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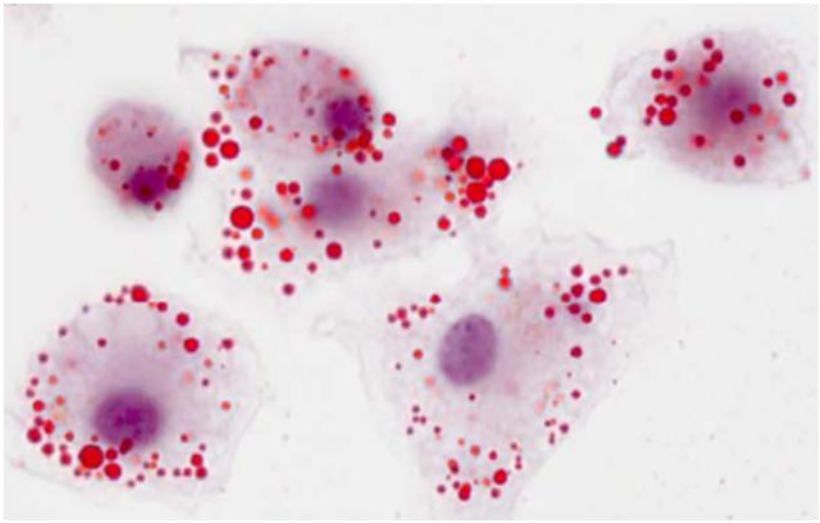

The impact of geranylgeranyltransferase type I (GGTase-I) deficiency in macrophages on the pathogenesis and development of inflammation related disorder/s

Master of Science Thesis in the Master Degree Program Biotechnology

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Lipid uptake by bone marrow derived macrophages from mice.

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ABSTRACT

Cardiovascular diseases are the leading cause of death in the industrial countries and the most common underlying pathology is atherosclerosis. Major risk factors of atherosclerosis are hypertension, diabetes, smoking, free radicals and high levels of cholesterol in the blood. The high cholesterol concentrations often lead to lipid deposits at the inner surface of coronary arteries that triggers an inflammatory process with the results of lipid cores and narrowing of the arteries. The mechanisms behind atherosclerosis are not yet fully understood and therefore it is important to focus on understanding the cellular processes and underlying mechanisms of the disease.

Statins are a group of cholesterol lowering drugs that target the cholesterol pathway by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase. Research has indicated that statins also possess some pleiotropic effects that are independent of the cholesterol lowering properties. It is thought that the pleiotropic effects are connected to statins ability to block the synthesis of the isoprenoid intermediates geranylgeranyl-pyrophosphate and farnesylpyrophosphate, which are used by gernaylgeranyltransferase type I (GGTase-I) and farnesyltransferase (FTase) in the post-translational modifications of CAAX-proteins.

The aim of this thesis was to define the impact of GGTase-I deficiency in macrophages and connect the findings to the pathogenesis of atherosclerosis. In this study it is shown that GGTase-I deficiency has striking effects on the progression of lipid lesions in the aortas of mice, with a reduction of around seventy percentage in males. The blood lipids were not altered, which point at that the positive effects do not depend on a decrease of cholesterol in the blood. Expression of scavenger receptors Sr-A1, Sr-B1, Cd36 and Marco were reduced and a decrease in cytokine Interleukin 10 was also demonstrated. It was further shown that treatment with lipopolysaccharides caused a stronger upregulation of scavenger receptors in the knockout cells compared to the controls, which indicates that these cells are more sensitive for inflammatory stimulation. The overall conclusion is that GGTase-I deficiency is strongly connected to the atherosclerotic process in mice, but the research in this area needs to continue to connect these findings in a broader perspective.

Keywords: atherosclerosis, GGTase-I, macrophages, acLDL, scavenger receptors

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INTRODUCTION AND BACKGROUND

Atherosclerosis - an inflammation related disorder

More than one million people in Sweden suffer from cardiovascular disease and this is the leading cause of death in the industrial countries (Asplund, 2009). The underlying pathology of most cardiovascular diseases is atherosclerosis (Asplund, 2009). Atherosclerosis is a chronic inflammatory disorder that is characterized by the presence of T-lymphocytes and macrophages in atherosclerotic lesions, proliferation of smooth muscle cells and neovascularisation (Vaughan, et al., 1999). This can be seen as thickening of the arterial wall that causes narrow arteries and changes in blood flow or even occlusion of the blood flow (Asplund, 2009). Major risk factors of atherosclerosis are hypertension, diabetes, smoking and free radicals (Ogura, et al., 2009). Another factor that increases the risk of atherosclerotic lesions is high concentration of cholesterol in the blood, which often leads to lipid deposits at the inner surface of coronary arteries (Mathews, et al., 2000). Atherosclerosis is a slow and silent progressing disease and can be present already in early life in small children and the development to a severe form of the disease can take several years (Ståhlman, 2010). The mechanisms behind atherosclerosis is not fully understood and therefore it is important to focus on understanding more about the cellular processes and underlying mechanisms, also to be able to identify therapeutic targets (Asplund, 2009).

Inflammation

Inflammation is a normal process of wound healing and includes removal of cellular and tissue debris from the wound, destruction of bacteria, microorganisms and other foreign materials, and secretion of different types of chemoattractants (Dee, et al., 2002). Many of the hematopoietically derived cells, including lymphatic leukocytes that form T-cells, B-cells and natural killer (NK) cells, and nonlymphatic leukocytes, which differentiate into basophils, eosinophils, neutrophils and monocytes, are important in the immune response. The monocytes are immature cells until they enter a tissue and become macrophages, which have the main function of phagocytosing (“eating”) foreign objects and organisms (Dee, et al., 2002).

Macrophages in the atherosclerotic process

Atherosclerosis starts with an accumulation of lipid deposits in the intima of the artherial wall (Gustafsson, 2004) (*Figure 1*). The lipids are trapped by proteoglycans that causes retension of the lipids (Asplund, 2009). Inside the intima the lipoproteins are modified in different ways, enzymatically or non-enzymatically, and are also often oxidized (Ståhlman, 2010). The accumulation of modified lipids leads to recruitment of circulating monocytes which migrate into the tissue, differentiate into macrophages and start to phagocytose the modified lipids (Gustafsson, 2004). Due to imbalance in the lipid metabolism the macrophages accumulate a lot of modified lipids, forming so called foam cells, and the accumulation of these cells form fatty streak lesions which have potential to develop to advanced lesions (Asplund, 2009). The foam cells express growth factors, cytokines, enzymes and reactive oxygen species (ROS) that start an inflammation process and promote modification of the lipids and proliferation and migration of smooth muscle cells into the lesion (Asplund, 2009). With time the lesion form a lipid core, which contains necrotic foam cells, and a fibrous cap is formed on the outside which causes the narrowing of the artery (Gustafsson, 2004).

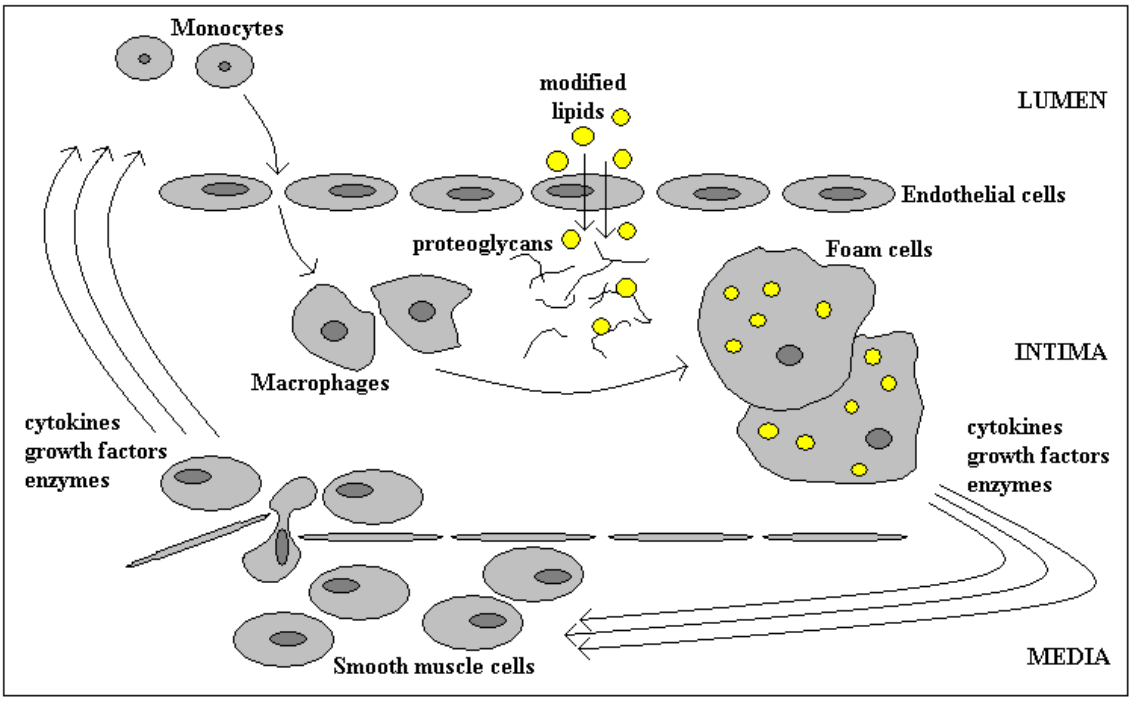

Figure 1. In the atherosclerotic process monocytes are recruited to the artherial wall and differentiate to macrophages. The macrophages phagocytose accumulated modified lipids and become foam cells. The foam cells release cytokines, growth factors and enzymes, which stimulate smooth muscle cells to migrate into the intima. The smooth muscle cells recruit more monocytes from the blood flow and an atherosclerotic lesion has started to form. (Modified from Asplund (2009) and Gustafsson (2004)).

Cytokines

Patients with a severe form of atherosclerosis have been shown to have elevated levels of circulating cytokines in the blood (Hansson, 2009). Different types of immune cells, like activated macrophages, T-cells and mast cells, secrete cytokines and these molecules can be involved in the destabilization of atherosclerotic lesions (Hansson, 2009).. Interferon γ (IFN-γ) is a major proatherogenic cytokine secreted by type 1 helper T-cells (Th1) that promotes macrophages and endothelial cells to produce adhesion molecules, cytokines, chemokines, radicals, proteases and coagulation factors, and also inhibits cell proliferation, cholesterol efflux and collagen production (Hansson, 2009). Tumor necrosis factor α (TNF-α) is produced by Th1 cells, macrophages and NK cells, and is a pro-inflammatory and cytotoxic cytokine that inhibits metabolic enzymes including the lipoprotein lipase (Hansson, 2009).. Interleukin 10 (IL-10) is an anti-inflammatory cytokine secreted by several immune cells, which inhibits secretion of certain cytokines and is immunosuppressive (Hansson, 2009). Interleukin 12 (IL-12) is a multifunctional cytokine that induces cellular immunity by promoting proliferation, IFN- γ production and activation of NK and Th1 cells (DelVecchio, et al., 2007). Interleukin 1β (IL-1β) is an important proinflammatory cytokine that is included in the cellular recruitment to sites of injury or infection and in the regulation of sleep, appetite and body temperature (Scroder & Tschopp, 2010). Interleukin 6 (IL-6) induces maturation of B-lymphocytes into plasma cells and is synthesized by monocytes, fibroblasts, endothelial cells, T-cells and B-cells (Yap & Lai, 2010). Keratinocyte-derived chemokine (KC), also called CXCL1, is expressed by macrophages, is involved in the accumulation of these cells in fatty streak lesions and contributes to endothelial regeneration (Zernecke, et al., 2008).

Blood lipids and cholesterol

Most of the lipids in an organism are in the form of triglycerides (Mathews, et al., 2000). When lipids are transported in the blood they need to be in a complex with proteins to form soluble aggregates, and these complexes are called lipoproteins. The families of lipoproteins are classified due to their density and include chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density-lipoprotein (LDL) and high-density-lipoprotein (HDL) (Mathews, et al., 2000). Of the lipoproteins the LDL particles are the richest in cholesterol. The LDL is responsible for the transport of cholesterol to tissues while the HDL is responsible in the returning of excess cholesterol from the tissues to the liver for metabolism or excretion (Mathews, et al., 2000). The LDL particle has the form of a sphere and contains a core of cholesteryl ester molecules surrounded by a lipid monolayer of phospholipids and unesterified cholesterol molecules (Alberts, et al., 2002). These cholesterol molecules constitute about two-thirds of the total plasma cholesterol (Mathews, et al., 2000). Each lipoprotein class contains its own characteristic apolipoproteins (Mathews, et al., 2000). The main apolipoprotein in LDL is called apoB-100 and it mediates the binding between the particle and the LDL receptor (Alberts, et al., 2002). It is also this specific protein that is responsible for the retention of the LDL-particle in the arterial wall in the atherosclerotic process by interacting with the arterial proteoglycans (Sigurdardottir, 2005).

LDL receptor

The human LDL receptor (LDLr) is a transmembrane glycoprotein that is involved in the uptake of normal LDL (Alberts, et al., 2002). The LDLr binds the LDL particle through recognition of the B-100 apoprotein and the entire molecule is phagocytosed (Mathews, et al., 2000). The cholesterol esters are then hydrolyzed to free cholesterol and the apolipoproteins are hydrolyzed to amino acids. The LDLr is recycled, it fuses with the plasma membrane and takes up a new LDL particle (Mathews, et al., 2000). A defect in the gene encoding the LDLr disrupts the pathway that regulates the uptake of cholesterol and this causes high levels of blood cholesterol, which leads to development of premature atherosclerosis (Alberts, et al., 2002).

Modified low density lipoproteins

Native LDL particles can freely enter the intima and are taken up by vascular cells via the LDLr (Siegel-Axel et al., 2008). These lipids do not initiate an inflammatory response, are not phagocytosed by macrophages and do not initiate atherosclerosis (Siegel-Axel et al., 2008). Modification of LDL particles in the arterial wall, particularly by oxidation, is crucial to the cellular uptake of LDL by macrophages in the early state of atherosclerosis (Navarra, et al., 2010). The modification, usually oxidation or acetylation, initiates an inflammatory response, is chemotactic for and induces migration of monocytes, alters the endothelium, induces differentiation of monocytes to macrophages and causes rapid uptake of the lipid by macrophages via scavenger receptors (Siegel-Axel et al., 2008). Acetylated LDL (acLDL) is a type of modified LDL (mLDL) that is extensively usedin *in vitro* foam cell formation assays, while oxidized LDL (oxLDL) is a form of mLDL that is found in atherosclerotic plaques (McLaren, et al., 2010).

Scavenger receptors

During the differentiation of monocytes to macrophages in the atherosclerotic process scavenger receptors and Toll-like receptors are upregulated (Hansson, 2009). The Toll-like receptors initiate signaling cascades that lead to inflammatory activation (Hansson, 2009). Scavenger receptors mainly mediate the uptake of mLDL, but they also have the capacity to bind a number of other ligands of biological importance and are important in tissue homeostasis, innate immunity and apoptosis (Greaves & Gordon, 2005) Several different classes of scavenger receptors have been identified and many of them are only present during pathologic conditions (Valiyaveettil & Podrez, 2009). Macrophages express at least six structurally different types of scavenger receptors and their mLDL uptake contributes to foam cell formation and atherosclerosis (Greaves & Gordon, 2005). The modified lipid uptake by macrophages via scavenger receptors is the main source in the formation of foam cells (Greaves & Gordon, 2005).

CD36 is a class B scavenger receptor that recognizes a number of distinct ligands including fatty acids and oxLDL (Valiyaveettil & Podrez, 2009). It is expressed by endothelial cells, adipocytes, skeletal muscle cells, dendritic cells, platelets, erythroid precursors as well as cells of the monocyte/macrophage lineage (Greaves & Gordon, 2005). Scavenger receptor B1 (SR-B1) is another class B scavenger receptors that binds a variety of ligands including native, oxidized or acetylated lipoproteins (Greaves & Gordon, 2005). SR-B1 mediates the transport of cholesteryl esters from HDL and also promotes the efflux of free cholesterol between cells and lipoproteins (Valiyaveettil & Podrez, 2009). Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) is a class E scavenger receptor that is expressed by macrophages, endothelial cells, smooth muscle cells and platelets (Valiyaveettil & Podrez, 2009). It has been shown to be involved in the pathogenesis of atherosclerotic lesions and binds among other ligands oxLDL, acLDL, bacteria, neutrophils, activated platelets and apoptotic cells (Navarra, et al., 2010). Scavenger receptor A1 (SR-A1) is involved in cell adhesion, apoptosis, antigen processing, antimicrobial immunity and atherogenesis (Greaves & Gordon, 2005). It is responsible for the main uptake of acLDL and together with CD36 responsible for up to 90% of total macrophage uptake of both oxLDL and acLDL. A structurally related receptor to SR-A1 is MARCO (macrophage receptor with collagenous structure) which has been shown to bind bacteria (Greaves & Gordon, 2005).

Statins

Statins are a group of cholesterol lowering drugs that target the cholesterol pathway by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Mathews, et al., 2000) (*Figure 2*). Statins have been shown to cause regression of atherosclerosis and to reduce the mortality in cardiovascular related diseases (Hofnagel, et al., 2007). It has been demonstrated that the drug lowers the LDL amount in blood by 19% to 60%, decreases the concentration of triglycerides and increases the levels of HDL (Takemoto & Liao, 2001). These effects on the lipid concentrations are believed to be the reason for the decrease of atherosclerosic plaques, because the disease is partly mediated by the uptake of modified LDL (Takemoto & Liao, 2001). Additionally to its cholesterol-lowering effects, research indicates that statins also have pleiotropic effects that are cholesterol-synthesis-independent (Hofnagel, et al., 2007). These effects include decrease in vascular inflammation, stabilizing of atherosclerotic plaques, decrease in oxidative stress and improvement and restoring of endothelial function (Takemoto & Liao, 2001). It is thought that many of the pleiotropic effects are connected to statins

ability to block the synthesis of the isoprenoid intermediates farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GTPP). (Takemoto & Liao, 2001).

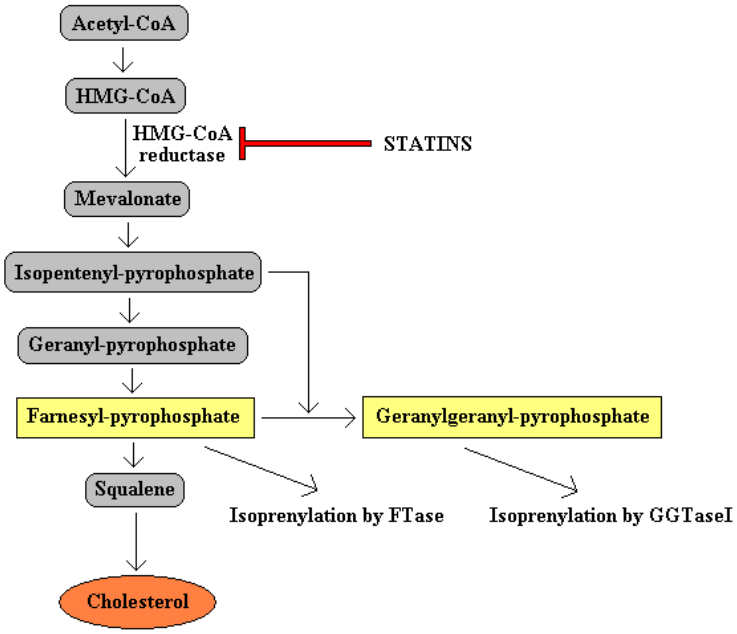

Figure 2. The cholesterol pathway. Statins inhibit HMG-CoA reductase and thereby inhibit the cholesterol pathway including the synthesis of the isoprenoid intermediates farnesylpyrophosphate and geranylgeranylpyrophosphate. (Modified from Sjögren, 2009).

Isoprenylation of CAAX proteins

CAAX proteins are proteins that include a CAAX motif at the C-terminal in which the C is a cysteine residue, AA are two aliphatic amino acids and X represents any amino acid depending on the substrate specificity (Gao, et al., 2009). The CAAX proteins are involved in cellular functions such as differentiation, proliferation, nuclear stability, embryogenesis, metabolism and apoptosis (Gao, et al., 2009).

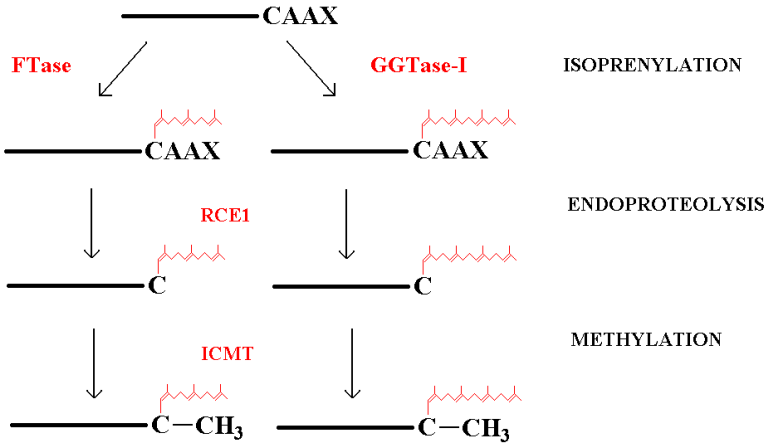

Figure 3. Post-translational modifications of CAAX-proteins including isoprenylation by FTase or GGTase-I, endoproteolysis by RCE1 and methylation by ICMT. (Modified from Sjögren, 2009).

The CAAX-proteins need to be post-translationally modified to function properly and this process includes three important steps; isoprenylation, endoproteolysis and methylation (Sjögren, 2009) (*Figure 3*). Isoprenylation is the attachment of a 15-carbon-farnesyl (FPP) or a 20-carbon-geranylgeranyl (GGPP) isoprenoid lipid group by the protein farnesyltransferase (FTase) respective geranylgeranyltransferase type-I (GGTase-I) (Long, et al., 2001). After isoprenylation the –AAX amino acids are cleaved off by the endoprotease RAS-converting enzyme 1 (RCE1) and the cysteine is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT) (Sjögren, 2009). The CAAX proteins that are post-translationally modified in this way include GTP-binding RAS and RAS-like proteins such as RHO, RAB, RAC, RAL and RAP, the γ-subunit of heterotrimeric G-proteins, nuclear lamins and centromeric proteins (Long, et al., 2001; Veillard, et al., 2006). The post-translational modification is important for the anchoring to the plasma and nuclear membranes (Gao, et al., 2009).

Statins and isoprenylation

Statins have been shown to prevent the synthesis of the isoprenoid intermediates GGPP and FPP of the cholesterol pathway by the inhibition of L-mevalonic acid synthesis (Veillard, et al., 2006). This inhibits the CAAX-proteins to be modified by GGTase-I and FTase, which uses the GGPP and FPP as substrates at the isoprenylation step. The inhibition affects particularly the small GTP-binding proteins, RAS and RHO, whose proper function and membrane localization are dependent on the post-translational modification, and this causes accumulation of the proteins in the cytoplasm (Takemoto & Liao, 2001). RAS and RHO are small GTP-binding proteins that cycle between an inactive GDP-bound state and an active GTP-bound state. RAS is modified by FTase while RHO is modified by GGTase-I (Takemoto & Liao, 2001).

RHO is a major target of the geranylgeranylation by GGTase-I and therefore the inhibition of this pathway by statins can be an important mechanism behind the pleiotropic effects of statins on the vascular wall (Takemoto & Liao, 2001). The RHO protein family is a subgroup of the RAS superfamily and includes among others the proteins CDC42, RAC and RHO (Alberts, et al., 2002). CDC42 is involved in the polymerization of actin filaments to form protrusion of the cell membrane and filopodia, RAC is included in the lamellipodium formation and RHO promotes the formation of focal contacts and stress fibers (Alberts, et al., 2002). This suggests that changes in functional RHO proteins can affect the intracellular transport, membrane trafficking, mRNA stability and gene transcription and the inhibition of RHO isoprenylation may affect not only the vascular wall cells but also leukocytes and bone (Takemoto & Liao, 2001).

Background of different techniques used in the study

Several techniques have been used in this thesis, which all are important in the studies of molecular mechanisms, and what follows is a short summary for each of the main techniques.

Mouse models and conditional gene targeting

Gene targeting is when a gene is altered in an intact cell causing an *in vivo* mutated gene (Wilson & Walker, 2000). This makes it possible to analyze a specific gene in a living organism or introduce a foreign gene (Wilson & Walker, 2000). To produce a so called "knockout" animal, for example a knockout mouse, a vector including the desired

mutant gene is introduced into an embryonic stem cell from the mouse and cultured to be able to undergo homologous recombination (Alberts, et al., 2002). Cells including the gene replacement are then injected into an early mouse embryo and a mouse with a transgene is produced. Mice with this transgene are then bred to produce a male and a female, both heterozygous (having one normal and one mutant allele) for the gene, which can mate and get homozygous (both alleles are mutated) progeny. Conditional gene targeting is when a gene is knockout in a specific tissue at a certain time in development (Alberts, et al., 2002). Cre/lox is a common technique to accomplish this and in the technique the transgenic mouse has been altered with a normal copy of the specific gene flanked by a pair of short DNA sequences, called *lox sites* that are recognized by the Cre recombinase protein. The mouse, which is phenotypically normal, is then mated with a transgenic mouse that expresses the Cre recombinase gene under the control of an inducible promoter. When the Cre is switched on the specific gene is knocked out (Alberts, et al., 2002).

Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique that makes it possible to create multiple copies of a DNA molecule by selective enzymatic amplification of a target DNA sequence (Brown, 2001). Two short oligonucleotides act as primers and hybridize to the borders of the DNA molecule strands and amplification is usually carried out by the thermostable DNA polymerase enzyme from *Thermus aquaticus*, the Taq polymerase. The newly synthesized DNA is then denatured and used as a template for the next round of amplification. The cycle of denaturation, hybridization and synthesis is repeated 25-30 times which results in several hundred million copies of the DNA sequence (Brown, 2001). Each cycle includes transition between three different temperatures, including a denaturation temperature of 94°C, at which the strands dissociate from each other, a hybridization temperature, which is calculated from the different nucleotides content of the primers, and an extension temperature, which is usually set at 74°C, just below the optimum for Taq polymerase. Agarose gel electrophoresis is usually used for analyzing the DNA sample, which is shown as a band with a specific length on the gel after staining with ethidium bromide (Brown, 2001).

Quantitative polymerase chain reaction

A modification of the PCR technique is quantitative PCR (qPCR), which enables identification of the initial concentrations of the template DNA and is very useful for measurement of mRNA for a protein expressed in abnormal amounts in a disease process (Wilson & Walker, 2000). Early methods involved comparison between standard DNA templates amplified simultaneously as the target DNA. Other methods include radiolabeling of the primers or nucleotides. An alternative method is the TaqMan assay in which the oligonucleotide probe is labeled with a fluorescent reporter and quencher molecule at each end. When the primers bind, the 5'-exonuclease activity of Taq polymerase releases the reporter from the quencher and a fluorescent signal can be detected. The signal increases in direct proportion to the amount of starting material (Wilson & Walker, 2000). Sample-sample variations can be corrected by measuring simultaneously the levels of an RNA that is present at constant levels during all stages of development, called a housekeeping gene (Tricarico, et al., 2002). A commonly used housekeeping gene is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a key enzyme in glycolysis (Tricarico, et al., 2002).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) is a technique to detect proteins in a sample by allowing the proteins to coat the surface of a well and then let unbound antibodies bind to them (Wilson & Walker, 2000). These antibodies are then detected with the help of labeled secondary antibodies (Wilson & Walker, 2000). A strong signal is achieved by using an unlabeled primary antibody and a labeled secondary antibody because the primary antibody is recognized by several molecules of the secondary antibody (Alberts, et al., 2002).

Aim of the thesis

The overall aim of this master thesis is to define the impact of GGTase-I deficiency in bone marrow macrophages (BMMs) and how this is connected to the pathogenesis of inflammation related disorders. More specifically, to investigate if the pleiotropic effects of statins are due to decreased geranlygeranylation of Rho proteins (CAAX proteins that are geranylgeranylated) and to define the impact of GGTase-I deficiency in macrophages on the development and progression of atherosclerosis in mice.

MATERIALS AND METHODS

Mouse breeding and genotyping

Breedings were put up to get conditional mice with the desired genotypes. Mice used in these experiments were homozygous for a conditional knockout allele of the β subunit of GGTase-I (*Pggt1b*^{fl}) and heterozygous or homozygous for the lysozyme M-Cre (LC) knock-in allele (*Pggt1b*$^{fl/fl}$*LC*). As controls littermate *Pggt1b*$^{fl/+}$*LC LDLr*$^{-/-}$ and *Pggt1b*$^{+/+}$*LC LDLr*$^{-/-}$ mice were used. Mice were from mixed genetic background, but backcrossed five times to get a pure *C57BL/6* background. Tail biopsies for DNA analysis were taken when the mice were 14 days old. Each sample were lysed in lysis buffer (25mM NaOH, 0.2mM EDTA, pH~12), heated to 95°C in 20 minutes, cooled down to 4° C and neutralization buffer (40mM Tris-HCl, pH~5) was added.

Pggt1b$^{fl/fl}$*LC LDLr*$^{-/-}$ mice for lipid lesion, body weight, blood lipid and cytokine analyses experiments, were put on high fat western type diet (R638, Lantmännen) when the mice were 6 or 7 weeks old. All other mice were given normal diet. The mice experiments were approved by the animal research ethics committee in Gothenburg.

The *Pggt1b* allele was genotyped by PCR amplification. A master mix specific for the genotype, including HotSTAR (alternatively titanium Taq DNA polymerase, 10xPCR buffer, dNTP), H_2O, primers and DNA was prepared and the DNA samples were added. For *Pggt1b*, forward primer 5'-CCTGAATGCAGATCTGTGGA-3' and reverse primer 3'-CCTATGAAAGCAGCACGACA-5' (Sigma Genosys), were used (primers for the other genes (Sigma Genosys) can be seen in *Table 1*). The DNA was amplified with GeneAmp® PCR System 9700 (Applied Biosystems) in programs specific for the genotypes. The program used for *Pggt1b* was the following; 95°C 5 minutes, (95°C 30 seconds, 68°C 30 seconds, 72°C 45 seconds) x5, (95°C 30 seconds, 64°C 30 seconds, 72°C 45 seconds) x5, (95°C 30 seconds, 58°C 30 seconds, 72°C 45 seconds) x30 and 72°C for 10 minutes. Similar programs were used for the other genotypes. The amplified DNA product was detected with QIAxcel® system, BioCalculator 3.0 (Qiagen).

Table 1. Primers used in PCR.

Gene	Primers	Sequence
Pggt1b	GGTFlx-F1 GGTFlx-R1	5'-CCTGAATGCAGATCTGTGGA-3' 3'-CCTATGAAAGCAGCACGACA-5'
LDLr$^{-/-}$,wt	FT-1 FT-2	5'-ACCCCAAGACGTGCTCCCAGGATGA-3' 3'-CGCAGTGCTCCTCATCTGACTTGT-5'
LDLr$^{-/-}$,KO	FT-3 FT-4	5'-AGGATCTCGTCGTGACCCATGGCGA-3' 3'-GAGCGGCGATACCGTAAAGCACGAGG-5'
Lys M-Cre	MLys1 LLys2 Cre8:	5'-CTTGGGCTGCCAGAATTTCTC-3' 3'-TTACAGTCGGCCAGGCTGAC-5' 5'-CCCAGAAATGCCAGATTACG-3'

Quantification of lipid lesions

Aortas were collected from *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice that were put on high fat western type diet, for an early time point of 12 weeks and a late time point of 24 weeks. Photos were taken of each aorta with a Nikon Coolpix 4500 digital camera and lipid lesions were quantified blindly as percentage of total area of the aorta, using Image J 1.42q

(Java1.6.0_10) software and Excel. Males and females were divided into separate groups.

Body weight statistics

Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$ mice put on high fat western type diet were weighted every second week with start at an age of 6 or 7 weeks. Mean weight was calculated for each time point with males and females in separate groups. The age differences between groups were adjusted by linear approximation Calculations were done in Excel.

Blood lipid and cytokine analysis

Serum was collected from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice put on high fat western type diet for an early time point of 12 weeks and a late time point of 24 weeks. Cytokine concentrations were measured with the use of Mouse ProInflammatory 7-plex Ultrasensitive Kit in a SECTOR 2400 Imager (Meso Scale Discovery). Blood lipid concentrations were measured by Triglycerides kit, Cholesterol kit, HDL-cholesterol Plus kit respective LDL-cholesterol kit, all from Konelab™ T Series in a Konelab 20 chemistry analyzer (Thermo Scientific).

Generation of BM-derived macrophages

Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$ mice were sacrificed and tibia and femur were collected. All extra tissues were removed and the bones were cleaned in PBS and put in eppendorf tubes. The bones were cut open and centrifuged at 4400rpm for 8 minutes at 4°C. The pellet, including the BM cells, was resuspended in 1 ml PBS/EDTA (10mM) in a 50 ml falcon tube and centrifuged at 1000rpm for 5 minutes at 4°C. The suspension was removed, the pellet was resuspended in 2 ml ACK solution (H_2O, NH_4Cl (0.15M), $KHCO_3$ (1.0M), EDTA-Na_2 (0.1mM)) in 7 minutes to lyse the red blood cells, 25 ml PBS/EDTA (10mM) was added and the tube was once more centrifuged at 1000rpm for 5 minutes at 4°C (alternatively deionized water was added for 15 seconds). The pellet was resuspended in 1 ml high-glucose DMEM supplemented with 10% fetal calf serum, 1% gentamycin, 1% glutamine, 1% HEPES, 0.01% β-mercaptoethanol, and 10% whole supernatant of CMG14-12 cells, which overexpress mouse macrophage colony-stimulating factor (M-CSF) (BMM medium). The cells were plated (approx. 10 million cells/100mm plate) and cultured at 37°C in a humid chamber with 5% CO_2. The medium was changed every third day. Cell counting and cell size measuring were done with the use of Vi-cell™ XR-Cell viability analyzer (Beckman Coulter), software Vi-cell XR 2.03.

LDL production

Human blood was given from two volunteer donors on 12 hour overnight fasting. Tubes with the blood containing EDTA were centrifuged at 2500rpm for 20 minutes at 4°C in an AVANTI J-25 centrifuge with rotor JA-20.1. The plasma was collected with Pasteur pipette in Falcon tubes and the density was set to 1.02 g/ml by adding potassium bromide. The suspension was added to tubes for ultracentrifugation and centrifuged in a Beckman ultracentrifuge at 37 000rpm for 20 hours at 4°C using a 90 TI rotor. The top fraction containing VLDL was discarded with a syringe and the rest was collected in Falcon tubes. The density was set to 1.05 by addition of potassium bromide and the suspension was added to tubes for ultracentrifugation and centrifuged at 43 000rpm for 20 hours at 4°C in a Beckman centrifuge using a 90 TI rotor. The top fraction was

collected in a Falcon tube, the density was set to 1.063 by the addition of potassium bromide. The suspension was added to tubes for ultracentrifugation and centrifuged at 43 000rpm for 20 hours at 4°C in a Beckman centrifuge using a 90 TI rotor. 30 minutes after centrifugation the top fraction containing the LDL was collected with a 2 ml syringe and the LDL was filtered through a 0.22µm pore-size centrifuge filter with low protein bindning (Millipore Ultrafree® MC).

Acetylation of LDL

The LDL concentration was determined with a Bio-Rad DC protein assay (BIO-RAD) and quantified in a Spectra Max® Plus 384 spectrophotometer, Softmax® version 5 (Molecular Devices) to 4.8mg/mL. One time saturated sodium acetate was added to 2µL LDL. Acetic acid anhydride was added in 2µL portion every 15 minute in a total amount of 1.5 times the protein mass. The solution was cooled (4°C) and stirred during the whole acetylation procedure with an extra stirring time of 30 minutes after the last addition of acetic acid anhydride. The solution was then desalted by a PD-10 desalting column.

AcLDL treatment of BMMs

BM macrophages (10^5) from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice were plated and cultured on coverslips in 12-wells plates in BMM medium for 2 hours. The cells were then treated with 50µg/ml acLDL and cultured overnight. Untreated cells were used as controls. Cells were stained with Oil red O, which colors uncharged lipids red. The medium was discarded and the wells were washed with PBS. Ice cold 3.7% formaldehyde in PBS was added and the plate was incubated for 10 minutes. The wells were washed once with PBS and then washed with 60% iso-propanol. 1.5 mL 0.5% Oil red O was added to each well and left for 20 minutes. The cells were washed for 30 seconds with 60% iso-propanol and hematoxylin was added. After 2 minutes the wells were rinsed with streaming cold water. The coverslips were mounted on an object slide with mowiol. The cells were photographed with a 40 times magnification by the use of an Axio Imager MI (Zeiss) light microscope with AxioCam HRc digital camera (AxioVision 40 software version 4.6.3.0, Zeiss) and quantified with Biopix iQ software (version 2.1.8. Biopix).

Determination of IL-1β secretion by ELISA

BM macrophages (10^5) from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice were treated with 50µg/ml acLDL in BMM medium overnight. Serum from the cells was analysed by ELISA (88-7013-22, BD Biosciences) to detect changes in the cytokine IL-1β secretion. A Corning Costar 9018 ELISA plate was coated with purified anti-mouse IL-1β capture antibody at a concentration of 100µL/well and incubated over night at 4°C. The wells were washed 5 times (wash buffer: 1x PBS, 0.05% Tween-20), blocked with 200µL/well of 1x assay diluent and incubated at room temperature for 1 hour. The wells were washed 5 times and 100µL/well of the serum samples including a standard was added. After incubation for 2 hours at room temperature the wells were washed 5 times, incubated for 1 hour at room temperature with 100µL/well of detection antibody (biotin-conjugated anti-mouse IL-1β (polyclonal)) and washed for another 5 times. 100µL/well of Avidin-HRP diluted in 1x assay diluents was added and the plate was incubated for 30 minutes at room temperature. The wells were washed 7 times, 100µL/well of substrate solution (1x TMB solution) was added, the plate was incubated for 15 minutes at room temperature and 50µL/well of substrate stop solution (1M H_2SO_4) was added. The plate was analyzed at 450 nm with a Spectra Max® Plus 384, Softmax® version 5 (Molecular Devices).

Determination of scavenger receptor expression by qPCR

Pggt1b$^{fl/fl}$*LC LDLr*$^{-/-}$ macrophages cultured for 8 days were plated on 6-well-plates ($5*10^5$cells/well) and stimulated with 10ng/mL lipopolysaccharides (LPS), molecules found in the outer membrane of bacteria, to create an inflammatory response. RNA was isolated from these cells and also from unstimulated cells by the use of RNeasy Mini Kit (Qiagen) 12 hours after the LPS stimulation. cDNA was synthesized from the RNA samples by the use of RT First Strand Kit (C-03, SuperArray, SABiosciences). Gene expression for scavenger receptors Sr-A1, Sr-B1, Cd36, Lox-1 and Marco was analysed with TaqMan quantitative RT-PCR (7900HT Fast Real-Time PCR system (Applied Biosystems)) using different TaqMan probes (Mm00432403 (Cd36), Mm00440265 (Marco), Mm00446214 (Sr-A1), Mm00450234 (Sr-B1), Mm00454586 (Lox-1), Mm004308313 (Gapdh), Applied Biosystems). The program used was the following: 95°C 10 minutes, (95°C 15 seconds, 60°C 1 minute)x40, 60°C 1 minute and 95°C 15 seconds. The result was analyzed with SDS 2.2.2 software and fold change were calculated in Excel where the Gapdh gene was used as a reference or so called housekeeping gene. RNA and cDNA concentrations and purity were measured in a NanoDrop® ND-100 Spectrophotometer, software version 3.5.2.

Graphs and Statistics

Data are plotted as mean ±SEM. Differences between groups were determined by 2-tailed Student *t* test. Significant values were considered when $P<0.05$. Graphs were made with Excel and Adobe Illustrator.

RESULTS

Atherosclerosis is decreased in *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice

To investigate the affect of GGTase-I deficiency on atherosclerosis, *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice were put on high fat western type diet followed by measurement of lipid lesions in the aortas for an early time point of 12 weeks and a late time point of 24 weeks after initiation of the diet. At the early time point there are no significant differences in amount of lipid lesion between the knockout mice and the controls, but there is a tendency of a decrease in the knockout males (*Figure 4*). At the late time point there are significant changes in both the male and female group. The *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* males have a reduction in aortic lesions of about 70% and the females of around 30%.

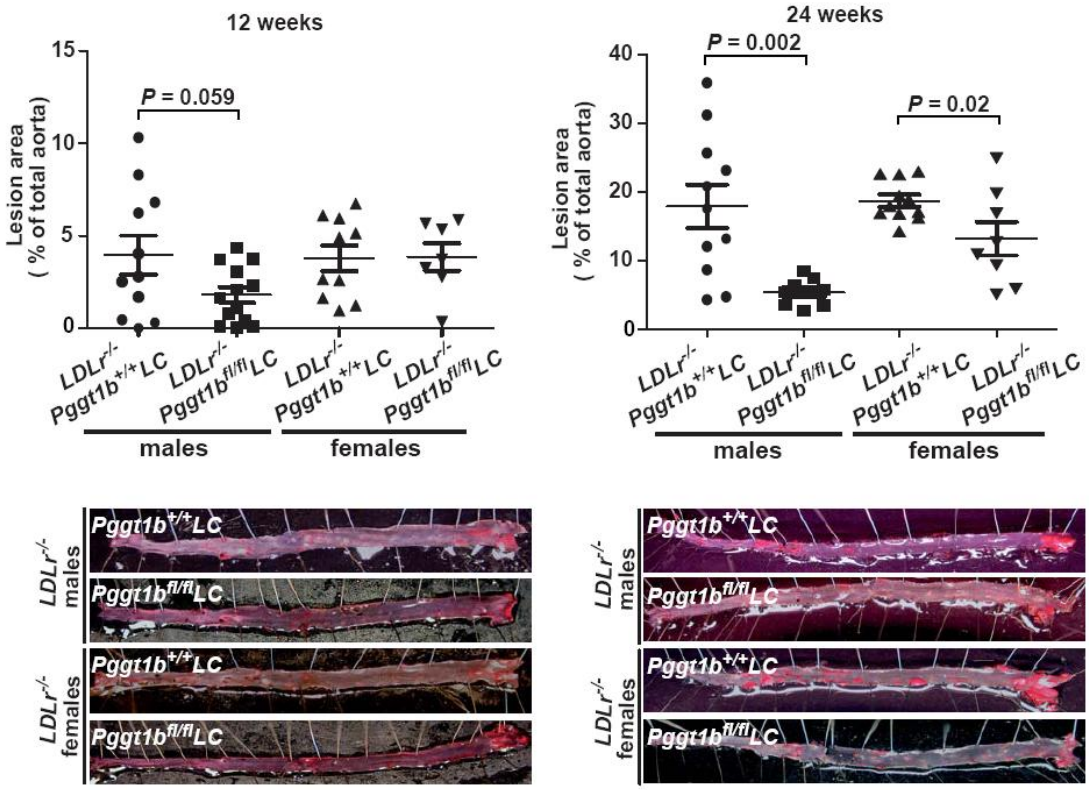

Figure 4. Aortic lesions in *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* and *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice at an early time point of 12 weeks and a late time point of 24 weeks after initiation of high fat western type diet. Data for males (for early respective late time point 13 *Pggt1b$^{fl/fl}$LC* and 11 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice) and females (for early and late time point 7 respective 8 *Pggt1b$^{fl/fl}$LC* and 10 respective 11 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice) are shown separately.

Body weights of *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice are not affected

The body weights of *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice were determined every second week after initiation of high fat western type diet to investigate how the knockout of *Pggt1b* affects the health of the mice. The males show no changes in the body weight of the knockout mice compared to the controls (*Figure 5*). In the female mice there are no significant differences in body weight except for four measured time points.

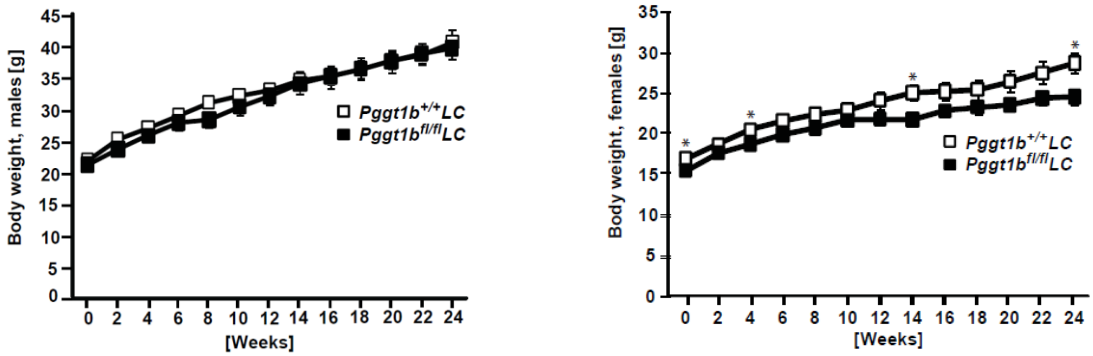

Figure 5. Body weights of *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice after initiation of high fat western type diet at an age of 6 to 7 weeks. Data from 10 *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ and 14 *Pggt1b*$^{+/+}$*LC LDLr*$^{-/-}$ males respective 11 *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ and 15 *Pggt1b*$^{+/+}$*LC LDLr*$^{-/-}$ females are shown. *P<0.05 versus controls.

No alteration of blood lipids in *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice

To elucidate the effects of *Pggt1b* knockout on blood lipids in *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice serum was collected from the mice and analyzed for cholesterol, triglycerides, HDL and LDL. No significant changes were seen in any of the blood lipid levels (*Figure 6*).

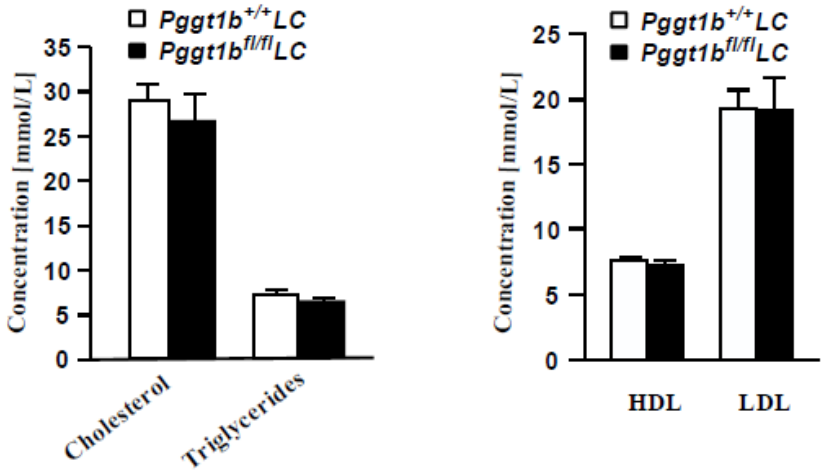

Figure 6. Analyses of blood lipid concentrations, including cholesterol, triglycerides, HDL and LDL, in serum from 20 *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ and 22 *Pggt1b*$^{+/+}$*LC LDLr*$^{-/-}$ mice.

GGTase-I deficiency causes reduced secretion of IL-10 in mice

In order to determine the impact of GGTase-I deficiency on the secretion levels of cytokines, the concentration of seven different cytokines were analyzed in serum from *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice on high fat western type diet. The values for IL-10 concentration show a significant reduction in secretion of about 65% in the knockout mice compared to controls (*Figure 7*). The levels of IFN-γ, IL-12, IL1β, IL-6, TNF-α and KC show no significant alteration, but there is a trend of decreased secretion of all the cytokines except for KC.

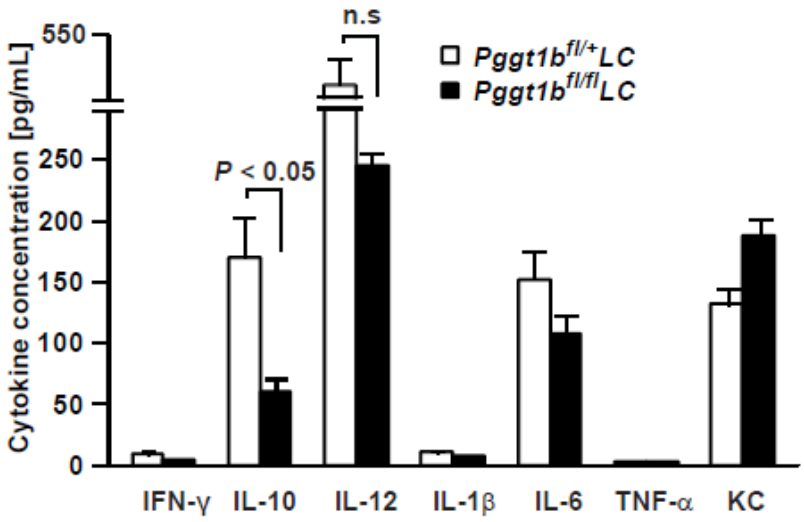

Figure 7. Cytokine concentrations, including IFN-γ, IL-10, IL-12, IL1β, IL-6, TNF-α and KC, in serum from 20 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* and 22 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice on high fat western type diet. Data is shown for serum taken at the late time point of 24 weeks.

Lipid uptake is not effected in *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* macrophages

To further investigate the impact of GGTase-I deficiency in mice on the uptake of lipids, cultured macrophages were treated with acLDL. Pictures taken from these experiments were used to quantify the lipid uptake using two different approaches (*Figure 8*). The BMMs from *Pggt1b* mice looked small and rounded when they were attached to the plate and therefore the average diameters of the cells in solution were measured. No significant differences in average diameter could be seen between these cells and the controls (result for three *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* cell lines and three controls, data not shown).

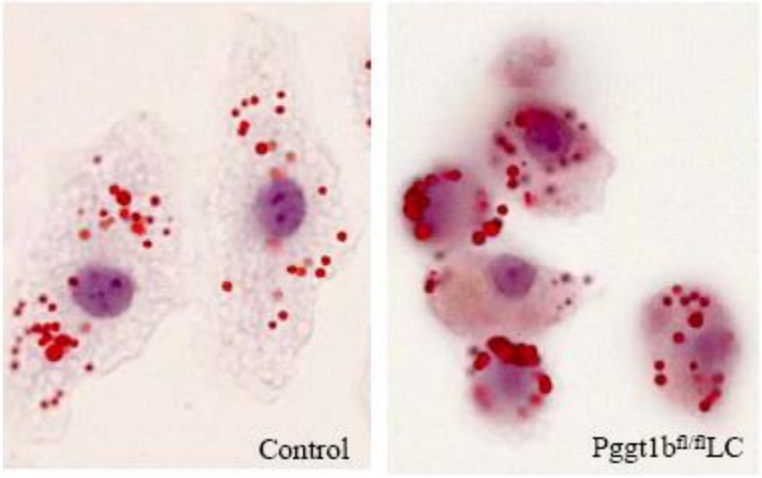

Figure 8. Uptake of acLDL into bone marrow macrophages from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* mice and controls. The lipid droplets are stained with Oil red O and the nuclei with hematoxylin. Photos are taken in a light microscope with a 40 time magnification.

Since the knockout macrophages had spreading deficiency the lipid uptake was quantified per cell number using Biopix iQ software and then normalized to lipid uptake

per cell surface area. There is no difference in lipid uptake between the knockout macrophages and controls when the lipid uptake is normalized to cell surface area (*Figure 9*). Another approach that is more commonly applied to this type of assay is to normalize the lipid uptake by dividing it with the cell number to get lipid uptake/ cell number. With this approach a 40% reduction in lipid uptake in GGTase-I deficient macrophages is found.

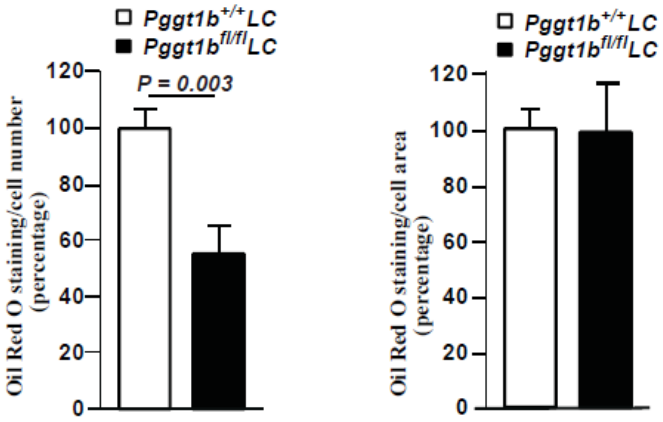

Figure 9. Uptake of acLDL into bone marrow macrophages from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* and *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice. The data are shown as total area of Oil red O-stained lipid droplets per cell number respective cell area and are from 20 randomly selected micrographs from 6 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* and 7 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice.

No changes in IL-1β levels in acLDL treated *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* macrophages

Medium was collected from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* BMMs and controls treated with acLDL to detect changes in the cytokine IL-1β secretion due to the treatment. The medium was analyzed by ELISA. No significant alterations in the secretion could be observed (*Figure 10*).

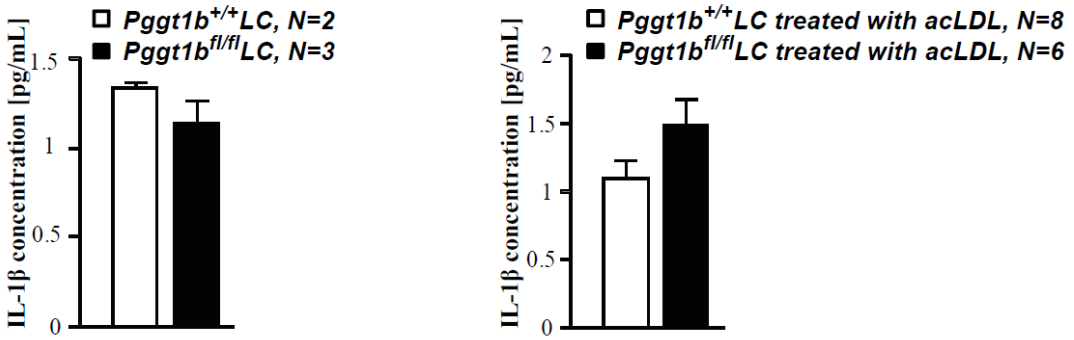

Figure 10. IL-1β secretion from untreated and acLDL-treated bone marrow macrophages. Data from 3 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* respective 2 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* untreated mice and 6 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* respective 8 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* acLDL treated mice is shown.

Decreased expression of scavenger receptors in *Pggt1b$^{fl/fl}$LC LDL$^{-/-}$* mice, but LPS stimulation causes an increased expression

To investigate if GGTase-I deficiency in mice affects the expression of scavenger receptors in macrophages RNA was isolated from *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* macrophages and analyzed by qPCR. A significant decrease is seen in the expression of Sr-A1, Sr-B1, Cd36 and Marco in the *Pggt1b* mice (*Figure 11*). No or undetectable expression was seen in Lox-1 for both the knockout cells and the controls.

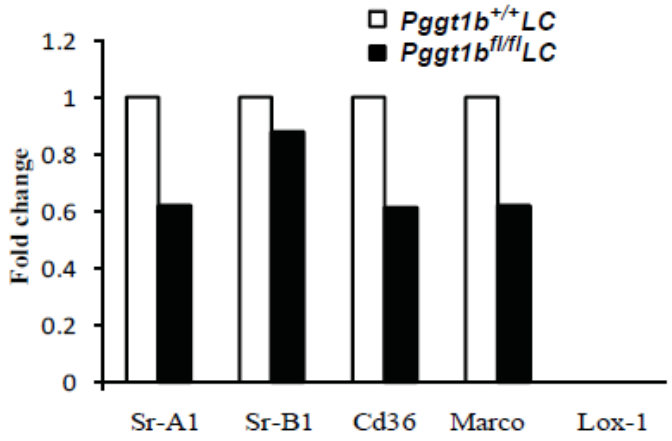

Figure 11. Fold change of scavenger receptor expressions, including Sr-A1, Sr-B1, Cd36, Marco and Lox-1, in bone marrow derived macrophages from 3 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* respective 3 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice is shown. The expressions were analyzed by qPCR.

RNA was also isolated from cultured macrophages stimulated with LPS with the reason to look at the expression of scavenger receptors during an inflammatory response. All the scavenger receptor values except Sr-B1 show a stronger upregulation in the knockout cells compared to the controls with the most significant increase in Lox-1 (*Figure 12*).

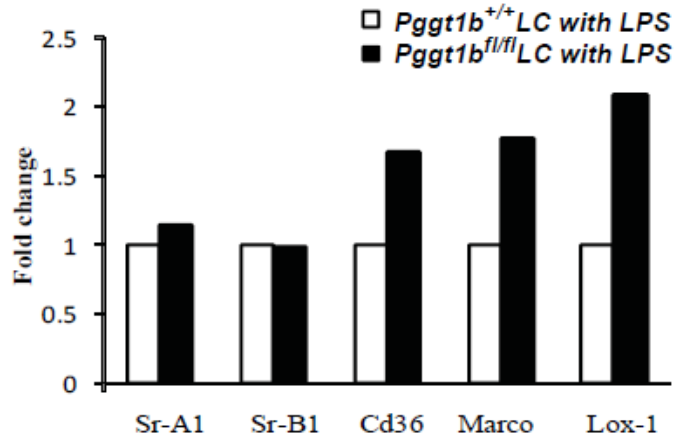

Figure 12. Fold change of scavenger receptor expressions, including Sr-A1, Sr-B1, Cd36, Marco and Lox-1, in bone marrow derived macrophages from 3 *Pggt1b$^{fl/fl}$LC LDLr$^{-/-}$* respective 3 *Pggt1b$^{+/+}$LC LDLr$^{-/-}$* mice treated with LPS.. The quantifications were done by qPCR.

DISCUSSION / SUMMARY

In this study it has been shown that GGTase-I deficiency in mice lacking LDLr has striking effects on the progression of lipid lesions in the aortas of these mice. With a decrease of around 70 % in males and 30 % in females of lipid lesions it is suggested that the knockout of the *Pggt1b* gene in macrophages reduces atherosclerosis. There is also a trend that the onset of the disease is delayed. Most of this thesis has been focused on trying to understand the underlying reason of these findings. It was shown that there were no alterations in body weights of the mice, except for a few measured time points within the female group, which implicate that the mice were in general healthy.

It was further shown that the knockout of *Pggt1b* in macrophages from mice does not affect the blood lipid concentrations. This elucidate that the positive effects that the knockout has on aortic lesion development does not depend on a decrease of cholesterol in the blood and that it is instead connected to some other cellular and molecular effects.

The cytokine concentrations were analyzed on the basis of the fact that atherosclerosis is an inflammatory disease and patients with a severe form of atherosclerosis have been shown to have elevated levels of circulating cytokines in blood (Hansson, 2009). Understanding the inflammatory signaling will give more clarity in the disorder process. Mice with *Pggt1b* knockout in macrophages showed reduced levels of IL-10 in serum. IL-10 is an anti-inflammatory cytokine that suppresses the immune response and may also suppress some other cytokines (Hansson, 2009). The decreased level of IL-10 would point at an increased immune response and thereby an increased amount of lipid lesions based on the fact that the immune response is upregulated in the atherosclerotic process. However, the knockout mice in this study have less atherosclerotic lesions and an increased level of IL-10 was expected. Even though no significant differences were shown for the other cytokines, there was a trend of reduction in all cytokines except for KC. IFN-γ, TNF-α, IL-12, IL-1β and IL-6 are all proinflammatory cytokines that promote an inflammatory response by upregulation of different cytokines and recruitment of inflammatory cells to the site of injury (DelVecchio, et al., 2007; Hansson, 2009; Scroder & Tschopp, 2010; Yap & Lai, 2010). A downregulation of these cytokines would indicate less inflammation and thereby less lipid lesions and that is what is seen in this study. From these opposing results the conclusion can be drawn that this must be examined further.

The small and rounded BMM phenotype of the *Pggt1b* mice indicates that the knockout cells had problems with attachment and spreading on the plate. This could be connected to inhibition of geranylgeranylation in some important CAAX proteins, like Cdc42, Rac and Rho that are involved in filipodia, lamellipodium, focal contacts and stress fiber formation (Alberts, et al., 2002), and the alteration in function of these proteins. If the *Pggt1b* knockout macrophages also have problems to adhere to the vessel wall in the living animal it could result in less accumulation of macrophages and therefore also less atherosclerotic lesions. The small and rounded phenotype of the *Pggt1b* mice caused the initial way of quantifying the lipid uptake in BMM per cell number to be questioned. If the knockout cells had the same volume but did not spread out as much the quantification by analyzing photos taken with light microscopy would neglect more lipid droplets in these cells compared to the controls. Therefore a new approach was used were the lipid droplets were quantified due to cell area, which should be a more truthful way to analyze the uptake. The new result demonstrated that there was no

difference in lipid uptake. Even if the second approach was more correct there are still some factors to take in account. The lipid droplets that are taken up have a range of different sizes. When these droplets are quantified by measuring the area on a photography it is not taken in account that the droplets have volumes that are not linearly proportional to the area. This means that if there is a difference in the average volume of the lipid droplets between the knockout cells and the controls the phenotype with the smaller droplets would be overestimated in lipid uptake, so there is still a need for another quantification method.

Previous studies have shown that macrophages may secrete IL-1β in response to acLDL treatment, but IL-1β was not affected by treatment with acLDL. The treatment did not trigger an upregulation of the cytokine, which has been seen when treating GGTase-I deficient cells with the immune response triggering molecule LPS (Khan, et al., 2010), which means that these macrophages are not sensitive to acLDL treatment.

Data for the scavenger receptor expressions show a decreased expression for the scavenger receptors Sr-A1, Sr-B1, Cd36 and Marco in the BMM from *Pggt1b*$^{fl/fl}$*LC LDLr*$^{-/-}$ mice. This result may explain why the macrophages have a reduced uptake of blood lipids, which further explains the reduced amount of lipid lesions in the aortas. This conclusion is drawn on the basis of the fact that the lipid uptake via scavenger receptors is the main reason for the formation of foam cells (Greaves & Gordon, 2005). That no expression was seen at all for Lox-1 in the knockout and the controls is probably due to that some scavenger receptors only are present during pathological conditions (Valiyaveettil & Podrez, 2009), which is supported by the fact that this receptor was highly upregulated when the cells were stimulated with LPS. The stimulation with LPS caused a higher upregulation of Sr-A1, Cd36, Marco and Lox-1 in the knockout cells compared to the controls. This indicates that these cells are more sensitive to the foreign material for some unknown reason. Lox-1 has been shown to be involved in the pathogenesis of atherosclerotic lesions (Navarra, et al., 2010) and the very strong upregulation of this receptor may indicate that the GGTase-I deficiency can be connected to the atherosclerotic process.

Within this thesis it has been shown that the knockout of *Pggt1b* in macrophages is strongly connected to the atherosclerotic process in mice, but still there is a need for further experiments and more understanding in this research area to be able to draw any final conclusions and to conclude that the pleiotropic effects of statins are due to inhibition of geranylgeranylation.

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