Defining the Role of GGTase-I in the Development of Rheumatoid Arthritis and Atherosclerosis

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2012

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ISBN 978-91-628-8483-3
Printed in Gothenburg, Sweden 2012
Printed by Kompendiet

ABSTRACT

RHO family proteins control cell movement and intracellular signaling by cycling between active GTP-bound and inactive GDP-bound states. Aberrant signaling of RHO GTPases has been implicated in many diseases including cancer and inflammation. Geranylgeranyltransferase type I (GGTase-I) attaches a 20-carbon geranylgeranyl lipid to a carboxyl-terminal *CAAX* motif of most RHO family proteins. Geranylgeranylation is viewed as essential for membrane targeting and activation of RHO proteins. Consequently, inhibiting GGTase-I to interfere with RHO protein lipidation and activity has been proposed as a strategy to treat cancer and inflammatory disorders. Moreover, statins – widely prescribed cholesterol-lowering drugs – possess anti-inflammatory properties that are independent of their cholesterol-lowering effects. These pleiotropic statin effects are thought to be mediated by reduced synthesis of geranylgeranyl lipids and reduced geranylgeranylation and inhibition of RHO family proteins. Despite the therapeutic interest in GGTase-I, no studies have yet defined the impact of inactivating GGTase-I in mouse models of inflammation using genetic strategies.

Paper I of this thesis shows that mice lacking GGTase-I in macrophages develop joint inflammation and bone erosions similar to rheumatoid arthritis. The disease was initiated by GGTase-I-deficient macrophages which accumulated high levels of GTP-bound RAC1, CDC42, and RHOA, and RAC1 remained associated with the plasma membrane. Moreover, GGTase-I deficiency led to robust activation of p38 MAPK and NF-κB, and increased production of proinflammatory cytokines. This effect was caused by non-geranylgeranylated GTP-bound RAC1. Thus, rather than being an anti-inflammatory drug target, GGTase-I protects mice from inflammation and arthritis development.

In Paper II, we tested if GGTase-I deficiency in macrophages would affect the development of atherosclerosis in LDL receptor–deficient mice. We hypothesized that aortic lesions would be enhanced due to local and systemic inflammation and the presence of rheumatoid arthritis – a disease that carries a high risk of atherosclerosis development in humans. Contrary to our expectations, GGTase-I deficiency markedly reduced atherosclerosis development. Cellular analyses revealed impaired foam cell formation due to high levels of cholesterol efflux. Molecular analyses revealed increased COX2 and PPARγ activity and expression of the scavenger receptors CD36 and SR-B1. The pathway was triggered by RHOA which accumulated in the active GTP-bound state in the GGTase-I-deficient macrophages.

This thesis challenges the current dogma that geranylgeranylation is essential for RHO protein activation and suggest that this posttranslational modification may actually inhibit RHO protein function. The thesis also sheds new light on the role of RHO family proteins in macrophage inflammatory signaling and cholesterol homeostasis and mechanisms underlying pleiotropic statin effects.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **GGTase-I deficiency hyperactivates macrophages and induces erosive arthritis in mice.**

Omar M. Khan, Mohamed X. Ibrahim, Ing-Marie Jonsson, Christin Karlsson, Meng Liu, Anna-Karin M. Sjogren, Frida J. Olofsson, Mikael Brisslert, Sofia Andersson, Claes Ohlsson, Lillemor Mattsson Hultén, Maria Bokarewa, and Martin O. Bergo. *J. Clin. Invest*. 2011; **121**:628–639.

II. **Targeting GGTase-I activates RHOA, increases macrophage cholesterol efflux, and reduces atherosclerosis development in mice.**

Omar M. Khan, Murali K. Akula, Kristina Skålen, Christin Karlsson, Stephen G. Young, Jan Boren and Martin O. Bergo. *Submitted.*

ABBREVIATIONS

ApoA1	Apolipoprotein A1
AD	Alzheimer's disease
COX2	Cyclooxygenase-2
CVD	Cardiovascular disease
ES cells	Embryonic stem cells
FTase	Farnesyltransferase
FTI	FTase inhibitor
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GDI	Guanine dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GGTase-I	Geranylgeranyltransferase type I
GGTI	GGTase-I inhibitor
GTP	Guanosine triphosphate
HCV	Hepatitis C virus
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
ICMT	Isoprenylcysteine carboxyl methyltransferase
LDL	Low density lipoprotein
MAL	Myocardin related transcription factor A
MHC II	Major histo-compatibility class II
MMP	Matrix metalloproteinases
MS	Multiple sclerosis
NAC	N-acetyl-cysteine
RA	Rheumatoid arthritis
RCE1	RAS converting enzyme I
ROS	Reactive oxygen species

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"When hearing something unusual, do not preemptively reject it, for that would be folly. Indeed, horrible things may be true, and familiar and praised things may prove to be lies. Truth is truth unto itself, not because [many] people say it is."

Ibn Al-Nafis, (1213-1288 A.D.)

INTRODUCTION

CAAX proteins

Many eukaryotic proteins contain a carboxyl-terminal *CAAX* sequence ("*C*" is a cysteine, "*A*" is usually an aliphatic amino acid, and "*X*" can be any amino acid). The presence of a *CAAX* motif stimulates three posttranslational processing steps (Figure 1). First, the cysteine residue (*i.e.*, the "*C*" in *CAAX*) is farnesylated or geranylgeranylated by farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I); second, the last three amino acids (*i.e.*, the *–AAX*) are cleaved off by RAS converting enzyme 1 (RCE1); and finally, the newly exposed isoprenylcysteine residue is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT).

There are about 280 predicted members in the *CAAX* protein family [1]. But not all *CAAX* proteins undergo modifications via the prenylation pathway. This is either because their *CAAX* sequence is poorly identified by the enzyme or because the carboxyl-terminus is not accessible for other reasons [1]. With the help of modern structural biology techniques more than 100 mammalian *CAAX* proteins have been identified that are modified by a farnesyl or geranylgeranyl lipid and likely undergo the subsequent processing steps mediated by RCE1 and ICMT. Members of *CAAX* protein family includes the RAS and RAS homolog (RHO) family of small GTP-binding proteins, the gamma subunit of heterotrimeric G-proteins, and the nuclear lamins prelamin A and lamin B.

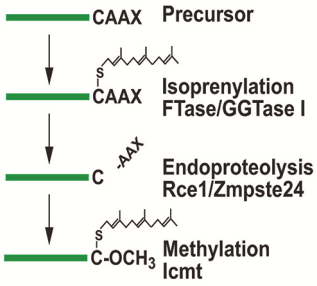

Figure 1. Postranslational modifications of *CAAX* proteins. The *CAAX* box sequence stimulates isoprenylation, which triggers subsequent endoproteolytic cleavage of the *-AAX* residues followed by methylation of the isoprenyl-cysteine residue. Prenylation stimulates protein-protein interactions and increases membrane affinity of *CAAX* proteins.

CAAX proteins are important in human diseases

Many *CAAX* proteins play important roles in human disease pathogenesis. The RAS proteins includes N-, K- and H-RAS which are by far the most thoroughly studied of the *CAAX* proteins. The RAS proteins transmit signals from cell-surface receptors which promote cell survival, cell growth, and cell proliferation. The RAS proteins are located along the inner surface of the plasma membrane. Targeting of RAS to the plasma membrane is dependent on the posttranslational modifications of the *CAAX* motif. Normally, the RAS proteins cycle between a GTP-bound active state and a GDP-bound inactive state. Somatic mutations in the RAS genes can cause the protein to remain in the GTP-bound state, resulting in constitutive signaling and oncogenic cell transformation. Oncogenic RAS

mutations are found in about 30% of all human cancers, 50% of colon cancers, and 90% of pancreatic cancers [2]. Oncogenic mutations in the *RAS* genes are also common in hematological malignancies such as myeloproliferative disorders and leukemia [3].

RHEB (RAS homolog enriched in brain) a small GTPase which regulates actin cytoskeletal rearrangements and proliferation, is over-expressed in human tumor cell lines [4].

Centromere associated proteins CENP-E and CENP-F, regulate the formation of kinetochore-microtubules during mitosis and have been associated with head and neck tumors [5]. RHOB, a small GTPase, inhibits transformation of human tumor cells and is downregulated in human tumors [6].

RHO family of proteins is another well-known family of GTP-binding *CAAX* proteins. Members of this RHO protein family including RHOA, RAC1 and RHOC are implicated in oncogenic transformation and upregulated in human cancers (Table 1).

A geranylgeranylated *CAAX* protein, FBL2, is essential for the replication of hepatitis C virus (HCV) RNA [7] and the activity of GGTase-I in *Candida albicans*—which causes most cases of fungal infections in immunocompromised patients [8].

Another *CAAX* protein Prelamin A is a precursor of lamin A, a key structural protein of the nuclear lamina, a proteinaceous meshwork lining the inner nuclear envelope. Prelamin A is involved in the pathogenesis of Hutchinson-Gilford progeria syndrome (HGPS), an accelerated aging disorder [9]. HGPS mutations result in the accumulation of the farnesylated and methylated form of prelamin A.

The activities and ability of all these *CAAX* proteins to contribute to disease pathogenesis is likely influenced by the posttranslational processing of the *CAAX* motif. Therefore, understanding the pathways of biochemical processing of *CAAX* proteins, their activation, and turnover is important in unraveling the underlying disease mechanisms associated with *CAAX* proteins and might help to identify potential drug targets for therapeutic intervention.

The goal of this thesis is to understand the basic biochemical importance of the posttranslational processing of *CAAX* proteins by GGTase-I and to define its role in the pathogenesis of inflammatory diseases.

Protein prenylation

As outlined earlier, *CAAX* proteins undergo covalent addition of an isoprenoid lipid on the cysteine residue at the carboxyl-terminal end. The reaction is catalyzed by FTase and GGTase-I. Either a 15-carbon farnesyl lipid is added by FTase – a process called farnesylation [10-14]; or a 20-carbon geranylgeranyl lipid is added by GGTase-I – a process called geranylgeranylation [14-19]. Protein farnesylation and geranylgeranylation are collectively called prenylation or isoprenylation.

A third enzyme called GGTase-II (or RAB-GGTase) catalyzes the transfer of two geranylgeranyl lipids on two cysteine residues of -CC or -CXC carboxyl-terminal sequences of RAB GTPases [20]. There is no substrate overlap between GGTase-I and GGTase-II.

Significance of protein prenylation

Posttranslational modifications of *CAAX* proteins is thought be extremely important for their overall function. Current dogma holds that *CAAX* protein prenylation and subsequent processing steps promote membrane and protein–protein interactions and are required for the activity of *CAAX* proteins. For example; if farnesylation of H-RAS is blocked, it fails to associate with the plasma membrane which leads to complete inhibition of H-RAS-mediated oncogenesis [21]. Similarly, geranylgeranylation facilitates membrane anchoring and is considered essential for the subcellular targeting and activation of RHO family proteins [22-23]. For example, when the geranylgeranyl cysteine residue of RAC1 is clipped off by the bacterial YopT protease or when the cysteine in its *CAAX* motif is mutated to serine, RAC1 localizes to the nucleus [2, 24-26]. Geranylgeranylation may also be important for protein–protein interactions, such as the binding of RHO proteins to RHO GTPase activating proteins (RHO-GAPs), which stimulate GTP hydrolysis and inactivation; RHO guanine nucleotide exchange factors (RHO-GEFs), which stimulate GDP/GTP exchange and activation; and RHO guanine-nucleotide dissociation inhibitor (RHO-GDI), which sequesters the GDP-bound inactive form of RHO proteins in the cytosol [2, 27-29]. Indeed geranylgeranylation regulates RHO protein stability and turnover by promoting their interactions with RHO-GDI [30]. Hence prenylation appears to be essential for *CAAX* protein activation, turnover, and targeting to membranes [1].

Most prenylated *CAAX* proteins undergo two additional processing steps, endoproteolytic cleavage by RCE1 and carboxyl methylation by ICMT. These processing steps are obviously less important than prenylation for membrane association, but are nevertheless required for full function of many *CAAX* proteins [31]. Therefore, inhibiting the processing of *CAAX* proteins, to interfere with their function, has been tested as strategies for treating diseases caused by mutant or hyperactive *CAAX* proteins, including cancer, inflammation, progeria, and other so-called laminopathies.

But *CAAX* protein processing plays a broader biological role. For example, RCE1-mediated proteolytic processing is required for the transport of the retinal *CAAX* protein [32]. Also, the ability of the bacterial toxin YopT to cleave and inactivate RHOA requires that RHOA has been processed by RCE1 but not ICMT. Furthermore, some drugs secondarily inhibit *CAAX* protein processing. Statins and bisphosphonates – used world-wide for lowering cholesterol and treating osteoporosis – inhibit the synthesis of geranylgeranyl lipids and lead to reduced geranylgeranylation of RHO proteins. Consequently, both beneficial and adverse effects of those drugs have been attributed to reduced geranylgeranylation and *inhibition* of RHO protein function.

FTase

The finding that RAS proteins require prenylation for their oncogenic activity during the early 1990s led to the identification and purification of mammalian protein farnesyltransferase from rat brain cytosol [33]. FTase is a heterodimer consisting of a 48-kDa α and a 45-kDa β subunit [33]. The β subunit is the active subunit of FTase and in humans is encoded by the *FNTB* gene. The yeast homolog of *FNTB* is *RAM1* which is not essential but its defiency causes growth defects [34]. FTase is a zinc (Zn^+) metalloenzyme which requires both Zn^+ and magnesium (Mg^+) for its activity. Zn^+ is not required for binding of the 15-carbon farnesyl lipid but it is important for substrate binding [35].

Substrates for FTase in mammalian cells include all the RAS proteins, nuclear lamins A and B, the gamma subunit of the retinal trimeric G protein transducin, rhodopsin kinase, and a peroxisomal protein of unknown function termed PxF [36-38].

GGTase-I

GGTase-I is a protein of 89-kDa and is composed of two distinct subunits [39]. It shares its α subunit with FTase and has a unique β subunit of 43-kDa [40] – which in humans are encoded by *FNTA* and *PGGT1B* genes respectively; the yeast homologues are *RAM2* and *CDC43* [41]. Soon after the discovery of FTase, mammalian protein GGTase-I was identified and purified from cytosolic extracts of bovine brain [42-43]. The crystal structure of mammalian GGTase-I, including substrate and product complexes, was solved in 2003 [44] and revealed that all protein prenyltransferases share a similar reaction mechanism and identified specific residues important for substrate specificity. Like FTase, GGTase-I requires Zn^+ for activity and point mutations in the β-subunit that alters any of the three amino acid residues that contribute to the Zn^+-binding results in complete loss of enzymatic activity [35].

GGTase-I is responsible for isoprenylating majority of the RHO family proteins, including RHOA, RAC1, CDC42 and RAP1, and most isoforms of the gamma subunit

of G proteins. A deficiency in GGTase-I activity—through mutations in either of the two subunits results in lethality in budding yeast *Saccharomyces cerevisiae*. This lethality could be overcome by overexpressing RHO1p and CDC42p, indicating that the only essential function of GGTase-I is the isoprenylation of these two *CAAX* proteins—at least in yeast. In the pathogenic yeast *Candida albicans*, GGTase-I is not essential for viability; knockout of the *CaCDC43* gene, resulted in viable cells in which there was an upregulation of the two critical substrates RHO1p and CDC42p. Based on these divergent results, it was impossible to predict the impact of GGTase-I deficiency in mammalian cells.

One of the aims of this thesis is to define the impact of inhibiting GGTase-I in mammalian cells on their viability and function.

Substrate specificity of GGTase-I and FTase

Much of the characterization of the substrate specificities of FTase and GGTase-I were performed in early 1990s and has confirmed the importance of the *CAAX* motif of the protein substrates for enzyme recognition [33, 35, 45-46]. The unique β subunits of FTase and GGTase-I contain the binding pockets for *CAAX* residues of the substrate proteins. It has been shown that the carboxyl-terminal residue in the *CAAX* sequence (*i.e.* the "*X*") is crucial for substrate specificity: When "*X*" is alanine, glutamine, methionine, serine, or phenylalanine, the cysteine residue is farnesylated by FTase; when the "*X*" residue is leucine, the cysteine is geranylgeranylated by GGTase-I. For example, the *CAAX* sequence of RHOA, *–CLIL*, is always geranylgeranylated by GGTase-I whereas the *–CVLS* sequence of H-RAS is always farnesylated by FTase. Replacing the *CAAX* sequence of RHOA with the H-RAS sequence by site-directed mutagenesis results in farnesylated RHOA [47].

GGTase-I and FTase are quite specific for their substrates with few exceptions. For example; N-RAS, K-RAS, and RHOB are normally farnesylated by FTase but can be efficiently geranylgeranylated by GGTase-I when FTase activity is blocked [48].

RHO protein family

The RHO GTPases is a subgroup of 20 proteins within the *CAAX*-protein family. The most well studied RHO proteins are RHOA, RAC1 and CDC42 [29, 49-51]. RHO proteins control the dynamics of cell shape and cell movement and many important cellular functions by acting as "molecular switches". RHO proteins bind GTP in their active form, a reaction catalyzed by RHO-GEFs, and to GDP in their inactive form, catalyzed by RHO-GAPs (Figure 2). At steady state, the concentration of GDP in cells is higher than GTP which favors RHO-GDP binding. GDP-bound inactive RHO proteins are sequestered in the cytosol by RHO-GDIs in an inactive complex [52]. However once activated, RHO-GDI dissociates and RHO proteins regulate extracellular stimulus–mediated signaling networks that converge on the

organization and remodeling of the actin cytoskeleton, cell cycle regulation, proliferation, migration, and regulation of gene expression and the production of growth factors and cytokines [53-55].

The activity of RHO GTPases is tightly regulated by the opposing effects of RHO-GEFs and RHO-GAPs (Figure 2). Since RHO GTPases control many important cellular functions, aberrant signaling has clinical implications. Indeed, RHO GTPases are implicated in pathogenesis of cancer [50, 56] (Table 1) and inflammation [57]. Therefore, inhibiting RHO protein function is proposed as an interesting therapeutic strategy in the treatment of cancer and inflammation-related disorders such as atherosclerosis, Alzheimer's disease (AD), rheumatoid arthritis (RA) and multiple sclerosis (MS) [55-57].

RHO proteins and disease

Activating mutations or increased expression of RHO GTPases may result in constitutive signaling to downstream effectors. This might result in transformation of normal cells into cancerous cells with uncontrolled growth and proliferation or hyperactive inflammatory cells with production of proinflammatory molecules leading to severe inflammation. The involvement of RHO GTPases in tumor formation and metastasis is well established and summarized in table 1 together with their most widely known functions. This section will highlight the contribution of the most widely studied RHO GTPases to inflammatory diseases.

RHOA

RHOA is a small GTPase of 20-22 kDa and together with RHOB and RHOC was the first to be discovered in 1980s. It is one of the most studied RHO proteins and is shown to regulate many cellular functions which include regulation of cytoskeleton, formation of actin stress fibers, cell–cell contacts, and cell adhesion, polarity, and migration [58]. RHOA also regulates the transcription of serum response factor (SRF) by modulating actin monomers and allowing the nuclear transport of myocardin related transcription factor A (MAL) [59-60].

RHOA has been associated with inflammatory conditions. One of the most immediate downstream effectors of RHOA is RHO-associated coiled-coil–containing protein kinase (ROCK). In fact, RHOA involvement in different cellular processes and diseases is often assessed indirectly by obtaining effects with pharmacologic inhibitors of ROCK. The ROCK inhibitor Y27632 inhibits RAS transformation [61], cardiac muscle contraction [62], MCP1-mediated chemotaxis of THP-1 cells [63], inflammation and coronary atherosclerosis [64], and NF-κB-induced production of inflammatory cytokines [65].

Table 1. RHO GTPase function and their association to cancer[1].

RHO GTPases	Functions	Involvement in diseases
RHOA	Cell migration, microtubule stability, cell-cell adhesion, cytokinesis, phagocytosis	Promotes transformation, oncogenesis, invasion, and metastasis. Upregulated in human tumors.
RHOB	Formation of stress fibers, and Endosomal transport	Inhibits tumor growth. Downregulated in tumors.
RHOC	stress fibers, focal adhesions, cell-cell adhesion	Promotes transformation and oncogenesis. Upregulated in tumors.
RAC1	Formation of lamellipodia, Cell migration, vesicle trafficking. Phagocytosis and NADPH oxidase activation.	Promotes transformation and oncogenesis. Upregulated in tumors; Rac1b splice variant expressed in tumors.
RAC2	Formation of lamellipodia, NADPH oxidase activation, and cell migration.	Upregulated in tumors.
RAC3	Formation of lamellipodia, focal complexes.	Hyperactive or upregulated in breast cancer. Neurite outgrowth.
CDC42	Formation of filopodia. Vesicle trafficking, cell polarity, migration, cytokinesis, phagocytosis.	Upregulated in breast cancer.

[1] Adopted from Snapshot: RHO family GTPAses, Cell 129, June 29, 2007.

Increased RHOA activity is associated with Crohn's disease and lung inflammation [65-66]. Inhibiting RHOA function by a ROCK inhibitor reduced intestinal inflammation in rats and N-acetylcysteine (NAC), a reactive oxygen species (ROS) scavenger, reduces lung inflammation in mice [65-66]. Overexpression of RHOA is associated with autoimmune neuritis in rats [67]. Inhibiting RHOA function by blocking its prenylation either by statins or GGTIs is a proposed mechanism for increase in peroxisome proliferater-activated receptor gamma (PPARγ) activity, a nuclear receptor protein regulating the transcription of genes involved in cellular differentiation, and carbohydrate, protein, and lipid metabolism [68]. The increase in PPARγ activity might be beneficial in regulating cholesterol transport and efflux from macrophages and peripheral tissues to the liver for excretion and thus presents an interesting target for treating cardiovascular diseases (CVD) [69-70].

Thus interfering with RHOA function by either blocking its downstream effectors or by blocking its prenylation might be useful in treating different conditions ranging from cancer to atherosclerosis.

RAC

RAC1, RAC2, RAC3 and RHOG constitute a subfamily of RHO GTPases. RAC1 is ubiquitously expressed and is the most widely studied. RAC2 is expressed exclusively in cells of hematopoietic origin [71-72] while RAC3 is expressed in cells of the nervous system [73-74]. RAC1 regulates lamellipodia formation during migration and phagocytosis while both RAC1 and RAC2 are part of NADPH oxidase component in macrophages and neutrophils. RAC1 promotes oncogenic transformation by RAS and is also upregulated in many different forms of tumors [50, 75-77]. Leukocytes activities including migration into the site of infection, production of proinflammatory cytokines and ROS, and clearance of apoptotic cells and pathogens are important functions during inflammation. RAC GTPases contribute to many of those functions. RAC1 activates NF-κB directly via its downstream effector p21 activated kinase (PAK) and indirectly via p38-mitogen activated protein kinase (MAPK) [78-79].

NF-κB plays a key role in regulating the immune response to infection. Deregulation of NF-κB activity is linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and aberrant immune development [80-87]. Therefore constitutively active RAC1 might result in increased activation of NF-κB and robust inflammatory response. Similarly, both RAC1 and RAC2 are implicated in Tumor necrosis factor alpha (TNFα) mediated Interleukin-6 (IL6) and Interleukin-8 (IL8) production [54] and increase in RAC1 activity may contribute to TNFα-mediated myocardial endotoxemia [88].

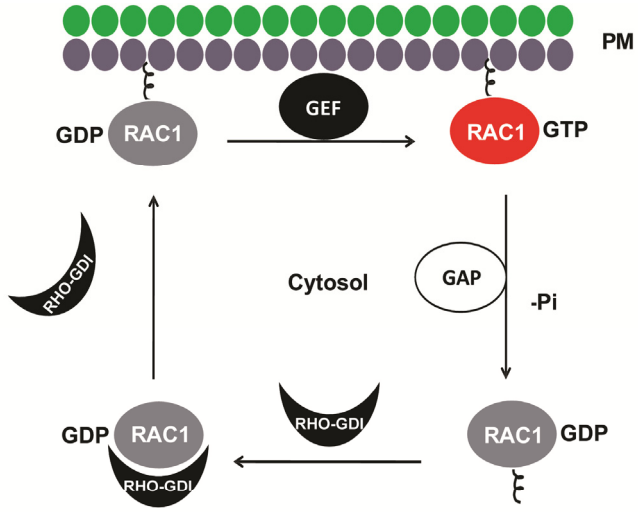

Figure 2. Activation and inactivation cycle of RAC1. Prenylation is considered essential for the RAC1 interaction with RHO-GDI. RHO-GDI sequesters GDP-bound RAC1 in the cytosol. This association is considered important for keeping inactive RAC1 stabilized in a GDP-RAC1-RHO-GDI complex. The opposing effects of two forms of regulatory proteins, RHO-GEFs and RHO-GAPs, regulate RAC1 GTPase activity. Upon extracellular stimulus, RHO-GDI dissociates from RAC1 and RAC1 is targeted to the plasma membrane (PM) for its activation to RAC1-GTP by the RHO-GEF. Prenylation is believed to be important for the activation and targeting of RAC1 to the membrane. Once activated, RAC1-GTP interacts with effector proteins including PAK kinases and MAL, and regulates signal transduction, cell proliferation, migration, and shape. Once the signal is withdrawn, RAC1-GTP is hydrolyzed to RAC1-GDP by RHO-GAPs. The interaction of RHO-GAP with RAC1 is also believed to be prenylation dependent. Thus protein prenylation may regulate many aspects of RAC1 biology.

The involvement of RAC GTPases in ROS production and phagocytosis by macrophages and neutrophils is well established [55]. Increased ROS production is involved in pathogenesis of autoimmune diseases [89-92]. Consequently, inhibiting RAC1 in mouse models of arthritis has shown promising results [93-94]. Thus targeting RAC1 provides an interesting approach in treating immunoinflammatory disorders like rheumatoid arthritis [95].

CDC42

CDC42 is another member of RHO family which regulates cell polarity and migration [96]. CDC42 stimulates the formation of filamentous actin–like bundles called filopodia. Filopodia are important for sensing environment-based cell–cell contacts and movements [53, 97-99]. CDC42 in yeast regulates mating and budding [100]. The role of CDC42 in human tumors is not well established. However, CDC42 is important for regulating cholesterol efflux in macrophages [101-103]. Similarly, CDC42 is downregulated in Tangier's disease – a disorder characterized by lipid retention in macrophages and other tissues [104].

RAP1

RAP1 is an essential modulator of NF-κB mediated pathways [105]. NF-κB is involved in pathogenesis of many diseases [80-87]. Therefore blocking pathways leading to activation of NF-κB is of paramount interest for therapeutic intervention. One study showed that knockout of RAP1 prevents NF-κB activation. Interestingly, the authors also found that increased NF-κB activity correlated with increased RAP1 expression in human breast cancer cells. When RAP1 activation was blocked in those cells, the cells developed susceptibility to apoptotic stimuli [105]. The role of RAP1 in inflammation and autoimmunity is yet to be explored but given the importance of NF-κB in regulating the events of immune system and inflammation, RAP1 may pose an interesting target for therapeutic intervention.

The development of GGTase-I inhibitors

During the 1980s and early 1990s it was discovered that *CAAX* proteins require prenylation for their activity and membrane targeting [1]. The finding that RAS proteins require farnesylation for their membrane targeting and oncogenic activity [13] stimulated interest in developing FTase inhibitors (FTIs). Studies in K- and N-RAS transgenic mouse models have shown promising results of FTIs in inhibiting tumor growth [106] and even tumor regression in mice with H-RAS induced cancer [107]. Consequently, FTIs were tested in a large number of clinical trials and despite their specificity in blocking *CAAX* protein farnesylation little success was achieved [108-111].

The most likely explanation why FTIs had little success in clinical trials is the ability of GGTase-I to alternately prenylate K- and N-RAS, but not H-RAS, in the setting of FTase inhibition. Indeed, FTIs showed a greater impact in blocking tumor formation in H-RAS–induced cancer in mice compared to K- and N-RAS–induced cancer. Unfortunately, *HRAS* mutations are rare in human cancers. This fuelled the interest in developing GGTase-I inhibitors (GGTIs). Since RHO GTPases contribute to RAS-induced transformation during tumor growth and metastasis, it was proposed that blocking GGTase-I activity might be an effective strategy to prevent tumor growth. Since then, several GGTIs have been synthesized and shown to inhibit the growth of

tumor cells [112]. Our group validated GGTase-I and FTase as drug targets in the treatment of K-RAS-induced cancer with genetic strategies in mouse models [47, 113]. There is concern, however that inhibition of GGTase-I would be too toxic, partly because geranylgeranylated proteins are quite numerous in cells. Along those lines, one study showed that treatment with a GGTI was lethal in mice and caused apoptosis of cultured cells [114]. However, other studies have suggested that targeting GGTase-I may not be particularly toxic [112]. Consequently, the success seen with targeting GGTase-I in mouse models of cancer has led to the evaluation of one GGTI in clinical trials [115].

However, blocking GGTase-I might also affect the functioning of inflammatory cells by interfering with the activity of RHO GTPases. For example; the activities of RHO family proteins are important for the ability of macrophages and lymphocytes to migrate into tissues, respond to inflammatory stimuli, and trigger ROS production, phagocytosis, NF-κB signaling, and cytokine production [116]. Because of this, blocking prenylation of RHO GTPases is proposed as an interesting strategy to prevent the development of autoimmune diseases including AD, MS and RA [117-125]. Inhibiting RHO proteins prenylation may also increase cholesterol efflux by increasing PPARγ activity in macrophages and reducing foam cell formation [69-70] which is a hallmark of atherosclerosis – a chronic inflammatory disease.

Because both existing and proposed new therapies are directed against *CAAX* protein processing, it is important to understand the role of the three posttranslational processing steps for the activity, stability, and membrane and protein interactions of the different classes of *CAAX* proteins. These studies are typically performed with more or less specific inhibitors and exogenously over-expressed *CAAX* proteins in different cell types in different laboratories and despite the growing interest in blocking RHO protein prenylation in treating different autoimmune and inflammation-related disorders, the role of GGTase-I in inflammatory cells have never been evaluated with genetic strategies.

This thesis serves to fill this void by evaluating the impact of knocking out GGTase-I in macrophages and to define the consequences of this intervention for the development of RA and atherosclerosis.

Statins

Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase – the rate-limiting enzyme in the cholesterol biosynthetic pathway (Figure 3). These widely prescribed cholesterol-lowering drugs efficiently reduce plasma cholesterol levels and prevent the risk of morbidity and mortality associated with cardiovascular disease. However, statins posses anti-inflammatory and immunomodulatory properties that are unrelated to their cholesterol-lowering effects. The first evidence that statins posses beneficial properties independent of cholesterol

lowering came from the clinical trials **CARE** [126], **LIPID** [127] and **HPS** [128] with unanticipated responses from the patients on statin therapy. Most importantly, despite overlapping cholesterol levels, statins had an overall beneficial effect on the incidence of heart disease and patients on statins showed an increased survival rate.

Since then, a large number of studies have established that statins have beneficial effects that are independent of cholesterol lowering. For example, statins are beneficial in conditions that are not even related to high cholesterol levels. Moreover, in many cases of CVD, statins improve overall prognosis even before the effect on plasma cholesterol level is observed [129-130] and statins improve endothelial barrier – a proprety that a similar non-statin lipid-lowering agent ezetimibe lacks [131-135].

Inflammation is considered central to atherosclerosis and CVDs [136-139] and statins in humans have shown anti-inflammatory effects. Statins reduce levels of C-reactive protein (CRP) – a common inflammatory marker in humans [140-141]. Statins in experimental settings have shown positive effects in autoimmune diseases like RA, MS and AD and also in cancer [120, 123, 142-148]. The precise mechanisms behind these so-called pleiotropic effects of statin therapy are essentially unknown. However, much of the pleiotropic effects are attributed to the fact that statins, by blocking the production of mevalonate, not only lowers the production of cholesterol but also lowers the production of geranylgeranyl pyrophosphate (GGPP) – the 20-carbon isoprenyl lipid used by GGTase-I to modify RHO proteins; and to a lesser extent farnesyl pyrophosphate (FPP) – the 15-carbon isoprenyl lipid used by FTase to modify the RAS proteins and prelamin A [1] (Figure 3). Indeed, there is overwhelming evidence that statins exert anti-inflammatory and immunomodulatory effects by blocking RHO protein prenylation [121, 141, 145, 148-149].

Statins also have some serious side effects. The most common is myositis and muscle and joint pain. The myositis in statin-treated patients can accelerate into myopathy and further into rhabdomyolysis, a severe skeletal muscle condition, which can lead to kidney failure and death [150-154]. Ironically, despite several studies proposing that statin use might be beneficial in treating RA and related disorders, muscle pain and sometimes joint pain are common side effects of statin therapy [150-155]. It is entirely possible that both positive and negative side-effects of statin therapy are mediated by reduced prenylation of small GTPases.

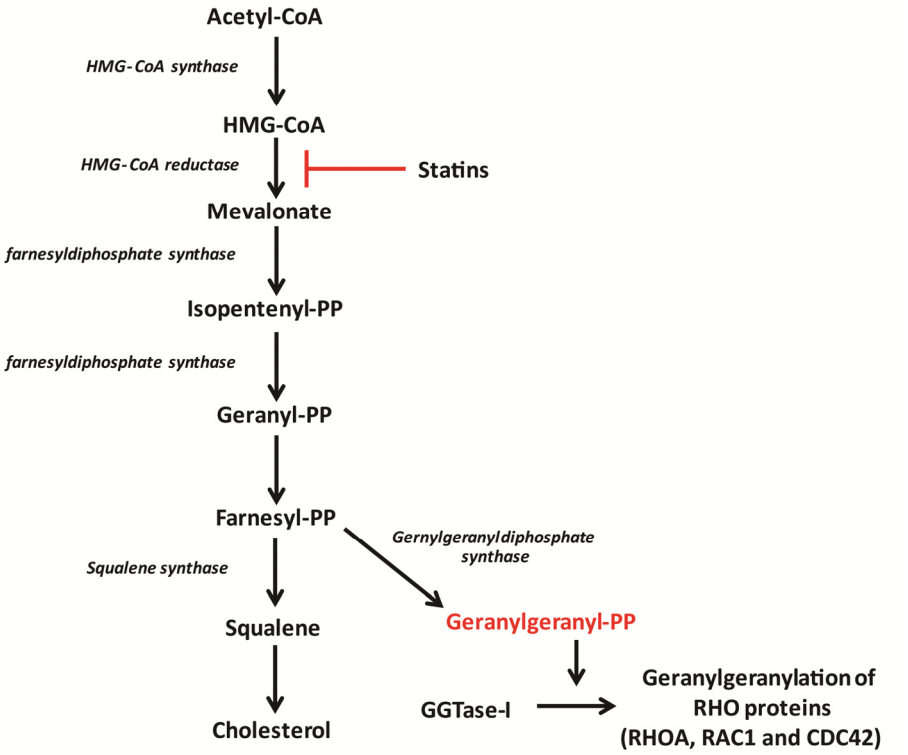

Figure 3. The cholesterol biosynthetic pathway. The rate-limiting step, the target of statins, in the synthesis of cholesterol is the conversion of HMG-CoA to mevalonate by HMG-CoA reductase. Mevalonate is subsequently converted into isopentenyl pyrophosphate (PP) by phosphorylation and decarboxylation. Isopentenl-PP is converted into geranyl-PP which condenses with one molecule of isopentenyl-PP and forms farnesyl-PP (FPP). Then, two molecules of FPP are condensed to give rise to squalene which is converted to cholesterol. In a second reaction, FPP is converted to GGPP by the action of geranylgeranyl diphosphate synthase. GGPP is used as a lipid substrate by GGTase-I for the posttranslational modifications of *CAAX* proteins. Statin therapy reduces the production of GGPP and to a lesser extent FPP and can result in reduced prenylation of RHO family proteins.

Most studies that have addressed the pleiotropic effects of statins used primary or established cell lines that have been treated in culture with various statins. Then, phenotypes, such as the proliferation and migration of smooth muscle cells and cancer cells or the uptake and efflux of lipids by macrophages have been quantified. The observed phenotypes can usually be restored by the addition of mevalonate or GGPP, but not by FPP or cholesterol, indicating that geranylgeranylated proteins among others, RHOA and RAC1, are involved. Based on those types of findings, it has been proposed that the mechanism of the pleiotropic effects of statin therapy involves the inhibition of protein prenylation and in particular RHO protein geranylgeranylation.

Thus blocking RHO protein prenylation provides an interesting strategy for therapeutic intervention in immunoinflammatory disorders. Importantly, the reduced prenylation of RHO proteins have always been thought to lead to their *inactivation*.

Importance of studying GGTase-I function in macrophages

Macrophages regulate many inflammatory processes. They migrate into tissues, secrete inflammatory cytokines, recruit neutrophils and lymphocytes into the site of infection, suppress T cell–mediated responses by ROS, cross talk with cells of the innate and adaptive immune system, phagocytose and kill pathogens and present their antigens to T cells, remove apoptotic cells and cell debris, and ingest modified forms of LDL [156-158]. Thus macrophages are crucial to inflammation and form a nexus of events together with other cells of the innate and adaptive immune system in regulating many aspects of acute and chronic inflammation. Macrophages are therefore often associated with pathological conditions. For example macrophages and macrophage-like synovial fibroblasts are prominent in synovial tissues during joint inflammation in rheumatoid arthritis [159-162]; macrophage foam cell formation is the hallmark of atherosclerosis which characterize the formation of early lesions called fatty streaks in the vessel walls [163]; mutations in the macrophage cholesterol efflux gene ABCA1 causes Tangier disease in humans [164]; macrophages help cancer cells proliferate and migrate by producing cytokines like TNFα and IL6 [165-166]; and macrophages may also serve as host cells for infectious pathogens and contribute to diseases like tuberculosis and Leishmaniasis [167].

Many macrophage functions are regulated by the RHO family proteins. Therefore inhibiting RHO proteins function by interfering with their posttranslational prenylation is proposed as an interesting therapeutic target. Along those lines, many studies have proposed that the pleiotropic statin effects to be mediated by blocking RHO protein prenylation [117, 119, 148-149, 168]. Several studies have proposed blocking RHO protein grenylgeranylation to be beneficial in treating RA and inflammation [118, 120, 122]. But statins also block protein farnesylation and geranylgeranylation of RAB GTPases and under those conditions it is difficult to determine the impact of only blocking RHO protein prenylation. There were several important questions in relation to inhibition of RHO protein prenylation in macrophages that were not addressed before, for example: Is GGTase-I dispensable for macrophage viability, differentiation and function? Would GGTase-I inhibition blunt the macrophage inflammatory response?

Targeting GGTase-I with genetic strategies in mice would address that issue.

Importance of validating GGTase-I as a target in mouse models of inflammation

As I write this paragraph, the professional footballer Fabrice Muamba collapses on the field with sudden cardiac arrest during a football match. What causes this sudden cardiac arrest is still a mystery but it is even more surprising that super-fit professionals like football players encounter this sort of cardiovascular complication. Today atherosclerosis together with rheumatoid arthritis poses a major risk factor for human morbidity. Atherosclerosis is one of the leading causes of death in US, Europe, Japan and Asia, and RA is a major cause of disability throughout the world. In many advanced cases RA patients may experience morbidity and mortality due to cardiovascular disease. Clearly inflammation in RA is not restricted to joints. Our knowledge about how inflammation in vessel walls contributes to atherosclerosis and how T and B cells become self-destructive is constantly evolving. RA and atherosclerosis are multifactorial diseases which makes it complicated to predict the precise mechanism for therapeutic intervention.

We believed that it would be useful to explore the impact of inhibiting RHO protein prenylation in animal models of inflammation and to use genetic strategies to inactivate GGTase-I. Mouse models provide an exciting opportunity to study the impact of inhibiting RHO protein function by blocking their prenylation.

Inhibiting RHO protein prenylation by inhibiting GGTase-I function in a cell type-specific manner in macrophages, has given me an opportunity to answer several important questions that are related to inhibiting protein prenylation during inflammation.

Inflammation

Everyone in their life experiences inflammation. The word inflammation is derived from the Latin word *inflammare* which means "to set fire on". Most common symptoms of inflammation are: 1) redness 2) swelling 3) burn and 4) pain[2]. Inflammation is a highly coordinated response of immune system of an organism against an injury and serves the purpose of healing and protecting the tissue from further damage [169]. Uncontrolled inflammation may have severe pathological consequences including tissue damage and body harm instead of wound healing and tissue repair.

Inflammation can be classified in two different forms. 1) Acute inflammation, characterized by infiltration of leukocytes (macrophages and neutrophils) to the site of tissue damage [170] and 2) Chronic inflammation, characterized by infiltration of

[2] Spencer WG, editor. Celsus. De Medicina 1935.

T cells and plasma cells to the site of tissue damage, leading to tissue remodeling and repair [171-172]. Regardless of the type of inflammation, macrophages have been classified as the main mediator of inflammation because of their ability to migrate into the site of injury from the vessel wall, secrete inflammatory cytokines including IL1-β, TNFα, IL6, IL-10, and IL-12, trigger ROS production, phagocytose dead cells and pathogens, and cross talk with cells of adaptive (T and B cells) and innate immunity (neutrophils and dendritic cells) [173].

Inflammation is tightly regulated and despite its importance and significance in wound healing, prolonged inflammation may lead to pathological conditions including acne vulgaris, asthma, atherosclerosis, cancer, hay fever, inflammatory bowel diseases, intestinal cystitis, periodontitis, and rheumatoid arthritis.

Rheumatoid Arthritis

RA is a progressive inflammatory and autoimmune disorder characterized by deformed joints of hands and feet [174-176]. It affects 1% of world's population and is a leading cause of disability [177]. The cause of arthritis is not fully elucidated, however it is believed that the early events involve formation of immune complexes [178-179], production of self-reactive antibodies [180], infiltration of lymphocytes and macrophages into the synovium, and hyperplasia of intimal lining resulting from the presence of macrophage- like synovial fibroblasts [176]. Typically, synovium is a single cell–layered structure but during RA lymphocytes and macrophages infiltrate inside the joint cavity and form a complex network of lymphoid aggregates and produce cytokines, chemokines and degradative enzymes resulting in joint inflammation and bone destruction [176].

T cells contribute to RA pathogenesis [181]. Different subsets of T cells including $CD4^+$, $CD8^+$, and T-helper cells play important roles in the progression of RA [182]. There is growing evidence that $CD4^+$ T cells play a dominant role. For example; activated $CD4^+$ T cells can be found in the inflammatory exudates of the rheumatoid synovium [183]. Secondly, data from animal model studies show that inflammatory arthritis and tissue-damaging autoimmunity can be induced by transfer of $CD4^+$ T cells from sick animals into healthy recipients [184]. Thirdly, clinical therapies directed against T cells and their products have shown great benefit in treating RA [185-188]. Finally the strongest evidence for the role for $CD4^+$ T cells in the onset of RA comes from the association of aggressive forms of the disease with particular major histo-compatibility class (MHC) II molecules including HLA-DR (1, 2, 4, 5, 8, 12, 14, and 15). HLA-DR is a cell surface receptor encoded by human leukocyte antigen (HLA) which is a ligand for T-cell receptor (TCR) activation. HLA takes part in graft-versus-host disease where a tissue transplant is rejected by the host on the basis of mismatched HLA. The exact significance of HLA-DR in the pathogenesis of RA is not known but it is hypothesized that these self antigens are presented to T-cells and

the activated T cells then start an array of classical immune response which marks the onset of leukocyte infiltration and local inflammation.

Macrophages are primarily considered to be effector cells in the pathogenesis of RA [189]. Macrophages produce inflammatory mediators and interact with other cells and extra cellular-matrix proteins to promote joint inflammation and bone destruction. Monocytes from blood can infiltrate into the joint cavity and differentiate into synovial tissue macrophages. These synovial macrophages express adhesion molecules, chemokines, cytokines, and degradative enzymes like matrix metalloproteinases (MMPs) which contribute to the cycle of inflammation, leukocyte infiltration, cartilage destruction and angiogenesis. Recently, one study showed the importance of macrophage oxidative burst (ROS) in suppressing the T-cell mediated immune response [157]. ROS is important in antimicrobial defense mechanism in the host [190]. However, increased amount of ROS produced by macrophages and neutrophils is often associated with immunopathologic conditions like RA [91]. The authors showed that macrophage ROS production suppressed arthritis development by blocking the activation of T cells and suggested an immunosuppresive role for macrophage ROS [157].

What determines the specificity of the self-reactive T cells for invading the joints is poorly understood. RA is a chronic disease but it carries an increased risk of mortality due to atherosclerosis and other CVDs [191]. Hence, inflammation in joints affects other organs including blood vessel walls.

Atherosclerosis

As outlined before, atherosclerosis is a chronic inflammatory disease [136-139, 192] – characterized by the deposition of cholesterol and formation of fatty streaks in the coronary arteries [192]. It is the leading cause of death in USA, Europe, and some parts of Asia [193-194]. In Sweden, about one million people suffer from CVDs, which are in most cases associated with atherosclerosis. Despite the high mortality rate associated with atherosclerosis and CVD the mechanisms underlying the disease onset and progression are still debatable. According to the so called 'response-to-injury' hypothesis, the earliest event in atherosclerosis is the initiation of an inflammatory cascade in response to endothelial dysfunction or injury in the arteries which is followed by retention of modified lipids, primarily low density lipoprotein (LDL) cholesterol, in the arterial wall [195-197]. Another hypothesis suggests that lipid retention comes first and endothelial injury later. This hypothesis is supported by the findings of fatty streaks in the absence of inflammation [198-199]. However, the role of local and systemic inflammation in the progression of atherosclerosis is widely accepted. Regardless of which event comes first, a host of risk factors might contribute to atherosclerosis including elevated levels of LDL and ROS, hypertension, diabetes mellitus, and viral infections [192].

Macrophages are crucial to the process of atherosclerosis. The retention of LDL by proteoglycans in the arterial wall marks the onset of atherosclerosis, according to the second hypothesis described above. The entrapped LDL molecules are then modified by different mechanisms [200-201]. These modified lipid molecules may cause endothelial dysfunction which is followed by an inflammatory cascade. Blood monocytes migrate to the site of lipid retention and endothelial dysfunction and differentiate into macrophages. Macrophages start phagocytosing modified LDL and turn into foam cells. Foam cells have a very strong potential for producing chemokines, cytokines, and ROS – all of which contribute to the ongoing inflammation by recruiting more leukocytes. Foam cell formation is thus a hallmark of atherosclerosis and blocking foam cell formation is often regarded as interesting target for therapeutic intervention in CVDs [201-205].

The role of T and B cells is well established in atherogenesis. Deficiency of both T and B cells inhibits atherosclerotic lesion development in mice [206]. T cells are commonly found in the lesions of atherosclerotic mice and the transfer of $CD4^+$ T cells from atherosclerotic mice to *SCID* mice increases lesion development [207] which highlights the importance of T cells in the pathogenesis of atherosclerosis. More recent work suggests atheroprotective roles of subsets of regulatory T cells. One study showed that naturally arising T regulatory cells ($CD4^+CD25^+$) inhibit atherosclerotic lesion development in mice. T regulatory cells maintain immunologic tolerance to self and non-self antigens and therefore provide new therapeutic approaches by modulating their function [208].

The importance of T cells and macrophages signifies the importance of the immune system during atherosclerosis development. Whether atherosclerosis can be blocked despite ongoing inflammation is not known. Very few studies have reported increased atherosclerosis despite reduced inflammation. However, a recent study, using RAG1-knockout mice, demonstrated uncoupling of atherosclerosis and inflammation in this fashion [209]. RA is an immunoinflammatory disorder which is strongly associated with increased cardiovascular risks in humans. Indeed, most premature deaths among RA patients result from atherosclerosis like CVDs [191], further highlighting the importance of ongoing inflammation in atherosclerosis development. Therefore studying the molecular and cellular mechanisms regulating inflammation is important in understanding the underlying mechanisms and pathogenesis of RA and atherosclerosis.

AIMS AND HYPOTHESES

The overall aim of this thesis is to define the biochemical and medical role of GGTase-I and to evaluate the impact of GGTase-I deficiency on the development of inflammatory diseases in mice.

- In **paper I** we tested the hypothesis that blocking GGTase-I would inhibit inflammatory activities of macrophages.

- In **paper II** we tested the hypothesis that hyperactive GGTase-I-deficient macrophages and the underlying RA would accelerate the development of atherosclerosis in mice.

EXPERIMENTAL STRATEGY

This section covers the genetically modified mouse models that I used for evaluating the role of GGTase-I in macrophage viability, function and inflammation in mice. For detailed description of techniques and experiments refer to the materials and methods sections of the two manuscripts.

Transgenic mice

The mouse has been used as a model system for a long time. But the use has dramatically increased over the last two decades due to the emergence of recombinant DNA technology. The introduction of exogenous DNA in the mouse genome was first made possible by viral infection and by microinjection of exogenous DNA into the pronulcei of mouse zygotes [210-211]. Since then, the so-called transgenic mouse has been used extensively for defining the role of different genes in development, health, and disease. For example; mice expressing human apoB and human isoforms of apoE are used for studying atherosclerosis development [212-213]. However the method of random integration of DNA and the use of exogenous promoter for expression makes it difficult to study the true impact of the manipulated gene. To overcome this limitation, gene targeting in mouse embryonic stem (ES) cells was developed. Gene targeting allows the modification of an endogenous gene within the ES cells by introducing a plasmid encoding a foreign DNA element that is targeted to the locus-of-interest by homologous recombination. Upon successful integration in the ES cell genome, the construct results in heterozygous targeted ES cells. The targeted ES cells are then injected into early embryo blastocysts and implanted into the uterus of a pseudopregnant female mouse. The pups born from this implantation are called chimeras. Male chimeras are bred and if the mutation is present in the germ line it can be transmitted to offspring, indefinitely. If a particular gene is modified by the introduction of a null mutation then the resultant mouse is called a 'knock out' mouse. The knockout mouse has allowed researchers to study the role of hundreds of genes involved in embryonic development and in health and disease.

Conditional gene targeting

The *Cre-lox*P system allows genetic manipulation of a mouse by direct recombination of genomic DNA. *Cre* recombinase is bacterial endonuclease (from bacteriophage P1) which recognizes and binds to specific *lox*P (locus of "x" crossing over) sites. The *lox*P sequence is a specific 34-base pair (bp) DNA sequence consisting of two 13-bp inverted repeats with one 8-bp spacer region in between [214]. *Cre* recombinase cleaves the DNA in the spacer regions of two *lox*P sites and the sequence in between that is "flanked by *lox*P sites" (floxed), is excised (Figure 4). In this study we used the *Cre-loxP* technique to knock out exon 7 of the GGTase-I β subunit [47] (Figure 5). Exon 7 of the *Pggt1b* gene, which contains the Zn^{2+}-binding residues essential for catalytic activity, was flanked by *lox*P sites and cloned

in to a gene targeting vector with long stretches of homology arms upstream and downstream.

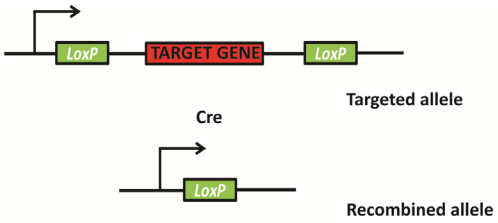

Figure 4. *Cre-loxP* gene targeting. The targeted gene is flanked by *lox*P sites. *Cre* recombinase recognizes and binds to *lox*P sites resulting in DNA recombination with elimination of the DNA sequence between the *lox*P sites.

After successful targeting and selection of the genetically modified clones, two chimeric mice (*Pggt1b*$^{fl/+}$) were generated which transmitted the mutation to the offspring.

To generate mice with macrophage-specific knockout of GGTase-I we bred *Pggt1b*$^{fl/fl}$ mice with mice expressing *Cre* recombinase under the control of lysozyme-M promoter. Mice from the resultant offspring have a knockout of exon 7 and completely lack GGTase-I activity in cells of myeloid origin. The macrophage-specific GGTase-I knockout mice are designated *Pggt1b*$^{\Delta/\Delta}$ in paper II and throughout this thesis; in paper I the mice are designated *Pggt1b*$^{flx/flx}$*LC*.

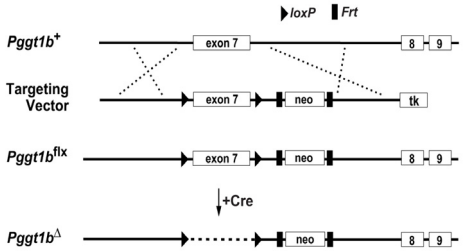

Figure 5. Targeting *Pggt1b*. A gene-targeting vector where exon 7 of the *Pggt1b* gene is flanked by *lox*P sites (*i.e., Pggt1b*^{fl}, "GGT-flox") was used to generate conditional *Pggt1b* knockout mice. Expression of *Cre* recombinase results in the excision of exon 7, which produces a null allele (*i.e., Pggt1b*$^{\Delta}$, "GGT-delta").

In paper I, we studied the impact of GGTase-I knockout in macrophage function during inflammation in *Pggt1b*$^{\Delta/\Delta}$ mice.

In paper II, we backcrossed *Pggt1b*$^{\Delta/\Delta}$ mice six times with C57Bl/6 inbred LDL receptor knockout mice, fed them a high-fat diet for up to 24 weeks, and studied the impact of GGTase-I knockout in macrophages on the development and progression of atherosclerosis.

SUMMARY OF RESULTS

Paper I: In this paper we studied the impact of inhibiting GGTase-I, using conditional gene targeting in a cell-type–specific manner on macrophage function during inflammation. We hypothesized that inhibiting GGTase-I in macrophages would reduce inflammation in mice. Contrary to our hypothesis, mice lacking GGTase-I in macrophages developed spontaneous erosive arthritis, RHO GTPases accumulated in active GTP-bound form, and macrophages produced high levels of proinflammatory molecules including cytokines and ROS.

GGTase-I deficiency induced spontaneous erosive arthritis in mice

GGTase-I deficiency in macrophages resulted in spontaneous erosive polyarthritis in mice (Figure 6A). The disease originated in the bone marrow (BM) and was transplantable in BM transplant experiments (Figure 6B). Depleting circulating monocytes with etoposide reduced joint inflammation by 70% and mean erosion score by 30 % (Figure 6C).

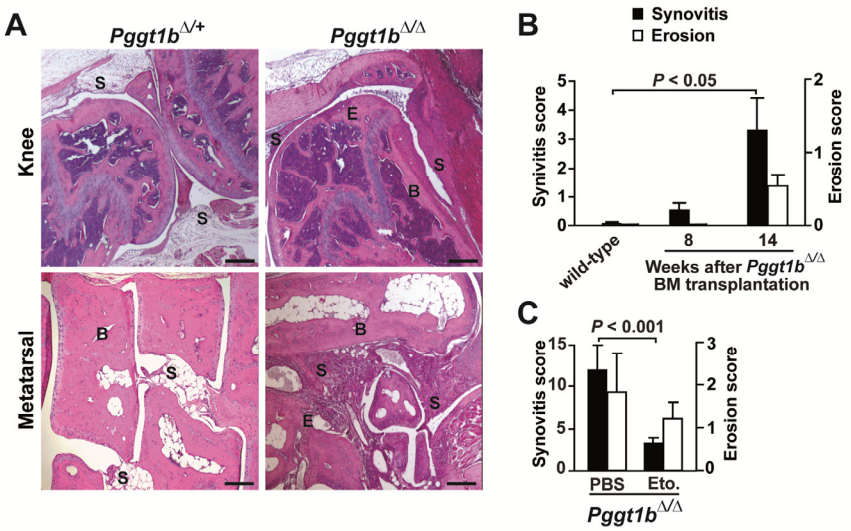

Figure 6. Knockout of GGTase-I in macrophages induces erosive arthritis in mice. (**A**) Hematoxylin and eosin–stained sections of joints from 12-week-old mice. S, synovium; B, bone; E, erosion. Scale bars: top panels, 400 µm; bottom panels, 200 µm. (**B**) Synovitis and bone erosion in joints of wild-type control mice and two groups of wild-type mice (*n* = 9–10/group) irradiated at 8 weeks-of-age and transplanted with *Pggt1b*$^{\Delta/\Delta}$ BM cells. (**C**) Synovitis and bone erosion in joints of 12-week-old *Pggt1b*$^{\Delta/\Delta}$ mice injected with etoposide (10 mg/kg, s.c., *n* = 14) or vehicle (PBS, *n* = 6) for 8 weeks.

GGTase-I deficiency causes accumulation of GTP-bound RAC1, CDC42 and RHOA

After establishing that GGTase-I deficiency is dispensable for macrophage viability, differentiation and function (paper I), we sought to identify the impact of GGTase-I knockout on RHO GTPase biology as prenylation is considered essential for the activity, stability, and membrane targeting of RHO proteins. First, we used affinity-precipitation to determine levels of active GTP-bound forms of RAC1, CDC42 and RHOA. To our surprise there was a robust accumulation of GTP-bound forms of all three isoforms (Figure 7A). To confirm that RHO GTPases in *Pggt1b*$^{\Delta/\Delta}$ macrophages were indeed non-prenylated – and not prenylated by FTase or by residual GGTase-I activity – we performed *in vitro* prenylation assays. In this assay, we used recombinant human GGTase-I and tritium-labeled geranylgeranylpyrophosphate (^{3}H-GGPP) and incubated them with cell extracts from *Pggt1b*$^{\Delta/\Delta}$ and control macrophages. In this assay, the non-prenylated RHO GTPases are covalently labeled with radioactive GGPP by the recombinant GGTase-I and can be visualized after Tris-HCL polyacrylamide gel electrophoresis (PAGE) and exposure to film. We observed robust total labeling and labeling of immunoprecipitated RAC1 and RHOA in extracts of *Pggt1b*$^{\Delta/\Delta}$ macrophages. No label was incorporated into proteins of control extracts because all the RHO proteins in those extracts had already prenylated by cellular GGTase-I (Figure 7B).

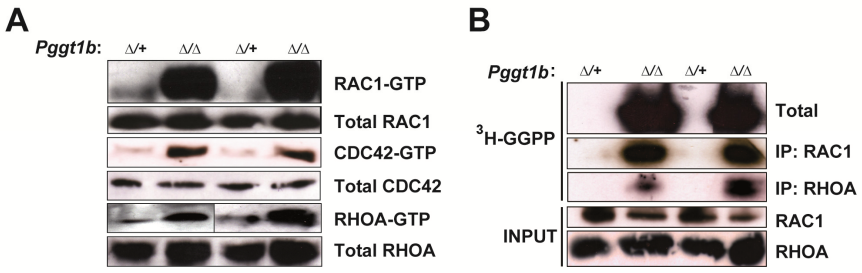

Figure 7. Knockout of GGTase-I results in accumulation of GTP-bound RHO family proteins. (**A**) Western blots showing the levels of GTP-bound and total RAC1, CDC42, and RHOA in lysates from BM macrophages. (**B**) Accumulation of unprocessed GGTase-I substrates susceptible to in vitro prenylation by ^{3}H-GGPP and recombinant GGTase-I. The experiment was repeated twice with similar results.

We next determined whether the increase in levels of GTP-bound RHO proteins had the potential to trigger an inflammatory cascade. *Pggt1b*$^{\Delta/\Delta}$ macrophages showed increased phospho-p38 MAPK and NF-κB activity and produced markedly increased levels of proinflammatory cytokines including TNFα, IL-1β and IL6 in response to LPS (Figure 8A–C).

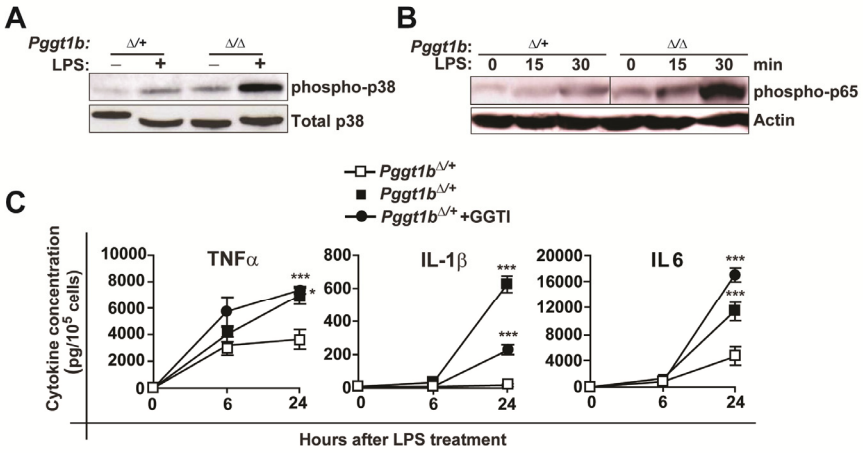

Figure 8. GGTase-I-deficient macrophages exhibit increased activation of pro-inflammatory signaling pathways. (**A**) Western blots of lysates from BM macrophages showing basal and LPS-stimulated phosphorylation of p38. (**B**) Western blot of lysates from basal and LPS-stimulated BM macrophages with an antibody to phosphorylated p65 subunit of NF-κB. Actin was used as a loading control. (**C**) Cytokine concentrations in medium of BM macrophages before and after LPS stimulation (n = 4–6/genotype assayed in duplicate). * $P < 0.05$, *** $P < 0.001$ (compared to *Pggt1b*$^{\Delta/+}$).

Blocking RAC1 reduced IL1-β and TNFα production and TNFα blocker reduced joint inflammation in mice

Pharmacological blocking of RAC1 reduced IL-1β and TNFα production in *Pggt1b*$^{\Delta/\Delta}$ macrophages (Figure 9A, B), confirming that RAC1 play a crucial role in the proinflammatory activities of macrophages. TNFα is a proinflammatory cytokine and together with IL-1β known to contribute to inflammatory arthritis. To show that uncontrolled TNFα production by *Pggt1b*$^{\Delta/\Delta}$ macrophages might be responsible for joint inflammation, we treated mice for eight weeks with the TNFα inhibitor Enbrel. Inhibiting TNFα in *Pggt1b*$^{\Delta/\Delta}$ mice reduced joint inflammation but had no effect on bone erosions (Figure 9C).

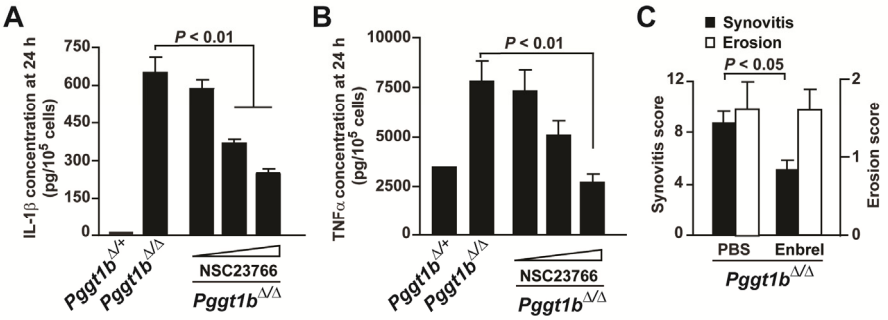

Figure 9. Inhibiting RAC1 reduces IL-1β and TNFα secretion of *Pggt1b*$^{\Delta/\Delta}$ BM macrophages and TNFα inhibition in vivo reduces joint inflammation. (**A, B**) Concentration of IL-1β (**A**) and TNFα (**B**) in medium of BM macrophages incubated with a RAC1 inhibitor (NSC23766; 10, 50, and 100 μM). (**C**) Synovitis and bone erosion in 20-week-old *Pggt1b*$^{\Delta/\Delta}$ mice treated with vehicle (PBS, *n* = 11) or Enbrel (Etanercept, 100 mg/kg/week, i.p., *n* = 9) for 8 weeks.

Paper II: In this paper we defined the role of inhibiting GGTase-I in macrophages on the development and progression of atherosclerosis. Here, we tested the hypothesis that the hyperactive *Pggt1b*$^{\Delta/\Delta}$ macrophages would worsen atherosclerosis development in mice. Surprisingly, *Pggt1b*$^{\Delta/\Delta}$ mice showed reduced atherosclerosis development despite increased local and systemic inflammation.

GGTase-I deficiency in macrophages reduced atherosclerosis in mice

Pggt1b$^{\Delta/\Delta}$ mice fed high-fat diet for 12 and 24 weeks had reduced lipid lesions compared to *WT* controls (Figure 10A and data not shown). Consistent with increased inflammation seen in chow diet-fed mixed background *Pggt1b*$^{\Delta/\Delta}$ mice, the number of T cells/mm^2 of aortic lesion was greater in mice fed high-fat diet. Moreover, *Pggt1b*$^{\Delta/\Delta}$ macrophages incubated with minimally modified LDL (mmLDL) produced exaggerated amounts of cytokines and chemokines (Figure 10B, C).

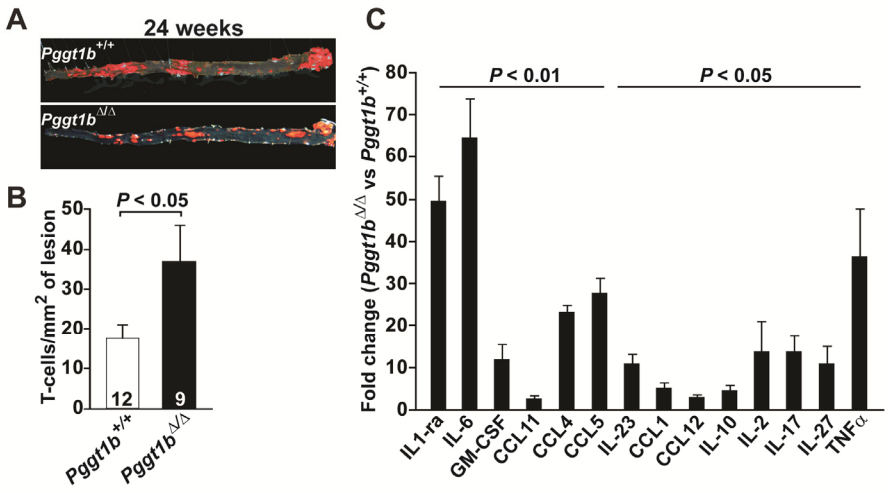

Figure 10. Knockout of macrophage GGTase-I reduces atherosclerosis development in *Ldlr*-deficient mice. (**A**) Sudan IV staining of lipid lesions in aortas of *Pggt1b*$^{+/+}$ and *Pggt1b*$^{\Delta/\Delta}$ mice fed a high-fat diet (HFD) for 24 weeks. (**B**) Quantification of Immunohistochemical analyses of the T cell composition in aortic root lesions of mice on high-fat diet for 12 weeks. (**C**) Levels of cytokines in medium of *Pggt1b*$^{+/+}$ and *Pggt1b*$^{\Delta/\Delta}$ IP macrophages (*n* = 5/genotype) incubated with mmLDL for 24 h.

GGTase-I deficiency reduced foam cell formation and increases cholesterol efflux in macrophages

We found that the reduced atherosclerosis in *Pggt1b*$^{\Delta/\Delta}$ mice was due to increased macrophage cholesterol efflux and reduced foam cell formation (Figure 10A). *Pggt1b*$^{\Delta/\Delta}$ macrophages exhibited a marked increase in basal and HDL-mediated cholesterol efflux (Figure 11A–C). ApoA1, another lipid acceptor, had a significant impact on cholesterol efflux in *Pggt1b*$^{\Delta/\Delta}$ macrophages, but the effect was less than with HDL. The increase in macrophage cholesterol efflux was regulated by RHOA: knockdown of RHOA, but not RAC1 and CDC42, using shRNA, significantly reduced cholesterol efflux (Figure 11D).

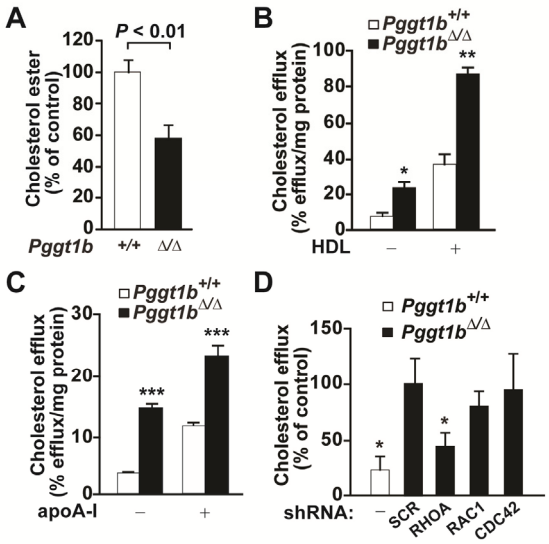

Figure 11. GGTase-I deficiency increases macrophages cholesterol efflux. (**A**) Macrophage cholesterol ester levels after a 36-h incubation with acLDL normalized to total cell protein content and expressed as percent of *Pggt1b*$^{+/+}$ (*n* = 7/genotype). (**B, C**) Basal and (**B**) apoA1- and (**C**) HDL-stimulated cholesterol efflux in BM, *n* = 4/genotype. (**D**) Basal cholesterol efflux in BM macrophages incubated with short hairpin RNA (shRNA) directed against RHOA, RAC1 and CDC42, and containing scrambled (SCR) sequence, *n* = 4-6. * *P* < 0.05, ** *P* < 0.01, and *** *P* < 0.001. (compared to *Pggt1b*$^{+/+}$).

DISCUSSION

In paper I we showed that inhibiting GGTase-I function in macrophages induced RA in mice. In paper II we showed that inhibiting GGTase-I in macrophages increased cholesterol efflux and reduced atherosclerosis development in mice. These studies demonstrate the biochemical and medical importance of inhibiting GGTase-I on macrophage function in the development and progression of inflammation-related disorders in mice and shed new light on important aspects of RHO GTPase biology, Our data challenge the widely-held view regarding *CAAX* protein biology which holds prenylation as important for RHO protein activation.

Impact of knocking out GGTase-I on macrophage function

Macrophages are considered crucial to inflammation [156]. In this thesis I evaluated the role of GGTase-I in macrophage viability and differentiation and function during inflammation. I showed that *Pggt1b*$^{\Delta/\Delta}$ macrophages are viable but less in number after 7 days in culture compared to controls (paper I). Also, *Pggt1b*$^{\Delta/\Delta}$ macrophages were small and rounded and exhibited increased production of proinflammatory molecules including cytokines and ROS. Surprisingly, inhibiting GGTase-I in macrophages not only increased levels of active GTP-bound RHO proteins including RAC1, CDC42 and RHOA but also stimulated the NF-κB activity via phosphorylation of p38 MAPK. Rheumatoid arthritis is initiated by T cells, and macrophages are only considered as effector cells in the pathogenesis of joint inflammation [182, 189]. However, our data challenge that widely held view and demonstrated that macrophages can initiate RA in *Pggt1b*$^{\Delta/\Delta}$ mice. Our finding was supported by the facts that *Pggt1b*$^{\Delta/\Delta}$ macrophages produced exaggerated amount of proinflammatory cytokines and ROS; the phenotypes of joint inflammation and bone erosions were transplantable in BM transplantation experiments; depletion of monocytes in *Pggt1b*$^{\Delta/\Delta}$ mice by etoposoid treatment blocked the progression of arthritis; and inhibiting TNFα, a major macrophage cytokine – by Enbrel treatment reduced joint inflammation in *Pggt1b*$^{\Delta/\Delta}$ mice.

Together with this, targeting GGTase-I in macrophages resulted in reduced atherosclerosis development in mice. *Pggt1b*$^{\Delta/\Delta}$ mice fed high fat diet for 12 and 24 weeks had reduced lipid lesions. Atherosclerosis is a chronic inflammatory disease and inflammation plays crucial role during different phases of atherosclerosis development. The smaller lesions in *Pggt1b*$^{\Delta/\Delta}$ mice challenge this view for many reasons. First, *Pggt1b*$^{\Delta/\Delta}$ macrophages were hyperactive and produced exaggerated amount of proinflammatory molecules which had the potential of accelerating the development of atherosclerosis. Second, the increased infiltration of $CD4^+$ and $CD8^+$ T cells in the smaller aortic lesions of *Pggt1b*$^{\Delta/\Delta}$ mice are consistent with increased inflammation. Finally, increased plasma CXCL1, a proinflammatory cytokine, and reduced IL-10, an anti-inflammatory cytokine, in *Pggt1b*$^{\Delta/\Delta}$ mice strengthens the finding that GGTase-I depletion in macrophages increased both local and systemic inflammation. GGTase-I deficient macrophages

showed four-to-five fold basal and HDL-mediated cholesterol efflux compared to controls. This increased efflux was mediated by a COX2-regulated increase in PPARγ activity. PPARγ regulates cholesterol efflux by either increasing LXRα-mediated expression of ABCA1 and ABCG1; or by directly increasing the expression of class B scavenger receptors CD36 and SR-B1 [215-218]. However in the case of *Pggt1b*$^{\Delta/\Delta}$ macrophages, the increased efflux was likely mediated by a PPARγ-mediated increase in CD36 and SR-B1 as HDL-stimulated efflux was significantly greater compared to apoA1-stimulated efflux. Consistent with that ABCA1 and ABCG1 expression in *Pggt1b*$^{\Delta/\Delta}$ macrophages remained unchanged.

Impact of knocking out GGTase-I on RHO protein function and localization

We showed that inhibiting GGTase-I in macrophages caused sustained activation of RHO GTPases RAC1, CDC42 and RHOA and that non-prenylated RAC1 is localized to the plasma membrane (paper I). Previous studies have shown that prenylation is important for RHO GTPase function and proper membrane targeting [219-221]. Some studies have shown that non-prenylated RAC1 is localized to the nucleus [26]. Our findings significantly differ from those studies. One potential reason is that before the development of the GGTase-I knockout allele, many studies used pharmacological inhibition of geranylgeranylation either by statins or GGTIs. But statins also block the synthesis of FPP and might affect farnesylated proteins as well as the geranylgeranylation of RAB GTPases. Moreover, GGTIs might have off-target effects. Apart from that, cell type and culture conditions used in those experiments might also explain the divergent results as well as the use of exogenously overexpressed proteins. For example, some studies have used GFP-tagged non-prenylated RAC1 mutant (RAC1-SAAX) where the cysteine in the *CAAX* box of RAC1 is replaced with serine by site-directed-mutagenesis, so RAC1 can no longer be prenylated. Those studies then used GFP-tagged RAC1-SAAX constructs for tracking the localization of non-prenylated RAC1. It is well known that GFP alone is localized to the nucleus and might not be suitable for studying the subcellular localization of *CAAX* proteins [222]. Regardless of the differences, we only analyzed endogenous GGTase-I substrates in these studies.

We evaluated the GTP-binding ability of RHO GTPases and their ability to interact with the main inhibitor RHO-GDI in the *Pggt1b*$^{\Delta/\Delta}$ macrophages. First, we affinity-purified active forms of RAC1, CDC42 and RHOA from *Pggt1b*$^{\Delta/\Delta}$ cell lysates and showed that despite little or no differences in total protein levels, RHO GTPases accumulated in GTP-bound active form in the GGTase-I deficient macrophages. Second, RHO GTPases are held in a soluble complex in cytosol by RHO-GDI which inhibits conversion of inactive GDP-bound to active GTP-bound states [52]. Our data showed that although both RAC1 and CDC42 bind less efficiently to RHO-GDI, the RHOA interaction with RHO-GDI was not affected. At steady state levels within a cell, RHO-GDI concentration is rate limiting and RHOA, RAC1 and CDC42 compete for RHO-GDI binding [30]. Therefore under the settings of less RAC1 and CDC42 binding, RHO-GDI can efficiently bind to non-prenylated RHOA. Third, we showed

that non-prenylated RAC1 is localized to the plasma membrane. Indeed, the GTP loading of RAC1 increases its affinity for the plasma membrane [223]. However, previous studies have demonstrated that blocking protein prenylation causes *CAAX* proteins to be mislocalized from the plasma membrane [219-221]. We showed both by immunoflourscence and by western blotting of membrane, cytosol and nuclear extracts that non-prenylated RAC1 is found in the plasma membrane in *Pggt1b*$^{\Delta/\Delta}$ macrophages. Recently one interesting study showed reversible palmitoylation of RAC1 which is required for its membrane organization; also RAC1 has a strong polybasic region which is usually considered essential for membrane targeting as mutations in the polybasic region mislocalize RAC1 away from plasma membrane [224-225]. Whether palmitoylation of non-prenylated RAC1 targets it to the plasma membrane is yet to be determined however, we believe that the strong polybasic region of RAC1 might be enough for membrane targeting. Fourth, to exclude the possibility of residual GGTase-I activity or alternate prenylation by FTase we performed *in vitro* prenylation of RHO GTPases. Indeed, RHO proteins including RAC1 and RHOA accumulated in non-prenylated form in *Pggt1b*$^{\Delta/\Delta}$ macrophages (*i.e.*, they could be prenylated in vitro by recombinant GGTase-I). Our data therefore provide strong evidence that prenylation is not required for the activation and membrane targeting of RHO GTPases, at least not in macrophages.

Which RHO proteins are involved in the phenotypes seen in GGTase-I-deficient macrophages?

RHO GTPases are involved in many cellular functions. For example, RAC GTPases regulate the NF-κB activity and ROS production via PAK1 and p38-MAPK [78, 220, 226]. But the fact that inhibiting RHO protein prenylation would activate this pathway was not known before. We showed that sustained activation of RAC1 in *Pggt1b*$^{\Delta/\Delta}$ macrophages led to increased p38-MAPK–NF-κB pathway signaling. Inhibiting RAC1 activity using a RAC1-specific shRNA or a pharmacological inhibitor, NSC23766, reduced the production of proinflammatory cytokines in LPS-treated *Pggt1b*$^{\Delta/\Delta}$ macrophages. Consistent with this, an inhibitor of PAK1 (PAK18) – an immediate downstream effector of RAC1 also dose-dependently reduced TNFα and IL-1β production in *Pggt1b*$^{\Delta/\Delta}$ macrophages. Increased cytokine production by macrophages and cells of the adaptive immunity drives different phases of arthritis, TNFα is a cytokine mainly produced by macrophages which is crucial for inflammatory activities of macrophages and lymphocytes. Indeed, blocking TNFα has shown promise in reducing inflammatory arthritis [227-238]. Our data suggest that this TNFα production by *Pggt1b*$^{\Delta/\Delta}$ macrophages partly drive the disease phenotypes in *Pggt1b*$^{\Delta/\Delta}$ mice. Indeed, TNFα inhibition in *Pggt1b*$^{\Delta/\Delta}$ mice treated with enbrel significantly reduced joint inflammation but had no effect on bone erosion. However, TNFα is also produced by other cells of the innate (neutrophils and dendritic cells) and adaptive immune system (T and B cells) albeit to a lower extent than macrophages. Nevertheless, at this stage it is impossible to rule out the contribution of TNFα produced by those cells in disease phenotypes of *Pggt1b*$^{\Delta/\Delta}$ mice.

Previous studies have suggested that inhibiting RHO protein activation by inhibiting their prenylation, either by statins or by GGTIs increase macrophage cholesterol efflux [70, 217]. Our data disagree with those studies and demonstrated that active GTP-bound RHOA is responsible for the increased macrophage cholesterol efflux. First, active GTP-bound RHOA accumulated in the absence of GGTase-I in macrophages. Second, a shRNA targeted against RHOA reduced macrophage cholesterol efflux. And third, RHOA targeting but not RAC1 reduced the mRNA levels of *Lxra*, *PPARg*, *CD36* and *Scarb1* – all of which contribute to macrophage cholesterol efflux [215-218].

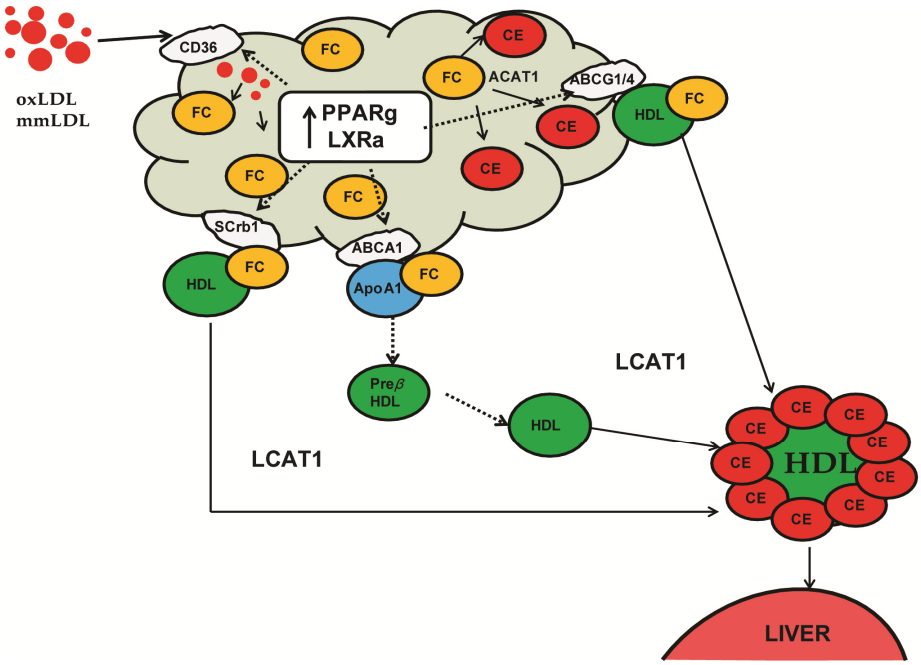

Figure 12. Macrophage cholesterol efflux and reverse cholesterol transport. Modified LDL particles (oxLDL, mmLDL) taken up by macrophages are either converted to free (FC) or esterified cholesterol (CE) by acetyl-CoA acyltransferase-1 (ACAT1) which results in foam cell formation. PPARγ opposes this effect by increasing cholesterol efflux by upregulating the gene expression of LXRα, CD36, SR-B1 and ATP-binding cassette transporters, ABCA1 and ABCG1. FC is effluxed via different mechanisms, **1)** Through ABCA1 by binding to apolipoprotein-A1 **2)** by interacting with HDL particles bound either to SR-B1 or ABCG1 or **3)** it is desorbed through the plasma membrane to an exogeneous lipid acceptor. The effluxed cholesterol is loaded on HDL, esterified by lecithin-cholesterol acyl transferase 1 (LCAT1) and transported to the liver for further processing.

Uncoupling of inflammation and atherosclerosis in *Pggt1b*$^{\Delta/\Delta}$ mice

Inflammation and lipid retention are closely coupled during different phases of atherosclerosis development [136-138]. Our findings provided a new insight on atherosclerosis development in mice. We demonstrated that reducing foam cell formation by increase in macrophage cholesterol efflux can dramatically reduce the development of atherosclerotic lesions in mice even in the presence of massive ongoing inflammation. In this study, I have identified a unique mechanism where macrophages despite their proinflammatory activities showed increased cholesterol efflux and reduced foam cell formation which was accountable for reduced subendothelial lipid lesions in *Pggt1b*$^{\Delta/\Delta}$ mice. We believe that reduced lipid lesions observed in *Pggt1b*$^{\Delta/\Delta}$ mice were due to increased macrophage cholesterol efflux which leads to efficient reverse cholesterol transport (RCT). RCT is a process where cholesterol from macrophages and peripheral tissues is made available to liver for excretion by high density lipoprotein (HDL). Indeed, increase HDL levels inversely correlate with atherosclerosis like CVDs (Figure 12) [239] and therapies targeting macrophage foam cell formation, and increasing macrophage cholesterol efflux and plasma HDL are considered atheroprotective [239-245]. One important mediator of cholesterol metabolism is nuclear receptor PPARγ which regulates the transcription of several genes involved in cholesterol transport including *LXRα*, *ABCA1*, *ABCG1*, *CD36* and *SCARB1* and enhances the macrophages cholesterol efflux [215, 217-218, 246-247]. Our data demonstrated that *Pggt1b*$^{\Delta/\Delta}$ macrophages had increased PPARγ activity and increased mRNA and protein levels of COX2, PPARγ, LXRα, CD36 and SR-B1. Increased COX2-PPARγ-CDC36-SR-B1 might be involved in increased macrophage cholesterol efflux. Consistent with those findings *Pggt1b*$^{\Delta/\Delta}$ mice had reduced plasma cholesterol and triglyceride levels and slightly reduced LDL levels on plasma cholesterol profile.

Thus, our data suggest that macrophage cholesterol efflux is uncoupled to its inflammatory activities and that this can be atheroprotective even in the setting of local and systemic inflammation.

Perspective

In paper I, our findings provided a very strong link between non-prenylated active GTP-bound RAC1 and increased cytokine production by *Pggt1b*$^{\Delta/\Delta}$ macrophages leading to inflammatory arthritis in mice. These surprising results raise an array of new questions: Is geranylgeranylation really dispensable for RHO protein function in all cell types? If so, why is this pathway conserved throughout evolution? How does non-prenylated RAC1 bind to membranes? Is the purpose of prenylation to inhibit rather activate RHO protein activity? Could non-prenylated RAC1 alone trigger rheumatoid arthritis development? Is non-prenylated/hyperactive RAC1 involved in RA in humans? Could targeted therapies against RAC1 be beneficial in reducing arthritis and related inflammatory disorders?

DISCUSSION

In paper II we propose a new mechanism by which active GTP-bound RHOA stimulates macrophage cholesterol efflux via increased signaling through the COX2-PPARγ-CD36-SR-B1 pathway. The increase in macrophage cholesterol efflux was potentially responsible for reduced lipid lesions in *Pggt1b*$^{\Delta/\Delta}$ mice despite the fact that these mice had massive ongoing joint inflammation. These results have clinical implications and raise several new questions: Is inflammation secondary to lipid retention and macrophage foam cell formation during the development of atherosclerosis? Are inflammation and foam cell formation completely dissociated processes? Might pharmacological activation of RHOA stimulate reverse cholesterol transport and affect atherosclerosis development in humans?

CONCLUSIONS

These studies allowed me to answer several important questions regarding the role of GGTase-I activity in macrophage function during inflammation and to shed new light on the biochemical and medical importance of this posttranslational processing step.

I can now conclude that:

- GGTase-I deficiency causes hyperactive macrophages and spontaneous erosive arthritis in mice.

- GGTase-I is dispensable for macrophage viability and function.

- Knockout of GGTase-I in macrophages causes sustained activation of RAC1, CDC42 and RHOA, suggesting that one function of RHO protein prenylation is to inhibit their activity, at least in macrophages.

- GGTase-I deficiency increases macrophage cholesterol efflux and reduces atherosclerosis in mice.

- RHOA activation increases COX2-PPARγ-regulated cholesterol efflux in macrophages which is atheroprotective in mice despite the underlying local and systemic inflammation.

ACKNOWLEDGMENTS

I would want to thank my main supervisor **Martin Bergo** who has had a great impact on my personality and work ethics. Martin's contribution in my development as a scientist is extremely valuable, irreplaceable and hard to put in words. In short, I consider Martin as second to my father and always look upon him as a role model of work ethics. Without Martin it would have been extremely difficult for me to be where I am today both as a person and as a scientist.

Bergo lab: I would like to thank **Christin Karlsson** who helped me with basically everything including projects and work-related matters. I will remember **Anna-Karin Sjogren** for being a great colleague and help with setting up experiments. A special thanks to **Mohamed Ibrahim** for helping me in many different experiments and to **Martin Dalin** for stimulating discussions and new ideas. Thanks to **Murali**, my exceptionally outstanding master student for his help on my projects. Thank you **Frida Olofsson** and **Frida Larsson** for help with mouse breeding. I also thank **Jaroslaw, Volkan,** and **Anna** for raising important questions and giving constructive criticism on my projects during lab meetings and **Meng Liu**, **Karin Andersson**, **Annika Wahlström**, and **Briony Cutts** and all former members of our lab who were great workmates.

I would like to thank **Maria Bokarewa** for being an outstanding collaborator and for helping me on my projects and **Ing-Marie Jonsson** and **Mikael Brisslert** from Maria's Lab for help with countless experiments.

I would like to sincerely thank my co-supervisor **Jan Borén** for an extremely stimulating collaboration on the atherosclerosis project and people from his group especially **Kristina Skålen** who taught me everything about mouse athero studies but also **Pernilla Jirholt** and **Maria Hayden** for helping with mouse breeding, IHC and cell culture.

People at wlab and CCS: I would like to thank **Galia Nystöm** for very valuable help with FACS and **Heidi Hedman** my twin (blond) sister for a wonderful time at wlab. Thanks also to **Lillemor M-H.** and **Noor** for help with experiments and culturing human primary macrophages.

My friends: I would like to thank my close friends specially **Bjarni** and **Arbab** for always being there for me and helping me through difficult times in life and making me feel home. Some close friends in Pakistan who always motivated me (**Fawad, Haris**, and **Khalid**)

In the end I would like to thank **my family** for their support and understanding and inspiring me to achieve one of the biggest goals of my life. My **dad** has always been an inspiration and gave me the never-give-up attitude. I owe special thanks to my **mom** for waking up the in the middle of the nights to pray for my success and well being. I want to thank my brothers and sister for their support all the way through.

In the end, I would like to thank all the people who helped me to write and proofread this thesis.

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