In Vitro Analysis of the Extracellular Expression of the Renin Angiotensin System on T Lymphocyte Populations

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ABSTRACT

Hypertension is a disease characterized by increased activity of the Renin Angiotensin System (RAAS) and immune related vascular dysfunction. Angiotensin II (Ang II) is the final effector molecule of the RAAS and has numerous biologic activities that perpetuates vascular remodeling and inflammation. Ang II signaling of inflammatory cells may be due the presence of RAAS components on T lymphocytes. Many studies have shown the importance of pro-inflammatory T cell phenotypes, through cytokine analysis, in hypertensive models. However, the specific characterization of the RAAS on these phenotypes has yet to be determined. We sought to establish the expression of RAAS components on naïve T cell subsets and compare that to changes in expression that may be seen with AngII treatment and anti-CD3/28 stimulation. Here we find that AngII and anti-CD3/28 treatments significantly increase the expression of RAAS components on T cell populations.

INTRODUCTION

The systolic blood pressure is the most important independent predictor of adverse cardiovascular events (1) (2). Blood pressure is the product of flow and resistance, where flow depends on cardiac output and blood volume; and resistance is influenced by the contractile state of the vascular sytem (mostly arteries). These components of blood pressure are subject to a range of regulatory mechanisms (Figure A). Therefore, defining the cause of diseases like hypertension requires considerations of many systems that control blood pressure homeostasis including: the central and sympathetic nervous sytem, the kidney, renin-angiotensin-aldosterone system (RAAS), the vasculature, and the numerous hormonal regulators of blood pressure. Interactions between these systems participates in blood pressure elevation and mediates end-organ damage. These regulatory systems are also the current targets for anti-hypertensive therapy (3).

Despite our understanding of the individual regulators involved in blood pressure maintenance, their molecular interactions in pathologic states remain unsolved, making it difficult to develop optimal therapy and predict prognosis (3). Of particular concern, is the interactions that occur between the RAAS and vascular remodeling, as this is a primary pathway to chronic elevations in blood pressure and subsequently fatal cardiovascular diseases. The significance of the RAAS in the pathogenesis of vascular dysfunction and stiffness is widely accepted (4).

The RAAS is the principal homeostatic regulator of intravascular volume, systemic blood pressure and tissue perfusion. This system responds to changes in blood pressure, blood volume, intravascular sodium and water and any clinical situation associated with altered hemodynamic stability that compromises organ blood flow. However, during chronic elevations in blood pressure, this system becomes persistent and dysregulated. Its final effector molecule angiotensin II (AngII), is the most important mediator in pathologic states. Through a series of hormonal cascades, angiotensin converting enzyme (ACE) cleaves Angiontensin I (AngI) to Angiotensin II (AngII), which is released into general circulation causing acute and chronic elevations in blood pressure. Acutely, it has a rapid pressor response via direct vasoconstriction, increased sympathetic outflow, and release of catecholamines. Over time, a chronic rise in blood pressure occurs through constant sodium reabsorption from the kidney and structural remodeling in the vasculature (1). It is these chronic maladaptive changes that progress to more fatal cardiovascular disorders.

Cardiovascular structural remodeling in hypertension is caused by AngII and leads to irreversible vascular dysfunction and chronic inflammation. Many studies have questioned the ability of systemically circulating AngII to maintain this chronic pathologic state and believe that it's synthesis and production by the local cells is a more probable cause. Evidence stating that components of the RAAS, including AngII, are locally produced by adventitia, smooth muscle cells and endothelial cells of the vasculature, have fueled these theories (5) (6). AngII can alter gene transcription in favor of vascular remodeling and hypertrophy by binding to its receptor, Angiotensin Type 1

Receptor (AT1R) (7). The effects seen from this binding include production of growth factors, extracellular matrix proteins, and inflammatory mediators. Specifically, vascular smooth muscle cells (VSMC) grow and proliferate, there is enhanced extracellular matrix deposition and there is stimulation of vascular adhesion molecules. (7) (8) (9). The increased expression of adhesion molecules like vascular adhesion molecule (VCAM-1) (7), stimulates the recruitment of monocytes, dendritic cells, natural killer cells (NK), and B and T lymphocytes in the layers of the vascular wall (9). This accumulation of inflammatory cells not only participates in the mechanical alterations of the vascular wall, but appears to be a necessary component in AngII induced hypertension. Studies have found that without this characteristic inflammatory response, hypertensive states do not persist (10) (11).

The association between the RAAS and inflammation is present in other disease processes as well. By preventing the synthesis and or the effects of AngII many studies have demonstrated alleviation of diseases like coronary graft disease in a murine model of heart transplantation, experimental autoimmune myocarditis and chronic allograft nephropathy (12) (13) (14). In particular, treatment with angiotensin-converting enzyme (ACE) inhibitors was shown to suppress pro-inflammatory mediators while increasing inflammatory regulators, involved in experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis. This effect was sufficient enough to reverse paralytic EAE (15). Furthermore, these mechanisms may not be limited to animal models. It was shown that HIV positive males, with depleted CD4+ T cell populations are significantly less prone to developing hypertension compared to those that are not

(11). Recent studies have strengthened the idea of hypertension as an immunological disease by eliminating the array of potential immune mediators that could be responsible. Guzik et al. in 2007 discovered that the adoptive transfer of T cells, not B cells into RAG $^{-/-}$ mice treated with Ang II, induced a full hypertensive response (10). Evidence from these studies has shed light on the importance of the altered inflammatory response that participates in the maintenance of hypertension.

T lymphocytes that accumulate in the perivascular space during hypertension release cytokines that affect adjacent vascular cells. These cytokines help to maintain the deleterious environment that occurs in hypertension. Several studies support that an imbalance of specific cytokines released from $CD4^+$ T-helper cell lineages maintain a pro-inflammatory environment. For example, Shao J. et al found there to be an increase in the cytokine interferon-gamma (IFNy), a pro-inflammatory cytokine characteristically secreted by the T helper 1 (Th1) subset of $CD4^+$ lymphocytes; and a decrease in interleukin 4 (IL-4), an immune-suppressing cytokine characteristically secreted by the T helper 2 (Th2) subset of $CD4^+$ lymphocytes (16). However, it seems that the most recently characterized lineage of the $CD4^+$ T lymphocyte population, T helper 17 (Th17) cells play more of a pathologic role in vascular dysfunction. Madhur MS. et al, described IL-17, the characteristic cytokine produced by Th17 cells, as a primary mediator necessary for the development of Ang II-induced hypertension. They showed that $IL17^{-/-}$ mice do not sustain hypertension and lack the vascular accumulation of T lymphocytes caused by Ang II (17). In addition to Th17 cells, another subset of $CD4^+$ cells are the T regulatory (T regs) cells. This subset functions to maintain self-tolerance and anti-

inflammatory properties in part through its released cytokine, IL-10 and counter-active effects on the Th17 subset. Treg deficiency is associated with autoimmune diseases in humans and in mice (18), and on the other hand it's presence has been shown to decrease cardiac inflammation, hypertrophy and fibrosis caused by chronic AngII-induced hypertension (19). Tregs have been shown to prevent AngII-induced hypertension, vascular damage and immune cell infiltration including cytokines IFN-gamma, TNF-alpha, and IL-6 (20).

Cells of the adaptive immune system may possess express RAAS components on their surface. Recent studies have found the Angiotensin type 1 receptor (AT1R) to be expressed on various leukocyte subsets including T lymphocytes, macrophages and B cells (21) (22). Other RAAS components including the AT1R has also been shown to be expressed and (23) required for the normal development and function of dendritic cells (DCs) (24). Jurewicz M et al. in 2007 found human $CD8^+$ T cells and NK cells to express renin, the renin receptor, angiotensinogen and angiotensin-converting enzyme through mRNA analysis. They concluded that these immune cells were capable of producing and delivering AngII to sites of inflammation (25). Similarly Hoch NE., et al. found T cells to possess an endogenous RAAS. Their T cells expressed angiotensinogen, angtiotensin converting enzyme (ACE), renin and produced physiologic levels of Ang II (26). Studies with similar findings have investigated the effect AngII causes by binding to its receptor in immune cells. For example, AngII, acting through the AT1R on immune cells, triggered the proliferation of splenic lymphocytes through a calcineurin-dependent pathway (21). Conversely, another report shows that AngII alone has no effect on NK

and T cell proliferation, but in the presence of the mitogens (phytohemagglutinin or anti-CD3), the addition of AngII can exerts a co-stimulatory effect on T cell proliferation (25). Silva-Filho in 2011 discovered that after 48-h exposure to exogenous AngII, produced no changes in the acute activation markers CD25 and CD69 in both $CD4^+$ and $CD8^+$ lymphocyte subsets in relation to cells treated with anti-CD3 alone. However, these activation markers were significantly decreased when Losartan (AngII type 1 receptor antagonist) was added indicating that endogenous production of AngII could mediate anti-CD3 activation of $CD4^+$ and $CD8^+$ T lymphocytes (27). It is clear that the chronic vascular inflammatory process that occurs in hypertension may be perpetuated by the presence of RAAS components on immune cells.

In summary, hypertension is a disease characterized by the activity of the RAAS and immune related vascular dysfunction. These seemingly unrelated systems seem to be working in concert to perpetuate cardiovascular dysfunction in hypertension through the presence of RAAS components on immune cells including T lymphocytes. Many studies have shown the importance of specific immune cells through cytokine analysis in hypertensive models, however the specific characterization of RAAS components on T lymphocyte subsets has yet to be determined. With an understanding of the differential expression with in these subsets, we may be able to show that the RAAS has the propensity to propel one specific phenotype and therefore target one lymphocyte lineage.

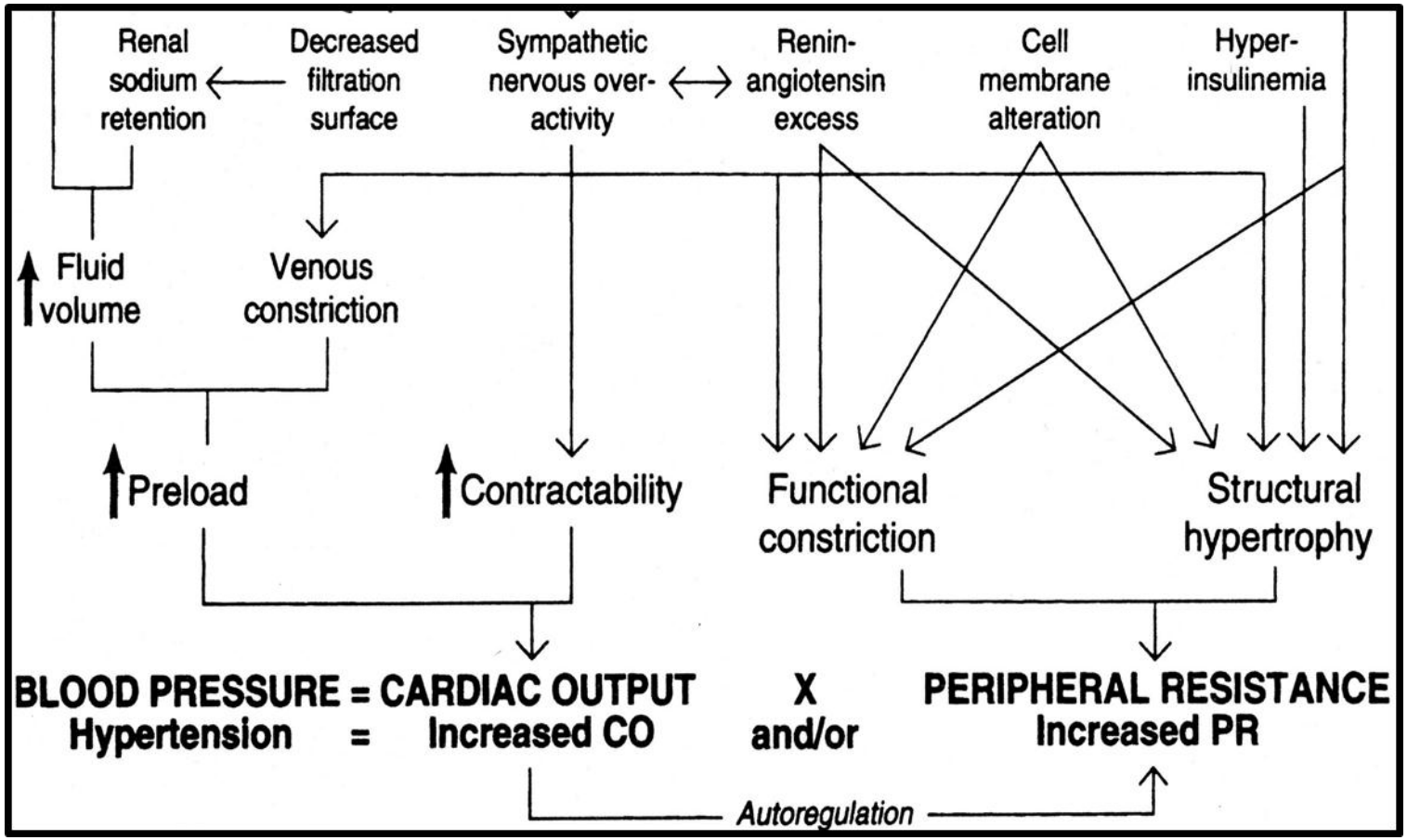

Figure A. Regulatory Mechanisms in Blood Pressure Maintenance (1)

The Renin Angiotensin System

Components

The renin-angiotensin aldosterone hormonal cascade is initiated by the synthesis of renin from the juxtaglomerular cells (JG) located in the afferent arteriole of the renal glomerulus. Upon stimulation, prorenin is cleaved to form mature renin and is released from the granules of the JG cells. In addition, the kidney also releases pro-renin which accounts for 70-90% of the immune-reactive renin in circulation (5). The secretion of renin is regulated by 4 mechanisms: (1) renal baroreceptors in the afferent arteriole that senses changes in the renal perfusion pressure, (2) changes in the delivery of NaCl in the macula densa cells of the distal tubule (together with the JG cells they make up the JG apparatus), (3) sympathetic nerve stimulation via beta-1 adrenergic receptors and (4) negative feedback by direct action of the AngII on the JG cells. Renin secretion is stimulated under circumstances in which perfusion pressure or NaCl declines and sympathetic activity increases. Renin can also be synthesized in other tissues including: brain, adrenal gland, ovary, and visceral adipose tissue and perhaps heart and vascular tissue. The mechanisms involved in its synthesis in these other tissues are not as well understood. Renin secretion is the key enzymatic regulator in the rate-limiting step of the RAAS.

Once renin is released into systemic circulation, it cleaves the N-terminal portion of angiotensinogen (AGT) to form the biologically inactive Angiotensin I (AngI). The main source of circulating AGT is the liver however its mRNA expression has been shown in cells of the kidney, brain, heart, vascular, adrenal gland, ovary, placenta and adipose tissue (28). Generally plasma levels remain constant since it is secreted constitutively, however it's synthesis can rise in response to glucocorticoids, sex steroids, thyroid hormone, inflammatory cytokines

(IL-1 and tumor necrosis factor), and AngII (28). Long-term rises in AGT have been suggested to be a risk factor for hypertension, although there is data suggesting long-term stimulation of AGT can be compensated by decreased renin secretion.

Angiotensin converting enzyme (ACE) hydrolyzes the inert AngI compound yielding the active form, AngII. ACE is membrane-bound exopeptidase that exists on the plasma membranes of vascular endothelial cells, microvillar brush border epithelial cells (renal proximal tubule cells), and neuroepithelial cells. ACE also exists in the soluble form in plasma, however it is the membrane-bound form that is thought to be physiologically important. ACE metabolizes a number of other peptides, including the vasodilator peptides bradykinin and kallidin, to inactive metabolites (29). Thus, ACE participates in increased vasoconstriction and decreased vasodilation.

Subsequent cleavages of AngI and II yield various metabolites shown to be biologically active. Ang III and IV are breakdown products of the N-terminus AngII localized especially in the brain and kidney. AngIII is present in the central nervous system (CNS), where it is suggested to play a primary role in basal blood pressure maintenance and in hypertension. AngIV is thought to have a cooperative role with AngII in the brain. For example, AngIV increases blood pressure only in the presence of AngII on AT1R's (30). Peptides cleaved from the C-terminus of AngII have also shown to have biologic activity. For instance, one heptapeptide fragment cleaved from AngII yields a molecule with significant structural homology to ACE (often referred to as ACE2). However unlike ACE it cannot convert AngI to AngII and it's activity cannot be antagonized by ACE inhibitors (29). ACE2 has the ability to cleave a single amino acid from the C-terminus of AngI to form Ang-(1-9), a peptide with no known functions at the present time.

As described earlier, AngII is the primary effector of the various RAAS-induced physiological and pathophysiological actions. AngII exerts it's actions through binding to it's specific receptors. AngII type 1 receptor (AT1R) is responsible for most of AngII's noted physiologic and deleterious effects. AT1R mediates these effects through its presence on the following tissue systems: the cardiovascular system (vasoconstriction, increased blood pressure, increased cardiac contractility, vascular and cardiac hypertrophy), kidney (renal tubular sodium reabsorption, inhibition of renin release), sympathetic nervous system and adrenal cortex (stimulation of aldosterone synthesis) (29). The AT1R also participates in AngII effects on cell growth, and proliferation, inflammatory responses and oxidative stress (31). The AT1R belongs to the family of receptors more generally called G-protein-coupled receptors known for containing 7 membrane-spanning sequences.

Angiotensin type 2 receptor (AT2R) exists primarily in the brain, kidney and other sites during early embryologic development. It's presence in adult tissue is markedly decreased, however its functions appear to oppose that of the AT1R by mediating vasodilation, antiproliferative and apoptotic effects in vascular smooth muscle. In the heart AT2R have been shown to inhibit growth and remodeling and in the kidney is has been suggested they mediate proximal tubule sodium reabsorption and stimulate the conversion of prostaglandin E2 to prostaglandin F2. (29) (5). The relevance of AT2R in adult tissues remains unclear.

Angiotensin type 4 receptors are thought to be responsible for the release of plasminogen activator inhibitor 1 by AngII and by the cleaved N-terminal metabolites (AngIII and AngIV), however the function of a type 3 receptor is unknown (32). The C-terminal peptides of AngI known to participate in vasodilation, natriureses, antiproliferation, and cardioprotection, are thought to be mediated by a unique receptor that does not bind AngII (32). Similar types of receptors have also be shown to bind both renin and prorenin in several tissues including the

heart, brain, placenta and kidney with localization to glomerular seangium and subendothelial vascular smooth muscle. One of these receptors that has been characterized and shown to cause reversible activation of bound prorenin and to enhance the catalytic activity of bound renin. Data indicates this receptor can initiate intracellular signaling, independent of Ang peptide synthesis, leading to activation of mitogen-activated kinases ERK1 and ERK2 (33). These findings lead to the possibility of AngII-independent effects on cellular growth responses by renin and prorenin.

AngII, via the AT1R also stimulates the production of aldosterone from the adrenal cortex. Aldosterone is a major regulator of sodium and potassium balance thus mediating extracellular volume. It has the ability to increase the reabsorption of sodium and water in the distal tubules and collecting ducts facilitating the excretion of potassium (and hydrogen ions) (34). AngII and extracellular postassium are the primary regulators of aldosterone. AngII synthesis can be stimulated by adrenocorticotrophic hormone (ACTH), norepinephrine, endothelin, and serotonin and inhibited by atrial natriuretic peptide and nitric oxide (NO).

Classic Pathway of Angiotensin Biosynthesis

A reduction in renal perfusion pressure due to decreased circulating blood volume, a reduction in tubular sodium chloride concentration sensed at the macula densa and increases in sympathetic discharge to the kidney triggers the release of renin from the stretch-sensitive JG cells lining the afferent arterioles. Renin, released into the extracellular space cleaves the biologically inert compound, AngI, which is further cleaved to it's active form, AngII by ACE. Conventional understanding of AngII as a circulating endocrine hormone is misleading. Both AngI and AngII have short half-lives and are probably synthesized relatively close to their site of action. The numerous actions of AngII through it's binding to the AT1R functions to restore circulatory homeostasis. AngII stimulates the release and production of aldosterone from

the adrenal cortex, promotes the constriction of renal and systemic arterioles and the reabsorption of sodium in the proximal portion of the nephron. The subsequent rise in blood pressure and restoration of renal perfusion inhibits further renin release. This inherent system plays an important role in maintaining organ perfusion at suboptimal blood volume levels, however it becomes deleterious when it is inappropriately activated in circumstances like hypertension and heart failure.

Alternative Angiotensin II biosynthesis pathways including a tissue RAAS

Advances in the understanding of the RAAS has lead to the concept of a fully functional tissue paracrine/autocrine RAAS independent of the classic circulating system. Data suggesting AngII production from several tissues, characterization of various angiotensin receptors and signal transduction pathways, existence of many angiotensin peptides with possible unique actions and the identification of cell surface receptors for renin and prorenin have supported the concept of a tissue RAAS. Renin and/or AGT taken up from circulation have been suggested to initiate AngII biosynthesis in certain tissues including: heart, peripheral blood vessels, kidney,brain, adrenal glands, pituitary, adipose tissue, testes, ovaries and skin (29) (5). Serine proteases like kallikrein, cathepsin G, chymase are thought to be alternative enzymes that contribute to AngII formation in the tissue RAAS (35). It is thought that non-ACE pathways exist in the kidney and are responsible for 40% of AngII generation. In the heart, coronary arteries and atherosclerotic aorta, chymase it suggested to be the primary enzyme converting AngI to AngII (35) (36) (37) (5). Conclusions from these studies suggest that dysregulation of a tissue RAAS may contribute to the pathogenesis of cardiovascular disease regardless of the presence of the circulating system (38). Despite overwhelming evidence for alternative enzymatic pathways

generating AngII in various tissues, many of the studies lack supportive evidence from in vivo models and direct correlations.

The role a tissue RAAS in cardiac tissue has been described under physiologic and pathologic conditions. In physiologic conditions, it maintains cellular balance by inhibiting and inducing cell growth, and proliferation and mediation of adaptive responses to myocardial stretch (39). It appears that AngI is produced locally thereby converting to AngII, instead of the uptake of peptides in the general circulation (29) (5). However, other studies argue that circulating renin and AGT migrate through endothelial barriers and are taken up by cardiac tissue where they exert their effects locally (40) (5). AngII's functions in cardiac tissue include: inotropic support, myocyte hypertrophy via AT1R and cardiac remodeling (39). In pathologic states, the cardiac RAAS seems to feed on a local upregulation of ACE and has been proposed to contribute to development and maintenance of left ventricular hypertrophy (38).

Other tissues thought to generate AngI and AngII include: vascular smooth muscle, endothelial and endocardial cells similarly through the uptake of circulating renin (29). Unlike cardiac tissue, the vascular RAAS is thought to contribute to maintenance of cardiovascular homeostatis and mediate the long-term effects on vascular remodeling by stimulating vascular smooth muscle cells and fibroblasts via both the AT1R and the AT2R (39). Activation of a vascular ACE may alter functions like: vascular smooth muscle growth, and the inflammatory and oxidative state of the vessel wall (38). The presence of AngII in the vessel wall has been shown to contribute to reactive oxidative species and associated with inflammation, atherosclerosis, hypertrophy, remodeling and angiogenesis (39).

Tissue RAAS's similarly exist in the kidney, brain and adrenal cortex. Overactivation of this system in the kidney may contribute to sodium-retaining diseases like hypertension and congestive heart failure (29). And in the brain, it is evident that a local RAAS

participates in a more central control of blood pressure since AngII cannot cross blood brain barrier and is isolated from the circulating system (39). Supporting studies have administered AngII in to the brain and found it to cause increased blood pressure via vasopressin release, sympathetic nervous system activation, and inhibition of baroreflexes (41) (39). Similarly, mRNA of the adrenal gland is positive for renin and AGT. And AngII has been shown to be formed at the zona glomerulosa (29). In total, over 90% of adrenal AngII is produced locally (42). It still needs to be determined if the adrenal RAAS functions as a paracrine or autocrine system or if it has a pathophysiologic role.

Dysregulation of the RAAS in Cardiovascular Disorders

Current understanding of the dysregulation of the RAAS in clinical hypertension has only focused on the role of the circulating system. Thus the pathophysiologic contributions of the tissue RAAS remains uncertain. It should be noted that plasma levels of RAAS components vary greatly between hypertensive cases and is therefore classified into groups including: normal plasma renin activity (PRA), mild to moderate increases in PRA, and high PRA. The majority of essential hypertensive cases have been classified as "normal" PRA , however this has faced scrutiny since normal renin levels should suppress further renin secretion. Therapeutic agents that block the RAAS have served as the primary evidence that this system in fact contributes to blood pressure elevation in essential hypertension.

Disease associated, maladaptive activation of RAAS promotes and worsens pathologic changes in several organ systems including: heart, kidney, and vasculature. The disease states include hypertension, diabetic and non-diabetic nephropathies and

heart failure. These pathologic processes lead to progressive kidney damage and cardiovascular remodeling. As the active molecule of the RAAS, Angiotensin II has numerous systemic and molecular effects that contribute to these disease states and will be considered as the main contributor in context of the RAAS's role in cardiovascular disorders.

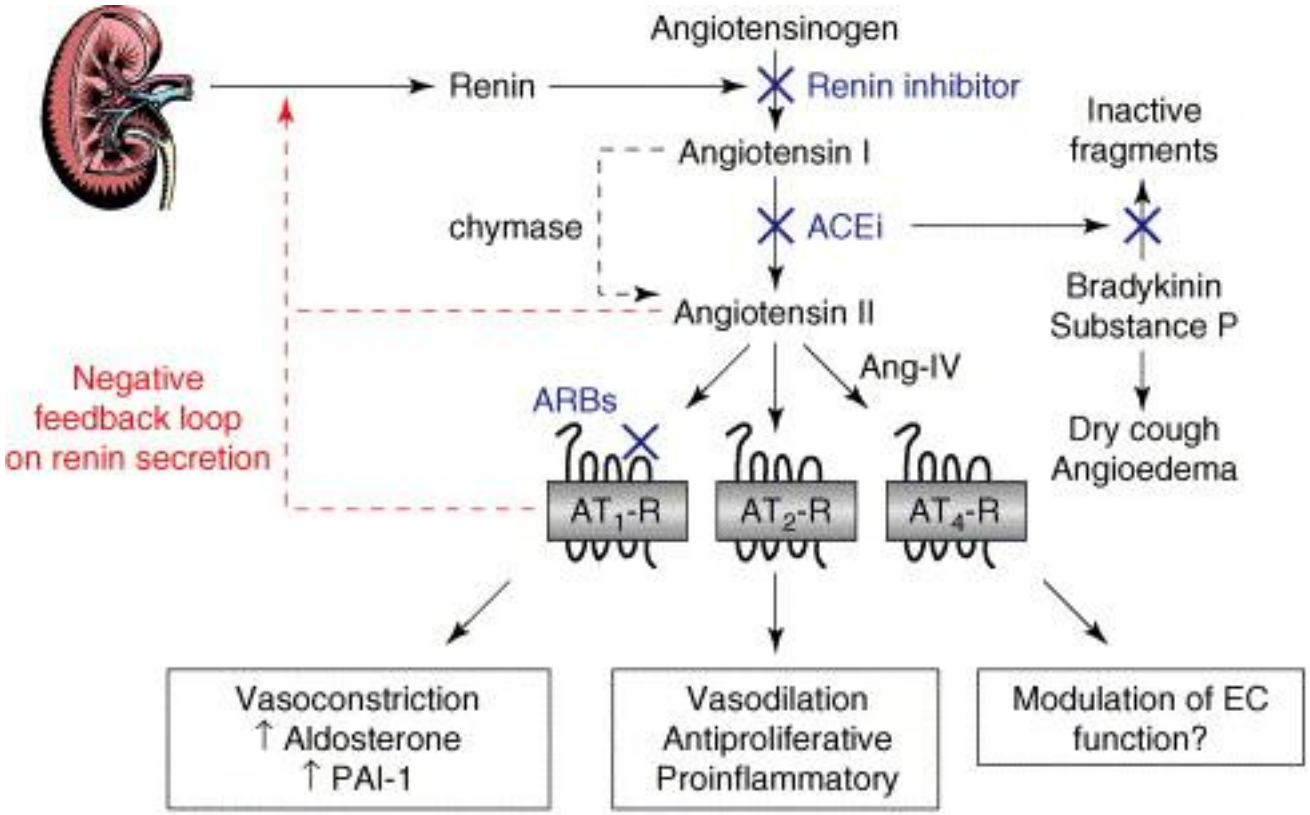

Figure B. The Renin Angiotensin System Cascade (43)

T Cell Mediated Adaptive Immunity

The Immune System

The immune system has two branches, the innate and adaptive immunity, in which both have their distinctive features and functions. An innate immune response is known for its rapid, nonspecific effector functions. It has evolved to sense a wide range of pathogens through germ-line encoded recognition receptors. However, an innate response is not effective against the overwhelming variability of antigenic structures and ability of pathogens to mutate. In contrast to the innate immune response, the adaptive immune system is highly specific and its response depends on unique receptors that have been selected through a process of somatic recombination of a large array of gene segments. After adaptive immune cells have mounted an initial response to a specific pathogen, these cells can survive in the host indefinitely providing immunological memory and faster protection if re-exposure occurs (44).

The innate and adaptive immune systems are crucial to host defense against microbial infections and the maintenance of immune homeostasis. Unfortunately, these immune responses are capable of causing tissue injury and disease. Dysregulation of the adaptive immune system leads to autoimmune and immune-mediated disease pathogenesis. Hyper activation of self-antigen or microbial-antigen-specific effector T and B cells often coincide with defects in the regulatory arm of the adaptive immune system. This results in the breakdown of immune homeostasis and the development of immune-mediated diseases. Diseases elicited by T lymphocytes are typically associated with chronic inflammation. Because inflammation plays a primary role in many T-cell mediated diseases, these are often referred to as immune-mediated inflammatory disease. The T cells that cause tissue injury may be autoreactive or specific for foreign protein antigens that are present in or bound to cells or tissues. Many organ specific

autoimmune diseases are caused by auto-reactive T cells including: type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and recently hypertension (45).

Adaptive Immunity and T cell Activation

The adaptive immune system is characterized by the activation of two types of specialized lymphoyctes, T and B cells. T lymphocytes, defined by their cell mediated effector responses, develop in the thymus; while antibody-producing B lymphocytes develop in the bone marrow. These highly mobile cells travel from their sites of origin (primary lymphoid organs) to the lymph nodes and spleen (secondary lymphoid organs) where they are exposed to circulating antigens. Innate immune system signals facilitate the activation and migration of the adaptive immune cells out of the secondary lymphoid organ and to sites of pathogen invasion. Navigation to these sites is regulated by adhesion molecules and chemokine receptors (44).

Mature naïve T cells are activated by the interaction of their T cell receptor (TCR) with antigenic peptides presented on MHC molecules. These MHC peptides are generally produced from proteins translated within the cells. $CD8^+$ T cells interact with peptides expressed on MHC class I, usually in response to infecting viruses or other pathogens replicating intracellularly. Conversely, the TCR's of $CD4^+$ T cells respond to peptides presented on MHC class II molecules in response to extracellular pathogens. Unlike the MHC class I expression, which is present on all nucleated cells, MHC class II molecules are present on antigen presenting cells (APC's) and are induced by innate immune stimuli, including ligands for Toll-like receptors (TLRs). APCs are classified as innate immune cells that sample environmental antigens and are usually concentrated at sites where pathogen encounter is likely, like skin and mucosal tissues. Through phagocytosis or endocytosis, APCs process exogenous proteins. Their activation constitutes not only their

expression of MHC II on their surface, but migration to secondary lymphoid organs where interaction with T cells can occur (44).

$CD4^+$ T-cell activation is initiated when the TCR recognizes a peptide/MHC complex on an APC. This results in a rapid congregation of TCR-associated molecules at the physical interface between T cells and APCs, also known as the immunological synapse. The T cell side of the synapse is focused around a central cluster of CD3 and the TCR, which bind to the peptide/MHC complex. The synapse is stabilized by adhesion molecules called integrins. The aggregation of these molecules initiates TCR signaling. Full activation of the T cell cannot occur without subsequent costimulation signals. Costimulation, involves B7 ligands on the APC (CD80 and CD86) with CD28 on the T cell. Other costimulatory molecules include members of the tumor necrosis factor (TNF) family (46), and others that maintain T cell activation of B cells (47). Upon stimulation and activation, the T cell then proliferates, produces cytokines and alters the expression of its surface receptors that induces its navigation from secondary lymphoid organs to sites of inflammation. Similar to $CD4^+$ cells, $CD8^+$ cell are activated much the same way except activated $CD8^+$ cells, also called cytotoxic T cells, release perforin and granzymes capable of killing adjacent cells. $CD8^+$ cells also produce cytokines that participate in pathophysiologic processes (48).

T-cell effector subsets

Although T cells develop and activate in much the same way, their effector functions are diverse and specific. T cells can function as helper cells, providing cytokine signals that enhance further B cell and T cell responses, or function in direct elimination of pathogens by killing infected target cells. Lastly, T cells can function by regulating immune responses and limiting tissue damage incurred by inflammatory immune responses (44).

The largest group of T cells in the $CD4^+$ T-helper population. T cells that have been designated to serve as helpers are of a naïve phenotype until activation in which they can differentiate into various effector phenotypes, which differ in the cytokines they produce and thus their function (Figure C). Initial studies of this population described 2 main categories of T-helper cells, T-helper-1 (Th1) and Th2 cells, each producing mutually exculsive cytokines (49). The Th1 cells were characterized by their exclusive secretion of IFN-y and IL-2 and were shown to differentiate from a naïve T-helper phenotype in the presence of IL-12 and IFN-y. This unique phenotype could be identified by the T-box expressed T-cell transcription factor (T-bet) (50). It was noticed that their cytokines participated in activating mononuclear phagocytes, natural killer cells, and cytolytic T cells for killing intracellular microbes and virally infected cells (51). In contrast, the Th2 cells produced IL-4, IL-5, IL-10 and IL-13, and their phenotypic development involved the presence of IL-4 and the transcription factor GATA-3 (52). Their effector functions were involved in enhancing antibody production, and hypersensitivity and parasite-induced immune responses (49). The concept of these effector $CD4^+$ subsets being mutually exclusive has since been disproved when it was shown that T-box transcription factor expression with IFN-y production can be induced in some Th2 cells (53). Even though the mutually exclusive paradigm of the Th1 and Th1 relationship has exceptions, immune responses to pathogens or immunologically mediated diseases are generally considered Th1 or Th2 mediated.

Since the discovery of Th1 and Th2 phenotypes of the $CD4^+$ T cells, strong evidence has lead to additional T helper diversity. The IL-17 producing Th17 lymphocyte subset was first described in 2005 (54). Th17 lymphocytes maturation is driven by the presence of transforming growth factor beta (TGF-B), IL-6 and IL-1, or TGF-B and IL-21 followed by IL-23. Th17s produce IL-17A-F, IL-21 and IL-22. It has been characterized for its protective role against extracellular bacteria and fungi (55). Th17 lymphocytes can be identified through their expressed

transcription factor RORyT (retinoic acid receptor related orphan receptor γt) (52). Th17's active cytokine IL-17a has been widely studied and has been implicated in the pathogenesis of autoimmune and inflammatory diseases like rheumatoid arthritis (RA), psoriasis, multiple sclerosis, asthma, inflammatory bowel disease and periodontal disease (56). Madhur et al found in 2010 that Ang II infusion increased IL-17a production from T cells and IL-17 is required for the maintenance of Ang II induced hypertension. In addition their work with IL-17 -/- mice displayed preserved vascular function and reduced T cell infiltration in response to Ang II (17).

The critical function of regulating T-cell innflammatory responses is governed by a T cell phenotype that also belongs to the CD4+ family of T cells. The T regulatory (Treg) population suppresses T-cell mediated immune responses. The T regulatory lymphocyte population is mediated by TGF-B and IL-10, direct cell-cell contact or CTLA-4-mediated inhibition, or by production of extracellular adenosine (50). They express the transcription factor forkhead box P3 (FoxP3) and cell surface receptors: CD25 and CD4. Treg lymphocytes are immune suppressants acting to turn off immune reactions and promote anti-inflammatory properties. IL-6 is an inhibitor of Treg development and when it is present the differentiation scheme will likely be directed towards the Th17 pathway (50).

$CD8^+$ T cells represent another family of circulating T cells. Unlike the $CD4^+$ T cells, they function to remove cells harboring intracellular pathogens, including viruses and transformed cells. $CD8^+$ T cells primarily recognize cytosolic antigenic peptides presented on MHC class I complexes. Because of this, these cells are also called cytolytic T lymphocytes (CTLs) and target host cells in a contact-dependent manner. In the brief time this CTL is in contact with its target infected cell, it activates apoptotic death. In this process, CTL granules are rapidly mobilized to its surface, where they fuse with the cell membrane of the target cell and

inject their contents, including perforin and granzyme. The granzymes are serine proteases that initiate apoptosis (44).

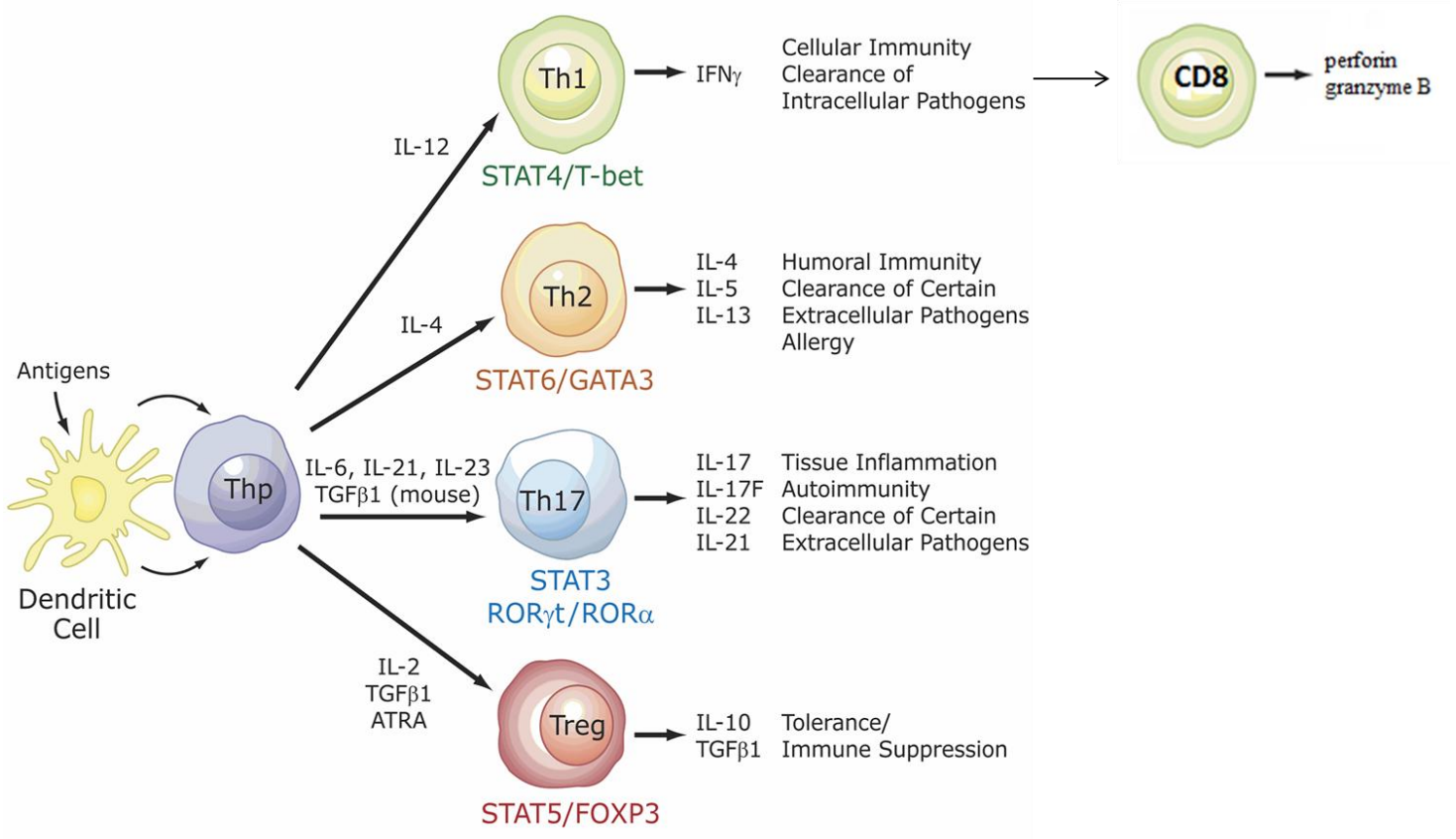

FIGURE C. Differentiation profile of T lymphocyte Subsets

HYPOTHESIS

There is a differential expression of RAAS components on T cell subsets that directs their function.

Experiment 1

Determine the extracellular expression of RAAS components angiotensin type-1 receptor (AT1R), angiotensin type-2 receptor (AT2R), angiotensin converting enzyme (ACE), angiotensinogen (AGT) and Renin on naïve, Angiotensin II stimulated and anti-CD3/28 stimulated $CD4^+$ lymphocyte subsets (Th1, Th2, Th17, and Tregs).

Experiment 2

Determine the extracellular expression of RAAS components (AT1R, AT2R, ACE, AGT, and Renin) on naïve, Angiotensin II stimulated and anti-CD3/28 stimulated $CD8^+$ T lymphocytes.

MATERIALS AND METHODS

Animals

Mice were maintained in animal facility under standard conditions with regards to light-dark cycles, room temperature, and diet. Experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). All mice were from a C57BL/6 background.

Splenic T cell isolation and culture

Male C57BL/6 male mice were used as splenic lymphocyte donors. Mouse spleens were aseptically removed and placed in 1640 RPMI medium supplemented with 10mM non-essential amino acids (Invitrogen 11140-050), 110.04mg/L sodium pyruvate (Invitrogen 11360-070), 20U/mL penicillin/ 20µg/mL streptomycin (Sigma P0781), 0.25µg/mL amphotericin B and 10% heat-inactivated fetal bovine serum (FBS). Spleens were passed through a 22µm nylon screen then spun at 1200rpm for 6 min. Total blood leukocytes were isolated using an osmotic lysis of excess red blood cells. Cells were washed with culture medium (described above) and centrifuged at 300g for 6 min. For an enriched lymphocyte population, the cell suspension was layered on Ficoll-Hipaque (Mediatech, Inc. Manassas, VA), and centrifuged 1450rpm for 20 min. The interphase of mononuclear leukocytes was carefully aspirated, washed in culture medium and centrifuged at 1200rpm for 6 min. For removal of B lymphocytes, Goat anti-mouse IgG antibody (100µg/mL, Jackson ImmunoResearch Labs 115-005-003) panning was performed for 2hrs with 5% CO2 at 37ºC. This preparation procedure, yields a ratio of 7 lymphocytes to 1 dendritic cell.

Cell count of the isolated lymphocytes were measured with trypan blue dye and confirmed >90% viability by Countess Cell Counter (Invitrogen Labs, Carlsbad, CA). Cells were fractionated into groups of 10^6 and incubated or not on pre-coated anti-CD3/28 mAb plates (2µg/mL; BD Pharmigen 553294, 553058) or with Angiotensin II and maintained for 12 h in a humidified atmosphere with 5% CO2 at 37ºC. Cells not placed on stimulation plates were immediately stained with fluorescent antibodies for flow cytometry as naïve population.

Real time PCR

Real time PCR was used to determine time and dose of AngII. Following various incubation times and concentrations of AngII lymphocytes were harvested from culture. Cultured lymphocytes were harvested in Trizol (Invitrogen Labs 15593-018, Carlsbad, CA) and chloroform was used to lyse cells and digest tissue while maintaining mRNA integrity. Isopropyl alcohol was added to cause the mRNA to precipitate at the bottom of the tube followed with ethanol to wash the mRNA. A Qiagen quantitect reverse transcription kit (Qiagen 205311, Valencia, CA) and gradient thermocycler (MJ Research, PTC-200, Ramsey, Minnesota) was used to convert the mRNA to cDNA. For the RT-PCR process the following primers were used: β-actin (as housekeeping gene in order to quantify the target gene expression), IL-17a, and FoxP3. Dilute cDNA accumulated during PCR was amplified with SYBR green. The Qiagen Quantitect SYBR green master mix (Qiagen 204143, Valencia, CA) was used and loaded into the PCR

tubes along with the primer mix and PCR water. The tubes were then loaded onto the Corbett Gene Roto-3000 72-well rotor plate and RT-PCR was performed with the Rotor Gene 6000 software (ver. 1.7). The thermal cycler on the rotor consists of three phases: DNA is heated to 95ºC for 15 seconds, cooled to 58ºC for 15 seconds, and then heated slightly to 72ºC for 20 seconds.

Flow Cytometry staining and acquisition

After T cell isolation and harvest,10^6 cells were stained for 20-30 min at 4ºC with antibodies in various combinations. After staining cells were washed twice with 0.5% FBS or fixed (formaldehyde containing solution) and permeabilized (saponin-containing buffer) (Fixation/Permeabilization Solution Kit purchased from BD Biosciences) .

Extracellular identification markers were used to attach antibodies conjugated to various flourochrome stains. Primary antibodies, purchased from Abcam Inc, Cambridge MA, used for extracellular staining were as follows: AT1R, AT2R, ACE, AGT and Renin. PE conjugated F(ab') Donkey anti-rabbit IgG secondary antibody, Pacific Blue conjugated anti-CD3, FITC conjugated anti-CD4, PE conjugated anti-CD4, FITC conjugated anti-CD8a, and APC conjugated anti-CD25 (purchased from eBioscience, SanDiego, CA) were also used for extracellular staining. For extracellular expression, cells were first washed twice with PBS, 0.5% FBS and stained with primary antibodies purchased. Following incubation for 30min at 4ºC, cells were washed again in 0.5% FBS and stained for anti-CD3, anti-CD8, anti-CD4, anti-CD25 and secondary antibodies (see above for flourochrome conjugates). Controls were stained with just secondary antibody

following extracellular methods and were analyzed for nonspecific binding. Following staining, cells were stored in fixation solution (BD Cytofix/Cytoperm Fixation Solution, BD Biosciences) for 30min at 4ºC.

Intracellular staining was performed to identify target cell transcription factor or cytokine or both using a fixation/permeabilization kit (BD biosciences). Antibodies used for intracellular staining were as follows: APC conjugated IFNγ, PerCp conjugated Tbet, PerCp conjugated IL17-A, APC conjugated IL-4 and PerCp conjugated FoxP3, all purchased from eBioscience, SanDiego, CA. Cells were washed with permeabilization buffer containing 0.1% saponin and 0.009% sodium azide (BD Biosciences) and spun at 6,000rpm for 4 min. Subsequently, the cells were stained with antibodies mentioned for intracellular identification, and incubated for 30min at 4ºC. Following staining cells were washed with permeabilization buffer and spun at 6,000rpm for 4min. Cells were then stored in formaldehyde containing fixation solution until flow cytometry acquisition.

Following extracellular and intracellular immunostaining procedures, cells were acquired immediately on an BD LSR-II flow cytometer with FACS Diva software. Data was further analyzed with Gatelogic software.

Flow cytometric analysis and gating strategy for expression of RAAS on T-lymphocytes

Flow cytometric analyses were performed on an LSR-II flow cytometer for the excitation of Pacific Blue, FITC, APC, PerCyp5.5 and PE, and for the forward angled (FS) and side-scattered (SS) light signals from illuminated cells. Fluorescence signals were collected on a log scale. A minimum of 100,000 events of labeled cells were

analyzed from each sample, and results were expressed as percentage of positive cells (%).

The gating strategy utilized to evaluate expression of RAAS components on T-lymphocytes was initiated by first gating on a negative sample, where fluorescent stains were not applied. All samples thereafter with fluorescent indicators beyond the negative applied gate would be positive for that particular fluorescent stain. To select for the T-lymphocyte populations of interest, samples were first labeled with the anti-CD3-PB antibody, a T-lymphocyte marker, then selection of $CD4^+$ or $CD8^+$ lymphocytes can easily be obtained and the resulting signals can be plotted in a $CD4^+$ or $CD8^+$ vs SS dot-plot. After further selecting for anti-$CD4^+$ -FITC antibody, various $CD4^+$ subsets (Tbet-PerCyp5.5, IL-4-APC, IL-17a-PerCyp5.5 or FoxP3-PerCyp5.5) can be gated according to their RAAS component-PE fluorescence intensity.

Statistical Methods

Data results were collected in duplicates and expressed as the mean % ± SEM. Values from stimulation groups were compared to naive groups using Student's T test. The level of significance was set at $p<0.05$.

RESULTS

Expression of RAAS components on naïve, AngII treated and anti-CD3/28 treated $CD4^+$ lymphocytes

To examine the effects of AngII treatment on CD4+ lymphocytes extracellular expression of RAAS, Tcells were isolated from spleens and cultured or not in plates with AngII treatment or anti-CD3/28 stimulation treatment. Lymphocytes not cultured were immediately stained for flow cytometry analysis as naïve population. In preliminary studies of spleen-derived lymphocytes, we determined AngII dose and time response based on ratio of IL-17a to FoxP3 expression using Real Time-PCR. AngII 10^{-9}M showed the greatest response after 12 hours in culture (Figure1).

Angiotensin Type-1 Receptor (AT1R)

Using flow cytometry, the AT1R was examined for its expression among naïve, AngII treated and anti-CD3/28 stimulated lymphocyte subsets. AT1R's were identified on all $CD4^+$ lymphocyte populations, however their pattern of expression as demonstrated by flow cytometry plots (Figure 2), varied with in the subsets and between stimulation groups. AngII treatment, and to a larger extent anti-CD3/28 stimulation, significantly upregulated the AT1R compared to naïve cells. Tbet had the highest expression of the AT1R in all groups. FoxP3 displayed the lowest expression when treated with AngII (5.05 ± 0.15%) and anti-CD3/28 (8.95 ± 0.23%). However, without stimulation (naïve cells) IL-17a labeled lymphocytes expressed the least amount of AT1R (1.11 ± 0.33%). These data indicate that the Th1 subset has a higher expression of AT1R constitutively and when stimulated compared to other $CD4^+$ lineages. Conversely, the

Treg subset responds the least to stimulation in regards to its expression of AT1R, compared to other subsets.

Angiotensin Type 2 Receptor (AT2R)

The AT2R is similarly expressed on all $CD4^+$ lymphocyte subsets (Figure 3). Its basal expression in the naïve group was one of the lowest compared to other RAAS components (Tbet= 5.42 ± 0.21%; IL-4= 1.19±0.1 %; IL-17a=1.41± 0.1% and FoxP3= 0.77±0.03%). However, when stimulated with anti-CD3/28, it appeared the AT2R significantly increased, similar to other components (Tbet= 80.0±0.5%, IL-4= 34.27±0.6%, IL-17a= 52.07±1.7% and FoxP3= 5.24±0.2%). Like the AT1R, the AT2R was expressed highest in Tbet labeled lymphocytes (Naïve= 5.42±0.2%, AngII=11.36±0.3% and anti-CD3/28= 80.0±0.5%), while its lowest expression was in FoxP3 labeled lymphocytes (Naïve= 0.77±0.03%, AngII= 2.2±0.1%, and anti-CD3/28= 5.24±0.2%). In response to AngII stimulation AT2R expression increased significantly among the subsets (Figure 3).

Angiotensin Converting Enzyme (ACE)

ACE expression was present in naïve and treated $CD4^+$ lymphocyte subsets (Figure 4). Like the AT2R, its expression in naïve $CD4^+$ T cells was among the lowest (Tbet= 4.12±0.04%, IL-4=0.58±0.1%, IL-17a= 1.39±0.02% and FoxP3= 0.67±0.3%). AngII treated $CD4^+$ subsets made small increases in their expression of ACE. All increases were significant ($p< 0.05$) except in FoxP3 labeled lymphocytes ($p= 0.11$). When stimulated with anti-CD3/28 all $CD4^+$ lymphocyte subsets increased their

expression significantly ($p<0.05$). Tbet labeled lymphocytes had the highest expression in naïve and stimulated groups (naïve= 4.12±0.03%, AngII= 8.96±0.2% and anti-CD3/28=64.98±0.3%). FoxP3 had the least increase in expression of the AT2R in response to stimulation (Figure 4).

Angiotensinogen (AGT)

$CD4^+$ lymphocyte subsets had similar expression of AGT in naïve and stimulated states (Figure 5). Interestingly, baseline expression was generally the same among subsets, with Tbet labeled lymphocytes expressing highest basally (8.82±0.1%). AGT's expression increased significantly in AngII treated ($p <0.05$) and anti-CD3/28 stimulated ($p <0.01$) $CD4^+$ lymphocytes. AGT expression increased the most in Tbet labeled lymphocytes stimulated with anti-CD3/28 treatment (69.15 ± 0.21%). Its expression increased the least on IL-4 labeled lymphocytes treated with AngII (from 2.5±0.2% in naïve group to 5.6±0.3% in AngII treated).

Renin

Lastly, Renin was characterized on the surface of $CD4^+$ T cell subsets. Its presence the naïve and stimulated groups was similar to other RAAS components studied (Figure 6). A significant increase in its expression is shown when AngII and anti-CD3/28 stimulation is applied (Figure 6). Cells labeled by transcription factor Tbet showed highest expression in all groups, showing 8.6±0.2% in the naïve, 16.9±0.3% in the AngII treated group and 72.5±0.8% in the anti-CD3/28 treated group. In the naïve and AngII stimulated groups the lowest expressers of Renin were nearly matched by cells labeled

with IL-17a (naïve= 1.5±0.1% and AngII=4.0±0.01%) and FoxP3 (naïve=1.6±0.03% and AngII= 4.1±0.2%). The lowest expression of Renin in the anti-CD3/28 group was held by FoxP3 labeled cells at 9.3 ± 0.125% (Figure 6).

Expression of RAAS components on naïve, AngII treated and anti-CD3/28 treated $CD8^+$ lymphocytes

RAAS components were further characterized for their expression on $CD8^+$ T lymphocytes. Activated $CD8^+$ cells were identified through their expression of CD25, marker of activation from a $CD3^+$ population. Like the $CD4^+$ lymphocyte subsets, this family of T cells also expressed RAAS components on their surface. Constitutively, this group appears to express most RAAS components at a higher level compared to $CD4^+$. ACE expression in the naïve group is lowest among the RAAS components at baseline (naïve) and with stimulation. Like the $CD4^+$ family, stimulation of $CD8^+$ T cells with AngII or anti-CD3/28 significantly increased their expression of all RAAS components (Figure 7).

FIGURES

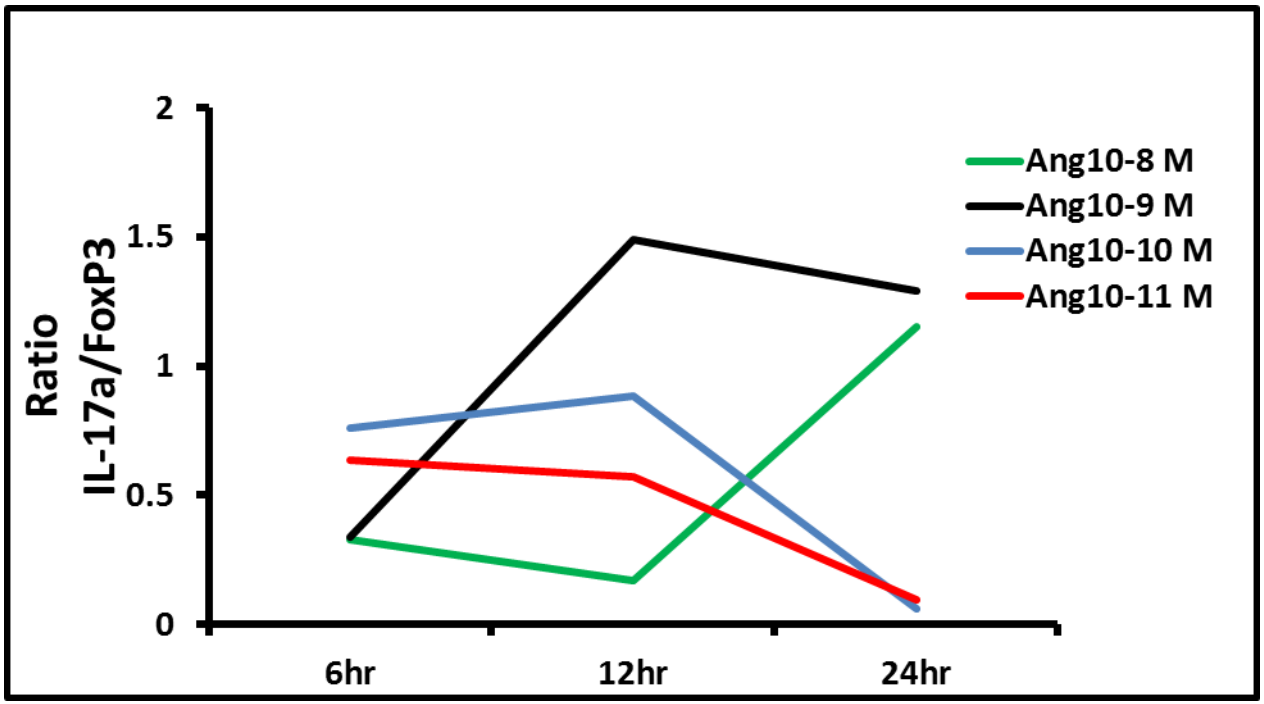

Figure 1. AngII Dose and Time Response using RT-PCR. AngII dose and time response was conducted using RT-PCR analysis of four treatments at three time courses and determined based on the ratio of Th17 to Tregs using markers IL-17a and FoxP3. AngII 10^{-9}M concentration showed the greatest response at the 12hr time point.

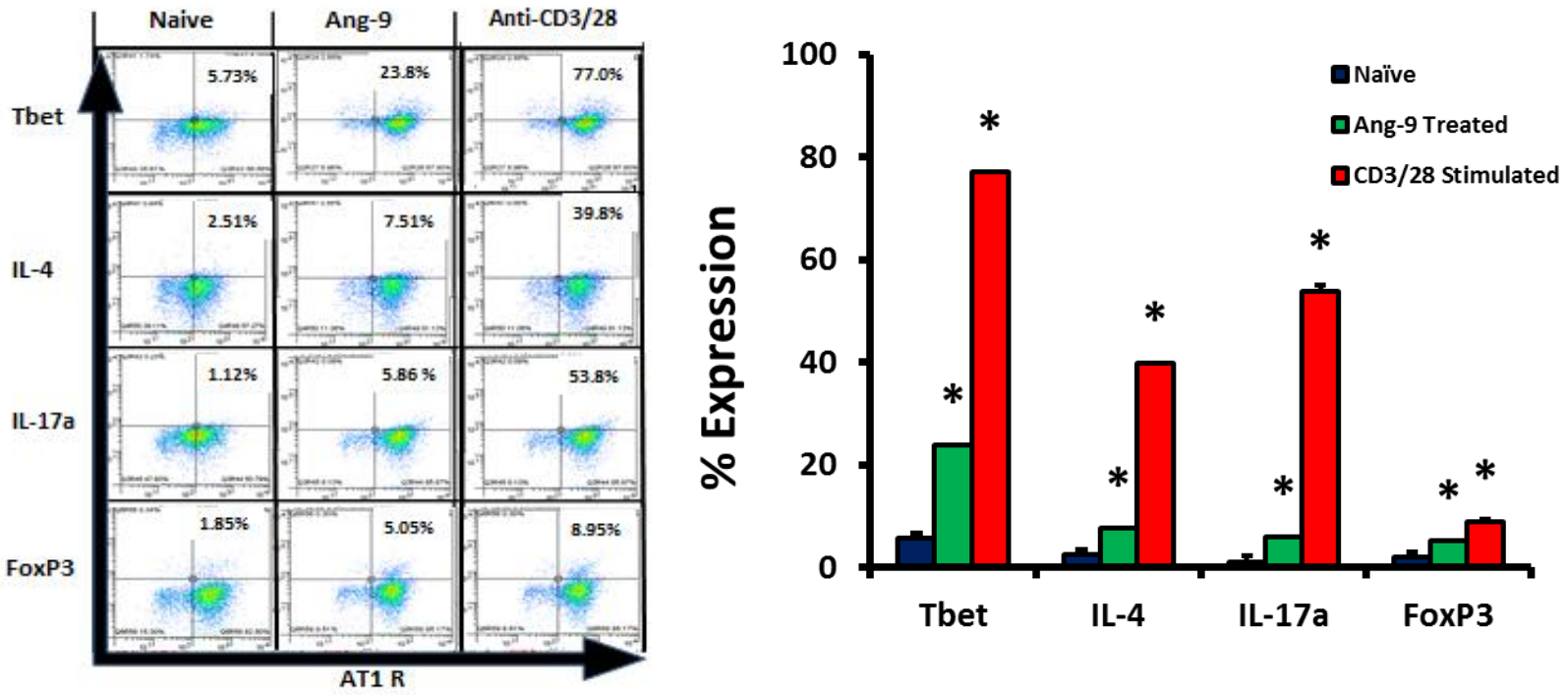

Figure 2. AT1R Expression in CD4$^+$ T cell Subsets. Using flow cytometry CD4$^+$ lymphocyte subsets were characterized for their extracellular expression of AT1R. After selecting for the CD4$^+$ population of lymphocytes, quadrant gates were applied to select for both CD4$^+$ subset markers and AT1R expression. AT1Rs were identified on all CD4$^+$ lymphocyte subsets. Treatment groups (AngII and anti-CD3/28) significantly increased the expression of AT1Rs on all CD4$^+$ lymphocytes ($p < 0.05$ compared to naïve group).

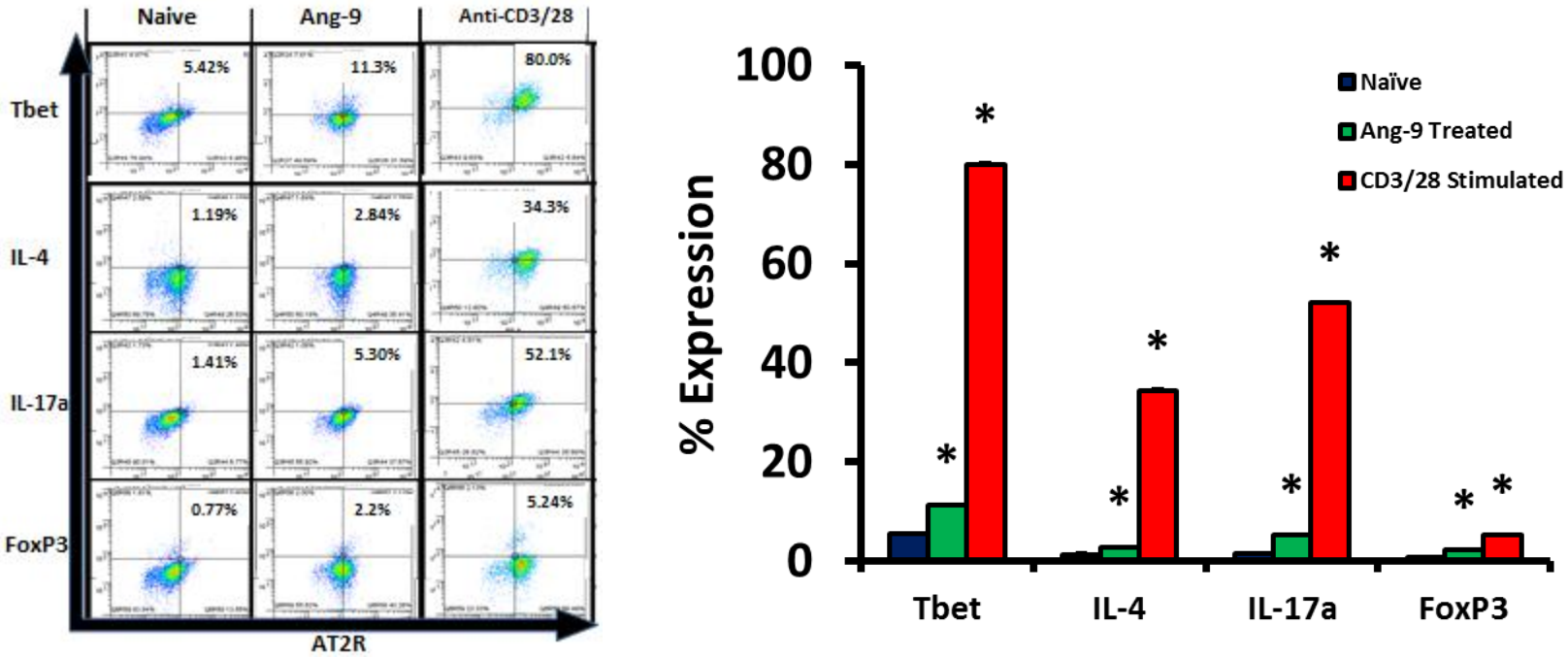

Figure 3. AT2R Expression on CD4$^+$ T cell Subsets. Flow Cytometry analysis shows AT2R expression on all CD4$^+$ lymphocyte subsets. Significant increases in the expression of this receptor is seen with treatment using AngII and anti-CD3/28 ($p < 0.05$ compared to naïve).

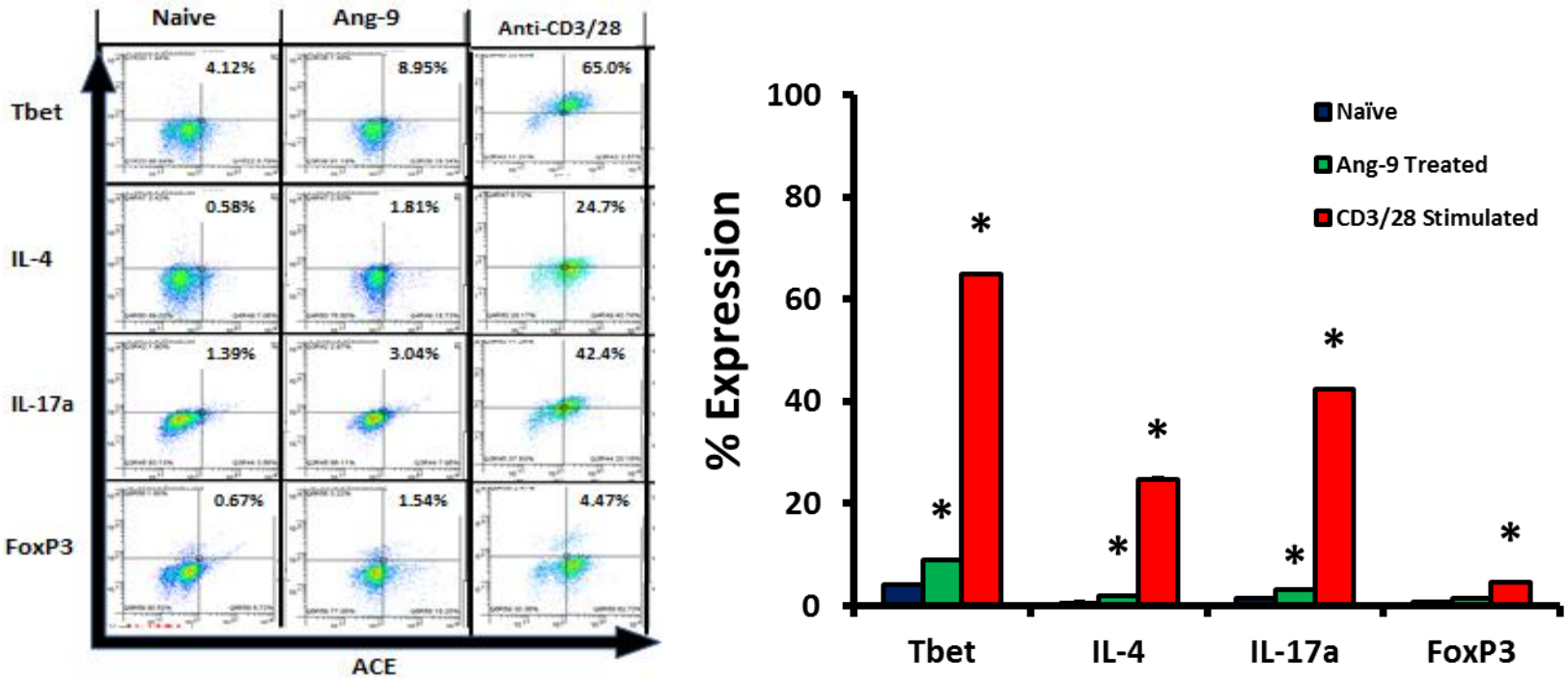

Figure 4. ACE Expression on CD4$^+$ T cell Subsets. Flow cytometry analysis of ACE shows its expression on all CD4$^+$ lymphocyte subsets. Significant increases were seen in all subsets with treatment except FoxP3 labeled lymphocytes treated with AngII. Tbet labeled and IL-17a labeled lymphocytes show greatest expression of ACE in naïve and treated groups (based on $p<0.05$ level significance compared to naïve).

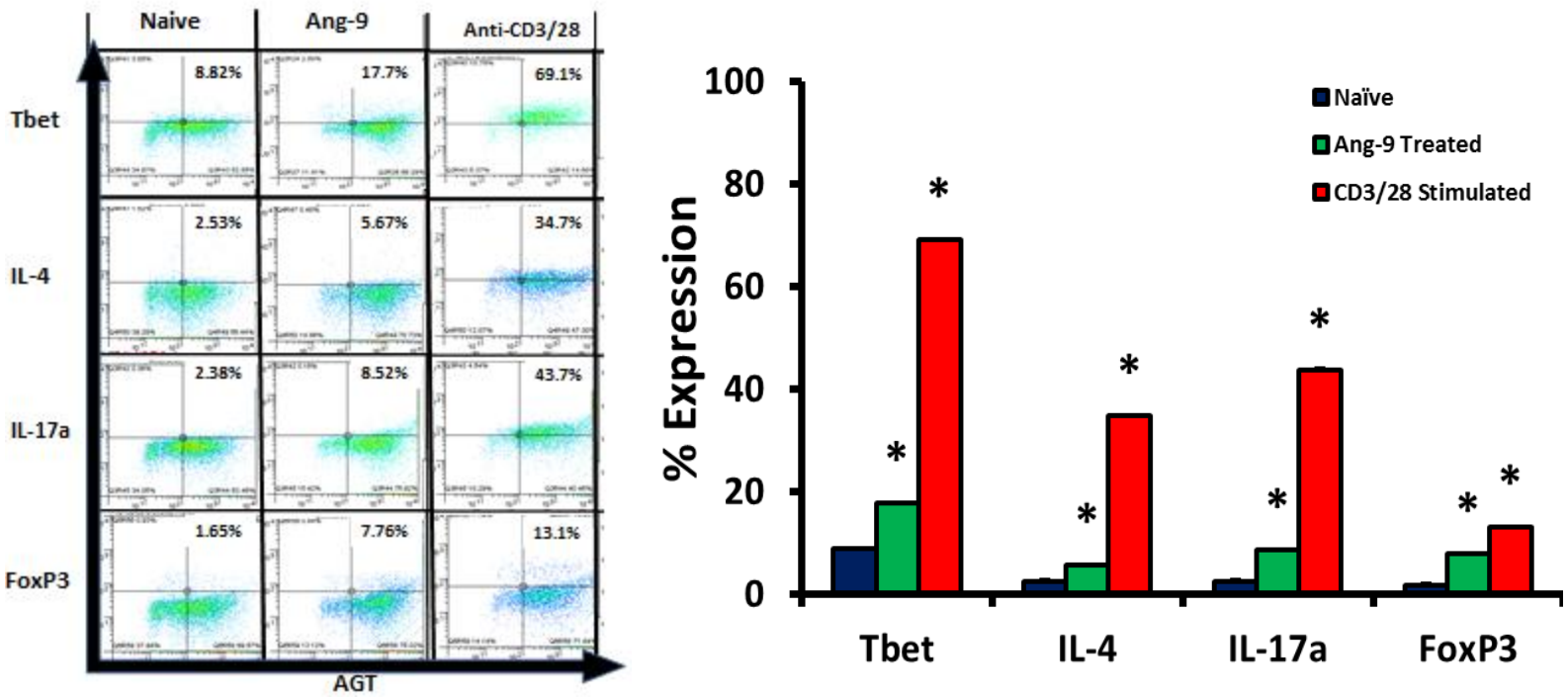

Figure 5. **AGT Expression in CD4$^+$ T cell Subsets**. Angiotensinogen expression on CD4$^+$ lymphocytes subsets is shown using flow cytometry analysis. All subsets significantly increased their expression with treatment with AngII and Anti-CD3/28. Tbet and IL-17a labeled lymphocytes had the largest increases with treatment, while Fox P3 was generally the lowest ($p < 0.05$ compared to naïve).

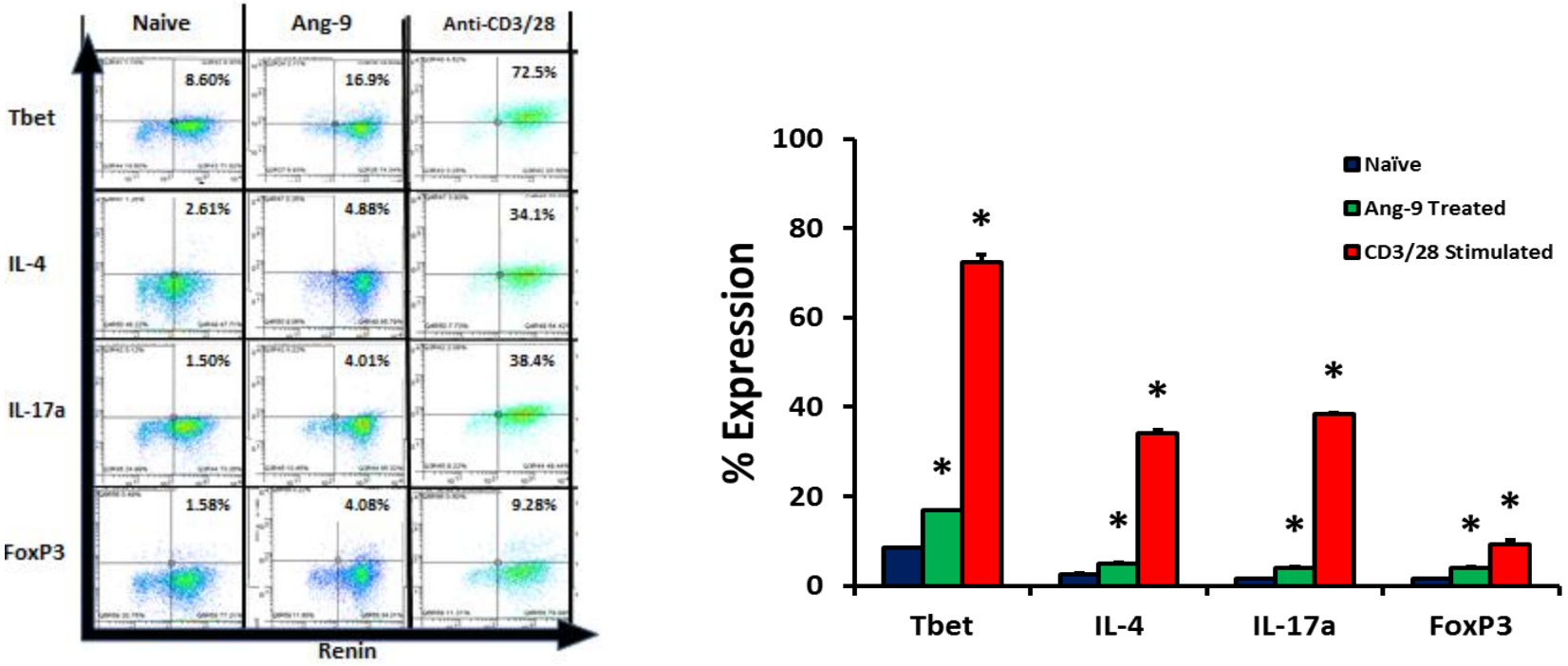

Figure 6. Renin Expression on CD4$^+$ T cell Subsets. Renin expression is shown on all CD4$^+$ lymphocytes subsets depicted in flow cytometry plots. Renin expression increased significantly in treatment groups compared to naïve. Tbet and IL-17 labeled cells show greatest expression with treatment. FoxP3 labeled cells show lowest expression, ($p < 0.05$ compared to naïve).

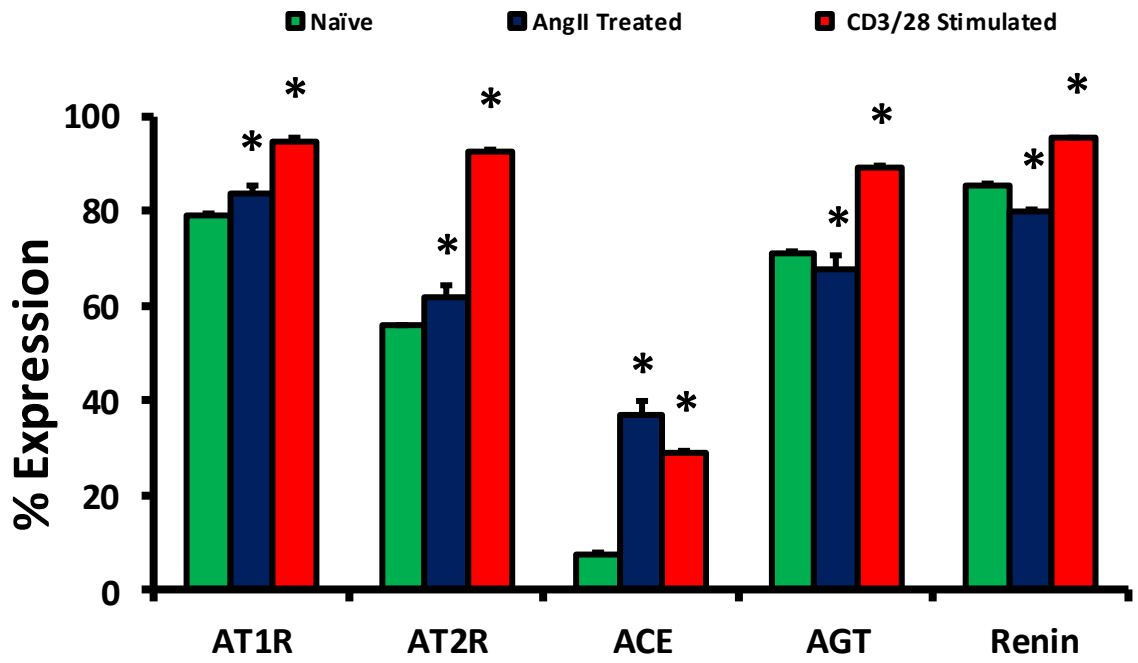

Figure 7. Expression of RAAS Components on CD8$^+$ T cells. Extracellular expression of RAAS components were examined on CD8$^+$, CD25$^+$ T cells. High basal expression is shown in naïve cells. Increased expression of AT1R, AT2R and ACE is seen with AngII and anti-CD3/28 treatment. However, treatment with AngII appears to decrease the expression of AGT and Renin on CD8$^+$ cells, while anti-CD3/28 increases their expression. ACE had the least expression on CD8$^+$ T cells, ($p < 0.05$ compared to naïve).

DISCUSSION

Angiotensin (ANG) II is a potent vasoactive peptide, and its actions are deleterious in pathologic states. Ang II participates in maintaining elevated blood pressure levels via the renin-angiotensin aldosterone system and is a powerful mediator of inflammation. Its inflammatory signaling allows for maladaptive remodeling in the vasculature seen in cardiovascular disease (50). The presence of AngII, perhaps through its synthesis and production in the local environment (5), specifically initiates a cell mediated adaptive immune response. As Guzik and his collegeas have shown, T cells are necessary for the progression of AngII induced hypertension (10). Specifically, it is the pro-inflammatory cytokines, IL-17 and IFNy, produced by distinct $CD4^+$ T cell lineages contributing to chronic immune mediated pathologies (48). It is likely that an overproduction these pro-inflammatory cytokines overwhelm the local environment and suppress the production of cytokines necessary for immune regulation (20). The ability of AngII to initiate production of these specific cytokines may be due to the presence of RAAS components on T-lymphocytes (21).

AngII is capable of augmenting an ongoing immune response (15) (27). However, mechanisms by which AngII mediates the regulation of T cell functions is not fully understood. In the present study, we found that exogenous treatment with AngII for 12hours activated the expression of RAAS components on $CD4^+$ and $CD8^+$ T cells. However, we also found that exposure of T cells to anti-CD3/28 antibodies for the same length of time produced a similar and more elaborated response. Both treatments

significantly increased T-cells expression of RAAS components. Other studies have explored the functions of T cells in response to co-stimulatory molecules including AngII. It has been shown that AngII alone does not cause proliferation of T cells, suggesting it is unable to provide a co-stimulatory signal without additional stimulation (25). However, AngII together with a T-cell receptor specific stimulant like anti-CD3 potentiates the proliferative effects of the T cell (25). Various functions of T-cells including proliferation may not be induced by AngII treatment, however it appears AngII is able to drive the expression of other RAAS components on $CD4^+$ T cell phenotypes, perhaps without proliferation. Future studies are needed to speculate on further increases in the expression RAAS components on T-cell subsets treated with both AngII and T-cell specific co-stimulatory molecules.

Cytokines produced by T-cells contribute to hypertension and other immune-mediated diseases. More specifically cytokines produced by $CD4^+$ T cell lineages, including IL-17 produced by the Th17 lineage and IFNy produced by the Th1 lineage, have been shown to play pivotal roles in immune related pathology (20). For example, Th1 are associated with immune response in arthrogenesis (57). Similarly, IL-17a, a cytokine produced by the Th17 lymphocytes has been described for its role in a variety of immune related diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and airway inflammation (58). Furthermore, $IL17^{-/-}$ mice lack the ability to sustain hypertension (17). In the present work, we find that pro-inflammatory lineages like Th1 and Th17 significantly increase their expression of RAAS components in response to AngII. This data supports the role of pro-inflammatory lineages in AngII

mediated immune responses. Furthermore, this data may indicate that these lineages are more likely to respond to and produce RAAS components in the local environment.

The overwhelming presence of pro-inflammatory cytokines in auto-immune responses is largely due to their ability to inhibit immune regulatory cells. $CD4^+$ T cells are also able to differentiate into the Th2 lineage and T regulatory lineage. The Th2 lineage functions in opposition to the Th1 lineage. The ratio of IFNy: IL-4 is thought to be more important to bringing about changes in cytokine signaling, as Th1 and Th2 cytokines are mutually inhibitory (59) (60). IL-4 is a cytokine produced by the Th2 lineage. This data is consistent with present data showing IL-4 labeled $CD4^+$ T lymphocytes express a lesser amount of RAAS components compared to the Th1 lineage when subject to AngII treatment. Another subset of the $CD4^+$ T-helper lymphocytes, the regulatory T lymphocytes (Tregs), suppress innate and adaptive immune responses, including proinflammatory effects of other lymphocytes, macrophages, dendritic cells and neutrophils. Tregs express CD4, interleukin (IL) 2 receptor α-subunit (CD25), and the transcription factor FoxP3, their functions is mediated largely through its released cytokine, IL-10. Deficiencies in Tregs are associated with autoimmune diseases in humans and mice (61) and it has recently been demonstrated that the adoptive transfer of Tregs prevents AngII-induced hypertension, vascular damage, and immune cell infiltration (20). We were able to support this data by showing that lymphocytes originating from $CD4^+$ lineage and positive for transcription factor FoxP3 expressed the lowest amount of RAAS components on their surface compared to other $CD4^+$ lineages. Tregs may exert their immuno-suppressive effects partly because they lack expression of

RAAS components both on their surface and intracellularly. Together with previous studies on T effector cells, specifically Th1 and Th17, this data supports the antagonistic relationship of Tregs and pro-inflammatory lymphocytes. The implications of this data also underline a potential for immune-modulatory therapy for AngII-induced hypertension.

Increasing evidence supports the idea that the RAAS, possibly through its local production (5), exerts its deleterious effects through the induction of inflammatory responses (21). Recent studies have investigated the presence of RAAS components on T lymphocytes. Hoch et al (2009) showed that splenic T cells express lower levels of renin, angiotensinogen, ACE and AngII, compared to circulating Tcells and that the addition of AngII did not change splenic $CD4^+$ T cell activation (26). In another study, mRNA analysis revealed human T cells express ACE, the renin receptor, renin but not angiotensinogen (25). Using flow cytometry, they went on to show AT1R expression extracellularly was negligible and AT2R showed slight expression. Intracellular staining indicated AT2R to be predominately expressed intracellularly (25). Full characterization of the $CD4^+$ T lymphocyte subsets expression of RAAS components has never been evaluated until this study. Using flow cytometric analysis, our data show that $CD4^+$ lymphocyte subsets express renin and ACE which further supports data on these components expression. Furthermore, our data indicate the presence of Angiotensinogen on the T-lymphocytes, which is not consistent with previous studies. When we looked at the expression of AngII receptors, we show a large percentage of T-lymphocytes expressing both the AT1R and the AT2R. Other RAAS components like the renin

receptor, AT4 Receptor and various AngII metabolites have been identified (5). Their roles in pathology are unclear, but they may participate in perpetuating inflammatory states. Future studies characterizing these components on T-lymphocytes could further describe potential functions for these components. The RAAS clearly exists on T lymphocyte populations and is capable of changing expression depending on the presence of local mediators. Continuing studies looking at the effect of RAAS antagonists like angiotensin receptor blockers or ACE inhibitors are necessary for further understanding.

$CD8^+$ effector T lymphocytes participate in immune responses just as $CD4^+$ T lymphocytes. However, their functional role is not as much production of cytokines as production of cytotoxic molecules like perforin and granzyme for direct cell killing (62). Phenotypic classification of $CD8^+$ T lymphocytes are: naive, memory and effector Tcells. Their differentiation pathway is less understood than the $CD4^+$ populations. However one study does show $CD8^+$ cells to produce IL-17. It's contribution was less than the Th17 subset of the $CD4^+$ lymphocytes. This study suggests that IL-17 producing $CD8^+$ cells differentiate from the same precursors that differentiate into IFNy-producing $CD8^+$ T cells (62). It is unclear whether these $CD8^+$ cell participate in AngII induced inflammation. Our data show $CD8^+$ to have a vast expression of RAAS components on their surface. The presence of RAAS components on $CD8^+$ T cell may implicate a role for them in local synthesis and production of AngII, however, future studies are needed to further determine the phenotype of $CD8^+$ T cell subsets, perhaps through their production of already known inflammatory cytokines, IL-17 or IFNy.

In this study we support the growing body of evidence illustrating a role of inflammation and immunity in hypertension and cardiovascular disease. We propose that inflammation and immune activation in a primary response with the onset of vascular dysfunction and high blood pressure. Inherent mechanisms controlling blood pressure and inflammation may participate in each other's dysregulation and pathology. Our data and others demonstrate this through the presence of RAAS components on pro-inflammatory T cell mediators. Implications of this data lead us to believe that immunotherapy might be useful in the treatment of essential and resistant hypertension. And on the other hand, perhaps antagonists of the RAAS like angiotensin receptor blockers or ACE inhibitors would be useful to treat autoimmune diseases.

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