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The Effect of Aging on the Innate Immune Response of Vascular Smooth Muscle

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THE EFFECT OF AGING ON THE INNATE IMMUNE RESPONSE OF VASCULAR SMOOTH MUSCLE

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As our understanding of atherosclerosis has become more sophisticated, a picture of the disease has emerged which emphasizes the role of inflammation in the pathogenesis of the disease, including signaling cascades mediated by toll-like receptors (TLRs). Data has also emerged suggesting that the process of aging plays an important role in atherogenesis, through pathways at least partially mediated by changes in the function of vascular smooth muscle cells. The purpose of this study was to elucidate the role that aging might play in the TLR-dependent innate immune response of vascular smooth muscle. Ex vivo cell and tissue culture models were used, with cytokine and chemokine production in response to stimulation with TLR agonists measured by ELISA assays. Our study demonstrated differences between aged and young cells and tissue specimens in the production of the pro-inflammatory cytokines interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1), with greater production of IL-6 and less production of MCP-1 seen in the aged specimens than in the young specimens. We discuss these results in the context of aging-related alterations in the innate immunity of vascular smooth muscle.

Epidemiology:

Heart disease is the leading cause of death for both men and women in the United States, accounting for nearly 30% of all US deaths in 2002 [1]. Coronary heart disease is the most common type of heart disease, with a lifetime risk of 49% for men and 32% for women at age 40. Even among adults who have reached age 70 without CHD, the lifetime risk is 35% for men and 24% for women [2]. Although the incidence and mortality of CHD and cardiovascular disease overall have decreased over the last 20 years with the advent of new therapies and lifestyle modifications including smoking reduction, the changing demographics of the American population suggest that CHD will remain a significant threat to public health for years to come.

Among the most important demographic shifts in the American population with regards to coronary heart disease is the population's advancing age. CDC estimates indicate that the proportion of Americans aged 65 or older is expected to increase from 12.4% in 2000 to 19.6% in 2030. The absolute number of Americans aged 65 or older is expected to rise even more dramatically, doubling from 35 million in 2000 to 71 million in 2030 [3]. The number of Americans over age 80 is expected to double during this time period as well, from 9.3 million in 2000 to 19.5 million in 2030. These demographic shifts in the American population are significant, as over 83% of deaths due to coronary heart disease occur in patients aged 65 or older, accounting for over 40% of deaths in that age group. Indeed, age per se is thought to be the most important risk factor for the development of coronary heart disease [4].

Pathogenesis of Atherosclerosis:

To understand why the risk of coronary heart disease increases so dramatically with age, it is important to understand the pathogenesis of the disease. The primary event leading to the development of coronary artery disease, as well as other types of vascular diseases including cerebral and peripheral artery diseases, is atherosclerosis. Atherosclerosis primarily affects the elastic and large muscular arteries of the body (including the aorta, carotid, and coronary arteries), and becomes symptomatic when the diameter of these large arteries becomes sufficiently narrowed to compromise the blood flow to end organs such as the brain, kidneys, or the heart. In the heart, the inability of the coronary blood supply to match the heart's demand due to arterial narrowing leads to angina, and the complete cessation of blood flow, usually caused by rupture of the atherosclerotic plaque, leads to myocardial infarction.

As the understanding of atherosclerosis has evolved, the notion of the disease as a simple problem of plumbing has become outdated, and in its place has emerged a picture of a complex inflammatory disease characterized by intense immunological activity. The primary event in the development of atherosclerosis is injury to the vascular endothelium, a single cell layer that serves as a barrier between the circulation and the internal components of the vessel wall. The culprits of endothelial injury are wide-ranging, and include hyperlipidemia, toxins including those present in tobacco smoke, bacteria and viruses, immune reactions, and hemodynamic disturbances including those arising from hypertension.

Injury to the vascular endothelium leads to impairment of endothelial function, and alterations of the endothelium's normal hemostatic properties. As a result, injured endothelium has increased adhesive properties with respect to leukocytes and platelets, as well as increased permeability. This increased adhesiveness is mediated by an increase in the endothelial expression of adhesion molecules, including vascular cell adhesion molecule-1 and P-selectin [5]. These cell-surface molecules interact with circulating monocytes and lymphocytes, slowing their progress through the bloodstream, and allowing them to respond to chemotactic stimuli in the nearby environment. One such chemoattractant factor produced by cells in the vessel wall in response to modified lipoproteins is monocyte chemoattractant protein 1 (MCP-1). MCP-1 and other chemoattractant factors direct the migration of monocytes through the endothelial layer and into the vascular wall. Other functional changes induced by injury to the vascular endothelium include the formation of vasoactive molecules, as well as assorted cytokines and growth factors [5].

Macrophage colony stimulating factor, which is overexpressed in inflamed intima, directs the maturation of monocytes into macrophages once they reach the intima [6]. These macrophages then augment their expression of scavenger receptors and engulf modified lipoproteins through receptor-mediated endocytosis. The end result of this process is the lipid-laden foam cell, the characteristic inhabitant of the early atherosclerotic lesion. In addition to their phagocytic role, these macrophages also secrete numerous growth factors and cytokines, including TNF-α and IL-1β, which amplify the ongoing inflammatory response [5].

The adaptive immune system plays a crucial role in the development of atherosclerosis as well, primarily through the work of CD4+ T cells. These T cells, which recognize fragments of antigens presented by cells expressing major histocompatibility complex class II molecules, are also known as helper T cells. The cytokine milieu in the atherosclerotic lesion, including IL-12 and IL-18 produced by macrophages and smooth muscle cells in plaques, promotes the induction of a Th-1 response among these T cells, and the differentiation of these T cells into Th-1 effector cells [7]. These Th-1 cells amplify the localized immune response through the production of proinflammatory cytokines including IFN-γ and TNF, as well as the CD-40 ligand. These Th-1 cytokines promote the development of atherosclerosis in a variety of ways. IFN-γ activates macrophages, leading to increased production of nitric oxide, pro-inflammatory cytokines, vasoactive and pro-thrombotic mediators. IFN-γ also inhibits the proliferation of endothelial cells and the differentiation of vascular smooth muscle cells, and SMC collagen production [8], [9]. It is thought that decreasing the cellular and collagenous content of the cap's fibrous plaque decreases the plaque's stability, leading to greater risk of thrombosis [10].

Tumor necrosis factor (TNF) contributes to inflammation by activating the NF-κB signaling pathway, leading to the generation of reactive oxygen species, proteolytic enzymes, and tissue factor by endothelial cells[11]. In addition to its pro-inflammatory effects, TNF has significant metabolic effects, including the suppression of lipoprotein lipase, which results in the accumulation of lipoproteins rich in triglycerides in the blood. Elevations in TNF levels, and the accumulation of such lipoproteins have been associated with the development of heart disease in clinical studies.

The first stage of atherosclerosis, the so-called fatty streak, is a purely inflammatory lesion, composed primarily of foam cells, with some T-lymphocytes. These lesions are not raised significantly from the vessel wall, and thus do not cause any disturbances in arterial blood flow. They have been seen in children as young as 1 year old, and occur in all children by the time they reach age 10 [12]. While the fatty streak is thought to be the precursor of the atherosclerotic plaque, their universal prevalence in the arteries of all humans, and their frequent location in arteries and populations not typically prone to atherosclerosis indicates that not all fatty streaks are destined to become atheromas, and that additional events are necessary for the development of a more complex atherosclerotic lesion.

The definitive event that differentiates a fatty streak from a more advanced atherosclerotic lesion is the deposition of fibrous tissue in the plaque. The majority of the extracellular matrix that is laid down comes from smooth muscle cells (SMCs). During this phase of plaque formation, SMCs migrate from the tunica media into the intima, under the influence of platelet-derived growth factor secreted by endothelial cells and activated macrophages [13]. This migration is facilitated by the secretion of MMP-9 and other proteases secreted by the SMCs, which degrade the existing extracellular matrix. Once the SMCs have arrived in the intimal layer, under the influence of various growth factors including transforming growth factor β and platelet-derived growth factor, they begin to proliferate and secrete extracellular matrix proteins. It is this migration and secretion of ECM proteins by SMCs that causes the transformation of the purely inflammatory lesion of the fatty streak into a fibrotic and ultimately calcified plaque that can have functional consequences for downstream arterial flow [5].

Histologically, this primary lesion of atherosclerotic disease, the atheroma, is a raised focal lesion within the vessel intima, with a soft lipid core primarily composed of cholesterol and associated esters, and a firm fibrous cap. There are three principal components of these plaques: cells, including smooth muscle cells, macrophages, and other leukocytes, extracellular matrix, and intracellular and extracellular lipid. The superficial cap of the plaque is typically composed of SMCs and collagen. Beneath and around the cap, in a cellular area known as the "shoulder" of the plaque, can be found a particularly high concentration of macrophages and T lymphocytes, as well as SMCs [14]. This is the area from which much of the plaque's growth occurs. Other cell types found within plaques include dendritic cells, mast cells, a few B lymphocytes, and natural killer T cells [10]. Deep to this cellular area is a disorganized mass of lipid and debris known as the necrotic core of the plaque. The cellular component of the necrotic core of the atherosclerotic lesion is primarily foam cells. As previously mentioned, these large cells, laden with cholesterol and other lipids, are primarily derived from migratory macrophages, although smooth muscle cells can also engulf lipid to become foam cells. The atherosclerotic plaque is a dynamic lesion, evolving with time as cell death and degeneration, as well as calcification and the synthesis and remodeling of extracellular matrix lead to enlargement of the plaque, with consequent narrowing of the arterial lumen and disruption of blood flow to downstream organs.

On top of this chronic process of plaque evolution and enlargement, acute focal plaque rupture, ulceration, or erosion can lead to the exposure of highly thrombogenic elements of the plaque to the circulation, and the superimposition of intravascular thrombosis on the luminal narrowing caused by the extravascular plaque. It is these

acute-on-chronic disturbances, leading to severe obstruction or complete occlusion of the vasculature, that leads to the most feared complication of atherosclerosis, myocardial infarction (MI). Interestingly, in many cases of MI, the lesion whose rupture was ultimately responsible for the thrombotic vascular occlusion causing the MI did not produce a critical arterial narrowing prior to rupture [15]. In other words, the lesions responsible for chronic symptomatic coronary artery disease (i.e. angina) are not necessarily the same lesions which ultimately lead to those patients' deaths. Recent studies suggest that it is the activation of inflammatory pathways, rather than simply the degree of stenosis, that places plaques at risk for rupture and leads to thrombosis and resultant ischemia and infarction [16].

As mentioned earlier, one aspect of the inflammatory response that predisposes plaques to rupture is the production of IFN-γ by resident T cells, which denudes the fibrous cap of its cellular and collagen components, decreasing its stability. Other T cell controlled changes that decrease plaque stability include release of IL-1 and CD40 ligand, which induce macrophages to release interstitial collagenases, such as MMP-1, MMP-8, and MMP-9. Plaque analysis has revealed that MMP-9 is catalytically active, and that overexpression of MMP-9 may promote the production of tissue factor and lead to tissue factor-mediated activation of the coagulation cascade [17].

The rupture of an unstable plaque leading to acute coronary syndrome is typically precipitated by the physical disruption of the plaque's fibrous cap, either due to frank cap rupture or erosion of the endothelium. Contact between the blood and the thrombogenic substances in the lipid core and elsewhere in the plaque initiates the formation of

thrombus. Once formed, a thrombotic occlusion can have sudden and catastrophic effects on blood flow to tissue downstream of the occlusion, and ultimately lead to death.

Role of Toll-Like Receptors:

As the picture of atherosclerosis as a chronic inflammatory disease has evolved over the past few decades, so too has the understanding of the immune system as a whole. One of the most dramatic areas of growth in our understanding of this complex system is the discovery and study of the Toll-like receptor (TLR). The Toll gene was first discovered by the German scientist Nusslein-Volhard in Drosophila in the mid-1980s, and the vertebrate homolog was discovered only in 1997. Toll-like receptors are a family of pattern recognition receptors that are now known to play a key role in both the innate and adaptive immune systems. To date, 11 distinct mammalian TLRs have been identified, each of which interacts with a distinct array of conserved microbial molecular sequences.

TLRs are type 1 membrane-spanning receptors, with leucine-rich repeat motifs and signaling motifs similar to those found in the IL-1 and IL-18 receptors. Most TLRs are cell-surface receptors. The exceptions are TLR 3, 7, 8, & 9, which are involved in viral recognition, and are found largely in the endosomal compartment [18]. All four are involved in viral recognition [19]. In binding to their ligands, TLRs form either homo or heterodimers, with each dimer having a different ligand specificity. TLRs 1, 2, 4, 5 & 6 are specialized to recognize unique bacterial products that are not made in the host, including the gram-negative bacterial outer membrane component lipopolysaccharide (TLR-4), flagellin (TLR-5), lipoteichoic acid and peptidoglycan (TLR-2), and lipoproteins. In addition to the unique microbial patterns that they recognize, TLRs have been shown to recognize endogenous molecules, including fibrinogen, heat shock

proteins, oxidized lipids, fibronectin, and surfactant protein A [20]. In addition to these endogenous molecules, TLRs have been shown to recognize degradation products of endogenous macromolecules, such as heparin sulfate and hyaluronan. This intriguing discovery indicates that, via TLRs, the extracellular matrix can stimulate the innate immune response when it has been altered as a result of tissue destruction [18]. It is thought that surveillance for such degradation products, released by injured or infected tissues, may be an integral part of the TLRs role in "keeping watch" for tissue damage [21].

Once TLRs have recognized and bound to their ligands via molecular pattern recognition, they tend to cluster, recruit accessory proteins and set in motion the complex NF-κB signaling cascade that ultimately leads to the translocation of NF-κB into the nucleus, and activation of gene transcription. The genes transcribed as a result of NF-κB signaling typically code for pro-inflammatory cytokines, including IL-1, IL-6, and IL-8. TLR signaling also induces the production of antimicrobial molecules, such as nitric oxide. This response enables macrophages to engulf and eliminate invading microorganisms [18]. The activation of TLRs expressed on dendritic cells stimulates their maturation, which allows them to stimulate the expansion and differentiation of T cells, providing a link between TLR function and activation of the adaptive immune system. TLR signaling has also been shown to induce the production of chemokines, polyreactive antibodies, and costimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) that can lead to sustained activation of the adaptive immune system [19, 22]. Toll-like receptors are also involved in the activation of complement, coagulation, phagocytosis, and apoptosis.

Over the past decade, a role for Toll-like receptors as surveillance receptors with substantial influence over the immune response has emerged. As this picture has evolved, new research has gotten underway to elucidate what role these receptors might play in the development of the chronic inflammatory disease of atherosclerosis. One possible link between TLR signaling and atherosclerosis has been postulated to occur through chronic infection. For twenty years, epidemiologic data has been available linking seropositivity for Chlamydia TWAR and acute MI and chronic coronary heart disease [23]. Furthermore, studies have shown that many human atherosclerotic plaques show signs of infection with Chlamydia pneumoniae [24].

This discovery raised the tantalizing possibility that Chlamydia species acted as an infectious vector capable of stimulating atherogenesis. Such a link would provide a clear mechanistic role for Toll-like receptor signaling in the pathogenesis of atherosclerosis, and would also provide a clear target for drug therapy aimed at eradicating Chlamydia infection. Limited data from animal studies have indicated that Chlamydia infection can accelerate atherosclerosis and treatment with azithromycin can prevent this acceleration [25]. Unfortunately, a large randomized prospective trial of weekly azithromycin treatment for a period of 1 year in patients with documented stable coronary artery disease failed to demonstrate any mortality benefit for patients taking azithromycin when compared to a placebo [26]. Other studies looking at atherosclerotic lesions in the carotids and peripheral arteries have similarly failed to demonstrate a benefit from antibiotic treatment. However, these studies were limited by the advanced age of the participants and the presumably long lag time between inoculation and antibiotic treatment. While firm evidence linking infection with the atherosclerotic

process has not been produced to date, the results of these studies are far from conclusive, and do not necessarily exclude the possibility of a role for infectious agents in the pathogenesis of atherosclerosis.

More intriguing evidence for the role of Toll-like receptor signaling in the pathogenesis of atherosclerotic disease comes from analysis of genetic polymorphisms coding for TLRs. Of all the polymorphisms thus far identified, two mutations in TLR-4 have been studied the most extensively – Asp299Gly and Thr399Ile. These polymorphisms were initially thought to be related to hyporesponsiveness to lipopolysaccharide (LPS), the gram-negative bacterial outer membrane component recognized by TLR-4. The initial study examined a physiologic response (decline in FEV1) in response to LPS inhalation [27]. Another study demonstrated that Asp299Gly was statistically overrepresented among adult patients admitted to the ICU with septic shock, suggesting that the polymorphism may predispose patients to the development of septic shock [28]. However, a subsequent study examining the in vitro response of donor monocytes to LPS demonstrated no difference between either polymorphism and wild-type monocytes in terms of response to LPS, and the significance of these polymorphisms in terms of host defense is not yet clear [29].

Initial studies of the effect of these polymorphisms on the risk of developing atherosclerotic disease seemed to indicate that these alleles were protective. A study on carotid intima-media thickness as measured by Doppler ultrasound indicated that patients carrying these alleles had less intima-media thickening, and a lower risk of developing carotid atherosclerosis [30]. Several larger studies, however, were unable to reproduce this result [31]. Research into the effects of these polymorphisms on the risk of MI has

so far been inconclusive as well. While most studies have reported a reduced risk of MI in patients carrying one of the polymorphisms, the largest such study, which included nearly 5,000 individuals, found no association between cardiac risk and either of the polymorphisms [18, 32]. In a meta-analysis of 7 of these studies, the pooled odds ration for MI was 0.9 (95% CI 0.68-1.19), suggesting some protective role for the alleles against myocardial infarction, but lacking the power to demonstrate statistical significance [18]. Larger studies will be needed in the future to determine conclusively whether the TLR-4 polymorphisms convey protection against the development of atherosclerotic disease.

The idea that TLRs are involved in the pathogenesis of atherosclerotic disease is further supported by correlations seen in recent clinical studies. One such study, which examined the peripheral circulating monocytes in patients immediately post-MI, found that these patients exhibited elevated levels of pro-inflammatory cytokines, most notably TNF-α and IL-6, which positively correlated with elevated expression of TLR-4 on the patients' monocytes [33]. Other studies have corroborated this result, and have suggested that upregulation of TLR-4 sensitizes monocytes, while downregulation of TLR-4 decreases their ability to mount a pro-inflammatory response [34]. The results of these studies have generated ongoing interest and further research aimed at the elucidation of a possible role for TLR-4 signaling in the inflammatory state of acute MI.

Experimental data on inflammatory signaling pathways in atheromas have supported the proposed role for TLR signaling in the development of atherosclerosis. Expression levels of TLR-1, -2, and -4 have been shown to be markedly elevated (by semi-quantitative PCR) among the resident cells of human atherosclerotic plaques [35], particularly among macrophages and endothelial cells. Furthermore, TLR-4 expression

can be upregulated by the presence of proatherogenic oxidized LDL, while TLR-2 expression can be downregulated by laminar, non-turbulent blood flow [18].

Studies using murine knockout models of TLR genes have provided convincing further evidence of a role for these receptors in the development of atherosclerosis. Because normal mice have more rapid hepatic LDL clearance than do humans, they do not develop atherosclerosis unless fed with high cholesterol diets for long periods of time [36]. Therefore, in order to develop a useful mouse model of atherosclerosis, it was necessary to develop a strain of mice deficient in a gene product involved in cholesterol metabolism – apolipoprotein E (ApoE). ApoE functions as a ligand for receptors that clear very low-density lipoprotein (VLDL) and chylomicron remnants. Mice deficient in the gene coding for ApoE have exceedingly high total cholesterol levels, particularly when fed Western diets, and develop atherosclerosis at an accelerated pace that makes them a workable experimental model for the pathogenesis of the disease [37].

A cross between *Tlr4* knockout mice and *ApoE* knockout mice yielded double homozygous progeny with a reduced burden of atherosclerosis when compared to the *ApoE* -/- controls, despite similar lipid profiles [38]. Studies of mice deficient in MyD88, an adaptor protein critical for TLR signal processing, found similar reductions in atherosclerotic burden. When compared to *ApoE* knockout mice, *MyD88/ApoE* double knockout mice had 60% fewer atherosclerotic lesions [38].

In addition to the strong evidence linking TLR-4 signaling to atherosclerotic disease, evidence also exists of a role for TLR-2 signaling in the development of atherosclerosis. The double homozygous progeny of *Tlr2* knockout mice with LDL receptor knockout mice (*Ldlr*) showed a modest reduction in atherosclerosis when

compared to controls. On the other hand, administration of a TLR-2 agonist led to dramatically increased atherosclerosis in *Ldlr* knockout mice when compared with *Ldlr/Tlr2* double knockouts [39]. While the body of evidence linking TLR-2 signaling to atherosclerosis is not as robust as that linking TLR-4, there does seem to be a link, and further studies to strengthen this link are ongoing.

As evidence for the role of TLR signaling in the development of atherosclerosis has continued to mount, there has remained some confusion as to the role of TLR signaling in vascular smooth muscle cells. As mentioned previously, vascular smooth muscle cells are known to play a crucial role in the development of atherosclerosis through their migration and production of ECM, as well as their ability to act as phagocytic cells. Until recently, it was thought that VSMCs did not express TLRs [10]. However, recent data suggests that vascular smooth muscle cells do in fact express both Toll-like receptors, and that activation of TLR-4 receptors on VSMCs can induce the production of pro-inflammatory cytokines that may play a role in the immune response that leads to the development of atherosclerosis.

In a paper published in the American Journal of Physiology (Heart and Circulatory Physiology) in 2005, Yang et al demonstrated that the mRNA coding for TLR-4, as well as the mRNA for several key coreceptor proteins, could be isolated from both murine and human VSMCs [40]. Furthermore, they showed that the pro-inflammatory cytokines MCP-1 and IL-6 were expressed at high levels by VSMCs stimulated with LPS. To strengthen the evidence that this production was due to TLR-4 signaling, they infected mice with a recombinant adenovirus engineered with a dominant negative form of the murine TLR-4 gene. When compared with VSMCs from mice that

had been infected with a control adenovirus that did not affect the TLR-4 gene, VSMCs from these mice showed markedly reduced production of the pro-inflammatory cytokine MCP-1 in response to LPS stimulation (mice with intact TLR-4 signaling showed a 33-fold increase in MCP-1 production upon LPS stimulation, while mice with TLR-4 deficiency showed no elevation from baseline signaling [40]. The findings of this study, within the context of the existing evidence linking inflammation and TLR-4 signaling to atherosclerosis, suggest a role for TLR-4 signaling within vascular smooth muscle cells as part of the inflammatory response leading to the development of atherosclerosis.

Monocyte chemoattractant protein 1 (MCP-1) is a chemokine thought to play an important role in the recruitment of monocytes into the subendothelium of damaged arteries. Extensive evidence exists linking this chemokine to this process. All of the cellular components of the arterial wall, including endothelial cells, smooth muscle cells, and fibroblasts, have been shown to secrete MCP-1 in response to known atherogenic stimuli, including modified low density lipoprotein and fluid shear stress [41, 42]. In addition, MCP-1 expression has been shown to occur in the arterial wall of rabbits and non-human primates in vivo in response to hypercholesterolemia [43, 44]. Furthermore, two murine knockout studies crossing MCP-1 and CCR-2 (the MCP-1 receptor) deficient mice demonstrated markedly decreased lipid plaque formation when compared with controls. This data strongly supports a key role for the chemokine MCP-1 in the development of atherosclerosis.

IL-6 is an important cytokine inflammatory mediator that might play a role in the pathogenesis of acute coronary syndromes. Like CRP, IL-6 has been found to become markedly elevated during acute coronary syndromes, and the degree of elevation is

associated with prognosis. A study showed it to be undetectable in the blood of controlled subjects, but elevated in 21% of patients with stable angina, 61% of patients with unstable angina, and 83% of patients with unstable angina requiring a prolonged hospitalization without troponin or CK-MB leaks [45]. Furthermore, IL-6 elevation has been shown to predict the risk of future cardiac events, with a 44% relative risk of future MI for each quartile increase of IL-6 concentration from baseline [46].

IL-6 has also been shown to stimulate the growth of vascular smooth muscle cells in vitro [47], and IL-6 gene transcripts have been demonstrated in human atherosclerotic lesions [48], suggesting a possible role for IL-6 in the pathogenesis of atherosclerosis. However, vascular injury studies using IL-6 knockout mice have thus far failed to demonstrate significant differences between these mice and those in control groups, [49, 50]. This failure raises the question of whether IL-6 is in fact a direct mediator of atherosclerosis, or more simply a marker of the inflammatory response. Research into the role that IL-6 might play in the pathogenesis of atherosclerosis is ongoing. Given the evidence linking TLR-4 signaling to atherosclerosis, the direct activation of production of IL-6 by TLR-4 stimulation, and the role of IL-6 in promotion of the inflammatory response, a role for IL-6 may ultimately be demonstrated in the vascular biology of atherosclerosis, although its role is unclear at this point.

Effect of Aging:

As previously discussed, atherosclerotic disease, the leading cause of death in the United States, is largely a disease of the elderly. Age has long been known to be the single most important risk factor for the development of cardiovascular disease. A traditional explanation for the increased disease burden among older patients is that atherosclerotic lesions develop slowly over time, as the result of the accumulation of large numbers of small vascular insults, leading to the progressive development of more, larger, and less stable atheromas as patients age, which simply increases the risk of developing clinically significant lesions. However, a body of evidence is emerging which suggests that the effects of aging cannot be explained simply by the accumulation of injuries over time, and that the vascular immune response may undergo some intrinsic change with the process of aging that predisposes it to the development of atherosclerosis.

Much of the evidence for the role of aging in the inflammatory response of the vasculature comes from the field of transplant biology. Although advances in immunosuppressive protocols have decreased the rates of acute transplant rejection, almost half of all vascularized organ grafts are still lost at a certain point post-transplant. The most common cause of chronic transplant rejection is transplant arteriosclerosis [51]. Research into the cause of this arteriosclerosis has revealed a role for both the age of the vessel, and the age of the host, in promoting arteriosclerosis, suggesting that aging is a complex process with effects both locally and systemically that promote accelerated arteriosclerosis.

Experimental data from rabbits, primates, mice and rats has demonstrated that aged animals exhibit a greater degree of neointimal hyperplasia than do their young counterparts when subjected to a mechanical endothelial injury [52, 53]. This difference is thought to stem from changes in the vascular smooth muscle cells induced by aging including increased proliferation due to increased responsiveness to growth factors such as platelet-derived growth stemming from increased expression of the PDGF receptor [53-55], as well as decreased susceptibility to apoptosis [56], and increased deposition of extracellular matrix [57].

In addition to these local effects of aging on the vascular response to injury, evidence is emerging of systemic effects of aging that increase inflammation and atherogenesis as well. Research in transplant biology has demonstrated that the age of the recipient is the most important determining factor in predicting renal allograft rejection outcome [58]. An animal model of aortic transplantation, in which aortas from aged rats were denuded of endothelium and transplanted into young rats and vice versa, demonstrated that the degree of intimal hyperplasia seen in the vascular graft was more a function of recipient age than donor age [58]. This is consistent with data that demonstrates higher systemic levels of pro-inflammatory cytokines including TNF-α associated with aging [59]. As research in this field continues, further evidence of the role of aging on local and systemic inflammatory signaling will continue to emerge, giving us a more sophisticated picture of the role of aging on the pathogenesis of atherosclerosis.

Summary:

In summary, a large body of evidence has emerged in the field of vascular biology painting a picture of atherosclerosis as an inflammatory disease. Toll-like receptor signaling, particularly signaling with TLR-4, appears to play a key role in the pathogenesis of atherosclerosis, through the production of inflammatory cytokines. Vascular smooth muscle cells have been demonstrated to express pro-inflammatory cytokines and chemokines, including MCP-1 and IL-6, in response to stimulation with the TLR-4 agonist lipopolysaccharide. Furthermore, the process of aging may accelerate atherogenesis through alterations in the activity of vascular smooth muscle cells. In this context, this project sought to explore the role that aging might play in the innate immune response of vascular smooth muscle cells in response to TLR stimulation, using ex-vivo cell and tissue culture techniques, and measuring the cellular and tissue response to stimulation through ELISA-based assays of pro-inflammatory cytokine production.

Statement of Purpose/Specific Aims:

The purpose of this study was to evaluate the effect of aging on the innate immune response of vascular smooth muscle, using a murine model and ex vivo cell and tissue culture techniques. This was done using TLR stimulation, with assays measuring the production of pro-inflammatory cytokines as the measure of the cellular and tissue immune response.

Hypothesis:

Our hypothesis was that aged vessels would exhibit increased production of pro-inflammatory cytokines when compared to young vessels in response to stimulation with Toll-like receptor 4 agonists.

Materials & Methods:

Mice:

Cell cultures were isolated from young and aged mice from a CBA background, with 3-4 mice pooled per group. For the tissue culture experiment, all mice were from a CBA background. Aged mice were 19-20 months old. The first experiment used 3 aged and 3 young mice, with supernatants collected from tissue cultures from each mouse at 1 time point each (1 aged and 1 young mouse was stimulated for 6 hours, 1 each for 24 hours, and 1 each for 48 hours). The second experiment used 3 young and 6 aged mice, with supernatants collected from each cultured vessel at 4 separate time points (12, 24, 48, and 72 hours). To test the role of T cells we depleted two of the aged mice in this experiment with CD4 (clone GK.15) and CD8 (clone 2.43) depleting T cells.

Vascular smooth muscle cell isolation:

Mice were euthanized with ketamine overdose. A large incision was then made in the midline of each mouse, extending from the pelvis to the suprasternal notch, and their rib cages were removed, exposing the heart and other internal organs. The descending aortas were then isolated and stripped of fat tissue and adventitia, and placed in a 20ml of ice-cold Dulbecco's Modified Eagle's Medium (DMEM). 3-4 aortas were pooled for each age group. The 3-4 aortas were then placed in collagenase type II and incubated at 37 degrees for 15 minutes. At this point, remaining adventitia was removed, and aortas

were transferred to a dish containing collagenase I/elastase, and cut into small pieces with a scalpel blade. Next, these pieces were incubated at 37° Celsius for 60 minutes, with forceful pipetting of the mixture containing the pieces done every 15 minutes to ensure dispersal of cells. The digestion was then stopped by the addition of 5ml of mouse aorta growth medium (MAGM) which included 20% fetal bovine serum, 1% penicillin/streptomycin, 1% mixed non-essential amino acids, and 1% L-glutamine. Cells were then spun at 1500 rpm for 8 minutes at room temperature. The supernatant was then removed, and the cells were spun again in 2 ml of MAGM after resuspension. Cells were then resuspended in 2ml MAGM and plated in a 6 well plate coated with fibronectin (Biocoat).

This step of the project was completed prior to the student's beginning work on the project.

Cell culture:

Once the vascular smooth muscle cells were isolated, they were cultured in flasks in MAGM. Media was changed twice weekly, and cells were allowed to grow until convergence, at which point they were trypsinized and regrown in a new flask to prevent overgrowth.

This was done by the student.

Vascular smooth muscle cell phenotyping – Alpha SMC actin staining:

Cells from culture flasks were trypsinized and transferred to large plates containing glass slides, and cultured to confluence in MAGM Slides coated with cells were then washed in PBS twice, and fixed for three minutes in ice-cold methanol. Slides were then allowed to air dry. Slides were then incubated for 45 minutes with 10% normal goat serum in phosphate buffered saline at room temperature. Cells were then incubated for 90 minutes at 37° Celsius with Sigma mouse anti-SMC actin (IgG2) 1:400 in PBS/2% Bovine Serum Albumin (BSA). Cells were then rinsed once and washed with PBS 3 times for 5 minutes each. Cells were then incubated for 45 minutes in the dark with Alexa flour 488 goat anti-mouse IgG (1:500 in PBS/2% BSA). Cells were then rinsed once, and washed with PBS 3x for 5 minutes each. Cells were then placed in Hoechst 1:10,000 for 3 minutes. Next, cells were washed twice with PBS. Cells were then mounted in glycerol/PBS and visualized.

This step was completed by the student under the supervision of lab technician Sofya Rodov, although data presented below is from a separate trial in which the student was not involved, as cells were better visualized on this second attempt.

Cell stimulation:

For the purposes of cell stimulation, cells were trypsinized and counted under microscopic visualization in order to ensure equal distribution. To each well of a 48 well plate, 10,000 cells were added, and incubated in 500 μl of 1% FBS in DMEM media for 24 hours to coordinate their progress through the cell cycle at G0. Cells were then incubated for 6 to 72 hours in 0.25% FBS in DMEM media. To this media, 0.5 ng/ml of LPS was added to cells in the LPS stimulation group, matched with unstimulated controls. Other TLR agonists were added to separate samples with concentrations of 0.5 ng/ml (peptidoglycan), 2ng/ml (poly I:C), 2ng/ml (flagellin), and 10ng/ml (CpG). All media was removed from each well at the indicated time point, and ELISA assays were run per the manufacturer's protocol to determine the concentration in each cell group.

Some trials of this step were completed by the student, although data presented below is from work done prior to student's arrival.

Tissue culture model:

For the second phase of the project, an ex-vivo tissue culture model was used, comparing vessels obtained from aged and young mice. Mice were euthanized by isofluorane inhalation, followed by cervical dislocation. A large incision was then made in the midline of each mouse, extending from the pelvis to the suprasternal notch, and their rib cages were removed, exposing the heart and other internal organs. Preceding the

tissue isolation, a small hole (~ 2mm) was cut in the right atrium of each mouse's heart. Next, each mouse's left ventricle was pierced with a 22 gauge needle, and injected with approximately 10ml of phosphate-buffered sodium, in order to flush out the blood from the mice's left-sided circulation. The mice's lungs, esophagus, and inferior vena cavas were then removed. The descending aortas, made clearly visible by this dissection, were isolated from both young and aged mice under direct and microscopic visualization, and care was taken during this process to remove the adventitia and peri-aortic fat, lymph nodes, and connective tissue surrounding the vessels. The vessels were then transected at their approximate mid-point, and each vessel was washed in 1ml of phosphate-buffered saline solution. Following the completion of these dissections, the vessels pieces were transferred into 96-well plates containing 200 μL of M199 media containing 20% fetal bovine serum, as well as 1% penicillin/streptomycin, and 1% l-glutamine. These vessels were then incubated at 37° Celsius for 4 hours. Following incubation, 50 μl of media was added to each well for a total of 250 μl per well. For one vessel piece per mouse, 0.5ug/ml of LPS was added to the media. Media was then removed from the tissue as follows: For the first group of mice, all media was removed at one time point for each mouse – at 6 hours, 24 hours, and 48 hours, respectively. For the second group of mice, 50 μl of media was taken off from each vessel piece at 12 hours, 24 hours, 48 hours, and 72 hours. ELISA assays were run per the manufacturer's protocol to determine the concentration in each supernatant.

This step was completed by the student.

Results:

Cultured cells display the phenotype of vascular smooth muscle cells:

In order to demonstrate that the cytokines measured from our stimulation experiments were indeed produced by vascular smooth muscle cells in response to TLR stimulation, we first needed to demonstrate that we had successfully isolated and cultured vascular smooth muscle cells. To that end, cultured cells isolated from both young and aged mice were stained via immunofluorescence for smooth muscle cell specific actin. Cells from both young and aged mice from both cell lines demonstrated the presence of this cytoskeletal protein in abundance, indicating that all, or least the vast majority, of cells used for the stimulation experiment, were indeed vascular smooth muscle cells.

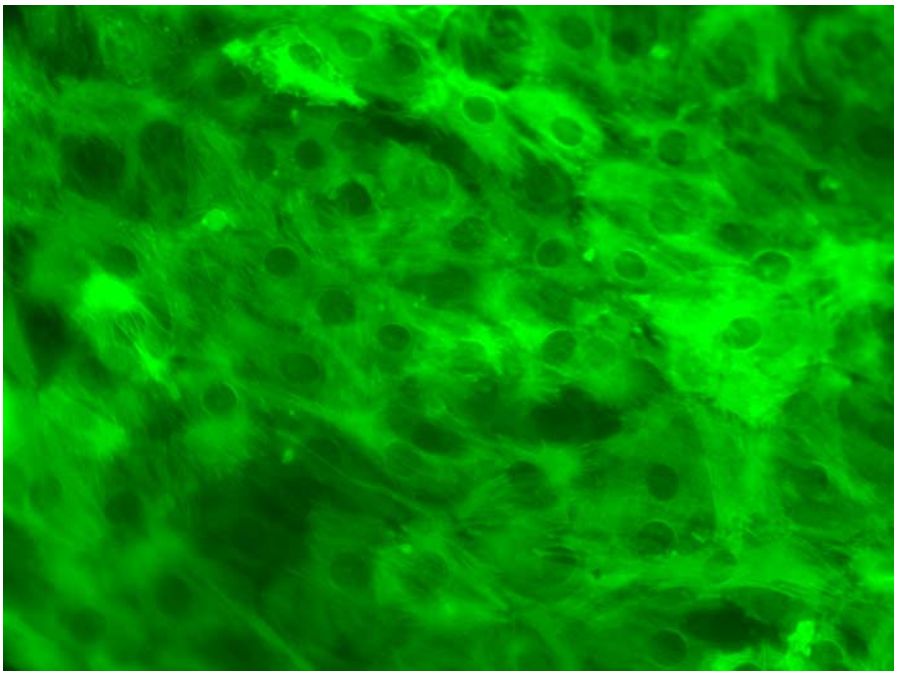

Figure 1: Immunofluorescence staining of cells derived from aged CBA mice

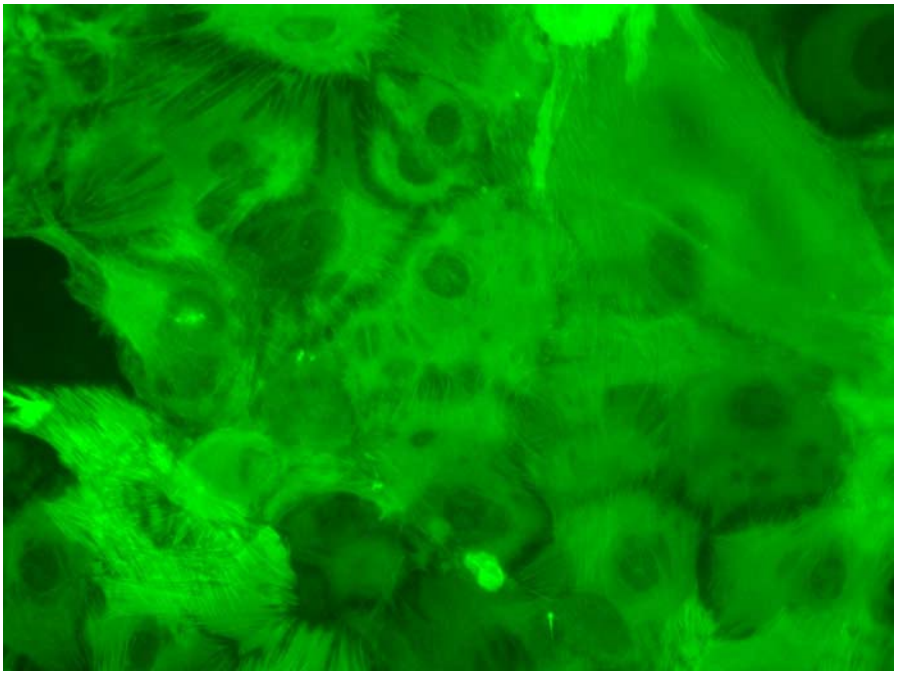

Figure 2: Immunofluorescence staining of cells derived from young CBA mice

Cell culture experiment: IL-6 production is elevated and MCP-1 production decreased in aged cells when compared to young cells in an ex vivo cell culture model

We stimulated the cultured cells with various TLR agonists, including LPS (TLR-4), peptidoglycan (TLR-2), Poly I:C (TLR-3), CpG (TLR-9) and flagellin (TLR-5), and measured their expression of the pro-inflammatory cytokine IL-6 and the chemokine MCP-1. The results of these experiments are shown in figures 3-16 below. The production of both MCP-1 and IL-6 was markedly elevated following LPS stimulation when compared to the other TLR agonists, a finding consistent with the documented role of TLR-4 signaling in the development of atherosclerosis, and the documented presence and activity of the TLR-4 receptor on vascular smooth muscle cells. Stimulation with

flagellin (TLR-5) and peptidoglycan (TLR-2) led to marked elevations of cytokine production above baseline as well, while the other TLR agonists did not markedly elevate the production of these pro-inflammatory cytokines (figures 8-9, 15-16).

During this first series of experiments, IL-6 production was markedly elevated in aged cells when compared to young cells undergoing stimulation with LPS, flagellin, and peptidoglycan, a finding consistent with our hypothesis that aging enhances the innate immune response of vascular smooth muscle via TLR-dependent pathways. MCP-1 production, however, was decreased in the aged cells when compared to the young cells across all three TLR agonists, a finding contrary to this hypothesis.

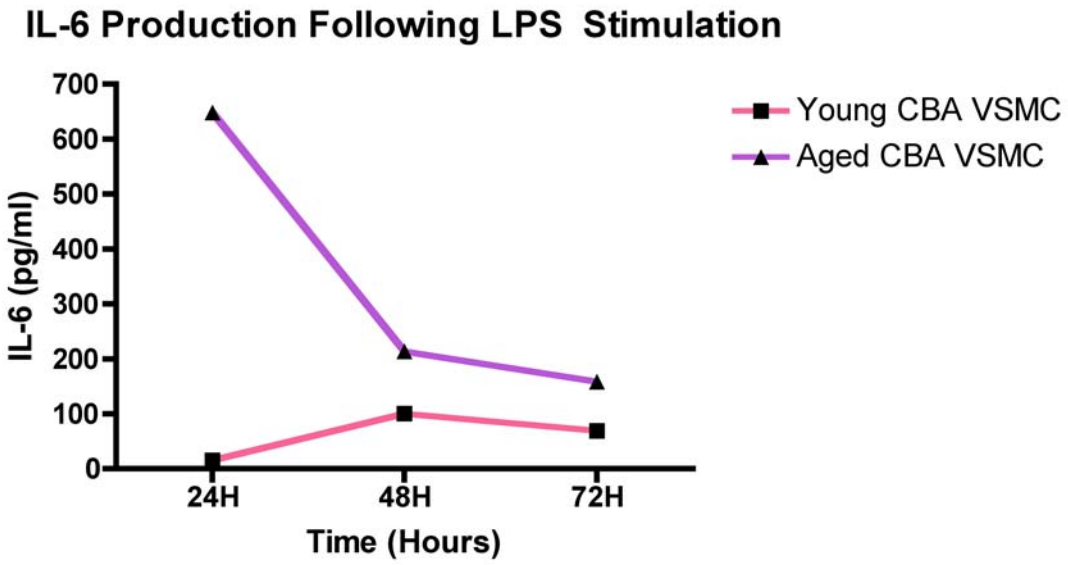

Figure 3: Production of IL-6 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-4 agonist LPS

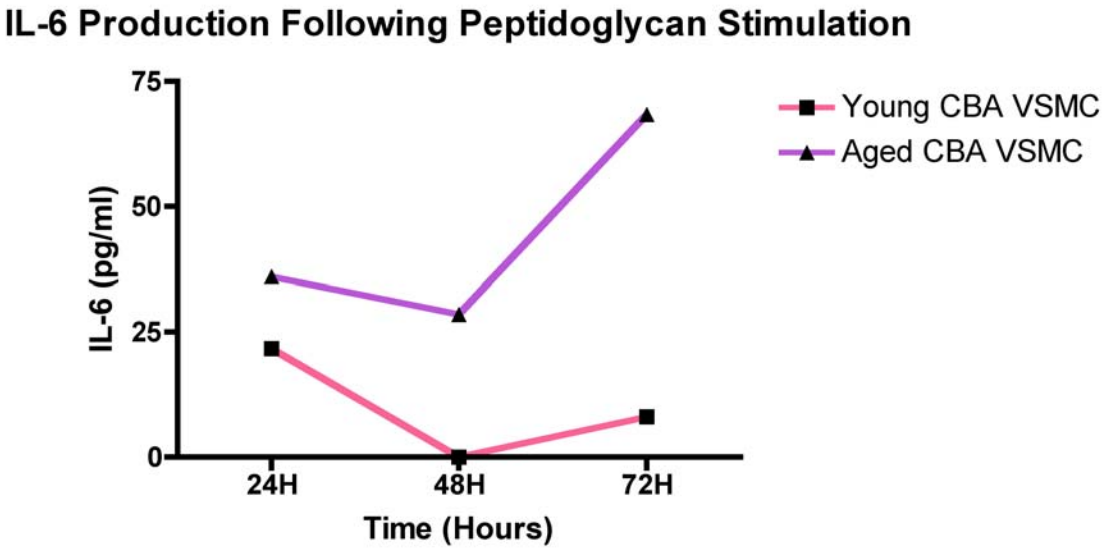

Figure 4: Production of IL-6 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-2 agonist peptidoglycan

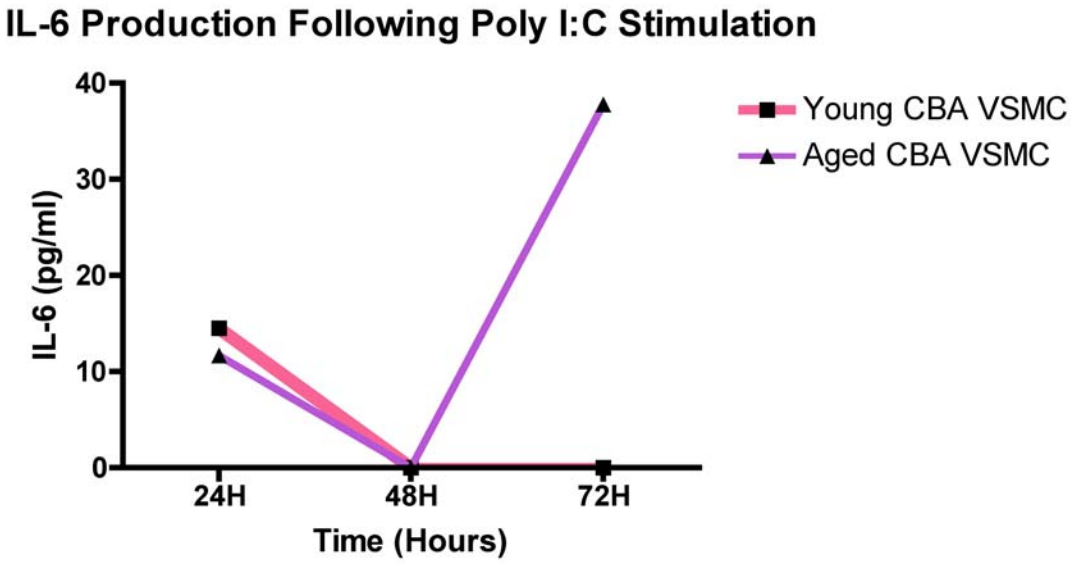

Figure 5: Production of IL-6 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-3 agonist poly I:C

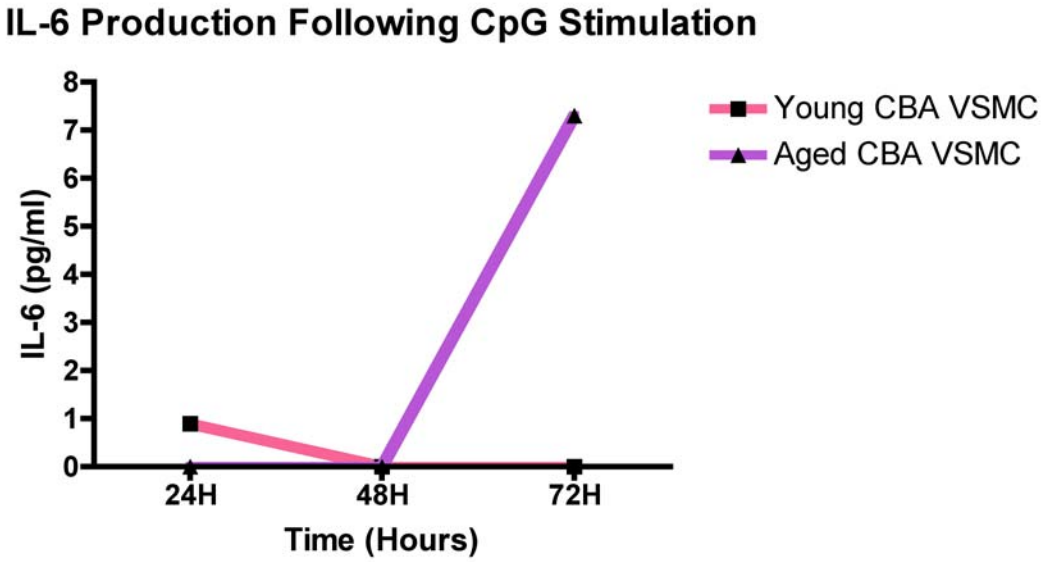

Figure 6: Production of IL-6 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-9 agonist CpG

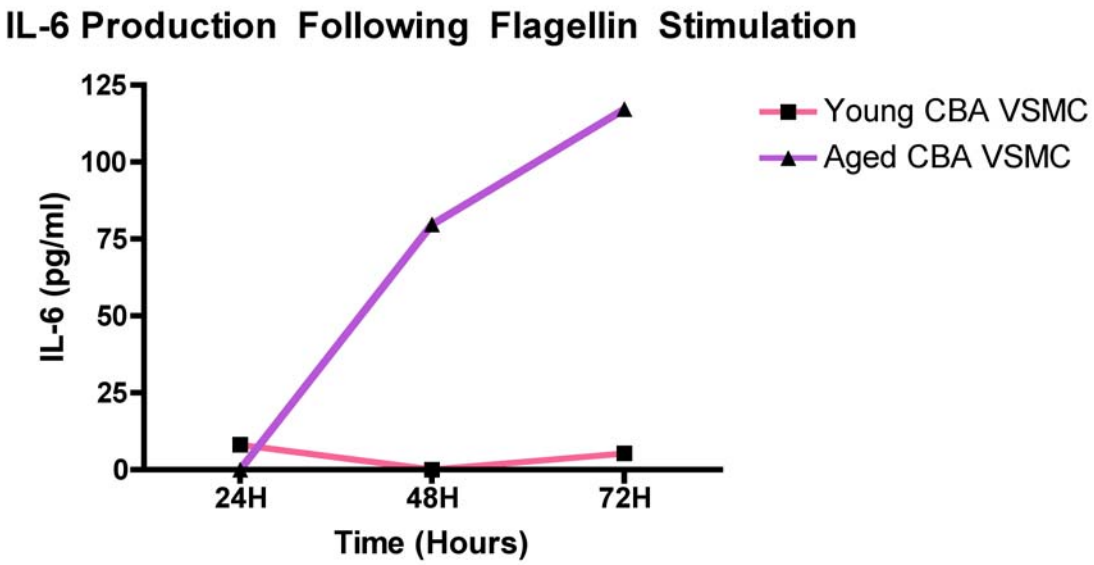

Figure 7: Production of IL-6 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-5 agonist flagellin

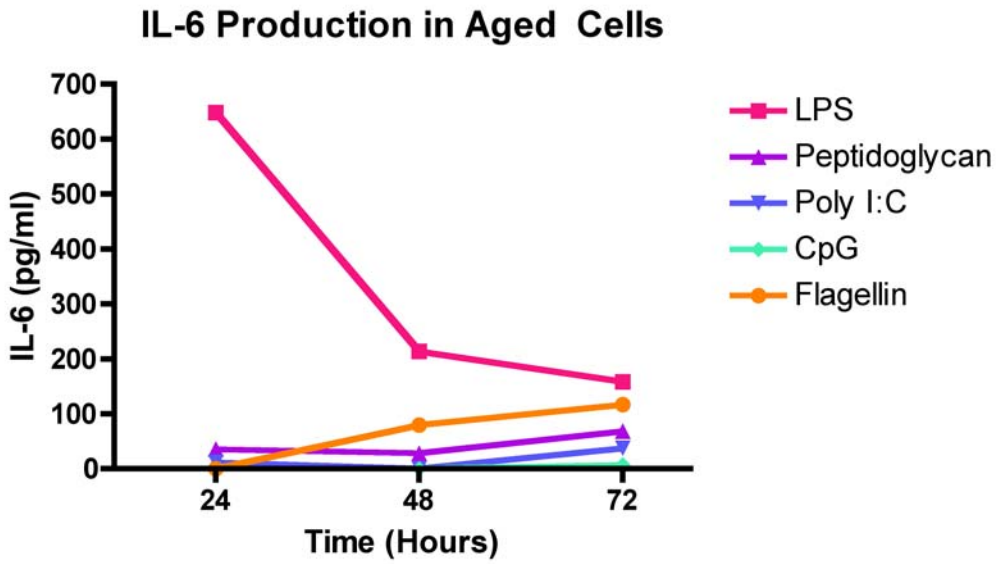

Figure 8: Production of IL-6 by vascular smooth muscle cells derived from aged CBA mice following stimulation with various TLR agonists

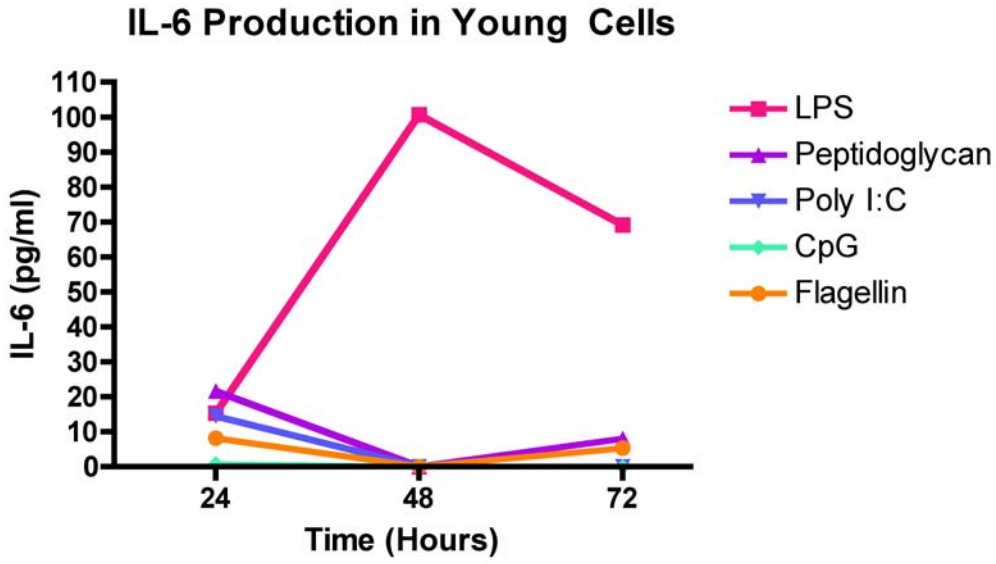

Figure 9: Production of IL-6 by vascular smooth muscle cells derived from young CBA mice following stimulation with various TLR agonists

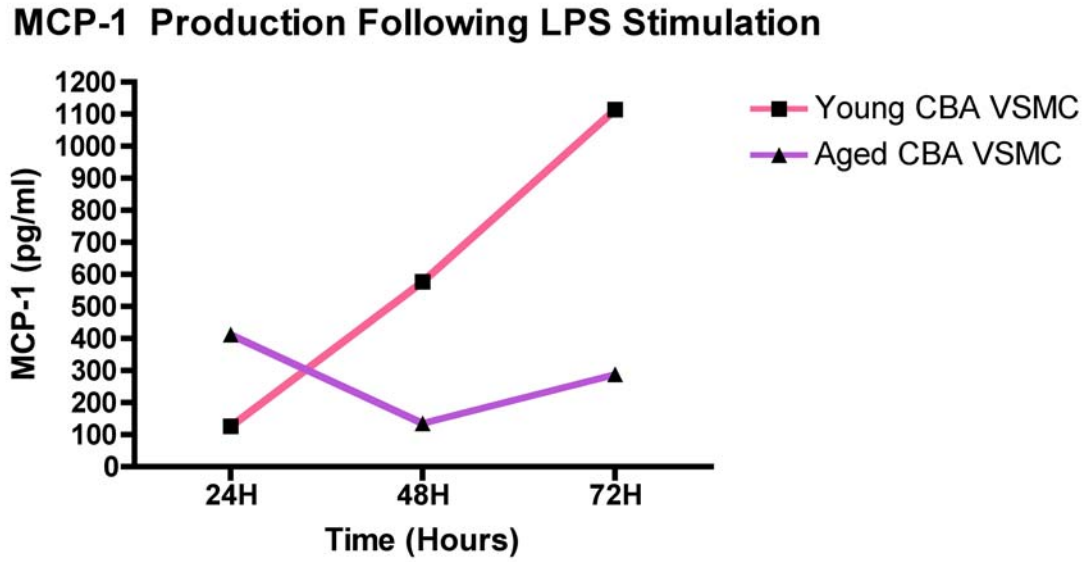

Figure 10: Production of MCP-1 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-4 agonist LPS

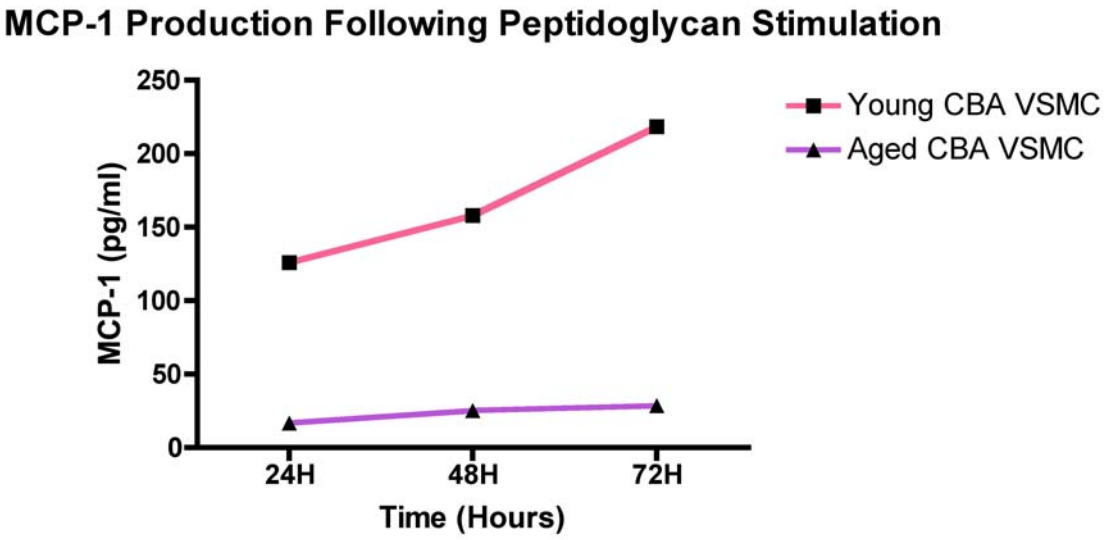

Figure 11: Production of MCP-1 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-2 agonist peptidoglycan

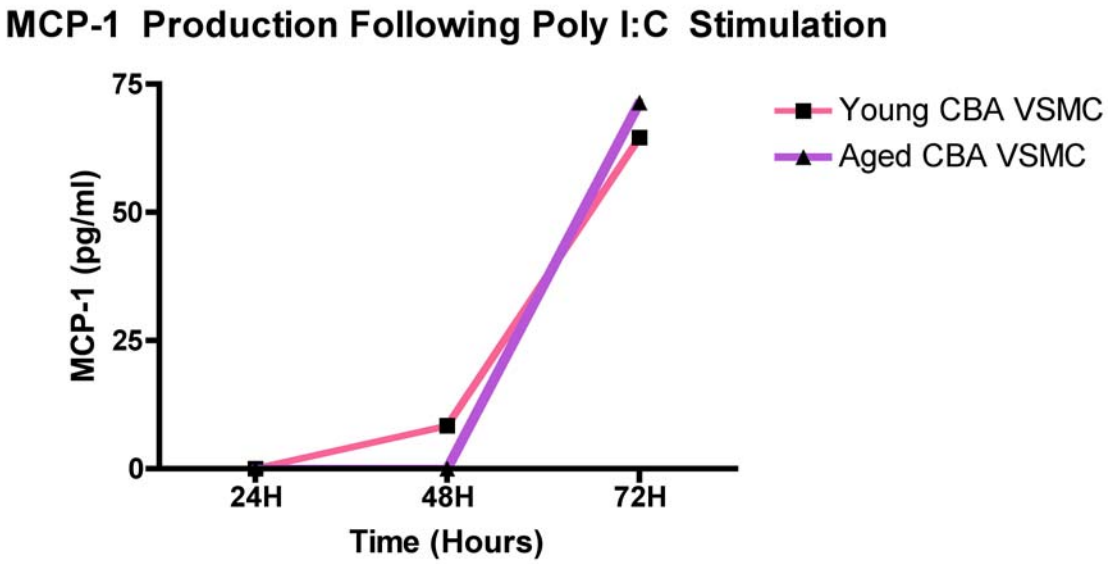

Figure 12: Production of MCP-1 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-3 agonist Poly I:C

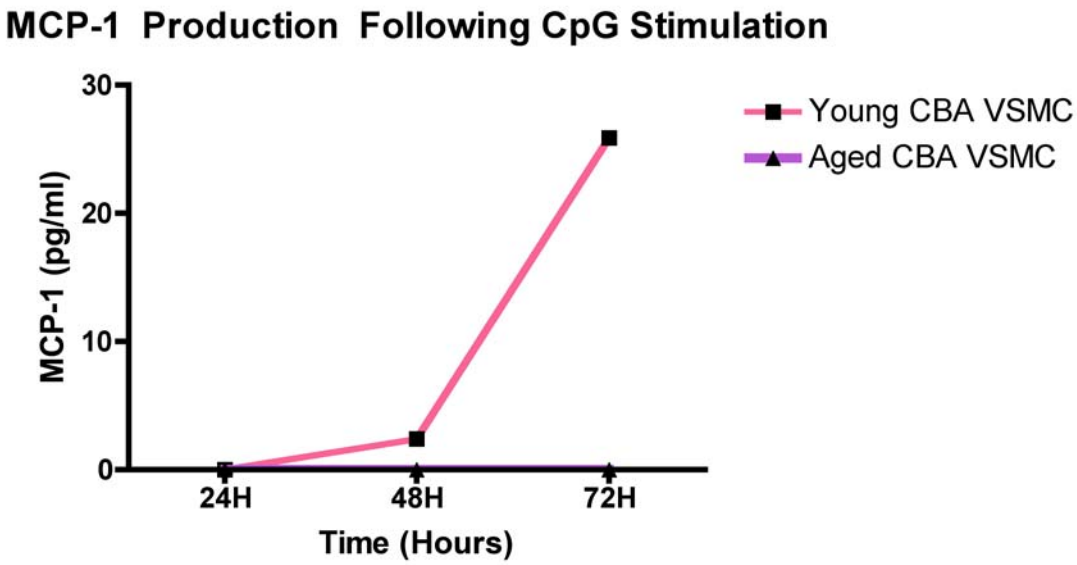

Figure 13: Production of MCP-1 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-9 agonist CpG

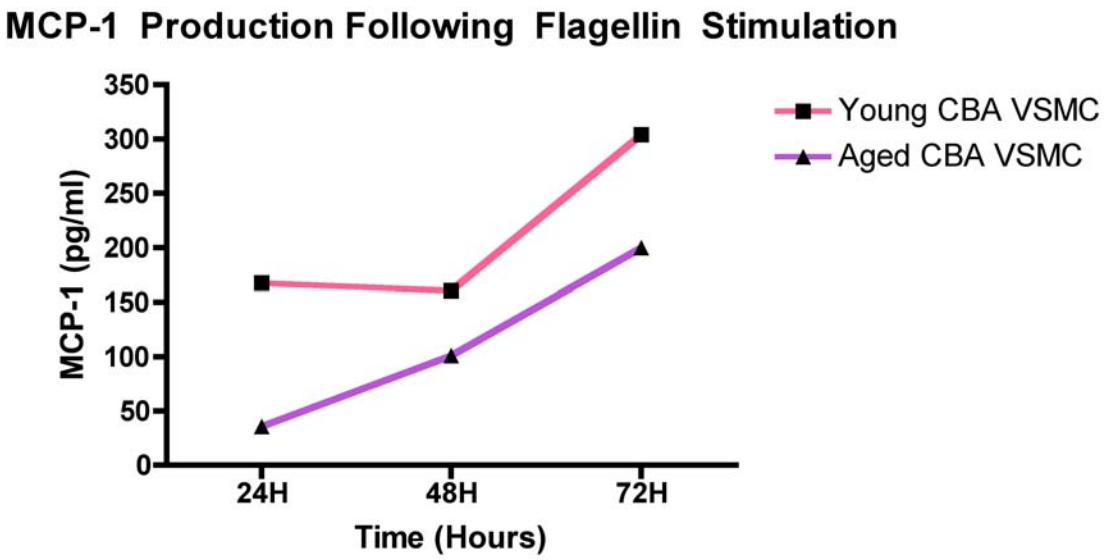

Figure 14: Production of MCP-1 by vascular smooth muscle cells derived from CBA mice following stimulation with the TLR-5 agonist flagellin

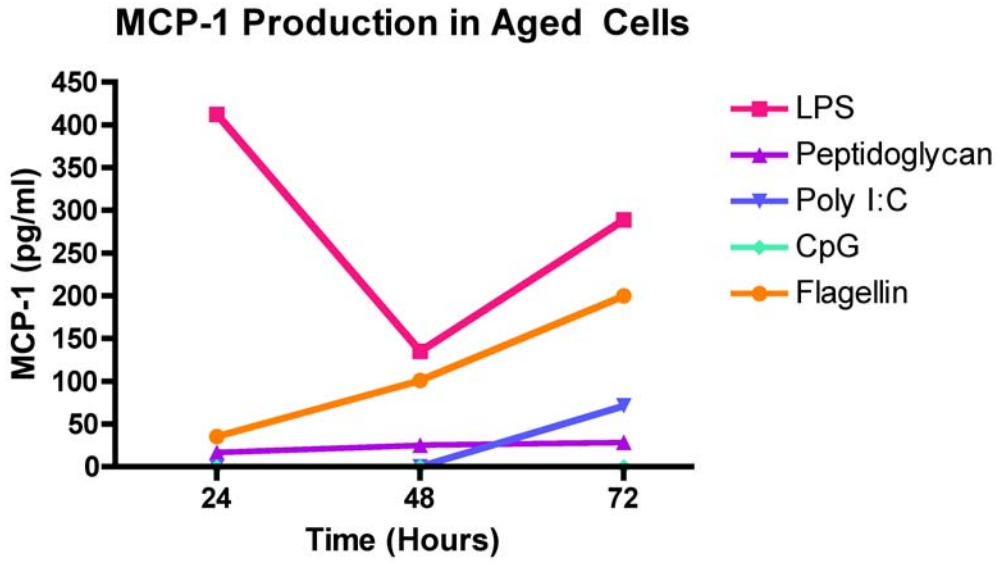

Figure 15: Production of MCP-1 by vascular smooth muscle cells derived from aged CBA mice following stimulation with various TLR agonists

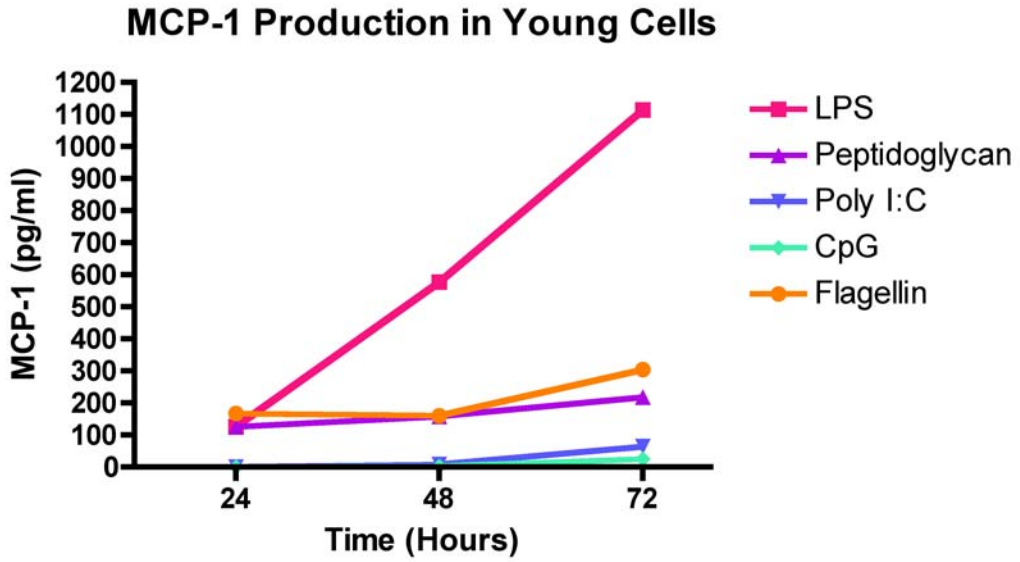

Figure 16: Production of MCP-1 by vascular smooth muscle cells derived from young CBA mice following stimulation with various TLR agonists

Tissue culture experiment: IL-6 production may be elevated in aged vessels stimulated with LPS compared to baseline and young stimulated and unstimulated vessels, MCP-1 production differences are also seen

Based on the differences seen in the vascular smooth muscle cell culture model between aged and young cells, further study was done using a tissue culture model in an attempt to replicate the data seen in the cell culture in a more physiologic system. Given the success of LPS stimulation when compared with stimulation by other TLR agonists during the initial cell culture experiment and the large body of evidence suggesting a role for TLR-4 signaling in the pathogenesis of atherosclerosis, a decision was made to use LPS stimulation for this next phase of the experiment. During the initial experiment, 3 aged and 3 young vessels were cultured from mice with a CBA background with

supernatants being taken only once from each vessel (i.e. 1 aged and 1 young vessel were stimulated for 6 hours, 1 each for 24 hours, and 1 each for 48 hours). The results of this experiment are shown in figures 17 and 18 below.

During this initial tissue culture experiment, IL-6 production in aged vessels was notably elevated when compared to baseline production at all time points, while IL-6 production in young vessels was elevated above baseline only at 48 hours. In addition, the production of IL-6 in aged vessels was notably elevated above that of young vessels at all time points, supporting our hypothesis that aging enhances the TLR-4 dependent innate immune response of vascular smooth muscle cells.

The MCP-1 data from this experiment did not clearly favor the immune response of aged or young vessels. At 6 and 24 hours, MCP-1 production in aged vessels was slightly higher than in young vessels, although at 6 hours production was near baseline in both stimulated vessels. At 48 hours, young vessels produced significantly more MCP-1 than did the aged vessels. Although the aged vessels produced more MCP-1 than did their your counterparts at two of three time points, the data was not as clear cut as that seen with IL-6, and it was difficult to draw any meaningful conclusions from this assay, given the variation and the relatively small differences seen at 6 and 24 hours.

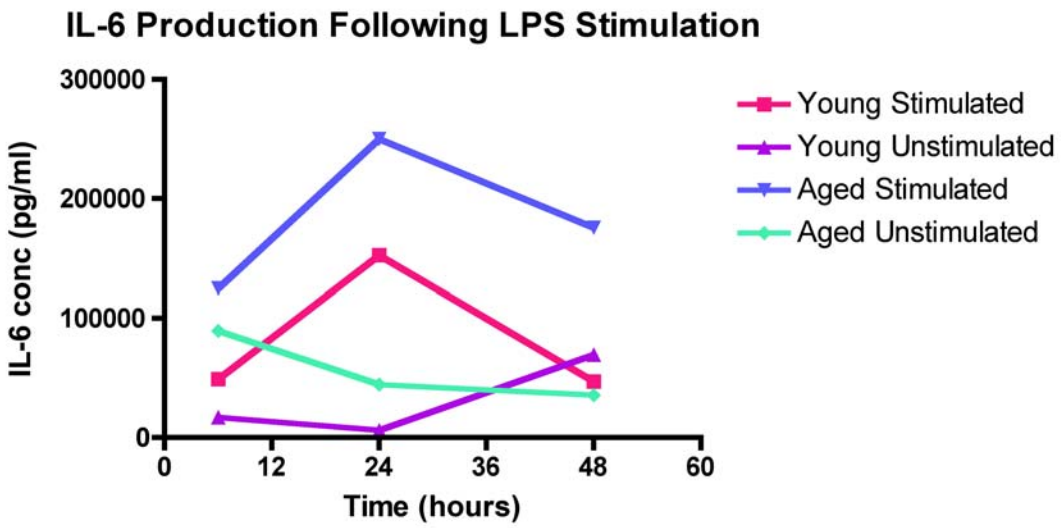

Figure 17: Production of IL-6 by tissue culture of stimulated and unstimulated young and aged vessels of CBA mice

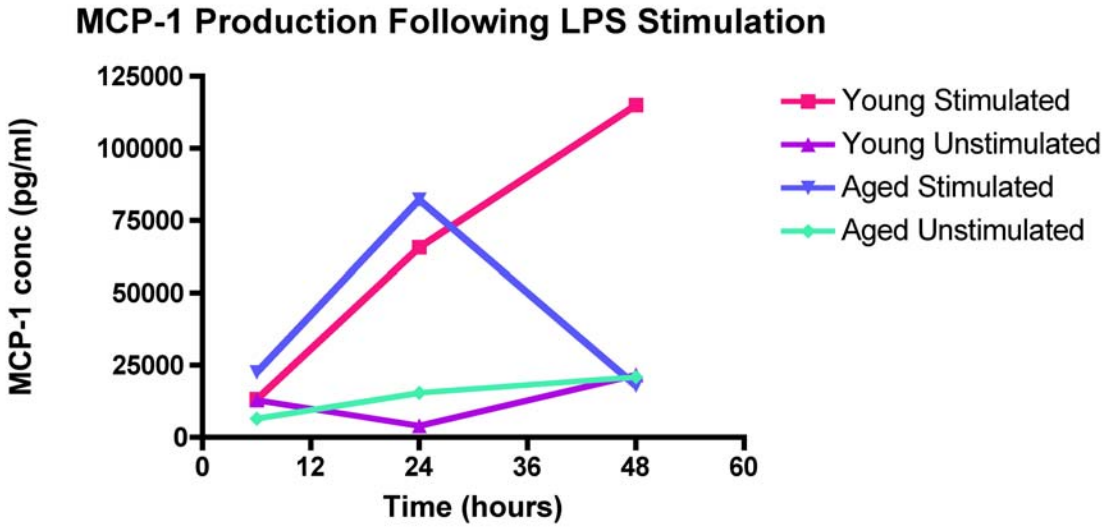

Figure 18: Production of MCP-1 by tissue culture of stimulated and unstimulated young and aged vessels of CBA mice

In order to gain a better understanding of the differences seen in these tissue cultures, the experiment was run again, this time using 3 young and 6 aged mice per group, again from a CBA background. For this second iteration of the experiment, data points were taken from each mouse at 12 hours, 24 hours, 48 hours, and 72 hours respectively, in order to maximize the amount of data available at each time point to increase the statistical power of the study. Data is shown below in figures 19-22. As was seen in the first tissue culture experiment, IL-6 production was increased in aged mice at early time points (12 and 24 hours). Significant heterogeneity of results was seen at 48 and 72 hours, and while IL-6 production was greater in the young vessels than in the aged vessels at these time points, these differences were not statistically significant.

In an attempt to provide further mechanistic data regarding the production of IL-6 by the tissue culture in response to TLR-4 stimulation with LPS, the aged mice that had undergone T cell ablations were separated from those that had not (figure 20). If the production of IL-6 was mediated by resident T cells, we would expect to see a significant reduction of IL-6 production in the T cell ablated mice when compared to the normal aged mice. A notable difference was seen at 48 hours between the ablated and normal mice (with less IL-6 production in the ablated mice) although this difference did not rise to the level of statistical significance. At other time points, IL-6 production was comparable in both sub-groups. Furthermore, IL-6 production in the ablated mice was significantly elevated above baseline levels of production. This suggests that the production of IL-6 in the tissue culture model is not mediated by T cells resident in the vasculature.

It is difficult to draw conclusions about the effect of aging on the TLR-4 dependent production of IL-6 in vascular smooth muscle based on this data, given the significant heterogeneity of the results and the inconsistent results, particularly at late time points. It may be that cell death begins to occur at the later time points, leading to the heterogeneity of inconsistency of the results. From these preliminary results, a trend has emerged which suggests the possibility that aged cells produce more IL-6 than do young cells in response to TLR-4 stimulation, particularly as measured at early time points (6-24 hours), although this is far from certain.

Similarly, it is difficult to draw conclusions from the production of MCP-1 seen in this second experiment. At early time points (12 and 24 hours), the aged and young cells produced very similar amounts of MCP-1. At the late time points (48 and 72 hours), the young cells produced more MCP-1 than did the aged cells, although again, there was significant heterogeneity of the results, which did not rise to the level of statistical significance. As with IL-6, there was no significant difference between normal aged mice and mice s/p T cell ablation (figure 22), suggesting that the production of MCP-1 in response to stimulation with the TLR-4 agonist LPS is not mediated by T-cells resident in the vessel wall. Given the variability seen between the experiments and the heterogeneity of the results, it is difficult to draw conclusions regarding the effect of aging on production of MCP-1 of vascular smooth muscle in response to TLR-4 stimulation.

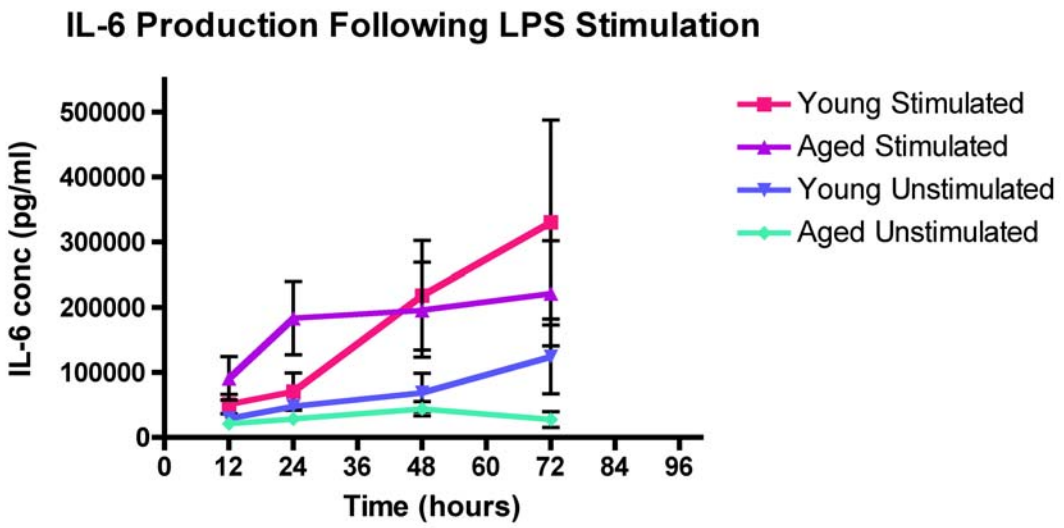

Figure 19: Production of IL-6 by tissue culture of stimulated and unstimulated young and aged vessels of CBA mice

Time	**Young-Stimulated**		**Aged-Stimulated**		**Young-Unstimulated**		**Aged-Unstimulated**	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
12H	51,322	14,954	91,129	33,433	29,275	7,321	21,501	10,537
24H	70,761	28,648	183,490	56,443	48,092	7,266	28,510	7,638
48H	218,277	83,911	196,162	72,562	69,091	29,499	44,311	11,091
72H	330,165	157,349	221,421	80,432	124,629	57,568	27,509	11,987

Table 1: Mean values and Standard Error of Mean for Graph in Figure 19

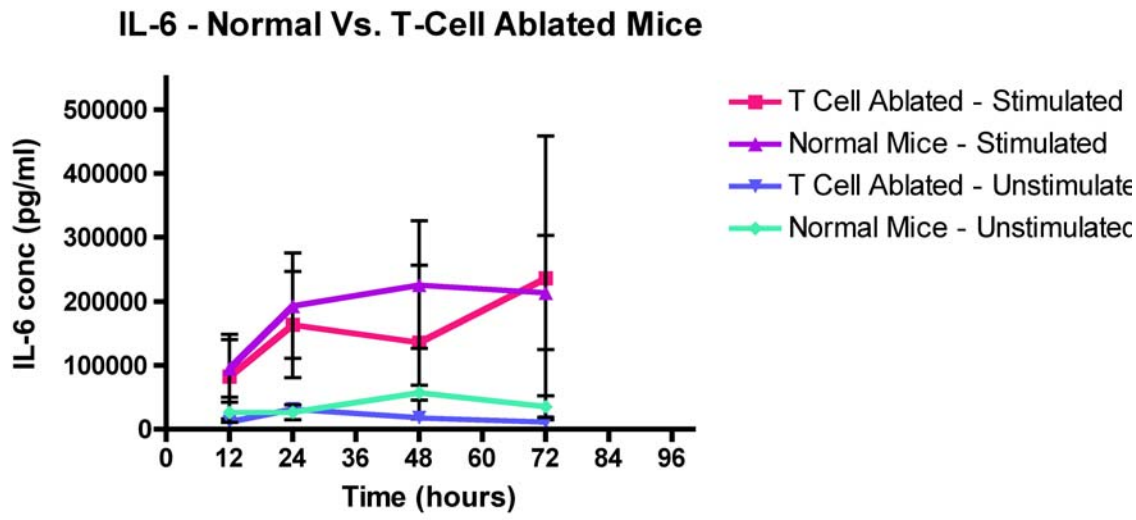

Figure 20: Production of IL-6 by tissue culture of stimulated and unstimulated aged vessels of CBA mice – normal mice vs. T cell ablated mice

Time	**Ablated-Stimulated**		**Normal-Stimulated**		**Ablated-Unstimulated**		**Normal-Unstimulated**	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
12H	82,592	66,232	95,397	45,224	11,514	1,857	26,495	15,877
24H	164,020	83,251	193,225	81,944	32,019	6,700	26,756	11,632
48H	136,019	119,837	226,234	99,325	18,216	872	57,358	11,713
72H	236,668	221,887	213,798	88,936	11,218	5,545	35,655	16,964

Table 2: Mean values and Standard Error of Mean for Graph in Figure 20

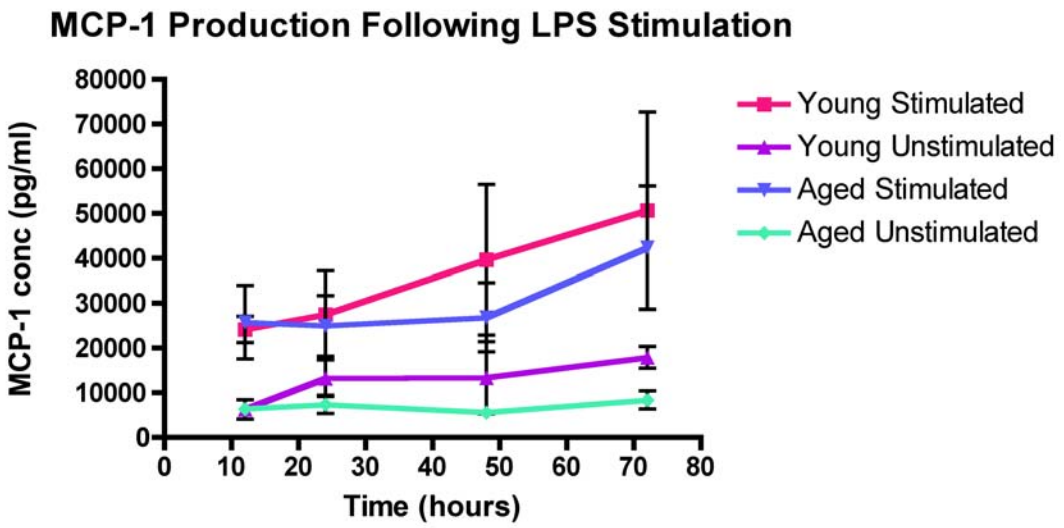

Figure 21: Production of MCP-1 by tissue culture of stimulated and unstimulated young and aged vessels of CBA mice

Time	**Young-Stimulated**		**Aged-Stimulated**		**Young-Unstimulated**		**Aged-Unstimulated**	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
12H	24,183	2,937	25,785	8,189	6,282	2,150	6,346	1,371
24H	27,508	9,709	24,953	6,758	13,261	4,088	7,384	2,036
48H	39,698	16,812	26,865	7,678	13,354	8,048	5,584	990
72H	50,703	21,977	42,398	13,797	17,933	2,400	8,383	2,029

Table 3: Mean values and Standard Error of Mean for Graph in Figure 21

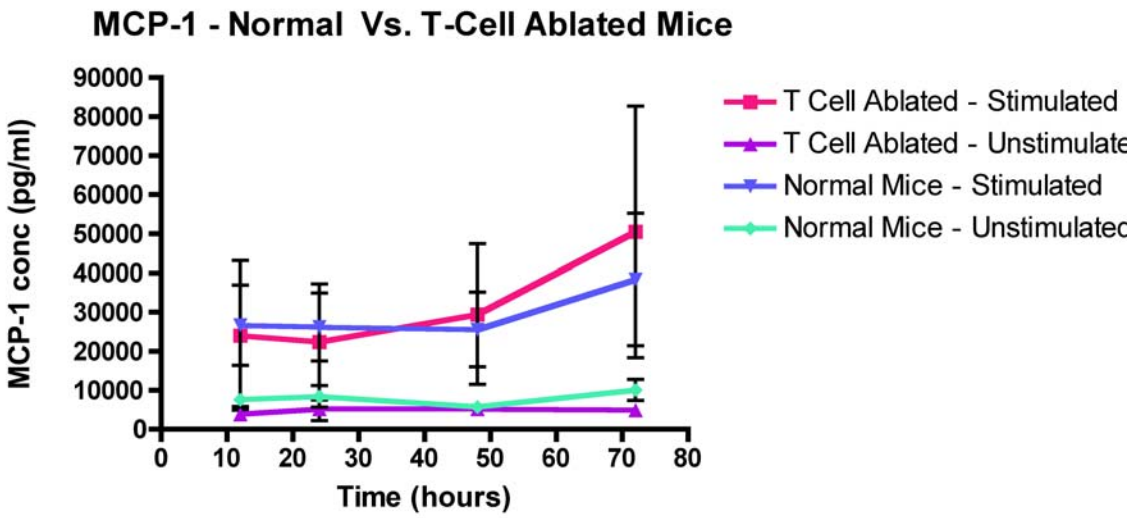

Figure 22: Production of MCP-1 by tissue culture of stimulated and unstimulated aged vessels of CBA mice – normal mice vs. T cell ablated mice

Time	**Ablated-Stimulated**		**Normal-Stimulated**		**Ablated-Unstimulated**		**Normal-Unstimulated**	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
12H	24,034	19,153	26,661	10,283	3,866	33	7,586	1,779
24H	22,388	14,830	26,236	8,711	5,232	3,003	8,460	2,775
48H	29,473	17,966	25,562	9,586	5,214	1,615	5,770	1,408
72H	50,521	32,199	38,336	16,930	4,927	654	10,110	2,690

Table 4: Mean values and Standard Error of Mean for Graph in Figure 24

Discussion:

Given the significant heterogeneity and inconsistency of the results of these experiments and the limited number of samples included in the analysis, it is difficult to draw any meaningful conclusions about the effect of aging on the TLR mediated immune response of vascular smooth muscle cells from this study.

Presence of various Toll-like receptors:

Our experiments with the vascular smooth muscle cell culture supports previous work done on the subject, in that stimulation with TLR-2 and TLR-4 agonists generated an immune response in the form of significant elevations in the production of MCP-1 and IL-6 [40]. Stimulation with the TLR-3 and -9 agonists Poly I:C and CpG did not yield a significant response. Interestingly, stimulation with flagellin, a TLR-5 agonist, did yield a response, with elevations in the production of MCP-1 and IL-6 comparable to that seen with peptidoglycan stimulation. In fact, among the various TLR agonists used in the cell culture experiment, flagellin stimulation produced the most clearly divergent curves between aged and young cells with respect to IL-6 production. In contrast with TLR-2 and -4, a literature search revealed no documentation of TLR-5 expression in vascular smooth muscle cells and no evidence of cross-reactivity of flagellin with the TLR-2 or -4 receptor.

Early studies of toll-like receptor signaling demonstrated activation of TLR-2 with LPS, although later studies demonstrated that purification of the commercial LPS

preparation negated this effect, suggesting that this cross-reactivity was due to a biological contaminant [60]. It is possible that the effect on VSMCs seen in our experiment is due to a similar contamination, although it is also possible that VSMCs do in fact express TLR-5, and that stimulation of the TLR-5 receptor by flagellin-bearing pathogens may play a role in the induction of an immune response in these cells, although further study is necessary to elucidate what, if any, role TLR-5 stimulation plays in this process. Experiments with TLR4 knockout VSCMs would evaluate whether contamination with TLR4 agonists is contributing to the activation of VSMCs with the flagellin mixture.

Limitations of the Cell and Tissue Culture Models:

A significant limitation of the cell culture model is that it is unclear to what degree aged and young cells retain their phenotypic differences through the process of proliferation, growth, division and passage in an in vitro setting. It is possible that in the artificial environment of a culture flask, this process results in a reversion of the aged cells to a younger phenotype. This limitation of the cell culture model was part of the impetus for the development of the tissue culture model.

Another significant limitation of the cell culture model and the first trials of the tissue culture model is in the limited number of samples analyzed in each experiment. Using only one time point from each cell and tissue culture makes it difficult to determine whether the differences seen between the aged and young cells represent true significant differences or merely statistical anomalies. In both cell and tissue culture models, decreased levels of cytokine production were seen at later time points when

compared with earlier time points in some experiments (IL-6, figure 3, MCP-1, figure 18). Because these time points do not reflect analysis of the same samples, it cannot be determined whether this decrease was due to cytokine instability and degradation over time, or simply due to an anomaly in one of the samples that was not present in the others, such as the presence of differing levels of endothelial cells or fibroblasts in addition to the smooth muscle cells, which affected the production of these cytokines. Multiple time points were examined for each sample in the second tissue culture experiment in order to correct for this limitation.

One significant limitation of this study that may have led to the generation of inaccurate results is that in the tissue culture model, results were not adjusted for vessel weight. As a result, the seemingly elevated production of IL-6 in the aged vessels may simply have been due to the presence of more tissue in these cultures. More recent studies have corrected this limitation.

IL-6 Production:

In summary, in both the cell and tissue culture models, IL-6 production was elevated in aged cells when compared to young cells following stimulation of TLR-4 with LPS. However, this effect was the least pronounced in the third experiment, in which the most data was generated. In this third experiment, a clear increase was found only at one of the four time points seen (24 hours). Furthermore, more recent studies, completed after the student finished working in the lab, have shown the opposite effect, with IL-6 production decreased in aged cells when compared to young cells (data not

shown). The heterogeneity of our results, combined with the recent contradictory results, make it difficult to draw any meaningful conclusions about the role that aging might play in the magnitude of the TLR-4 dependent immune response of VSMCs, and further study is ongoing to determine what, if any, differences exist between aged and young vessels in regard to this signaling pathway.

MCP-1 Production:

Like IL-6, the data regarding MCP-1 production is inconclusive. In the majority of time points in the majority of the experiments, the young cells and vessels produced more MCP-1 than did the aged cells in response to TLR-4 stimulation, although these differences did not rise to the level of statistical significance, and there was considerable variability and heterogeneity of the results. Our hypothesis, based on the studies discussed earlier, was that both MCP-1 and IL-6 would be produced in higher concentrations in the aged cells than in the young cells. As discussed, it has been shown that aging accelerates the process of atherosclerosis, that TLR-4 dependent signaling is involved in the process of atherosclerosis, that VSMCs produce the pro-inflammatory cytokines MCP-1 and IL-6 in response to TLR-4 stimulation, and that at least part of the accelerations in atherogenesis seen in aged vessels is due to differences in the function of VSMCs. For those reasons, it seemed likely that TLR stimulation would lead to increased production of IL-6 and MCP-1 in aged vessels and vascular smooth muscle cells when compared to young vessels and cells. Our data on MCP-1 production does not support this hypothesis. One possible explanation for the different levels of production of

MCP-1 and IL-6 in response to TLR stimulation is that promoters of the MCP-1 and IL-6 genes may be differentially occupied (e.g. by repressors) in various subsets and/or ages of cells. However, our data is far from conclusive in regards to this differential production, and further studies are ongoing to determine what, if any, role aging may play in the production of MCP-1 and IL-6 in response to TLR stimulation.

Conclusion:

In conclusion, our study generated preliminary data suggesting that aged vascular smooth muscle produces more of the pro-inflammatory cytokine IL-6 and less of the pro-inflammatory chemokine MCP-1 than does young vascular smooth muscle. However, the significant heterogeneity and inconsistency of these results makes it difficult to draw meaningful conclusions from our data, and further studies are ongoing to further elucidate what role aging might play in this inflammatory response.

Future Goals/Experiments:

Further study is warranted to determine what differences exist between aged and young cells in terms of the production of pro-inflammatory cytokines in response to TLR stimulation. Further tissue culture experiments with LPS stimulation will be performed, in which samples are normalized for weight. This normalization and repetition should increase the statistical power of this study, and allow for more meaningful conclusions to be made.

In addition, further study is warranted on the role that TLR-5 stimulation with flagellin may play in the immune response of VSMCs, as well as the role that aging might play in the pathogenesis of that response.

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