Is there a role for myeloid cell-derived versican in diabetes-accelerated atherosclerosis?

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Abstract

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Cardiovascular diseases, caused by atherosclerosis or hardening of the arteries, are the number one cause of death in the world, causing over 30% of deaths. Diabetes is also becoming an increasingly common disease with nearly 10% of Americans diagnosed with diabetes. Diabetes is known to increase macrophage expression and inflammatory activation leading to increased risk of death by 50%. Macrophages are important inflammatory response cells that are present in every stage of atherosclerosis and may become a target for emerging strategies to combat atherosclerosis. Versican is an extracellular matrix protein that is found in higher levels in diabetic individuals and is associated with increased atherosclerosis. Therefore, it is hypothesized that if the versican gene is knocked out, there will be fewer apoptotic cells, less inflammation, and slower atherosclerotic progression. To determine how versican and diabetes affect lipid loading, inflammation, and apoptosis, a combination of cholesterol assays; versican staining; qPCR for *Il1b, Il6, Ccl2,* and *Il110;* TUNEL; and necrosis/macrophage viability was utilized. Overall, diabetes increased necrotic core and cholesterol loading, but did not alter lesion size. Diabetes also increased expression of *Il1b, Ccl2,* and *Il10.* While versican reduced necrotic core size under diabetic conditions, it did not alter lipid or cholesterol loading nor inflammatory cytokine expression for *Il1b, Il6, Ccl2,* or *Il10.* In addition, there was no significant effect of diabetes or versican on the number of

apoptotic cells associated with macrophages or free within the plaque. Finally, diabetes, but not versican-deficiency, increased necrosis in isolated peritoneal macrophages. Altogether, these results suggest that diabetes has a greater effect on lipid loading and inflammation. The presence of versican in diabetic and inflammatory conditions has been observed, but its role in the progression of these diseases is still not fully understood. Additional research is needed to further evaluate the impact of versican on efferocytosis and atherosclerotic progression.

Introduction

Cardiovascular diseases are the number one cause of death in the world, causing over 30% of deaths globally and steadily increasing. The most common of these diseases, coronary heart disease (heart attack) and stroke, account for over 75% of these deaths (3). Diabetes, another major disease of concern globally, caused over 1.5 million deaths in 2014 (4). The global prevalence of diabetes has increased from 4.7% in 1980 to 8.5% in 2014 and is estimated to increase 48% globally by 2045 (5, 6). In the United States, over 30 million people had diagnosed diabetes in 2014, leading to over 7 million hospital visits and costing more than $245 billion dollars in direct and indirect medical costs (6, 7). Additionally, diabetes puts people at a 50% increased risk of death from other causes, such as cardiovascular disease (7). Diabetes increases the risk of cardiovascular diseases by accelerating the underlying process, known as atherosclerosis.

Atherosclerosis is the underlying pathology resulting in cardiovascular disease

Atherosclerosis, the underlying pathology of cardiovascular disease, is a chronic inflammatory disease that is characterized by progressive plaque buildup inside the arteries. The exact causes of atherosclerosis are not entirely known, but diet, lack of exercise, elevated blood pressure, diabetes, smoking, age, and genetics are contributing risk factors for development (8). Untreated, atherosclerosis can lead to blockage of arteries causing heart attack, stroke, or death (9).

Atherosclerotic initiation, the key first step in atherogenesis, predominately occurs in areas that have turbulent blood flow, such as artery branch points or curves, where apolipoprotein B (apoB)-containing lipoproteins enter the subendothelial space, bind to proteoglycans of the extracellular matrix (ECM), become retained, and accumulate. Accumulation triggers a low-grade inflammatory response (8-10) (Figure 1A). These particles are prone to modifications by proteases, lipases, or reactive oxygen species that cause these modified lipoproteins to activate nearby endothelial cells and macrophages, upregulating adhesion molecule and chemokine secretion, and monocyte recruitment (11, 12). Bone marrow-derived monocytes transmigrate to the area of inflammation and, under macrophage colony-stimulating factor (M-CSF) and

granulocyte-macrophage stimulating factor (GM-CSF), differentiate into macrophages (12). Monocyte recruitment also leads to platelet adhesion and chemokine secretion, which stimulates migration, accumulation, and proliferation of vascular smooth muscle cells (SMC) and leukocytes, causing progression of atherosclerosis (13).

Monocyte-differentiated macrophages can ingest trapped lipoproteins via phagocytosis, scavenger receptor-mediated uptake or pinocytosis (Figure 1B)(14, 15). Within the macrophage, lipoproteins are degraded, and the cholesteryl esters are hydrolyzed to cholesterol. Cholesterol is either effluxed out of the cell or re-esterified into cholesteryl ester and stored within specialized cytosol organelles, known as lipid droplets, forming foam cells (9). Foam cells are named for their characteristic foamy appearance that develops when the cytosol becomes cholesterol-rich (16). Accumulation of foam cells in the lesion can lead to the development of a fatty streak, the earliest form of an atherosclerotic lesion.

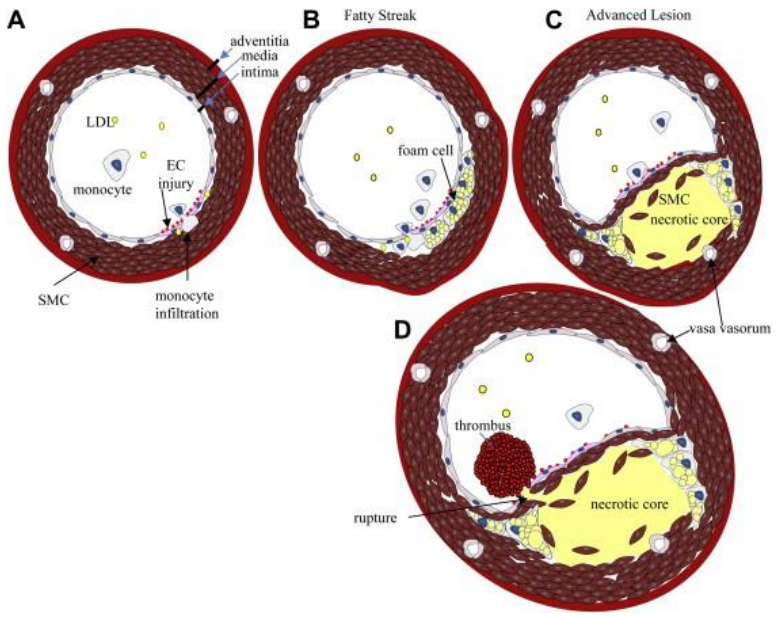

Figure 1.**Schematical representation of lesion formation** (2). **A**. Lesion Initiation: Endothelial cell (EC) injury and LDL infiltration leads to monocyte recruitment. **B**. Lesion Progression: Monocytes differentiate into macrophages, engulf modified LDL, and turn into foam cells forming a fatty streak. **C**. Fatty streak develops into an advanced lesion with an area of necrosis and fibrous cap. **D**. Plaque Rupture: Thinning fibrous cap results in thrombus formation. Adapted from *Zeaden et al*.

Later, smooth muscle cells migrate to the area, stimulating release of growth factors, pro-inflammatory cytokines, and T-cell activation. Among these are tumor necrosis factor (TNF)-α, interleukin (IL)-2, M-CSF, platelet-derived growth factor (PDGF), and IL-1 (9, 16). As the inflammatory response persists, the fatty streak develops into an intermediate lesion, which leads to the formation of a SMC fibrous cap on the luminal side of the lesion (Figure 1C). The fibrous cap acts as an overlying cover to the injured area (16). In addition, trapped macrophages become apoptotic, releasing cholesterol and other lipids into the lesion. As debris accumulates, an area of necrosis forms within the lesion (16).

In advanced atherosclerosis, the number of smooth muscle cells in the fibrous cap begins to decline causing thinning of the cap, which leads to rupture and thrombosis, usually at the edges (Figure 1D) (16). A thrombus, or blood clot, can lead to clinical manifestations of atherosclerosis, such as myocardial infarction or stroke (17).

Macrophages play a key role in atherosclerosis progression.

Macrophages are present in every stage of atherosclerosis and are key mediators in both the promotion and resolution of inflammation (14, 15). As mentioned above, macrophage accumulation and retention are a key first step in atherogenesis (13). While the mechanism is still unknown, many studies are focusing on how cholesterol loading and early lesion development alter macrophage phenotype to a more pro-inflammatory phenotype (15).

Advanced atherosclerosis is characterized by a large area of necrosis, a thinning fibrous cap, and a non-resolving inflammatory state (15). Reduced efferocytosis, or clearing of dead cells (discussed below), and large-scale macrophage apoptosis create a growing necrotic area within the lesion. The cause of macrophage apoptosis is likely caused by a variety of factors, such as oxidized lipoproteins, oxidized phospholipids, and excess accumulation of lipoprotein-derived cholesterol in the endoplasmic reticulum (18).

A major function of macrophages is efferocytosis (phagocytosis of dead cells). Under normal circumstances macrophages respond and mobilize rapidly to remove dead cells from tissues before they are broken down causing the release of anti-inflammatory cytokines and pro-resolving lipid mediators. In atherosclerosis, persistent inflammation causes many lesion cells to become apoptotic (9, 19). It is unknown if there is reduced efferocytosis in atherosclerosis, or if there are more apoptotic cells than can be cleared (19). Recent studies suggest that it is a combination of these mechanisms that cause reduced apoptotic cell clearance and necrotic core expansion (19-22). Efferocytosis also stimulates release of anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-10, and pro-resolving lipid mediators, as well as decreases expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-8 (19, 23, 24). Efficient efferocytosis has been shown to lead to decreased macrophage accumulation, smaller plaque size, and reduced plaque necrosis (25).

In addition, macrophages secrete matrix metalloproteinases (MMPs), enzymes that degrade extracellular matrix proteins, likely leading to thinning of the fibrous cap (26, 27). Other studies have shown that macrophages are more highly located on the

edges of the fibrous cap, where rupturing is more likely, further suggesting macrophages function in fibrous cap thinning and plaque rupture (27).

Versican's role in atherosclerosis.

As the atherosclerotic lesion progresses, there is also increased expansion of the ECM. The ECM, located within the artery walls, is composed of several different proteoglycans and the glycosaminoglycan, hyaluronan. One of the proteins in the ECM is versican. Versican is a large chondroitin sulfate proteoglycan that has been found abundantly in regions that are prone to lesion development. Accumulation of versican begins early in atherosclerosis when lesion expansion, intimal thickening, and SMC proliferation leads to expansion of a proteoglycan-rich ECM (28). In addition, monocyte-to-macrophage differentiation leads to increased expression of versican (29). A versican-rich ECM influences the activation and retention of leukocytes including macrophages, furthering initiation and progression of atherosclerosis. Specifically, versican can interact with macrophage receptors to stimulate pro-inflammatory cytokine expression, such as TNF-α and IL-6, which promote atherogenesis (28).

In addition, *Llorente-Cortés et al.* found that versican-LDL interactions cause a structural change in LDL, leading to increased binding to the LDL receptor, and inducing LDL internalization within vascular SMCs. This causes higher cholesteryl ester accumulation in vascular SMC compared to native LDL particles (30). Other studies have shown that versican expression is high at the edges of the necrotic core and near deposited lipoproteins, further suggesting versican has a role in lipid retention in atherogenesis (28). Recently, Chang and colleagues demonstrated that macrophages produce versican in response to inflammatory stimuli, and that myeloid cell-derived versican is important in a model of acute lung injury suggesting that versican can have immune-modulatory effects (31).

Effects of diabetes on atherosclerosis.

Diabetes mellitus is a chronic disease in which the pancreas either does not produce sufficient insulin or does not efficiently utilize insulin. Because diabetes creates

an environment with low insulin availability, it is characterized by hyperglycemia, or high blood sugar levels, which can lead to organ damage if untreated, especially to blood vessels and the nervous system. Diabetes is a major cause of micro- and macro-vascular complications that can lead to blindness, kidney failure, heart attacks, stroke, and amputation of lower limbs (32). Coronary artery disease is the major cause of death in individuals with type 1 diabetes (33). Additionally, individuals with diabetes have been shown to be at a 2- to 4- fold increased risk of atherosclerosis compared to non-diabetic individuals (33, 34). There have been several proposed factors that contribute to this increased risk, including hyperglycemia, hypertriglyceremia, hyperinsulinemia, accelerated formation of advanced glycation end-products (AGEs), increased oxidative stress and increased inflammatory potential (Figure 2) (33).

Hyperlipidemia, although more common in type 2 diabetes, has been shown to contribute to larger lesion size, differential effects on extracellular matrix proteins, including an increasing trend for versican expression, and reduced elastin secretion. Together this contributes to lesion instability and accelerated atherogenesis under diabetic conditions (35). In addition, diabetes has been linked with impaired reverse cholesterol transport out of the lesion compared to nondiabetic individuals. This contributes to increased lesion size, as well as, impaired lesion regression (1).

In a 3-way comparison of individuals that were healthy, diabetic, or had metabolic syndrome, *Marso et al.* found that diabetic individuals have a higher frequency of asymptomatic lesions that are significantly greater in area. In both asymptomatic and cardiovascular event-related lesions, the necrotic core area was larger in diabetic individuals. In diabetic individuals, the asymptomatic lesions contained more high-risk features within these lesions that could lead to a future cardiovascular event compared to non-diabetic individuals (36). This study shows larger necrotic cores form under diabetic conditions and that these lesions have a higher risk of causing a future major cardiovascular event.

Studies have shown that diabetes is associated with increased production of proinflammatory cytokines, such as C-reactive protein (CRP), IL-6, IL-1β, and TNF-α (37). Consistently, a study by *Bradshaw et al.* showed that there was a significant increase in circulating IL-1β and IL-6-secreting monocytes in individuals with recent-

onset type 1 diabetes compared to healthy, age-matched controls or type 2 diabetic individuals. Furthermore, they also demonstrated that the gene expression in monocytes of major proinflammatory cytokines such as IL-1β, IL-6, TNF-α, are significantly increased in type 1 diabetes, even in the absence of exogenous stimuli, confirming that diabetes results in a monocyte and macrophage phenotype that favors a pro-inflammatory state (38). We have previously demonstrated that this state of heightened inflammatory capacity in myeloid cells can be mimicked in a mouse model of type 1 diabetes-accelerated atherosclerosis, and the interference of this results in reduced diabetes-accelerated atherosclerosis (39).

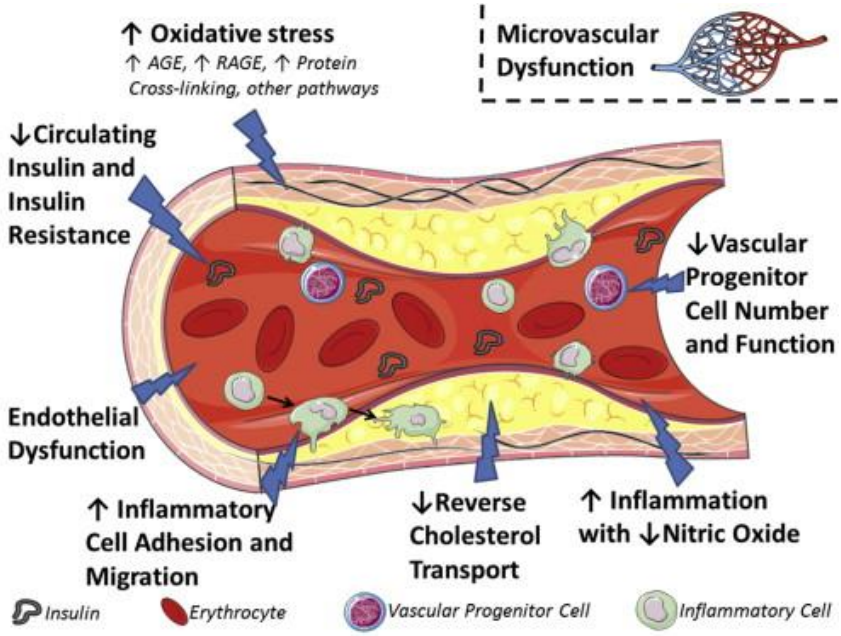

Figure 2. (1) **Schematic of proposed risk factors that might accelerated atherosclerosis under diabetic conditions**. Diabetes risk factors include reduced insulin levels, cholesterol transport, progenitor cell number, and nitric oxide release in addition to increased oxidative stress and inflammation are risk facts that increase risk of atherosclerosis development. Adapted from *Kovacic et al*.

Efferocytosis, phagocytosis and clearance of dead cells, has been shown to be impaired in diabetes through reduced macrophage engulfing activity (40). In addition, diabetes leads to increased apoptosis of cells causing increased cell death and necrosis within the lesion (40). Together these further increases the likelihood of developing chronic inflammatory diseases in diabetes.

Gaps in the Literature

Diabetes accelerates atherosclerosis, at least in part, via changes in monocyte and macrophage behavior and phenotype, however the mechanisms underlying this altered monocyte/macrophage phenotype are unknown. Based on recent data in lung injury models and preliminary data, we hypothesized that versican might be one factor that could alter macrophage phenotype under diabetic conditions, potentially altering both macrophage inflammatory potential and lipid loading. Preliminary data from hyperlipidemic, diabetic mice indicated that myeloid cell-derived versican-deficiency prevents diabetes-induced necrotic core expansion; therefore, this study aims to examine how versican influences necrotic core formation in atherosclerotic lesions. We hypothesize that myeloid cell-derived versican accelerates advanced lesions formation under diabetic conditions through a pathway that involves trapping of LDL, resulting in increased lipid loading and therefore increased cell death and necrotic core formation. Thus versican-deficiency, selectively in macrophages, will result in less lipid loading and slower plaque progression.

Methods

Animals, induction of diabetes and tissue harvest

All animal work was approved by the University of Washington Institutional Animal Care and Use Committees. Female *Ldlr*$^{-/-}$*;Gp*$^{+}$ mice were fed a high-fat diet (40% of calories from fat, 1.25% cholesterol) for 12 weeks to induce atherosclerosis (Figure 3A). In this model, a viral glycoprotein (GP; from the lymphocytic choriomeningitis virus; LCMV) is expressed under control of the rat insulin promoter (RIP-GP). Injection of LCMV results in an immune response that targets the GP-expressing β-cells, resulting in cell death and development of diabetes within 7-10 days. Mice without the GP transgene do not become diabetic after LCMV infection. After 12 weeks on the high-fat diet, the mice were switched to chow diet, lethally irradiated (10Gy) and bone marrow transplanted with either wild type (*Vcan*$^{fl/fl}$) or myeloid-selective deletion of versican (*Vcan*$^{fl/fl}$*/LysMCre*$^{+/+}$; Lyz2-Cre driven deletion of loxP-flanked exon 4 in the versican gene) (reference PMID:28912382). Following bone marrow transplantation, mice were allowed to recover for 5 weeks before diabetes was induced with one intraperitoneal injection of LCMV (1x10^{4} PFU/mouse). At onset of

diabetes the mice were switched to a low-fat, semi-purified diet and were maintained for an additional 4 weeks. At the end of the study, the hearts were collected, fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO), embedded in OCT and sectioned at the level of the aortic sinus. All animal work was carried out by others in the laboratory prior to initiation of this study.

Macrophage isolation

At the end of the study, resident peritoneal macrophages were harvested by peritoneal lavage and a macrophage-enriched population was allowed to adhere for 30 minutes before being stimulated for 6 hours with 10 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) or vehicle control. Alternatively, in another cohort of mice thioglycolate-elicited macrophages were harvested (thioglycollate injected 4 days prior) for cholesterol/cholesteryl ester quantification and propidium iodide (PI) and annexin V staining by flow cytometry (Apoptosis detecting kit; Sigma-Aldrich). Total protein levels in macrophages were quantified using Pierce™ BCA Protein Assay Kit microplate procedure (Thermo Scientific, Rockford, IL, USA). Absorbance was measured at 562 nm on a plate reader (BioTek Instruments, Highland Park, VT, USA). Free and total cholesterol were quantified using the Amplex® Red Cholesterol Assay kit and protocol (Invitrogen, Molecular Probes, Eugene, OR). Samples were analyzed for florescence at 590 nm on a microplate reader. Cholesterol concentration was normalized to protein concentration prior to analysis.

mRNA sampling

Total RNA was isolated from the peritoneal macrophages using the Macherey-Nagel NuceloSpin® RNA Plus protocol (Macherey-Nagel, Bethlehem, PA). The column was discarded and the flow-through was reverse transcribed to make cDNA using Thermo Scientific RevertAid Reverse Transcriptase protocol (Thermo Scientific, Waltham, MA). Samples were mixed with 100 pmol of a random hexamer primer followed by cDNA synthesis cocktail (4µL 5x Reaction Buffer, 20U RiboLock RNase Inhibitor, 1mMdNTP mix, 200U RevertAid Reverse Transcriptase) and incubated for 10

min at 25°C followed by 60 min at 42°C then 10 min at 70°C to terminate the reaction. The resulting cDNA was stored at -20℃ for later testing.

Real-time quantitative Polymerase Chain Reaction (qPCR) was used to assess expression of *versican*, *Il1b*, *Il6*, *Il10*, and *Cccl2* using the Maxima SYBR Green/ROX qPCR Master Mix protocol (Applied Biosystems, Foster City, CA). qPCR samples (7.4 µL 2x Maxima SYBR Green/ROX qPCR Master Mix, 0.3µM forward primer, 0.3 µM reverse primer, 4 µL cDNA in nuclease-free H_2O) were run on cycling heat for: 1 cycle at 95℃ for 10 min for initial denaturation, and 40 cycles of: 95℃ for 15s for denaturation, 60℃ for 60s for annealing/extension.

All samples were normalized to *Rn18s* concentration and converted to ΔΔCt to express values as fold-over control samples prior to analysis. All samples were run in duplicates and statistical analysis was performed on $2^{-(\Delta Ct)}$ values. Primer sequences are listed in Appendix A: Supplemental Regent Table.

Immunohistochemistry and TUNEL

The analysis of lesion size and necrotic core formation was carried out on the first section of the aortic sinus containing all three leaflets for all animals. Adjacent sections were stained for versican and MAC-2. Sections were stained with primary antibodies overnight at 4°C using a rabbit anti-mouse versican GAG beta domain polyclonal antibody (1:50 dilution, EMD Millipore Corporation, Temecula, CA) or purified anti-mouse MAC-2 monoclonal antibody (1:1000 dilution, Cedarlane, Burlington, Canada) for versican and MAC2, respectively. Negative controls included non-immune rabbit IgG antibody and anti-rat IgG2a antibody for versican and MAC-2, respectively. A goat anti-rabbit IgG antibody (1:10000 dilution, Life Technologies, Carlsbad, CA) or goat anti-rat IgG (H+L) antibody (1:250000 dilution, Southern Biotech, Birmingham, AL) were used as secondary antibodies for versican or MAC2, respectively. Staining was visualized with DAB chromogen (Thermo Scientific, Fremont, CA) and counterstained with methyl green. Sections were analyzed using Image J software. The cross-sectional area (um^2) and percent of the lesion was calculated using the total lesion area, as determined by Movat staining.

To test for the presence of apoptosis and efferocytosis within the atherosclerotic lesion, we used the TUNEL stain using the *in-situ* cell death detection kit, TMR Red protocol for difficult tissues (Sigma-Aldrich, St. Louis, MO) in conjugation with anti-CD68, a macrophage-associated protein, and cell nuclei (using DAPI). Samples were stained for CD68 using rat anti-mouse CD68 (1:75 dilution, Bio Rad, Hercules, CA) as the 1° antibody and Alexa Fluor^{TM} 488 goat anti-rat IgG (G+L) (1:250 dilution, Invitrogen, Carlsbad, CA) as the 2° antibody. Images of TUNEL (red fluorescence), CD68 (green fluorescence), and DAPI (blue fluorescence) were overlaid to determine “macrophage associated” versus “free” apoptotic cell. The ratio of “macrophage associated” to “free” apoptotic cells was calculated to determine the efferocytosis index.

Oil Red O staining was done following the IHC World protocol to quantify neutral lipid accumulation within the lesion. Sections were counterstained with Harris haematoxylin prior to mounting. Samples were quantified by counting the pixels stained for lipids (red) and compared to total lesion size to determine the area of the lesion that contained lipids. Sections were analyzed using Image J software. The percent of the total lesion and area (um^2) of the lesion was calculated using the total lesion area, as determined by Movat staining.

Analysis

The data was analyzed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA) and expressed as mean ± S.E. Two-way Anova was used to analyze for multiple comparisons.

Results

Myeloid cell-derived versican prevents diabetes-augmented necrotic core formation

To begin to test if diabetes affects advanced atherosclerotic lesions, and whether myeloid cell-derived versican influences the development of these lesions, female *Ldlr*$^{-/-}$*;Gp*$^{+}$ mice were fed a high-fat diet for 12 weeks to establish atherosclerosis. At this point the mice received either bone marrow from wildtype (WT) mice or from mice with myeloid cell selective deficiency in versican (VCAN$^{MC-/-}$) to generate bone marrow chimeras, diabetes was induced, and the mice were allowed to be diabetic for 4 weeks (Figure 3A). At the end of the four weeks, the diabetic mice were hyperglycemic and modestly hypercholesterolemic, and levels were not altered by myeloid cell versican-deficiency (Figure 3B-C). To confirm that the versican gene was knocked out in macrophages, we examined versican mRNA gene expression in isolated macrophages from the peritoneal cavity (Figure 3D). Versican expression was reduced by 80% in the macrophages isolated from the nondiabetic mice transplanted with VCAN$^{MC-/-}$ bone marrow, and to 95% reduced in macrophages from diabetic mice transplanted with VCAN$^{MC-/-}$ bone marrow. Consistent with versican being induced under inflammatory conditions (31) and diabetes being a state of heightened inflammatory status, diabetes resulted in increased macrophage expression of versican.

To begin to address the role of myeloid cell-derived versican in atherosclerosis under diabetic conditions, the extent of atherosclerosis was analyzed in the aortic sinus, a site known to develop advanced atherosclerosis in mice. Neither diabetes nor myeloid cell versican-deficiency altered the extent of atherosclerosis (Figure 3E). Much of the extracellular matrix is believed to be deposited by smooth muscle cells, although myeloid cell versican-deficiency reduced the extent of versican staining (Figure 3F-G). It was not co-localized with macrophage staining, suggesting that macrophages are not expressing the majority of the versican.

Although, diabetes did not alter the size of the atherosclerotic lesion, diabetes resulted in larger necrotic cores (Figure 3H), consistent with data on atherosclerotic lesions from human diabetics (36). Interestingly, myeloid cell versican-deficiency reduced necrotic core size selectively under diabetic conditions.

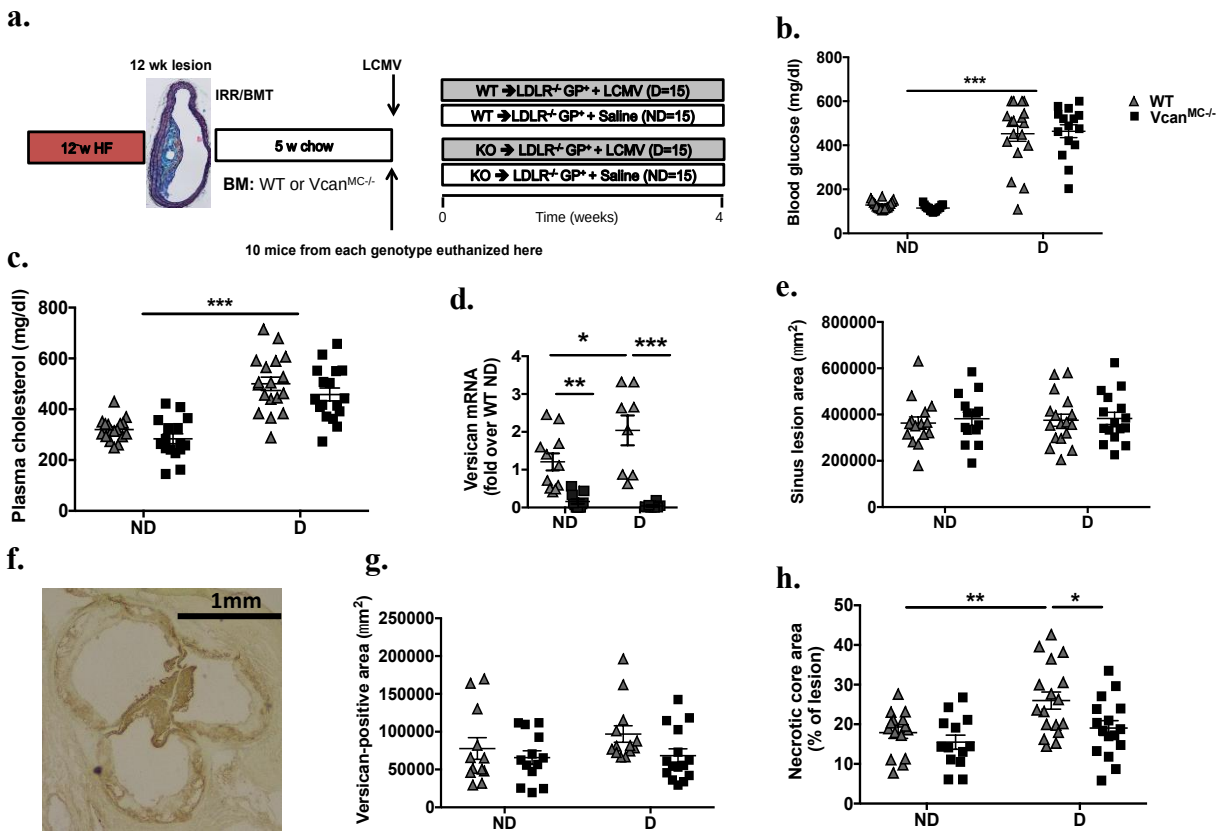

Figure 3. **Effect of versican and diabetes expression within lesions**. A. Mouse model set-up procedure with 12 weeks on a high-fat (HF) diet followed by bone marrow transplant of either wild-type or Vcan$^{MC-/-}$ bone marrow with 5 weeks to recover on a normal diet before injection with LCMV to induce diabetes. After 4 weeks, tissues were collected. B. Blood glucose levels measured at time of tissue collection, C. Plasma cholesterol levels measured at time of tissue collection, D. Expression of versican in isolated macrophages displayed as fold over WT ND, E. Sinus lesion area measured by Movat stain, F. image of an aortic sinus section immuno-stained for versican, G. Area of versican-positivity measured by pixilation of versican stained area, H. Area of necrotic core, expressed as a percent. *p<0.05, **p<0.01, ***p<0.001

Diabetes results in increased macrophage cholesterol loading but not increased atherosclerotic lipid deposition.

To indirectly determine if cholesterol loading of peritoneal macrophages was associated with lipid deposition within the lesion, we compared Oil Red O staining of lipids in the lesion to total and free cholesterol isolated from peritoneal macrophages. Neither diabetes nor versican-deficiency altered lipid deposition (Figure 4A). Both total

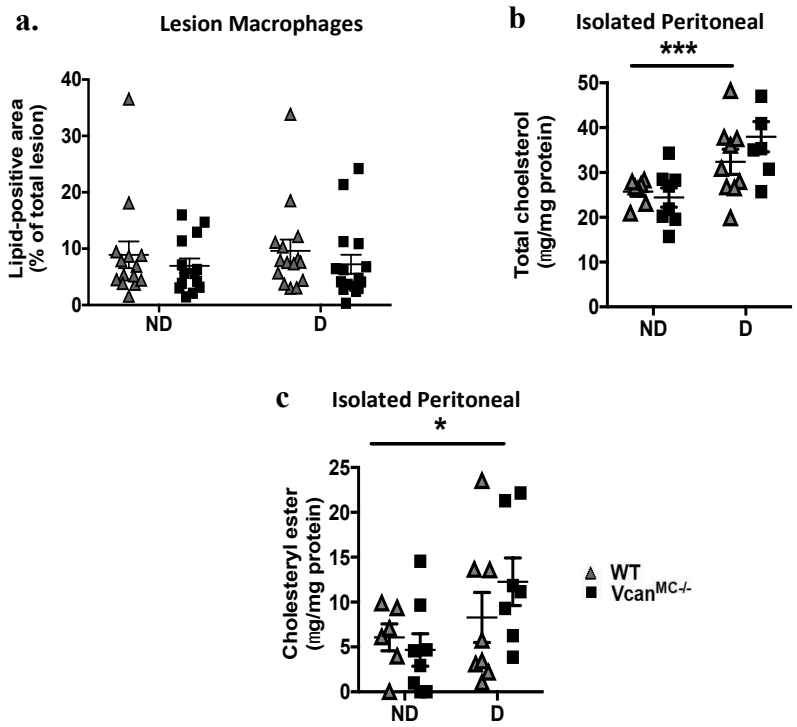

Figure 4. **Role of lipids within lesion** A. Area of lipid-positivity within the lesion as measured by Oil Red O staining, B. Area of macrophage-positivity within the lesion as measured by Mac-2 staining, C. amount of lipid loading within the lesion, measured by cholesteryl esters present within macrophages. *p<0.05

cholesterol and cholesteryl ester concentrations within the macrophage were greatly increased under diabetic conditions but were unaffected by versican-deficiency (Figure 4B-C). Altogether, this suggests that cholesterol loading is influenced under diabetic conditions, but it does not equate to increased lipid deposition.

Diabetes results in an increased pro-inflammatory macrophage phenotype, however this is unaltered by versican-deficiency

To determine if diabetes or versican-deficiency affected inflammation, we examined expression of known inflammatory cytokines using qPCR. Isolated macrophages were either stimulated with LPS or remained unstimulated prior to extraction of mRNA and reverse transcription to cDNA. Expression for *Il1b* was increased under diabetic conditions in both unstimulated and stimulated cells showing that increased inflammation is present in diabetes (Figure 5A). In addition, *Ccl2* and *Il10* expression was increased under diabetic conditions when stimulated with LPS further showing that inflammation is present (Figure 5B-C). Versican-deficiency did not appear to impact inflammation through expression of *Il1b, Ccl2,* or *Il10.* Lastly, expression of *Il6* was unaltered under both diabetic and versican-deficient conditions.

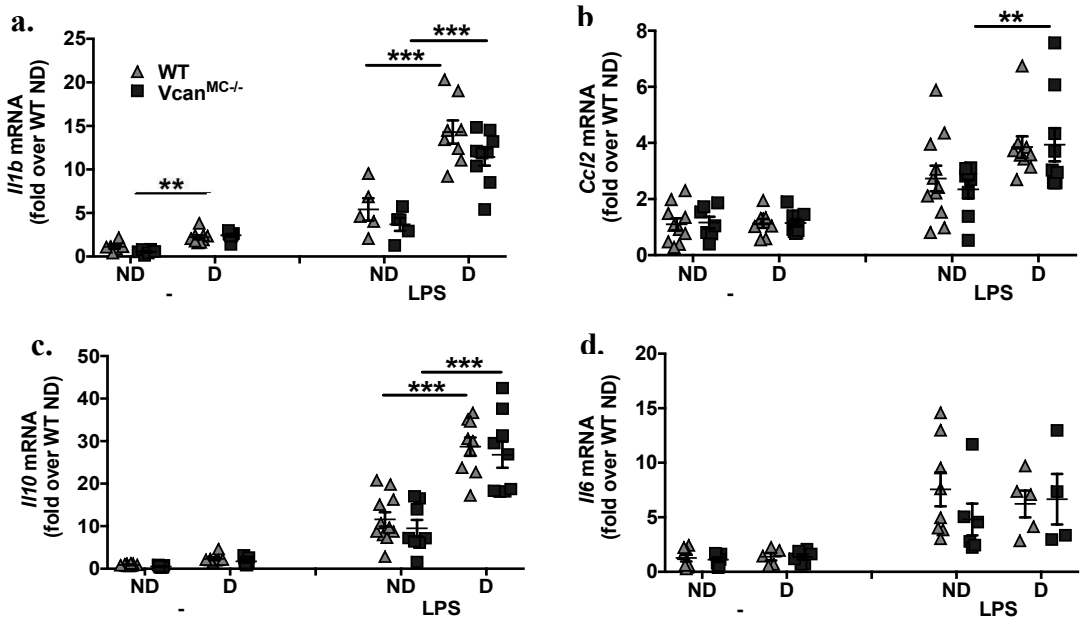

Figure 5. **Pro- and anti-inflammatory cytokine production in Vcan$^{MC-/-}$ macrophages under non-diabetic and diabetic conditions in unstimulated and LPS-stimulation (10ng/mL)**. A. *Il1b* mRNA B. *Ccl2* mRNA C. *Il10* mRNA D. *Il6* mRNA. *p<0.05, **p<0.01, *** p<0.001

Neither diabetes nor versican-deficiency alter in situ apoptotic cells or efferocytosis

Because both increased apoptosis or reduced efferocytosis could contribute to increased lesion development, we used TUNEL staining to test how if these pathways contributed to the increased necrotic core area observed under diabetic conditions and whether this was affected by versican-deficiency. Figure 6A shows a lesion that is stained and overlaid with TUNEL (red), CD68 (green), and nuclei (blue) and figure 6C shows a close-up of a region of the lesion that contains both a free and macrophage-associated apoptotic cell. Diabetes did not increase the number of apoptotic cells within the lesion, as evident by TUNEL-staining despite evidence that diabetes increases the size of the necrotic core (Figure 6B). Similarly, myeloid-selective deficiency of versican did not alter the number of apoptotic cells. Furthermore, neither diabetes nor versican affected *in situ* efferocytosis (Figure 6D).

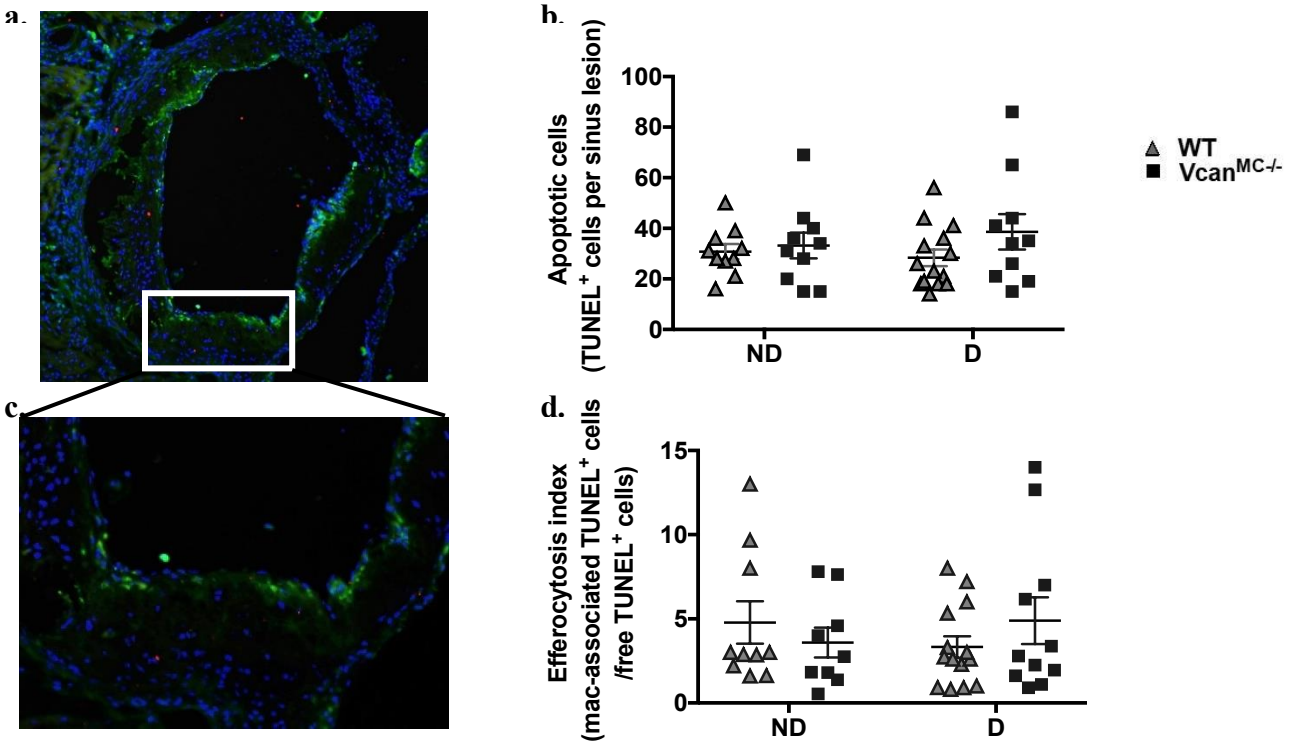

Figure 6. **Apoptosis and efferocytosis within sinus lesions** A. Image of sinus lesion overlaid with nuclei (blue), CD68 (green), and TUNEL (red) with, C. enhanced subsection of the lesion. B. No difference was observed in both apoptosis or, D. efferocytosis under diabetic nor versican-deficient conditions.

Increased necrosis and reduced presence of live macrophages under diabetic conditions suggest other mechanisms are driving apoptosis.

To test how macrophages were trapped within the lesion and how diabetic or versican-deficient conditions affect this, Mac-2 staining was done on adjacent sections to the versican staining. Diabetes, but not versican-deficiency, resulted in fewer

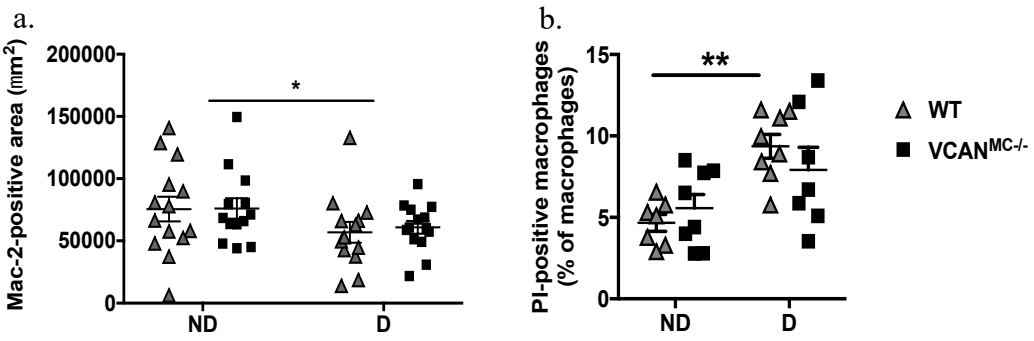

Figure 7. **Necrosis and macrophage viability within sinus lesions**. A. Area of Mac-2 staining of aortic sinus cross-section of lesion, B. Cell viability (necrosis) expressed as the percent of total macrophages.

macrophages within the lesion (Figure 7A). In addition, versican staining was not colocalized with the macrophage staining. Together, these results suggest that macrophages are supporting some, but not the majority of versican expression *in vivo*.

To address how cell viability was impacted by either diabetic or versican-deficient conditions, PI staining was done on isolated macrophages via flow cytometry. Diabetes, but not versican-deficiency, resulted in increased necrosis of macrophages (Figure 7B). Altogether, these results suggest that other mechanisms are driving apoptosis and atherogenesis.

Discussion

In this study, we show that in a mouse model, type 1 diabetes results in larger necrotic cores even though lesion area and lipid loading are unaffected by diabetic conditions and that versican deficiency can reduce this effect. We also show that the amount of versican expressed within the lesion is trending down under diabetic conditions, although this is not significant. Meanwhile the number of macrophages present in the lesion is reduced under diabetic conditions. This suggests that macrophages partially contribute to versican expression within the lesion, but that most of versican expression is not from macrophages. Finally, we show that expression of *Il1b*, *Ccl2*, and *Il10* are significantly increased under diabetic conditions confirming that diabetes increases myeloid cell inflammation.

Many studies have shown that inflammatory cytokine expression is increased under diabetic conditions. For example, *Bradshaw et al.* showed that IL-6, IL-1β, TNF-α production was higher in both recent onset and long-term type 1 diabetic individuals compared to healthy controls, although the difference between the number of IL-6- and IL-1β-secreting monocytes was not as drastic in long-term diabetic individuals compared to recent onset type 1 diabetes (38). This could partially explain why we did not observe a difference in *Il6* expression. Similarly, *Bradshaw et al.* examined the number of circulating monocytes secreting IL-6 and IL-1β in human blood samples. Our study examined mRNA expression in isolated macrophages. Similarly, our findings mirror what we have found previously in diabetic macrophages, where expression of *Il1b* and *Ccl2* is increased in macrophages under diabetic conditions (38, 39).

A paper by *Chang et al.* found that versican deficiency within acute lung disease significantly reduced IFN-β, CCL2, IL-10, IL-6, and TNF-α in poly(I:C) stimulated cells isolated from wildtype or knockout cells or bronchoalveolar fluid (31). ur study did not find a difference between wild-type and versican deficient macrophages, although expression of *Ccl2, Il10, Il6,* and *Il1b* are trending downwards under stimulated non-diabetic conditions. *Chang et al.* examined lung tissue samples, whereas we examined peritoneal macrophages, which could partially explain this difference.

Efferocytosis is an important mediator in the resolution of atherosclerotic plaques. Our results differ from *Suresh Babu et al*, who found that diabetes impairs

efferocytosis (40). While methodology was vastly different, one would expect the same effect. Therefore, more research is needed to confirm how efferocytosis and versican deficiency affect efferocytosis and apoptosis.

Versican's role in atherosclerosis was discovered relatively recently and has not been extensively studied, especially in *in vivo* models. Therefore, a strength of this study is its ability to contribute to the small pool of knowledge on versican expression in macrophages in both diabetic and non-diabetic conditions. Another strength of this study is the large sample size. This adds significance and power of our findings.

Several limitations exist for the study that should be mentioned, including the sensitivity of the TUNEL stain to test for apoptosis. The TUNEL stain provides a snapshot, or cross-sectional view, of a single moment in time making it a less sensitive measure of apoptosis, therefore it may not have captured complete apoptosis. Including cleaved caspase-3 in conjugation with TUNEL would provide a more complete view of apoptosis within the lesion. Caspase-3 is a protein that has a role in the cell signaling pathway that, when cleaved, leads to the execution of cell death. Therefore, including this protein in our apoptosis analysis would allow us to tag cells that are about to undergo apoptosis. Furthermore, this approach is not able to distinguish between "macrophage-associated" cells. These cells could be apoptotic macrophages or macrophages that have phagocytosed an apoptotic cell (efferocytosis). We have assumed that they are phagocytosed apoptotic cells thereby concluding that "macrophage-associated" apoptotic cells are associated with improved efferocytosis. It is also possible that that cellular materials were degraded following cell death preventing recognition of what may have originally been an apoptotic cell through the TUNEL assay. In addition, we used the assumption that cholesterol loading and PI-staining from isolated peritoneal macrophages were comparable to macrophages within the lesion. These macrophages may not have the same phenotype as lesion macrophages, so comparisons between these macrophages this assumption must be taken into consideration. Finally, atherosclerotic or diabetic mouse models may not be completely generalizable to humans due to differences in biology or mechanisms.

Future research should include analysis of versican from SMC which would help to assess if SMC versican affects apoptosis and necrotic core formation (41). Whereas,

the macrophages that were utilized in this study were bone marrow-derived monocytes that differentiated into macrophages, under inflammatory conditions, cell signals, such as PDGF, TGF-β, MMPs, and reactive oxygen species, can stimulate SMCs to differentiate into macrophages (41). Our data suggests that versican and macrophages are not colocalized within the lesion, and that macrophage area is reduced under diabetic conditions while necrosis remains high. Therefore, we hypothesize that bone marrow-derived macrophages are not expressing the majority of the versican. In addition, it has been suggested that SMC-derived proteoglycans have 3 times higher affinity for binding LDL compared to quiescent cells and that more cholesteryl ester is retained via LDL-versican interactions (30, 42). Therefore, comparing SMC-derived versican would provide valuable information. Finally, comparing the amount of lipids present only in the necrotic core could provide evidence to how versican deficiency and diabetes affect lipid loading and necrosis.

In conclusion, diabetes increases expression of *Il1b, Ccl2,* and *Il10* confirming that diabetes has an effect on the immune system. Myeloid cell-derived versican deficiency does not appear to alter cytokine expression under the conditions we tested. In addition, apoptosis does not appear to be affected by diabetes nor myeloid cell-derived versican deficiency even though diabetes increases necrotic core size and versican deficiency reduces it, suggesting that other pathways of cell death, such as necrosis, are drivers of atherosclerosis, not apoptosis, or that rapid degradation after cell death impairs assay recognition of apoptosis. Other forms of cell death, such as necroptosis (inflammatory cell death), autophagy (cellular component breakdown), or oncosis (passive cell death) may also affect cell death within atherosclerotic lesions. These results show that future research is needed to assess the impact of versican within sinus lesions and to investigate the mechanism of how diabetes accelerates atherosclerosis progression.

Appendices

A. Supplemental Reagent Tables

Primers for Real-Time PCR

Gene	Primer Sequences	Source
Versican	F 5'-TGATGCAGGCGTCTACCGATGT-3' R 5'-TGCTGGTGGCTGCCCTGTAGTG-3'	Invitrogen
Il1b	F 5'-GGGCTGCTTCCAAACCTTTG-3' R 5'-TGATACTGCCTGCCTGAAGCTC -3'	Invitrogen
Il6	F 5'-TAGTCCTTCCTACCCCAATTTCC-3' R 5'-TTGGTCCTTAGCCACTCCTC-3'	Invitrogen
Il10	F 5'-GCTCTTACTGACTGGCATGAG-3' R 5'-CGCAGCTCTAGGAGCATGTG-3'	Invitrogen
Ccl2	F 5'-TTAAAAACCTGGATCGGAACCAA-3' R 5'-GCATTAGCTTCAGATTTACGGGT-3'	Invitrogen

Antibodies for immunohistochemistry

Antibody	Staining	Description	Source
1° Versican	Versican	polyclonal rabbit anti-mouse Versican GAG beta domain	EMD Millipore
2° versican	Versican	Biotinylated goat anti-rabbit IgG (H+L) DS Grd	Life Technologies
1° Mac-2	Macrophages	purified anti-mouse MAC-2 monoclonal	Cedarlane
Negative Control for Mac-2	Macrophage	Purified Rat IgG2a Isotype Control	Cedarlane
2° Mac-2	Macrophages	Biotinylated goat anti-rat IgG (H+L) mouse ads-BIOT	Southern Biotech
TUNEL	TUNEL	Terminal deoxynucleotidyl transferase from calf thymus	Roche
CD68	TUNEL	Rat anti-mouse CD68	Bio Rad
2° CD68	TUNEL	Alexa Fluor™ 488 goat anti-rat IgG (G+L)	Invitrogen
Nuclei	TUNEL	ProLong™ Diamond Antifade Mountant with DAPI	Invitrogen

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