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ANGIOTENSIN II INDUCTION OF REGIONAL EFFECTS IN MURINE VASCULATURE

Albert Phillip Owens III

University of Kentucky

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ANGIOTENSIN II INDUCTION OF REGIONAL EFFECTS IN MURINE VASCULATURE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine, Graduate Center for Toxicology at the University of Kentucky

By
Albert Phillip Owens III
Lexington, Kentucky

Director: Dr. Alan Daugherty, Professor of Internal Medicine
Lexington, Kentucky
2008
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ABSTRACT OF DISSERTATION

ANGIOTENSIN II INDUCTION OF REGIONAL EFFECTS IN MURINE VASCULATURE

The renin angiotensin system (RAS) exerts many diverse physiological functions throughout the body, mediated by its effector peptide, angiotensin II (AngII). AngII has been linked with a variety of different functions ranging from the initiation of severe vascular pathologies, such as atherosclerosis and abdominal aortic aneurysm (AAA), to mundane physiological processes of fluid homeostasis, vascular contraction, and regulation of blood pressure. To provide a potential link between these functions, an in-depth analysis of regional effects of AngII on aortic vasculature was performed.

The studies presented in this dissertation tested the overall hypothesis of whether regional changes exist in the vasculature in response to angiotensin II (AngII). We first infused AngII into C57BL/6 animals and studied the aortic morphology in detail. On first glance, we detected a thickening throughout the aorta, with no overt changes from region to region. However, upon further analysis, it was demonstrated that there was a region-specific aortic arch hyperplasia, versus the hypertrophy in the remainder of the aorta. Through a series of experiments, this hyperplasia was linked to the redox-mediated protein Id3. Further analysis of the vasculature demonstrated AngII exerted aortic contractions which were limited to the infrarenal aorta. These contractions were mediated by the AT1b receptor subtype in the RAS. We also demonstrate that AngII leads to suprarenal specific formation of AAA, which can be attenuated by the deletion of specific innate immune mediator proteins, such as MyD88 and TLR4. Overall, these data suggest many region-specific roles for AngII in the aortic vasculature and provide many novel findings as to the cause of these effects.
ANGIOTENSIN II INDUCTION OF REGIONAL EFFECTS IN MURINE VASCULATURE

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December 3, 2008
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DISSERTATION

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ACKNOWLEDGMENTS

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Chapter One: Angiotensin II Effectiveness in Vascular Smooth Muscle Cells

The renin angiotensin system (RAS) is a very complex system that controls such physiological functions as blood pressure regulation, sodium balance, and water retention. These actions are mediated via the RAS effector octapeptide angiotensin II (AngII). AngII activation of angiotensin receptors (AT) induces many actions in various tissues and organ systems, with the subsequent signaling processes being tightly controlled and extremely complex in nature. This peptide has emerged as a critical regulatory hormone controlling such systems as the heart, brain, vasculature, and kidneys. Repeated and aberrant signaling through these AT receptors can lead to pathological results in various cell types. One of the most extensively studied cell types, with regard to AngII, is the vascular smooth muscle cell (VSMC). This review will encompass a basic overview of the RAS and the role of AngII in regulation of VSMC growth and proliferation.

The Renin Angiotensin System

A. System Overview

The RAS is one of the most diverse and physiologically relevant processes in the body. Most actions of the RAS are mediated through the octapeptide hormone AngII. This eight amino acid peptide is a powerful dipsogen and thus regulates thirst responses, as well as numerable other responses, including: blood pressure, sodium balance, and sympathetic nervous activity. The classic production of systemic AngII is mediated by the angiotensinogen (Agt) to angiotensin I (AngI) to AngII pathway. Agt is formed in the liver and is the only known precursor of the RAS. Agt is cleaved to the decapeptide, AngI, by a circulating aspartyl protease renin, which is formed and
secreted by granular cells in the juxtaglomerular apparatus of the kidney. This AngI is subsequently cleaved to AngII via the dipeptidyl carboxypeptidase angiotensin converting enzyme 1 (ACE1), which is localized to lung endothelial cells. AngII can then be subsequently degraded via many pathways involving aminopeptidases forming the products angiotensin III and IV (AngIII, AngIV).1-4

Many other functional peptides exist in the RAS. These peptides consist of the vasodilatory Ang1-7 and precursor peptide Ang1-12. The 1-12 peptide is an intermediate molecule derived directly from Agt and is thought to be another substrate for the formation of Ang peptides.5,6 The vasodilatory peptide, Ang1-7, is formed from conversion of AngI via the tissue specific endopeptidases neprilysin, thimet oligopeptidase, and prolyl oligopeptidase.7,8 Furthermore, the recently discovered carboxypeptidase enzyme angiotensin converting enzyme 2 (ACE2) has the ability to cleave one amino acid from AngII, resulting in conversion and down regulation of AngII and subsequent up regulation of the vasodilatory peptide Ang1-7.9 This data suggests many pathways in the RAS compete with/for the production of AngII and also suggests that levels of AngII are therefore directly controlled by the balance of endopeptidases, ACE, and ACE2.10

While the above overview might seem simplistic, the RAS is much more complex than originally described. The classic production of systemic AngII, represented above, is also combined with local production of AngII in tissue compartments. This local tissue RAS is present in the vasculature and is suggested to contain many components of the RAS system, with the controversial exception of renin.11,12 Studies have demonstrated ACE and Agt protein and mRNA are present in vascular smooth muscle cells, endothelium, and adventitial perivascular fat.11,13-17 These data suggest a prominent role of smooth muscle cells in the propagation of the RAS.

B. Heterogeneity of Type I (AT1) Angiotensin Receptor
The AT1 receptor is of the seven transmembrane superfamily of G-protein coupled receptors (GPCRs) and mediates virtually all known physiological functions of AngII in numerous cell targets distributed throughout the body. In humans, the AT1 receptor contains 359 amino acids, is mapped to chromosome 3, and shares approximately 95% homology to rodent and bovine receptors.\textsuperscript{18-20} Reports suggested that another AT1 receptor existed in the human genome with differences in 10 out of the 359 amino acids.\textsuperscript{21,22} Although the human AT1 receptor has an alternative splicing of exons 3 and 5, resulting in a functional receptor with a 32 amino acid N-terminal extension,\textsuperscript{23} subsequent studies have not confirmed the existence of a second AT1 receptor.\textsuperscript{24,25}

While the human genome only contains one AT1 receptor, this receptor is split into two subtypes in rats and mice. These receptor subtypes share 92% homology at the nucleotide level and 95% homology of amino acid residues and are characterized as AT1a (chromosome 17) and AT1b (chromosome 2).\textsuperscript{26-28} The similarity in homology and localization to different chromosomes is hypothesized to be the result of a gene duplication in evolutionary development.\textsuperscript{29} The two subtypes have demonstrated similar ligand binding and activation properties and are functionally and pharmacologically indistinguishable.\textsuperscript{30} However, differences between the two receptors are evident in regard to tissue distribution, chromosome location, and potential regulation.

AT1a and AT1b mRNAs transcripts have been identified in rat: kidney, liver, lung, testes, adrenal, heart, aorta, brain, and pituitary glands. While the AT1a is the critical receptor in a majority of these tissues, the AT1b is the major subtype expressed in brain, testes, and adrenal gland.\textsuperscript{31-34} AT1a receptor expression in mouse tissues are similar to the organ lists above with the addition of ovaries and adipose. However, the mouse has a more punctate localization of AT1b receptors showing transcripts confined to adrenal glands, testis, and brain.\textsuperscript{35} Recent data also demonstrates a high concentration of AT1b receptors in vascular smooth muscle, specifically the abdominal aorta and small resistance vessels.\textsuperscript{36-38} The homology of these two receptor subtypes makes it difficult, if
not impossible, to develop antibodies for protein differentiation of the AT1a versus the AT1b receptor. Several investigators have developed dozens of antibodies without successful discrimination of the two receptors (unpublished data, personal communications).

Genetic ablation studies of AT1a and AT1b receptors have yielded some insights into the similar and divergent functions of these homologous subtypes. The ablation of AT1a leads to substantially lower basal systolic blood pressure (SBP), and infusion with AngII results in undetectable increases in SBP. However, while this data does not suggest a role for the AT1b receptor in SBP, when dose-dependent increases in SBP were induced in AT1a ablated mice, subsequent administration of the AT1 antagonist losartan or candesartan significantly decreased SBP 8mmHg, compared to controls. These data suggest that while AT1a is the main mediator of blood pressure, the AT1b receptor contributes minimally to pressor effects. Similarly, many studies with AT1a receptor ablation suggest a compensatory role for the AT1b receptor. AT1b receptors also compensate for the lack of AT1a in regards to smooth muscle cell calcium mobilization. These studies are supported by the AT1a/b double deficient animal, which demonstrates even lower SBP and a severe pathological kidney dysfunction compared to mice only lacking the AT1a receptor. One of the major differences of the AT1a and AT1b receptors is particularly evident in the role of AngII-mediated vascular contractions. AngII-induced aortic contractions are limited to the abdominal aorta, where significant amounts of AT1b receptor transcripts are localized. Furthermore, AT1a receptor deficiency contracts similarly to proficient mice, which is ablated by the addition of losartan. This and recent data, showing AT1b receptor ablation significantly down regulates AngII abdominal contraction, demonstrates a role of AT1b as the sole mediator of aortic and resistance vessel contractions. Other studies show differences between the AT1a and AT1b receptors in regards to glucocorticoids, estrogen, dietary sodium regulation of AT1a receptors, and superoxide production, with the AT1a subtype demonstrating the majority
of changes versus the AT1b receptor.

Despite the aforementioned similarities and differences, definitive roles of the AT1a and AT1b receptors remains incomplete and poorly understood in current literature. A prominent amino acid difference exists in the cytoplasmic tail of the AT1a and AT1b receptors, which could explain some of the differences in function between the two subtypes. A role was delineated for the final intracellular loop and cytoplasmic tail, of the AT1 GPCR, which required the specific amino acid sequence (position 312-14) of tyrosine-phenylalanine-leucine for essential coupling of the Gq-mediator protein to the cytoplasmic tail. This tyrosine is though to be a critical site of phosphorylation for receptor associated interactions. Specifically, a critical difference is found in amino acid 312 of the C-terminus which is present as an argenine in AT1b receptors and a tyrosine in AT1a receptors. This Tyr to Arg switch leads to a ineffective Gq coupling and activation site in AT1b receptors versus the AT1a subtype. This, and other, known substitutions may represent the differences in the functionality of the receptor subtypes, however, further examination of this hypothesis is required.

C. Type II (AT2) Angiotensin Receptors

The AT2 receptors, a second major isoform of AT receptors, are localized to the X chromosome and are composed of 363 amino acids. Unlike the homology between the AT1a and AT1b receptor subtypes, the AT2 receptor is only 32% homologous to the AT1 receptors. Expression levels of AT2 are maximal during fetal development, specifically developing fetal adrenal medulla, brain, connective tissues, and the skeletal system. The expression levels of AT2 dramatically decrease at and after the time of birth and are expressed in limited amounts in the medial vasculature, brain, heart, kidneys, adventitia, and adrenal glands.

While AT1 receptors perform most of the physiological signaling of the RAS, AT2 receptors bind AngII and exhibit anti-proliferative, pro-apoptotic, and
vasodilatory effects in VSMCs, which can antagonize the actions of the AT1 receptor. Furthermore, AT2 receptor signaling activates tyrosine and serine/threonine phosphatases, which can inhibit AT1 receptor signaling pathway activation via dephosphorylation. It has also been demonstrated that AT1 antagonism occurs through interaction and dimerization of the AT1 and AT2 receptors resulting in dysfunctional AT1 receptor signaling.

These data suggest the AT2 receptor can act as a potent antagonist to the actions of AT1. However, numerous conflicts exist in the interpretation of AT2 studies and the function of this receptor in the RAS. One such example demonstrated AT2 could initiate cardiac hypertrophy, independent of AngII, and furthermore could not antagonize AT1 receptor initiation of similar hypertrophic disease. Other reports suggest AT2 receptor degradation can be prevented independent of AngII via a yet unidentified ligand, suggesting promiscuous ligand binding sites. Therefore, more experimentation is needed before a specific role for this receptor can be ascertained after fetal development.

Physiological Effects of AngII in Vascular Smooth Muscle Cells

A. Effects on Vascular Contraction

AT receptor activation via AngII activates a plethora of signaling pathways. However, in the most simple terms, AngII signaling occurs through five major pathways: GPCR pathways, NADPH oxidase production of reactive oxygen species (ROS), Mitogen-Activated Protein Kinase pathways (MAPK), Receptor tyrosine kinases, and nonreceptor tyrosine kinases. For the induction of vascular contraction, the GPCR activation pathway is considered to be the most important, while the remaining pathways are involved in more pathophysiological functions, and will be discussed in the next section.

AngII vascular contraction is an extremely rapid event, which utilizes many signaling cascades. When the GPCR AT1 receptors are activated by the agonist
AngII, subsequent coupling to the Gα (q/11 and 12/13) and Gβγ complexes\textsuperscript{67} initiates the downstream effector phospholipases (PL), such as PLA\textsubscript{2}, PLC, and PLD.\textsuperscript{68} Specifically, activation of PLC is considered one of the earliest and most rapidly detectable events resulting from AngII stimulation of SMCs. Activation of PLC leads to immediate up regulation of membrane bound diacylglycerol (DAG) and soluble inositol-1,4,5-triphosphate (IP\textsubscript{3}) via hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP\textsubscript{2})\textsuperscript{69-72}. This effect is dependent upon AT1 receptors, as the selective antagonist Losartan, inhibits hydrolysis of PtdInsP\textsubscript{2}. The sarcoplasmic reticulum contains many IP\textsubscript{3} receptors, which are stimulated by the increase in IP\textsubscript{3}, leading to the opening of channels allowing for calcium to efflux into the cytoplasmic space. Binding of this free cytoplasmic calcium to calmodulin leads to activation of myosin light chain kinase (MLCK), which subsequently phosphorylates myosin light chain resulting in actin and myosin interaction and subsequent smooth muscle cell contractions.\textsuperscript{73-77} This IP\textsubscript{3}-mediated calcium release is considered a first transient burst that is short lasting, producing transient contractions. The signal is propagated by AngII-mediated transmembrane calcium influx, which is speculated to occur via voltage-gated channels and leads to sustained AngII-induced vasoconstriction of the smooth muscle.\textsuperscript{78-82}

The other important mediator for propagation of contractions is the membrane bound DAG molecule, described above. The release of calcium, combined with AngII upregulations of DAG and the presence of phosphatidylerine, leads to the activation of protein kinase C (PKC). PKC is implicated in both AngII-mediated vascular contractions and VSMC growth via activation of Na+H+ exchanger, resulting in intracellular alkalinization, a potent mediator of actin and myosin association.\textsuperscript{83-86} This alkalinization induces vasoconstriction via increasing concentrations of sodium and calcium and by sensitizing contractile machinery to calcium signals.\textsuperscript{77,87-90} It is important to note that both AT1a and AT1b receptors are both implicated in the AngII induction of intracellular calcium.\textsuperscript{43} Many other pathways exist in the initiation and
propagation of contractions, such as AngII-mediated formation of leukotrienes and cyclooxygenase-derived prostaglandins via PLA2 production of arachidonic acid. However, it appears the IP3 and DAG-mediated contractions are the most prominent.

The signaling pathways, listed above, lead to the final consequence of SMC contraction. AngII effects on aortic contractile responses have provided insight that the octapeptide has region-specific effects. Initial studies by Zhou et al. demonstrated AngII-mediated contractions to be confined to the abdominal aorta and the femoral artery, with weakened contraction in the carotid artery and almost no signal in the thoracic aorta. Further, these studies demonstrated that AT2 antagonism with PD123319 resulted in no change in AngII contractility, while AT1 antagonism with Losartan resulted in full ablation of contraction. This effect was correlated to an increase in AT1 mRNA expression in the abdominal aorta. In a subsequent experiment, Zhou et al. provided a connection of this contractile effect to the AT1b receptor using genetically ablated AT1a receptor mice. The results demonstrated no change in contraction using the deficient AT1a mice, however, the administration of Losartan ablated the contractile signal. Further, mRNA analysis confirmed the up regulation of AT1b in the femoral and abdominal aorta versus the thorax. Therefore, in mouse aortic rings, AngII does not induce a contraction in thoracic tissue, but generates a large transient contraction in abdominal tissue. Recent verification of this pharmacological inhibition has been shown with the use of AT1b receptor deficient animals displaying minimal to no AngII-induced contractions.

Small resistance vessels have also been analyzed for their contractile properties to stimulation with AngII. These resistance vessels are considered crucial for mediation of vasoconstriction induction of hypertension to AngII. Similar results to abdominal aortic contraction and femoral contractions Zhou et al. demonstrated AngII-induced contractions in small mesenteric arteries and found that the AT1b receptor accounted for 98% of AT1 expression. The efferent and afferent arterioles of the renal microvasculature have also been
studied utilizing an in vitro measurement of afferent and efferent arteriolar diameters (AAD and EAD). Results demonstrated no change in the AAD with the ablation of the AT1a receptor, suggesting a role for the AT1b receptor. However, AT1a deletion resulted in failure to elicit any change in EAD, when compared to proficient animals, demonstrating a role for the AT1a receptor. These results were later confirmed using AT1b receptor deficient animal demonstrating functional expression of AT1a and AT1b on the afferent arterioles, and sole functional expression of AT1a on the efferent arterioles. Combined with results from complete AT1 deficient animals (double deficient AT1a and AT1b), these results indicate that AT1a can also exhibit a contractile response in the vasculature of the kidney. Therefore, a disparity still exists in the understanding of AT1a and AT1b mediation of regional contractions, and further study is needed.

As previously noted, aortic segments incubated with the AT2R antagonist PD123319 resulted in normal AngII abdominal contractions. However, two independent studies demonstrate an increased AngII induced contraction in AT2R-/- animals. To confirm these results we obtained AT2R-/- animals and incubated all four regions with AngII (unpublished data). We demonstrate no difference in AngII infrarenal contraction with the AT2R-/- animals verifying the studies of Zhou et al. Akishita et al. used thoracic aortas for their studies and variable genetic background of their mice. Furthermore, Tanaka et al also demonstrated increased contraction in AT2R-/- thoracic aortas. These results are somewhat confounding since many groups demonstrate no reactivity to AngII in thoracic aortic segments, including our results in three different genotypes.

B. Effects of AngII on Vascular Remodeling

Numerous studies are devoted to the study of AngII and subsequent physiological and pathological effects on SMCs, both in vitro and in vivo. These studies demonstrate AngII to be a powerful growth factor leading to mitogenic,
hypertrophic, apoptotic, and differentiation phenotypes in VSMCs. The mediation of these various phenotypes is attributed to the cytokines or growth factors present and the location of the VSMCs. These region-specific effects may be due to the phenotypic diversity of aortic SMCs that arise as a consequence of heterogeneity of embryological origin, as well as the cytokines and growth factors present. SMCs in the ascending aortic regions are primarily of neural crest origin, which extends from the aortic root to just distal of the subclavian artery. This same lineage also extends to the carotid arteries. Conversely, thoracic and abdominal aortic SMCs are derived from somite and splanchnic mesoderm lineages, respectively. This may represent many of the differences noted in the literature.

Most studies on cultured aortic SMCs have isolated cells from the thoracic region of rats. In these cells, AngII consistently promotes hypertrophy, with a lesser number of studies demonstrating a proliferation response. The relative effects of AngII on hypertrophy versus proliferation, in vitro, appear to depend on culture conditions, such as confluency and the co-incubation with specific cytokines. The expression of the cyclin-dependent kinase inhibitor, p27kip1, in cultured cells is also a factor determining the relative effect on hypertrophy versus proliferation. The AngII-induced suppression of the cyclin dependent kinase (CDK) 2 lead to high levels of p27kip1, leading to G1-phase arrest and cellular hypertrophy. Alternatively, recent data also suggests AngII regulates inhibitor of differentiation (Id) proteins 2 and 3 via superoxide release. These Id proteins depress the activity of p21waf1/cip1, p27kip1, and p53, allowing for proliferation responses. Studies demonstrate that the Id3 protein is highly overexpressed in vascular injury models resulting in uncontrolled VSMC proliferation. These data suggest that AngII can modulate hypertrophic situations by suppressing the cell cycle and mitogenic conditions by allowing unimpeded cell-cycle progression.

Many AngII-induced responses on SMCs are also due to stimulation of NADPH oxidase with the subsequent generation of ROS. These include
AngII-induced hypertrophy and proliferative responses, as noted above. NADPH oxidase is a multimeric complex. SMCs express specific isoforms of gp91phox, with nox1 being the principal regulator of AngII-induced responses.\textsuperscript{118-121} The activity of nox1 is regulated by the presence of p47phox.\textsuperscript{122} Consistent with the nox1-p47phox axis, deficiency of p47phox decreases AngII-induced increases in systolic blood pressure.\textsuperscript{123}

Lesser number of studies have chronically infused AngII in vivo to determine effects on medial dimensions.\textsuperscript{124-126} SMC proliferative responses to AngII infusion have been demonstrated in rat carotid and mesenteric arteries.\textsuperscript{127-130} In contrast, the predominance of studies have demonstrated AngII increased medial thickness through hypertrophy of rat\textsuperscript{124,125,127} and mouse\textsuperscript{121,126,131} aortas. The location of aortic sections used in these studies has not been commonly stated, but is likely to be the thoracic region. Recently unpublished data from our laboratory presents region-specific aortic arch hyperplasia, versus hypertrophy in the thoracic and suprarenal/infrarenal abdomen. These results were performed in a mouse model of AngII infusion, and are consistent with the report of rat studies in the carotid artery. Similar to this report, this effect was not due to pressure, as norepinephrine infusion did not result in similar proliferative responses to AngII. As previously stated, aortic arch and carotid SMCs are derived from a neural crest lineage, while thoracic and abdominal cells are derived from a somite and splanchnic mesoderm line of cells. This difference in embryologic origin could explain the differences of SMC responsiveness to AngII.

**Conclusions**

The RAS is a complex collection of receptors that lead to the initiation of numerous signaling cascades resulting in many physiological and pathological consequences. Even with the vast knowledge of AngII signaling cascades and resultant vascular functions and dysfunctions, these conflicting data suggest many underlying processes involved in medial thickening and that the exact
mechanisms of arterial remodeling are as yet still undetermined. However, consistently recurring themes seem to be the origin, the surrounding environment, and regulation of cell cycle progression of AngII on VSMC to determine the outcome of remodeling.

2.1 SYNOPSIS

Angiotensin II (AngII) promotes many changes in aortic tissue. The aim of this study was to determine the nature of aortic medial changes during AngII infusion and determine the role of blood pressure and oxidant mechanisms. AngII (1,000 ng/kg/min) infusion for 28 days into mice increased systolic blood pressure (SBP) and promoted medial expansion of equivalent magnitude throughout the entire aorta. Both effects were ablated by AT1a receptor deficiency. Similar increases in blood pressure by administration of norepinephrine promoted no changes in aortic medial thickness. Increased medial thickness was due to smooth muscle cell (SMC) expansion attributable to hypertrophy in most aortic regions, with the exception of hyperplasia of the ascending aorta. Deficiency of the p47phox component of NADPH oxidase ablated AngII-induced medial expansion in all aortic regions. Analysis of mRNA and protein throughout the aorta revealed a much higher abundance of the inhibitor of differentiation 3 (Id3) in the ascending aorta compared to all other regions. A functional role was demonstrated by Id3 deficiency inhibiting AngII-induced SMC hyperplasia of the ascending aorta. In conclusion, AngII promotes both aortic medial hypertrophy and hyperplasia in a region-specific manner via an oxidant mechanism. The ascending aortic hyperplasia is dependent on Id3.
2.2 INTRODUCTION

Angiotensin II (AngII) has many physiological and pathological effects on smooth muscle cells (SMCs) of aortic tissue both in vitro and in vivo. Most studies on cultured aortic SMCs have isolated cells from the thoracic region of rats.\textsuperscript{97,98,101} In these cells, AngII consistently promotes hypertrophy,\textsuperscript{97,101} with a lesser number of studies demonstrating a proliferation response.\textsuperscript{98} The relative effects of AngII on hypertrophy versus proliferation, \textit{in vitro}, appear to depend on culture conditions, such as confluence and the co-incubation with specific cytokines.\textsuperscript{98} The expression of the cyclin-dependent kinase inhibitor p27\textsuperscript{kip1}, in cultured cells, is a factor determining the relative effect on hypertrophy versus proliferation.\textsuperscript{104}

Many AngII-induced responses on SMCs are due to stimulation of NADPH oxidase with the subsequent generation of reactive oxygen species (ROS).\textsuperscript{115-117} These include AngII-induced hypertrophy and proliferative responses. NADPH oxidase is a multimeric complex. SMCs express specific isoforms of gp91\textsuperscript{phox}, with nox1 being the principal regulator of AngII-induced responses.\textsuperscript{118,119,121} The activity of nox1 is regulated by the presence of p47\textsuperscript{phox}.\textsuperscript{122} Consistent with the nox1-p47\textsuperscript{phox} axis, deficiency of p47\textsuperscript{phox} blunts AngII-induced increases in systolic blood pressure.\textsuperscript{123} More recently, the dominant-negative helix-loop-helix protein (dnHLH), inhibitor of differentiation 3 (Id3), has been invoked as a regulator of redox-mediated AngII induced proliferation.\textsuperscript{112,113}

AngII effects on aortic contractile responses have provided insight that this octapeptide has region-specific effects. AngII does not induce contractions of mouse aortic rings isolated from thoracic tissue, but generates a large transient contraction in abdominal tissue \textit{in vitro}.\textsuperscript{132,133} Contractions of aortic rings are mediated by AT1b receptors despite the presence of AT1a receptors, which may be indicative of divergent signaling mechanisms stimulated by these subtypes.\textsuperscript{15,16} Region-specific effects on aorta have been noted in response to other bio-activators such as transforming growth factor-beta.\textsuperscript{134,135} These region-
specific effects may be due to the phenotypic diversity of aortic SMCs that arise as a consequence of heterogeneity of embryological origin. AngII infusion, in vivo, is well known to promote changes in medial aortic SMCs via mechanisms that are independent of increased SBP. The aim of the present study was to determine whether AngII infusion in vivo promoted heterogenous effects on SMC growth and/or proliferation throughout the entire aorta. Despite previously described differences in contractile activity of AngII on thoracic versus abdominal aorta, we were unable to demonstrate morphological differences in these two regions during AngII infusion. However, the ascending aorta has a different response than the rest of the aorta with a medial expansion that was attributable to hyperplasia, versus hypertrophy in other regions. Aortic medial expansion throughout the aorta was ablated by deficiency of p47phox, while deficiency of Id3 inhibited the hyperplasia induced by AngII in the ascending aorta.

2.3 Materials and Methods

Mice:

AT1a receptor deficient mice (N=10; B6.129P2-Agtr1atm1Unc; stock no. 002682), C57BL/6 (stock no. 000664), and p47phox mutant mice (B6(Cg)-Ncf1m1J/J, stock no. 004742; backcrossed N ≥10 C57BL/6; C57p47/p47) were purchased from the Jackson Laboratory. Littermate controls were utilized for all experiments. Id3 -/- mice were a generous gift from Dr. Yuan Zhang (Duke University), and were N ≥10 for the C57BL/6 background. Mice were housed in a pathogen-free environment and fed a normal diet (Harlan Teklad catalog No. 2918) and water ad libitum. All studies were performed using male mice. Genotyping for AT1a receptor-/- mice, C57p47/p47, and Id3-/- mice was performed as described previously. All procedures were approved by the University of Kentucky IACUC.
**Alzet Pump Implantation:**

At 8 to 10 weeks of age, C57BL/6, AT1a receptor-/-, C57p47/p47, and Id3/- male mice were implanted with Alzet mini-osmotic pumps (Model 2004, Durect Corp), subcutaneously in the right flank and infused with either sterile saline or AngII (Sigma cat# A9525; 1,000 ng/kg/min) for 28 days (n = 5-10), as described previously. In addition, norepinephrine (L-(−)-norepinephrine bitartrate salt (NE), Sigma cat# A9512; 5.6 mg/kg/day) was infused into C57BL/6, AT1a receptor-/-, and C57p47/p47 mice (n=5 each group). NE was dissolved in L-ascorbic acid/saline (Fisher cat# A61; 0.2% wt/vol) as described previously. Nitro-L-arginine methyl ester hydrochloride (Sigma cat# N5751) was introduced via the drinking water at a concentration of 1.5 mg/ml (~100 mg/kg/day, changed daily).

**Systolic Blood Pressure Measurement:**

SBP was measured noninvasively on conscious mice using the Visitech tail cuff system (BP-2000 Visitech Systems). Mice were habituated 1.5 weeks prior to pump implantations and SBP was measured 5-6 times a week, at the same time of day, throughout the entire infusion period, as described previously.

**Tissue Preparation:**

Mice were anesthetized with ketamine/xylazine, blood was collected with EDTA (0.2% wt/vol) from the right ventricle, and exanguination was performed via an incision in the right atria. Aortas were perfused via the left ventricle with phosphate-buffered saline and then perfusion fixed with paraformaldehyde (4% wt/vol) under physiological pressure for 30-45 minutes. Organs were removed and aortas were filled, via the left cardiac ventricle, with low melting point
agarose (Promega cat# V2111; 3% wt/wt) and colored with a green tissue dye (Polysciences, Inc. cat# 24110), as described previously.\textsuperscript{143} Ascending aortic sections were located 1 to 3 mm distal to termination of valve leaflet stubs. Thoracic sections were located between 5 mm and 7 mm posterior of the left subclavian artery. Suprarenal abdominal aortic sections were flush with the superior mesenteric artery and 2 mm anterior. Infrarenal abdominal aortic sections were 2 mm cut from the left renal branch to the posterior. These aortic sections are schematically represented (Figure 2.1). Aortic sections were placed in OCT (Tissue-Tek cat# 4583) and cut serially in 10 μm increments (posterior to anterior) with 9 sections per slide and a total of 8 slides. With the exception of ascending arch aortas (no branch points), all branch points and aortic vessels were discarded, leading to the final coverage of approximately 900 to 1500 μm of aorta per cross-sectional slide.

**Morphometric Analyses:**

Sections were stained with hematoxylin-eosin, mounted with glycerol/gelatin (Sigma), and captured on a Nikon Optiphot-2 with a Nikon DXM camera. The inner elastic lamina was traced and subtracted from the outer elastic lamina to measure medial areas. Medial thickness was calculated from means of 4 orthogonal measures of the distances between the internal and external elastic lamina. Mean medial areas and thicknesses were measured from 5 to 15 sections per mouse. Measurements were performed using Image Pro-Plus 5.0 software (Media Cybernetics).

**Nuclei Counting:**

Frozen sections were fixed in paraformaldehyde (4% wt/vol). Propidium iodide staining (Molecular Probes, P-3566, 1 μg/ml) was performed, subsequently to RNA digestion for 15 min at 37°C. The nuclei counts were
quantitated on 2 aortic segments of equivalent length (0.25 mm) per serial section (minimum of 6 sections per slide).

**Aortic Immunostaining and Histology:**

Histological analysis was performed on paraformaldehyde fixed frozen sections using Movat’s pentachrome (PolyScientific, cat# K042). Immunostaining was performed on frozen serial sections as described previously. An α-actin SMC antibody (5 μg/ml; Abcam, cat# ab5694) was incubated for 30 minutes at 37°C. Subsequent application of a goat antirabbit biotinylated antibody (1:500; Vector) was incubated for 30 minutes at 37°C. Positive reactive areas were visualized via application of an ABC kit (10 minutes 37°C) and subsequent detection with AEC chromogen (2 applications of 10 minutes 37°C; Vector). Several controls were used, including: no primary antibody, no primary and secondary antibodies, and non-immune sera or IgG when appropriate. Images were captured with a 20x objective lens using a Nikon Eclipse E600 scope and a Nikon DXM1200F digital camera.

**SMC Isolation:**

Aortas from 8-10 week old male C57BL/6J mice (Jackson Laboratory) were isolated and dissected free of adventitia in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM). Aortas were divided into four sections: ascending aorta (above the heart to left subclavian artery), thoracic aorta (left subclavian artery to last intercostal artery), suprarenal aorta (last intercostal artery to right renal artery), and infrarenal aorta (left renal artery to iliac bifurcation). SMCs were isolated via chemical digestion using type III porcine pancreatic elastase (250 μg/ml, Sigma) and type I collagenase (1 μg/ml, Worthington), as described previously. SMCs were maintained in DMEM with fetal bovine serum (20% vol/vol; FBS, Equitech, Inc.) and penicillin and streptomycin (1% wt/vol, Gibco) in
a 37°C incubator with 5% CO₂. SMC phenotype was determined via visualization with Cy3 labeled α-actin clone 1A4 (Sigma).

**Cellular Immunofluorescence and Confocal Microscopy:**

SMCs were isolated as described above, and grown on glass Lab-Tek chamber slides (Nunc) (n=6 separate samples each section). At 70% confluence, cells were washed, and subsequently incubated with serum-free media. After 72 hours, wells were rinsed with PBS, and incubated with either saline or AngII (1 μM) for 1, 2, 12, and 24 hours. Slides were then rinsed with PBS and fixed with paraformaldehyde (4% wt/vol) for 10 minutes. Cells were permeabilized with Triton X-100 (0.5% vol/vol) in PBS for 10 minutes at 37°C. Non-specific binding was blocked via incubation with normal goat serum (15 μg/ml) and BSA (1% wt/vol) for 30 minutes at 37°C. An rabbit monoclonal Id3 primary antibody (5 μg/ml; Cal Bioreagents, clone 6-1) was incubated overnight in a humidity chamber at 4°C. After washing 3 times in PBS, secondary goat antirabbit labeled Cy2 (1:250 dilution, Jackson Immuno) and Cy3-labeled α-SMC actin (1:100 dilution, clone 1A4, Sigma) were added for 30 minutes at 37°C. Chambers were subsequently washed 3 times with PBS and then incubated with Hoechst 33342 (1 μg/ml; Invitrogen) for 5 minutes. Chambers were removed, slides were mounted with aqueous mounting media (Biomeda Corp), and cover slips were fixed in place using clear nail polish. Controls were included using no addition of primary antibody, no addition of either primary or secondary antibodies, and rabbit IgG control (5 μg/ml). Slides were visualized and captured using an Olympus BX61WI confocal microscope and FluoView software.

**SMC Proliferation Analyses:**

SMCs were isolated, as described above, counted using a hemacytometer, and seeded onto 35 x 10 mm culture wells at 10,000 cells/well.
Separate primary harvests from 5 different groups of 8-10 week old male C57BL/6 mice (Jackson Laboratory) were performed. Cells were immediately serum-starved for 24 hours and then incubated with saline, AngII (1 μM), or PDGF-BB (25 ng/ml; Sigma) for 24, 48, or 72 hours. Saline, AngII, or PDGF-BB were incubated with cells in the presence of FBS (2.5% vol/vol; which did not induce changes in cell numbers). Wells were washed 3 times using PBS and cells removed using trypsin-EDTA (0.25% wt/vol; Gibco), quenched with FBS, and subsequently counted using a hemacytometer. Cell numbers were also verified on a Hemavet 950 (Drew Scientific) cell counter.

**Dichlorodihydrofluorescein diacetate (DCF-DA) Analyses:**

SMCs were isolated, as described above, plated on glass Lab-Tek chamber slides, and grown to ~70-80% confluence in DMEM with FBS (20% vol/vol; n=5 separate samples each section). Cells were then serum-starved for 24 hours in DMEM, and subsequently incubated with AngII (1 μM), AngII (1 μM) + losartan (1 μM), saline, H₂O₂ control (1 μM), or saline no DCF-DA blank control. DCF-DA (Invitrogen) reagent was incubated with cells after 24 hours of incubation. Chamber slides were subsequently visualized using a Nikon Eclipse E600 scope with excitation sources and filters appropriate for fluorescein (FITC) detection.

**Amplex Red Analyses:**

SMCs were isolated, as described above, plated on 6-well culture plates, and grown to ~70-80% confluence in DMEM with FBS (20% vol/vol; n=4 separate samples each section). Cells were serum-starved for 24 hours and incubated with saline, AngII (1 μM), or AngII (1 μM) + PEG-catalase (100 U/ml, Sigma) for an additional 24 hours. Amplex red assay (Invitrogen) was performed using modifications described previously in the protocol. Fluorescence
intensity was determined using a microplate reader (Molecular Devices, SpectroMax M2) in the excitation range of 530-560 nM and emission detection at 590 nm. SMCs were incubated with standard lysis buffer, protein was extracted, and examined using a Bradford Assay (Bio-Rad). Fluorescent readings were subsequently normalized to cellular protein content.

**Renin Analyses:**

Blood was collected in syringes filled with EDTA (0.2 M) and centrifuged at 2,000 rpm for 20 minutes for plasma collection. Renin concentrations in plasma (8 μl) were measured by incubation with an excess of angiotensinogen (0.4 μM; Sigma) in the presence of EDTA (0.02 M) for 30 min at 37°C. AngI generated was quantified by RIA using a commercially available kit (Diagnostic Systems Laboratories).

**Cell Culture and Western Blot Analyses:**

SMCs were isolated, as described above, and plated on 6-well culture plates in DMEM with FBS (20% vol/vol). Separate primary harvests from 3 different groups of 8-10 week old male C57BL/6 mice (Jackson Laboratory) were performed. At ~70-80% confluence, wells were washed 3 times with PBS and serum-starved for 72 hours, as described previously. Cells were then incubated with either saline or AngII (1 μM) for 1, 2, 12, or 24 hours and total cell lysates were extracted using 1x cell lysis buffer (cat: 9803; Cell Signaling) with addition of protease inhibitor cocktails. Protein content was measured using a Bradford Assay (Bio-Rad) and subsequently equal amounts of protein (20 μg) were resolved using SDS-PAGE (12.5% wt/vol), and transferred via electrophoresis (50 V for 90 minutes) to PVDF membranes (Millipore). Membranes were blocked at room temperature with nonfat milk (5% wt/vol) in Tris-buffered saline, containing tween 20 (0.5% vol/vol; TBS-T). Membranes
were cut at the 25 kD molecular weight marker and the lower section was incubated with an antibody against Id3 (0.5 μg/ml, clone 6-1, Cal Bioreagents). The upper section was incubated with a β-actin antibody (1:1000, Sigma) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hour and immune complexes were visualized by a Supersignal West Pico Chemiluminescence Kit (Pierce) and quantified using a Kodak Imager.

**Real Time Polymerase Chain Reaction:**

Ascending (aortic sinus to left subclavian), thoracic (left subclavian to last intercostal), suprarenal (last intercostal to right renal), and infrarenal (left renal to iliac bifurcation) sections of aortas were harvested from thirty 8 week old male C57BL/6 and placed in RNA Later (Ambion, cat# 7024), and adventitia was removed. Aortas were combined, in groups of 5, for a total of 6 independent samples for each section. Aortas were ground with a glass mortar and pestle in the presence of lysis buffer (Rneasy Fibrous Minikit Isolation System, Qiagen, cat# 74704). All samples were incubated with Turbo DNA-free (Ambion, cat# AM1907) to remove all DNA contamination. RNA concentrations were quantified using a UV spectrophotometer (Beckman DU530). Reverse transcription of RNA was performed with i-script (Bio-Rad, cat# 170-8891) with a concentration of 100 ng total RNA using an i-cycler (Bio-Rad). Real-time analysis of cDNA amplifications were performed using a Bio-Rad i-cycler with Id3 Taqman probes (Applied Biosystems, catalog# Mm00492575-m1) and IQ supermix (Bio-Rad, cat# 170-8860). These results were compared to an 18s rRNA endogenous control (Applied Biosystems, cat# 4352930E). Results were extrapolated using the standard curve method with kidney serving as the Id3 standard. Negative controls consisted of no RT controls and Id3-/- tissue cDNA, which was not detectable with these primers. Another separate run was performed and stopped in the middle of the sigmoidal real-time curve. These samples were run on a agarose gel (3% wt/vol) diluted with a 6x blue-green running dye and molecular
weight was analyzed with a PGEM DNA marker (Promega).

Statistics:

All statistical analyses were performed using either SigmaStat (SPSS Inc.) or version 8.2 of SAS (SAS Institute). All measurements are represented as means ± SEM. One Way ANOVA was performed on measurements, where indicated, with a Holm-Sidak post hoc test. For two group comparison of parametric data, a Student’s t-test was performed. Repeated measures data were analyzed with SAS fitting a linear mixed model expressing the temporal trend in systolic blood pressure as a quadratic polynomial in time for each treatment. A Bonferroni-adjusted pairwise comparison was performed within similar genotypes among treatments.

2.4 RESULTS

**AngII infusion uniformly increased aortic thickening via AT1a receptors, independent of pressure**

AngII infusion (1,000 ng/kg/min) into C57BL/6 mice led to a sustained increase in SBP (~40 mmHg) during the 28 days of delivery (Figure 2.2A). AngII infusion also led to increased medial thickness and area to a similar percent in all aortic regions (Figure 2.2B; Table 2.1). To determine the contribution of pressure per se to medial expansion, NE was administered to male C57BL/6 mice at doses that increased SBP by the same magnitude as AngII (approximately 40-50 mmHg). Contrary to AngII infusion, NE administration led to no significant changes in medial thickness compared to age-, gender-, and strain-matched mice infused with saline. Equivalent data were also obtained by increasing blood pressure through administration of N\(^6\)-nitro-L-arginine methyl ester (L-NAME, data not shown).
To define whether AngII-induced medial thickening was mediated through AT1a receptors, similar experiments were performed in AT1a receptor -/- mice. Plasma renin concentrations were markedly increased in saline-infused AT1a receptor -/- mice compared to C57BL/6 wild type controls (Table 2.1). Interestingly, AngII infusion reduced plasma renin concentrations in both C57BL/6 and AT1a receptor -/- mice. To determine if AT1b receptors mediated effects of AngII to reduce plasma renin concentrations in AT1a receptor -/- mice, we co-infused AngII and losartan. AngII-mediated reductions in plasma renin concentrations were abolished in AT1a receptor -/- mice administered losartan. AngII infusion failed to change SBP in AT1a receptor -/- mice (Figure 2.3A). Moreover, infusion of AngII did not change aortic medial thickness or area in AT1a receptor -/- mice (Figure 2.3B, Table 2.1). Infusion of NE increased SBP in AT1a receptor -/- mice (Figure 2.3). However, NE infusion had no effect on plasma renin concentrations or medial dimensions in any aortic region of AT1a receptor -/- mice (Figure 2.3).

The basis of AngII-induced medial thickening differed among aortic regions

Medial thickening with AngII infusion was associated with a marked expansion of the intra-laminar space in all aortic regions (Figures 2.4-2.5). This expansion may potentially be attributable to contributions from extracellular matrix expansion, and SMC hypertrophy or hyperplasia. To determine the contribution of extracellular matrix (ECM), aortas were stained with Movat’s pentachrome to permit visualization of elastin, collagen, and proteoglycans (Figure 2.4). There were no overt differences in staining for ECM components. Serial sections from all regions were immunostained for α-actin or stained with propidium iodide to access expansion of cell size and number, respectively (Figure 2.5). Immunostaining for α-actin demonstrated uniform reactivity throughout the intra laminar spaces (Figure 2.5A). Negative controls demonstrated no overt staining (Figure 2.6). Nuclei counts were normalized to a
standard section length, since the medial expansion confounded the utilization of vessel area. AngII infusion did not significantly affect nuclei density in both thoracic and abdominal aortic regions (Figure 2.5B). When combined with data from immunostaining, this was consistent with the medial expansion being due to increased volume of SMCs. Unexpectedly, AngII promoted a marked increase in nuclei density in tissue sections from the ascending aorta. Since this entire region immunostained positively for SMCs, this was consistent with medial expansion due to hyperplasia. AngII also exerted region-specific effects on cultured SMCs. In agreement with the observations in vivo, AngII promoted a proliferation of SMCs cultured from ascending aortas, but not in SMCs isolated from other aortic regions (Figure 2.7). PDGF-BB promoted similar proliferation in SMCs cultured from all aortic regions. Therefore, the basis for AngII-induced medial expansion differed among aortic regions.

**p47phox deficiency ablated AngII-induced medial thickness**

Given the role of AngII-induced ROS on SMC hypertrophy and hyperplasia, and the above region-specific data, we examined whether concentrations of superoxide and hydrogen peroxide differed along the aorta. In SMCs cultured from different aortic regions, there was uniform expression of ROS in all aortic regions examined (Figure 2.8). To determine whether inability to generate ROS resulted in functional changes in vivo, saline, AngII, or NE was infused into mice with a spontaneous loss of function mutation for p47phox. Basal blood pressure was not different between p47phox deficient mice compared to wild type (C57BL/6). AngII infusion did not increase blood pressure initially in p47phox functionally deficient mice (Figure 2.9), but did result in a belated increase during week 4 of infusion. Plasma renin concentrations were unchanged in saline-infused p47phox deficient compared to wild type mice, and infusion of AngII led to similar reductions in plasma renin concentrations in p47phox deficient mice. The functional deficiency of p47phox had no effect on the ability of NE infusion to
increase SBP (Figure 2.9A). The absence of functional \( p47^{phox} \) abolished AngII-induced increases in medial thickness for all regions of the aorta (Figure 2.9B).

**AngII initiated Id3 translocation to nuclei**

To provide a basis for AngII-induced medial SMC hyperplastic response that was limited to the ascending portion of aortas, we defined the expression of Id3. Real-time PCR analysis of Id3 mRNA demonstrated a significantly increased (3.5 fold) abundance in ascending aortas versus other aortic regions (Figure 2.10A). Western blotting of Id3 protein in cultured SMCs revealed similar regional differences in the aorta (Figure 2.10B-D). As noted previously,\(^{149}\) Id3 abundance fluctuated over time in the presence of serum, but was always greater in cells isolated from the ascending region. Furthermore, AngII incubation of SMCs isolated from ascending aortas resulted in Id3 colocalization within nuclei at 1 and 24 hours, with cycling to the cytoplasmic compartment at 12 hours (Figures 2.11-2.12). This effect was not observed in other aortic regions (Figures 2.13). These data demonstrate AngII initiates cyclic Id3 localization to the nuclei compartment, in the ascending aorta, suggesting a novel mechanisms whereby id3 mediates the regional hyperplastic effects of AngII.

**Id3 deficiency ablated ascending aortic hyperplasia**

To determine the *in vivo* contribution of Id3 to AngII-induced ascending aortic hyperplasia, Id3/- mice were infused with either saline or AngII. AngII markedly increased SBP in Id3/- mice (Figure 2.14A). Furthermore, Id3 deficiency did not abolish AngII-induced increases of medial thickness in the ascending and abdominal regions (Figure 2.14B). Similar to Id3+/+ mice, immunostaining of aortas from Id3/- mice with \( \alpha \)-actin demonstrated uniform reactivity throughout the intralaminar spaces and no overt matrix deposition by Movats Pentachrome staining. Moreover, plasma renin concentrations were
similarly reduced by AngII infusion in Id3-/- mice, compared to other mouse strains (Table 2.1). SMC nuclei density was unchanged in other aortic regions compared to wild type mice (data not shown). However, AngII-induced increases in ascending aortic SMC nuclei density were strikingly reduced by Id3 deficiency (Figure 2.15).
Figure 2.1: Schematic diagram of analyzed regional aortic segments. The left subclavian, superior mesenteric, and left renal arteries represent landmarks for standardization between animals. Dashed yellow lines indicate cuts in the aorta, while arrows represent direction of sectioning from start to finish.
Figure 2.2: AngII-induced uniform medial expansion throughout the aorta, independent of increased SBP. (A) SBP was measured prior to pump implantation and every week during drug administration. Points represent the mean of weekly observations (n=5) and bars represent SEM. * Denotes P < 0.0001 for AngII, NE, and L-NAME versus saline using repeated measures with Bonferroni post-hoc. # Denotes P < 0.003 AngII versus NE and L-NAME week 4. (B) Aortic medial thickness measured in arch, thoracic, suprarenal, and infrarenal sections of C57BL/6 mice with saline, AngII, NE, and L-NAME. Histobars are means of 5 - 10 mice and bars represent SEM. * Denotes P < 0.001 in AngII infusion versus all other groups (one-way ANOVA with Holm Sidak post hoc).
Figure 2.3: AngII infusion induced medial thickening via interaction with AT1a receptors. (A) SBP was measured prior to pump implantation and every week after drug administration in AT1a receptor -/- mice infused with saline, AngII, or NE. Points represent the mean of weekly observations (n=5) and bars represent SEM. * Denotes P < 0.0001 for NE versus saline or AngII using repeated measures with Bonferroni post-hoc. (B) Medial thickness in 4 aortic regions. Histobars represent means and bars represent SEM.
Figure 2.4: AngII infusion results in no overt deposition of extracellular matrix. Movat’s pentachrome staining of sections of aortas from mice infused with either saline or AngII. The following denotes specific cellular content and the visual color associated: muscle (red), ground substance (blue/green), elastin fibers (black), collagen and reticulum fibers (yellow).
Figure 2.5: AngII infusion induced medial thickness by either hypertrophy or hyperplasia. (A) Representative C57BL/6 aortic sections of immunostaining for α-actin. Tissue sections from ascending, thoracic, suprarenal, and infrarenal aortic sections, infused with saline or AngII, were immunostained with a rabbit anti-α-actin (1:200 dilution). (B) Nuclei density was determined by counting, and normalized to aortic section length. Circles represent means of individual mice, diamonds represent means of 5-10 mice in each group, and bars are SEM. * Denotes P < 0.001 difference between saline or AngII-infused mice using a Student’s t test. Examples of C57BL/6 saline (C) and AngII (D) infused aortic arch sections stained with propidium iodide for nuclei identification.
Figure 2.6: Control tissues for the SMC α-actin immunostaining. (Upper Panel) No primary antibody control incubated with secondary antibody (1:500 dilution) only, (Middle Panel) no primary and no secondary antibody control, (Lower Panel) Rabbit IgG control 5 μg/ml.
Figure 2.7: AngII induces cellular proliferation in SMCs harvested from ascending aortas. SMCs harvested from specific regions were seeded at day 0 with 10,000 cells/well, serum starved for 24 hours, and subsequently incubated for 72 hours with saline, AngII (1 μM), or PDGF-BB (25 ng/ml). Histobars are means of 5 individual experiments and bars represent SEM. * Denotes P < 0.001 for SMCs from ascending aortas incubated with either AngII or PDGF-BB versus saline, and thoracic, suprarenal, and infrarenal PDGF-BB versus saline and AngII (one-way ANOVA with Holm Sidak post hoc).
Figure 2.8: AngII induces similar concentrations of ROS in SMCs from all aortic regions. Ascending aortic SMCs were incubated with (A-B) saline, (C) AngII (1 μM), or (D) AngII (1 μM) + losartan (1 μM) for 24 hours and subsequently stained for DCF-DA (except for A). (E) Regional release of H$_2$O$_2$ was detected using an Amplex Red assay in SMCs incubated with saline, AngII (1 μM), or AngII (1 μM) + PEG-catalase (100 U/ml) for 24 hours. Histobars are means of 4 individual experiments and bars represent SEM. * Denotes P < 0.001 in AngII incubated cells versus all other groups (one-way ANOVA with Holm Sidak post hoc).
Figure 2.9: Functional deficiency of p47phox attenuated both AngII-induced hypertrophy and hyperplasia. (A) SBP was measured prior to pump implantation and every week during infusion with saline, AngII, or NE. Points represent means of weekly observations (n=5) and bars represent SEM. * Denotes P < 0.0001 for NE versus saline or AngII using repeated measures with Bonferroni post-hoc. # denotes P < 0.01 for AngII versus saline infused mice. (B) Effects of infusions on medial thickness in p47phox deficient mice in 4 aortic regions. Histobars are means of 5 mice and bars represent SEM.
Figure 2.10: *Id3* was most abundant in SMCs of the ascending aortic region. (A) *Id3* mRNA abundance was quantified by real-time PCR. Histobars are means of 3 mice and bars are SEM. * Denotes $P < 0.001$ for abundance in ascending aorta versus all other regions (one-way ANOVA). Ascending, thoracic, suprarenal, or infrarenal SMCs were serum-starved for 72 hours and incubated with either saline or AngII (1 μM) for 1 (B), 12 (C), or 24 (D) hours. Histobars represent *Id3* protein abundance normalized to $\beta$-actin and are means of 3 individual experiments with bars represent SEM. * Denotes $P < 0.001$ for ascending aortic SMCs versus all other regions, while ** denotes $P < 0.05$ for ascending aortic SMCs incubated with AngII versus saline at the 12 hour interval (one-way ANOVA with Holm Sidak post hoc).
Figure 2.11: Detection of Id3 mRNA and protein. (A) Gel electrophoresis of Id3 mRNA the 4 aortic regions as indicated. Examples show mRNA resolved on a 3% gel with Id3 (top) and 18s rRNA (bottom) from a qRT-PCR stopped in the middle of the sigmoidal curve. Growth arrested SMCs from (B) ascending, (C) thoracic, (D) suprarenal, and (E) infrarenal aortic segments were incubated with either saline or AngII (1 μM) for 1, 2, 12, or 24 hours. Blots are representative examples from each regional segment, with Id3 analysis (top) and β-actin internal control (bottom). Id3-/- spleen lysate was used for specificity analysis of the Id3 antibody.
Figure 2.12: Control immunofluorescent staining in SMCs. Ascending aortic SMCs were serum-starved for 72 hours, fixed, and visualized. Upper images represent no Cy2 control, while the lower images represent basal ascending Id3 abundance after serum starvation. SMCs were stained for nuclei (Hoechst, blue), Id3 (green, right column only), and α-SMC actin (red). Merged images are in the bottom panels with Id3 and α-SMC actin colocalization represented by yellow.
Figure 2.13: AngII induced nuclei localization of Id3 in SMCs harvested from ascending aortas. Ascending aortic SMCs were serum-starved for 72 hours and incubated with AngII (1 μM) for 1 (upper images), 12 (middle images), or 24 (lower images) hours. SMCs were stained for nuclei (Hoechst, blue), Id3 (green), and α-SMC actin (red). Images were merged, with Id3 and nuclei colocalization represented by pale blue and Id3 and α-SMC actin colocalization represented by yellow.
Figure 2.14: Id3 is minimally expressed in thoracic and abdominal SMCs. Thoracic (upper images), suprarenal (middle images), and infrarenal (lower images) aortic SMCs were serum-starved for 72 hours and incubated with AngII for 24 hours. SMCs were stained for nuclei (Hoechst, blue), Id3 (green), and α-SMC actin (red). Merged images are represented in the bottom panels, with Id3 and α-SMC actin colocalization represented by yellow.
Figure 2.15: Id3 deficiency did not attenuate AngII-induced increases in SBP and medial thickness. (A) SBP was measured during AngII infusion in Id3 +/+ and -/- mice. Points represent means of weekly observations (n = 5) and bars represent SEM. * Denotes P < 0.0001 for saline versus AngII infused mice using repeated measures with Bonferroni post-hoc. (B) Aortic medial thickness was measured in ascending and suprarenal aortic sections of Id3 deficient mice infused with saline or AngII. Histobars are means of 6-7 mice and bars represent SEM. * Denotes P < 0.001 for AngII versus saline infusion (one-way ANOVA with Holm Sidak post hoc).
2.16: Deficiency of Id3 ablated AngII-induced hyperplasia in ascending aortas. (A) Nuclei density in ascending aortas from Id3 +/- and -/- mice. Circles represent means of individual mice, diamonds represent means of 4 - 5 mice in each group, and bars are SEM. * Denotes P < 0.001 for ascending aortas from Id3 +/- mice infused with AngII versus saline by Student’s t test. Examples of saline (B) or AngII (C) infused Id3-/- mice ascending aortic sections stained with propidium iodide.
### Table 2.1: Renin, body weight, and medial area of morphology studies

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Treatment Groups</th>
<th>Body Weight Change (g)</th>
<th>Plasma Renin Concentration (ng/ml/30 min)</th>
<th>Ascending Aorta Medial Area (mm²)</th>
<th>Thoracic Aorta Medial Area (mm²)</th>
<th>Suprarenal Aorta Medial Area (mm²)</th>
<th>Infrarenal Aorta Medial Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Saline</td>
<td>2.8 ± 1.4</td>
<td>9.6 ± 1.3*</td>
<td>0.105 ± 0.007</td>
<td>0.0775 ± 0.005</td>
<td>0.0559 ± 0.002</td>
<td>0.0390 ± 0.002</td>
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<tr>
<td>C57BL/6</td>
<td>AngII</td>
<td>2.4 ± 1.2</td>
<td>1.2 ± 0.8</td>
<td>0.204 ± 0.020†</td>
<td>0.129 ± 0.009†</td>
<td>0.101 ± 0.006†</td>
<td>0.0632 ± 0.005†</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>NE</td>
<td>2.7 ± 1.4</td>
<td>12.3 ± 1.1*</td>
<td>0.120 ± 0.020</td>
<td>0.0737 ± 0.002</td>
<td>0.0560 ± 0.005</td>
<td>0.0381 ± 0.001</td>
</tr>
<tr>
<td>AT1aR-/-</td>
<td>Saline</td>
<td>1.3 ± 0.7</td>
<td>46.2 ± 2.5*</td>
<td>0.126 ± 0.009</td>
<td>0.0702 ± 0.002</td>
<td>0.0570 ± 0.003</td>
<td>0.0363 ± 0.001</td>
</tr>
<tr>
<td>AT1aR-/-</td>
<td>AngII</td>
<td>1.8 ± 0.0</td>
<td>24.8 ± 3.3</td>
<td>0.126 ± 0.006</td>
<td>0.0649 ± 0.004</td>
<td>0.0561 ± 0.005</td>
<td>0.0319 ± 0.001</td>
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<tr>
<td>AT1aR-/-</td>
<td>Losartan</td>
<td>1.3 ± 0.7</td>
<td>50.7 ± 11.2*</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td>AT1aR-/-</td>
<td>AngII + Losartan</td>
<td>1.2 ± 0.2</td>
<td>46.0 ± 6.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C57Bl/6Hut</td>
<td>Saline</td>
<td>2.6 ± 1.3</td>
<td>12.6 ± 0.5*</td>
<td>0.120 ± 0.009</td>
<td>0.0869 ± 0.002</td>
<td>0.0623 ± 0.002</td>
<td>0.0381 ± 0.001</td>
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<td>C57Bl/6Hut</td>
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<td>0.0603 ± 0.003</td>
<td>0.0331 ± 0.002</td>
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<tr>
<td>C57Bl/6Hut</td>
<td>NE</td>
<td>2.0 ± 1.4</td>
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<td>0.0609 ± 0.002</td>
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<tr>
<td>Idd-/-</td>
<td>Saline</td>
<td>1.4 ± 0.7</td>
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<td>0.137 ± 0.003</td>
<td>ND</td>
<td>0.0562 ± 0.002</td>
<td>ND</td>
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<tr>
<td>Idd-/-</td>
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<td>0.244 ± 0.020†</td>
<td>ND</td>
<td>0.118 ± 0.008†</td>
<td>ND</td>
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</table>

Values are represented as mean ± SEM.

Body weight change calculated as difference in weight from day 0 to day 28 of infusion.

ND: not determined.

* Indicates P<.004 in plasma renin groups compared to unmarked categories within mouse strains.

One-Way ANOVA with Holm-Sidak Post Hoc.

† Indicates P<.001 in AngII vs all other groups within mouse strains. One-Way ANOVA with Holm-Sidak Post Hoc.
2.5 DISCUSSION

This study demonstrated that AngII promoted a uniform medial expansion throughout the aorta. Despite the uniformity of the expansion, the underlying cause differed in a region-specific manner. SMC hyperplasia occurred in the ascending aorta, but SMC hypertrophy in all other aortic regions. This effect was due to stimulation of AT1a receptors, but independent of increases in SBP. AngII-induced medial expansion was ablated in all aortic regions in mice with functional deficiency of p47phox, while the hyperplastic response localized to the ascending aorta was inhibited by deficiency of Id3.

AngII-induced medial expansion has the potential to be attributable to both cellular and extracellular components. While AngII is known to increase several ECM proteins, including collagen\textsuperscript{150,151} and proteoglycans\textsuperscript{152}, these did not appear to provide a major contribution to medial expansion. Therefore, this study focused on the contributions of increases in cellular components to medial expansion. Many studies have demonstrated that AngII promotes hypertrophy\textsuperscript{97,101} of cultured aortic SMCs, with a lesser number demonstrating proliferation.\textsuperscript{98} Most of these studies have been performed with SMCs that have been derived from rat thoracic aorta. A lesser number of studies have chronically infused AngII in vivo to determine effects on medial dimensions.\textsuperscript{124-126} SMC proliferative responses to AngII infusion have been demonstrated in rat carotid and mesenteric arteries.\textsuperscript{127-130} In contrast, the predominance of studies have demonstrated AngII increased medial thickness in vivo through hypertrophy of rat\textsuperscript{124,125,127} and mouse\textsuperscript{121,126,131} aortas. The location of aortic sections used in these studies has not been commonly stated, but is likely to be the thoracic region. Thus, the description of medial expansion being attributable to hypertrophy in the thoracic aorta is consistent with the present study. However, heterogeneity of AngII-induced responses within the aorta has not been described previously. Our studies demonstrated that AngII-induced proliferation was limited to SMCs cultured from the ascending aorta versus other aortic
regions. However, the present study did not discern whether the in vivo increase in nuclei was attributable to proliferation or polyploidization, although the magnitude of the increase in nuclei in the ascending aorta is much larger than has been described for polyploidization.\textsuperscript{124,153-155}

The basis for the heterogeneous cause of AngII-induced medial expansion in different regions of the aorta may be attributable to the diversity of embryonic origin of SMCs with potential functional differences.\textsuperscript{99} SMCs in the ascending aortic regions are primarily of neural crest origin, which extends from the aortic root to just distal of the subclavian artery.\textsuperscript{100} This same lineage also extends to the carotid arteries. Conversely, thoracic and abdominal aortic SMCs are derived from somite and splanchnic mesoderm lineages, respectively. Regional differences in aortic SMCs have been described previously.\textsuperscript{99}

Deficiency of AT1a receptors ablated AngII-induced medial expansion in all regions. Previous studies have highlighted the role of the AT1b receptor subtype in aortic function.\textsuperscript{43,132} Although both “a” and “b” subtypes of AT1 receptors are expressed in the mouse aorta, deficiency of the “b” subtype substantially reduced AngII-induced contractions that are restricted to the abdominal aorta.\textsuperscript{133} Although the two subtypes are highly homologous, the differences are predominantly in the final intracellular loop and the cytoplasmic tail of this seven membrane spanning protein.\textsuperscript{156} These amino acid substitutions have predictive differences on intracellular signaling.\textsuperscript{157,158} Thus, in future studies, the relative differences between the subtypes will provide insight into the definition of the intracellular pathway that leads to medial expansion.

As with previous studies, absence of AT1a receptors had no effect on basal SBP in mice on a C57BL/6 background.\textsuperscript{41,136,159} Moreover, AT1a receptor deficiency increased plasma renin concentrations, demonstrating removal of AngII-mediated negative feedback on renin synthesis and secretion.\textsuperscript{44} This maintenance of pressure is probably due to the continued presence of AT1b receptors, since compound deficiency of both subtypes promotes a severe phenotype as occurs with deficiency of angiotensinogen, renin, or ACE.\textsuperscript{160} The
presence of functional AT1b receptors can be inferred in the present study by the downregulation of plasma renin concentrations during AngII infusion into AT1a receptor deficient mice and its reversal during concomitant infusion of the AT1 receptor antagonist, losartan.

Numerous studies have demonstrated a functional role of ROS, specifically superoxide (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)), in both hypertrophic and hyperplastic responses of SMCs.\(^{105,112,113,131}\) AngII-induced production of \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) has the potential to increase redox signaling pathways. However, we were unable to demonstrate any regional differences in ROS production. A major source of ROS during AngII stimulation of SMCs is via augmentation of NADPH oxidase activity as demonstrated both in studies in cultured SMCs\(^{105,115,161}\) and in vivo.\(^{121,126,131}\) NADPH oxidases are multimeric complexes with components that differ in a cell-specific manner. In regard to AngII-induced responses in SMCs, these are mediated by a complex containing nox1 in which p47\(^{\text{phox}}\) is a critical component for activation.\(^{122}\) The importance of this coupling has been shown using gp91ds-tat which is a peptide that inhibits the assembly of Nox1 with p47\(^{\text{phox}}\).\(^{162}\) Gp91ds-tat inhibits AngII-induced increases in SBP and medial expansion of carotid arteries. Conversely, deficiency of p47\(^{\text{phox}}\) attenuates AngII-induced increases in SBP.\(^{123,163}\) In contrast to results from the present study, a previous study demonstrated that p47\(^{\text{phox}}\) deficiency increased basal blood pressure, even though AngII-induced increases in SBP were attenuated.\(^{164}\) Moreover, those p47\(^{\text{phox}}\) deficient mice show basal increases in renin,\(^{164}\) which we were not able to demonstrate. Differences in results may arise from the mode of interference with p47\(^{\text{phox}}\), since the previous study used genetically engineered p47\(^{\text{phox}}\) deficient mice, while the present study used p47\(^{\text{phox}}\) deficient mice that arose from a spontaneous mutation.\(^{165}\) However, although the spontaneous and engineered mutations are on exon 8 and 7, respectively, there is no obvious basis for these mice exhibiting different manifestations of deficiency. The engineered p47\(^{\text{phox}}\) deficient mice have been shown previously to exhibit no change in basal SBP. However, other studies have demonstrated a greater
This greater efficacy of AngII-induced increases in SBP may be a consequence of the use of the [Val5] variant of AngII which has a greater efficacy at stimulating AT1 receptors compared to the AngII sequence of human and mouse peptides. Irrespective of these differences among studies, the current study demonstrated that deficiency of p47phox ablated aortic medial expansion in all regions.

AngII-induced proliferation of cultured SMCs occurs via activation of NADPH oxidase with generation of superoxide production that subsequently induces the expression of the dnHLH protein, Id3. Id3 dimerizes with the bHLH factor E47, inhibits E47-induced activation of expression of cyclin dependent kinase inhibitor p21WAF1/Cip1, and promotes SMC proliferation. This mitotic effect is mediated via nuclei translocation of Id3, from the cytoplasm, via an E47 nuclear localization signal (NLS). Id3 has also been implicated in proliferative responses to carotid artery injury. As noted earlier, the carotid artery shares common embryonic origin with cells in the ascending aorta. In agreement with the disparity of the ascending aorta versus other aortic regions, Id3 mRNA and protein abundance was much greater in this region. In contrast to previous reports using rat thoracic SMCs, AngII had no observed effects in upregulation of Id3 protein levels in mouse SMCs. However, we demonstrate that AngII signaling can initiate Id3 localization to the nuclei compartment at 1 and 24 hours. Previous data demonstrated Id3 was upregulated and phosphorylated at these intervals in correlation with cell cycle progression. Furthermore, direct evidence of a role of Id3 in the hyperplastic response was derived from mice with genetic deficiencies of this protein. Although there is the potential for Id3 deficiency to lead to compensatory increases of other Id proteins, the total ablation of AngII-induced hyperplasia of the ascending aorta provided a striking demonstration of the specific requirement for Id3. Moreover, effects of Id3 deficiency on AngII-induced proliferative responses were observed despite a marked increase in the blood pressure response to AngII, supporting pressure-independent effects of AngII on SMC proliferation.
In summary, this study demonstrated that AngII infusion promotes aortic medial expansion by disparate mechanisms in a region-specific manner. A major difference was the demonstration of the disparity of responses in the ascending aorta compared to other regions. Unique responses of this region in aortic lumenal expansion have also been demonstrated in mice harboring fibrillin-1 mutations that were infused with AngII. Although this study defined AngII-induced changes in SMCs, this effect could be directly on SMCs or indirectly from another cell type. For example, endothelial cells secrete a wide range of products that directly affect SMC function. Recent data implicate PDGF-DD secretion from endothelial cells as a moderator of SMC phenotypic modulation, particularly in areas of disturbed blood flow, such as the aortic arch. Thus, subsequent studies will determine the contribution of SMC and endothelial cells to the observed phenotypes by using Cre-Lox technology to promote cell-specific AT1a receptor deficiency.

3.1 Synopsis:

Background – Angiotensin II (AngII) promotes region-specific contraction in the abdominal aorta. The aim of this study was to definitively determine the receptor mediating this response and to further define this regional contractile response. Materials and Methods – C57BL/6 aortas were segmented into 4 regions (aortic arch, thoracic aorta, suprarenal aorta, and infrarenal aorta) and treated with physiological agonists: potassium chloride (KCl, 80mM), 5-hydroxytryptamine (5-HT, 1\mu M), and AngII (1\mu M). Equivalent contractile responses were demonstrated with KCl and 5-HT, while AngII only induced a contraction in the infrarenal abdominal aorta. Angiotensin receptor subtype 1A (AT1aR), angiotensin receptor subtype 1b (AT1bR), and angiotensin receptor subtype 2 (AT2R) mRNA expression was examined via real-time PCR. All subtypes were concentrated in the infrarenal abdominal aorta, with AT1bR demonstrating a 19 fold increase over the other two receptor subtypes. To verify the receptor responsible for infrarenal-specific contraction of AngII, AT1aR deficient mice (AT1aR-/-), AT1bR-/-, and AT2R-/- mice were examined. The absence of the AT1aR and AT2R did not change AngII induction of abdominal contraction. However, AT1bR-/- mice demonstrated complete ablation of infrarenal contraction to AngII.

Conclusions: – AngII induces infrarenal-specific abdominal aortic contractions mediated exclusively via the AT1bR.
3.2 Introduction:

Angiotensin II (AngII) is the main effector peptide of the renin angiotensin system, regulating blood pressure and fluid/electrolyte homeostasis. Several receptor subtypes exist for engaging AngII, most notably AT1 and AT2 receptors. In particular, the AT1 receptor is subdivided into two homologues in rodent models: AT1a receptor and AT1b receptor. These two receptors share 94% homology, however, have been shown to demonstrate different functional properties as well as differential expression throughout the vasculature.

Genetically manipulated models have been used to ascertain the role of Ang receptors in murine models. AT1a receptor deficient (AT1aR-/-) mouse studies show the preponderance of AngII-induced vascular pathology and blood pressure regulation is mediated through this receptor subtype. The AT1b receptor (AT1bR) performs a minimal role in these functions, however, roles for this receptor subtype have been indirectly shown for AngII-induced vascular smooth muscle cell contractile responsiveness. These responses are region-specific and located in the abdominal aortic region and small resistance vessels. The large aortic vessel displays embryologic heterogeneity complicating the specificity of this effect. Furthermore, the divergent responses mediated through these two receptor subtypes may contribute to these region-specific effects.

This study defines regional contractile responses along the large vessel of the aorta and ascertains the specific receptor subtypes responsible. In order to definitively link the AT1bR to this abdominal contractile response, we used aortas obtained from AT1bR-/- animals.
3.3 Materials and Methods:

Mice:

Male AT1aR-/- mice in a C57BL/6 (B6) background (B6.129P2-Agr1atm1Unc, stock no. 002682) and male C57BL/6 (stock no. 000664) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The AT1b receptor -/- (AT1bR-/-) mice were a generous gift from Dr. Thomas Coffmann (Duke University). The AT2 receptor -/- (AT2R-/-) mice were a generous gift from Dr. Inagami (Vanderbuilt University). All mice were 10 times backcrossed in a C57BL/6 background and were housed in a pathogen-free environment and fed a normal diet (Harlan Teklad catalog No. 2918) and water ad libitum. Genotyping for AT1aR-/-, AT1bR-/-, and AT2R-/- mice was performed as previously described.44,95,145

Solutions and Chemicals:

Two solutions were prepared for incubation with aortic sections. Krebs Henseleit physiologic solution was composed of: 123mM NaCl, 15.5mM NaHCO₃, 1.2mM MgSO₄·7H₂O, 1.25mM CaCl₂, 11.5mM Dextrose, and 4.7mM KCl. Krebs Henseleit KCl contraction solution was similar to the physiologic solution, listed above, except NaCl was excluded, and KCl was increased to 80mM. All solutions were contained in heated reservoirs (37°C) and were aerated with oxygen. The addition of CaCl₂ was performed after 20 minutes of incubation at 37°C, with aeration, as this chemical was difficult to dissolve. AngII (Sigma and Baxter) and 5-hydroxytryptamine (5-HT, Sigma) was dissolved in sterile deionized water (1mM) and 5μl was added to 50ml incubation baths of physiologic solution for a final concentration of 1μM. All chemicals were purchased from Fisher Scientific or Sigma and were of the highest grade commercially available.46,174
**Tissue Preparation:**

Mice were anesthetized with ketamine/xylene and exanguinated via ligation of the right atria. Aortas were perfused, via the left ventricle, with phosphate-buffered saline, removed, and then adventitia was carefully dissected free. Measurement of contractile activity was performed using aortic rings as described previously. Aortas were stripped free of endothelial cells by passing a pair of serrated forceps over the aortic lumen. Aortic arch (3 mm), upper thoracic aorta (4 mm), middle thoracic aorta (4 mm), lower thoracic aorta (4 mm), suprarenal abdominal aorta (4 mm), and infrarenal abdominal aorta (4 mm) segments were mounted by passing two tungsten wires through the arterial lumen and immersed in Krebs Henseleit solution. Tension (1 gm) was maintained continuously and recorded on a Micro-Med instrument. After 30 min for equilibration, tissues were incubated with KCl (80 mM, Sigma), 5-HT (1 μM, Sigma), and AngII (1 μM, Sigma) with 30 minutes of recovery between each agonist. Studies with the AT1aR-/- (n=10) and AT2R-/- (n=8) mice were conducted in tandem with B6 controls allowing for no deviation in regard to buffer or sample preparation. Studies with the AT1bR-/- (n=5) mice were conducted in tandem with littermate controls, bred in-house.

**Real Time Polymerase Chain Reaction:**

Ascending (aortic sinus to left subclavian), thoracic (left subclavian to last intercostal), suprarenal (last intercostal to right renal), and infrarenal (right renal to iliac bifurcation) sections of aortas were harvested from 30 C57BL/6 and placed in RNA Later (Ambion, cat# 7024), and adventitia was removed. Aortas were combined, in groups of 3, for a total of 10 independent samples for each section. Aortas were ground with a glass mortar and pestle in the presence of lysis buffer (Rneasy Fibrous Minikit Isolation System, Qiagen, cat# 74704). All samples were incubated with Turbo DNA-free (Ambion, cat# AM1907) to remove
all DNA contamination. RNA concentrations were quantified using a UV spectrophotometer (Beckman DU530). Reverse transcription of RNA was performed using i-script (Bio-Rad, cat# 170-8891) with a concentration of 100 ng total RNA on a i-cycler (Bio-Rad). Real-time analysis of the cDNA was performed using a Bio-Rad i-cycler and AT1aR taqman probe (Applied Biosystems, catalog# Mm00616371_m1), AT1bR taqman probe (Applied Biosystems, catalog # Mm01701115_m1), AT2R taqman probe (Applied Biosystems, catalog # Mm01341373_m1) and IQ supermix (Bio-Rad, cat# 170-8860). These results were compared to an 18s rRNA endogenous control (Applied Biosystems, cat# 4352930E). Results were extrapolated using the standard curve method with adrenal glands serving as the standards. Negative controls consisted of no RT controls.

**Statistics:**

All statistics were performed using SigmaStat (SPSS Inc.). All measurements are represented as the mean ± SEM. One Way ANOVA was performed on all measurements with a Holm-Sidak post hoc test.

### 3.4 Results:

**AngII incubation induced region-specific contractions in the infrarenal abdominal aorta**

Literature on the regional role of AngII aortic contractions is limited to the broadly described thoracic and abdominal segments. To further define the contribution of AngII in regional contractile responses, B6 aortas were segmented into four regions (arch, thorax, suprarenal, and infrarenal abdomen). Similar responses to KCl (80mM) and 5-HT were observed in all regions of the aorta (Figure 3.1A-E). However, AngII (1µM) treatment of aortic sections
resulted in a marked contraction in only the infrarenal aorta (Figure 3.1D-E). As previously noted, AngII-induced contractions were transient resulting in a tachyphylactic response in all aortic regions (Figure 3.1A-D).

**Angiotensin receptor expression is basally concentrated in the infrarenal aorta**

To provide a basis for AngII-induced contractions, which were limited to the infrarenal aorta, AT1a receptors, AT1b receptors, and AT2 receptors were analyzed by real-time PCR. Analysis of AT receptor subtypes demonstrated augmented mRNA expression in the abdominal (suprarenal and infrarenal) regions of the aorta (Figure 3.2A-C). AT1aR expression in the infrarenal aorta was increased 13 (arch), 34 (thoracic), and 2.5 (suprarenal) fold compared to the other regions of the aorta (Figure 3.2A). AT1bR infrarenal mRNA was upregulated 35, 9, and 3 fold (Figure 3.2B), while infrarenal AT2R expression was upregulated 6, 22, and 4 fold versus the other regions of the aortic vasculature (Figure 3.2C). The expression of AT1bR mRNA was increased 19 fold over AT1aR and AT2R in the infrarenal aorta.

**AT1bR is the sole mediator of AngII-induced regional contractions**

The above results suggest the AT1bR is highly concentrated in the infrarenal segment of the aorta versus other receptor subtypes. This, and other correlative data indirectly links AT1b receptors to AngII-induced contractility. To definitively determine whether this receptor subtype is responsible for AngII-mediated contraction, AT1aR-/-, AT1bR-/-, and AT2R-/- mice aortic regions were assessed. When extrapolated to 80mM KCl, AT1aR-/- mice were not significantly different from wild-type control animals when treated with agonists 5-HT or AngII (Figure 3). The infrarenal aortic segments in AT1aR+/+ (78 ± 13%) and -/- (74 ± 14%) mice produced a robust and similar contractile response. Similarly, the AT2R/-/- animals were unchanged from AT2R+/+ mice when treated
with these agonists, including the AngII-induced infrarenal contraction (+/+: 116 ± 28%; -/-: 105 ± 14%). However, while the response to 5-HT was unchanged between AT1bR genotypes, the AngII-induced infrarenal contraction was completely ablated in the AT1bR/- mice (0%) versus +/- animals (99 ± 18%, Figure 3.4).
Figure 3.1: Angiotensin II induced region-specific contractions in the infrarenal aorta. Representative recordings of maximal 80mM KCl contractions, and percent maximal contraction to KCl for 5-HT and AngII for (A) aortic arch, (B) thoracic aorta, (C) suprarenal abdominal aorta, and (D) infrarenal abdominal aorta. I-bars underneath graph represent point of agonist stimulation (left bar) and washing (right bar) of aortic tissues. (E) 5-HT (1μM) and AngII (1μM) values expressed as percentage of maximal contraction compared to 80mM KCL. Histobars are means of 14 mice and bars represent SEM. *Denotes P<.001 in AngII infrarenal aorta versus all other AngII aortic groups (one-way ANOVA with Holm Sidak post hoc).
Figure 3.2: AT receptors are regionally concentrated in the infrarenal abdomen. (A) AT1a, (B) AT1b, (C) AT2 receptor real-time mRNA expression levels in the four regions of the aorta. Values were extrapolated from an adrenal gland standard curve, where final values for specific receptor subtypes were extrapolated from an 18s rRNA internal control. Histobars represent the means of 10 samples and bars are SEM. *Denotes P<.008 in infrarenal aorta versus all other aortic groups (one-way ANOVA with Holm Sidak post hoc). **Denotes P<.001 in suprarenal AT1b expression versus aortic arch and thoracic aortic regions.
Figure 3.3: AngII-induced regional contractions are unchanged in AT1aR-/- aortas. 5-HT (1μM) and AngII (1μM) values expressed as percentage of maximal contraction compared to 80mM KCL within genotype controls. Histobars are means of 5 mice and bars represent SEM. *Denotes P<.001 in AngII infrarenal AT1aR+/+ and -/- aortas versus all other AngII aortic groups (one-way ANOVA with Holm Sidak post hoc). No difference existed between genotypes in the AngII-treated infrarenal aorta.
Figure 3.4: AngII-induced regional contractions are ablated by AT1bR deficiency. 5-HT (1μM) and AngII (1μM) values expressed as percentage of maximal contraction compared to 80mM KCL within genotype controls. Histobars are means of 5 mice and bars represent SEM. *Denotes P<.001 in AngII infrarenal AT1bR+/+ aortas versus all other AngII aortic groups (one-way ANOVA with Holm Sidak post hoc).
Figure 3.5: AngII-induced regional contractions are unchanged in AT2R deficiency. 5-HT (1μM) and AngII (1μM) values expressed as percentage of maximal contraction compared to 80mM KCL within genotype controls. Histobars are means of 5 mice and bars represent SEM. *Denotes P<.001 in AngII infrarenal AT2R+/+ and -/- aortas versus all other AngII aortic groups (one-way ANOVA with Holm Sidak post hoc). No difference existed between genotypes in the AngII-treated infrarenal aorta.
3.5 Discussion:

This study demonstrated that AngII promotes region-specific contractions limited to the infrarenal aortic vasculature. To examine the molecular basis of this effect, angiotensin receptors were quantified and found to be concentrated in the infrarenal aorta, with AT1bRs being the most abundant. AngII-induced infrarenal contractions were ablated only in the AT1bR-/- mouse defining this receptor subtype as the sole mediator of murine aortic contractions.

Studies have linked AngII-induction of contractions to the abdominal region of the aorta, however, an in-depth analysis of contractile function along the aorta has not yet been examined. Therefore, the aorta was divided into four regions: aortic arch, thoracic aorta, suprarenal aorta, and infrarenal aorta. Contraction to physiological agonists KCL and 5-HT were unchanged in all aortic segments, suggesting no generalized decrease in smooth muscle function between different regions. However, AngII incubation mediated only infrarenal-specific contractions, when extrapolated to KCL. In order to verify this effect, the aorta was divided into 7 aortic segments, consisting of: arch, upper thoracic, middle thoracic, lower thoracic, suprarenal, infrarenal, and suprailiac aortic vessels (unpublished). AngII-induced aortic contractions were limited to the infrarenal and suprailiac aortas, suggesting sole localization of contractions below the right renal vessel.

AngII abdominal contractile response has been indirectly linked to AT1bRs by using pharmacological inhibition of AT1 receptors with the antagonist losartan in AT1aR-/- animals. AT1aR-/- had a similar response to AT1aR+/+ animals demonstrating this subtype does not mediate AngII abdominal contraction, in accordance with previous literature. To directly link AT1bRs to AngII-induced abdominal contraction, AT1bR-/- animals were obtained and aortic function examined. Contraction to KCl and the agonist 5-HT were unchanged between aortic regions in AT1bR+/+ and -/- animals demonstrating no loss of smooth muscle cell function between genotypes. However, incubation of AT1bR-/-
infrarenal aortas with AngII resulted in total ablation of the contractile response elicited in the other genotypes, verifying the studies using pharmacologic inhibition. Interestingly, the AT1aR, AT1bR, and AT2R mRNA expression was most abundant in the infrarenal aorta, with AT1bR being the most highly expressed. Abundance of AT1aR in the infrarenal suggests presence of the receptor and combined with AT1aR/-/- results definitively demonstrates this receptor does not mediate AngII contractions. However, conflicting data exists for the AT2R in AngII aortic contractility.\textsuperscript{94,95}

Previous studies with aortic segments incubated with the AT2R antagonist PD123319 resulted in normal AngII abdominal contractions.\textsuperscript{46} However, two independent studies demonstrate an increased AngII induced contraction in AT2R/-/- animals.\textsuperscript{94,95} To confirm these results we obtained AT2R/-/- animals and incubated all four regions with AngII. We demonstrate no difference in AngII infrarenal contraction with the AT2R/-/- animals verifying the studies of Zhou et al. Akishita et al. used thoracic aortas for their studies and mice with variable genetic backgrounds.\textsuperscript{94} Furthermore, Tanaka et al also demonstrated increased contraction in AT2R/-/- thoracic aortas.\textsuperscript{95} These results are somewhat confounding since many groups demonstrate no reactivity to AngII in thoracic aortic segments, including the results in three different genotypes presented here.

Definitive roles of the AT1a and AT1b receptors remains incomplete and poorly understood in current literature. Numerous studies demonstrate disparate roles for these two receptor subtypes, which share 94% amino acid sequence homology.\textsuperscript{172} Despite the similarities in these receptor subtypes, the differences are predominantly found in the final intracellular loop and cytoplasmic tail of this G-protein coupled receptor. A critical difference is found in amino acid 312 of the C-terminus which is present as an argenine in AT1b receptors and a tyrosine in AT1a receptors.\textsuperscript{52} This Tyr to Arg switch leads to a ineffective Gq coupling and activation site in AT1b receptors versus the AT1a subtype. This, and other, known substitutions may represent the differences in the functionality of the
receptor subtypes.

While this manuscript was being prepared, Swafford et al. reported similar ablation of AngII-induced contractions with AT1bR-/- animals.\textsuperscript{47} Unlike previous publications by Zhou et al., this study was forthcoming with aortic ring locations being near the iliac branch of the abdominal aorta.\textsuperscript{47} On the contrary, our studies focus on the region-specificity of this effect being localized to the entire region of the abdominal aorta, below the renal arteries, thereby reporting a novel regional effect.

Although this study defined a specific role for the AT1bR in AngII-induced contraction, the mechanism and signaling underlying this response is undefined. Functional evidence suggests AT1a receptors mediate effects through direct action on smooth muscle cells (postjunctional), while AT1b receptors mediate effects through indirect facilitation of norepinephrine on smooth muscle.\textsuperscript{175} Other studies provide a possible cross-talk pathway between alpha and beta adrenergic receptors, which mediate norepinephrine signaling, and AT1 receptors.\textsuperscript{176} Thus, subsequent studies will examine the role of alpha/beta adrenergic receptors and their ability to cross-link with AT1b receptors in AngII-induced murine aortic contractions.

In summary, this study demonstrated that AngII incubation of aortic sections lead to a robust and regional contraction localized solely to the infrarenal vasculature. This response was directly propagated from smooth muscle cells and did not involve endothelial cells. This study provides direct evidence that AT1bRs mediate this contraction through disparate mechanisms to the homologue, AT1a.
Chapter Four: Involvement of Innate Immunity in Angiotensin II-induced Vascular Disease

Synopsis

Angiotensin II (AngII) infusion into hyperlipidemic animals results in the formation of atherosclerosis and abdominal aortic aneurysm (AAA). As these two vascular disease processes initiate and progress, they accumulate a variety of inflammatory cell infiltrates mainly representative of the innate immune system. Ablation of macrophages, mast cells, neutrophils, and natural killer cells has been examined in one or both of these models and results consistently demonstrate attenuation of disease progression. This is a comprehensive analysis of these ablation studies.

Introduction

Atherosclerosis and AAAs are both characterized as chronic inflammatory diseases resulting from the accumulation of immune cells, most notably macrophages, at the site of injury. The identification and subsequent characterization of these immune cells has led to the overall hypothesis of immune modulation of atherosclerosis, to which the same conclusions can applied to AAAs. Experiments using genetically altered animals and manipulations of cultured cells have confirmed this process of immune modulation of disease. To date, immune responses have been described in every stage of atherosclerosis and AAAs, in both mice and men. This review will attempt to summarize AngII-induced vascular disease (focused on atherosclerosis and AAA) processes and the subsequent involvement of innate immunity in the initiation of pathology.

Angiotensin II Vascular Disease (Special focus on atherosclerosis and
Atherosclerosis

Atherosclerosis is the leading cause of death in the western world. This chronic disease is characterized by lesion formation, composed of lipids, platelets, smooth muscle cells, macrophages (foam cells), T and B lymphocytes, and matrix material that accumulates slowly over time. This cellular accumulation leads to a narrowing of the blood vessels causing restricted blood flow, which can lead to thrombus, ischemic stroke, or coronary occlusion.

Angiotensin II (AngII) is the primary bioactive peptide of the renin-angiotensin system (RAS) and plays physiologic roles in regulation of blood pressure and fluid balance. AngII activates AT1 receptors (AT1a in mice) to induce most of its physiological effects. Infusion of AngII into hyperlipidemic mice has recently been shown to augment atherosclerotic pathology. This AngII-induced atherosclerosis is rapid and consists mainly of lipid-laden macrophages and lymphocyte accumulation, similar to the diet-induced pathology. A possible complication of this effect is the noted increase in systolic blood pressure induced by AngII. However, concomitant studies with norepinephrine and subpressor doses of AngII, have demonstrated these effects of AngII are not pressor dependent.

Abdominal aortic aneurysm (AAA)

AAA is a devastating and silent pathology that effects 5-10% of the male population over the age of 65 and is listed as the 13th leading cause of death in the United States. AAA, defined as a permanent localized dilation in the arterial wall with a diameter greater than 50% of normal, is an inflammatory disease that can result in the dissection of the aortic wall and eventual rupture. Surgical intervention at late stages of expansion is currently the only utilized treatment, as no pharmacological therapy is currently available.
It has recently been demonstrated that infusion of AngII into hyperlipidemic mice induces the formation of AAAs localized to the suprarenal portion of the aorta. In comparison to human AAA data, the AngII model is similar in elastin fiber destruction, intimal stability, proteolytic destruction of medial connective tissues, inflammation, and increased incidence in the male gender.\textsuperscript{148,177,190,191} An interesting difference is in the location of the aneurysm, which is suprarenal in mice, but infrarenal in humans. However, murine AAA formation is also localized to the suprarenal aorta, independent of AngII infusion, in genetic ablation studies with LRP and iNOS, and in long term hyperlipidemic animals.\textsuperscript{188,192-195} Therefore, this suggests that mouse AAA localization to the suprarenal aorta may be due to hemodynamic factors in relation to the quadruped nature of mice versus the biped nature of humans.\textsuperscript{188} This effect, however, has been demonstrated to be independent of blood pressure using subpressor doses of AngII and hydralazine-induced pressure ablation studies.\textsuperscript{185,190,196} AngII-induced AAA formation is remarkably consistent and has been reproduced by many laboratories.\textsuperscript{183,185,196,197}

The progression of AngII-induced AAA formation has been described in detail by examination of tissue sections at predetermined intervals.\textsuperscript{177} The first observable event is the accumulation of macrophages into the tunica media of the suprarenal aorta occurring between 1 to 3 days after infusion. This accumulation is speculated to give rise to inflammatory cytokines and matrix degrading enzymes, as medial elastin destruction and Lumenal expansion soon ensues. The subsequent break in medial elastin appears to be contained by a rapidly thickened adventitial layer surrounding the vessel. This containment forms the rapid development of an intramural thrombotic region, which becomes fibrous and accumulates macrophages and other inflammatory cell types over time. The aneurysm then gradually expands over time, and eventually leads to regions of thinning and changes in histology.\textsuperscript{198}

\textbf{Comparing and contrasting atherosclerosis and AAA}
Atherosclerosis and AAA share similar beginnings in regards to arterial expansion. For instance, both disease states initially expand the vessel in a process referred to as outward arterial remodeling. In atherosclerotic disease, this outward expansion is a compensatory response for increased accumulation of macrophages, lipids, and other inflammatory cells (plaques) in the lumen. Eventually, this outward remodeling achieves an as yet unidentified limit, at which point the vessel expansion turns inward leading to progressive occlusion of the vessel. In contrast, aneurysmal expansion of the vessel does not turn inward, but instead continues expanding outward in what is usually deemed an uncontrolled or exaggerated manner. Differences are also observed at the terminal points of these disease pathologies. While AAA leads to progressive outward remodeling and eventual erosion and rupture to the outside of the vessel, atherosclerotic disease leads to inward occlusion of vessels via plaque ruptures and subsequent thrombolytic responses. These effects are not only disease dependent, but also appear to be location dependent. For instance, the formation of AAAs are most common in the aortic and cerebral vessels, but very rarely develop in the carotid, coronary, or femoral arteries, which are the most common sites for atherosclerosis development.

A consistent similarity in these disease processes is macrophage accumulation in the development of both atherosclerotic and AAA pathology. However, the mechanism of recruitment differs between these two diseases, as is shown with intimal accumulation of macrophages in atherosclerotic disease, and medial layer accumulation of macrophages in AAAs prior to dissection and accumulation in adventitial layers. This difference may be associated with the response to injury hypothesis in atherosclerotic prone areas, leading to lipid accumulation, and subsequent infiltration of macrophages to engulf said lipid, while AAAs are not initiated in this manner. However, the exact reason for this difference is not known.

Numerous animal studies have also demonstrated differences in these two AngII-induced vascular diseases. For example, administration of the broad
spectrum matrix metalloproteinase (MMP) inhibitor doxycycline significantly reduces the formation of AAAs, but demonstrates no effect on the formation of atherosclerosis.\textsuperscript{208} Another pivotal experiment involving AT1a receptor deficiency on bone marrow-derived cells demonstrated a modest reduction in AngII-induced atherosclerosis, but no effect on AAA formation.\textsuperscript{41} Further experiments also demonstrated orchidectomizing male hyperlipidemic mice led to profound reductions in AngII-induced AAA and resultant increases in atherosclerosis versus sham controls.\textsuperscript{148} These studies provide an experimental basis for separation of atherosclerosis and aneurysm disease phenotypes. However, one consistent theme demonstrated repeatedly is the involvement and necessity of innate immune processes for both initiation and progression of atherosclerotic and AAA disease processes.

**Overview of Innate Immune Responses**

**General innate immune response**

Innate immune cells represent the first line of defense against an invading microorganism or circulating antigen. The main effectors of this arm of immunity are of the phagocytic lineage, including monocyte/macrophages, dendritic cells, and neutrophils. However, granular and leukocytic natural killer cells (NK cells), mast cells, basophils, and eosinophils are also innate effectors. Pathogens contain antigens not normally found in host tissues. The innate immune cells recognize these antigens via pathogen-associated molecular patterns (PAMPs), which subsequently lead to inflammatory responses, unlike adaptive immune B and T lymphocytes, which are usually specific for a single antigen. Pattern recognition receptors (PRRs) then recognize these PAMPs, are subsequently activated, and rapidly signal through NF-κB and Map kinase pathways, leading to up regulation of gene products.\textsuperscript{209-211} These products elicit paracrine and autocrine cytokine and chemokine signals leading to mobilization of the other
members of the innate immune system in a matter of minutes to hours.\textsuperscript{209-212}

The majority of the PRRs relevant to vascular pathology are the scavenger receptors (SR) and Toll-like receptors (TLRs). The most commonly recognized PAMP is lipopolysaccharide (LPS), which is recognized by SR-A and TLR4.\textsuperscript{209-211,213} Subsequent recognition of ligands leads to ligation of these receptors. However, it is important to note that SR ligation leads to endocytosis and eventual lysosomal degradation of the recognized antigen, while TLR recognition leads to signaling via transmembrane pathways. Therefore, the remainder of this section will examine the TLR pathway, which, when activated, demonstrates augmented vascular disease processes.

\textbf{Pattern Recognition Receptors (PRRs)}

The most commonly associated PRRs with vascular disease and inflammatory processes are the toll-like receptors (TLRs). The TLRs are classified as Type I transmembrane proteins containing unique extracellular leucine rich repeats (LRR) as well as a conserved intracellular Toll-interleukin-1 receptor (TIR) domain.\textsuperscript{214,215} The LRR domains are highly conserved, however, they can recognize unique ligands that share no structural similarity.\textsuperscript{212,216} The conserved TIR domain serves as a scaffold for signaling and is present in all TLRs.\textsuperscript{217} To date, there have been 11 members of the toll family discovered in mammals, including the interleukin 1 (IL-1) and IL-18 cytokine receptors, which share the conserved TIR domain.\textsuperscript{217,218} Signaling for the TIR based TLRs is via the MyD88 pathway, resulting in NF-κB, map kinase (MAPK), and AP-1 activation, resulting in increased inflammatory responsiveness.\textsuperscript{219} A MyD88-independent pathway exists for TLR3 and 4, in which signaling is through interferon responsive elements, leading to the activation of type I interferons α and β.\textsuperscript{220}

The MyD88 signaling subunit and its receptor complexes are expressed in a variety of cell types including: SMCs,\textsuperscript{221,222} endothelial cells,\textsuperscript{223,224} fibroblasts,\textsuperscript{225},
B cells, dendritic cells, and monocyte-derived cells mainly consisting of macrophages, and, to a lesser extend, mast cells and neutrophils. Activation of TLRs, via endogenous or exogenous ligands, can enhance the production of matrix metalloproteinase-2 (MMP-2) and MMP-9, monocyte chemoattractive protein-1 (MCP-1), interleukin 1 (IL-1), IL-6, IL-12, and interferons (α, β, and γ), all of which have been linked to both atherosclerosis and aneurysm pathologies.

**TLRs in atherosclerosis and aneurysm**

Atherosclerosis is attenuated in IL-1β, TLR2, TLR4, and IL-18 deficient mice on a hyperlipidemic background. All of these receptor types are dependent on the MyD88 pathway for function, however, TLR4 also has a MyD88 independent pathway. Recent studies have also demonstrated that MyD88 deficiency, in apoE-/- mice, significantly reduced atherosclerotic lesion formation in both the aortic sinus, and the intimal surface of the aorta. Furthermore, there was a concomitant reduction in inflammatory cytokines (IL-12p40 and IL-1β), chemoattractive cytokines (MCP-1 and MIPs), and lipid accumulation in MyD88 deficient mice. These two independent studies also demonstrate a 75-80% reduction in the amount of macrophages found in the atherosclerotic lesions in the aortic sinus. Furthermore, the elicitation of mouse peritoneal macrophages (MPMs), in MyD88 deficient mice, is reduced 45% when compared to wild-type. These results show MyD88/-/- mice secrete less cytokines and chemokines, and have a possible defect in macrophage function. Furthermore, these results suggest that the deficiency of innate immune pathways in macrophages function can attenuate atherosclerotic disease, similar to the osteopetrotic (op) mouse studies, which are monocyte-depleted. A paucity of data exists with regard to the toll system in the pathogenesis of AAA. The only existing report linking TLRs and AAAs is a gene array of human aneurysm tissues showing up regulation of TLR1, TLR2, TLR6,
TLR7, TLR8, MyD88, and CD14 mRNA levels compared to control tissues. This study also highlights an enormous role for immunity in the development of AAA. Recently, the TLR pathway in the progression of AAA formation has recently been described by our laboratory, showing that MyD88 and TLR4 deficiency significantly attenuate the formation of this disease (Chapter 5).

Translational studies, involving TLR4, also exist in the medical literature. TLR4 is highly expressed in atherosclerotic lesions, colocalized to macrophages. Furthermore, twelve polymorphisms have been discovered in the human TLR4 receptor. Two common missense mutations are D299G and T399I. The T399I is associated with only a mild disruption of signaling. The D299G mutation is the most biologically relevant and has been linked to lipopolysaccharide hyporesponsiveness, causing an ablation of TLR4 signaling and cytokine production. The Bruneck clinical study suggests the D299G polymorphism is associated with a decreased: intimal-medial carotid thickness, carotid atherosclerosis, and inflammatory plasma markers IL-6 and C-reactive protein. These findings were further confirmed by Ameziane et al., which demonstrated patients with D299G polymorphisms had significant decreases in fibrinogen and vascular adhesion molecule 1, which are both linked to atherosclerosis. However, many conflicting studies have also been reported, suggesting no changes in atherosclerosis progression over time. These data suggest more analysis of the TLR pathway needs to be performed before a definitive statement can be made on their role in human disease.

**Associations of Innate Immunity in AngII-induced Vascular Disease**

Macrophages are the most abundant inflammatory cells accumulating in the subendothelial space in experimental atherosclerotic lesions. Op mice, that lack macrophage colony stimulating factor (M-CSF), are monocyte-depleted and have markedly reduced atherosclerotic lesions in either apoE-/- or LDL receptor-/- backgrounds, with long-term fat feeding, suggesting a crucial role in the
development of disease pathology.\textsuperscript{239,240} Recent studies have also demonstrated AngII-induced atherosclerosis is also reduced, similar to the previously described studies, in an apoE-/- background.\textsuperscript{174} However, the apoE-/- littermates, that were wild type for M-CSF, failed to develop a significant number of AAAs. This result was confounded by the background strain of the mice, which is resistant to AAA formation. Also, op mice have many defects leading to difficulties in generating these mice and may also engender interpretive difficulties. The contribution of macrophages in the formation of AAA remains to be elucidated.

Recently, our laboratory has attempted to answer this question of macrophage involvement in AAA pathology using the MyD88 deficient mouse model, which has been attributed to demonstrate macrophage dysfunction.\textsuperscript{146} Our results demonstrate the deficiency of MyD88 has a profound reduction in both atherosclerosis and AAAs (Figures 5.4 and 5.5). This effect was attributed to signaling through the innate immune receptor, TLR4 (Figure 5.7). Furthermore, up regulation of macrophages via high fat diet and AngII infusion is significantly attenuated in these studies (Figure 5.8). We also demonstrate this effect is due to bone marrow-derived cells expressing the MyD88 protein (Figure 5.9). These results indicate that the innate immune effector MyD88, as well as the involvement of macrophages, is critical toward the formation of AngII-induced atherosclerosis and AAA pathology.

Recent reports have also elucidated a role for mast cells in the pathogenesis of atherosclerosis and AAA.\textsuperscript{248,249} Mast cells represent another innate immune mediator, that induce a proinflammatory profile usually associated with immediate hypersensitivity and chronic allergenic reactions. These studies show mast cell-deficient KitW-sh/kitW-sh mice are important participants in the pathogenesis of both atherosclerosis and AAA. Furthermore, they demonstrate mast cell-specific upregulations of IL-6 and IFN-\textgamma cytokines are responsible for this effect using bone marrow-derived mast cell populations from mice deficient in the aforementioned cytokines.\textsuperscript{248,249} These studies were not conducted using the AngII model of vascular disease, but instead fat fed-induced atherosclerosis
and the calcium chloride and elastase model of AAA formation. While similarities exist in diet-induced and AngII-induced atherosclerosis; the elastase and calcium chloride models are not similar in that they mainly result in adventitial thickening and not true aneurysm formation, in regard to lumenal expansion (as shown in figure 2B Sun et al.). Nonetheless, these studies demonstrate a mechanism for mast cell deficiency to decrease vascular disease.

The neutrophil, which is the earliest innate-mediator cell to arrive at sites of inflammation and injury, has been demonstrated to mediate the formation of AAA in the elastase model. Studies using elastase-induced AAAs in rats demonstrated L-selectin up regulation and the association of said up regulation with macrophage and neutrophils. These studies were confirmed in L-selectin ablated mice, resulting in less macrophage and neutrophil accumulation and significantly attenuated AAA formation. This L-selectin ablation study was also confirmed with neutropenia induced using anti-PMN antibody injections resulting in significant attenuation of AAA size and incidence. However, the L-selectin and neutropenia studies also have a concomitant down regulation in macrophages, and therefore it is not clear whether the actions are being mediated by neutrophils or macrophages. This will require subsequent investigations to separate out the two cell types. While neutrophils are most certainly present in atherosclerotic disease, studies involving neutrophil depletion have yet been examined. Interestingly, a study involving the deficiency of myeloperoxidase (MPO) demonstrated an increase in atherosclerotic lesions in fat-fed animals. MPO is a heme enzyme produced and secreted by activated phagocytic cells, which upregulates a host of oxidative processes. More importantly, MPO makes up 5% of neutrophil protein and is considered the main weapon in the neutrophil's arsenal. Therefore, this study begs the question whether the neutrophil is a friend or a foe in atherosclerosis.

NK cells also perform a substantial role in the pathogenesis of vascular disease. Transgenic mice with overexpression of the inhibitory major histocompatibility complex (MHC) class I-specific receptor, Ly49A, prevent the
activation of NK cells, and thus these mice have the absence of functional NK cells.\textsuperscript{257} Recent bone marrow transplantation studies with these Ly49A mice, in an AngII-infusion model of atherosclerosis, demonstrated a 70\% reduction in the size of aortic sinus lesions and a 38\% reduction in en face analysis of the aortic arch.\textsuperscript{258} However, these results are complicated by conflicting studies using lyst-beige mutant mice, which also demonstrate decreased NK cell function.\textsuperscript{132,133,140} These beige mice demonstrate either no change in mice fed a atherogenic ‘paigen’ diet,\textsuperscript{156} or minimally, but significantly, upregulated atherosclerosis in an LDL receptor-/- model.\textsuperscript{259} The analysis in this lyst-beige model are complicated by phenotypic abnormalities, such as defects in macrophages and T/B lymphocytes, and further, do not have a complete defect in NK cells.\textsuperscript{132,133,140} This, therefore, questions the validity of the lyst-beige studies in regard to specificity to NK cells, when compared to specific knock down of NK cells, as is shown in the Ly46 studies. A more recent study crossed the beige mutant mice into an apoE-/- background and found a reduction in chow-fed atherosclerosis and an increase in diet-induced atherosclerosis dependent on both resident and marrow cells.\textsuperscript{160} These results demonstrate a complex phenotype in these beige mutants. Currently, the role of NK cells in AngII-induced AAAs has not yet been elucidated.

**Summary**

AngII administration to hyperlipidemic mice results in the reproduceable vascular pathology of atherosclerosis and AAAs. These vascular disease processes differ in their progression, however, are similar in initiation involving key mediators of the innate immune system. To date, studies have ascertained a role for macrophages, mast cells, and NK cells in atherosclerosis, while macrophages, mast cells, and neutrophils have been examined in AAAs. However, it is not currently understood how depletion of one cell type leads to reductions/enhancements in pathology that is not compensated by another cell
type. This is an ongoing quandry of these cumulative examinations of genetic ablation studies. To conclude, innate immunity demonstrates a critical role in initiation of atherosclerosis and AAA pathogenesis.
Chapter Five: Angiotensin II and Region-specific Vascular Disease: Toll-like Receptor 4 Attenuates Angiotensin II-induced Atherosclerosis and Suprarenal Abdominal Aortic Aneurysms via a Myeloid Differentiation Factor 88-dependent Mechanism.

5.1 Synopsis:

Angiotensin II (AngII) infusion augments atherosclerosis and induces abdominal aortic aneurysms (AAAs), which are both characterized by macrophage infiltration. This study ascertained the effects of MyD88 deficiency on AngII-induced vascular pathologies and the toll receptor (TLR) responsible for this effect. AngII incubation of primary cultures upregulated TLR2, TLR4, CD14, and MyD88 mRNAs. Further, AngII-induced atherosclerotic lesions and AAAs stain positive for TLR4 and MyD88. Male mice with or without deficiency in MyD88, TLR2, and TLR4, on a low density lipoprotein receptor (LDLr) deficient background, were fed a fat-enriched diet and infused with AngII (1,000ng/kg/min) for 28 days. Deficiency of MyD88, TLR2, or TLR4 attenuated AngII-induced augmentation of atherosclerosis. However, only MyD88 and TLR4 deficiency attenuated diameter of the suprarenal abdominal aorta and AAA formation. To determine the cell type responsible for this effect, bone marrow transplantation studies were performed. LDLr-/- male mice were irradiated and repopulated with bone marrow from MyD88 (+/+ vs. -/-) or TLR4 (+/+ vs. -/-) mice, respectively. Repopulation of MyD88+/+ animals with deficient bone marrow-derived cells similarly reduced AngII-induced vascular pathology to whole body ablation. However, TLR4+/+ repopulation with -/- bone marrow-derived cells did not show a change in AngII-induced vascular disease. The present study defines an important link between AngII-induced vascular pathology and innate immunity and suggests alternate mechanisms for initiation and progression of disease.
5.2 Introduction:

Angiotensin II (AngII) infusion into hyperlipidemic mice enhances atherosclerosis and induces abdominal aortic aneurysms (AAAs).\textsuperscript{139,181} Although AngII promotes both vascular diseases, their pathologies imply they are generated via different mechanisms. Pharmacological, surgical, and genetic manipulations can have differential effects on the severity of these diseases. A major difference is the intimal macrophage accumulation throughout the development of AngII-induced atherosclerosis, while macrophage accumulation in the media of the aorta and progression to the adventitia is the characteristic of the initial to the late stages, respectively, of AngII-induced AAA development.\textsuperscript{139,177}

Macrophages are the most abundant cells in experimental atherosclerotic lesions. The functional role of this cell type in the development of lesions has been demonstrated in monocyte-depleted mice. Osteopetrotic (op) mice, that lack macrophage colony stimulating factor (M-CSF), have reduced circulating monocytes and markedly reduced atherosclerotic lesions on either apolipoprotein E deficient (apoE-/-) or LDLr-/- backgrounds.\textsuperscript{239,240} Macrophages are present throughout the initiation and maturation stages of AngII-induced AAA formation, although their functional role has not been defined in this disease.\textsuperscript{177} Previously published results used apoE-/- op mice to study the contribution of macrophages to AAA formation.\textsuperscript{174} However, the apoE-/- littermates, that were op+/+ for M-CSF, failed to develop a significant number of AAAs. This may have been attributable to the mixed strain background of the op mice, which differs from the C57BL/6 background used for all other studies. Thus, the contribution of macrophages to AAA formation has not been determined using mice with macrophage depletion.

Recently, a considerable attention has been drawn in myeloid differentiation factor 88 (MyD88) as a major regulator in the innate immune signaling pathways. This mediator protein is the signaling component for the toll-
like receptors (TLRs).\textsuperscript{211,212} Numerous studies have demonstrated that MyD88 deficiency, as well as TLR1, TLR2, TLR4, and IL-18 deficiency, decreases the size of atherosclerotic lesions.\textsuperscript{146,147,235-238} MyD88 deficiency produces multiple defects in macrophage functions.\textsuperscript{146,237} However, MyD88 is expressed in all cells involved in vascular pathology and the contribution of macrophage specific deficiency to lesion formation has not been determined.

The aim of the present study was to determine whether MyD88 deficiency in a hyperlipidemic background provides a model for determining the role of functionally deficient macrophages on AngII-induced atherosclerosis and AAA formation. MyD88 deficiency on either an apoE-/- or LDLr-/- background resulted in a profound reduction in AngII-induced atherosclerosis and AAA formation. Further, this effect was dependent upon bone marrow-derived cells. TLR2 and TLR4 deficiency were examined as receptor mediators of this MyD88 effect. Both receptors significantly attenuated atherosclerosis formation, however, only TLR4 deficiency resulted in ablation of AAA formation.

5.3 Materials and Methods:

Experimental animals and diet

Male apoE-/- mice (originating from the Jackson Laboratory) that were MyD88+/+ (n=42) and +/- (n=28) were a kind gift of Mason Freeman and Kathryn Moore (Harvard U).\textsuperscript{146} These mice were 8x backcrossed on a C57BL/6 background and additionally crossed into apoE-/- background (10x) for a total of 9x backcrossing into C57 background. Additionally, the MyD88 mice were bred out of an apoE-/- background and into a LDLr-/- (+/-: 19; +/-: 23) background (originating from the Jackson Laboratory) resulting in a full 10x backcrossing into the C57BL/6. Additionally, TLR2+/+ (n=9), TLR2-/- (n=11), TLR4+/+ (n=14) and TLR4-/- (n=20) mice in an LDLr-/- background and backcrossed 10x C57BL/6 were a kind gift of Linda Curtiss (Scripps Inst.), Peter Tobias (Scripps Inst.), and
These mice were all bred as littermate controls, and were housed in a pathogen-free barrier facility. All MyD88 mice were given sterile autoclaved antibiotic water (Sulfatrim 0.2%) ad libitum, while the TLR2 and TLR4 mice were given normal reverse osmosis water ad libitum. All apoE-/- mice were fed a normal mouse laboratory diet throughout experimentation. LDLr-/- animals were fed a normal laboratory diet, until induction of hypercholesterolemia, when the diet was supplemented with saturated fat (milk fat 21% wt/wt) and cholesterol (0.15% wt/wt, diet number TD88137; Harlan Teklad). All studies were performed with the written approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

Osmotic minipump implantation

At 8 to 12 weeks of age, mice were implanted with Alzet osmotic minipumps (model 2004, Durect Corporation), subcutaneously into the right flank, and infused with AnII (1,000 ng/kg/min, Bachem) for a period of 28 days, as described previously.177

Genotyping by polymerase chain reaction

MyD88 genotyping used the following primers: MyD88 forward 5′–TGGCATGCCTCCATCATAGTTAACC–3′, MyD88 reverse 5′–GTCAGAAACAACCACCACCATGC–3′, and MyD88 neo 5′–ATCGCCTTCTATCGCCTTCTTGACG–3′. All primers were combined into one solution and the resultant wild-type and deficient allele bands were 550 and 600 basepairs (bp), respectively. TLR4 genotyping used the following primers: HC-26 forward 5′–TGTTGCCCTTCAGTCACAGAGACTCTG–3′, HC-27 reverse 5′–CGTGTAAACCACCAGAGCCAGGTTTTGAAGGC–3′, and HC-28 neo 5′–ATCGCCTTCTATCGCCTTCTTGAGAG–3′. HC-26 and 27 were combined in one reaction for wild-type allele bands at 1300 bp, while HC-26 and 28 were
combined in another reaction for deficient allele bands at 1300 bp. TLR2 genotyping used the following primers: HC-23 forward 5′–TTGGATAAGTCTGATAGCCTTGCCTCC–3′, HC-24 reverse 5′–GTTTAGTGCCCTGCATCCAGTCAGTGCG–3′, and HC-25 neo 5′–ATCGCCTTTAGGCCTTTCTTGACGAG–3′. HC-23 and 24 were combined in one reaction for wild-type allele bands at 900 bp, while HC-23 and 25 were combined in another reaction for deficient allele bands at 900 bp. All mice were genotyped and this screen was verified by two people. All bone marrow experiments had femurs genotyped at time of euthanasia to verify successful repopulation of recipient cells.

**Aortic tissue and plasma collection**

Twenty-eight days after pump implantation, mice were sedated with a mixture of ketamine and xylazine. Blood was drawn into a 23 gauge needle containing 0.2% EDTA from the left ventricle of the heart and immediately centrifuged 2,000 rpm for 20 minutes to collect plasma. Mice were then euthanized via ligation of the right atrial septum. Aortas were perfused with sterile saline and subsequently extracted from the mouse and placed into 10% formalin overnight. These aortas were then carefully cleaned free of all extraneous adventitia and stored until photography and atherosclerosis analysis.

**Quantification of atherosclerosis**

Atherosclerosis was quantified using en-face analysis, or the cutting of the aortic root down to the aortic aspect, in order to visualize lesions on the intimal surface of the aorta from the aortic arch to the upper thorax (approximately 3mm posterior to aortic aspect). Thoracic en face atherosclerosis was quantitated in a similar manner (3mm posterior to aortic aspect to last intercostal artery). Lesions on the intimal surface and aortic arch and thoracic area were traced.
using Image-pro software. Atherosclerosis is represented as the sum of the lesion areas divided by the area of the vessel for each region.

**Abdominal aortic measurements**

Abdominal aortas were pinned on wax and multiple magnified photographs were taken of the arch, thoracic, and abdominal regions using a millimeter ruler as a calibrator. Maximum width of abdominal aortas were measured using computerized morphometry (Image-Pro Software, ). Suprarenal abdominal diameter was quantified using external diameters to classify incidence of AAA (defined as a dilation greater than 50% of the normal vessel diameter of 0.80mm).

**Ultrasound Imaging**

High resolution ultrasound was performed on all animal subjects prior to pump implantation and on day 28 of infusion. This measurement was used to confirm external abdominal aortic measurements were based on lumenal dilatation and not adventitial thickening. Two-dimensional imaging using real-time microvisualization was performed using a Visualsonics Vevo 770, as previously described.

**Irradiation and bone marrow repopulation**

Mice were provided antibiotic water (Sulfratrim 0.2%) one week prior to irradiation. Recipient LDLr-/- mice (TLR4+/+: n=23; MyD88+/+: n=22) were irradiated with two separate doses of 450 Rads from a cesium source, delivered 4 hours apart. Bone marrow-derived cells from MyD88+/+ or -/- x LDLr/-/- and TLR4+/+ or -/- x LDLr/-/- donor mice were obtained from the femurs and then subsequently injected into the tail vein of 7-8 week old irradiated recipient mice.
(1x10^7 cells per animal). These recipient mice were maintained on antibiotic water for the next four weeks, and were then returned to normal reverse osmosis water before beginning experimentation.41

White blood cell analysis

White cell counts in whole blood were immediately sampled, at time of collection, on a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific, Waterbury, CT).

Blood monocyte determination with flow cytometry

MyD88-/x LDLr-/-, TLR4-/x LDLr-/-, and LDLr-/- mice were either fed high fat diet for 1 month (n=5 each) or fed a fat enriched diet for 3 weeks with 2 weeks AngII infusion (n=5 each). Peripheral blood monocyte populations were determined via fluorescence-activated-cell sorter (FACS) using fluorescently labeled antibodies against CD115 (phycoerythrin, eBioscience catalog 12-1162-83), F4/80 (biotinylated, Serotec catalog MCA497B), or Gr-1 (PerCp-Cy5.5, BD Pharmingen catalog 552093). The biotinylated antibody was detected using secondary streptavidin-APC (eBioscience). Briefly, blood was collected, as previously described above, and 200μl of whole blood was placed into Szilvassey’s red blood cell (RBC) lysis solution. RBC lysis solution was quenched with BSA-rich Hank’s buffered saline solution (HBSS). FACS staining buffer was composed of 5ml 1x phosphate buffered saline, and 5ml HBSS, with the addition of 333μl 30% BSA and 100μl normal rabbit and normal mouse serum. Primary antibodies were incubated 1:200 for 30-40 minutes on ice, washed, and then placed in 1:400 secondary antibody. Double positive CD115 and F4/80 monocyte populations were classified as Ly-6C low, intermediate, and high, as described previously. Staining protocols were generously provided by Dr. Gwendalyn Randolph (Mt. Sinai, NY).260
Thioglycollate elicitation of peritoneal macrophages

MyD88-/- (n=10), TLR4-/- (n=5), and LDLr-/- (n=10) mice were fed a high fat western diet for one week. These mice were then injected with 1ml 3% thioglycollate media resuspended in sterile saline. After 4 days of incubation, mice were sedated and subsequently cleared of all blood. The abdominal region was cleaned with 75% ethanol, cleared of all fur, and peritoneal macrophages were extracted using 5ml sterile saline via an 18 gauge needle. Cells were incubated with RBC lysis solution and then centrifuged 1,000 rpm for 5 minutes. Cells were resuspended in 200µl of sterile saline, of which 20µl was used for white blood cell calculations, while 1µl was diluted 1:20 and counted on a hemacytometer. The remaining solution was stained with fluorescently labeled CD68-FITC (Serotec, MCA1957F) 1:10 and analyzed via FACS for percent macrophages in the peritoneum.

Measurement of plasma lipids

Plasma cholesterol concentrations were analyzed using a commercially available Cholesterol E enzymatic kit (Wako Chemical Company, Richmond, VA). Lipoprotein cholesterol distribution was performed by fast protein liquid chromatography (FPLC), which separates lipoprotein fractions by size exclusion, on a double superose 6 column. Collected fractions were assayed with the Cholesterol E enzymatic kit (Wako).

Blood pressure measurements

Systolic blood pressure (SBP) was measured noninvasively in tail arteries of conscious mice using the Coda 8 Kent blood pressure system (Kent Scientific Corp, Torrington, Connecticut). Mice were measured 5 days prior to pump implantation, and during the last 5 days of drug administration.
**Immunohistochemistry**

Frozen atherosclerotic and AAA sections were fixed in 4% paraformaldehyde for 10 minutes. Sections were stained for macrophages (Accurate Chemical), MyD88 (Abcam), and TLR4 (Santa Cruz). Tissue sections were blocked with nonimmune rabbit serum. Immunohistochemical analysis was conducted using a Microprobe system (Fisher Scientific) and Vectastain Elite ABC kit (Vector). Negative controls were no primary, no primary no secondary, and isotype-matched irrelevant antibodies. Immunoreactivity was visualized by using 3-amino 9-ethyl carbazole (Biomed Corp), which is visualized by a red precipitate. Tissue sections were then counterstained with aqueous hematoxylin (Biomed).177

**Real-time polymerase chain reaction**

Vascular smooth muscle cells and thioglycollate-elicited mouse peritoneal macrophages were harvested from C57BL/6 mice and plated. These cells were treated with saline (24 hours), AngII (1μM, bachel, 24 hours), lipopolysaccharide (1μg/ml, TLR4 agonist, invivogen, 3 hours), and Pam3CSK4 (1μg/ml, TLR2 agonist, invivogen, 3 hours). Samples were washed and then RNA was harvested using the Qiagen All Prep RNA/protein kit. All samples were incubated with Turbo DNA-free (Ambion, cat# AM1907) to remove all DNA contamination. RNA concentrations were quantified using a UV spectrophotometer (Beckman DU530). Reverse transcription of RNA was performed using i-script (Bio-Rad, cat# 170-8891) with a concentration of 100 ng total RNA. Real-time analysis of the cDNA was performed using a Bio-Rad i-cycler with TLR2 (Applied Biosystems (AB) catalog Mm01213946_g1), TLR4 (AB catalog Mm00445273_m1), MyD88 (AB catalog Mm00440339_g1), and CD14 (AB catalog Mm00438094_g1) taqman probes and IQ supermix (Bio-Rad, cat# 170-8860). These results were compared to an 18s rRNA endogenous control.
(Applied Biosystems, cat# 4352930E). Results were extrapolated using the delta Ct method. Negative controls consisted of no RT controls.

**Statistics**

All statistical analyses were performed using either SigmaStat (SPSS Inc.) or version 8.2 of SAS (SAS Institute, Cary, NC). All measurements are represented as the mean ± S.E.M. One Way ANOVA was performed on measurements, where indicated, with a Holm-Sidak post hoc test. A Student’s t-test was performed on the nuclei counts. Repeated measures data was analyzed with SAS fitting a linear mixed model expressing the temporal trend in systolic blood pressure as a quadratic polynomial in time for each treatment. A Bonferroni-adjusted pairwise comparison was performed within similar genotypes among treatments.

**5.4 Results:**

**AngII augments innate immune Toll pathway components *in vitro* and *in vivo***

Numerous studies have associated innate immunity with AngII-induced pathologies, however, a paucity of data exists connecting this pathology to the toll pathway. Therefore, we first examined whether AngII mediates components of the toll pathway *in vitro* with direct treatment of primary cell cultures or *in vivo* via vascular pathology models. Elicited peritoneal macrophages (Figure 5.1a) or VSMCs (Figure 5.1b) treatment with AngII significantly increased the mRNA expression levels of TLR2, TLR4, and the adaptor molecule CD14 similar to a TLR2 (Pam3CSK4) or TLR4 (LPS) agonist. MyD88 was upregulated in cultured macrophages, but unchanged in cultured VSMCs.

To provide a basis for these molecules in AngII-induced vascular
pathologies, immunostaining was performed on atherosclerotic lesions and AAA tissues. Both MyD88 and TLR4 were present in AngII-induced atherosclerotic lesions (Figure 5.2) and AAA tissues (Figure 5.3) colocalized with macrophages. Therefore, AngII can upregulate Toll pathway components, and these proteins are present in AngII-induced vascular pathologies.

**Deficiency of MyD88 prevents AngII-induced atherosclerosis and AAAs**

MyD88 deficiency abolishes signaling of all toll receptors, with the exception of the TLR4 and TLR3 MyD88-independent signaling pathways. To determine the contribution of MyD88 to AngII-induced atherosclerosis and AAA pathology, MyD88+/+ and -/- mice, on an apoE-/- background, were infused with AngII (1000 ng/kg/min) for 28 days. Since many pro-inflammatory effects are mediated by deficiency of apoE, MyD88+/+ and -/- mice were also examined on an LDLr-/- background and fed a high fat-enriched diet. Deficiency of MyD88, on either an apoE-/- or LDLr-/- background, had no effect on plasma cholesterols or SBP (pre and post infusion, Table 5.1). No difference was detected between MyD88 genotypes, on the apoE-/- background, with regard to body weight. However, after fed a fat-enriched diet for 5 weeks, MyD88-/- mice had a 13% increase in body weight (Table 5.1).

AngII-induced atherosclerosis was quantified on the intimal surface of the aortic arch and thoracic aorta. MyD88 deficiency ablated atherosclerosis in both aortic regions on the apoE-/- (arch: 82.3%; thorax: 79.1% decrease) and the LDLr-/- (arch: 64%; thoracic: 87% decrease) backgrounds (Figures 5.4a and 5.5a).

Infusion of AngII into MyD88+/+ mice led to a significant increase of suprarenal aortic diameters (apoE-/-: 1.5 ± 0.17mm; LDLr-/-: 2.0 ± 0.2mm; Figures 5.4b and 5.5b, P<0.001), while MyD88 deficiency attenuated abdominal aortic expansion (apoE-/-: 0.97 ± 0.08mm; LDLr-/-: 0.99 ± 0.04mm). Furthermore, MyD88 deficiency also significantly attenuated arch aneurysm and
area, AAAs, and rupture-induced death versus proficient animals (Table 5.2, P<.001).

**TLR2 and TLR4 attenuate AngII-induced atherosclerosis, while only TLR4 mediates AngII-induced AAAs**

To establish the toll receptor mediating the MyD88 effects, TLR2 receptor deficiency was examined. TLR2+/+ and -/- mice, on an LDLr-/- background, were fed a high fat-enriched diet for 5 weeks and infused with AngII (1000 ng/kg/min) for 28 days. No differences were detected in plasma cholesterol levels, body weight, or SBP (Table 5.1). Atherosclerotic lesions in both aortic arch (73% decrease) and thoracic aorta (87% decrease) were attenuated in TLR2-/- mice (Figure 6a, P<0.001). However, TLR2 deficiency did not alter AngII-induced increases in suprarenal aortic diameters (+/+: 2.0 ± 0.22mm; +/-: 1.8 ± 0.2mm, Figure 5.6b), aortic arch area or aneurysm (+/+: 44%; +/-: 36%), AAAs (+/+: 89%; +/-: 82%), or rupture-induced death (+/+: 22%; +/-: 36%, Table 2). This demonstrates MyD88 mediated aneurysmal formation is not through the TLR2-MyD88 axis.

It has been reported that TLR4 mediates vascular pathologies and signals through the MyD88 pathway. Therefore, we also examined TLR4+/+ and -/- mice, on an LDLr-/- background, in the development of atherosclerosis and AngII-induced aneurysm formation. TLR4 genotypes had similar body weights and plasma cholesterol levels. However, TLR4 deficiency significantly reduced both pre and post infusion SBP levels by approximately 12mmHg (P<0.005). Ablation of TLR4 significantly attenuated AngII-induced atherosclerosis in both the aortic arch (55%) and thoracic aorta (66%, Figure 5.7a, P<0.001). Deficiency of TLR4 also significantly decreased suprarenal aortic diameters (+/+: 2.23 ± 0.37mm; +/-: 1.03 ± 0.05mm, Figure 5.7b, P<0.001), AAAs (+/+: 64%; +/-: 25%) and abdominal aortic aneurysm (+/+: 100%; +/-: 25%, Table 5.2, P<.01). However, TLR4 deficiency had no effect on rupture-induced death or aortic arch...
area. These data are consistent with TLR4 being the major receptor for MyD88-induced effects on AngII-induced aneurysmal formation.

**MyD88 deficiency results in dysfunctional monocyte/macrophage populations.**

AngII infusion significantly increased white blood cells, neutrophils, and monocytes (Figure 5.8a, P<0.001). However, while MyD88+/+ and -/- resulted in similar levels of WBCs prior to AngII infusion, WBCs in deficient mice remained unchanged after 28 days of infusion.

Blood monocyte populations were examined in male MyD88-/- x LDLr-/-, TLR4-/- x LDLr-/-, and LDLr-/- mice. TLR4-/- and LDLr-/- mice had an increase of 447% and 390%, respectively, after 28 days of fat feeding, while deficiency of MyD88 only increased blood monocyte levels 82% (Figure 5.8b, P<.001). Moreover, MyD88 deficiency elicited 62% fewer CD68+ macrophages than LDLr-/- mice (Figure 5.8c, P<.001). This data is consistent with MyD88 deficiency having defective regulation of monocyte/macrophage populations.

The aforementioned monocytes were further categorized into 3 subpopulations of Ly-6C (Ly-6C<low>, Ly-6C<int>, and Ly-6C<hi>), which are associated with distinct chemokine receptor profiles. MyD88 deficiency significantly increased Ly-6C<low> and decreased Ly-6C<hi> monocyte populations with 4 weeks of high fat diet versus TLR4-/- and LDLr-/- mice (Figure 5.9a, P<.007) Further, AngII infusion significantly increased the inflammatory profile of monocytes in LDLr-/- mice, while MyD88 deficiency remained unchanged when compared to saline (Figure 5.9b). TLR4 deficiency augmented Ly-6C<hi> monocyte populations, independent of treatment.

**MyD88 donor genotype is the major determinant of vascular diseases in chimeric mice infused with AngII, independent of TLR4 signaling**
Upon the demonstration of a critical role for MyD88 and TLR4 in the development of AngII-induced vascular pathologies and the general impairment of monocytes in peripheral blood of MyD88-/- mice, we used bone marrow transplantation to define the involvement of hematopoietic cells. First, male MyD88+/+ x LDLr-/- mice were irradiated and subsequently repopulated with bone marrow-derived cells from MyD88+/+ or -/- animals. Genotype was verified from femur-derived bone marrow at time of sacrifice (unpublished).

Recipients of MyD88+/+ or -/- bone marrow had no change in body weights, plasma cholesterols, blood pressures, or WBCs (Table 3). Intimal atherosclerotic lesions were significantly attenuated in both aortic arch (35%) and thoracic aorta (77%, Figure 5.10a, P<.006) in MyD88+/+ mice repopulated with MyD88-/- donor cells, compared to those repopulated with MyD88+/+ bone marrow-derived cells. Similar to the results of whole body deletion, deficiency of MyD88 in bone marrow-derived cells significantly attenuated suprarenal aortic width (repopulated with +/+ donor cells: 1.51 ± 0.12 mm; with -/- donor cells: 1.02 ± 0.07mm, Figure 5.10b, P<0.001). Infusion of AngII into MyD88+/+ mice repopulated with MyD88+/+ bone marrow-derived cells resulted in a 68% incidence of AAA versus only 9% (P<0.001) in those repopulated with MyD88-/- bone marrow-derived cells. Furthermore, arch area (+/+ repopulation: 22.3 ± 0.81 mm; -/- repopulation: 18.9 ± 0.46 mm) and arch aneurysm (+/+ repopulation: 59%; -/- repopulation: 13%) was also significantly decreased when comparing MyD88+/+ to MyD88-/- donors in recipient mice. No difference was found in rupture-induced deaths (4% each donor genotype).

To determine whether this bone marrow cell-derived effect was mediated by TLR4 in the same cell type, mice that were TLR4+/+ x LDLr-/- were irradiated and repopulated with TLR4+/+ or -/- bone marrow-derived cells. Donor cell repopulation was confirmed as described in the MyD88 study (unpublished). Body weight, SBPs, plasma cholesterol, and white blood cells (except platelet numbers, which were significantly lower in the recipient mice repopulated with TLR4-/- bone marrow-derived cells) were unchanged between recipients (Table
5.3). Intimal atherosclerotic lesions were unchanged between the TLR4+/+ recipients repopulated with TLR4+/+ and -/- donors (Figure 5.11a). Surprisingly, the suprarenal diameter (+/+ donors: 1.4 ± 0.09mm; -/- donors: 1.52 ± 0.11mm) and incidence of AngII-induced AAAs (+/+ donors: 55%; -/- donors: 48%) were not significantly altered by repopulation with TLR4/- bone marrow-derived cells into +/+ recipients (Figure 5.10b). In addition, no differences were observed in aortic arch area (+/+ donor: 21.9 ± 0.69 mm; -/- donor: 22.0 ± 0.80 mm) and aneurysm incidence (+/+ donor: 50%; -/- donor: 43%) or rupture-induced death (+/+ donor: 0%; -/- donor: 13%).
Figure 5.1: AngII increases innate immune mRNA expression in vascular and hematopoietic cells. MyD88, TLR2, TLR4, and CD14 real-time mRNA expression levels in (A) thioglycollate-elicited mouse peritoneal macrophages and (B) abdominal aortic smooth muscle cells treated with saline, AngII (1μM), LPS (1μg/ml), or Pam₃CSK₄ (1μg/ml). Values were extrapolated using the delta delta Ct method with saline treatment as control and 18s rRNA as the internal control. Histobars represent the means of 6 samples and bars are represented as SEM. *Denotes P<.004 in AngII, LPS, and Pam₃CSK₄ treatments versus saline. #Denotes P<.002 in LPS and/or Pam₃CSK₄ treatments versus AngII.
Figure 5.2: AngII-induced atherosclerotic lesions express MyD88 and TLR4 colocalized to macrophages. Atherosclerotic sinus sections from AngII infused LDLr/- mice were stained for control nonimmune rabbit serum (A), rabbit IgG, (C and E), macrophage (B), MyD88 (D), or TLR4 (F). Positive staining was represented by red coloration. Magnification = 200x.
Figure 5.3: MyD88 and TLR4 colocalize with macrophage staining in AngII-induced AAAs. AAA sections from AngII infused LDLr/- mice were stained for control nonimmune rabbit serum (A), rabbit IgG (C and E), accurate macrophage (B), MyD88 (D), or TLR4 (F). Positive staining was represented by red coloration. Magnification = 200x.
Figure 5.4: MyD88 deficiency attenuates AngII-induced vascular pathology in apoE-/- mice. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area, respectively. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (MyD88+/+) and gray circles (-/-) represent individual mice, diamonds represent means, and bars are SEMs. *P<.001 Mann-Whitney Rank Sum analysis MyD88+/+ vs -/- arch and thoracic atherosclerosis. #P<.001 Fisher’s Exact Test MyD88+/+ vs -/- abdominal aortic width.
Figure 5.5: MyD88 deficiency attenuates AngII-induced vascular pathology in LDLr−/− mice. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (MyD88+/+) and gray circles (−/−) represent individual mice, diamonds represent means, and bars are SEMs. *P<.001 Mann-Whitney Rank Sum analysis MyD88+/+ vs −/− arch and thoracic atherosclerosis. #P<.001 Fisher’s Exact Test MyD88+/+ vs −/− abdominal aortic width.
Figure 5.6: TLR2 deficiency attenuates AngII-induced atherosclerosis, but did not change AAAs in LDLr-/- mice. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (TLR2+/+) and gray circles (-/-) represent individual mice, diamonds represent means, and bars are SEMs. *P<.001 Mann-Whitney Rank Sum analysis TLR2+/+ vs -/- arch and thoracic atherosclerosis.
Figure 5.7: TLR4 deficiency attenuates AngII-induced vascular pathology in LDLr⁻/⁻ mice. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (TLR4+/+) and gray circles (-/-) represent individual mice, diamonds represent means, and bars are SEMs. *P<.001 Mann-Whitney Rank Sum analysis TLR4+/+ vs -/- arch and thoracic atherosclerosis. #P<.001 Fisher’s Exact Test TLR4+/+ vs -/- abdominal aortic width.
Figure 5.8: AngII augmentation of WBCs, HFD-induced monocytes, and elicitation of macrophages are attenuated by MyD88 deficiency. (A) AngII infused MyD88+/+ (n=19) and -/- (n=23) mice (on high fat diet and LDLr-/- background) whole blood examined on hemavet analyzer. (B) LDLr-/- (n=10), MyD88-/- (n=10), and TLR4-/- (n=10) comparison of whole blood monocyte double positive populations of CD115 (M-CSF receptor 1) and F4/80 before and after 1 month high fat diet. (C) LDLr-/- (n=10), MyD88-/- (n=10), TLR4-/- (n=5) thioglycollate elicited CD68+ peritoneal macrophages. Genotypes represented as follows: LDLr-/- open bars, MyD88-/- hatched bars, TLR4-/- filled bars. *Represents P<0.001 Mann-Whitney Rank Sum analysis MyD88-/- versus LDLr-/- or TLR4-/-.
Figure 5.9: MyD88 deficiency augments Ly-6C\textsubscript{low} and attenuates Ly-6C\textsubscript{hi} subpopulations of peripheral blood monocytes. (A) LDLr\textsubscript{-/-} \((n=8)\), TLR4\textsubscript{-/-} \((n=7)\), and MyD88\textsubscript{-/-} \((n=8)\) mice were fat fed for 1 month and peripheral blood monocytes (CD115\textsuperscript{*} and F4/80\textsuperscript{*}) were divided into Ly-6C low, intermediate, and high subpopulations. Genotypes represented as follows: LDLr\textsubscript{-/-} open bars, TLR4\textsubscript{-/-} hatched bars, MyD88\textsubscript{-/-} filled bars. *Represents \(P<0.001\) One Way ANOVA on ranks MyD88\textsubscript{-/-} versus LDLr\textsubscript{-/-} or TLR4\textsubscript{-/-} within subpopulation. (B) LDLr\textsubscript{-/-}, TLR4\textsubscript{-/-}, and MyD88\textsubscript{-/-} mice were infused with saline \((n=5)\) or AngII \((n=5-10)\) for 2 weeks while being fat-fed. Peripheral blood monocytes were subdivided as described above. *\(P<0.01\) LDLr\textsubscript{-/-} saline versus AngII Mann-Whitney rank sum.
Figure 5.10: Development of AngII-induced atherosclerosis and AAAs are attenuated by MyD88 deficiency in bone marrow-derived cells. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (MyD88+/+ bone marrow donor) and gray circles (−/− bone marrow donor) represent individual mice, diamonds represent means, and bars are SEMs. *P<.006 Mann-Whitney Rank Sum analysis MyD88+/+ vs −/− arch and thoracic atherosclerosis. #P<.001 Fisher’s Exact Test MyD88+/+ vs −/− abdominal aortic width.
Figure 5.11: Development of AngII-induced vascular pathology does not require the presence of TLR4 in bone marrow-derived cells. (A) Atherosclerotic lesion area was measured on aortic arch and thoracic intimal surfaces and taken as a percentage of total arch and thoracic area. (B) Measurements of maximal external width of abdominal aortas (mm). Clear circles (TLR4+/+) and gray circles (-/-) represent individual mice, diamonds represent means, and bars are SEMs.
Table 5.1: Metabolic effects of AngII-infusion into multiple genotypes

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Number of mice</th>
<th>Plasma cholesterol (mg/dL)</th>
<th>Pre-infusion SBP (mmHg)</th>
<th>Post-Infusion SBP (mmHg)</th>
<th>Body weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88+/+ x apoE-/-</td>
<td>42</td>
<td>320 ± 32</td>
<td>112 ± 5</td>
<td>171 ± 4*</td>
<td>29.1 ± 0.4</td>
</tr>
<tr>
<td>MyD88-/- x apoE-/-</td>
<td>27</td>
<td>306 ± 23</td>
<td>116 ± 3</td>
<td>173 ± 3*</td>
<td>29.0 ± 0.3</td>
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<tr>
<td>MyD88+/+ x LDLr-/-</td>
<td>19</td>
<td>1174 ± 57</td>
<td>127 ± 2</td>
<td>174 ± 4*</td>
<td>27.7 ± 0.9</td>
</tr>
<tr>
<td>MyD88-/- x LDLr-/-</td>
<td>23</td>
<td>1250 ± 72</td>
<td>135 ± 10</td>
<td>164 ± 5*</td>
<td>31.2 ± 0.5†</td>
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<tr>
<td>TLR2+/+ x LDLr-/-</td>
<td>9</td>
<td>1334 ± 57</td>
<td>115 ± 2</td>
<td>157 ± 4*</td>
<td>26.9 ± 1.5</td>
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<tr>
<td>TLR2-/- x LDLr-/-</td>
<td>11</td>
<td>1275 ± 62</td>
<td>118 ± 4</td>
<td>150 ± 9*</td>
<td>26.5 ± 3.1</td>
</tr>
<tr>
<td>TLR4+/+ x LDLr-/-</td>
<td>14</td>
<td>1257 ± 62</td>
<td>142 ± 2†</td>
<td>168 ± 3†</td>
<td>26.5 ± 0.6†</td>
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<tr>
<td>TLR4-/- x LDLr-/-</td>
<td>19</td>
<td>1180 ± 50</td>
<td>131 ± 2</td>
<td>158 ± 3*</td>
<td>27.7 ± 0.5†</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM.
N/A: Information not available.
*Indicates P<.001 post-infusion systolic blood pressures versus pre-infusion, within genotypes. Repeated measures analysis with Bonferroni.
† Indicates P<.005 in MyD88-/- x LDLr-/- weight measures versus +/+ Mann-Whitney Rank Sum.
‡ Indicates P<0.025 in TLR4+/+ x LDLr-/- systolic blood pressures versus -/- in both pre and post infusion values. Two-way ANOVA with Repeated Measures.
Table 5.2: Arch area, arch and abdominal aneurysm incidence, and rupture rates

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Number of mice</th>
<th>Arch area (mm²)</th>
<th>Arch Aneurysm</th>
<th>Abdominal aneurysm</th>
<th>Rate of rupture</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyO88+/+ x apoE−/−</td>
<td>42</td>
<td>20.8 ± 0.7</td>
<td>24/42 (57%)</td>
<td>33/42 (79%)</td>
<td>14/42 (33%)</td>
</tr>
<tr>
<td>MyO88+/− x apoE−/−</td>
<td>27</td>
<td>16.0 ± 0.4*</td>
<td>1/27 (4%)</td>
<td>2/27 (7%)</td>
<td>1/27 (4%)</td>
</tr>
<tr>
<td>MyO88+/+ x LDL−/−</td>
<td>19</td>
<td>23.2 ± 0.9</td>
<td>10/19 (52%)</td>
<td>17/19 (92%)</td>
<td>8/19 (42%)</td>
</tr>
<tr>
<td>MyO88−/− x LDL−/−</td>
<td>23</td>
<td>19.1 ± 0.8*</td>
<td>2/23 (9%)†</td>
<td>3/23 (13%)†</td>
<td>1/23 (4%)†</td>
</tr>
<tr>
<td>TLR2+/+ x LDL−/−</td>
<td>9</td>
<td>22.8 ± 1.1</td>
<td>4/9 (44%)</td>
<td>8/9 (89%)</td>
<td>2/9 (22%)</td>
</tr>
<tr>
<td>TLR2−/− x LDL−/−</td>
<td>11</td>
<td>23.0 ± 1.0</td>
<td>4/11 (36%)</td>
<td>9/11 (82%)</td>
<td>4/11 (36%)</td>
</tr>
<tr>
<td>TLR4+/+ x LDL−/−</td>
<td>14</td>
<td>21.7 ± 1.0</td>
<td>9/14 (64%)</td>
<td>14/14 (100%)</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>TLR4−/− x LDL−/−</td>
<td>19</td>
<td>22.4 ± 0.8</td>
<td>5/20 (25%)</td>
<td>5/20 (25%)†</td>
<td>4/20 (20%)</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM.
* Indicates P < .001 MyO88+/+ vs −/− aortic arch area. Mann-Whitney Rank Sum analysis.
† Indicates P < .009 in MyO88+/+ vs −/− arch and abdominal aneurysm and rupture rate; TLR4+/+ vs −/− abdominal aneurysm. Fisher's Exact Test.
Table 5.3: Effects of AngII-infusion into LDLr-/- mice chimeric for MyD88 and TLR4

5.5 Discussion:

This study examined the link between the main effector of the renin angiotensin system, AngII, and the toll pathway in regards to vascular pathology. We show that AngII can similarly upregulate TLR2, TLR4, and CD14 mRNA expression in both vascular smooth muscle cells and macrophages. The data also demonstrates up regulation of a crucial intracellular adaptor protein, MyD88, localized to macrophages, but not vascular cells. Unpublished results also confirm AngII up regulation of these 4 toll pathway components in mature dendritic cells. The up regulation of MyD88 expression in activated macrophages and dendritic cells may suggest this adaptor protein has increased activity in cells of hematopoietic origin versus vascular-derived cells. TLR4 and MyD88 proteins were also found to be colocalized to macrophages in AngII-induced atherosclerotic lesions and AAAs. Previous data shows colocalization of TLR2 and TLR4 to macrophages in human and murine atherosclerotic lesions, however, this is the first link to AAAs. These data suggest AngII can upregulate toll pathway components and that these proteins are present in AngII-induced vascular disease.

This study also demonstrated significant attenuation of AngII-induced aortic arch and thoracic atherosclerosis using gene-targeted deletions of the toll pathway receptors TLR2 (Figure 5.6A) and TLR4 (Figure 5.7A), as well as their major signaling subunit MyD88 (Figures 5.4A and 5.5A). These results confirm numerous demonstrations of MyD88, TLR2, and TLR4 attenuating the progression of atherosclerosis in long-term fat feeding studies, using an apoE-/- background. However, apoE is known to have many anti-atherogenic and anti-inflammatory effects, possibly mediated through regulation of plasma cholesterol homeostasis and immunomodulation. Deficiency of apoE also upregulates responsiveness and activation, specifically of TLR4 and TLR3. Therefore, we bred out the apoE component and crossed the MyD88 mice onto an LDLr-/- background to confirm our results. All results obtained in the apoE-/-
strain were also demonstrated in the LDLr-/- strain, suggesting no complications in regards to data interpretation. We also show no changes in TLR2 or TLR4 attenuations of atherosclerosis in the LDLr-/- strain, confirming a previous fat feeding study in TLR2 x LDLr animals. These data also show that the AngII accelerated model of atherosclerosis induces similar correlative results to long term diet studies in the short span of 5 weeks.

The present study also demonstrates the novel involvement of the toll pathway in the development of AngII-induced AAAs. Specifically, deletion of MyD88, in both an apoE-/- and LDLr-/- background, significantly attenuates the formation of abdominal dilation, aortic arch area increases, aortic arch aneurysm, AAAs, and incidence of rupture-induced death (Figure 5.4 and 5.5, Table 5.1 and 5.2). We further show that TLR4 deficiency also significantly attenuates the formation of suprarenal abdominal dilation, aortic arch aneurysm, and AAAs (Figure 5.7 and Table 5.2). However, TLR4 deficiency had no effect on rupture-induced death, or aortic arch area. Furthermore, infusion of 19 TLR4-/- mice with AngII led to one mouse with AAA at the end of 28 days. Out of the five TLR4-/- AAAs, four of them were rupture-induced deaths occurring rapidly in the first week of infusion. As shown in Table 5.3, TLR4-/- bone marrow results in a significant deficit in platelet numbers, suggesting these mice may develop thrombocytopenia contributing to the inability to withstand aneurysm rupture in these mice. Other studies have confirmed lower basal platelet numbers in TLR4 deficient animals, however, basal levels have not been verified in our deficient animals. TLR2 deficiency, while having a role in atherosclerosis, did not affect a change in AngII-induced aneurysms (Figure 5.6 and Table 5.2). These data suggest that TLR4 mediates signaling of MyD88 to induce arch aneurysm and AAA formation.

Recently published studies have suggested TLR4 mediates insulin resistance and lipid-induced activation of inflammatory pathways. Results have shown fat-fed animals have increases in both expression and signaling of toll receptors in adipose tissue. It has also been demonstrated that TLR4-/- mice
develop diet-induced obesity, to a higher extent than wild type animals, without the up regulation in inflammatory cytokines, adipose macrophages, or complications of diabetes.\textsuperscript{269,270} Our results confirm this data, insomuch as fat-fed TLR4-/- mice have a trend for increased body and fat pad weights. The mice in this study were fed a 21% saturated fat-enriched diet for 5 weeks, which could account for the difference in results. However, we show that MyD88-/- animals, fed a high fat diet, have a significant increase in body and fat pad weight, resulting in over a 100% increase in adiposity index. Despite this increase in fat mass, blood monocyte and inflammatory cell levels (Figure 5.8), and AngII-induced pathologies remain at low levels. This study did not specifically examine the role of diabetes or insulin resistance, however, insulin levels were recorded at time of termination (unpublished data). These levels suggest a trend for decrease in MyD88-/- (+: 101mg/dL, -: 91mg/dL) and TLR4-/- (+: 93mg/dL, -: 83mg/dL) mice, despite increased body weight and fat masses. This suggests the MyD88 adaptor protein may also play an important role in insulin resistance and lipid induced activation of inflammatory pathways.

MyD88 deficiency resulted in stagnant levels of white cells after AngII infusion, and significantly attenuated levels of blood monocytes after fat feeding (Figure 5.8). This data supports a hypothesis presented by Bjorkbacka et al., suggesting MyD88 deficiency has defects in macrophage activation.\textsuperscript{146} This also supports a role for MyD88 in circulation of monocytes and macrophages, which are key mediators of atherosclerosis and aneurysm formation. Therefore, we ascertained the role of the hematopoietic cells by performing a bone marrow transplantation using MyD88+/+ irradiated recipients injected with MyD88+/+ and -/- bone marrow-derived donor cells. The results demonstrate that MyD88+/+ repopulation with MyD88-/- cells leads to a significant decrease in aortic arch and thoracic atherosclerosis (Figure 5.9). However, the decrease in aortic arch atherosclerosis, in MyD88-/- bone marrow-derived cells (34%), is much less than the whole body deficiency of MyD88 (apoE-/- 82%; LDLr-/- 64%), suggesting a possible role for recipient MyD88, which was not examined in this study. Similar
to whole body deficiency experiments, MyD88-/- bone marrow derived-cells significantly attenuated the formation of suprarenal aortic diameter, arch area increases, arch aneurysm incidence, and AAA incidence. These data suggest MyD88, in hematopoietic populations, are important to the formation of AngII-induced atherosclerosis and critical to aneurysm formation.

To confirm whole body experiments, TLR4 was also examined via bone marrow transplantation using TLR4+/+ irradiated recipients injected with TLR4+/+ and -/- bone marrow-derived donor cells. Surprisingly, TLR4-/- donor cells did not recapitulate the whole body studies, as there was no change in regards to AngII-induced atherosclerosis or aneurysm formation. Previous studies have demonstrated TLR2 deficiency also had no effects on atherosclerosis when TLR2+/+ recipients were irradiated and injected with TLR2-/- marrow-derived cells. Other studies suggest that endothelial cell TLR2 is responsible for the reductions in atherosclerosis. In a published abstract, Mullick et al. also demonstrates TLR4-/- bone marrow has no effect on atherosclerosis in a long-term fat feeding study, which confirms our data. These data suggest that TLR4 does not mediate the bone marrow-induced effects of MyD88.

Further work will be necessary to characterize these differences. However, it is possible that MyD88 is important on hematopoietic cells, such as monocytes and macrophages, which are critical for initiation of these pathologies. With the defects in macrophage populations we speculate that the function of these cells are diminished and therefore a reduction in vascular pathology ensues. However, it is very important to note that TLR4 deficiency does not share these decreases in monocyte populations with MyD88. This, therefore, suggests that TLR4 might play a critical role in endothelial or smooth muscle cells for further progression of atherosclerosis and aneurysm disease processes. Therefore, we speculate that macrophages deficient in MyD88 do not require TLR4 due to their inability to become initially activated. However, without TLR4, it appears MyD88 can still become activated and progress disease on monocyte/macrophages. It remains to be tested whether deficiency of TLR4 on
endothelial and SMCs would ablate the progression of these diseases.

In conclusion, the present study demonstrated the presence of the adaptor protein MyD88 in marrow-derived cells is critical for induction of AngII-induced vascular disease. However, studies with TLR4 deficiency only reciprocate experiments with whole body deficient MyD88. Future studies will define the relative role of MyD88 in marrow-derived cells in the initiation of disease, and TLR4 in resident cells in the progression of pathology.
Chapter Six: Discussion and Future Directions

Natural progression of thesis (Disclaimer):

The purpose of this thesis was to try and ascertain the relative regional effects of Angiotensin II on the aortic vasculature. The rationale for this was to try and determine a morphological causality for the formation of AAA localized to the suprarenal aorta. However, during the course of the examination of the vasculature, we determined there to be no obvious difference between the suprarenal aorta and anterior thoracic aortic sections in regard to morphological assembly in vivo. This resulted in the production of a new project, to analyze the various portions of the aorta, in a more in-depth manner and characterize these results in the literature. During this documentation process, we tried to characterize contractility in these various segments, only to find that AngII did not lead to any significant contractions in either the thoracic or suprarenal aorta. This was a surprising result, as it has been reported more than a few times that AngII caused abdominal contractions. We then extended this study to the infrarenal aorta, when we found that this area of the vasculature is what was interpreted as ‘abdominal aorta’ in the literature. This led to a segmentation of these two projects and an expansion in aortic characterizations to include the entire aorta (aortic arch and infrarenal aortas were now included in the analysis). This inclusion of the aortic arch led to the discovery of regional hyperplasia, versus the remainder of the aortas hypertrophic response to AngII. In searching for a mechanism, I happened to catch a lecture at the ATVB meeting in 2005 by Coleen McNamara, about the role of AngII and redox control of Id3. We subsequently obtained the Id3/- mice and the result turned into our major finding of the paper. Concurrent with these ‘morphology’ studies, I was also interested in the main focus of the laboratory, that being AngII induction of atherosclerosis and abdominal aortic aneurysm. With the reading of recent papers out of Moshe Arditi’s UCLA group and Mason Freeman’s Harvard group describing the role of
MyD88 and Toll receptors in fat-fed atherosclerosis, I thought there were many similarities with this innate immune system and the causalities of AAA formation. Then, and even to date, very few publications have examined the role of innate immunity in aneurysm. The opportunity was given to me to present my ideas involving MyD88 and AAA as a grant for my qualifying examination in order to get the project, and luckily, I must have struck a cord somewhere, for soon after I was writing an AHA grant on the subject and we were in the process of obtaining MyD88-/- mice from Harvard.

While it is a difficult task to link these three unique and different projects, I eventually came to the conclusion that although separate, this thesis is connected by the original hypothesis of regional differences in regard to AngII. The morphology project (Chapter 2) has regional effects of AngII in the aortic arch leading to hyperplasia versus the hypertrophy found in the remainder of the aorta. The contractility project (Chapter 3) finds regional induction of AngII contraction highly localized to the infrarenal aorta. The MyD88 atherosclerosis and aneurysm project (Chapter 5) finds a characterized reduction of aneurysm in the suprarenal aorta. This project connects three disparate studies into the regional effects of AngII.

A. Chapter 2 Discussion and Future Directions.

Chapter 2 demonstrated that AngII promoted a uniform medial expansion throughout the aorta. The following characterization of this expansion led to the underlying finding of the paper: SMC hyperplasia in the ascending aorta, and SMC hypertrophy in all other aortic regions. This effect was due to stimulation of AT1a receptors, but independent of increases in SBP. AngII-induced medial expansion was ablated in all aortic regions in mice with functional deficiency of p47phox, while the hyperplastic response localized to the ascending aorta was inhibited by deficiency of Id3.

The novelty of this chapter is in the demonstration that AngII infusion
promotes aortic medial expansion by disparate mechanisms in a region-specific manner. A major difference was the demonstration of the disparity of responses in the ascending aorta compared to other regions. Unique responses of this region in aortic lumenal expansion have also been demonstrated in mice harboring fibrillin-1 mutations and during AngII infusion.\textsuperscript{170} Although this study defined AngII-induced changes in SMCs, this effect could be directly on SMCs or indirectly from another cell type. For example, endothelial cells secrete a wide range of products that directly affect SMC function. Thus, subsequent studies will delete the AT1a receptor in either endothelium or SMCs to determine whether AngII is exerting its effects by an action directly on SMCs.

We have recently found that AT1a SMC depletion using cre-lox technology demonstrated no changes in AngII-induced medial thickening. These mice show uniform medial expansion similar to the normal C57BL/6 aortas dissected and analyzed for Chapter 2. This result leads us to believe that endothelial-specific AT1a might be responsible for medial thickening. This would be a most interesting finding, since it is the SMC in the aortic wall affected by AngII infusion in the current study. When re-examining tissue sections, it is near impossible to detect any difference in the small amount of endothelia that remains on the sections. This may be due to the continual perfusion or fixation process that was undertaken for nearly 30 minutes of constant flow. Therefore, the deletion of endothelial AT1a, using a Tie2 promoter, will hopefully give us insight into the formation of this SMC pathology. However, there is also a possibility that the endothelial specific depletion might also yield us no results. If this is the case, then an examination of the adventitial AT1a might be warranted. Another possibility exists that this effect requires a combination of many different cell types acting in unison for this SMC effect. In that rare case, it might be possible to tease out the effects using cell culture systems using transmembrane cultures of endothelial cells and SMCs and varying depletion of AT1a in either cell type to detect changes in SMCs when treated with AngII.

Another interesting area to examine further would be the interaction of
AngII and Id3. When this paper is published, it will only represent the third paper in the literature connecting the RAS with Id3. An interesting question that has not yet been answered is why is Id3 so concentrated in the aortic arch of C57BL/6 mice? Another interesting result, that will go unreported, was that of the eight Id3-/- mice infused with AngII, one of them died of AAA rupture, and two others developed extremely large AAAs. With the recent publication of p21 involvement in AngII-induced AAA, and Id3 being intimately connected with the regulation of p21, this may be an interesting area of study.271

B. Chapter 3 Discussion and Future Directions.

Chapter 3 demonstrated that AngII promotes region-specific contractions limited to the infrarenal aortic vasculature. To examine the molecular basis of this effect, angiotensin receptors were quantified and found to be concentrated in the infrarenal aorta, with AT1bRs being the most abundant. AngII-induced infrarenal contractions were ablated only in the AT1bR-/- mouse defining this receptor subtype as the sole mediator of murine aortic contractions.

The novelty of this study is the demonstration that AngII incubation of aortic sections can lead to a robust and regional contraction localized solely to the infrarenal vasculature. This response was directly propagated from smooth muscle cells and did not involve endothelial cells. This study provides direct evidence that AT1bR’s mediate this contraction through disparate mechanisms of the homologue, AT1a.

One interesting area of future study involves a connection I made in the literature in Chapter 3’s discussion section. This difference was between amino acid 312 of the C-terminus which is present as an argenine in AT1b receptors and a tyrosine in AT1a receptors.52 This Tyr to Arg switch possibly leads to an ineffective Gq coupling and activation site in AT1b receptors versus the AT1a subtype. This represents only one reason why AT1a and AT1b receptors differ between each other, and also a major gap in the literature. It is still not yet clear
why there are differences between these very homologous receptors, and I believe this to be an interesting area of study.

Another result suggested AT1a receptors mediate effects through direct action on smooth muscle cells (postjunctional), while AT1b receptors mediate effects through indirect facilitation of norepinephrine on smooth muscle. Other studies providing a possible cross-talk pathway between $\alpha/\beta$ adrenergic receptors and AT1 receptors. An interesting hypothesis might be that AT1b concentration in the infrarenal aorta leads to activation by AngII and subsequent release of norepinephrine release resulting in aortic contraction. The test of this hypothesis would be simple enough and would require alpha/beta adrenergic antagonism before addition of AngII into the culture bath. Further, if this hypothesis is correct, the addition of adrenal glands in close proximity to the aorta in the contraction bath would lead to a highly significant increase in contractile properties. This would possibly prove that the infrarenal aorta proximity to the adrenal glands could lead to even more stimulation of contraction.

This is my chance to express my wild theories about the possible involvement of AT1b in the role of AngII-induced AAA, so I’m going to take it and run. I have been trying to associate a role of in vivo contraction with the production of AngII-induced AAA localized to the suprarenal aorta. Our results suggest that AngII-induction of contraction is all but absent in the suprarenal aorta, and thus likely mediates minimal effects in abdominal aortic aneurysm formation, in the AngII infusion model. However, another extremely interesting result is the noticed formation of aneurysms everywhere throughout the aorta (arch, thorax, suprarenal) with the exception of an absolute lack of AAA formation to the infrarenal aorta, the sole mediator of AngII-induced contraction. It might be that AT1b mediated contractions are creating more turbulent flow in a region that probably already has plenty due to the enormity of branch points. An interesting way to test whether AT1b mediation of contraction is mediating the concentration of AAAs to the suprarenal aorta would be to take AT1bR-/- mice and infused
them with AngII, or backcross them onto a hyperlipidemic background and compare the results to the AT1a studies.

C. Chapter 5 Discussion and Future Directions

Chapter 5 examined the link between the main effector of the renin angiotensin system, AngII, and the toll pathway in regards to vascular pathology. We demonstrated that AngII can similarly upregulate TLR2, TLR4, and CD14 mRNA expression in both vascular cells and macrophages, verifying and expanding a paucity of studies.\textsuperscript{263,264} This study also demonstrated significant attenuation of AngII-induced aortic arch and thoracic atherosclerosis using gene-targeted deletions of the toll pathway receptors TLR2 (Figure 4.6) and TLR4 (Figure 4.7), as well as their major signaling subunit MyD88 (Figures 4.4 and 5). These results confirm numerous demonstrations of MyD88, TLR2, and TLR4 attenuating the progression of atherosclerosis in long-term fat feeding studies, using an apoE-/- background.\textsuperscript{146,236,237} Attenuation of AngII-induced AAA was demonstrated by the ablation of TLR4 and MyD88, while TLR2 was ineffective at attenuating pathology. Furthermore, we demonstrated a link between the MyD88 gene and obesity studies in the literature examining TLR4, suggesting these obesity characteristics are MyD88 dependent. Lastly, we found a role for MyD88 deficiency in bone marrow-derived cells in the induction of both AngII-induced pathologies, while TLR4 deficiency in marrow-derived cells had no effect.

Therefore, the present study novelly demonstrated the presence of the adaptor protein MyD88 in marrow-derived cells is critical for induction of AngII-induced vascular disease. However, studies with TLR4 deficiency only reciprocate experiments with whole body deficient MyD88. Future studies will define the relative role of MyD88 in marrow-derived cells in the initiation of disease, and TLR4 in resident cells in the progression of pathology.

Further work will be necessary to characterize the differences in the MyD88 and TLR4 bone marrow studies. It is possible that MyD88 is important on
hematopoietic cells, such as monocytes and macrophages, which are critical for initiation of these pathologies. With the defects in macrophage populations we speculate that the function of these cells are diminished and therefore a reduction in vascular pathology ensues. However, it is very important to note that TLR4 deficiency does not share these decreases in monocyte populations with MyD88. This, therefore, suggests that TLR4 might play a critical role in endothelial or smooth muscle cells for further progression of atherosclerosis and aneurysm disease processes. Therefore, we speculate that macrophages deficient in MyD88 do not require TLR4 due to their inability to become initially activated. However, without TLR4, it appears MyD88 can still become activated and progress disease on monocyte/macrophages. This therefore suggests that the mechanism of initiation involves macrophage/monocyte populations, but also needs the presence of endothelial or SMCs. This hypothesis has been somewhat verified in the studies by Cassis et al., where AT1a receptor deficiency on bone marrow derived cells has no effect on the inhibition of AAA formation, but has minor effects on atherosclerosis. Further, this study shows that the ablation of AT1a in recipient cells attenuates atherosclerosis and AAA independent of marrow-derived cells, suggesting a role for both cell types in atherosclerosis. This may be similar to the result we are observing, with the exception that MyD88 deficient bone marrow derived cells might have nothing to do with the MyD88 pathway, but instead with defective macrophages. A reverse bone marrow transplantation needs to be performed to verify the hypothesis that the toll pathway initiates atherosclerosis and AAA via endothelial cells and SMCs and not via marrow-dependent cells. Further studies should also be conducted in regard to AngII regulation of blood monocytes and macrophages, and their subsequent role in vascular disease.

D. Discussion Summary

This thesis describes the novel findings of region-specific effects in AngII-
induced morphological, contractile, and pathological changes. These studies provide evidence that the aortic arch may have altered embryological origins versus the remainder of the aorta, leading to region-specific hyperplasia. Further, that the infrarenal aorta contains highly localized concentrations of AT1b receptors, which potentially leads to consistent up regulation in AngII contractions. Finally, that AngII may induce region-specific suprarenal AAA formation via mediation of the innate immune toll pathway. With all science, however, much more work is needed to expand our knowledge of these phenomena and to further characterize the always elusive question: why?
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PUBLICATIONS
3. Alan Daugherty, Debra L. Rateri, A. Phillip Owens III, Israel F. Charo,


ABSTRACTS

2. A. Phillip Owens III, Deborah A. Howatt, and Alan Daugherty. Toll-like Receptor 4 Deficiency Attenuates Angiotensin II-induced Atherosclerosis and Abdominal Aortic Aneurysm via a MyD88-dependent Mechanism. 11th Annual Gill Heart Institute Cardiovascular Research Day; October, 2008, University of Kentucky, Lexington, KY.

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