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The role of Toll-Like Receptor 2, 4 and 9 in atherosclerosis

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1 Introduction

Cardiovascular Disease (CVD) is the number one cause of death in the world (WHO, June 2011). Historically thought of atherosclerosis as lipid storage dysfunction focusing on low-density lipoprotein (LDL), it is now considered a disease with a complex construct including the vascular, immune and metabolic system and their components. While blood cholesterol (Ross and Harker, 1976) remains a key player, chronic inflammation now equally underpins our understanding and the therapy of atherosclerosis and leading diseases (Hansson and Libby, 2006). For example, macrophages were the first cells of the innate immune system to be identified as major components of an atherosclerotic lesions in swine (Gerrity et al., 1979).

Named after the first discovered Toll receptor in the fruit fly *Drosophila melanogaster,* Toll-Like Receptors (TLRs) are the important proteins in the innate immune system. Their function is to recognize pathogen-associated molecular patterns (PAMPs), first identified by the Russian scientist Ilya Mechnikov in 1883 (O'Neill, 2004). They are responsible for the early response to viral and bacterial infections. Recent studies have suggested a link between TLRs and atherosclerosis. The lack of Myeloid Differentiation primary-response gene 88 (MyD88), an intracellular protein of the TLR signaling cascade, resulted in a declining extent of atherosclerosis (Bjorkbacka et al., 2004) in Apolipoprotein E (ApoE) deficient mice.

2 Literature review

2.1 Atherosclerosis

Atherosclerosis is a complex, multigenic disease in medium and large arteries involving the vascular, metabolic and immune system in development, processing and outcome of atherosclerotic plaque.

The term “arteriosclerosis” was first stated in the unfinished Traité d’ anatomie pathologique, 1829, by Jean Lobstein (Lobstein, 1833). During that time, the pathogenesis of arteriosclerosis remained unclear and two opponents argued about the role of the inflammation. Rudolf Virchow claimed that “cellular pathology” (Virchow, 1860) or cellular inflammatory changes in the wall of the vessel were the primary reason. By contrast, “humoral pathology” (Rokitansky, 1849) was proposed by Carl von Rokitansky, whereby inflammation was only secondary in nature and atheroma is a result of degenerated blood elements like fibrin (W. Slijkhuis, 2009). Later on, in 1908 Ignatovski found that special diets like milk and egg yolks cause arteriosclerosis in rabbits (Dock, 1958). However, it was Anichkov and Chalatov, who discovered the role of cholesterol in atherogenesis in 1913 (Igor E. Konstantinov, 2006). Decades later and based upon Virchow’s theory, Russell Ross postulated in his hypothesis that arteriosclerosis, as a multigenic disease, is the result of endothelial injury, which leads to dysfunction and inflammation in the arterial wall (F. H Epstein, 1999). These theories, based upon Virchow’s “cellular pathology”, are accepted and opened the origin of today’s research.

Major processes in an atherosclerotic lesion are the accumulation and modification of lipids, inflammation, cell death and fibrosis. Fibrosis, driven by smooth muscle cells, prevents the blood flow from coming into contact with the prothrombotic lipid core in the plaque. Rupture of the plaque can lead to myocardial infarction, stroke and other related diseases.

2.1.1 Epidemiology

Cardiovascular Disease (CVD) is the leading cause of death in Germany, with 40.2 %, being responsible for 342.233 deaths in 2011 (Statistisches Bundesamt, 2011). The costs of CVD were about 36.973 billion Euros in 2008, leading the list of most costly diseases with 14.5 % (Statistisches Bundesamt, 2008).

The lifetime risk of contracting a CVD varies depending on several risk factors like blood pressure, diabetes, current smoking and total cholesterol (Berry et al., 2012).

2.2 Mechanisms of atherosclerosis

2.2.1 Fatty streaks

The development of so-called fatty streaks starts early in life (1993). Endothelial dysfunction (Mudau et al., 2012) in areas with disrupted shear stress (Prado et al., 2008, Ridger et al., 2008) leads to a binding of apolipoprotein- B100 on the extra cellular matrix in the intima of the vessel. This leads to a concentration-dependent invasion and accumulation of Low-density Lipoprotein (LDL) particles in the arterial wall (Steinberg et al., 1989). In the first stage, the trapped LDL is oxidized to mildly oxidized LDL by the oxidative waste of the arterial wall cells. Endothelial cells change to start expressing P-Selectin (Vora et al., 1997), VCAM-1 (Vascular Cell Adhesion Molecule-1) (Li et al., 1993) and other receptor ligand mediated processes in combination with cytokines, which in turn leads to monocytes adhesion. The trapped oxidized lipids promote the tethering of monocytes by MCP-1/CCL2 (monocyte chemotactic protein-1/ Chemokine Ligand 2) (Cushing et al., 1990), the activation to macrophages by M-CSF (macrophage colony-stimulating factor) (Rajavashisth et al., 1990) and arresting by GRO (growth-related oncogene) (Schwartz et al., 1994, Smith et al., 2005), induced by endothelial cells and monocytes itself (Figure 1). Monocytes are also responsible for forming foam cells by taking up and accumulating highly oxidized LDL (oxLDL). Further cells of the innate immune system also migrate into the arterial wall; for example, neutrophils (Weber and Noels, 2011). The local macrophages also act as a link between the innate and adaptive immune response.

In parallel, TLRs, expressed on immune cells, become activated by factors such as Heat Shock Protein 60 and oxLDL initiates a signal cascade leading to the activation of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells). NF-κB

controls the expression of genes of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, MCP-1, Tumour-necrosis Factor (TNF)-α and adhesion molecules.

Minimally modified Low-density Lipoprotein (mmLDL) is turned into highly oxidized LDL by the oxidative capacity of macrophages, by reactive oxygen species. There is now a change in the receptor expression of the macrophages away from LDL receptors to scavenger receptors (ScRs)' for example, CD (Cluster of Differentiation) 36, and oxidized LDL receptors, partly driven by M-CSF (Clinton et al., 1992). This leads to a loss of controlled cellular uptake of LDL (Brown and Goldstein, 1990) by the macrophages.

By contrast, HDL (high-density lipoprotein) protects the LDL against oxidation by metal ions (Hessler et al., 1979, Parthasarathy et al., 1990) by the endothelial and vascular smooth muscle cells. Additional HDL has anti-inflammatory properties; for example, HDL inhibits of the expression VCAM-1 (Besler et al., 2012), IL-8 and MCP-1 (Navab et al., 2011) (Navab et al., 1991). Furthermore, HDL plays a role in the reverse Cholesterol transport (Hafiane and Genest, 2013) mediated by ATP (Adenosine-5'-triphosphate) -binding cassette transporters.

T-Lymphocytes, mainly $CD4^+$ and localized on the shoulder region, reflect the vast minority of inflammatory cells in the fatty streak (Jonasson et al., 1986). Different subpopulations of T cells represent the different function that they play in the immune system. T Helper 1 (T_h1) cells promote inflammation by producing Interferon-gamma (IFN-γ), TNF-α and activating macrophages. This T cell promoted inflammation plays an important role in progression and the advanced atherosclerotic lesion.

By comparison, T helper 2 (T_h2) cells show an inhibiting effect on the inflammation, driven by the production of IL-4, IL-5, and IL-13. It has been shown that T-cells play a regulatory role in the inflammatory process, by Transforming Growth Factor (TGF)-β and IL-10 (Gorelik and Flavell, 2002). IL10 has an immunosuppressive effect and inhibits the secretion of cytokines like IFN-γ.

The role of T cells in progression of atherosclerosis is illustrated by the fact that activated T cells are particularly high in plaque in acute coronary syndrome (ACS) (Hosono et al., 2003).

Dominated by macrophages, this fatty streak can disappear without causing symptoms or is able to progress into a more complicated atheroma lesion (Hansson and Libby, 2006).

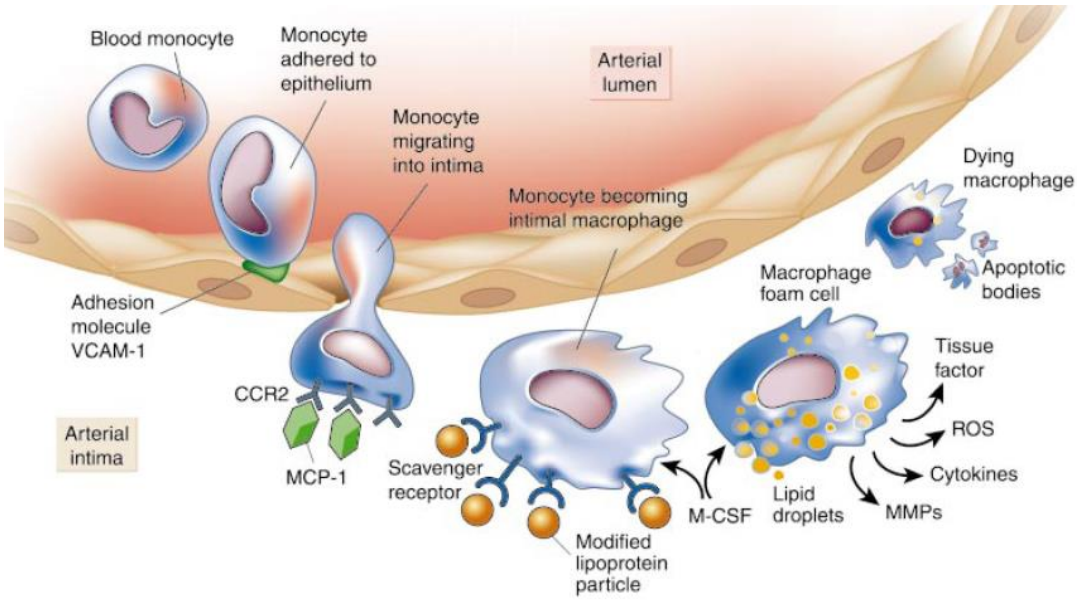

Figure 1 Monocytes in atherogenesis (Libby et al., 2010)

Monocyte adhesion, migration and foam cell formation in the arterial wall. Adhesion is mainly driven by VCAM-1, MCP-1 as ligand on CCR2 is responsible, together with M-CSF, for tethering and activation. The uncontrolled uptake of modified LDL by increased expression of scavenger receptors leads to forming foam cells with apoptosis as a final result.

2.2.2 Progression

The uncontrolled lipid accumulation in the macrophages with following cell death and enlargement of the necrotic core leads to a proliferation of smooth muscle cells into the arterial wall. This process causes irreversible progression and the manifestation of the fatty streak into a more complicated atheroma lesion.

Lesion progression has its origin in the cell death of foam cells. Apoptosis can be triggered by high concentrations of cytokines like TNF-α (Canault et al., 2006), highly oxidized LDL (Reid and Mitchinson, 1993) and oxidant stress (Dickhout et al., 2005). Undergoing cell exacerbates an inflammatory response and as a result of apoptosis and accumulation to a necrotic core. The lesion starts growing by inflammatory action and reaction by immune cells predominantly in the shoulder region of the plaque (Rosenfeld et al., 1987).

There are several growth factors and chemoattractants for smooth muscle cells (SMCs). Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), IL-1 and TNF-α, often produced by SMCs themselves, are major players in the multiplication of SMCs in the lesion. The source of some cytokines is oxidized LDL stimulated macrophages (Ross, 1993). In addition SMCs produce Macrophage Inhibitory Factor (MIF), IFN-γ and MCP-1 (Doran et al., 2008). SMCs have the ability

to switch between a “contractile” and “synthetic” phenotype in response to the inflammatory environment, like in an atherosclerotic lesion (Doran et al., 2008). While the “contractile” type is steadier, the “synthetic” SMC can synthesize collagen at a higher rate. Similar to macrophages, the “synthetic” SMCs express scavenger receptors and LDL receptors, while they are also able to form foam cells (Campbell and Campbell, 1994) in a significance number (Stary et al., 1994). Extracellular matrix is mainly produced by SMCs and accumulate in the lesion during progression (Geng and Libby, 2002) and can stabilize the plaque.
All this leads to an abluminal expansion of the arterial wall and a luminal restriction.

2.2.3 Rupture of Atherosclerotic Plaques

Various mechanisms can lead to a thrombotic event, often resulting in complete occlusion of the diseased vessel. Around two-thirds of the overall thrombotic events are mediated by plaque rupture. Approximately one-third of thrombotic events are the result of superficial erosion of the plaque (Virmani et al., 2000).
The first plaque rupture was reported in 1844 on an autopsy of a sudden heart death, where the vessel wall contained “several atheromatous plaques, one of which quite clearly had ulcerated, pouring the atheromatous mass into the arterial lumen” (Virchow, 1860).
Plaque rupture is a multigenic event with several factors influencing the development of rupture and their outcome. For example, it is remarkable that even large plaques can cause no symptoms as long as they do not restrict the blood flow (Brown et al., 1993). Indeed, the interaction of a large necrotic core, a thin fibrous cap, the activity of immune, inflammatory cells and their products causes plaque rupture. As a common phenomenon in atherosclerotic lesion, intraplaque haemorrhage significantly contributes to the enlargement of the plaque and also has a negative influence towards plaque vulnerability (Kolodgie et al., 2003). By contrast, a stable plaque typically has a thick fibrous cap, which separates the blood stream from the thrombotic core of the plaque. This stability results from a matrix rich collagen type 1 and 3 produced by SMCs.
IFN-γ, an inflammatory mediator, plays an important role by down-regulating type 1 and 3 collagen genes in SMCs (Amento et al., 1991). Produced by activated T-lymphocytes, IFN-γ lowers the integrity and stability of the fibrous cap of the plaque.

Furthermore, plaques with high levels of free cholesterol, mainly of apoptotic foam cells, show an significant increase in plaque rupture (Felton et al., 1997).

Key players in destabilization are MMPs (Matrix Metalloproteinase), cysteine proteases and chymases (Liu et al., 2004) (Lindstedt and Kovanen, 2004, Jones et al., 2003). MMPs, released by macrophages, which predominate over SMCS this stage of disease, have the ability to degrade the extracellular matrix are partly responsible for a remodeling of the atherosclerotic plaque.

The rupture of the fibrous cap leads to the release of the content of the lipid-rich core, as well as tissue factor, which subsequently results in the thrombotic occlusion of the vessel (Wilcox et al., 1989).

Superficial erosion of plaques is another possible mechanism of plaque-associated thrombosis. The cell death of endothelial cells (Bombeli et al., 1997) or SMCs (Flynn et al., 1997) can promote the thrombotic activation and exposition of thrombogenic collagen (Farb et al., 1996). This can occur as a response to the inflammatory process of the atherosclerotic lesion. It is reported that Myeloperoxidase and MMPs, produced by immune cells, play an important role in the death of endothelial cells (Niccoli et al., 2010, Libby, 2008).

Plaque rupture can also be subclinical and not cause any symptoms (Libby, 2008). Such a subclinical thrombotic event can cause remodeling of the plaque, leading to progression in plaque growth and therapy worsening of the stenosis of the vessel.

2.3 Treatment

2.3.1 Lifestyle interventions

Lifestyle changes can significantly reduce cardiovascular risk. Diet, including moderate alcohol consummation, physical activity and stress reduction, can positively influence cardiovascular risk. This outcome is mediated by anti-oxidative and anti-inflammatory effects on the vascular system. State of art physical activity should be recommend to every person (Wexler et al., 2012). Beyond this lifestyle intervention, patients should also be recommended to stop smoking.

2.3.2 Medical

Unfortunately, the currently established therapy, including statins, is unable to prevent 50-70 % of all clinical events. Therefore, there is a large medical need to develop new therapeutic options to treat atherosclerosis and thereby reduce cardiovascular mortality and morbidity (Klingenberg and Hansson, 2009).

2.3.2.1 Established therapy

Statins are an established and tested drug class in the treatment of Coronary Artery Disease (CAD). Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) inhibit the cholesterol synthesis and also have anti-inflammatory effects; for example, by down-regulation of NF-κB and MAPK (Mitogen-Activated Protein Kinases) pathways and inhibiting proinflammatory cytokine production (Tian et al., 2012, Antonopoulos et al., 2012). There is indication of the direct effects on stability and the size of the plaque (Libby and Aikawa, 2002), although no convincing study has proven a significant effect in primary prevention to date (Abramson J, 2007).

Treatment with niacin significantly reduces the incidence of CAD by changing the cholesterol profile (Phan et al., 2013, Philpott et al., 2013).

Another category of therapy is therapy with Non-steroidal Anti-inflammatory Drugs (NSAIDs). Aspirin as the only irreversible anti-platelet drug, is in secondary prophylaxes an established therapy for CAD (Anand and Yusuf, 1999). The effect seems likely to be a predominant anti-platelet effect rather than an anti-inflammatory effect.

Beta-blockers have been shown in clinical tests to reduce the mortality of Myocardial infarction (MI) and decelerate the progression of coronary atherosclerosis (Sipahi et al., 2007). The inhibition of the Renin Angiotensin system, either with Angiotensin-Converting Enzyme (ACE)-inhibitors or Angiotensin-receptor blockers, shows a significant reduction in coronary events, not only by lowering blood pressure (Yusuf et al., 2008).

2.3.2.2 Emerging

New therapeutic options are currently being tested in clinical phases. Different strategies like HDL mimetics (Joy, 2012), IL-1 receptor antagonist (Klingenberg and Hansson, 2009) or immunosuppressive (Ridker, 2009) drugs have showed promising results to date. Large clinical trials testing the hypothesis that anti-inflammatory drugs

reduce cardiovascular events are currently ongoing (for example the “Cardiovascular Inflammation Reduction Trial (CIRT)” with methotrexate).

2.3.2.3 Experimental

There are many experimental studies about atherosclerosis and their treatment. For example, the blocking of CCR5 (Chemokine Receptor 5) or CCR2 has shown promising results in trials (Weber and Noels, 2011). Immunization, as a vaccination, is a promising strategy in primary and secondary prevention by effecting T cell subtypes and the induction of regulatory T cells (Hansson and Nilsson, 2009).

2.3.3 Revascularization

There two main established invasive therapies for CAD patients: percutaneous coronary intervention (PCI) and coronary artery bypass surgery (CABG). Both modalities are effective and safe treatment options for patients with multi-artery diseases, although there remains a lack of new randomized studies comparing each option.

The major limitation for PCI is restenosis of the coronary artery. The leading processes underlining this restenosis include the immune system, endothelial disruption and platelet activation. One result of these complex processes is a proliferation of smooth muscle cells (Adams, 2013). Therefore, drug eluted stents, coated in the first generation with paclitaxel or sirolimus, have been developed. A disadvantage of these stents is the higher risk of thrombosis, requiring anti-platelet therapy for 12 months. Therefore, bare-metal stents remain a better choice in some conditions, such as bleeding problems or operations. Next generation stents, eluted with everolimus, have been shown to reduce the probable stent thrombosis rate from 3.9 % with first generation stents to 0.9 % after two years (Smits et al., 2011).

The long-term outcome in survival differs between PCI and CABG (Bravata et al., 2007), although the latter offers an advantage in the complete relief of angina over five years and revascularization was not so often necessary. However, CABG showed an increase in procedural strokes (1.2 % vs. 0.6 %). Other studies suggest that CABG only offers a benefit in survival and symptom relief in three vessel disease against medical treatment (Soran et al., 2009).

3 The Innate Immune system

The innate immune system plays an important role during the first defense against pathogens in the human body. Therefore, pattern-recognition receptors (PRRs) identify exogenous pathogen-associated molecular patterns (PAMPs), initiating a downstream immune response. PRRs are able to recognize a broad range of pathogen related compounds, including lipids, carbohydrates and nucleic-acid structures stemming from microorganisms, and especially viral and bacterial origins.

In addition to PAMPS, PRRs have been shown to recognize endogenous damage associated molecular patterns (DAMPS) (Bianchi, 2007). For example, oxLDL, extracellular matrix or heat-shock proteins can play an important role in acute and chronic inflammation (Chtarbanova and Imler, 2011). Nonetheless, it should be mentioned that these endogenous ligands remain somewhat controversial due to the possibility of contamination with exogenous ligands (Trinchieri and Sher, 2007).

3.1 Toll-Like Receptor family

The protein Toll was originally discovered as a protein responsible in the development of Drosophila embryo for dorsoventral polarity in 1988. Later on, it could be demonstrated that in Toll regulates the ability to act against fungal infection the Drosophila fly. The Russian scientist Medzhitov and Charles Janeway reported a human homolog in 1991. Toll-Like Receptors are the best described and explored receptors in the PRR family and expressed on various cells of the innate and adaptive immune system, involving dendritic cells, leucocytes, B and T lymphocytes. Besides non-immune system cells express TLRs, for example endothelial cells. They demonstrate a NF-κB and NF-κB – dependent gene activation of this “Toll-Like” protein (O'Neill, 2004).

To date, 13 TLRs have been discovered in human and mice, although only TLR1-9 are expressed and functional in both species, whereas TLR10 is not functional and TLR11-13 are only expressed in mice. TLRs are type 1 transmembrane proteins and all structured in a leucine-rich ectodomain domain for ligand recognition, a transmembrane domain that defines their cellular localization and an intracellular domain. This intracellular domain, so-called toll-interleukin 1 receptor (TIR), is required for downstream signaling in the cell (Seneviratne et al., 2012).

Human TLRs can be divided into cell surface located TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) and intracellular TLRs (TLR3, TLR7, TLR8, TLR9) located on vesicles or organelles. Furthermore, TLRs form homodimers, with the exception of TLR2 and TLR4. TLR2 requires TLR1 or TLR6 and form a heterodimer, while TLR4 can cooperate with TLR6 (concerted with co-receptor CD36) for dimerization (Stewart et al., 2010). Different TLRs cooperate with each other (Trinchieri and Sher, 2007), as first demonstrated by Whitmore et al. They show that the stimulation of mouse macrophages with a TLR3 ligand and a TLR9 ligand induce TNF, IL-6 and IL12p40 in a higher level than simply an expected additive level (Whitmore et al., 2004).

3.1.1 TLR Signaling Pathways

TLR signaling mainly ends in a NF-κB dependent or interferon-regulatory factor dependent down streaming pathway and it is also responsible for the production of antimicrobial molecules and proinflammatory cytokines, like IL-6, TNF-α and lL-1β (Hodgkinson and Ye, 2011).

After the activation of TLR adaptor molecules being recruited by the TIR domain and with the exception of TLR3, all TLRs use MyD88 (Myeloid Differentiation primary-response gene 88). Further adapter molecules are TRIF (TIR-domain-containing adapter-Inducing Interferon-β), TRAM (TRIF-related Adaptor Molecule) and TIRAP (TIR domain containing Adaptor Protein), also called MAL (MyD88-adaptor like). Complex downstream signaling leads to proinflammatory transcription factor and MAPK activation (Figure 2).

Both TLR4 and TLR3 have been associated with signaling via a MyD88 independent pathway, using TRIF as an adaptor molecule. Activation of this pathway leads to the activation of transcription factor IRF3 (Interferon Regulatory Factor 3) and NF-κB, as well as the induction of proinflammatory cytokines and type 1 interferon (Kawai and Akira, 2010).

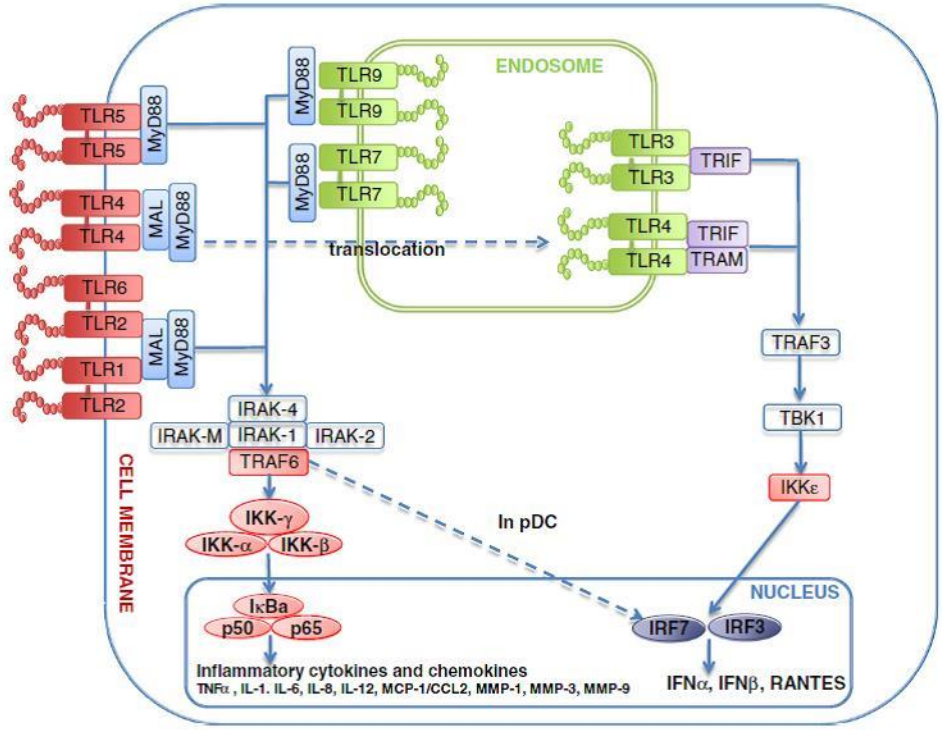

Figure 2: Signaling pathways of TLR (Seneviratne et al., 2012)

Toll-like Receptor signaling leads to the activation of several transcription factors, including NF-κB and interferon regulatory factors (IRFs). All TLRs - with the exception of TLR3 - recruit MyD88, which is responsible for downstream signaling via NF-κB, directly by the IκB Kinase (IKK) complex, activation. Recruitment of TRIF by TLR3 or TLR4 leads to an IFN activation. MAL: MyD88-adaptor like; TRAF: Tumor necrosis factor receptor-associated factor; TRIF: TIR domain-containing adaptor inducing interferon β; TRAM: TRIF-related adaptor molecule.

3.1.2 Toll-Like Receptor ligands

Ligands can be categorized as lipid-based particles (detected by TLR2, TLR4, TLR6), proteins (TLR5) and nucleic acids (TLR3, TLR7, TLR8, TLR9) (Mann, 2011). A further common classification is to distinguish between exogenous and endogenous ligands (Figure 3).

Exogenous, or PAMPS, can be bacterial, viral, protozoa or fungi origin. For example, bacterial Lipopolysaccharides (LPS) or viral DNA (Deoxyribonucleic Acid) signal via TLR2, TLR4, TLR9 (Ionita et al., 2010).

Endogenous ligands, or DAMPs, are especially heat shock proteins (HSPs), modified LDL, HMGB1 (High-Mobility Group Protein B1), fibronectin and other damage released molecules. They can all emerge from damaged cells and the environment. Even self DNA can be modified under inflammatory conditions and recognized by TLR7 and TLR9.

For the recognition of some ligands, co-receptors and binding molecules are required. Accordingly, TLR4 can form a complex with CD14, Myeloid Differentiation factor 2 (MD2) and Lipopolysaccharide-Binding Protein (LBP) for recognition of LPS. By heterodimerisation with TLR1 or TLR6, the specificity of TLR2 to detect ligands is increased (Seneviratne et al., 2012).

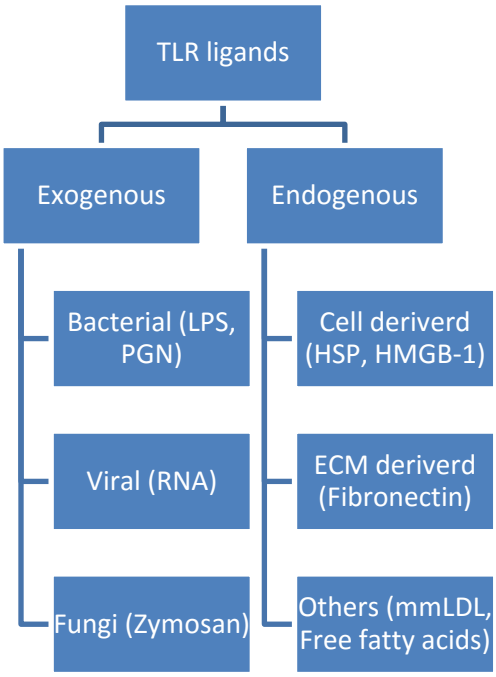

Figure 3: TLR ligands

The figure shows a schematic representation of possible TLR ligands (modified from (Ionita et al., 2010). LPS: Lipopolysaccharides; PGN: Peptidoglycan; RNA: Ribonucleic acid; HSP: Heat shock proteins; ECM: Extracellular matrix; mmLDL: minimally modified low-density lipoprotein

3.1.3 TLRs and atherosclerosis

The hypothesis that TLRs play an important role in atherosclerosis is mainly supported by experimental mouse studies, the expression of TLRs in atherosclerosis, epidemiological studies and studies with TLR gene polymorphism.

First of all, immune cells that migrated into atherosclerotic plaques show an expression of different TLRs. TLR1, TLR2, TLR4 and TLR5 have been shown to be expressed in the atherosclerotic lesion. For example, TLR4 is mainly expressed in the shoulder region of the plaque (Mann, 2011), which is a sensitive area in terms of stability and growth. TLRs play an important role in the composition of plaque, inflammatory cytokine release and the stability of plaque.

Epidemiological studies also support an important role for TLRs in atherosclerosis, suggesting a link between bacterial infection and atherosclerosis through the activation of TLR dependent signaling and pro-inflammatory processes (Saikku et al., 1988). For example, Chlamydia pneumonia has been detected in atherosclerotic lesions and is a possible ligand for TLRs (Frantz et al., 2007).

Finally, the polymorphism of genes encoding TLRs offer a potential causal connection of TLR in atherosclerosis. Unfortunately, studies in this area are inconsistent. While several studies point towards a risk reduction for myocardial infarction (mainly with a TLR4 Asp299Gly Polymorphism), the largest study in this field did not concur (Frantz et al., 2007).

Furthermore, in clinical studies, patients with unstable plaques and acute myocardial infarction show higher amounts of TLR4 positive circulating monocytes (Frantz et al., 2007).

Experimental studies with mice also suggest a link between TLRs and atherosclerosis in the two main mouse models of atherosclerosis.

Previous studies with deficiency of Apolipoprotein E (ApoE) as a background:

TLR/co-molecules	**Mouse Model**	**Effect**	**Period**	**Reference**
TLR1	Double knockout ApoE-/-:TLR1-/-	No effect	10 and 14 weeks HFD plus 1.25 % cholesterol	(Curtiss et al., 2012)
TLR2	Double knockout ApoE-/-:TLR2-/-	Reduction of atherosclerosis	5 and 7 month chow diet	(Liu et al., 2008)
TLR2	Double knockout ApoE-/-:TLR2-/-	Reduction of atherosclerosis just after 3 weeks chow diet	3 and 36 weeks chow diet	(Higashimori et al., 2011)
TLR3	Double knockout ApoE-/-:TLR3-/-	Increase of atherosclerosis	15 weeks chow diet	(Cole et al., 2011)
TLR3	TLR3 agonist	Increase of atherosclerosis	7 weeks HFD plus 1.25 % cholesterol	(Zimmer et al., 2011)
TLR4	Double knockout ApoE-/-:TLR4-/-	Reduction of atherosclerosis	3 and 36 weeks chow diet	(Higashimori et al., 2011)
TLR4	Double knockout ApoE-/-:TLR4-/-	Reduction of atherosclerosis	6 months on Western diet	(Michelsen et al., 2004b)
TLR6	Double knockout ApoE-/-:TLR6-/-	No effect	10 and 14 weeks HFD plus 1.25 % cholesterol	(Curtiss et al., 2012)
TLR7	Double knockout ApoE-/-:TLR7-/-	Increase of atherosclerosis	10, 18 and 26 weeks chow diet	(Salagianni et al., 2012)
MyD88	Double knockout ApoE-/-:MyD88-/-	Reduction of atherosclerosis	6 months on western diet	(Michelsen et al., 2004b)
MyD88	Double knockout ApoE-/-:MyD88-/-	Reduction of atherosclerosis	10 and 14 weeks western diet	(Bjorkbacka et al., 2004)
CD14 (Co-receptor of TLR4)	Double knockout ApoE-/-:CD14-/-	No effect	10 and 14 weeks western diet	(Bjorkbacka et al., 2004)

Table 1: Influence of TLRs in atherosclerosis in ApoE-/- mice

Western diet: Including 0.15 % cholesterol; HFD: High-fat diet

Furthermore the influence of TLR on atherosclerosis in mice with knockout of Low-density Lipoprotein receptor (LDLr) as background:

TLR/co-molecules	**Mouse Model**	**Effect**	**Period**	**Reference**
TLR2	Bone Marrow transfer of TLR2-/-	Reduction of atherosclerosis	8 weeks on Western diet	(Hasu et al., 2011)
TLR2	Bone Marrow transfer of TLR2-/-	Reduction of atherosclerosis mediated just from cell not of BM origin	10 and 14 weeks HFD plus 1.25 % cholesterol	(Mullick et al., 2005)
TLR3	Bone Marrow transfer of TLR3-/-	Reduction of atherosclerosis	7-8 weeks HFD plus 1.25 % cholesterol	(Lundberg et al., 2013)
TLR4	Double knockout LDLr-/-:TLR4-/-	Reduction of atherosclerosis after 24 weeks diabetogenic diet, yet not after 24 weeks chow diet	24 weeks on chow or diabetogenic diet (saturated fatty acids-rich, and carbohydrate-rich diet)	(Ding et al., 2012)

Table 2: Influence of TLRs in atherosclerosis in LDLr-/- mice
Western diet: Including 0.15 % cholesterol; HFD: High-fat diet

3.1.3.1 Toll-Like Receptor 2

TLR2 is able to detect a large range of PAMPs, including bacterial (lipopeptides, peptidoglycan and lipoteichoic), fungi (zymosan) and viral (hemagglutinin protein) (Kawai and Akira, 2010). As previously mentioned, by forming heterodimers with TLR1 or TLR6, TLR2 recognizes both tricylated and diacylated lipoproteins of gram negative and positive bacteria, as well as mycoplasma (Kawai and Akira, 2010). Pam3CSK4 is a specific agonist of TLR2-TLR1 heterodimer. Furthermore, TLR2 has the ability to enhance their ligand repertoire by using the co receptor CD36 (Jimenez-Dalmaroni et al., 2009).

TLR2 shows a reduction of atherosclerosis in LDL knockout mice and increased lesion size with a TLR2 agonist. Interestingly, the cells responsible for the proatherogenic effect are not of bone marrow (BM) origin; in fact, non-BM origin cells are responsible for the reduction of lesion size (Mullick et al., 2005). Endothelial cells are one cell type considered responsible for the proatherogenic effect of TLR2; however, an *in vitro* link remains to be established in studies concerning the effect of laminar shear stress and endothelial TLR2 expression (Michelsen et al., 2004a).

Despite the described proatherogenic effect of TLR2 signaling, TLR2 has also been reported to induce T_h2 cell differentiation (Redecke et al., 2004). Furthermore, TLR2 has been linked to an increase in regulatory T cells (Cole et al., 2010), possibly via dendritic cell mediated promotion (Manicassamy et al., 2009). This is controversial given the hypothesized proatherogenic effect of TLR2 signaling, due to the described paradoxical athero-protective role of T_h1 suppression (Mallat et al., 2003). The long-term effect of the TLR2 gene deletion in mice on a Western diet remains unclear, with one study by Hasu et al. (Hasu et al., 2011) reporting a possible loss of the proatherogenic effect of TLR2.

3.1.4 Toll-Like Receptor 4

TLR4 works in a receptor multimer, including two copies of TLR4 and a MD2 subunit and the main ligand LPS. CD14 is also involved as a co-receptor and the soluble LPS-binding protein (Kawai and Akira, 2010).

TLR4 is the only TLR that is able to use the MyD88-and the TRIF pathway for signaling. Normally located on the cell surface, TLR4 is able to traffic to an endosome and uses the TRIF pathway. This gives the possibility for an early initiation of NF-κB and MAPK

activation, as well as late phase activation by TRIF. However, TLR4 requires both pathways for a sufficient cytokine production (Kawai and Akira, 2010).

Oxidized LDL and mmLDL can up regulate TLR4 expression, but not unmodified LDL (Doherty et al., 2006). As a result, studies suggest that TLR4 is proatherogenic, through a suggested increased systemic inflammation, with increased MCP-1 and IL12 levels (Michelsen et al., 2004b). However, a lack of CD14 in ApoE knockout mice could not show an effect on atherosclerosis (Bjorkbacka et al., 2004). While TLR4 is generally accepted to play an important role in the development of early atherosclerosis (Higashimori et al., 2011), the long-term effect of TLR4 deficiency is remains to be elucidated.

3.1.5 Toll-Like Receptor 9

TLR9 is an endosomally-located TLR that recognizes unmethylated Cytosine-phosphate-Guanosine (CpG) sequences in DNA, which are relatively rare in vertebrate genomes yet abundant in bacteria. Multiple cells in the atherosclerotic plaque have an accumulation of TLR9, including macrophages, plasmacytoid- and conventional- dendritic cells and B cells (Boonstra et al., 2003, Boonstra et al., 2006). However, their exact role for TLR9 remains controversial with evidence supporting both a pro- and anti-atherogenic role. TLR9 activation leads *in vitro* to an increase foam cell formation, indicating a possible pro-atherogenic effect of TLR9 (Sorrentino et al., 2010). Furthermore, the herpes simplex virus as an ligand for TLR9 has been shown to promote atherosclerosis in ApoE knockout (KO) mice (Curtiss and Tobias, 2009). In studies with human plaque, it was shown that TLR9 is expressed on plasmacytoid dendritic cells in fibrofatty lesions (Niessner et al., 2006). *In vitro* stimulation of atherosclerotic lesion tissue with Oligonucleotides (ODNs), TLR9 agonists, increases the secretion of IFN-α and increases the cytotoxicity of $CD4^+$ T cells towards SMCs (Niessner et al., 2006). By contrast, other studies support the hypothesis that TLR9 may be anti-atherogenic. The activation of TLR9 stimulates *in vitro* IL-10 by conventional dendritic cells, which can inhibit the expression of IFN-α by plasmacytoid dendritic cells (Waibler et al., 2008). Furthermore, the stimulation of B cells through TLR9 activation was observed, as well as an increase of IL-10 production, which has separately been shown to have the ability to reduce $CD4^+CD25^-$ T cell proliferation (Bouaziz et al., 2010).

4 Hypotheses

TLR2, TLR4 and TLR9 have the potential to modulate atherosclerotic plaque size, lesion progression and stability.
Determining the role and possible mechanisms of TLR signaling in the progression of atherosclerosis will allow developing novel therapeutic strategies for the treatment of atherosclerosis.

5 Aims

The aim of this study is to investigate the influence of the innate immune system, primarily TLR2, TLR4 and TLR9, on the development of atherosclerosis and plaque composition, specifically foam cells, Lipids, collagen and adhesion molecules.

6 Methodology

6.1 Study Design

To investigate the relationship between Toll-Like Receptor 2, 4, 9 and atherosclerosis, the histology of double knockout (DKO) mice fed a high fat diet for 8 or 15 weeks was carried out (Figure 4). Basic histology (H&E and ORO outlined below) was used to examine the plaque size and lipid content in the atherosclerotic plaques. Samples found to exhibit differences were further investigated using advanced histology (IHC), including the investigation of collagen, foam cells, adhesion molecules and plasma cholesterol.

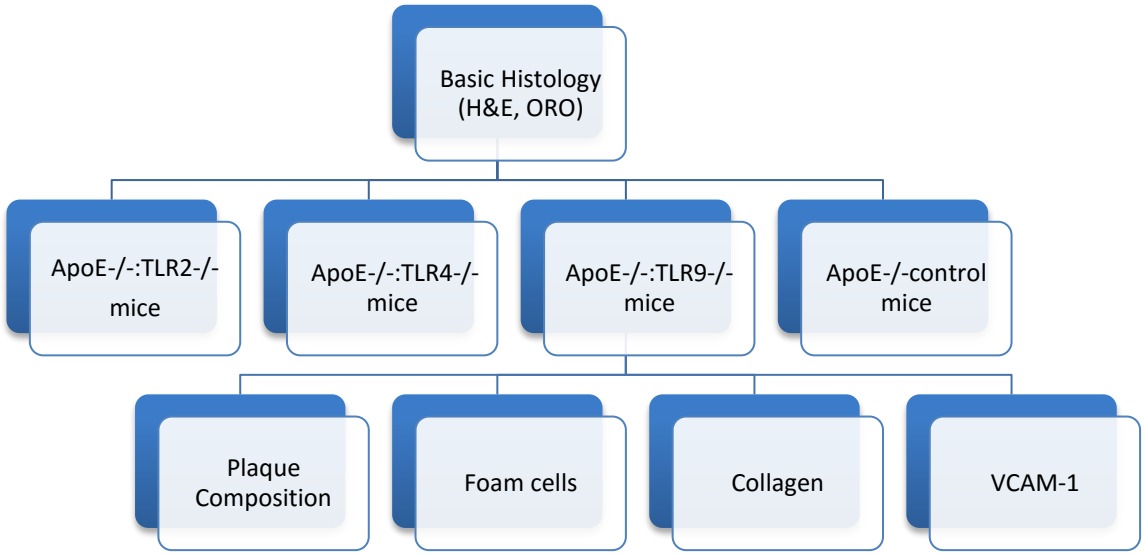

Figure 4: Study design of the TLR Study

This figure represents the first study design of the thesis.

6.1.1 Atherosclerosis Animal model

For this study, male Apolipoprotein E (ApoE) deficient mice were chosen. The mice were bred on a C57BL/6J background and supplied from the Animal Resource Centre (Western Australia, Australia). They were fed a Western high fat diet, containing 22 % fat and 0.15 % cholesterol (SF00-219, Specialty Feeds, Western Australia, Australia) as well as tap water for the experimental period. This animal model is widely used and highly regarded.

6.1.2 Toll-Like Receptor Knockout

To generate double knockout (DKO) mice, TLR2-/-, TLR4-/- or TLR9-/- mice on a C57Bl/6 background were crossed with ApoE-/- mice. The F1 generation was interbred to generate the F2 generation. F2 mice were genotyped using Polymerase Chain Reaction (PCR) for confirmation of the double knockout mice.

6.1.3 Oligonucleotide injection

8 week-old ApoE-/-:TLR9+/+ and ApoE-/-:TLR9-/- male mice were injected with Type B CpG oligonucleotide (ODN) 1668, a specific TLR9 agonist (Obermeier et al., 2005), (Integrated DNA Technologies; Coralville; USA) with the sequence: T*C*CA*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T (10 µg/kg , i.v; intravenous) or vehicle control (Phosphate Buffered Saline; PBS) for a period of 8 weeks. In addition, all mice received a Western diet for the duration. Upon conclusion, the mice were culled and the tissue removed.

6.1.4 CD4-depleting antibody injection

8 week-old ApoE-/-:TLR9+/+ and ApoE-/-:TLR9-/- male mice were injected with CD4-depleting antibody or isotype control. 300 µg of antibody was injected weekly i.v. in the tail vein. In addition, all mice received a Western diet for the duration. Upon conclusion, the mice were culled and the tissue removed. Blood was collected for lipids analysis, paraaortic and inguinal lymph nodes and spleen were collected for FACS (Fluorescent Activated Cell Sorting) analysis and aortic sinus was collected for lesion analysis. The function of CD4-depleting antibody and isotype control antibody was evaluated by FACS analysis (Figure 5).

6.1.5 Tissue preparation

Animals were sacrificed humanely with anesthetic, comprising Ketamine (KETELAR; Pfizer, New Zealand, 20 mg/ml diluted in saline) and xylazine hydrochloride (ILIUM XYLAZIL; Troy Laboratories Pty Ltd; Australia, diluted 10 mg/ml in saline) via i.p. (intraperitoneal) injection. After the collection of blood (900 µl) from the vena cava inferior, 10 ml PBS, pH 7.4 under physiological pressure was perfused via a catheter placed in the left ventricle for cholesterol analysis. After perfusion, the aortic root and right carotid artery were embedded in optimal cutting temperature compound (OCT) (Sakura Finetechnical), frozen and stored at -80 °C until sectioning. 6 µm thick latitudinal cryosections were prepared using a cryostat (Leica, CM1950 or Zeiss MICROM HM 550). Samples were cut until the three aortic valves were visible and 15 slides with two samples per slide were collected.

6.1.6 Ethics

Animal ethics was obtained for all animal experiments, approved by the AMPREP Animal Ethics Committee. Furthermore, an accredited supervisor taught the correct handling and monitoring of the mice, while regulation of the Baker IDI Occupational Health and Safety Policy was obeyed.

6.1.7 Histology

Tissue sections of the aortic roots were stained using standard histology techniques to identify plaque size, lipids, foam cells, adhesion molecules and collagen in double knockout and ApoE deficient control mice.

6.1.7.1 Haematoxylin and eosin staining

Frozen sections of aortic roots and right carotid arteries were thawed at room temperature (RT) for 30 mins, before being immersed in Mayer's haematoxylin for 8 mins to stain the nuclei of the cells (for Mayer's Haematoxylin: 5 g of haematoxylin and 50 g of aluminum ammonium sulphate were dissolved and heated in 700 ml of distilled water (dH_2O). Once the solution was cooled, 30 ml of 1 % sodium iodate, 20 ml of glacial acetic acid and 300 ml of glycerol were added. The slides were subsequently washed with tap water, Scott's Tap Water Substitute (8.75 g of sodium bicarbonate, 50 g of magnesium sulphate dissolved in 2500 ml of dH_2O) and then tap water again. The slides were then rinsed 10 times in 95 % alcohol and counterstained in eosin (0.25 % eosin, 8 mM sodium acetate, 85 mM acetic acid in 95 % ethanol, all stock

solutions were also made up in 95 % ethanol) for 8 mins. The slides were then dehydrated by dipping 30 times into absolute alcohol and cleared in xylene (Labscan, Gliwice, Poland) for 10 mins, before being mounted in Gurr DePex (DPX; VWR International, Dublin, Ireland). All products were from Sigma, Missouri, USA, unless stated otherwise.

6.1.7.2 Oil Red O staining

Oil Red O (ORO) staining was used to stain the lipids in the plaque. Frozen sections of aortic roots and right carotid arteries were thawed at RT for 30 mins and then fixed in 10 % buffered formalin (SIGMA, Missouri, USA) diluted in PBS for 4 mins. The slides were washed in PBS for 4 mins and subsequently dipped into 60 % isopropanol for 25 secs. The slides were stained with freshly prepared ORO solution (120 ml of 0.5 % Oil Red O (O-0625; SIGMA, Missouri, USA) (in isopropanol) with 80 ml dH_2O) for 10 mins. To wash off the unspecific stain, the slides were immersed in 60 % Isopropanol for 40 secs, and then dH_2O for 2 mins. To get a better contrast, the slides were immersed for 15 secs in Mayer's haematoxylin, followed by three washing steps: tap water (under running tap water in a container until water turns clear), Scott's tap water (30 secs) and tap water again (3 mins). The slides were mounted with aquamount (Aquatex, Merck, Darmstadt, Germany) and stored in the dark.

6.1.7.3 Masson's Trichrome stain

To stain TLR impact on collagen, Masson's trichrome staining was used. Frozen sections of aortic roots and right carotid arteries were thawed for 30 mins at RT and subsequently placed in Bouin's fluid at 56 ^{o}C for 1 hour for mordanting. The slides were allowed to cool for 4 mins before being washed under running tap water and then stained with Weigert's Haematoxylin for 10 mins. The slides were washed in 80 % alcohol and then water, stained with Biebrich Scarlet solution for 2 mins and washed again in water before being immersed in phosphomolybdic/phosphotungstic acid solution for 15 mins. The slides were again washed in water, stained with Aniline Blue for 5 mins, washed in water and treated with 1 % glacial acetic acid for 5 mins. After a final washing step in water, the slides were dehydrated in absolute alcohol four times for 1 min, cleared in xylene twice for 2 mins and subsequently mounted in Gurr DePex (VWR International, Dublin, Ireland). All reagents were from Australia Biostain Pty Ltd, Traralgon, Australia.

6.1.7.4 Immunohistochemistry

The vectastain ABC kit (Avidin-Biotin-Complex; Vector Laboratories, Burlingame, USA) was used to stain frozen sections of aortic roots and right carotid arteries. The slides were thawed at room temperature (RT) and fixed in acetone for 30 mins at - 20 ^{o}C. The slides were washed twice in PBS-Tween (PBS with 0.01 % Tween 20) for 5 mins, before endogenous peroxidase activity was blocked by treatment with 3 % hydrogen peroxide (in methanol) for 20 mins. The slides were subsequently washed as before and blocked for an hour with 10 % rabbit serum, 15 mins with avidin, and then 15 mins with biotin, rinsing with PBS after each blocking step. Primary antibody (Table 3) was diluted in PBS and incubated overnight at 4 ^{o}C. The slides were washed as before, incubated with secondary antibody (Table 3) for 30 mins, washed again, incubated with ABC reagent for 30 mins, washed again and then stained with 3,3'-diaminobenzidine (DAB) substrate kit for peroxidase. As soon as the sections turned brown, the slides were immersed in dH_2O to stop the reaction. The slides were subsequently washed twice in water for 5 mins and counterstained with Mayer's haematoxylin for 15 secs. The slides were cleared under running tap water and washed in 95 % alcohol for 2 mins, absolute alcohol three times for 6 mins and xylene for 10 mins. The slides were then mounted in Gurr DePex (VWR International, Dublin, Ireland).

Primary Antibody	Target	Dilution	Source	Incubation	Secondary Antibody	Dilution
CD68	Macrophages Foam Cells	1:100	AbD Serotec, Kidlington, United Kingdom	4 °C overnight	Anti-Rat IgG (Vectastain, Vector Laboratories, Burlingame, USA)	1:400
VCAM-1	Adhesion Molecule	1:100	Santa Cruz Biotechnology, Santa Cruz, USA	4 °C overnight	Anti-Goat IgG (Vectastain, Vector Laboratories, Burlingame, USA)	1:200
MOMA-2	Macrophages	1:100	AbD Serotec, Kidlington, United Kingdom	4 °C overnight	Anti-Rat IgG (Vectastain, Vector Laboratories, Burlingame, USA)	1:400
33D1	Dendritic cells	1:50	eBioscience, Inc (San Diego, USA)	4 °C overnight	Anti-Rat IgG (Vectastain, Vector Laboratories, Burlingame, USA)	1:500
CD86	Mature dendritic cells	1:100	eBioscience, Inc (San Diego, USA)	4 °C overnight	Anti-Rat IgG (Vectastain, Vector Laboratories, Burlingame, USA)	1:200

Table 3: List of Antibodies used for immunohistochemistry

MOMA-2: Macrophages/Monocytes Antibody; IgG: Immunoglobulin G

6.1.8 Visualization of samples

Images were taken using the Olympus BX50 microscope with QCapture Pro software (QImaging, Surrey, Canada) and analyzed using OPTIMAS 6.2 VideoPro-32 software (Bedford Park, Australia). To measure plaque area, H&E stained plaque was measured in μm^2. For immunohistochemistry of CD68, CD86, MOMA-2, 33D1 and VCAM-1 and ORO histology, the areas of interests were determined and the percentage area per total area of plaque was calculated. To ensure comparison between samples, slides with the same distance from the aortic root were used.

6.1.9 Plasma Cholesterol

Mouse blood (900 µl) was collected from the vena cava inferior and centrifuged (10 mins, 1500 g). Plasma was diluted 1:10 in water and analyzed by the COBAS Integra 400 Plus Analyzer (Roche Diagnostics Limited, Forrenstrasse, Switzerland). Total Cholesterol, HDL, VLDL (Very-low-density Lipoprotein)/LDL and triglycerides were measured.

6.1.10 $CD4^+$ T cell cytokine secretion

Spleens were taken from 8 ApoE-/-:TLR9-/- mice and 8 ApoE-/-:TLR9+/+ mice as a control group. Spleens were crushed between two microscope slides and 5 ml Lysing solution (BD FACS, BD Biosciences, San Jose, USA) was added. The spleen samples were centrifuged (5mins, 1500 g) down, washed with 20 ml PBS with Ca/Mg +0.1 % BSA (Bovine Serum Albumin), centrifuged again and re-suspended in 2 ml PBS +0.1 % BSA. The $CD4^+$ T cells of the spleens were isolated by a commercial kit (MACS, $CD4^+$ T cell isolation kit II).

Thereafter, cells were adjusted to a total cell number of 10^7 cells and centrifuged at 300 g for 10 mins. Supernatant was pipetted of and cell pallet was re-suspended with 40 µl PBS with Ca/Mg +0.1 % BSA. 10 µl of Biotin-Antibody Cocktail (containing biotin conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR (T Cell Receptor) γ/δ and Glycophorin A were added mixed and incubated for 10 mins on ice. Additionally, 30 µl PBS with Ca/Mg +0.1 % BSA was added together with 20 µl of Anti-Biotin MicroBeads. After mixing and incubating for 15 mins on ice, the cells were washed with 1 ml PBS with Ca/Mg +0.1 % BSA und centrifuged (300 g, 10 mins). The pallet was re-suspended in 500 µl PBS with Ca/Mg +0.1 % BSA and $CD4^+$ cells were separated with BD Influx™ cell sorter.

Separated $CD4^+$ T cells were incubated at a concentration of 10^6 cells/ml cell culture medium (RPMI-1640, 10 % fetal calf serum). For stimulation, anti-CD3 (BD Biosciences, USA) was pre-coated onto wells (2.5 µg/well). After 48 h, supernatant were collected. The secreted levels of TNF-α and IL-10 by $CD4^+$ T cells were measured by Enzyme Linked Immunosorbent Assays (ELISA) (eBioscience).

For ELISA (all compounds and solutions from eBioscience), MaxiSorp 96 well plate was coated with 100 µl of capture antibody (diluted in PBS) at 4 °C overnight. After incubation, the wells were aspirated and each well was washed three times with 300 µl PBS-Tween (PBS with 0.05 % Tween 20). Each well was blocked for 1 hour with 200 µl Assay Diluent, and washed twice with PBS-Tween. To generate a standard curve, 100 µl of recombinant cytokine in different dilutions (diluted in provided Assay Diluent) was added to the two top rows of well. 100 µl of supernatant from activated $CD4^+$ T cells was added to the appropriate wells. Sealed plate was incubated overnight at 4 °C. After washing the wells five times as described, 100 µl of biotin-conjugated detection antibody (diluted in Assay Diluent) was added and incubated for 1 hour at room temperature. 100 µl of Avidin-HRP (Horseradish Peroxidase; diluted in Assay Diluent) was added and incubated for 30 mins at room temperature. After washing seven times, 100 µl of Substrate Solution (Tetramethylbenzidine) was added and incubated for a further 15 mins at room temperature. 50 µl of Stop Solution (1 M H_3PO_4) was added to each well. The absorption was measured at 450 nm using a BioRAD BenchMARK PLUS microplate with Microplate Manager 5.2.1 software.

6.1.11 Evaluation of cell types in flow cytometry

To evaluate the different subsets of monocytes, a Ly6C analysis of $CD115^+/CD11b^+$ cells flow cytometry has been performed.

The blood and spleen of 16 week-old ApoE-/-:TLR9-/- or ApoE-/- :TLR9+/+ mice were analyzed. The mice were fed with normal chow diet. 1 ml blood each were taken from the inferior cava, stored with 100 µl heparin, lysed with 20 ml Lysing solution and incubated on RT for 15 mins. The spleen was crushed between two microscope slides and 5 ml Lysing solution (BD FACS, BD Biosciences, San Jose, USA) was added to lyse red blood cells. The blood and spleen samples were centrifuged (3 mins, 1500 g), washed with 20ml PBS containing Ca/Mg (1mM each) +0.1 % BSA, centrifuged (3 mins, 1500 g) again, whereby the cell pellet of the blood was re-suspended in 200 µl,

the pellet of the spleen with 2 ml, PBS+0.1 % BSA. Cells of each sample were counted using the improved Neubauer Hemocytometer, adjusted to 2 million cells and centrifuged (3 mins, 1500 g) again. Cells were incubated with the antibodies or isotype controls for 30 mins on ice in the dark. Cells were centrifuged (3 mins, 1500 g) and re-suspended with 250 ml Cellfix (Becton Dickinson Benelx, Erembodegem, Belgium) in FACS tubes (5 ml polystyrene tubes; BD Falcon, Fransklin Lakes, USA).

For the analysis of Ly6C high/intermediate/low positive cells:

Antibody	**Marker**	**Flourchrome**	**Source**
CD115	Macrophage colony stimulating factor	APC	BD Biosciences (USA)
CD11b	Activated Monocytes	FITC	BD Biosciences (USA)
Ly-6C	´Inflammatory´ Monocytes	Pacific Blue	BD Biosciences (USA)

Table 4: Antibodies used for macrophages subsets
APC: allophycocyanin; FITC: Fluorescein Isothiocyanate

For the others lymphocytes:

Antibody	**Marker**	**Flourchrome**	**Source**
CD22	B cells	PE	BD Biosciences (USA)
CD4	T cells	Pacific Blue	Caltag Laboratories (USA)
CD8	T cells	PerCP	BD Biosciences (USA)
Nk1.1	Natural killer cells T cells	Pecy7	BD Biosciences (USA)
TCRb	T cells	FITC	eBioscience, Inc (San Diego, USA)
CD5	B1 cells	APC	BD Biosciences (USA)

Table 5: Antibodies used for splenic lymphocytes quantification
PE: Phycoerythrin; PerCP: Peridinin Chlorophyll Protein; PE-Cy7: Phycoerythrin and Cyanine; Nk: Natural Killer cells TCRb: T Cell Receptor beta chain

Cells positive for the various cell markers were identified by FACS. The FACS output shows the percentage of cells that bind with high/medium or low affinity to Ly6C, in CD11b and CD115 double positive population of cells, for macrophages subset quantification (Table 4). Lymphocytes were gated with their particular marker (Table 5).

The function of the CD4-depleting antibody or isotype control antibody was also evaluated by FACS (Figure 5).

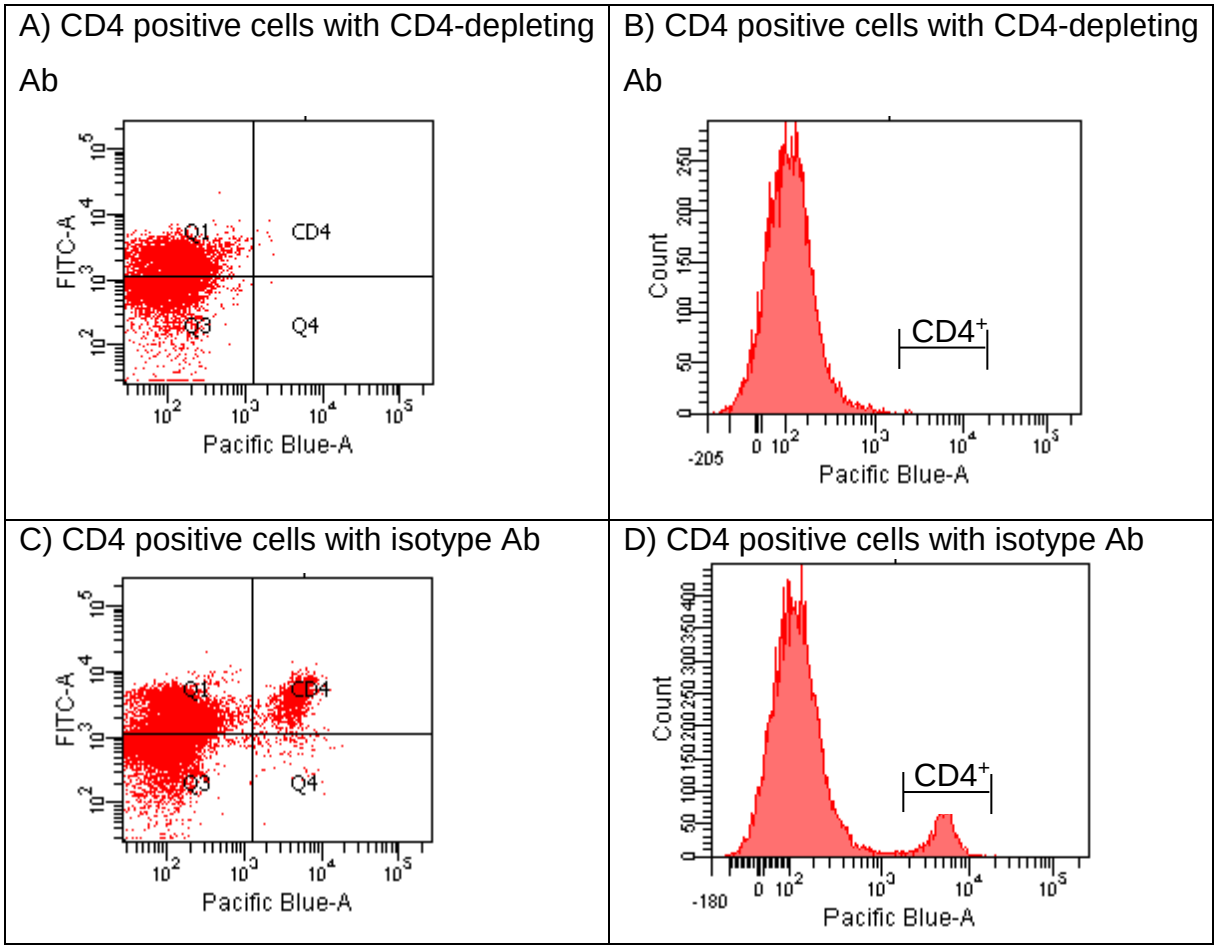

Figure 5: Function of the CD4-depleting Ab

CD4⁺ cells were tagged with TCRb-FITC-antibody and CD4-Pacific Blue-antibody in mice receiving CD4-depleting antibody (A, B) or isotype control (C, D). Double positive cells (in the quadrant labeled 'CD4'), in A) and C) representing the portion of CD4 cells as well as marked single staining with CD4-Pacific Blue-antibody (B, D).

6.1.11.1 Evaluation of regulatory T cells

To evaluate $CD4^+CD25^+Foxp3^+$ cells, regulatory T cells, in ApoE-/-:TLR9-/- mice flow cytometry was performed.

Splenic cells of 16 weeks old ApoE-/-:TLR9-/- or ApoE-/- :TLR9+/+ mice were prepared as in the section above. However, the surface of the cells was first stained with CD4 and CD25 antibodies or isotype control. Cells were incubated 20 mins on ice and centrifuged (3 mins, 1500 g). The cell pellet was re-suspended with 1 ml of freshly prepared Fixation/Permeabilization working solution (eBioscience, Inc ,San Diego, USA) to each sample. The cells were incubated for 30 mins on ice and centrifuged (3 mins, 1500 g). The cell pellet was re-suspended with 1 ml Permeabilization Buffer (eBioscience, Inc ,San Diego, USA), incubated again for 30 mins on ice and centrifuged (3 mins, 1500 g). The cells were washed with 5 ml PBS +0.1 % BSA and resuspended with 100 µl Permeabilization Buffer. Anti-mouse Foxp3 antibody or isotype control was added and incubated for 30 mins on ice. The cells were re-suspended with 250 ml Cellfix (Becton Dickinson Benelx, Erembodegem, Belgium) in FACS tubes (5 ml polystyrene tubes; BD Falcon, Fransklin Lakes, USA).

Antibody	**Flourchrome**	**Source**
CD4	FITC	Caltag Laboratories (USA)
CD25	PE	BD Biosciences (USA)
Foxp3	APC	eBioscience, Inc (San Diego, USA)

Table 6: Antibodies used for splenic regulatory T cells quantification

The cells were gated and subsequently cells gates based upon the positive antibody stain against the isotype control were used. First of all, lymphocytes were gated for $CD4^+$ cells and then of these cells $CD25^+$ cells. The $CD4^+CD25^+$ lymphocytes were gated in $CD4^+/CD25^+/Foxp3^+$ cells. The total cell number of $CD4^+CD25^+Foxp3^+$ cells was calculated.

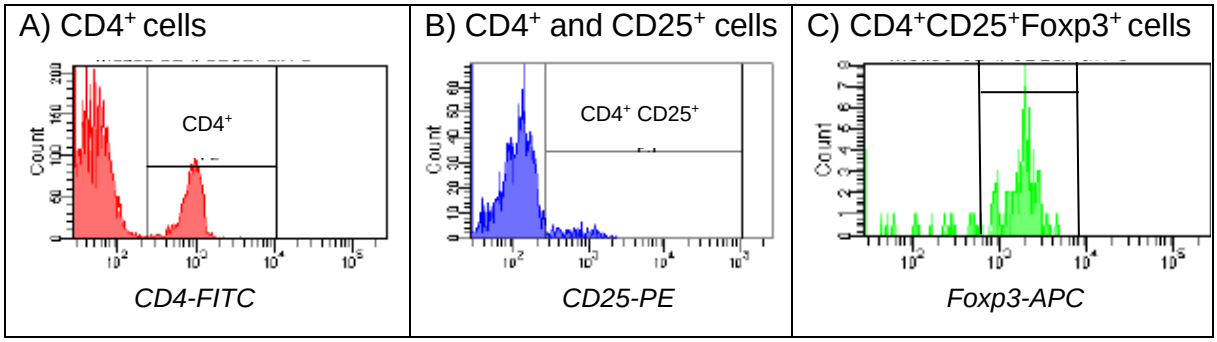

Figure 6: Gates used to measure regulatory T cells

6.2 Statistical Analysis

Statistical analysis was calculated and analyzed with Graph Pad Prism Version 5 (California, USA). A Mann-Whitney-U test was performed to compare two different groups. Multiple groups were analyzed with one-way ANOVA. The data was considered significant if P-values were 0.05 or less.

7 Results

7.1 Primary findings

Following a Western diet for 15 weeks, double knockout mice of ApoE and either TLR2 or TLR4 did not show a difference in atherosclerotic plaque size (Figure 7) or a change of lipid composition (Figure 9), compared to the ApoE single knockout control mice. There was a significant increase in the atherosclerotic lesion size of the ApoE-/-:TLR9-/- group compared to the control group (Figure 8). Furthermore, the plaque lesions of ApoE-/-:TLR9-/- group exhibited increased lipid composition (Figure 10) and reduced collagen (Figure 11) compared to control mice. In addition, there were an increased number of foam cells (Figure 12; Figure 13) and mature dendritic cells (Figure 14) in these lesions.

To evaluate the anti-atherogenic effect of TLR9 during the plaque progression, ApoE-/-:TLR9-/- and ApoE-/- mice were examined after 8 weeks of a Western diet. Both lesion size (Figure 16) and lipid composition (Figure 17) were significantly increased in the ApoE-/-:TLR9-/- with more dendritic cells (Figure 21) compared to ApoE-/- mice.

The LDL/HDL ratio was not altered in all double knockout experiments (Figure 31-34). The administration of a specific TLR9 agonist ApoE-/-:TLR9-/- and ApoE-/- mice for 8 weeks was associated with significantly reduced atherosclerotic lesions (Figure 24) compared to ApoE-/- mice without agonist.

In search of a possible mechanism, a $CD4^+$ cell depleting antibody was applied and plaque was analyzed. The antibody significantly reduced atherosclerotic plaque size in ApoE-/-:TLR9-/- mice compared to ApoE-/-:TLR9-/- mice receiving an isotype control antibody (Figure 28). Besides ApoE-/-:TLR9-/- mice were found to have increased levels of TNF-α and decreased levels of IL-10 secreted by $CD4^+$ T cells (Figure 30).

7.2 Histology 15 weeks

7.2.1 ApoE-/-:TLR2-/- or ApoE-/- :TLR4-/- mice do not have an altered plaque size

The double knockout of ApoE and either TLR2 or TLR4 did not show a difference in atherosclerotic plaque size compared to the ApoE single knockout control in 15 weeks Western diet mice.

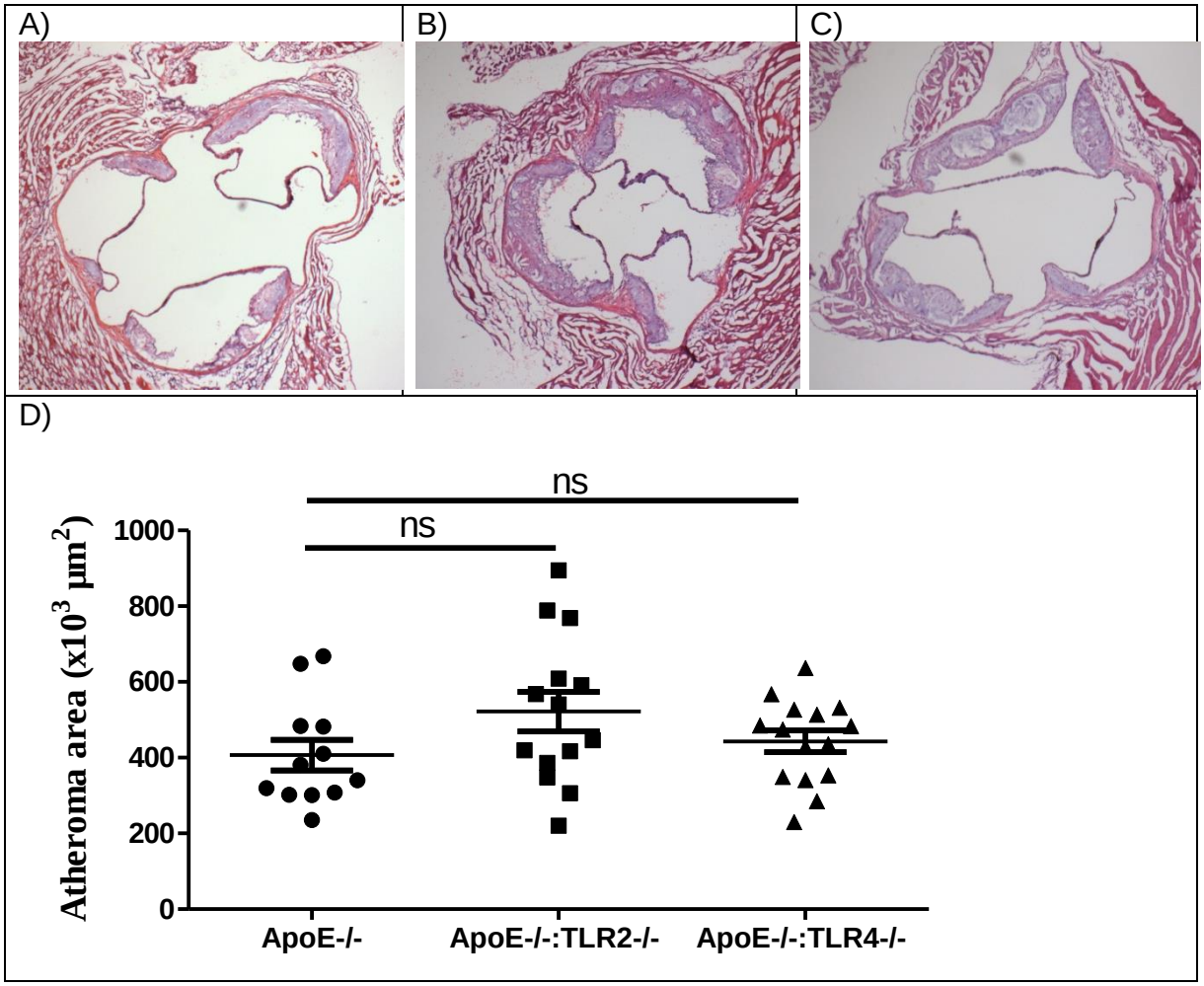

Figure 7: ApoE-/-:TLR2-/- or ApoE-/-:TLR4-/- mice do not show an altered plaque size

H&E stained aortic root sections from ApoE deficient (A), ApoE-/-:TLR2-/- (B) or ApoE-/-:TLR4-/- (C) mice fed a Western diet for 15 weeks. The purple atherosclerotic plaque area, in the three valves, was quantified. The mean atheroma area (x10³ µm²) ± SEM are shown (D).

7.2.2 ApoE-/-:TLR9-/- mice have a significant increase in plaque size

Aortic root sections from 15 week-old Western diet mice were stained with H&E. There was a significant increase in the purple plaque area of the ApoE-/-:TLR9-/- group compared to the control group.

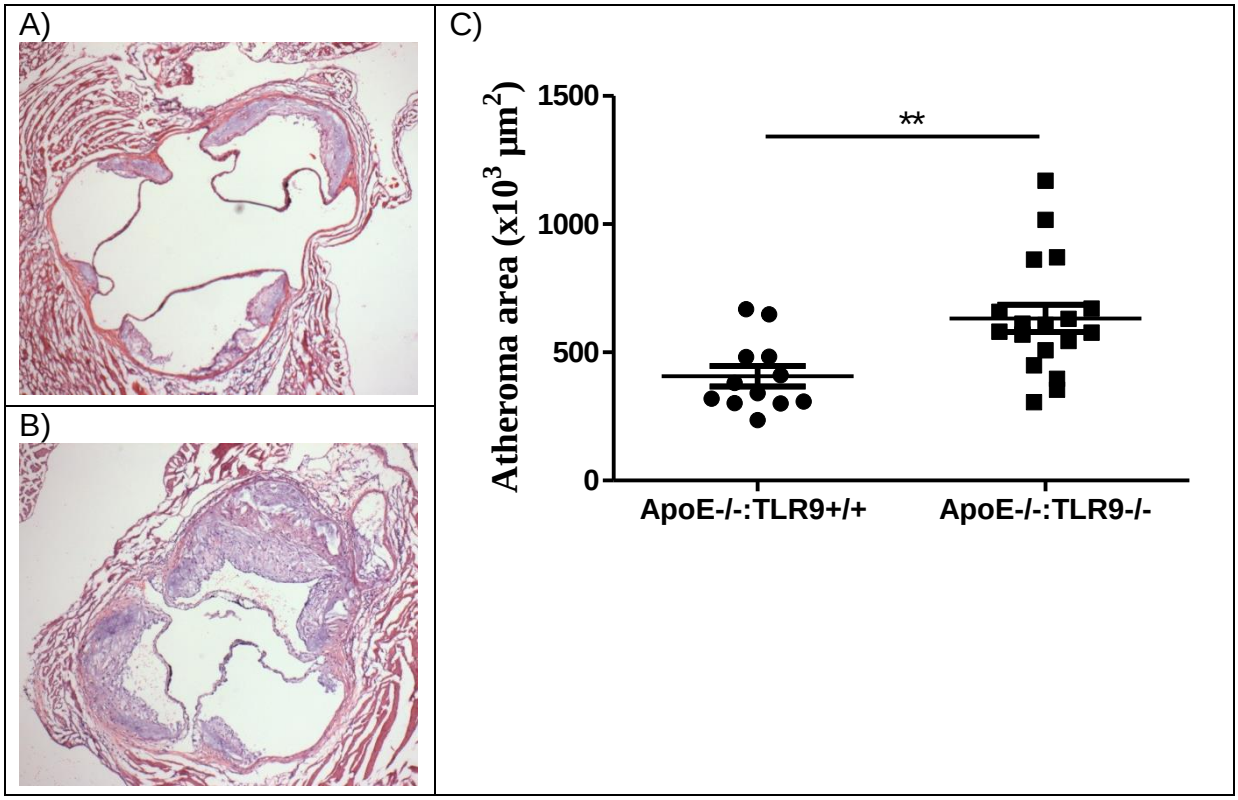

Figure 8: ApoE-/-:TLR9-/- mice have a significant increase in plaque size

*Aortic root sections of ApoE-/-:TLR9-/-(A) and ApoE-/- control mice (B) fed a Western diet for 15 weeks were stained with H&E. The plaque was quantified and analyzed. The mean atheroma area (x10^3 µm^2) ± SEM are shown (**p = 0.0049) (C).*

7.2.3 ApoE-/-:TLR2-/- or ApoE-/- :TLR4-/- mice do not show a change in lipid content

Given that foam cells predominantly comprise lipids, Oil Red O histology was performed to quantify the lipid content in the aortic root of ApoE-/-:TLR2-/- and ApoE-/-:TLR4-/- mice fed a Western diet for 15 weeks. There was no effect on the percentage of lipids; neither ApoE-/-:TLR2-/- nor ApoE-/-:TLR4-/- showed significant differences compared to ApoE deficient mice.

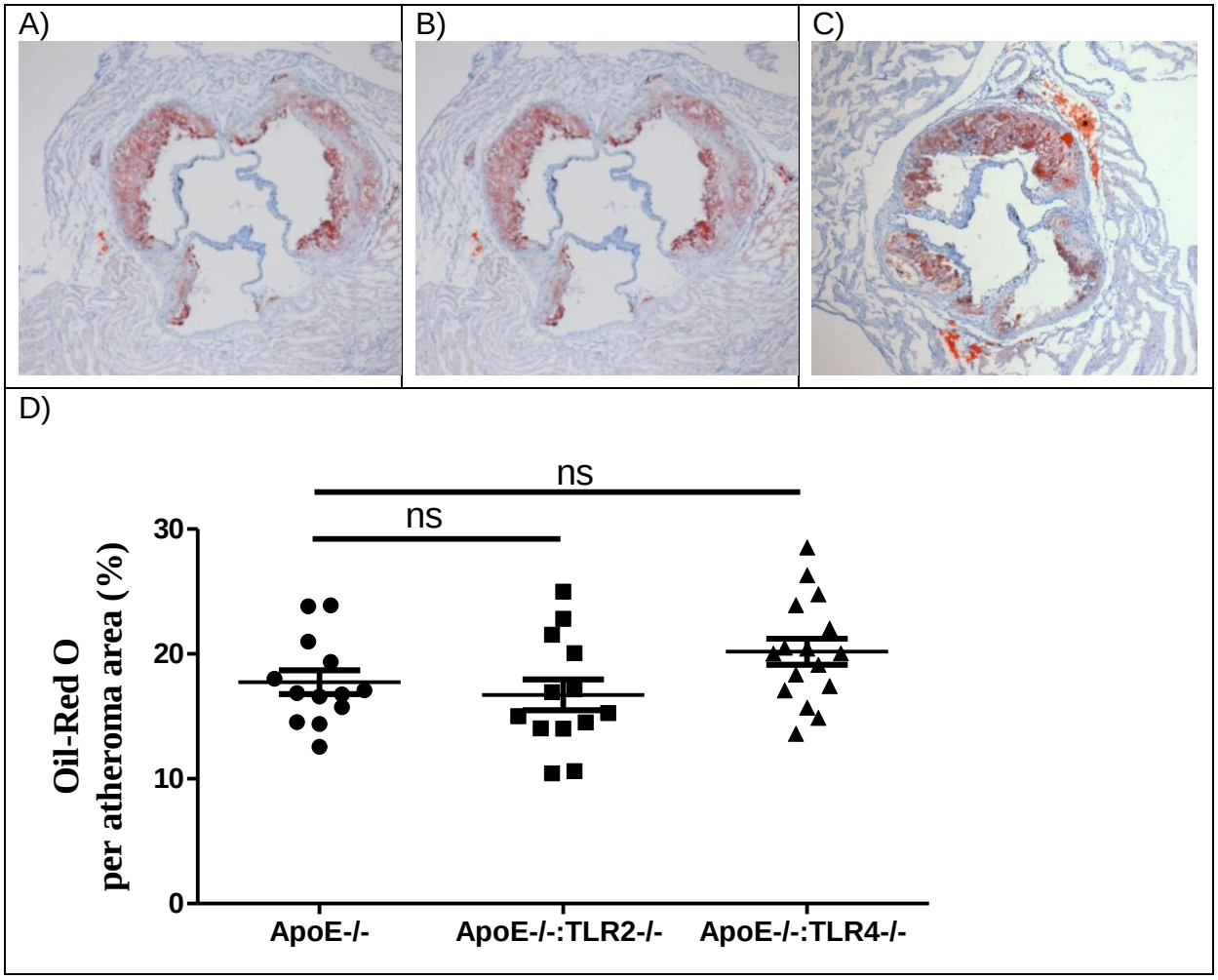

Figure 9: ApoE-/-:TLR2-/- or ApoE-/-:TLR4-/- mice do not show a change in lipid content in atherosclerosis

Aortic root sections of ApoE-/- control mice (A), ApoE-/-:TLR2-/- (B) and ApoE-/-:TLR4-/- mice (C) fed a Western diet for 15 weeks were stained with ORO. Lipids were identified by red signal intensity and percentage area of the plaque ± SEM was calculated (D).

7.2.4 ApoE-/-:TLR9-/- mice show a significant increase in lipid content in atherosclerosis

To also identify the fraction of lipids in the atherosclerotic plaque of ApoE-/-:TLR9-/- mice fed a Western diet for 15 weeks, Oil Red O histology of aortic roots was performed. A significantly higher percentage of lipid content was found in ApoE-/-:TLR9-/- mice compared to ApoE deficient mice. An increase in lipid content in plaques is associated with a decrease in plaque stability.

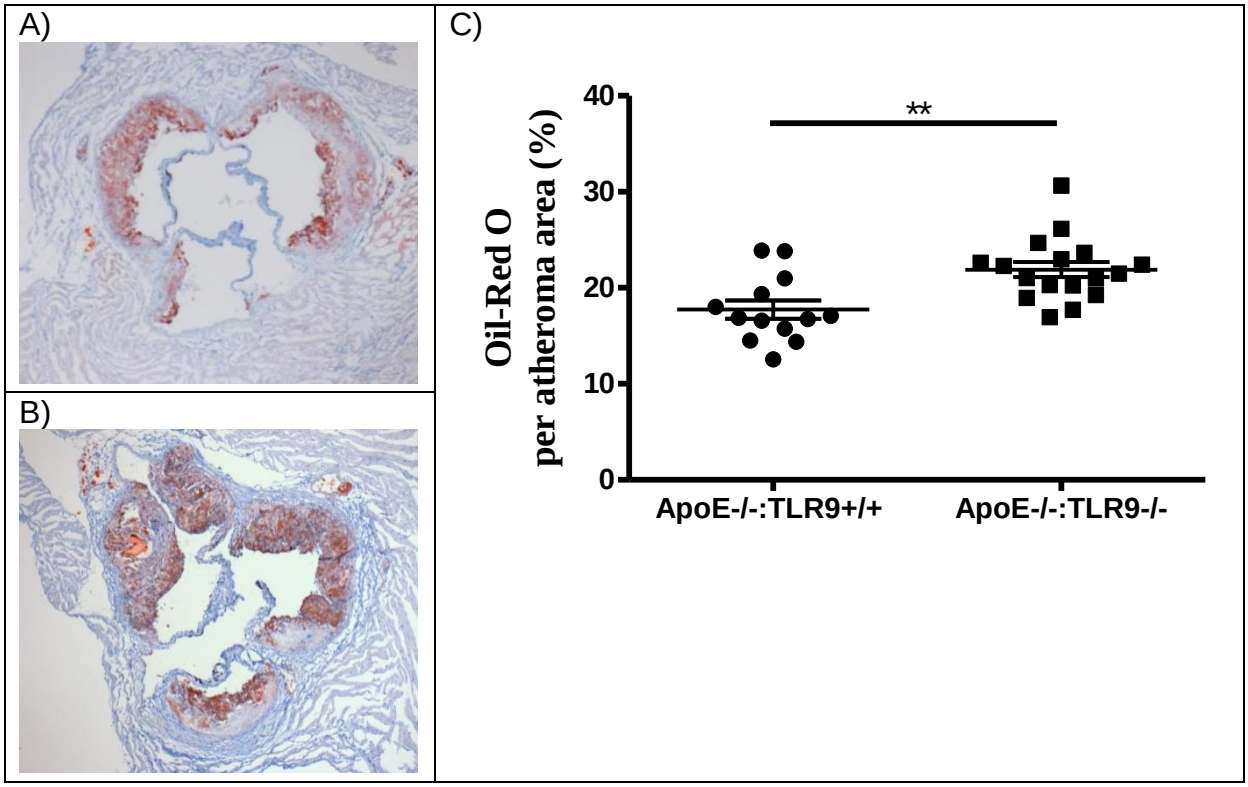

Figure 10: ApoE-/-:TLR9-/- mice show an increase in lipid content in atherosclerosis

*Aortic root sections of ApoE-/- control mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were stained with ORO. Lipids were identified by red signal intensity and percentage area of plaque ± SEM was calculated (**p = 0.0039) (C).*

7.2.5 ApoE-/-:TLR9-/- mice show a significant reduction of collagen in atherosclerosis

Given that collagen plays a key role in plaque stability, Masson's Trichrome staining was used to investigate collagen in atherosclerotic lesions in terms of plaque stability. A significant reduction of collagen in ApoE-/-:TLR9-/- mice compared to ApoE deficient control mice was found.

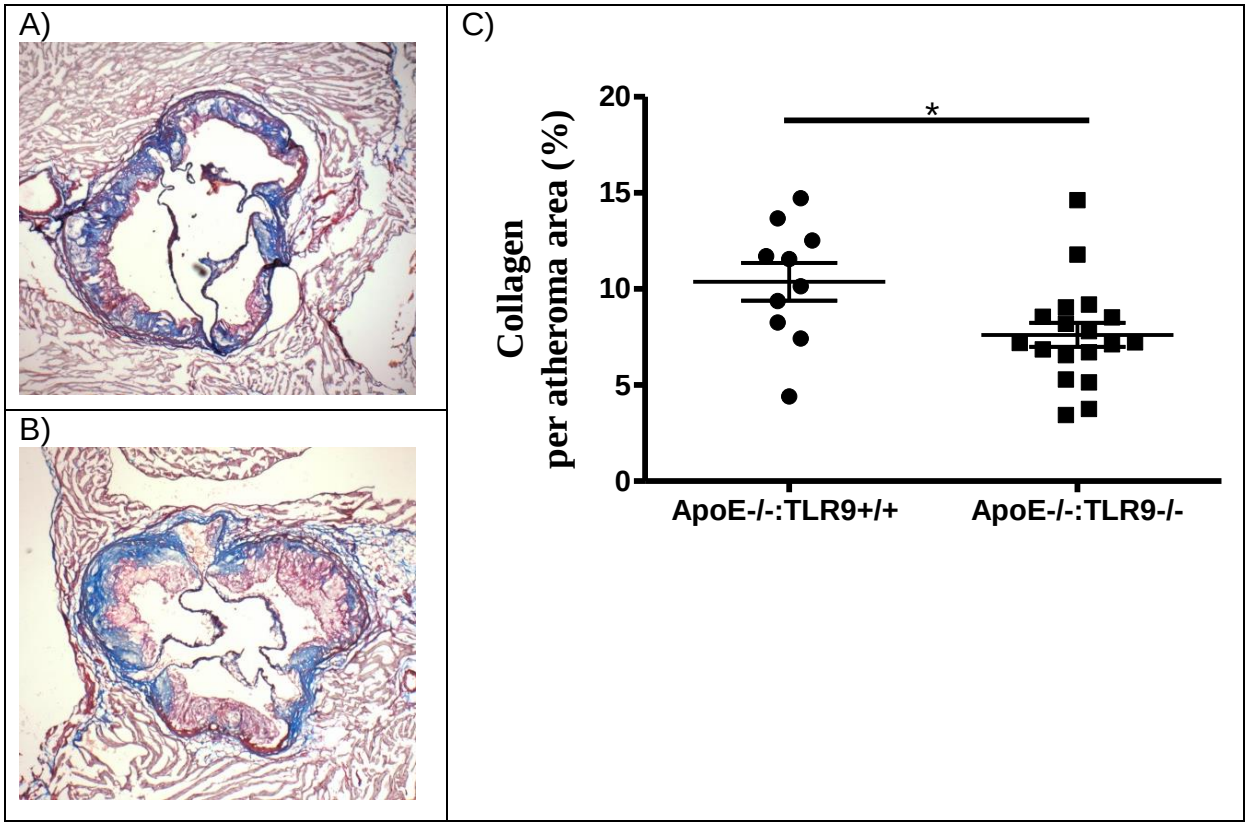

Figure 11: ApoE-/-:TLR9-/- reduces collagen in atherosclerosis

*Aortic root sections of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were stained and blue signal intensity was detected. Percentage area of plaque ± SEM was calculated and analyzed (*p = 0.0176) (C).*

7.2.6 ApoE-/-:TLR9-/- mice show a significant increase in foam cells

To further explore plaque stability, Immunohistochemistry (IHC) using anti-CD68 antibody was undertaken to determine the presence of foam cells. The knockout of both TLR9 and ApoE causes a significant increase of CD68 positive foam cells (Figure 12). To confirm this initial finding of an increase in foam cells, IHC with anti-MOMA-2 antibody was performed with a similar significant increase in foam cells (Figure 13). Control stains without primary antibody did not show a peroxidase-based signal. Taken together, all of these results indicate that ApoE-/-:TLR9-/- mice demonstrate an increase in plaque progression and development, as well as a reduction in plaque stability.

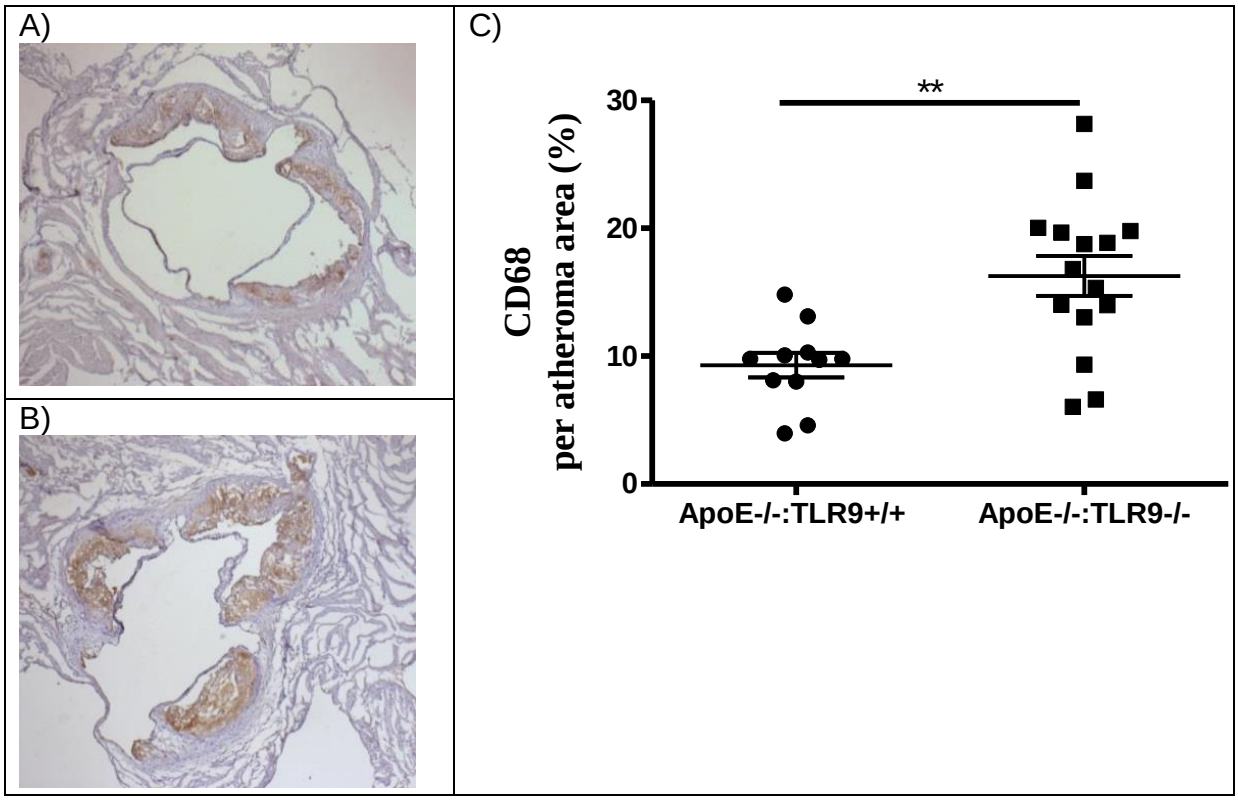

Figure 12: ApoE-/-:TLR9-/- significantly increases CD68 positive foam cells in atherosclerosis

*Aortic root sections of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were stained with IHC CD68 and brown signal intensity was detected. Percentage area of plaque ± SEM was calculated and analyzed (**p = 0.0059) (C).*

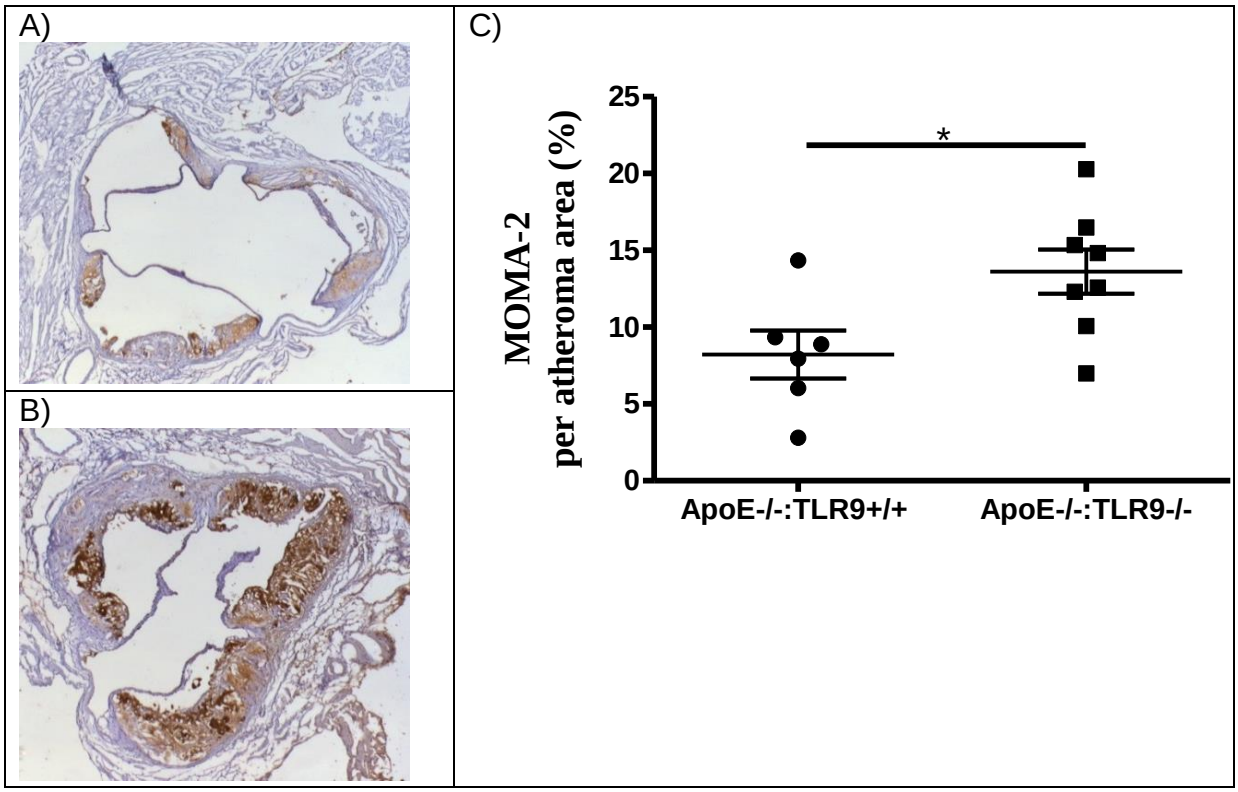

Figure 13: ApoE-/-:TLR9-/- significantly increases MOMA-2 positive foam cells in atherosclerosis

*Aortic root sections of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were stained with MOMA-2 and brown signal intensity was detected. Percentage area of plaque ± SEM was calculated and analyzed (*p = 0.0293) (C).*

7.2.7 ApoE-/-:TLR9-/- mice show a significant increase in mature dendritic cells

Dendritic cells (DCs) play an important regulatory role in the immune system and T cell differentiation (Banchereau and Steinman, 1998). Phagocytosis of pathogens leads to a maturation of the dendritic cells and an expression of characteristic co-stimulatory molecules, including CD86. Mature DC stimulate T cell proliferation by inducing differentiation of naive T cells to T effector cells (Dietel et al., 2013). Here, we examine the maturation marker CD86 in ApoE-/-:TLR9-/- in comparison to ApoE-/-:TLR9+/+ mice, finding a significant increase of mature dendritic cells in ApoE and TLR9 double knockout mice. Control stains without primary antibody did not show a peroxidase-based signal.

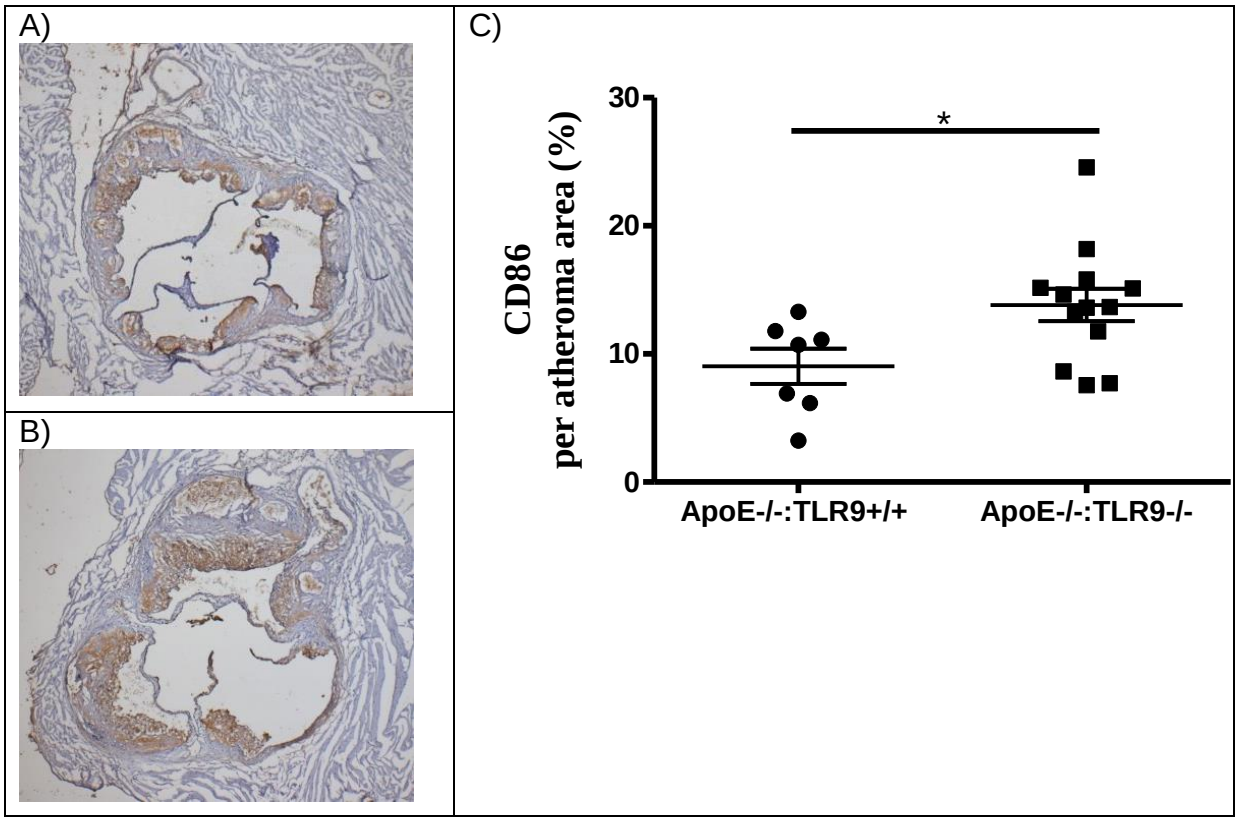

Figure 14: ApoE-/-:TLR9-/- mice show a significant increase mature dendritic cells

*Aortic root sections of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were stained with IHC CD86 and brown signal intensity was detected. Percentage area of plaque ± SEM was calculated and analyzed (*p = 0.0140) (C).*

7.2.8 ApoE-/-:TLR9-/- mice do not show a difference in VCAM-1 expression in atherosclerosis

To explore inflammation in the ApoE-/-:TLR9-/- mice, IHC was performed using anti-VCAM-1 antibody, a known marker of inflammation. No significant difference was detected between the ApoE-/-:TLR9-/- and ApoE deficient mice, although a trend for increased VCAM-1 was observed.

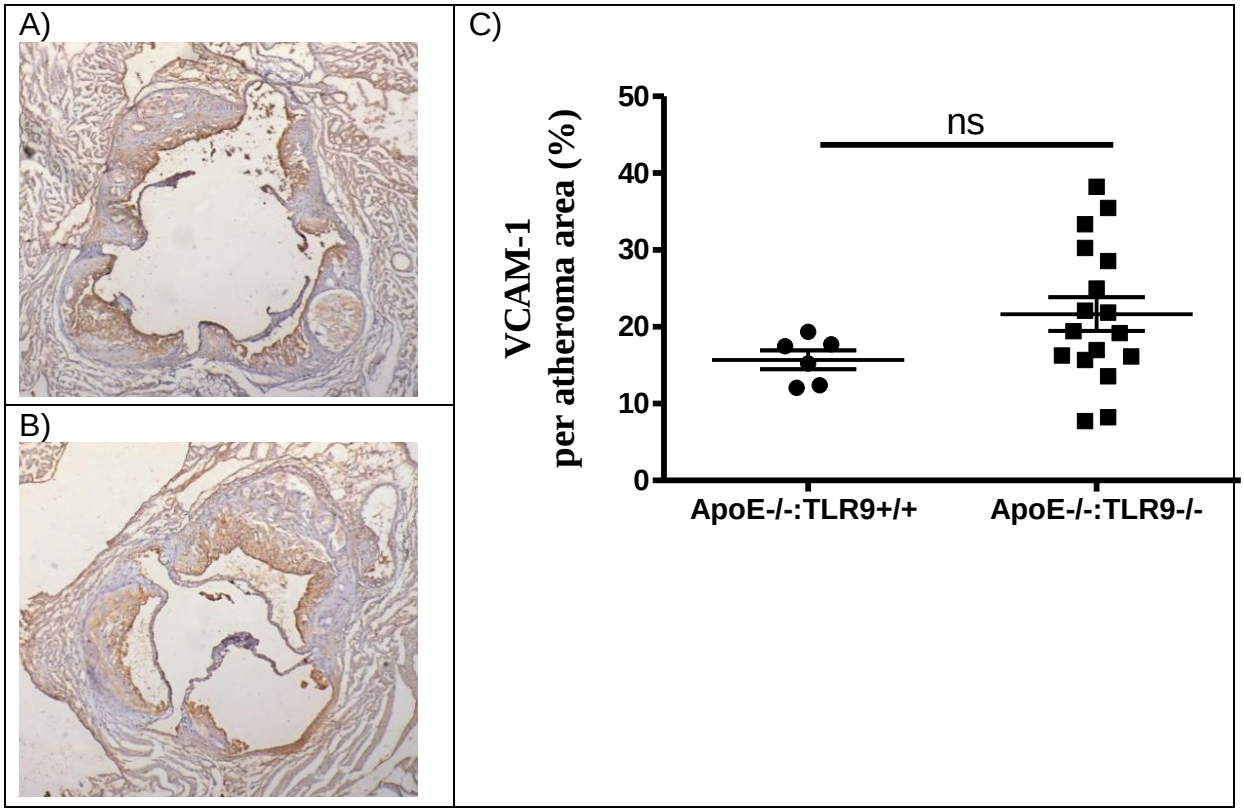

Figure 15: ApoE-/-:TLR9-/- mice do not show a difference in VCAM-1 expression in atherosclerosis

VCAM-1 stains from the aortic root of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 15 weeks were produced and brown signal intensity was detected. Percentage area of the plaque ± SEM was calculated and analyzed (C).

7.3 Histology 8 weeks

To examine the effect of TLR9 in an early stage of atherogenesis, ApoE-/-:TLR9-/- mice and their control ApoE-/-:TLR9+/+ mice were fed for 8 weeks with a Western diet. Lesion size and plaque composition was histological quantified and measured as previously described.

7.3.1 ApoE-/-:TLR9-/- mice show a significant increase in atherosclerosis in the early state

Aortic root sections from mice fed a Western diet for 8 weeks were stained with H&E. There was a significant increase in the plaque area of the ApoE-/-:TLR9-/- group compared to the control group.

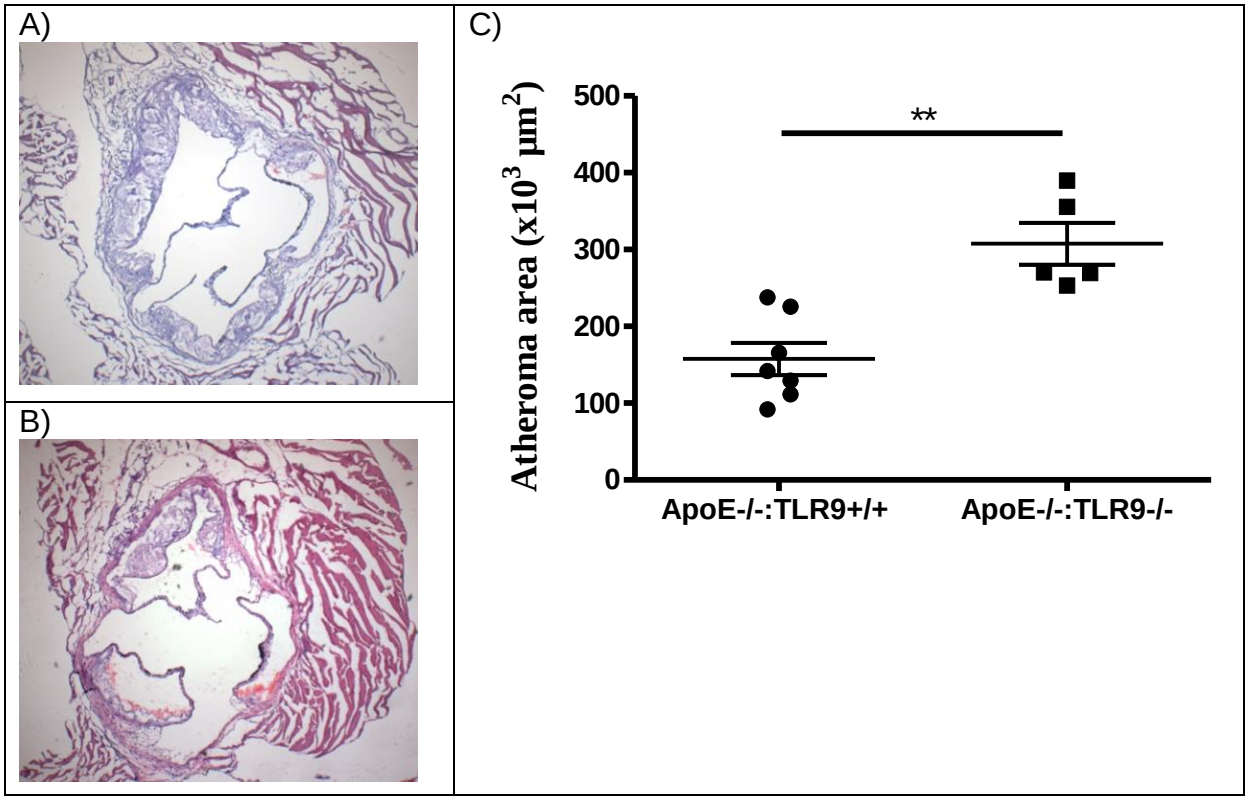

Figure 16: ApoE-/-:TLR9-/- mice show a significant increase in atherosclerosis in the early state

*Aortic root sections of ApoE-/-:TLR9-/- (A) and ApoE-/- control mice (B) were stained with H&E. The plaque was quantified and analyzed. Data shows the mean atheroma area (x10^3 µm^2) ± SEM (**p = 0.0025) (C).*

7.3.2 ApoE-/-:TLR9-/- mice show a significant increase in lipid content in early atherosclerosis

Oil Red O histology was carried out to quantify lipids in the atherosclerotic plaque area. ApoE-/-:TLR9-/- mice have a significant increase in the lipid content in the aortic roots compared to ApoE deficient control mice. These results indicate that ApoE-/-:TLR9-/- mice have an increase in atherosclerotic lesions and a decrease in plaque stability.

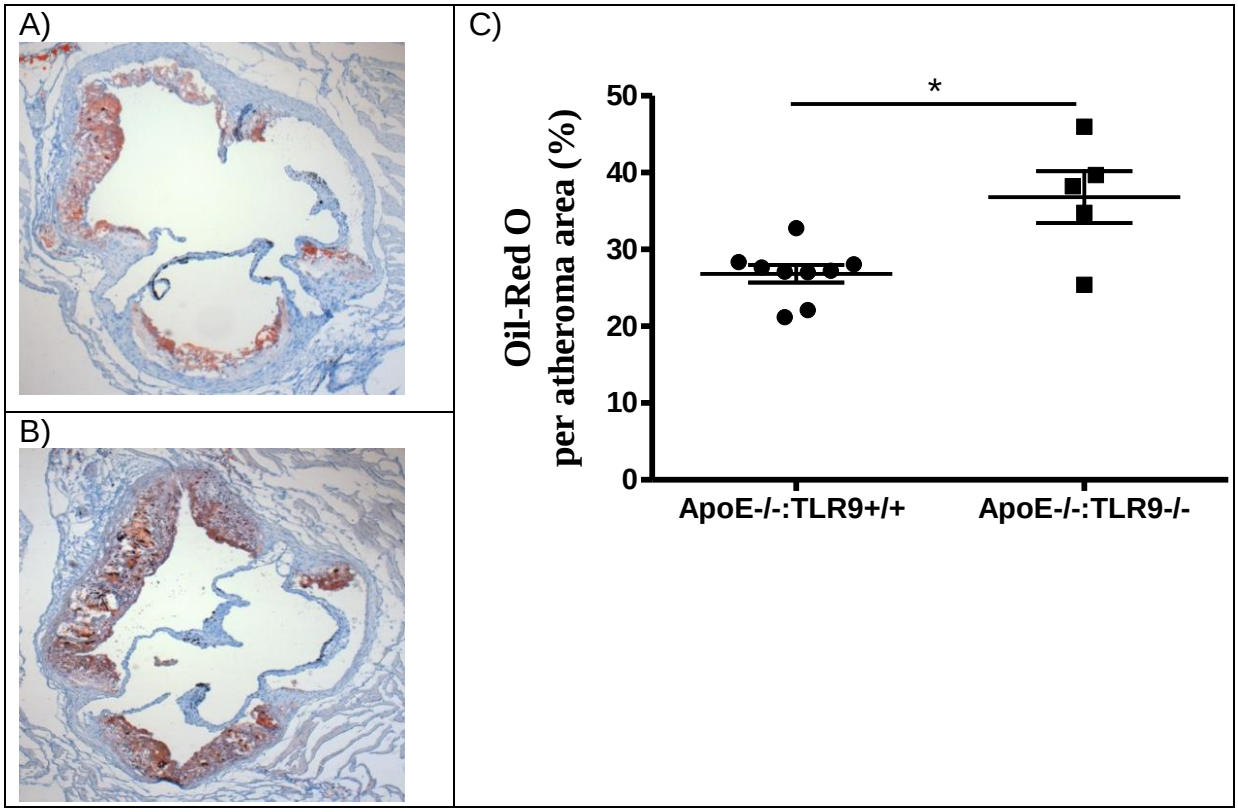

Figure 17: ApoE-/-:TLR9-/- mice show a significant increase in lipid content in early atherosclerosis

*ORO histology was performed to identify the lipids in the atherosclerotic plaque of the aortic roots of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 8 weeks. Lipids were identified by red signal intensity and percentage area of plaque ± SEM was calculated (*p = 0.0420) (C).*

7.3.3 ApoE-/-:TLR9-/- mice do not show a change in collagen in early atherosclerosis

To investigate plaque stability, Masson's Trichrome was performed to enable quantification of collagen content in the atherosclerotic plaque area. TLR9 knockout does not significantly reduce collagen in mice fed a Western diet for 8 weeks, although a trend was observed (p= 0.067).

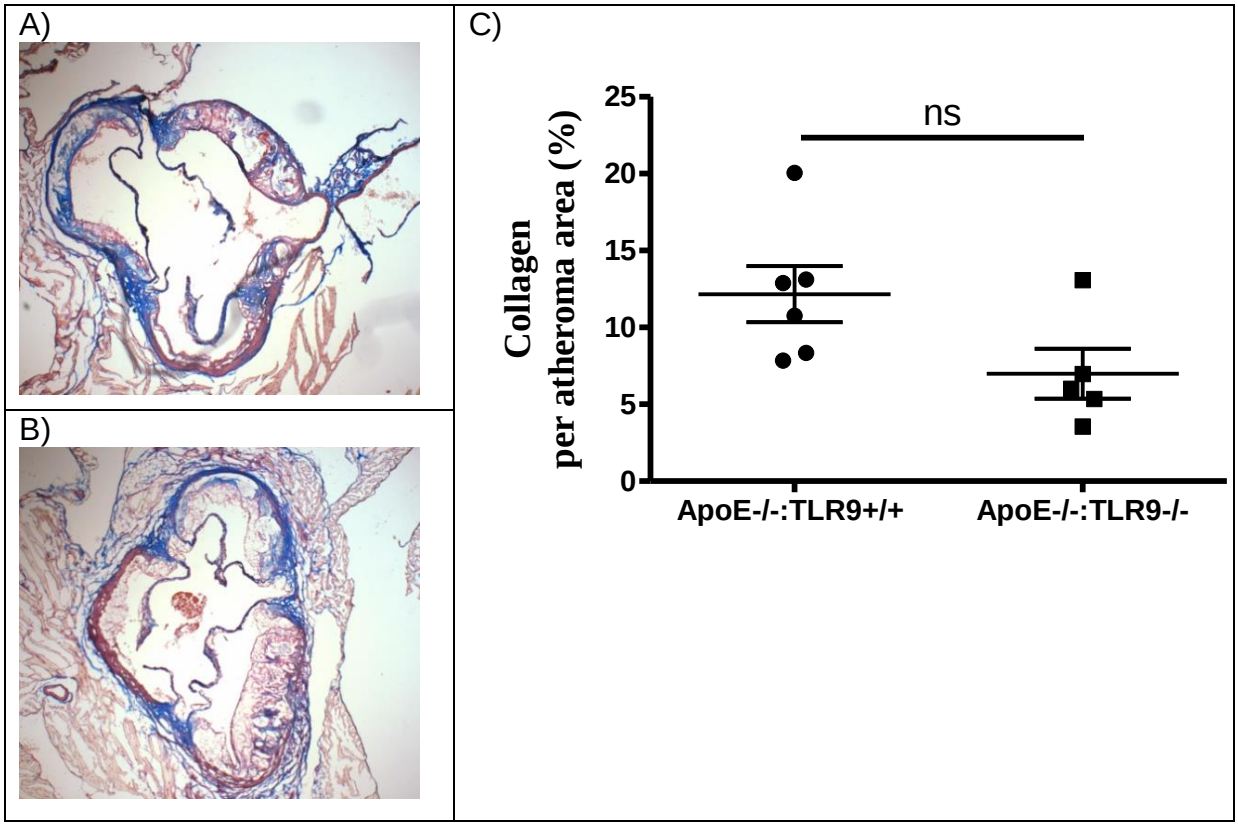

Figure 18: ApoE-/-:TLR9-/- mice do not show a change in collagen in early atherosclerosis

Masson's Trichrome was used to investigate collagen in atherosclerotic lesions in aortic roots of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 8 weeks. Blue signal intensity was detected and percentage area of the plaque ± SEM was calculated and analyzed (C).

7.3.4 ApoE-/-:TLR9-/- mice do not show a significant affect increase in foam cells in early atherosclerosis

CD68 specific staining was used to investigate the presence of foam cells in the plaque area of the aortic roots. There was a non-significant trend of increased CD68 staining in the ApoE-/-:TLR9-/- compared to the ApoE-/-:TLR9+/+ groups fed a Western diet for 8 weeks.

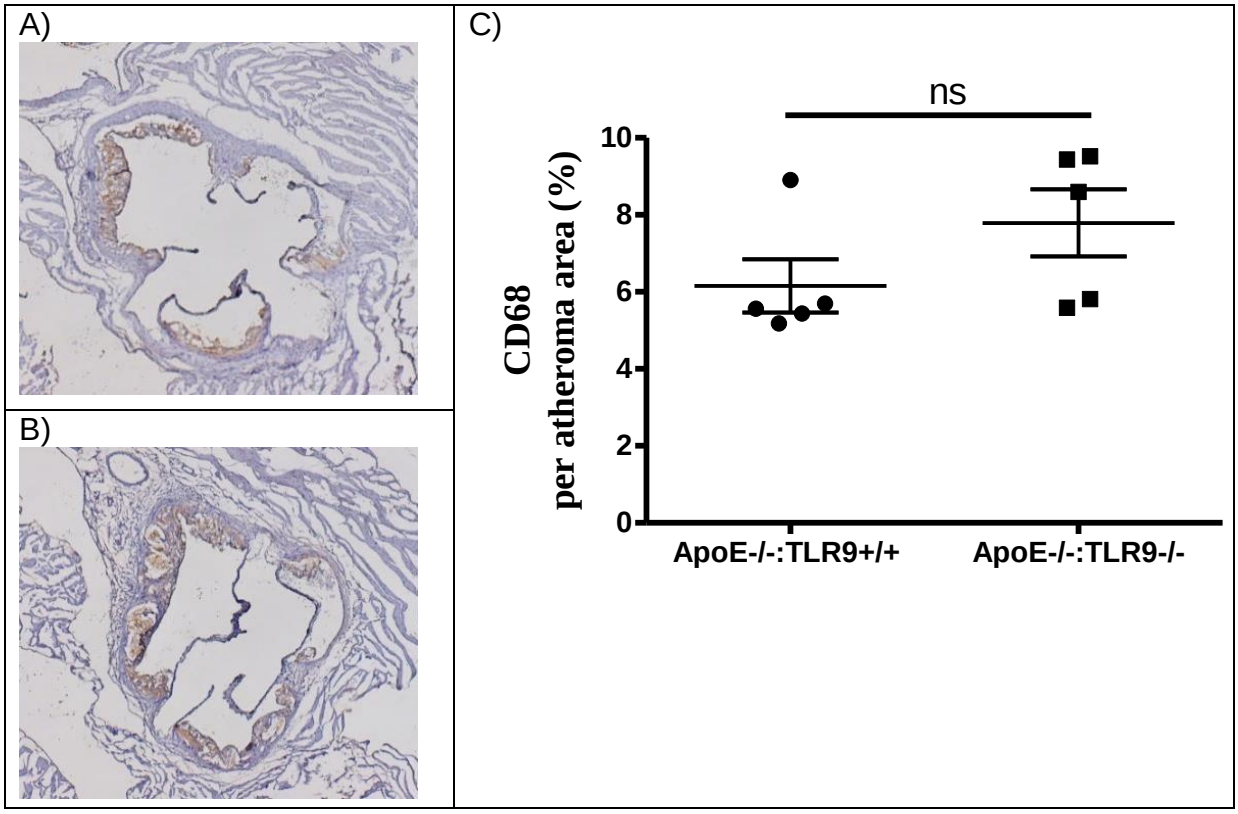

Figure 19: ApoE-/-:TLR9-/- mice do not show a significant increase in foam cells in early atherosclerosis

Aortic roots of ApoE-/- mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 8 weeks were stained with IHC CD68 and brown signal intensity was detected. Percentage area of the plaque ± SEM was calculated and analyzed (C).

7.3.5 ApoE-/-:TLR9-/- mice do not show a change in VCAM-1 expression in early atherosclerosis

There are no significant differences in VCAM-1 expression between the ApoE-/-:TLR9-/- and ApoE deficient mice fed a Western diet for 8 weeks.

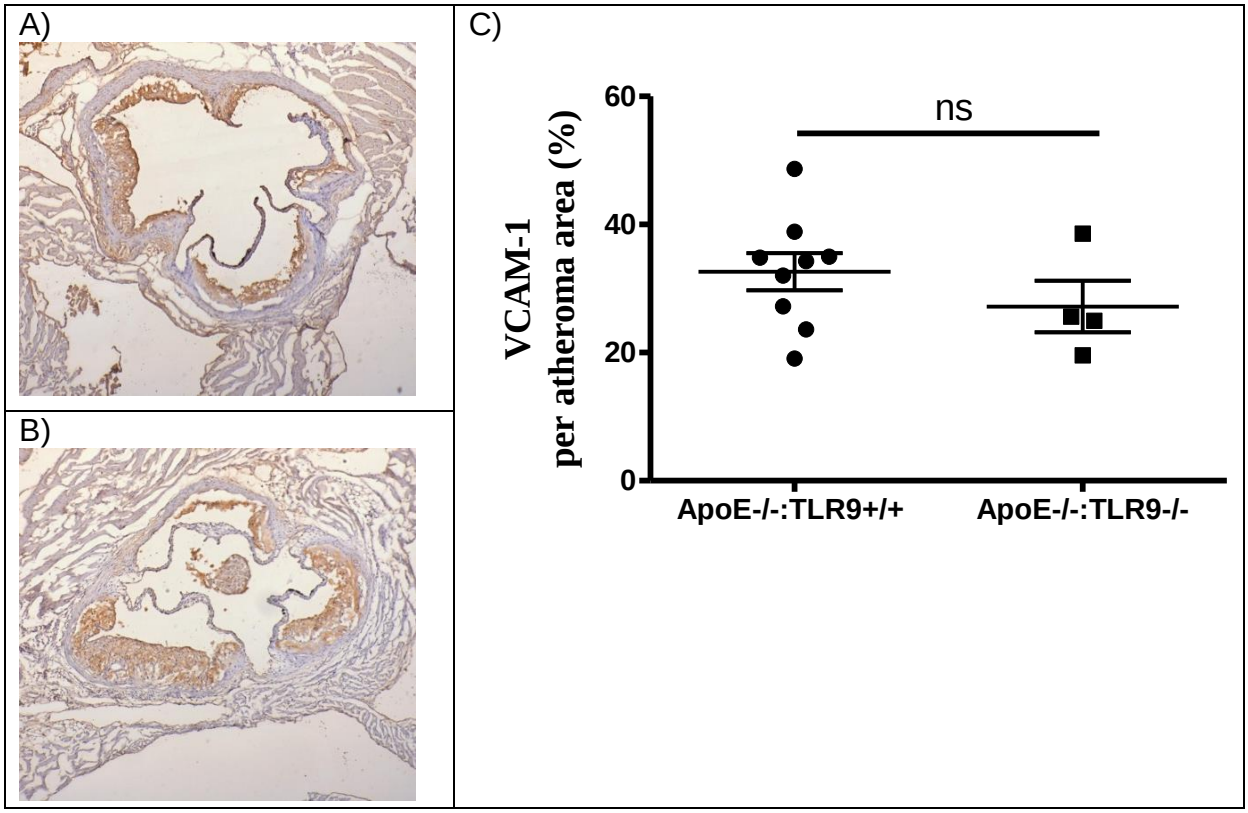

Figure 20: ApoE-/-:TLR9-/- mice do not show a change in VCAM-1 expression in early atherosclerosis

VCAM-1 stains from the aortic root of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 8 weeks. Brown signal intensity was detected and percentage area of the plaque ± SEM was calculated and analyzed (C).

7.3.6 ApoE-/-:TLR9-/- mice show a significant increase in dendritic cells in early atherosclerosis

TLR9 is expressed by dendritic cells, which play an important role in the development of atherosclerosis. Furthermore, they may affect the accumulation of $CD4^+$ T cells (Niessner et al.) in atherosclerotic plaque.

A dendritic cell marker (33D1) was used to explore the presence of dendritic cells in the atheroma area. IHC was performed to indicate these dendritic cells in the aortic roots of double knockout of ApoE and TLR9 in comparison to single knockout of ApoE. A significant increase of dendritic cells was found present in the plaques of TLR9 knockout mice.

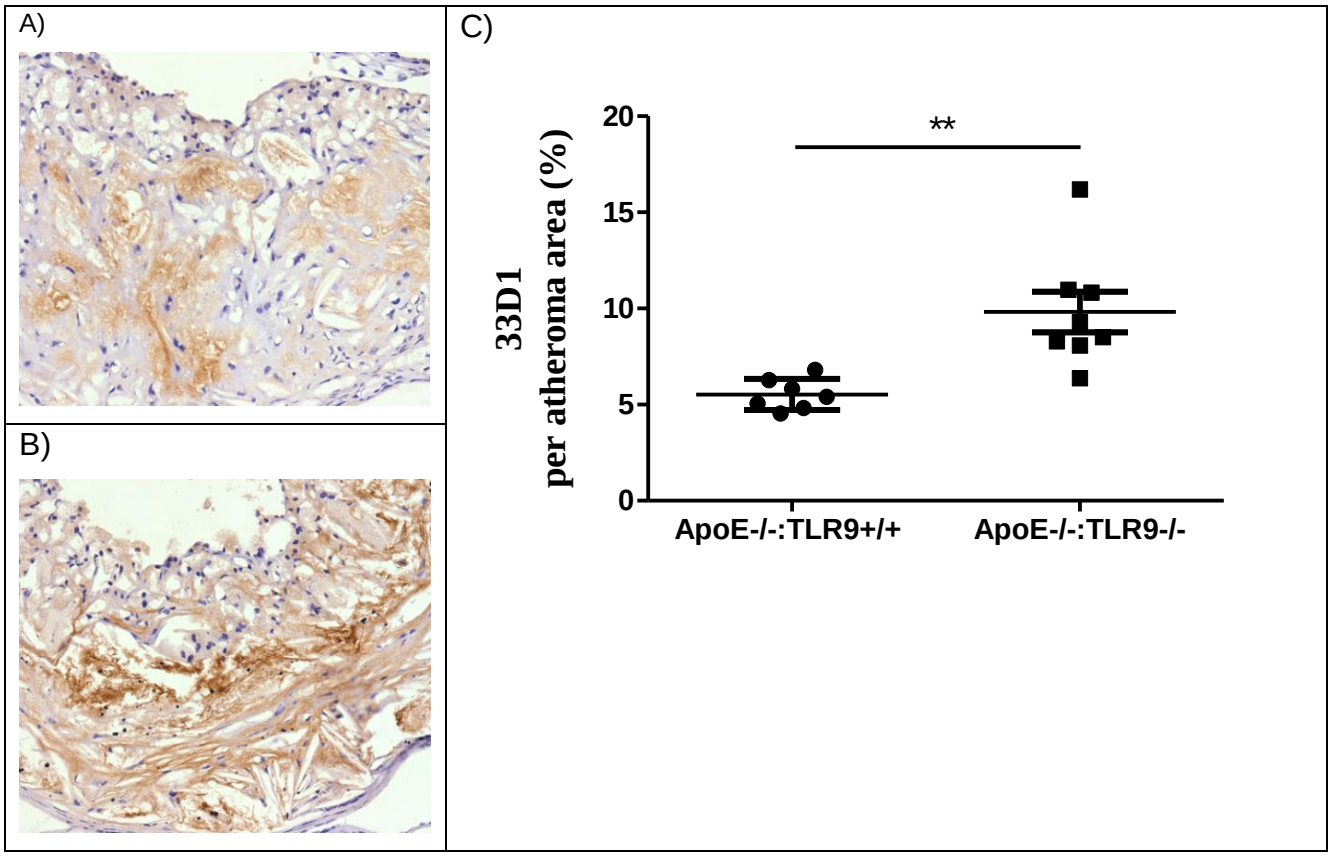

Figure 21: ApoE-/-:TLR9-/- mice show a significant increase in 33D1 receptor positive dendritic cells

Aortic root sections of ApoE deficient mice (A) and ApoE-/-:TLR9-/- mice (B) fed a Western diet for 8 weeks were stained with IHC the dendritic cell marker 33D1 and brown signal intensity was detected. Percentage area of plaque ± SEM was calculated and analyzed (p = 0.0028) (C).

7.4 Knockout of TLR9 does not change levels of monocytes subsets in ApoE KO mice

To examine the effect of deletion of TLR9 in ApoE deficient mice on monocytes subsets, FACS was used to differentiate $CD115^+CD11b^+$ monocytes into $Ly6C^{high}$, $Ly6C^{intermediate}$ and $Ly6C^{low}$ positive cells. There were no differences in subset proportions between ApoE-/-:TLR9-/- and ApoE-/-:TLR9+/+ mice (each subset was analyzed with Mann-Whitney-U test).

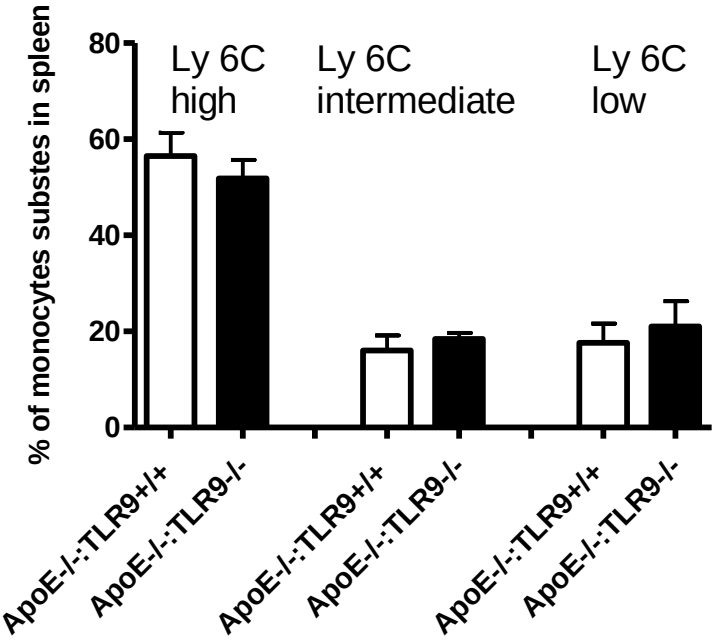

Figure 22: Knockout of TLR9 does not change levels of monocytes subsets in ApoE KO mice

Mice with knockout of TLR9 and ApoE exhibit similar levels of monocytes/macrophages in comparison to only ApoE deficient mice on high fat diet. Therefore $CD11b^+$ and $CD115^+$ double positive cells of the spleen of 12 weeks old ApoE-/-:TLR9-/- and ApoE-/-:TLR9+/+ mice were gated and afterwards sub-gated in $Ly6C^{hi}$, $Ly6C^{int}$ and $Ly6C^{lo}$ monocytes subsets. Results present the mean percentage of $CD115^+CD11b^+$ monocyte subsets ± SEM (N=8, each group).

7.5 Knockout of TLR9 does not change numbers of regulatory T cells in ApoE KO mice

To examine the effect of deletion of TLR9 in ApoE deficient mice regulatory T cells, FACS was used to measure $CD4^+CD25^+Foxp3^+$ cells. There was no differences in cell numbers between ApoE-/-:TLR9-/- and ApoE-/-:TLR9+/+ mice.

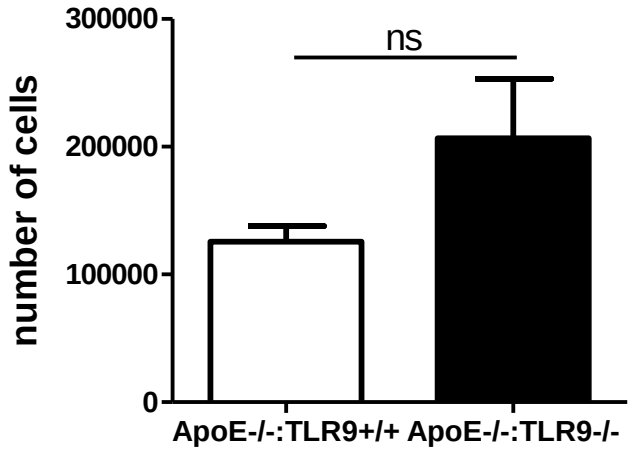

Figure 23: Knockout of TLR9 does not change number of regulatory T cells in ApoE KO mice

Knockout of TLR9 in ApoE-/- mice does not significantly change the number of splenic $CD4^+CD25^+Foxp3^+$ regulatory T cells compared to knockout of ApoE alone. Results present the mean number of $CD4^+CD25^+Foxp3^+$ cells ± SEM (N=5, each group)

7.6 Treatment with the TLR9 agonist ODN 1668

7.6.1 ODN 1668 treatment reduces plaque size in ApoE-/-:TLR9+/+ mice

Weekly treatment of ApoE-/-:TLR9+/+ with the TLR9 agonist ODN 1668 over 8 weeks whilst on a high fat diet. ApoE-/-:TLR9+/+ mice that received ODN 1668 exhibited a significant reduction in plaque size compared to the vehicle treated mice or ApoE-/-:TLR9-/- mice receiving ODN 1668.

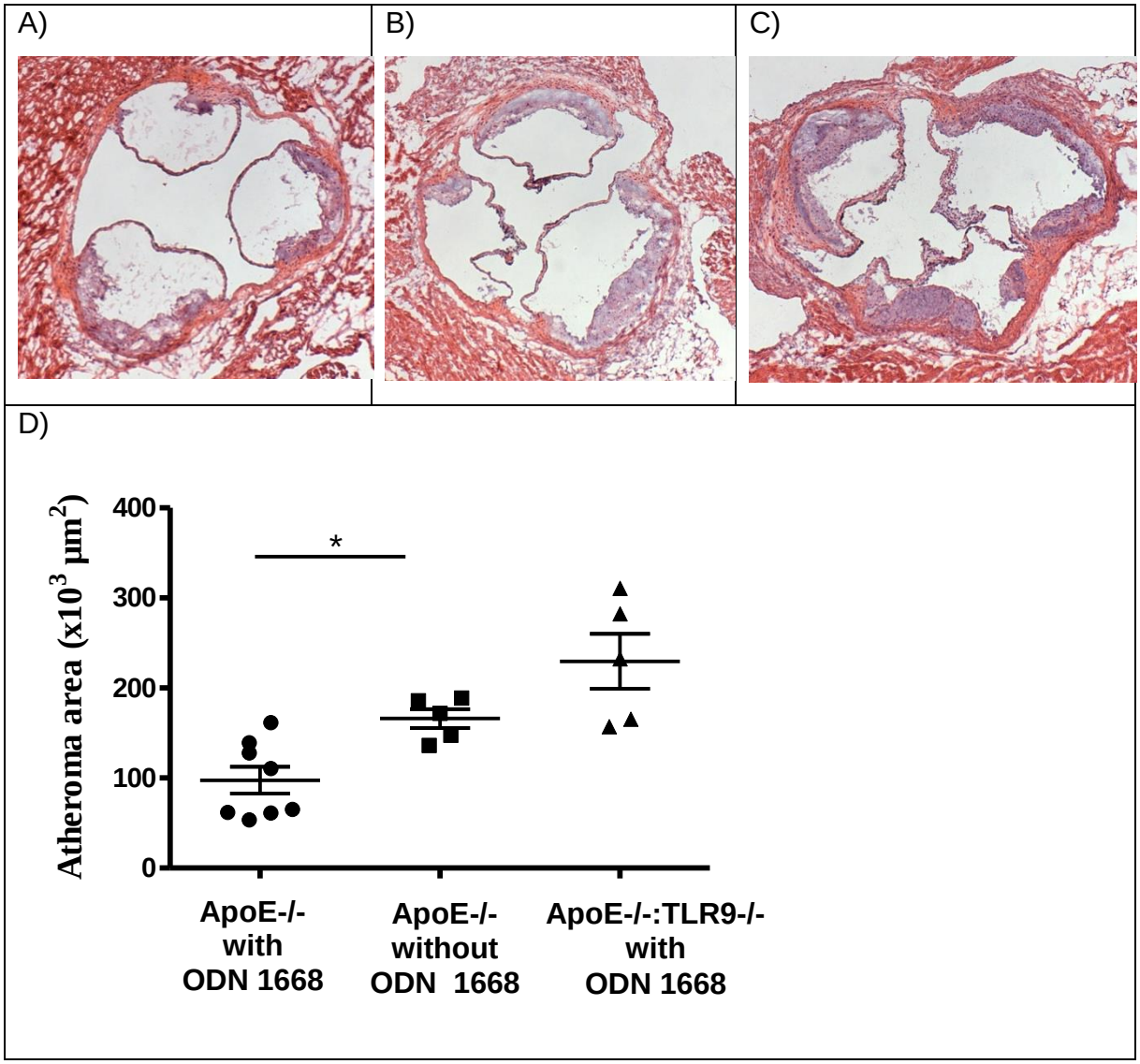

Figure 24: ODN 1668 treatment reduces plaque size in ApoE-/-:TLR9+/+ mice

*H&E stained aortic root sections from (A) vehicle treated (B) ODN 1668 treated ApoE -/- or (C) ApoE-/-:TLR9-/- mice fed a Western diet for 8 weeks. The atherosclerotic plaque area (purple), in the three valves, was quantified. The mean atheroma area (x10^3 µm^2) ± SEM are shown (*p=0.0109) (D).*

7.6.2 ODN 1668 treatment does alter lipids in early atherosclerosis

Because ODN 1668 affects atherosclerosis, ORO histology was performed to explore a possible mechanism. Thereby, the lipid fraction in the atherosclerotic plaque of ApoE-/-:TLR9-/-, ApoE-/-:TLR9+/+ mice fed a Western diet for 8 weeks with a weekly injection of ODN 1668 was carried out. ODN 1668 treatment was associated with a reduced percentage of lipids in the atheroma area compared to vehicle treated ApoE-/- mice, although this level was found to be significant compared to lipid levels in ODN 1668 treated ApoE-/-:TLR9-/- mice.

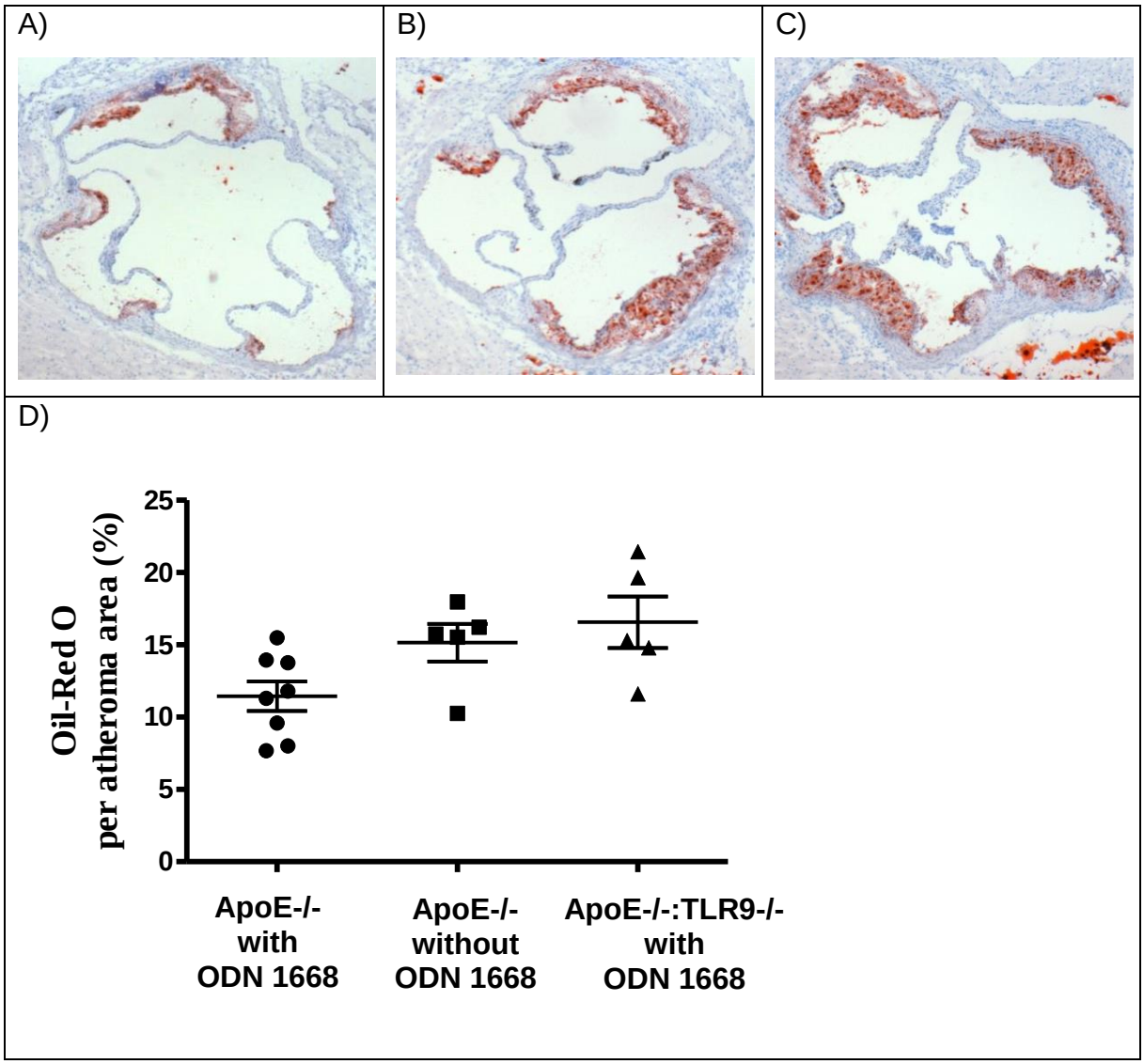

Figure 25: ODN 1668 treatment does alter lipids in early atherosclerosis

Lipid composition of atherosclerotic plaques, as determined by ORO histology. ApoE -/- mice treated with (A) ODN 1668, (B) vehicle control or (C) ApoE-/-:TLR9-/- treated with ODN 1668 fed a Western diet for 8 weeks. Lipids were identified by red signal intensity and percentage area of plaque ± SEM was calculated (D).

7.6.3 ODN 1668 treatment does alter collagen in early atherosclerosis

To investigate plaque stability, Masson's Trichrome was performed to identify collagen in the atherosclerotic plaque area. ODN1668 treatment does not significantly reduce collagen in ApoE-/-:TLR9 +/+ or ApoE-/-:TLR9 -/- mice fed a Western diet for 8 weeks.

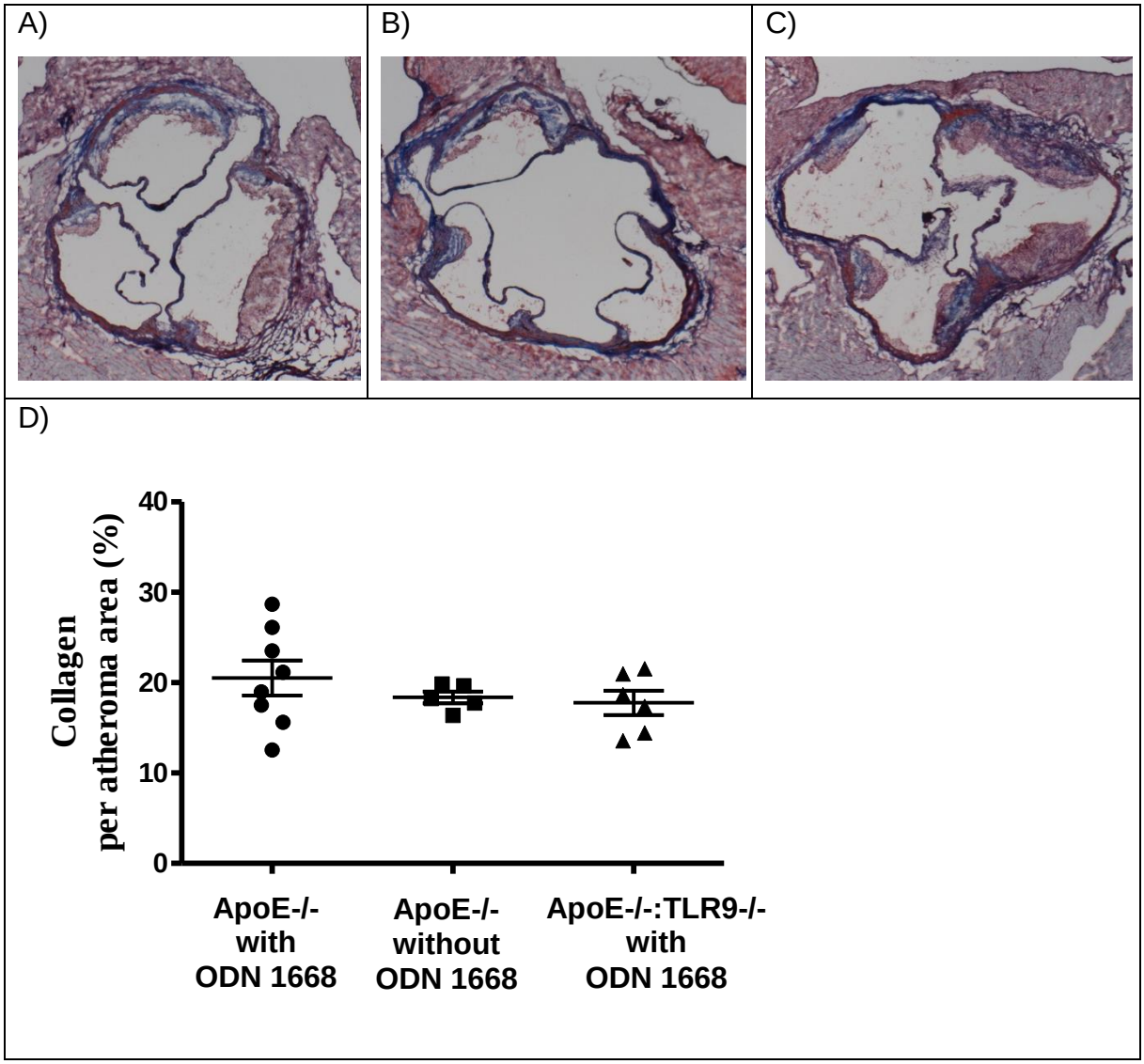

Figure 26: ODN 1668 treatment does not alter collagen in early atherosclerosis

Collagen content in atherosclerotic plaques, as determined by Masson's Trichrome histology. ApoE -/- mice treated with (A) ODN1668, (B) vehicle control or (C) ApoE-/-:TLR9-/- treated with ODN1668 fed a Western diet for 8 weeks. Collagen was identified by blue signal intensity and percentage area of plaque ± SEM was calculated (D).

7.7 Application of an CD4-depleting antibody

7.7.1 The administrated CD4-depleting antibody reduce significantly CD4 positive cells

To confirm the effect of the CD4-depleting antibody, lymphocytes of the spleens of ApoE-/-:TLR9-/- mice receiving CD4-depleting antibody or isotype antibody were analyzed using flow cytometry. The result demonstrates a significant reduction of the CD4-depleting antibody receiving group compared to the isotype antibody receiving control group.

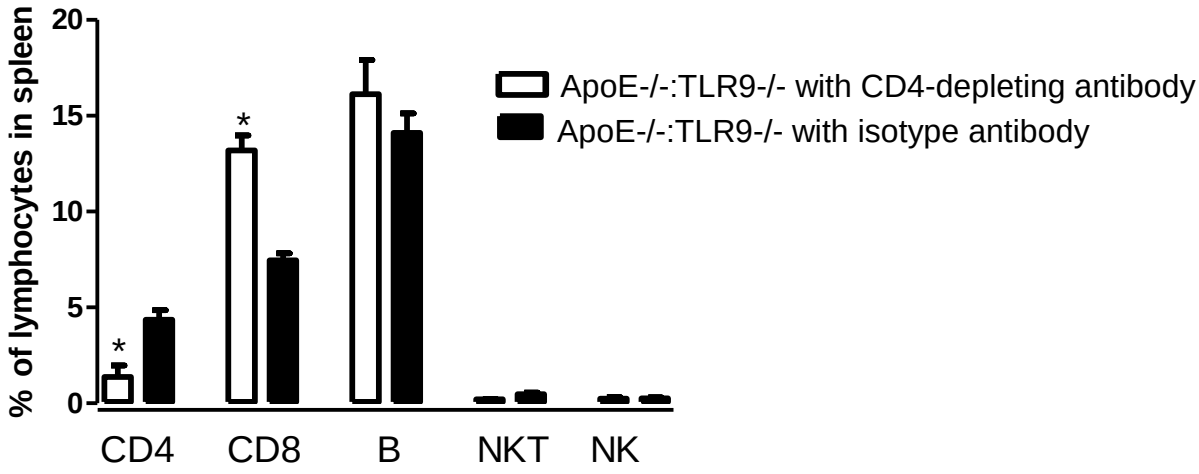

Figure 27: The administrated CD4-depleting antibody reduce significantly CD4 positive cells

The $CD4^+$ cells are significantly reduced in spleens of ApoE-/-:TLR9-/- mice after weekly i.v. injection of 300 µg CD4-depleting antibody. Bar graphs represent the proportion of lymphocytes subsets of the spleen after 8 weeks treatment and Western diet. Results are assessed by flow cytometry analysis and demonstrate a significant reduction in $CD4^+$ T cells in the CD4-depleting antibody-treated group compared to isotype antibody-treated control group (1.4±0.6 % vs. 4.4±0.5 %). $CD8^+$ T cells were significantly increased in CD4-depleting antibody group compared to isotype control group (13.2±0.8 % vs. 7.5±0.4 %). Other lymphocytes were not affected (NK: Natural killer cells; NKT: Natural killer T cells). Results present mean percentage of splenic lymphocytes subsets ± SEM (N=8, each group).

7.7.2 CD4-depleting antibody reduces plaque size in early atherosclerosis

Because our group found a higher accumulation of $CD4^+$ cells in the atherosclerotic plaque (Koulis et al., 2014) in ApoE-/-:TLR9-/- mice, the next step was to assign the role of these CD4 positive cells on the development of atherosclerotic lesions. Therefore, a CD4-depleting antibody or an isotype control was administrated over a period of 8 weeks to ApoE-/-:TLR9-/- mice. The function of this antibody was confirmed by FACS (Figure 27). Aortic root sections from 8-week Western diet mice were stained with H&E.

A significant increase in the plaque area of the CD4-depleting antibody ApoE-/-:TLR9-/- group compared to the isotype control group was observed. The result indicates that $CD4^+$ cells play a key role in atherosclerosis.

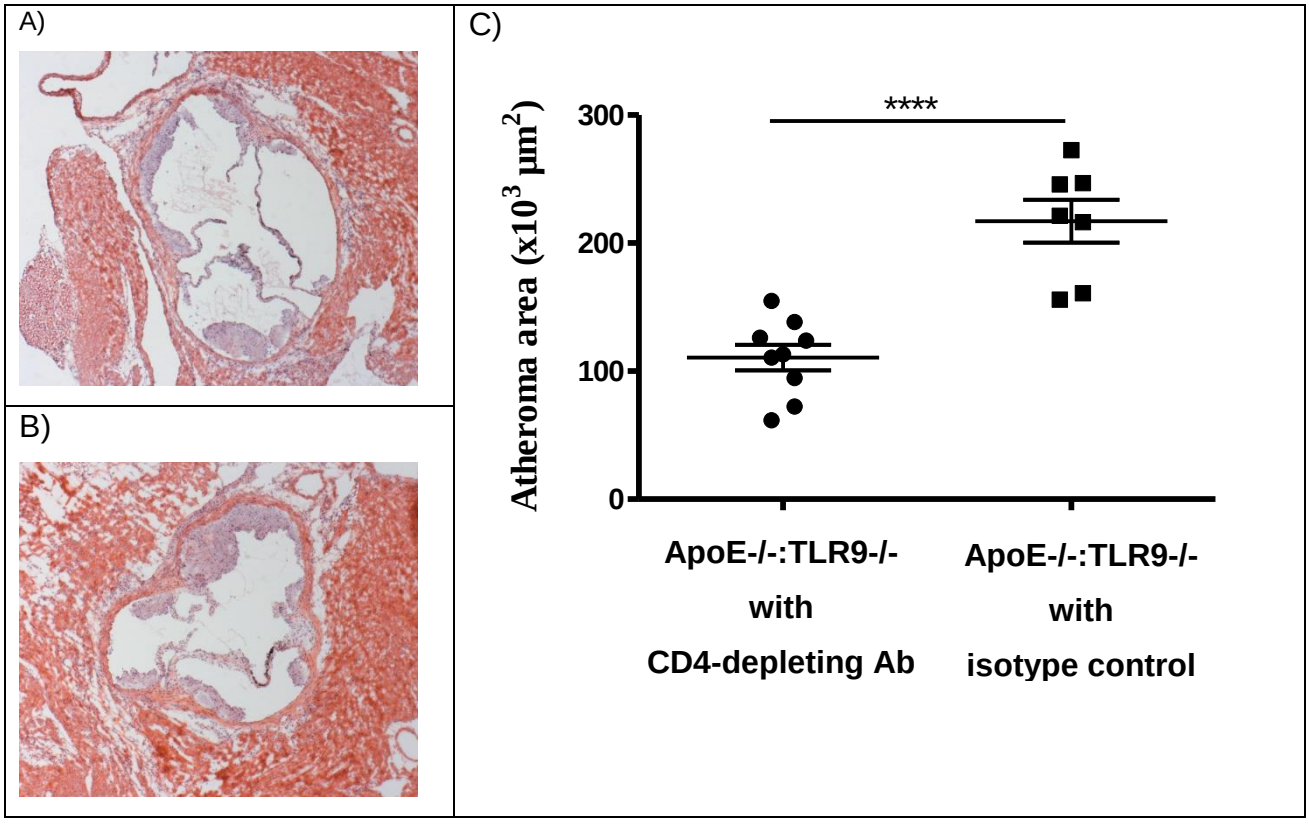

Figure 28: CD4-depleting Antibody reduces plaque size in early atherosclerosis

Aortic root sections of ApoE-/-:TLR9-/- mice receiving (A) CD4-depleting antibody or (B) Isotype control fed a Western diet for 8 weeks and stained with H&E. The plaque was quantified and analyzed. The mean atheroma area ($x10^3$ μm^2) ± SEM are shown (C).

7.7.3 CD4-depleting antibody does not alter lipids in early atherosclerosis

To examine the influence of CD4-depleting antibody on the content of lipids in the atherosclerotic plaque of ApoE-/-:TLR9-/-, ApoE-/-:TLR9+/+ mice were fed a Western diet for 8 weeks with the administration of CD4-depleting antibody. Oil Red O histology of aortic roots was performed. No significant difference in the percentage of lipid positive staining (Oil-Red O) of the atheroma area was detected between ApoE-/-:TLR9-/- mice receiving the CD4-depleting antibody or the isotype control antibody.

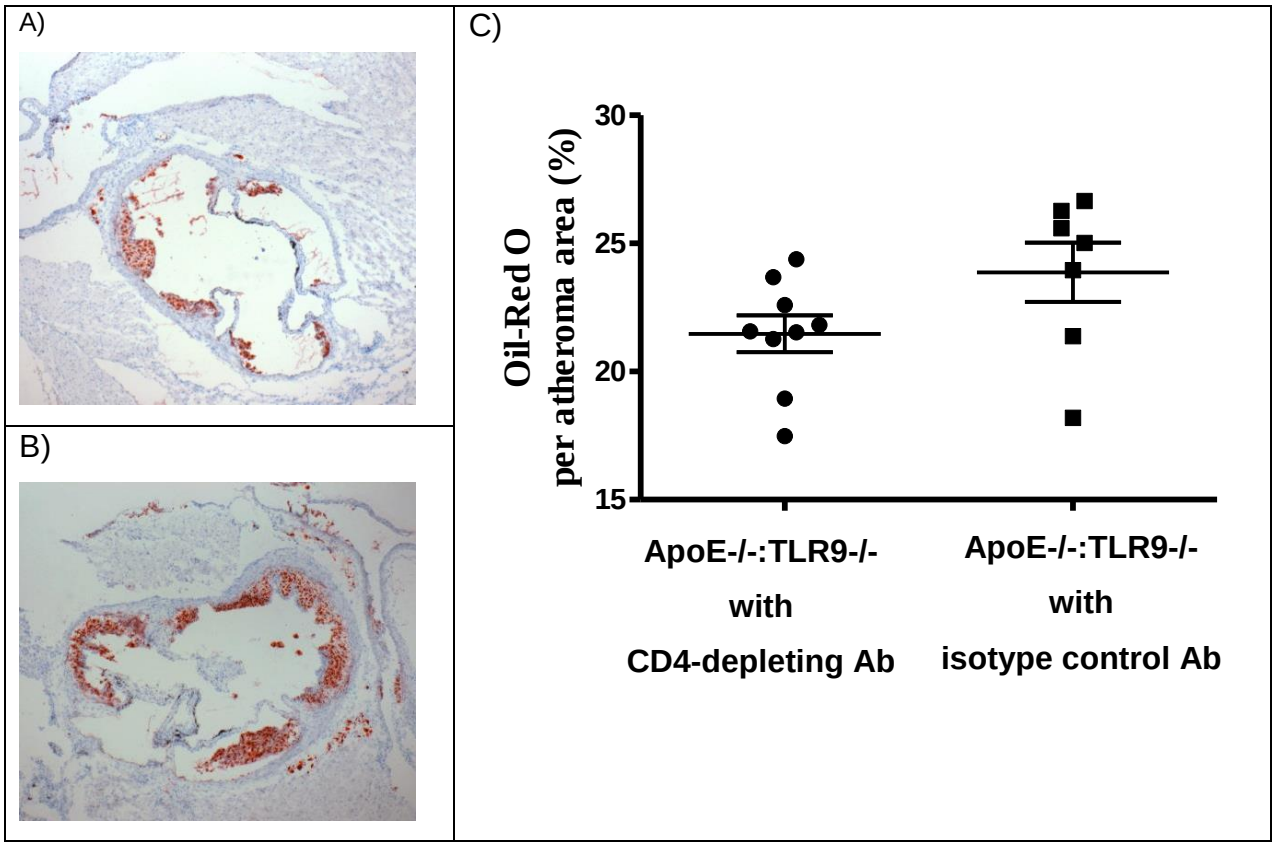

Figure 29: CD4-depleting antibody does not alter lipids in early atherosclerosis

Aortic root sections of ApoE-/-:TLR9-/- receiving (A) CD4-depleting antibody (Ab) and (B) isotype (ISO) control fed a Western diet for 8 weeks were stained with H&E. Lipids were identified by red signal intensity and percentage area of plaque ± SEM was calculated (C).

7.7.4 ApoE-/-:TLR9-/- knockout changes levels of TNF-α and IL-10 secreted by CD4+ T cells

To evaluate the role of CD4+ T cells from ApoE-/-:TLR9-/- mice in the development of atherosclerotic plaque, anti- and pro-inflammatory cytokines of CD4+ T cells were measured. Therefore, CD4+ T cells of ApoE-/-:TLR9-/- and ApoE-/-:TLR9+/+ mice were stimulated with anti-CD3 for 48h and TNF-α and IL-10 levels were measured. TNF-α was significantly increased in the supernatant of stimulated CD4+ T cells of ApoE-/-:TLR9-/- mice compared to the ApoE-/-:TLR9+/+ control mice, while IL-10 was significantly reduced in ApoE-/-:TLR9-/- mice compared to the control group.

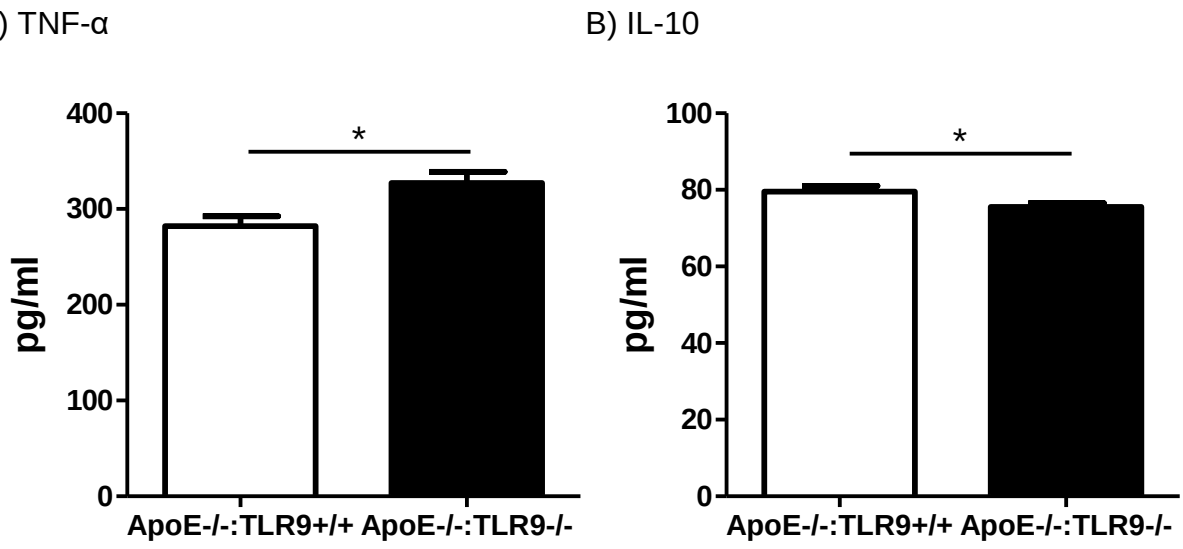

Figure 30: ApoE-/-:TLR9-/- knockout changes levels of TNF-α and IL-10 secreted by CD4+ T cells

Splenic CD4+ T cells of ApoE-/-:TLR9-/- mice secrete elevated levels of pro-inflammatory TNF-α (A) (p=0.0319). Furthermore ApoE-/-:TLR9-/- mice secrete reduced levels of anti-inflammatory IL-10 (B) (p=0.0355). Control group were splenic CD4+ T cells of ApoE-/-:TLR9+/+ mice. Results present mean of secreted levels of cytokines ± SEM (N=8, each group).

7.8 Plasma Cholesterol Evaluation

The effect of the various treatments (ODN 1668 or CD4-depleting antibody) or genetic manipulations (TLR9 knockout) on plasma cholesterol was determined using COBAS technology.

Neither the ODN nor antibody treatments were found to significantly alter the cholesterol plasma levels. The absence of the TLR9 gene was not found to significantly alter the plasma cholesterol levels within the first 8 weeks of growth, although by 15 weeks a significant increase in total cholesterol, HDL and VLDL/LDL levels were observed. However, despite this increase, the VLDL/LDL:HDL ratio was not significantly altered, nor was the total triglycerides levels.

7.8.1 TLR9 does not alter LDL/HDL Ratio after 15 weeks western diet

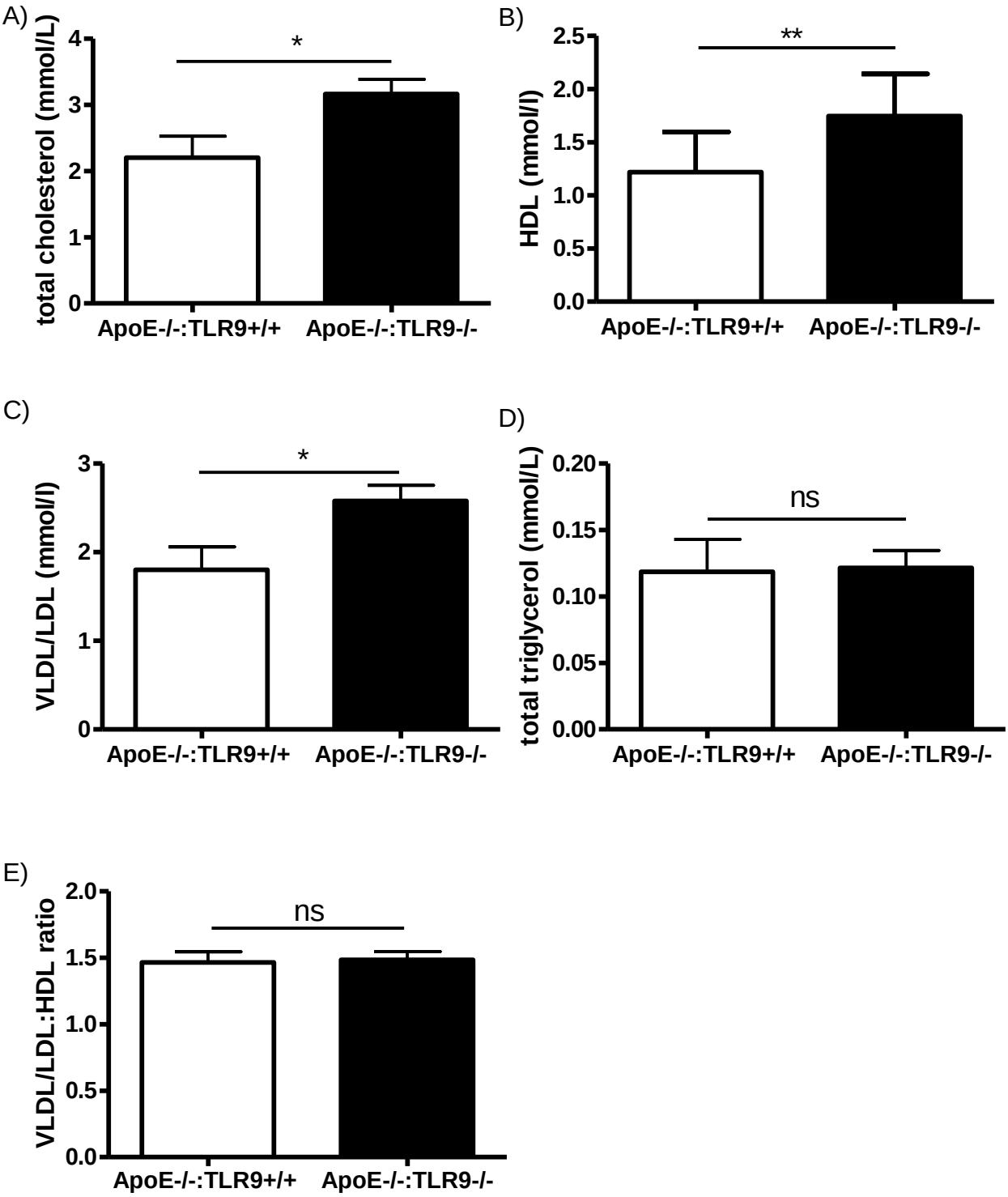

Figure 31: Knockout of TLR9 does not alter LDL/HDL Ratio after 15 weeks western diet

COBAS based-measurement plasma cholesterol (mmol/L) of ApoE-/-:TLR9+/+ and ApoE-/-:TLR9-/- mice after 15 weeks Western diet. Detailed plasma analysis presented as Total cholesterol (A) (p=0.0221), HDL (B) (p=0.0085), VLDL/LDL (C) (p=0.0212), total triglycerol (D) and VLDL/LDL:HDL ratio (E). Data presented as mean ± SEM (N≥12 each group).

7.8.2 TLR9 does not alter LDL/HDL Ratio after 8 weeks western diet

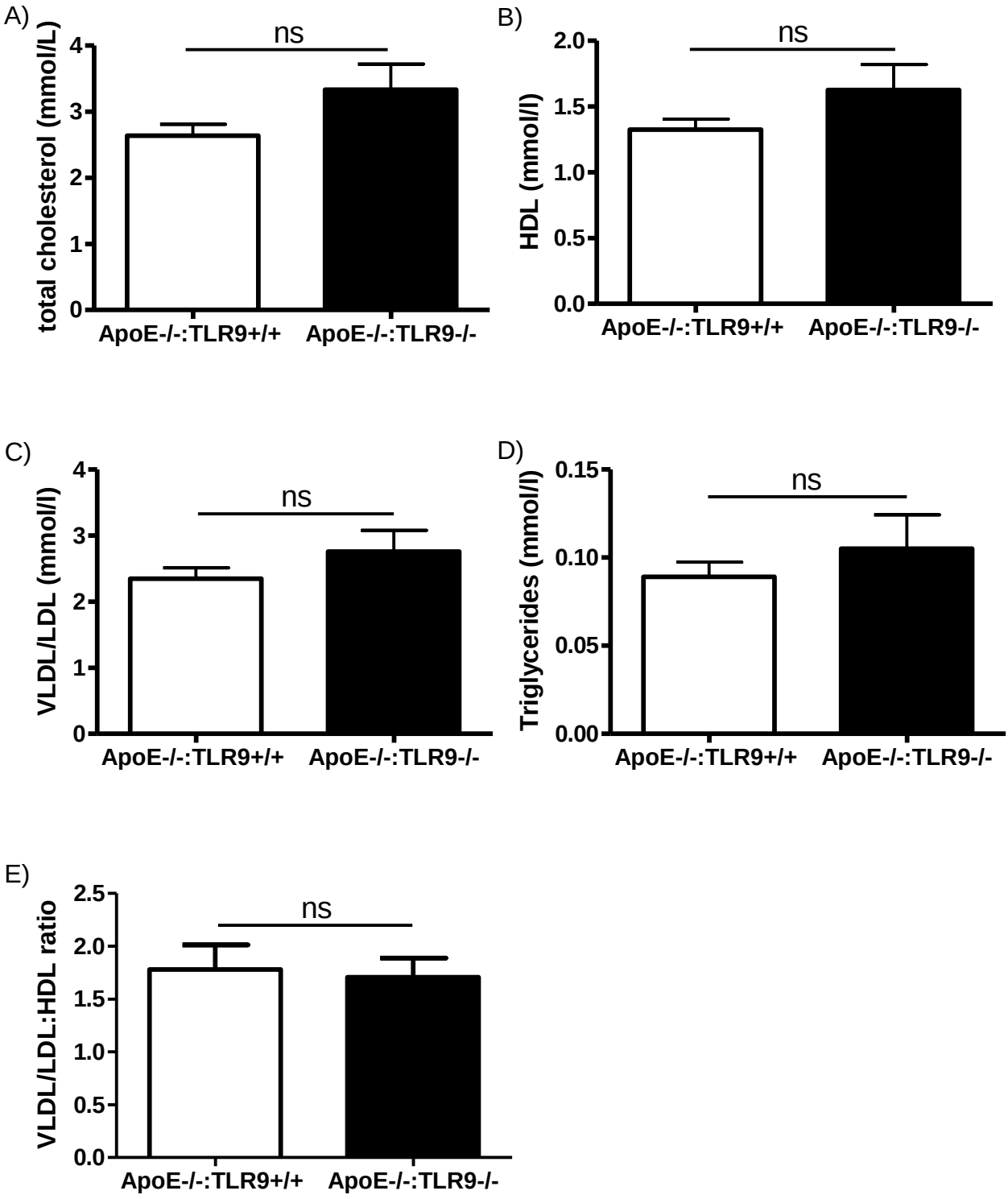

Figure 32: Knockout of TLR9 does not alter LDL/HDL Ratio after 8 weeks western diet

COBAS based-measurement plasma cholesterol (mmol/L) of ApoE-/-:TLR9+/+ and ApoE-/-:TLR9-/- mice after 8 weeks Western diet. Detailed plasma analysis presented as Total cholesterol (A), HDL (B), VLDL/LDL (C), total triglycerol (D) and VLDL/LDL:HDL ratio (E). Data presented as mean ± SEM (N≥12 each group).

7.8.3 ODN administration does not alter Plasma Cholesterol

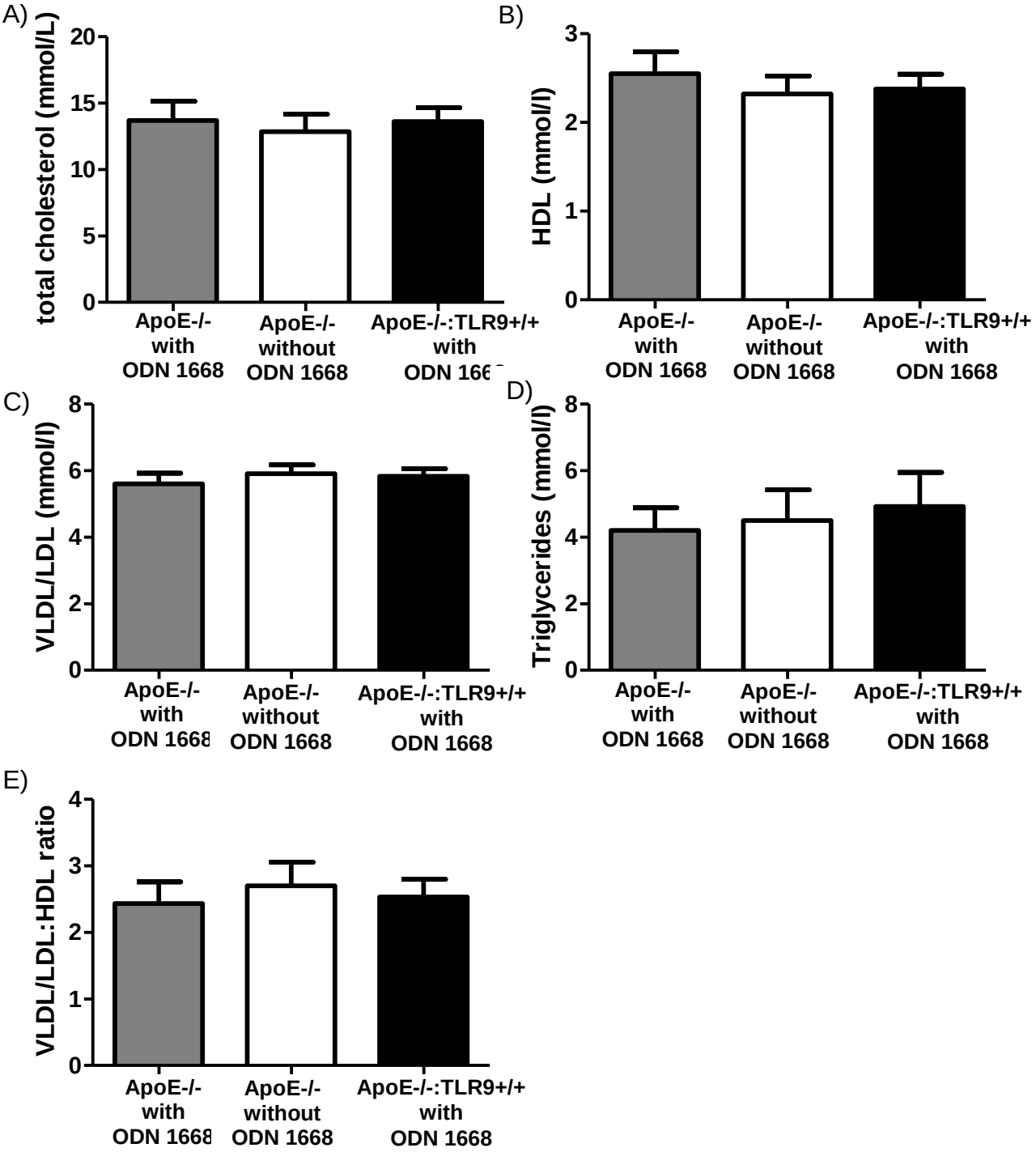

Figure 33: Knockout of TLR9 does not alter LDL/HDL Ratio after 8 weeks western diet and ODN 1668 injection

COBAS based-measurement plasma cholesterol (mmol/L) of ApoE-/-:TLR9+/+ and ApoE-/-:TLR9-/- mice receiving vehicle or ODN treatment after 8 weeks Western diet. Detailed plasma analysis presented as Total cholesterol (A), HDL (B), LDL (C), total triglycerol (D) and VLDL/LDL:HDL ratio (E). No significant difference between plasma total cholesterol, HDL, VLDL/LDL, triglycerides or VLDL/LDL ratio could be obtained. Data presented as mean ± SEM (N=8, each group).

7.8.4 CD4 depletion does not alter Plasma Cholesterol

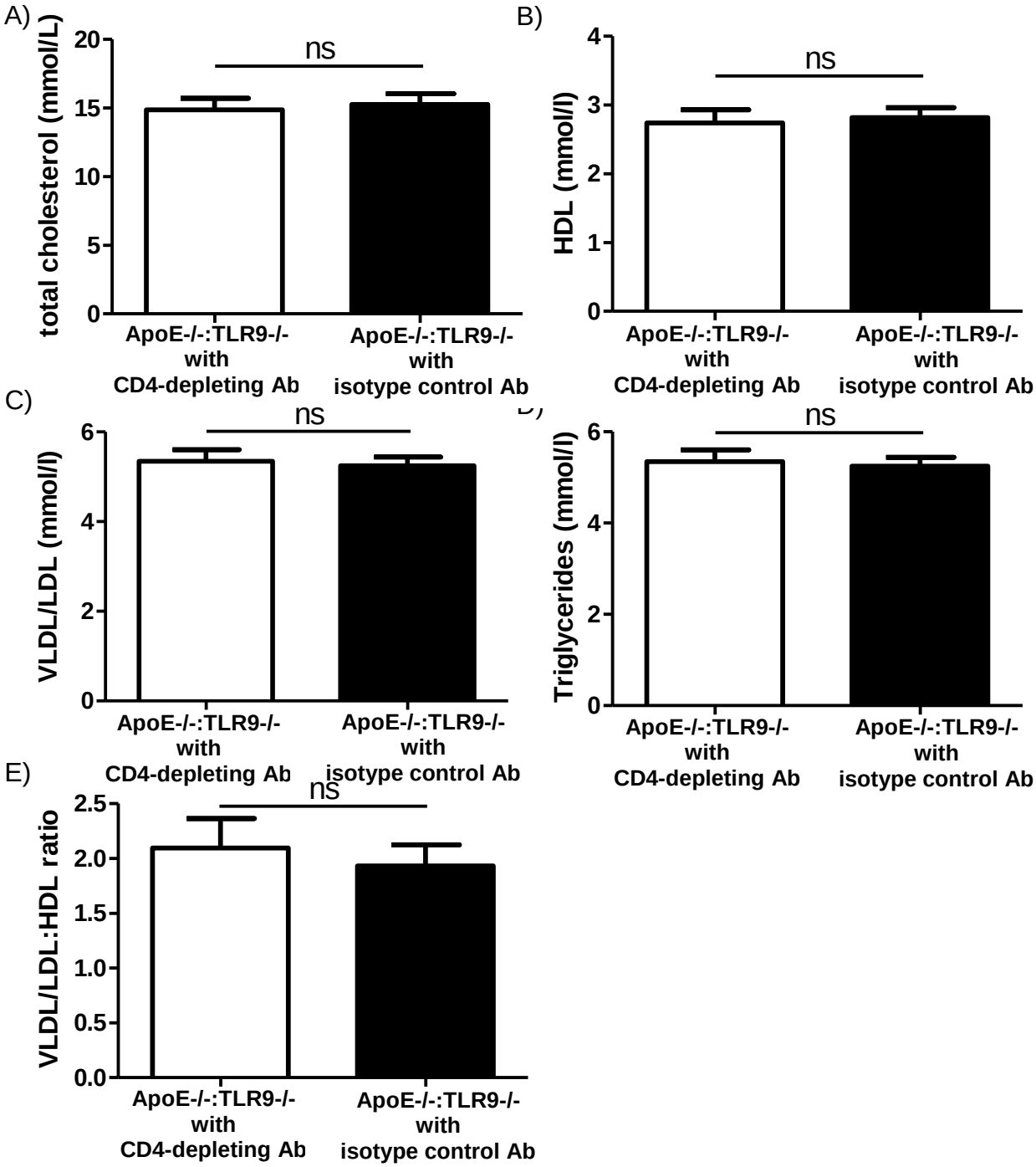

Figure 34: Knockout of TLR9 does not alter LDL/HDL Ratio after 8 weeks western diet and CD4-depleting antibody treatment

COBAS based-measurement plasma cholesterol (mmol/L) ApoE-/-:TLR9-/- mice receiving CD4-depleting antibody or isotype control after 15 weeks Western diet. Detailed plasma analysis presented as Total cholesterol (A), HDL (B), LDL (C), total triglycerol (D) and VLDL/LDL:HDL ratio (E). Data presented as mean ± SEM (N=8, each group).

8 Discussion

8.1 TLR2 knockout did not affect atherosclerosis

To study the influence of TLR2 in an atherosclerotic mouse model, a genetic knockout of TLR2 in ApoE deficient mice was performed. To evaluate the long-term effect, mice were fed for 15 weeks with a Western diet. After this period, plaque size and lipid content were analyzed. The lesions did not show significant differences compared to single knockout ApoE mice.

A negative relationship has previously been demonstrated between TLR2 and atherosclerosis progression. In their paper, Liu et al. (Liu et al., 2008) reported a reduction of atherosclerotic lesion size, less lipids, less F4/80^{+} macrophages infiltration and less MCP-1 expression (in lesion and systemic) in 5 and 7 month mice on a normal chow diet. Interestingly, they could not find a TLR2 involvement in Ac-LDL uptake or foam cell formation.

Mullick et al. (Mullick et al., 2005) reported a reduction of lesion size in aortic sinus after 10 and 14 weeks on high fat diet (HFD) and total aortic lesion after 10 weeks but not after 14 weeks. In this study, the mice received a HFD with 1.25 % cholesterol and 15.8 % fat, starting at the age of 12-15 weeks in the non-bone marrow transplantation (BMT) group and 14-16 weeks in the BMT study group. Therefore, 20 week-old female low-density Lipoprotein receptor deficient mice (LDLr-/-) were reconstituted with bone marrow (BM) from TLR2 lacking mice (TLR2-/-) or control mice (TLR2+/+). To evaluate the impact of BM derived cells, they engraft in 20 week-old female mice TLR2 lacking BM or wild type control. After 4 weeks recovery and 16 weeks on HFD, atherosclerotic lesions were measured. Their data illustrate that only the loss of TLR2 in non-BM derived cells increased atherosclerosis, while TLR2 deficient BM derived cells had no impact on atherogenesis.

Hasu et al. (Hasu et al., 2011) studied a similar hypothesis to Mullick et al., with a major difference being the age of the mice studied (younger BM recipients, 8 vs. 20 weeks), diet (lower content of cholesterol 0.15 % vs. 1.25 %) and end point (8 weeks vs. 16 weeks). They conducted BM transfer in 8 week-old mice and started HFD after 6 weeks recovery. The 8 week-old TLR2-/- mice with BM transplant showed a significant reduction of atherosclerosis in the aortic root and arch in both genders with a lower

lipid and macrophage content. Their study suggests an impact of BM derived cell lacking TLR2 on early atherosclerosis. Upon the first viewing, this result is contrary to Mullick et al.'s study. Therefore, Hasu et al. repeated the experiment with the hypothesis that diet, age and feeding period could have an important impact on atherosclerosis development. With the modified protocol, they observed an increased lesion area in the aortic root. Interestingly, they only found increased lesions in the aortic arch in female mice, and not in male mice. With this protocol, similar to Mullick's, no significant difference in lesion formation was observed in the aortic root or aortic arch between WT (wildtype) BM derived mice and TLR2-/- BM derived mice. In conclusion, they supported the hypothesis that TLR2 plays a proatherogenic effect of BM derived cells in early lesion formation in both genders, although they point to a possible loss of this affect in later formation.

The central hypothesis of the influence of TLR2 in atherosclerosis was tested in this study, using knockout of TLR2 in a background of ApoE deficient mice. Our results do not support this proposed relationship as neither plaque size nor lipid content was found to be significantly different after 15 weeks of HFD. In order to understand this disparate result, the methodological and inherent bias present in each study must be discussed.

Firstly, the composition of the high fat diet may play an important role in plaque development as differences can be found in the diet used between the aforementioned studies and ours. The T_h1/T_h2 ratio has been suggested to correlate with the plasma cholesterol level, with high cholesterol levels induced by a HFD suspected of inducing an abnormal switch from T_h1 cells to T_h2 cells in ApoE knockout mice (Zhou et al., 1998). In turn, this switch can potentially lead to an abnormal immune response in ApoE mice and mask immunological effects.

The study of Hayes et al. is an example of where diet can induce an immunological effect. Hayes et al. explored the effect of RAG-1 (Recombinase Activating Gene 1) in ApoE deficient mice. They could find a 40 % percent reduction of atherosclerosis in ApoE-/-:RAG-1-/- mice compared to ApoE-/- only mice when fed with a normal chow diet. However, this effect was absent when the study was repeated using mice fed with a Western diet (0.15 % cholesterol) (Dansky et al., 1997).

A similarly important consideration is the duration of the different diets (normal chow vs. HF). To rule out the influence of a high fat diet and the induced hypercholesterolemia, Liu et al. used a normal chow diet for the study. However, the

time required for atherosclerosis to develop in these mice is considerably longer, at 5 and 7 months, which raise questions about the pathways involved in the development. For instance, components of innate immune system can be time dependent, with $CD11b^+$ macrophages reported as being important for atherogenesis but they do not appear to play a role in established plaque (Stoneman et al., 2007). Hasu et al. identified an influence of TLR2 in early atherosclerosis (8 weeks on HFD) dependent upon BM derived cells, although this was lost after 16 weeks HFD, similar to the result of Mullick et al. and the conclusion of our study. We examined a duration of 15 weeks using a high fat diet comprising 22 % fat and 0.15 % cholesterol. It is possible that the duration of our study or the induced hypercholesterolemia and potential abnormal immune response masked the proatherogenic effect of TLR2.

Interestingly, Liu et al. (Liu et al., 2008) reported their female ApoE KO mice to have more plaque accumulation than their matched male mice. Given that we used male mice in our study, it is possible that small changes could not be determined. However, past studies agree that the main effects of TLR2 in atherosclerosis are gender-unspecific.

Moreover, the role of non-bone marrow derived cells, like endothelial cells, are important in the development of atherosclerosis. Mullick et al. demonstrated TLR2 in non-BM derived cells as being responsible for the proatherogenic effect of TLR2 in progression and late-stage atherosclerosis. However, as acknowledged by the author in that study, the question of TLR2 expression in endothelial cells is controversial. Dutzendorfer et al. demonstrated that TLR2 expression is increased under “disturbed flow” conditions (as present in atherosclerotic prone areas) (Dunzendorfer et al., 2004). However, this finding remains controversial with studies such as Liang and colleagues reporting an increase in TLR4 and not TLR2 expression in endothelial cells during exposure to flow shear stress (Liang et al., 2002). Furthermore, some reports have even failed to detect TLR2 in primary human aortic endothelial cells (Walton et al., 2003) or murine and human atherosclerotic plaque (Xu et al., 2001).

Ultimately we cannot rule out a negative effect of TLR2 on atherosclerosis, even when we couldn’t find significant differences neither in plaque size nor in lipid content.

The contradictory outcomes of several studies on this topic suggest that time, diet and other factors are influencing TLR2's effect in atherosclerosis. Further studies are required to understand these differences and potentially interesting mechanisms.

8.2 TLR4 knockout did not affect atherosclerosis

The role of TLR4 has become prominent in hypotheses since the innate immune system was reported as being central to atherosclerosis. The ability to recognize different pathogens of bacteria, with downstream NF- κB and MAPK activation, and the induction of inflammation has under-pinned the interest in TLR4. The hypothesis of an infectious trigger of atherosclerosis has not been definitively proven, although the potential contribution of pathogens such as Chlamydia pneumoniae, Porphyromonas gingivalis and Helicobacter pylori (Rosenfeld and Campbell, 2011) continues to attract attention. Despite the continued failure of large-scale trials using antibiotics for secondary prophylaxes of CVD to show a significant protective effect (O'Connor et al., 2003, Grayston et al., 2005, Cannon et al., 2005), TLR4 remains an interesting potential therapeutic target to address chronic inflammatory diseases such as atherosclerosis.

Our study did not show a link between TLR4 and atherosclerosis with neither lesion size nor lipid content significantly different between ApoE-/-:TLR4+/+ mice and ApoE-/-:TLR4-/- mice after 15 weeks on a Western diet. This result is consistent with the study of Wright and colleagues, who found that deletion of TLR4 did not alter the development of atherosclerotic lesions (Wright et al., 2000), despite studying several time points up to a total age of 27 weeks. Furthermore, they found no impact of TLR4 on plasma and cholesterol levels, although they only measured aortic cholesteryl ester as a parameter for atherosclerotic progression.

By contrast, Michelsen et al. reported a reduction of atherosclerotic lipid content, infiltration of macrophages and serum MCP-1 concentration in male and female ApoE and TLR4 deficient mice on a Western diet (0.15 % cholesterol) for 6 months. The deletion of MyD88 or TLR4 did not alter serum cholesterol or the lipid profile. This report by Michelsen is supported by Higashimori et al., who suggest that TLR4 promotes atherosclerosis especially in an early stage by early foam cell accumulation and lesional expression of IL-1α and the proinflammatory mediators MCP-1 and VCAM-1. They found that the deletion of TLR4 in ApoE deficient mice is predominantly relevant reducing early atherosclerosis (3 weeks HFD and 20 week-old mice with a chow diet) and only a moderate change after 36 weeks chow diet compared to ApoE single knockout mice. They also reported no impact on cholesterol or lipid profile.

In their study, Ding et al. found decreased atherosclerosis in 24 weeks on diabetogenic diet (diabetogenic high, saturated fatty acids-rich and carbohydrate-rich) in LDLr-/-:TLR4-/- mice compared to LDLr-/-:TLR4+/+ control group. In contrast to other reports, the decrease in atherosclerotic lesion was found be correlate with increased plasma cholesterol in LDLr-/-:TLR4-/- mice. Additionally, triglycerides levels were also increased. Furthermore, they reported no significant differences in chow diet fed mice after 24 weeks. Interestingly, they found that TLR2 expression was increased owing to TLR4 deletion (Ding et al., 2012).

Studies show that TLR4 is up regulated by flow shear stress in endothelial cells (Liang et al., 2002). Hence, TLR4 is only expressed in aortic atherosclerotic lesions of ApoE deficient mice, but not in C57BL/6J mice without deficiency of ApoE. Despite human coronary, plaques showed expression of TLR4 (Xu et al., 2001).

CD14 is an important cofactor for TLR4 in recognition of LPS. Björkbacka et al. did not find any differences in CD14 and ApoE knockout mice compared to ApoE single knockout mice (Bjorkbacka et al., 2004) up to 8 and 10 weeks on HFD. This finding argues against an involvement of the CD14/TLR4 complex in atherogenesis.

After all, our study did not show an impact of TLR4 on atherosclerosis after 15 weeks of high fat diet. Though other studies suggest, consistent to our result, that TLR4 could influence early atherogenesis but may lose its role during further development of atherosclerosis. As discussed for the role of TLR2, other factors, for example diet, can also have a major effect on the role of TLR4 on atherogenesis.

8.3 A dominant role of TLR9 in atherosclerosis

8.3.1 The role of TLR9 outside of atherosclerosis

TLR9, an endosomally-located Toll-Like Receptor, is expressed in various cell types of the immune system and is important for the recognition of CpG sequences in DNA. CpG motifs are relatively rare in vertebrate DNA compared to viral and bacterial DNA, with approximately 1% of the whole DNA. We demonstrate a protective effect of TLR9 in atherosclerosis, thus providing novel data linking TLR9 function with effects on the immune system and atherosclerosis.

Previous studies have indicated both pro- and anti-inflammatory effects of the Toll-Like Receptor 9, leading to a controversy over the function and contribution of TLR9 to disease. A proinflammatory effect was reported by Niessner et al., indicating that TLR9 activation leads to an inflamed plaque type by induced IFN-α production (Niessner et al., 2006). Hoque et al. demonstrated a significant role of TLR9 in the development of acute Pancreatitis in a mouse model (Hoque et al.), in which the TLR9 dependent effect was associated with a rapid recruitment and increased portion of neutrophils and elevated interleukin-1β expression. In addition, a TLR9 antagonist can inhibit these effects. Reports support these previous studies by suggesting that an increased number of TLR9 deficient mice survive polymicrobial sepsis in contrast to WT mice. These TLR9-/- mice generally exhibit less inflammation, lower bacterial colonization in blood and peritoneal lavage fluid and decreased levels of the inflammation cytokines TNF, IL-10, MCP-1 and IL-10 (Plitas et al., 2008). TLR9 also promotes glomerular leukocytes recruitment, which leads to glomerular nephritis (Summers et al.). Similarly, Krysko et al. reported that TLR9 is required for neutrophil recruitment in doxorubicin-induced sterile inflammation in mice (Krysko et al., 2011), while TLR9 has been separately described as a promoter of liver fibrosis (Miura et al.) and acute hepatoxicity in mouse models (Imaeda et al.).

In contrast to the discussed pro-inflammatory properties of TLR9, pre-conditioning with a specific agonist of TLR9, CpG ODN, reduced myocardial ischemia/reperfusion injury by increased levels of the anti-inflammatory Interleukin-10 (Markowski et al., 2013). Furthermore, pretreatment with ODN 1668, a specific TLR9 agonist, attenuated the inflammation response and cardiac remodeling in a mouse model (Velten et al., 2012). In liver ischemia/reperfusion injury, a model of sterile inflammation, conventional DCs produce anti-inflammatory IL-10 in a TLR9 dependent manner. This production has the

ability to reduce the secretion of TNF, IL-6 and Reactive Oxygen Species from inflammatory monocytes (Bamboat et al., 2010).

Similarly, lack of TLR9 leads to the increased production of proinflammatory cytokines like IL-6 and IL-17A in experimental autoimmune diabetes models. Treatment with CpG ODN protects wild type mice from induced diabetes, mediated by a TLR9 dependent increase of indoleamine 2,3-dioxygenase from dendritic cells (Fallarino et al., 2009). Additionally, TLR9 is protective in the development of murine Lupus (Wu and Peng, 2006), even with some controversies (Christensen et al., 2005).

The central role of TLR9 in inflammatory diseases makes the receptor interesting for investigations concerning the role in atherosclerosis. However, opposing reports about the anti-or pro-inflammatory nature of TLR9 make it difficult to hypothesize the specific role of TLR9 in the development and progression of atherosclerosis. This is particularly the case given the described an athero-protective effect of intracellular located TLR3 (Cole et al., 2011) and TLR7 (Salagianni et al., 2012) or a possible proatherogenic effect of surface located TLR2 and TLR4 (Ding et al., 2012, Mullick et al., 2005).

8.3.2 Effect of TLR9 deletion on atherosclerotic lesion size and characteristics in ApoE-/- mice

In vitro data that TLR9 is involved in the formation of foam cells (Gu et al., 2010, Lee et al., 2008, Sorrentino et al., 2010) leads to the hypothesis that TLR9 may accelerate the development of atherosclerosis.

Our *in vivo* study does not support this, given that we found a significant increase in atherosclerotic burden in early-stage and later atherosclerosis in ApoE-/-:TLR9-/- mice. The athero-protective effect of TLR9 on atherosclerosis is emphasized by a significantly higher lipid content and a higher proportion of $CD68^+$ macrophages in TLR9 lacking mice. Macrophages expressing CD68 are responsible for lipid uptake and forming foam cells (Ball et al., 1995). We confirmed this observed increased macrophage migration with MOMA-2, another marker for mouse macrophages. Together, a significant reduction in collagen in the composition of the plaque in ApoE and TLR9 lacking mice was observed, demonstrating characteristic properties of an unstable plaque (Libby and Aikawa, 2002). VCAM-1 was not found to be affected by TLR9.

Our *in vivo* results indicating an athero-protective effect of TLR9 contrast with previously reported *in vitro* studies suggesting a pro-inflammatory role of TLR9. However, like TLR9, TLR7 is an endosomally-located TLR that has reported to be athero-protective (Salagianni et al., 2012) and thus provides some precedence for our results. Recent findings suggest an athero-protective role of endosomally-located TLRs, namely TLR3, TLR7 and TLR9 thus far, whilst TLRs located on the surface of the cells, TLR2 and TLR4, demonstrate proatherogenic effects (Salagianni et al., 2012, Cole et al., 2011, Hasu et al., 2011, Vink et al., 2002).

Moreover, similar discrepancies have been observed between *in vitro* data and *in vivo* data regarding TLR3. *In vitro* data has led to a proinflammatory attitude of TLR3, while *in vivo* data has revealed a protective role of TLR3 from arterial injury in hypercholesterolemia mice (Cole et al., 2011).

A possible explanation for the opposing conclusions is the multiple appearance and expression of TLR in various cell types of the immune system and non-immune system cells, like endothelial cells. *In vitro* data can demonstrate the specific effect of an agonist or antagonist on one cell type, although *in vivo* experiments are required to investigate the complex system with inhibiting and activating processes.

Furthermore, the true action of Toll-Like Receptors might differ under disease environments; for example, the blocking of TLR7 and TLR9 leads to post-interventional vascular remodeling in mice (Karper et al., 2012). In contrast to this disease model is the genetic deficiency of TLR7, as reported by Salagianni et al. (Salagianni et al., 2012), or TLR9, as we report here, leading to a significant increase of atherosclerosis. While *in vivo* studies have primarily been carried out in mice models, such findings can be translated to humans. Stenotic valves of humans hearts are known to express lower levels of TLR9 compared to healthy control valves, while TLR2 and TLR4 have an increased expression (Lopez et al., 2012).

Our study has also shown a significant increase in dendritic cell count after 8 weeks on an atherogenic diet. The complex role of plasmacytoid DCs is marked by a described pro- and anti-atherogenic effect. Döring et al. describe a proatherogenic effect of plasmacytoid DCs via INF-α (Doring et al., 2012), while Daissormont et al. demonstrated an anti-atherogenic role by modulation of T cells (Daissormont et al., 2011). Moreover, DC are known to play a crucial role in several autoimmune and neuroinflammatory disease, such as diabetes, rheumatoid arthritis and multiple sclerosis (Manuel et al., 2007). Our findings demonstrate that mature DCs, detected by the maturation marker CD86, are significantly increased in atherosclerotic plaque of ApoE-/-:TLR9-/- mice. The blocking of DC maturation can suppress T_h1 specific immune response in a mouse model of multiple sclerosis (Zinser et al., 2004). Moreover, mature DCs correlate with a vulnerable plaque phenotype (Dietel et al., 2013, Yilmaz et al., 2004) and by clustering with T cells they lead to the activation and proliferation of T cells. Activated T cells result in the acceleration of inflammation by increased inflammatory cytokine secretion, including TNF-α and IFN-γ (Ranjit and Dazhu, 2006).

The finding of a proatherogenic role of TLR9 provides a further link between the innate immune system and atherosclerosis.

8.3.3 A TLR9 agonist reduces atherosclerotic lesion development

The reduction of atherosclerosis after oligodeoxynucleotide (ODN) administration in ApoE mice supports a possible new range of therapeutic applications for synthetic TLR9 agonists. However, controversy remains about the pro- and/or anti-inflammatory effect about CpG motifs; TLR9 ligands. For example, in inflammatory bowel diseases, the influence of bacteria in development and progression are a point of interest, as in atherosclerosis. Obermeier et al. pointed out an exacerbation of established intestinal inflammation with CpG-containing ODN treatment in murine colitis models (Obermeier et al., 2002). In contrast, they were also able to find a highly protective effect role of CpG ODN when given in a prophylactic manner (Obermeier et al., 2003, Rachmilewitz et al., 2004). These data suggest possible explanations for the protective effect in the epithelial cell-dependent mechanism of the intestinal mucosa; for example, by inhibition of TNF, IL-8 secretion (Ghadimi et al., 2010) and induction of Prostaglandin E2 (Rachmilewitz et al., 2002). However, they describe a more important mechanism for the protective effect of prophylactic administrated CpG ODNs. Pretreatment of $CD4^+CD62L^+$ cells with CpG ODN significantly reduced intestinal inflammation in a SCID transfer model of colitis. Recipients of these pretreated $CD4^+$ T cells had less histological signs of colitis, as well as decreased IL-6 and IFN-γ levels, while IL-10 levels were increased. Conclusive to this, $CD4^+CD62L^+$ cells of TLR9-/- mice secreted significant higher levels of IFN-γ, IL-5, IL-6 and reduced levels of IL-10 (Obermeier et al., 2005), by a possible switch of these T cells to adaptive regulatory T cells (Bluestone and Abbas, 2003).

Three classes of CpG ODN, with different characteristics, have been discovered/developed thus far. While their properties and therapeutic potential differs between the classes, all three classes require TLR9 for signaling (Krieg, 2012). The therapeutic potential is particularly based upon the TLR9-induced immune response. This response appears to be important to stimulate protective immunity against intracellular pathogens, such as Hepatitis C Virus, and in vaccination approaches, for example against Cytomegalovirus or Hepatitis B Virus. Several clinic studies with CpG ODN TLR9 agonists indicate an ontological effect with antitumor activity. In particular, melanoma and other skin cancers are points of interest in the therapeutic approach of CpG ODNs (Krieg, 2012).

The CpG oligodeoxynucleotide class (ODN 1668, class B) that we used has been demonstrated to enhance arthritis development (Wu et al., 2007), as well as intestinal

inflammation (Bleich et al., 2009) in mouse models. This class was also demonstrated to protect against myocardial ischemia/reperfusion injury in a mouse model (Cao et al., 2013). As previously mentioned, Niessner et al. demonstrated a proinflammatory effect of human plasmacytoid DCs, with increased production of INF-α, resulting in a vulnerable plaque. Interestingly, they could demonstrate increased INF-α production *in vitro* when stimulated with the B-class TLR9 ligand, ODN2006. In contrast to Niessner et al. *in vitro* experiments, there are *in vivo* experiments suggesting a suppressive effect of TLR9 ligands in atherosclerosis. Next to our study of ODN 1668 treatment of ApoE-/- mice, Cheng et al. also described an anti-atherosclerotic effect of ODN (ODN A151) with decreased levels of IFN-γ and TNF-α level in ApoE-/- mice (Cheng et al., 2008). Moreover, there are several CpG ODNs in human trials against cancer, asthma and hepatitis C and other diseases as promising drugs (Krieg, 2012).

8.3.4 TLR9 deletion and serum lipid levels

TLR9 and ApoE deficient mice had significantly higher serum levels of VLDL/LDL. These cholesterol rich particles are associated with atherosclerosis development. Ding et al. have recently proposed increased plasma cholesterol levels as a major contributor of the proatherogenic effect of TLR4 (Ding et al., 2012). In our study, we discovered a parallel increase in HDL levels in ApoE-/-:TLR9-/- mice. HDL has the ability to regress progression of atherosclerosis, partly by changing the inflammatory properties of $CD68^+$ macrophages (Feig et al., 2011). The anti-atherosclerotic effect of HDL has been demonstrated in mice (Choudhury et al., 2004), as well as humans (Rye et al., 2009). Therefore, we hypothesized that the increased pro-atherogenic VLDL/LDL serum levels are compensated by the increased anti-atherogenic HDL serum levels. Studies suggest that the VLDL/LDL:HDL ratio is a better predictor for atherosclerotic progression and cardiovascular risk than the values themselves (Fernandez and Webb, 2008, Enomoto et al., 2011). In our study, the VLDL/LDL:HDL ratio between TLR9-/- and TLR9+/+ groups remained unchanged. A cholesterol-independent, anti-inflammatory effect of TLR9 is further supported by the CD4-depleting antibody study or the administration of a TLR9 agonist, whereby the anti-inflammatory effect was present in the absence of changes of plasma cholesterol levels. However, further studies are required to evaluate the influence of TLR9 on the cholesterol profile.

8.3.5 Contribution of $CD4^+$ T cells to atherosclerosis in ApoE-/-:TLR9-/- mice

$CD4^+$ T cells represent the major population of lymphocytes in atherosclerotic plaque (Benagiano et al., 2003). Reports about increased T cells in unstable plaque increase their potential importance (Liuzzo et al., 2000). Nevertheless, the stimulation of plasmacytoid dendritic cells in a TLR9 dependent manner induces the differentiation of $CD4^+$ T cells into IL-10 producing immunosuppressive regulatory T cells, characterized by $CD4^+CD25^+Foxp3^+$ (Moseman et al.). Regulatory T cells have been shown to be athero-protective in various studies (Mor et al., 2007, Dinh et al., 2012, Klingenberg et al., 2013). However, previous studies have also shown that regulatory T cells were not affected in ApoE-/-:TLR9-/- compared to ApoE-/-:TLR9+/+ control mice. Separately, splenic lymphocytes have been shown to comprise an increase in $CD4^+$ and $CD8^+$ T cells in ApoE-/-:TLR9-/- mice (Koulis et al., 2014). Lymphocytes are major contributors in atherogenesis (Song et al., 2001). Activated T cells are expressed in higher numbers in atherosclerotic lesions from patients with unstable angina or acute myocardial infarction compared to those with chronic stabile or stabilized unstable angina pectoris (Hosono et al., 2003). The critical role of $CD4^+$ T cells is underlined by a similar increased portion of $CD4^+$ T cells in the atherosclerotic lesions in ApoE-/-:TLR9-/- mice. Zhou et al. elegantly demonstrated the important role of $CD4^+$ T cells in atherosclerosis when they transferred $CD4^+$ T cells into immunodeficient ApoE-/- mice. These immunodeficient and atherosclerotic plaque-forming mice are generated by crossing ApoE-/- with the SCID (Severe Combined Immunodeficiency) strain, and are characterized by the absence of T and B cells. They observed a 164 % increase of atherosclerotic lesions in ApoE(-/-)scid/scid mice with transferred $CD4^+$ cells compared to ApoE(-/-)/scid/scid mice without transferred $CD4^+$ cells (Zhou et al., 2000). Knockout of $CD4^+$ cells in ApoE deficient mice leads to a reduction of atherosclerosis lesions and an increase of $CD8^+$ cells (Zhou et al., 2005). We observed the same effect when ApoE-/-:TLR9-/- mice were treated with CD4-depleting antibody. However, Tyznik et al. have proposed that the increase of $CD8^+$ is simply a compensatory effect caused by the lack of $CD4^+$ T cells (Tyznik et al., 2004).

Furthermore, Kyaw et al. demonstrate a pro-atherogenic role of $CD8^+$ cell in ApoE mice (Kyaw et al., 2013). One possible explanation could be a change in function of $CD8^+$ T cells after CD4 depletion (Tyznik et al., 2004). However, this hypothesis concerning

the specific role of $CD8^+$ cells in ApoE-/-:TLR9-/- mice requires further experimentation and investigation.

$CD4^+$ T cells play a critical role in vascular remodeling in a mouse model of vascular injury (Cuttica et al., 2011). To examine the functional contribution of $CD4^+$ T cells in our context, we administrated a CD4-depleting antibody or an isotype control antibody to ApoE-/-:TLR9-/- mice. A lack of $CD4^+$ T cells lead to a significant reduction of atherosclerosis in ApoE-/-:TLR9-/- mice, compared to ApoE-/-:TLR9-/- mice receiving an isotype control antibody. This indicates a major role of $CD4^+$ T cells in the plaque development of ApoE-/-:TLR9-/- mice. A potential mechanistic link between this lymphocyte subpopulation and plaque development is provided by our finding that splenic $CD4^+$ T cells from in ApoE-/-:TLR9-/- secrete more TNF-α and less lL-10 than splenic $CD4^+$ T cells from ApoE-/-:TLR9+/+ mice. IL-10 is an anti-inflammatory cytokine and reduces atherosclerosis and improves plaque stability (Caligiuri et al., 2003). The pro-inflammatory cytokine TNF-α has long been known for its crucial role in macrophages proliferation (Branch et al., 1989), which plays the major cellular role in plaque progression (Robbins et al., 2013).

8.3.6 TLR9 is not influencing macrophages subsets

Recent studies implicate different subsets of monocytes as being important during atherogenesis, with $CD115^+CD11b^+Ly6c^{high}$ circulating cells particularly associated with increased atherosclerosis. These monocytes have a higher inflammatory potential with increased expression of CCR2, the MCP-1 receptor, and are potentially important cells in the development of atherosclerosis (Tacke et al., 2007, Swirski et al., 2007). They are more likely to adhere and accumulate in the plaque and become macrophages. In comparison, despite being critical in the development of atherosclerosis $CD11b^+$ cells are less so in already established lesions (Stoneman et al., 2007). The study of Salagianni et al. demonstrated a protective role of TLR7, also a endosomally-located TLR, in atherosclerosis (Salagianni et al., 2012), which is attributed to increased MCP-1 levels, secreted by peritoneal macrophages in ApoE-/-:TLR7-/- mice, and with increased macrophage accumulation and activation. They observed a difference in morphological shape in subsets of macrophages associated with an increased “inflammatory” subset of macrophages ($Ly6c^{high}$). In our study, we were unable to identify a change in subsets of macrophages, suggesting that

the anti-atherosclerotic effect of TLR9 is not mediated by changes in macrophages subtypes.

9 Limitations

We examined the development of atherosclerosis in mice and not in humans, a species where genetic deficiency of ApoE or LDLr is a pre-requisite for atherosclerotic plaque development. However, it is important to remember that such genetic defects were originaly discovered in humans and were then translated to genetic deficiencies in mice, this is particularly true for homozygous familial hypercholesterolemia. Similarly, the process of atherogenesis in human occurs over decades, while such mouse models have accelerated development in a matter of weeks. Furthermore, the composition of plaque differs between mice and humans, with inflammation being the major component of murine atherosclerotic plaques, while in humans inflammatory cells constitute approximately 2-5 % of the atherosclerotic lesion (Finn et al., 2010). Moreover, lesion initiation and early atherosclerosis in mice is believed to be driven by macrophages and their pro-inflammatory products. However, in humans, the initial mechanism is understood to be intimal thickening, defined by SMCs, proteoglycans, collagen and extracellular lipids (Nakashima et al., 2008).

Despite such differences, mouse models are highly valuable models for atherosclerotic development and progression as they aid the identification of physiological mechanisms leading to atherosclerosis and can also be used as a test-bed for preventive medicine. Importantly, however, the most negative complication in humans is that of unstable plaques that lead to rupture and thrombogenic events. The term ‘vulnerable plaque’ is now commonly used within the atherosclerosis research community. However, mice plaque models are de facto not vulnerable and extremely rarely lead to rupture. Past research has assumed that specific plaque compositions are correlated with a higher risk of plaque rupture in humans. Our group has recently developed a mouse model with instable plaques (Chen et al., 2013) to better aid our understanding. Moreover, in the greater context of gene deletion, redundancy is likely to exist where expression patterns of other proteins are altered in response to TLR deficiency, potentially masking effects on atherosclerosis. For example, the deletion of TLR4 in ApoE deficient mice leads to an increased expression of TLR2 in these mice (Nakashima et al., 2008).

Finally, the size of test groups was limited by technical issues with breeding and thus the statistical power of our study was somewhat limited for certain disease parameters.

10 Future Directions

The study presented here demonstrates an athero-protective effect of TLR9. While exploring some fundamental aspects of this relationship, further experiments are required to more fully examine the influence of TLR9 on atherosclerosis. To assess the role of bone marrow and non-bone morrow cells, a bone marrow transplantation mouse model could be designed whereby bone marrow cells of mice ApoE-/-:TLR9-/- could be transplanted into ApoE-/-:TLR9+/+ mice and thus plaque severity could be measured.

A better understanding of the effector cell types that mediate the protective effect of TLR9 would greatly support our findings and further experiments focused on understanding the role of dendritic cells are currently being planned.

Another possible effector cell type are B cells since TLR9 is a stimulus for them (Li et al., 2013), and they are thought to protect against atherosclerosis by producing antibodies against oxLDL (Major et al., 2002). However, the B2 B cell subset ($CD22^{+}CD5^{-}$) has been identified as B cell population with proatherogenic potential (Kyaw et al., 2010).

Another avenue of research would be the principal nature of the role of TLR9. It is difficult to differentiate between 'primary' effects of TLR9 on atherosclerosis and 'secondary' lipid induced inflammatory effect in our *in vivo* focused studies. This secondary effect may be investigated by considering oxLDL mediated T cell proliferation (Stemme et al., 1995) *in vitro*, using foam cell formation and phagocytosis assays. Similarly, comparing ApoE-/-:TLR9-/- and ApoE-/-:TLR9+/+ macrophages could clarify the lipid-induced effect and their impact on plaque development in ApoE-/-:TLR9-/- mice. Further experiments are required to resolve the increase in VLDL/LDL and HDL in ApoE-/-:TLR9-/- mice.

Additional further experiments should be performed with type B CpG oligodeoxy-nucleotide ODN 1668, especially because this drug group belongs to a novel and promising therapeutic and/or preventive approach in humans (Krieg, 2012). Obermeier et al. demonstrated a different effect of ODNs in intestinal bowel diseases, dependent on the time point of drug administration. ODNs were found to accelerate intestinal bowel disease when given as a therapeutic approach, whereas inflammation was inhibited when given preventively (Obermeier et al., 2005, Obermeier et al., 2003). Therefore, possible experiments could include assessing ODN injection before a high

fat diet is begun compared to administration in existent atherosclerotic lesions, with the influence of progression or regression being assessed.

11 Summary

Inflammation is a critical and complex component in the orchestra of multiple mechanisms that cause atherosclerosis and its complications. Therefore, the innate immune system with its pattern recognition receptors, especially the Toll-Like Receptors (TLRs), is under special investigation. To investigate the role of TLRs in the development and progressing of atherosclerosis, knockout mice of TLR2, TLR4 or TLR9 were crossed with knockout of ApoE to generate double knockouts. These mice were fed a high fat diet for 8 or 15 weeks and plaque size and composition was examined.

Neither double knockout mice of TLR2 nor TLR4 were found to show a significant difference in size and/or lipid content in their atherosclerotic lesions. This result was surprising because previous studies have indicated TLR2 and TLR4 to be pro-atherogenic. However, important experimental aspects such as the time point, diet and genetic background all vary between these previous studies. It may be the case that a long-term 15-week high fat diet induces mechanisms to compensate and negate TLR2 and TLR4 effects.

In vitro studies have suggested that TLR9 have a pro-atherogenic potential, although the *in vivo* studies presented here demonstrate an anti-atherogenic effect of TLR9. Beyond plaque size and lipid content, cellular composition, inflammatory status and plasma lipids were also assessed. To evaluate the influence of TLR9 on early atherosclerosis, ApoE-/-:TLR9-/- mice and their controls were fed with a high-fat diet for only 8 weeks and then atherosclerotic plaques were analyzed. For both stages of atherosclerosis, an exacerbation of atherosclerotic lesion severity was found with an increased plaque size, lipid content, accumulation of macrophages and dendritic cells, while the collagen content was reduced. Circulating VLDL/LDL were increased in our ApoE-/-:TLR9-/- mice, although HDL was similarly increased and thus accordingly, the VLDL/LDL:HDL was not altered. Furthermore, we found TLR9 to differ from previous TLR7 focused studies in that TLR9 deficiency did not cause a shift in monocyte subsets towards an inflammatory subtype.

Our studies indicate changes in the number of $CD4^+$ cells as potential contributors to TLR9 dependent changes in atherosclerosis. The depletion of $CD4^+$ cells via antibody treatment in ApoE-/-:TLR9-/- mice, resulted in a significant reduction of plaque size compared to control mice. Additionally, we found $CD4^+$ cells of ApoE-/-:TLR9-/- mice

to exhibit a proinflammatory phenotype with increased secretion of TNF-α and decreased IL-10.

Finally, given the newly described anti-atherogenic role of TLR9, we investigated the therapeutic potential of TLR9 agonists. The treatment of ApoE-/- mice with class B CpG oligodeoxynucleotide ODN 1668 led to a reduction in lesion severity, indicating that ODN 1668 is a novel, promising therapeutic and/or preventive approach in atherosclerosis.

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13 Abbreviations

Ab	Antibody
ABC	Avidin-Biotin-Complex
ACS	Acute Coronary Ayndrome
APC	Allophycocyanin
ApoE	Apolipoprotein E
ATP	Adenosine-5'-triphosphate
bFGF	basic Fibroblast Growth Factor
BM	Bone Marrow
BMT	Bone Marrow Transplantation
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Surgery
CAD	Coronary Artery Disease
CCL	Chemokine (C-C motif) Ligand
CCR	Chemokine (C-C motif) Receptor
CD	Cluster of Differentiation
CpG	Cytosine-phosphate-Guanosine
CVD	Cardiovascular Disease
DAB	3,3’-diaminobenzidine
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cells
dH_2O	Distilled water
DKO	Double Knockout
DNA	Deoxyribonucleic Acid
DPX	DePex
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbent Assays
FACS	Fluorescent Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
GRO	Growth-Related Oncogene
H&E	Haematoxylin and Eosin
HDL	High-density Lipoprotein

HFD	High-Fat diet
HMGB1	High-Mobility Group Protein B1
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
IHC	Immunohistochemistry
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
IRF	Interferon Regulatory Factor
i.p.	intraperitoneal
i.v.	intravenous
KO	Knockout
LBP	Lipopolysaccharide-Binding Protein
LDL	Low-density Lipoprotein
LDLr	Low-density Lipoprotein receptor
LPS	Lipopolysaccharides
MAL	MyD88-Adaptor Like
MAPK	Mitogen-Activated Protein Kinases
MCP-1	Monocyte Chemotactic Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MD2	Myeloid Differentiation factor 2
MI	Myocardial Infarction
MIF	Macrophage Inhibitory Factor
mmLDL	Minimally modified Low-density Lipoprotein
MMP	Matrix Metalloproteinase
MOMA-2	Macrophages/Monocytes Antibody
MyD88	Myeloid Differentiation primary-response gene 88
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cells
NKT	Natural Killer T cells
NSAIDs	Non-steroidal Anti-inflammatory Drugs
OCT	Optimal Cutting Temperature compound

ODN	Oligonucleotide
ORO	Oil Red O
oxLDL	Oxidised Low-density Lipoprotein
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
PE-Cy7	Phycoerythrin and Cyanine
PerCP	Peridinin Chlorophyll Protein
RGN	Peptidoglycan
PRR	Pattern-recognition receptor
RAG-1	Recombinase Activating Gene 1
RNA	Ribonucleic Acid
RT	Room Temperature
SCID	Severe Combined Immunodeficiency
ScR	Scavenger Receptor
SEM	Standard Error of Mean
SMC	Smooth Muscle Cells
TCR	T Cell Receptor
TGF	Transforming Growth Factor
T_h1	T Helper 1
T_h2	T Helper 2
TIR	Tollinterleukin 1 Receptor
TLR	Toll-Like Receptor
TNF	Tumour-necrosis Factor
TRAF	TNF Receptor Associated Factor
TRIF	TIR-domain-containing adapter-Inducing Interferon-β
TRAM	TRIF-related Adaptor Molecule
TIRAP	TIR domain containing Adaptor Protein
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very-low-density Lipoprotein
WT	Wildtype

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17 Curriculum vitae

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18 Publications

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