ABSTRACT

Title of Thesis: ROLE OF TRPV4 CALCIUM CHANNEL IN *PORPHYROMONAS GINGIVALIS*-INDUCED EXACERBATION OF OXIDIZED LDL-MEDIATED MACROPHAGE FOAM CELL FORMATION.

Nabyendu Gupta, Master of Science, 2018

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Atherosclerosis, a chronic vascular disease accounts for major deaths worldwide. Associations between atherosclerosis and periodontitis have been documented by epidemiological studies. The process underlying *P. gingivalis* (Pg) infection induced atherogenesis however, is unclear. Macrophage 'foam cells' formation is a hallmark for in-vivo atherosclerosis progression. Our studies indicate that a calcium channel, Transient Receptor Potential channel of the vanilloid family 4 (TRPV4) mediates foam cell generation on Pg lipopolysaccharide (PgLPS) stimulation. Also, we observed increased TRPV4 activity (Ca^{2+} influx) with PgLPS treatment. Genetic deletion of TRPV4 or inhibition by TRPV4 specific chemical antagonist impeded PgLPS-triggered aggravated oxidized LDL (oxLDL) uptake and foam cell formation. Our results mechanistically showed that i) TRPV4 is required for oxLDL uptake, but not its cell surface binding ii) Decreased foam cell formation is independent of CD36 expression. Altogether, our results demonstrate that TRPV4 plays a crucial role in PgLPS-induced exacerbation of oxLDL-mediated macrophage foam cell formation.

ROLE OF TRPV4 CALCIUM CHANNEL IN *PORPHYROMONAS GINGIVALIS*-INDUCED EXACERBATION OF OXIDIZED LDL-MEDIATED MACROPHAGE FOAM CELL FORMATION

by

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List of Abbreviations

- BMDM – Bone Marrow Derived Macrophage
- CD36 – Cluster of Differentiation 36
- CVD – Cardiovascular Disease
- DiI – 1,1'–Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine Perchlorate
- DMEM – Dublecco's Modified Eagle 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
- PgLPS – *Porphyromonas gingivalis* lipopolysaccharide
- ROS – Reactive Oxygen Species
- SDS – Sodium Dodecyl Sulphate
- SR-A – Scavenger Receptor A
- TRPV4 – Transient Receptor Potential Cation Channel Subfamily V Member 4
- WT – Wild Type

Chapter 1: Introduction

A chronic arterial disease, atherosclerosis, is one of the leading causes of death throughout the world [1-4] Cardiovascular disease (CVD) is characterized by atherosclerosis as the underlying pathology. 17.9 million lives are lost each year due to CVD, contributing to 31% of worldwide deaths [70]. Despite accounting for the massive loss of lives, the molecular mechanisms of this disease are not well understood.

A defining event in atherogenesis initiation and progression is macrophage foam cell formation [1]. Macrophagic recognition and ingestion of oxidized low-density lipoproteins (oxLDL) is believed to be a critical pathogenic occurrence in atherogenesis [3]. Monocytic scavenger receptor such as CD36 has a crucial role in the uptake of oxLDL leading to foam cell formation [4, 5]. However, several discrepancies have been reported in scavenger receptor mediated foam cell formation, suggesting additional mechanisms in this process.

Origination of reactive oxygen species (ROS) and heightened oxidative stress has been distinctly associated with atherosclerosis [1-7]. The surface of LDL particles is oxidized by increased ROS production to generate oxLDL which causes its endocytosis by scavenger receptors like CD36 [3-5]. Internalization of oxLDL may also increase ROS generation in a positive feedback loop, ultimately leading to atherosclerotic lesion progression[1-7].

Macrophage differentiation, apoptosis, adhesion, proliferation, migration and inflammatory response is mediated by Ca^{2+} signalling (48, 49). Atherosclerotic lesion generation has been associated with calcium overload (50, 51). Transient Receptor

Potential channel subfamily Vanilloid member 4 (TRPV4) is a cation channel ubiquitously expressed in a variety of cells including macrophages (56-64, 40-47). Recent finding from others as well as our group has shown TRPV4 to be allied with various cellular and pathological conditions (40-47, 56-64).

Association between periodontitis and atherosclerosis has been correlated by epidemiological studies (17-22). Despite limiting the traditional cardiovascular disease (CVD) risk factors, atherogenesis in animal models has been linked to *P. gingivalis* spawned periodontitis infection (23-26). However, the mechanisms underlying periodontitis induced atherosclerosis is not clearly defined. Thus, we hypothesize that TRPV4 has an essential function in regulating PgLPS mediated foam cell formation.

The objective of this study was to test the role of TRPV4 calcium channel in *P. gingivalis*-induced exacerbation of oxidized LDL-mediated macrophage foam cell formation. To investigate the role of this calcium channel we have carried out several in vitro and in vivo experiments utilizing bone marrow derived murine macrophages, resident murine macrophages, macrophage cell lines (RAW cells). We made several key observations: i) our findings elucidated that PgLPS induced exacerbation of oxLDL mediated foam cell formation is TRPV4 dependent; ii) PgLPS influenced TRPV4 specific calcium influx is dependent on matrix stiffness; iii) Treatment of PgLPS causes increased expression of TRPV4 protein; and iv) Foam cell formation is independent of CD36 expression levels. TRPV4 is essential for oxLDL uptake.

Chapter 2: Literature Review

Atherosclerosis represents the predominant cause underneath heart disease and stroke. Early lesions of atherosclerosis are characterized by endothelial dysfunction followed by cholesterol rich low-density lipoprotein (LDL) retention [1, 2]. Inflammation in the arterial intima causes oxidation of LDL (oxLDL). Scavenger receptor such as CD36 present on the surface of macrophages recognizes these modified LDL and subsequent engulfment ensues, resulting in "lipid-loaded macrophage foam cell" [1, 3]. Continuous deposition of foam cells, calcium, necrotic debris and cellular waste products leads to atheroma progression and ultimately culminates in development of atherosclerosis related vascular pathologies [3, 6, 7].

A variety of inflammatory secretions generating from macrophages makes them critical regulators of atherogenesis [3, 4]. In previous studies it has been shown that a distinctive feature of atherosclerotic murine model is macrophagic proliferation and accumulation in advanced lesions [8]. Decrease in foam cell formation or reduced oxLDL uptake in mice has been attributed to CD36 or SR-A deficient macrophages [9-12]. Nonetheless, several discrepancies have been reported regarding the role of scavenger receptors in atherosclerosis [13]. ApoE knock out mice for example lacking CD36 or SR-A resulted in an increased risk of atherosclerosis and foam cell formation [14]. Altogether, these reports indicate complex nature of atherogenesis and also suggest that additional mechanisms may be involved in atherosclerotic lesion development and progression.

Chronic inflammation coupled by oxidative environment escalates the generation of reactive oxidative species (ROS). Thus, this process can increase overall

oxidative stress. Atherogenesis and oxidative stress has been distinctly correlated [1-7]. Increased ROS production causes heightened generation of oxidized phospholipids on the surface of LDL particles forming atherogenic oxLDL which is specifically recognized and internalized by macrophages with the help of SR-A and CD36 [3, 5]. Heightened ROS production and oxLDL uptake may cause mitochondrial damage leading to apoptosis in macrophages as well as necrotic core formation [1-7].

Recent studies have suggested that P. *gingivalis* infection might be associated with atherosclerosis [15-26]. Nearly 100 million people are affected in the United States by periodontitis [17-22]. Despite controlling traditional risk factors of Cardiovascular disease (CVD), associations between periodontitis and atherosclerosis have been supported by epidemiological studies and animal models [17-22]. Arterial stiffness and periodontitis have been linked in other studies as well [27-31]. Macrophages can directly interact with their surrounding mechanical environment [32-38]. Interestingly, bacterial LPS is shown to increase macrophage rigidity and vascular stiffness [35, 39].

Various macrophagic functions such as apoptosis, cell differentiation, adhesion, proliferation, migration and inflammation involve Ca^{2+} signalling [40, 41]. Increased calcium influx is identified with atherosclerotic lesion generation, macrophage foam cell formation and phagocytosis [42-44]. Several channels are present in macrophages to regulate Ca^{2+} homeostasis [45].

Ca^{2+} permeable membrane channel, TRPV4, is ubiquitously expressed in various cells including macrophages [45-53]. TRPV4 has been linked to various diseases such as motor neuropathies and skeletal dysplasia [47-49]. This channel has

also been linked to eNOS activation in endothelial cells, as well as preventing monocyte adhesion to these cells, suggesting an atheroprotective role [50]. Increased occurrence of endothelial dysfunction and vascular disease is observed with impaired TRPV4 channel [51, 54, 55]. In this study we demonstrate that *Porphyromonas gingivalis* LPS-induced increase in oxLDL uptake and macrophage foam cell formation is TRPV4 dependent.

Chapter 3: Research Objectives

Epidemiological studies have shown that there is a link between *Porphyromonas gingivalis* (Pg) infection, causative agent of Periodontitis and Atherosclerosis. However, it is not well known how Pg infection advances this cardiovascular disease. Here, we have elucidated the **role of TRPV4 calcium channel in Pg-induced macrophage foam cell formation, a critical process in atherogenesis.**

Research Aims

1. Examine whether *P. gingivalis* lipopolysaccharide-induced oxLDL mediated macrophage foam cell formation is reliant on TRPV4.
2. To determine TRPV4-dependent Ca^{2+} influx in response to *P. gingivalis* lipopolysaccharide.
3. Investigate if lack of foam cell formation in TRPV4 deficient cells is due to lack of expression of CD36.
4. Determine if TRPV4 protein expression is modulated by PgLPS treatment.
5. Determine whether TRPV4 is required for oxLDL uptake.

Impacts

TRPV4 has been indicated as a novel plasma membrane Ca^{2+} channel involved in foam cell formation, a critical event in atherosclerosis. Comprehending the underlying pathology of periodontitis fostered atherosclerosis, might assist us in uncovering disease pathogenesis mechanisms and assessing fatalities. Hence, these findings can have a major impact in the discovery of selective therapeutics to reduce the disease burden around the globe.

Chapter 4: Materials and methods

Cell culture and Animals:

TRPV4 knockout (TRPV4KO) mice had been originally generated by Dr. Suzuki (Jichi Medical University, Tochigi, Japan) on C57BL/6 [52] and given to us by Dr. Zhang (Medical College of Wisconsin, Milwaukee, WI). Congenic Wild Type (WT) C57BL/6 mice were bought from Charles River Laboratories (Wilmington, Massachusetts, USA). Animal experimentation were conducted by guidelines issued by Institutional Animal Care and Use Committee and approved by University of Maryland review committee. Previously described protocols [12, 56] were followed to collect Murine Resident Macrophages (MRM) and Bone Marrow Derived Macrophages (BMDMs). PBS was injected into mouse peritoneal cavity to collect MRMs. Bone Marrow cells yielded BMDMs by harvesting the cells in RPMI1640 media with M-CSF supplementation for 7-8 days and incubated at 37^{o}C and 5% CO_2.

Agonists, Antagonists, Antibody and Reagents:

TRPV4 agonist GSK1016790A (GSK101) and antagonist GSK2193874 (GSK219), DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and calcium ionophore A23187 (A23) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRPV4 antibody were obtained from Alomone Labs (Jerusalem, Israel) and anti-β-actin antibody was supplied by Santa Cruz Biotechnology (Dallas, TX, USA). CD36 antibody was purchased from BD Pharmingen. LDL was incubated with $CuSO_4$ (5μM) for 12h at 37^{o}C to produce copper-oxidized LDL (oxLDL) [12]. Macrophage Colony Stimulating Factor (M-CSF) was obtained from R & D. Molecular

Devices (Sunnyvale, CA, USA) provided us FLIPR Calcium 6 assay kit. Western Blot Reagents were bought from Bio-Rad Laboratories (Hercules, CA, USA). RPMI1640 media as well as other cell culture reagents were purchased from Gibco. Varying stiffness (0.5-50 kPa) easy coat polyacrylamide hydrogels had been obtained from Martigen Life Technologies (Brea, CA, USA). InvivoGen (San Diego, CA, USA) was the manufacturer of PgLPS.

Intracellular Calcium measurement:

Measurement of calcium influx responses in BMDM cells was done with FLIPR 6 Calcium Assay Kit. Collagen coated 96 well clear bottomed plates with 10% serum containing RPMI media and 25 ng/ml M-CSF were used for seeding cells. 6-7 days were given for cellular differentiation and adherence. Media was changed to 0.5% serum containing RPMI and 500ng/ml PgLPS to appropriate wells. After incubation for 24hours, cells were again incubated for 90 minutes in FLIPR Calcium 6 dye in 1X HBSS, 20mM HEPES (pH 7.4) and 2.5 mM probenecid. GSK101 was used as a specific TRPV4 agonist (increasing cytosolic calcium) and GSK219 was used as TRPV4 antagonist. Relative Fluorescence Unit (RFU) was used as a means of measuring cytosolic Ca^{2+} influx [57-59].

Foam Cell Assay:

WT and TRPV4KO MRMs were seeded on collagen coated (10µg/ml) glass coverslips (in 12 well plates) or on polyacrylamide hydrogels of varying stiffness (0.5kPa and 8kPa). Cells were incubated for 48 hours in 10% serum containing RPMI. Addition of 50µg/ml native LDL (nLDL) as control and oxidized LDL (oxLDL) in the

presence or absence of 500ng/ml PgLPS followed by 20 hours of incubation [57-59]. Cells were fixed using 10% formalin for 10 minutes and stained with Oil Red O and counted by Zeiss Axio Observer (40x) microscope [12, 58].

Western Blot Analysis:

BMDM cells incubated in the presence or absence of PgLPS (0, 250, 1000 ng/ml) for 48 hours were washed with ice cold PBS and lysis buffer composed of 20mM Tris-HCL (pH 7.5), 1mM EGTA, 1mM EDTA, 150mM NaCl, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1% NP-40, 1mM NP-40, 2.5mM $Na_4P_2O_7$ 1µg leupeptin and 1mM Na_3VO_4 [12]. Same amounts of protein lysate were resolved in a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane which was probed with TRPV4 and CD36 antibodies. Signals were detected using an ECL kit (Thermo Fischer Scientific) and quantified using Image J software.

Real Time quantitative polymerase chain reaction (qRT-PCR):

RNeasy Micro Kit (Qiagen) was used to extract total RNA from WT and TRPV4KO cells. One step qRT-PCR analysis done by QuantiNova SYBR Green qRT-PCR Kit (Qiagen). Following the manufacturer's instructions, qRT-PCR was performed using TRPV4, CD36 and GAPDH primers (TaqMan gene expression assay, Applied Biosystems). TRPV4 and CD36 expression was measured as the amount of TRPV4 or CD36 relative to mRNA for GAPDH by comparative C_T method outlined in ABI 7900 HT sequence detection system user bulletin.

oxLDL uptake:

For investigating uptake assay, MRMs were seeded on glass coverslips in the presence or absence of 500ng/ml PgLPS and incubated with DiI-labeled oxLDL (DiI-oxLDL) (5µg/ml) at 37^{o}C for 30 minutes[12, 44, 56]. Zeiss Axio Observer Microscope (63x) used to visualize fluorescence. Quantification done through ImageJ software.

Statistical Analysis:

All data are represented as mean ± SEM. Statistical comparison experimental and control groups were performed using Student's t-test or ANOVA using SigmaPlot or Prism software. $p<0.05$ was considered statistically significant.

Chapter 5: Results

PgLPS-induced oxLDL mediated macrophage foam cell formation is reliant on TRPV4

Ca^{2+} signalling is vital in macrophage foam cell formation [36, 42-44]. Gram negative bacterial outer membrane is composed of highly inflammatory lipopolysaccharide (LPS) molecules that induce Ca^{2+} influx and generate foam cells [60-62]. Our hypothesis was TRPV4 might be involved in PgLPS influenced ingestion of oxLDL to form foam cell. We tested our hypothesis by comparing foam cell formation in TRPV4 KO and WT MRMs with or without PgLPS. A 4-fold increase in foam cell generation was observed in oxLDL-treated versus nLDL (native LDL)-treated WT MRMs (Fig. 1A & B). PgLPS and oxLDL when administered together compounded foam cell production with respect to untreated controls (Fig. 1A & B). Genetic deletion of TRPV4 significantly reduced macrophage foam cell formation, irrespective of oxLDL treatment alone or in combination with PgLPS (Fig. 1A & B). Pharmacological inhibition of TRPV4 channel by GSK219 reduced foam cell formation (Fig. 2A & B). These results correlate TRPV4 as a critical player in PgLPS-induced oXLDL-mediated macrophage foam cell formation.

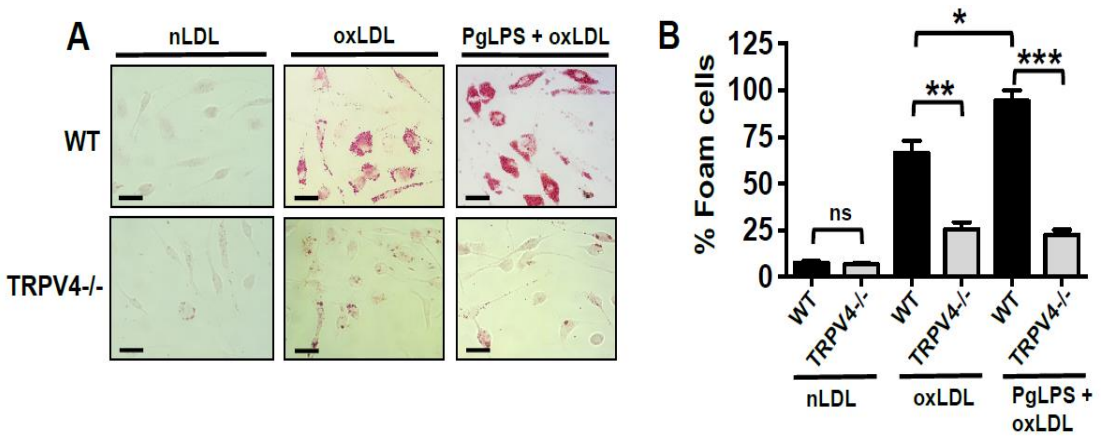

Figure 1. **PgLPS-induced oxLDL mediated macrophage foam cell formation is reliant on TRPV4. (A)** Murine resident macrophages from WT and TRPV4 KO mice were isolated and incubated for 48 hours in the presence or absence of PgLPS (500ng/ml), followed by a subsequent 20 hours incubation with native low-density lipoproteins (nLDL, 50µg/ml) or oxidized low-density lipoprotein (oxLDL, 50µg/ml). Macrophage foam cell formation was measured by fixing the cells and staining them with Oil Red O. Representative images shown were obtained from five different fields per condition (40X original magnification). Results expressed as mean ± SEM. ***p<0.001 for oxLDL treated WT vs TRPV4 KO cells with PgLPS, **p<0.01 for oxLDL treated WT vs TRPV4 KO cells and *p<0.05 oxLDL treated vs untreated WT cells without PgLPS; n = 500 cells per condition (**B)** Results quantified from 1A.

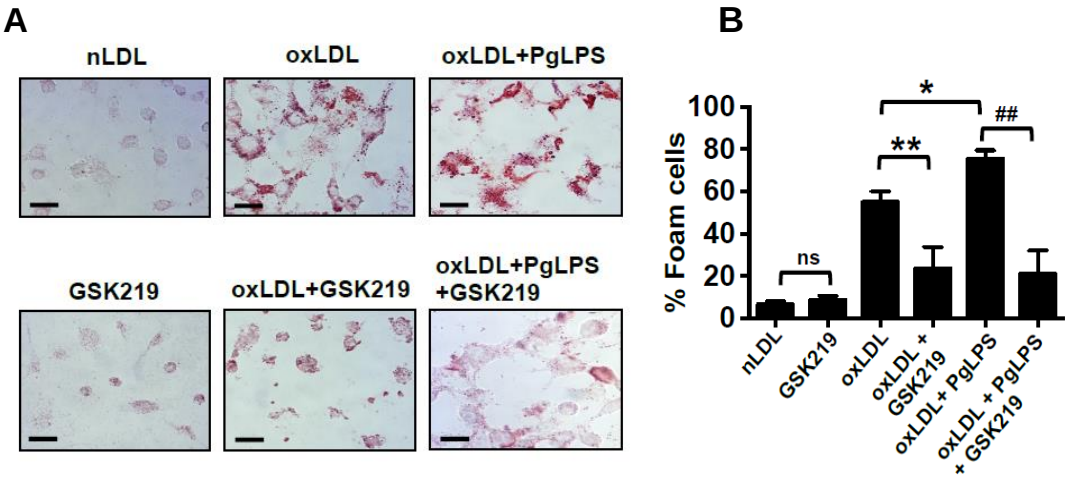

Figure 2. **PgLPS-induced oxLDL mediated macrophage foam cell formation is inhibited by TRPV4-specific inhibitors. (A)** Cells were cultured similarly as above and treated with TRPV4 specific antagonist, GSK219 and nLDL (50µg/ml) or oxLDL (50µg/ml). Oil Red O stained cells. Representative images shown were obtained from five different fields per condition (40X original magnification). **(B)** Results quantified from 2B. Results expressed as mean ± SEM. ##p<0.001 for oxLDL, PgLPS and GS219 treated cells vs oxLDL and PgLPS cells, **p<0.01 for oxLDL treated vs oxLDL plus GSK219 treated cells and *p<0.05 for oxLDL treated vs PgLPS plus oxLDL treated cells; n = 500 cells per condition.

TRPV4 dependent Ca^{2+} influx is induced by PgLPS and dependent on matrix stiffness

TRPV4 specific agonist GSK101 was used to measure Ca^{2+} influx in BMDMs in presence or absence of PgLPS [53]. We observed increased influx of Ca^{2+} in PgLPS treated cells in comparison to untreated cells. (Fig. 3A & B). TRPV4 KO BMDMs on the other hand did not show Ca^{2+} influx. GSK101 induced Ca^{2+} however, was blocked by GSK219, a TRPV4 selective antagonist in PgLPS treated BMDMs [53] (Fig. 4A). These observations indicate that the loss of TRPV4 function either by pharmacological inhibition or genetic deletion blocks PgLPS induced Ca^{2+} influx. Our results also showed that PgLPS induced TRPV4 channel activity was increased on increasing the matrix stiffness when BMDMs were plated on stiffer matrices (1-25kPa). However, TRPV4 KO cells showed abrogated Ca^{2+} influx (Fig. 4B). These data confirm the induction of TRPV4 specific Ca^{2+} influx on effect of PgLPS and matrix stiffness.

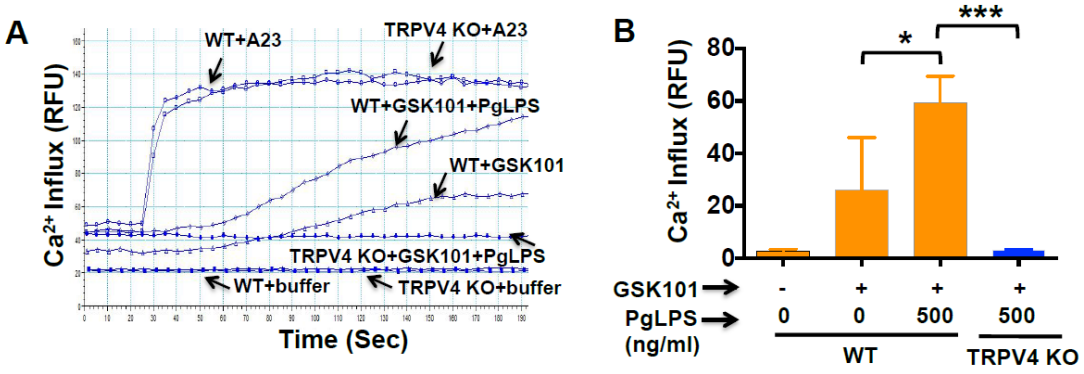

Figure 3. TRPV4 dependent Ca^{2+} influx is induced by PgLPS. (A) BMDMs loaded with Calcium 6 dye, showing effect of GSK101 (10nM) on Ca^{2+} influx in TRPV4Ko and WT cells in the presence or absence of PgLPS. **(B)** Results quantified from 3A. *p<0.05 for PgLPS treated WT vs untreated WT cells, ***p<0.001 for PgLPS treated WT vs TRPV4 KO cells.

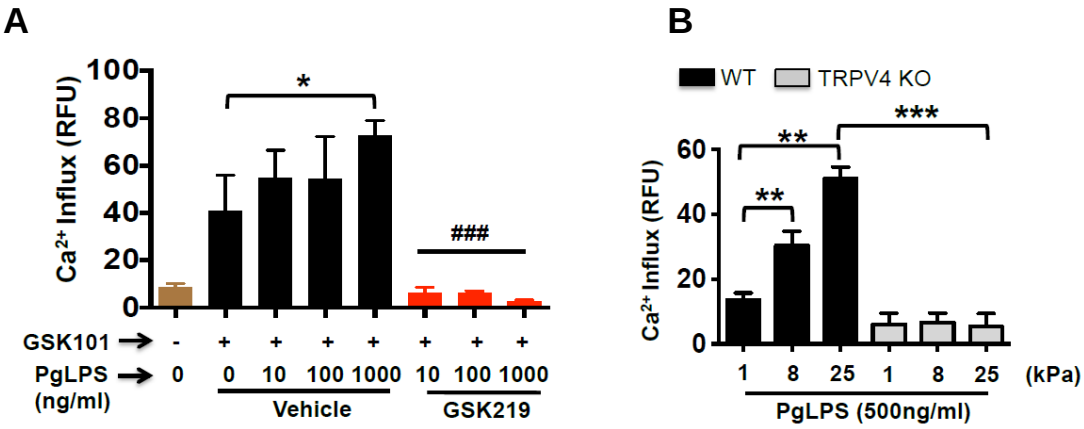

Figure 4. TRPV4 dependent Ca^{2+} influx is induced by PgLPS and dependent on matrix stiffness. (A) Blocking PgLPS induced Ca^{2+} influx by TRPV4 specific GSK219. *p<0.05 for PgLPS treated WT vs untreated WT cells, ###p<0.001 for PgLPS treated WT vs PgLPS plus GSK219 treated WT cells. **(B)** TRPV4 KO and WT MRMs were plated on variable stiffness (1, 8 and 25kPa) hydrogels. GSK101 (10nM) mediated Ca^{2+} influx was assessed. **p<0.01 for soft (1kPa) vs stiff (8kPa and 25kPa) hydrogel grown cell, ***p<0.001 for WT vs TRPV4 KO cells grown on 25kPa hydrogels. A23187 (A23) is a non-specific Ca^{2+} calcium ionophore. Experiments were replicated three times.

CD36 expression does not play a role in decreased foam cell formation in TRPV4 absent cells

CD36 is considered to be a major scavenger receptor involved in macrophage foam cell formation. Thus, we tested the expression levels of CD36 by qRT-PCR and western blot analysis in WT and TRPV4Ko macrophages. Expression levels of CD36 mRNA as well as CD36 protein were equal in WT and TRPV4 KO cells which were treated with or without PgLPS for 24 hours (Fig. 5A & B).

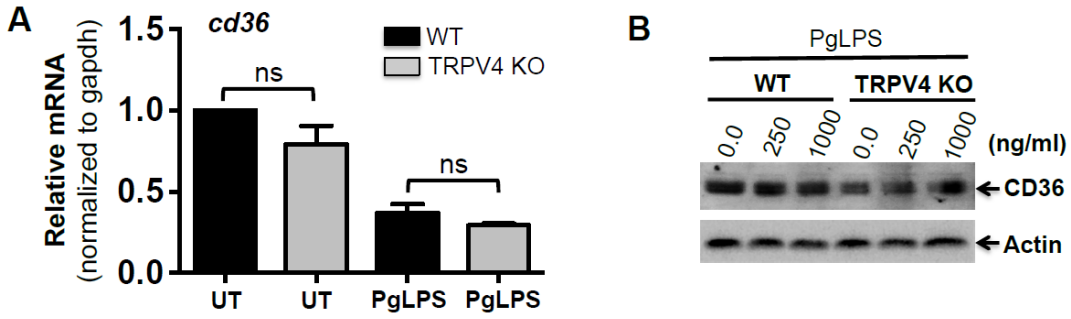

Figure 5. CD36 expression does not play a role in decreased foam cell formation in TRPV4 absent cells. (A) CD36 expression level in WT and TRPV4 KO MRMs determined by qRT-PCR analysis. TaqMan Gene Expression Assay used. C_T values normalized to GAPDH. **(B)** CD36 protein expression was shown by representative immunoblots from three independent experiments conducted in WT MRMs in the presence or absence of PgLPS treatment. Actin was used as loading control.

TRPV4 protein expression is increased with increased PgLPS stimulation

TRPV4 expression level was measured to deduce the role of TRPV4 in macrophage foam cell formation. We observed similar TRPV4 mRNA expression levels in the presence or absence of PgLPS for 24 hours (Fig. 6A). Protein expression of TRPV4 was increased with PgLPS treatment in a dose dependent manner (Fig. 6B & C).

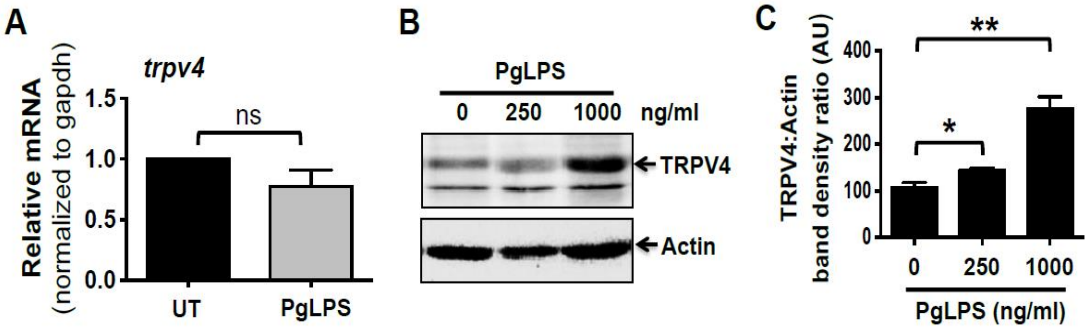

Figure 6. TRPV4 protein expression is increased with increased PgLPS stimulation. (A) TRPV4 levels in WT MRMs with or without PgLPS were measured by qRT-PCR analysis using TaqMan Gene Expression Assay. C_T values normalized to GAPDH. **(B)** TRPV4 protein expression depicted by representative immunoblots from three independent experiments conducted in WT MRMs with or without treatment of PgLPS. Actin was used as loading

control. **(C)** Quantification of results from 6B. Results expressed as mean ± SEM. *p<0.05 for 250 ng/ml PgLPS treated vs untreated cells **p<0.01 for 1000 ng/ml PgLPS treated vs untreated cells.

Macrophage oxLDL uptake is TRPV4 dependent

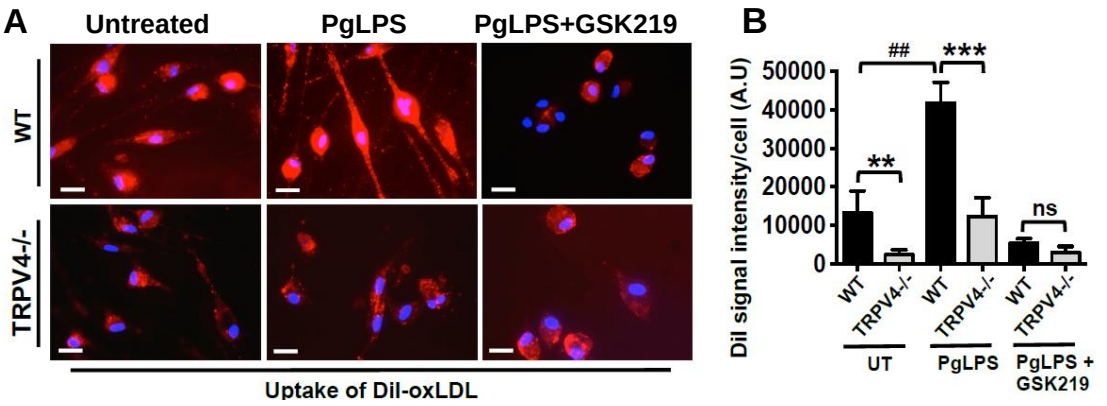

Figure 7. Macrophage oxLDL uptake is TRPV4 dependent. (A) MRMs isolated from WT and TRPV4 KO mice were incubated with DiI labelled oxLDL (5µg/ml) at 37°C for 30 minutes. Representative images shown were obtained from five different fields per condition (40X original magnification). Uptake of oxLDL was measured by red fluorescence. **(B)** Quantification results from 7A depicted by bar graph showing mean DiI fluorescence intensity (mean ± SEM) (ImageJ software, NIH). **p<0.01 for TRPV4 KO vs WT untreated cells, ##p<0.01 for PgLPS treated WT vs WT untreated cells, ***p<0.001 for PgLPS treated WT vs PgLPS treated TRPV4 KO cells; n = 20 cells per condition.

Chapter 6: Discussion

The results of this study concluded that i) macrophage foam cell formation by PgLPS stimulation is reliant on TRPV4; and ii) PgLPS treatment increases TRPV4-mediated Ca^{2+} influx in a matrix stiffness dependent manner. We also showed that i) decreased foam cell formation in TRPV4 deficient cells is independent of CD36 expression; and ii) increased PgLPS stimulation increases TRPV4 protein expression. In addition, we demonstrated that oxLDL uptake in PgLPS-treated macrophages is dependent on TRPV4.

Individuals with chronic periodontal disease have the immunomodulatory molecule *Porphyromonas gingivalis* lipopolysaccharide present in their blood stream. Prior findings have reported that PgLPS exposure augments binding and uptake of oxidized LDL, initiates macrophage foam cell formation, leads to M1 macrophage infiltration and macrophage regulated inflammation in the affected tissues [15-26, 62]. Our group and others previously have shown an association between foam cell formation in macrophages and oxLDL uptake [12, 43, 44]. A variety of cellular processes are governed by Ca^{2+}, including macrophage phagocytosis and foam cell formation [42, 44]. Studies have shown that Ca^{2+} influx and foam cell formation is aggravated by oxLDL [42, 44]. These effects however, were negated by non-specific Ca^{2+} channel inhibitors [44, 59, 63]. Recently, we demonstrated the requirement of TRPV4 in oxLDL mediated macrophage foam cell formation [58]. This current study specifically addresses role of TRPV4 in PgLPS and matrix stiffness-mediated macrophage foam cell formation.

TRPV4 has been documented to have an atheroprotective role whereby, the channel activation inhibits monocyte adhesion and causes eNOS activation in endothelial cells [50]. On the other hand, TRPV4 channel mis regulation has been linked to reduced macrophage foam cell generation, vascular diseases and endothelial dysfunction [51, 54, 55, 58]. Yet, the mechanism underlying TRPV4-mediated macrophage foam cell formation by PgLPS stimulation is not known. This study discovered that TRPV4 protein expression and Ca^{2+} intake is increased with increased PgLPS stimulation. TRPV4 dependent calcium influx activity was increased on exposure of PgLPS to cells in a matrix stiffness-dependent manner. Our findings are consistent with a previous report showing sensitivity of macrophage inflammatory responses to matrix stiffness [36]. We have also shown that uptake of oxLDL is TRPV4 dependent. Pharmacological inhibition (GSK219) or genetic deletion of TRPV4 (TRPV4 KO) nullifies calcium intake and oxLDL internalization when BMDMs are grown on a stiff matrix substrate.

TRPV4 cation channel is expressed ubiquitously in different cell types. Activity of this protein is influenced by numerous biological and mechanical stimulus [46-53, 55, 57-59, 64-67]. TRPV4 deficient mice have been reported to have various pathological conditions including abnormal osmosensing, bone development, vasodilatory responses and lung edema [46-53, 55, 57-59, 64-67]. Our group has shown that TRPV4 null mice are protected from lung and skin fibrosis induced by bleomycin [57, 68]. In another study LPS treatment has been linked to increasing stiffness of lung tissue in mice [39]. Combined clinical and experimental data indicates a connection between atherosclerotic burden and arterial stiffness [27-31]. Latest studies related to

atherogenesis have pointed out the critical role of mechanical factors, e.g. matrix stiffness, and biochemical factors (lipopolysaccharide, LPS) in disease development and progression [27-38, 69]. These results put forward a potential mechanism to augment TRPV4 activity during PgLPS influenced atherogenesis.

Previously, results from our laboratory and others showed matrix stiffness induces several atherogenic macrophagic functions such as motility, proliferation and phagocytosis [32-38]. The current study has identified TRPV4, a plasma membrane Ca^{2+}-permeable channel as having a crucial role in regulating pro-atherogenic responses associated with pathogenesis of periodontitis-induced atherosclerosis. Additional studies however, are required for understanding the exact mechanisms underlying periodontitis-influenced atherogenesis to establish novel therapeutic medications.

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