ABSTRACT

Title of Document: THE PROATHEROGENIC ROLE OF THE TRPV4 CALCIUM-PERMEABLE CHANNEL IN MACROPHAGES

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Directed By: Professor Shaik O. Rahaman
Department of Nutrition and Food Science

Atherosclerosis is a multi-faceted chronic disease and one of the leading causes of cardiovascular diseases which attribute to 28.2% of all-cause mortality worldwide. Central tenets of atherogenesis are macrophage passage and transformation to foam cells. It is known that numerous macrophage membrane proteins regulate this process by controlling properties such as migration and binding and uptake of oxidized lipids. The focus of this study is to identify mechanisms by which the transient receptor potential (TRP) channel of the vanilloid subfamily, TRPV4, a calcium-permeable channel, regulates proatherogenic macrophage functions. The findings show TRPV4 is expressed and functional in macrophages, TRPV4 modulates macrophage migratory characteristics, oxidized low density lipoprotein (oxLDL) uptake and foam cell formation is reliant on TRPV4-elicited Ca^{2+} influx, and the physiological inflammatory stimulus of lipopolysaccharide can mediate TRPV4 function. These results identified previously unknown components to macrophage migration and foam cell development. Collectively, these discoveries associate the TRPV4 channel with atherogenesis by identifying new regulators for macrophage phagocytosis and migration that are essential to atherosclerosis development.

THE PROATHEROGENIC ROLE OF THE TRPV4 CALCIUM-PERMEABLE CHANNEL IN MACROPHAGES

By

Michael Merth

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Advisory Committee:
Professor Shaik O. Rahaman, Chair
Professor David K. Y. Lei
Professor Debabrata Biswas

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Chapter 1: Introduction

Cardiovascular disease (CVD) is the number one cause of death in the US, and atherosclerosis is the most dominant underlying pathology (1). The impacts of atherosclerosis are far-reaching, but the two major forms of cardiovascular disease affected by atherosclerosis include ischemic heart disease and cerebrovascular disease. Together, these diseases are the leading causes of death worldwide, representing 84.5% of cardiovascular-related deaths and 28.2% of all-cause mortality (2).

Over the last few decades, preventative and corrective methods with tenuous and often risky therapeutic drug trials have exposed the need for a better understanding of the innate immune system's role in atherosclerosis. Despite the profound impacts of atherosclerosis on Western societies, many of the underlying mechanisms of atherogenesis are poorly understood. One particular area of study includes identifying the contributory role of macrophages in this complex pathogenesis.

Studies have shown that atherosclerosis is closely related to macrophage infiltration, sequestration, and the development of foam cells (3-9). Macrophage phagocytosis of oxidized low density lipoprotein (oxLDL) causes the formation of lipid-laden foam cells that are typically abundant during the development of atherosclerosis (3-6). This process has shown to be exacerbated by the presence of physiological inflammatory stimuli, such as lipopolysaccharides (LPS), yet the exact mechanisms whereby LPS can contribute to atherosclerosis remain elusive (10-12).

Multiple macrophage functions require Ca^{2+} influx, including migration and phagocytosis (9, 13-15). The presence of macrophages and foam cells are vital to atherogenesis (5); therefore, macrophage migration and subsequent foam cell formation via phagocytosis are critical calcium-

dependent atherogenic processes. A significant gap in the research is a comprehensive understanding of the role calcium channels play in macrophage foam cell formation and migration.

Recent pathological findings revealed interesting properties of the transient receptor potential cation channel subfamily V member 4 (TRPV4) calcium-permeable channel, which may indicate a fundamental role in the regulation of atherosclerotic macrophage functioning (12, 16-22). It primarily functions as a Ca^{2+} influx channel activated by mechanosensitive and chemosensitive signaling pathways. TRPV4 is well-documented to be ubiquitously expressed in many different cell types, including macrophages, and operates in various functional and pathological models (12, 16). Through the role of TRPV4, macrophage intracellular Ca^{2+} flux may be modulated by unknown chemical or endogenous stimuli or changes to the arterial stiffness; therefore, we intend to investigate the channel's role in atherogenesis. We will test the hypothesis that the TRPV4 channel contributes multiple proatherogenic properties by determining a role for TRPV4 in atherosclerotic macrophage migration and foam cell formation.

To support the hypothesis, in vitro experimentation was performed on RAW cell line mouse macrophages. In vitro mouse cell models were chosen for the initial trials in order to build a strong foundation for future research. The potential outcomes from this study design can guide forthcoming in vivo and human cell models.

Chapter 2: Literature Review

Atherosclerosis

Atherosclerosis is a chronic vascular disease of the arterial wall and a major precursor to a variety of serious cardiovascular diseases such as coronary artery disease, stroke, and peripheral artery disease. The atherosclerotic process, also known as atherogenesis, develops slowly over decades, through an assorted sequence of inflammatory and biochemical events resulting in the narrowing of the arterial lumen (3). The disease can affect nearly any part of the arterial network but are most frequently observed in high-pressure vessels such as the coronary, renal, femoral, cerebral, and carotid arteries (23). The atherosclerotic lesions develop and progress through a complex inflammatory process involving the corroboration of immune cells, blood components, and lipoproteins within the arterial wall (24-26). In general, the process is associated with a state of heightened oxidative stress leading to the sequestration, oxidation, and modification of low-density lipoprotein (LDL) within the arterial walls and subsequent foam cell formation (4); this process results in atheromata and vascular dysfunction (27).

The precise pathological progression that leads healthy arteries to atherosclerotic lesion formation is multifaceted and not fully understood. Pioneering theories purported "response-to-injury" hypothesis. The supposition of this model states an initial insult (e.g., smoking, hypertension, dyslipidemia) to the vascular endothelium with successive membrane dysfunction is required in promoting atherogenesis (28, 29). A revised and more modern perspective, the "response-to-retention hypothesis," argues that subendothelial retention of atherogenic lipoproteins are critical to the pathological instigation opposed to permeability alone (30). Although known sources of endothelial insult alter permeability and can play a contributory role,

atherogenesis requires that the permeated lipoproteins are retained in the subendothelial space (31-33).

Low-Density Lipoprotein (LDL)

Damage to the endothelium is associated with the infiltration and accumulation of the atherogenic blood plasma LDL into the subendothelial space (30, 34). LDL is a water soluble transport particle with a surface monolayer primarily of phospholipids and apolipoprotein-B (ApoB) and functions as a major transporter of nonpolar lipids in the hydrophobic core (35). Circulating LDL can vary in size. As very low-density lipoprotein (vLDL) is converted to LDL through the hydrolysis of internal triglycerides by lipoprotein lipase (LPL), the release of free fatty acids results in a larger percentage of internalized cholesterol, which in turn increases the particle density. As this process continues, there is an increase in the production of small dense LDL (sdLDL) particles which have a greater susceptibility to the passage, sequestration, and oxidation between the endothelium monolayer (36-38).

After passage into the subendothelial space, LDL can become trapped in the extracellular matrix (ECM) where it is highly prone to oxidation through an assortment of biochemical modifications to form the extremely atherogenic oxidized LDL (oxLDL) (24, 39). It's worth noting that the pathologic lipoprotein oxidation primarily occurs only after the subendothelial retention because serum LDL is markedly protected by plasma protein and/or recycled quickly to the liver if modified (40, 41).

Mediators inducing LDL oxidation include reactive oxygen species (ROS) as well as lipases and enzymes secreted by endothelial cells and immune cells such as macrophages including sphingomyelinase (SMase), lipoprotein-associated phospholipase A2 (Lp-LpA2), and myeloperoxidase (MPO) (42, 43). The oxLDL is a forceful promoter of inflammatory signaling

and an inhibitor of nitric oxide (NO) production within the vessel (44). The oxLDL correspondingly induces endothelial cells to secrete growth factor macrophage colony stimulating factor (M-CSF) and the chemotactic protein monocyte chemoattractant protein-1 (MCP1) to recruit and transmigrate circulating plasma monocytes into the subendothelial space (35). As the monocytes pass into the subendothelial space they are further signaled by M-CSF to differentiate and transform into macrophages (45).

Macrophages

Macrophage Type

Macrophages are an essential component to atherosclerosis progression. Early stages of atherosclerosis are characterized by macrophage migration into the tunica intima, the innermost layer of an artery or vein, followed by an increase of proinflammatory cytokine expression (26). This state of chronic inflammation hinges on the interactions between the monocyte-derived macrophages and the lesion environment. Throughout this process, two types of macrophages, M1 and M2, emerge, but their regulation, phenotype, and contribution to cholesterol deposition are still under investigation (46).

Macrophages show a degree of plasticity and may differentiate interchangeably between M1 and M2 phenotypes under different environmental surroundings (47). M1 macrophages, also known as “killer macrophages,” may be activated by lipopolysaccharide (LPS) and interferon gamma (INFγ) which subsequently promotes further inflammation and exacerbates atherosclerosis (48). Their proatherogenic role chiefly involves the secretion of high levels of inflammatory cytokines such as interleukin-12 (IL-12) (48).

M2 macrophages typically have a constructive role throughout immune functions; however, they can also contribute to atherogenesis. M2 macrophages are primarily involved in

wound healing processes and tissue repair but are also participating in the production of the anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) to abrogate the initiation of immune system damage (46). M2 macrophages can reduce plaque buildup through the removal of cholesterol in the arterial wall, but during the uptake of oxLDL, as often the case in the atherogenic environment, M2 macrophages transform to foam cells, ultimately contributing to the atheroma lesion (46, 49).

Macrophage Signaling

Many inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleuikin-1β (IL-1β), and macrophage inflammatory protein-1α (MIP1α), are released from activated macrophages to stimulate additional monocyte recruitment in the blood stream to the subendothelial space of the arterial walls (50). These cytokines stimulate endothelial cell expression of chemokines that signal to the associated monocyte receptor (50). The endothelial derived chemokines, vascular-cell-adhesion molecule-1 (VCAM-1) and intracellular-adhesion molecule-1 (ICAM-1), and their associated monocyte integrins, integrin α4β1 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1), are critical signaling pathways to facilitate the binding and passage (diapedesis) of monocytes during early atherogenesis (51).

Macrophage Migration

Monocytes and macrophages are extremely motile cells able to move along 2-dimentional (e.g., vessel walls) and 3-dimensional (e.g., extracellular space) complex environments with the abilities to alternate between amoeboid or mesenchymal-like migratory methods (52, 53). Microscopy has shown that macrophage migration follows an actin-dependent cycle of events beginning with an actin-rich membrane protrusion, followed by an integrin-

mediated attachment of the stabilized protrusion to the extracellular substrate, ending with myosin contracting on the actin filaments and rear adhesion site release (54, 55).

Before detachment, the intracellular actin-myosin motors produce contractile forces that are counter-balanced at the anchored location of the substrate (54, 55). The major macrophage protrusion is the lamellipodia, a sheet-like membrane extension, which is highly organized with branched actin filaments and the main site of cell-substrate adhesions (56). Actin polymerization at the plasma membrane is facilitated through the Arp2/3 complex and is essential for protrusive forces and persistent directional migration (56-59). The Arp2/3 complex usually remains in an inactive state and requires dynamic signaling through nucleation-promoting factors (NPFs) to initiate the construction of new filamentous F-actin structures (60, 61).

Macrophage migration requires traction force generation through a combination of exterior adhesiveness to the substrate and interior tension within the cytoskeleton (62). Rho proteins have shown a significant role in cell contractility and adhesion during macrophage migration through regulating integrin expression and myosin activity (63, 64). Efficient migration of macrophages requires directional persistence towards the attractant (e.g., chemoattractant or stiffness gradient). Macrophages obtain their persistence by partially limiting crosswise membrane extensions and promoting an actin-rich leading edge protrusion and adhesion stabilization (65).

Monocyte and macrophage chemotaxis involves cellular migration through the sensation of an external signal (e.g. chemoattractants) and the responding controlled formation of internal cytoskeletal mechanical forces. Chemotaxis can be complicated due to the role of specific cell surface receptors in the detection of an external chemical gradient and the subsequent intracellular signaling. In this process macrophages can detect specific chemoattractants and

migrate towards a positive external gradient (66). Atherosclerotic lesions secrete numerous inflammatory cytokines that recruit circulating plasma monocytes and macrophages to the injured area. When detected by the membrane receptors, specific cytokines stimulate downstream intracellular signaling to promote cytoskeletal remodeling and extension of the actin-rich protrusions towards the chemoattractant (67).

Similarly, cellular mechanotaxis guides cell movement at the cell-substrate interface but through increasing substrate stiffness gradients (68). Substrate stiffness appears to dramatically mediate macrophage cell shape and function by influencing the cytoskeleton network (69). Changes in morphology are driven by the interactions of the membrane adhesion sites and actin-myosin contractile forces upon the substrate (70, 71). As substrate stiffness increases, macrophages turn from rounded to polarized in what may be a mechanism to better probe a gradient environment of stiffness fluctuations (72). The substrate stiffness regulates, to some degree, the macrophage force generation during migration, and although the basic towing mechanics have been identified, there are still unknown factors affecting migration in the atherosclerotic environment (72, 73). Since TRPV4 mediates remodeling of actin-myosin structures and its activity is upregulated by increasing matrix stiffness, we hypothesize that macrophage migration may be dependent on TRPV4 activity.

Macrophage Phagocytosis

Macrophages are primarily known for their role in the innate immune system. Their principal functions include migration and phagocytosis. These processes require swift actin cytoskeletal remodeling to produce membrane protrusions and contractile forces (74). Macrophage phagocytosis is an essential mechanism during atherogenesis used to engulf and internalize oxLDL and free lipid material (75). This process yields an abundance of cholesteryl

esters that are later hydrolyzed in endosomes resulting in internalized cholesterol, free fatty acids, and many cytoplasmic vesicles (75).

The recognition and internalization of oxLDL by specific plasma membrane scavenger receptors is a complex and critical component to atherosclerosis development (76, 77). The process involves synchronized coordination between scavenger receptors, oxLDL, and the surrounding extracellular matrix (ECM) to appropriately rearrange the macrophage cytoskeleton for particle engulfment (68, 72, 78). oxLDL is first recognized and taken up by macrophages through three primary scavenger receptors: SR-A, CD36, and CD68 (79). CD36 and SRA are the primary receptor proteins accounting for the largest proportion of macrophage uptake of oxLDL (80).

CD36 is an integral membrane protein on the surface of monocytes and macrophages that serve as a multi-ligand scavenger receptor essential for the endocytosis of oxLDL and subsequent foam cell formation (81). Past studies have implicated CD36 signal transduction pathways in involving the non-receptor tyrosine kinases of the src family and serine/threonine kinases of the mitogen-activated protein (MAP) kinase family (82-84). More recently it's been shown that the cytoplasmic carboxy-terminal of CD36 associates with the Lyn and MEKK2 signaling complex during macrophage phagocytosis (15). Furthermore, CD36-dependent activation of JNK has proved to be necessary for oxLDL phagocytosis, while the inhibition of JNK and Src pathways decreases c-Jun phosphorylation and prohibits oxLDL uptake and foam cell formation (15).

Macrophage Calcium Signaling

Macrophages have an intricate system of ion channels and membrane pumps that are responsible for regulating cellular Ca^{2+} flux (85). Although cytoplasmic Ca^{2+} signals can develop

through the release of intracellular ER stores, plasma membrane channel access dominates the potential ion pool (86).

Ca^{2+} second messenger signaling is vital for several macrophage functions, including cell differentiation and proliferation, apoptosis, migration, adhesion, and mediating inflammatory responses (8, 9). Moreover, recent research has shown specific Ca^{2+}-dependent intracellular signaling to associate with phagocytosis and foam cell formation (9, 14, 15). The process is commonly initiated by direct ligand-receptor binding on the membrane surface followed by a receptor activation inducing channeled Ca^{2+} influx (74). Regarding macrophage functions, the flow of Ca^{2+} allows NPF stimulation of Arp2/3 that triggers actin polymerization (F-actin) and cytoskeleton remodeling; processes that permit for the construction of F-actin dominant engulfment and motility structures, such as lamellipodia or phagocytic cups (60, 61, 74). Interestingly, decreases in protrusion size and persistence lengths were shown from a loss of the S100A4 protein in macrophages (87). S100A4 is a Ca^{2+}-dependent molecular regulator of myosin during macrophage chemotaxis (87).

Macrophage Stiffness

Atherosclerosis results in the hardening of the arterial walls and thus increases the microenvironment stiffness (88). Monocytes and macrophages are both capable of migrating through the blood vessel endothelium, and it is likely arterial stiffness affects the transmigration efficiency (89). Interestingly, it's been observed that macrophage phagocytosis proficiency is mediated by different substrate stiffness – preferring engulfment in stiff environments – and thus encouraging foam cell development (90).

A surmounting inflammatory cascade along with targeted macrophage and monocyte recruitment is characteristic of developing atherosclerosis and provides a dynamic

microenvironment for arterial wall structural remodeling (91). The endothelial monolayer is known to have an elasticity stiffness of ~0.5 kPa (92), but new atherosclerotic research using atomic force microscopy has shown a range of arterial stiffness that depends on the stage and location of the plaque. Tracqui et al. subdivided plaques into three distinct regions and measured the area stiffness. They concluded that the hypocellular fibrosis area with robust fibrotic material averaged a stiffness of 59.4 kPa, the cellular rich area colonized by smooth muscle cells (SMC) averaged a stiffness of 10.4 kPa, and the lipid-rich area abundant with foam cells and extracellular lipids averaged a stiffness of 5.5 kPa (88).

The stiffness of the cellular microenvironment has shown to regulate an assortment of fundamental cell functions such as migration, differentiation and proliferation, and recent experimental studies indicate changes in macrophage morphology in accordance to substrate stiffness (68, 72, 93). With increased stiffness, macrophages exhibit improved polarization, more protruding filopodia, more defined F-actin, and a larger presence of αV integrins (68, 72). In addition, macrophage activation via LPS has shown to increase specific inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) under stiffer matrices (12, 68, 72). These results indicate that macrophage phagocytosis and migration is likely contingent on environmental ECM stiffness, but the mechanosensing signaling mechanisms are yet to be determined.

Macrophage Foam Cells

The continual uptake of oxLDL by macrophages produces lipid-laden foam cells. In an atherogenic environment, foam cells continue to grow through the persistent uptake of oxLDL and lipid material until oxidative stress, death receptor activation, prolonged activation of the ER stress pathways, or nutrient deprivation facilitates apoptotic signals and cell destruction (5, 6). Free cholesterol in the foam cell influences the ER to down-regulate LDL receptor production

and endogenous cholesterol synthesis through the suppression of the sterol-regulatory element binding pathway (SREBP) (94). In contrast, reducing ER stress has shown to shift macrophage differentiation from M2 toward the inflammatory M1 phenotype through the induction of high-density lipoprotein (HDL) and apoA-1 cholesterol efflux receptors (46).

The development and perseverance of foam cells may be mitigated by the removal of the fatty contents by high-density lipoprotein (HDL), but in atherogenic conditions foam cell quantity and size overcome HDLs efferocytosis capacity (5). Free cholesterol, released from macrophage lysosomes or rehydrolyzed from cholesteryl esters, can migrate to the plasma membrane and efflux out of the cell via reverse cholesterol transport receptors, primarily ABCA1 and ABCG1 (95). But despite normal cholesterol esterification processes, to some degree, free cholesterol enriches the plasma membrane and enhances further inflammatory signaling (96).

The foam cell destruction leaves behind its lipid-laden contents (e.g., cholesterol, triglycerides, membrane remnants, and cholesterol esters) in the arterial wall, contributing to what is known as a “fatty streak” (7). This destruction further recruits additional monocytes to the injured location and continues the engulfment-death cycle. The continual accumulation of both active and remnant dead foam cells, combined with defective efferocytosis, form a growing lipid pool that over time becomes the foundation of an atherosclerotic plaque (5).

Atherosclerotic Plaques

As macrophages and foam cells continue to proliferate, they secrete higher concentrations of cytokines that induce the propagation and migration of smooth muscle cells (SMCs) from the tunica media to the tunica intima (50). SMC lipid ingestion and secretion of collagen, elastin, and proteoglycans produce a protective cap of a fibrous matrix over the fatty streak, known as a

plaque (97). The atherosclerotic inflammatory process at low levels is relatively benign since the arterial lumen has other preservation methods; however when the plaque buildup exceeds the phagocytosis capacity, the lesions grow, and a necrotic core persists (98). Proatherogenic oxLDL and TNF-α further promotes the death of macrophages, foam cells, and other plaque cells which rapidly advances the necrotic core expansion (3, 99). A mature plaque will thicken the endothelial lining, protrude into the lumen, reduce elasticity, and in some cases obstruct arterial blood flow (stenosis) (100). Over time, calcium accumulates into deposits and crystallizes in the outer layer of the plaque between the atheroma and the vessel wall, significantly hardening the artery (101).

Atherosclerotic plaques can be stable or unstable depending on the amount and strength of the SMC and ECM (102). Stable plaques are rich in ECM and SMC and tend to be relatively asymptomatic, while unstable plaques are abundant in macrophages and foams cells with a weak ECM foundation making it susceptible to rupture (103). Neighboring macrophages secrete TNF-α and NO that induces SMC apoptosis and ultimately weakening the plaque (104). Macrophages are further involved in thinning the fibrous cap by the release of protease-activated enzymes, macrophage-derived matrix metalloproteinases (MMPs), that degrade specific ECM proteins (105).

A vulnerable plaque has the potential to rupture into a thrombus that can lead to an acute arterial occlusion. Any vascular occlusion has the potential to cause downstream oxygen and nutrient tissue starvation, causing serious cardiovascular events such as myocardial infarction and stroke (24, 35, 106). Post-rupture, collagen and tissue factors induce a clotting cascade of platelet activation, adhesion, and aggregation at the rupture site that potentially creates an occlusive thrombus clot through coagulation (39).

Porphyromonas gingivalis LPS-induced Atherosclerosis

Current epidemiological studies and experimental research have shown an association between periodontal disease and atherosclerotic cardiovascular disease, particularly with Porphyromonas gingivalis (Pg) infection (10, 11, 107). Pg is a gram-negative bacterium responsible for human periodontal disease in subgingival plaques (108). The bacterium can release powerful virulent factors, such as LPS, that can enter blood circulation and travel away from the periodontal cavity throughout the body (109). pgLPS actively induces immune system responses and activates inflammatory signaling pathways in a variety of cells (110); however, pgLPS mostly utilizes Toll-like Receptor 2 (TLR2) which allows some evasion of host immune recognition (111).

Experimental studies have given some plausible mechanisms that link pgLPS infection to atherosclerosis, but the precise role is still under investigation. These studies have shown that pgLPS infection increases oxidative modification of LDL, builds induction of MMPs, raises production of inflammatory cytokines, exacerbates endothelial cell death, and promotes lipid deposition within the arterial wall (110, 112-115). More current studies have shown that LPS dose-dependently increases macrophage oxLDL uptake and foam cell formation, an early event in atherosclerotic plaque development (12, 107, 115-117).

TRPV4

Macrophage scavenger receptor proteins have an integral role in atherosclerosis, but additional membrane proteins are also likely to affect macrophage-dependent atherogenesis. The Transient Receptor Potential Cation Channel, Subfamily V, Member 4 (TRPV4) is a Ca^{2+} permeable non-selective cation channel widely expressed in a variety of different cell types and tissues (16). The channel is involved in numerous physiological processes and cellular functions

which may suggest a pathological role in a dysfunctional environment (16); however, its contribution to atherosclerosis is not well understood.

TRPV4 Structure

In humans, the TRPV4 gene is located on the 12q23-q24.1 chromosome with five possible splice variants (118). The TRPV4 protein consists of six transmembrane traversing α-helices and a permeable cation pore recognized to be between the fifth and sixths transmembrane passage (118). TRPV4 is also involved in various regulatory domains and protein-interaction sites including phosphoinositide 3-kinase (PI3K) and Src homology 2 (SH2) recognition domains, putative protein kinase C (PKC) phosphorylation sites, and putative PDZ domains (16, 119). The transmembrane protein has an intracellular N-terminal believed to have six ankyrins (ANK) capable of protein-protein interactions, with the first ANK repeat in the near vicinity to a proline-rich domain responsible for the channels mechanosensing properties (16, 120-122). On the other end, the intracellular C-terminus contains many calmodulin (CAM) binding sites putatively involved in the Ca^{2+} dependent channel activation (122, 123).

TRPV4 Activation

Activation of TRPV4 opens the membrane channel allowing the passage of extracellular Ca^{2+} into the cell. The process is possible through multiple stimulators, which include physical, chemical, mechanical, thermal, and endogenous stimuli (16). Synthetic pharmacologic TRPV4 agonists and antagonists have also been created over the last decade. Highly specific synthetic agonists used in research include 4α-phorbol 12,13-didecanoate, 4α-phorbol 12,13-dihexanoate, and the exceedingly potent GSK1016790A (GSK101) (18, 124). In the past, TRPV4 antagonists were relatively limited and unspecific, but recently small-molecule GSK2193874 (GSK219) has

shown to be a reliable, selective TRPV4 antagonist. GSK219 blocks TRPV4-mediated calcium influx as well as inhibits channel activation of recombinant TRPV4 currents (125).

TRPV4 activation through membrane stretching via hypotonicity, polarization, or shear stress is a slow progression but has proven to behave as a cellular mechanosensor (126). The TRPV4 mechano-stimuli are believed to be detected, in part, by intracellular phospholipase (PLA_2), leading to the creation of arachidonic acid that is later metabolized to epoxyeicosatrienoic acids (127). Endogenous lipids, primarily the long chain polyunsaturated fatty acid endocannabinoid metabolite arachidonic acid, can trigger typical whole-cell currents in TRPV4 expressing cells (127). The activation by arachidonic acid has shown, through pharmacological blockers, the signaling cascade directly involves the epoxygenase pathway with epoxyeicosatrienoic acids stimulating TRPV4 in a membrane-delimited fashion (127).

Intracellular Ca^{2+} can activate or inhibit TRPV4 activity depending on the internal ion concentration (123, 124). Inhibition arises with an increase of intracellular Ca^{2+} that initiates a Ca^{2+} dependent negative feedback and channel inactivation, but in low internal Ca^{2+} environments activation remains possible (123).

TRPV4 Functional Pathways

There is an incomplete understanding of the TRPV4 functional signaling pathway. It has been shown that TRPV4 functionally interacts with PACSIN3, a protein that modulates the subcellular localization of TRPV4 and is responsible for blocking dynamin-mediated endocytosis during vesicle trafficking (128). It has also been demonstrated that microtubule-associated protein 7 (MAP7) interacts with the TRPV4 C-terminus to form the mechanosensitive protein complex by connecting the channel to cytoskeletal microtubules (129). Furthermore, recent studies have observed that F-actin colocalizes with TRPV4 in dynamic membrane structures, and

variations in matrix stiffness induce cytoskeletal remodeling as well as TRPV4-dependent Ca^{2+} flux (22, 130, 131).

Chapter 3: Research Objectives

A relationship between TRPV4 and macrophage-mediated atherosclerosis has not been previously demonstrated, and the associated specific molecular mechanisms have not been determined. This study will test the hypothesis that the TRPV4 channel contributes multiple proatherogenic properties by determining a role for TRPV4 in atherosclerotic macrophage migration and foam cell formation.

Research Aims

1. Determine whether TRPV4 is expressed and functional in macrophages.
    a. Covering figures 1A-1D.

2. Determine whether TRPV4 modulates macrophage migration.
    a. Covering figures 2A, 2B, 3A-3E, 4A-4C, 5A-5E, and 6A-6C.

3. Determine if TRPV4 modulates macrophage foam cell formation.
    a. Covering figures 7A, 7B, 8A, 8B, 9A, and 9B.

4. Identify possible mechanisms by which inflammatory stimuli may regulate TRPV4-elicited Ca^{2+} influx.
    a. Covering figures 10A and 10B.

Impacts

Determining an association between TRPV4 properties and mechanisms and the regulation of macrophage migration and foam cell formation will advance our knowledge of atherogenesis and have significant therapeutic implications.

Chapter 4: Materials and Methods

Cell Culture and Reagents

The initial cell culture models used RAW cells obtained from ATCC (Manassas, VA, USA). RAW cells are leukemia virus-transformed mouse macrophages which are frequently used in atherosclerosis research models. Cell culture growths and incubations were conducted at 37° C and 5% CO_2 for 24 hours using RPMI-1640 culture media (Gibco) unless noted otherwise.

Small molecules GSK2193874 (GSK219), GSK1016790A (GSK101), and A23187 (A23) were purchased from Sigma-Aldrich (St. Louis, MO, USA) to act as TRPV4 agonists and antagonists. For all RAW cell experimentation, the specific antagonist GSK219 was used as a TRPV4 channel inhibitor and GSK101 as a TRPV4 channel stimulator.

The FLIPR Calcium 6 Assay kit, pgLPS, and Human LDL was purchased from Molecular Devices (Sunnyvale, CA, USA), InvivoGen (San Diego, CA, USA), and Stemcell Technologies (Vancouver, BC, Canada) respectively. Gibco cell culture media related reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA) while all other chemicals were purchased from Sigma-Aldrich.

Intracellular Calcium Ion Influx

Ca^{2+} influx was measured by the FlexStation system (Molecular Devices) using the FLIPR Calcium 6 Assay Kit (Molecular Devices). RAW cells were initially prepped and incubated with 0.5% SCM-RPMI. The following day media was replaced by 1% BSA serum-free RPMI with select wells treated with/without various concentrations of oxLDL (10, 50, 100 µg/mL) and pgLPS (10, 100, 1000 ng/mL) prior to a second incubation. On the final day, select samples were treated with GSK219 and GSK101 following the addition of FLIPR Calcium 6 dye

and 2.5 mM probenecid. The calcium sensitive dye is absorbed into the cell's cytoplasm during incubation. When the target is activated, intracellular calcium is released, binding with the dye, and creating a fluorescence signal. FlexStation analysis measured cytosolic Ca^{2+} fluctuations in relative fluorescence units and evaluated the results using ΔF/F (max-min) according to Tsien et al. (132).

Scratch Assay

Cell motility in relation to blocking the TRPV4 channel was first investigated regarding cell migration into an artificial wound introduced onto a confluent monolayer of RAW cells. A vertical scratch was made by scraping the middle of the cell monolayer with a p10 pipette tip. Designated wells were treated with GSK219 at various concentrations (NT, 1 µM, 10 µM, 25 µM). The cell migration was viewed using Zeiss Axio Observer microscope (10x phase contrast) after the initial scratch and again after 24 hours. Cells were kept at 37° C and 5% CO_2 between viewings. The subsequent quantitative analysis was performed by counting a total number of migrated cells into the scratch after 24 hours under each condition.

Time-Lapse Microscopy

Time-lapse microscopy was used to monitor individual cell migration on variable stiffness (0.5 kPa and 8.0 kPa) and with or without the presence of GSK219 (2 µM and 10 µM). RAW cells were cultivated to approximately 75% confluency on a polyacrylamide coverslip or customized stiffness gel-coated coverslips. Cell images were captured at 10-minute intervals using a temperature and CO_2 controlled microscope chamber with 20x objective over a 6-hour period. Cell tracking was gained through a manual tracking plugin from ImageJ software (National Institutes of Health), and further analysis of cell migration, to include velocity, distance, and trajectory was carried out using MatLab software (Natick, MA).

Oil Red O Foam Cell Assay

Oil Red O (ORO) staining was used to observe and quantify the role of TRPV4 in the internalization of lipid. ORO is a fat-soluble dye that stains lipids red and can indicate macrophage internalization of oxLDL, and therefore, the formation of foam cells. First, RAW cells were treated with/without GSK219 (5 µM) and oxLDL (50 µg/mL) and incubated overnight. The following day the cells were washed and then fixed with 10% phosphate-buffered formalin before staining with ORO (0.2%) until saturation. Images were examined using Zeiss Axio Observer with the 40x objective to determine the percent foam cell development for each control and experimental group.

oxLDL Binding and Uptake Assay

DiI-fluorescently labeled oxLDL (DiI-oxLDL) was observed in models with/without GSK219 (10 and 50 µM) and DiI-oxLDL (5 µg/mL) to assess whether TRPV4 modulates the binding of oxLDL in RAW cells. RAW cells were seeded and incubated with select wells treated with GSK219 at the various concentrations. Then, on ice, DiI-oxLDL was added to selected wells and remained on the ice for 2 hours before fixation with 10% formalin. Fluorescent images were viewed with Zeiss Axio Observer at 40x and quantified using ImageJ software.

DiI-oxLDL was similarly used to assess whether TRPV4 regulates the uptake of oxLDL in RAW cells. RAW cells were seeded and incubated with select wells treated with GSK219 (20 µM). Then, at room temperature, DiI-oxLDL (5 µg/mL) was added to all wells and returned to the incubator. At specific intervals (10 min, 30 min, 60 min), select wells were washed with PBS and fixed with 10% formalin. Fluorescent images were viewed with Zeiss Axio Observer at 40x and quantified by ImageJ software.

Statistics

All data is presented as means ± SEM. The Student's t-test was used to relate the control and experimental groups statistically. SigmaPlot 13 software (Systat Software, San Jose, CA) was used with p-values of ≤ 0.05 to determine statistical significance.

Chapter 5: Results

TRPV4 is expressed and functional in RAW Cells

Recent publications (12), confirmed in-house through immunostaining, examined the expression profile of TRPV4 in RAW cells to better understand the contributory role of this calcium channel in macrophage foam cell formation. In addition to confirming RAW cell TRPV4 expression, pgLPS was used as a physiological inflammatory stimulus during immunostaining in order to observe a potential channel regulator. As a result, the pgLPS provided an increased expression of TRPV4 in a dose-dependent manner (data not shown). Taken together, research shows TRPV4 is expressed in RAW cells, and there is a channel upregulation in the presence of pgLPS.

Determining the functional extent of the TRPV4 calcium channel was conducted through assays in the presence of TRPV4 agonists and antagonists. Measurements of Ca^{2+} influx induced by the selective TRPV4 agonist GSK101 were analyzed to test whether TRPV4 channel activation functionally induces Ca^{2+} influx in RAW cells (18). A rapid (within 20-50 seconds) concentration-dependent increase in intracellular Ca^{2+} influx in RAW cells in response to GSK101 stimulation (EC_{50} = 2.5 nM) was detected (Fig. 1A, 1B), supporting earlier research (12). However, it was also observed that Ca^{2+} influx is significantly inhibited in RAW cells when under pretreatment with selective TRPV4 antagonist GSK219 (Fig. 1C, 1D). This effect shows a dose-dependent inhibition of the TRPV4 channel function when compared to the untreated model (EC_{50} = 2.5nM), and exposed the inhibitory effect while under GSK101 concentrations four times greater than the EC_{50} at 10 nM (Fig. 1C, 1D). These results indicate that the TRPV4 channel is not only expressed but is highly functional in RAW cells as evidence by the mediation of cytosolic Ca^{2+} influx.

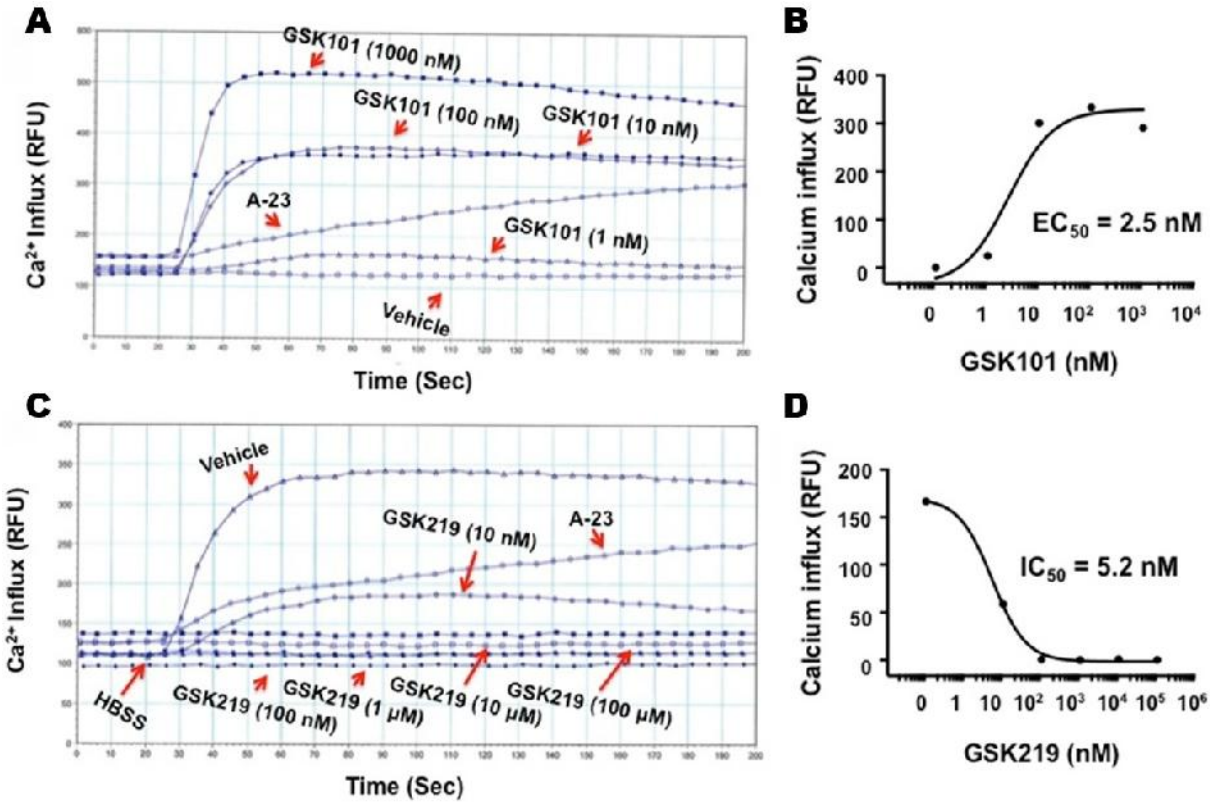


Figure 1: TRPV4 Ca^{2+} influx functional response in RAW cells. *RAW cells were compared for their Ca^{2+} influx response to TRPV4 specific agonist and antagonists over 200 seconds. A) GSK101 (1, 10, 100, 1000 nM) treatments show a dose-dependent increase in Ca^{2+} influx. B) Quantitation of the Ca^{2+} influx response resulted in a dose-dependent response with EC_{50} = 2.5nM. C) GSK101(10 nM) causes Ca^{2+} influx in RAW cells which is inhibited by its antagonist, GSK219 (10 nM, 100 nM, 1 µM, 10 µM,100 µM). D) Quantitation of Ca^{2+} influx in the presence of GSK101 and GSK219 exhibits an IC_{50} = 5.2 nM. Calcium ionophore A23187 (A-23) was used as a positive control.*

TRPV4 modulates macrophage migration over 24 hours

Atherosclerosis requires macrophage migration from circulating plasma through the vessel walls. As such, a scratch assay was performed to determine if functional TRPV4 influences RAW cell migration by blocking the TRPV4 channel via chemical antagonist. After 24 hours migrated cells were counted and adjusted for the variance in scratch width. Illustrations at 40x magnification show after 24 hours a dose-dependent decrease according to the pretreatment concentration of TRPV4 antagonist GSK219 (Fig. 2A). Quantitation of migrated cells from the scratch assay further exposes that blocking the TRPV4 channel with GSK219 will significantly abrogate RAW cell migration over 24 hours (Fig. 2B). This scratch assay shows

that a functional TRPV4 channel is essential for RAW cell migration and that blocking the channel significantly inhibits cell movement on plastic surface.

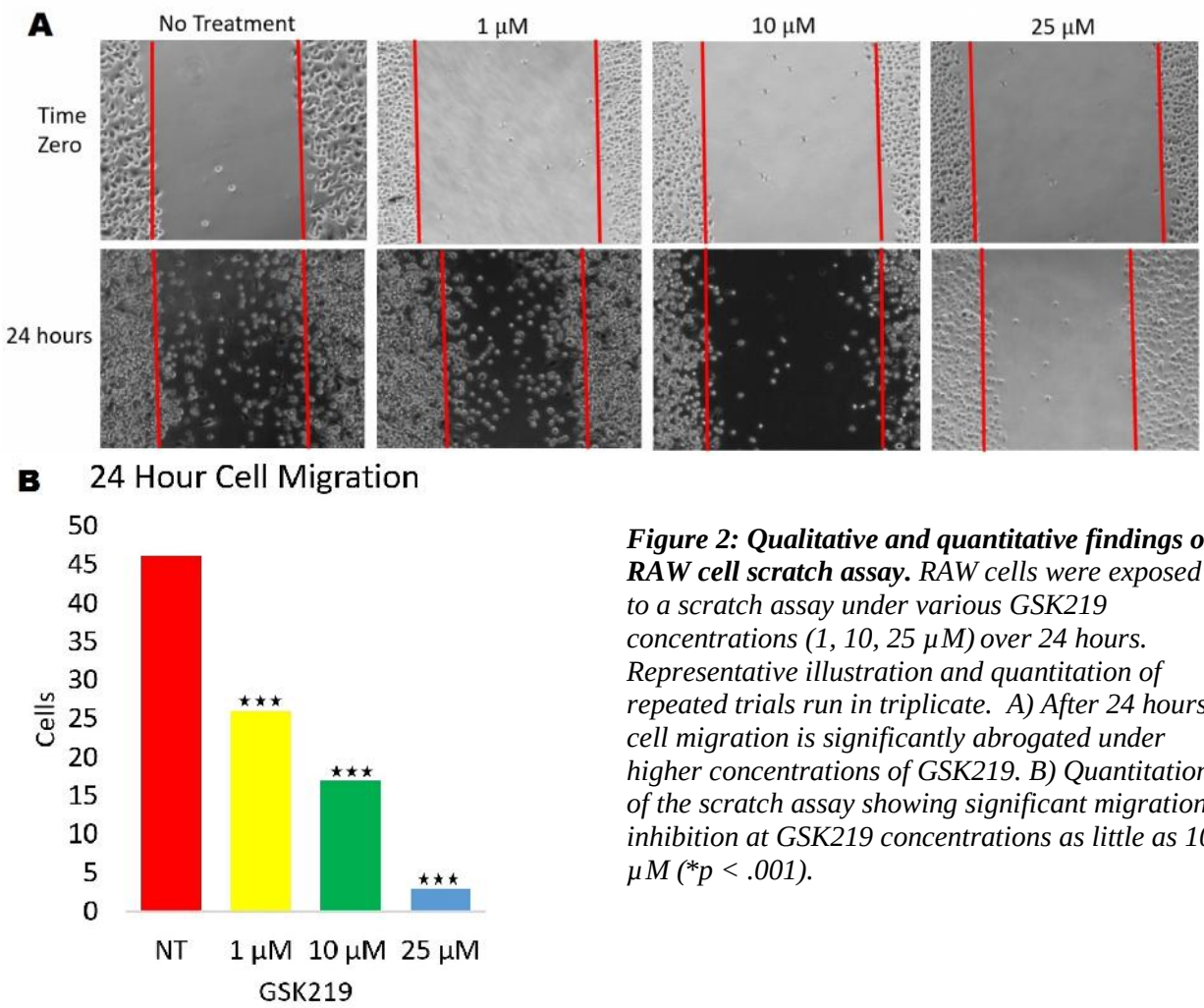
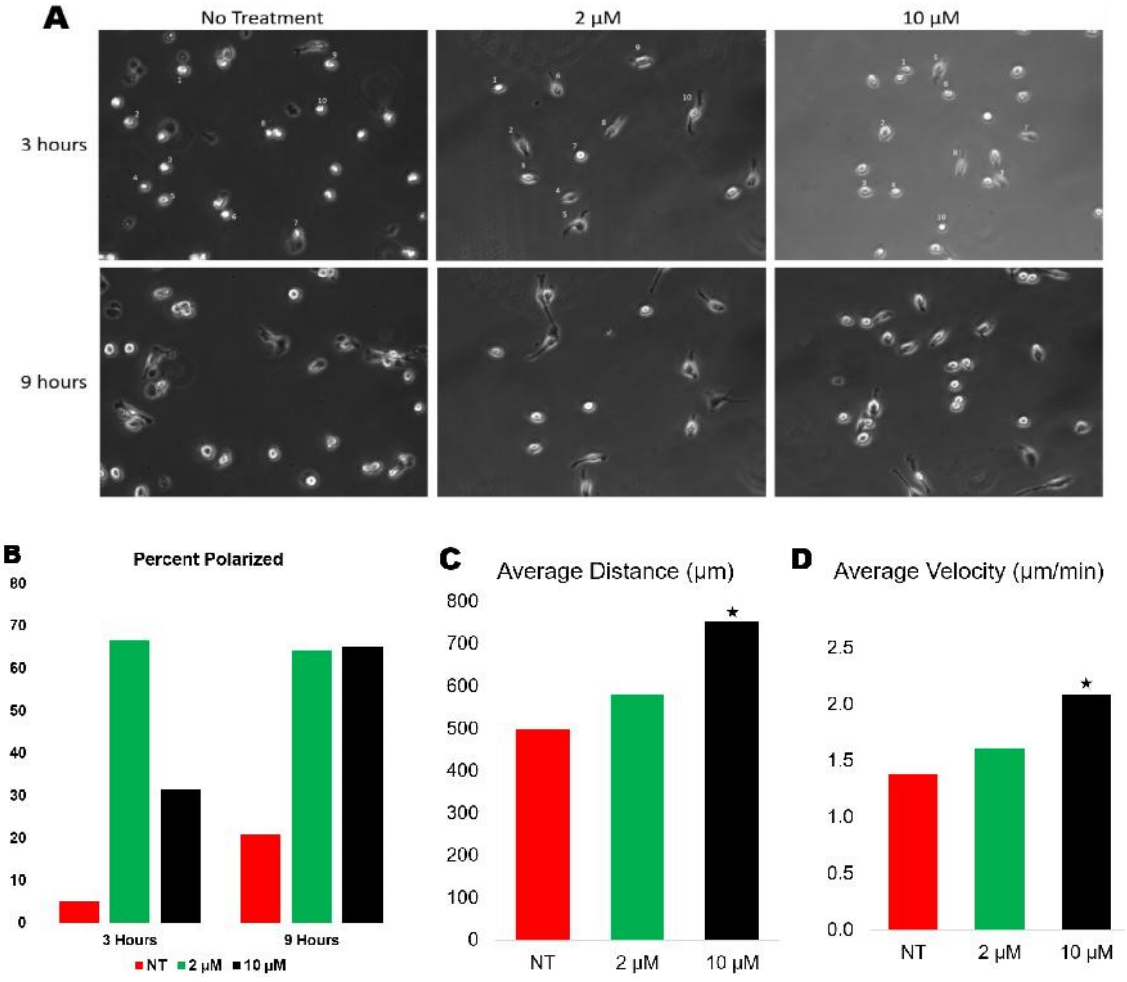


Figure 2: Qualitative and quantitative findings of RAW cell scratch assay. *RAW cells were exposed to a scratch assay under various GSK219 concentrations (1, 10, 25 µM) over 24 hours. Representative illustration and quantitation of repeated trials run in triplicate. A) After 24 hours cell migration is significantly abrogated under higher concentrations of GSK219. B) Quantitation of the scratch assay showing significant migration inhibition at GSK219 concentrations as little as 10 µM (*p < .001).*

TRPV4 modulates macrophage migratory characteristics over 6 hours

Individual macrophage migration characteristics were observed through microscopic time-lapse analysis. At least ten cells were tracked under each condition of various concentrations of TRPV4 antagonist GSK219. Observations at 10x magnification began 3 hours after seeding, capturing images every 10 minutes, for a total of 6 hours (Fig. 3A). The process allowed the determination of a variety of macrophage migration characteristics affected by TRPV4 functionality. Polarized cells, as an indication of cell movement, were visually counted

before and after the 6-hour migration (Fig. 3A). Quantitative analysis of the percentage of polarized cells per field before and after migration revealed an increase in cell polarization in samples treated with 10 µM GSK219 (Fig. 3B). Average distance and velocity were also measured in each of the ten tracked cells for each treatment condition. Analysis revealed that at earlier time points (up to 6 hours) the mean velocity and distance traveled by each cell increases accordingly to greater concentrations of GSK219 (Fig. 3C, 3D). Taken together, it appears that blocking the TRPV4 channel allows a rise in total cell polarization as well as critical migration functions such as distance and velocity.



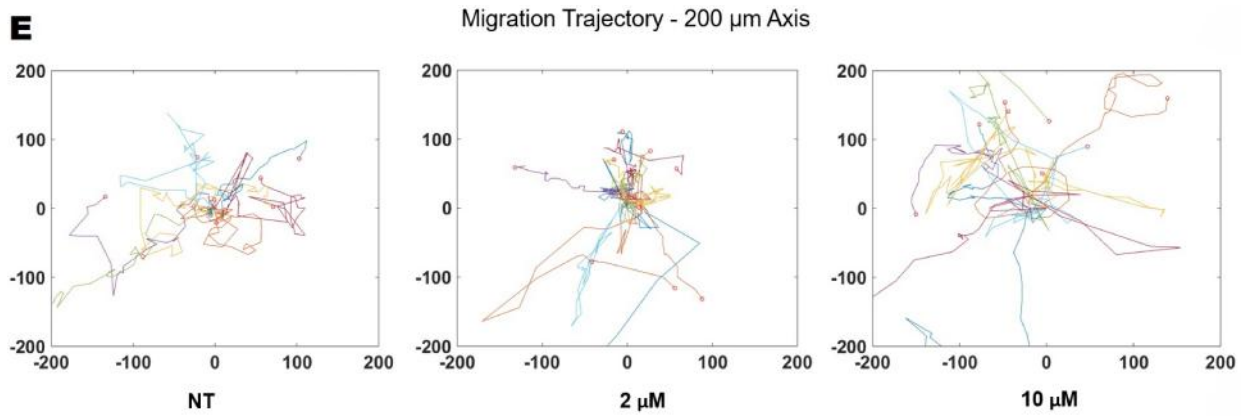
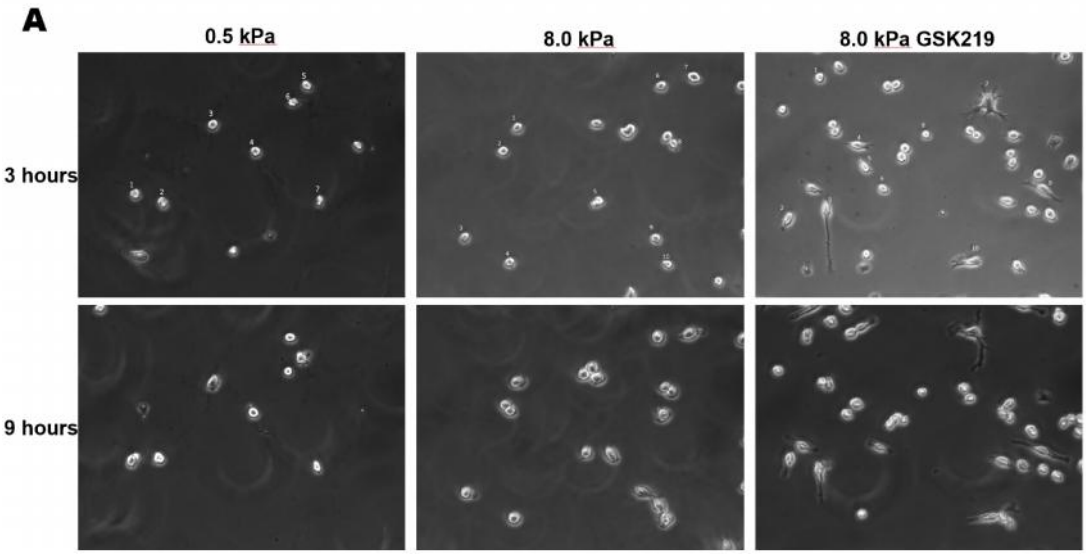


Figure 3: Raw cell migration characteristics influenced by TRPV4 inhibition. *RAW cells were observed and analyzed under various TRPV4 antagonist concentrations using time-lapse microscopy. A) Samples were pretreated with GSK219 (2 µM and 10 µM) and compared to the untreated control (NT) after 6 hours. B) Percent cell polarization was visually inspected and quantified. Though there were notable increases in cell polarization after 6 hours in the untreated and the 10 µM GSK219 groups, the effect was not seen in the 2 µM GSK219 group. The analysis remains inconclusive, and no statistical conclusions could be made. C) Cell coordinates were tracked every 10 minutes to determine the group pretreated with 10 µM GSK219 traveled statistically further distance (µm) than the untreated group ($p < 0.05$). D) The average distance traveled for each tracked cell over 6 hours determined the average velocity (µm/min). Similar to the average distance traveled, the average velocity was statistically greater in the group pretreated with 10 µM GSK219 as compared to the untreated group (*$p < 0.05$). E) Raw cell 6-hour migration 2-dimentional trajectories give a visual perspective of how far each cell travels outside of its original x,y coordinate.*

TRPV4 influences macrophage migration patterns under varied stiffness matrices

Since atherosclerosis causes changes in arterial matrix stiffness and TRPV4 is a matrix stiffness sensitive (mechanosensitive) ion channel, the role of TRPV4 in macrophage migration under variable substrate stiffness was examined. Individual RAW cell migration patterns under various stiffness matrices with or without TRPV4 antagonist GSK219 was observed through microscopic time-lapse analysis. 7-10 cells were tracked under each condition of 0.5 kPa stiffness, 8.0 kPa stiffness, and 8.0 kPa stiffness with GSK219 (10 µM). Observations at 10x magnification began 3 hours after seeding, capturing images every 10 minutes, for a total of 6 hours (Fig. 4A). The process allowed the determination of a variety of macrophage migration patterns affected by substrate stiffness and GSK219 TRPV4 inhibition. Polarized cells were visually counted before and after the 6-hour migration (Fig. 4A). No cellular polarization was

observed in the lowest stiffness (0.5 kPa) before or after the trial (Fig. 4A). Quantitative analysis of the percentage of polarized cells per field before and after migration was statistically inconclusive potentially due to a low number of cells counted (Fig. 4B). The average distance and velocity were also measured in each treatment condition. Analysis revealed that the mean velocity and distance traveled by each cell is statistically higher in the 8.0 kPa sample pretreated with GSK219 when compared to the untreated 8.0 kPa sample (Fig. 4C, 4D). There was also a significant difference in velocity and distance traveled between the two untreated stiffness matrices; over 6 hours the cells grown on the lower stiffness had a greater average velocity and distance traveled (Fig. 4C, 4D). Altogether, the results show that blocking the TRPV4 channel potentiates an increase in migration within the first 6 hours of adherence to 8.0 kPa substrate; furthermore, this data suggests a slight increase in migration distance and velocity by decreasing substrate stiffness from 8.0 kPa to 0.5 kPa.



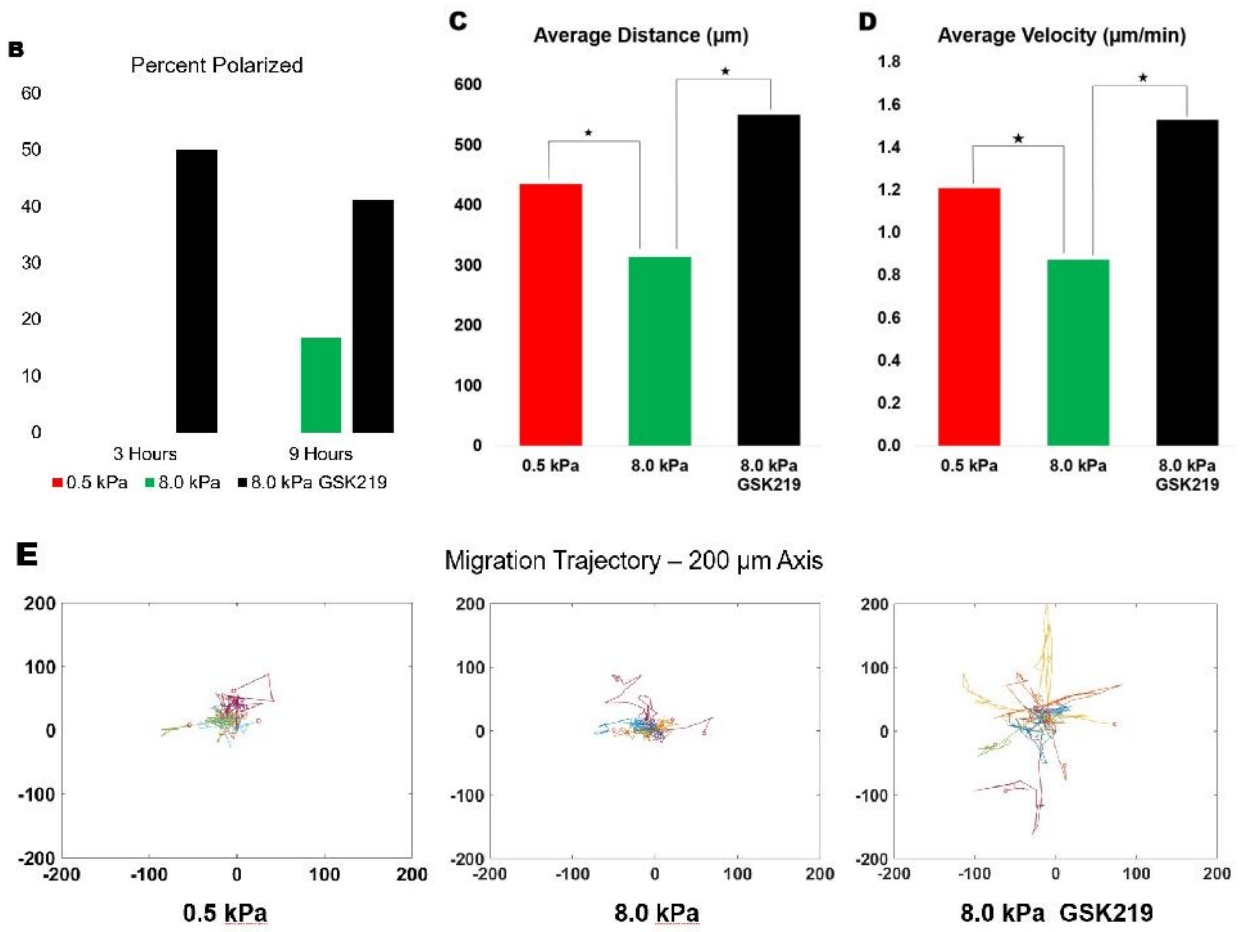


Figure 4: Raw cell migration characteristics influenced by substrate stiffness. *RAW cells were observed and analyzed under various stiffness matrices using time-lapse microscopy. A) RAW cells were grown on different stiffness gels (0.5 kPa and 8.0 kPa) with one sample pretreated with GSK219 (10 μM) and observed over 6 hours. B) Percent cell polarization was visually inspected and quantified. No cellular polarization was found at any time for the 0.5 kPa sample, and only a small increase occurred in the untreated 8.0 kPa sample. The polarization analysis remains inconclusive, and no statistical conclusions could be made. C) Cell positions were tracked every 10 minutes to determine that the 8.0 kPa stiffness with GSK219 (10 μM) group traveled a further distance (μm) than the untreated 8.0 kPa group (*p < 0.05). A significant difference in average total distance traveled was observed between substrates 0.5 kPa and untreated 8.0 kPa (*p < 0.05). D) The average distance traveled for each tracked cell over 6 hours determined the average velocity (μm/min). Similar to the average distance traveled, the average velocity was statistically greater between the treated 8.0 kPa group as compared to the untreated 8.0 kPa group as well as the 0.5 kPa group and untreated 8.0 kPa group (*p < 0.05). E) Raw cell 6-hour migration 2-dimentional trajectories give a visual perspective of how far each cell travels outside of its original x,y coordinate.*

Macrophage foam cell formation is reliant on TRPV4-elicited Ca^{2+} influx

It has been shown that TRPV4 can influence force-dependent cytoskeletal modifications (22); therefore, it was hypothesized that TRPV4 likely has a contributing role in macrophage

phagocytosis and foam cell formation. RAW cells were stained with Oil Red O (which stains the neutral lipids) after pretreatment with oxLDL and TRPV4 specific antagonist GSK219 to block the channels function. The objective was to quantify the internalization of lipid under each condition. The assay shows foam cell formation in the presence of oxLDL as indicated by the intensity of red staining, and it is apparent that the intensity declines and foam cell development is abrogated when treated with GSK219 (Fig. 5A). Quantitation of lipid internalization shows a 60% decline between oxLDL and oxLDL+GSK219 samples (Fig. 5B). Collectively these findings indicate TRPV4-dependent Ca^{2+} influx is a contributing function to foam cell formation.

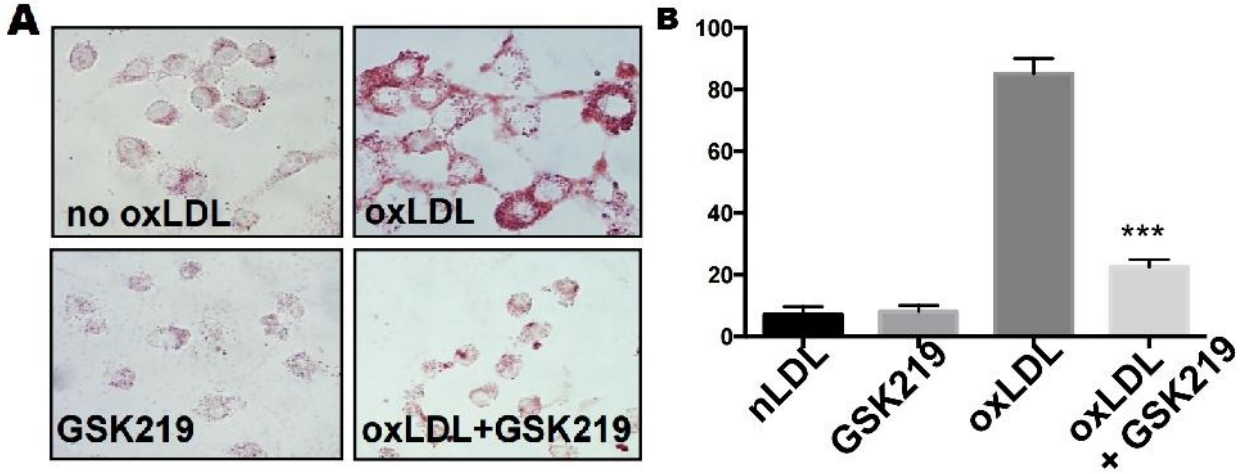


Figure 5: TRPV4 functional phagocytosis Oil Red O stain assay. *RAW cells were plated on plastic wells ± oxLDL (50 µg/mL) ± GSK219 (5 µM) for 24 hrs. A) Representative Oil Red O stained cells showing inhibition of foam cell formation by TRPV4 antagonism. B) Quantitation of foam cells presenting a significant decrease when treated with GSK219 (*p < 0.001).*

TRPV4-elicited Ca^{2+} influx in not required for oxLDL binding to macrophages

Ligand-receptor binding is required for macrophage scavenging. To assess the role of TRPV4 in oxLDL binding, RAW cells were exposed to a fluorophore (DiI)-tagged form of oxLDL (DiI-oxLDL) and examined under confocal fluorescence microscopy. Visualization of DiI-oxLDL on the macrophage membrane surface was used to determine the binding effect. There were no apparent differences in oxLDL binding in samples inhibited by GSK219 when compared to the untreated controls as evidence by the outer DiI-oxLDL surface ring in each

sample condition (Fig. 6). As a result, binding of oxLDL in RAW cells is not dependent on TRPV4 channel activity as seen by pre-treatment with selective TRPV4 antagonist GSK219.

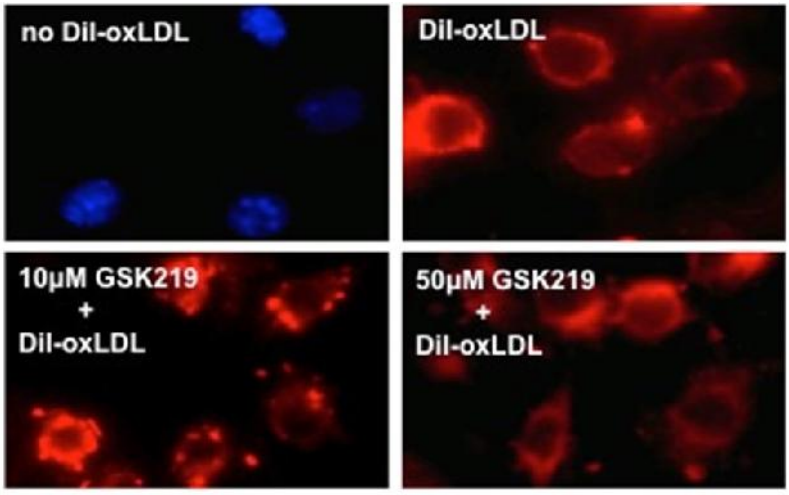


Figure 6: Effects of TRPV4 antagonist on RAW cell DiI-oxLDL binding. *RAW cells were pretreated under select conditions at 4° C to assess the role of TRPV4 in oxLDL binding: no DiI-oxLDL, DiI-oxLDL (5 μg/mL), DiI-oxLDL + GSK219 (10 uM), DiI-oxLDL + GSK219 (50 uM). Fluorescence images at 63x are shown. Red indicates the DiI-oxLDL while blue represents DAPI nuclear staining. Outer binding rings are displayed in each condition treated with DiI-oxLDL indicating that binding is not affected by TRPV4 functions.*

TRPV4-elicited Ca^{2+} influx regulates oxLDL uptake in macrophages

oxLDL uptake is the pillar of macrophage phagocytosis of modified lipids. RAW cells were pretreated with or without GSK219 and incubated with DiI-oxLDL at 37° C to assess the role of TRPV4 in the oxLDL uptake. Representative fluorescence microscopy images visualize the internalization of DiI-oxLDL as indicated by the red infiltration; furthermore, fluorescence intensity analysis through ImageJ quantified the labeled lipids. Macrophages treated with GSK219 showed significantly less DiI-oxLDL internalization after 10, 30, and 60 minutes compared to the untreated samples (Fig. 7A, 7B, 7C). The results indicate that TRPV4 is required for the uptake of oxLDL in RAW cells as seen by pre-treatment with selective TRPV4 antagonist GSK219.

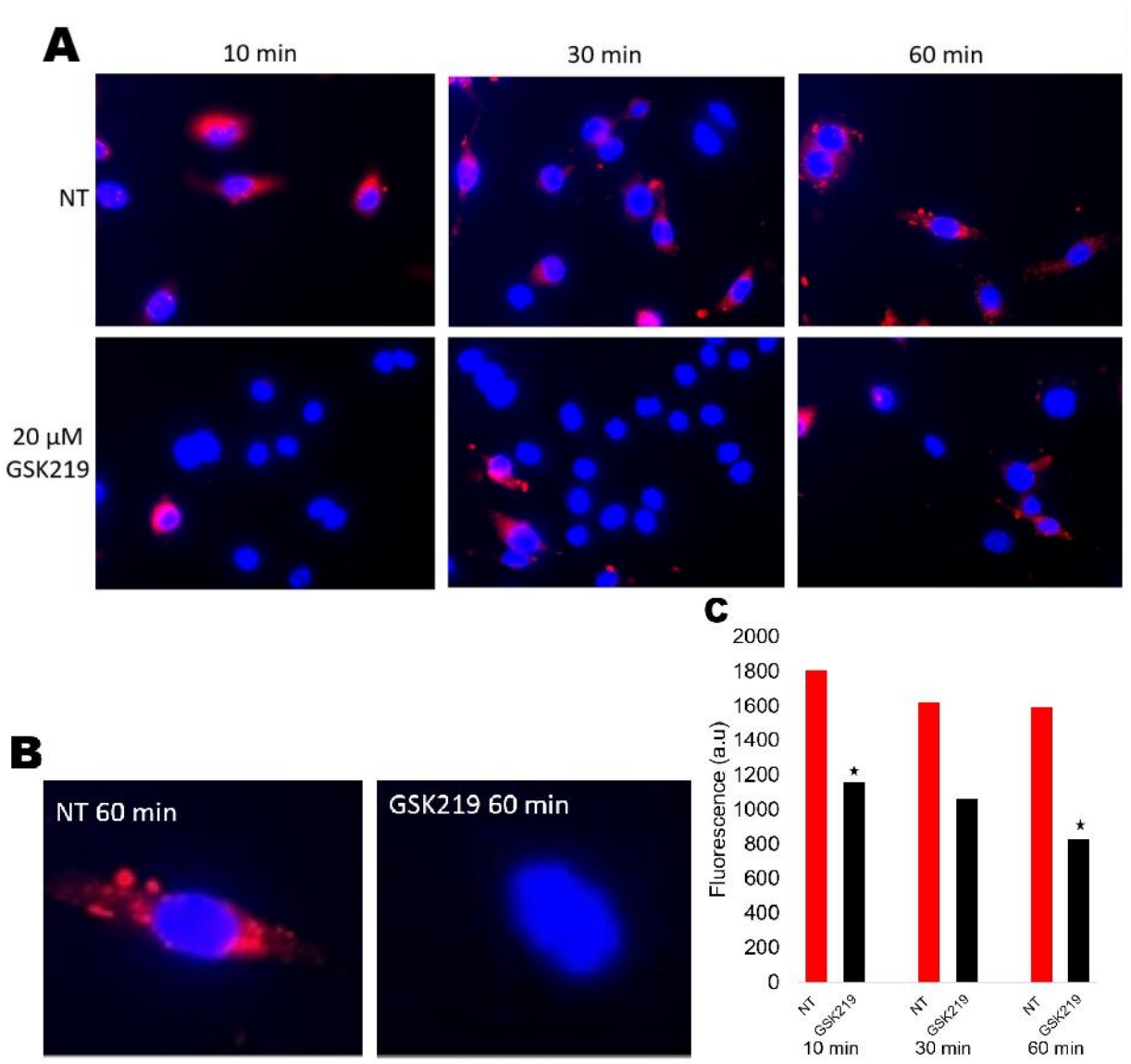


Figure 7: Effects of TRPV4 antagonist on RAW cell DiI-oxLDL uptake. *RAW cells were pretreated with DiI-oxLDL (5 µg/mL) ± GSK219 (20 uM) to assess the role of TRPV4 in oxLDL uptake. Fluorescence images were captured at 40x after 10, 30, and 60 minutes of 37° C incubation. Red indicates the DiI-oxLDL while blue represents DAPI nuclear staining. A) Red fluorescence cellular infiltration is prominently displayed in untreated groups compared to GSK219 treated groups. The absence of DiI-oxLDL uptake in treated macrophages indicates uptake is affected by TRPV4 functions. B) Single macrophage representations of the treated and untreated 60-minute groups, closely illustrating DiI-oxLDL uptake. C) The bar graph exhibits mean cellular fluorescence of DiI-oxLDL uptake. Fluorescence intensity was analyzed using ImageJ software. Five random representative cells of each group were used for quantitation. The untreated macrophages had significant increases in fluorescence at 10 and 60 minutes compared to the GSK219 treated groups (*p < 0.05).*

pgLPS stimulates TRPV4-mediated Ca^{2+} influx

pgLPS (LPS derived from P. gingivalis) was used to test whether a physiological inflammatory stimulus affects TRPV4-dependent Ca^{2+} influx. RAW cells were exposed to

various concentrations of pgLPS to measure TRPV4 cytosolic Ca^{2+} influx. Significant increases were detected in the treated cells as compared to the untreated group. pgLPS potentiates TRPV4-dependent Ca^{2+} influx in RAW cells in a dose-dependent manner (Fig. 8A, 8B). Samples were also treated with TRPV4 antagonist GSK219 alongside pgLPS. Results show that changes in intracellular Ca^{2+} are undetectable for samples treated with GSK219 as compared to the buffer control (Fig 8A, 8B). Antagonism of the TRPV4 channel with GSK219, selectively nullified pgLPS potentiation (Fig. 8A, 8B). These findings confirm that pgLPS stimulation potentiates TRPV4-dependent Ca2+ influx in RAW cells.

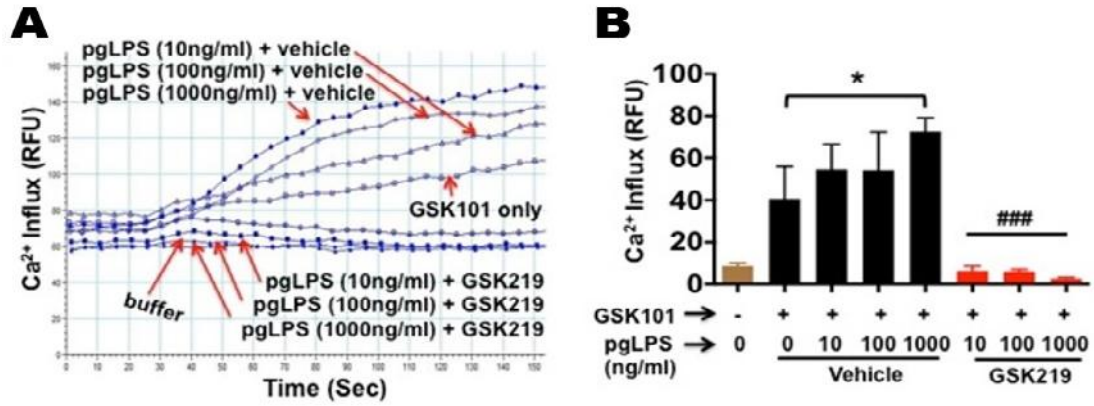


Figure 8: RAW cell Ca2+ influx with and without the presence of pgLPS and GSK219. *FlexStation measurements of RAW cell Ca^{2+} influx in the presence of pgLPS (10, 100, 1000 ng/mL) and GSK219 (100 nM) to determine the regulatory role of pgLPS on TRPV4 Ca^{2+}. A) pgLPS pretreatment potentiates TRPV4-dependent Ca^{2+} influx in RAW cells. Antagonist GSK219 inhibits this effect. B) Quantitation of Ca^{2+} influx in the presence of GSK101 (5 nM) and GSK219 (100 nM), and pgLPS (10, 100, 1000 ng/mL). A significant increase in Ca^{2+} influx was observed between samples with 1000 ng/mL pgLPS and those without pgLPS (*$p < 0.05$).*

Chapter 6: Discussion

In this study, novel proatherogenic roles for TRPV4 are defined in macrophages. The findings show TRPV4 is expressed and functional in macrophages, TRPV4 modulates macrophage migratory characteristics, oxLDL uptake and foam cell formation is reliant on TRPV4-elicited Ca^{2+} influx, and the physiological inflammatory stimulus of pgLPS can mediate TRPV4 function. Collectively, these discoveries, for the first time, associate the TRPV4 channel with atherogenesis by identifying new regulators for macrophage oxLDL phagocytosis and migration that are essential to atherosclerosis.

Recent efforts have demonstrated TRPV4 expression and function in mouse macrophages (12). In addition, Scheraga et al. has shown TRPV4-elicited Ca^{2+} influx exacerbation after E. coli LPS treatment. Work here, in the form of functional calcium influx assays, using pgLPS, a physiologically relevant proatherogenic stimulus, support recent research (Figs. 1 and 8). Since it is known that pgLPS is a strong inflammatory stimulator (110) and is associated with atherosclerotic cardiovascular disease (10, 11, 107), its relationship to TRPV4 was investigated. Similar to Scheraga et al. findings, it was found that TRPV4-dependent Ca^{2+} influx is potentiated by pgLPS stimulation.

Macrophages travel by a calcium-dependent process to the vascular lesion during atherogenesis (5, 8, 9, 14). Data presented here reveals, through time-lapse microscopy, a relationship between TRPV4 activity (calcium influx) and macrophage migration. The findings suggest that blocking TRPV4 function via GSK219 may increase macrophage polarization, distance, and velocity in the short-term, but may inhibit long-term motility (Figs. 2 and 3). We speculate that inhibition of TRPV4 activity may reduce the number of “trapped macrophages” in intimal areas during atherogenesis. This effect was replicated on a significantly lower stiffness of

8.0 kPa (Fig. 4). Together the results suggest a role for TRPV4 in macrophage migration but to which extent will require additional research.

It is known that macrophage phagocytosis of oxLDL and subsequent foam cell development is critical to atherogenesis (3-9). It is shown here, through Oil-Red-O staining and DiI-oxLDL assays, that macrophage oxLDL uptake and foam cell formation can be abrogated when exposed to TRPV4 antagonist GSK219 (Figs. 5 and 7); however, the same effect was not observed for oxLDL binding to macrophages (Fig. 6). Collectively, the data demonstrates TRPV4 has a contributing role in atherogenesis by regulating the development of foam cells possibly by regulating the uptake oxLDL but not the binding of oxLDL to cell surface.

This study persuasively shows the contributory role of TRPV4 in multiple macrophage atherogenic functions, but it is not without limitations. Most significant of which is the sole use of RAW cell in in vitro experimentation. RAW cells are an immortal, highly proliferative cell line derived from cancer cells. Although they can serve as a foundational model for mechanistic studies, there are significant differences between cultured cell line models and those harvested for ex vivo studies. Primary macrophages, harvested from bone marrow or peritoneum and differentiated and activated in vivo, would serve as a more relevant atherosclerosis model. Future studies from this laboratory will adopt an in vivo TRPV4 knockout model to reinforce these initial findings. Furthermore, this laboratory intends to develop a TRPV4 ApoE double knockout mouse model to translate previous research to dietary models better using high-fat diets. Work reported here mechanistically connects TRPV4 to atherosclerosis, but dietary influences on this model remain to be determined.

In summary, multiple in vitro assays was used to uncover a novel, atherogenic role of TRPV4 in macrophages. It was identified that the functions of the TRPV4 channel modulate

macrophage oxLDL phagocytosis and migration – two critical processes during atherogenesis. Further investigation of this connection could have profound therapeutic implications.

Chapter 7: Submitted Abstracts

1. University of Maryland, Bioscience Day 2015; TRPV4 Calcium Channels Regulate Macrophage Foam Cell Formation

2. University of Maryland, Research Day 2016; Role of TRPV4 Ion Channels in Atherogenesis

3. University of Maryland, AGNR Annual Meeting 2016; Role of TRPV4 Ion Channels in Atherogenesis; Best Poster Award

4. University of Maryland, Bioscience Day 2016; Role of TRPV4 Ion Channels in Atherogenesis

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