Thesis

In presenting this dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I agree that the Library of the University shall make it available for inspection and circulation in accordance with its regulations governing materials of this type. I agree that permission to copy from, or to publish, this dissertation may be granted by the professor under whose direction it was written, or, in his absence, by the Dean of the Graduate School when such copying or publication is solely for scholarly purposes and does not involve potential financial gain. It is understood that any copying from, or publication of, this dissertation which involves potential financial gain will not be allowed without written permission.

_____

Derick Okwan-Duodu

Modulation of Macrophage Inflammatory Response by Angiotensin Converting Enzyme

By

Derick Okwan-Duodu
Doctor of Philosophy

Program in Immunology and Molecular Pathogenesis

Kenneth E. Bernstein, M.D.
Advisor

Ifor R. Williams, M.D., PhD
Committee Member

Charles A. Parkos, M.D., PhD
Committee Member

Aron E. Lukacher, M.D., PhD
Committee Member

James C. Zimring, M.D., PhD
Committee Member

Accepted:

Dean of the Graduate School

April 4, 2013

Date

Modulation of Macrophage Inflammatory Response by Angiotensin Converting Enzyme

By

Derick Okwan-Duodu
B.Sc., Middlebury College, 2005

Advisor: Kenneth E. Bernstein, M.D.

A dissertation submitted to the Faculty of the Graduate
School of Emory University in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Program in Immunology and Molecular Pathogenesis

2013

Abstract

The renin angiotensin system (RAS), critical for blood pressure control, is emerging as an important modulator of the immune response. Angiotensin-converting enzyme (ACE) is a key member of the RAS, in that it participates in the generation of the effector octapeptide angiotensin II from angiotensinogen. To understand the role of ACE in the immune response, we substituted control of the ACE gene from its endogenous promoter to c-*fms* promoter, with a resulting over-expression of ACE in myelomonocytic lineage cells such as neutrophils, monocytes and macrophages. These mice, referred to as ACE 10/10, are resistant to tumor growth and bacterial infections (*Listeria monocytogenes* and methicilin-resistant *S. aureus*). The upregulation of iNOS and other pro-inflammatory cytokines (TNF-α, IL-12) were critical to the immune changes in these mice. *In vitro,* ACE 10 macrophages potently destroyed tumor cells and bacteria, exhibiting strong hallmarks of the classically –activated M1 macrophage phenotype. We found that the biochemical changes were associated with increased expression of C/EBPα, a transcription factor crucial for development, maturation and differentiation of myeloid cells. Because C/EBPα has been shown to drive myelomonocytic cell maturation, it is likely that the increased resistance to tumors was in part due to a diminution of immature myelomonocytic cells that participate in tumor-induced myeloid-mediated immunosuppression. In both resistance to tumors and bacterial infection, the phenotype of ACE 10/10 mice was transferable by bone marrow transplantation to recipient WT animals, suggesting immune-mediated effects, rather than mere alteration of ACE in other tissues. Pharmacological ACE inhibition reversed the phenotypes observed and suggested that the catalytic activity of ACE was an important mediator of the

inflammatory responses. However, pharmacological and genetic blockade of angiotensin II (Ang II) and its receptor AT-1 (AT_1R) had minimal effects in the ACE 10/10, suggesting an ACE-mediated phenomenon independent of Ang II, the most well described bioactive product of ACE activity. Altogether, these data reveal remarkable ability of the blood pressure regulating peptidase ACE to potently modify the behavior of myelomonocytic lineage cells for anti-tumor and bacterial immunity.

Modulation of Macrophage Inflammatory Response by Angiotensin Converting Enzyme

By

Derick Okwan-Duodu
B.Sc., Middlebury College, 2005

Advisor: Kenneth E. Bernstein, M.D.

An Abstract of
A dissertation submitted to the Faculty of the Graduate
School of Emory University in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Program in Immunology and Molecular Pathogenesis

2013

Acknowledgements

I am eternally grateful for the assistance, support and inspiration of several people in my life throughout the course of this work. My first gratitude goes to Dr Ken Bernstein. Many mentors stay in their comfort zone, but in fully supporting this work, which is significantly different from his years of expertise and research in renal physiology, we have together been intrigued, challenged and edified in the process. That infectious, daring spirit to venture the road less traveled is shared by several people in the Bernstein lab, including Drs Sebastien Fuchs, Frank Ong, Sandrine Billet, Chentau Lin and Gregory (Xiao) Shen, all from whom I learned a lot as a person and as a young scientist. I reserve special thanks to Dr Shen, whose original findings in the lab encouraged me to undertake my studies. He has been an ever-present mentor and friend, and I am indebted to him for all the extra hours and energy he spent on me to hone my scientific thinking and technique. Without him, no progress would have been made. Ellen Bernstein, for her motherly support, dedication to detail and science, and everything life-related, also deserves special praise.

Those who know me appreciate my need for a vibrant, well-rounded life outside of the laboratory to fuel my academic and intellectual undertaking. Many thanks to my Atlanta friends who, even from a distance, provided enormous support. My amazing California friends assisted me in making a new home in Los Angeles. Last, but not least, this work is dedicated to my mother Brigid, my father Isaac, and my siblings Felix, Deborah and Samuel. Although we are far apart, I feel your presence every day. Loving, and supporting as I continue to grow as an independent man, you are the reason I strive for excellence.

Table of Contents

FIGURES AND TABLES

INTRODUCTION

The History of the Renin-Angiotensin System

The renin-angiotensin system (RAS) is a complex cascade of enzymes and peptides traditional associated with the regulation of blood pressure (1, 2). It comprises sequential catalysis of angiotensinogen by renin and angiotensin converting enzyme (ACE) to produce the bioactive octapeptide angiotensin II, which exerts effects on vasoconstriction, fluid homeostasis and renal function (1, 3). As early as 1898, Tiergerstedt and Bergman identified 'renin' as the pressor agent in crude rabbit kidney extracts responsible for transient increases in blood pressure of experimental animals (4, 5). Several decades later, Goldblatt clipped canine renal arteries, with a concomitant increase in blood pressure, thus reviving the link between the kidney and blood pressure (6). Subsequently, after contribution by others, renin was purified and confirmed as an enzyme.

The product of the enzymatic activity of renin, angiotensin, was next to be discovered (7). Years later, Skegg and his co-workers observed that angiotensin existed in two forms: an inactive decapeptide and a biologically active octapeptide, with the latter being a product of chloride-sensitive enzymatic degradation of the former. The octapeptide was called angiotensin II, and the enzyme that accounted for this was the hypertension-converting enzyme, now called angiotensin converting enzyme (ACE) (8-11). The first synthesis of angiotensin II that opened the discovery into the biology of the RAS and its interconnecting relationship with aldosterone and other physiological players in the kidney to maintain blood pressure, fluid balance and electrolyte homeostasis. The period in which these discoveries were made spanned over 60 years, and still remain the building blocks of our understanding of blood pressure regulation and more (Figure 1-1).

Renin Angiotensin System

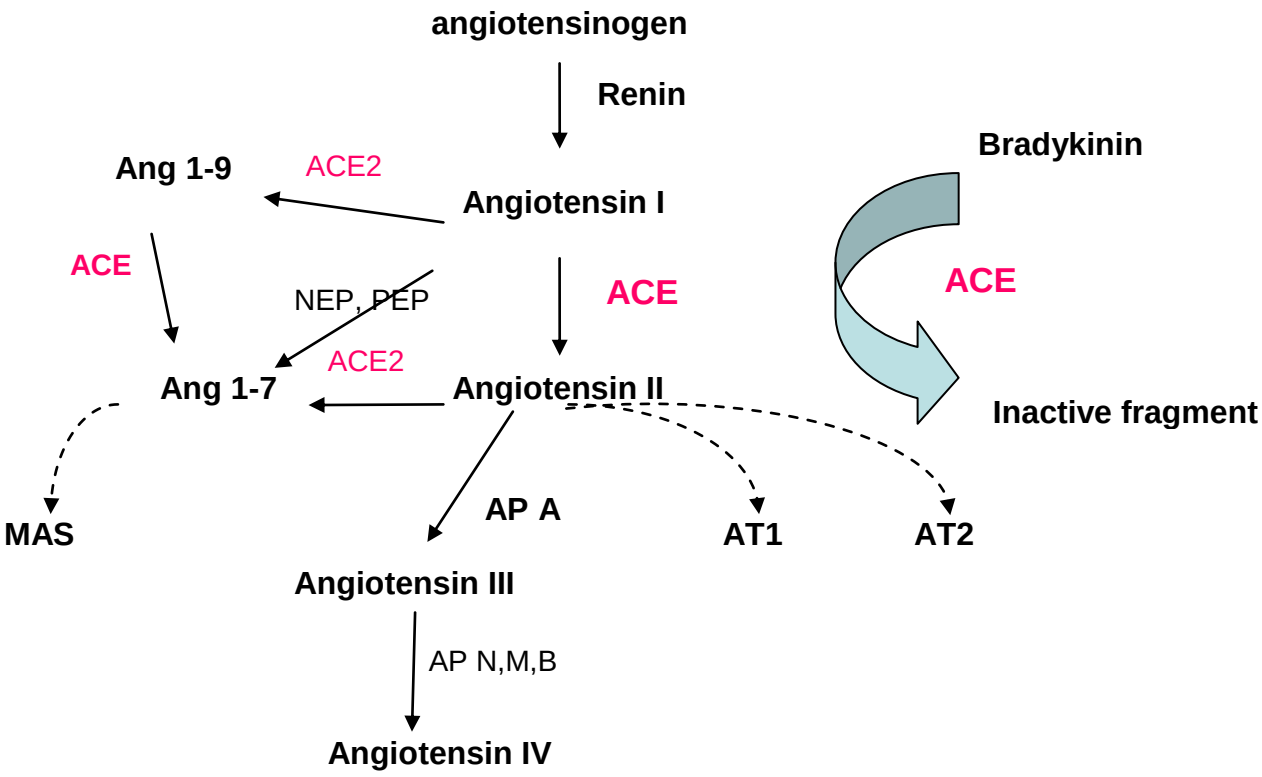


Figure 1-1. The Renin-Angiotensin System (RAS). In the RAS, renin cleaves angiotensinogen to form the inactive decapeptide angiotensin 1. ACE then converts angiotensin I to angiotensin II. As the key effector of the RAS, angiotensin II acts on its receptors (AT1 and AT2) to exert its diverse functions including vasoconstriction, salt retention and a concomitant increase in blood pressure. In other organ systems, angiotensin II can be further converted into active fragments (angiotensin III and angiotensin IV) by aminopeptidases N, M and P (AP N, M, B). ACE is central to the RAS in that it generates the vasoconstrictor angiotensin II; it also degrades the vasodilator bradykinin. Angiotensin I and II are also substrates of ACE2, a homologue of ACE.

Angiotensin converting enzyme (ACE) - biochemistry and physiology

ACE is a 150 to 180 kD dipeptidyl carboxypeptidase, existing as a single polypeptide chain of two homologous domains containing the zinc-binding motif His-Glu-Met-Gly-His (HEMGH) (see Figure 1-2) (12). The backbone itself is 147 kD, but differential glycosylation of its amino acids account for the variation in its overall molecular weight (13). The two domains of ACE are catalytically active, but have varying substrate specificity. For example, although both termini cleave angiotensin I (14), the C- terminus is the main cite of angiotensin I cleavage and subsequent angiotensin II generation *in vivo* (15). On the other hand, the N-terminus is solely responsible for the cleavage of the bioactive tetrapeptide AcSDKP (14). Other substrates of ACE include bradykinin, substance P, leutenizing hormone releasing hormone and enkephalins. Indeed, ACE is not only synthesized and expressed within the vascular endothelium and renal epithelium, but also in intestinal epithelium, regions of the brain, activated monocytes/macrophages, and cells of the bone marrow. Thus, potentially, ACE could affect a variety of physiological processes beyond blood pressure regulation.

A 90 kD testis specific isoform of ACE has been described, which is transcribed from an intragenic promoter located in intron 12 of the ACE gene (16). This isoform, called testis ACE, is expressed in developing germ cells and mature sperms (17). Our group and others have found that male mice lacking ACE have reduced fertility, owing to the absence of the catalytic activity of this ACE isoform (18, 19). Sperm-specific expression of the testis ACE, even in the absence of somatic ACE, restores male fertility (20). Interestingly, male angiotensin deficient mice have normal fertility (21), suggesting that the substrate of ACE activity mediating fertility is not angiotensin peptide. However, the specific physiologic substrate remains to be identified.

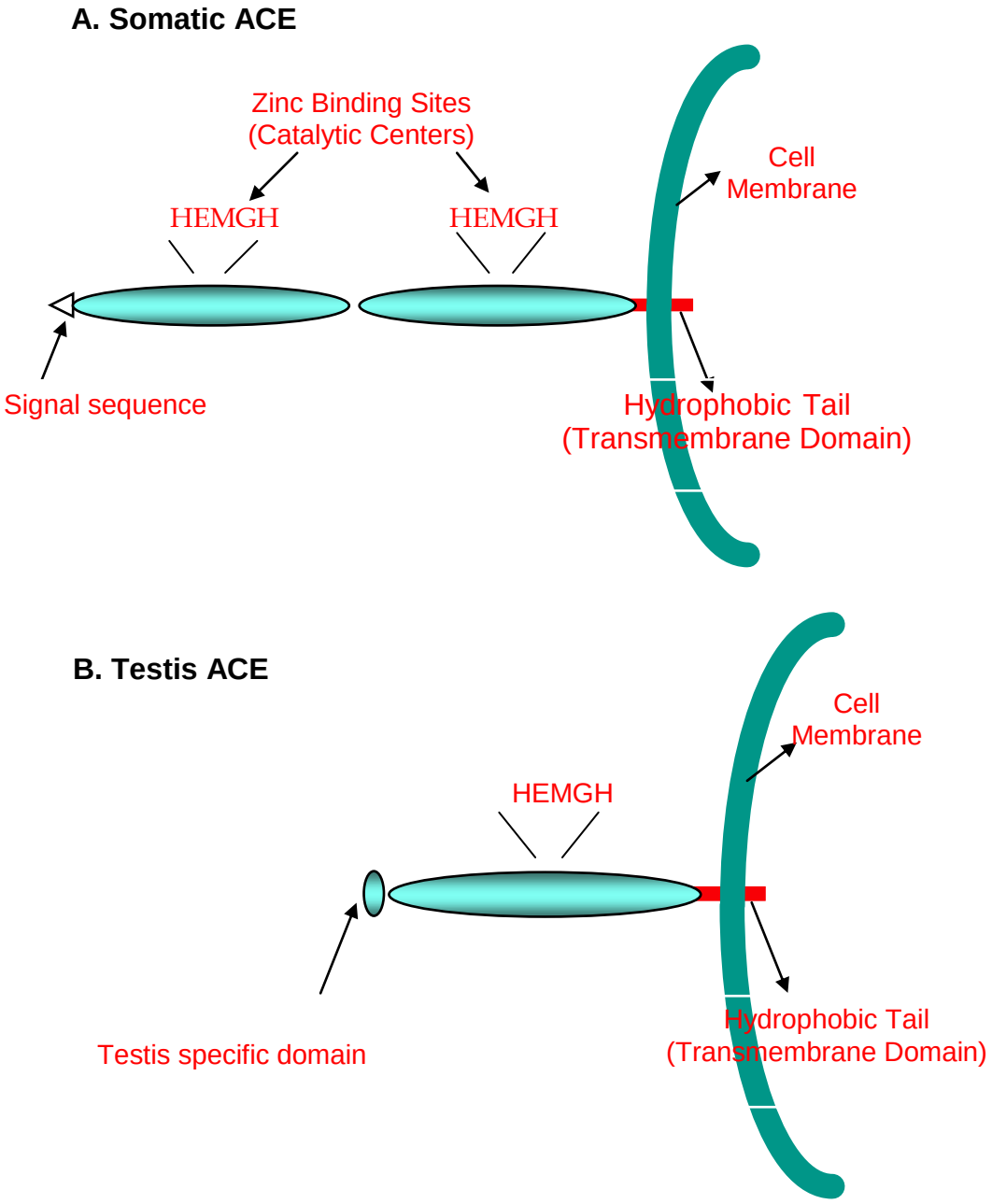


Figure 1-2. Somatic ACE and testis ACE structure. (A) Somatic ACE contains an amino terminal signal sequence, two catalytic domains and a carboxyl terminal hydrophobic tail that anchors the protein to the plasma membrane. HEMGH represents the consensus zinc-binding sites His-Glu-Met-Gky-His. (B) Testis ACE contains only three domains: a 66 amino acid domain, only one catalytic (carboxyl terminal) domain and a transmembrane anchor.

Angiotensin II – physiology

Angiotensin II, as the name suggests, is a classic vassopressor. Intravenous administration of this peptide causes a rapid spike in systolic blood pressure.

In addition to this direct vasoppressor effect, angiotensin II stimulates aldosterstone release, which further results in retention of sodium and extracellular fluid volume in various compartments of the kidney. Furthermore, angiotensin II increases thirst sensation by stimulating the sobfornical organ of the brain, potentiates activity of the sympathetic nervous system, and directly stimulates reabsorption of sodium in the kidney tubules. Together, these mechanisms sustain cardiovascular and electrolyte homeostasis.

Angiotensin II does not only regulate cardiovascular and fluid balance. It induces gluconeogenesis in the liver, and acts as a mitogen, stimulating growth of a number of cells types including cardiomyocytes, renal mesangium, renal tubular epithelium and vascular smooth muscles. There are several lines of evidence demonstrating effect of angiotensin II on fibrosis of the heart, lungs, kidneys and several other organs (22, 23). In line with this, angiotensin II antagonism ameliorates organ fibrosis (24). It is important to note that the effect of angiotensin II on these systems is not exclusively due to its pressor effect. For example, when Weiss et al reported that atherosclerosis is augmented in Apo-E knockout mice infused with angiotensin II, they elegantly showed that norepinephrine infusion, which equivalently increased systolic blood pressure as angiotensin II treatment, did not have any such effect (25).

Angiotensin Receptors

The two major receptors cloned for angiotensin II are the AT-1 and AT-2 (26-28). Although most species have a single AT-1 receptor, rodents express two AT-1 receptors (AT-1A and AT-1B) that are >90% homologous. AT-1 receptors mediate vasoconstriction, aldosterone release, thirst stimulation and other major biological functions of angiotensin II, whereas AT-2 acts in opposition to these effects. Thus, a current paradigm holds that the AT-2 receptor functions to modulate many of the physiologic activities mediated by the AT-1 receptor. Nonetheless, by far the most important, dominant and well-understood receptor of angiotensin II is the AT-1. For example, the adult human and mouse kidney comprises over 90% of the AT-1 subtype (29, 30).

Structurally, AT-1 proteins are members of the seven transmembrane receptors that couple to G-proteins which initiate a signaling cascade to activate protein kinase C and ultimately increase intracellular calcium levels (28). The AT-1 receptor can also signal via tyrosine-kinase pathways, although it lacks the intrinsic kinase activity classically observed in cytokine receptor signaling. Our laboratory was first to demonstrate that angiotensin II activation of AT-1 receptor rapidly increases tyrosine phosphorylation and activation of the Jak/STAT pathway, and in so doing, gave a mechanistic basis for the role of angiotensin II as a mitogen (31, 32). It is worth nothing that the tyrosine kinase signaling can be retained without being coupled to G-proteins, suggesting independence of these two pathways (33) .

Local RAS

The central role of the RAS in blood pressure regulation is evidenced by the effectiveness of pharmacological inhibitors of this system for several hypertensive patients. Indeed, ACE inhibitor use effectively manages hypertension and decreases incidence of recurrent myocardial infarction, development of heart failure and death (34-36). Furthermore, qualitative or quantitative alterations in genes encoding the RAS components result in cardiovascular pathologies (21, 37). Despite these observations, in general, most patients that present with hypertension have normal systemic markers of the RAS components.

Several years after the discovery of the RAS, it was thought that the plasma was the principal site of angiotensin II production, and that the RAS was merely a systemic endocrine network. However, Ng and Vane astutely observed that plasma ACE activity was insufficient to account for all the angiotensin II generated *in vivo*, and thus proposed the existence of pulmonary tissue-bound ACE (38, 39). This seminal finding ushered in a new era of the investigations of local, tissue-specific RAS. Our group and others have used the gene targeting approach to study the contribution of these local RAS to blood pressure control, with fascinating outcomes that suggest that these local RAS may be sufficient to maintain blood pressure (40-42). To date, components of the RAS have been described in a myriad of tissues and organs, from brain to testis, and the RAS is now thought to affect several physiological and pathological processes, from fertility, through cognition and memory, to cancer and inflammation (40, 43-47).

Transgenic Models to Investigate Tissue RAS

To understand the various roles of the RAS on physiology and biology, it has been necessary to devise new tools by which to either ablate completely the expression of various components of the RAS or specifically limit its expression to one region or tissue. There are three essential techniques used to produce genetically altered mice, and all have been used to study the RAS. The first technique involves microinjection of DNA into the pro-nucleus of a fertilized ovum to produce a 'transgenic' animal, with the possibility of multiple copies of the DNA integrating into the host DNA. The introduced transgene can be designed to target specific tissues (using a promoter that is active and unique to the tissue) or can be systemic. In this technique, in addition to the exogenous DNA that gets encoded, the resulting animal also retains its normal complement of its genome. This allows studies on the gain of function of a particular protein product of the gene.

The advent of techniques to culture embryonic stem (ES) cells in vitro gave birth to the second approach by which to genetically alter mice, that is, the targeted homologous recombination of genes to produce a knockout animal (48). To knockout a gene, a targeting construct is produced which contains the desired genetic changes. This contruct, flanked by homologous DNA from the locus of interest, is introduced to the ES cells, which then undergoes homologous recombination, allowing for the incorporation of this construct into the genome. Several approaches allow for the selection of the ES cells that underwent the appropriate recombination. They are then implanted into the blastocyst of a pseudopregnant mother, leading to the generation of a chimeric animal. This powerful technique allows one to study biology and physiology in the absolute absence of a particular gene, and thus is more reliable than pharmacological inhibition. However, it can be beset with the disadvantage that loss of several

proteins from birth may result in embryonic lethality. Furthermore, the absence of a gene from birth may not allow useful study of complex processes as it may not be compatible with life. This makes it necessary to devise pharmacological or alternative conditional ablation of gene and their activity.

The need for temporary and conditional expression of a gene has led to the technology of the Cre-Lox system. Cre-Lox recombination involves flanking a gene with loxP sites (49), and then targeting that specific sequence of DNA to be spliced with the help of a Cre recombinase enzyme in a specific tissue. Thus, this recombination only knocks out a specific gene in a specific tissue at a specific time to generate conditional knockouts. Using these various techniques, our group and others have made major advances on the role of the RAS in the circulation and specific tissues.

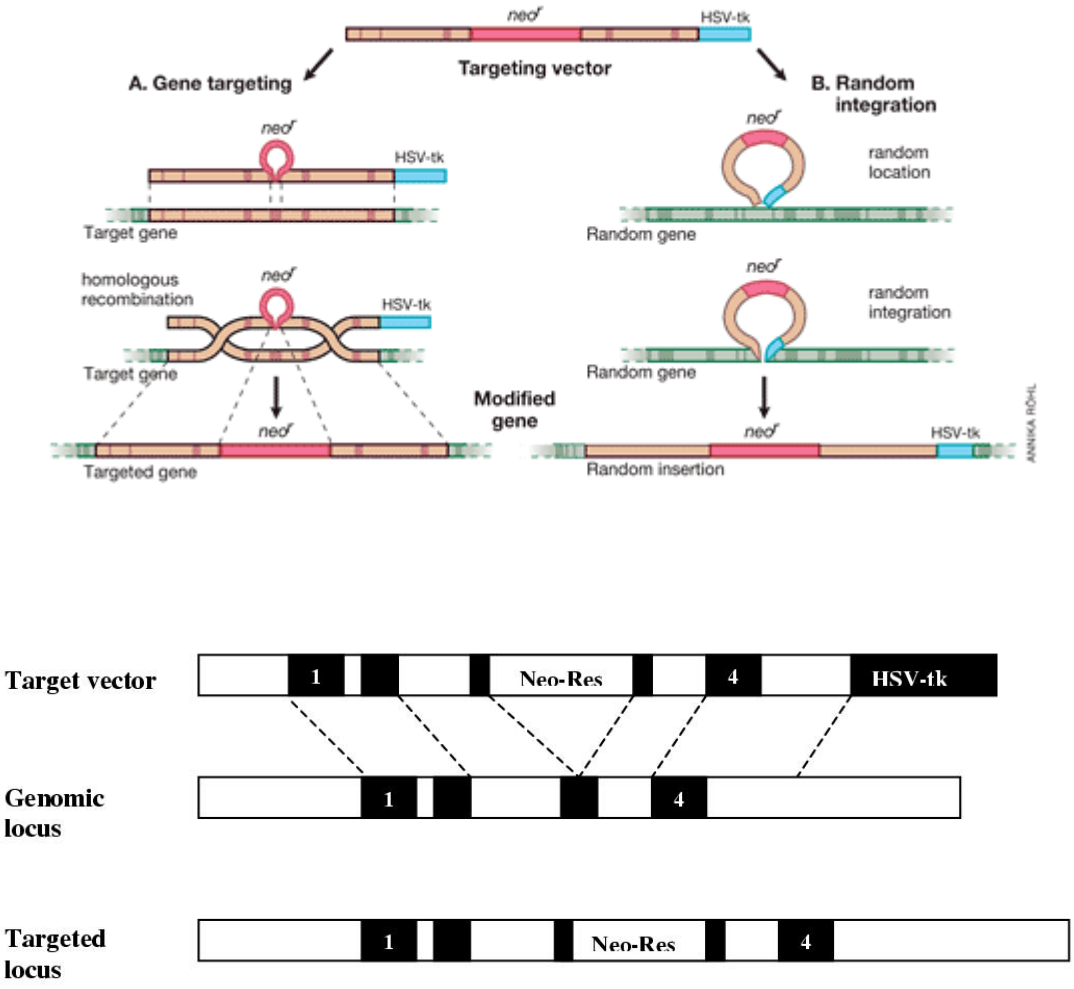


Figure 1-3. Genetic Manipulation by a Transgenic or Knockout Approach. To produce a transgenic mouse, a gene of interest is coupled to a promoter and this construct DNA is injected into the pronucleus of a fertilized ovum. A single or multiple copies of this DNA inserts into the recipient genome by random integration. To generate a knockout mice, a vector is constructed to contain cloned fragment of a homologous gene, a neomycin resistance (neo^r) cassette and the herpes simplex thymidine kinase gene (HSV-tk). Proper homologous recombination (bottom shematics) allows for the insertion of the neo^r cassette but not the HSV-tk. This permits the positive and negative selection of properly targeted embryonic stem cell clones. In this knockout approach, only the homologous genetic locus is modified.

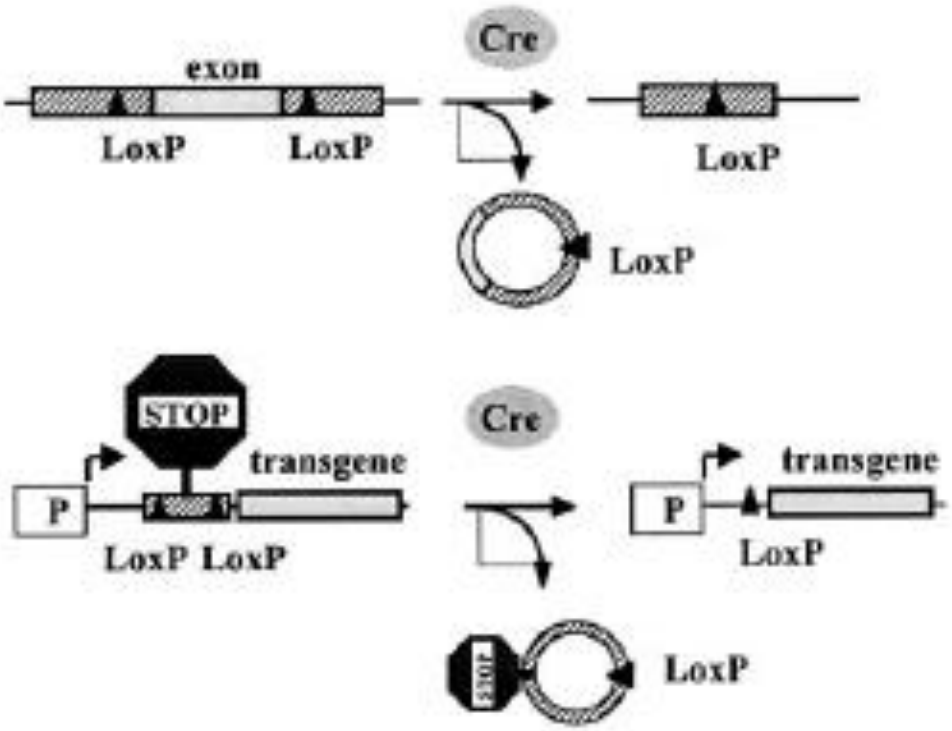


Figure 1-4. The Cre-lox recombination system. The Cre recombinase can be isolated from P1 phages of E. coli. It induces recombination between site-specific sequences called loxP, allowing for excision of the DNA flanked by these sequences. In transgenic mice, the expression of the Cre recombinase can be under the control of a tissue-specific or ubiquitous promoter. A floxed stop sequence can be placed between the promoter and the cDNA of interest. Thus, this cDNA cannot be expressed by tissues or cells lacking that Cre-recombinase. However, Cre recombinase expression excises the floxed stop sequence, allowing the cDNA to be expressed.

ACE KNOCKOUT MICE (ACE 1)

In our laboratory, these animals were generated by using targeted homologous recombination to insert a neomycin cassette into the 12th intron of the somatic ACE, and simultaneously disrupting the start codon for testis ACE. This strategy completely eliminated somatic and testis ACE, in an animal model we have termed ACE 1. As expected, these mice are hypotensive, have major renal impairment, with inability to concentrate urine in accordance with the role of the RAS in blood pressure and fluid balance. However, at the time, there were additional, unexpected phenotypes that were observed, including male fertility, mild anemia, and reduced fat to lean mass ratio (18, 50, 51). As will be discussed later, the ACE 1 mice also showed immune changes that implicated ACE in myelomonocytic cell maturation and function, as well as T cell function.

ACE 2

The phenotype of the complete ACE knockout mice was similar to that of the angiotensinogen or AT-1 deficient mice reported by other groups. Thus, it suggested the critical role of ACE as a key enzyme responsible for the generation of angiotensin II. However, as indicated earlier, male KO were also sterile, making breeding arduous since it required heterozygous males. This complete knockout model did not give any insight into which catalytic domain was central to the roles of ACE. In addition, the phenotype was too complex and multi-faceted when compared to wild-type mice. Furthermore, it did not distinguish the contribution of tissue bound versus secreted ACE in the phenotypes. To address some of these challenges, a second line of mice, called ACE 2, was produced in the laboratory. This strain was created by inserting a cDNA element containing exons 13-24 of the ACE gene into the 12th intron of the ACE 1 construct, in

front of a neomycin cassette (Figure 1-4). This technique maintains disruption of the testis ACE and truncates the N terminal active site and also ablates the carboxyl terminal hydrophobic tail required for anchoring the ACE protein to the cell membrane. In essence, resulting mice would only have somatic ACE, and this ACE would only be shed into circulation without being tissue-bound. This model allowed the investigation of the contribution of plasma versus tissue ACE in the functional physiology of the RAS. Analysis of this mouse showed systolic blood pressure that was identical to that of the ACE 1, or any of the angiotensinogen or AT-1 deficient strains, suggesting that circulating ACE was important in regulating blood pressure. The inference was that the reduction in the blood pressure was due to absent endothelial and/or renal ACE. It was also possible that the reduction in the total ACE protein alone was responsible for the renal phenotype in the ACE 2.

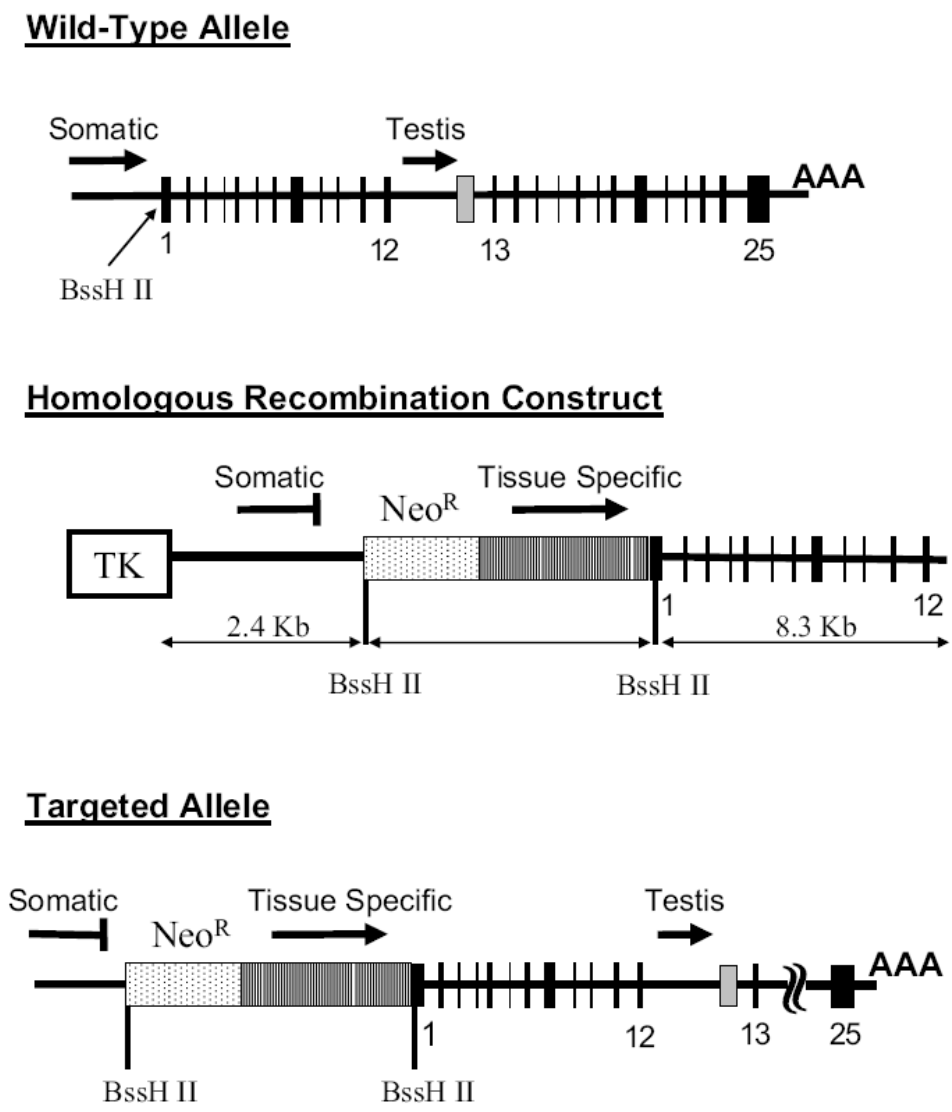


Figure 1-5. Gene targeting strategy. The top portion of the figure shows normal organization of the ACE allele, with somatic and testis promoters indicated by arrows. ACE exons 1–25 are shown by black rectangles. A unique BssHII restriction site is located between the transcription start site and the translation start site of somatic ACE. The start site for testis ACE transcription is indicated by a gray box between the 12th and 13th exons of somatic ACE. A targeting construct used for homologous recombination (middle diagram) contains 10.7 kb of homologous genomic DNA organized into left and right arms of 2.4 and 8.3 kb. Between these arms, we

incorporate the 3.1 kb KT3NP4 neomycin resistance cassette (NeoR) followed by a novel tissue-specific promoter such that the structural portion of the ACE gene would now be under the control of this new promoter. Effects of the somatic ACE promoter are minimized by positioning the neomycin cassette such that any transcripts generated by this promoter would terminate within the neomycin cassette. While not indicated in the diagram, this strategy does not affect the testis ACE promoter. A thymidine kinase (TK) cassette is positioned 5′ to the 2.4 kb homology arm. With proper homologous recombination, this will not be incorporated within the modified ACE allele (bottom diagram). (adapted from (52)). Essentially, this strategy can be employed to direct ACE expression specifically to the liver (using an albumin promoter), macrophages (using *c-fms* promoter) or any other tissue or organ of interest.

ACE 3

To address the issue of which source of ACE is the most important contributor to blood pressure regulation, our laboratory has taken advantage of gene targeting to express ACE in specific tissues. One simple idea was to create an animal that lacked endothelial ACE and yet made ACE in a tissue-specific manner. Thus, a strain of mice, called ACE 3, was generated in which an albumin promoter was used to drive ACE expression (Figure 1-6). These mice, by virtue of tissue-restricted expression, did not express endothelial (renal, pulmonary, etc) ACE. However, they did express testis ACE as the ACE gene is not disrupted in any way. Analysis of the ACE 3 surprisingly revealed normal blood pressure and renal function suggesting that baseline normotension was possible in the absence of endothelial ACE (41).

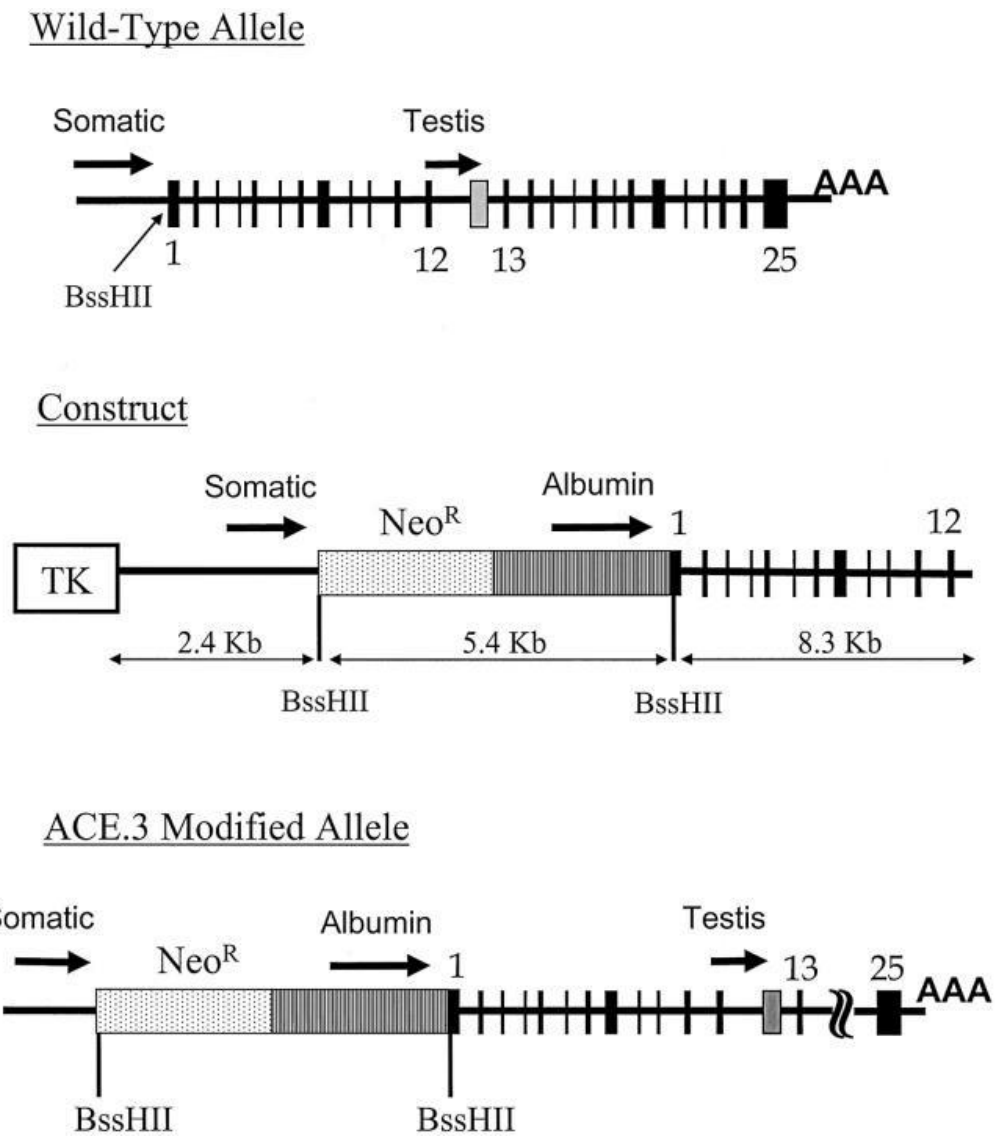


Figure 1-6. Targeting for liver-specific ACE expression (ACE 3). In the ACE, a properly targeted ACE allele (ACE.3 Modified Allele) positions a neomycin cassette to block the somatic ACE promoter and the albumin promoter to control somatic ACE expression. TK is a thymidine kinase resistance cassette used for negative selection of ES cells. Notice that somatic promoter, which directs expression to somatic tissues, and the testis promoter, which controls the expression of testis ACE in male germ cells are unaffected.

ACE 10

The ACE 3 model is a powerful model that demonstrates the redundancy of endothelial ACE for baseline blood pressure and fluid homeostasis. However, the ACE 3 is grossly artificial, as the liver of wild type mice does not express ACE. We used a *c-fms* promoter, specific for myelomonocytic cells to drive express ACE expression (Figure 1-7). This model, called ACE 10, was destined to serve two major purposes. First, it was a closer approximation than the ACE 3 in terms of physiology, since macrophages and some immune cells express ACE. Second, and perhaps more importantly, there has been reported upregulation of tissue ACE in cardiovascular diseases such as atherosclerosis, where ACE is markedly increased in foam cell-forming macrophages *in vivo*. Thus, the ACE 10 model allows not only an understanding of a non-endothelial (vascular and/or renal) requirement of ACE for blood pressure control, but also how alterations in tissue (specifically macrophage) ACE could impact cardiovascular disease. Of note, the ACE 10 mice presented with normal blood pressure (53). Since macrophages naturally express ACE albeit at low levels, we anticipated that the ACE 10 may be useful tool in delineating the role of ACE, if any, in myeloid differentiation and function.

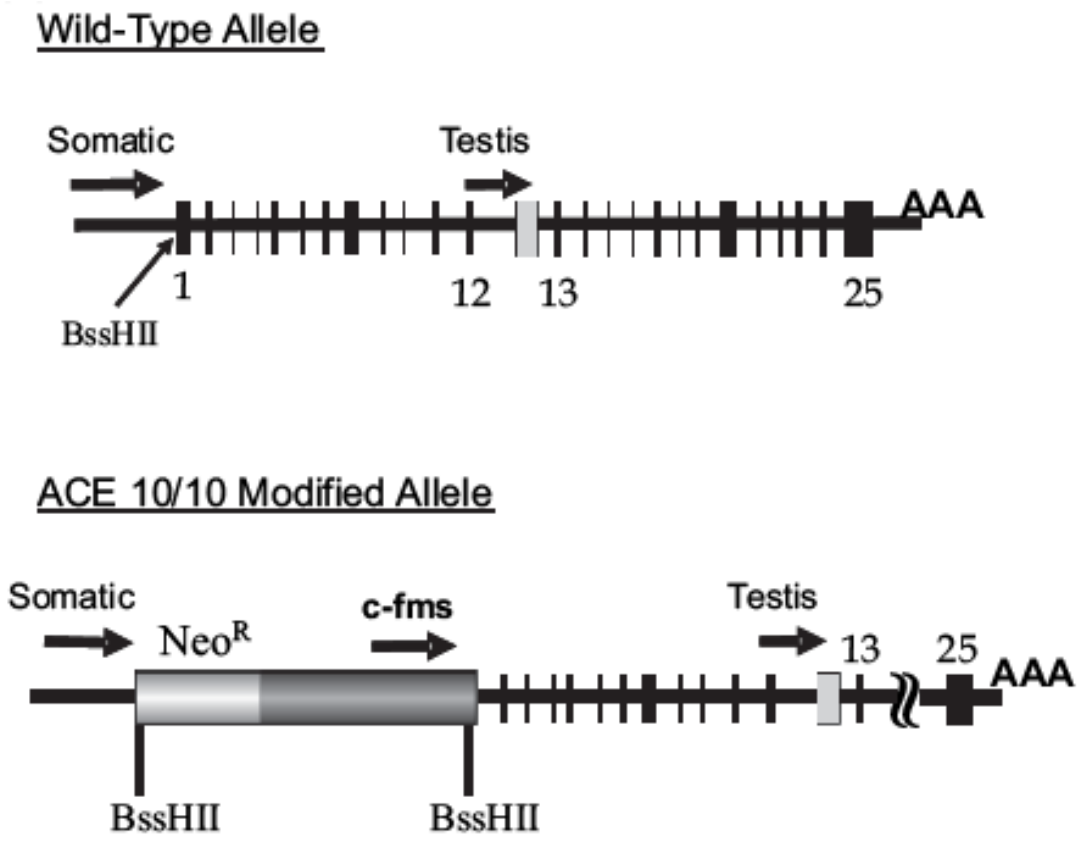


Figure 1-7. Targeting for macrophage-specific ACE. The top of the figure shows the wild-type organization of the ACE locus. Both the somatic ACE promoter and the testis ACE promoter are indicated with **arrows**. In the ACE 10/10 gene (bottom), homologous recombination was used to position a neomycin resistance cassette (Neo^R) and a 7.2-kb *c-fms* promoter cassette such that the structural portion of the ACE gene is now under the control of the *c-fms* promoter.

The RAS in immunity

Components of the RAS, pivotal for vascular tone and blood pressure control, are fittingly expressed in the kidney and the vasculature. However, a growing body of evidence has implicated the existence of a hematopoietic niche of the RAS. The significance of this local RAS in cardiovascular disease is bolstered by current discoveries that inflammatory cells play crucial role in hypertension, atherosclerosis and end-organ damage, mediated in part by their sensitivity to angiotensin II. The evolutionary preservation of the RAS in almost all immune cells also suggests that it may mediate the immune response beyond cardiovascular contexts. Indeed, the RAS may modify behavior of several immune cell types, including dendritic cell maturation and function, macrophage heterogeneity, T cell differentiation and function, and neutrophil recruitment to sites of injury. A careful review of the rapidly expanding new role of the RAS in inflammation may help unveil novel therapeutic targets in diseases such as cancer, as well as broaden our knowledge of the inter-relationship between inflammation and cardiovascular disease.

Systemic RAS influences Innate Immune response to Acute Inflammation

For years, it has been suspected that benefits of ACE inhibitor use in cardiovascular disease extended beyond blood pressure reduction. This observation has been supported by the accumulating findings that systemic RAS influences recruitment, phenotypic changes and behavior of several immune cell types during acute and chronic inflammation. For example, it is now known that RAS inhibition affects recruitments of inflammatory cells to the site of cardiac injury. Swirski et al demonstrated elegantly that the splenic reservoir of monocytes are deployed during acute cardiac injury migrate only in response to RAS signaling (54), and that ACE inhibitors prevent the release of these inflammatory cells into the circulation, which may explain

part of the benefits of this therapy in cardiovascular disease (55). During pulmonary inflammation, it has been observed that ACE inhibition influences migration of neutrophils and monocytes/macrophages to the lung via bradykinin and angiotensin modulation (56, 57). These observations are also replicated in neurological models, whereas AT1 receptor blockade inhibits recruitment and adhesion of PMN's to impact endothelial function of brain microvessicles (58, 59). Thus cardiac, pulmonary and neurological models of acute inflammation substantiate the critical role of the RAS in recruitment of inflammatory cells to these sites. These demonstrate that the systemic hormonal RAS influences immune cell responses. In all likelihood, it is the ability of these cells to respond to angiotensin II via their AT1 receptors that mediate these responses.

Local, immune-specific RAS influences systemic cardiovascular disease

There is now little doubt that all the components of the RAS are expressed by most immune cells, including T cells, B cells, dendritic cells, macrophages, NK cells and neutrophils. While these local RAS components seem dispensable for baseline regulation of blood pressure, there is evidence that the immune RAS could prove crucial during cardiovascular diseases such as hypertension. For example, RAG or SCID mice, both of which have defective T cells, have normal blood pressure (60, 61), suggesting that T cells do not seem to play any role in maintaining baseline normotension. However, angiotensin II induced hypertension is blunted in RAG or SCID (60, 61). More remarkably, while adoptive transfer of T cells restored Ang II sensitivity to Ang II hypertension, RAG mice transferred with T cells from AT1 deficient mice were still unresponsive to angiotensin II-induced hypertension (61). This suggests that T cells not only play an important role in cardiovascular disease, but that their AT1 expression status is what equips them to influence hypertension.

In addition to T cell RAS influencing hypertension, several groups have also shown that RAS of other immune cells, particularly monocytes and macrophages influence systemic angiotensin II-mediated cardiovascular pathologies. For example, bone marrow transplantation of mice RAS component knockout significantly reduces progression of atherosclerosis and other Angiotensin II-mediated cardiovascular disease. For example, Fukuda, and Tsubakimoto separately showed that atherosclerosis was reduced in ApoE mice that received bone marrow from AT1 deficient mice, but not bone marrow from from AT1 wild type mice (62, 63) . It is worth noting that these findings are seemingly in conflict with findings of other groups, in which absence of AT1 receptors on bone marrow cells actually exacerbates angiotensin II-induced hypertension (64) or atherosclerosis (65). Indeed, other groups found no difference in atherosclerotic lesion development, regardless of AT1 expression status of donor bone marrow cells (66). While further work is required to resolve the apparent discrepancies between these studies, these data together still suggest an important role of bone marrow RAS on cardiovascular disease, and may point to novel, finer therapeutic targets in these diseases.

Local RAS in immune responses beyond blood pressure

The RAS is fully expressed on a wide variety of immune cells. Clearly, this local RAS is dispensable for baseline blood pressure regulation (41, 64, 67). Is it possible then that this immune RAS has been evolutionarily conserved to mediate immune response outside of blood pressure and cardiovascular disease? We now consider role of RAS in T cell, DC and macrophage function.

The RAS in T cell function

One interesting observation has been the finding that T cells express all components of the RAS (68, 69). Endogenous RAS potentiate TNF and ROS generation by T cells (69), as well as CD69, a marker of T cell maturation. Perhaps, the full effect of RAS on T cell function was brought to bear in an autoimmune disease model, where ACE inhibitors and reduced Th1 and Th17 T cells, while inducing CD4+D25+FOxP3+ regulatory T cells (70, 71). Indeed, in the absence of AT1 receptors, spleen T cell proliferation is impaired, and this dampens the inflammatory response, permitting cardiac transplantation without overt host graft-host reaction (72). The availability of RAS gene knockouts (ACE, AT1, Agt knockouts) should facilitate understanding of the true extent of endogenous RAS on T cell function.

The RAS in Dendritic Cell function

Because T cell behavior is shaped, in part, by the antigen presenting cells with which they interact, perhaps it is not surprising that RAS can also influence dendritic cell function. Nahmod et al have shown that the RAS autocrine loop in DC is important for their differentiation, maturation, and function (73, 74). Since DC's are crucial cells in several immunotherapies, the extent of RAS agonists/antagonists on DC-mediated inflammatory responses it remains to be defined. Our unpublished observations indicate that an animal model in which the CD11c promoter has been used to drive ACE expression markedly augments antibody production when these mice are challenged by CFA-OVA.

The RAS in Neutrophil Function

Components of the RAS are expressed in both human and mouse neutrophils. Importantly, the expression of several proteases, including chymases, ensures ample generation

of angiotensin II, even separate from ACE activity (75). It is thought that endogenous Ang II can promote the differentiation of the myeloid cell line PLB-985 cells towards neutrophil or monocyte lineages (76) . It can also induce a dose-dependent activation of NADPH oxidase in neutrophils (76). Ang II stimulates neutrophil migration, which may have an effect on pulmonary injury and repair. Indeed, systemic ACE inhibition limits LPS-induced lung injury by interfering with bradykinin and AngII -regulated neutrophil recruitment (57). However, Ang II has been recently shown to enhance shedding of homing receptors (CD62L) on human neutrophils (77). Furthermore, there is evidence that ACE inhibitors could facilitate degranulation of PMN's (78). Thus, although it is clear that the RAS modulates neutrophil function, the complex dynamics are incompletely understood.

The RAS in NK cell function

Human NK cells do possess a functional RAS (79). Apparently, Angiotensin II induces mitogen and anti–CD3-stimulated NK cell proliferation (79). The relevant biological implications *in vivo* remain to be tested. In addition, murine studies have yet to confirm expression of RAS components by NK cells.

The RAS in Monocyte/Macrophages Function

Several lines of evidence suggested that ACE may play a critical role in shaping macrophage/monocyte function. First, granulomas such as sarcoidosis, are complex pro-inflammatory immune state and are notorious for high ACE expression (80). Second,

atherosclerotic plaque formation, in which macrophages play essential role, is highly enriched in ACE expression. ACE expression is increased in lipid laden macrophages, and co-localizes with CD68 positive monocytes/macrophages at the site of rupture of coronary atherosclerotic plaques (81, 82). Third, ACE inhibitors have proven beneficial for several diseases, ranging from stroke, diabetes, myocardial infarcts, glumerulonephritis, adriamycyn-induced nephrosis, and arthritis (45, 83, 84). The hallmark of all these pathologies states is an increased production of pro-inflammatory cytokine milieu, which may contribute to end-organ damage. This suggests that the benefits of ACE inhibitors extend beyond blood pressure. Indeed, *in vitro*, ACE inhibition and AT-1 receptor blockade reduce pro-inflammatory cytokine production by primary macrophages, as well as transformed macrophage cell lines, such as RAW 264.7 cells (85). It is thought that the reduction in these cytokines may in part explain the benefits of ACE inhibitors in a number of cardiovascular diseases.

Macrophages are a heterogenous group of mononuclear cells that exhibit extreme diversity, plasticity and functional adaptivity (86-88). In a simplified parallel to the Th1 and Th2 dichotomy of T cell polarization, macrophages can be polarized to mount M1 or M2 functional programs. Classical or M1 macrophage activation, in response IFN-g or microbial products (such as LPS and CpG), is characterized by high capacity to present antigen; high IL-12, low IL-10, and high production of nitric oxide (NO) and reactive oxygen intermediates. M1 macrophages are thus generally considered potent effector cells that kill intracellular micro-organisms and tumor cells. In contrast, alternative activation of macrophage (M2) is promoted by various signals such as IL-4 and IL-13, and these macrophages orchestrate resolution of the inflammatory response, adaptive Th2 immunity, and scavenge debris (86, 87, 89).

Beyond the external cytokine cues and their signaling pathways, the actual regulation of monocyte/macrophage commitment to and transition from these different phenotypes remains to be fully defined. It is unknown whether specific subsets of monocytes are pre-ordained to polarize to unique macrophage subsets. However, it is thought that macrophage heterogeneity is orchestrated by differentiation-related mechanisms and that the generated heterogenous macrophage phenotypes are then maintained by systemic in vivo or environmental cues (90). Further, transcriptional profiling has identified that genes associated with metabolic activities are a prominent feature of macrophage differentiation and polarization (91). In support of this concept, PPARγ is markedly elevated during monocyte differentiation into macrophages, and has been described as key regulator of the alternative activation of macrophages (92-94). The CCAAT family of transcription factors also demonstrates unique temporal expression patterns during development of bone marrow progenitor cells into monocytes and macrophages (95). Consequently, C/EBPα is crucial for macrophage maturation and function (96); C/EBPβ has been recently described to orchestrate the M2 program (97), while C/EBPδ is a potent amplifier of the NF-kβ signaling, enhancing the pro-inflammatory M1 response (98). Thus, the importance of early developmental process in the shaping of macrophage heterogeneity has been long noted (90).

Although ACE is traditionally associated with blood-pressure regulation, it is markedly increased during monocyte differentiation into macrophage (99, 100). In some *in vitro* reports, ACE is upregulated up to a 100 fold during differentiation of human monocytes to macrophages (101). This suggests that ACE may have an impact on macrophage polarization and heterogeneity. Recently it has been shown that the bone marrow AT1 receptors can mediate differentiation of monocyte lineage progenitors from hematopoietic stem cells. In that work, it was found that angiotensin II is required for TNF-α synthesis and release by stromal cells which

was important for M-CSF induced differentiation of myelomonocytic cells (102). Furthermore, using the model of anti-glomerular basement membrane (GBM) glomerulonephritis, Aki *et al.* have recently showed that the activation of RAS increases the inflammatory M1 macrophage infiltrate into the kidney during disease progression, and that AT-1 inhibition promoted the switch from M1 towards the anti-inflammatory, reparative M2 phenotype, thereby improving disease outcome (103).

While the above studies are informative, they are somewhat circumstantial and association studies, and do not provide direct evidence for the real role of ACE in macrophage behavior. To gain further insight into the mechanism by which ACE shapes monocyte/macrophage heterogeneity, we utilized the mouse model in which ACE is over-expressed in myelomoncytic cells, called the ACE 10. Immunological analysis of these mice showed that they were resistant to melanoma growth, with an increased CD8 T cell response. Central to the enhanced anti-tumor specific CD8 T cell response were macrophages that were polarized towards the so-called M1 inflammatory state with increased production of nitrites, IL-12 and a reduced production of IL-10 (53). Subsequently, we found that ACE also dramatically changes the antigen presenting capacity of these macrophages, enabling cells to process MHC class I antigens more effectively than their wild type counterparts. ACE inhibition also reduced antigen presentation (104). Thus, ACE may affect macrophage behavior, including their ability to present antigens and produce cytokines in response to various stimuli.

These remarkable effects of ACE over-expression on macrophages were powerfully complemented when we evaluated the immune functions of the ACE deficient (KO) mice. Earlier studies on the ACE KO had focused solely on their cardiovascular and hypotensive phenotype. However, we observed that ACE KO mice were characterized by poor, defective

myelopoiesis in the bone marrow, necessitating compensatory extramedullary myelopoiesis. C/EBPα, necessary for steady state myelopoeisis was lowly expressed. Mature macrophages from these mice also showed reduced ability to produce pro-inflammatory cytokines, in contrast to the enhanced cytokine production in the ACE 10 (105). Angiotensin II supplementation restored C/EBPα expression and improved the cytokine defects, suggesting that the RAS was critical for the macrophage development and function. We believe that the findings from this genetic ACE over-expression and inhibition provide the strongest but incomplete evidence yet for the direct role of ACE in shaping macrophage differentiation, polarization and function.

References

1. Corvol, P., X. Jeunemaitre, A. Charru, Y. Kotelevtsev, and F. Soubrier. 1995. Role of the renin-angiotensin system in blood pressure regulation and in human hypertension: new insights from molecular genetics. *Recent Prog Horm Res* 50:287-308.
2. Corvol, P., T. A. Williams, and F. Soubrier. 1995. Peptidyl dipeptidase A: angiotensin I-converting enzyme. *Methods Enzymol* 248:283-305.
3. Timmermans, P. B., P. C. Wong, A. T. Chiu, W. F. Herblin, P. Benfield, D. J. Carini, R. J. Lee, R. R. Wexler, J. A. Saye, and R. D. Smith. 1993. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* 45:205-251.
4. Inagami, T. 1998. A memorial to Robert Tiegerstedt: the centennial of renin discovery. *Hypertension* 32:953-957.
5. Yamakawa, T., S. Eguchi, Y. Yamakawa, E. D. Motley, K. Numaguchi, H. Utsunomiya, and T. Inagami. 1998. Lysophosphatidylcholine stimulates MAP kinase activity in rat vascular smooth muscle cells. *Hypertension* 31:248-253.
6. Goldblatt, H., J. Lynch, R. F. Hanzal, and W. W. Summerville. 1934. Studies on Experimental Hypertension : I. The Production of Persistent Elevation of Systolic Blood Pressure by Means of Renal Ischemia. *J Exp Med* 59:347-379.
7. Braun-Menendez, E., J. C. Fasciolo, L. F. Leloir, and J. M. Munoz. 1940. The substance causing renal hypertension. *J Physiol* 98:283-298.
8. Skeggs, L. T., Jr., K. E. Lentz, H. Hochstrasser, and J. R. Kahn. 1963. The purification and partial characterization of several forms of hog renin substrate. *J Exp Med* 118:73-98.

9. Skeggs, L. T., Jr., J. R. Kahn, K. Lentz, and N. P. Shumway. 1957. The preparation, purification, and amino acid sequence of a polypeptide renin substrate. *J Exp Med* 106:439-453.

10. Skeggs, L. T., Jr., J. R. Kahn, and N. P. Shumway. 1956. The purification of hypertensin II. *J Exp Med* 103:301-307.

11. Skeggs, L. T., Jr., W. H. Marsh, J. R. Kahn, and N. P. Shumway. 1954. The purification of hypertensin I. *J Exp Med* 100:363-370.

12. Bernstein, K. E., B. M. Martin, A. S. Edwards, and E. A. Bernstein. 1989. Mouse angiotensin-converting enzyme is a protein composed of two homologous domains. *J Biol Chem* 264:11945-11951.

13. Weiss, S., T. Doan, K. E. Bernstein, and N. Dascal. 2004. Modulation of cardiac Ca2+ channel by Gq-activating neurotransmitters reconstituted in Xenopus oocytes. *J Biol Chem* 279:12503-12510.

14. Fuchs, S., H. D. Xiao, J. M. Cole, J. W. Adams, K. Frenzel, A. Michaud, H. Zhao, G. Keshelava, M. R. Capecchi, P. Corvol, and K. E. Bernstein. 2004. Role of the N-terminal catalytic domain of angiotensin-converting enzyme investigated by targeted inactivation in mice. *J Biol Chem* 279:15946-15953.

15. Fuchs, S., H. D. Xiao, C. Hubert, A. Michaud, D. J. Campbell, J. W. Adams, M. R. Capecchi, P. Corvol, and K. E. Bernstein. 2008. Angiotensin-converting enzyme C-terminal catalytic domain is the main site of angiotensin I cleavage in vivo. *Hypertension* 51:267-274.

16. Howard, T. E., S. Y. Shai, K. G. Langford, B. M. Martin, and K. E. Bernstein. 1990. Transcription of testicular angiotensin-converting enzyme (ACE) is initiated within the 12th intron of the somatic ACE gene. *Mol Cell Biol* 10:4294-4302.

17. Langford, K. G., Y. Zhou, L. D. Russell, J. N. Wilcox, and K. E. Bernstein. 1993. Regulated expression of testis angiotensin-converting enzyme during spermatogenesis in mice. *Biol Reprod* 48:1210-1218.

18. Esther, C. R., Jr., T. E. Howard, E. M. Marino, J. M. Goddard, M. R. Capecchi, and K. E. Bernstein. 1996. Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Lab Invest* 74:953-965.

19. Krege, J. H., S. W. John, L. L. Langenbach, J. B. Hodgin, J. R. Hagaman, E. S. Bachman, J. C. Jennette, D. A. O'Brien, and O. Smithies. 1995. Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* 375:146-148.

20. Ramaraj, P., S. P. Kessler, C. Colmenares, and G. C. Sen. 1998. Selective restoration of male fertility in mice lacking angiotensin-converting enzymes by sperm-specific expression of the testicular isozyme. *J Clin Invest* 102:371-378.

21. Kim, H. S., J. H. Krege, K. D. Kluckman, J. R. Hagaman, J. B. Hodgin, C. F. Best, J. C. Jennette, T. M. Coffman, N. Maeda, and O. Smithies. 1995. Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A* 92:2735-2739.

22. Mezzano, S. A., M. Ruiz-Ortega, and J. Egido. 2001. Angiotensin II and renal fibrosis. *Hypertension* 38:635-638.

23. Crawford, D. C., A. V. Chobanian, and P. Brecher. 1994. Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. *Circ Res* 74:727-739.

24. Powell, J. S., J. P. Clozel, R. K. Muller, H. Kuhn, F. Hefti, M. Hosang, and H. R. Baumgartner. 1989. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* 245:186-188.

25. Weiss, D., J. J. Kools, and W. R. Taylor. 2001. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* 103:448-454.

26. Murphy, T. J., R. W. Alexander, K. K. Griendling, M. S. Runge, and K. E. Bernstein. 1991. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature* 351:233-236.

27. Nakajima, M., M. Mukoyama, R. E. Pratt, M. Horiuchi, and V. J. Dzau. 1993. Cloning of cDNA and analysis of the gene for mouse angiotensin II type 2 receptor. *Biochem Biophys Res Commun* 197:393-399.

28. Mukoyama, M., M. Nakajima, M. Horiuchi, H. Sasamura, R. E. Pratt, and V. J. Dzau. 1993. Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. *J Biol Chem* 268:24539-24542.

29. Chung, O., and T. Unger. 1998. Pharmacology of angiotensin receptors and AT1 receptor blockers. *Basic Res Cardiol* 93 Suppl 2:15-23.

30. Chung, O., H. Kuhl, M. Stoll, and T. Unger. 1998. Physiological and pharmacological implications of AT1 versus AT2 receptors. *Kidney Int Suppl* 67:S95-99.

31. Marrero, M. B., B. Schieffer, W. G. Paxton, L. Heerdt, B. C. Berk, P. Delafontaine, and K. E. Bernstein. 1995. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature* 375:247-250.

32. Schieffer, B., W. G. Paxton, M. B. Marrero, and K. E. Bernstein. 1996. Importance of tyrosine phosphorylation in angiotensin II type 1 receptor signaling. *Hypertension* 27:476-480.

33. Doan, T. N., M. S. Ali, and K. E. Bernstein. 2001. Tyrosine kinase activation by the angiotensin II receptor in the absence of calcium signaling. *J Biol Chem* 276:20954-20958.

34. Yusuf, S., C. J. Pepine, C. Garces, H. Pouleur, D. Salem, J. Kostis, C. Benedict, M. Rousseau, M. Bourassa, and B. Pitt. 1992. Effect of enalapril on myocardial infarction and unstable angina in patients with low ejection fractions. *Lancet* 340:1173-1178.

35. Pfeffer, M. A., E. Braunwald, L. A. Moye, L. Basta, E. J. Brown, Jr., T. E. Cuddy, B. R. Davis, E. M. Geltman, S. Goldman, G. C. Flaker, and et al. 1992. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. *N Engl J Med* 327:669-677.

36. Cambien, F., O. Poirier, L. Lecerf, A. Evans, J. P. Cambou, D. Arveiler, G. Luc, J. M. Bard, L. Bara, S. Ricard, and et al. 1992. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 359:641-644.

37. Billet, S., S. Bardin, S. Verp, V. Baudrie, A. Michaud, S. Conchon, M. Muffat-Joly, B. Escoubet, E. Souil, G. Hamard, K. E. Bernstein, J. M. Gasc, J. L. Elghozi, P. Corvol, and E. Clauser. 2007. Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *J Clin Invest* 117:1914-1925.

38. Ng, K. K., and J. R. Vane. 1968. Fate of angiotensin I in the circulation. *Nature* 218:144-150.

39. Ng, K. K., and J. R. Vane. 1967. Conversion of angiotensin I to angiotensin II. *Nature* 216:762-766.

40. Bernstein, K. E., H. D. Xiao, K. Frenzel, P. Li, X. Z. Shen, J. W. Adams, and S. Fuchs. 2005. Six truisms concerning ACE and the renin-angiotensin system educed from the genetic analysis of mice. *Circ Res* 96:1135-1144.

41. Cole, J., D. L. Quach, K. Sundaram, P. Corvol, M. R. Capecchi, and K. E. Bernstein. 2002. Mice lacking endothelial angiotensin-converting enzyme have a normal blood pressure. *Circ Res* 90:87-92.

42. Massiera, F., M. Bloch-Faure, D. Ceiler, K. Murakami, A. Fukamizu, J. M. Gasc, A. Quignard-Boulange, R. Negrel, G. Ailhaud, J. Seydoux, P. Meneton, and M. Teboul. 2001. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J* 15:2727-2729.

43. Fuchs, S., K. Frenzel, C. Hubert, R. Lyng, L. Muller, A. Michaud, H. D. Xiao, J. W. Adams, M. R. Capecchi, P. Corvol, B. D. Shur, and K. E. Bernstein. 2005. Male fertility is dependent on dipeptidase activity of testis ACE. *Nat Med* 11:1140-1142; author reply 1142-1143.

44. Bernstein, K. E. 2006. Views of the renin-angiotensin system: brilling, mimsy, and slithy tove. *Hypertension* 47:509-514.

45. Leehey, D. J., A. K. Singh, N. Alavi, and R. Singh. 2000. Role of angiotensin II in diabetic nephropathy. *Kidney Int Suppl* 77:S93-98.

46. Metzger, R., R. M. Bohle, P. Chumachenko, S. M. Danilov, and F. E. Franke. 2000. CD143 in the development of atherosclerosis. *Atherosclerosis* 150:21-31.

47. George, A. J., W. G. Thomas, and R. D. Hannan. 2010. The renin-angiotensin system and cancer: old dog, new tricks. *Nat Rev Cancer* 10:745-759.

48. Capecchi, M. R. 1994. Targeted gene replacement. *Sci Am* 270:52-59.

49. Sauer, B., and N. Henderson. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 85:5166-5170.

50. Esther, C. R., Jr., T. E. Howard, Y. Zhou, M. R. Capecchi, M. B. Marrero, and K. E. Bernstein. 1996. Lessons from angiotensin-converting enzyme-deficient mice. *Curr Opin Nephrol Hypertens* 5:463-467.

51. Esther, C. R., Jr., E. M. Marino, and K. E. Bernstein. 1997. The role of Angiotensin-converting enzyme in blood pressure control, renal function, and male fertility. *Trends Endocrinol Metab* 8:181-186.

52. Xiao, H. D., S. Fuchs, K. Frenzel, L. Teng, P. Li, X. Z. Shen, J. Adams, H. Zhao, G. T. Keshelava, K. E. Bernstein, and J. M. Cole. 2004. The use of knockout mouse technology to achieve tissue selective expression of angiotensin converting enzyme. *J Mol Cell Cardiol* 36:781-789.

53. Shen, X. Z., P. Li, D. Weiss, S. Fuchs, H. D. Xiao, J. A. Adams, I. R. Williams, M. R. Capecchi, W. R. Taylor, and K. E. Bernstein. 2007. Mice with enhanced macrophage angiotensin-converting enzyme are resistant to melanoma. *Am J Pathol* 170:2122-2134.

54. Swirski, F. K., M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J. L. Figueiredo, R. H. Kohler, A. Chudnovskiy, P. Waterman, E. Aikawa, T. R. Mempel, P. Libby, R. Weissleder, and M. J. Pittet. 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325:612-616.

55. Leuschner, F., P. Panizzi, I. Chico-Calero, W. W. Lee, T. Ueno, V. Cortez-Retamozo, P. Waterman, R. Gorbatov, B. Marinelli, Y. Iwamoto, A. Chudnovskiy, J. L. Figueiredo, D. E. Sosnovik, M. J. Pittet, F. K. Swirski, R. Weissleder, and M. Nahrendorf. 2010. Angiotensin-converting enzyme inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. *Circ Res* 107:1364-1373.

56. Yao, S., D. Feng, Q. Wu, K. Li, and L. Wang. 2008. Losartan attenuates ventilator-induced lung injury. *J Surg Res* 145:25-32.

57. Arndt, P. G., S. K. Young, K. R. Poch, J. A. Nick, S. Falk, R. W. Schrier, and G. S. Worthen. 2006. Systemic inhibition of the angiotensin-converting enzyme limits lipopolysaccharide-induced lung neutrophil recruitment through both bradykinin and angiotensin II-regulated pathways. *J Immunol* 177:7233-7241.

58. Ito, H., K. Takemori, and T. Suzuki. 2001. Role of angiotensin II type 1 receptor in the leucocytes and endothelial cells of brain microvessels in the pathogenesis of hypertensive cerebral injury. *J Hypertens* 19:591-597.

59. Yamakawa, H., M. Jezova, H. Ando, and J. M. Saavedra. 2003. Normalization of endothelial and inducible nitric oxide synthase expression in brain microvessels of spontaneously hypertensive rats by angiotensin II AT1 receptor inhibition. *J Cereb Blood Flow Metab* 23:371-380.

60. Crowley, S. D., Y. S. Song, E. E. Lin, R. Griffiths, H. S. Kim, and P. Ruiz. 2010. Lymphocyte responses exacerbate angiotensin II-dependent hypertension. *Am J Physiol Regul Integr Comp Physiol* 298:R1089-1097.

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

62. Fukuda, D., M. Sata, N. Ishizaka, and R. Nagai. 2008. Critical role of bone marrow angiotensin II type 1 receptor in the pathogenesis of atherosclerosis in apolipoprotein E deficient mice. *Arterioscler Thromb Vasc Biol* 28:90-96.

63. Cassis, L. A., D. L. Rateri, H. Lu, and A. Daugherty. 2007. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol* 27:380-386.

64. Crowley, S. D., Y. S. Song, G. Sprung, R. Griffiths, M. Sparks, M. Yan, J. L. Burchette, D. N. Howell, E. E. Lin, B. Okeiyi, J. Stegbauer, Y. Yang, P. L. Tharaux, and P. Ruiz. 2010. A role for angiotensin II type 1 receptors on bone marrow-derived cells in the pathogenesis of angiotensin II-dependent hypertension. *Hypertension* 55:99-108.

65. Kato, H., J. Ishida, K. Nagano, K. Honjo, T. Sugaya, N. Takeda, F. Sugiyama, K. Yagami, T. Fujita, M. Nangaku, and A. Fukamizu. 2008. Deterioration of atherosclerosis in mice lacking angiotensin II type 1A receptor in bone marrow-derived cells. *Lab Invest* 88:731-739.

66. Koga, J., K. Egashira, T. Matoba, M. Kubo, Y. Ihara, M. Iwai, M. Horiuchi, and K. Sunagawa. 2008. Essential role of angiotensin II type 1a receptors in the host vascular wall, but not the bone marrow, in the pathogenesis of angiotensin II-induced atherosclerosis. *Hypertens Res* 31:1791-1800.

67. Crowley, S. D., S. B. Gurley, M. I. Oliverio, A. K. Pazmino, R. Griffiths, P. J. Flannery, R. F. Spurney, H. S. Kim, O. Smithies, T. H. Le, and T. M. Coffman. 2005. Distinct roles for the kidney and systemic tissues in blood pressure regulation by the renin-angiotensin system. *J Clin Invest* 115:1092-1099.

68. Rasini, E., M. Cosentino, F. Marino, M. Legnaro, M. Ferrari, L. Guasti, A. Venco, and S. Lecchini. 2006. Angiotensin II type 1 receptor expression on human leukocyte subsets: a flow cytometric and RT-PCR study. *Regul Pept* 134:69-74.

69. Hoch, N. E., T. J. Guzik, W. Chen, T. Deans, S. A. Maalouf, P. Gratze, C. Weyand, and D. G. Harrison. 2009. Regulation of T-cell function by endogenously produced angiotensin II. *Am J Physiol Regul Integr Comp Physiol* 296:R208-216.

70. Stegbauer, J., D. H. Lee, S. Seubert, G. Ellrichmann, A. Manzel, H. Kvakan, D. N. Muller, S. Gaupp, L. C. Rump, R. Gold, and R. A. Linker. 2009. Role of the renin-angiotensin system in autoimmune inflammation of the central nervous system. *Proc Natl Acad Sci U S A* 106:14942-14947.

71. Platten, M., S. Youssef, E. M. Hur, P. P. Ho, M. H. Han, T. V. Lanz, L. K. Phillips, M. J. Goldstein, R. Bhat, C. S. Raine, R. A. Sobel, and L. Steinman. 2009. Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity. *Proc Natl Acad Sci U S A* 106:14948-14953.

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

73. Nahmod, K., C. Gentilini, M. Vermeulen, L. Uharek, Y. Wang, J. Zhang, H. P. Schultheiss, J. Geffner, and T. Walther. 2010. Impaired function of dendritic cells deficient in angiotensin II type 1 receptors. *J Pharmacol Exp Ther* 334:854-862.

74. Nahmod, K. A., M. E. Vermeulen, S. Raiden, G. Salamone, R. Gamberale, P. Fernandez-Calotti, A. Alvarez, V. Nahmod, M. Giordano, and J. R. Geffner. 2003. Control of dendritic cell differentiation by angiotensin II. *FASEB J* 17:491-493.

75. Reilly, C. F., D. A. Tewksbury, N. M. Schechter, and J. Travis. 1982. Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *J Biol Chem* 257:8619-8622.

76. Hazan-Halevy, I., T. Levy, T. Wolak, I. Lubarsky, R. Levy, and E. Paran. 2005. Stimulation of NADPH oxidase by angiotensin II in human neutrophils is mediated by ERK, p38 MAP-kinase and cytosolic phospholipase A2. *J Hypertens* 23:1183-1190.

77. Vega, A., R. El Bekay, P. Chacon, I. Ventura, and J. Monteseirin. 2010. Angiotensin II induces CD62L shedding in human neutrophils. *Atherosclerosis* 209:344-351.

78. Miselis, J., T. Siminiak, and H. Wysocki. 1994. Evidence for stimulation of neutrophil degranulation by selected angiotensin converting enzyme inhibitors in vitro. *J Hum Hypertens* 8:565-569.

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

80. Silverstein, E., J. Friedland, H. A. Lyons, and A. Gourin. 1976. Markedly elevated angiotensin converting enzyme in lymph nodes containing non-necrotizing granulomas in sarcoidosis. *Proc Natl Acad Sci U S A* 73:2137-2141.

81. Ribichini, F., F. Pugno, V. Ferrero, G. Bussolati, M. Feola, P. Russo, C. Di Mario, A. Colombo, and C. Vassanelli. 2006. Cellular immunostaining of angiotensin-converting enzyme in human coronary atherosclerotic plaques. *J Am Coll Cardiol* 47:1143-1149.

82. Komohara, Y., J. Hirahara, T. Horikawa, K. Kawamura, E. Kiyota, N. Sakashita, N. Araki, and M. Takeya. 2006. AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. *J Histochem Cytochem* 54:763-771.

83. Dalbeth, N., J. Edwards, S. Fairchild, M. Callan, and F. C. Hall. 2005. The non-thiol angiotensin-converting enzyme inhibitor quinapril suppresses inflammatory arthritis. *Rheumatology (Oxford)* 44:24-31.

84. Katragadda, S., and R. R. Arora. 2010. Role of angiotensin-converting enzyme inhibitors in vascular modulation: beyond the hypertensive effects. *Am J Ther* 17:e11-23.

85. Hagiwara, S., H. Iwasaka, S. Matumoto, S. Hidaka, and T. Noguchi. 2009. Effects of an angiotensin-converting enzyme inhibitor on the inflammatory response in in vivo and in vitro models. *Crit Care Med* 37:626-633.

86. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

87. Mantovani, A., A. Sica, and M. Locati. 2005. Macrophage polarization comes of age. *Immunity* 23:344-346.

88. Stout, R. D., C. Jiang, B. Matta, I. Tietzel, S. K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 175:342-349.

89. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 25:677-686.

90. Witsell, A. L., and L. B. Schook. 1991. Macrophage heterogeneity occurs through a developmental mechanism. *Proc Natl Acad Sci U S A* 88:1963-1967.

91. Martinez, F. O., S. Gordon, M. Locati, and A. Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177:7303-7311.

92. Bouhlel, M. A., B. Derudas, E. Rigamonti, R. Dievart, J. Brozek, S. Haulon, C. Zawadzki, B. Jude, G. Torpier, N. Marx, B. Staels, and G. Chinetti-Gbaguidi. 2007.

PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* 6:137-143.

93. Chinetti, G., S. Griglio, M. Antonucci, I. P. Torra, P. Delerive, Z. Majd, J. C. Fruchart, J. Chapman, J. Najib, and B. Staels. 1998. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 273:25573-25580.

94. Odegaard, J. I., R. R. Ricardo-Gonzalez, M. H. Goforth, C. R. Morel, V. Subramanian, L. Mukundan, A. Red Eagle, D. Vats, F. Brombacher, A. W. Ferrante, and A. Chawla. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 447:1116-1120.

95. Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman. 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood* 80:1725-1735.

96. Heath, V., H. C. Suh, M. Holman, K. Renn, J. M. Gooya, S. Parkin, K. D. Klarmann, M. Ortiz, P. Johnson, and J. Keller. 2004. C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood* 104:1639-1647.

97. Ruffell, D., F. Mourkioti, A. Gambardella, P. Kirstetter, R. G. Lopez, N. Rosenthal, and C. Nerlov. 2009. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A* 106:17475-17480.

98. Litvak, V., S. A. Ramsey, A. G. Rust, D. E. Zak, K. A. Kennedy, A. E. Lampano, M. Nykter, I. Shmulevich, and A. Aderem. 2009. Function of C/EBPdelta in a regulatory

circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 10:437-443.

99. Danilov, S. M., E. Sadovnikova, N. Scharenborg, I. V. Balyasnikova, D. A. Svinareva, E. L. Semikina, E. N. Parovichnikova, V. G. Savchenko, and G. J. Adema. 2003. Angiotensin-converting enzyme (CD143) is abundantly expressed by dendritic cells and discriminates human monocyte-derived dendritic cells from acute myeloid leukemia-derived dendritic cells. *Exp Hematol* 31:1301-1309.

100. Okamura, A., H. Rakugi, M. Ohishi, Y. Yanagitani, S. Takiuchi, K. Moriguchi, P. A. Fennessy, J. Higaki, and T. Ogihara. 1999. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. *J Hypertens* 17:537-545.

101. Ohishi, M., M. Ueda, H. Rakugi, A. Okamura, T. Naruko, A. E. Becker, K. Hiwada, A. Kamitani, K. Kamide, J. Higaki, and T. Ogihara. 1997. Upregulation of angiotensin-converting enzyme during the healing process after injury at the site of percutaneous transluminal coronary angioplasty in humans. *Circulation* 96:3328-3337.

102. Tsubakimoto, Y., H. Yamada, H. Yokoi, S. Kishida, H. Takata, H. Kawahito, A. Matsui, N. Urao, Y. Nozawa, H. Hirai, J. Imanishi, E. Ashihara, T. Maekawa, T. Takahashi, M. Okigaki, and H. Matsubara. 2009. Bone marrow angiotensin AT1 receptor regulates differentiation of monocyte lineage progenitors from hematopoietic stem cells. *Arterioscler Thromb Vasc Biol* 29:1529-1536.

103. Aki, K., A. Shimizu, Y. Masuda, N. Kuwahara, T. Arai, A. Ishikawa, E. Fujita, A. Mii, Y. Natori, Y. Fukunaga, and Y. Fukuda. 2010. ANG II receptor blockade enhances anti-inflammatory macrophages in anti-glomerular basement membrane glomerulonephritis. *Am J Physiol Renal Physiol* 298:F870-882.

104. Shen, X. Z., A. E. Lukacher, S. Billet, I. R. Williams, and K. E. Bernstein. 2008. Expression of angiotensin-converting enzyme changes major histocompatibility complex class I peptide presentation by modifying C termini of peptide precursors. *J Biol Chem* 283:9957-9965.

105. Lin, C., V. Datta, D. Okwan-Duodu, X. Chen, S. Fuchs, R. Alsabeh, S. Billet, K. E. Bernstein, and X. Z. Shen. 2010. Angiotensin-converting enzyme is required for normal myelopoiesis. *FASEB J.*

CHAPTER 2: RESPONSE OF ACE10/10 MICE TO INFECTION

The major physiological characterization of the ACE 10 mice had been completed by Dr Xiao Shen when I joined the Bernstein Lab. Despite myeloid-cell specific expression of ACE- and none in the endothelium-, these mice had normal blood pressure. However, it was the unexpected immunological response in the ACE 10 that intrigued me the most. Even more captivating, these effects seem independent of Angiotensin II, the major bioactive product of ACE. That raised the possibility of the identification of a novel, perhaps Angiotensin-II –independent roles of ACE in immunity. The experimental tumor model is quite artificial. Hence, we wanted to extrapolate the tumor finding to an infection model, in order to fully understand the ACE 10, and also make a stronger case for a role of ACE in immunity. If our prediction of the enhanced M1 macrophage response by the ACE 10 was accurate, certainly these mice should elicit a robust response to an infectious model. I started studying the response of the ACE 10 to *Listeria monocytogenes* infection, with the help of Pablo Penaloza, a PhD friend in Rafi Ahmed's lab (Emory University). However, at the inception of this project, the Bernstein laboratory re-located to Cedars-Sinai Medical Center. After a few months of re-establishing ourselves, we formed a strong partnership with Dr George Liu, an expert on immunity to Staphyloccocal infection at the Immunology Institute at Cedars-Sinai Medical Center. I was also fortunate to partner with Dr Vivekanand Datta, a fellow at the Pathology Department at Cedars-Sinai Medical Center, and an experienced microbiologist as well. What followed from our discussion and deliberation was a set of studies in which we identified a remarkable ability of ACE over-expression in myeloid cells to shape the inflammatory response to bacterial infections. This work was published in The Journal of Biological Chemistry 2010;285(50):39051-60, and the complete text of this manuscript follows. I am indebted to my

co-authors for their tireless contribution to completing this work. In particular, Dr Anand advanced the quality of this work with his input on the *S. aureus* studies. He was instrumental, tireless, and pleasant to work with. Dr Liu was an ever-present mentor and extremely generous with his time, and Dr. Helen Goodridge played no small role with her help on some of the *in vitro* studies.

ACE OVER-EXPRESSION IN MOUSE MYELOMONOCYTIC CELLS AUGMENTS RESISTANCE TO *LISTERIA* AND METHICILLIN RESISTANT *S. AUREUS*.*

Derick Okwan-Duodu[1,4], Vivekanand Datta[2], Xiao Z. Shen[1,2], Helen S. Goodridge[1], Ellen A. Bernstein[1], Sebastien Fuchs[1,2], George Y. Liu[1,3], Kenneth E. Bernstein[1,2]

From [1]Department of Biomedical Sciences, [2]Department of Pathology and Laboratory Medicine, [3]Department of Pediatrics, Cedars-Sinai Medical Center, Los Angeles, California, 90048 USA

[4] Emory University School of Medicine, Atlanta, GA, 30322 USA

Running Head: ACE augments bacterial innate immunity via increased iNOS

Address correspondence to:

Kenneth E. Bernstein, M.D.,

110 N. George Burns Rd, Davis 2021

Los Angeles, CA 90048, USA.

Telephone: 310-423-7562;

Fax: 310-423-7331;

Email: kbernst@cshs.org

The abbreviations used are: ACE, angiotensin converting enzyme; RAS, renin angiotensin system; TPM, thioglycollate elicited peritoneal macrophages

Abstract

Gene targeting in ES cells was used to substitute control of angiotensin converting enzyme (ACE) expression from the endogenous promoter to the mouse c-fms promoter. The result is an animal model called ACE 10/10 in which ACE is over expressed by monocytes, macrophages and other myelomonocytic lineage cells. To study the immune response of these mice to bacterial infection, we challenged them with L. monocytogenes or methicillin resistant S. aureus (MRSA). ACE 10/10 mice have a significantly enhanced immune response to both bacteria *in vivo* and *in vitro*. For example, 5 days after Listeria infection, the spleen and liver of ACE 10/10 mice had 8.0- and 5.2-fold less bacteria than wild type mice (WT). In a model of MRSA skin infection, ACE 10/10 mice had 50-fold less bacteria than WT mice. Histologic examination showed a prominent infiltrate of ACE positive mononuclear cells in the skin lesions from ACE 10/10. Increased bacterial resistance in ACE 10/10 is directly due to over-expression of ACE, as it is eliminated by an ACE inhibitor. Critical to increased immunity in ACE 10/10 is the over-expression of iNOS and reactive nitrogen intermediates, as inhibition of iNOS by the inhibitor 1400W eliminated all in vitro and in vivo differences in innate bacterial resistance between ACE 10/10 and WT mice. Increased resistance to MRSA was transferable by bone marrow transplantation. The over-expression of ACE and iNOS by myelomonocytic cells substantially boosts innate immunity and may represent a new means to address serious bacterial infections.

Introduction

The classical renin-angiotensin system (RAS) is a complex cascade of enzymes and peptides associated with the regulation of blood pressure (1). RAS regulates production of the vasoconstrictor angiotensin II from angiotensinogen via sequential catalysis by renin and angiotensin-converting enzyme (ACE). While renin is precise in substrate specificity, ACE is a somewhat promiscuous peptidase that cleaves angiotensin I, substance P, AcSDKP, β-endorphins, and several other physiologic peptides. Since ACE has variety of substrates, it may affect several physiologic and pathologic processes such as hematopoiesis, fertility, atherosclerosis, multiple sclerosis and inflammation (2-5).

Several groups have presented evidence highlighting diverse roles for ACE and the RAS in the immune response, including production of reactive oxygen species by angiotensin II (6-8). Recently, Platten *et al.* showed that the RAS plays a major role in autoimmunity, as indicated by their analysis of multiple sclerosis and experimental autoimmune encephalitis (9). Studies have also found a role for RAS in the recruitment of inflammatory cells into tissues through the regulation of adhesion molecules and chemokines by resident inflammatory cells (7). ACE regulates bradykinin, which is critical for dendritic cell maturation and TH1 cell development during *T. cruzi* infection (10). Clinically, ACE is a useful marker for several cytokine-mediated inflammatory diseases (11).

Our group has recently described a mouse model called ACE 10/10 in which gene targeting in ES cells was used to substitute control of ACE expression from the endogenous promoter to the mouse *c-fms* promoter (12). The result is an animal model in which ACE is over expressed by monocytes, macrophages and other myelomonocytic lineage cells. These mice demonstrated a robust immunological response when challenged with mouse models of melanoma and lymphoma. Specifically, ACE 10/10 mice resisted tumor growth far better than

wild-type (WT) mice. This was correlated with increased numbers of anti-tumor $CD8^+$ T-cells. Further, when compared to WT cells, ACE 10/10 macrophages showed an increased pro-inflammatory phenotype, characterized by increased infiltration of the tumors, increased production of pro-inflammatory cytokines, and superior processing of tumor antigens (13). While this phenotype was eliminated by ACE inhibitors, the production of angiotensin II was probably not crucial in tumor resistance. Rather, ACE over-expression in myelomonocytic cells seemed to trigger a more profound immune response in which the animals responded more vigorously than WT mice to immunologic challenge.

While the finding that ACE 10/10 mice resist the growth of tumors better than WT mice is exciting, tumor growth models in mice are somewhat artificial. This led us to ask whether the enhanced immune response observed in ACE 10/10 mice would also be present when these animals were exposed to two models of bacterial infection: *Listeria monocytogenes* (*Listeria*), a gram positive rod shaped bacterium that is a much studied intracellular pathogen, and methicillin resistant *Staphylococcus aureus* (MRSA), an aggressive extracellular microorganism that is increasing in prevalence and incidence (14, 15). The spread of community associated (CA) MRSA is an important public health concern because these bacteria can be particularly virulent. Here, we report that ACE 10/10 mice have a substantially enhanced innate immune response to both *Listeria* and MRSA *in vivo* and *in vitro*. For example, in a model of skin infection with MRSA, ACE 10/10 mice had a 50-fold lower load of bacteria than WT mice. The increased resistance to bacteria was directly due to over-expression of catalytically active ACE by myelomonocytic cells as it was eliminated by an ACE inhibitor. Further, we found that the over-expression of iNOS was critical to the mechanism of increased innate immunity, since a specific iNOS inhibitor, 1400W, reduced the resistance to bacteria of ACE 10/10 mice to WT levels. Thus, our data show that the manipulation of ACE in myelomonocytic cells markedly increases

the immune response not only to mouse models of tumor but to aggressive forms of bacterial infection. These data offer new approaches for increasing innate resistance to serious bacterial infections such as CA-MRSA.

Experimental Procedures

Ethics- All experimental procedures were pre-approved by the Cedars-Sinai Institutional Animal Care & Use Committee (CSMC IACUC) and were performed according to the guidelines of the Cedars-Sinai Division of Comparative Medicine and the CSMC IACUC.

Materials- Lipopolysaccharide (LPS) (Escherichia coli 055:B5) was from Sigma-Aldrich (St. Louis, MO). Murine IL-6, IFN-γ, TNF-α and IL-12/p40 ELISA kits were purchased from eBioscience (San Diego, CA). Griess assay reagents were from Promega (Madison, WI). ACE activity was measured using the ACE-REA kit from American Laboratory Products Company, Ltd. (ALPCO, Windham, NH) as previously described (16).

Mice- ACE 10/10 mice have been previously described (12). These mice were back-crossed at least 10 generations to C57BL/6 mice (Jackson Laboratory, Bar Harbor ME). All mice were maintained in micro isolator cages.

Bacterial Infection- *L. monocytogenes* strain EGD was a gift from Dr Rafi Ahmed (Emory University, GA). Bacteria were propagated overnight on brain heart infusion. This was then diluted 1:100 in fresh media and incubated at 37°C with shaking at 250 rpm until A540 of about 2.5. The bacteria were then washed in phosphate buffered saline (PBS) and stored in PBS with 30% glycerol at -80^{o}C at a concentration of 1X10^{8}/ml. For each experiment, a fresh vial was thawed and bacteria were washed in PBS and diluted appropriately. Mice were injected intravenously with 100 µl of bacteria in PBS. Livers and spleens from infected mice were

harvested at different time points after infection, and CFUs were calculated by plating serial dilutions of tissue homogenates on brain heart infusion plates (Hardy Diagnostics, CA).

S. aureus USA 300, strain SF8300, was obtained from Dr Binh Diep (UCSF, CA). Bacteria were routinely cultured in Todd-Hewitt broth at 37°C with shaking at 250 rpm. An overnight bacterial culture was diluted 1:1000 in pre-warmed media and incubated at 37°C with shaking at 250 rpm until A540 of about 2.5. Bacteria were immediately pelleted by centrifugation at 4000 rpm for 10 min at 4°C, washed twice with equal volume of PBS, and resuspended in PBS at a concentration of 2 x 10^{10} CFU/ml. This was mixed with an equal volume of PBS containing dextran microbeads (Cytodex) suspension at 50 mg/100 ml. 100 µL of this suspension was injected subcutaneously in each flank of mice. Skin lesions were defined by darkened areas of necrosis, and lesion size was quantified over the course of 4 days by a protocol previously described (17). Briefly, the length and width of the lesion was multiplied, and irregularly-shaped lesions were broken down into smaller symmetrical pieces and measured. On day four, mice were euthanized; skin lesion was measured, excised, weighed and homogenized in 1 ml of PBS for CFU determination by serial dilutions.

Inhibitors- ALZET osmotic minipumps (Cupertino, CA) were placed subcutaneously, following manufacturer's instructions. ACE inhibitors ramipril (Roxane Laboratories, OH) or lisinopril (Merck, NJ) was dosed at 16 mg/kg/day, losartan (LKT Laboratories, MN) was dosed at 30 mg/kg/ day, and hydralazine (Sigma MO) was administered at 40 mg/kg/day for 1 week in the drinking water. Tail cuff blood pressure measurements were performed as previously described to verify effectiveness of the drugs in reducing blood pressure (*42*). The iNOS specific inhibitor 1400W (Cayman, MI) was administered at 50 µg/ml in drinking water. Treatment was initiated on the day of infection and was maintained until the termination of the experiment.

Bone Marrow Transplantation- Enhanced green fluorescent protein transgenic mice (TgN(beta-act-EGFP)04Obs) (18) which have been back-bred at least 10 generations to a C57BL/6 background (Jackson Laboratories, ME) were a gift from Dr David Archer (Emory University, GA). Homozygous ACE 10/10 mice expressing GFP (ACE 10/10-GFP) were obtained by a classical breeding scheme. For bone marrow transplantation, donor mice were 8-week-old ACE 10/10-GFP or littermate WT/ WT-GFP; recipients were 8-week-old WT C57BL/6 that did not express GFP. Bone marrow was obtained from donor mice by flushing femurs and tibias with RPMI 1640 medium. Nucleated cells were counted, and the bone marrow was resuspended at a concentration of 2 x 10^7/ml. Recipient mice were irradiated with 1100 rads and immediately reconstituted with either 2 x 10^6 ACE 10/10-GFP or WT/ WT-GFP bone marrow cells, as previously described (12). After 8 weeks, the recipient mice were analyzed by FACS for blood leukocyte expression of GFP, ACE and CD45.

Macrophage Isolation and in vitro Bacterial Infection- To collect TPMs, 6-8 week old mice were injected intraperitoneally with 2 ml aged thioglycollate broth. Four days later, macrophages were harvested from the peritoneal cavity by lavaging with sterile Hank's phosphate-buffered saline. The cells were centrifuged at 400 x g for 10 min at 4°C and resuspended in complete media: RPMI 1640 (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Mediatech). 10^5 macrophages were distributed into wells of 96-well plates. After 2h incubation at 37°C, nonadherent cells were washed away, leaving wells with confluent adherent macrophages. After 18hr culture in complete media with or without 10 ng/ml IFN-γ, cells were infected with 10^6 bacteria (multiplicity of infection of 10) in 100 µL antibiotic-free media. After 1 hr of phagocytosis, non-incorporated bacteria were removed by washing, and complete media was

supplemented with 5 μg/ml gentamicin to kill any residual extracellular bacteria. In some experiments, macrophages were treated with 10 μM of the specific iNOS inhibitor 1400W at the time of the priming with IFN-γ. At different time points, cells were washed, then lysed with 0.01% Triton in PBS, and viable bacteria ascertained by dilution and plating on brain heart infusion plates.

Nitrite accumulation- Nitrite anion production by macrophages was determined with the Griess assay using the manufacturer's instructions (Promega, Madison, WI). Briefly, macrophage monolayers were stimulated with 10 ng/ml IFN-γ for 18 hrs and supernatants were harvested. In some experiments, the macrophage monolayers were stimulated with 10 ng/ml IFN-γ and then infected with *L. monocytogenes* at a multiplicity of 10 or 50 bacteria per cell. A total of 50 μl of culture supernatant was combined with an equal volume of the Griess reagent, and the samples were incubated at room temperature for 10 minutes before quantifying the absorbance at 540 nm. The nitrite production was determined using a standard curve and normalized to total cell number in each sample.

Western Blotting- 10^7 thioglycollate-elicited peritoneal macrophages were stimulated with 1 μg/ml LPS overnight. Cells were washed with ice cold PBS and lysed in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1% NP-40 for 20 min. The lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatants were collected. After protein quantification using the Pierce protein assay, 40 mg of protein were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were then washed in Tris-Tween-buffered saline (TTBS, 20 mM Tris-HCl buffer, pH 7.6, 137 mM NaCl and 0.05% (vol/vol)

Tween 20), and blocked overnight with 5% (wt/vol) nonfat dry milk solution in TTBS. Subsequently, the membrane was probed with a 1:600 dilution of polyclonal iNOS antibody (a kind gift of Dr David Harrison, Emory University, GA) or a 1:1000 dilution of anti-arginase 1 antibody (BD Transduction, KY) in 5% (wt/vol) nonfat milk in TTBS. The blot was revealed by enhanced chemiluminescence (GE Healthcare UK Limited). The membranes were probed with β actin (Cell Signaling, Danvers, MA) to verify loading.

Immunohistochemistry- Tissue was embedded in paraffin using standard techniques. 5 micron sections were cut and steamed for 30 min in buffer, pH 6.1 (Dako #S1699). They were then stained in a Dako automated stainer with a 1:200 dilution of a rabbit anti-ACE antibody (19). Secondary antibody (Dako #K4003) and DAB chromogen (Dako #3468) were performed according to the manufacture's instructions.

Statistics- All data are expressed as the arithmetic means +/- SEM. Comparisons between two groups of animals or treatments were made by one-way ANOVA or student's T test. Values of $p<0.05$ were considered statistically significant.

Results

Increased Resistance to L. monocytogenes Infection in ACE 10/10 Mice.

We tested the response of ACE 10/10 mice to *Listeria* infection *in vivo* and *in vitro*. To test *in vivo* bacterial burden, WT and ACE 10/10 mice were injected intravenously (i.v.) with 4 X 10^3 CFU of *Listeria*, strain EGD. The mice were sacrificed three or five days after infection, and bacterial counts were determined in spleen and liver. In both spleen and liver, the ACE 10/10 mice consistently had substantially fewer bacteria (Fig. 1a). At day 3 after infection, the bacteria burden in the spleen and liver of WT mice was 6.5- and 5.5-fold more than that of the equivalent ACE 10/10 tissues (n=at least 9, $p<0.01$). At day 5, the spleen and liver of WT mice had an 8.0- and 5.2-fold greater bacterial load than those of the ACE 10/10 mice (n=at least 9, liver: $p<0.002$; spleen: $p<0.003$).

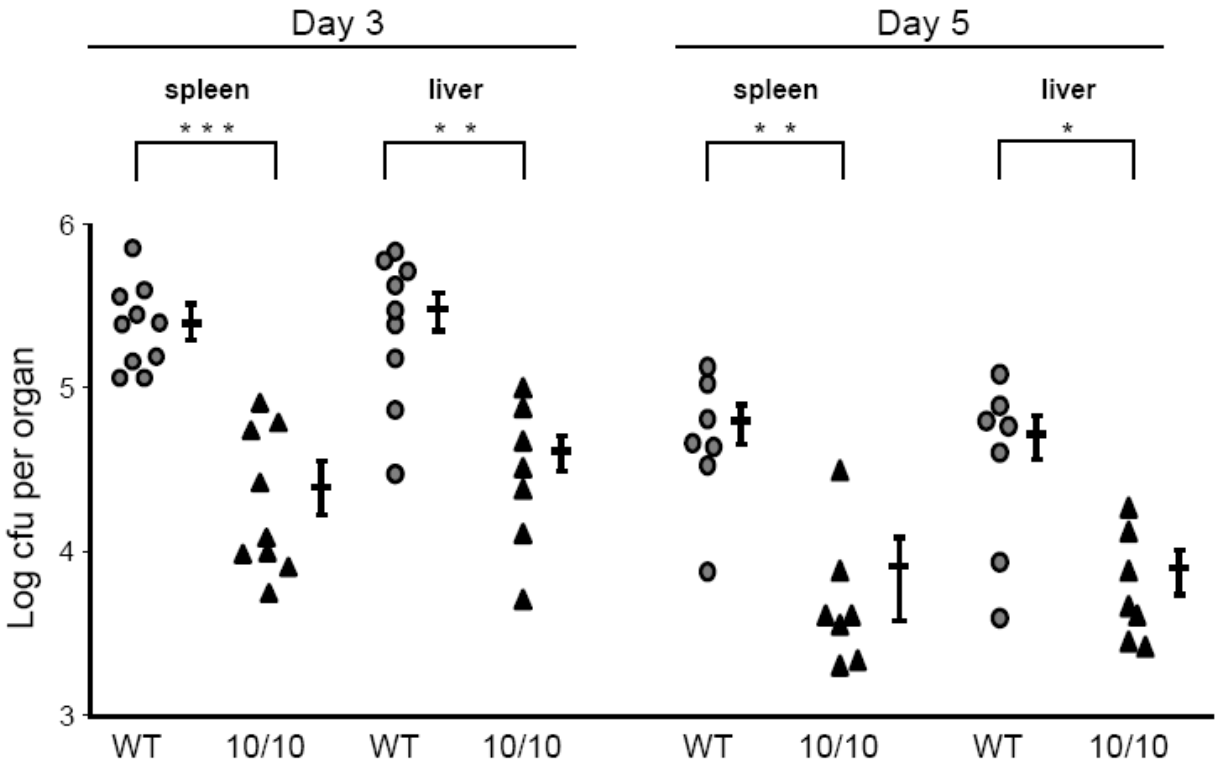
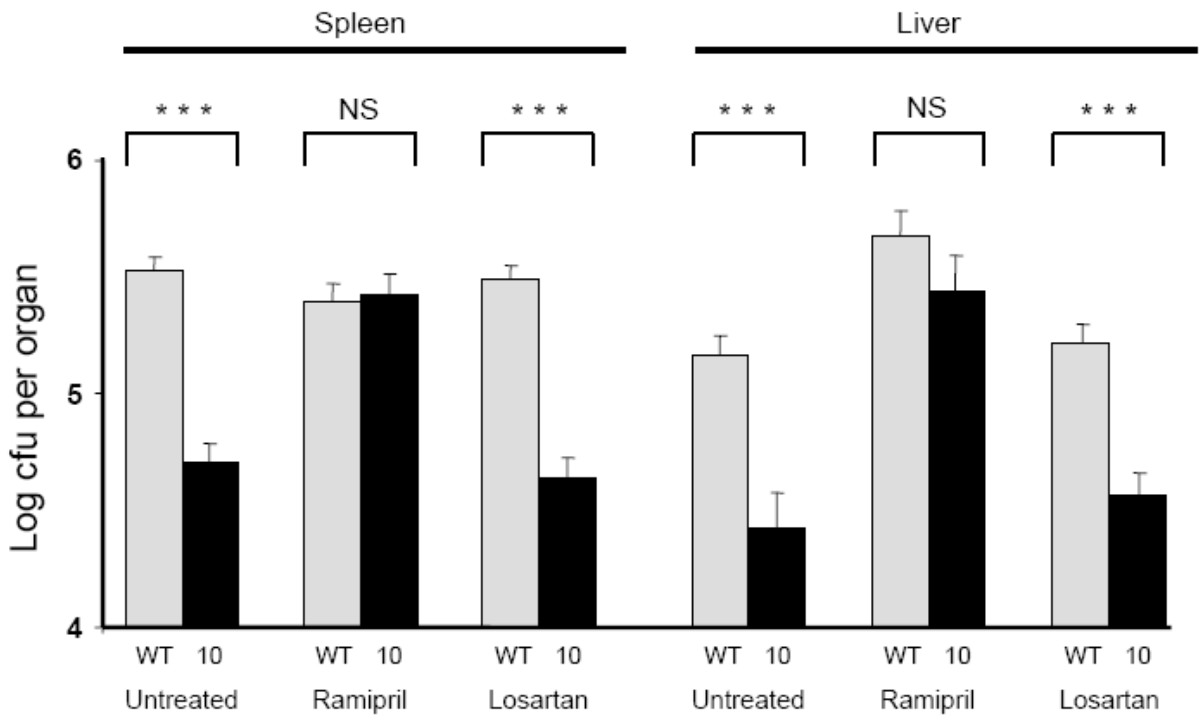
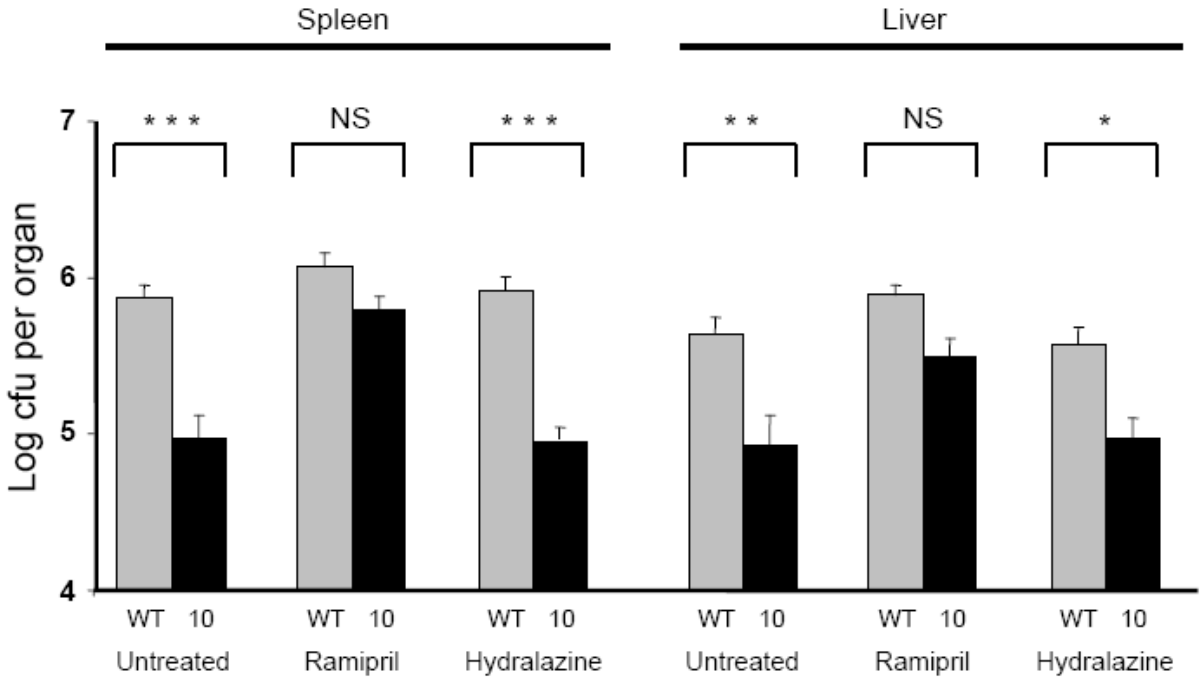


Figure 2-1. *In Vivo* challenge with *L. monocytogenes*. (**A**) Wild type (WT) and ACE 10/10 (10/10) mice were inoculated I.V. with 4 $X10^3$ *L. monocytogenes*, strain EGD. Groups of mice were sacrificed 3 days or 5 days after inoculation and the number of colony forming units (CFU) in the spleen and liver was determined. Values for individual WT (circles) and ACE 10/10 (triangles) mice are shown, as well as the group means and SEM. *p<0.05 **p<0.005, ***p<0.0005. ACE 10/10 have a significantly lower bacterial burden than WT.



(**B**) Wild type (WT, grey bars) and ACE 10/10 (10, black bars) were implanted with an osmotic mini-pump and treated with either saline, the ACE inhibitor ramipril or the AT1 receptor antagonist losartan. After 7 days, the mice were challenged with 4 X 10^3 *Listeria* i.v. Three days later, the number of bacteria in the spleen and in the liver was determined. Ramipril eliminated the difference between WT and ACE 10/10 while losartan had no significant effect. n=6



(**C**) Wild type (WT) and ACE 10/10 (10) were implanted with osmotic mini-pumps and treated with either ramipril or hydralazine. Mice then received 3 X 10^4 *Listeria* i.v. Five days after inoculation, the bacterial counts in the spleen and liver were determined. Ramipril eliminated differences between ACE 10/10 and WT mice but no such effect was found with hydralazine. n=6

To understand the increased resistance to infection in ACE 10/10, we also tested additional groups of WT and ACE 10/10 mice that were treated with either the specific ACE inhibitor ramipril or the angiotensin II AT1 receptor antagonist losartan delivered via osmotic minipump. Three days after infection, mice were sacrificed and bacterial burden was determined in the spleen and liver (Fig. 1b). These data showed that the treatment of ACE 10/10 mice with ramipril consistently eliminated significant differences between ACE 10/10 and WT, indicating that the catalytic activity of ACE is directly responsible for the reduced bacterial burden in ACE 10/10 mice. In contrast, treatment of mice with the AT1 antagonist did not alter the bacterial resistance noted in ACE 10/10 mice. For example, in spleen, ACE 10/10 and WT mice treated with losartan averaged 4.3×10^4 and 3.0×10^5 CFU respectively ($n=6$, $p<0.0005$), which was essentially identical to the results in the absence of losartan.

Using a higher inoculum of *Listeria* (3×10^4), similar differences were observed between ACE10/10 and WT mice. Following a 5 day i.v. challenge, we recovered just less than a log lower colony count in both spleen and liver compared to WT (Fig. 1c). For example, in the spleen, ACE 10/10 averaged 9.3×10^4 CFU while WT averaged 7.4×10^5 ($n=7$, $p<0.003$). Similar to the previous experiment, this effect was negated by addition of an ACE inhibitor, but non-specific lowering of blood pressure by hydralazine, which works independently of RAS, had no such effect.

ACE 10/10 Macrophages have Enhanced Listeriocidal Activity. Macrophages are a well-characterized cell host for *Listeria* replication. To directly assess the ability of ACE 10/10 macrophages to kill *Listeria*, an *in vitro* killing assay was performed using thioglycollate elicited peritoneal macrophages (TPMs), in the presence or absence of interferon-γ (IFN-γ). *Listeria* was added at a multiplicity of infection (MOI) of 10 and phagocytosis was allowed to proceed for 1 hr. Non-incorporated bacteria were then removed and the media was supplemented with gentamicin to kill residual extracellular bacteria. After further incubation such that the total time since first exposure to bacteria was 2 to 8 hrs, CFUs were determined by standard methods. In the absence of IFN-γ priming, there was no significant difference between the ability of ACE 10/10 and WT macrophages to kill *Listeria* (Fig. 2, top two lines). ACE 10/10 macrophages express abundant surface ACE (13), and thus these data demonstrated that ACE has no direct bactericidal effect (Fig. 2). Neither is there a difference in phagocytosis, since flow cytometric analysis of engulfed FITC-labeled *Listeria* by IFN-γ primed TPMs from WT and ACE 10/10 was similar (Supporting Fig. S1). However, once primed with IFN-γ, there was substantially better killing of *Listeria* by the ACE 10/10 cells. The data are significant by 4 hrs ($p<0.005$); by 8 hrs, ACE 10/10 macrophages have 11.6 fold less bacteria than WT macrophages ($n=8$, $p<0.001$). We also performed an identical experiment but now mice were treated with an ACE inhibitor for one week prior to isolating TPM. Macrophages were then treated with IFN-γ overnight in media also containing ACE inhibitor. ACE inhibition eliminated all differences in *Listeria* killing between ACE 10/10 and WT macrophages after 4 or 8 hours of incubation (4 hrs: ACE 10/10 = 1.4×10^5 CFU/10^5 macrophages, WT = 1.2×10^5 CFU/10^5 macrophages; 8 hrs: ACE10/10 = 4.1×10^4 CFU/10^5 macrophages, WT = 7.2×10^4 CFU/10^5 macrophages. n=at least 5, $p<0.6$). Thus, both *in vivo* and *in vitro* data show significantly better killing of *Listeria* in ACE 10/10 mice as compared to WT. This is dependent on the increased ACE activity in the ACE 10/10 model.

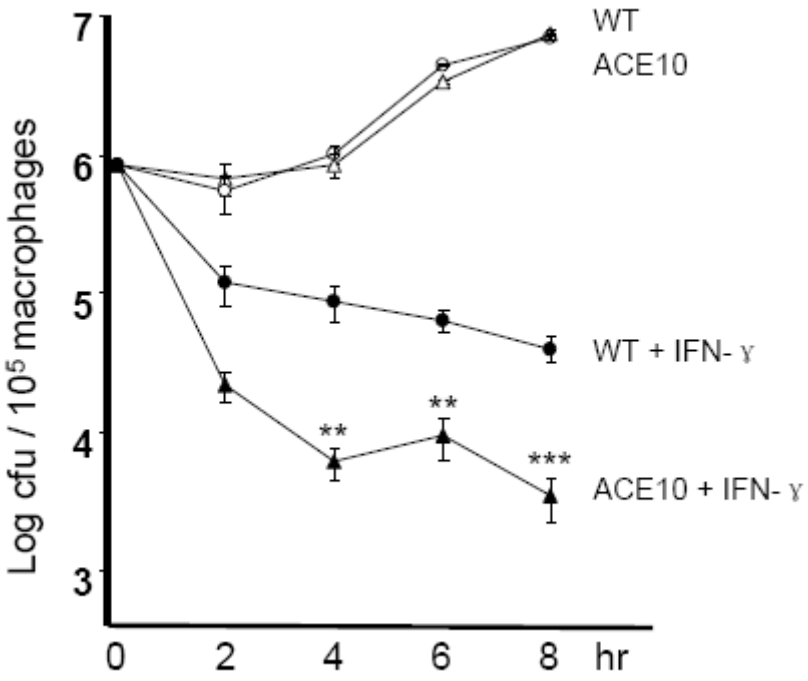


Figure 2-2**.** *In vitro* killing of Listeria by macrophages. TPMs were cultured overnight with either IFN-γ (ACE 10/10: filled triangle; WT: filled circle) or without IFN-γ priming (ACE 10/10: open triangle; WT: open circle). At time 0, cells were infected with *Listeria* at a multiplicity of 10 for 1 hr. Then, after washing, media containing gentamicin was added to kill the remaining extracellular bacteria. After further incubation such that the total time since first exposure to bacteria was 2 to 8 hrs, the antibiotic was removed, the cells were lysed and CFUs were determined. In the presence of IFN-γ priming, there is substantially better killing of *Listeria* by ACE 10/10 macrophages than by WT cells. n=8 mice, **p<0.005, ***p<0.001.

Increased Nitrite Production is Essential to the ACE 10/10 Phenotype. Substantial previous work has demonstrated that the killing of *Listeria* is dependent upon the generation of reactive oxygen species (ROS) by NADPH oxidase (Nox2) and by the generation of reactive nitrogen intermediaries, predominantly nitric oxide (NO) by inducible nitric oxide synthase (iNOS) (20). Our group has investigated each of these systems in ACE 10/10 mice.

To study burst ROS generation, TPM from ACE 10/10 and WT mice were isolated and cultured overnight with or without 10 ng/ml of IFN-γ. *Listeria* was then added and ROS were quantified over 80 min using a standard luminescence assay (21) . While more than 10 mice per group were examined, there was no consistent difference between ACE 10/10 and WT mice. Representative data are shown in supporting Fig. 2.

To investigate the role of nitrites, TPM were isolated and then primed overnight with or without 10 ng/ml IFN-γ. Some groups of cells were also exposed to *Listeria* at an MOI of either 10 or 50 (Fig. 3a). In the absence of IFN-γ priming, there was no difference in nitrite production between macrophages from ACE 10/10 or WT. In contrast, TPM from ACE 10/10 consistently showed a 2.2- to 2.9-fold increased production of nitrite, as compared to identically treated cells from WT mice. Even in the absence of stimulation with *Listeria*, IFN-γ primed cells from ACE 10/10 made 2.4-fold more nitrite than WT cells (ACE 10/10: 16.3 μM; WT: 6.8 μM, n=6, $p<0.005$).

Macrophages make NO via the up-regulation of iNOS (22). To evaluate this, TPMs were cultured for 18 hours in the presence of lipopolysaccharide (LPS,1 ug/ml). Culture supernatants were removed and cell lysates were analyzed for iNOS expression by Western blot analysis (Fig. 3b). Samples were also probed for L-arginase expression, as this competes with iNOS for arginine. To control for loading, samples were probed for β-actin levels and, after band density analysis, iNOS levels were normalized for the levels of the housekeeping gene (Fig. 3c). This

study showed a 2-fold increase in iNOS protein expression in macrophages from ACE 10/10 mice as compared to WT (ACE 10/10: 0.95 +/- 0.2; WT: 0.47+/- 0.14, n=5 mice, $p<0.005$). In contrast, we saw no difference in L-arginase expression between the two groups.

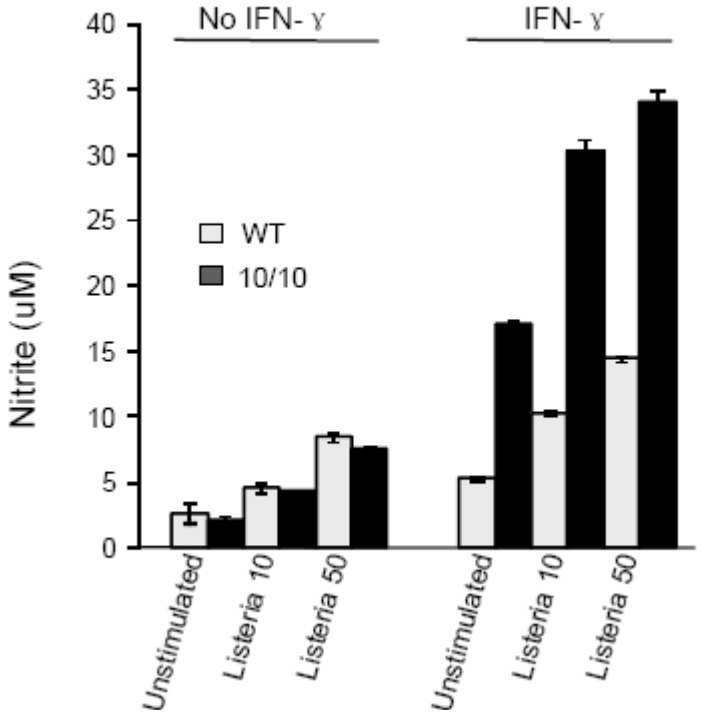
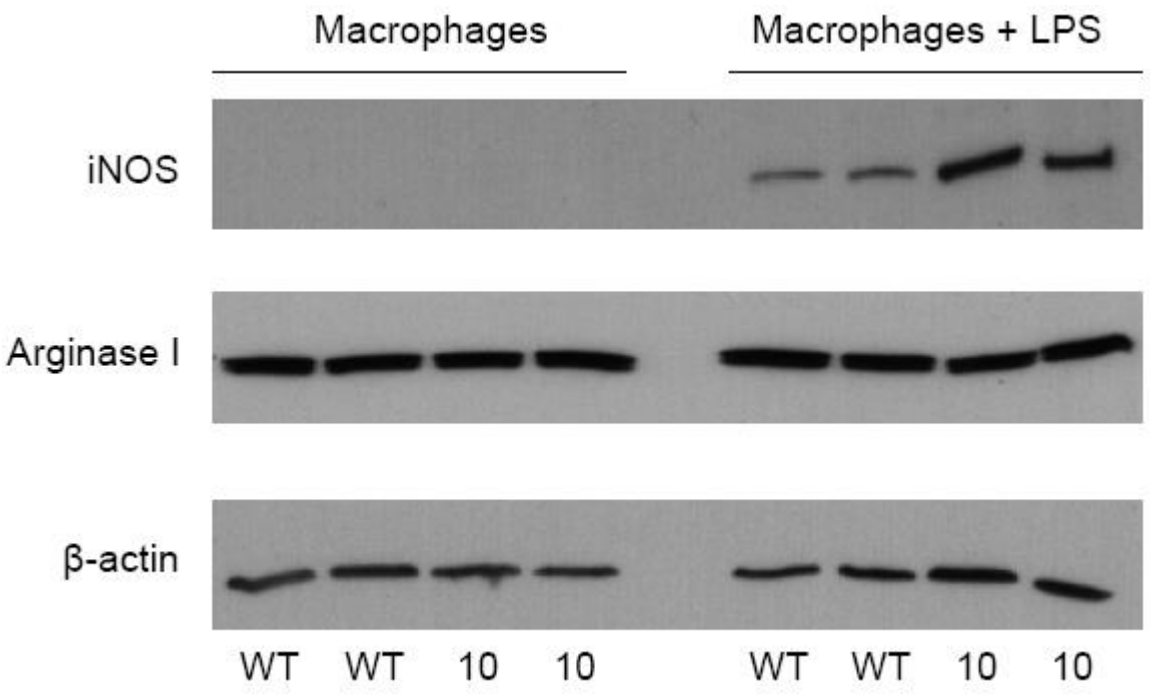
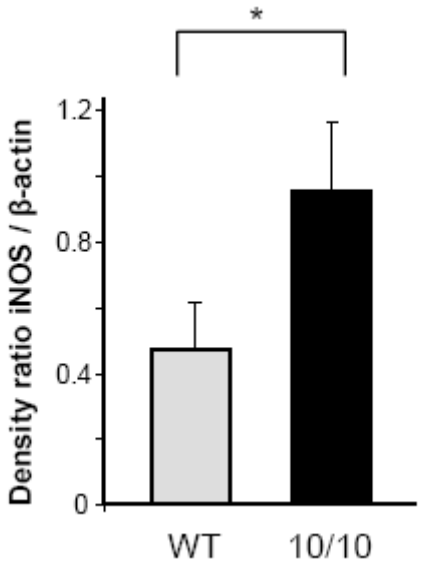


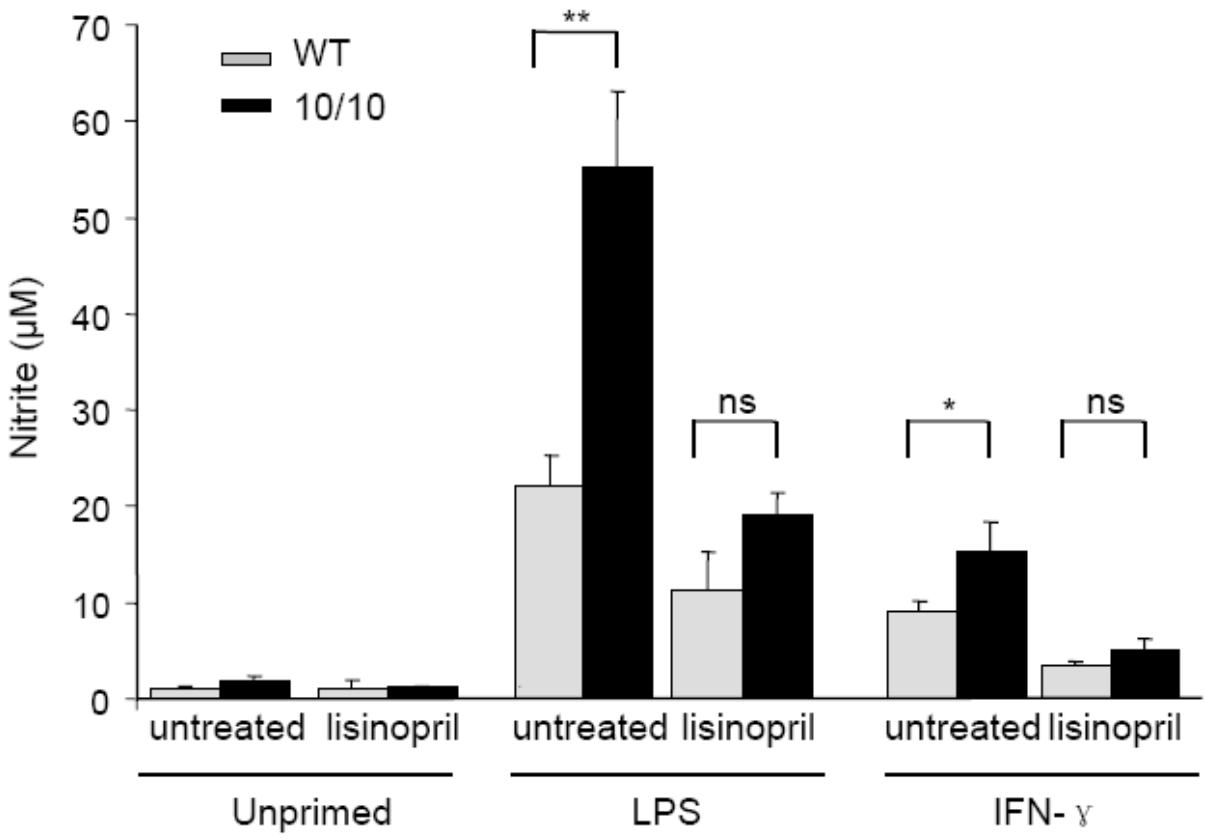
Figure 2-3. Macrophage production of nitrites and iNOS. **(A)**. TPMs from WT (light grey) or ACE 10/10 (black) mice were cultured overnight with or without IFN-γ. After 18 hrs, groups of cells were treated with *Listeria* at a multiplicity of either 10 or 50. Separate groups of cells were left untreated. Four hours later, the culture supernatant was collected and nitrite levels were measured. In the absence of IFN-γ priming, there was no significant difference in nitrite levels between cells from WT and ACE 10/10. However, with IFN-γ treatment, ACE 10/10 macrophages made from 2.2 to 2.9-fold more nitrites than identically treated cells from WT mice ($n=6$, $p<0.005$ for all groups of IFN-γ treated cells comparing ACE 10/10 to WT).



(**B**) TPMs from WT and ACE 10/10 (10) were cultured for 18 hrs with or without 1 µg/ml of LPS. Cell lysates were then probed for iNOS, arginase I and β-actin by Western blot.



(**C**) Densitometry was used to quantitate the average density ratio of iNOS relative to the expression of β-actin. There is increased expression of iNOS by ACE 10/10 macrophages. n=5, *$p<0.05$



(**D**) WT (gray bars) and ACE 10/10 (black bars) mice were treated with lisinopril for 7 days. TPMs from these mice and from untreated mice were then stimulated with either LPS or IFN-γ for 18hrs. Nitrite accumulation in the supernatant was then determined by Griess assay. ACE inhibition abrogates the elevated nitrite production by ACE 10/10 macrophages. n=5 mice per group with or without inhibitor, * $p=0.05$, ** $p<0.01$).

We showed that the increased *in vivo* resistance of ACE 10/10 mice to *Listeria* is eliminated by treatment with an ACE inhibitor. If, in fact, increased bacterial killing is also due to increased activity of iNOS, then ACE inhibitor treatment should be associated with reduced nitrite production. To study this, WT and ACE 10/10 mice were treated with the ACE inhibitor lisinopril for 1 week. TPMs were then collected, primed with LPS or IFN-γ, and assessed for nitrite production. Indeed, treatment of the mice with lisinopril resulted in a marked diminution of nitrite levels in the ACE 10/10 cells such that there was no significant difference in nitrite production between IFN-γ or LPS stimulated WT and ACE 10/10 cells (Fig. 3d)

Given both the increased production of iNOS and nitrites by ACE 10/10 cells, we directly investigated the role of iNOS in the killing of *Listeria* by performing an *in vitro* killing assay (Fig. 4a). TPMs were incubated overnight with or without IFN-γ. In this experiment, we also included groups of IFN-γ primed macrophages that were incubated with the compound 1400W, a specific inhibitor of iNOS (23). While 1400W had virtually no effect on WT cells, there was a marked effect on cells from ACE 10/10 mice; in the presence of 1400W, these cells killed *Listeria* equivalent to either WT cells with or without 1400W. The combination of data showing enhanced *in vitro* production of nitrites by ACE 10/10 cells, and the effects of the iNOS inhibitor 1400W in eliminating *in vitro* differences in killing between ACE 10/10 and WT, implies that a critical part of the enhanced immune response observed in ACE 10/10 mice is IFN-γ stimulated production of iNOS.

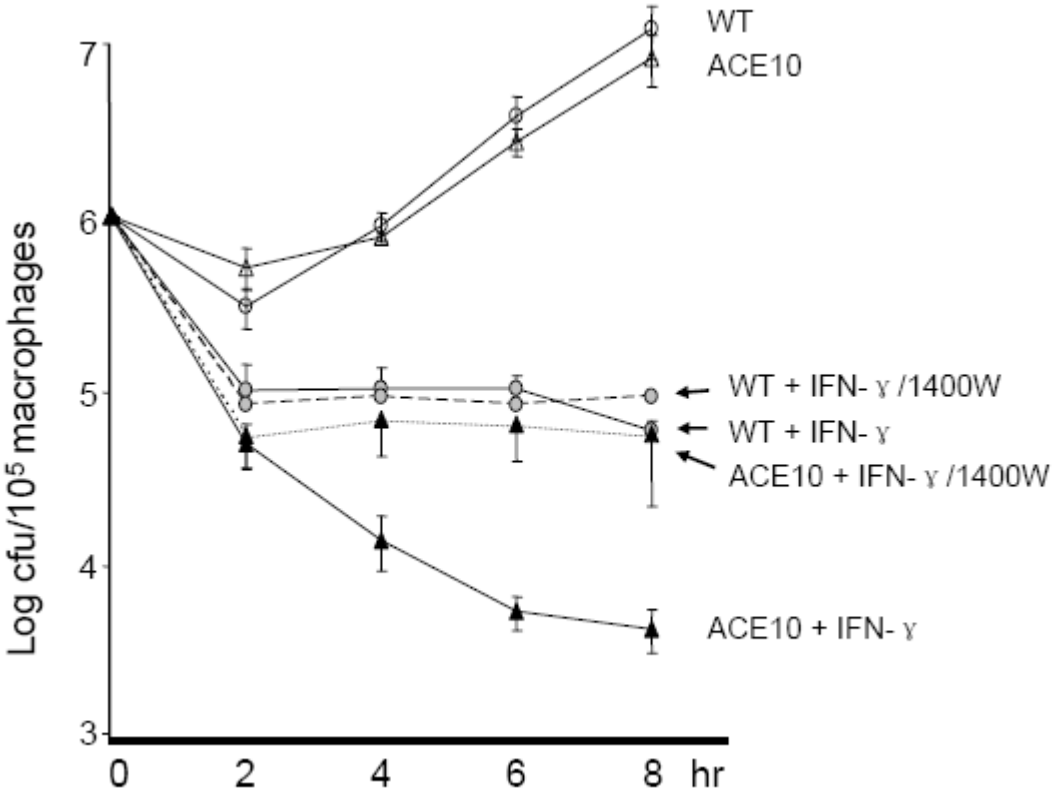


Figure 2-4. Effect of iNOS inhibition on bacteria killing

(**A**) TPMs from ACE 10/10 (triangles) or WT (circles) mice were cultured overnight. Some cells were cultured with IFN-γ or the combination of IFN-γ plus 1400W, as indicated in the figure. After 18 hrs, cells were mixed with *Listeria* at a multiplicity of 10 for 1 hr. After washing, media containing gentamicin was added to kill the remaining extracellular bacteria. At the indicated times after the first addition of *Listeria*, the gentamicin was removed, the cells were lysed and CFUs were determined. While inhibition of iNOS has no significant effect on the WT cells, treatment of ACE 10/10 cells renders these cells equivalent to WT in the killing of *Listeria* (n=5 mice. ACE 10/10:IFN-γ + 1400W vs WT:IFN-γ + 1400W, $p>0.4$ at all time points).

To investigate the role of iNOS *in vivo*, WT and ACE 10/10 mice were inoculated i.v. with 3 x 10^4 CFU of *Listeria*, strain EGD. Beginning on the day of infection, a group of mice were treated with water containing 50 μg/ml 1400W and were maintained on the drug throughout the duration of the experiment (Fig. 4b). Five days after infection, mice were sacrificed and the burden of *Listeria* in the spleen and liver was determined. In the absence of the iNOS inhibitor, ACE 10/10 mice showed significantly less bacteria in the spleen and liver, similar to data presented in Fig. 1. With the administration of the iNOS inhibitor, all groups of mice showed a significant increase in the burden of *Listeria*. This is consistent with published data showing an important role for iNOS in the control of *Listeria* infection (20, 24). In addition, the administration of 1400W to ACE 10/10 mice rendered these mice functionally equivalent to WT in that there was no longer any significant difference in bacterial burden.

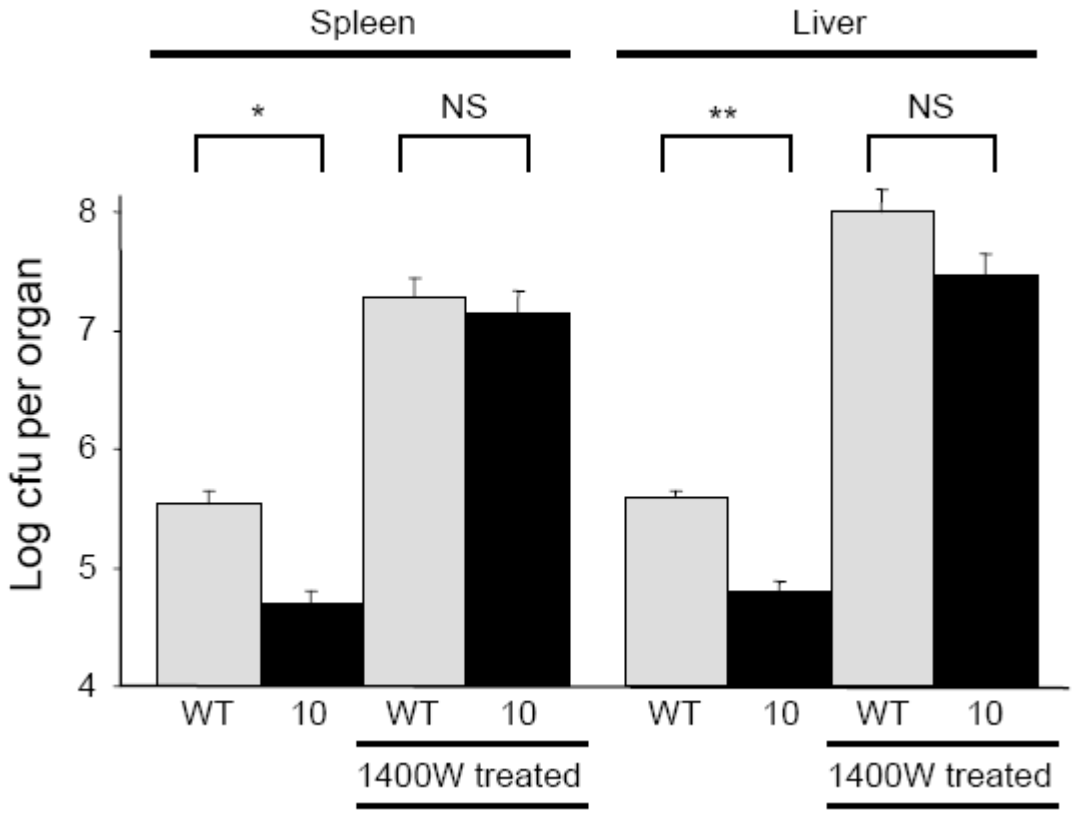


Figure 2-4(**B**). WT (grey bars) and ACE 10/10 mice (10, black bars) were inoculated i.v. with 3 x 10^4 *Listeria*. On the day of infection, some mice were treated with the iNOS inhibitor 1400W in the drinking water. The mice were sacrificed on day 5 and the bacterial burden in the spleen and liver was determined. Inhibition of iNOS with 1400W increased the bacterial burden in all groups. In the presence of 1400W, the difference between WT and ACE 10/10 mice was eliminated (n=6, *$p<0.05$, **$p<0.005$).

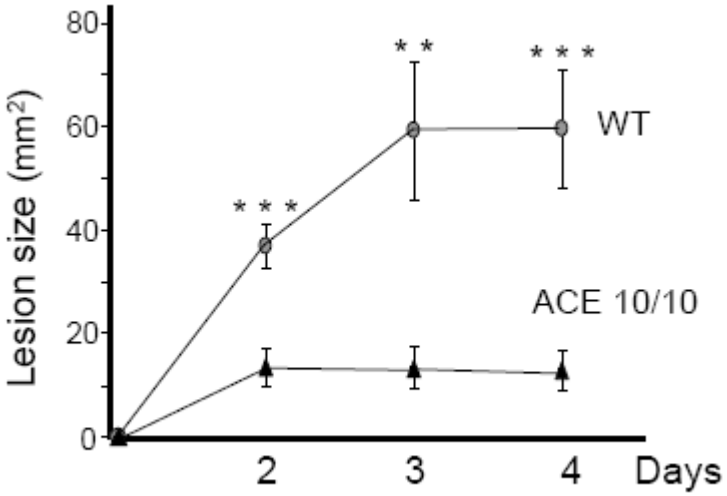
ACE 10/10 Mice Demonstrate Increased Immunity to MRSA.

While *Listeria* is a traditional model for understanding the innate immune response to bacteria, we also wanted to study the response of ACE 10/10 mice to the challenging clinical problem of MRSA. Utilizing a necrotizing fasciitis model, ACE 10/10 and WT mice were inoculated subcutaneously with $1X10^9$ CFU MRSA USA 300, strain SF8300 (25, 26). On days two, three and four after bacterial inoculation, the size of the indurated and inflamed skin lesion was measured (Figs. 5a,b). ACE 10/10 mice developed substantially smaller lesions compared to WT mice. At 4 days, WT mice had an average lesion of 60 mm^2 while ACE 10/10 averaged only 13 mm^2 ($n=8$, $p<0.005$). The mice were sacrificed on day 4, the lesional skin was excised, and the bacterial load of *S. aureus* was determined by serial dilution. Data from 4 separate experiments showed that there was a marked difference in the skin burden of bacteria (Fig. 5c). While WT mice averaged 6.2×10^7 CFU, ACE 10/10 animals had only 1.2×10^6 (n = at least 14, $p<0.001$).

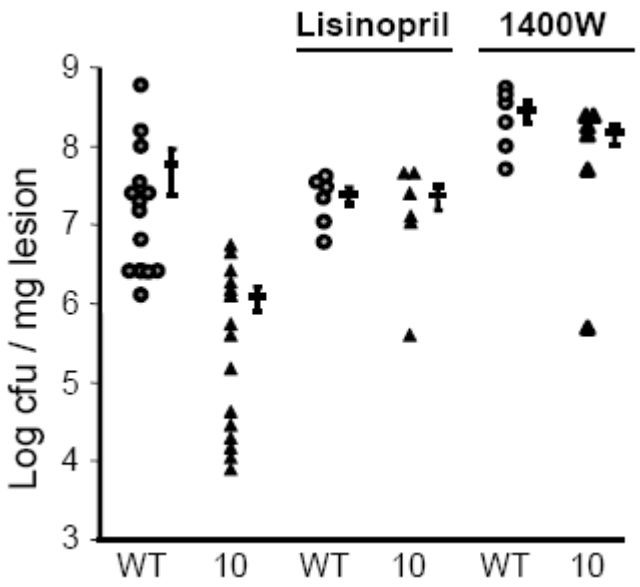
To determine if the increased resistance of ACE 10/10 mice to MRSA was dependent upon ACE catalytic activity, mice were treated with an ACE inhibitor for 1 week before inoculation of bacteria. The mice were then infected with MRSA, sacrificed on day 4 and the skin bacterial burden determined (Fig. 5c). Similar to what we saw with *Listeria*, inhibition of ACE activity rendered the ACE 10/10 mice as sensitive to MRSA as WT.

Previously, we showed that the enhanced resistance of ACE 10/10 mice to *Listeria* was very dependent on the functional activity of iNOS. To investigate whether this was true for resistance to MRSA, groups of WT and ACE 10/10 mice were treated with the iNOS inhibitor 1400W during the course of infection with *S. aureus* (Fig. 5c). Treatment of ACE 10/10 mice with 1400W increased their bacterial burden and eliminated any differences seen between these mice and similarly treated WT mice (WT: 2.8×10^8; ACE 10/10: 1.45×10^8, $n=6$, $p=0.16$).

Figure 2-5. Skin infection with methicillin-resistant *S. aureus* (MRSA). Mice were infected subcutaneously with $1X10^9$ MRSA, clone USA 300, in the hind flanks. (**A**) A representative comparison of skin lesions present in ACE 10/10 and WT mice 4 days after MRSA infection.



(**B**) Lesion size of WT (circles) and ACE 10/10 (10, triangles) were measured on days 2, 3 and 4 after infection, n ≥ 14 mice per group, **p<0.005, ***p<0.0005).



(**C**) Four days after skin infection, WT (circles) and ACE10/10 (triangles) were sacrificed, and bacterial counts in the lesion were determined. ACE 10/10 mice averaged over 50-fold less bacteria within lesions ($n \geq 14$, $p<0.001$). These differences were eliminated when mice were treated with either the ACE inhibitor lisinopril or the iNOS inhibitor 1400W

Four days after infecting mice with MRSA, skin lesions from both WT and ACE 10/10 mice were histologically examined. As anticipated, WT mice showed skin ulceration with a marked neutrophilic infiltrate and abscess formation (Fig. 6a). Occasional clumps of bacteria were observed. There was also extension of the lesion into the underlying muscle with focal muscle necrosis. ACE 10/10 mice also showed a neutrophilic infiltrate with abscess formation. Distinguishing the lesion in the ACE 10/10 was the increased presence of mononuclear cells, particularly at the periphery of the necrosis. A particularly marked example of this is shown in Fig. 6b-6d where a central region of necrosis is surrounded by a cuff of mononuclear cells. These cells were positive for ACE expression by immunohistochemical straining with an anti-ACE antibody.

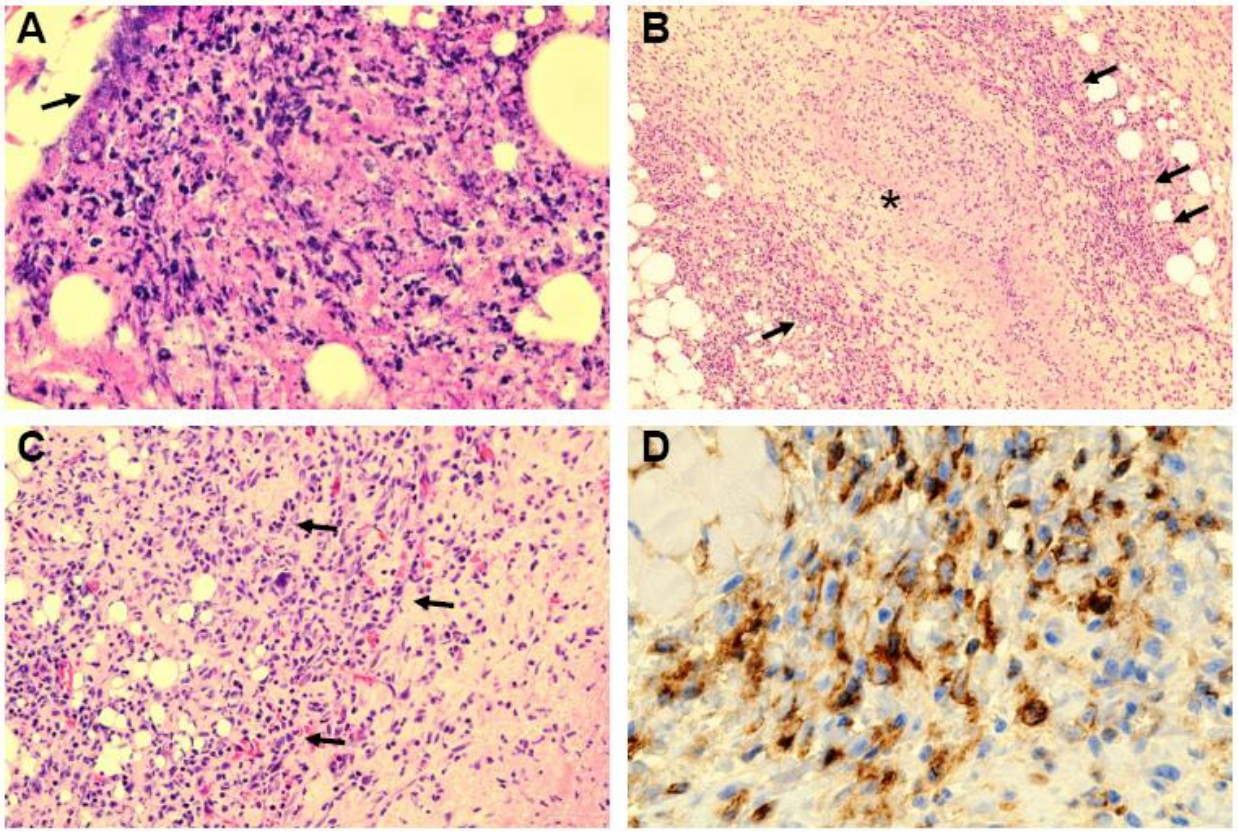


Figure 2-6. Histology of skin after MRSA infection. Four days after subcutaneous infection with MRSA, mice were sacrificed and histologic sections of the lesions were prepared. (**A**) WT mice show extensive acute inflammation within the superficial and deep dermis. There is abundant polymorphonuclear leukocytes with karyorrhexis, tissue necrosis and pus formation. Occasional masses of bacteria are present (arrow). (**B**) ACE 10/10 mice also had areas of necrosis and pus formation. However, at the periphery of the necrosis, there was a mononuclear cell infiltrate. Pictured in panels B is a marked example where a central region of necrosis (indicated by *) is surrounded by a cuff of mononuclear cells (indicated by arrows). (**C**) A higher power of the mononuclear infiltrate (arrows) is shown. (**D**) Immunohistochemical staining of the tissue sections with an anti-ACE antibody showed that these cells were positive for ACE expression.

If the enhanced resistance of ACE 10/10 mice is due to their altered myelomonocytic cells, then this should be transferable by bone marrow transplantation. Thus, we lethally irradiated WT C57BL/6 mice and then immediately transplanted bone marrow from either ACE 10/10 or littermate WT mice following a previously published protocol (12). Donor mice were on a C57BL/6 background and also carried a transgene for GFP expression. After 8 weeks, successful bone marrow engraftment was verified by FACS analysis of GFP expression. Mice were then challenged with a subcutaneous dose of 4 $X10^8$ MRSA. Four days after infection, the mice were sacrificed, and the number of bacteria within the skin was determined (Fig. 7). These data show that WT mice, transplanted with bone marrow from ACE 10/10 mice, have a significantly better ability to resist MRSA growth than WT mice transplanted with WT bone marrow (WT: 1.5×10^7; ACE 10/10: 2.4×10^6, $n=5$, $p<0.05$).

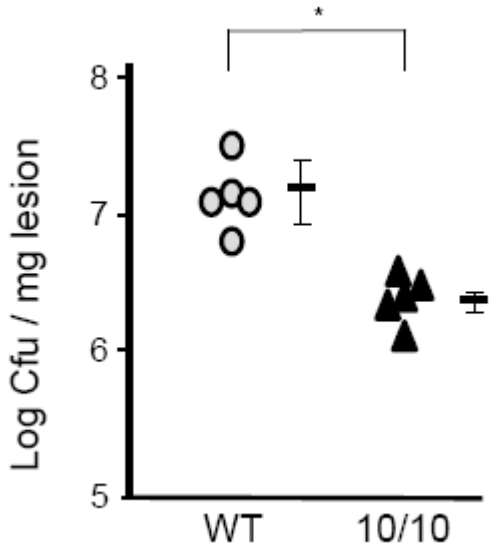


Figure 2-7. Transplantation of ACE10/10 bone marrow into WT mice confers increased resistance to MRSA. Recipient C57BL/6 mice were lethally irradiated and then immediately transplanted with bone marrow from either ACE 10/10 or littermate wild-type (WT) mice. After engraftment, the mice were challenged subcutaneously with 1 X 10^9 MRSA. Four days later, mice were sacrificed and bacterial burden in the skin lesion was determined. WT mice receiving bone marrow from ACE 10/10 mice (triangles) showed a significantly reduced bacteria burden in the skin lesion, compared to WT mice receiving WT bone marrow (circles). n=5, *$p<0.05$.

Discussion

In the ACE 10/10 model, the endogenous ACE promoter has been supplanted such that the control of ACE gene expression is now regulated by the *c-fms* promoter. The result is that tissues that normally express c-fms, such as monocytes, macrophages and other myelomonocytic cells now over-express ACE. Phenotypically, ACE 10/10 mice have a normal basal blood pressure. As was found in several different models, the expression of any appreciable quantity of ACE, irrespective of tissue location, is sufficient to allow homeostatic regulation of the renin-angiotensin system with resulting normal blood pressure (27). Thus, any phenotype identified in this model cannot be attributed to the many secondary effects of low or high blood pressure. ACE 10/10 mice resist growth of tumor models far better than WT mice and this increased resistance is associated with changes in adaptive immunity, such as increased numbers of tumor specific $CD8^+$ T cells. However, tumor models suffer from the intrinsic problem that such models in mice are somewhat artificial. These considerations led us to investigate how ACE 10/10 mice respond to infection with *L. monocytogenes*, a traditional model to study innate immunity, and with MRSA. Both models examined the behavior of the mice within only a few days of infection, during which the innate immune response plays a dominant role. With both infections, ACE 10/10 mice showed a significantly enhanced ability to resist bacterial growth. This was particularly so with resistance to skin infection with MRSA; not only was there 50-fold less bacteria in the lesions, but the reduction in the amount of tissue necrosis and abscess formation in ACE 10/10 mice is strong proof of the differences between these animals and WT.

Similar to what was found with tumor resistance, the inhibition of ACE enzymatic activity eliminated the enhanced immune response of the ACE 10/10 mice to bacterial infection. Thus, the catalytic activity of ACE, and not just the expression of ACE protein, is the critical

feature explaining the enhanced innate response to bacteria. Further, the *in vitro* killing data presented indicates that ACE catalytic activity is not what is directly killing bacteria. Rather, *in vitro*, macrophages need to be primed by IFN-γ to observe the ACE 10/10 effect. Most likely, such priming also occurs *in vivo* as part of the infectious process. We previously demonstrated that ACE is catalytically active and able to cleave peptides within the ER compartment of ACE 10/10 macrophages (13). Thus, it is unclear if it is ACE action here, or whether the ACE present on the cell surface creates a unique local environment that is critical for explaining the enhanced response of the ACE 10/10 mice. However, we presume that it is the enhanced production or degradation of one or even several peptides that is the ultimate explanation for the differences between ACE 10/10 and WT mice. Angiotensin II, though, does not appear to be a critical factor in the ACE 10/10 model. This is strongly suggested by our finding that an AT1 receptor antagonist had no apparent effect on resistance to *Listeria*. While we did not study a genetic model in which the production of angiotensin II was impossible due to a genetic change, these experiments were performed in the study of tumor resistance and confirmed the lack of apparent involvement of angiotensin II (12).

Substantial previous analysis established that two of the major mechanisms responsible for innate immunity to bacteria are the generation of reactive oxygen intermediates (ROI), as a result of NADPH oxidase activation, and the generation of reactive nitrogen intermediates (RNI) as a result of iNOS expression and activity (20, 28, 29). We found no consistent difference in ROI generation between ACE 10/10 macrophages and WT. In contrast, several lines of data showed significant differences in RNI generation between ACE 10/10 and WT mice, suggesting that this plays a major role in the increased immune response of the ACE 10/10 model. These include the increased nitrite and iNOS protein synthesized in response to LPS, the increase in nitrite production in response to IFN-γ priming and perhaps most importantly, the finding that

the specific iNOS inhibitor 1400W eliminates all differences in bacterial killing between ACE 10/10 and WT.

Richardson *et al.* have discussed that nitric oxide (NO) is a pro-inflammatory molecule critical for clearance of a wide variety of pathogens including viral, fungal, bacterial and parasitic microorganisms (30). It is an important effector of host innate immunity because of its immunomodulatory roles, as well as its direct antimicrobial activity. NO can also be oxidized into potent nitrogen species, such as peroxynitrite, that have bacteriostatic or bacteriocidal effects (31-33). Mice deficient in iNOS show increased susceptibility to *L. monocytogenes* (20, 34). iNOS deficiency is also associated with increased mortality from *S. aureus* infection (35, 36). Indeed, the aggressiveness of MRSA is partly due to their ability to neutralize the lethality of NO generation via several inducible genes that increase the efficiency of MRSA to detoxify nitrites (30, 37). Here, our studies are different in emphasis in that we show that, not only does NO play a role under natural circumstances, but that the over-expression of ACE induces iNOS and nitrites by myelomonocytic cells to markedly improve the immune response to MRSA and ameliorate infection.

Recently, others have stressed the phenotypic behaviors of different subgroups of activated macrophages (38-40). Specifically, 'classically activated macrophages' have been implicated to have anti-bacterial and anti-tumor properties while 'alternatively activated macrophages' are postulated to promote tumor growth and suppress the extent of inflammation. We previously published that the over-expression of ACE in the ACE 10/10 model appears to tilt macrophage toward a classically activated phenotype. Our data here are consistent with this idea in that iNOS expression is one of the phenotypes of a classically activated macrophage. Further work will be necessary to investigate precisely how many of the phenotypic differences in ACE 10/10 mice can be explained as secondary to iNOS induction. Thus, our data support a central

role of iNOS in bacterial resistance; whether iNOS plays a similar central role in other immune changes found in the ACE 10/10 mouse, such as resistance to tumor and adaptive immunity, remain to be explored.

Chambers and DeLeo have recently described the history of antibiotic resistance in *S. aureus* as a series of waves (14) beginning in the 1940s with penicillin resistant *S. aureus* in hospitals. The introduction of methicillin and the 1961 report of *S. aureus* resistant strains, marked the second wave. By the late 1970s, new strains of MRSA became endemic, but were usually found in immunocompromised patients or in a hospital setting. In 1997, the emergence of new aggressive strains marked a new wave of infection that has continued to increase in incidence (41), infecting healthy adults and sensitive to very few antibiotics (42, 43). Given the history of MRSA evolution, the implication for the future is that new approaches are needed to address MRSA and other forms of aggressive infection. Several groups have published attempts to regulate iNOS expression as a means of enhancing resistance to bacteria. In particular, the use of microspheres containing iNOS has been investigated as a therapeutic approach to decrease subcutaneous abscess area, reduce bacterial burden in lesions, and induce cytokine expression by the host in the face of *S. aureus* infection (44, 45). Our work lends credence to the idea that ACE manipulation is a potential new approach to increase innate resistance in the face of a serious bacterial infection.

References

1. Corvol, P., T. A. Williams, and F. Soubrier. 1995. Peptidyl dipeptidase A: angiotensin I-converting enzyme. *Methods Enzymol* 248:283-305.

2. Bernstein, K. E., H. D. Xiao, K. Frenzel, P. Li, X. Z. Shen, J. W. Adams, and S. Fuchs. 2005. Six truisms concerning ACE and the renin-angiotensin system educed from the genetic analysis of mice. *Circ Res* 96:1135-1144.

3. Leehey, D. J., A. K. Singh, N. Alavi, and R. Singh. 2000. Role of angiotensin II in diabetic nephropathy. *Kidney Int Suppl* 77:S93-98.

4. Metzger, R., R. M. Bohle, P. Chumachenko, S. M. Danilov, and F. E. Franke. 2000. CD143 in the development of atherosclerosis. *Atherosclerosis* 150:21-31.

5. Stegbauer, J., D. H. Lee, S. Seubert, G. Ellrichmann, A. Manzel, H. Kvakan, D. N. Muller, S. Gaupp, L. C. Rump, R. Gold, and R. A. Linker. 2009. Role of the renin-angiotensin system in autoimmune inflammation of the central nervous system. *Proc Natl Acad Sci U S A* 106:14942-14947.

6. Hoch, N. E., T. J. Guzik, W. Chen, T. Deans, S. A. Maalouf, P. Gratze, C. Weyand, and D. G. Harrison. 2009. Regulation of T-cell function by endogenously produced angiotensin II. *Am J Physiol Regul Integr Comp Physiol* 296:R208-216.

7. Ruiz-Ortega, M., C. Bustos, M. A. Hernandez-Presa, O. Lorenzo, J. J. Plaza, and J. Egido. 1998. Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol* 161:430-439.

8. Brown, D. I., and K. K. Griendling. 2009. Nox proteins in signal transduction. *Free Radic Biol Med* 47:1239-1253.

9. Platten, M., S. Youssef, E. M. Hur, P. P. Ho, M. H. Han, T. V. Lanz, L. K. Phillips, M. J. Goldstein, R. Bhat, C. S. Raine, R. A. Sobel, and L. Steinman. 2009. Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity. *Proc Natl Acad Sci U S A* 106:14948-14953.

10. Scharfstein, J., A. C. Monteiro, V. Schmitz, and E. Svensjo. 2008. Angiotensin-converting enzyme limits inflammation elicited by Trypanosoma cruzi cysteine proteases: a peripheral mechanism regulating adaptive immunity via the innate kinin pathway. *Biol Chem* 389:1015-1024.

11. Montecucco, F., A. Pende, and F. Mach. 2009. The renin-angiotensin system modulates inflammatory processes in atherosclerosis: evidence from basic research and clinical studies. *Mediators Inflamm* 2009:752406.

12. Shen, X. Z., P. Li, D. Weiss, S. Fuchs, H. D. Xiao, J. A. Adams, I. R. Williams, M. R. Capecchi, W. R. Taylor, and K. E. Bernstein. 2007. Mice with enhanced macrophage angiotensin-converting enzyme are resistant to melanoma. *Am J Pathol* 170:2122-2134.

13. Shen, X. Z., A. E. Lukacher, S. Billet, I. R. Williams, and K. E. Bernstein. 2008. Expression of angiotensin-converting enzyme changes major histocompatibility complex class I peptide presentation by modifying C termini of peptide precursors. *J Biol Chem* 283:9957-9965.

14. Chambers, H. F., and F. R. Deleo. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol* 7:629-641.

15. Zenewicz, L. A., and H. Shen. 2007. Innate and adaptive immune responses to Listeria monocytogenes: a short overview. *Microbes Infect* 9:1208-1215.

16. Cole, J., D. L. Quach, K. Sundaram, P. Corvol, M. R. Capecchi, and K. E. Bernstein. 2002. Mice lacking endothelial angiotensin-converting enzyme have a normal blood pressure. *Circ Res* 90:87-92.

17. Tseng, C. W., P. Kyme, J. Low, M. A. Rocha, R. Alsabeh, L. G. Miller, M. Otto, M. Arditi, B. A. Diep, V. Nizet, T. M. Doherty, D. O. Beenhouwer, and G. Y. Liu. 2009. Staphylococcus aureus Panton-Valentine leukocidin contributes to inflammation and muscle tissue injury. *PLoS One* 4:e6387.

18. Okabe, M., M. Ikawa, K. Kominami, T. Nakanishi, and Y. Nishimune. 1997. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 407:313-319.

19. Langford, K. G., S. Y. Shai, T. E. Howard, M. J. Kovac, P. A. Overbeek, and K. E. Bernstein. 1991. Transgenic mice demonstrate a testis-specific promoter for angiotensin-converting enzyme. *J Biol Chem* 266:15559-15562.

20. Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* 10:29-38.

21. Dahlgren, C., and A. Karlsson. 1999. Respiratory burst in human neutrophils. *J Immunol Methods* 232:3-14.

22. MacMicking, J., Q. W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-350.

23. Babu, B. R., and O. W. Griffith. 1998. Design of isoform-selective inhibitors of nitric oxide synthase. *Curr Opin Chem Biol* 2:491-500.

24. Nathan, C., and M. U. Shiloh. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* 97:8841-8848.

25. Diep, B. A., A. M. Palazzolo-Ballance, P. Tattevin, L. Basuino, K. R. Braughton, A. R. Whitney, L. Chen, B. N. Kreiswirth, M. Otto, F. R. DeLeo, and H. F. Chambers. 2008. Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. *PLoS One* 3:e3198.

26. Tenover, F. C., and R. V. Goering. 2009. Methicillin-resistant Staphylococcus aureus strain USA300: origin and epidemiology. *J Antimicrob Chemother* 64:441-446.

27. Bernstein, K. E. 2006. Views of the renin-angiotensin system: brilling, mimsy, and slithy tove. *Hypertension* 47:509-514.

28. Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation. Its role in resistance to Listeria monocytogenes. *J Immunol* 150:888-895.

29. MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. W. Xie, K. Sokol, N. Hutchinson, and et al. 1995.

Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641-650.

30. Richardson, A. R., P. M. Dunman, and F. C. Fang. 2006. The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol* 61:927-939.

31. Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman. 1991. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288:481-487.

32. Wink, D. A., K. S. Kasprzak, C. M. Maragos, R. K. Elespuru, M. Misra, T. M. Dunams, T. A. Cebula, W. H. Koch, A. W. Andrews, J. S. Allen, and et al. 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254:1001-1003.

33. Wink, D. A., and J. B. Mitchell. 1998. Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med* 25:434-456.

34. Boockvar, K. S., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, Jr., and R. L. Kurlander. 1994. Nitric oxide produced during murine listeriosis is protective. *Infect Immun* 62:1089-1100.

35. Sakiniene, E., T. Bremell, and A. Tarkowski. 1997. Inhibition of nitric oxide synthase (NOS) aggravates Staphylococcus aureus septicaemia and septic arthritis. *Clin Exp Immunol* 110:370-377.

36. Sasaki, S., T. Miura, S. Nishikawa, K. Yamada, M. Hirasue, and A. Nakane. 1998. Protective role of nitric oxide in Staphylococcus aureus infection in mice. *Infect Immun* 66:1017-1022.

37. Richardson, A. R., S. J. Libby, and F. C. Fang. 2008. A nitric oxide-inducible lactate dehydrogenase enables Staphylococcus aureus to resist innate immunity. *Science* 319:1672-1676.

38. Benoit, M., B. Desnues, and J. L. Mege. 2008. Macrophage polarization in bacterial infections. *J Immunol* 181:3733-3739.

39. Mantovani, A., A. Sica, and M. Locati. 2005. Macrophage polarization comes of age. *Immunity* 23:344-346.

40. Porta, C., M. Rimoldi, G. Raes, L. Brys, P. Ghezzi, D. Di Liberto, F. Dieli, S. Ghisletti, G. Natoli, P. De Baetselier, A. Mantovani, and A. Sica. 2009. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc Natl Acad Sci U S A* 106:14978-14983.

41. 1999. From the Centers for Disease Control and Prevention. Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus--Minnesota and North Dakota, 1997-1999. *JAMA* 282:1123-1125.

42. Kobayashi, S. D., and F. R. DeLeo. 2009. An update on community-associated MRSA virulence. *Curr Opin Pharmacol* 9:545-551.

43. Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 13:1510-1514.

44. Han, G., L. R. Martinez, M. R. Mihu, A. J. Friedman, J. M. Friedman, and J. D. Nosanchuk. 2009. Nitric oxide releasing nanoparticles are therapeutic for Staphylococcus aureus abscesses in a murine model of infection. *PLoS One* 4:e7804.

45. Martinez, L. R., G. Han, M. Chacko, M. R. Mihu, M. Jacobson, P. Gialanella, A. J. Friedman, J. D. Nosanchuk, and J. M. Friedman. 2009. Antimicrobial and healing efficacy of sustained release nitric oxide nanoparticles against Staphylococcus aureus skin infection. *J Invest Dermatol* 129:2463-2469.

FOOTNOTES

*We thank Drs. Rafi Ahmed, Ifor William, and David Archer, Emory University, Atlanta, GA for reagents and advice. We also thank Ms. Fai Chung for help with immunohistochemistry and Dr. Xu Chen for help with mouse husbandry. The work was supported by NIH grants R01 K039777 (KB), R01 DK051445 (KB), R01 AI074832 (GL), and R00 HL088000 (SF).

CHAPTER 3: MECHANISMS OF THE ACE 10

At this point, the response of the ACE 10 in both the tumor and bacterial infection model had confirmed that the ACE 10 certainly exerted a markedly different immunological response, consistent with the hypothesis of a M1 macrophage phenotype. However, several questions still remained. How does over-expression of an exopeptidase drastically transform the behavior of myelomonocytic cells? What are the molecular mechanisms that are responsible, for example, for the enhanced NOS protein levels to increase nitric oxide synthesis in the ACE 10? Is there an intrinsic change in these cells, such that the ACE 10 cells would behave differently and distinctly separate from wild type macrophages, even in a wild type microenvironment? In other words, we had not established beyond doubt if ACE over-expression induces a bona-fide change in these myelomonocytic cells independent of the milieu of the ACE 10. One other possibility was that the ACE 10 macrophage is a factor of environmental milieu within the ACE 10 mice itself, by virtue of the fact that these mice have no endothelial ACE. For example, the absence of endothelial ACE may augment Angiotensin I levels in other to maintain normal pressure. In addition, the tetra-peptide AcSDKP, which may have its effects on macrophage/monocyte behavior, could also be increased in the absence of endothelial ACE, as was the case in the ACE 10 model.

To address these some of these issues, we decided to go back to the tumor model and study the behavior of these myelomonocytic cells. The tumor model was an ideal one, in part because tumor progression has a direct impact on the behavior of myelomonocytic cells. We hoped that using this model, we would understand the mechanisms by which ACE overexpression influences the behavior of these cells. Our

work describing the changes in the myelomonocytic cells as a result of ACE overexpression is presented in the ensuing chapter in the form of a draft manuscript in preparation for submission. Surely, there will be revisions, including anticipated requirements for new experiments and data prior to eventual publication of this study. Special praise must go to Dr Shen, whose knowledge and expertise of the tumor model from his previous publication was key to this work.

Over-expression of Angiotensin Converting Enzyme in Myelomonocytic Cells Alters Myeloid Mediated Tumor-Induced Immunosuppression

Running Title: ACE alters myeloid immunosuppression

Derick Okwan-Duodu[1,3], Sandrine Billet[1,2], Frank S Ong[1,2], Chentao Lin[1,2], Ellen Bernstein[1], Nico van Rooijen[4,] Xiao Z. Shen[1,2] and Kenneth E. Bernstein[1,2]

[1]Department of Biomedical Sciences, [2]Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048

[3]Emory University School of Medicine, Atlanta, Georgia 30322

[4]Department of Molecular Cell Biology, Vrije Universiteit Medical Center, 1081 BT Amsterdam, The Netherlands.

Correspondence:

Kenneth E. Bernstein, M.D.,

110 N. George Burns Rd, Davis 2021

Los Angeles, CA 90048, USA.

Telephone: 310-423-7562;

Fax: 310-423-7331;

Email: kbernst@cshs.org

Abstract

Tumor-induced immune dysfunction impairs myelomonocytic cell maturation, creating an immunosuppressive environment fortified by immature CD11b+Gr-1+(int) myeloid derived suppressor cells (MDSCs) that impede classically activated (M1) macrophage and CD8 T cell anti-tumor responses. Mice with enhanced expression of angiotensin converting enzyme (ACE) in myelomonocytic cells, called ACE 10, markedly resist tumor growth with an increased CD8 T cell response. However, the behavior of myelomonocytic cells in these animals is unknown. We now show a marked diminution in the number of MDSCs that accumulate in the spleen of the ACE 10 during tumor challenge. The contraction of immature myeloid cells in the ACE 10 was associated with increased levels of the myeloid differentiation transcription factor C/EBPα in mature macrophages as well as immature differentiating precursors when compared to WT cells of equivalent stage. ACE inhibition eliminated the enhanced tumor immunity in the ACE 10 with a concomitant increase in splenic MDSCs and down-regulation of C/EBPα expression. In contrast to macrophages of WT tumor-bearing mice, ACE 10 macrophages maintained C/EBPα expression, and sustained their anti-tumor M1 response within the tumor microenvironment. Accordingly, *in vitro*, ACE 10 macrophages killed tumor cells more efficiently than WT cells in an NO-dependent manner. Thus, ACE through C/EBPα, potently modifies the behavior of myelomonocytic cells. Our data identifies a novel approach to breach the immunosuppressive state associated with myeloid cells in the tumor micro-environment.

Introduction

Despite major advances in dendritic cell immunotherapy and vaccination (46), cancers are still a leading cause of death in western societies. This is partly because the tumor microenvironment is immunosuppressive, characterized by immature myeloid derived suppressor cells (MDSCs) and alternatively activated (M2) macrophages (47-50). MDSCs are a heterogeneous group of immature CD11b+Gr+(int) myelomonocytic cells that collaborate with alternatively activated M2 macrophages to suppress effective CD8 T cell responses and impede antigen-presentation and tumor killing by other immune cells (51-53). While M2 macrophages, instructed by IL-4, express arginase-1, IL-10, and angiogenic factors to support tumor growth (50, 54), the classically activated M1 macrophage, in response to LPS or IFN-γ, synthesize pro-inflammatory cytokines such as IL-12, TNFα, and nitrites, and consequently kill tumor cells (55, 56). Reduction of MDSCs and enhancement of M1 macrophages improve anti-tumor immunity (57). However, by mechanisms that largely remain undefined, the tumor microenvironment suppresses M1 phenotype and expands the M2 macrophage and MDSC populations. Thus, these myeloid lineage cells represent a major hurdle in the development of anti-tumor immunity.

A current paradigm posits that MDSCs and M2 macrophages originate from common myeloid precursors, and that immune dysfunction in tumor-bearing mice expands these two populations (49, 58). Notably, CD11b(+)Gr-1(int) cells comprise less than 2% of the naïve spleen, but they increase to as much as 10-25% in the spleen of tumor bearing mice (49). The mechanisms of accumulation of these myeloid immunosuppressive cells are not fully understood, but they are thought to involve altered

macrophage differentiation and tumor-mediated defects in myelopoiesis, instigated by transcription factors and genes that play important roles in myeloid cell development (49, 58). Among these transcription factors is the C/EBP family, which exerts a remarkable influence on myelopoiesis, monocyte differentiation, and macrophage development (59-61). In line with this, the tumor microenvironment restricts generation of potent effector M1 macrophages by down-regulating C/EBPα (58, 62, 63). Furthermore, C/EBPβ favors polarization of M2 macrophages (64) and has been recently implicated in myelomonocytic cell-mediated tumor-induced immune tolerance (65). Thus, because impaired myeloid cell maturation is a hallmark of cancer progression (66), manipulation of myelomonocytic cells could hold therapeutic promise.

We have previously described a mouse model in which angiotensin converting enzyme (ACE), the critical peptidase of the blood pressure regulatory renin angiotensin system (RAS), is over-expressed in myelomonocytic lineage cells (12) . We have shown that these mice, referred to as ACE 10, elicit an increased immune resistance to tumor growth and bacterial infections (12, 67). The response against B16-F10 melanoma in the ACE 10 was associated with an increased CD8 T cell response as well as an increased infiltration of myeloid cells within the tumors, which is paradoxical, because macrophage infiltration is a marker for severity, metastasis, and poor prognosis for an overwhelming number of tumors (47, 68, 69). Having recently shown that ACE affects myelopoiesis (70), we were prompted to investigate how ACE can alter myeloid cell-mediated immunosuppression during tumor development. Here, we demonstrate that increased expression of ACE is associated with increased C/EBPα levels which promotes myeloid cell maturation. Consequently, fewer MDSCs accumulate in the spleen of the ACE 10

during tumor challenge. Compared to markedly suppressed C/EBPα expression in WT tumor bearing mice, ACE 10 macrophages maintained expression of C/EBPα and sustained anti-tumor M1 response. Thus, we unveil a novel approach by which to breach the immunosuppressive barrier that is associated with myeloid lineage cells during tumor progression.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS) (Escherichia coli 055:B5) and All *trans*-retinoic acid (ATRA) was from Sigma-Aldrich (St. Louis, MO). Griess assay reagents for nitrite determination were from Promega (Madison, WI). IL-4 and GM-CSF were purchased from Peprotech. Liposome chlodronate were obtained from Dr Nico van Rooijen (Vrije Universiteit, Amsterdan, Netherlands).

Mice

Mice over-expressing myelomonocytic ACE (called ACE 10) mice have been previously described (12). ACE 10/10 mice were back-crossed at least 10 generations to C57BL/6 mice (Jackson Laboratory, Bar Harbor ME). All mice were maintained in micro isolator cages, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Cedars Sinai Medical Center.

Melanoma Cells and Tumor Model

The B16-F10 melanoma cell line American Type Culture Collection (ATCC, Manassas, VA), were maintained as previously described (12). Tumor cell concentration was adjusted to 1 x 10^7 cells/ml, and 100 µl of the suspension was injected intradermally into the dorsal skin using a 26-gauge needle under anesthesia. The size of the tumor was measured using a caliper. The tumor volume was calculated according to the formula (V = [L x W^2] x 0.52), where V is volume, L is length, and W is width (length is greater than

width). In vitro killing assays were performed by seeding tumor cells (target) and macrophages (effector) at a 10: 1 E:T ratio. Some groups of effectors were primed with 100 ng/ml LPS overnight prior to co-culture with B16-F10. After overnight incubation, tumor cell death was analyzed by LDH release assay, performed by manufacturer's instruction (Promega).

Thioglycollate Elicited Macrophage Isolation and Culture

Cells were harvested after routine 2% thioglycollate gel injection, and cultured as previously described (12). In brief, 6-8 week old mice were injected intraperitoneally with 2 ml aged thioglycollate broth. Four days later, peritoneal exudate cells were isolated by lavaging the peritoneal cavity with ice-cold HBSS. After 2 h of incubation at 37°C, adherent cells were used as peritoneal macrophages.

Tumor Associated Macrophage Isolation

Tumor associated macrophages were isolated as previously described (12). In brief, tumor bearing mice were injected i.p with tumor cells. For in vitro killing assays, tumor bearing mice were injected i.p with 2ml aged thioglycollate broth. Four days later, peritoneal exudates cells were harvested by lavage, F4/80 positive cells were sorted by flow cytometry and used for subsequent studies.

***In vivo* Macrophage Depletion with Liposome Chlodronate**

Macrophages were depleted by injection of 1mg of chlodronate encapsulated liposome every 72hrs as by manufacturer's instructions (71). Control mice were injected with

liposome-PBS. Flow cytometry confirmed depletion of macrophages and other CD11b+ myeloid cells as reported by several other groups.

Flow Cytometry

Myeloid derived suppressor cells were analyzed by staining with APC conjugated Gr-1 monoclonal antibody (eBioscience, 1:100), and PE conjugated CD11b monoclonal antibody (eBioscience, 1:100). Thioglycollate elicited macrophage and tumor associated macrophage expression for C/EBPα was performed by staining surface of cell by FITC conjugated F4/80 monoclonal antibody (eBioscience 1:100). Cells were intracellularly stained using monoclonal anti-rabbit C/EBPα (Cell Signalling, 1:100) using standard fixation and permeabliziation protocol (eBioscience) as recommended by manufacturer.

Western Blotting

Protein lysates were performed as previously described (67). Briefly, primary antibodies included rabbit anti-mouse C/EBPα (Cell Signaling Technology), rabbit anti-mouse β actin (Sigma), Secondary antibodies, each of which was conjugated to horseradish peroxidase, included rabbit anti-mouse IgG (Sigma) and goat anti-rabbit IgG (Calbiochem, Temecula, CA) diluted 1:10,000. Cellular protein lysates were electrophoresed on a 10–20% tris-glycine polyacrylamide gel (Invitrogen) and transferred to 0.2-μM nitrocellulose membranes (BioRad Laboratories, Hercules, CA), blocked in 5% dry milk, and incubated with primary antibody at the indicated dilutions (4°C, overnight). After washing three times, membranes were incubated with respective

secondary antibodies (room temperature, 1 hour). Immunoreactive proteins were visualized by enhanced chemiluminescence, (GE Healthcare UK Limited).

In vitro Expansion of Bone Marrow Myeloid Derived Suppressor Cells (MDSCs)

Bone marrow cells were obtained from the femurs and tibias. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 ng/ml GM-CSF, 10 ng/ml IL-4, and 50 µM 2-ME. The cultures were maintained at 37°C in 5% CO_2-humidified atmosphere in 6-well plates. After 3 days of culture, floating cells were gently removed, and cells were collected and analyzed by flow cytometry for expression of CD11b and Gr-1.

Statistics

All data are expressed as the arithmetic means +/- SEM. Comparisons between two groups of animals or treatments were made by one-way ANOVA or by two tailed student's T test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Differential effect of myeloid cells on the tumor response in ACE 10 mice

ACE 10 mice significantly limit B16-F10 melanoma growth *in vivo* with enhanced myelomonocytic cell infiltration within the tumors (12).That F4/80+ and CD11b+ cells correlated with tumor reduction in the ACE 10 is intriguing, because WT myeloid cells support tumor growth, as they are polarized by the tumor micro-environment towards a tumor-promoting MDSC and the alternatively activated M2 phenotype (47). To investigate this, we depleted F4/80+ and CD11b+ myeloid cells using clodronate-encapsulated liposomes, a well-described reagent that effectively eliminates macrophages and MDSCs (72). Control groups were administered empty liposome containing PBS. Flow cytometric analysis confirmed that liposome clodronate depleted F4/80+ and CD11b+ cells effectively and equivalently in both WT and ACE 10 mice (Fig 1b). In accordance with previous reports (72), liposome clodronate significantly reduced tumor growth in WT by nearly 50% (Fig 1a), suggesting that WT myeloid cells negatively impact anti-tumor immunity. Interestingly, depletion of myeloid cells in the ACE 10 mice did not significantly alter tumor volume (Fig 1a). The inability to further reduce tumor volume in the ACE 10 mice after myeloid cell depletion may be because these mice already reject tumors, such that elimination of MDSCs does not further improve this immunity. One other possibility, although unlikely, is that myeloid cells do not play any role in the enhanced ACE 10 anti-tumor immunity. An alternate hypothesis is that depletion of pro-tumoral MDSCs and anti-tumoral M1 macrophages offset each other.

To gain more insights into the ACE 10 myeloid cells, we treated a separate cohort of ACE 10 mice with the ACE inhibitor captopril for several days prior to tumor implantation. At the day of tumor implantation, the macrophages of some of these mice were depleted with clodronate liposome. As previously reported, ACE inhibition blunts the enhanced ACE expression in the ACE 10 and increases tumor size in the ACE 10 mice (Fig 1a). This result suggests that myelomonocytic cell ACE over-expression is an important component of the anti-tumor immunity in the ACE 10. Depletion of myeloid cells after ACE inhibition in the ACE 10 resulted in a reduced tumor burden (Fig 1a). All together, these data argue for a role of myeloid cells in the ACE 10 tumor immunity. Specifically, they suggest that while myeloid cells in WT mice are detrimental to anti-tumor immunity, those with increased ACE expression have an altered phenotype, and thus do not contribute significantly to the myeloid-mediated immunosuppression.

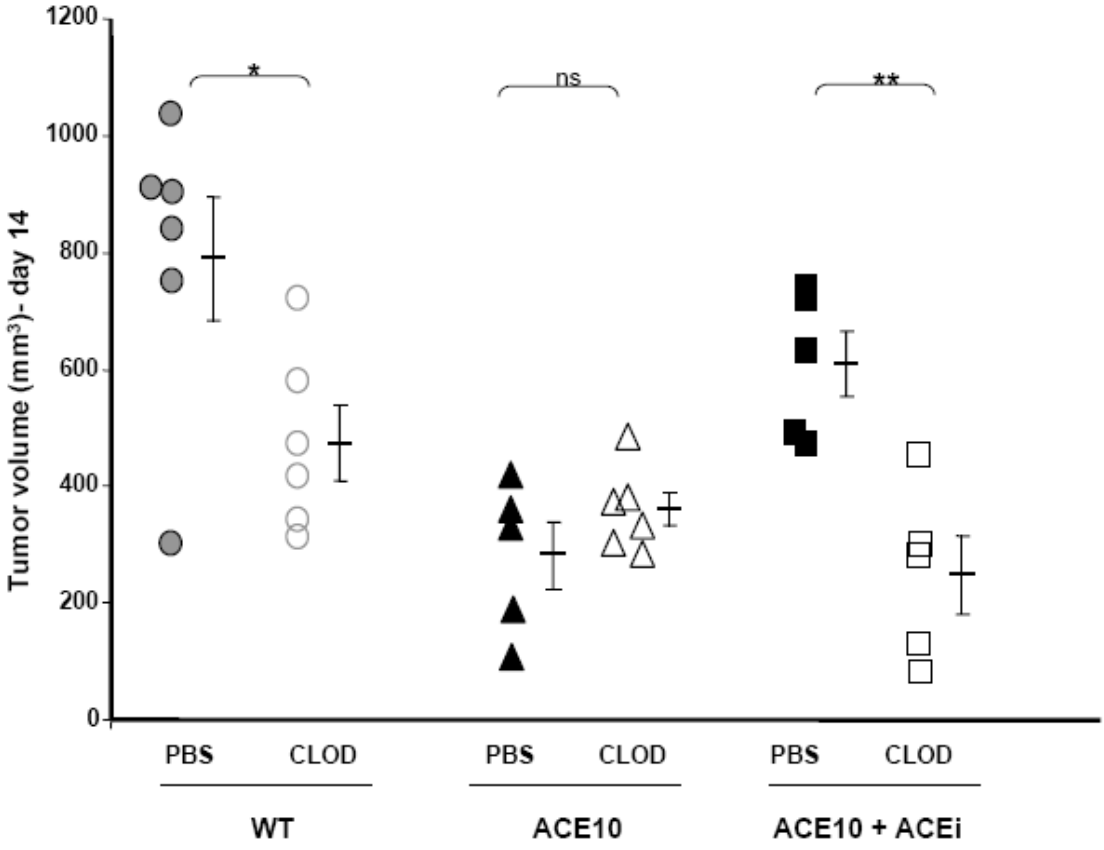


Figure 3-1A. Effect of myeloid cell depletion on tumor growth

WT and ACE 10 mice were challenged by an intradermal injection of $1X10^6$ B16-F10. Mice were depleted of macrophages *in vivo* by intraperitoneal injection of 1 mg liposome chlodronate beginning the day of tumor challenge (day 0), and then every 72 hrs (n=6). Control groups were injected with liposome-PBS (n=6). A group of ACE 10 mice (n=12) were treated with ACE inhibitors, and were maintained on ACE inhibitors throughout the course of the studies. Of this group, some mice were depleted of macrophages by chlodronate (n=6). Fourteen days later, tumor volume was measured. *$p< 0.05$, ** $p<0.02$, ns = not significant. Data are pooled from 3 independent experiments.

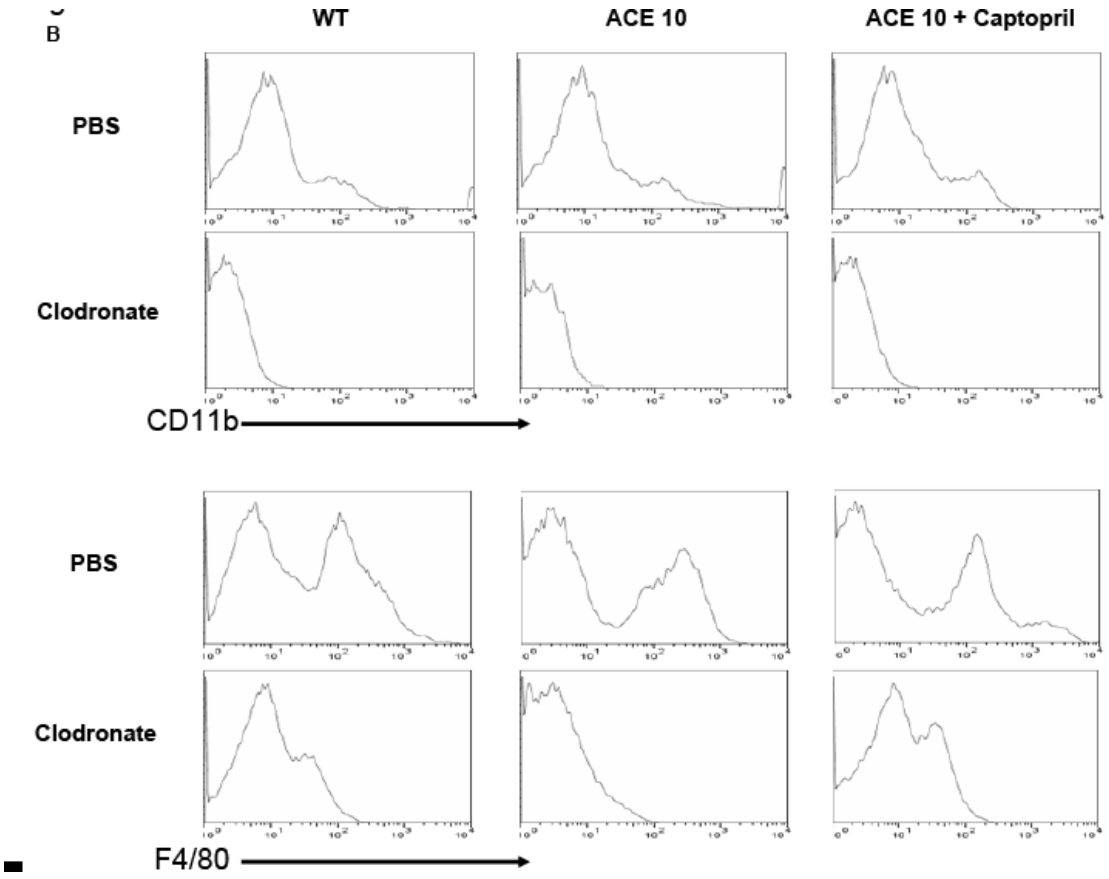


Figure 3-1B. A representative flow cytometric analysis to confirm depletion of C11b+ and F4/80+ myeloid cells.

Reduced Myeloid Derived Suppressor Cells (MDSCs) in the ACE 10 mice

To understand the behavior of myeloid cells in the ACE 10, we first examined myeloid derived suppressor cells (MDSCs). During tumor progression, dysfunctional myelopoiesis rapidly expands and accumulates MDSCs in the spleen. Thus, we asked if the quantity of MDSCs differed *in vivo* between tumor-bearing WT and ACE 10 mice. Certainly, tumor-bearing mice increase splenic numbers of MDSCs (Fig 2a). Remarkably, we found low numbers of MDSCs in the spleen of ACE 10 tumor bearing mice. A representative flow cytometric analysis of CD11b+Gr+(int) cells in the spleen is shown in Fig 2a. Whereas these immature cells comprised nearly 15% of splenocytes of WT tumor bearing mice, they only made up < 7% of the ACE 10 splenocytes (Fig 2a,b). The reduced MDSCs in the spleen of ACE 10 tumor-bearing mice were not due to diminished cellularity of the ACE 10 splenocytes (Fig 2b, bottom panel). Furthermore, ACE 10 mice maintained on ACE inhibitor through the course of the tumor growth increased MDSC population in the spleen (Fig 2 a,b). Thus, these data suggest that *in vivo*, the presence of ACE in the myelomonocytic cells promotes myeloid cell maturation and consequently suppresses the ability to generate immunosuppressive MDSCs that accumulate in the spleen.

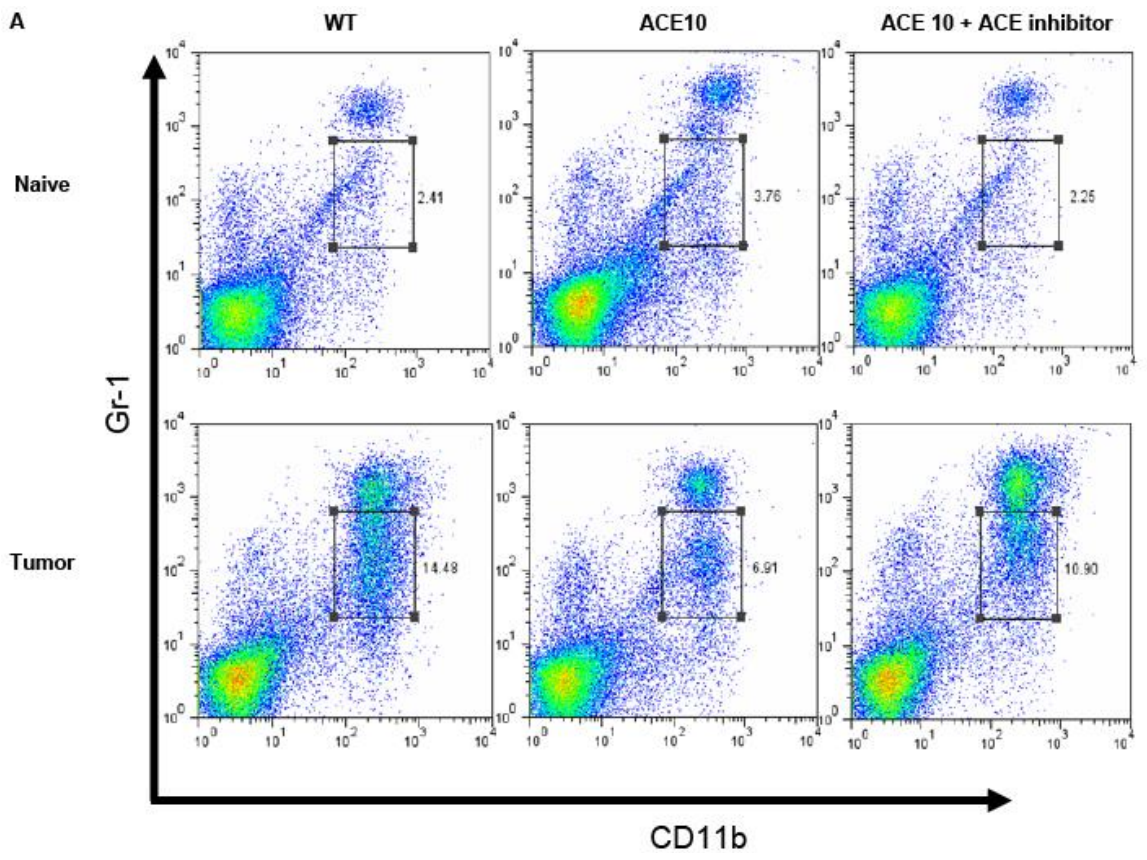


Figure 3- 2A: *In vivo* analysis of myeloid derived suppressor cells (MDSCs)

Spleen cells from naïve and tumor bearing WT, ACE 10, and ACE 10 mice treated with ACE inhibitor were stained for CD11b (X-axis) and and Gr-1 (Y-axis). Representative flow cytometric analysis is shown. There is reduced number of CD11b+Gr-1+(int) cells in the spleen of tumor-bearing ACE 10 mice, compared to equivalently treated WT mice. Treatment with ACE inhibitor increased the number of the MDSCs in the spleen of ACE 10 mice. A representative FACS analysis is shown.

(=6 mice per group).

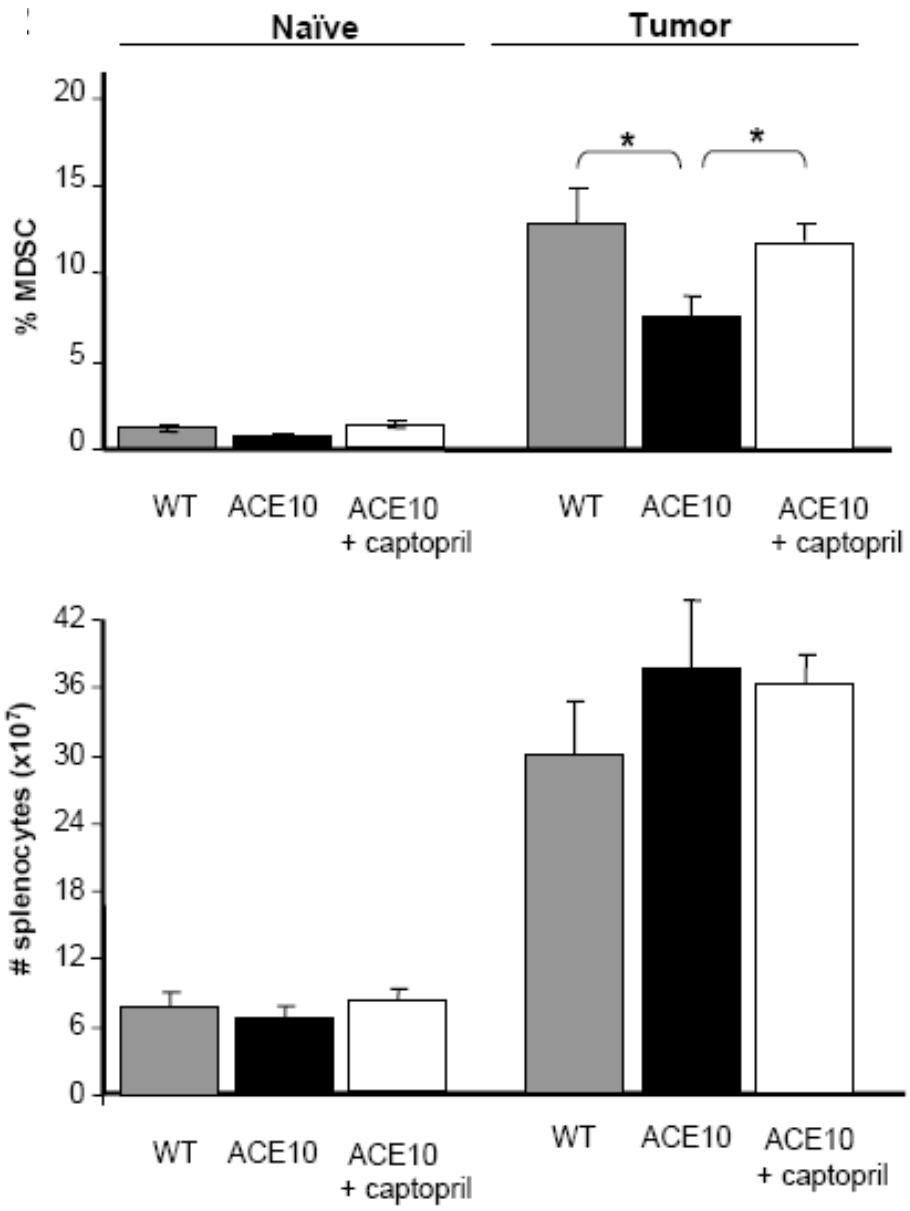


Figure 3- 2B. Percentage of immature cells in the spleen from ACE 10 and WT tumor bearing mice after 14 days (n=6 per group). $*$ $p < 0.05$. Total splenocytes in tumor-bearing mice of WT and ACE 10 mice (n=6 per group), There were no significant differences in the total spleen cells for equivalently treated groups of mice (bottom panel).

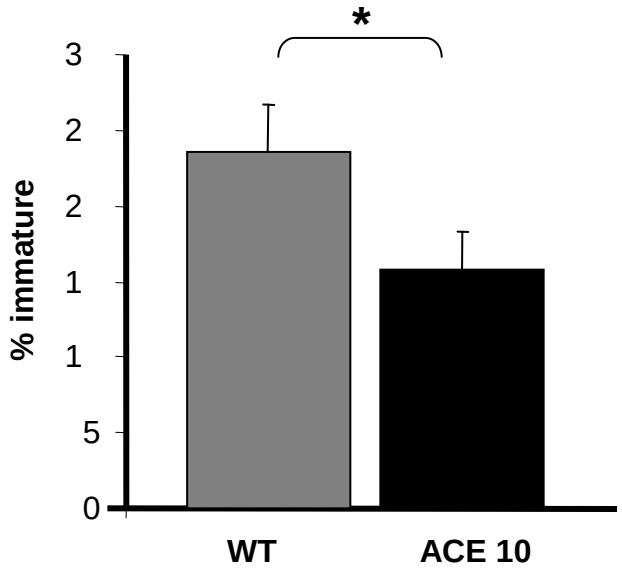


Figure 3-3A. *In vitro* analysis of myeloid derived suppressor cells (MDSCs)

Bone marrow progenitor cells of WT and ACE 10 were cultured with GM-CSF and IL-4 (10 ng/ml each). Three days later, equal number of cells were stained for CD11b and Gr-1 and analyzed by flow cytometry. Percentage of CD11b+Gr-1+(int) immature cells was determined.

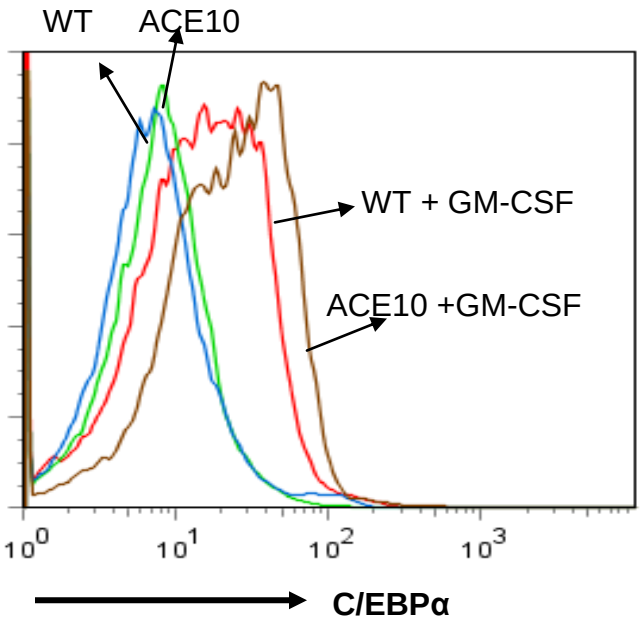


Figure 3-3B. C/EBPα expression in progenitor cells were measured by intracellular cytokine staining after 24 hours of culture as in (a). All experiment was performed three independent times. *p< 0.05.

Increased C/EBPα in ACE 10 Myeloid Cells

We have recently indicated that physiological ACE, through C/EBPα, regulates myelopoiesis. Thus, to understand the mechanisms by which ACE 10 maintain reduced immature cells, we hypothesized that C/EBPα would mediate myeloid maturation in the ACE over-expressing myeloid cells. First we set up *in vitro* cultures to compare C/EBPα expression in bone marrow progenitors of the ACE 10 and WT (Fig 3). Our experiment also revealed that after 3 days of culture, whereas immature CD11b+Gr-1(int) cells comprised 23% ± 2.4% in the WT, they only made up 16% ± 2.1% of cells in the ACE 10 (Fig 3a). Furthermore, we found that GM-CSF-induced C/EBPα expression was augmented in the ACE 10 cells, as shown in Fig 3b. Thus, *in vitro*, there is a reduced number of MDSCs associated with ACE over-expression. These findings suggest that ACE, through C/EBPα, drives myeloid maturation with a consequent reduction of tumor-promoting immature cells *in vivo*. This is in accordance with our recent report showing impaired C/EBPα induction in the absence of ACE (70).

All *trans*-retinoic acid (ATRA) suppresses MDSCs via C/EBPα

We have so far demonstrated that the over-expression of ACE induces C/EBPα which drives myeloid maturation, thus reducing the expansion of immature cells. Although C/EBPα is widely accepted to be critical for myeloid differentiation, its involvement in regulating the expansion of MDSCs during tumor progression is relatively unknown. To establish this as a generalized phenomenon beyond the ACE 10 model, we tested the ability of a known agent that suppresses MDSCs to induce C/EBPα

in myeloid cells. All *trans*- retinoic acid (ATRA) induces complete remission in patients with acute pro-myelocytic leukemia by promoting differentiation of immature myeloid blasts and suppressing the expansion of MDSCs (73, 74). We hypothesized that this may be because it induces myeloid cell maturation via C/EBPα. To assess this, we differentiated WT bone marrow progenitors in the presence of 10 ng/ml GM-CSF and 10 ng/ml IL-4, with or without 1μM ATRA. This experiment confirmed that ATRA indeed markedly reduces the percentage of immature cells, similar to what we found in the ACE 10 (Fig 4a). Notably, ATRA induced increased expression of C/EBPα in the myeloid progenitor cells after 24 hrs of culture (Fig 4b). Thus, we demonstrate in the ACE 10, and corroborate with an independent model using ATRA, that the induction of C/EBPα may represent a novel mechanism by which to promote myeloid maturation and suppress MDSCs in the tumor micro-environment.

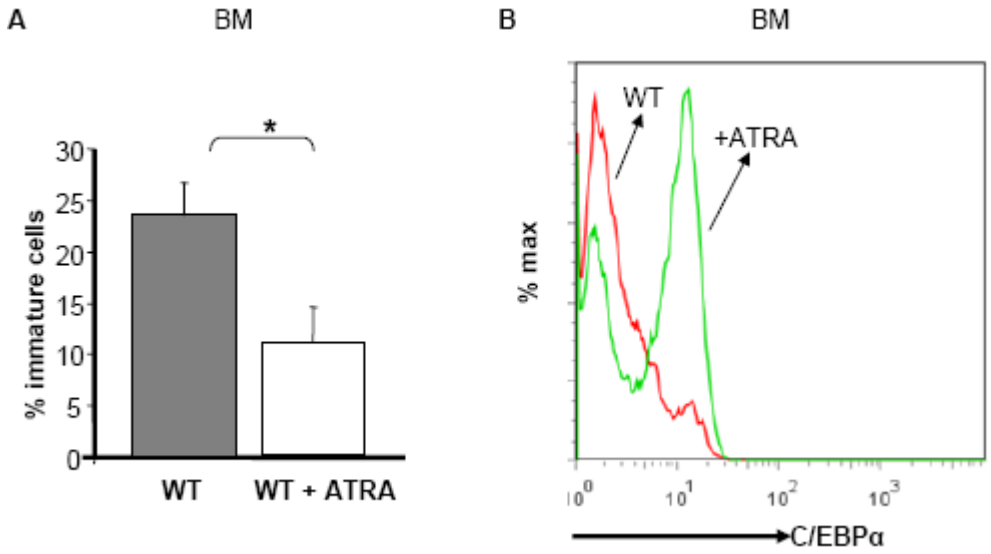


Figure 3-4: All *trans*- retinoic acid (ATRA) limits expansion of MDSCs via C/EBPα

(A) Bone marrow progenitors were differentiated *in vitro* with 10 ng/ml GM-CSF and IL-4 and 50 µM 2-beta mercaptoethanol. Some groups of cells were co-cultured with 1µM ATRA. After 3 days of culture, cells were collected, labeled with anti-Gr-1 and anti-CD11b Abs, and analyzed by flow cytometry. *$p < 0.05$ for statistically significant differences between WT and ACE 10. Experiment was performed 2 independent times (n=5 per group). (B) Cells were cultured as in (a), and C/EBPα expression was measured after 24 hrs. (c) WT mice were administered a total of 5 mg of ATRA by minipump delivery. Thioglycollate-elicited macrophages were analyzed for C/EBPα expression (n=5 per group). Experiment was performed 2 independent times.

Sustained C/EBPα expression in ACE 10 macrophages

So far, we have shown that C/EBPα expression in the ACE10 enables myeloid cells to properly differentiate, thus reducing immature pro-tumor MDSCs. Thioglycollate-elicited peritoneal macrophages (TPMs) are a simple window by which to analyze bone marrow (BM), since these macrophages readily develop from the BM (70). Thus, to study if the enhanced C/EBPα occurs in the ACE 10 *in vivo*, we looked at the expression of C/EBPα in TPMs. Western blotting and densitometric analysis showed that naïve ACE 10 TPMs expressed significantly higher levels of C/EBPα protein compared to WT (Fig 5a,b).

Among several mechanisms, down-regulation of C/EBPα has been implicated as a major means by which the tumor micro-environment impairs effective M1 response (62, 63, 75). Thus, we sought to determine if C/EBPα expression is altered in these cells in the tumor-microenvironment (Fig 5). To obtain these tumor-associated macrophages (TAMs), tumor-bearing mice were injected with thioglycollate to recruit cells to the peritoneum (63). Following a peritoneal lavage, cells were collected and F4/80 + cells were analyzed for C/EBPα expression by flow cytometry. This experiment confirmed that C/EBPα is down-regulated in WT TAMs compared to TPMs (Fig 5c,d). However, we noted that both naïve and tumor-bearing ACE 10 macrophages expressed higher amounts of C/EBPα compared to equivalently-treated WT (Fig 5c). Furthermore, ACE inhibition reversed the enhanced C/EBPα expression in the ACE 10 (Fig 5 c,d). Altogether, these results reveal that ACE manipulation affects C/EBPα expression in both naïve and tumor-associated macrophages.

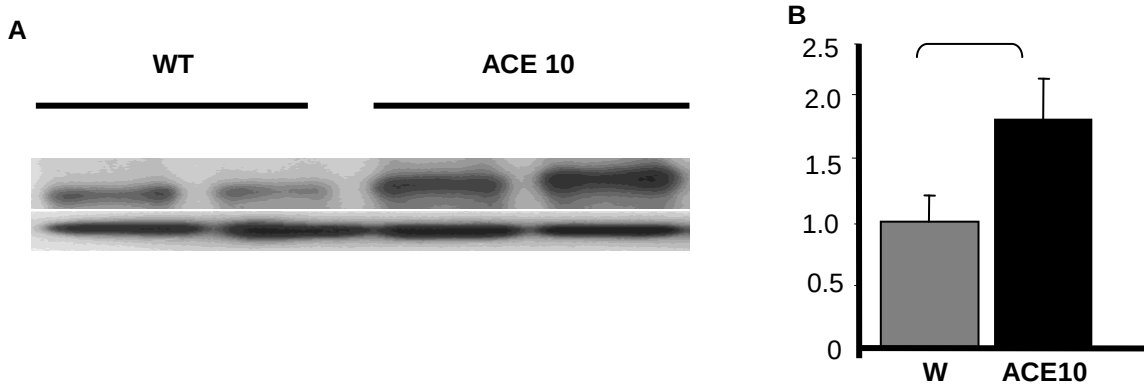


Figure 3-5: Analysis of C/EBPα induction and functional assays of ACE 10 macrophages (A) C/EBPα expression in naïve ACE 10 and WT TPM was determined by western analysis. A representative blot of two mice per group is shown (B) Densitometry was used to quantitate amount of C/EBPα relative to β actin expression (n=6 mice per group).

Enhanced Tumor Killing by ACE 10 Macrophages

If a reduced C/EBPα expression is a means by which tumors suppress effective M1 response, then a sustained C/EBPα expression in the ACE 10 might promote anti-tumor response in these macrophages. To study this, TPMs were harvested from WT and ACE10 mice, and co-cultured with B16-F10 tumor cells in an *in vitro* killing assay (Fig 5e). We observed that ACE 10 TPMs primed with LPS killed tumor cells more efficiently than WT by nearly two-folds (Fig 5e). Previous work has shown that nitric oxide is the most potent mediator of macrophage tumor cytotoxicity (56, 76).To test the effects of nitrite production on the enhanced tumoricidal ability of ACE 10 TPMs, we treated primed cells with the iNOS specific inhibitor 1400W prior to co-culture with tumor cells. As seen in Fig 5e, nitric oxide blockade renders ACE10 TPM indistinguishable from WT TPM. This data is in accordance with our earlier reports showing that augmented nitrite production by the ACE 10 macrophages plays crucial role in their immune phenotype (12, 67).

We also tested the ability of TAMs to directly kill tumor cells (Fig 5f). Cells from peritoneal lavage of tumor-bearing mice were seeded into 96-well plate for 2hrs, allowing macrophages (>95%) to adhere. It is of note that LPS-activated TAMs kill tumor cells less efficiently than TPMs (Fig 5f vs 5e). However, ACE10 TAMs primed with LPS maintain a significant advantage over their WT counterparts in their ability to kill tumor cells. The reduced tumor killing by WT TAMs is due to inability to produce iNOS as a result of decreased sensitivity to LPS (56). To further demonstrate the retained robust response to LPS by ACE 10, we exposed TPMs to supernatant from B16-F10 tumor cells (called MCM) prior to stimulating with LPS (Fig 5g). WT TPMs exposed to MCM had a

reduced response to LPS, but those of ACE 10 maintain a significant ability to produce nitrites (WT 1.1 ± 0.7 μM, ACE 10; 6.8 ± 1.3μM, $p < 0.005$). Indeed, we have previously shown that, *ex vivo*, ACE 10 tumor induced macrophages produce nearly 10 folds the nitrites levels of WT (12). Altogether, these data show that over-expression of ACE in m promote retention of the M1 response through enhanced C/EBPα expression.

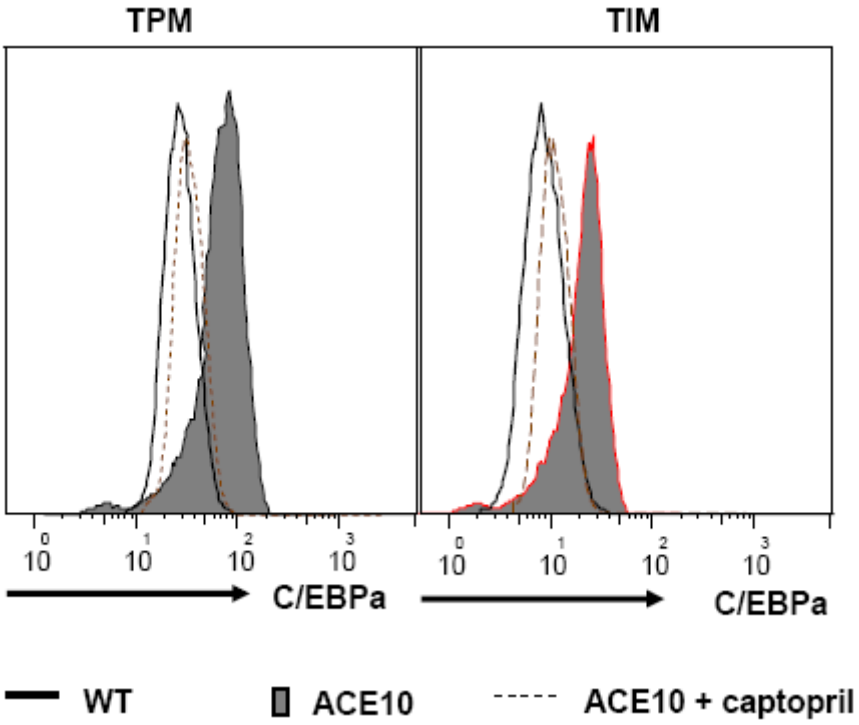
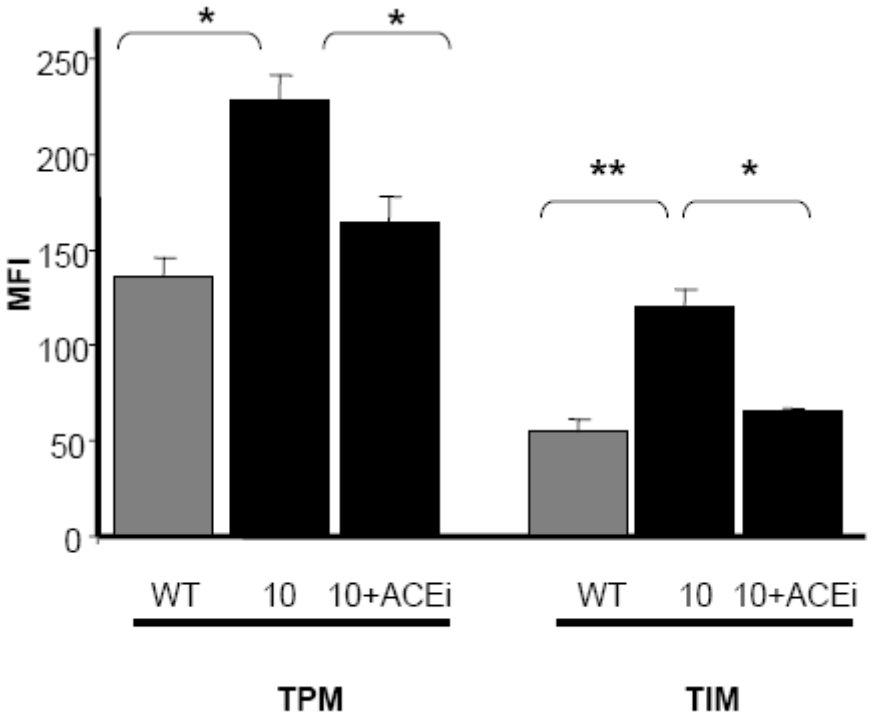


Figure 3-5 (C,D). FACS analysis of TPMs and TAMs for intracellular C/EBPα expression. F4/80 positive cells collected from peritoneal wash of naïve or tumor-bearing WT, ACE 10, and ACE 10 mice treated with ACE inhibitor. Cells were stained intracellularly for C/EBPα. A representative data is shown in (C).



Mean fluorescence intensity (MFI) of C/EBPα intracellular staining is shown for WT, ACE 10, and ACE 10 mice treated with ACE inhibitor (n= 5-6 animals per group). Data is presented as mean ± SEM. *$p<0.05$, **$p<0.02$

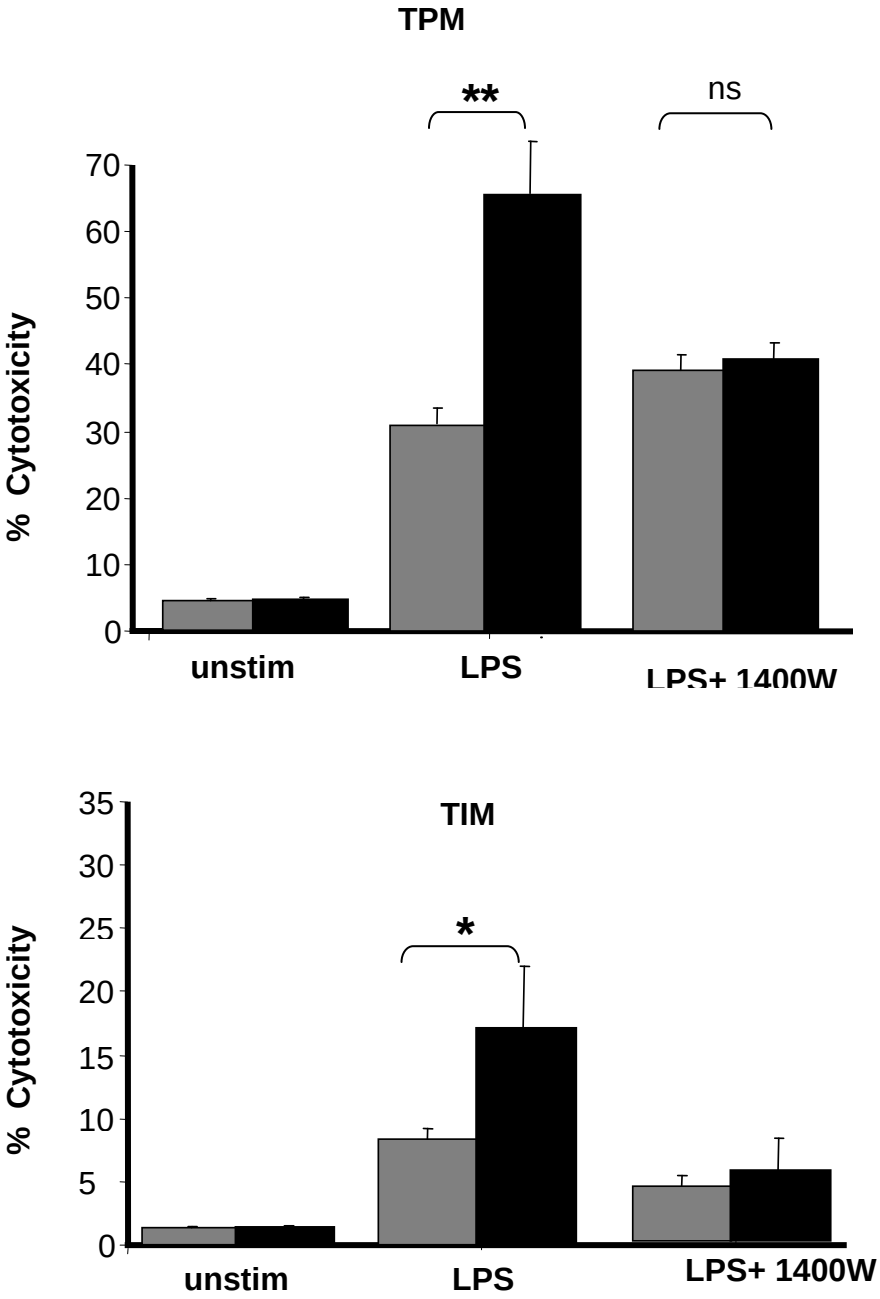


Figure 3-5 (E,F). TPMs (e) or TAMs (f) were seeded at $2X10^5$ in 96-well plates. Triplicate wells were primed overnight with 100ng/ml LPS. Some wells of cells were co-cultured with 1 µM of the iNOS inhibitor 1400W. The next day, all wells of cells were co-incubated with $2X10^4$ tumor cells. 18 hours later, tumor cell death was evaluated by measuring optical density of LDH released in the supernatant.

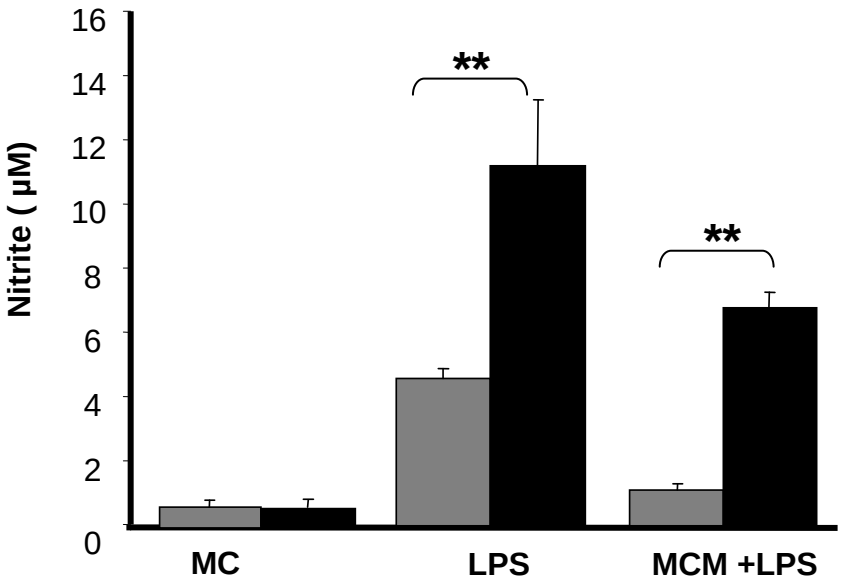


Figure 3-5G. Nitrite production in the supernatant was measured by Griess assay. TPM were incubated with 1:1 melanoma conditioned media: complete DMEM media supplemented with FCS. Six hours later, cells were thoroughly washed in PBS, and cultured with 1 µg/ml for 18 hrs. Nitrite release in the supernatant was then measured by Griess assay. Experiments were repeated three independent times. *p< 0.05, ** p<0.02, ns = not significant.

In conclusion, we elucidate a novel mechanism by which ACE modifies the behavior of myelomonocytic cells during tumor growth. In WT mice, tumor-induced myelopoiesis generates immature MDSCs that are educated by the micro-environment to become ineffective M1 macrophages, in part by down-regulating C/EBPα. However, the over-expression of ACE in myelomonocytic cells induces C/EBPα which interferes with tumor-induced immune dysfunction by a two-prong approach: first by driving myeloid cell maturation and thereby reducing immature MDSCs, and second by maintaining an effective M1 response. Because MDSCs promote regulatory T cells at the expense of CD8 T cell responses, their diminution allows for effective CD8 T cell and M1 macrophage response in the ACE 10 (see model in Fig 6). This finding may underline a novel therapeutic target for anti-tumor immunity.

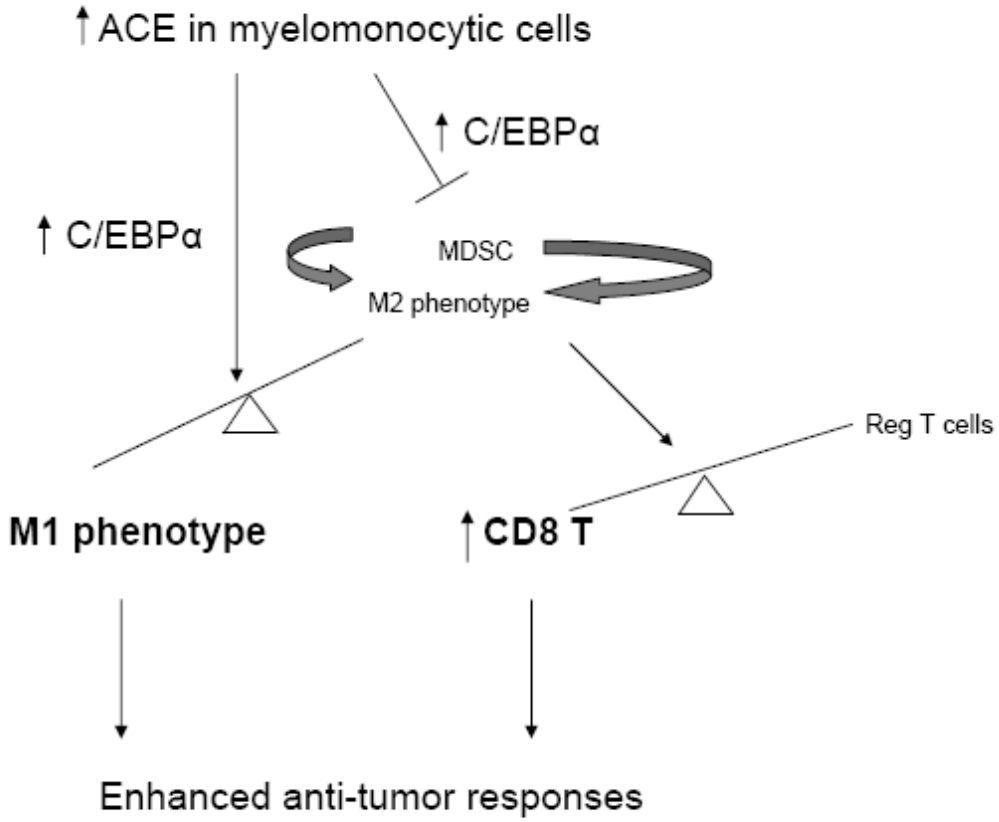


Figure 3-6: model of reduced myeloid-mediated immunosuppression in the ACE 10

ACE over-expression induces C/EBPα in myelomonocytic cells. Enhanced C/EBPα promotes myeloid maturation, decreasing the pro-tumor M2/MDSCS population. Because M2/MDSCS myeloid cells induce regulatory T cells, their diminution allows for increased CD8 T cells. C/EBPα also allows ACE 10 to maintain a robust anti-tumor M1 response. The M1 macrophages and CD8 T cells, combined, improve anti-tumor immunity in the ACE 10 mice.

ACKNOWLEDGEMENT

The authors wish to thank Drs. Charles Parkos and Ifor Williams (Emory University School of Medicine, Atlanta GA), and George Liu (Cedars Sinai Medical Center, Los Angeles, CA) for helpful discussions and advice. This work was supported by NIH grants R01 DK039777 (KB) and R01 DK051445 (KB).

DOD, KEB and XZS designed experiments

DOD, XZS and KEB wrote paper

DOD, CL, EB, FSO and XZS performed experiment

DOD, XZS and KEB contributed to study design

References:

1. Rosenberg, S. A., J. C. Yang, and N. P. Restifo. 2004. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 10:909-915.

2. Qian, B. Z., and J. W. Pollard. 2010. Macrophage diversity enhances tumor progression and metastasis. *Cell* 141:39-51.

3. Ostrand-Rosenberg, S., and P. Sinha. 2009. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182:4499-4506.

4. Gabrilovich, D. I., and S. Nagaraj. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 9:162-174.

5. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 23:549-555.

6. Sinha, P., V. K. Clements, S. K. Bunt, S. M. Albelda, and S. Ostrand-Rosenberg. 2007. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol* 179:977-983.

7. Sinha, P., V. K. Clements, S. Miller, and S. Ostrand-Rosenberg. 2005. Tumor immunity: a balancing act between T cell activation, macrophage activation and tumor-induced immune suppression. *Cancer Immunol Immunother* 54:1137-1142.

8. Sinha, P., V. K. Clements, and S. Ostrand-Rosenberg. 2005. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. *Cancer Res* 65:11743-11751.

9. Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

10. Mantovani, A., A. Sica, P. Allavena, C. Garlanda, and M. Locati. 2009. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum Immunol* 70:325-330.

11. Dinapoli, M. R., C. L. Calderon, and D. M. Lopez. 1996. The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduce expression of the inducible nitric oxide synthase gene. *J Exp Med* 183:1323-1329.

12. Sinha, P., V. K. Clements, and S. Ostrand-Rosenberg. 2005. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol* 174:636-645.

13. Sica, A., and V. Bronte. 2007. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 117:1155-1166.

14. Heath, V., H. C. Suh, M. Holman, K. Renn, J. M. Gooya, S. Parkin, K. D. Klarmann, M. Ortiz, P. Johnson, and J. Keller. 2004. C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood* 104:1639-1647.

15. Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman. 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood* 80:1725-1735.

16. Witsell, A. L., and L. B. Schook. 1991. Macrophage heterogeneity occurs through a developmental mechanism. *Proc Natl Acad Sci U S A* 88:1963-1967.

17. Torroella-Kouri, M., J. C. Keith, M. Ivanova, and D. M. Lopez. 2003. IL-11-induced reduction of C/EBP transcription factor binding may contribute to the IL-12 downregulation in tumor-bearing mice. *Int J Oncol* 22:439-448.

18. Torroella-Kouri, M., X. Ma, G. Perry, M. Ivanova, P. J. Cejas, J. L. Owen, V. Iragavarapu-Charyulu, and D. M. Lopez. 2005. Diminished expression of transcription factors nuclear factor kappaB and CCAAT/enhancer binding protein underlies a novel tumor evasion mechanism affecting macrophages of mammary tumor-bearing mice. *Cancer Res* 65:10578-10584.

19. Ruffell, D., F. Mourkioti, A. Gambardella, P. Kirstetter, R. G. Lopez, N. Rosenthal, and C. Nerlov. 2009. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A* 106:17475-17480.

20. Marigo, I., E. Bosio, S. Solito, C. Mesa, A. Fernandez, L. Dolcetti, S. Ugel, N. Sonda, S. Bicciato, E. Falisi, F. Calabrese, G. Basso, P. Zanovello, E. Cozzi, S. Mandruzzato, and V. Bronte. 2010. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity* 32:790-802.

21. Kusmartsev, S., and D. I. Gabrilovich. 2003. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol* 74:186-196.

22. Shen, X. Z., P. Li, D. Weiss, S. Fuchs, H. D. Xiao, J. A. Adams, I. R. Williams, M. R. Capecchi, W. R. Taylor, and K. E. Bernstein. 2007. Mice with enhanced macrophage angiotensin-converting enzyme are resistant to melanoma. *Am J Pathol* 170:2122-2134.

23. Okwan-Duodu, D., V. Datta, X. Z. Shen, H. S. Goodridge, E. A. Bernstein, S. Fuchs, G. Y. Liu, and K. E. Bernstein. 2010. Angiotensin converting enzyme over-expression in mouse myelomonocytic cells augments resistance to listeria and methicillin resistant S. Aureus. *J Biol Chem*.

24. Bingle, L., N. J. Brown, and C. E. Lewis. 2002. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 196:254-265.

25. Steidl, C., T. Lee, S. P. Shah, P. Farinha, G. Han, T. Nayar, A. Delaney, S. J. Jones, J. Iqbal, D. D. Weisenburger, M. A. Bast, A. Rosenwald, H. K. Muller-Hermelink, L. M. Rimsza, E. Campo, J. Delabie, R. M. Braziel, J. R. Cook, R. R. Tubbs, E. S. Jaffe, G. Lenz, J. M. Connors, L. M. Staudt, W. C. Chan, and R. D. Gascoyne. 2010. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* 362:875-885.

26. Lin, C., V. Datta, D. Okwan-Duodu, X. Chen, S. Fuchs, R. Alsabeh, S. Billet, K. E. Bernstein, and X. Z. Shen. 2010. Angiotensin-converting enzyme is required for normal myelopoiesis. *FASEB J*.

27. Zeisberger, S. M., B. Odermatt, C. Marty, A. H. Zehnder-Fjallman, K. Ballmer-Hofer, and R. A. Schwendener. 2006. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br J Cancer* 95:272-281.

28. Kusmartsev, S., F. Cheng, B. Yu, Y. Nefedova, E. Sotomayor, R. Lush, and D. Gabrilovich. 2003. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res* 63:4441-4449.

29. Degos, L., and Z. Y. Wang. 2001. All trans retinoic acid in acute promyelocytic leukemia. *Oncogene* 20:7140-7145.

30. Torroella-Kouri, M., R. Silvera, D. Rodriguez, R. Caso, A. Shatry, S. Opiela, D. Ilkovitch, R. A. Schwendener, V. Iragavarapu-Charyulu, Y. Cardentey, N. Strbo, and D. M. Lopez. 2009. Identification of a subpopulation of macrophages in mammary tumor-bearing mice that are neither M1 nor M2 and are less differentiated. *Cancer Res* 69:4800-4809.

31. Chang, C. I., J. C. Liao, and L. Kuo. 2001. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. *Cancer Res* 61:1100-1106.

32. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83-93.

33. van Rooijen, N. 1994. Liposome mediated modulation of macrophage functions. *Adv Exp Med Biol* 355:69-74.

CHAPTER 4: Conclusions and Future Directions

ACE and myelomonocytic cell function

Components of the RAS are undoubtedly involved in the regulation of the immune response. The ACE 10 model demonstrates that the carboxypeptidase ACE can modify the behavior of myelomonocytic cells, enhancing their capacity as antigen presenting cells (1), and promoting the propensity for an effective pro-inflammatory response (2, 3). At least in the bacterial infection models, nitric oxide production seemed most critical in the ACE 10 immune phenotype, as inhibition of this cytokine rendered the ACE 10 identical to WT mice. Whereas the enhanced immunity to both tumors and bacterial infections were abrogated by ACE inhibition, AT1 receptor blockade had minimal effect. Thus, these phenotypes, while dependent on the catalytic activity of ACE, are not mediated by angiotensin II, the most well known and active effector of the RAS. It must be noted that several carboxypeptidases have been described to influence the inflammatory response. For example, the kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells (4). The upregulation of carboxypeptidase-D also influences nitric oxide synthesis in RAW 264.7 macrophage cell line (5). Furthermore, mast cells possess a wide array of peptidases in their granules that mediate their inflammatory response and host defense (6, 7) .

Not only do peptidases play a role in cytokine production, but they can also mediate bacterial killing, as demonstrated by the direct anti-microbial capacity of the non-catalytic carboxy terminal domain of the metallopeptidase MMP-12 (8). Thus, it is

conceivable that the ACE 10 represents a model in which ACE acts independent of the RAS, providing additional arsenal as a carboxypeptidase by which to cleave tumor and bacterial antigens and process them, as well as ensure the ability of the myelomonocytic cells to mount an appropriate inflammatory response. In fact, our own unpublished observations suggest that ACE is significantly upregulated by WT monocytes/macrophages, particularly in the face of an inflammatory challenge such as infection with *Listeria monocytogenes*, suggesting that ACE, and perhaps other peptidases may play a key role in normal physiological immune responses. In line with this, previous work has also shown that monocytes differentiating into macrophages or dendritic cells markedly augment ACE levels (9, 10). Furthermore, chronic inflammation, such as granulomas (sarcoids) and atherogenesis induce ACE expression in CD68+ and F4/80+ macrophages. Thus, ACE could be involved in myeloid cell maturation by processing certain substrates required for myelopoiesis, as well as supporting the function of mature myeloid cells during an inflammatory challenge.

In our analysis of the molecular changes that result from ACE over-expression in myelomonocytic cells, we have focused on differentiation- mediated mechanisms. In this search, we have found significant increase in the levels of the transcription factor C/EBPα in ACE 10 macrophages. C/EBPα is CCAAT-binding protein whose role in steady-state myelopoiesis is well-recognized (11, 12). However, there is evidence that they are markedly down-regulated in macrophages of tumor-bearing mice (13-15). We found that this transcription factor is sustained in the ACE 10, perhaps driving the ability of the ACE 10 tumor-associated macrophages to sustain effective tumor antigen processing and direct killing. The modification of C/EBPα in the ACE 10 is nicely

complemented by our recent discovery of immune dysfunction and myelopoiesis defects in the ACE KO mice (16). We found that the defective myelopoiesis and impaired macrophage function in the ACE KO was in part due to diminished C/EBPα levels, which impairs myeloid maturation and consequently increases immature blasts in extramedullary sites such as the spleen. Thus, there seem to be a link between ACE expression and myeloid differentiation through C/EBPα. Further work is required to dissect this previously unknown inter-relationship. For example, to fully appreciate the role of C/EBPα in the phenotypes of the ACE KO and the ACE 10, we can take advantage of small interfering RNA (siRNA) to suppress the expression of C/EBPα in the ACE 10 cells, or lentivirus to force expression of C/EBPα in the ACE KO cells. In theory, these techniques should restore the phenotypes of these strains to WT cells.

The finding that ACE modifies myeloid maturation and function has important implications in tumor immunity. Tumor-induced myeloid dysfunction generates immature, ineffective myeloid cells that accumulate in secondary lymphoid organs-particularly the spleen- and supplant anti-tumor immunity. Furthermore, it has been recently described that angiotensin II/AT1 signaling axis is necessary for the deployment of the splenic reservoir of myelomonocytic cells that mediate control and repair of inflammatory injuries (17, 18). Thus, one could imagine a situation in which despite the reduction in blood pressure, and probably reduced angiogenesis in the absence of RAS, the compensatory expansion of immature myeloid cells in the spleen of the ACE KO mice would result in an unpredictable response to tumor challenge. The revelation of the role of the RAS in splenic myeloid cell trafficking also suggests that, perhaps contrary to our previous assumption that WT mice treated with ACE inhibitors have a reduction in

tumor volume because of sudden drop in blood pressure (3), it may be that ACE inhibition blocks the deployment of immature cells to the tumor micro-environment, in the same way ACE inhibition blocks deployment of monocytes to injured heart sites (18). This hypothesis is supported by our in unpublished data (n=5 mice per group), where treatment of WT mice with hydralazine did not reduce tumor volume as efficiently as treatment with an ACE inhibitor or AT1 receptor blocker, although all treatments equivalently reduced blood pressure. By extension, that ACE plays a role in deployment of inflammatory cells to injured tissues may partly explain the reduction in blood pressure of WT mice treated with ACE inhibitors in angiotensin-II infused hypertension models, since inflammatory monocytes are critical to the pathophysiology of renal injury and hypertension (19, 20). To maximize therapeutic opportunities, more work is necessary to dissect the impact of the RAS on inflammatory cells outside of its role in tumor angiogenesis and hemodynamics.

The other aspect of the inflammatory response that has not been addressed so far in the ACE 10 is the response to the alternative activation (M2) program. How do the ACE 10 cells respond to stimulation with IL-4 or IL-13? In other words, are the ACE 10 cells simply hyperactive to any stimuli, or that they are preferentially polarized towards the M1 state? This can be easily addressed by stimulating the cells with IL-4 and/or IL-13 to measure known markers of the M2 activation such as the mannose receptor (CD 206), Arginase-1, Ym-1, and several others. We could also investigate ability of ACE 10 macrophages to restore sensitivity to LPS or the M1 cue after exposure to the M2 cue, and vice-versa. Most of the macrophage polarization programs occur at the expense of the other. For example, SHIP proteins favor M1 polarization by repressing M2 program.

Thus it will be of interest to test of the ACE polarization is uniquely one-dimensional, that is, that it favors M1 response without altering M2 response (21). These studies could be performed together with the ACE KO, and modern technologies such as microarrays analysis could be included to fully appreciate the extent of changes to macrophages by virtue of ACE over-expression or inhibition.

C/EBPα: the potential link between RAS and Obesity

Our data so far suggests an intriguing correlation between ACE and C/EBPα.. In the ACE KO mice, angiotensin II infusion restored the myeloid differentiation defects and mature macrophage function, with a concomitant increase in C/EBPα expression (16). C/EBPα is a key transcription factor that modulates differentiation of various cells and tissues, including adipogenesis (22-24). In fact, the role of C/EBPα role in adipose differentiation was widely known before its role in myeloid cell differentiation was described (11). Interestingly, components of the RAS are also abundantly expressed in adipose tissue (25, 26), and angiotensin II stimulates adipose tissue differentiation (27), similar to adipose differentiation by C/EBPα. Several experimental groups and clinical data also suggest that RAS inhibition may improve metabolic syndrome and help prevent adiposity in both humans and mice. Furthermore, some of the metabolic phenotype of the ACE and angiotensinogen deficient mice -increased lean mass, reduced adiposity, and improved glucose clearance (28, 29) - mirrors the expected metabolic phenotype of C/EBPα downregulation (22, 30-32). Clues from the role of C/EBPα in the myelopoiesis defects in ACE KO mice may shed light on the mechanism by which the RAS mediate

adiposity and improves metabolic syndrome of obesity, diabetes, insulin insensitivity, and dyslipidemia.

Challenges of the ACE 10 mice

Despite the many answers we have obtained from the complex ACE 10 mice, there remain several unresolved questions. For example, in the ACE 10 mice, our unpublished data shows that the absence of systemic ACE does increase the tetrapeptide AcSDKP. We do not know the full extent of the importance of the increased amounts of this molecule in the ACE 10 mice. This is a salient issue, given our unpublished observation that indicates that an animal model in our laboratory- called ACE 7, with inactivated N-terminus of the ACE gene and thus increased accumulation of AcSDKP- shows a markedly different inflammatory phenotype compared to the wild-type mouse. Nonetheless, a preliminary experiment in which the ACE 10 mice were treated with a daily injection of the S17092 compound (an inhibitor of the oligopeptidase POP responsible for formation of AcSDKP) showed that the enhanced inflammatory response, at least to tumors *in vivo,* was still preserved.

The issue of AcSDKP is part of a bigger challenge in understanding the ACE 10 model, viz the absence of ACE expression in the endothelium and traditional places. Is the ACE over-expression in macrophages by itself responsible for the changes in the ACE 10, or is it the milieu in the ACE 10 mice, by virtue of the absence of ACE in traditional places, that plays a role? For example, systemic Angiotensin 1-7, which is important for myelopoiesis, may also be elevated in the ACE 10 (33). Our bone marrow transplantation of ACE 10 into WT recipients suggests that the ACE 10 milieu is

irrelevant to the inflammatory response. However, to settle this matter beyond doubt, we do have another animal model, called Jabl, which is a transgenic mouse with ACE over-expression in myelomonocytic cells. This strain preserves normal ACE expression in the endothelium and traditional places. Thus, this model is a closer approximation to WT, with the exception of an increase myelomonocytic ACE. If this model repeats the phenotype in the ACE 10, it will support our notion that it is not the accumulation of ACE substrates -AcSDKP, Angiotensin 1-7, and others- that is critical for the ACE 10 phenotype, but rather the mere over-expression of ACE in the myelomonocytic cells that is important. It will also essentially eliminate the possibility that it is the absence of a myriad of ACE products accounting for the ACE 10 phenomenon, since these products cannot accumulate *in vivo* in the presence of endothelial and other tissue ACE. *In vitro* models can also be established, where lentiviral vectors deliver ACE into myelomonocytic cells, allowing for further understanding of the mechanisms by which ACE influences differentiation and function of myeloid cells. This experiment will be beleaguered with the challenge of poor transfection efficiency of macrophages and myeloid cells, but it is worth the effort if we are to translate lessons from the ACE 10 into clinic.

The proposed future *in vivo* and *in vitro* studies outlined above do not lie to rest the possible ability of local angiotensin II to mediate some of the ACE 10 phenotype. Both genetic and pharmacological elimination of angiotensin peptides have no effect on the enhanced immunity in the ACE 10 (3). In theory, however, it is plausible that local, macrophage angiotensin II could be elevated in the ACE 10. A sustained local angiotensin II could desensitize the AT1 receptors, thus nullifying the impact of any

pharmacological AT1 receptor blockade. However, as discussed earlier, it is possible that a new, undescribed substrate of ACE in the ACE 10 is responsible for some of the ACE 10 phenotype. It is also possible that ACE acts merely on its own, independent of Angiotensin II or any substrate, since signaling by ACE, although controversial, has been reported (34-36). However, since ACE inhibitor treatment abrogates essentially every aspect of the ACE 10 we have studied, it suggests the catalytic activity – and hence a product- of ACE is involved. In light of the fact that angiotensin II infusion into ACE KO mice restores the myeloid defects, we can only conclude that angiotensin II plays a role in macrophage-mediated immunity, but that it is not critical to the ACE 10 phenomenon.

ACE 10 and Atherosclerosis

Our discovery of the immune changes offers exciting potential studies into understanding the role of the RAS in a variety of other inflammatory diseases. We had originally generated the ACE 10 mice to understand the role of monocyte/macrophage RAS in cardiovascular disease, particularly atherosclerosis. I believe that question is still relevant, its resolution is still crucial, as it may broaden our understanding of not only the role of inflammation and macrophages in atherosclerosis and cardiovascular disease, but may also help elucidate the contribution of polarized macrophages to these pathologies. It is widely known that genetic or pharmacological elimination of several pro-inflammatory cytokines (TNF-α, IL-12, IFN-γ) improved atherosclerosis, while elimination of anti-inflammatory cytokines (IL-10) worsens this disease (37). Thus, the concept has been that M1 macrophages are deleterious, whereas M2 macrophages are beneficial. While this is generally true, a growing body of evidence suggests that it may be an inaccurate

oversimplification of the role of inflammation in atherogenesis. Consider IL-6, a well known pro-inflammatory cytokine and a systemic marker of cardiovascular disease such as myocardial infarction and plaque rupture. Although administration of recombinant IL-6 exacerbates atherosclerosis in both normal and apolipoprotein (ApoE deficient) mice (38), IL-6 deficient mice on these two backgrounds surprisingly also developed worse serum cholesterol levels and subsequent atherosclerotic lesion formation than their WT counterparts (39, 40), suggesting an apparently contradictory beneficial role for this cytokine. Another example is IL-4, a well know anti-inflammatory cytokine. As a Th2 cytokine that promotes anti-inflammatory M2 polarization, one would predict that its absence would worsen atherosclerosis, yet the opposite has been reported (41). These examples suggest that it is not the mere presence of a cytokine that is critical, but rather the timing and stage of disease, among other complex dynamics, that dictates the role of the inflammatory milieu in atherosclerosis.

Recently, in contrast to the one dimensional relationship between macrophage polarization state and their impact on atherogenesis, the plasticity of macrophages is rather gaining grounds (42, 43). For example, the previously considered ‘proinflammatory’ macrophages that form foam cells in atherosclerotic plaques – with marked expression of ACE and angiotensin II (44) - have now also been identified with high expression of PPARγ (45, 46), a nuclear receptor protein essential for M2 polarization (47). In fact, there is evidence that the M2 milieu can initiate atherogenesis (46), and that depending on the stage and timing of disease, PPARγ agonists can promote, reduce, or have no impact on atherosclerotic plaque formation and destabilization (48-50). Thus, whether the expression of the M1 or M2 markers is

causative or a consequence of atherosclerosis is not as clearly understood as previously thought. Studying ACE 10 will contribute meaningfully to understanding the pathophysiology of atherogenesis, since the ACE 10 macrophages are preferentially biased towards an M1 phenotype. It is not unthinkable that a robust inflammatory M1 response is actually required to control initial plaque formation. Preliminary data generated on ACE 10 mice fed with high cholesterol were troubling and seemingly contradictory to widely accepted dogmas, but in the face of current knowledge, the results are arguably reconcilable. Further work is required to dissect these findings.

ACE 10 and granuloma

It is interesting to note that the atherosclerotic plaque phenotype has been compared to granulomatous state (51, 52). True granulomas, are a melting pot of several immune cell types coordinated by phagocytic giant macrophage-like cells at the interphase of an inflammatory insult. Granulomas such as sarcoids, like atherosclerosis, are notorious for high ACE expression in macrophage-like giant cells. Unfortunately, animal models designed to study granulomas do not express ACE, thus confounding the role of ACE in granuloma formation. In that regard, the ACE 10 mouse provides an excellent model, because it preserves the over-expression of ACE in myeloid cells, which is a major feature of the disease in humans.

ACE 10, the kidney and hypertension

Another intriguing finding about the ACE 10 has been their normal blood pressure. In a separate animal model generated in the lab, called ACE 3, the albumin promoter drives ACE expression, which limits its production to the liver. The large size of the liver ensures that enough ACE is made and eventually shed into the circulation, thus maintaining normal blood pressure in these mice. Detailed analysis of the ACE 3 led to the conclusion that endothelial ACE is not required for normal blood pressure(53). Thus, although the ACE 10 may simply be a mere representation of this concept, it must be noted that serum ACE is lower in the ACE 10 compared to WT (evidenced by high plasma AcSDKP level). Furthermore kidney and cardiac-specific ACE expression models in our laboratory resulted in slight but significant reduction in blood pressure (54, 55). In addition, unlike the liver, a hematopoietic niche of the RAS does exist. Thus, it raises the question whether the hematopoietic niche of the RAS, without ACE over-expression, is capable of regulating blood pressure. In other words, does normal expression of ACE or the RAS in the bone marrow alone restore blood pressure? To address this question, we performed a preliminary experiment in which bone marrow of WT was transferred into ACE deficient mice. Bone marrow transplantation restored blood pressure in the ACE KO, suggesting that this store of ACE may be able to regulate blood pressure. In light of the fact that several hypertensives present with normal systemic RAS components and yet respond to RAS inhibition, it would be interesting to demonstrate in detail that the bone marrow reservoir of the RAS could affect blood pressure. There are animal models in which components of the RAS, such as Angiotensin II, are constitutively activated. If adoptive transfer of immune cells, or bone marrow transplantation into WT mice raises blood pressure, we would be the first to demonstrate

that hematopoietic alterations could influence blood pressure significantly. Alternatively, Jabl mice could be treated with ACE inhibitor to demonstrate if upregulation of macrophage ACE alone is sufficient to manipulate blood pressure.

ACE 10 and the brain

The other possibility for the ACE 10 mice is in neuro-inflammation and neurodegenerative diseases. In other words, because ACE is expressed in all monocyte/macrophage lineage cells, there should be increased ACE expression in the microglia. There is substantial debate on the contribution of ACE polymorphism and ACE inhibitor treatment on cognition and Alzheimer's disease. Higher peripheral ACE activity has been recently associated with late onset of the disease (56). Furthermore, *in vitro*, it has been demonstrated that amyloid β42, the pathological peptide that accumulates in the brain of Alzheimer's patients is a substrate of ACE (57). Thus the ACE 10 model, equipped with enhanced immune response and ACE-rich microglia, could serve a powerful tool to study the role of ACE in this disease.

Inflammation is tightly regulated in the brain, with myeloid derived suppressor cells playing a key role. In fact, normal human monocytes exposed to glial micro-environment acquire myeloid derived suppressor cell-like features (58). In an intracrial glioma model, anti-tumor vaccination was impaired because of infiltrating myeloid derived suppressor cells that obstructed T cell responses (59). Because we have demonstrated a reduced tendency to develop myeloid derived suppressor cells in the ACE 10, it could represent a relevant model in understanding the contribution of

immunosuppression in neuro-inflammation and brain tumors such as glioblastomas. Perhaps, ACE over-expression in myeloid cells can breach immunosuppression in the brain during tumor development, allowing for improved responses against a wide array of brain tumors for which therapeutic advances are desperately needed.

Reference

1. Shen, X. Z., A. E. Lukacher, S. Billet, I. R. Williams, and K. E. Bernstein. 2008. Expression of angiotensin-converting enzyme changes major histocompatibility complex class I peptide presentation by modifying C termini of peptide precursors. *J Biol Chem* 283:9957-9965.

2. Okwan-Duodu, D., V. Datta, X. Z. Shen, H. S. Goodridge, E. A. Bernstein, S. Fuchs, G. Y. Liu, and K. E. Bernstein. 2010. Angiotensin converting enzyme over-expression in mouse myelomonocytic cells augments resistance to listeria and methicillin resistant S. Aureus. *J Biol Chem*.

3. Shen, X. Z., P. Li, D. Weiss, S. Fuchs, H. D. Xiao, J. A. Adams, I. R. Williams, M. R. Capecchi, W. R. Taylor, and K. E. Bernstein. 2007. Mice with enhanced macrophage angiotensin-converting enzyme are resistant to melanoma. *Am J Pathol* 170:2122-2134.

4. Sangsree, S., V. Brovkovych, R. D. Minshall, and R. A. Skidgel. 2003. Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B1 receptor agonists. *Am J Physiol Heart Circ Physiol* 284:H1959-1968.

5. Hadkar, V., and R. A. Skidgel. 2001. Carboxypeptidase D is up-regulated in raw 264.7 macrophages and stimulates nitric oxide synthesis by cells in arginine-free medium. *Mol Pharmacol* 59:1324-1332.

6. Trivedi, N. N., and G. H. Caughey. 2010. Mast cell peptidases: chameleons of innate immunity and host defense. *Am J Respir Cell Mol Biol* 42:257-267.

7. Mallen-St Clair, J., C. T. Pham, S. A. Villalta, G. H. Caughey, and P. J. Wolters. 2004. Mast cell dipeptidyl peptidase I mediates survival from sepsis. *J Clin Invest* 113:628-634.

8. Houghton, A. M., W. O. Hartzell, C. S. Robbins, F. X. Gomis-Ruth, and S. D. Shapiro. 2009. Macrophage elastase kills bacteria within murine macrophages. *Nature* 460:637-641.

9. Lapteva, N., M. Nieda, Y. Ando, K. Ide, Y. Hatta-Ohashi, G. Dymshits, Y. Ishikawa, T. Juji, and K. Tokunaga. 2001. Expression of renin-angiotensin system genes in immature and mature dendritic cells identified using human cDNA microarray. *Biochem Biophys Res Commun* 285:1059-1065.

10. Okamura, A., H. Rakugi, M. Ohishi, Y. Yanagitani, S. Takiuchi, K. Moriguchi, P. A. Fennessy, J. Higaki, and T. Ogihara. 1999. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. *J Hypertens* 17:537-545.

11. Witsell, A. L., and L. B. Schook. 1991. Macrophage heterogeneity occurs through a developmental mechanism. *Proc Natl Acad Sci U S A* 88:1963-1967.

12. Porse, B. T., T. A. Pedersen, X. Xu, B. Lindberg, U. M. Wewer, L. Friis-Hansen, and C. Nerlov. 2001. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. *Cell* 107:247-258.

13. Torroella-Kouri, M., J. C. Keith, M. Ivanova, and D. M. Lopez. 2003. IL-11-induced reduction of C/EBP transcription factor binding may contribute to the IL-12 downregulation in tumor-bearing mice. *Int J Oncol* 22:439-448.

14. Torroella-Kouri, M., X. Ma, G. Perry, M. Ivanova, P. J. Cejas, J. L. Owen, V. Iragavarapu-Charyulu, and D. M. Lopez. 2005. Diminished expression of transcription factors nuclear factor kappaB and CCAAT/enhancer binding protein underlies a novel tumor evasion mechanism affecting macrophages of mammary tumor-bearing mice. *Cancer Res* 65:10578-10584.

15. Torroella-Kouri, M., R. Silvera, D. Rodriguez, R. Caso, A. Shatry, S. Opiela, D. Ilkovitch, R. A. Schwendener, V. Iragavarapu-Charyulu, Y. Cardentey, N. Strbo, and D. M. Lopez. 2009. Identification of a subpopulation of macrophages in mammary tumor-bearing mice that are neither M1 nor M2 and are less differentiated. *Cancer Res* 69:4800-4809.

16. Lin, C., V. Datta, D. Okwan-Duodu, X. Chen, S. Fuchs, R. Alsabeh, S. Billet, K. E. Bernstein, and X. Z. Shen. 2010. Angiotensin-converting enzyme is required for normal myelopoiesis. *FASEB J*.

17. Swirski, F. K., M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J. L. Figueiredo, R. H. Kohler, A. Chudnovskiy, P. Waterman, E. Aikawa, T. R. Mempel, P. Libby, R. Weissleder, and M. J. Pittet. 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325:612-616.

18. Leuschner, F., P. Panizzi, I. Chico-Calero, W. W. Lee, T. Ueno, V. Cortez-Retamozo, P. Waterman, R. Gorbatov, B. Marinelli, Y. Iwamoto, A. Chudnovskiy, J. L. Figueiredo, D. E. Sosnovik, M. J. Pittet, F. K. Swirski, R. Weissleder, and M. Nahrendorf. 2010. Angiotensin-converting enzyme inhibition

prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. *Circ Res* 107:1364-1373.

19. Ricardo, S. D., H. van Goor, and A. A. Eddy. 2008. Macrophage diversity in renal injury and repair. *J Clin Invest* 118:3522-3530.

20. Gonzalez-Villalobos, R. A., R. Satou, D. M. Seth, L. C. Semprun-Prieto, A. Katsurada, H. Kobori, and L. G. Navar. 2009. Angiotensin-converting enzyme-derived angiotensin II formation during angiotensin II-induced hypertension. *Hypertension* 53:351-355.

21. Rauh, M. J., V. Ho, C. Pereira, A. Sham, L. M. Sly, V. Lam, L. Huxham, A. I. Minchinton, A. Mui, and G. Krystal. 2005. SHIP represses the generation of alternatively activated macrophages. *Immunity* 23:361-374.

22. Darlington, G. J., S. E. Ross, and O. A. MacDougald. 1998. The role of C/EBP genes in adipocyte differentiation. *J Biol Chem* 273:30057-30060.

23. Rosen, E. D., C. J. Walkey, P. Puigserver, and B. M. Spiegelman. 2000. Transcriptional regulation of adipogenesis. *Genes Dev* 14:1293-1307.

24. Lane, M. D., Q. Q. Tang, and M. S. Jiang. 1999. Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem Biophys Res Commun* 266:677-683.

25. Thatcher, S., F. Yiannikouris, M. Gupte, and L. Cassis. 2009. The adipose renin-angiotensin system: role in cardiovascular disease. *Mol Cell Endocrinol* 302:111-117.

26. Massiera, F., M. Bloch-Faure, D. Ceiler, K. Murakami, A. Fukamizu, J. M. Gasc, A. Quignard-Boulange, R. Negrel, G. Ailhaud, J. Seydoux, P. Meneton, and M.

Teboul. 2001. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J* 15:2727-2729.

27. Jones, B. H., M. K. Standridge, and N. Moustaid. 1997. Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* 138:1512-1519.

28. Jayasooriya, A. P., M. L. Mathai, L. L. Walker, D. P. Begg, D. A. Denton, D. Cameron-Smith, G. F. Egan, M. J. McKinley, P. D. Rodger, A. J. Sinclair, J. D. Wark, H. S. Weisinger, M. Jois, and R. S. Weisinger. 2008. Mice lacking angiotensin-converting enzyme have increased energy expenditure, with reduced fat mass and improved glucose clearance. *Proc Natl Acad Sci U S A* 105:6531-6536.

29. Weisinger, H. S., D. P. Begg, G. F. Egan, A. P. Jayasooriya, F. Lie, M. L. Mathai, A. J. Sinclair, J. D. Wark, and R. S. Weisinger. 2008. Angiotensin converting enzyme inhibition from birth reduces body weight and body fat in Sprague-Dawley rats. *Physiol Behav* 93:820-825.

30. Kimura, T., V. M. Christoffels, S. Chowdhury, K. Iwase, H. Matsuzaki, M. Mori, W. H. Lamers, G. J. Darlington, and M. Takiguchi. 1998. Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme genes in C/EBPalpha knockout mice. *J Biol Chem* 273:27505-27510.

31. Massiera, F., J. Seydoux, A. Geloen, A. Quignard-Boulange, S. Turban, P. Saint-Marc, A. Fukamizu, R. Negrel, G. Ailhaud, and M. Teboul. 2001. Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity. *Endocrinology* 142:5220-5225.

32. Rolland, V., X. Le Liepvre, M. L. Houbiguian, M. Lavau, and I. Dugail. 1995. C/EBP alpha expression in adipose tissue of genetically obese Zucker rats. *Biochem Biophys Res Commun* 207:761-767.

33. Heringer-Walther, S., K. Eckert, S. M. Schumacher, L. Uharek, A. Wulf-Goldenberg, F. Gembardt, I. Fichtner, H. P. Schultheiss, K. Rodgers, and T. Walther. 2009. Angiotensin-(1-7) stimulates hematopoietic progenitor cells in vitro and in vivo. *Haematologica* 94:857-860.

34. Kohlstedt, K., C. Gershome, M. Friedrich, W. Muller-Esterl, F. Alhenc-Gelas, R. Busse, and I. Fleming. 2006. Angiotensin-converting enzyme (ACE) dimerization is the initial step in the ACE inhibitor-induced ACE signaling cascade in endothelial cells. *Mol Pharmacol* 69:1725-1732.

35. Kohlstedt, K., R. Kellner, R. Busse, and I. Fleming. 2006. Signaling via the angiotensin-converting enzyme results in the phosphorylation of the nonmuscle myosin heavy chain IIA. *Mol Pharmacol* 69:19-26.

36. Fleming, I. 2006. Signaling by the angiotensin-converting enzyme. *Circ Res* 98:887-896.

37. Tedgui, A., and Z. Mallat. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 86:515-581.

38. Huber, S. A., P. Sakkinen, D. Conze, N. Hardin, and R. Tracy. 1999. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 19:2364-2367.

39. Schieffer, B., T. Selle, A. Hilfiker, D. Hilfiker-Kleiner, K. Grote, U. J. Tietge, C. Trautwein, M. Luchtefeld, C. Schmittkamp, S. Heeneman, M. J. Daemen, and H.

Drexler. 2004. Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation* 110:3493-3500.

40. Elhage, R., S. Clamens, S. Besnard, Z. Mallat, A. Tedgui, J. Arnal, A. Maret, and F. Bayard. 2001. Involvement of interleukin-6 in atherosclerosis but not in the prevention of fatty streak formation by 17beta-estradiol in apolipoprotein E-deficient mice. *Atherosclerosis* 156:315-320.

41. King, V. L., S. J. Szilvassy, and A. Daugherty. 2002. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler Thromb Vasc Biol* 22:456-461.

42. Mantovani, A., C. Garlanda, and M. Locati. 2009. Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arterioscler Thromb Vasc Biol* 29:1419-1423.

43. Johnson, J. L., and A. C. Newby. 2009. Macrophage heterogeneity in atherosclerotic plaques. *Curr Opin Lipidol* 20:370-378.

44. Potter, D. D., C. G. Sobey, P. K. Tompkins, J. D. Rossen, and D. D. Heistad. 1998. Evidence that macrophages in atherosclerotic lesions contain angiotensin II. *Circulation* 98:800-807.

45. Bouhlel, M. A., B. Derudas, E. Rigamonti, R. Dievart, J. Brozek, S. Haulon, C. Zawadzki, B. Jude, G. Torpier, N. Marx, B. Staels, and G. Chinetti-Gbaguidi. 2007. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* 6:137-143.

46. Khallou-Laschet, J., A. Varthaman, G. Fornasa, C. Compain, A. T. Gaston, M. Clement, M. Dussiot, O. Levillain, S. Graff-Dubois, A. Nicoletti, and G.

Caligiuri. 2010. Macrophage plasticity in experimental atherosclerosis. *PLoS One* 5:e8852.

47. Odegaard, J. I., R. R. Ricardo-Gonzalez, M. H. Goforth, C. R. Morel, V. Subramanian, L. Mukundan, A. Red Eagle, D. Vats, F. Brombacher, A. W. Ferrante, and A. Chawla. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 447:1116-1120.

48. Thorp, E., G. Kuriakose, Y. M. Shah, F. J. Gonzalez, and I. Tabas. 2007. Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice. *Circulation* 116:2182-2190.

49. Nakaya, H., B. D. Summers, A. C. Nicholson, A. M. Gotto, Jr., D. P. Hajjar, and J. Han. 2009. Atherosclerosis in LDLR-knockout mice is inhibited, but not reversed, by the PPARgamma ligand pioglitazone. *Am J Pathol* 174:2007-2014.

50. Meisner, F., D. Walcher, F. Gizard, X. Kapfer, R. Huber, A. Noak, L. Sunder-Plassmann, H. Bach, C. Haug, M. Bachem, T. Stojakovic, W. Marz, V. Hombach, W. Koenig, B. Staels, and N. Marx. 2006. Effect of rosiglitazone treatment on plaque inflammation and collagen content in nondiabetic patients: data from a randomized placebo-controlled trial. *Arterioscler Thromb Vasc Biol* 26:845-850.

51. Boyle, J. J., H. A. Harrington, E. Piper, K. Elderfield, J. Stark, R. C. Landis, and D. O. Haskard. 2009. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. *Am J Pathol* 174:1097-1108.

52. Komohara, Y., H. Yano, S. Shichijo, K. Shimotohno, K. Itoh, and A. Yamada. 2006. High expression of APOBEC3G in patients infected with hepatitis C virus. *J Mol Histol* 37:327-332.

53. Cole, J., D. L. Quach, K. Sundaram, P. Corvol, M. R. Capecchi, and K. E. Bernstein. 2002. Mice lacking endothelial angiotensin-converting enzyme have a normal blood pressure. *Circ Res* 90:87-92.

54. Gonzalez-Villalobos, R. A., S. Billet, C. Kim, R. Satou, S. Fuchs, K. E. Bernstein, and L. G. Navar. 2010. Intrarenal Angiotensin-Converting Enzyme Induces Hypertension in Response to Angiotensin I Infusion. *J Am Soc Nephrol*.

55. Xiao, H. D., S. Fuchs, D. J. Campbell, W. Lewis, S. C. Dudley, Jr., V. S. Kasi, B. D. Hoit, G. Keshelava, H. Zhao, M. R. Capecchi, and K. E. Bernstein. 2004. Mice with cardiac-restricted angiotensin-converting enzyme (ACE) have atrial enlargement, cardiac arrhythmia, and sudden death. *Am J Pathol* 165:1019-1032.

56. Akatsu, H., N. Ogawa, T. Kanesaka, A. Hori, T. Yamamoto, N. Matsukawa, and M. Michikawa. 2011. Higher activity of peripheral blood angiotensin-converting enzyme is associated with later-onset of Alzheimer's disease. *J Neurol Sci* 300:67-73.

57. Zou, K., T. Maeda, A. Watanabe, J. Liu, S. Liu, R. Oba, Y. Satoh, H. Komano, and M. Michikawa. 2009. Abeta42-to-Abeta40- and angiotensin-converting activities in different domains of angiotensin-converting enzyme. *J Biol Chem* 284:31914-31920.

58. Rodrigues, J. C., G. C. Gonzalez, L. Zhang, G. Ibrahim, J. J. Kelly, M. P. Gustafson, Y. Lin, A. B. Dietz, P. A. Forsyth, V. W. Yong, and I. F. Parney.

2010. Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. *Neuro Oncol* 12:351-365.

59. Jia, W., C. Jackson-Cook, and M. R. Graf. 2010. Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma + vaccination model. *J Neuroimmunol* 223:20-30.