challenge in asthmatic patients (Erpenbeck et al., 2003). Studies in a mouse model of allergic disease also emphasize the importance of IL-9 in anaphylaxis (Soroosh & Doherty, 2009). However, more research needed to classify Th9 as a distinct subset from Th2.

Follicular helper T cells (Tfh) migrate into B cell follicles and provide cognate help during the processes of somatic hypermutation, class switch recombination, and affinity maturation of activated B cells to produce plasma cells that secrete high affinity antibodies (King, 2009). Differentiation of Tfh involves IL-6 and IL-21 but unlike Th17, TGF- $\beta$  or ROR $\gamma$ t does not participate in the process. STAT3 as well as Bcl6 are expressed in Tfh and shown to play an important role in the downstream signaling (Luckheeram et al., 2012)

#### *Role of CD4 lymphocytes in hypertension*

In Ang II-infused hypertensive rats with kidney injury, there is an increase in production of Th1 cytokine IFN $\gamma$  and a decrease in production of Th2 cytokine IL-4. The number of IFN $\gamma$ -secreting T cells is also elevated with Ang II infusion. Administration of the AT1 receptor blocker, olmesartan, ameliorates the disease manifestation and restores the Th1/Th2 balance to normal. However, administration of non-specific vasodilator, hydralazine, does not affect the Th balance, despite comparable blood pressure reduction.

These results suggest that Ang II through AT1 receptor directly stimulates Th1, which may participate in the pathogenesis of Ang II induced kidney injury (Shao et al., 2003). Another study in hypertensive kidney injury also showed that Ang II induced Th1/Th2 imbalance is not restricted to splenocytes, since there is an accumulation of CD4 T cells and an increased IFN $\gamma$  expression in the kidneys of mice infused with Ang II (Crowley et al., 2008). Using a Cre-lox gene targeting strategy to specifically delete AT1 receptors from T lymphocytes, Zhang et al., 2012 showed that deficiency of AT1R on T cells exacerbates kidney injury during Ang II induced hypertension. In this study, the AT1 receptors on T cells seem to suppress Th1 differentiation in the setting of hypertension. Splenic T lymphocytes from mice lacking AT1 receptors on T cells have an increased expression of IFN $\gamma$  and TNF $\alpha$  as well as enhanced Tbet/GATA3 expression ratios during Ang II infusion, suggesting an induction of Th1 cells in these specific knockout mice (J. Zhang et al., 2012).

In Ang II-induced cardiac damage model, IFN $\gamma$  receptor knockout mice have reduced cardiac hypertrophy, cardiac macrophage and T cell infiltrates, and electric remodeling induced by Ang II infusion compared with wild type mice. However, inhibition of Th17 signaling with IL-23 receptor antibody or IL-17A antibody does not protect Ang II-induced cardiac damage and electrical remodeling in this study (Marko et al., 2012).

In the autoimmune disease multiple sclerosis and its animal model, experimental autoimmune encephalitis (EAE), the AT1 receptor is induced in myelin-specific CD4 T

cells during development of neuroinflammation. Blocking the effects of Ang II with ACE inhibitors or AT1R blockers suppresses Th1 and Th17 responses and stimulates suppressive CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells. Treatment with ACE inhibitors also prevents signs of EAE when administered prior to the EAE induction and reverses paralysis when administered during established EAE (Platten et al., 2009). These data indicate a significant role of Ang II in Th1/Th17-mediated autoimmune disease.

The role of Th17 in Ang II-induced hypertension and vascular dysfunction was also emphasized in a study using IL-17-deficient mice (Madhur et al., 2010). Ang II infusion leads to an increased IL-17 production from T cells as well as IL-17 protein levels in the aortas. IL-17-deficient mice have reduced blood pressure compared with wild type mice after 4 weeks of Ang II infusion; however, the initial hypertensive response is similar between two groups. The aortas of IL-17 deficient mice exhibit reduced vascular inflammation with a decrease in superoxide production and T cell infiltration in response to Ang II. Moreover, this study examined the serum level of IL-17 in diabetic patients and reported a significantly higher level in the patients with hypertension compared to those with normotension. Another clinical study in rheumatoid arthritis patients also indicates IL-17 as a potential predictor of microvascular function and arterial compliance (Marder et al., 2011). These previous studies suggest a crucial and yet complicated role of Th1 and Th17 in mediating Ang II-induced hypertension and target-organ damage.

*Protective roles of regulatory T cells in hypertension*

Regulatory T cells (Tregs) suppress immune activation, maintain immunological self-tolerance, and participate in autoimmune diseases, graft rejection, and tumor immunity. Recently, several studies suggest that Tregs may also have a role in hypertension and cardiovascular disease.

Kvakan et al., 2009 showed that adoptive transfer of isolated Tregs reduces Ang II-induced cardiac hypertrophy and fibrosis as well as improves susceptibility to ventricular arrhythmias induced by chronic Ang II exposure. Cardiac infiltrating macrophages, CD4 and CD8 T cells, and activated CD69+ cells, resulting from Ang II infusion, are also reduced with adoptive transfer of Tregs. However, blood pressure in response to Ang II infusion is not reduced with transferred Tregs in this study (Kvakan et al., 2009).

The potential roles of Tregs in vascular disease are supported by another publication in which adoptive transfer of Tregs prevents Ang II-induced hypertension and vascular injury (Barhoumi et al., 2011). In this study, mice received 3 adoptive transfers of Tregs (CD4+CD25+) at 2-week intervals prior to Ang II infusion. Ang II induced a progressive increase in systolic blood pressure that was reversed by adoptive transfer of Tregs. Adoptive transfer of Tregs also reduced impairment of artery endothelium-dependent relaxation, mesenteric artery stiffness, and oxidative stress, induced by Ang II infusion. Consistent with the cardiac effects, adoptive transfer of Tregs reduced macrophages and T cells infiltration in the aortas. The similar benefits of transferred Tregs are shown in coronary arteriolar endothelial dysfunction in Ang II-dependent

hypertensive mice (Matrougui et al., 2011). One proposed mechanism of Treg-mediated improvement of endothelial dysfunction is their ability to secrete the anti-inflammatory cytokine, IL-10, which in turn inhibits NADPH oxidase activity, causing accumulation of oxidative stress and vascular endothelial dysfunction (Kassan, Galan, Partyka, Trebak, & Matrougui, 2011). These studies together suggest the immunosuppressive effect of Tregs as a potential therapeutic target and a new direction to investigate vascular disease in hypertension.

#### *Immune system and hypertensive renal injury*

Hypertension is intimately related to the kidney. Dysregulation of kidney function is reported to be either a cause or consequence of hypertension. In salt-sensitive hypertension, subtle kidney injury induced by recurrent stimulation of the renin-angiotensin system (RAS) results in impaired sodium excretion and increased blood pressure (Oparil, Zaman, & Calhoun, 2003). Ang II is a crucial mediator that controls renal sodium excretion and blood pressure. The renal tissues also express a local RAS, which seems to be a major source of Ang II that regulates renal function. Increasing intrarenal Ang II production causes an increase in systemic blood pressure regardless of systemic Ang II level (Carey & Siragy, 2003). Accordingly, treatment of hypertension with ACE inhibitors or AT1 receptor antagonists produces pleiotropic effects on kidney beyond blood pressure reduction (Van Buren & Toto, 2013; X. Zhang et al., 2013). Therefore, it follows that the renal function in Ang II-dependent hypertension is of interest to investigators. Several immunological studies using Ang II-induced

hypertension have also addressed the pathogenic role of immune cells infiltrated to the kidney during the development of hypertension.

Crowley et al., 2008 showed that chronic administration of Ang II to uninephrectomized mice induces severe blood pressure elevation and kidney injury. Treatment with the immunosuppressive agent mycophenolate mofetil (MMF) significantly reduced urinary albumin excretion, ameliorated glomerulosclerosis, and reduced T cell infiltration into the renal interstitium. However, MMF did not show benefits on the severity of hypertension and cardiac hypertrophy in this study (Crowley et al., 2008). A subsequent study from the same group done in immunodeficient (scid) mice also indicates that the absence of lymphocytic activity protects the heart and kidney from Ang II dependent hypertension (Crowley et al., 2010). The scid mice have reduced hypertensive response to Ang II infusion and less cardiac hypertrophy. Also, during Ang II administration the lymphocyte deficiency decreases urine albumin excretion and increases sodium excretion, possibly by stimulation of endothelial nitric oxide synthase (eNOS), and cyclooxygenase-2 (COX-2) dependent pathways.

### *Conclusion*

Vascular remodeling and stiffening is directly correlated with hypertension. Vasculatures, including large elastic arteries, adapt themselves to sustained elevation of blood pressure by changing their geometry, structure, and function. This is to restore the arterial wall stress to homeostatic level and adjust arterial stiffness to an optimal value. Angiotensin (Ang) II, a main effector molecule in the renin-angiotensin system, plays a

crucial role in blood pressure regulation. In addition to regulating vascular tone, sodium balance, cell growth and hypertrophy, and fibrosis, Ang II activates adaptive immunity, promotes immune cell infiltration into target organs, and stimulates inflammatory responses. It has been increasingly appreciated that chronic inflammation and immune activation are principal features of hypertension and its complexity. Different subsets of T lymphocytes, including Th1, Th2, Th17 and suppressive regulatory T cells have been shown to be involved in arterial remodeling and target organ damage including cardiac hypertrophy and renal injury during the course of Ang II-dependent hypertension. Genetic immunocompromise, immunosuppressive treatment, and adoptive transfer of regulatory T cells have been shown to modulate the hypertensive response to Ang II, directly by preventing vascular remodeling or indirectly by ameliorating kidney injury. Understanding the underlying mechanism of immune cells in the pathogenesis of hypertension and vascular remodeling could lead to novel therapeutic targets for clinical treatment of hypertension and vascular diseases.

## **Explanation of the dissertation format**

Three original research papers prepared for publication are included in this dissertation as appendices. The overall objective is to define the roles of CD4<sup>+</sup> T lymphocytes as specific regulators of Ang II-induced vascular remodeling and stiffening based on the central hypothesis that proinflammatory T helper subsets, Th1 and Th17, are responsible for a stiffer vascular system by stimulating fibrosis and extracellular matrix alteration. In contrast, suppressive regulatory T cells counter this effect and prevent the vasculature remodeling process.

The same animal model of Ang II-induced hypertension was used in the three papers. An osmotic mini-pump was implanted subcutaneously to continuously release Ang II at 490 ng/kg/min for specific period of time. In order to assess vascular stiffness, the aortas from hypertensive mice were subjected to biaxial mechanical testing.

The first paper investigates the engagement of adaptive immune system in aortic remodeling using RAG1 knockout immunodeficient mice which produce no T and B cells. In this paper, the roles of each CD4 subsets are determined by rendering mice deficient in specific transcription factors for each CD4 subsets (Tbet for Th1, and ROR $\gamma$ t for Th17) as well as characterizing lymphocytic infiltrates in the aortas during the time course of Ang II infusion. To emphasize that the remodeling of large arteries is dependent on enhanced adaptive immunity, the second paper determines the role of regulatory T cells in preventing vascular remodeling. Regulatory T cells were stimulated and expanded *in vivo* by injecting IL-2 cytokine immune complexes prior to and during

the infusion of Ang II and the hypertensive and vascular responses to Ang II were determined.

Since renal homeostasis is also tightly regulated by Ang II we examined the kidney function in the Ang II-hypertensive mice. Surprisingly, in the wild type mice, infusion of Ang II increased glomerular filtration rate (GFR) as a predictive compensatory mechanisms; however, the GFR of the RAG1<sup>-/-</sup> mice was markedly decreased. This finding led us to further investigate the roles of lymphocytes in renal hemodynamic response to Ang II; the detail of this research is in the third paper.

## CHAPTER 2: PRESENT STUDY

The details of the research are presented in the appended papers (Appendix A, B, and C), and this chapter provides a summary of the methods, results, and significant findings from these papers.

### **Summary of paper 1: Role of CD4<sup>+</sup> Lymphocytes in Angiotensin II-Induced Arterial Hypertension, Remodeling, and Stiffness**

Potential roles of T lymphocytes in the process of cardiac and aortic remodeling have been suggested. This study, therefore, aimed to investigate a role of CD4<sup>+</sup> T lymphocyte subsets in this process. Wild type C57BL/6 (WT) and immunodeficient RAG1<sup>-/-</sup> (C57BL/6 background) mice were treated with [Val<sup>5</sup>] angiotensin II at 490 ng/kg/min via subcutaneously implantable osmotic mini-pumps. The mice were then sacrificed at 0, 3, 7, 14, and 21 days for analysis. Ang II infusion increased axial aortic stiffness measured by biaxial mechanical testing within 7 days of the treatment in the WT mice, but failed to do so in the RAG1<sup>-/-</sup> mice. Ang II-induced vascular remodeling defined as increased wall thickness and increased collagen accumulation was also reduced in the RAG1<sup>-/-</sup> mice, comparing to the WT. Characterizing T cell subsets in the peri-aortic infiltrates during the time course of Ang II infusion revealed a sequential activation of Th1 and Th17 responses. To define the specific roles of these T cell subsets, we used Tbet<sup>-/-</sup> and RORγt<sup>-/-</sup> mice, which are deficient in Th1 and Th17, respectively. There was no significant difference in hypertensive response to Ang II between the WT and the CD4-subset knockout mice. However, the aortic stiffness of the Tbet<sup>-/-</sup> at baseline

was significantly lower than that of the WT and did not increase after 7 days of Ang II infusion. The baseline aortic stiffness of the  $ROR\gamma t^{-/-}$  mice was comparable to that of the  $Tbet^{-/-}$ . Infusion of Ang II for 7 days, however, significantly increased the stiffness value of the Th17 deficient mice but the increased magnitude was much lower than the WT. In conclusion, this study has shown that remodeling of large elastic arteries is dependent on the adaptive immune system. In the Ang II-induced hypertension model, there is a temporal and sequential activation of Th1 and Th17 in the peri-vascular aortic infiltrates, which is associated with vascular stiffness and vascular structural and material alterations.

**Summary of paper 2: Modulation of Ang II-Induced Vascular Remodeling by *In Vivo* Stimulation of Regulatory T Cells with IL-2 Cytokine Complex**

Enhanced adaptive immunity is associated with vascular pathology and dysfunction in Ang II-induced hypertension. Inhibition of immune activation using pharmacological immunosuppressive agents or adoptive transfer of regulatory T cells (Tregs) has shown benefits in Ang II-induced hypertension and target-organ damage. In this study, we applied an alternative approach to stimulate proliferation of Tregs *in vivo* using cytokine immune complexes of IL-2 and anti-IL-2 monoclonal antibody clone JES6-1, abbreviated as IL-2/ $\alpha$ IL-2 in this paper. The IL-2/ $\alpha$ IL-2 mAb complex at a total dose of 6  $\mu$ g (5 $\mu$ g  $\alpha$ IL-2 mAb + 1  $\mu$ g IL-2) was injected intraperitoneally once a day for 5 consecutive days. [Val5] angiotensin II was then infused via osmotic mini-pumps on day 7 for two weeks. To maintain the expanded population of Tregs throughout the 14 days of

Ang II infusion, the IL-2/ $\alpha$ IL-2 complex was repeatedly given to the mice 3 times weekly. Treating mice with the IL-2/ $\alpha$ IL-2 complex markedly induced Tregs expansion in the spleens with no effect on total CD4<sup>+</sup> and CD8<sup>+</sup> cell numbers. Ang II infused mice that received the IL-2/ $\alpha$ IL-2 complex had significantly decreased vascular remodeling and stiffening compared with mice receiving Ang II alone. Stimulation of Tregs with the IL-2/ $\alpha$ IL-2 complex suppressed the Th1 and Th17 responses in the lymphoid organs and also reduced immune cell infiltration into the perivascular aortic infiltrates. This study provides data that support the protective roles of Tregs in vascular remodeling and stiffening and the use of IL-2/ $\alpha$ IL-2 mAb complex as a new potential therapeutic in Ang II-induced vascular diseases.

### **Summary of paper 3: The Effect of Angiotensin II on Renal Function is Modulated by CD4 Lymphocytes**

The essential roles of Ang II in regulating renal hemodynamics have been well established. Previous studies have indicated that lymphocytes are associated with renal injury in Ang II-induced hypertension. Consequently, in this study, we determined whether the CD4<sup>+</sup> T lymphocyte subsets play a role in the regulation of glomerular filtration rate (GFR) in response to Ang II. The GFR was progressively increased in the WT mice after 14 days of Ang II infusion as Ang II constricts renal arterioles with a higher sensitivity to efferent arterioles as well as stimulates secretion of vasodilatory substances. However, in the mice lacking T and B lymphocytes (RAG1<sup>-/-</sup>), the GFR in response to Ang II markedly decreased. The decreased GFR in the RAG1<sup>-/-</sup> was

associated with decreased urinary prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and increased thromboxane B<sub>2</sub> (a stable metabolite of thromboxane A<sub>2</sub>) levels, as compared with the WT mice. Adoptive transfer of CD4<sup>+</sup> T cells into the RAG1<sup>-/-</sup> mice restored their GFR and PGE<sub>2</sub> levels to that of the WT mice. Interestingly, the Th1-deficient (Tbet<sup>-/-</sup>) mice showed an increased GFR similar to the WT, whereas the Th17-deficient (RORγt<sup>-/-</sup>) mice exhibited a reduced GFR after Ang II infusion, similar to the RAG1<sup>-/-</sup>. The urinary PGE<sub>2</sub> levels were also decreased in the Th17-deficient mice but remained similar to the WT in the Th1-deficient mice. These results suggest a crucial role of Th17 in regulating renal hemodynamic response to Ang II, possibly by controlling secretion of vasodilatory substances such as PGE<sub>2</sub>.

### CHAPTER 3: GENERAL CONCLUSIONS AND PERSPECTIVES

It is conventionally accepted that hypertension produces vascular stiffening by inducing structural changes due to hemodynamic over-load. However, a recent Framingham cohort study done by Kaess et al., 2012 suggests that initial blood pressures are not associated with aortic stiffness and vascular stiffness is a precursor rather than a result of hypertension (Kaess et al., 2012). Similarly, in our study, infusion of angiotensin II (Ang II) into C57BL/6J wild type mice led to an immediate increase in aortic stiffness (at Day 7), which occurred prior to detectable vascular structural changes. The vascular remodeling, as evidenced by increased collagen accumulation and increased aortic wall thickness, then took place later to compensate for the hemodynamic stress and restore the increased stiffness toward baseline level. Interestingly, administration of Ang II failed to induce aortic stiffness and remodeling in the immunodeficient mice lacking T and B lymphocytes (RAG1<sup>-/-</sup>), suggesting that the vascular structural and mechanical responses to Ang II are dependent upon functional lymphocytes. Existing studies also indicate that lymphocytes, macrophages, and other immune cells are present in the vasculature and contribute to the formation of atherosclerotic lesions as well as remodeling of blood vessels (Schiffrin, 2012) and T lymphocytes play a crucial role in blood pressure regulation. Further studies regarding the precise mechanism of how immune cells affect the gene expression and protein levels of vascular connective tissues will be helpful to elucidate this concept.

In the Ang II-hypertensive model, there was a sequential activation of Th1 and Th17 in the perivascular aortic infiltrates that was temporally associated with vascular stiffness and vascular remodeling. Additionally, the initial increased stiffness induced by Ang II was prevented in the Th1-deficient (Tbet<sup>-/-</sup>) mice, whereas the compensatory structural remodeling was inhibited in the Th17-deficient (RORγt<sup>-/-</sup>) mice but the initial stiffness during the first week of Ang II infusion was preserved. These findings suggest that the Th1 and Th17 have a temporal and distinct association with the progression of vascular stiffness and remodeling respectively. In the autoimmune disease, multiple sclerosis, and its animal model, experimental autoimmune encephalitis (EAE), where Th1 and Th17 are critical in the pathogenesis, blocking the effects of Ang II with angiotensin-converting enzyme (ACE) inhibitor, lisinopril, or AT1 receptor blocker (ARB), candesartan, suppresses Th1 and Th17 responses and stimulates suppressive regulatory T cells. Treatment with lisinopril or candesartan also successfully prevents EAE and reduces severity of the disease in mice (Platten et al., 2009). It follows, therefore, that Ang II may be involved in the Th1/Th17-mediated vascular stiffening and remodeling in a similar fashion to other organ-specific autoimmune diseases.

ACE inhibitors and ARBs are commonly used by millions worldwide to lower blood pressure and slow the progression of cardiovascular and renal diseases. However, the drugs do not completely prevent the complications or reverse the pathology. As the previous clinical study by Kaess et al., 2012 and the presenting study suggest that vascular stiffening may precede hypertension, larger studies on ACE inhibitors and ARBs in Th1/Th17-mediated vascular stiffening would lead to a new therapeutic regimen for

these drugs as a prophylactic that may prevent the development of hypertension and its complications.

Previous studies have shown that increasing the number of suppressive regulatory T cells (Treg) in the circulatory system by adoptive transferring prevents Ang II-induced hypertension and cardiovascular complications (Kvakan et al., 2009). In this current study, *in vivo* stimulation of Treg lymphocytes with IL-2 cytokine complexes also ameliorated Ang II-induced vascular stiffness and remodeling, partly by inhibiting Th1 and Th17 responses. The IL-2 cytokine complex treatment effectively increased expansion of Tregs in the lymphoid compartment and the same protocol has been reproducible in other autoimmune diseases. Therefore, it is a promising technique that can be used to study the roles of Treg lymphocytes in other vascular diseases in which adaptive immunity may be involved in the pathology, such as Ang II-mediated microvascular thrombosis (Senchenkova, Russell, Kurmaeva, Ostanin, & Granger, 2011).

Although Treg lymphocytes clearly show benefits in Ang II-dependent hypertension, the underlying molecular mechanism has still been unclear. Treg lymphocytes suppress other immunocompetent cells by various molecular and cellular mechanisms. One proposed mechanism is the ability of Treg lymphocytes to secrete anti-inflammatory cytokine, IL-10, which inhibits oxidative stress cascades and vascular endothelial dysfunction (Matrougui et al., 2011). However, in another Ang II-induced hypertension study, plasma levels of IL-10 were not up-regulated with adoptive transfer

of Tregs (Barhoumi et al., 2011), suggesting that other mechanisms including cell-to-cell contact-dependent suppression may also be involved in the process.

Beside their critical role in the vascular response to Ang II, the present study provides additional evidence that lymphocytes also affect the renal hemodynamic response to Ang II-induced hypertension. The results of this study suggest that CD4<sup>+</sup> T lymphocytes, more specifically Th17 cells, play a critical role in regulating the renal response to Ang II by modulating the release of vasodilatory molecule, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). This evidence may help explain the mechanism whereby suppression of T cell activation with cyclosporine induces a decrease in GFR in both human and animal models and provides a cautionary notion that Th17 may have a protective role in renal function under conditions where renal perfusion pressure needs to be maintained.

In conclusion, the current study provides substantial evidences to extend the concept that the adaptive immune system contributes largely to the pathogenesis of hypertension and its complications. A better understanding in this concept would lead to a new potential target of treatment not only to adjust the hemodynamic response but to prevent and reverse the disease pathology.

### **Future directions**

As the present study indicates that Ang II-induced aortic stiffening is mediated by T lymphocytes, the future directions will be to elucidate the cellular and molecular mechanisms of this finding. More specifically, two future studies are proposed.

**1) Define the cellular mechanism of lymphocytes-mediated vascular stiffness in Ang II-hypertensive model.**

It has been understood that extracellular matrix proteins (ECM) including collagen and elastin, which are deposited in the artery wall, account for the majority of its mechanical properties (Wagenseil, J. E., & Mecham, R. P. 2009). However, in this study the aortic stiffening occurred prior to the accumulation of ECM. Therefore, it follows that different cell types in the vasculature, such as fibroblasts, myofibroblasts, and smooth muscle cells (VSMCs) may regulate the initial stiffness. A recent study suggested that increased intrinsic VSMC stiffness directly attributes to increased aortic stiffness in aging (Qiu, H. et al., 2010). It has been shown that Ang II induces expression of adhesion molecules on vascular smooth muscle cells, which contribute to lymphocytes accumulation (Braun, Pietsch, Schrör, Baumann, & Felix, 1999; Tummala et al., 1999). In addition, T-lymphocytes are then shown to induce VSMCs phenotypic change and stimulate their proliferation (Rolfe, Campbell, Smith, Cheong, & Campbell, 1995). Therefore, it could be hypothesized that aortic T cell infiltrates induced by Ang II modulate VSMCs phenotype, resulting in increasing their intrinsic stiffness, which is associated with the initial increased aortic stiffness.

In addition, Th17 deficient mice have reduced vascular remodeling induced by Ang II without lower blood pressure compared with WT mice, suggesting that immune-mediated Ang II induced vascular remodeling may be independent to blood pressure. To further define that vascular remodeling mediated by T lymphocytes is not due to

elevation of blood pressure, one proposed experiment is using non-specific vasodilator, hydralazine, which reduce blood pressure without affecting the immune balance. If the vascular remodeling induced by Ang II is not associated with hypertension, administration of hydralazine during Ang II infusion would not prevent the progression of remodeling.

**2) Determination whether the activation of adaptive immune system during the course of Ang II-induced hypertension is antigen-specific or a result of a direct effect of Ang II on T lymphocytes.** This question could be addressed by using antigen-specific transgenic mouse strains. This future study might as well lead to identifying a novel antigen that trigger the immune response during Ang II-induced hypertension in a similar manner to oxidized LDL and heat shock proteins that have been identified as major candidate antigens that gives rise to autoimmune responses in atherosclerosis (Shi, 2010).

## REFERENCES

- Abbas, Abul K., Lichtman, Andrew H., & Pillai, Shiv. (2007). *Cellular and molecular immunology*. Philadelphia: Saunders Elsevier.
- Alberts, B. (2002). *Molecular biology of the cell*. New York: Garland Science.
- Barhoumi, T., Kasal, D. A., Li, M. W., Shbat, L., Laurant, P., Neves, M. F., & Schiffrin, E. L. (2011). T regulatory lymphocytes prevent angiotensin II–Induced hypertension and vascular injury. *Hypertension*, *57*(3), 469-476.
- Belmadani, S., Zerfaoui, M., Boulares, H. A., Palen, D. I., & Matrougui, K. (2008). Microvessel vascular smooth muscle cells contribute to collagen type I deposition through ERK1/2 MAP kinase,  $\alpha$ v $\beta$ 3-integrin, and TGF- $\beta$ 1 in response to ANG II and high glucose. *American Journal of Physiology. Heart and Circulatory Physiology*, *295*(1), H69-76.
- Benetos, A., Adamopoulos, C., Bureau, J. M., Temmar, M., Labat, C., Bean, K., & Guize, L. (2002). Determinants of accelerated progression of arterial stiffness in normotensive subjects and in treated hypertensive subjects over a 6-year period. *Circulation*, *105*(10), 1202-1207.
- Benetos, A., Laurent, S., Asmar, R. G., & Lacolley, P. (1997). Large artery stiffness in hypertension. *Journal of Hypertension. Supplement : Official Journal of the International Society of Hypertension*, *15*(2), S89-97.

- Berry, C. L., Greenwald, S. E., & Rivett, J. F. (1975). Static mechanical properties of the developing and mature rat aorta. *Cardiovascular Research*, 9(5), 669-678.
- Braun, M., Pietsch, P., Schror, K., Baumann, G., & Felix, S. B. (1999). Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovascular Research* 41(2) 395-401.
- Carey, R. M., & Siragy, H. M. (2003). Newly recognized components of the renin-angiotensin system: Potential roles in cardiovascular and renal regulation. *Endocrine Reviews*, 24(3), 261-271.
- Castoldi, G., Di Gioia, C. R., Pieruzzi, F., D'Orlando, C., Van De Greef, W. M., Busca, G., & Stella, A. (2003). ANG II increases TIMP-1 expression in rat aortic smooth muscle cells in vivo. *American Journal of Physiology. Heart and Circulatory Physiology*, 284(2), H635-43.
- Chatziantoniou, C., Boffa, J. J., Ardaillou, R., & Dussaule, J. C. (1998). Nitric oxide inhibition induces early activation of type I collagen gene in renal resistance vessels and glomeruli in transgenic mice. role of endothelin. *The Journal of Clinical Investigation*, 101(12), 2780-2789.
- Che, Z. Q., Gao, P. J., Shen, W. L., Fan, C. L., Liu, J. J., & Zhu, D. L. (2008). Angiotensin II-stimulated collagen synthesis in aortic adventitial fibroblasts is mediated by connective tissue growth factor. *Hypertension Research : Official Journal of the Japanese Society of Hypertension*, 31(6), 1233-1240.

Chirinos, J. A. (2012). Arterial stiffness: Basic concepts and measurement techniques.

*Journal of Cardiovascular Translational Research*, 5(3), 243-255.

Crowley, S. D., Frey, C. W., Gould, S. K., Griffiths, R., Ruiz, P., Burchette, J. L., &

Coffman, T. M. (2008). Stimulation of lymphocyte responses by angiotensin II promotes kidney injury in hypertension. *American Journal of Physiology. Renal Physiology*, 295(2), F515-24.

Crowley, S. D., Song, Y. S., Lin, E. E., Griffiths, R., Kim, H. S., & Ruiz, P. (2010).

Lymphocyte responses exacerbate angiotensin II-dependent hypertension. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 298(4), R1089-97.

Dernellis, J., & Panaretou, M. (2005). Aortic stiffness is an independent predictor of

progression to hypertension in nonhypertensive subjects. *Hypertension*, 45(3), 426-431.

Dong, M., He, J., Wang, Z., Xie, X., & Wang, H. (2005). Placental imbalance of Th1-

and Th2-type cytokines in preeclampsia. *Acta Obstetrica Et Gynecologica Scandinavica*, 84(8), 788-793.

Erpenbeck, V. J., Hohlfeld, J. M., Discher, M., Krentel, H., Hagenberg, A., Braun, A., &

Krug, N. (2003). Increased expression of interleukin-9 messenger RNA after segmental allergen challenge in allergic asthmatics. *Chest*, 123(3 Suppl), 370S.

- Fleenor, B. S. (2012). Large elastic artery stiffness with aging: Novel translational mechanisms and interventions. *Aging and Disease*, 4(2), 76-83.
- Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *Nature Immunology*, 4(4), 330-336.
- Fontenot, J. D., & Rudensky, A. Y. (2004). Molecular aspects of regulatory T cell development. *Seminars in Immunology*, 16(2), 73-80.
- Franklin, S. S. (2005). Arterial stiffness and hypertension: A two-way street? *Hypertension*, 45(3), 349-351.
- Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., & Borden, W. B. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2013). Heart disease and stroke statistics-2013 update: A report from the american heart association. *Circulation*, 127(1), e6-e245.
- Gomez-Garre, D., Ruiz-Ortega, M., Ortego, M., Largo, R., Lopez-Armada, M. J., Plaza, J. J., & Egido, J. (1996). Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension*, 27(4), 885-892.
- Graham, R. M. (1994). Cyclosporine: Mechanisms of action and toxicity. *Cleveland Clinic Journal of Medicine*, 61(4), 308-313.

Greenwald, S. E., Moore, J. E., Jr, Rachev, A., Kane, T. P., & Meister, J. J. (1997).

Experimental investigation of the distribution of residual strains in the artery wall.

*Journal of Biomechanical Engineering*, 119(4), 438-444.

Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D., & Alexander, R. W. (1994).

Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circulation Research*, 74(6), 1141-1148.

Grinnell, F. (1994). Fibroblasts, myofibroblasts, and wound contraction. *The Journal of*

*Cell Biology*, 124(4), 401-404.

Guzik, T. J., Hoch, N. E., Brown, K. A., McCann, L. A., Rahman, A., Dikalov, S., &

Harrison, D. G. (2007). Role of the T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. *Journal of Experimental Medicine*, 204(10), 2449-2460.

Haque, M. Z., & Majid, D. S. A. (2011). High salt intake delayed angiotensin II–Induced

hypertension in mice with a genetic variant of NADPH oxidase. *American Journal of Hypertension*, 24(1), 114-118.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K.

M., & Weaver, C. T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology*, 6(11), 1123-1132.

- Hayashi, K., & Naiki, T. (2009). Adaptation and remodeling of vascular wall; biomechanical response to hypertension. *Journal of the Mechanical Behavior of Biomedical Materials*, 2(1), 3-19.
- Herrera, J., Ferrebuz, A., MacGregor, E. G., & Rodriguez-Iturbe, B. (2006). Mycophenolate mofetil treatment improves hypertension in patients with psoriasis and rheumatoid arthritis. *Journal of the American Society of Nephrology : JASN*, 17(12 Suppl 3), S218-25.
- Humphrey, J. D., Eberth, J. F., Dye, W. W., & Gleason, R. L. (2009). Fundamental role of axial stress in compensatory adaptations by arteries. *Journal of Biomechanics*, 42(1), 1-8.
- Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., & Littman, D. R. (2006). The orphan nuclear receptor ROR $\gamma$ t directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*, 126(6), 1121-1133.
- Kaess, B. M., Rong, J., Larson, M. G., Hamburg, N. M., Vita, J. A., Levy, D., Benjamin, E. J., Vasan, R. S., Mitchell, G. F. (2012). Aortic stiffness, blood pressure progression, and incident hypertension. *JAMA: the journal of the American Medical Association*, 308(9), 875-881.
- Kagan, H. M., & Sullivan, K. A. (1982). Lysyl oxidase: Preparation and role in elastin biosynthesis. *Methods in Enzymology*, 82 Pt A, 637-650.

- Kassan, M., Galan, M., Partyka, M., Trebak, M., & Matrougui, K. (2011). Interleukin-10 released by CD4(+)CD25(+) natural regulatory T cells improves microvascular endothelial function through inhibition of NADPH oxidase activity in hypertensive mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(11), 2534-2542.
- King, C. (2009). New insights into the differentiation and function of T follicular helper cells. *Nature Reviews Immunology*, *9*(11), 757-766.
- Kohu, K., Ohmori, H., Wong, W. F., Onda, D., Wakoh, T., Kon, S., & Satake, M. (2009). The Runx3 transcription factor augments Th1 and down-modulates Th2 phenotypes by interacting with and attenuating GATA3. *The Journal of Immunology*, *183*(12), 7817-7824.
- Kvakan, H., Kleinewietfeld, M., Qadri, F., Park, J. K., Fischer, R., Schwarz, I., & Elitok, S. (2009). Regulatory T cells ameliorate angiotensin II-induced cardiac damage. *Circulation*, *119*(22), 2904-2912.
- Li, Z., Froehlich, J., Galis, Z. S., & Lakatta, E. G. (1999). Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension*, *33*(1), 116-123.
- Liao, D., Arnett, D. K., Tyroler, H. A., Riley, W. A., Chambless, L. E., Szklo, M., & Heiss, G. (1999). Arterial stiffness and the development of hypertension. the ARIC study. *Hypertension*, *34*(2), 201-206.

- Lopez, B., Querejeta, R., Gonzalez, A., Beaumont, J., Larman, M., & Diez, J. (2009). Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure. *Hypertension*, *53*(2), 236-242.
- Luckheeram, R. V., Zhou, R., Verma, A. D., & Xia, B. (2012). CD4(+)T cells: Differentiation and functions. *Clinical & Developmental Immunology*, *2012*, 925135.
- Madhur, M. S., Lob, H. E., McCann, L. A., Iwakura, Y., Blinder, Y., Guzik, T. J., & Harrison, D. G. (2010). Interleukin 17 promotes angiotensin II–Induced hypertension and vascular dysfunction. *Hypertension*, *55*(2), 500-507.
- Marder, W., Khalatbari, S., Myles, J. D., Hench, R., Yalavarthi, S., Lustig, S., & Kaplan, M. J. (2011). Interleukin 17 as a novel predictor of vascular function in rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *70*(9), 1550-1555.
- Marko, L., Kvakan, H., Park, J. K., Qadri, F., Spallek, B., Binger, K. J., & Muller, D. N. (2012). Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage. *Hypertension*, *60*(6), 1430-1436.
- Matrougui, K., Zakaria, A. E., Kassan, M., Choi, S., Nair, D., Gonzalez-Villalobos, R. A., & Partyka, M. (2011). Natural regulatory T cells control coronary arteriolar endothelial dysfunction in hypertensive mice. *The American Journal of Pathology*, *178*(1), 434-441.

- Messerli, F. H., Williams, B., & Ritz, E. (2007). Essential hypertension. *Lancet*, 370(9587), 591-603.
- Montezano, A. C., & Touyz, R. M. (2012). Molecular mechanisms of hypertension--reactive oxygen species and antioxidants: A basic science update for the clinician. *The Canadian Journal of Cardiology*, 28(3), 288-295.
- Nataraj, C., Oliverio, M. I., Mannon, R. B., Mannon, P. J., Audoly, L. P., Amuchastegui, C. S., & Coffman, T. M. (1999). Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *The Journal of Clinical Investigation*, 104(12), 1693-1701.
- Olivetti, G., Anversa, P., Melissari, M., & Loud, A. V. (1980). Morphometry of medial hypertrophy in the rat thoracic aorta. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, 42(5), 559-565.
- Oparil, S., Zaman, M. A., & Calhoun, D. A. (2003). Pathogenesis of hypertension. *Annals of Internal Medicine*, 139(9), 761-776.
- Owens, G. K., Rabinovitch, P. S., & Schwartz, S. M. (1981). Smooth muscle cell hypertrophy versus hyperplasia in hypertension. *Proceedings of the National Academy of Sciences of the United States of America*, 78(12), 7759-7763.
- Platten, M., Youssef, S., Hur, E. M., Ho, P. P., Han, M. H., Lanz, T. V., & Strominger, J. L. (2009). Blocking angiotensin-converting enzyme induces potent regulatory T

cells and modulates TH1- and TH17-mediated autoimmunity. *Proceedings of the National Academy of Sciences of the United States of America*, 106(35), 14948-14953.

Qin, Z. (2008). Newly developed angiotensin II-infused experimental models in vascular biology. *Regulatory Peptides*, 150(1-3), 1-6.

Qiu, H., Zhu, Y., Trzeciakowski, J. P., Gansner, M., Depre, C., Resuello, R. R., Natividad, F. F., Hunter, W. C., Genin, G. M., Elson, E. L., Vatner, D. E., Meininger, G. A., & Vatner, S. F. (2010). Short communication: vascular smooth muscle cell stiffness as a mechanism for increased aortic stiffness with aging. *Circulation Research*, 107(5), 615-619.

Rengarajan, J., Szabo, S. J., & Glimcher, L. H. (2000). Transcriptional regulation of Th1/Th2 polarization. *Immunology Today*, 21(10), 479-483.

Roach, M. R., & Burton, A. C. (1957). The reason for the shape of the distensibility curves of arteries. *Canadian Journal of Biochemistry and Physiology*, 35(8), 681-690.

Rodriguez-Iturbe, B., Pons, H., Quiroz, Y., Gordon, K., Rincon, J., Chavez, M., & Johnson, R. J. (2001). Mycophenolate mofetil prevents salt-sensitive hypertension resulting from angiotensin II exposure. *Kidney International*, 59(6), 2222-2232.

- Rolfe, B. E., Campbell, J. H., Smith, N. J., Cheong, M. W., & Campbell, G. R. (1995). T lymphocytes affect smooth muscle cell phenotype and proliferation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *15*(8), 1204-1210.
- Ruiz-Ortega, M., Lorenzo, O., Rupérez, M., Esteban, V., Suzuki, Y., Mezzano, S., & Egido, J. (2001). Role of the renin-angiotensin system in vascular diseases: Expanding the field. *Hypertension*, *38*(6), 1382-1387.
- Safar, M. E., Blacher, J., Mourad, J. J., & London, G. M. (2000). Stiffness of carotid artery wall material and blood pressure in humans: Application to antihypertensive therapy and stroke prevention. *Stroke*, *31*(3), 782-790.
- Saito, S., Sakai, M., Sasaki, Y., Tanebe, K., Tsuda, H., & Michimata, T. (1999). Quantitative analysis of peripheral blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia. *Clinical and Experimental Immunology*, *117*(3), 550-555.
- Saito, S., Umekage, H., Sakamoto, Y., Sakai, M., Tanebe, K., Sasaki, Y., & Morikawa, H. (1999). Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with preeclampsia. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, *41*(5), 297-306.
- Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., & Nomura, T. (2006). Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews*, *212*, 8-27.

- Sakaguchi, S., Yamaguchi, T., Nomura, T., & Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell*, *133*(5), 775-787.
- Seaberg, E. C., Munoz, A., Lu, M., Detels, R., Margolick, J. B., & Riddler, S. A. (2005). Association between highly active antiretroviral therapy and hypertension in a large cohort of men followed from 1984 to 2003. *AIDS (London, England)*, *19*(9), 953-960.
- Schiffrin, E. L. (2012). Immune modulation of resistance artery remodeling. *Basic & Clinical Pharmacology & Toxicology*, *110*(1), 70-72.
- Senchenkova, E. Y., Russell, J., Kurmaeva, E., Ostanin, D., & Granger, D. N. (2011). Role of T lymphocytes in angiotensin II-mediated microvascular thrombosis. *Hypertension*, *58*(5), 959-965.
- Shadwick, R. E. (1999). Mechanical design in arteries. *The Journal of Experimental Biology*, *202*(Pt 23), 3305-3313.
- Shao, J., Nangaku, M., Miyata, T., Inagi, R., Yamada, K., Kurokawa, K., & Fujita, T. (2003). Imbalance of T-cell subsets in angiotensin II–Infused hypertensive rats with kidney injury. *Hypertension*, *42*(1), 31-38.
- Shevach, E. M. (2009). Mechanisms of Foxp3+ T regulatory cell-mediated suppression. *Immunity*, *30*(5), 636-645.

- Song, L., Leung, C., & Schindler, C. (2001). Lymphocytes are important in early atherosclerosis. *The Journal of Clinical Investigation*, *108*(2), 251-259.
- Soroosh, P., & Doherty, T. A. (2009). Th9 and allergic disease. *Immunology*, *127*(4), 450-458.
- Staessen, J. A., Wang, J., Bianchi, G., & Birkenhager, W. H. (2003). Essential hypertension. *Lancet*, *361*(9369), 1629-1641.
- Tan, C., & Gery, I. (2012). The unique features of Th9 cells and their products. *Critical Reviews in Immunology*, *32*(1), 1-10.
- Tao, X., Constant, S., Jorritsma, P., & Bottomly, K. (1997). Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *Journal of Immunology (Baltimore, Md.: 1950)*, *159*(12), 5956-5963.
- Tone, Y., Furuuchi, K., Kojima, Y., Tykocinski, M. L., Greene, M. I., & Tone, M. (2008). Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nature Immunology*, *9*(2), 194-202.
- Van Buren, P. N., & Toto, R. D. (2013). The pathogenesis and management of hypertension in diabetic kidney disease. *Medical Clinics of North America*, *97*(1), 31-51.
- van der Slot, A. J., van Dura, E. A., de Wit, E. C., De Groot, J., Huizinga, T. W., Bank, R. A., & Zuurmond, A. M. (2005). Elevated formation of pyridinoline cross-links by

profibrotic cytokines is associated with enhanced lysyl hydroxylase 2b levels.

*Biochimica Et Biophysica Acta*, 1741(1-2), 95-102.

van der Slot, A. J., Zuurmond, A. M., van den Bogaardt, A. J., Ulrich, M. M., Middelkoop, E., Boers, W., & Bank, R. A. (2004). Increased formation of pyridinoline cross-links due to higher telopeptide lysyl hydroxylase levels is a general fibrotic phenomenon. *Matrix Biology : Journal of the International Society for Matrix Biology*, 23(4), 251-257.

van Popele, N. M., Grobbee, D. E., Bots, M. L., Asmar, R., Topouchian, J., Reneman, R. S., & Witteman, J. C. (2001). Association between arterial stiffness and atherosclerosis: The rotterdam study. *Stroke; a Journal of Cerebral Circulation*, 32(2), 454-460.

van Varik, B. J., Rennenberg, R. J., Reutelingsperger, C. P., Kroon, A. A., de Leeuw, P. W., & Schurgers, L. J. (2012). Mechanisms of arterial remodeling: Lessons from genetic diseases. *Frontiers in Genetics*, 3, 290.

Wagenseil, J. E., & Mecham, R. P. (2009). Vascular extracellular matrix and arterial mechanics. *Physiological Reviews*, 89(3), 957-989.

Waller, B. F., Orr, C. M., Slack, J. D., Pinkerton, C. A., Van Tassel, J., & Peters, T. (1992). Anatomy, histology, and pathology of coronary arteries: A review relevant to new interventional and imaging techniques--part I. *Clinical Cardiology*, 15(6), 451-457.

- Watts, S. W., Rondelli, C., Thakali, K., Li, X., Uhal, B., Pervaiz, M. H., & Fink, G. D. (2007). Morphological and biochemical characterization of remodeling in aorta and vena cava of DOCA-salt hypertensive rats. *American Journal of Physiology. Heart and Circulatory Physiology*, 292(5), H2438-48.
- Wohlfahrt, P., Krajcoviechova, A., Seidlerova, J., Galovcova, M., Bruthans, J., Filipovsky, J., & Cifkova, R. (2013). Lower-extremity arterial stiffness vs. aortic stiffness in the general population. *Hypertension Research : Official Journal of the Japanese Society of Hypertension*, 31(6), 1233-1240
- Zhang, J., Patel, M. B., Song, Y., Griffiths, R., Burchette, J., Ruiz, P., & Crowley, S. D. (2012). A novel role for type 1 angiotensin receptors on T lymphocytes to limit target organ damage in hypertension. *Circulation Research*, 110(12), 1604-1617.
- Zhang, X., Eirin, A., Li, Z. L., Crane, J. A., Krier, J. D., Ebrahimi, B., & Lerman, L. O. (2013). Angiotensin receptor blockade has protective effects on the poststenotic porcine kidney. *Kidney International*,
- Zhu, J., & Paul, W. E. (2008). CD4 T cells: Fates, functions, and faults. *Blood*, 112(5), 1557-1569.
- Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L., & Paul, W. E. (2006). GATA-3 promotes Th2 responses through three different mechanisms: Induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Research*, 16(1), 3-10.

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations\*. *Annual Review of Immunology*, 28(1), 445-489.

Zieman, S. J., Melenovsky, V., & Kass, D. A. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(5), 932-943.

**APPENDIX A - ROLE OF CD4<sup>+</sup> LYMPHOCYTES IN ANGIOTENSIN II  
INDUCED ARTERIAL HYPERTENSION, REMODELING, AND STIFFNESS**

“Paper was prepared to submit to Hypertension”

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## ABSTRACT

Potential roles of T lymphocytes in a process of cardiac and aortic remodeling have been suggested. This study, therefore, aimed to investigate the engagement of CD4<sup>+</sup> T lymphocyte subsets in this process. Wild type C57BL/6 (WT) and immunodeficient RAG1<sup>-/-</sup> mice were administered with [Val<sup>5</sup>] angiotensin II (Ang II) via osmotic minipumps. Ang II infusion caused increased vascular stiffness within 7 Days in WT mice and demonstrated no change in the immunodeficient RAG1<sup>-/-</sup> mice. We saw an increase in Th1-lymphocytes that was associated with an initial increase in vascular stiffness and a subsequent activation of Th17 which was associated with the development of vascular remodeling. To define the specific roles of these T cell subsets, we used Tbet<sup>-/-</sup> and RORγt<sup>-/-</sup> mice, which are deficient in Th1 and Th17, respectively. There was no significant difference in hypertensive response to Ang II between the WT and the CD4-subset knockout mice. The initial increase in vascular stiffness on day 7 was prevented in the Ang II infused Tbet<sup>-/-</sup> mice. In the RORγt<sup>-/-</sup> (Th17-deficient) mice, the Ang II induced vascular structural remodeling was inhibited but the peak stiffness observed on day 7 was preserved. In conclusion, this study has shown that remodeling of large elastic arteries is dependent on the adaptive immune system. In the Ang II-induced hypertension model, there is a temporal and sequential activation of Th1 and Th17 in the peri-vascular aortic infiltrates, which is associated with vascular stiffness and vascular structural and material alterations.

## 1. INTRODUCTION

It is generally accepted that arterial hypertension is directly associated with vascular remodeling, which is dependent upon a multicellular network including vascular smooth muscle cells (VSMC), vascular extracellular matrix, and vascular endothelial cells (VEC). The biomechanical properties of remodeled vessels, in particular the stiffness of larger arteries, are dependent on the structural and material properties of the vessel. Vascular fibrosis has been related to renin-angiotensin-aldosterone system (RAAS) through the angiotensin II (Ang II) receptor type 1 (AT1 receptor). The AT1R is known to induce collagen production by the VSMCs (Ford et al., 1999) and adventitial fibroblasts (Che et al., 2008) through stimulation of TGF- $\beta$  and its downstream mediator connective tissue growth factor (CTGF) (Rodriguez-Vita et al., 2005). Therefore vascular remodeling is an active process of structural and material changes that involves alterations in cellular processes, including growth, apoptosis, migration, inflammation, and production of extracellular matrix (ECM) proteins.

Vasculature adaptations to hypertension involve predictable adaptive remodeling in order to restore the vascular biomechanical stress and strain relationships to the ideal values (Hayashi and Naiki, 2009). Also, hemodynamic forces alone have been reported to modulate the size and thickness of blood vessels through induction of structural alterations of the vessel wall (Langille and Dajnowiec, 2005) which may take days. However cytoskeletal and cellular modifications and rearrangements of ECM may occur in minutes to hours (Humphrey, 2008b). The fibroblast actin cytoskeleton provides tissue

traction through attachment to the ECM to enable the ECM adaptive remodeling (Tomasek et al., 2002). Therefore vascular remodeling involves matrix to matrix, cell to cell, and cell to matrix interactions that collectively alter the biomechanical properties in response to hemodynamic over-load.

Existing evidence supports the central roles of adaptive immune system, particularly T-lymphocytes, in cardiovascular pathology. In Ang II-induced hypertension, immunodeficient mice have a blunted hypertensive response (Guzik et al., 2007) and adoptive transfer of suppressive regulatory T (Treg) lymphocytes reduces hypertension (Barhoumi et al., 2011). Besides, T-lymphocytes express AT1 receptors and also produce Ang II which may in turn relate to the lymphocyte cardiovascular dependency (Hoch et al., 2009). In terms of remodeling, previous studies have also shown that selective stimulation of CD4<sup>+</sup> lymphocytes induces cardiac remodeling (Yu et al., 2010) and adoptive transfer of Treg lymphocytes reduces cardiac remodeling (Kvakan et al., 2009). In the Ang II-induced hypertension study contained herein, we determined the role of CD4<sup>+</sup> lymphocyte subsets in vascular structural remodeling and vascular stiffness. We revealed a sequential activation of Th1 and Th17 in the perivascular aortic infiltrates that was temporally associated with vascular stiffness and vascular remodeling.

## **2.0 MATERIALS AND METHODS**

### **2.1. Animals**

C57BL/6, RAG1<sup>-/-</sup>, and RORγt<sup>-/-</sup> male 10 week old mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All the RAG1<sup>-/-</sup> and RORγt<sup>-/-</sup> mice used in this

study were C57BL/6 background. This study was approved by the University of Arizona Animal Care Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the mice were maintained in the animal facility of the University of Arizona and fed with NIH-31 Modified Open Formula Mouse/Rat Sterilize Diet from Harlan Laboratories. The mice were treated with [Val<sup>5</sup>] angiotensin II (Sigma Chemical Co) at 490 ng/min/kg via subcutaneously implantable osmotic pumps (model 1004; Alzet, Palo Alto, Calif., USA) and sacrificed at 0, 3, 7, 14, or 21 days after angiotensin II infusion. For 3 day treatment, we used catheter applications with Alzet pumps that permitted a seven day delay in the administration of angiotensin II to allow a surgical recovery.

## **2.2. Determination of blood pressure**

Systolic and diastolic blood pressure was monitored during Angiotensin infusion 2 times a week before the sacrificing dates by the tail cuff method using Hatteras system.

Twenty-four hour continuous blood pressure was also measured in a separate group of mice using a biotelemetry device (Data Sciences International, St. Paul, MN). The biotelemetry mice were anesthetized with isoflurane and the transmitter was positioned at the right flank. The mice were allowed to recover for 7 days before treated as described above and then the blood pressure was recorded daily throughout the treatment.

## **2.3. Perfusion Fixation and Histological Staining**

Before perfusion fixation, the mice were anti-coagulated with subcutaneously injection of 100  $\mu$ l of 1,000 USP units/ml Heparin Sodium. Thirty mL of saline was infused to remove vascular blood, followed by the administration of 30 mL of 3% glutaraldehyde in 10% formaldehyde solution at a constant perfusion pressure of 40 mmHg to prevent fixation contraction. The tissues were paraffin embedded, cut at 5  $\mu$ m, and stained with H&E, Masson's Trichrome, Verhoeff's elastin stain and Picrosirius red collagen stains. Image analysis was performed using NIH ImageJ software to quantify aortic dimension and the tissue collagen related to the angiotensin II treatments.

#### **2.4. Flow cytometry**

Subsequent to isolation of aortic lymphocytes with lymphocyte gradient separation medium (Mediatech Inc, Herndon, VA), lymphocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) 10 ng/ml, ionomycin 1  $\mu$ g/ml and Brefeldin A 10  $\mu$ g/ml for 5 h. Surface staining was performed before permeabilization using perm/wash buffer (BD biosciences, san Diego, CA). Permeabilized cells were subsequently incubated with transcription factors antibodies. Efluor 450-conjugated anti-CD3, FITC-conjugated anti-CD4, Percp-Cy5.5-conjugated anti-CD8a, APC-conjugated anti-CD25, PE-conjugated anti-FOXP3, Percp-Cy5.5-conjugated anti-tbet, PE-conjugated anti-Gata3 were purchased from eBioscience. PE-conjugated anti-ROR $\gamma$ T was from R&D system. The BD LSR II with BD FACSDiva software Flow cytometry system and Gatelologic software were used to analyze data.

#### **2.5. Aortic tissue in filtrate flow cytometry**

Aortas were isolated, flushed with PBS, and cut into pieces. Liberase® collagenase was used to digest the tissues and release the cellular infiltrates. The aortic lymphocytes were then isolated using Lymphocyte Gradient Separation medium and then phenotyped with intracellular flow cytometry as described above.

## **2.6. Biomechanics**

Biomechanical analyses of the thoracic aortas were performed using the microbiaxial optomechanical device (MOD), previously demonstrated by Keyes *et al* (Keyes et al., 2011). Aortas were cannulated with glass microcapillaries, secured with cyanoacrylate, and fixed onto the testing MOD device. Aortas were tested at 37°C in a Ca<sup>2+</sup> free solution, and subjected to cyclic increases of axial strains up to 15% of its maximum stretch while being held at an intraluminal pressure of 0, 70, and 130 mmHg.

Correspondingly, circumferential analysis of the vessels was acquired with pressurizes from 0 to 130 mmHg cyclically while held at axial strains of 0, 15, and 30%. Axial force, axial strain, circumferential intraluminal pressure, and circumferential outer diameter were acquired over the course of testing and the second Piola Kirchhoff stress and Green's strains were calculated as previously demonstrated (Keyes et al., 2011). The maximum tangential modulus was taken as the maximum slope of each stress-stretch as reported (Di Martino et al., 2006).

## **2.7. Statistics**

ANOVA with multicomparison procedures was used to test the difference among the defined groups with SPSS version 11.5. Values obtained from treatment groups were

compared with control values using Student's t-test. Comparable non-parametric tests (Kruskal-Wallis and the rank sum test) were substituted when test for normality and equal variance failed. All data are reported as means  $\pm$  SE.

### 3.0 RESULTS

#### 3.1. RAG1<sup>-/-</sup> mice have reduced vascular stiffness induced by Ang II.

Descending thoracic to suprarenal aortic segments were harvested from the mice and immediately subjected to biaxial analysis to derive the axial and circumferential stress-strain relationships. These stress-strain responses represent the passive properties of the aortas since the aortas are immersed in calcium free saline. Figure 1A and 1B show representative axial stress-strain plots of WT and RAG1<sup>-/-</sup> mice after Ang II infusion at four defined time points. Similarly samples of circumferential stress-strain plots from the WT and the RAG1<sup>-/-</sup> are shown in figures 1C and 1D respectively. Axial and circumferential Young elastic modulus or vascular stiffness values were derived from the peak stress-strain plots. Figure 1E shows that the mean axial elastic modulus in the WT increased by 65% at 3 days and by 2-fold on Day 7. After Day 7 the axial elastic modulus progressively declines toward control levels by Day 21. The axial stiffness pattern in the RAG1<sup>-/-</sup> remained constant throughout the 21 day study period. The circumferential stiffness remained unchanged with Ang II infusion in the WT and slightly increased in the RAG1<sup>-/-</sup> mice (Fig. 1F). These data suggest that the Ang II initially induces vascular stiffness which is later restored toward homeostatic levels by the increase in aortic remodeling. However, this compensatory adaptation was impaired in

the immunodeficient mice as we observed no significant changes in vascular stiffness or remodeling in response to Ang II in the RAG1<sup>-/-</sup> mice.

### **3.2. Hemodynamic and vascular remodeling in response to 21 days of Ang II-induced hypertension: A comparison of WT and RAG1<sup>-/-</sup>**

Next, we compared the hemodynamic effect of [Val<sup>5</sup>]-angiotensin II (Ang II), the AT<sub>1</sub> receptor agonist (Zou et al., 1998), in WT and RAG1<sup>-/-</sup> mice. Using tail cuff measurements, both strains of mice developed hypertension however at 20 days, the RAG1<sup>-/-</sup> peak systolic pressures were 20 mmHg lower (Fig 2A) and the diastolic pressures were 33 mmHg lower (Fig 2B) than the WT. Biotelemetry of unrestrained mice supports the hypertensive states measured by tail cuff method (Fig 2C).

Aortic remodeling in response to Ang II infusion in the RAG1<sup>-/-</sup> mice were then determined, compared with the WT mice. Figure 2D shows a time dependent increase in adventitial thickness in the WT group which increased 4-fold above control on day 21, whereas less than 1.5-fold increase was observed in the RAG1<sup>-/-</sup> mice over the same period of time. Similarly, there was a 2-fold increase in medial thickness in the WT mice compared with a less than 1.5-fold increase in the RAG1<sup>-/-</sup> (Fig 2E). Inner aortic diameter measurement showed the aortic dilation in the RAG1<sup>-/-</sup> in response to Ang II, compared to the WT (Fig 2F). Figure 2G shows representative histological examples of the aortas from WT and RAG1<sup>-/-</sup> mice infused with Ang II over 21 days. In summary, AT<sub>1</sub> receptor stimulation induced structural changes in WT mice defined by increases in vascular

remodeling and collagen deposition. These effects were unquestionably reduced in immune-deficient mice.

### **3.3. Ang II stimulation induces an early Th1 response with a latter Th17 response in peri-aortic lymphocytic infiltrates**

The above findings prompted us to determine the time-dependent immune response to Ang II. Since others have shown that adoptive transfer of T but not B lymphocytes restores hypertensive response in immunodeficient mice (Guzik et al., 2007), we focused on characterizing T-cells responses in our study. Lymphocytes harvested from aortic infiltrates at set time points of Ang II infusion were analyzed with flow cytometry. The lymphocytes were gated on CD3<sup>+</sup> cells and from CD3<sup>+</sup> population to CD4<sup>+</sup> and CD8<sup>+</sup> (Fig 3A). In the aortic infiltrates, there was an increase in CD4<sup>+</sup> and a substantial decrease in the aortic CD8<sup>+</sup> lymphocytes infiltrates spanning days 3 to 14 of Ang II infusion (Fig 3B). We next characterized CD4 subsets in the aortic infiltrates during Ang II treatment using their respective transcriptional factors. On day 3 of Ang II infusion, there was a brief but striking increase in Th1 (CD4<sup>+</sup>T-bet<sup>+</sup>) lymphocytes, from 20 to 58% in the aortic tissues (Fig 3C), with no significant change in aortic Th2 (CD4<sup>+</sup>Gata-3<sup>+</sup>) lymphocytes (Fig 3D). This pattern of Th1 expression suggests an immediate induction of a pro-inflammatory as a consequence of the Ang II infusion. The Th17 (CD4<sup>+</sup> RORγt<sup>+</sup>) lymphocytes revealed a second and gradual response to Ang II infusion with about 2.5-fold increase in the percentage of CD4<sup>+</sup>RORγt<sup>+</sup> cells in the aortic lymphocytic infiltrates on day 21 (Figure 3E). Interestingly, the Treg lymphocytes

(CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) remained constant in the aortic infiltrate (Figure 3F). Samples of flow cytometric histograms of each CD4 subsets are shown in Fig 3G and 3H. These results suggest that there is an early and chronic induction of pro-inflammatory CD4<sup>+</sup> lymphocytes mediated sequentially by the Th1 and Th17 lymphocytes in response to Ang II infusion.

#### **3.4. Tbet<sup>-/-</sup> (Th1 deficient) mice lack the Day 7 increase in stiffness**

Due to the observed increase in both the vascular stiffness and aortic CD4<sup>+</sup>Tbet<sup>+</sup> lymphocytic infiltrates on Days 3 and 7, we determined if the stiffness was dependent upon the CD4<sup>+</sup>Tbet<sup>+</sup> lymphocytic infiltrates. Using Tbet<sup>-/-</sup> mice with a C57BL/6J background, figure 4A shows that the systolic blood pressure response to Ang II infusion of the Tbet<sup>-/-</sup> mice was equivalent with the WT (Fig 4A). The increased vascular elastic modulus on Day 7 was prevented in the Ang II infusion Tbet<sup>-/-</sup> mice, which suggests that this vascular stiffness is Th1 lymphocytes dependent (Fig. 4B).

#### **3.5. RORγt<sup>-/-</sup> (Th17 deficient) mice become hypertensive with chronic Ang II stimulation however without aortic tissue remodeling.**

We observed that the Th17 (CD4<sup>+</sup>RORγt<sup>+</sup>) lymphocytes were chronically increased during the second and third week period of Ang II-induced hypertension (Fig. 3E) which coincided with the period of vascular remodeling (Fig. 2). Additionally, IL-17<sup>-/-</sup> mice develop Ang II-induced hypertension however like the RAG1<sup>-/-</sup>, the IL-17<sup>-/-</sup> mice are less hypertensive at 2 and 3 weeks compared with the control mice (Madhur et al., 2010). Therefore, we sought to determine the effect of Ang II infusion on vascular

remodeling in the  $ROR\gamma t^{-/-}$  mice, which lack tissue-infiltrating Th17 cells and have attenuated autoimmune diseases (Ivanov et al., 2006). We observed that the  $ROR\gamma t^{-/-}$  became hypertensive similar to that of the WT (Fig 5A). Unlike the  $Tbet^{-/-}$  mice, infusion of Ang II for 7 days, significantly increased the axial elastic modulus of the  $ROR\gamma t^{-/-}$  mice. However, the increased magnitude was significantly lower than the WT (Fig. 5B). Interestingly, the  $ROR\gamma t^{-/-}$  mice had marked reduced levels of medial hyperplasia (Fig 5C) and adventitial thickening (Fig 5D) compared with the WT. This experiment implies that the Th17 lymphocyte is an essential cell type involved in vascular remodeling induced by Ang II.

#### 4.0 DISCUSSION

Previous studies suggest that the integrity of the immune system is necessary for AT1R induction of hypertension (Guzik et al., 2007; Kasal et al., 2012; Madhur et al., 2010). Also  $CD4^{+}$  lymphocyte is critical in various models of cardiac remodeling (Yu et al., 2010; Kvakan et al., 2009). It follows therefore, that the  $CD4^{+}$  lymphocyte may be essential in the vascular remodeling processes and the resulting stiffness as well. The primary objective of this research is to examine the relationship among the  $CD4^{+}$  lymphocyte subsets in the molecular, mechanical, and physical properties of AT1R mediated vascular remodeling. We have shown that remodeling of aorta resulting in vascular stiffness is dependent upon the adaptive immune system. More specifically, chronic administration of the angiotensin II type 1 receptor (AT1R) agonist causes both vascular structural and material remodeling and altered biomechanical properties that are

dependent upon the sequential infiltration of Th1 and Th17 lymphocytes into aortas. This investigation is clinically meaningful since arterial stiffness is significantly and independently associated with end organ damage and cardiovascular diseases as reviewed by Ziemann et al (Ziemann et al., 2005).

Classically, vascular remodeling is understood to be an adaptive response of the vessel wall to compensate for hemodynamic stresses. The altered material and structural modifications occurring in the course of vascular remodeling takes place in order to reestablish ideal biomechanical stress or strain values (Humphrey, 2008a). Though the structural factors that control vascular remodeling are inadequately understood; the three cells types located in the vasculature, namely; the vascular endothelial cell, smooth muscle cell, and adventitial fibroblast, have the capacity to respond to hormonal stimulation and/or mechanical stretch. The material changes are dependent on the relative concentrations of primarily collagen and elastin scaffolding proteins. Ang II, through the AT1 receptor, causes the production of collagen type I by the vascular smooth muscle cell (Ford et al., 1999), adventitial fibroblast (Che et al., 2008) and stimulates TGF- $\beta$  which also elicits collagen synthesis. However, we observed that the aortas of RAG1<sup>-/-</sup> immunodeficient mice failed to demonstrate histologic, biochemical, and biomechanical evidence of aortic remodeling as contrasted with the WT mice even though both strains of mice were hypertensive, suggesting that without the presence of functional lymphocytes the structural and material remodeling induced by Ang II was inhibited

It was observed that there is a temporally related remodeling in the terms of a biphasic mechanical properties of the aortas. First we found that the WT aortas became stiff after the first seven days of AngII infusion and subsequently the stiffness, defined as the slope of stress to strain relationship, decreased in parallel with the measured increase in structural vascular remodeling. The acute increase in stiffness between days 0 and 7 in the WT mice occurred prior to detectable compensatory remodeling, as evidenced by a latter phase of increased ECM protein mRNA expression, collagen content, collagen cross-linking, and medial and adventitial thickness. Most notably, immune competence appears to be obligatory for vascular stiffening in the context of this hypertension model since the RAG1<sup>-/-</sup> mice failed to show any change in axial and circumferential stiffness over 21 days of Ang II infusion.

In the arterial vasculature, Ang II stimulation of aortic fibroblasts induces fibroblast proliferation and proinflammatory factors, particularly MCP-1 and IL-6 (Tieu et al., 2011). The adventitial derived MCP-1 in turn recruits monocytes which may support lymphocytic infiltration. Evidence also shows that not only Ang II but also its precursors are capable in stimulation of T lymphocytes and NK cells proliferation (Jurewicz et al., 2007). In addition, the CD4<sup>+</sup> lymphocytes possess renin-angiotensin system including types I and II receptors, ACE, and angiotensinogen which also regulate lymphocyte activation, IFN- $\gamma$ , monocyte chemoattractant protein (MCP-1)(Han et al., 2012), chemotaxis (Silva-Filho et al., 2011), NADPH oxidase activity (Hoch et al., 2009) and IL-18 secretion (Sahar et al., 2005). As we have shown, AT1 receptor stimulation causes an immediate Th1 response followed by a chronic Th17 reaction in vivo.

The vascular stiffness relates to the temporally related lymphocytic infiltration. The marked increase in aortic Th1 infiltrates preceded the increased vascular stiffness. Thereafter a progressive increase of Th17 lymphocytic infiltrates paralleled the compensatory structural and material remodeling. There are similar reports suggesting a sequential order of CD4<sup>+</sup> subset infiltrates in other inflammatory disease conditions. During experimental autoimmune encephalomyelitis, Th1 cells accumulate initially in the CNS and subsequently allow for the entry of Th17 cells (O'Connor et al., 2008). Conversely there is also a reversal of the Th1 and Th17 cell recruitment order and their potential interaction, where the Th17 lymphocytes accumulate first followed by Th1 in kidney during glomerulonephritis (Paust et al., 2012). Therefore this differential tissue preference for the sequential Th1 and Th17 infiltration is likely to be a consequence of the target tissue response and the specific to the pathologic stimulus.

We also provided additional confirmatory studies utilizing the Tbet<sup>-/-</sup> (Th1-deficient) mouse. The increased stiffness on day 7 is prevented in the Th1-deficient mice. Also with the RORγt<sup>-/-</sup> (Th17-deficient) mouse, the compensatory structural remodeling is inhibited but peak stiffness observed on day 7 is preserved. These findings occurred despite the fact both the Tbet<sup>-/-</sup> and RORγt<sup>-/-</sup> mice were similarly hypertensive relative to the WT mice. Therefore we provide evidence that there is a temporal order and separate mechanisms related to the Th1 and Th17 cellular aortic infiltrates that are associated with vascular stiffness and compensatory remodeling.

Kroenke et al suggests that Th1 lymphocytic infiltration results in macrophage tissue intrusion through a CXCL9, 10, and 11 chemokine secretory profile and in addition it is recognized that Ang II also promotes adventitial fibroblasts to secrete the chemokine, MCP-1, which in turn recruits monocytes/macrophage infiltrates. Even though our focus was the CD4<sup>+</sup> lymphocyte subsets, these perivascular macrophages likewise appear to be a central contributor in vascular remodeling (Zhou et al., 2010).

The material remodeling processes occurred in our study after 7 days of AngII administration temporally coincided with the infiltrations of Th17 lymphocytes into the aortas. It follows therefore, that Th17 may mediate the vascular remodeling process. In a cardiac model IL-17 has been demonstrated to activate cardiac fibroblast proliferation via an Akt/miR-101/MKP-1-dependent p38 MAPK and ERK1/2 pathway (Valente et al., 2012) and fibrosis through PKC $\beta$ /Erk1/2/NF- $\kappa$ B signaling pathway (Liu et al., 2012). It was shown that the fibroblasts constitutively express the IL-17 receptor and IL-17A stimulated fibroblast proliferation and migration (Valente et al., 2012). Consequently there is support for Th17/IL-17 cytokine pathway for remodeling and fibrosis.

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## **DISCLOSURES**

None declared.

## References

- Amano, M., Y.Fukata, and K.Kaibuchi. 2000. Regulation and functions of Rho-associated kinase. *Exp. Cell Res.* 261:44-51.
- Bakker, E.N., C.L.Buus, J.A.Spaan, J.Perree, A.Ganga, T.M.Rolf, O.Sorop, L.H.Bramsen, M.J.Mulvany, and E.VanBavel. 2005. Small artery remodeling depends on tissue-type transglutaminase. *Circ. Res.* 96:119-126.
- Barhoumi, T., D.A.Kasal, M.W.Li, L.Shbat, P.Laurant, M.F.Neves, P.Paradis, and E.L.Schiffrin. 2011. T regulatory lymphocytes prevent angiotensin II-induced hypertension and vascular injury. *Hypertension* 57:469-476.
- Bradshaw, A.D., C.F.Baicu, T.J.Rentz, A.O.Van Laer, J.Boggs, J.M.Lacy, and M.R.Zile. 2009. Pressure overload-induced alterations in fibrillar collagen content and myocardial diastolic function: role of secreted protein acidic and rich in cysteine (SPARC) in post-synthetic procollagen processing. *Circulation* 119:269-280.
- Che, Z.Q., P.J.Gao, W.L.Shen, C.L.Fan, J.J.Liu, and D.L.Zhu. 2008. Angiotensin II-stimulated collagen synthesis in aortic adventitial fibroblasts is mediated by connective tissue growth factor. *Hypertens. Res.* 31:1233-1240.

Chung, A.C., and H.Y.Lan. 2011. Chemokines in renal injury. *J. Am. Soc. Nephrol.* 22:802-809.

Di Martino, E.S., A.Bohra, J.P.Vande Geest, N.Gupta, M.S.Makaroun, and D.A.Vorp. 2006. Biomechanical properties of ruptured versus electively repaired abdominal aortic aneurysm wall tissue. *J. Vasc. Surg.* 43:570-576.

Edvardsen, T., S.Urheim, H.Skulstad, K.Steine, H.Ihlen, and O.A.Smiseth. 2002. Quantification of left ventricular systolic function by tissue Doppler echocardiography: added value of measuring pre- and postejection velocities in ischemic myocardium. *Circulation* 105:2071-2077.

Ford, C.M., S.Li, and J.G.Pickering. 1999. Angiotensin II stimulates collagen synthesis in human vascular smooth muscle cells. Involvement of the AT(1) receptor, transforming growth factor-beta, and tyrosine phosphorylation. *Arterioscler. Thromb. Vasc. Biol.* 19:1843-1851.

Grinnell, F. 1994. Fibroblasts, myofibroblasts, and wound contraction. *J. Cell Biol.* 124:401-404.

Guzik, T.J., N.E.Hoch, K.A.Brown, L.A.McCann, A.Rahman, S.Dikalov, J.Goronzy, C.Weyand, and D.G.Harrison. 2007. Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J. Exp. Med.* 204:2449-2460.

Han, Y.L., Y.L.Li, L.X.Jia, J.Z.Cheng, Y.F.Qi, H.J.Zhang, and J.Du. 2012. Reciprocal interaction between macrophages and T cells stimulates IFN-gamma and MCP-1 production in Ang II-induced cardiac inflammation and fibrosis. *PLoS. ONE.* 7:e35506.

Hayashi, K., and T.Naiki. 2009. Adaptation and remodeling of vascular wall; biomechanical response to hypertension. *J. Mech. Behav. Biomed. Mater.* 2:3-19.

Hirota, K., H.Yoshitomi, M.Hashimoto, S.Maeda, S.Teradaira, N.Sugimoto, T.Yamaguchi, T.Nomura, H.Ito, T.Nakamura, N.Sakaguchi, and S.Sakaguchi. 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J. Exp. Med.* 204:2803-2812.

Hoch, N.E., T.J.Guzik, W.Chen, T.Deans, S.A.Maalouf, P.Gratze, C.Weyand, and D.G.Harrison. 2009. Regulation of T-cell function by endogenously produced angiotensin II. *Am. J. Physiol Regul. Integr. Comp Physiol* 296:R208-R216.

Huaux, F., T.Liu, B.McGarry, M.Ullenbruch, Z.Xing, and S.H.Phan. 2003. Eosinophils and T lymphocytes possess distinct roles in bleomycin-induced lung injury and fibrosis. *J. Immunol.* 171:5470-5481.

Humphrey, J.D. 2008a. Mechanisms of arterial remodeling in hypertension: coupled roles of wall shear and intramural stress. *Hypertension* 52:195-200.

Humphrey, J.D. 2008b. Vascular adaptation and mechanical homeostasis at tissue, cellular, and sub-cellular levels. *Cell Biochem. Biophys.* 50:53-78.

Ivanov, I.I., B.S.McKenzie, L.Zhou, C.E.Tadokoro, A.Lepelley, J.J.Lafaille, D.J.Cua, and D.R.Littman. 2006. The orphan nuclear receptor ROR $\gamma$  directs the differentiation program of proinflammatory IL-17<sup>+</sup> T helper cells. *Cell* 126:1121-1133.

Jurewicz, M., D.H.McDermott, J.M.Sechler, K.Tinckam, A.Takakura, C.B.Carpenter, E.Milford, and R.Abdi. 2007. Human T and natural killer cells possess a functional renin-angiotensin system: further mechanisms of angiotensin II-induced inflammation. *J. Am. Soc. Nephrol.* 18:1093-1102.

Kasal, D.A., T.Barhoumi, M.W.Li, N.Yamamoto, E.Zdanovich, A.Rehman, M.F.Neves, P.Laurant, P.Paradis, and E.L.Schiffrin. 2012. T regulatory lymphocytes prevent aldosterone-induced vascular injury. *Hypertension* 59:324-330.

Keyes, J.T., D.G.Haskett, U.Utzinger, M.Azhar, and J.P.Vande Geest. 2011. Adaptation of a planar microbiaxial optomechanical device for the tubular biaxial microstructural and macroscopic characterization of small vascular tissues. *J. Biomech. Eng* 133:075001.

Khader, S.A., G.K.Bell, J.E.Pearl, J.J.Fountain, J.Rangel-Moreno, G.E.Cilley, F.Shen, S.M.Eaton, S.L.Gaffen, S.L.Swain, R.M.Lockley, L.Haynes, T.D.Randall, and A.M.Cooper. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4<sup>+</sup>

T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat. Immunol.* 8:369-377.

Kroenke, M.A., T.J.Carlson, A.V.Andjelkovic, and B.M.Segal. 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* 205:1535-1541.

Kvakan, H., M.Kleinewietfeld, F.Qadri, J.K.Park, R.Fischer, I.Schwarz, H.P.Rahn, R.Plehm, M.Wellner, S.Elitok, P.Gratze, R.Dechend, F.C.Luft, and D.N.Muller. 2009. Regulatory T cells ameliorate angiotensin II-induced cardiac damage. *Circulation* 119:2904-2912.

Langille, B.L., and D.Dajnowiec. 2005. Cross-linking vasomotor tone and vascular remodeling: a novel function for tissue transglutaminase? *Circ. Res.* 96:9-11.

Li, L., D.L.Zhu, W.L.Shen, and P.J.Gao. 2006. Increased migration of vascular adventitial fibroblasts from spontaneously hypertensive rats. *Hypertens. Res.* 29:95-103.

Liu, Y., H.Zhu, Z.Su, C.Sun, J.Yin, H.Yuan, S.Sandoghchian, Z.Jiao, S.Wang, and H.Xu. 2012. IL-17 contributes to cardiac fibrosis following experimental autoimmune myocarditis by a PKCbeta/Erk1/2/NF-kappaB-dependent signaling pathway. *Int. Immunol.*

Lopez, B., R.Querejeta, A.Gonzalez, J.Beaumont, M.Larman, and J.Diez. 2009. Impact of treatment on myocardial lysyl oxidase expression and collagen cross-linking in patients with heart failure. *Hypertension* 53:236-242.

Madhur, M.S., H.E.Lob, L.A.McCann, Y.Iwakura, Y.Blinder, T.J.Guzik, and D.G.Harrison. 2010. Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. *Hypertension* 55:500-507.

Meng, F., K.Wang, T.Aoyama, S.I.Grivennikov, Y.Paik, D.Scholten, M.Cong, K.Iwaisako, X.Liu, M.Zhang, C.H.Osterreicher, F.Stickel, K.Ley, D.A.Brenner, and T.Kisseleva. 2012. Interleukin-17 Signaling in Inflammatory, Kupffer, and Hepatic Stellate Cells Exacerbates Liver Fibrosis in Mice. *Gastroenterology*.

Nataraj, C., M.I.Oliverio, R.B.Mannon, P.J.Mannon, L.P.Audoly, C.S.Amuchastegui, P.Ruiz, O.Smithies, and T.M.Coffman. 1999. Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *J Clin. Invest* 104:1693-1701.

Paust, H.J., J.E.Turner, J.H.Riedel, E.Disteldorf, A.Peters, T.Schmidt, C.Krebs, J.Velden, H.W.Mittrucker, O.M.Steinmetz, R.A.Stahl, and U.Panzer. 2012. Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis. *Kidney Int.* 82:72-83.

- Platten, M., S. Youssef, E.M.Hur, P.P.Ho, M.H.Han, T.V.Lanz, L.K.Phillips, M.J.Goldstein, R.Bhat, C.S.Raine, R.A.Sobel, and L.Steinman. 2009. Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity. *Proc. Natl. Acad. Sci. U. S. A* 106:14948-14953.
- Rodriguez, C., B.Raposo, J.Martinez-Gonzalez, L.Casani, and L.Badimon. 2002. Low density lipoproteins downregulate lysyl oxidase in vascular endothelial cells and the arterial wall. *Arterioscler. Thromb. Vasc. Biol.* 22:1409-1414.
- Rodriguez-Vita, J., E.Sanchez-Lopez, V.Esteban, M.Ruperez, J.Egido, and M.Ruiz-Ortega. 2005. Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-beta-independent mechanism. *Circulation* 111:2509-2517.
- Sahar, S., R.S.Dwarakanath, M.A.Reddy, L.Lanting, I.Todorov, and R.Natarajan. 2005. Angiotensin II enhances interleukin-18 mediated inflammatory gene expression in vascular smooth muscle cells: a novel cross-talk in the pathogenesis of atherosclerosis. *Circ. Res.* 96:1064-1071.
- Schiffrin, E.L. 2012. Immune modulation of resistance artery remodelling. *Basic Clin. Pharmacol. Toxicol.* 110:70-72.

Shi, Y., M.Pieniek, A.Fard, J.O'Brien, J.D.Mannion, and A.Zalewski. 1996. Adventitial remodeling after coronary arterial injury. *Circulation* 93:340-348.

Silva-Filho, J.L., M.C.Souza, M.G.Henriques, A.Morrot, W.Savino, M.P.Nunes, C.Caruso-Neves, and A.A.Pinheiro. 2011. AT1 receptor-mediated angiotensin II activation and chemotaxis of T lymphocytes. *Mol. Immunol.* 48:1835-1843.

Stenmark, K.R., N.Davie, M.Frid, E.Gerasimovskaya, and M.Das. 2006. Role of the adventitia in pulmonary vascular remodeling. *Physiology. (Bethesda. )* 21:134-145.

Thorley, A.J., P.Goldstraw, A.Young, and T.D.Tetley. 2005. Primary human alveolar type II epithelial cell CCL20 (macrophage inflammatory protein-3 $\alpha$ )-induced dendritic cell migration. *Am. J. Respir. Cell Mol. Biol.* 32:262-267.

Tieu, B.C., X.Ju, C.Lee, H.Sun, W.Lejeune, A.Recinos, III, A.R.Brasier, and R.G.Tilton. 2011. Aortic adventitial fibroblasts participate in angiotensin-induced vascular wall inflammation and remodeling. *J. Vasc. Res.* 48:261-272.

Tomasek, J.J., G.Gabbiani, B.Hinz, C.Chaponnier, and R.A.Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell Biol.* 3:349-363.

Valente, A.J., T.Yoshida, J.D.Gardner, N.Somanna, P.Delafontaine, and B.Chandrasekar. 2012. Interleukin-17A stimulates cardiac fibroblast proliferation and migration via negative regulation of the dual-specificity phosphatase MKP-1/DUSP-1. *Cell Signal.* 24:560-568.

van der Slot-Verhoeven AJ, E.A.van Dura, J.Attema, B.Blauw, J.DeGroot, T.W.Huizinga, A.M.Zuurmond, and R.A.Bank. 2005. The type of collagen cross-link determines the reversibility of experimental skin fibrosis. *Biochim. Biophys. Acta* 1740:60-67.

Wilson, M.S., S.K.Madala, T.R.Ramalingam, B.R.Gochuico, I.O.Rosas, A.W.Cheever, and T.A.Wynn. 2010. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. *J. Exp. Med.* 207:535-552.

Yu, Q., R.Vazquez, S.Zabadi, R.R.Watson, and D.F.Larson. 2010. T-lymphocytes mediate left ventricular fibrillar collagen cross-linking and diastolic dysfunction in mice. *Matrix Biol.* 29:511-518.

Zhang, J.D., M.B.Patel, Y.S.Song, R.Griffiths, J.Burchette, P.Ruiz, M.A.Sparks, M.Yan, D.N.Howell, J.A.Gomez, R.F.Spurney, C.B.Wilson, T.M.Coffman, and S.D.Crowley. 2012. A Novel Role for Type 1 Angiotensin Receptors on T Lymphocytes to Limit Target Organ Damage in Hypertension. *Circ. Res.*

Zheng, L., C.C.Xu, W.D.Chen, W.L.Shen, C.C.Ruan, L.M.Zhu, D.L.Zhu, and P.J.Gao. 2010. MicroRNA-155 regulates angiotensin II type 1 receptor expression and phenotypic differentiation in vascular adventitial fibroblasts. *Biochem. Biophys. Res. Commun.* 400:483-488.

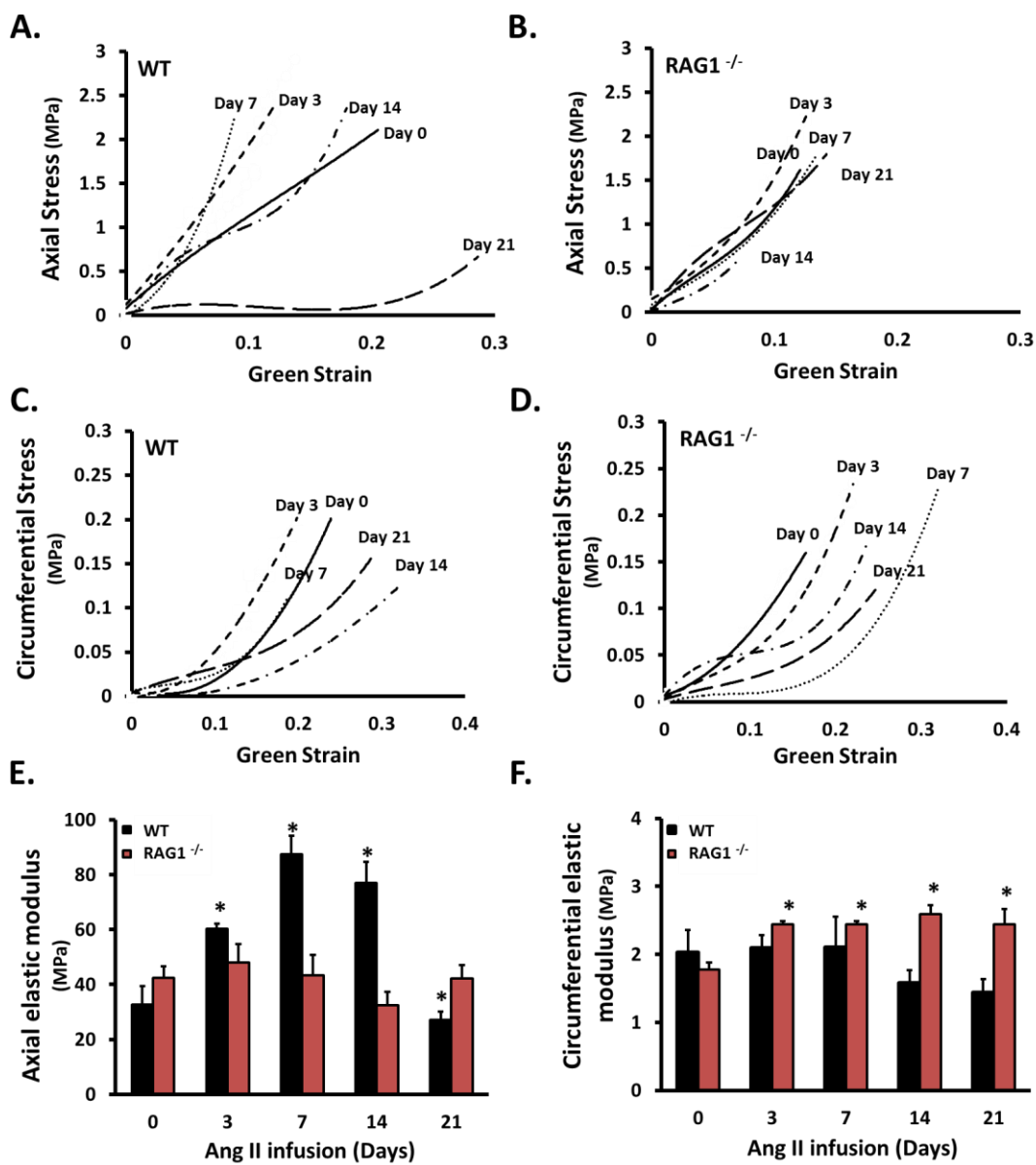
Zhou, J., P.C.Tang, L.Qin, P.M.Gayed, W.Li, E.A.Skokos, T.R.Kyriakides, J.S.Pober, and G.Tellides. 2010. CXCR3-dependent accumulation and activation of perivascular macrophages is necessary for homeostatic arterial remodeling to hemodynamic stresses. *J. Exp. Med.* 207:1951-1966.

Zieman, S.J., and D.A.Kass. 2004. Advanced glycation endproduct crosslinking in the cardiovascular system: potential therapeutic target for cardiovascular disease. *Drugs* 64:459-470.

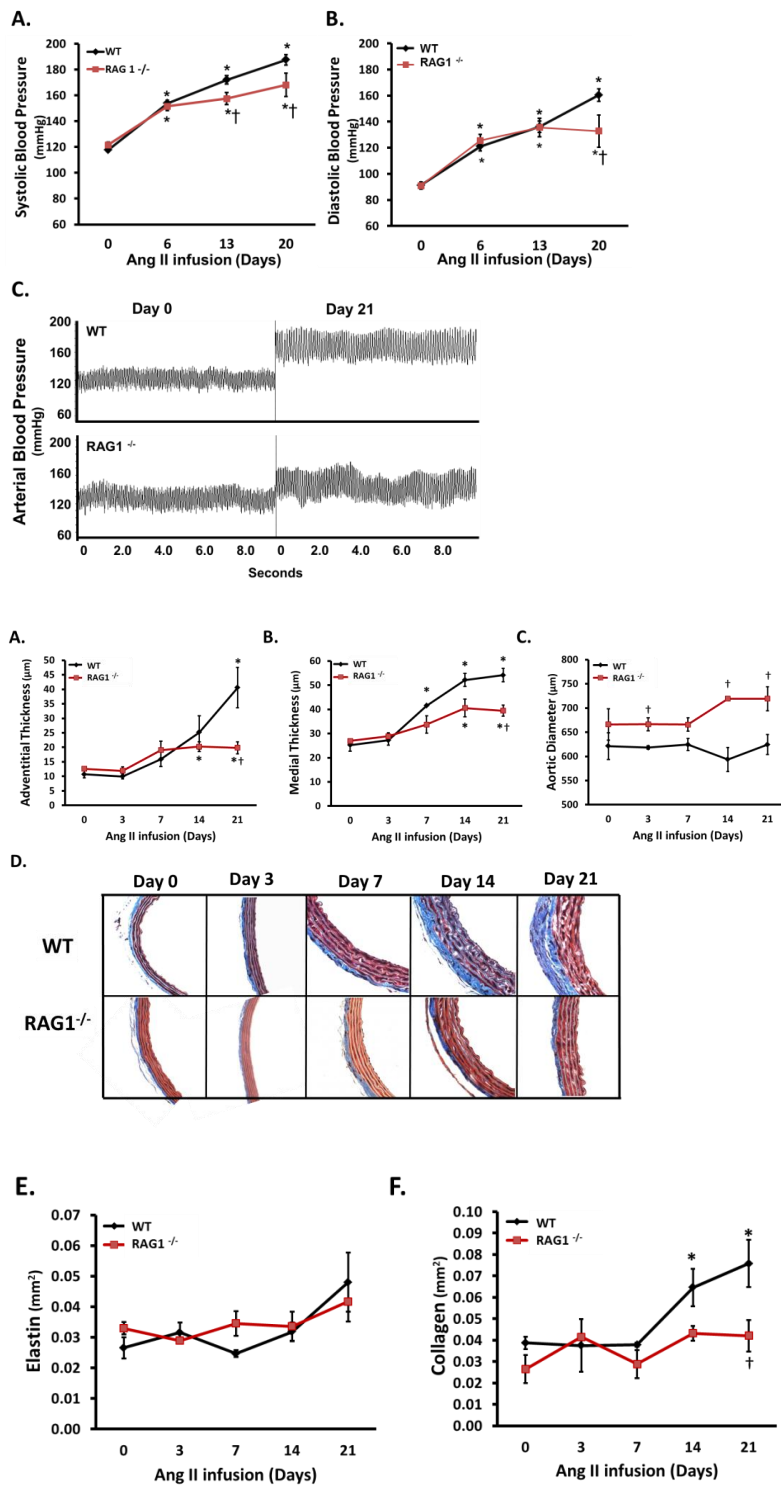
Zieman, S.J., V.Melenovsky, and D.A.Kass. 2005. Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler. Thromb. Vasc. Biol.* 25:932-943.

Zou, L.X., J.D.Imig, A.Hymel, and L.G.Navar. 1998. Renal uptake of circulating angiotensin II in Val5-angiotensin II infused rats is mediated by AT1 receptor. *Am. J. Hypertens.* 11:570-578.

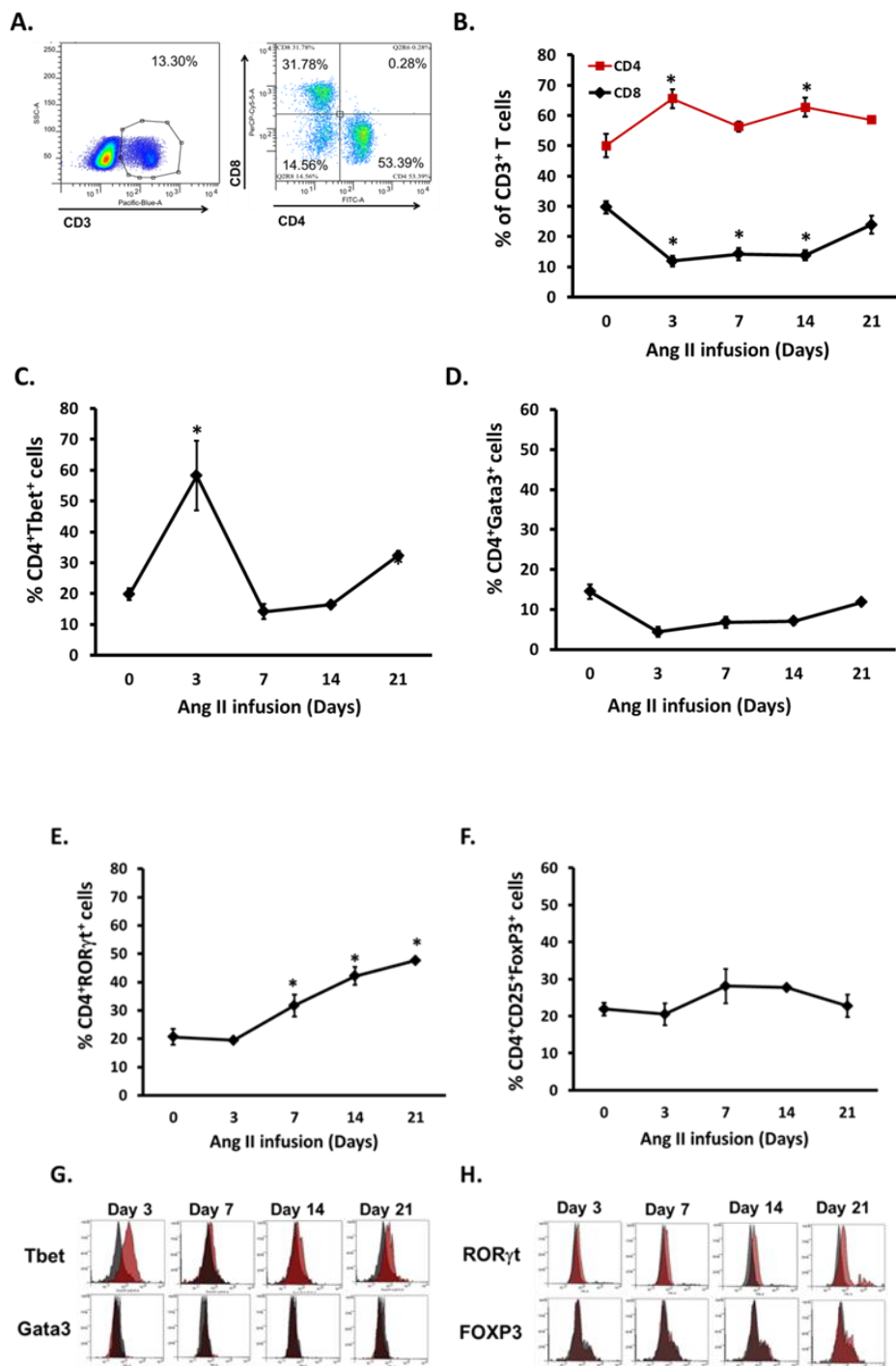
## FIGURES

Figure 1. RAG1<sup>-/-</sup> mice have reduced vascular stiffness induced by Ang II.

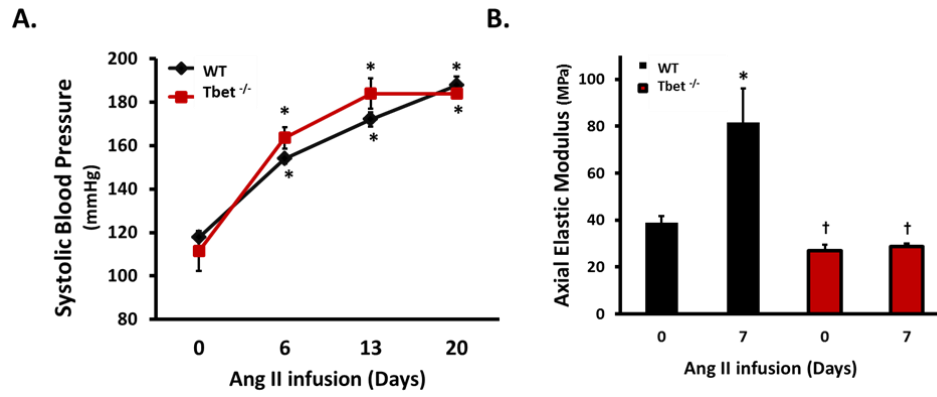
**FIGURE 2. Comparison of hemodynamic function and vascular structure.**



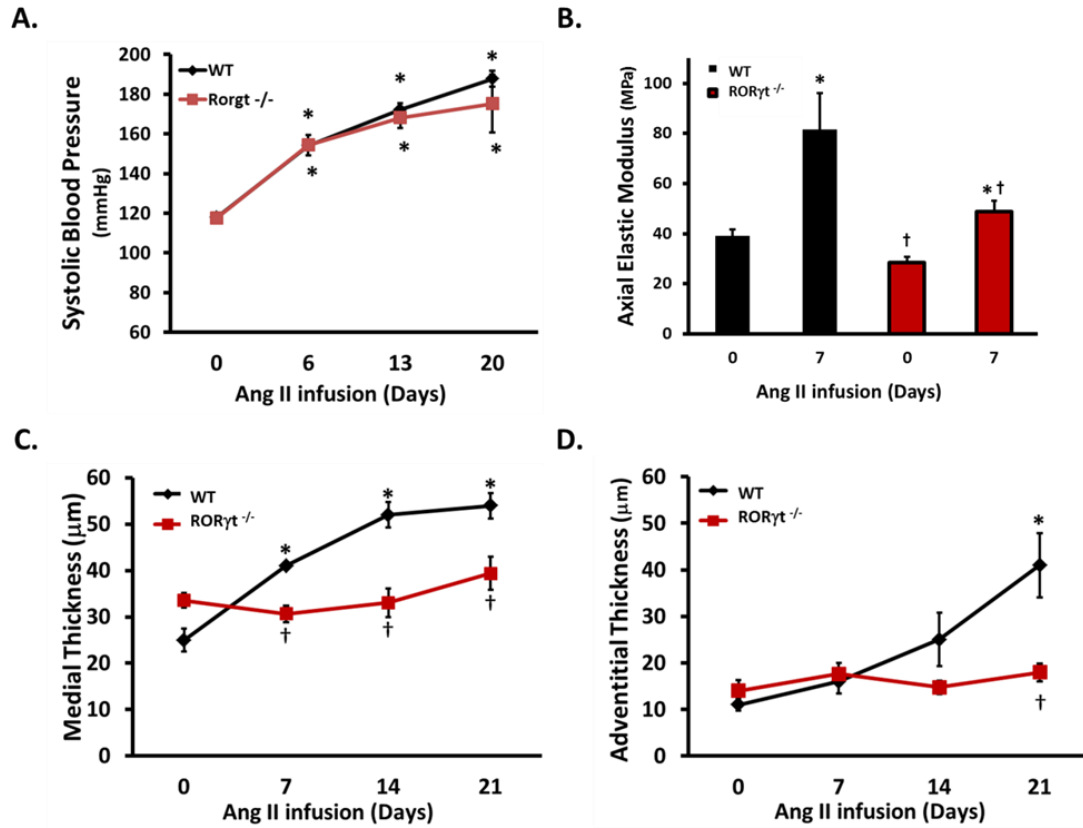
**FIGURE 3. Aortic immune response to Ang II infusion.**



**FIGURE 4. Th1 deficient mice have impaired vascular stiffness induced by AT1 receptor stimulation.**



**FIGURE 5. AT1 receptor stimulation induces vascular stiffness but not vascular remodeling in TH17 deficient mice.**



## 6.0. FIGURE LEGENDS

### **FIGURE 1. RAG1<sup>-/-</sup> mice have reduced AT1 receptor induced vascular stiffness.**

[Val<sup>5</sup>]-angiotensin II (Ang II) was infused subcutaneously at a rate of 490 ng/kg/min into WT and RAG1<sup>-/-</sup> mice with Alzet pumps for 21 days. Aortic stiffness was determined by *ex vivo* biomechanical analysis. The descending thoracic aortas harvested from WT and RAG1<sup>-/-</sup> were subjected to tubular biaxial test to derive axial and circumferential stress-strain responses. (A and B) Representative plots of axial stress-strain in WT (A) and RAG1<sup>-/-</sup>. (C and D) Representative plots of circumferential stress-strain in WT (C) and RAG1<sup>-/-</sup>(D). Mean axial (E) and circumferential (F) stiffness (elastic modulus) was calculated from the slopes taken at the peak stress-strain plots. (n=6 per group). Data are means ± SEM \*P <0.05 compared with respective day 0 and †P <0.05 compared with time matched WT.

**FIGURE 2. Comparison of hemodynamic function and vascular structure.** Blood pressure measurements were obtained from non-invasive Hatteras tail cuff system at day 0, 6, 13, and 20 after Ang II infusion. (A) The mean values of systolic blood pressure increase with Ang II infusion in both mouse strains but are significant lower in the RAG1<sup>-/-</sup> on the second and third weeks (n=12). (B) The mean values of diastolic blood pressure in the RAG1<sup>-/-</sup> are lower than the WT only on the third week (n=12). (C) Representative tracing of bio-telemetry system compares the blood pressures of unrestrained mice. WT and RAG1<sup>-/-</sup> mice were perfusion fixed at day 0, 7, 14, and 21 after Ang II infusion. The lower thoracic aortas were paraffin embedded and stained with

H&E, Masson's Trichrome, VerhoeffVan Gieson (VVG), and Picosirius Red (PCR) for histomorphometric analysis. (D-F) The mean adventitial thickness (D), medial thickness (E), and aortic diameter (F) were quantified using Image J program (n=4 per group). (G) Representative photomicrographs of Trichrome stained aortic transverse sections from the WT and the RAG1<sup>-/-</sup> on days 0, 7, 14, 21 post-Ang II infusion. (E) Quantification of elastin using VVG staining in the lower thoracic aortas from WT and RAG1<sup>-/-</sup> (n=4 per group). (F) Quantification of collagen levels using PCR staining (n=4 per group). Data are means  $\pm$  SEM \*P <0.05 compared with respective day 0 and †P <0.05 compared with time matched WT.

**FIGURE 3. Aortic immune response to Ang II infusion.** Lymphocytes isolated from aortic infiltrates of WT treated with Ang II were analyzed with flow cytometry. (A) Representative flow cytometry analyses of CD4<sup>+</sup> and CD8<sup>+</sup> cells. (B) The percentages of CD4<sup>+</sup> and CD8<sup>+</sup>T cells gated from CD3<sup>+</sup> population in the peri-aortic infiltrates. (C-F) The percentages of Th1 (T-bet<sup>+</sup>CD4<sup>+</sup>) cells (C), Th2 (Gata3<sup>+</sup>CD4<sup>+</sup>) cells (D), Th17 (ROR $\gamma$ t<sup>+</sup>CD4<sup>+</sup>) cells (E), and Treg (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) cells (F) in peri-aortic infiltrates. (G, H) Representative histogram of peri-aortic CD4<sup>+</sup> T cells expressing T-bet, GATA3, ROR $\gamma$ t and FoxP3 during the time course of AT1 receptor stimulation. The overlapping histograms represent the data from day 0 (grey) compared with the treatment on different days (red) as indicated. All intracellular flow cytometry analyses were gated on CD4<sup>+</sup> population. Data are mean  $\pm$  SEM of 6-8 mice per group \*P <0.05 compared with respective day 0 and †P <0.05 compared with time matched WT.

**FIGURE 4. Th1 deficient mice have impaired vascular stiffness induced by AT1 receptor stimulation.** (A) Systolic blood pressure measured by tail-cuff Hatteras system, compared between WT and Tbet<sup>-/-</sup> mice (n=6-10 per group). (B) The mean axial aortic stiffness after 7 days Ang II infusion between WT and Tbet<sup>-/-</sup> mice was determined by *ex vivo* biomechanical analysis (n=4-6 per group). Data are means ± SEM \*P <0.05 compared with respective day 0 and †P <0.05 compared with time matched WT

**FIGURE 5. AT1 receptor stimulation induces vascular stiffness but not vascular remodeling in TH17 deficient mice.** (A) Tail-cuff systolic blood pressure measurements showed no significant difference at any time point between the WT and RORγt<sup>-/-</sup> mice (n=8 per group). (B) The mean axial elastic modulus from RORγt<sup>-/-</sup> mice after 7 days of Ang II infusion, compared with the WT (n=6 per group) (C) Ang II mediated medial hyperplasia was not evident in the RORγt<sup>-/-</sup> mice over the 21 day period using histomorphometric analysis (n=4). (D) There was no increase in adventitial thickness in the RORγt<sup>-/-</sup> in response to Ang II (n=4). Data are means ± SEM \*P <0.05 compared with respective day 0 and †P <0.05 compared with time matched WT

**APPENDIX B - MODULATION OF ANG II-INDUCED VASCULAR  
REMODELING BY STIMULATION OF REGULATORY T CELLS WITH IL-2  
CYTOKINE COMPLEX**

“Paper was prepared to submit to Hypertension”

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**Abstract**

Enhanced adaptive immunity is associated with vascular pathology and dysfunction in Ang II-induced hypertension. Inhibition of immune activation using pharmacological immunosuppressive agents or adoptive transfer of regulatory T cells (Treg lymphocytes) has shown to reduce Ang II-induced hypertension and target-organ damage. In this study, we applied an alternative approach to stimulate proliferation of Treg lymphocyte *in vivo* using cytokine immune complexes of IL-2 and anti-IL-2 monoclonal antibody clone JES6-1. The IL-2/anti-IL-2 mAb complex at a total dose of 6  $\mu\text{g}$  (5  $\mu\text{g}$   $\alpha\text{IL-2}$  mAb + 1  $\mu\text{g}$  IL-2) was injected intraperitoneally once a day for 5 consecutive days. Ang II-infused mice that received the IL-2/anti-IL-2 complex had significant decreased vascular remodeling and stiffening compared with mice receiving Ang II alone without any differences in arterial blood pressure. This stimulation of Treg lymphocyte with the IL-2/anti-IL-2 complex suppressed the Th1 and Th17 responses in the lymphoid organs and reduced IFN $\gamma$  and IL-17 expression as well as T-cell and macrophage infiltrates in the aortas. This study provides data that support the protective roles of Treg lymphocyte in vascular remodeling and stiffening and the use of the IL-2/anti-IL-2 mAb complex as a new potential therapy in Ang II-induced vascular diseases.

## Introduction

Angiotensin II (Ang II), an essential mediator in the renin angiotensin system, plays a crucial role in regulation of arterial pressure and sodium homeostasis.<sup>1</sup> beyond its effect on vascular tone, Ang II stimulates cell growth and hypertrophy and participates in the inflammatory response, thereby contributing to changes in vascular structure and function.<sup>2,3</sup> In support of this concept, several studies have reported a role of Ang II in inflammation and immune activation. Guzik et al<sup>4</sup> showed that RAG1<sup>-/-</sup> immunodeficient mice have reduced Ang II-induced hypertension, vascular dysfunction and hypertrophy. Adoptive transfer of T but not B cells restores this pathology.<sup>4</sup> Ang II also induces a Th1/Th2 imbalance associated with hypertensive kidney injury<sup>5</sup> and inhibition of the Th1 cytokine, IFN $\gamma$ , reduces Ang II-induced cardiac damage.<sup>6</sup> More importantly, suppression of immune responses with regulatory T cell (Treg lymphocyte), a subset of CD4<sup>+</sup> T cells expressing IL-2 receptor  $\alpha$ -subunit (CD25) and transcription factor Foxp3,<sup>7</sup> has shown benefits in Ang II-hypertensive model.<sup>8</sup> Adoptive transfer of Tregs reduces Ang II-increased systolic blood pressure, attenuates vascular NADPH oxidase activity and improves vascular injury,<sup>9</sup> potentially by their ability to secrete anti-inflammatory cytokine, IL-10.<sup>10</sup>

Recent studies have reported that immune complexes of IL-2 and the anti-IL-2 monoclonal antibody (mAb) clone JES6-1 can induce rapid expansion of Treg lymphocyte with strong suppressive function *in vivo*.<sup>11-13</sup> These expanded Treg cells have been shown to prevent mice from experimental autoimmune encephalomyelitis

induction,<sup>11</sup> suppress collagen-induced arthritis,<sup>12</sup> and attenuate development of atherosclerosis.<sup>13</sup> Therefore, this immune complex could logically be used to assess the role of adaptive immune system on vascular remodeling and stiffness.

In the current study, we investigated the ability of IL-2/anti-IL-2 mAb JES6-1 complex to stimulate Treg lymphocyte and protect mice from Ang II-induced vascular remodeling. We have demonstrated that IL-2/anti-IL-2 mAb complex induces expansion of Treg lymphocytes and prevents vascular remodeling and stiffening induced by Ang II by inhibiting Th1 and Th17 responses. These results support the use of the IL-2/ anti-IL-2 mAb complex as a novel therapeutic target and provide strong clinical implications since arterial stiffening is considered an independent marker for increased cardiovascular diseases.<sup>14</sup>

## **Methods**

### **Animals and study design**

C57BL/6 male 10-week-old mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). This study was approved by the University of Arizona Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the mice were maintained in the animal facility of the University of Arizona and fed with NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet from Harlan Laboratories and randomly divided into placebo and three treatment groups.

Mice were administered IL-2/anti-IL-2 JES6-1 monoclonal antibody (5 µg of anti-IL-2 mAb + 1 µg of IL-2 (R&D system, Minneapolis, MN) via i.p. injection for 5 consecutive days. [Val<sup>5</sup>] angiotensin II (Ang II) (Sigma Chemical Co) at 490 ng/min/kg was then infused via subcutaneously implantable osmotic pumps (model 1004; Alzet, Palo Alto, Calif., USA) on day 7 for 14 days. During the 2 weeks of Ang II infusion, the mice also received the IL-2/anti-IL-2 complex in 3 doses weekly. The control group received the pumps filled with vehicle (PBS). Ang II treatment group received PBS injection. Four groups of mice were sacrificed after 14 days of Ang II infusion.

### **Blood Pressure Measurements**

All mice were trained with the tail cuff system and data were recorded weekly for Days 0, 7, and 14. Blood pressure values were measured with the tail cuff system while mice were placed on a heated platform (Hatteras Instruments, Cary, North Carolina). Blood pressure values recorded were from an average of ten consecutive measurements with a standard deviation lower than 10.

### **Perfusion Fixation and Histological Staining**

Before perfusion fixation, mice were anti-coagulated with a subcutaneous injection of 100 µl of 1,000 USP units/ml Heparin Sodium. Thirty mL of saline was infused to remove vascular blood, followed by the administration of 30 mL of 3% glutaraldehyde in 10% formaldehyde solution at a constant perfusion pressure of 40 mmHg. The tissues were paraffin embedded, cut at 5 µm, and stained with H&E, Masson's Trichrome, Verhoeff's elastin stain and Picrosirius red collagen stains. Image

analysis was performed using NIH ImageJ software to quantify aortic wall thickness and the tissue collagen related to the Ang II treatments.

### **Biomechanical analysis of aortic stiffness**

Biomechanical analyses of the thoracic aortas were performed using the microbiaxial optomechanical device (MOD), previously demonstrated by Keyes et al<sup>15</sup>. Aortas were cannulated with glass microcapillaries with cyanoacrylate and mounted into the testing device. Aortas were tested at 37<sup>0</sup>C in a calcium free solution, and subjected to cyclic increasing of axial strains up to 15% of their maximum stretch while held at an intraluminal pressure of 0, 70, and 130 mmHg. Correspondingly, the vessels were pressurized from 0 to 130 mmHg cyclically while held at axial strains of 0, 15, and 30%. Axial force, intraluminal pressure, axial strain, and outer diameter were acquired over the course of testing and the second Piola Kirchhoff stress and Green's strains were calculated as previously demonstrated.<sup>15</sup> The maximum tangential modulus was taken as the maximum slope of each stress-stretch as reported by Di Martino.<sup>16</sup>

### **Flow cytometry**

Subsequent to isolation of splenic lymphocytes with lymphocyte gradient separation medium (Mediatech Inc, Herndon, VA), lymphocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) 10 ng/ml, ionomycin 1 µg/ml and Brefeldin A 10 µg/ml for 5 h. Surface staining was performed before permeabilization using perm/wash buffer (BD biosciences, san Diego, CA). Permeabilized cells were subsequently incubated with antibodies against intracellular transcription factors and cytokines. Efluor

450-conjugated anti-CD3, FITC-conjugated anti-CD4, Percp-Cy5.5-conjugated anti-CD8a, APC-conjugated anti-CD25, PE-conjugated anti-FOXP3, Percp-Cy5.5-conjugated IL-17, and PE-conjugated IFN $\gamma$  were purchased from eBioscience. The BD LSR II with BD FACSDiva software flow cytometry system and Gatelologic software were used to analyze data.

### **Real-time RT PCR quantification**

Aortic tissues harvested from each of the 4 groups were homogenized in TRIzol for RNA extraction (Invitrogen Life Technologies, Carlsbad, CA). Using Rotor-Gene RG-3000 (Corbett Research, San Francisco, CA) the real time PCR was performed with SYBR Green in a 72 well rotor. Using custom designed primers synthesized by Integrated DNA Technologies the following genes were investigated: *F4/80* (forward, 5'-GCAGATACAGCAATGCCAAG-3', reverse, 5'-GACACTGGGGCACTTTTGT-3'), *CD3 $\epsilon$*  (forward, 5'-CCTGAAAGCTCGAGTGTGTG-3', reverse, 5'-TTGGCCTTCCTATTCTTGCT-3'), *IL-17a* (forward, 5'-GAGGTTGAAACCCTTCCAGA-3', reverse, 5'-TCAGCTGCATTTCTGTGTCC-3'), *IFN $\gamma$*  (forward, 5'-TTTGAGGTCAACAACCCACA-3', reverse, 5'-ATTGGGACAATCTCTTCCCC-3'), *Foxp3* (forward, 5'-TGGACTACTTCAAGTACCACAATATGC-3', reverse, 5'-GCGAACATGCGAGTAAACCAAT-3'). The gene cycle was normalized by the respective  $\beta$ -*actin* (BC040513) RNA expression. All data are reported as normalized threshold and fold change of treated groups compared with control.

## Statistics

All data are reported as means  $\pm$  SEM. Comparisons among the defined groups were analyzed by one-way ANOVA, followed by Bonferroni tests using SPSS. Analysis of time-course for blood pressure was done by two-way ANOVA. Values obtained from treatment groups were compared with control values using Student's t-test. A value of  $P < 0.05$  was considered statistically significant.

## Results

### **Expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells with IL-2/anti-IL-2 complex treatment.**

A previous study has shown that the total dose of 6  $\mu$ g per injection (5  $\mu$ g anti-IL-2 mAb + 1  $\mu$ g IL-2), which is equivalent to 1:2 molar ratios, gives maximal Treg lymphocyte expansion.<sup>11</sup> In this study the same dose of IL-2/anti-IL-2 complex or saline was administered via i.p. injection once a day for 5 consecutive days. [Val<sup>5</sup>] Angiotensin II (Ang II) was then infused via Alzet osmotic pumps on day 7. To maintain the expanded Treg population during the period of Ang II infusion, we also injected the complex 3 times weekly as previously described<sup>13</sup>. Mice were sacrificed after 14 days of Ang II infusion for flow cytometric analysis of splenocytes. Splenic lymphocytes were gated on a CD4<sup>+</sup> cell population. Figure 1A shows a representative flow cytometric analysis of splenic Treg lymphocytes gated from expression of CD25 and Foxp3. Administration IL-2/anti-IL-2 complex led to a 6-fold increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in splenic lymphocytes. Ang II infusion alone did not alter the percentage of Tregs. However, when the Ang II was given with the IL-2/anti-IL-2

complex the percentage of Treg lymphocytes increased by 4 fold, which is significantly lower than treating with the IL-2/anti-IL-2 complex alone ( $P=0.001$  vs IL-2/anti-IL-2; Figure 1B.) Total Treg cell numbers were also significantly increased in the spleens of mice treated with the IL-2/anti-IL-2 complex and chronic infusion of Ang II significantly diminished the effects of the IL-2/anti-IL-2 complex on Treg lymphocyte population ( $P<0.001$  vs IL-2/anti-IL-2; Figure 1C). Total  $CD4^+$  and  $CD8^+$  cell numbers were not altered by Ang II infusion or the IL-2/anti-IL-2 complex treatment (Figure 1D).

### **IL-2/anti-IL-2 complex decreases Ang II induced aortic remodeling**

Immunodeficient mice have blunted vascular hypertrophy in response to Ang II<sup>4</sup> and adoptive transfer of Treg lymphocytes prevents Ang II induced vascular dysfunction.<sup>9</sup> Therefore, we determined whether induction of immunosuppressive Treg lymphocyte with the cytokine complex attenuates Ang II induced aortic remodeling. After 2 weeks of Ang II infusion, the lower thoracic aortas harvested from the control and the treatment groups were paraffin embedded and stained with Verhoeff's Van Gieson (VVG) for elastin, Masson's Trichrome for evaluating the aortic dimension, and Picosirius Red (PSR) for collagen distribution and accumulation in the aortas. Representative histological aortic samples from four different groups showed vascular structural alterations in response to Ang II and the IL-2/anti-IL-2 complex (Figure 2A). There was a significant increase in the medial and adventitial thickness after 14 days of Ang II infusion ( $P<0.001$ ; Figure 2B and 2C). Administration of IL-2/anti-IL-2 complex significantly reduced the adventitial hyperplasia ( $P=0.046$  vs Ang II). The medial

thickness also decreased after IL-2/anti-IL-2 complex treatment but the number did not reach statistical significance ( $P=0.065$  vs Ang II). In addition, Ang II significantly increased aortic collagen accumulation. The treatment of IL-2/anti-IL-2 complex during the course of Ang II infusion reduced vascular collagen content to the level comparable with control (Figure 2D).

### **IL-2/anti-IL-2 complex therapy inhibits Ang II-induced aortic stiffening**

Aortic stiffness was determined using *ex vivo* biomechanical analysis. The stress-strain relationship of the descending thoracic aortas was measured. Figure 3A shows a representative stress-strain plot from mice treated with Ang II, IL-2/anti-IL-2, Ang II plus IL-2/anti-IL-2, comparing with control. The mean aortic stiffness, represented as elastic modulus was derived from a peak slope of collagen phase of the stress-strain plot (Figure 3B). Chronic administration of Ang II for 14 days led to an increase in mean axial aortic stiffness by 2-fold. However, stimulation of Treg lymphocytes with the IL-2/anti-IL-2 complex significantly reduced axial stiffness to a comparable level with the control level ( $P=0.03$  vs Ang II).

### **Expansion of Treg lymphocytes with the IL-2/anti-IL-2 complex does not affect hypertensive response to Ang II**

We next determined whether administration of IL-2/anti-IL-2 complex prevents hypertension in Ang II-infused mice. Using the noninvasive Hatteras tail cuff system, systolic and diastolic blood pressures were measured at baseline and weekly for 2 weeks. Infusion of Ang II significantly increased systolic blood pressure by 40 mmHg and

diastolic blood pressure by 60 mmHg on day 14. Treatment with the IL-2/anti-IL-2 complex did not impact baseline blood pressure. Giving the IL-2/anti-IL-2 concomitantly with Ang II did not reduce or alter the increased blood pressure caused by Ang II infusion in our study (Figure 4A and 4B).

### **IL-2/anti-IL-2 complex treatment inhibits Ang II-induced immune activation**

Proinflammatory Th1 and Th17 immune responses play crucial roles in Ang II-induced hypertension and target-organ damage.<sup>6,17</sup> Therefore we examined whether the IL-2/anti-IL-2 complex inhibits Ang II-induced Th1 and Th17 responses. Using flow cytometric analysis of splenocytes, we found that Ang II infusion increased the percentage of CD4<sup>+</sup> T cells producing IFN $\gamma$  (P=0.07; Figure 5A). Co-administration of IL-2/anti-IL-2 complex significantly reduced the percentage of IFN $\gamma$  secreting T cells induced by Ang II (P=0.001 vs Ang II). In addition, splenic CD4<sup>+</sup> T lymphocytes producing IL-17 also significantly increased with Ang II infusion. This effect was prevented by IL-2/anti-IL-2 complex treatment (Figure 5B). To examine Th1 and Th17 activation in the aorta, we used real time PCR to determine aortic gene expression of the cytokines IFN $\gamma$  and IL-17a. Ang II caused a significant increase in *IFN $\gamma$*  gene expression in the aortic tissues (P=0.04). IL-2/anti-IL-2 treatment was shown to reduce the expression of the up-regulated gene to the comparable level with the control (Figure 6A). We also found a significant up-regulation of aortic *IL-17a* mRNA with Ang II infusion (P=0.035), which was abolished by IL-2/anti-IL-2 complex treatment (Figure 6B). These data suggest that IL-2/anti-IL-2 complex suppressed immune activation of Th1 and Th17

in the lymphoid organ and the aorta. It has been shown that Ang II induces T-cell and macrophage infiltration into the vasculature, which contributes to vascular remodeling.<sup>4,9</sup> We used real time PCR to examine aortic expression of *CD3ε*, a signaling molecule of T lymphocytes and *F4/80*, a surface marker of macrophages after Ang II infusion and/or IL-2/anti-IL-2 complex treatment. There was an 11-fold increase in aortic *CD3ε* gene expression after 14 days of Ang II infusion. IL-2/anti-IL-2 complex treatment prevented the Ang II-mediated increase in aortic *CD3ε* gene expression ( $10.91 \pm 0.51$  vs  $2.22 \pm 0.74$ ; Figure 6C). Ang II infusion also caused a 6-fold increase in aortic *F4/80* mRNA, which was abolished by IL-2/anti-IL-2 complex treatment (Figure 6D). Administration of the IL-2/anti-IL-2 complex alone also led to a significant reduction of aortic *CD3ε* and *F4/80* expression compared with the control level. These data suggest that the IL-2/anti-IL-2 complex inhibits Ang II-enhanced immune cell accumulation in the vasculature.

Using immunohistochemistry, we examined expression of Foxp3, a key regulator of Treg lymphocytes. However, few Foxp3<sup>+</sup> cells were detected in the aortas. Administration of either IL-2/anti-IL-2 complex or Ang II did not alter the number of aortic Foxp3<sup>+</sup> cells (data not shown). The real time PCR of aortic *Foxp3* gene expression was 2-fold greater in the IL-2/anti-IL-2 complex treated group than in the control group ( $2.1 \pm 0.44$  vs  $1.0 \pm 0.58$ ). However, the data did not approach statistical significance.

## Discussion

Existing evidence supports the crucial roles of adaptive immune system, particularly T-lymphocytes, in Ang II dependent hypertension and cardiovascular

diseases.<sup>4,18,19</sup> In the present study, we support and extend this concept by demonstrating that *in vivo* stimulation of regulatory T cells (Treg lymphocytes) ameliorates Ang II-induced aortic stiffening, and remodeling.

Treg lymphocytes suppress activation and expansion of multiple innate and adaptive immunocompetent cells and play a critical role in maintaining self-tolerance and immune homeostasis.<sup>20</sup> Recent studies have suggested protective roles of Treg lymphocytes in hypertension and cardiovascular diseases. Adoptive transfer of isolated Treg lymphocytes reduces Ang II-induced cardiac hypertrophy, inflammation and fibrosis,<sup>18</sup> prevents Ang II-induced hypertension and vascular injury,<sup>9</sup> and improves coronary arteriolar endothelial dysfunction in Ang II hypertensive mice.<sup>21</sup> However, using Treg adoptive transfer as a therapy may be limited due to their low numbers present in lymphoid organs, requiring large numbers of donors or *in vitro* expansion to achieve sufficient numbers for transfer. In this study we employed an alternate approach to induce expansion of Treg lymphocytes *in vivo* using the IL-2 cytokine complex therapy as originally described by Boyman et al.<sup>22</sup>

IL-2 signaling is required for expansion, survival and homeostatic maintenance of regulatory T cells.<sup>23,24</sup> Immune complexes of IL-2 and one particular anti-IL-2 mAb, clone JES6-1 have been shown to induce selective expansion of Treg lymphocytes with little or no effects to other cells.<sup>11</sup> However, the underlying mechanism has not been fully elucidated. One possible explanation is the increase in biological half-life of IL-2 by association with the anti-IL-2 mAb.<sup>25</sup> In our study, sustained expansion of Treg

lymphocytes was accomplished by repeated injection of IL-2/anti-IL-2 JES6-1 complex during the course of Ang II infusion. IL-2/anti-IL-2 complex selectively increased Treg numbers in spleens without affecting total CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers. Our results are consistent with previous reports that IL-2 coupled with JES6-1 restricts stimulation of Treg lymphocytes.<sup>13,26,27</sup> Interestingly, although Ang II infusion did not alter the number of splenic Treg lymphocytes, we found that the percentage of expanded Treg lymphocytes induced by IL-2/anti-IL-2 complex treatment were significantly reduced with Ang II infusion. A possible mechanism is that Ang II increases production of TNF- $\alpha$ ,<sup>28</sup> which has been shown to impair differentiation and function of Treg lymphocytes through Akt/Smad3 pathway<sup>29</sup> and dephosphorylation of Foxp3.<sup>30</sup>

Sustained selective expansion of Treg lymphocytes in the lymphoid compartment after IL-2 complex treatment decreased aortic wall thickness as well as reduced collagen accumulation in the vasculatures, suggesting that the cytokine complex treatment is effective in inhibiting Ang II-induced vascular remodeling in our study. In addition, the IL-2/anti-IL-2 complex also prevented aortic stiffening induced by Ang II. In a previous Treg adoptive transfer study, Barhoumi et al<sup>19</sup> demonstrated similar protective roles of Treg lymphocytes in Ang II induced arterial stiffening in small peripheral arteries. In this adoptive transfer study, injection of Treg lymphocytes also prevents Ang II-induced blood pressure elevation, whereas in our study stimulation of Treg lymphocytes with the IL-2/anti-IL-2 complex had no effect on blood pressure response to Ang II. This inconsistency may be explained by the different degree of enhancing Treg lymphocytes population since another adoptive transfer study with lower dose of Treg lymphocytes

also showed that transfer of Treg lymphocytes ameliorates Ang II-induced cardiac hypertrophy and fibrosis without lowering blood pressure.<sup>18</sup>

Ang II-dependent hypertension and vascular remodeling is associated with immune cell infiltration and T cell activation. Consistent with previous reports,<sup>5,17</sup> our study demonstrated that infusion of Ang II led to an increase in Th1 and Th17 immune responses in both lymphoid compartment and aortic tissues. IFN $\gamma$ , a Th1 cytokine, and IL-17, a Th17 cytokine were up-regulated during Ang II infusion. Expanded Treg lymphocytes with IL-2/anti-IL-2 complex suppressed CD4<sup>+</sup> T cells producing IFN $\gamma$  or IL-17, consistent with a known mechanism of naturally occurring Treg lymphocytes to inhibit cytokine formation of CD4<sup>+</sup> cells. Also, IL-2/anti-IL-2 complex treatment reduced aortic T cell and macrophage infiltrates. This is possibly due to a reduced expression of adhesion molecules, MCP-1 and VCAM-1, for macrophage accumulation with IL-2 complex treatment,<sup>13</sup> and/or a result of Th1 inhibition as Th1 lymphocytes promote macrophage intrusion via CXCL-9, 10, and 11 chemokines<sup>31</sup> and blockade of IFN $\gamma$  signaling reduces cardiac macrophage and T cell infiltrates.<sup>6</sup>

A limitation of this study is that we were not able to detect Foxp3<sup>+</sup> Treg lymphocytes in the aortas with the technique used, possibly due to their naturally low expression in vasculature. Moreover, the potential mechanisms that Treg lymphocytes used to inhibit immune responses and vascular remodeling are still unclear. We cannot presume that the suppression of Th1 and Th17 we observed after IL-2 complex treatment is solely due to the ability of the complex to stimulate Treg lymphocytes without taking

into consideration that IL-2 also directly influences and regulates various immune cells; for example, IL-2 signaling through transcription factor STAT5 directly constrains IL-17 production and Th17 polarization.<sup>32</sup> Further studies will be necessary to clarify the effect of Treg lymphocytes in the vasculature during Ang II-induced hypertension.

### **Perspectives**

Our data extend the concept of adaptive immunity in Ang II-dependent hypertension and emphasize a protective role of regulatory T cells in preventing vascular complications induced by Ang II. The balance between suppressive regulatory T cells and responder T cells, particularly Th1 and Th17, may contribute to regulation of vascular structure and function in response to Ang II. Stimulation of Treg lymphocytes by IL-2/anti-IL-2 antibody complexes could be a new therapeutic approach in Ang II-induced vascular diseases.

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### **Disclosure**

None declared.

## References

1. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: From physiology to the pathobiology of hypertension and kidney disease. *Pharmacological Reviews*. 2007;59(3):251-287.
2. Mehta PK, Griendling KK. Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. *American Journal of Physiology - Cell Physiology*. 2007;292(1):C82-C97.
3. Ruiz-Ortega M, Lorenzo O, Rupérez M, et al. Role of the renin-angiotensin system in vascular diseases: Expanding the field. *Hypertension*. 2001;38(6):1382-1387.
4. Guzik TJ, Hoch NE, Brown KA, et al. Role of the T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204(10):2449-2460.
5. Shao J, Nangaku M, Miyata T, et al. Imbalance of T-cell subsets in angiotensin II–Infused hypertensive rats with kidney injury. *Hypertension*. 2003;42(1):31-38.
6. Marko L, Kvakan H, Park JK, et al. Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage. *Hypertension*. 2012;60(6):1430-1436.
7. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008;133(5):775-787.

8. Barhoumi T, Kasal DA, Li MW, et al. T regulatory lymphocytes prevent angiotensin II-Induced hypertension and vascular injury. *Hypertension*. 2011;57(3):469-476.
9. Kassan M, Galan M, Partyka M, Trebak M, Matrougui K. Interleukin-10 released by CD4(+)CD25(+) natural regulatory T cells improves microvascular endothelial function through inhibition of NADPH oxidase activity in hypertensive mice. *Arterioscler Thromb Vasc Biol*. 2011;31(11):2534-2542.
10. Webster KE, Walters S, Kohler RE, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: Induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *The Journal of Experimental Medicine*. 2009;206(4):751-760.
11. Lee S, Cho M, Oh H, et al. Interleukin-2/anti-interleukin-2 monoclonal antibody immune complex suppresses collagen-induced arthritis in mice by fortifying interleukin-2/STAT5 signalling pathways. *Immunology*. 2012;137(4):305-316.
12. Dinh TN, Kyaw TS, Kanellakis P, et al. Cytokine therapy with interleukin-2/Anti-Interleukin-2 monoclonal antibody complexes expands CD4+CD25+Foxp3+ regulatory T cells and attenuates development and progression of atherosclerosis. *Circulation*. 2012;126(10):1256-1266.
13. Zieman SJ, Melenovsky V, Kass DA. Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler Thromb Vasc Biol*. 2005;25(5):932-943.

14. Keyes JT, Haskett DG, Utzinger U, Azhar M, Vande Geest JP. Adaptation of a planar microbiaxial optomechanical device for the tubular biaxial microstructural and macroscopic characterization of small vascular tissues. *J Biomech Eng.* 2011;133(7):075001.
15. Di Martino ES, Bohra A, Vande Geest JP, Gupta N, Makaroun MS, Vorp DA. Biomechanical properties of ruptured versus electively repaired abdominal aortic aneurysm wall tissue. *J Vasc Surg.* 2006;43(3):570-6; discussion 576.
16. Madhur MS, Lob HE, McCann LA, et al. Interleukin 17 promotes angiotensin II–Induced hypertension and vascular dysfunction. *Hypertension.* 2010;55(2):500-507.
17. Kvakan H, Kleinewietfeld M, Qadri F, et al. Regulatory T cells ameliorate angiotensin II–induced cardiac damage. *Circulation.* 2009;119(22):2904-2912.
18. Barhoumi T, Kasal DA, Li MW, et al. T regulatory lymphocytes prevent angiotensin II–Induced hypertension and vascular injury. *Hypertension.* 2011;57(3):469-476.
19. Fontenot JD, Rudensky AY. Molecular aspects of regulatory T cell development. *Semin Immunol.* 2004;16(2):73-80.
20. Matrougui K, Zakaria AE, Kassan M, et al. Natural regulatory T cells control coronary arteriolar endothelial dysfunction in hypertensive mice. *The American journal of pathology.* 2011;178(1):434-441.

21. Boyman O, Surh CD, Sprent J. Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease. *Expert Opin Biol Ther.* 2006;6(12):1323-1331.
22. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med.* 2005;201(5):723-735.
23. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol.* 2005;6(11):1142-1151.
24. Finkelman FD, Madden KB, Morris SC, et al. Anti-cytokine antibodies as carrier proteins. prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. *The Journal of Immunology.* 1993;151(3):1235-1244.
25. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science.* 2006;311(5769):1924-1927.
26. Letourneau S, van Leeuwen EM, Krieg C, et al. IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor alpha subunit CD25. *Proc Natl Acad Sci U S A.* 2010;107(5):2171-2176.

27. Hoch NE, Guzik TJ, Chen W, et al. Regulation of T-cell function by endogenously produced angiotensin II. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 2009;296(2):R208-R216.
28. Zhang Q, Cui F, Fang L, Hong J, Zheng B, Zhang JZ. TNF- $\alpha$  impairs differentiation and function of TGF- $\beta$ -induced treg cells in autoimmune diseases through akt and Smad3 signaling pathway. *Journal of Molecular Cell Biology*. 2013;5(2):85-98.
29. Nie H, Zheng Y, Li R, et al. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF- $\alpha$  in rheumatoid arthritis. *Nat Med*. 2013;19(3):322-328.
30. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med*. 2008;205(7):1535-1541.
31. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*. 2007;26(3):371-381.

## Figures

### Figure 1.

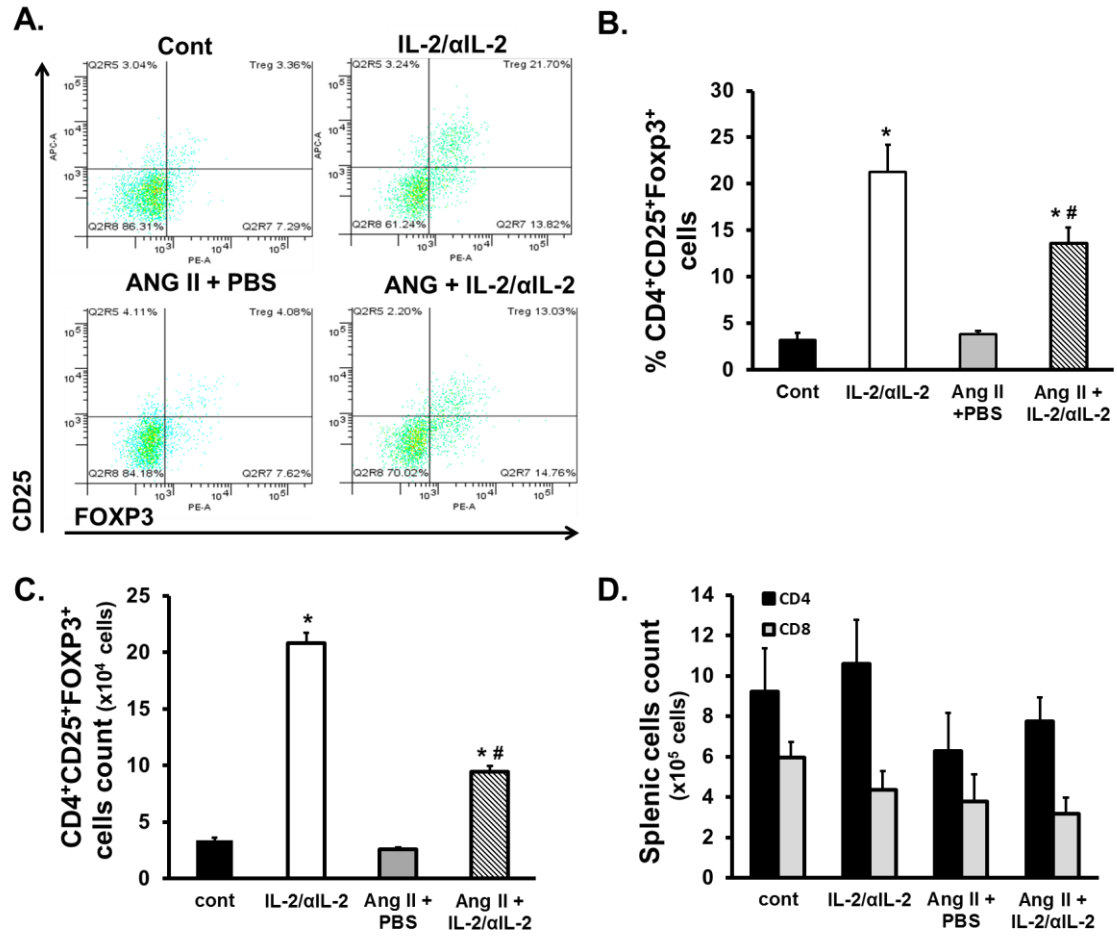


Figure 2.

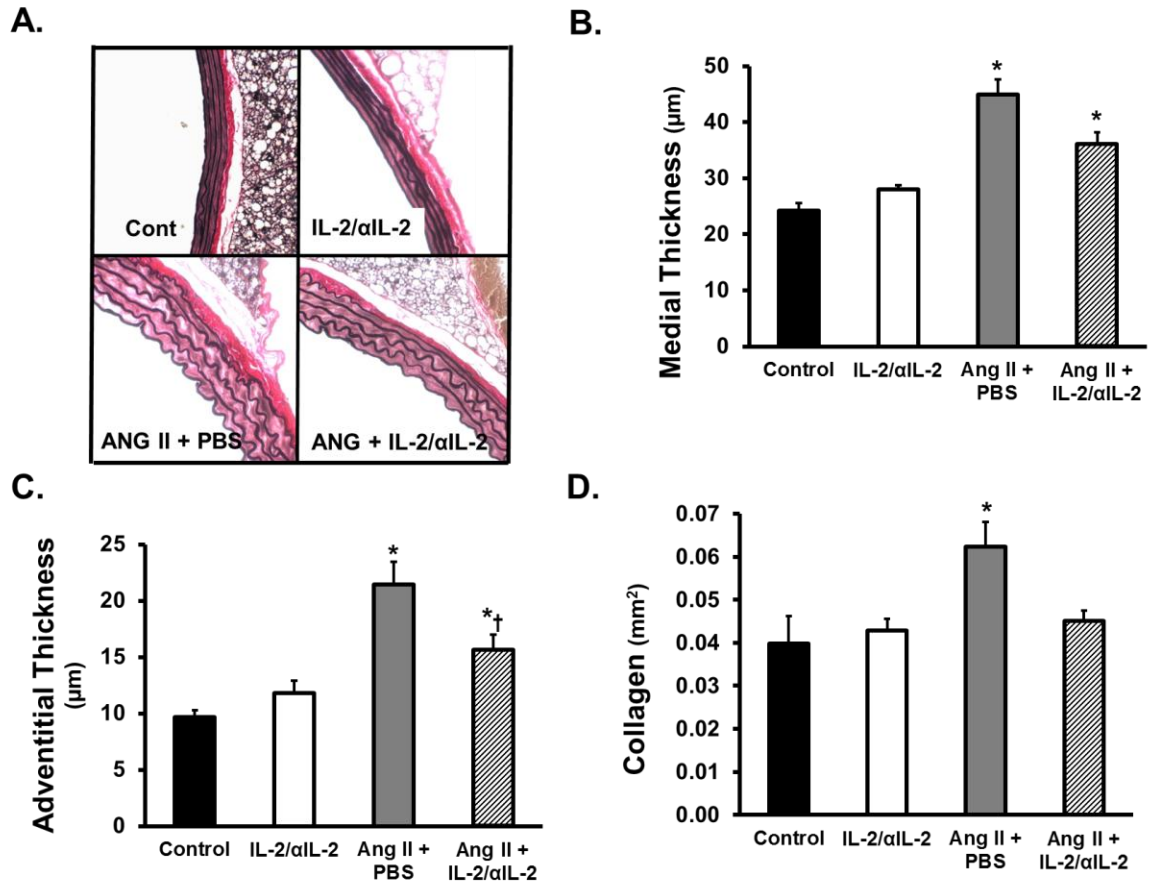


Figure 3.

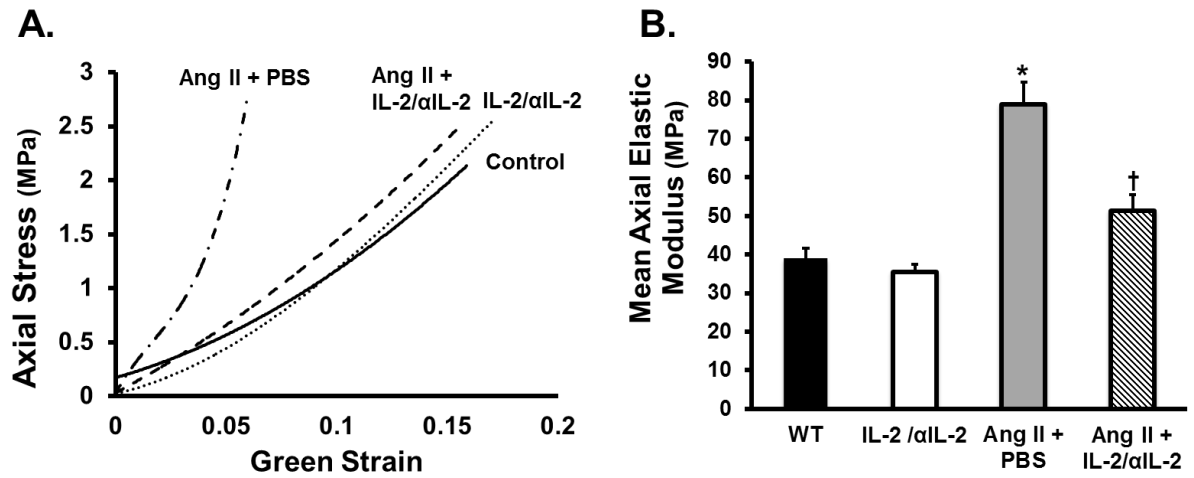


Figure 4.

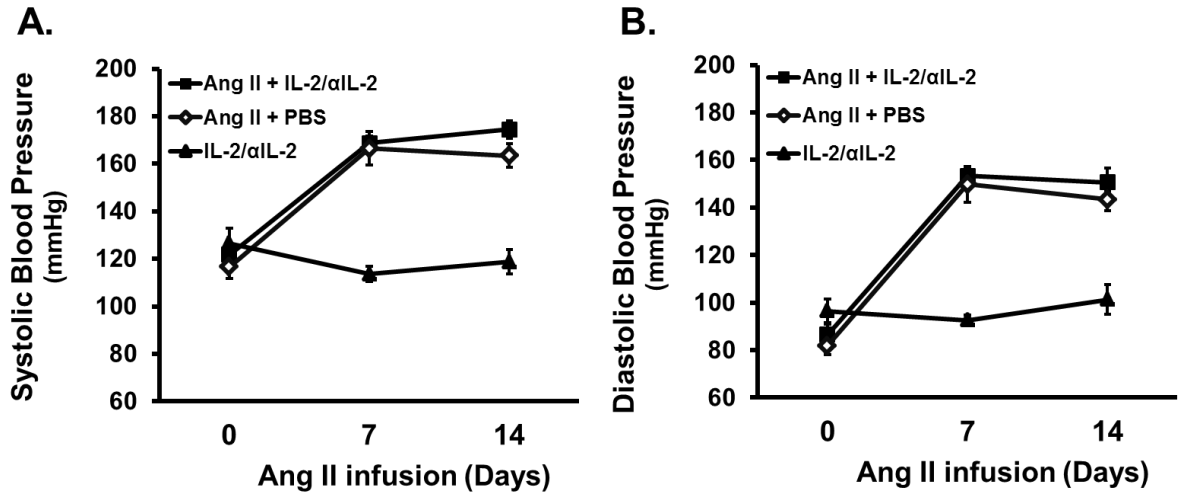


Figure 5.

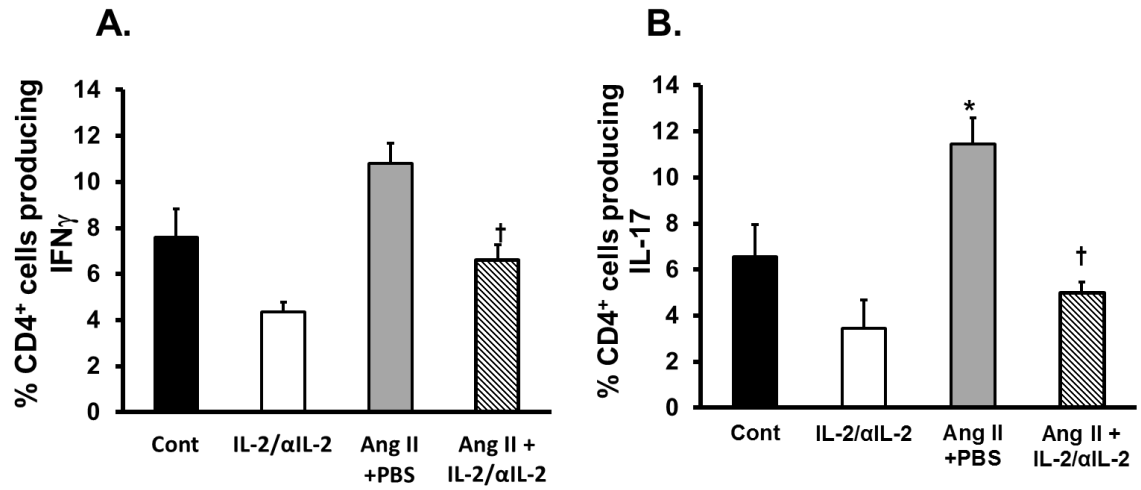
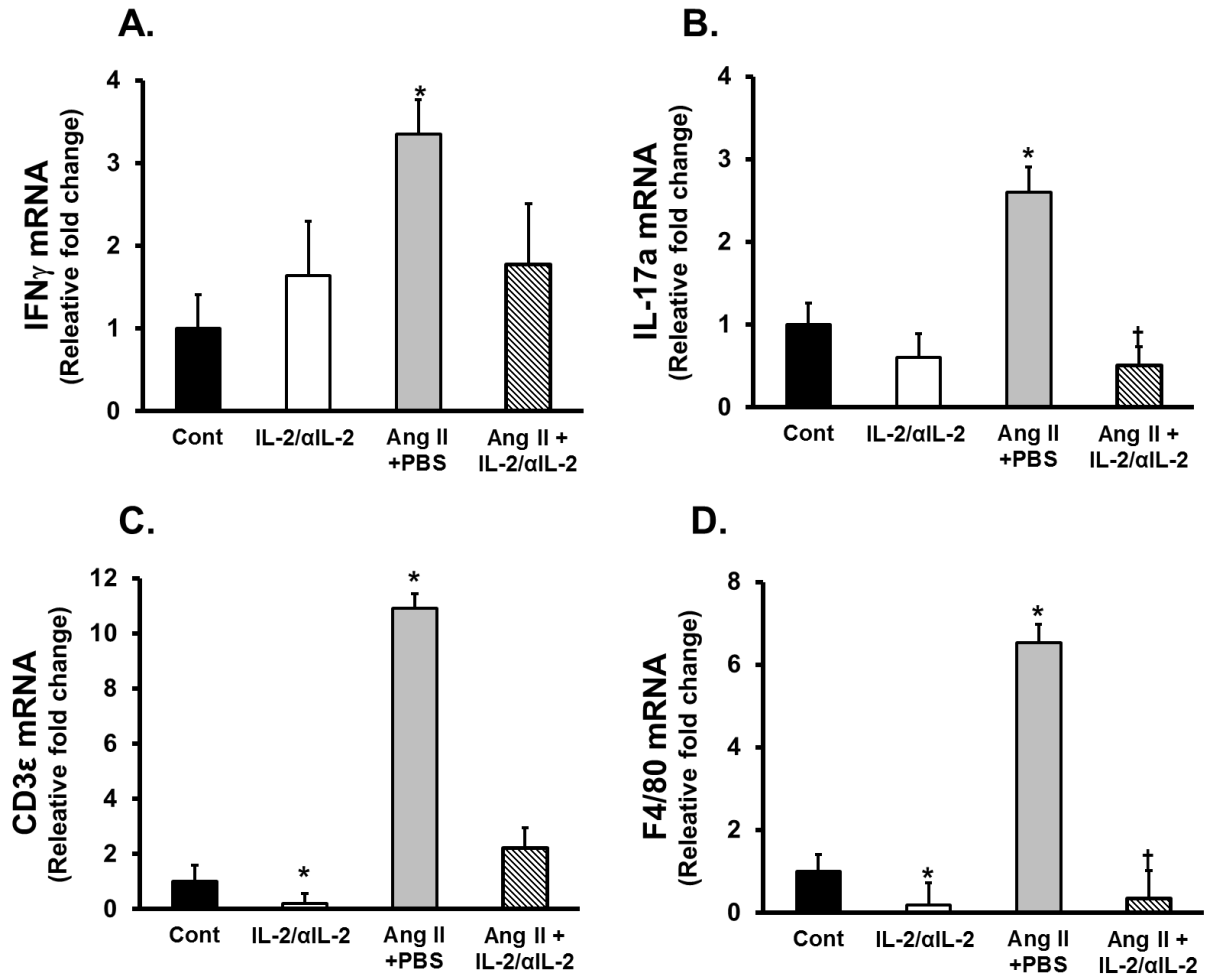


Figure 6



## Figure Legends

### Figure 1.

**Expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells with IL-2/anti-IL-2 complex treatment.** Mice were injected i.p. daily with IL-2/anti-IL-2 JES6-1 mAb (IL-2/ $\alpha$ IL-2) or PBS for 5 days. [Val<sup>5</sup>]-Angiotensin II (Ang II) was then infused via osmotic Alzet pumps on day 7. After that, the mice were received the IL-2/anti-IL-2 complex or PBS 3 times weekly throughout the 14 days of Ang II treatment. Mice received saline infusion serve as a baseline control. Lymphocytes isolated from the spleens were then analyzed with flow cytometry. (A) Representative flow analysis of Tregs which express CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> from the four groups of mice. (B) The mean percentage of Tregs gated from CD4<sup>+</sup> population shows a significant increase in Tregs population after IL-2/ $\alpha$ IL-2 complex treatment. (C) Total splenic CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> cells number. (D) Total splenic CD4<sup>+</sup> and CD8<sup>+</sup> cells number. N = 7-11 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and #P <0.05 for IL-2/ $\alpha$ IL-2 complex vs Ang II plus IL-2/ $\alpha$ IL-2.

### Figure 2.

**Expansion of Tregs with IL-2/anti-IL-2 complex impairs Ang II induced aortic remodeling.** 4 groups of mice were perfusion fixed at day 14 of Ang II infusion. The lower thoracic aortas were paraffin embedded and stained with Masson's Trichrome, VerhoeffVan Gieson (VVG), and Picosirius Red (PSR) for histomorphometric analysis. (A) Representative photomicrographs of VVG stained aortic transverse sections from the treatment groups compared with their littermate control are shown. (B) The mean aortic

medial thickness and (C) aortic adventitial thickness were quantified using Image J program. (D) The mean collagen density stained with PSR. N = 5 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and  $\dagger$ P <0.05 for Ang II vs AngII plus IL-2/ $\alpha$ IL-2.

### **Figure 3.**

#### **Expansion of Tregs with IL-2/anti-IL-2 complex prevented vascular stiffness**

**induced by Ang II.** Aortic stiffness was determined by *ex vivo* biomechanical analysis.

The descending thoracic aortas were subjected to microbiaxial test to derive stress-strain responses. (A) Representative plots of axial stress-strain shows a decreased aortic stiffness in Ang II infused mice after receiving IL-2/ $\alpha$ IL-2 treatment. (B) Mean axial stiffness shown as elastic modulus was calculated from the slopes taken at the peak stress-strain plots. N = 3-8 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and  $\dagger$ P <0.05 for Ang II vs AngII plus IL-2/ $\alpha$ IL-2.

### **Figure 4.**

**IL-2/anti-IL-2 complex does not affect Ang II induced hypertension.** Blood pressure measurements were obtained from non-invasive Hatteras tail cuff system at day 0, 7, and 14 after Ang II infusion. The mean values of systolic (A) and diastolic (B) blood pressure increased with Ang II infusion. Administration of IL2/ $\alpha$ IL-2 complex did not reduce the increased BP induced by Ang II. N = 6-12 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and  $\dagger$ P <0.05 for Ang II vs AngII plus IL-2/ $\alpha$ IL-2.

### **Figure 5.**

**IL-2/anti-IL-2 inhibits Ang II-induced Th1 and Th17 in splenocytes.** (A) Splenic lymphocytes were isolated and incubated with PMA, Ionomycin, and Brefeldin A for 5

hours for cytokines detection. The lymphocytes were gated on CD4<sup>+</sup> population. The mean percentage of CD4<sup>+</sup> T cells producing IFN $\gamma$  was shown. (B) The mean percentage of splenic CD4<sup>+</sup> T cells secreting IL-17a. N = 8-10 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and <sup>†</sup>P <0.05 for Ang II vs AngII plus IL-2/ $\alpha$ IL-2.

**Figure 6.**

**IL-2/anti-IL-2 suppresses immune cells infiltration and expression of IFN $\gamma$  and IL-17 in the aortas.** Relative genes expression measured by real time PCR of IFN $\gamma$  (A), IL-17a (B), CD3 $\epsilon$  (C), and F4/80 (D) in the aortic tissues. mRNA expression was normalized by  $\beta$ -actin expression and reported as relative fold change to control group in each panel. N = 4-11 per group. Data are means  $\pm$  SEM \*P <0.05 vs control and <sup>†</sup>P <0.05 for Ang II vs AngII plus IL-2/ $\alpha$ IL-2.

**APPENDIX C - THE EFFECT OF ANGIOTENSIN II ON RENAL FUNCTION IS  
MODULATED BY CD4 LYMPHOCYTES**

“Paper is pending publication in AJP-Regulatory, Integrative, and Comparative  
Physiology”

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Abstract; 228

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Figures; 3

**ABSTRACT**

Angiotensin II (Ang II) plays important roles in the regulation of vascular tone, renal function, and immune cell activation. To explore whether the adaptive immune system plays a role in the renal hemodynamic response to Ang II, we compared WT with T- and B-cell deficient RAG1<sup>-/-</sup> mice. Mice were challenged with an infusion of angiotensin receptor-1 agonist [Val<sup>5</sup>] angiotensin II and the renal function was determined weekly. As expected the GFR progressively increased after 14 days of Ang II infusion in the WT mice. However the GFR markedly decreased in the RAG1<sup>-/-</sup> mice. Adoptive transfer of CD4<sup>+</sup> lymphocytes to the RAG1<sup>-/-</sup> mice reestablished the GFR to that of the WT controls. We subsequently examined the CD4<sup>+</sup> lymphocyte subsets and found in Th1-deficient (Tbet<sup>-/-</sup>) mice that Ang II infusion appropriately increased the GFR but in Th17-deficient (RORγt<sup>-/-</sup>) Ang II infusion markedly decreased renal function. In addition, the decrease of the GFR in the RAG1<sup>-/-</sup> mice was associated with significantly decreased urinary PGE<sub>2</sub> and elevated TXB<sub>2</sub> levels, as compared with the WT mice. Adoptive transfer of CD4<sup>+</sup> T-cells into RAG1<sup>-/-</sup> mice restored PGE<sub>2</sub> levels to that of the WT mice. PGE<sub>2</sub> levels were also decreased in Th17-deficient mice but remained similar to WT in Th1-deficient mice with Ang II infusion. In conclusion, this study indicates that Th17 plays an obligate role in the renal homeostatic response to Ang II.

**Key words:** Angiotensin II, immunodeficiency, Th17, PGE<sub>2</sub>, glomerular filtration rate

## INTRODUCTION

The essential roles of the renin-angiotensin-aldosterone system (RAAS) in preserving renal hemodynamics especially during periods of hypotension have been well established. Angiotensin II (Ang II) regulates renal vascular resistance and aldosterone-mediated water and electrolyte homeostasis mainly through angiotensin type 1 receptor (AT1R) (2). It is known that activation of the AT1R in vitro constricts both the afferent and efferent renal arterioles with a higher sensitivity to efferent than afferent arterioles (1). However the afferent vasoconstriction is largely offset through local secretion of vasodilatory and vasoconstrictive substances which optimize glomerular capillary perfusion pressures and thereby filtration. Therefore, in vivo vasoconstriction induced by Ang II is predominantly of the efferent arteriole as Ang II is a powerful stimulus for the secretion of prostaglandins that preferentially vasodilate the afferent arterioles.

Ang II plays an important role in maintaining GFR under conditions of hypotension or hypovolemia by increasing perfusion pressure within the glomerular capillary bed for any given volume. It should be noted that this response can be abrogated by substances that interfere with COX -2 metabolism. In some experimental models Ang II can produce a maladaptive increase in glomerular hyperperfusion resulting in progressive glomerular sclerosis (5). In contrast, it is widely accepted that prolonged renal hypoperfusion and ischemia results in the development of acute kidney injury; however limited hypoperfusion is followed by renal functional recovery lacking histopathological changes (18). Central to maintenance of normal glomerular perfusion is autoregulation of the renal arteriolar resistance. It has been demonstrated that in an Ang

II-hypertension rodent model with an experimentally impaired autoregulation response the renal vascular functional abnormalities precede any evidence of pathological structural changes (23), which is also supported with clinical evidence (15). Therefore, it is important to understand the pathways that alter the autoregulatory control of renal arteriolar vasculature.

Existing evidence also supports the crucial roles of the adaptive immune system, particularly T lymphocytes, in Ang II-dependent arterial hypertension. Immunodeficient mice have about a fifteen percent lower hypertensive response to Ang II compared with WT mice (11) and adoptive transfer of suppressive regulatory T lymphocytes reduces Ang II-induced hypertension (3). It follows therefore that T lymphocytes may affect renal vascular function as well. Lymphocytes have been associated with renal vascular dysfunction in Ang II-hypertensive rodents (22). When the lymphocyte proliferative response is suppressed with mycophenolate mofetil, the renal vascular autoregulatory function is preserved in Ang II-hypertensive rats (10) and renal injury is diminished (7). However the role of the CD4<sup>+</sup> and more specifically CD4<sup>+</sup> subsets on renal function is unknown.

Here we define the role of the CD4<sup>+</sup> lymphocyte in the regulation of the glomerular filtration rate (GFR) in murine Ang II-induced hypertension. Using chronic infusion of an AT1R agonist, we first examined the effect of Ang II-hypertension on the renal GFR in T and B deficient (RAG1<sup>-/-</sup>), and adoptive transfer of CD4<sup>+</sup> lymphocytes to RAG1<sup>-/-</sup> mice. These studies were followed with the role of Th1 deficient (Tbet<sup>-/-</sup>), Th17 deficient (RORγt<sup>-/-</sup>) mice on the GFR in Ang II-hypertension. Collectively, our data

reveal the differential importance of the CD4<sup>+</sup> lymphocyte phenotypes in the GFR and highlight the role of the Th17 in regulation of the renal prostanoid PGE<sub>2</sub>, which is critical in the Ang II mediated autoregulation of glomerular perfusion.

## **MATERIALS AND METHODS**

### **Animals**

C57BL/6, RAG1<sup>-/-</sup>, RORγt<sup>-/-</sup> and Tbet<sup>-/-</sup> male 10 week old mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All of the RAG1<sup>-/-</sup>, RORγt<sup>-/-</sup> and Tbet<sup>-/-</sup> mice used in this study were of C57BL/6 background. This study was approved by the University of Arizona Animal Care Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the mice were maintained in the animal facility of the University of Arizona and fed with NIH-31 Modified Open Formula Mouse/Rat Sterilized Diet from Harlan Laboratories. The mice were treated with [Val<sup>5</sup>] angiotensin II (Sigma-Aldrich) at 490 ng/min/kg via subcutaneously implantable osmotic pumps (model 1004; Alzet, Palo Alto, Calif., USA). The mice were then sacrificed on days 0, 7, or 14 during AngII infusion.

### **Blood Pressure Measurements**

All mice were trained with the tail cuff system and data were recorded for Days 0, 6 and 13. Blood pressure values were measured with the tail cuff system while the mice were placed on a heated platform (Hatteras Instruments, Cary, North Carolina). Blood pressure values recorded were from an average of ten consecutive measurements.

Twenty-four hour continuous blood pressure was also measured in a separate group of mice using a biotelemetry device (Data Sciences International, St. Paul, MN). The biotelemetry mice were anesthetized with isoflurane and the transmitter was positioned at the right flank. The mice were allowed to recover for 7 days before treated as described above and then the blood pressure was recorded daily throughout the treatment. We programed our biotelemetry system to measure the arterial blood pressure only during the awake phases of the diurnal cycle rather than continuous 24 hour measurements in order for our values to be comparable with tail-cuff measurements.

#### **T cell isolation and adoptive transfer**

Enriched T cells were isolated from spleens and lymph nodes of  $CD8^{-/-}$  with C57BL/6 background mice using lymphocyte gradient separation medium (Mediatech Inc. Herndon, VA) followed with IgG panning. Flow cytometry was used to confirm the purity of  $CD4^{+}$  cells.  $2 \times 10^7$  isolated  $CD4^{+}$  T cells were injected intraperitoneally into each  $RAG1^{-/-}$  two weeks prior to Ang II administration.

#### **Perfusion Fixation and Histological Staining**

Before perfusion fixation, the mice were anti-coagulated with subcutaneously injection of 100  $\mu$ l of 1,000 USP units/ml Heparin sodium. Thirty mL of saline was infused to remove vascular blood, followed by the administration of 2:1 3% glutaraldehyde: 1% formaldehyde solution at a constant perfusion pressure of 40 mmHg. The kidney tissues were paraffin embedded, cut at 5  $\mu$ m, and stained with H&E and Masson's Trichrome for pathology and fibrosis assessment.

#### **Flow cytometry**

Subsequent to isolation of splenic lymphocytes with gradient separation, lymphocytes were strained with cell surface antibodies. Efluor 450-conjugated anti-CD3, FITC-conjugated anti-CD4, and Percp-Cy5.5-conjugated anti-CD8a were purchased from eBioscience. The BD LSR II with BD FACSDiva software Flow cytometry system and Gatelologic software were used to analyze data.

### **GFR and urinary PGE<sub>2</sub> and TXA<sub>2</sub> levels**

Urine was collected directly from the bladder and blood from right atrium. Plasma was then separated from cellular components by centrifugation at 10000 rpm for 10 minutes. Urine and plasma samples were stored at -80°C prior to analyses. Creatinine concentration in urine and plasma was measured using the Creatinine Assay Kit (ab65340) from Abcam (Cambridge, MA, USA) according to the manufacturer's directions. Creatinine clearance rate was calculated to estimate glomerular filtration rate (GFR). The mice were placed in metabolic cages for 48 hours before the sacrifice to obtain urine volume. The GFR was calculated using the formula,  $GFR = (\text{urine creatinine} \times \text{urine flow}) / \text{plasma creatinine} / \text{body weight}$ . Urinary PGE<sub>2</sub> and TXB<sub>2</sub> concentrations were measured using ELISA kits from R&D Systems according to the manufacturer's directions.

### **Statistics**

ANOVA with multi-comparison procedures was used to test the difference among the defined groups. Values obtained from treatment groups were compared with control values using Student's t-test. Comparable non-parametric tests (Kruskal-Wallis and the

rank sum test) were substituted when tests for normality and equal variance failed. All data are reported as means  $\pm$  SEM.

## RESULTS AND DISCUSSION

### Divergence of renal function in WT compared with RAG1<sup>-/-</sup> mice

We hypothesized that with Ang II-induced hypertension the CD4<sup>+</sup> lymphocytes regulate renal function. We first compared the renal function in response to Ang II between WT and RAG1<sup>-/-</sup> mice. The AT1R agonist, [Val<sup>5</sup>] Ang II, was continuously administered subcutaneously at a dose of 490 ng/kg/min with Alzet<sup>®</sup> osmotic pumps. Most notably, with Ang II infusion over 14 days, the GFR progressively increased greater than 2-fold in the WT mice and significantly declined greater than 2-fold in the RAG1<sup>-/-</sup> mice (Fig. 1A). The urine volumes consequently increased by 54% (P<0.05) in the WT group and decreased by 61% (P<0.05) in the RAG1<sup>-/-</sup> group compared with control values. The WT response to Ang II-induced hypertension demonstrated what one would expect with a state of hyperfiltration. We did not evaluate inulin clearance, neurohormonal responses, free water excretion and urinary concentrating ability, but it is unlikely that such information would materially modify our conclusions. The data, therefore, suggest that Ang II-induced hypertension produced divergent responses related to the mouse phenotype with a state of renal hyperfiltration in the WT and hypoperfusion in the RAG1<sup>-/-</sup> mice.

Histological evaluation of the kidney tissues showed no evidence of histopathology or infiltrates after two weeks of Ang II infusion in the WT or RAG1<sup>-/-</sup> mice (Fig. 1B). Supporting the histological results, the urine albumin levels did not differ

in the WT and RAG1<sup>-/-</sup> groups throughout the 14 day study (Fig.1C). Therefore our low dose of Ang II combined with the two week study period did not appear to induce renal structural and functional pathology. The renal pathologist performing the histological assessment was blinded to the interventions thus limiting a possible bias. The kidneys were microscopically normal and therefore presented no diagnostic challenge. However others have observed renal histopathology and infiltrates with Ang II but at twice the Ang II dose and for a longer duration of time (8). The Ang II dose used in our study, however, induced increased arteriolar resistance in both strains of mice as confirmed with increased systolic blood pressure (Fig. 1D and E) which was only ten percent lower in the RAG1<sup>-/-</sup> mice. Our measured blood pressures were higher than that of Guzik (11) since we programed our biotelemetry system to measure the arterial blood pressure only during the awake phases of the diurnal cycle rather than continuous 24 hour measurements in order for our values to be comparable with tail-cuff measurements. These data add additional evidence that strongly suggest either the T or B lymphocytes affect the renal response to Ang II-induced hypertension.

#### **Adoptive transfer of CD4<sup>+</sup> lymphocytes restores the RAG1<sup>-/-</sup> GFR to that of the WT**

To further investigate the role of lymphocytes in the Ang II-induced renal function, we reconstituted the RAG1<sup>-/-</sup> mice with CD4<sup>+</sup> T lymphocytes. The CD4<sup>+</sup> T cells were isolated from pooled spleens and lymph nodes of naïve CD8<sup>-/-</sup> mice with a C57BL/6 background. Administration of 2x10<sup>7</sup> enriched CD4<sup>+</sup> T cells to each of the RAG1<sup>-/-</sup> mice was performed two weeks prior to Ang II infusion. We observed a 10% reconstitution of CD4<sup>+</sup> per total lymphocytes in the recipient mice after 2 weeks adoptive transfer. This is

compared with 18% CD4<sup>+</sup> lymphocytes in the WT. Fig. 1F shows a representative flow cytometric analysis from the splenic CD3<sup>+</sup> versus CD4<sup>+</sup> T lymphocytes of the WT and CD4<sup>+</sup> adoptive transferred RAG1<sup>-/-</sup> mice. There was no detection of CD8 expression in the spleens of recipient mice (<0.2%), confirming the purity of our transferred CD4<sup>+</sup> T cells. The adoptive transfer of CD4<sup>+</sup> T cells restored the GFR of the RAG1<sup>-/-</sup> mice to a level equivalent to the WT mice (Fig. 1G). These adoptive transfer data reveal that the CD4<sup>+</sup> lymphocytes have a fundamental role in the regulation of the GFR in the presence of Ang II.

### **Renal function of Th1- and Th17-deficient mice responds to Ang II-induced hypertension differently**

It has been described that Ang II infusion alters the balance of helper T lymphocytes by increasing the Th1 cytokine, interferon- $\gamma$  and decreasing Th2 cytokine, interleukin-4 (20). Also, the Th17 cells producing the pro-inflammatory cytokine, interleukin 17 (IL-17), were described to promote Ang II-induced hypertension (13). We sought, therefore, to determine the effect of Ang II administration on renal function in the Th1- and Th17-deficient mice, Tbet<sup>-/-</sup> and ROR $\gamma$ t<sup>-/-</sup> respectively. We found that the GFR increased in the Tbet<sup>-/-</sup> mice, which paralleled that of the WT mice (Fig. 2A). Figure 2A also shows a 40% decrease in GFR in the ROR $\gamma$ t<sup>-/-</sup> mice after 14 days of Ang II infusion, which resembles the decreased GFR in the RAG1<sup>-/-</sup>. The Th1- and Th17-deficient mice developed comparable hypertensive responses to Ang II without renal histopathology (Fig. 2BC). These data suggest that Ang II infusion increases the GFR in WT and RAG1<sup>-/-</sup> with adoptive transfer of CD4<sup>+</sup> lymphocytes as well as the Th1 KO (Tbet<sup>-/-</sup>) mice.

However, Ang II infusion markedly decreases the GFR in RAG1<sup>-/-</sup> and Th17-deficient (RORγt<sup>-/-</sup>) mice. With our observed reduction of the GFR in the RAG1<sup>-/-</sup> and RORγt<sup>-/-</sup> mice with Ang II infusion and an increase in the WT and Tbet<sup>-/-</sup> mice, we determined to define a potential mechanism. We explored if an interaction between the T-lymphocyte subsets and Ang II affect the GFR through modulation of prostanoids, since the direct and preferential vasodilatory effect of PGE<sub>2</sub> serves as a compensatory regulator of afferent vascular resistance to Ang II.

**PGE<sub>2</sub> and TXA<sub>2</sub> levels relate with the renal functional differences observed in the immune deficient mice.**

Ang II stimulates the synthesis and release of different prostanoids including prostaglandin (PG)E<sub>2</sub> and to a lesser extent thromboxane (TX) A<sub>2</sub> (19). PGE<sub>2</sub> counters the Ang II mediated afferent arteriolar vasoconstriction by acting through the PGE<sub>2</sub> receptors; EP2 and EP4 (21) and thus resulting in increased GFR (4). Supporting this concept, it has been shown that NSAIDs decrease prostaglandin synthesis, thereby markedly diminishing the GFR when accompanied with Ang II infusion (4).

We determined the weekly differential changes in PGE<sub>2</sub> and the stable metabolite of TXA<sub>2</sub>, TXB<sub>2</sub>, between the WT and the RAG1<sup>-/-</sup> immunodeficient mice during the Ang II-induced hypertension. The PGE<sub>2</sub> levels remained unchanged in the WT after Ang II infusion. However, the urinary PGE<sub>2</sub> was significantly decreased by half in the RAG1<sup>-/-</sup> (Fig. 3A). The urinary TXB<sub>2</sub> was decreased in the WT mice on day 14 of Ang II infusion but remained unchanged in the RAG1<sup>-/-</sup> mice (Fig. 3B). Most strikingly, adoptive transfer of CD4<sup>+</sup> lymphocytes restored the PGE<sub>2</sub> to WT levels (Fig. 3C) and reduced the TXB<sub>2</sub>

levels (Fig. 3D). We next explored the enzymatic mRNA expressions under these conditions. However, renal gene expression of *prostaglandin E synthase* (Ptges) and *prostaglandin-endoperoxide synthase 2* (COX-2) measured with real time RT-PCR did not differ between the WT and RAG1<sup>-/-</sup> mice (data not shown). These results suggest that the CD4<sup>+</sup> lymphocyte affects a downstream pathway in the release of PGE<sub>2</sub> and TXA<sub>2</sub>.

We next determined which CD4<sup>+</sup> lymphocyte subtype might regulate renal PGE<sub>2</sub> production. Consistent with the GFR, the urinary PGE<sub>2</sub> levels were decreased only in the RORγt<sup>-/-</sup> mice but not in the Tbet<sup>-/-</sup> mice (Fig. 3E). There was no significant change in TXB<sub>2</sub> levels in both strains (Fig. 3F). These data indicate that Ang II induced PGE<sub>2</sub> production in the kidney is dependent on Th17 but not Th1 lymphocytes.

Literature supporting our observations shows that suppression of T-cell activation with cyclosporin inhibits Ang II–stimulated prostaglandin production (6) and induces a decrease in GFR in both human (9) and animal models (16). Therefore, it appears that the renal secretion of PGE<sub>2</sub> may be under the control of the adaptive immune system. COX-2, a key enzyme in the PGE<sub>2</sub> synthesis pathway, can be induced by activated T lymphocytes and pro-inflammatory cytokines in mesenchymal stem cells (12). Our results suggest that CD4<sup>+</sup> T lymphocytes participate in a critical role in regulating the hemodynamic response to Ang II by modulating the release of PGE<sub>2</sub> and perhaps TXA<sub>2</sub>.

## **Conclusions**

These data clearly support the novel concept that Ang II's modulation of GFR is dependent upon CD4<sup>+</sup> lymphocytes and adoptive transfer of CD4<sup>+</sup> lymphocytes restores Ang II mediated changes in renal function to that of the WT. We have suggested that the

prostanoid pathway is a mechanism for these observations. It is known that PGE<sub>2</sub> and TXA<sub>2</sub> serve as autoregulatory mediators of renal afferent vascular resistance to Ang II however our work does not exclude other mediators such as purinoceptors (10). The Th17 lymphocyte appears to be necessary to maintain renal vascular autoregulation through at least PGE<sub>2</sub>. However even though the precise mechanism has yet to be fully defined, this GFR-lymphocyte dependency, as confirmed with the RAG1<sup>-/-</sup> mice and CD4<sup>+</sup> lymphocyte adoptive transfer studies, has broad clinical implications in the immunodeficient patient.

Our RORγt<sup>-/-</sup> data are consistent with others in the concept that Th17 lymphocytes are protective under certain circumstances (14). However, most Th17 research has been linked to the pathogenesis of autoimmune disorders. This identified subset plasticity provides an appreciation of the pleiotropic nature of the Th17 secretory associated factors. Since the Th17 lymphocyte has been recently identified as a pivotal mediator of many disease processes, there is a broad effort to develop Th17 suppressive regimes. Our data provide a cautionary note that suppression of the Th17 may have a negative effect on renal function under conditions where Ang II is needed to maintain perfusion pressure. In summary, we suggest that PGE<sub>2</sub> release as a compensatory mechanism to protect the GFR during Ang II infusion is dependent upon the CD4<sup>+</sup> lymphocyte Th17 subset.

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### Reference List

1. **Arima S.** Role of angiotensin II and endogenous vasodilators in the control of glomerular hemodynamics. *Clin Exp Nephrol* 7: 172-178, 2003.
2. **Banday AA and Lokhandwala MF.** Oxidative stress-induced renal angiotensin AT1 receptor upregulation causes increased stimulation of sodium transporters and hypertension. *Am J Physiol Renal Physiol* 295: F698-F706, 2008.
3. **Barhoumi T, Kasal DA, Li MW, Shbat L, Laurant P, Neves MF, Paradis P and Schiffrin EL.** T regulatory lymphocytes prevent angiotensin II-induced hypertension and vascular injury. *Hypertension* 57: 469-476, 2011.
4. **Baylis C and Brenner BM.** Modulation by prostaglandin synthesis inhibitors of the action of exogenous angiotensin II on glomerular ultrafiltration in the rat. *Circ Res* 43: 889-898, 1978.
5. **Brenner BM, Lawler EV and Mackenzie HS.** The hyperfiltration theory: a paradigm shift in nephrology. *Kidney Int* 49: 1774-1777, 1996.
6. **Bunke M, Wilder L and Martin A.** The effect of cyclosporine on agonist-stimulated glomerular and mesangial cell vasodilatory prostaglandin production. *Transplantation* 52: 718-722, 1991.

7. **Crowley SD, Frey CW, Gould SK, Griffiths R, Ruiz P, Burchette JL, Howell DN, Makhanova N, Yan M, Kim HS, Tharaux PL and Coffman TM.** Stimulation of lymphocyte responses by angiotensin II promotes kidney injury in hypertension. *Am J Physiol Renal Physiol* 295: F515-F524, 2008.
8. **Crowley SD, Zhang J, Herrera M, Griffiths R, Ruiz P and Coffman TM.** Role of AT(1) receptor-mediated salt retention in angiotensin II-dependent hypertension. *Am J Physiol Renal Physiol* 301: F1124-F1130, 2011.
9. **Gossmann J, Radounikli A, Bernemann A, Schellinski O, Raab HP, Bickeboller R and Scheuermann EH.** Pathophysiology of cyclosporine-induced nephrotoxicity in humans: a role for nitric oxide? *Kidney Blood Press Res* 24: 111-115, 2001.
10. **Guan Z, Giddens MI, Osmond DA, Cook AK, Hobbs JL, Zhang S, Yamamoto T, Pollock JS, Pollock DM and Inscho EW.** Immunosuppression preserves renal autoregulatory function and microvascular P2X1 receptor reactivity in ANG II-hypertensive rats. *Am J Physiol Renal Physiol* 304: F801-F807, 2013.
11. **Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, Goronzy J, Weyand C and Harrison DG.** Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med* 204: 2449-2460, 2007.

12. **Hegy B, Kudlik G, Monostori E and Uher F.** Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. *Biochem Biophys Res Commun* 419: 215-220, 2012.
13. **Madhur MS, Lob HE, McCann LA, Iwakura Y, Blinder Y, Guzik TJ and Harrison DG.** Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. *Hypertension* 55: 500-507, 2010.
14. **O'Connor W, Jr., Zenewicz LA and Flavell RA.** The dual nature of T(H)17 cells: shifting the focus to function. *Nat Immunol* 11: 471-476, 2010.
15. **Palmer BF.** Disturbances in renal autoregulation and the susceptibility to hypertension-induced chronic kidney disease. *Am J Med Sci* 328: 330-343, 2004.
16. **Perico N, Zoja C, Benigni A, Ghilardi F, Gualandris L and Remuzzi G.** Effect of short-term cyclosporine administration in rats on renin-angiotensin and thromboxane A2: possible relevance to the reduction in glomerular filtration rate. *J Pharmacol Exp Ther* 239: 229-235, 1986.
17. **Ricci Z, Cruz D and Ronco C.** The RIFLE criteria and mortality in acute kidney injury: A systematic review. *Kidney Int* 73: 538-546, 2008.
18. **Saotome T, Ishikawa K, May CN, Birchall IE and Bellomo R.** The impact of experimental hypoperfusion on subsequent kidney function. *Intensive Care Med* 36: 533-540, 2010.

19. **Scharschmidt LA, Lianos E and Dunn MJ.** Arachidonate metabolites and the control of glomerular function. *Fed Proc* 42: 3058-3063, 1983.
  
20. **Shao J, Nangaku M, Miyata T, Inagi R, Yamada K, Kurokawa K and Fujita T.** Imbalance of T-Cell Subsets in Angiotensin II-Infused Hypertensive Rats With Kidney Injury. *Hypertension* 42: 31-38, 2003.
  
21. **Tang L, Loutzenhiser K and Loutzenhiser R.** Biphasic actions of prostaglandin E(2) on the renal afferent arteriole : role of EP(3) and EP(4) receptors. *Circ Res* 86: 663-670, 2000.
  
22. **Zhang JD, Patel MB, Song YS, Griffiths R, Burchette J, Ruiz P, Sparks MA, Yan M, Howell DN, Gomez JA, Spurney RF, Wilson CB, Coffman TM and Crowley SD.** A Novel Role for Type 1 Angiotensin Receptors on T Lymphocytes to Limit Target Organ Damage in Hypertension. *Circ Res* 2012.
  
23. **Zhao X, Cook AK, Field M, Edwards B, Zhang S, Zhang Z, Pollock JS, Imig JD and Inscho EW.** Impaired Ca<sup>2+</sup> signaling attenuates P2X receptor-mediated vasoconstriction of afferent arterioles in angiotensin II hypertension. *Hypertension* 46: 562-568, 2005.

**FIGURE CAPTIONS****Figure 1. CD4<sup>+</sup> lymphocytes regulate renal hemodynamic response to Ang II.**

The GFR decreases in RAG1<sup>-/-</sup> and increases in the WT mice during the first 14 days of AT1 receptor stimulation. [Val<sup>5</sup>]-angiotensin II (Ang II) was infused subcutaneously at a rate of 490 ng/kg/min into WT and RAG1<sup>-/-</sup> mice with Alzet pumps for 14 day. (A) Mice were placed individually in metabolic cages for 48 hours before the sacrificing dates to obtain urine volume. Glomerular filtration rate (GFR) of the WT and RAG1<sup>-/-</sup> in response to Ang II was calculated based on urinary and plasma creatinine concentrations (n=6-8 per group). (B) Representative histology of kidney section from the WT and the RAG1<sup>-/-</sup> mice after administration of Ang II shows no pathology during Ang II administration in the WT or the RAG1<sup>-/-</sup>. (C) Albumin:Creatinine ratio obtained from bladder urine from the WT and RAG1<sup>-/-</sup> at sacrificing dates. (D) The mean values of systolic blood pressure obtained from non-invasive Hatteras tail cuff system at day 0, 6, and 13 after Ang II infusion show hypertensive responses in both WT and RAG1<sup>-/-</sup> mice (n=4-20 per group). (E) Representative tracing of bio-telemetry system compares the 24 hour blood pressures of unrestrained mice. (F) Enriched CD4<sup>+</sup> T cells were isolated from pooled spleens and lymph nodes of naïve CD8<sup>-/-</sup> mice. The isolated cells were injected to the RAG1<sup>-/-</sup> at a dose 2x10<sup>7</sup> cells per mouse 2 week prior to Ang II infusion. Representative flow cytometry analysis of splenocytes from the naïve WT, RAG1<sup>-/-</sup> before and after the adoptive transfer to confirm the purity and viable of reconstituted cells are shown. (G) GFR of the RAG1<sup>-/-</sup> received Ang II and CD4<sup>+</sup> cells were analyzed compared to the baseline level and the RAG1<sup>-/-</sup> received Ang II alone for 14 days (n=3-8 per group). Data

are means  $\pm$  SEM \*P <0.05 compared with respective day 0 within strains and  $\dagger$ P <0.05 compared with time matched WT.

**Figure 2. Th17 but not Th1 is necessary to protect GFR during Ang II administration.**

(A) GFR obtained from ROR $\alpha$ t $^{-/-}$  and Tbet $^{-/-}$  mice treated with Ang II at 490 ng/kg/min for 14 days in comparison with the WT and RAG1 $^{-/-}$  (n=7-11 per group). (B) Representative histology of kidney section stained with H&E from the ROR $\alpha$ t $^{-/-}$  and the Tbet $^{-/-}$  mice after administration of Ang II. (C) The mean values of systolic blood pressure measured by non-invasive Hatteras tail cuff system (n= 6-20). Data are means  $\pm$  SEM \*P <0.05 compared with respective day 0.

**Figure 3. The CD4 $^{+}$  subset, Th17, is required for PGE $_2$  secretion.**

(A) Urinary prostaglandin E $_2$  (PGE $_2$ ) concentration was measured from the bladder urine of the WT and the RAG1 $^{-/-}$  during Ang II infusion (n=6-8 per group). (B) Urinary thromboxane A $_2$  (TXA $_2$ ) was measured as its stable metabolites, TXB $_2$  (n=6-8 per group). Urinary PGE $_2$  (C) and TXB $_2$  level (D) of the RAG1 $^{-/-}$  received Ang II and CD4 $^{+}$  cells were analyzed compared to the baseline level and the RAG1 $^{-/-}$  received Ang II alone (n=3-8 per group). Urinary PGE $_2$  level (E) and TXB $_2$  level (F) from the ROR $\alpha$ t $^{-/-}$  and Tbet $^{-/-}$  mice were analyzed as described and compared with the WT. Data are means  $\pm$  SEM \*P <0.05 compared with respective day 0 within strains and  $\dagger$ P <0.05 compared with time matched WT.

## FIGURES

Figure 1.

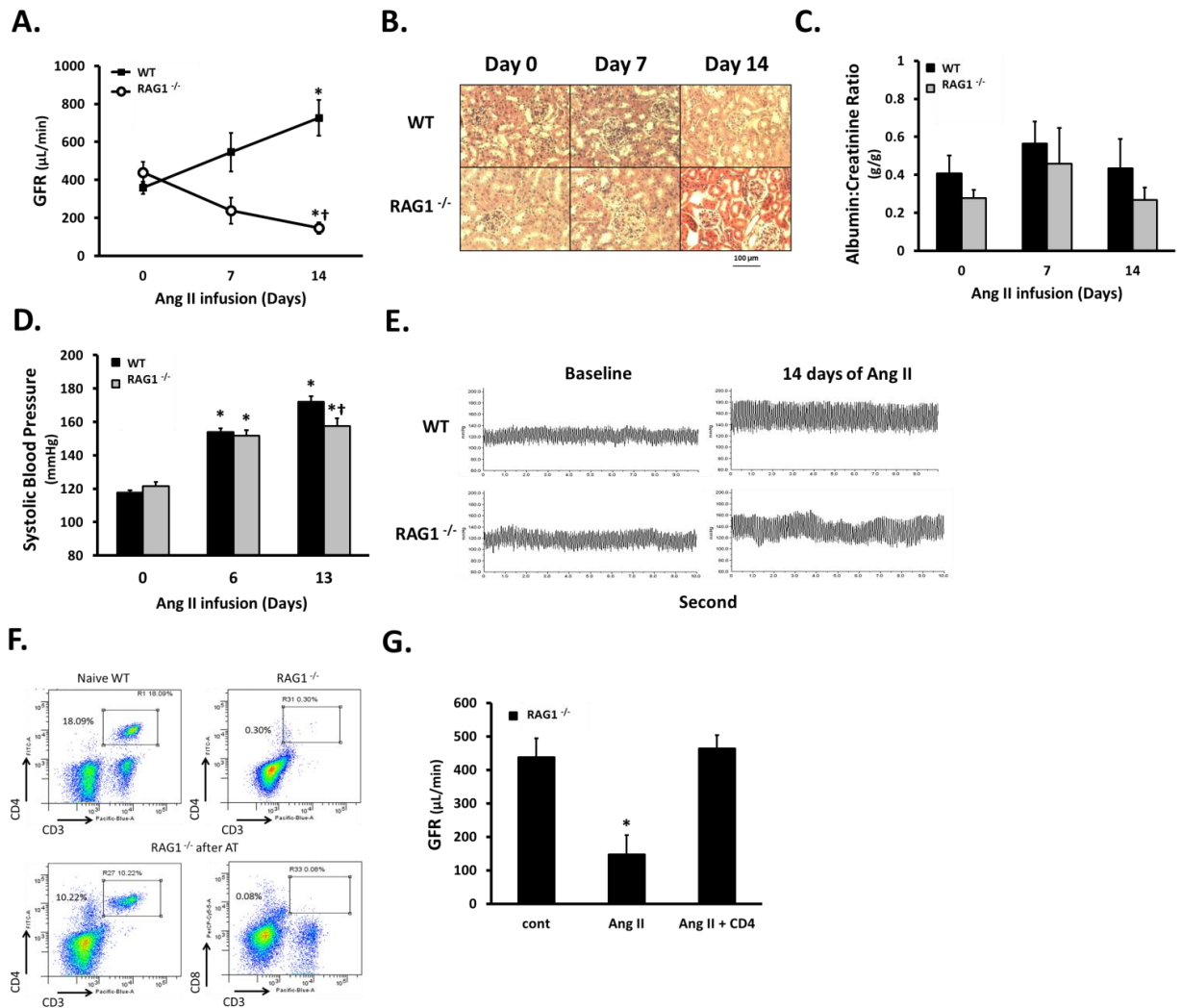


Figure 2.

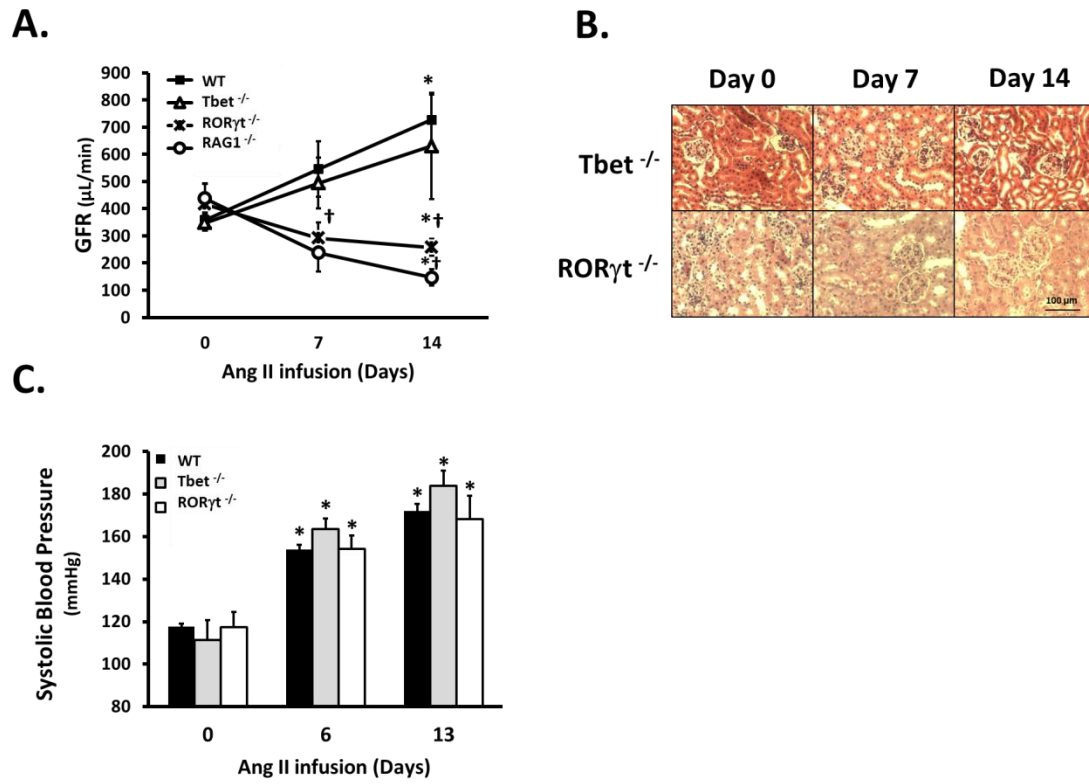


Figure 3.

