ABSTRACT

Title of Document: TRPV4, A CALCIUM-PERMEABLE CHANNEL, REGULATES OXIDIZED LDL-INDUCED MACROPHAGE FOAM CELL FORMATION

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Accumulation of lipid-laden "macrophage foam cell" in the arterial wall is the hallmark of atherosclerosis that leads to the highest number of cardiovascular disease-related deaths in United States. Membrane scavenger receptors such as SR-A, and CD36 play important role in controlling oxidized low-density lipoprotein binding and uptake, and, thereby, in macrophage foam cell formation. Recent studies also put emphasis on the role of mechanical factors, such as matrix stiffness, in the regulation of macrophage function and atherogenesis. However, the identity of a plasma membrane mechanosensor and the underlying mechanisms that may promote atherogenesis is yet to be identified. We have found that a calcium-permeable plasma membrane protein TRPV4, a mechanosensor, may play an essential role in regulating macrophage foam cell formation, a critical process in atherosclerosis. We have also found that TRPV4 is essential for oxLDL uptake, but not for its binding. Altogether, herein, we demonstrate that TRPV4 plays a critical role in macrophage-foam-cell formation by regulating oxLDL uptake in cells.

TRPV4, A CALCIUM-PERMEABLE CHANNEL, REGULATES OXIDIZED LDL-INDUCED MACROPHAGE FOAM CELL FORMATION

by

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List of Abbreviations

BMDM – mouse Bone Marrow Derived Macrophage

CD36 – Cluster of Differentiation 36

CVD – Cardiovascular Disease

DiI – 1,1'-Dioctadecyl-3,3,3',3'-tetramenthylindocarbocyanine per-chlorate

DMEM – Dulbecco's Modified Eagle's Medium

eNOS – endothelial Nitric Oxide Synthase

KO – Knock Out

M-CSF – Macrophage Colony Stimulating Factor

MRM – Murine Resident Macrophage

nLDL – native Low-Density Lipoprotein

oxLDL – oxidized Low Density Lipoprotein

PAGE – Polyacrylamide Gel Electrophoresis

RIPA – Radio-immunoprecipitation assay

ROS – Reactive Oxygen Species

SDS – Sodium Dodecyl Sulfate

SR-A – Scavenger Receptor A

TRPV4 – Transient receptor potential cation channel subfamily V member 4

WT – Wild Type

Chapter 1: Introduction

Atherosclerosis is a chronic inflammatory cardiovascular disease (CVD) caused in part by lipid-laden foam cell accumulation in arterial wall (1). Impacts of this disease are quite extensive but ischemic heart disease and cerebrovascular diseases are two major forms of CVD, mostly affected by atherosclerosis. Together these two diseases are representing 84.5% of CVD related deaths and 28.2% of all-cause mortality (68), hence, have become the most common cause of death in USA and in many advanced countries. Although this disease has huge negative impact in people life from advanced countries, the underlying mechanisms of this disease are poorly understood.

Foam cell formation and its infiltration/accumulation to vascular intimal layer is a hallmark of atherosclerosis (1). During atherogenesis, phagocytosis of oxLDL by macrophage and formation of lipid-laden foam cells are considered to be the major pathogenic processes. Macrophage cell membrane scavenger receptors such as CD36, is known to play leading role in internalization of oxLDL and subsequent foam cell formation (3-5). Although scavenger receptors are well characterized, downstream signaling pathways of this receptor in oxLDL binding and uptake is not well understood.

Increased reactive oxygen species (ROS) generation in atherosclerosis is well characterized phenomenon (1-7). ROS can oxidize phospholipids on the

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surface of LDL particles and transform them to oxLDL, which can be taken up by macrophage surface scavenger receptor such as CD36 (3-5). Uptake of oxLDL also increases ROS generation by feed-forward manner and plays a crucial role in the development and progression of atherosclerotic lesions (1-7).

Ca^{2+}-dependent signaling is required for different macrophage functions such as, cell proliferation, differentiation, apoptosis, migration, adhesion (15-16). Ca^{2+} signaling dependent actin remodeling is important for phagocytosis in macrophage (16, 19) and association of calcium overload with the genesis of atherosclerotic lesion is well established (17, 18). Recent data has also indicated that different mechanical factor such as matrix stiffness, can regulate numerous physiological function, such as, vascular elasticity, macrophage activation and atherogenesis (22-29). However, the identity of a plasma membrane mechanosensor and the potential mechanisms by which mechanotransduction signals can be transduced/propagate in macrophages to promote atherogenesis is not understood.

Emerging data from our group and others have provided evidence for the involvement of transient receptor potential cation channel subfamily V member 4 (TRPV4) in atherosclerotic macrophage functioning (12, 16-22). TRPV4 is a calcium-permiable, mechanotransductory membrane protein which is expressed ubiquitously in different cell types including macrophages (29-31). This channel is likned with different human diseases (45-47) and we hypothesize that TRPV4 may module induction of arterial stiffness or macrophage foam cell formation by changing the Ca^{2+} intake and promoting inflammation in cells.

In this study we tested the hypothesis that TRPV4, a calcium permiable channel, may play a role in macrophage foam cell formation. To test this hypothesis, numerous ex vivo/in vitro experimentation were performed using murine bone marrow derived macrphages and murine resident macrophages. We found that functional TRPV4 channels in macrophages is regulating macrophage foam cell formation in the presence of oxLDL. Matrix stiffness-dependent potentiation of oxLDL uptake but not binding in macrophages is also dependent on TRPV4. Mechanistically, we showed that i) Plasma membrane localization of TRPV4 is facilitated by increasing matrix stiffness, and ii) decrease foam cell formation in TRPV4 KO macrophages is not due to lack of expression of CD36, a major receptor for oxLDL. Altogether, our results suggest that TRPV4 is critically involved in oxLDL-induced macrophage foam cell formation by regulating oxLDL uptake in cells. Thus, targeting TRPV4 channels may be a selective theraputic target for atherosclerosis.

Chapter 2: Literature Review

The major cause of mortality and morbidity in today's world is atherosclerosis (1,2). Atherosclerosis is a chronic inflammatory disease and it is considered that atherosclerosis is initiated by endothelial dysfunction, which allows the retention of low-density lipoproteins (LDL) in the intima of inflamed arteries (1,3). An inflammatory response is triggered by the oxidative modifications of LDL (oxLDL) through enzymatic or non-enzymatic processes, which in turn activates endothelial cells, smooth muscle cells, and immune cells. This results in recruitment of monocytes into arterial intima (1,3–5). Monocyte-derived macrophage uptake of oxLDL through scavenger receptors such as CD36 contributes to formation of the primary component of atherosclerotic lesions, the lipid-laden macrophage "foam cells" (3–5). The development and progression of atheroma plaques and consequent development of atherosclerosis-related pathologies is caused by persistent accumulation of macrophage foam cells along with other cellular waste products, lipids, and calcium in the arterial intima (1–7).

Overall oxidative stress can be enhanced by chronic inflammatory condition, which dramatically augments the production of reactive oxygen species (ROS). There is a well-recognized association between atherogenesis and heightened oxidative stress (1–7). Phospholipids on the surface of LDL particles can get oxidized by increment of ROS and generate atherogenic oxLDL particles which can be taken up specifically by macrophage scavenger receptors like CD36 and SR-A (3–5). It has been shown that uptake of oxLDL can also

enhance ROS generation following mitochondrial dysfunction which can promote macrophage death, a process likely to contribute to the development of necrotic cores and progression of atherosclerotic lesions (1–7).

Macrophages are key player in atherogenesis by generating lipid-laden foam cells and by secreting inflammatory mediators (3–5). Previous studies also reported macrophage proliferation and accumulation in advanced lesions in atherosclerotic murine models (8). Deficiency of scavenger receptors in macrophages such as CD36 or SR-A blocked uptake of oxLDL and also reduced foam cell formation in mice (9–12). However, disparities have been observed regarding the role of scavenger receptors in foam cell formation. For example, ApoE null mice having a genetic deficiency in either CD36 or SR-A display increased atherosclerosis and increased numbers of macrophage foam cells (13,14). These findings suggest involvement of additional mechanisms in macrophage foam cell formation.

Ca^{2+}-dependent signaling is a critical component of numerous macrophage functions, including cell differentiation, proliferation, apoptosis, migration, adhesion, and mediating inflammatory responses (15,16). Calcium overload is associated with the genesis of atherosclerotic lesions (17,18) and phagocytosis and macrophage foam cell formation are also regulated by Ca^{2+} signaling (12,16,19,20). Macrophages have an intricate system of ion channels and membrane pumps that are responsible for regulating cellular Ca^{2+} homeostasis (21). Emerging data support a role for a mechanical factor, e.g.,

matrix stiffness, in regulation of numerous macrophage functions, vascular elasticity, and atherogenesis (22–29).

TRPV4 calcium-permeable channel, a member of the transient receptor potential vanilloid (TRP) superfamily, is ubiquitously expressed in various cell types including macrophages (29–31). Previous reports by our group and others have shown that a range of biochemical and physical stimuli in vitro and in vivo stimulates TRPV4 (29–44). Various gain- and loss-of-function mutations in TRPV4 have been linked to human diseases including skeletal dysplasia and sensory and motor neuropathies (45–47). TRPV4 has been linked to multiple physiological functions including osmolarity sensing in kidneys, sheer-stress detection in blood vessels, and control of osteoclast differentiation in bone (32–44). In mice, TRPV4 deficiency is associated with altered pressure/ vasodilatory responses, osmosensing, as well as lung and skin fibrosis (32–44). It has been reported that TRPV4 is a regulator of adipose oxidative metabolism, inflammation and energy homeostasis in obese mice models (48). TRPV4-null mice exhibit impaired endothelium-dependent relaxation induced by acetylcholine in vitro and in vivo (49). An athero-protective role of TRPV4 has been recently reported, whereby TRPV4 activation in endothelial cells induces eNOS activation and inhibits monocyte adhesion to endothelial cells (50). Thus, the role of TRPV4 in oxLDL-induced macrophage foam cell formation is critical to elucidate and important to determine the mechanism of its involvement in this process. It is hypothesized that TRPV4 calcium channels may play a role in macrophage foam cell formation because it is an important mechanosensor that

responds to changes in tissue stiffness by permitting Ca2+ influx into the cell (29–44) and changes in substrate stiffness affects macrophage phenotype and function (22–29). In this research project, the role of TRPV4 in the uptake of oxLDL and regulation of macrophage foam cell formation has been examined.

Chapter 3: Research Objectives

Arterial stiffness is one of the most important pathophysiological symptoms of atherosclerosis. However, it is unknown whether matrix stiffness plays any role in macrophage foam cell formation. Here, we will test the hypothesis that **TRPV4, a calcium-permiable channel, may play a role in macrophage foam cell formation.**

Research Aims

1. Assess whether macrophage foam cell formation is dependent upon TRPV4.
2. Determine whether deficiency of TRPV4 modulates matrix stiffness-induced macrophage foam cell formation.
3. Examine that TRPV4 is expressed and functional in mouse primary macrophages.
4. Test whether matrix stiffness facilitates expression and/or plasma membrane localization of TRPV4.
5. Determine mechanism of TRPV4-dependent foam cell formation

Impacts

TRPV4 is a mechanotransductor and Ca^{+2} permeable channel. It involves in macrophage foam cell formation, which is the most important hallmark of atherosclerosis. By dissecting the molecular mechanism of this complex process, we will advance our knowledge in understanding the underlying mechanism of atherogenesis. Thus, results of this project may contribute to the future development of targeted therapies for atherosclerosis.

Chapter 4: Materials and methods

Reagents and antibody

Antibody to TRPV4 was purchased from Alomone Labs (Jerusalem, Israel) and anti-CD36 antibody was purchased from BD Pharmingen. Goat-anti rabbit Alexa Flour 488 was purchased form Life Technologies. Calcium ionophore A23187 (A23), GSK2193874 (GSK219), GSK1016790A (GSK101), DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramenthylindocarbocyanine per-chlorate) were purchased from Sigma Aldrich (St. Louis, MO, USA). GSK219 worked as TRPV4 antagonist by blocking the TRPV4 mediated Ca^{2+} influx into the cell and GSK101 was used as an agonist by giving rise to Ca^{2+} influx in cell. FLIPR Calcium 6 assay kit was purchased from Molecular Devices (Sunnyvale, CA, USA). Macrophage Colony Stimulating Factor (M-CSF) was purchased from R & D. To prepare copper-oxidized LDL (oxLDL) we incubate LDL (Stem cell Technologies) with CuSO4 (5μM) for 12 h at 37°C as described by Rahaman et al. (2006) (12). Martigen Life technologies (Brea, CA, USA) was the manufacturer of Easy Coat hydrogels of various stiffness (0.5, 8, 50 KPa). We obtained RPMI 1640, DMEM media and cell culture related agents from Gibco.

Animal and Cell culture

TRPV4 knockout (TRPV4 ko) mice, which were originally generated by Dr. Suzuki (Jichi Medical university, Tochigi, Japan), were obtained from Dr. Zhang (Medical College of Wisconsin, Milwaukee, WI). C57BL/6 congenic Wild type mice were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA). Institutional Animal Care and Use committee guidelines

approved by the University of Maryland review committee were strictly followed to conduct all animal experiments. Animals were kept in a 12-hour dark/light cycle in a temperature and humidity controlled pathogen free environment and fed ad libitum. We followed previously described protocol (12, 20) to isolate murine resident macrophages (MRM) and bone marrow derived macrophages (BMDM). Peritoneal lavage was collected by PBS to isolate MRM while BMDM were harvested from mouse bone marrow cells by treating it with RPMI1640 medium with 7-8 days supplementation of M-CSF (50ng/mL) (12, 20, 52, 53). All cells were incubated at 37°C and 5% CO_2.

Intercellular Calcium influx assay

FLIPR Calcium 6 Assay kit (Molecular Device, Boston, USA) was used to measure changes in the BMDM cells. FlexStation system was used to detect signals (32, 34). About 10,000 cells were seeded into each well of 96-well plate with 10% serum containing RPMI supplemented with 50ng/mL M-CSF. Cells were incubated for 24 hours and allow them to adhere with the base. FLIPR Calcium 6 dye in 1X HBSS, 20mM HEPES (pH 7.4) and 2.5 mM probenecid were added after that and incubated for 90 minutes. GSK101, TRPV4 agonist, was used to induce calcium influx into the cell (35, 37) and systolic influx of the calcium was recorded by measuring $\Delta F/F$ (Max-Min) as we have published before (32, 34). Relative fluorescence units (RFU) was used to show the data.

Western Blot Analysis

BMDMs were lysed in modified RIPA buffer which contains 50mM Tris-HCl (pH 7.5), 150mM sodium chloride, 0.5% sodium deoxycholate, 1mM

EDTA, 1mM EGTA, 1% NP-40, 2.5mM Sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4 and 1 µg/mL leupeptide (34). Protein concentration was measured by Bradford method and equal volume of protein were separated in 12% SDS-PAGE gel and then transferred to ploy vinylidene difluoride membrane. Then the membrane was probed with CD36 antibody (1:1000), detected signal with ECL kit (Thermo Fischer Scientific) and an enhanced chemiluminescence system UVP Biospectrum (Upland, CA) was used to visualize and quantify.

Immunofluorescence Analysis

MRMs were seeded on variable stiffness hydrogel coverslip (1 KPa, 8 KPa, 50KPa) and fixed with 3% paraformaldehyde. 1% Triton X-100 was used to permeabilize the cells and 3% BSA/PBS was used as blocker. Samples were incubated with anti-TRPV4 (1:100) IgG followed by goat-anti rabbit Alexa flour 488 (1:300). Cells were mounted by using Prolonged Diamond Antifade DAPI containing reagent (Thermos Fischer Scientific). Cells were visualized at 63X by Zeiss Axio Observer microscope and ImageJ software from NIH was used to quantify immunofluorescence intensity.

Oil Red O Foam Cell Assay

Collagen coated (10µg/mL) glass coverslip or polyacrylamide hydrogels of varied stiffness (0.5 and 8KPa) coverslips were used for foam cell assay. MRM of both WT and TRPV4 ko were seeded on the coverslips in 10% serum containing RPMI 1640 and incubated for 48 hours. Floating cells were washed off and then added 50µg/mL of native LDL (nLDL) as control and oxidized LDL

(oxLDL) followed by 20 hours incubation. After incubation cells were fixed with 10% formalin for 10 minutes and stained with Oil Red O (12, 20) and observed by Zeiss Axio Observer (40X) microscope.

Binding and Uptake of oxLDL

To perform binding assay, 10% SC-RPMI was used to seed MRM cells on glass coverslip and incubate with DiI-oxLDL (5μg/mL) for 60 minutes at 4°C. Cells were washed after incubation and fixed with 10% formalin for 10 minutes. Fluorescent intensity was visualized at 63X by Zeiss Axio Observer microscope and quantified by ImageJ Software (NIH). To examine uptake assay MRMs were treated similarly and seeded on glass coverslip but incubate with DiI-oxLDL for three different time points; 10, 30 and 60 minutes at 37°C (12, 20, 52). Zeiss Axio observer was used to examine the cells (63X) and ImageJ software was used to quantify.

Statistical analysis

All data are expressed as mean ± SEM as indicate below. Statistical comparisons between control and experimental groups were performed with the two tailed Student's t-test or one-way ANOVA using SigmaPlot (Systat Software, San Jose, CA) or Prism software; $p \leq 0.05$ was considered statistical significance.

Chapter 5: Results

OxLDL-induced macrophage foam cell formation is dependent on TRPV4

Macrophage foam cell formation is critically dependent on cytoskeletal remodeling (54, 55). Previous studies have suggested that cytoskeletal modification is crucial for force generation, which is essential for cell migration. This remodeling is dependent on Ca^{2+} influx which is highly dependent on TRPV4. We hypothesized that TRPV4 may play important role in macrophage oxLDL-internalization and subsequently to form foam cells. To test our hypothesis, we compared the foam cell formation in WT and TRPV4 ko MRMs. We found a 7-fold increase in foam cell formation in oxLDL treated WT MRMs compared to nLDL treated cells. oxLDL-induced foam cell formation reduced significantly when TRPV4 is genetically deleted (Fig. 1A & B). This data suggests that presence of TRPV4 channels facilitate oxLDL-induced macrophage foam cell formation.

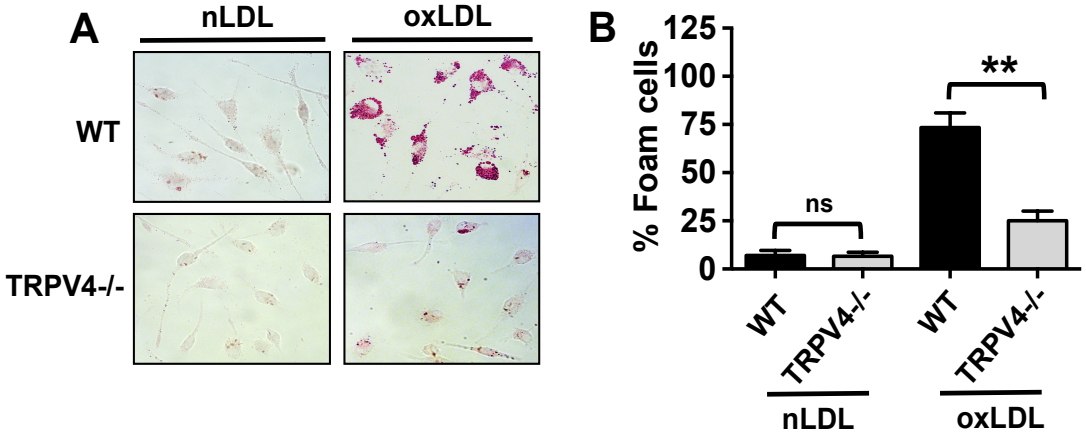


Figure 1. oxLDL-induced macrophage foam cell formation is TRPV4 dependent. **A.** WT and TRPV4 ko primary resident macrophages (MRM) were collected and cultured for 48h, followed by an additional 20h incubation with native low-density lipoprotein

(nLDL, 50μg/mL) or oxidized LDL (oxLDL, 50μg/mL). After fixing the cell, Oil Red O was used to stain and assess macrophage foam cell formation. Five different field per condition were examined and representative micrographs (original magnification 40X) are shown. **B.** Quantitation of results from 1A. Results are expressed as mean ± SEM. **p<0.01 for oxLDL treated TRPV4 ko vs WT cells; n = 500cells/condition.

TRPV4 deficiency protect against matrix stiffness-induced macrophage foam cell formation induced by oxLDL

Matrix stiffness play a critical role in various macrophage functions such as, phagocytosis, motility, cell proliferation and adhesion (22-29). However, the identity of a plasma membrane protein, which also acts as a mechanosensor and transduces the mechanical signal to operate different macrophage function, is not yet characterized. Recent data have shown that TRPV4 channel plays important role in transforming growth factor-β1- induced and matrix stiffness-induced lung and dermal fibroblast differentiation, thus affecting the development of pulmonary or skin fibrosis in mice (32-34). Here we observed that in higher stiffness (8KPa), amount of oxLDL induced foam cell formation in WT MRMs is 3-fold higher compare to low stiffness (0.5KPa). When we compared TRPV4 ko MRMs with WT MRMs, both cell types seeded on higher stiffness (8KPa), we found 2-fold reduction of foam cell formation in TRPV4 ko. No significant difference was observed when we compared WT MRMs with TRPV4 ko MRMs plated on 0.5KPa stiffness gel (Fig 2A & B). This data suggests that matrix stiffness-induced macrophage foam cell formation is dependent on TRPV4.

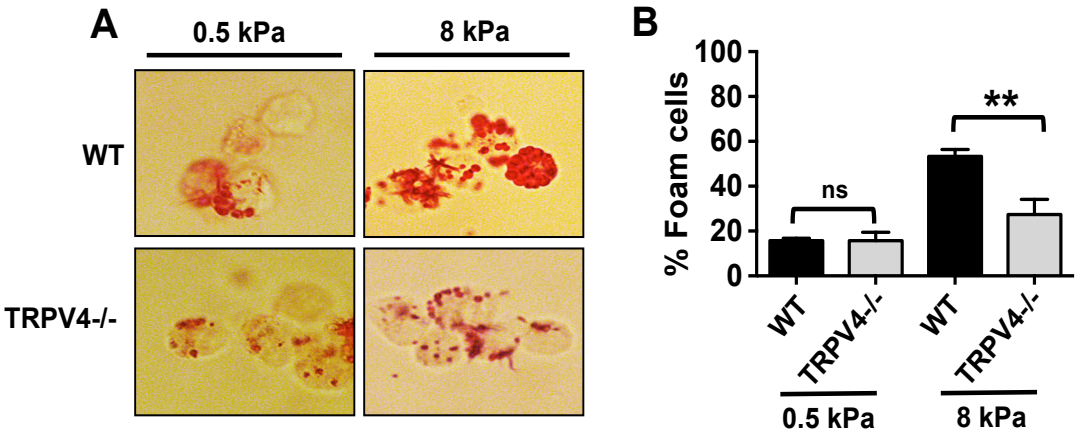


Figure 2. oxLDL-induced macrophage foam cell formation in response to stiffness is regulated by TRPV4. A. WT and TRPV4 ko MRMs were grown on soft (0.5KPa) and stiff (8KPa) hydrogels for 24h and then incubated with oxLDL (50µg/mL) for 24h. Cells were stained with Oil Red O and examined five different field per condition. Representative images (original magnification 40X) are shown. **B.** Quantitation of results from 2A. Results are expressed as mean ± SEM. **p<0.01 for oxLDL treated TRPV4$^{-/-}$ vs WT cells; n = 50cells/condition, Student's t-test.

TRPV4 protein is expressed and functional in mouse primary macrophages

GSK101, a selective TRPV4 agonist, (35) was used to detect the presence of TRPV4-mediated Ca^{2+} influx in macrophages. A rapid (within 20-50 seconds) rise in Ca^{2+} influx in WT BMDM was observed. This influx is concentration dependent, which means with increased dose of GSK101, the Ca^{2+} influx inside the macrophage would increase (Fig 3A). We found that half-maximal effective concentration (EC_{50}) of GSK101 on BMDM is 12nM (Fig 3B). In case of TRPV4 ko BMDM we did not observe any Ca^{2+} influx in presence of GSK101, as expected (Fig 3C). This data provided evidence that TRPV4 is expressed in macrophage and it is functionally active, as shown by previous report (12).

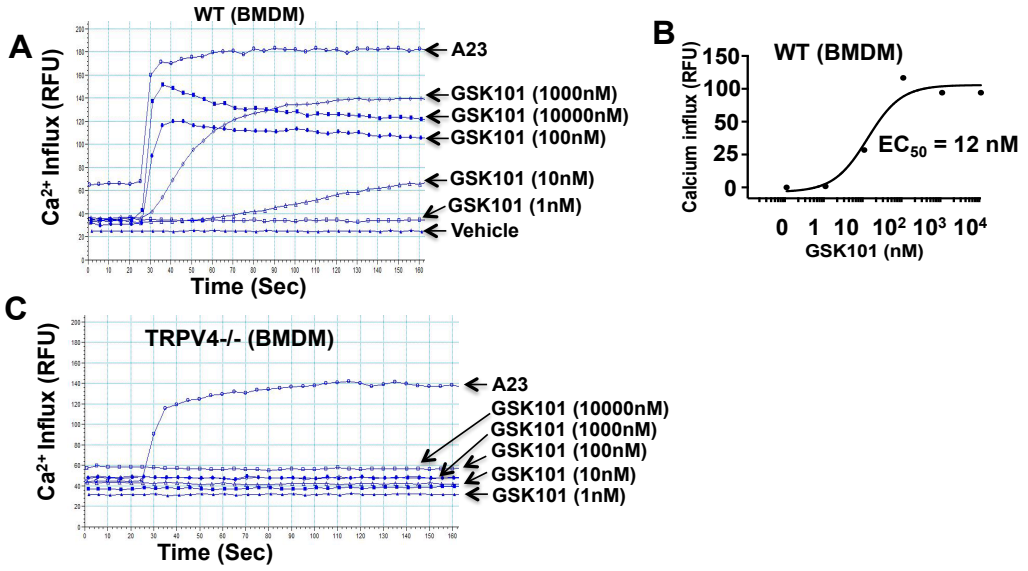
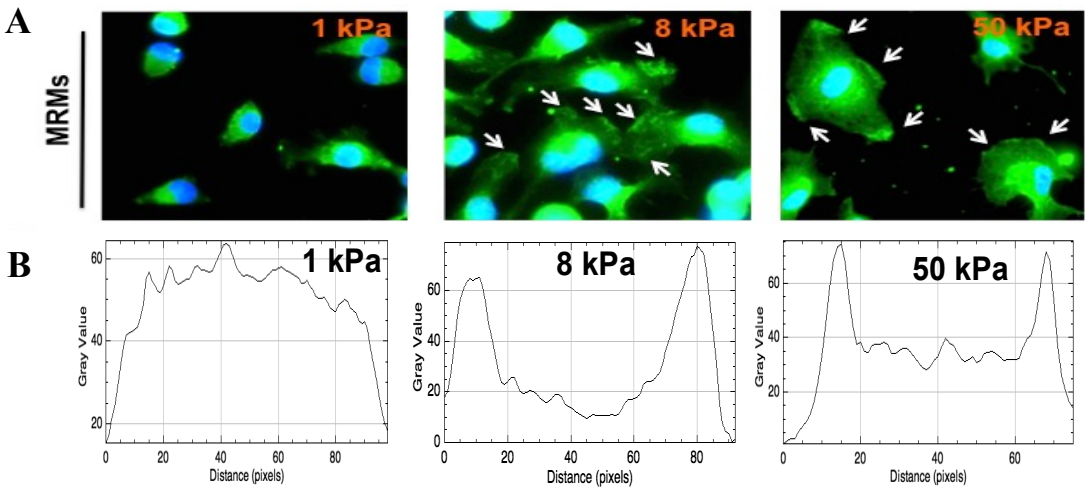


Figure 3. TRPV4 calcium channel is functional in primary normal bone marrow-derived macrophage (BMDM). A & C. BMDM cells from WT and TRPV4 ko mice were loaded with Calcium 6 dye and recorded calcium influx by FlexStation 3. Result showing effects of TRPV4 agonist GSK101 on Ca^{2+} influx of two different cell types. **B.** EC_{50} value of GSK101 for WT BMDM cells were measured. A23187 (A23) is a non-specific calcium ionophore used as a positive control.

Plasma membrane accumulation of TRPV4 is influenced by matrix stiffness

Plasma membrane localization of TRPV4 is influenced by various stimuli (56, 57). Previously, we found that with increasing matrix stiffness, TRPV4's ability to modulate foam cell formation was increased (Fig 2). To understand the mechanism, we examined the plasma membrane localization of TRPV4 with increasing stiffness in WT MRMs. TRPV4 protein was not localized on plasma membrane when we seeded cells on 1kPa matrices. TRPV4 proteins were distributed throughout the cytoplasm of the cell in this case. However, in higher stiffness, such as 8KPa and 50KPa, we found significantly augmented TRPV4 accumulation on plasma membrane. These data suggested that TRPV4 plasma



membrane localization could be facilitated by matrix stiffness. As we know from previous study that CD36 is a major receptor for oxLDL binding and uptake, we tested the expression level of CD36 in WT and TRPV4 ko MRMs by immunoblot analysis. We found no change in the level of CD36 protein expression (Fig 4 C). These data suggest that reduced macrophage foam cell formation in TRPV4 KO cells is not due to lack of CD36 expression.

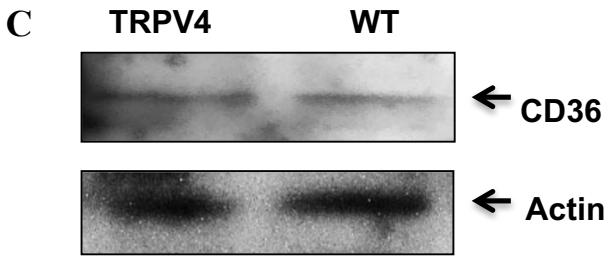


Figure 4. Increasing matrix stiffness enhance plasma membrane localization of TRPV4. A. WT MRM cells were seeded on various stiffness gels (1, 8 and 50 KPa) and incubated for 48h and then stained with anti TRPV4 IgG. Representative fluorescent images (original magnification was 40X; n = 10cells/condition) are shown TRPV4 (green) WT MRMs. **B.** Plot profile using Image J software from TRPV4 stained cells showing plasma membrane localization of TRPV4 grown on stiff (8 and 50KPa) matrix compared to soft (1KPa). **C.** Representative immunoblot from three independent experiments showed CD36 expression in WT and TRPV4 ko BMDM. Loading control was actin.

oxLDL uptake, but not binding, is dependent on TRPV4 in macrophages

In order to be engulfed by macrophages, lipid particles need to be first bound on the surface of macrophages and then subsequently following membrane remodeling lipid particles are taken inside the cell. We examined oxLDL binding and uptake by macrophages to assess the underlying mechanism of TRPV4-dependent macrophage-foam-cell formation. WT and TRPV4 ko MRMs were incubated with DiI-oxLDL for 1 hour at 4°C for binding assay. There was no significant difference in oxLDL binding in TRPV4 ko MRMs when we compare it with WT MRMs (Fig. 5A & B). For uptake assay, WT and TRPV4 ko MRMs were incubated with oxLDL for three different time points (10, 30, 60 minutes). TRPV4 ko MRMs showed significant reduction in DiI-oxLDL uptake compared to WT MRMs with increasing time (Fig. 6A and B). This data suggests that TRPV4 plays a critical role in oxLDL uptake by macrophages but not in its binding.

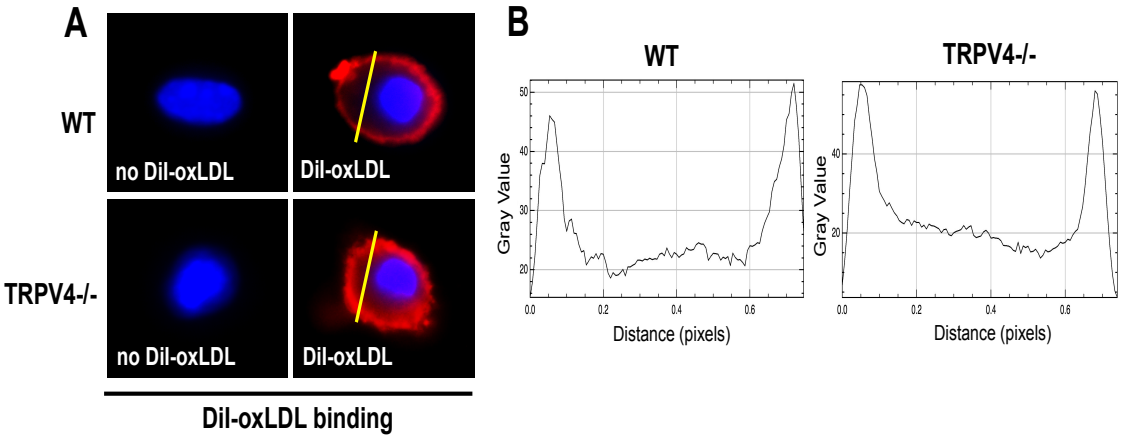


Figure 5. DiI-oxLDL binding to macrophage is not dependent on TRPV4. A. MRMs were collected from WT and TRPV4 ko mice and cells were incubated with or without DiI-oxLDL (2.5µg/mL) for 1h at 4°C to assess oxLDL binding. Representative images of oxLDL bindng on macrophage membrane(n = 10 cells/ condition) are shown. DiI-oxLDL binding indicated by red fluorescence. **B.** Plot profile by using ImageJ software of results of figure 5A.

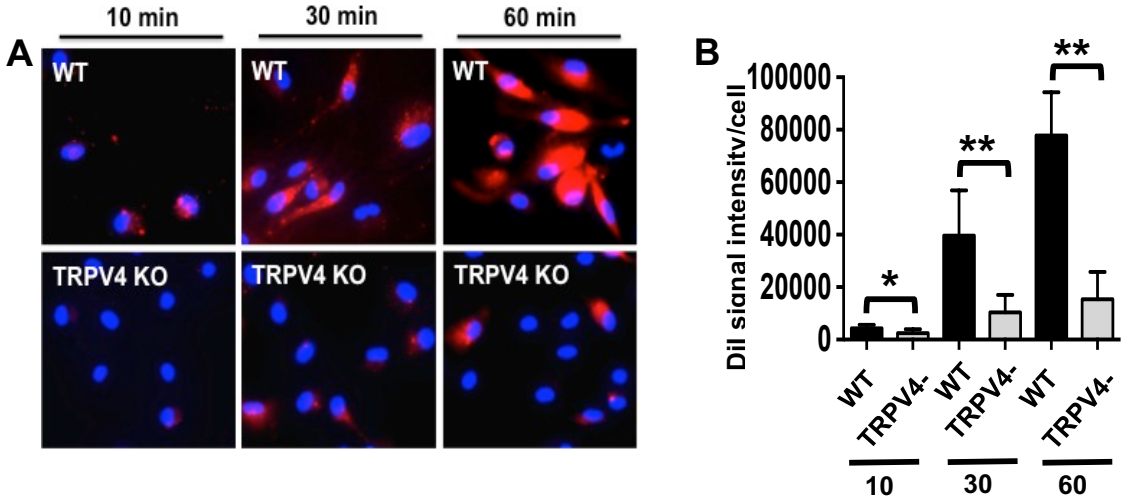


Figure 6. TRPV4 is required for DiI-oxLDL uptake in macrophage. A. MRMs were collected from WT and TRPV4 ko mice and cells were incubated with or without DiI-oxLDL (5μg/mL) for 10, 30 and 60 minutes at 37°C and uptake of oxLDL was assessed. Representative images from five different fields per condition were examined (orginal magnification, 40X). DiI-oxLDL uptake indicated by red fluorescence. **B.** Bar graphs show mean DiI fluorescence intensity (mean±SEM) analyzed using ImageJ software. *p < 0.05 and ***P < 0.001 for oxLDL uptake in TRPV4 ko vs WT cells at respective time points, n = 20 cells/condition.

Chapter 6: Discussion

Results from this study showed that: i) oxLDL-induced macrophage foam cell formation is regulated by TRPV4 calcium-permeable channel; ii) pathophysiologically-relevant stiff matrix increase oxLDL-induced foam cell formation in a TRPV4-dependent manner; iii) TRPV4-dependent calcium influx regulates uptake of oxLDL into macrophage but not its binding to macrophage surface. We also demonstrate that reduced foam cell production in the absent of TRPV4 is not due to lack of expression of CD36, a major receptor for oxLDL. Furthermore, our data showed that plasma membrane localization of TRPV4 is sensitive to matrix stiffness.

Previously, it has been shown that internalization and trafficking of oxLDL inside the macrophage is the main cause of lipid-laden foam cell formation. However, the identity and the nature of the molecular pathways regulating this process are still incompletely understood. Previous study from our laboratory and others have shown that uptake of oxLDL is a scavenger receptor-mediated process (3-5, 9-13). It has been well known that Ca^{2+} signaling plays an important role in various cellular processes, including phagocytosis in various cell types, such as macrophages. To engulf materials, macrophage needs to generate phagocytic cap, which requires extensive actin remodeling inside the cell. It has been reported that this process requires involvement of extracellular Ca^{+2} influx (58, 59). We have also reported in our previous study that oxLDL can increase macrophage foam cell formation in a calcium influx dependent manner (15-20).

Stiffness dependent alteration of gene expression and respective molecular signaling is found to be involved in numerous cellular processes such as, fibrogenesis and oncogenesis (60, 66). During atherosclerosis, arterial epithelial wall becomes stiffer which elevates the risk of atherogenesis and is considered as a marker of atherosclerosis (23-25). Macrophage, an essential cell type in atherogenesis, is known to respond to changes to their surrounding mechanical environment (22-29). As such, it is important to identify the macrophage plasma membrane receptor by which mechanical signal is transduced into cell to promote atherogenesis. As TRPV4 channels can be sensitized by changes in matrix stiffness (22-29), we tested, whether macrophage foam cell formation is dependent on TRPV4. Previous reports on matrix stiffness dependent pro-atherogenic functions such as, phagocytosis, motility and proliferation of macrophages, indicated the role of matrix stiffness in pro-atherogenic activity of macrophages in the context of atherosclerosis (22-29).

TRPV4 activity is known to be regulated by both mechanical (e.g., matrix stiffness) and soluble (e.g., TGFβ1) factors. This membrane protein is ubiquitously expressed in various cell types (29-44). TRPV4 deficiency has been linked to many pathophysiological processes in mice including vasodilatory responses, osmosensing, bone development, neurological functions and lung edema (29-44). Studies by our group recently have shown that TRPV4 knock out mice are protected from bleomycin induced lung and skin fibrosis (32, 33). Different human disease such as skeletal dysplasia and sensory and motor neuropathies have been linked with loss or gain of function of TRPV4 (45-47).

Osmolarity sensing in kidneys, sheer-stress detection in blood vessels, myofibroblast differentiation in lung and skin and control of osteoclast differentiation in bone are few examples where TRPV4's role is shown to be important (29-44). In a recent study, involvement of TRPV4 has been recognized in matrix stiffness sensing and mechanosensing in macrophage function and atherosclerosis (22-29). Here, we found that in response to pathophysiological increase in matrix stiffness, plasma membrane localization of TRPV4 was upregulated in macrophages. We also found that increasing matrix stiffness influenced TRPV4 dependent exacerbation of oxLDL-induced foam cell formation. This could be a potential mechanism how TRPV4 activity is related to atherogenesis. Emerging data suggests role of Ca^{2+} signaling in foam cell formation (15-20) but which Ca^{2+} channels are involved or the mechanism by which Ca^{2+} signals promote atherogenesis is not understood. Interestingly, biochemical signals and mechanical signals both are involved in macrophage activation (22-29). These observations are relevant to the pathophysiology of atherosclerosis, wound healing and fibrosis in general. All these disease phenomena involve injuries that trigger atherogenic/fibrotic responses to increase tissue stiffness, which may subsequently influence overall activation of different cell types including macrophages and fibroblast in a positive feed-forward manner.

Experimental and clinical data suggest that arterial stiffening and atherosclerotic burden has pathophysiological connection (22-29). Matrix remodeling, endothelial dysfunction and insulin resistance shares many

pathophysiological mechanisms with arterial stiffening and atherogenesis. In atherosclerosis, injuries trigger inflammatory responses to increase tissue stiffness, a very important pathophysiology of this disease. TRPV4 activation might be subsequently influenced by the tissue stiffness, which subsequently can increase macrophage foam cell formation in a positive feed-forward manner. The question of how different signaling events, such as matrix stiffness, hyperlipidemia, ROS and inflammation, might co-operate and/or integrate to affect atherogenesis remains to be elucidated. In our study, we found that oxLDL binding to the macrophage is not dependent on TRPV4. This could be due to the similar expression level of CD36, in macrophages. But in contrary, uptake of oxLDL in macrophage, which needs a robust remodeling of actomysin machinery, is significantly dependent on TRPV4. As previously shown, actomysin remodeling is critically dependent on TRPV4 dependent Ca^{2+} signaling during myofibroblast differentiation (32). Thus, in future studies it will be interesting to find out the mechanism by which actomysin modification-dependent processes potentiates oxLDL-induced macrophage foam cell formation in a TRPV4 dependent manner.

Being a ubiquitously expressed protein, TRPV4 has diverse role in different cell types (29-44). According to a recent report, TRPV4 agonist induced rise in cytosolic Ca^{2+} in endothelial cells inhibits vascular inflammation and atherosclerosis in mice model (50). There are evidences that TRPV4 is a modulator of adipose oxidative metabolism and inflammation (50) and deficiency of this protein in mice developed impaired endothelium-dependent

relaxation, induced by acetylcholine (49). All these different results suggest that cell type specific variation of roles of TRPV4 under different pathophysiological conditions. However, our results are consistent with a model that suggests TRPV4 mediated Ca^{2+} influx integrates matrix stiffness signals to promote macrophage foam cell formation, and hence, may contribute to development of atherosclerosis. Although, further studies are required to determine specific pro-atherogenic and inflammatory functions of macrophage TRPV4, herein, we suggest a novel role of TRPV4 calcium-permeable channels in oxLDL-induced macrophage foam cell formation. Our data indicates that TRPV4-dependent foam cell formation in macrophage is sensitive to matrix stiffness. Altogether, our data suggests a novel role of this TRPV4 in atherogenesis. Thus, therapeutic targeting of TRPV4 channel may be a selective approach for the treatment of atherosclerosis.

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