Role of TNFR I Signaling in Racial Differences in Endothelial Function: Potential Modulatory Effect of High Laminar Shear Stress

by

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Abstract

In the endothelium, TNF binding to TNF receptor I (TNFR I) instigate a cascade of inflammatory events that facilitate monocytes recruitment and consequently initiation of atherosclerosis. In addition, TNF promotes endothelial dysfunction by suppressing endothelial nitric oxide synthase (eNOS) activity and compromising NO bioavailability. Epidemiological and clinical evidence suggest higher prevalence of endothelial dysfunction and subclinical atherosclerosis in African American (AA) population. This is supported by limited in vitro evidence demonstrating heightened inflammatory response and atherogenic potential in endothelial cells (ECs) from AA donors. Moreover, atheroprotective effects of aerobic exercise are well-established. We have previously shown the effect of high laminar shear stress (HSS), as an exercise mimetic, on mitigating some aspects of racial differences in endothelial function on a cellular level. Therefore, we conducted the present study to examine possible racial differences in TNF-induced monocyte adhesion and transendothelial migration as well as TNFR I signaling complex expression/activity. We also investigated the effects of HSS on attenuating possible racial differences. THP-1 monocytes and human umbilical vein endothelial cells (HUVECs) from Caucasian Americans (CA) and AA donors were used in a co-culture system to examine racial differences in atherogenic potential. Additionally, a model of in vitro exercise mimetic was applied to investigate potential modulatory effect HSS. We report no significant racial differences in TNF-induced monocyte adhesion and migration, the expression of TNFR I signaling complex or TNF-induced activation of NF-κB. Application of HSS produced transient atheroprotective effects in AA HUVECs comparable to that in CA HUVECs.

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List of Abbreviations

TNF	Tumor necrosis factor
TNFR I	TNF receptor I
TRAF	TNFR associated factor
NF-κB	Nuclear factor of kappa B
MAP Kinase	Mitogen-activated protein kinase
eNOS	Endothelial nitric oxide synthase
NO	Nitric oxide
sTNFR	Soluble TNF receptor
HSS	High laminar shear stress
AA	African Americans
ECs	Endothelial cells
CA	Caucasian Americans
CVD	Cardiovascular diseases
FMD	Flow-mediated dilation
HUVECs	Human umbilical vein endothelial cells
SOD1	Superoxide dismutase 1
IL-6	Interleukin-6
TACE	TNFα converting enzyme
ADAM17	a disintegrin and metalloprotease 17
TNFR II	TNF receptor II
SODD	Suppressor of death domain

TRADD	TNFR I-associated death domain
FADD	Fas- associated death domain
RIP1	Receptor-interacting protein 1
cIAP1/2	Cellular inhibitor of apoptosis protein 1/2
LUBAC	Linear ubiquitin chain assembly complex
TAK1	Transforming growth factor β-activated protein kinase 1
IKK	IκB Kinase
NEMO	NF-κB essential modulator
NIK	NF-κB inducing kinase
IκBα	Inhibitor of kappa B α
JNK	c-Jun N-terminal kinases
ASK1	Apoptosis signal-regulating kinase 1
ERKs	Extracellular-signal-regulated kinases
AP-1	Activator protein 1
ATF-2	Activating transcription factor 2
PI3K	Phosphatidylinositol-3 kinase
TLR	Toll-like receptors
IL-1	Interleukin-1
cIMT	Carotid intima-media thickness
VCAM-1	Vascular cell adhesion molecule 1
ICAM-1	Intracellular adhesion molecule 1
MCP-1	Monocyte chemoattractant protein 1

ADMA	Asymmetric dimethylarginine
ROS	Reactive oxygen species
ET-1	Endothelin-1
LSS	Low laminar shear stress
PBMCs	Peripheral blood mononuclear cells

1.0 Introduction

Tumor necrosis factor (TNF) is a potent, pro-inflammatory cytokine produced predominately by activated leukocytes. One of the main targets of TNF is the endothelium where it initiates a cascade of events leading to endothelial dysfunction (1-3). The diverse effects of TNF are mediated primarily by the cell surface receptor TNF Receptor I (TNFR I) (4). Once activated by TNF binding, TNFR I forms a signaling complex with adaptor proteins and TNFR associated factors (TRAFs); and activate multiple signaling pathways like nuclear factor of kappa B (NF-κB) and mitogen-activated protein (MAP) kinase (5-8). This induces the expression of adhesion molecules and chemokines that cause monocytes recruitment and activation, consequently amplifying inflammation and initiating atherogenesis (9-11). Moreover, TNF has a detrimental effect on endothelial nitric oxide synthase (eNOS) promoter activity compromising nitric oxide (NO) bioavailability, a hallmark of endothelial dysfunction (12).

Efficacy of exercise training in improving endothelial function is well established (13-15). With aerobic training, soluble TNFR I (sTNFR I) levels improve (16, 17). sTNFR I binds to circulating TNF and neutralizes its activity (18). In vitro, application of high laminar shear stress (HSS) is a validated exercise mimetic model. Evidence suggests that HSS mitigates inflammation and induces atheroprotective effects. Specifically, HSS has been shown to inhibit some of the

TNF-induced downstream effects (19-21). Moreover, HSS induces a greater reduction in oxidative stress and inflammatory markers in African American (AA) endothelial cells (ECs) compared to Caucasian (CA) ECs (22).

AA have the highest prevalence of cardiovascular disease (CVD) (23). This may be partially attributed to endothelial dysfunction commonly seen in this population. Young and healthy AA have lower flow-mediated dilation (FMD) than CA (24, 25). Additionally, in vitro data show that AA ECs exhibit greater oxidative stress and heightened inflammation compared to CA ECs (22). AA human umbilical vein ECs (HUVECs) express higher levels of NADPH oxidase subunits and lower superoxide dismutase 1 (SOD1) activity than CA HUVECs (26). Moreover, AA HUVECs produce higher basal levels of interleukin (IL)-6, an inflammatory cytokine, than CA HUVECs in response to TNF stimulation (27).

Racial differences in EC responses to stimuli, particularly TNF, could play an important role in the promotion of endothelial dysfunction, plaque development, and consequently CVD. Despite the compelling data supporting higher prevalence of endothelial dysfunction and CVD in AA, it is predominantly of observational nature. Evidence explaining the mechanism(s) prompting this disparity is still lacking. Therefore, research exploring the underlying cellular mechanism(s) that can be targeted for treatment/prevention is needed and of high importance.

The aims of the proposed study are:

Aim 1: To investigate possible racial differences in TNF-induced monocyte adhesion and migration. We hypothesize that higher signaling activity of TNFR I signaling complex will promote higher atherogenic potential in AA ECs.

Aim 2: To examine possible racial differences in TNFR I signaling complex expression/activity, negative regulators, and downstream effects. We hypothesize that AA ECs will exhibit higher expression/activity of the TNFR I signaling complex and lower expression of modulators.

Aim 3: To examine the effects of HSS on mitigating possible racial differences. We hypothesize that HSS will attenuate some of the potential racial differences in TNF-induced downstream effects.

2.0 Literature Review

2.1 TNF

2.1.1 History and production

TNF is a potent, pleiotropic, pro-inflammatory cytokine. It is produced predominately by activated monocytes, macrophages, and other cell types. In addition to its role in mediating inflammation, TNF is an important regulator of cell differentiation, proliferation, survival and cytotoxicity (5). Named after one of its first identified effects, TNF was originally described as an endotoxin-induced glycoprotein that can cause tumor necrosis in animal models (4, 28). In 1985, the development of recombinant TNF led to adopting TNF in cancer treatment (4, 28). However, due its mitogenic and cytotoxic effects, the field has shifted towards implementing anti-TNF antibodies to control disease progression in various inflammatory conditions (4, 18).

TNF is initially produced as a membrane-bound, 26 kDa TNF-precursor (4, 29). TNFα converting enzyme (TACE), also known as a disintegrin and metalloprotease 17 (ADAM17), mediates the cleaving of the extracellular domain and the release of the 17 kDa, trimeric, soluble and biologically active TNF (4, 29). The importance of TACE and ectodomain shedding in regulating TNF function was demonstrated using genetically modified mouse line. These mice, wherein cleaving of TNF precursor is suppressed, exhibited a phenotype similar to TNF knockout mice (29).

2.1.2 Receptors

TNF induces its diverse effects by signaling through two cell surface receptors TNF Receptor I (TNFR I) and TNF Receptor II (TNFR II). These receptors are expressed in almost all nucleated cells and their differential expression depends on cell type and presence of disease (4, 18, 30). Like other TNF receptors superfamily, TNFR I and II receptors share the same extracellular ligand-binding domain that includes four cysteine-rich subdomains. However, their intracellular domains structures are distinct suggesting diverse functions (4, 18, 30). The intracellular domains of both receptors lack enzymatic activity. TNFR I contains a death domain allowing the recruitment of adapter proteins that contain death domains and involved in apoptosis signaling; while TNFR II does not contain a death domain, it can still recruit adaptor proteins facilitating other signaling events (30).

TNF pro-inflammatory and pro-apoptotic effects are mainly mediated by TNFR I, whereas functions of TNFR II are less identified. However, evidence has demonstrated TNFR II role in tissue repair and angiogenesis (4). Differences in the induced effects can be attributed to the type of ligand binding to the receptor. It has been shown that TNFR II can bind efficiently to membrane-bound TNF while soluble TNF primarily binds to TNFR I (1). In addition, according to the ligand passing concept, it has been suggested that TNFR II has higher affinity and dissociation rate than TNFR I (18, 31). Therefore, it can bind to soluble TNF at low concentrations and then pass it along to TNFR I for amplification and effective signaling (1, 18). Contradicting this concept, McFarlane et al. have demonstrated that TNFR II accounts for about 10% of TNFR-induced NF-κB activation (30).

Two main factors regulate the expression of TNF receptors, rate of synthesis and receptor shedding. Numerous cytokines and interleukins have been implicated in controlling transcription rate of both receptors (4). Additionally, TACE mediates the shedding of receptors' extracellular domain forming sTNFR in response to inflammatory stimuli, thereby decreasing the amount of active membrane-receptors (1, 4, 18). As a natural inhibitory mechanism, sTNFR bind to TNF neutralizing its biological effect. On the other hand, others suggest that this binding increases TNF half-life and improves its biological activity, serving as a circulating slow release reservoir (18). However, the correlation of TNF/sTNFR ratio in the serum with the disease severity is of clinical significance (31). Further, administering sTNFR is an effective treatment strategy to control inflammatory conditions (4).

2.2 TNFR I signaling complex

Binding of the TNF ligand to TNFR I induces receptor trimerization and initiates two cellular responses: gene activation and apoptosis (5, 6). Conformational change of the receptor leads to the release of the suppressor of death domain (SODD) and recruitment TNFR I-associated death domain protein (TRADD) (1). TRADD facilitates the recruitment of other death domain containing signaling proteins such as Fas-associated death domain protein (FADD). TNFR I/TRADD/FADD complex initiates a series of caspase activations starting with recruiting caspase-8 to the receptor complex leading to TNF-induced apoptosis (1, 5, 6).

Moreover, TRADD recruits receptor-interacting protein 1 (RIP1) and TRAF2/5, thereby activating gene induction signaling pathways, namely NF-κB and MAP kinase (1, 5, 6). TRAF2/5 then recruits E3 ligases cellular inhibitor of apoptosis protein (cIAP)1/2 to the receptor complex.

cIAP1/2 generate multiple ubiquitin linkages allowing the recruitment of linear ubiquitin chain assembly complex (LUBAC) to the TNFR signaling complex (6, 32). In his review, Walczak highlighted the significant role LUBAC plays in stabilizing the signaling complex. Further, LUBAC poly-ubiquinates RIP1 and the regulatory subunit of the IκB Kinase (IKK) complex, NF-κB essential modulator (NEMO), allowing the recruitment of IKK and transforming growth factor β-activated protein kinase 1 (TAK1) to the signaling complex. This ultimately leads to IKK and MAP kinase activation (6, 32). In addition, cIAP1/2 are negative regulators of the non-canonical NF-κB pathway by causing constitutive degradation of the NF-κB inducing kinase (NIK). The importance of cIAP1/2 is further demonstrated by the attenuated NF-κB signaling associated with cIAP1/2 knockouts (32). Signaling of TNFR I complex is depicted in Fig. 1.

2.3 TNF-induced gene activation signaling

In resting cells, the two heterodimers of NF-κB transcription factors (p50 and p65) are maintained in the cytosol by the inhibitory effect of inhibitor of kappa B α (IκBα) forming an inactive complex. TNF induces the activation of the canonical NF-κB pathway. As stated above, this activates IKK leading to IκBα phosphorylation and ultimately ubiquitination and degradation. As a result, NF-κB heterodimers are liberated and can translocate to the nucleus where they bind to DNA inducing transcription of several inflammatory mediators as well as regulators of TNFR I signaling (1, 32).

Moreover, three pathways of the MAP kinase signaling cascades have been implicated in TNF activation. p38 MAP kinases and the c-Jun N-terminal kinases (JNK) are activated mainly by TRAF2-induced activation of the upstream kinase, apoptosis signal-regulating kinase 1 (ASK1)

(1, 8). On the other hand, the activation of extracellular-signal-regulated kinases (ERKs) depends on LUBAC-induced activation of TAK1 and IKK2. MAP Kinase signaling result in activation of multiple transcription factors including: Activator protein 1 (AP-1), c-Jun and activating transcription factor 2 (ATF-2) (8).

TNF signaling is complicated by its biphasic nature. TNF induces an inflammatory response that initiates a secondary increased expression of TNF by target cells. Interaction of multiple transcription factors is required to induce complete activation of TNF promoter. Evidence suggests that type of transcription factors involved depends on cell type (8). In addition, TNF induces activation of phosphatidylinositol-3 kinase (PI3K) dependent kinase Akt. Pharmacological inhibition of Akt confirmed its role as a negative regulator of MAP kinase; however, it had minimal effect on NF-κB (1).

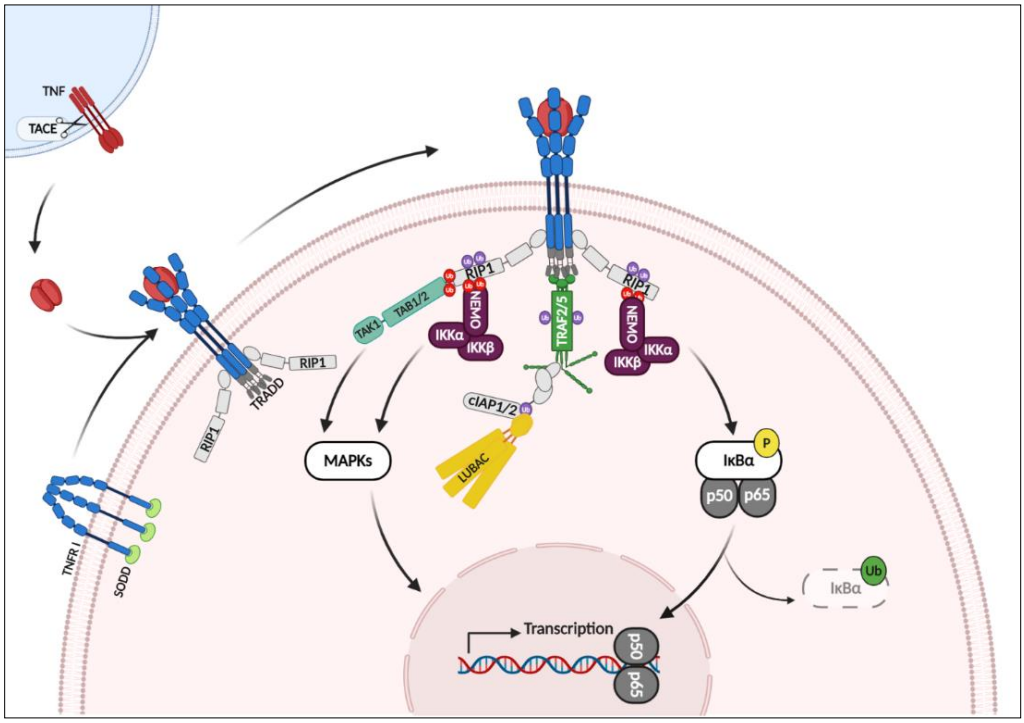

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Figure 1: TNFR I signaling complex and gene activation.

Binding of the TNF ligand to TNFR I induces a conformational change of the receptor intracellular domain that leads to the release of the SODD and the recruitment of TRADD and RIP1. TRADD recruits TRAF2/5 which recruits E3 ligases cIAP1/2 to the receptor complex. cIAP1/2 generate multiple ubiquitin linkages allowing the recruitment of LUBAC to the TNFR signaling complex. LUBAC poly-ubiquinate RIP1 and the regulatory subunit of the IKK complex, NEMO, allowing the recruitment of IKK and the TAB/TAK1 complex to the signaling complex. Consequently, IKK is activated leading to IκBα phosphorylation and degradation. As a result, NF-κB heterodimers are liberated and can translocate to the nucleus. Further, MAP kinases are activated thereby inducing transcription of several inflammatory mediators as well as regulators of TNFR I signaling.

(**TNF**, Tumor necrosis factor. **TNFR I**, TNF receptor I. **SODD**, suppressor of death domain. **TRADD**, TNFR I-associated death domain. **RIP1**, receptor-interacting protein 1. **TRAF2/5**, TNFR associated factor1/2. **cIAP1/2**, cellular inhibitor of apoptosis protein 1/2. **LUBAC**, Linear ubiquitin chain assembly complex. **IKK**, IκB Kinase. **NEMO**, NF-κB essential modulator. **TAB/TAK1**, TAK1-binding protein /Transforming growth factor β-activated protein kinase 1. **NF-κB**, Nuclear factor of kappa B. **MAP kinases**, Mitogen-activated protein kinase).

2.4 TRAFs

2.4.1 Structure and function

TRAFs are a family of intracellular adaptor proteins that function as signal transducers for the TNF receptor superfamily, Toll-like receptors (TLR), and interleukin (IL)-1 receptor family. There are six members identified to date, TRAF1-6 characterized by a carboxy-terminal domain that mediates their interaction with upstream receptors and other adaptor proteins; and an amino-terminal which contains zinc finger and RING finger domains that play a role in downstream signaling (33-35). All TRAFs, except TRAF1, have a RING finger motif enabling them to act as ubiquitin E3 ligases (33, 35).

TRAF1 is an inducible protein that interacts with TRAF2 and associate with the cytoplasmic domain of TNFR II forming a heterodimeric complex (33). Evidence suggests that TRAF1, together with TRAF2, act as a negative regulator of TNF-induced apoptosis (34). TRAF2 is the most ubiquitously expressed of the TRAFs family. As stated above, it plays a major role in activating NF-κB and AP-1 transcription factors. Further, data from knockout models demonstrated that TRAF2 is crucial to activate JNK while it can be dispensable for NF-κB activation (34). Meanwhile, TRAF3 cannot activate JNK and canonical NF-κB pathways, possibly due to its zinc sequence motif (36). However, TRAF3 forms a complex with TRAF2 and cIAP1/2 and negatively regulate non-canonical NFκ-B signaling. In unstimulated cells, TRAF3 constitutively mediates K48-linked polyubiquitination of NIK causing its proteasomal degradation. With ligand-induced receptor activation, TRAF2 mediates K63-linked polyubiquitination of cIAP1/2 enhancing its K48-linked ubiquitination towards TRAF3 leading to

its degradation and NIK liberation (35-37). Unlike other TRAFs, TRAF4 is located predominantly in the nucleus and evidence on its role as a cytosolic signal transducer is limited (33, 34). TRAF5 is comparable to TRAF2 in structure and function. Using TRAF2-deficient cells, the redundant role of TRAF5 in TNF-induced activation of NF-κB was highlighted. However, TRAF5 had no effect on JNK signaling activation (38). Finally, TRAF6 mediates CD40, TLR, and IL-1 receptors-induced activation of NF-κB and JNK (33-35).

2.4.2 TRAFs role in endothelial cells

TNF stimulation upregulates the expression of TRAF1, 5, and 6 in ECs while TRAF2 and 3 remain unaffected. TRAFs are of clinical relevance in ECs particularly in inflammatory conditions. Atherosclerotic arteries exhibit greater levels of all TRAFs compared to control arteries. Furthermore, TRAFs seem to play a role in plaque progression as TRAF2 and 3 are expressed in atheromatous plaques at a greater level than fibrous lesions wherein TRAF5 is the most expressed (39). Surprisingly, TRAF5 appears to be a negative regulator of atherogenesis. $TRAF5^{-/-}$ mice develop larger plaques than controls and exhibit higher levels of inflammatory markers and enhanced adhesion of monocytes to the ECs. This is further supported by clinical data of acute and chronic coronary artery disease patients having lower levels of TRAF5 than healthy controls which increased with recovery (40).

2.5 TNF and cardiovascular disease

Several lines of evidence have demonstrated TNF implication in CVD pathophysiology and complications. Higher circulating levels of TNF are associated with high blood pressure,

glucose intolerance, diabetes and subclinical atherosclerosis (41-44). A recently published meta-analysis concluded that circulating levels of TNF are associated with higher risk of coronary heart disease independent of conventional risk factors (45). Additionally, local and systemic levels of TNF correlate with heart failure and ischemic stroke severity (2, 46). Treating brain injury with recombinant sTNFR I significantly prevented stroke development in rats (46). These reports highlight the need to address TNF signaling in cardiovascular disease management.

Epidemiological data supports the beneficial effect of anti-TNF therapy on vascular measures in patients with inflammatory autoimmune diseases. In cohorts of rheumatoid arthritis, anti-TNF therapy induced a significant reduction in pulse wave velocity, a non-invasive measure of arterial stiffness, and carotid intima-media thickness (cIMT) (11). A reduction that was independent of disease duration or other factors, whereas improvements in augmentation index, a non-invasive measure of arterial compliance, was influenced by age (47).

2.6 TNF and endothelial dysfunction

One of the main targets of TNF is the endothelium. TNF is a potential mediator of endothelial dysfunction induced by insulin resistance in individuals with metabolic syndrome (48, 49). Intra-arterial infusion of TNF significantly reduced endothelium-dependent vasodilation in healthy and diseased populations (50). Whereas several studies have validated the efficacy of anti-TNF agents in improving FMD in patients diagnosed with rheumatoid arthritis and other inflammatory autoimmune diseases (2, 10, 11). Further, TNF expression was

significantly upregulated in small resistance arteries of obese individuals, implicating ECs as a source, not just a target, of TNF (9).

Endothelial dysfunction shifts the endothelium towards an activated state exhibiting a pro-inflammatory, pro-apoptotic, and pro-thrombotic profile. TNF induces ECs activation by upregulating factors involved in inflammation such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein 1 (MCP-1), and coagulation such as tissue factor and Plasminogen activator inhibitor-1. This facilitates recruitment and adhesion of monocytes to the site of injury and their subsequent migration to the subendothelial space. Further, activation of the endothelium increases its permeability to lipoproteins leading to their accumulation and modification by oxidation within the subendothelial space. Intimal monocytes start differentiating to macrophages that engulf modified lipoproteins forming foam cells and fatty streaks and consequently atherosclerotic plaque. Moreover, TNF facilitates proliferation of smooth muscle cells that migrate to the lesion site, and fibrous tissue formation resulting in arterial wall thickening. Therefore, TNF plays a central role in the inflammatory cascades implicated in the initiation and progression of atherogenesis (1-3, 9-11). Data from animal models of atherosclerosis further supports this theory. ApoE$^{-/-}$, TNF $^{-/-}$ double knockout mice develop less atherosclerosis than apoE$^{-/-}$ single knockout mice and exhibit decreased expression of ICAM-1, VCAM-1 and MCP-1 (10). Likewise, TACE protein expression is upregulated in human atherosclerotic plaques (51).

TNF is also associated with a decrease in NO bioavailability brought about by several factors (9, 10). TNF induces a reduction in eNOS promoter activity and mRNA destabilization (12). TNF also induces the production of arginase, a competitor of eNOS for the substrate L-arginine, and inhibits degradation of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, thus compromising NO bioavailability. Further, TNF activates NADPH oxidases leading to an increase in reactive oxygen species (ROS) production in ECs. Accumulation of ROS results in eNOS uncoupling and superoxide production that scavenges NO to produce peroxynitrite (9, 10). Finally, ROS activates NF-κB leading to enhanced expression of NADPH oxidases and inflammatory cytokines, creating this vicious cycle of inflammation and oxidative stress (10).

2.7 Racial differences in CVD and endothelial function

Racial differences in CVD are well established. Non-Hispanic blacks have the highest prevalence of CVD and associated risk factors compared to other racial/ethnic groups. Further, recent data from The Multi-Ethnic Study of Atherosclerosis (MESA) have demonstrated that AA had the highest common cIMT used as a measure of subclinical atherosclerosis (23). Several reports have attributed this discrepancy to impairments in endothelial function. In a review, Petal et al. described that endothelial dysfunction is manifested by lower FMD, thicker cIMT, and higher endothelin-1 levels (ET-1), a potent constrictor, in AA compared to their CA counterparts (52).

In healthy AA, FMD is significantly lower than CA (24, 25). Also, AA had an attenuated mental stress-induced vasodilation compared to CA. Administration of NO inhibitor blunted

vasodialatory response only in CA, indicating that the impairment observed in AA is NO-mediated (53). Interestingly, Perregaux et al. reported that the protective effect of gender is absent in AA (25). These reports are of concern since FMD is a valid predictor of cardiovascular events in some populations (54, 55). Additionally, in a population of healthy volunteers with a positive family history of hypertension, AA had high levels of ET-1 at baseline and in response to a cold stressor (56).

These well documented epidemiological and clinical observations have driven some researchers to investigate potential mechanism(s) underlying racial disparity in cell models. Kallinowski et al. concluded that an imbalance of the redox state in AA ECs may predispose AA to endothelial dysfunction. Indeed, AA ECs produce more superoxide leading to more peroxynitrite formation compromising NO bioavailability (57). These findings are consistent with Feariheller et al. who demonstrated that AA HUVECs express higher levels of NADPH oxidase subunits and lower SOD1 than CA HUVECs (26). Moreover, AA ECs display heightened inflammatory response. In response to TNF stimulation, AA HUVECs had an increase in endothelial microparticles generation, a marker of ECs activation, by 89% compared to 8% in CA, suggesting that AA ECs sustain a greater TNF-induced damage than CA (27).

2.8 Effect of exercise/shear stress

Exercise intervention is the most established non-pharmacological measure to treat and prevent CVD (58). Exercise training enhances endothelial function mainly by increasing NO bioavailability, improving antioxidant capacity, and inducing anti-inflammatory effect. Further, exercise induces atheroprotective effects by improving the metabolic profile, reducing plaque

development, stabilizing atherosclerotic lesions, and regressing stenosis (59). One of the main cytokines that play a role in vascular function and can be modified by exercise is TNF. Palmefors et al. provided high quality evidence supporting training-induced decrements in systemic TNF levels in patients with CVD risk (60). Moreover, after a 5-month moderate-intensity training program, TNF was significantly decreased in healthy obese women, and that reduction was independently associated with HbA_{1c} (61).

Nonetheless, reports on the effects of exercise on circulating TNF levels are inconsistent. This may be attributed to its short half-life; hence detecting changes in the systemic circulation is difficult (62). In addition, lower shedding of the TNFRs is related to disease development more than high TNF levels (63). Therefore, the more stable sTNFR I is used and has been recognized as an inflammatory marker. sTNFR I is negatively correlated with age, indicating that receptor shedding declines with aging (62). Additionally, athletes have higher levels of sTNFR I than sedentary individuals (64). In response to an acute bout of exercise, sTNFR I increase significantly (65, 66). This effect likely neutralizes the effects of TNF released from exercising muscle. Moreover, sTNFR I levels are improved after an aerobic training program in patients from osteoarthritis and diabetes cohorts (16, 17).

Up to 40% of the beneficial effects of exercise on vascular function cannot be explained by regulation of traditional risk factors. With training, repeated exercise bouts create a repetitive, laminar, and unidirectional shear stress on the vascular wall. HSS mediates functional and structural vascular adaptations associated with exercise training (58). In vitro, HSS inhibits TNF-induced activation of MAP Kinases, particularly JNK (19-21). This inhibitory

effect is mediated by blocking TNFR I and TRAF2 association in response to TNF activation (21). Moreover, HSS upregulates TRAF3 expression in HUVECs leading to suppressed CD40-induced AP-1 activation. Thus, TRAF3 is a possible mechanosensitive mediator of HSS atheroprotective effect.

As noted earlier, TNF, through TNFR I signaling, plays a critical part in the pathophysiology of endothelial dysfunction and atherogenesis. However, role of TNFR I signaling in racial differences in endothelial (dys)function has not been explored yet. Therefore, the proposed study aims to examine possible racial differences in TNFR I signaling complex expression/activity as well as TNFR I signaling role in potentiating atherogenesis using a cell model. We also plan to investigate the effects of HSS on attenuating possible racial differences.

3.0 Methods

3.1 Materials

Recombinant human TNF was purchased from R&D Systems (cat. No. 210-TA-020/CF, Minneapolis, MN). Nuclear Extraction Kits was purchased from Abcam (cat. No. ab113474, Cambridge, MA). Calcein-AM fluorescent dye was purchased from Corning (cat. no. 354216, Corning, NY). Primary antibodies used were: TNFR1 (cat. no. 3736), TACE (cat. no. 6978), TRAF2 (cat. no. 4724), TRAF3 (cat. no. 4729), and TRAF5 (cat. no. 41658) from Cell Signaling Technology (Beverly, MA). β-actin (cat. no. 3700) from Cell Signaling (Beverly, MA) was used as loading control for cytosolic extracts.

3.2 Cell culture

3.2.1 HUVECs

HUVECs were purchased from Lonza (Morristown, NJ) and cultured in the endothelial basal medium-2 medium containing 2% fetal bovine serum and growth supplements and used between passages 6 and 7. Cells from three AA and three CA donors were grown in parallel and the experiment was repeated three times. Cells were maintained at 37°C in a 5% CO_2 atmosphere, and cultured in gelatin coated 100 mm tissue culture dishes. When cells reached 80%–90% confluence, they were washed with Hank's Balanced Salt Solution (HBSS) buffer and serum starved for 2 hrs before stimulation or application of shear stress. A concentration of 30 ng/mL of TNF (cat. No. 210-TA-020/CF, Minneapolis, MN) was used and incubated for 6 hrs. This dose was chosen based on preliminary experiments.

3.2.2 THP-1

THP-1 monocytes (ATCC® TIB202™, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), 1% penicillin /streptomycin (VWR, Radnor, PA), and 2mM/L GlutaMAX (Gibco, Waltham, MA). Cells were cultured in T-75 flasks and maintained at 37°C in a 5% CO_2 atmosphere.

3.3 Cell adhesion assay

HUVECs were seeded in gelatin coated 24-well plates at a density of $5x10^4$ per well. Once confluent, HUVECs were washed with HBSS buffer and serum starved for 2 hrs and then activated with TNF (30 ng/mL, 6 hrs) or left untreated. THP-1 monocytes were labeled with 5 µM of Calcein-AM (Molecular Probe) and suspended in serum-free RPMI 1640 medium. After activation of HUVECs, labeled THP-1 cells were added to the HUVECs monolayer ($5x10^5$ per well) and incubated at 37°C with 5% CO_2 for 1 hr. Labeled THP-1 cells were added to empty wells as a background control. Nonadherent THP-1 monocytes were removed and washed with 1X phosphate buffer saline (PBS) three times. Adherent cells were then lysed with 200 µl of RIPA lysis buffer. To quantify cell adhesion, fluorescence intensity was measured with a fluorescence plate reader at excitation wavelength set at 494 nm and emission wavelength at 517 nm. Cell adhesion was determined as a percentage of the control (untreated HUVECs).

3.4 Transendothelial migration assay

The transendothelial migration assay was performed in a 24-well Transwell plates with 3.0 µm transparent PET Membrane (VWR, Radnor, PA). HUVECs ($5x10^4$) were seeded into upper Transwell inserts. Confluent HUVECs were washed with HBSS buffer and serum starved for 2 hrs then activated with TNF (30 ng/mL, 6 hrs) or left untreated. Subsequently, 700 µl of RPMI 1640 medium was added to lower chambers and Calcein-labeled THP-1 monocytes ($5x10^5$ in 300 µl serum-free RPMI 1640) were added to the upper well inserts and incubated for 24 hrs at 37°C under 5% CO_2. Cells that transmigrated to the lower chamber were pelleted and lysed with 200 µl of RIPA lysis buffer. To quantify cell migration, fluorescence intensity was measured with a fluorescence plate reader at excitation wavelength set at 494 nm and emission wavelength at 517 nm. Migrated cells were determined as a percentage of the control (untreated HUVECs).

3.5 Laminar shear stress

After 2 hrs of serum deprivation, confluent monolayers were exposed to two levels of unidirectional shear stress for 24 hrs with a rotating cone-in-plate instrument at a 0.5° angle, designed for 100 mm tissue culture dish. Cone-and-plate experimental models have the advantage of inducing flow with a moving upper conical boundary and thus, do not generate any pressure gradients that could alter cell function (67). The following three conditions were applied: 1. HSS (20 dyne/cm^2), 2. Low laminar shear stress (LSS) (5 dyne/cm^2.), and 3. HSS (20 dyne/cm^2) +TNF (30 ng/mL, 6 hrs).

3.6 Western blotting

Cell lysate was fractionized using the Nuclear Extraction Kit (cat. No. ab113474, Abcam, Cambridge, MA) following manufacturer's instructions with some modifications. Cytosolic extract was used for western blotting. SDS-PAGE was utilized to separate protein on 4-15% TGX gels (BioRad, Hercules, CA) according to molecular weight. 10- 15 µg protein was loaded into wells for each experimental condition, with the amount kept constant within each gel. All samples were run on duplicate gels. Both a pre-stained Novex Sharp (Life Technologies, Rockville, MD) and HRP-linked ladder MagicMark XP (Life Technologies, Rockville, MD) were loaded for molecular weight visualization. Proteins were transferred to PVDF membranes using a wet transfer process. Membranes were blocked with 5% non-fat dry milk (NFDM) for 1h at room temperature (RT). This was followed by incubation with primary antibodies of interest with gentle agitation overnight at 4-8°C. Primary antibodies used were: TNFR1 (cat. no. 3736), TACE (cat. no. 6978), TRAF2 (cat. no. 4724), TRAF3 (cat. no. 4729), and TRAF5 (cat. no. 41658) from Cell Signaling Technology (Beverly, MA). β-actin (cat. no. 3700) from Cell Signaling Technology (Beverly, MA) was used as loading control. Membranes were then washed with Tris-Buffered saline with Tween (TBST) and incubated with an appropriate secondary antibody conjugated with horseradish peroxidase. Protein was visualized by chemiluminescent detection using an HRP illumination substrate Luminata Forte (EMD Millipore, Billerica, MA). The UVP ChemiDoc-It2 imaging system was used to image each PVDF membrane and Vision Woks software was used to quantify protein expression by band densitometry analysis. Densities of selected proteins were expressed relative to a loading control for normalization.

3.7 Assays

Cell culture supernatant was collected centrifuged, aliquoted, and immediately stored at −80°C until analysis. sTNFR I (cat. no. DRT100, R&D Systems, Minneapolis, MN) Assay Kit was used to detect the sTNFR I production in HUVECs cell culture supernatant. NF-κB Transcription Factor Assay Kit (cat. no. 43296, Active Motif, Carlsbad, CA) was used to quantify NF-κB activation and DNA binding in nuclear extract.

3.8 Statistical analysis

All variables were checked for normality using Skewness-Kurtosis All test for normality and descriptive statistics were performed. A Mann-Whitney U test was used to examine racial difference in transendothelial migration. A two-way ANOVA was performed to examine potential race by condition interactions. Post hoc adjustments for multiple comparisons was done using the Bonferroni's test. Analysis was performed using SPSS version 26 (SPSS Inc., Chicago, IL). Data are expressed as mean ± *SE* and the level of significance was set at $p \leq 0.05$.

4.0 Results

4.1 THP-1 monocytes adhesion and transendothelial migration

THP-1 monocyte adhesion to untreated HUVECs was slightly higher in AA HUVECs compared to CA HUVECs. TNF treatment increased THP-1 monocyte adhesion by about 1.5-fold in both racial groups (p<0.001, Fig.2). However, there was no racial difference (p=0.77, Fig.2) or an interaction effect (p=0.12, Fig.2). Additionally, TNF increased the percentage of transmigrated THP-1 monocytes in both CA and AA HUVECs (58 % ±24.4 vs 130.8 % ± 44.5, respectively), however, there was no racial difference (p=0.7, Fig.3).

4.2 TNFR I signaling complex and HSS

TNFR I and TRAF3 were significantly upregulated in response to HSS compared to all other conditions (p<0.001, Fig.4 and p=0.03, Fig.5 (b)), whereas TRAF5 was downregulated in response to HSS, an effect that was not altered by TNF treatment (p=0.006, Fig.5 (c)). TRAF2 levels were not affected by any of the experimental conditions (p= 0.09, Fig.5 (a)). In addition, there was no racial difference or an interaction effect in the expression of TNFR I or TRAFs across all conditions.

4.3 TNFR I shedding and HSS

HSS induced an upregulation in TACE expression that was unaltered by a follow-up TNF treatment (p<0.001, Fig.6). Both LSS and HSS conditions increased sTNFR I levels by about 2 fold in CA and AA HUVECs, an increment that was diminished by TNF treatment (p<0.001, Fig.6).

Shedding of TNFR I was lower in AA HUVECs compared to CA HUVECs; however, this difference was not statistically significant (p=0.05).

4.4 NF-κB binding activity

NF-κB DNA binding activity tended to be higher in AA HUVECs in response to TNF treatment, nonetheless, it was not significant. Additionally, HSS had no protective effect against TNF-induced NF-κB activation (p<0.001, Fig.8).

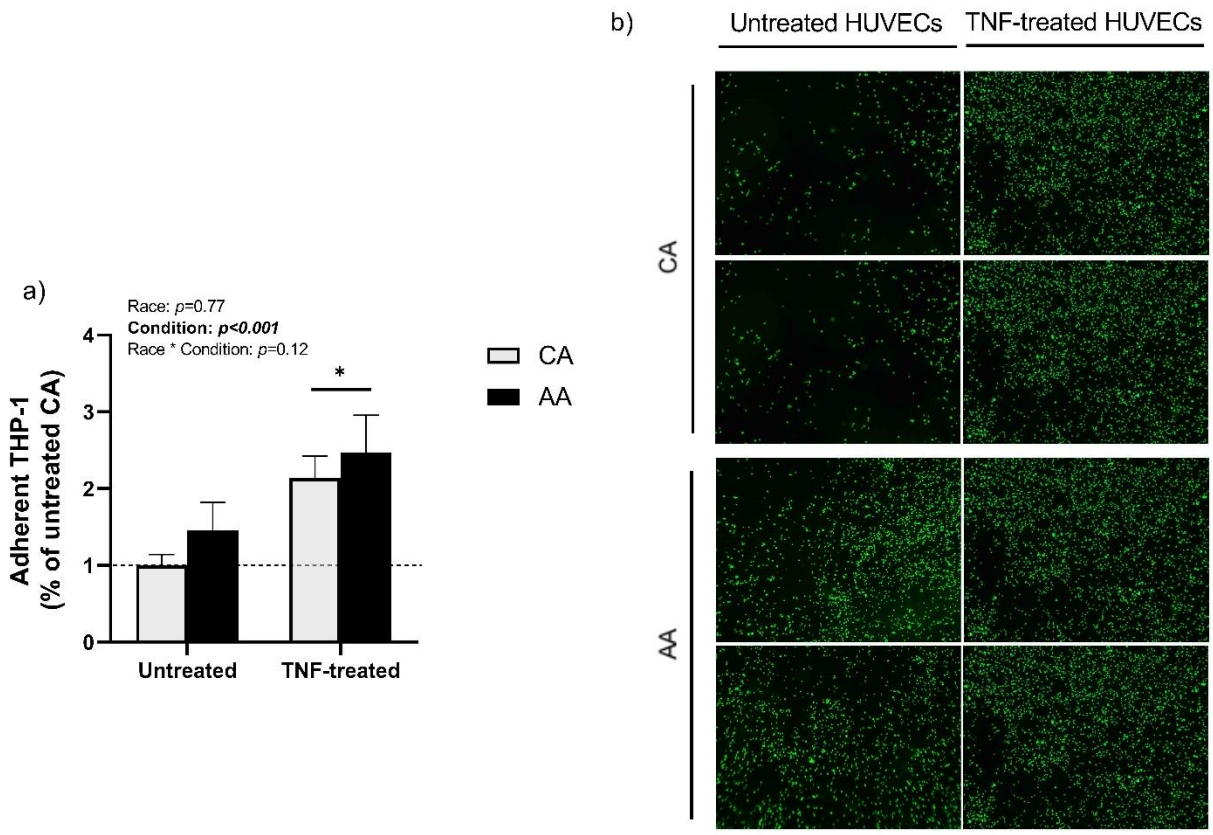

Figure 2: THP-1 monocyte adhesion on HUVECs.
(a) Adhesion of labeled THP-1 on TNF-treated HUVECs was quantified by fluorescence microplate reader. Net fluorescence is expressed relative to CA untreated HUVECs. (B) Representative images of labeled THP-1 adhesion on untreated and TNF-treated HUVECs. Images were taken by fluorescence microscopy. Data are represented as mean ± *SE* from 3 independent experiments.
* p< 0.001 compared to untreated.

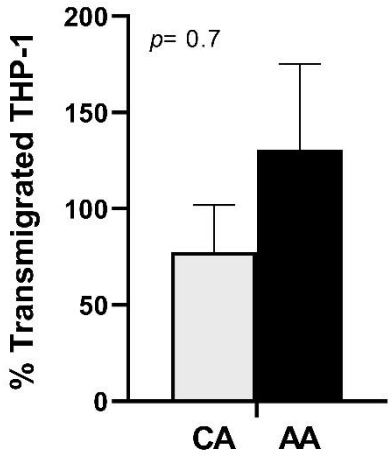

Figure 3: Transmigrated THP-1 monocytes in TNF-treated HUVECs.
Transmigration of labeled THP-1 in TNF-treated HUVECs was quantified by fluorescence microplate reader. Net fluorescence is expressed relative to untreated HUVECs. Data are represented as mean ± *SE* from one independent experiment.

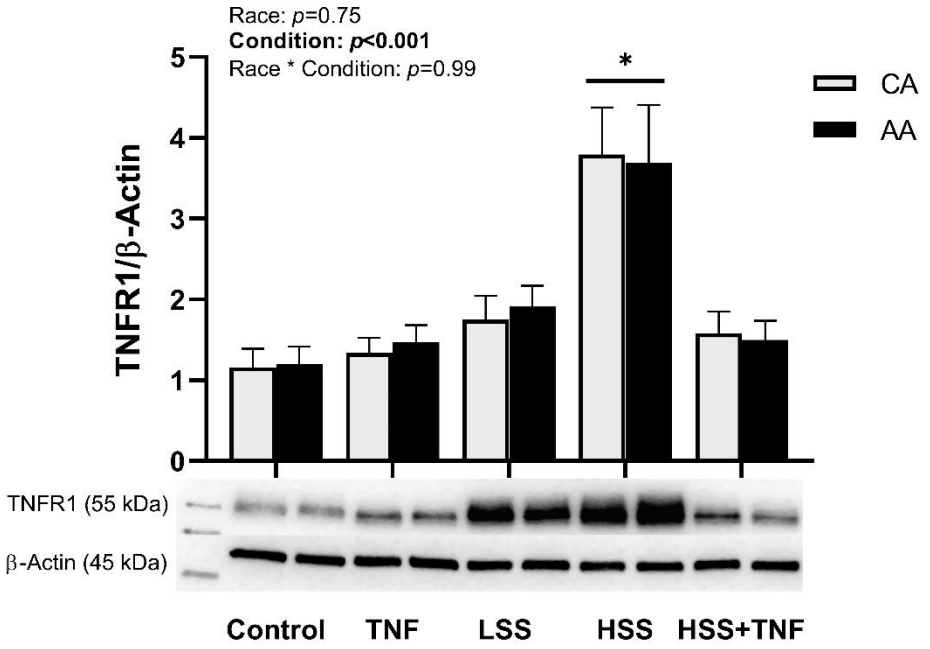

Figure 4: HSS upregulates TNFR1 expression in CA and AA HUVECs.
HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to LSS (5dyne/cm^2, 24hrs), HSS (20dyne/cm^2, 24hrs), or HSS (20dyne/cm^2, 24hrs) followed by TNF (30ng/mL, 6hrs). HSS significantly increased TNFR1 expression in both groups. Densitometric quantification was normalized to housekeeping protein (β-Actin). Data are represented as mean ± *SE* from 3 independent experiments.
* $p < 0.001$ compared to other conditions.

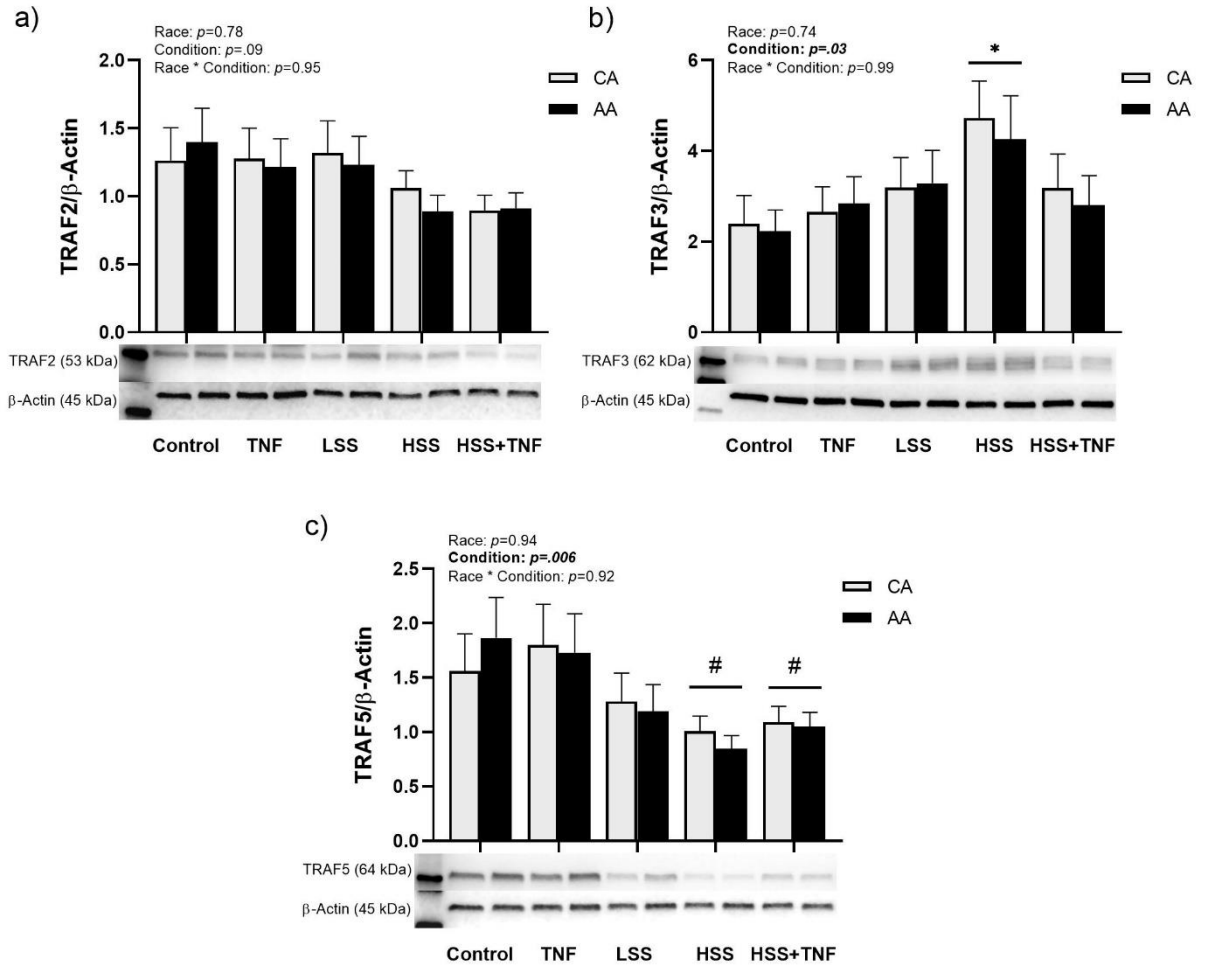

Figure 5: TRAFs expression under different experimental conditions.
HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to LSS (5dyne/cm^2, 24hrs), HSS (20dyne/cm^2, 24hrs), or HSS (20dyne/cm^2, 24hrs) followed by TNF (30ng/mL, 6hrs). HSS significantly upregulated TRAF3 expression (b) and downregulated TRAF5 (c). However, TRAF2 expression did not change across all conditions. Data are represented as mean ± *SE* from 3 independent experiments.
* *p*= 0.03 compared to other conditions.
# *p*= 0.006 compared to control, TNF, and LSS.

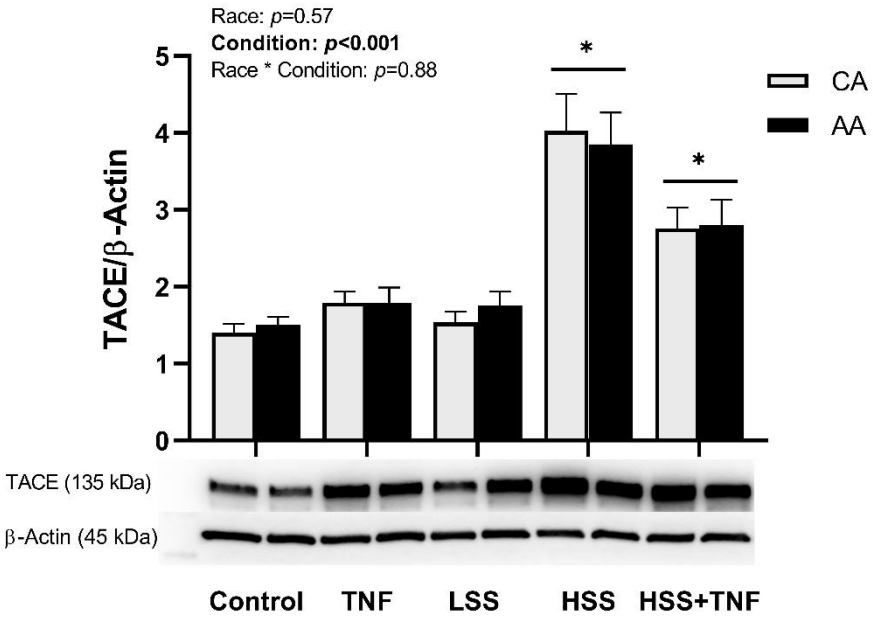

Figure 6: HSS upregulates TACE expression in CA and AA HUVECs.
HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to LSS (5dyne/cm^2, 24hrs), HSS (20dyne/cm^2, 24hrs), or HSS (20dyne/cm^2, 24hrs) followed by TNF (30ng/mL, 6hrs). Densitometric quantification was normalized to housekeeping protein (β-Actin). Data are represented as mean ± *SE* from 3 independent experiments.
* $p < 0.001$ compared to control, TNF, and LSS.

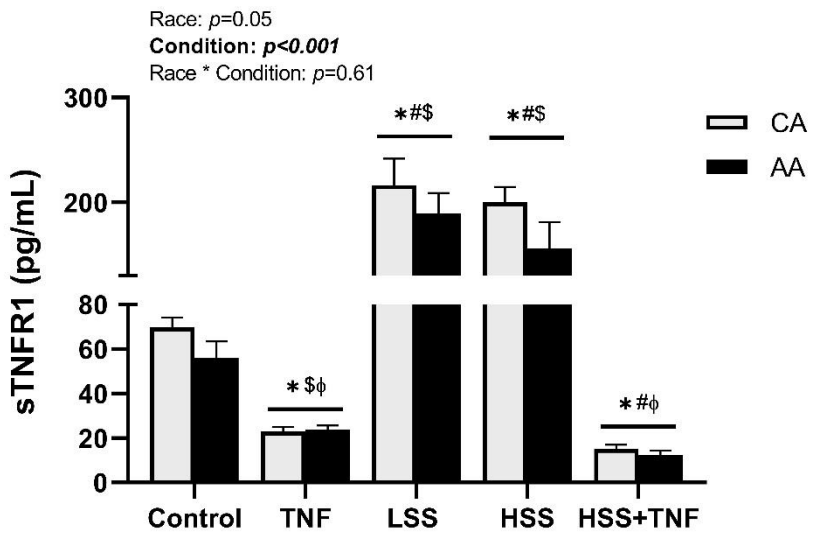

Figure 7: TNFR1 shedding under different experimental conditions.
HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to LSS (5dyne/cm^2, 24hrs), HSS (20dyne/cm^2, 24hrs), or HSS (20dyne/cm^2, 24hrs) followed by TNF (30ng/mL, 6hrs). Shedding of TNFR1 was quantified in cell culture supernatant. Shear stress-induced upregulation of sTNFR1 was significantly suppressed by TNF treatment. Data are represented as mean ± *SE* from 3 independent experiments.
* $p < 0.001$ compared to control.
# $p < 0.001$ compared to TNF.
Φ $p < 0.001$ compared to LSS, HSS.
$ $p < 0.001$ compared to HSS+TNF.

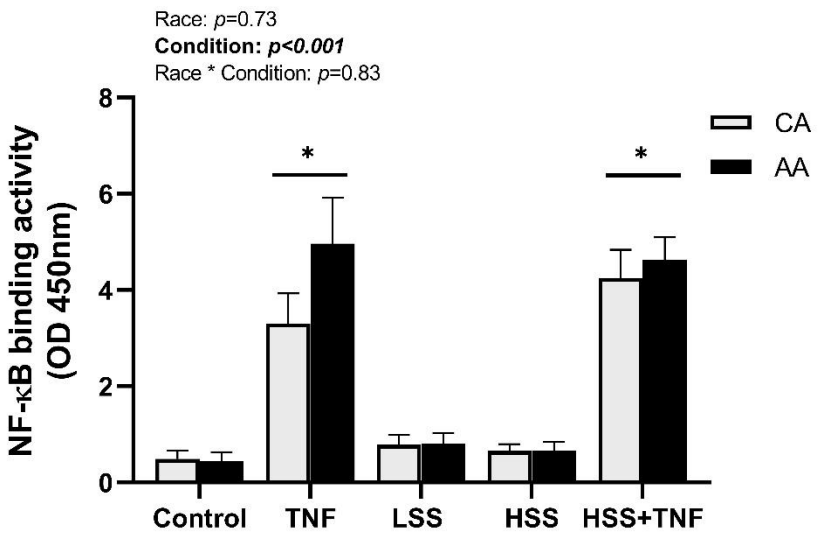

Figure 8: NF-κB binding activity under different experimental conditions.
HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to LSS (5dyne/cm^2, 24hrs), HSS (20dyne/cm^2, 24hrs), or HSS (20dyne/cm^2, 24hrs) followed by TNF (30ng/mL, 6hrs). NF-κB binding activity was quantified in nuclear extract. TNF-induced NF-κB activation was not altered by HSS in both groups. Data are represented as mean ± *SE* from 3 independent experiments.
* $p < 0.001$ compared to control, LSS, and HSS.

5.0 Discussion

The main findings of this study are the following: 1) There were no racial differences in TNF-induced monocytes adhesion or migration 2) There were no racial differences in TNF-induced activation of TNFR I signaling complex, 3) There were no racial differences in the modulatory effects of HSS on TNFR I signaling, and 4) Pre-shearing was not sufficient to mitigate TNF detrimental effects as evident by NF-κB binding activity and diminished shedding of TNFR I. These results do not support our proposed hypothesis of higher TNFR I signaling complex expression/activity and downstream effect in AA HUVECs, however, HSS-induced atheroprotective effect mitigated TNFR I signaling in both racial groups.

TNFR I activation by TNF binding induces endothelial dysfunction manifested by suppressed eNOS activity, higher oxidative stress levels and amplified expression of adhesion molecules. Adhesion molecules (such as VCAM-1, ICAM-1, MCP-1 and E-selectin) play a key role in recruiting monocytes and initiating atherogenesis (2, 10). Plasma levels of TNF and adhesion molecules are associated with subclinical atherosclerosis (44). Additionally, data from the Atherosclerosis Risk in Communities (ARIC) study highlighted adhesion molecules as independent molecular markers of coronary heart disease (68). Previous research from our lab have demonstrated heightened inflammatory response to TNF in ECs from AA donors (27). Further, basal and induced ICAM-1 and VCAM-1 levels are higher in AA HUVECs compared to CA HUVECs (unpublished observations). Therefore, we hypothesized TNF-induced monocytes adhesion and transmigration to be higher in AA HUVECs; however, our results failed to support this hypothesis. Research implementing co-culture systems to investigate molecular

mechanisms prompting racial differences in CVD is scarce. In our experiments, we used THP-1 cells, a validated monocyte cell model to investigate atherogenesis and other vascular inflammatory conditions (69). Nonetheless, we acknowledge the differences between THP-1 cells and primary monocytes and the limitations of THP-1 cells as a monocytes cell line model. Interestingly, recent evidence has shown that peripheral blood mononuclear cells (PBMCs) from young-normotensive AA exhibit higher resting oxidative stress levels (70). Thus, examining the interactions between PBMCs and AA HUVECs may be more relevant.

Contrary to our hypotheses, our results do not support differential expression/activity of TNFR I signaling between both races. Both CA HUVECs and AA HUVECs responded similarly to all experimental conditions. There was a trend towards higher NF-κB activation in response to TNF and lower shedding of TNFR I with HSS in AA HUVECs; however, these differences were not statistically significant. These observations may be explained by several factors. First, we did not measure the expression or activity of MAP Kinases nor other transcription factors such as AP-1 that my play a bigger role in prompting racial differences than NF-κB signaling. Second, most available assays, including the one we have used, are not sensitive enough to distinguish between free form of sTNFR I and that bound to TNF. Additionally, given that we did not measure TNF levels, we cannot speculate if sTNFR I levels in AA HUVECs that is comparable to CA are sufficient to neutralize the possibly higher TNF levels in AA HUVECs. Lastly, in addition to mediators of inflammation, NF-κB induces transcription of TNFR I signaling negative regulators that may be differentially expressed such as IκBα and A20. It has been shown that, despite the well-established suppressive effect of A20 on TNF-induced genes regulated by NF-κB, it does

not interfere with the nuclear translocation of the NF-κB heterodimers or DNA binding (71). If such is the case, we speculate lower expression/activity of negative regulators in AA ECs.

Several clinical reports have demonstrated higher shedding of TNFR I with aerobic exercise and its association with better vasodilation and functional outcomes (16, 17, 65). In the present study, we used a cone and plate viscometer that mimics the shear stress patterns the endothelium is exposed to in vivo during aerobic exercise. Our results support clinical data in that HSS, and surprisingly LSS, induced higher shedding of TNFR I. Unexpectedly, application of HSS prior to TNF treatment did not lessen TNF unfavorable effects. Yamawki et al. (21) have suggested that shear stress atheroprotective effects are mediated by inhibiting MAP kinase signaling as well as TRAF2-TNFR I interaction. They have shown that pre-exposing rabbit aortas to HSS significantly attenuated TNF-induced VCAM-1 expression by inhibiting MAP Kinase signaling while TNF-induced NF-κB activation remained unaffected. Collectively, these results suggest signaling pathway specificity in terms of atheroprotective effects of HSS.

In regards to TRAFs, our results are in agreement with Urbich et al. (72). HSS induced an upregulation of TRAF3 while TRAF2 levels remained unaffected. TRAF3 is an important negative regulator of NIK (35-37). Consistent with the anti-inflammatory effects of HSS, TRAF3 upregulation brought about by HSS is associated with inhibition of CD40-induced endothelial cells activation (72). On the other hand, while Urbich et al. (72) show no effect of HSS on TRAF5 levels in HUVECs, our results demonstrated a significant decrease in the expression of TRAF5 following the application of HSS. This finding could be due to differences in the doses of shear stress. Whereas they exposed HUVECs to 15 dyne/cm^2 of shear for 18 hrs, we used 20

dyne/cm^2 for 24 hrs. In vivo data suggest a negative regulatory role for TRAF5 as knocking out TRAF5 accelerated atherosclerosis in animal models. Additionally, higher systemic levels of TRAF5 are associated with recovery in patients with coronary heart disease (40). Therefore, as one of the atheroprotective effects of laminar shear, we expected TRAF5 levels to increase with HSS. Yet, they decreased. It is plausible that the role of TRAF5 as a pro- or anti-inflammatory mediator is cell specific.

There are limitations to the current study. First, our sample size for the transendothelial migration experiments was small. Increasing sample size may help decrease the wide range of variability we have encountered in our findings. Second, different concentrations of serum were used in HUVECs culture media for different conditions. Serum deprivation is recommended to prime cells when examining proinflammatory cytokines signaling. Therefore, we used serum free culture medium for the static conditions; however, we had to use full serum culture medium for the shear stress conditions. This is mainly because our cone and plate shear stress model is optimized for a limited range of medium viscosity. Nonetheless, we used only 2% fetal bovine serum which is considered on the lower range (73). Lastly, we used HUVECs as our endothelial cell model which is a validated cell model in the field of vascular physiology. It is often used by our group and others to investigate molecular mechanisms of vascular disease and, in particular, racial disparity in endothelial (dys)function (22, 26, 74). However, we acknowledge its drawbacks; hence our results should be interpreted with caution. We also agree with the remarks of Robinson et al. (75) that shed light on the disadvantages of using commercially available HUVECs and how vendors offer limited access to information

about the health and lifestyle of the mother that can lead to epigenetic and phenotypic changes of ECs.

In conclusion, our results do not support racial differences in the expression of TNFR I complex or its signaling activity, particularly NF-κB activation. Additionally, HSS can mitigate some aspects of TNFR I signaling similarly in ECs from both racial groups, yet, this modulatory effect was not long-lasting. Given the higher burden of subclinical atherosclerosis in AA and the central role that TNF plays in atherogenesis, we still maintain that the TNFR I signaling complex takes part in prompting racial differences in this context. Therefore, we propose other aspects of TNFR I signaling such as MAP kinase signaling and their associated down-stream effects as recommended targets for future research.

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