Defining the importance of protein geranylgeranylation in innate immunity

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Dedicated to my grandfather

ABSTRACT

RHO family proteins and other intracellular proteins are prenylated with a 20-carbon lipid—a product of the cholesterol synthesis pathway—by protein geranylgeranyltransferase type I (GGTase-I). Prenylation is widely believed to target proteins to membranes where they encounter effector molecules that stimulate GTP-binding and activation. However, my host group found that knockout of GGTase-I in mouse macrophages (*Pggt1b*$^{\Delta/\Delta}$) actually increases GTP-loading of RHO proteins such as RAC1, RHOA, and CDC42, and also increases proinflammatory signaling and cytokine production, and induces severe rheumatoid arthritis. These results suggest that prenylation may inhibit rather than stimulate RHO protein function. The mechanisms underlying increased GTP-loading and exaggerated innate immune responses in the absence of GGTase-I are not known. During my PhD, I have addressed these issues in two independent but interconnected projects.

In project 1, we found that there is an imbalance between inflammatory and anti-inflammatory cytokines produced by *Pggt1b*$^{\Delta/\Delta}$ macrophages. We also found that knockout of GGTase-I prevents the interaction between KRAS and PI3K catalytic subunit p110δ and that this reduces signalling through the PI3K-AKT-GSK3β pathway. Moreover, *Pggt1b*$^{\Delta/\Delta}$ macrophages exhibit increased caspase-1 activity that is directly responsible for the production of active interleukin IL-1β, and that this effect requires the *MEFV* (pyrin) inflammasome. Thus, we conclude that GGTase-I promotes an association between KRAS and p110δ and thereby controls major inflammatory pathways in macrophages.

In project 2, we tested the importance of RHO proteins in the development of arthritis in *Pggt1b*$^{\Delta/\Delta}$ mice. We found that knockout of *Rac1* (*i.e.*, in *Pggt1b*$^{\Delta/\Delta}$*Rac1*$^{\Delta/+}$ mice), but not *Rhoa* and *Cdc42*, markedly reduced inflammatory cytokine production and arthritis in *Pggt1b*$^{\Delta/\Delta}$ mice. We also found that non-prenylated RAC1 bound more strongly to the RAS GTPase-activating-like protein 1 (IQGAP1) – which facilitated RAC1 GTP-loading and activation. Knockout of *Iqgap1* in *Pggt1b*$^{\Delta/\Delta}$ mice abolished cellular phenotypes *in vitro* and inhibited arthritis *in vivo*. Thus, we conclude that blocking prenylation stimulates RAC1 effector interactions and activates wide-spread pro-inflammatory signaling. Thus, prenylation normally restrains innate immune responses by inhibiting RAC1 effector interactions.

Keywords: *CAAX* proteins, GGTase-I, RAC1. **ISBN:** 978-91-7833-233-5

SAMMANFATTNING

Enzymet geranylgeranyltransferas typ I (GGTase-I) kopplar på en fettmolekyl på ett 100-tal proteiner inne i celler i en process som kallas prenylering. En klass proteiner som prenyleras heter RHO-proteiner. RHO-proteiner är viktiga för funktionen hos inflammatoriska celler som aktiveras vid infektioner och skador. Man har länge tänkt att prenylering gör att RHO-proteinerna lättare kan binda till membran i cellen där de kommer i kontakt med proteiner som aktiverar RHO-proteinerna. När vi först studerade detta fann vi att om man knockar ut genen som kodar för GGTase-I i makrofager hos möss så hindras modifieringen av RHO-proteiner, som förväntat, men istället för att inaktiveras så ansamlade sig RHO-proteinerna i sin aktiva form; och makrofagerna blev hyperaktiva och orsakade inflammation och ledgångsreumatism. Vi är också intresserade av hur de kolesterolsänkande statinerna kan aktivera RHO-proteiner och stimulera produktion av inflammatoriska substanser. När statinerna hämmar kolesterolsyntesen så hämmas också produktionen av den fettmolekyl som kopplas på RHO-proteinerna. I denna avhandling har jag försökt svara på dessa frågor.

I det första projektet, fann vi att i makrofager som saknar GGTase-I aktiverar pyrin-inflammasomen och caspas-1 som leder till produktion av en inflammationsdrivande substans som heter interleukin 1-beta (IL-1β). I det andra projektet fann vi att RHO-proteinet RAC1 ensamt ansvarar för utvecklingen av inflammation och reumatism i mössen. Vi fann vidare att när RAC1 inte prenyleras, så blir det hyperaktivt på grund av att det får en kraftigt ökad förmåga att binda till proteinerna TIAM1 och IQGAP1 vilka stimulerar signalering till proteinerna NF-kB och som tidigare, till inflammasomen och caspas-1. För att bevisa inblandningen av RAC1 och IQGAP1 så knockade vi först ut genen för RAC1 och fann att inflammationen och reumatismen så gott som försvann; och när vi knockade ut genen för IQGAP1 fann vi att RAC1 (och övriga RHO-proteiner) återfick normal-aktivitet och att inflammationen och reumatismen botades. Vi fann också att statiner hämmar prenylering av RAC1 och ökar produktionen av IL-1β och att denna effekt beror av IQGAP1. Från dessa studier kan vi dra slutsatsen att när prenylering hämmas, så binder RAC1 till IQGAP1 och TIAM1, blir hyperaktivt och orsakar sen massiv inflammation. Detta betyder i sin tur att prenylering normalt sett fungerar som en broms för immunförsvaret genom att hämma RAC1-aktivering. I ett bredare perspektiv leder våra resultat till en ökad förståelse för prenylering i sig, men vi identifierar också IQGAP1, och till viss mån även RAC1, som nya potentiella måltavlor för läkemedel som kan användas för behandling av en ovanlig men allvarlig autoinflammatorisk sjukdom som heter mevalonate kinase deficiency (MKD).

List of papers

1. **Akula MK,** Shi M, Jiang Z, Foster CE, Miao D, Li AS, Zhang X, Gavin RM, Forde SD, Germain G, Carpenter S, Rosadini CV, Gritsman K, Chae JJ, Hampton R, Silverman N, Gravallese EM, Kagan JC, Fitzgerald KA, Kastner DL, Golenbock DT, Bergo MO, and Wang D. Control of the innate immune response by the mevalonate pathway. *Nature Immunology*. 2016; **17**: 922–929.

2. **Akula MK,** Ibrahim MX, Khan OM, Kumar TI, Erlandsson MC, Karlsson C, Xu XF, Brisslert M, Brakebusch CH, Bokarewa M, Wang D, and Bergo MO. Protein prenylation restrains innate immunity by limiting RAC1 effector interactions. *Submitted.*

Additional publications not included in this thesis

3. Le Gal K, Ibrahim MX, Wiel C, Sayin VI, **Akula MK,** Karlsson C, Dalin MG, Akyurek LM, Lindahl P, Nilsson J, and Bergo MO. Antioxidants can increase melanoma metastasis in mice. *Sci. Transl. Med.* 2015; **7**: 308re8.

4. Ibrahim MX, Sayin VI, **Akula MK,** Liu M, Fong LG, Young SG, and Bergo MO. Targeting isoprenylcysteine methylation ameliorates disease in a mouse model of progeria. *Science.* 2013; **340**: 1330–1333.

5. Khan OM, **Akula MK,** Skalen K, Karlsson C, Stahlman M, Young SG, Boren J, and Bergo MO. Targeting GGTase-I activates RHOA, increases macrophage reverse cholesterol transport and reduces atherosclerosis in mice. *Circulation.* 2013; **127**: 782–790.

CONTENTS

1 ABBREVIATIONS

AIM2	Absent in melanoma 2
AT1	Angiotensin type 1
CDC42	Cell division control protein 42 homolog
DAMP	Danger associated molecular patterns
eNOs	endothelial nitrous oxide synthase
FPP	Farnesyl pyrophosphate
FTase	Farnesyltransferase
FTI	farnesyltransferase inhibitor
GAP	GTPase activating protein
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factor
GGPP	Geranylgeranyl pyrophosphate
GGTase-I	Geranylgeranyltransferase type I
GGTI	Geranylgeranyltransferase type I inhibitor
GTP	Guanosine triphosphate
GTPase	Guanosine tri-phosphatase
GRD	GTPase activation protein-related domain
HIDS	Hyper-IgD syndrome

HMG-COA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
ICMT	Isoprenylcysteine carboxyl methyltransferase
IQGAP1	Ras GTPase-activating-like protein IQGAP1
IQGAP2	Ras GTPase-activating-like protein IQGAP2
JNK	c-Jun N-terminal kinases
LDL	Low-density lipoprotein
MAPK	mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MEFV	Pyrin
MKD	Mevalonate kinase deficiency
MS	Multiple sclerosis
MMP13	Matrix metallopeptidase 13
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP1	NACHT, LRR and PYD domains-containing protein 1
NLRC4	NLR family CARD domain-containing protein 4
PAK1	P21 activating kinase
PAMP	Pathogen associated molecular patterns
PDEδ	Phosphodiesterase-δ
PI3K	Phosphoinositol-3-kinase

PRR	Pathogen recognised receptors
RA	Rheumatoid arthritis
RAC1	Ras-related C3 botulinum toxin substrate 1
RAC2	Ras-related C3 botulinum toxin substrate 2
RAC3	Ras-related C3 botulinum toxin substrate 3
RCE1	Ras-converting enzyme 1
RHOA	Ras homolog gene family, member A
RHOGDI	RHO protein GDP dissociation inhibitors
ROS	Reactive oxygen species
ROCK1/2	RHO associated coiled-coil-containing protein kinase 1 and 2
SLE	Systemic lupus erythematous therapy
SmgGDS	small G-protein dissociation stimulator
TIAM1	T-cell lymphoma invasion and metastasis-inducing protein 1
ZMPSTE24	zinc metalloproteinase Ste24 homologue

2 INTRODUCTION

CAAX-PROTEINS

CAAX proteins are a group of proteins that contain a *CAAX* sequence at the carboxyl-terminal end where *C* is a cysteine; *A* are aliphatic amino acids; and *X* can vary. *CAAX* proteins undergo a three-step post-translational modification process: First, the *CAAX*-motif of a protein is recognized by either of two enzymes called geranylgeranyltransferase-I (GGTase-I) or farnesyltransferase (FTase) which are responsible for transferring a C_{20} geranylgeranyl lipid or a C_{15} farnesyl lipid (prenyl group) to the cysteine residue of the *CAAX*-motif, respectively. This process is collectively called protein prenylation. Second, the endoprotease RAS-converting enzyme (RCE1) cleaves off the terminal *–AAX* sequence. And third, the newly-exposed isoprenylcysteine residue is methyl-esterified by isoprenylcysteine carboxyl methyltransferase (ICMT) (Fig. 1). Prenylation is believed to be essential for proper function of *CAAX* proteins because it stimulates membrane targeting, interaction with effector proteins, and activation (1). Moreover, these modifications help to decide the localisation of proteins to specific parts of the cell, improving protein-protein interactions and modulating protein stability.

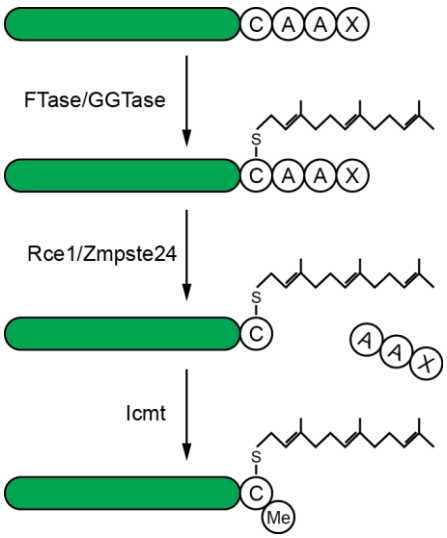

Fig. 1. Posttranslational modification of *CAAX* proteins. The cysteine residue of the *CAAX* box is prenylated by GGTase-I or FTase; the *–AAX* tripeptide is cleaved off by RCE1, and the newly-formed prenylcysteine residue is methylated by ICMT. (Picutre: Emil Ivarsson)

In the last three decades, prenylation has generated a broad interest in the research community due to the involvement of *CAAX* proteins in the pathophysiology of various diseases. Progeria is a well-known example of one such disease, where the toxic accumulation of prenylated prelamin-A drives the disease (2, 3), and where inhibitors of FTase and recently also ICMT are tested as therapeutic options. Cancer is another example; it has been found that prenylated RAS proteins are involved in the pathogenesis of at least 30% human cancers. Lots of research has focused on inhibiting CAAX protein processing enzymes as a strategy to block the activity of oncogenic RAS (4).

The history of protein prenylation

In the genome, there are several hundred proteins that terminate with a *CAAX* sequence. At least 100 of those are predicted to be farnesylated or geranylgeranylated (5-8). The first evidence of protein prenylation was discovered in the late 1970s, when the fungal mating factor rhodotorucine A was found to contain a farnesyl lipid attached to a cysteine residue close to the carboxyl terminus (9). More evidence of protein prenylation emerged in studies on statins, a group of drugs that inhibit HMG-CoA (3-hydroxy-3-methyl-glutaryl-Coenzyme A) reductase—the enzyme which catalyzes the committed step in cholesterol biosynthesis (10). A key finding came in a study where statin-induced proliferation arrest was not rescued by addition of sterols, which suggested that some intermediary by-products in the cholesterol pathway are involved in controlling cell proliferation (11, 12). In support of that, further evidence emerged in ^{3}H-mevalonate-labelling experiments in cells lacking HMG-COA reductase, in which researchers found incorporation of ^{3}H-mevalonate in cellular proteins, suggesting that these proteins were prenylated (13). Later, a nuclear protein Lamin B (a nuclear lamina protein) was discovered as the first prenylated protein in mammals, but it was not known at the time which prenyl moiety was attached (14). Finally, the interest in protein prenylation began to attract worldwide attention when it was discovered that RAS proteins are farnesylated, and that farnesylation was essential for the ability of mutant RAS to localize to the plasma membrane, interact with RAF and transform cells (15-17). Due to this finding, an intense race to develop farnesyltransferase inhibitors started.

Prenylation by GGTase-I and FTase-I

As outlined earlier, the isoprenoids used by GGTase-I and FTase are the cholesterol biosynthetic intermediates farnesyl pyrophosphate (FPP, 15 carbons) and geranylgeranyl pyrophosphate (GGPP, 20 carbons) produced from isopentenyl diphosphate. FTase and GGTase-I enzymatic activity involves attaching an FPP or a GGPP molecule, respectively, to the cysteine residue of the *CAAX* motif. The attachment is made with a thioether linkage (18). Both FTase and GGTase-I are comprised of heterodimers, and they reside in the cytosol. They share a common alpha subunit (encoded by the gene *FNTA*) but have distinct catalytic beta subunits

(encoded by *FNTB* and *PGGT1B*), which are vital to performing their functions (19-21).

The question of what factors determine whether a *CAAX* protein will undergo farnesylation or geranylgeranylation, received much attention in the early nineties. The research led up to the understanding that it is the `*X*` residue in the *CAAX* motif that dictates if the protein is farnesylated or geranylgeranylated (22-25). More specifically, *CAAX* proteins become farnesylated if the *X* residue is Methionine (M), Serine (S), Glutamine (Q), or Alanine (A); whereas proteins become geranylgeranylated by GGTase-I if `X` is either Leucine (L) or phenylalanine (F) (25). HRAS, KRAS, NRAS, prelamin A, and lamin B are the most studied substrates for FTase, whereas small GTP-binding proteins like RAS homolog gene family, member A (RHOA), Ras-related C3 botulinum toxin substrate 1 (RAC1), and Cell division control protein 42 homolog (CDC42) are the most studied substrates for GGTase-I (5, 23, 26). Although both enzymes are highly specific towards their target proteins, in some cases they compensate each other via cross-prenylation or alternative prenylation. Examples of this are KRAS and NRAS, two well-known isoforms of RAS are normally farnesylated, but studies found that those two isoforms are geranylgeranylated by GGTase-I in cells where FTase is inhibited (27). This cross prenylation phenomenon has been proposed as an explanation of how RAS-driven cancers manage to deal with farnesyltransferase inhibitor (FTI) treatment (28). This idea was also proven with genetic experiments in mouse models (29, 30).

The regulation of prenylation is not fully understood, but recent studies have identified interesting mechanisms that regulate prenylation through splice variants of SmgGDS (small G-protein dissociation stimulator), which is a nucleotide exchange factors specific for *CAAX* proteins that contain carboxyl-terminal polybasic region. They have been found to regulate the entrance and passage of *CAAX* proteins through the prenylation pathway (31). SmgGDS-558 and SmgGDS-607 are two splice variants that are involved in the regulation of prenylation by recognizing the *CAAX* sequence. SmgGDS-558 forms a complex with geranylgeranylated *CAAX* proteins that are processed by GGTase-I, while SmgGDS-607 associates with unprenylated *CAAX* proteins that eventually become geranylgeranylated by GGTase-I. These two then regulate trafficking of the *CAAX*

proteins to the plasma membrane (31, 32). This novel mechanism depicts one facet of how prenylation regulates the handling and trafficking of *CAAX* proteins to the plasma membrane.

Post-Prenylation processing by RCE1 and ICMT

After prenylation, the *CAAX* proteins are further modified by RAS converting enzyme (RCE1), an endoprotease that clips off the *-AAX* tripeptide sequence from *CAAX* box. RCE1 was first found in a yeast screen designed to identify genes involved in RAS protein processing. Subsequently, mammalian RCE1 was identified based on homology to the yeast RCE1 and is believed to proteolyze most isoprenylated *CAAX* proteins (33, 34). Further studies led to the discovery of another yeast protease, sterile24 (Ste24), which is responsible for proteolysis of the *–AAX* sequence from yeast a-factor (Ste24 also cleaves a-factor upstream of the fully processed farnesylmethylcysteine). Ste24 was identified in the late 90ies at UC Berkeley (35-37). The mammalian orthologue was subsequently given the cumbersome name zinc metalloproteinase Ste24 homologue (ZMPSTE24). The only mammalian substrate for ZMPSTE24 identified thus far is prelamin A which is an intermediate filament protein of the nuclear lamin (37, 38).

The final enzymatic processing step in the posttranslational maturation of *CAAX* proteins is carboxyl methylation of the isoprenylcysteine residue. The enzyme responsible for this step was also identified in yeast and named Ste24 (39). Its mammalian orthologue, isoprenylcysteine carboxyl methyltransferase (ICMT) was identified later (40, 41). This final processing step, together with prenylation, renders the carboxyl-terminal end of the protein hydrophobic and prone to associate with membranes (42, 43). Both RCE1 and ICMT resides in the endoplasmic reticulum that is responsible for the trafficking of farnesylated and geranylgeranylated proteins to the plasma membrane. Furthermore, RCE1 and ICMT have been explored as potential drug targets as they both process all the main RAS isoforms, and both the farnesylated and geranylgeranylated forms (44).

Importance of protein prenylation

Membrane targeting

The trafficking of *CAAX* proteins to their target location on membranes is controlled and tightly regulated by specific cytosolic proteins and chaperones. RHO protein GDP dissociation inhibitors (RHOGDIs) and 14-3-3 proteins, for instance, bind to geranylgeranylated forms of RHO family proteins and RND subfamily of RHO proteins, respectively (45, 46). Recent structural studies of these proteins have revealed the presence of hydrophobic pockets that can accommodate the prenyl moieties of *CAAX* proteins. For example, RHO-GDI1 has a pocket in which the geranylgeranyl group of RHOA fits perfectly (47). But interestingly, in GGTase-I-knockout cells, where RHOA is not prenylated, RHOA binds just as strongly as in wild-type cells (48). These pockets strengthen the association between them and help to restrict the release of *CAAX* proteins to further signaling (45, 49). Similar to the above, phosphodiesterase-δ (PDEδ) forms a complex with farnesylated RAS and restricts the release of RAS to oncogenic signaling (50, 51).

Cellular roles of prenylation

Farnesylation is required for RAS activation, and this phenomenon has caught the attention of the scientific community because 30% of human cancers have an activating mutation is a *RAS* gene. Thus, inhibiting farnesylation seemed in the early nineties to be the key to combat RAS-induced cancers, many of which have a high mortality rate (i.e., pancreatic, lung, and colon cancer). A race between academic laboratories and pharmaceutical companies ensued and several FTIs were developed. The FTIs inhibited HRAS-driven cancers quite effectively in mouse models and several FTIs produced anti-proliferative effects in different types of cancer cells via cell cycle arrest in the G1 or G2/M phase of the cell cycle (52). These preclinical studies raised the hope in the field that FTIs might indeed be used to stop RAS induced cancers in its tracks.

Contrary to expectations, clinical trials with FTIs showed no beneficial effect in the treatment of cancer. It was quickly discovered that when FTase is inhibited KRAS and NRAS can become geranylgeranylated by GGTase-I and continue to function normally. This was troubling as most RAS-mutant cancers involve mutations in KRAS and NRAS. HRAS mutations, which the preclinical studies relied on, is exclusively farnesylated. Moreover, the observed antiproliferative effects with FTIs could not be solely attributed to the inhibit activation of RAS, which indicates the

possible involvement of other *CAAX* proteins in the control of cell proliferation (26, 52). This argument was supported by genetic studies in our own laboratory: Knockout of Fntb caused cell proliferation arrest in both KRAS and HRAS mutant cells and improved survival in mice with KRAS-induced lung cancer—despite the fact that KRAS was geranylgeranylated and fully functional (29).

Further research revealed that centromere-associated protein E and F (CENP-E and CENP-F), whose activation are dependent on farnesylation, are involved in the control of cell cycle progression at metaphase (53, 54). Later, it was discovered that the nuclear proteins Lamin B1 and Lamin B2 are farnesylated proteins that are also involved in regulation of cell proliferation and senescence (55, 56). In addition to that, the lamin A (LMNA) precursor, prelamin A is involved in the progression of Hutchinson-Gilford progeria syndrome (HGPS), a rare ageing disorder caused by mutations in LMNA (2). Researchers have also identified two other important farnesylated proteins, RHEB GTPases and liver kinase B (LKB), that play a central role in cellular energy metabolism through mammalian target of rapamycin (mTOR) and AMP activated protein kinase (AMPK) signaling (57, 58). Taken together, it is hypothesized that inhibition of FTase may block other prenylated proteins (CENP-E, CENP-F, lamin A/C, lamin B, RHEB GTPases, and LKB) along with RAS to exert anti-proliferative effects.

The disappointing lack of progress with FTIs in clinical trials, cross prenylation of RAS, and the involvement of geranylgeranylated proteins (RALA, RALB, RHOC, RAC1 and CDC42) cancer progression, shifted the focus to GGTase-I as a potential target to combat cancer (28, 59-65). Unlike FTase, GGTase-I is essential for modification of many *CAAX* proteins, and thus blocking GGTase-I activity could potentially inhibit the function of many *CAAX* proteins at once. Similar to FTIs, GGTase-I inhibitors (GGTIs) cause cell cycle arrest, but here primarily at G0 and G1 phase and those effects might be mediated by downstream signaling of RHO proteins (66). Our lab has shown supporting evidence of this in a study where genetic inactivation of GGTase-I reduces KRAS-induced lung cancer and myeloproliferative disease (MPD) (29, 30).

Importantly, many *CAAX* proteins, including the RHO family proteins, are involved in multiple functions of inflammatory cells. During my PhD, I have focused on the

importance of GGTase-I-mediated geranylgeranylation of RHO family proteins and the impact of inhibiting this enzyme for the progression of inflammatory diseases.

RHO proteins

RHO family proteins are a subcategory of *CAAX* proteins that are low-molecular-weight (~21 kDa) GTP-binding proteins that act like molecular switches and cycle between inactive GDP (guanosine diphosphate) bound and active GTP (guanosine triphosphate) bound forms (Fig. 2) (2). Conversion of GDP to GTP (activation) is catalyzed by guanine nucleotide exchange factors (GEFs); GTP hydrolysis to GDP (inactivation) is stimulated by GTPase-activating proteins (GAPs). RHO protein GDP dissociation inhibitors (RHO-GDIs) are key regulatory enzymes that form complexes with the inactive GDP-bound form of RHO proteins and keep them sequestered in the cytosol (67). Different external signals and growth factors induce dissociation of RHO proteins from the RHO-GDI complex, which leads to the availability of RHO proteins for membrane targeting, GTP-binding, activation and further signaling.

Furthermore, other types of proteins stabilizes RHO proteins in their active GTP-bound form, *e.g.* RAS GTPase-activating like proteins IQGAP1 (IQGAP1) and RAS GTPase-activating like proteins IQGAP2 (IQGAP2) (68, 69). Balancing between active and inactive forms of RHO proteins is very important in order to regulate specific cellular functions such as actin cytoskeleton remodeling, cell cycle progression, proliferation, migration, production of growth factors, and cytokine release (70-72). Inappropriate signaling of RHO family GTPases contributes to the pathogenesis of cancer (73-75), inflammation (76), and cardiovascular diseases (77).

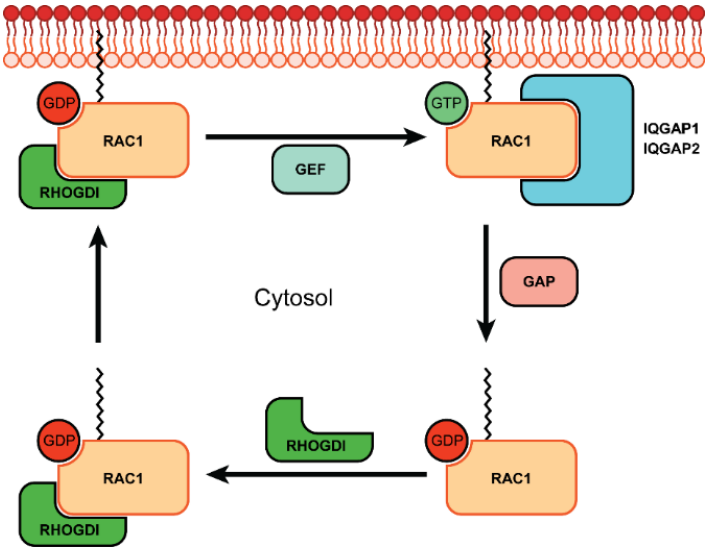

Fig. 2. Activation and inactivation of the RHO family protein RAC1. Prenylation is important for the interaction between RAC1 and RHO-GDI. RHO family proteins (including RAC1) form complexes with RHO-GDI in the cytosol in their inactive GDP-bound forms. RAC1-GEFs and RAC1-GAPs stimulate GTP binding and GTP hydrolysis, respectively, and thereby activate and inactivate RAC1. Growth factors and extracellular signals result in the release of RAC1-GDP from the RAC1-GDP-RHOGDI complex. RAC1 then transfers to the plasma membrane and is subsequently converted into its active GTP-bound form through the action of a GEF. Once RAC1 is activated, it interacts with different kinds of effectors (*e.g.*, IQGAP1, IQGAP2) to signal downstream to control cell proliferation, migration, and morphology. Eventually, GAPs assist in the hydrolysis of the GTP and RAC1 is inactivated. (Picture: Emil Ivarsson)

To date, 22 mammalian members of RHO family proteins have been identified, and they further subdivided into RAC subfamily GTPases, CDC42 subfamily GTPases, RHOA subfamily GTPases and other RHO GTPases (78). Among them, RAS-related C3 botulinum toxin substrate 1 (RAC1), RAS homolog gene family, member A (RHOA), and Cell division control protein 42 homolog (CDC42) are the most well-studied members of this group (78). The section below describes key molecular biology events involved in activation of RHO family proteins and their roles in the progression of inflammatory diseases.

RAC1

The RAC subfamily of GTP binding proteins consists of RAC1, RAC2, RAC3 and RHOG. RAC1 is the most well-studied among them due to its critical involvement in the regulation of a wide range of cellular functions, such as cytoskeletal modifications—especially lamellipodia formation—migration and invasion; generation of reactive oxygen species (ROS) through NADPH oxidase; and initiation of the inflammatory response (79-81). RAC1 is expressed ubiquitously in most cells in the body, while RAC2 and RAC3 expression are limited to hematopoietic cells and neural cells, respectively (82-84).

Similar to other RHO family proteins, RAC1 acts as a tightly controlled molecular switch between inactive GDP bound state, and active GTP bound state. TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein-1), VAV1 (Proto-oncogene VAV), VAV2 (Proto-oncogene vav) and β-pix (RHO guanine nucleotide exchange factor 7) are the most commonly known GEFs, which catalyze the release of GDP and recruitment of GTP in RAC1 leading to RAC1 activation (85). RAC-GAP1, cdGAP, and RICS are GAPs involved in GTP hydrolysis (86-88). In addition, some proteins induce stability to the GTP form of RAC1, including IQGAP1 (89, 90).

Several transmembrane receptors with the help of their ligands can stimulate the conversion of active RAC1 and then transmit signals to effectors that trigger a wide range of physiological outcomes. P21 activating kinase (PAK1) is the canonical effector downstream of RAC1 that transmits RAC1 downstream signals. Activated RAC1 binds to PAK and then stimulates PAK kinase activity, which in turn regulates cytoskeleton remodeling, adhesions, gene transcription via control of different signaling cascades like c-JUN N-terminal kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and canonical JNK regulated WNT signaling (91-96). RAC1 plays an essential role in activation of NADPH oxidase complex in immune cells including macrophages. Incorporation of activated RAC1 into NADPH complex is an essential requirement to generate ROS, and then it stimulates superoxide production to provide an immune response against invading pathogens (97, 98). Furthermore, RAC1 is required for immunoglobulin receptor–mediated phagocytosis to promote inflammatory

response via activation of MAPK (mitogen-activated protein kinase) and JNK (99). Overexpression of the active form of RAC1 has also been identified in many tumor types, and RAC1 is sometimes required for oncogene-induced transformation, including by RAS and TIAM1 (100-105).

Aberrant signaling of RAC-family GTPases, especially RAC1, is found in the progression of several inflammatory diseases including osteoarthritis (106, 107), Crohn´s disease (108), psoriasis (109) and mevalonate kinase deficiency (110). Active RAC1 plays an important role in eliciting an immune response against infection. ROS production (79, 111), and NF-κB activation (111, 112), are two key signaling pathways involved in RAC1-mediated inflammatory disease progression. Activated RAC1 uses those signaling pathways to trigger production of several inflammatory mediators, including interleukins (e.g., IL-6 and TNF-α) (113) and matrix metalloproteinase (MMP13) (106, 114) that drive disease progression. A few studies suggested that blocking RAC1 might be a strategy to treat inflammatory disorders such as arthritis and autoimmune disorders (115, 116).

RHOA

RHOA is a ubiquitously expressed RHO family GTPase protein involved in regulation of different cellular events including cytoskeleton modification, formation of stress fibers, focal adhesions, and cell to cell adhesions, cell to matrix adhesions and cell migration (117-119). RHOA-triggered cellular responses are mediated by a RHOA effector called RHO associated coiled-coil-containing protein kinase (ROCK). The ROCK inhibitor Y27632 is often used to block RHO mediated cellular responses. These include the inhibition of RAS-induced oncogene transformation (120), NFκB-dependent cytokine production in experimental colitis (121), MCP-1-induced chemotaxis (122), vasoconstriction (123), and cardiac hypertrophy (124, 125). Fasudil is the only ROCK inhibitor approved for clinical use, and is used to treat cerebral vasospasm due to its potent vasodilation effects (126).

Mounting evidence shows that increased RHOA signaling is a hallmark in pathogenesis of inflammatory diseases, including inflammatory Crohn's disease (121), ulcerative colitis (127), cardiac hypertrophy (128) and asthma (129). Statins, the drugs that lower cholesterol by blocking HMG-CoA reductase activity, display

beneficial cardiovascular effects that have been linked to reduced prenylation of RHOA. Blocking RHOA protects mice from cardiac hypertrophy-induced ischemia (130). Subsequently, a strategy was proposed to block RHO proteins signaling by statins or GGTIs to prevent progression of inflammatory diseases such as atherosclerosis. However, research in our lab showed that this was not accurate. We find that blocking RHOA prenylation, by knocking out GGTase-I, markedly increases RHOA-GTP levels and activity, increases macrophage reverse cholesterol transport which markedly reduced atherosclerosis in GGTase-I knockout mice (131). Another study showed that blocking RHOA signaling by reduced prenylation triggered intestinal inflammation in intestinal epithelial cells isolated from inflammatory bowel disease patients (132). Furthermore, one study showed that inactivation of RHOA triggers production of mature IL-1β via activation of pyrin inflammasome (*MEFV)* in Hyper-IgD syndrome (HIDS) patients (133). This overproduction of IL-1β is a major driving force for main pathological abnormalities in HIDS patients. As outlined in Paper 2, we believe the mechanism of hyperinflammation is related to RAC1 and not RHOA. Altogether these conflicting results needs clarification. On one hand active RHOA contributes regression of atherosclerosis, and on other side, inactivation of RHOA promotes inflammation in IBD and HIDS patients. These studies stresses the importance of exploring the biology of RHOA in specific tissues and cell types in more detail before proposing targeting RHOA or RHOA signaling in disease therapy.

CDC42

CDC42 is a ubiquitously expressed RHO family GTPase protein involved in the regulation of cytoskeleton remodeling and membrane trafficking that controls a wide range of physiological functions, including the formation of filopodia, cell motility, polarity, growth and cytokinesis (134, 135). Similar to RAC1, activated CDC42 is associated with several effectors, mainly PAK1, and IQGAP1. The majority of these effectors contain either a CDC42-RAC interacting (CRIB) domain or a p21-binding domain (PBD) in their structure that allows binding of CDC42GTP (136). Overexpression of CDC42 has been observed in lung cancer and melanoma, and this phenomenon is sometimes required for RAS-induced transformation (137). However, very few studies have described the role of CDC42 in inflammation, particularly in endothelial cell-associated inflammatory diseases.

CDC42 is responsible for senescence-associated inflammation in endothelial cells, via activation of the p53/p21 pathway, and can thereby promote plaque formation in atherosclerosis in $APOE^{-/-}$ mice (138). In addition, CDC42 is required for promoting endothelial cell function and regeneration, which are important during vascular repair in acute lung injury and acute respiratory distress syndrome (139).

IQ motif containing GTPase activating protein 1 (IQGAP1)

IQGAP1 does not belong to a *–CAAX* protein family or RHO family, but it is the common effector for the RHO family proteins, most importantly RAC1, RHOA and CDC42. IQGAP1 is a 190 kDa ubiquitously expressed scaffolding protein that plays an important role in cytoskeletal rearrangement (140), the mitogen-activated protein kinase pathway (141, 142), and in β-catenin-mediated transcription (143). IQGAP1 is originally named due to its sequence containing isoleucine (I)-glutamine (Q) domains (IQD) and a GTPase activation protein-related domain (GRD). In addition to that, it also contains a calponin homology domain (CHD), a coiled-coil domain (CCD), a tryptophan-tryptophan domain (WWD), and RAS GAP carboxyl-terminal domains (RGCTD). Several protein recognition motifs present in the multi-domain composition are responsible for wide array of IQGAP1 interactions (144). Although the structure of IQGAP1 contains a GRD, it is unable to execute GTP hydrolysis; instead, it is thought to stabilize GTP-bound proteins (145-147).

Even though IQGAP1 interacts with several proteins, no developmental or physiological defects were found in *Iqgap1*-knockout mice. The only phenotype observed was late-onset of gastric hyperplasia (148). Since IQGAP1 engages in interaction with multiple protein partners, it might be a clinically important target to combat diseases including cancer. For example, earlier studies reported that IQGAP1 is required for RAS-induced tumorigenesis via association with ERK1/2 and phosphoinositol-3-kinase (PI3K), and thereby activating scaffold-induced MAPK pathways responsible for tumorigenesis (149, 150). Disrupting or blocking IQGAP1-ERK1/2 or IQGAP1-PI3K interactions is considered a promising strategy to inhibit tumor progression. Elevated levels of IQGAP1 has been noticed in many different cancers, and this facilitates the formation of complexes with the GTP-bound forms of RAC1 and CDC42, which drive tumor progression (147). Earlier

studies reported that IQGAP1 is an attractive target against bacterial infections, because of the ability of bacteria to use IQGAP1 to modify cytoskeleton dynamics and form F-actin pedestals that are required for the entry of S*almonella typhimurium* and *Escheria Coli* into the host cell (151-154). All of these results put together suggest that IQGAP1 is an attractive target to treat cancer, bacterial infections, asthma and several other diseases.

When I began my studies, dogma held that prenylation is required for the membrane targeting or RHO family proteins and for the ability of RHO proteins to interact with GEFs, GAPs, and IQGAP1. In particular, the activation of RHO proteins was believed to require prenylation. However, some studies had shown, paradoxically, that statins, which reduce GGPP production, actually increases levels of GTP-bound RHO proteins, but the mechanisms underlying this effect was not known.

Statins

Statins are potent inhibitors of the cholesterol biosynthesis pathway by reversibly inhibiting key regulatory enzyme HMG-CoA (3-Hydroxy-3-methylglutaryl-CoA) reductase (Figure 3) (155). Due to their structural resemblance to the HMG-CoA moiety, statins occupy the HMG-CoA binding site in the reductase enzyme (it has a higher affinity for binding than HMG-CoA) and thereby inhibit its enzymatic activity (155). Statins are one of the most widely-prescribed drugs in the world, and it is used primarily to reduce serum cholesterol levels in response to hypercholesterolemia and thereby reduce the risk of future cardiovascular disease (156). Furthermore, statins possess some cholesterol-independent effects, also known as pleiotropic effects. These pleiotropic effects have in clinical trials been shown to be independent of cholesterol lowering and to include anti-inflammatory (157), antioxidant (158), anti-thrombogenic effects (159), and improved endothelial function effects (160). Statin pleiotropic effects have been noted in several clinical trials: MIRACL (161), PROVE-IT (162) CARE (163), PRINCE (164), HPS (165) and ASCOT (166). In these clinical trials, statin-treated individuals had a reduced risk of heart disease and increased survival rate despite the fact that the statins did not reduce cholesterol levels in a significant fashion (167).

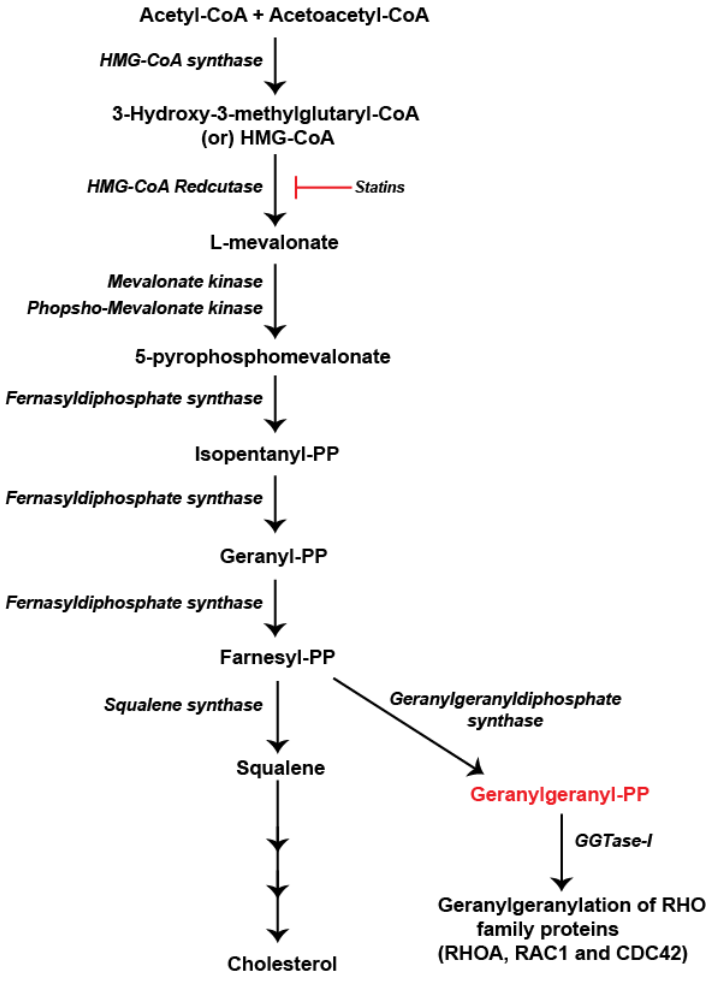

Fig 3: Biosynthesis of cholesterol and isoprenoid intermediates: Statins inhibit HMG-CoA reductase, a key enzyme in cholesterol biosynthesis regulation, by blocking synthesis of L-mevalonate in the mevalonate pathway. L-Mevalonate is converted to isopentenyl pyrophosphate (Isopentenyl-PP) by mevalonate kinase and phosphomevalonate kinase, respectively. Isopentenyl pyrophosphate is subsequently converted to geranyl pyrophosphate (Geranyl-PP) and farnesyl pyrophosphate (Farnesyl-PP) by farnesyl diphosphate synthase. Furthermore, farnesyl-PP is converted to geranylgeranyl pyrophosphate (GGPP) by geranylgeranyl diphosphate synthase. Finally, geranylgeranyltransferase-I (GGTase-I) uses GGPP to initiate lipid modifications of *CAAX* family proteins. Statin therapy reduces the synthesis of GGPP and thereby inhibit lipid modifications of CAAX proteins.

Several studies have cemented the view that pleiotropic statin effects—independent of cholesterol reduction—are mechanistically important. For example, statins

improve vascular endothelial function before significantly affecting serum cholesterol levels (160, 168, 169). The mechanism behind statins vasoprotective function is mainly due to the upregulation of endothelial nitrous oxide synthase activity (eNOS), which leads to increased synthesis and release of endothelial-derived nitrous oxide (NO) (170, 171). These vasoprotective effects of statins are absent in $eNOS^{-/-}$ mice, which indicates that endothelial-derived nitrous oxide production mediates at least some parts of the beneficial effects of statins on endothelial function (172). The antioxidant effect of statins is another potential mechanism to restore endothelial cell function. Statins reduce the generation of ROS via down regulation of the angiotensin type1 (AT1) receptor and NAD(P)H oxidized subunit p22Phox (173).

Some studies have reported that statins had a beneficial effect on other inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) dementia, atherosclerosis, and systemic lupus erythematosus (SLE) (174-177). Statins reduce levels of high-sensitivity C-reactive protein (hs-CRP), a clinical marker for inflammation usually elevated in individuals with high cardiovascular risk (157, 178, 179). Most of the pleiotropic effects of statins are believed to be caused by reduced synthesis of the isoprenoid intermediates FPP and GGPP, which are used in the prenylation reactions (180) (Figure 1). Thus, statins are—in light of the aforementioned dogma—thought to prevent sub-cellular localization, membrane targeting, and activation of RHO family proteins.

Until recently, several studies have reported on different pleiotropic effects of statins. Many of the pleiotropic effects observed in cell cultures were restored by adding of GGPP to the culture medium but not by adding FPP or LDL-cholesterol, suggesting that geranylgeranylation of RHO proteins, such as RAC1 and RHOA underlies the effects. One example of this is the statin-induced vasoprotective effects, which are reversed by addition of GGPP but not FPP or ox-LDL, suggesting that inhibition of geranylgeranylation of RHO proteins is responsible for the vasoprotective mechanisms (171, 181, 182). However, geranylgeranylation is also important for a large class of RAB proteins, which are prenylated by GGTase-II in a pathway that is entirely distinct from that mediated by GGTase-I. RAB proteins coat membranes of vesicles and are important for their correct targeting within the

cell. These findings indicate that RHO proteins or RAB proteins might be involved in mediating the beneficial effects of statin treatment.

However, despite having these beneficial side effects, statins can also cause some serious complications. A large proportion of statin-treated patients experience some form of muscle pain (myalgia) symptoms, which can range from mild to severe. In severe cases statins can cause rhabdomyolysis, which has been reported to occur in 0.44-0.54 cases per 10 000 person-years. Rhabdomyolysis is a disease in which skeletal muscle starts to break down, which in turn can cause kidney failure and death (95, 96). Cerivastatin was the first drug to be discontinued in the United States due to high rates of fatal rhabdomyolysis (183). The exact cause of statin induced myalgia and rhabdomyolysis remains elusive, but in light of findings in our group, we have hypothesized that statin-induced inhibition of prenylation of RHO family GTPases underlies both positive and negative pleiotropic effects.

Targeting GGTase-I in diseases

Cancer

RAS is one of the most well-studied oncogenes and is a notorious driver of progression in as much as 30% human cancers. Direct targeting of RAS has been extremely difficult due to its high affinity for the active GTP-bound state and the structural similarities with other GTPase proteins (184). Thus a strategy developed to use FTIs to combat RAS-induced cancers. As outlined earlier, clinical trials with FTIs failed due to alternative prenylation of KRAS and NRAS. GGTase-I inhibitors in combination with FTIs could have been an effective strategy but researchers tested GGTIs and also dual-prenylation inhibitors as potential anti-cancer drugs (185). In our lab, we have earlier shown that knockout of both FTase and GGTase-I completely blocked KRAS prenylation, rendered the protein soluble, and incapable of causing cancer (30). So far one GGTI (GGTI-248) went through clinical trials (26). But overall, the strategy of targeting both prenyltransferases in cancer therapy is likely to be too toxic as no cells can proliferate in the absence of these enzymes. Some cells clearly survive in the absence of both enzymes, such as macrophages and type II pneumocytes in the lung (29).

Inflammation

Explaining the importance of prenylation in the development of inflammatory diseases is particularly complicated due to a surprising discovery in our group. According to current dogma, one would expect that blocking prenylation would inhibit RHO protein activity and that this, in turn, would protect against inflammatory symptoms caused by RHO protein signaling. Surprisingly, however, we found that knockout of GGTase-I in macrophages hyperactivates RHO proteins, stimulates pro-inflammatory signaling pathways, enhances cytokine production in response to lipopolysaccharide (LPS) stimulation, and causes mice to develop severe erosive arthritis in all of their joints (48). This result not only challenges the long-held assumption that prenylation is required for *CAAX* protein function but suggests that prenylation might a negative regulator of RHO-GTPase activation. Furthermore, our lab showed that knockout of GGTase-I in macrophages increases active RHOA and that this mediates an increase in reverse cholesterol transport and a 60% reduction in atherosclerosis in LDL receptor-deficient mice (131). These discoveries not only challenge the widely-held view that prenylation is required for activation but they also shed light on the paradoxical findings of increased GTP-loading and cytokine production in statin-treated cells and provides an impetus for studying this further. How do RHO family proteins become GTP-bound when they are not prenylated? Which GGTase-I substrate drives inflammation in GGTase-I-deficient mice? Or are other mechanisms in place? What is the importance of RHO protein prenylation? There are many questions that need answers.

The current thesis focuses on defining the importance of GGTase-I in macrophage-induced innate immunity. Macrophages are crucial mediators for immune sensing. Macrophages migrate into damaged tissues and recruit other inflammatory cells, trigger inflammatory cytokine production, generate ROS, contribute to phagocytosis, pathogen killing, and remove apoptotic cells from the site of infection (186). These events altogether contribute to primary defence mechanisms for humans via unleashing a wide range of innate immune responses against infections (186). All these immune responses are responsible for controlling the signalling events of inflammation.

Innate immune system

The innate immune system is a first-line defence mechanism that protects the host from pathogens such as fungi, bacteria, and insects. The primary function of the innate immune system is to recruit immune cells to the site of infection. This results in the release of chemical mediators such as pro- and anti-inflammatory cytokines, activation of the complement cascade, and formation of antibody complexes. These factors contribute to a proper and well-balanced immune response against harmful pathogens (187).

Inflammation is a key protective mechanism in innate immune response driven by the recruitment of immune cells at the site of infection by harmful pathogens. Well-controlled inflammation protects the host from infection. However, a less well-controlled response, in particular an excessive one, is at the root of many chronic inflammatory and autoimmune diseases. Immune cells contain a special group of receptors on their cell membranes called pathogen recognized receptors (PRRs). The amount of innate immune response depends primarily on how fast the PRRs recognize pathogen-associated molecular patterns (PAMPs) generated in response to harmful pathogens and also how fast he PRRs recognize danger-associated molecular patterns (DAMPs) which generated in response to endogenous host stress (188). Activation of PRRs stimulates inflammatory signaling cascades that result in the production and secretion of chemical mediators such as interferons, pro-inflammatory and anti-inflammatory cytokines (189).

The inflammasome is a multi-domain complex which contains one or more caspases in their structure, and it is essential for the activation of inflammatory responses. Inflammasomes are expressed mainly in myeloid cells and act as immune sensors (receptors) controlling the activation of caspase-1 that further results in the production of the highly immune-active cytokines IL-1β and IL-18. To date, researchers have identified five receptors responsible for inflammasome formation. They are NACHT, LRR and PYD domains-containing protein 1 (NLRP1), NLRP3, NLR family CARD domain-containing protein 4 (NLRC4), absent in melanoma 2 (AIM2) and pyrin (MEFV) (190). Activation of inflammasome is a key event, mediated by innate immune response and; in the progression of several auto-inflammatory diseases (191). Interleukin-1 beta (IL-1β)

is an important inflammatory cytokine expressed in myeloid cells like macrophages and monocytes at the site of infection or injury.

Many cells produced IL-1β in response to bacterial toxins (lipopolysaccharide) via TLR4 signaling pathway. But, LPS alone is not enough to stimulate activation and release of IL-1β (192). These results led to emergence of two signal model for maturation and release of IL-1β in cells. In Signal I, LPS stimulate the synthesis of pro-IL-1β, whereas signal II is required for conversion of pro-IL1beta to its active form (P17). The exact mechanism for signal II is not clearly understood but earlier research shown that second signal mediated through activation of caspase-1 (193). This cytokine, and also IL-18, is secreted via a non-classical secretory pathway that doesn't involve the ER-golgi-plasma membrane vesicular transport machinery (194). Several bacterial toxins (e.g. lipopolysaccharide, LPS) can activate caspase-1 mediated signaling that triggers production of active IL-1β (195). Recent studies showed that activation of RHOA in response to bacterial toxins is essential to inhibit caspase-I mediated pyrin (one of the components of the inflammasome, MEFV) inflammasome signaling in macrophages (196).

Rheumatoid arthritis (RA)

RA is a chronic inflammatory autoimmune disease caused by progressive destruction of joints in the body (Fig. 4). RA is believed to originate from losing self-tolerance against immunogens which leads to inappropriate activation of immune reactions that affect structural cells and building blocks of bone, cartilage, and connective tissue in the joints (197, 198). RA is characterized by severe pain, stiffness and swelling of joints. RA affects 1% of the population globally, but the exact cause of RA remains unclear (199). Earlier studies reported that formation of immune complexes that activate immune cells, production of self-reactive antibodies (anti-citrullinated protein antibodies and C-reactive protein antibodies), infiltration of immune cells (lymphocytes and macrophages) into the synovium are key regulatory events that underlie the initiation and progression of disease (200). These effects lead to the aggressive development of synovium hyperplasia and subsequent damage of cartilage present in periarticular bone. Synovium is a single thin translucent membrane layer present in non-articular surfaces of the joint but, during RA, immune cells infiltrate inside the joint cavity, form immune complexes

to activate immune cells to produce cytokines, self-reactive antibodies and matrix metalloproteases (MMPs), causing the synovium to expand and this process goes hand-in-hand with to joint destruction (198).

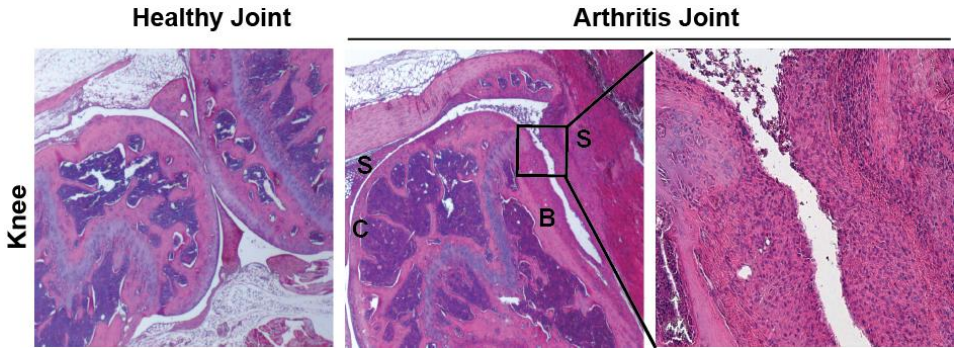

Fig 4: Photos of normal mouse joint and joint from mouse with arthritis: Hematoxylin and Eosin–stained sections of joints from 12-week-old mice. S, synovium;B, bone. Note thickened synovium in the square at right.

Among lymphocytes, T-lymphocytes, particularly, $CD4^+$, $CD8^+$ and effector T-lymphocytes are involved in the pathogenesis of RA (201). $CD4^+$ T-lymphocytes also called regulatory T-lymphocytes (Tregs), help to maintain self-tolerance by inhibiting pathological immune responses against immunogens (201). Defects in Tregs are often noticed in RA patients. Tregs isolated from RA patients produce high levels of inflammatory cytokines and suppress proliferation of effector T-lymphocytes that are responsible for the production of anti-inflammatory cytokines like TGF-β and IL-10 (202). Earlier studies found evidence that depletion of $CD4^+$ T cells reduced the severity of disease in peptidoglycan aggrecan (PG)-immunized mouse model (203).

Further evidence came from multiple studies showing that $CD4^+$ T-lymphocytes form complexes with aggressive forms of disease-contributing proteins encoded by *Hla-drb1*, a gene involved in the progression of inherited arthritis (204). *Hla-drb1* encodes proteins that are components of human leukocyte antigen-DR1 (HLA-DR1). HLA-DR1 is expressed by antigen-presenting cells (APCs) such as macrophages and acts as a ligand to activate $CD4^+$ T-lymphocytes and thereby trigger wide array of inflammatory responses (204). Several clinical studies

reported that targeting T-lymphocytes and their products have shown significant benefit in treating RA (205-207).

Monocytes/Macrophages also play a central role in the pathogenesis of RA. Typically, monocytes in blood infiltrate into the synovial membrane and differentiate into macrophages that increase the production of inflammatory mediators. The hypersecretion of inflammatory mediators such as cytokines and MMPs, leads to the destruction of extracellular matrix proteins which breaks down vital joint components. Earlier studies have shown that a population of $CD4^+CD25^+$ monocytes is elevated in RA patients (208). Also, activated monocytes promote bone resorption by differentiating into osteoclasts, which are only cells in the body that can perform the task of bone resorption (209). Depletion of monocyte populations has shown great benefit in treating inflammation in mice (210, 211). Macrophages act as APCs and present immunogenic peptides to $CD4^+$ T-lymphocytes to activate them and then trigger inflammatory responses as described above. In addition to that, earlier studies showed that ROS produced by macrophages and neutrophils contribute to cartilage destruction. ROS also promotes signaling pathways that contribute to inflammation (212).

There is no curative therapy for RA patients. Current therapies and drugs are mainly focused on reducing cytokine productions to alleviate joint pain but not on reversing cartilage function. This is a problem that needs further research to find better molecular targets or predictive biomarkers that can lead to treatment at an earlier stage to more effectively combat the disease.

Mevalonate kinase deficiency (MKD)

Mevalonate pathway—synonymous with the cholesterol biosynthetic pathway— is a key metabolic pathway responsible for the synthesis of cholesterol (180). Inhibiting the first step of this pathway with statins leads to reduced production of cholesterol. A few steps down in the pathway is where mevalonate kinase (MK) operates. MK is responsible for conversion of mevalonate to phosphomevalonate (180). Patients carrying loss of function mutations in the gene encoding the MK enzyme are prone to developing a host of inflammatory phenotypes including recurrent fever attacks, lymphadenopathy, arthralgia, skin lesions, and diarrhoea (213). Hyperimmunoglobulinemia D syndrome (HIDS), and mevalonate aciduria

(MA) are two rare hereditary diseases that occur due to the deficiency of MK (213). Collectively these disorders are called MK deficiency (MKD). Until now, less than 300 MKD cases have been detected globally (214). Increased serum levels of Immunoglobulin D (IgD), and increased excretion of mevalonate serve as specific biomarkers to identify MKD. In addition, increased levels of inflammatory markers such as C-reactive protein (CRP) and serum amyloid A (SAA) can frequently be detected in the patient's serum (215). Current therapies available for MKD patients are mainly focused on reducing IL-1β levels using IL-1β receptor antagonists such as Anakinra (216). However, what are the precise mechanisms underlying this disease?

Excessive production of IL-1β is a dominant force in the progression of MKD. Activation of NFκβ signaling is mainly involved in the production of IL-1β in MKD monocytes, and the conversion of pro-IL-1β to mature IL-1β is carried out via the inflammasome-dependent activation of caspase-1 (217). The exact molecular mechanisms for the development of inflammatory phenotypes are not clearly understood. However, it is thought that reduction of prenylation of RHO family proteins is involved in the development of MKD cellular phenotypes. This is because MKD leads not only to reduce cholesterol synthesis but also to the reduce production of GGPP and FPP. Moreover, incubating macrophages with statins—to inhibit HMG-CoA reductase upstream of MK—recapitulates the in vitro cellular phenotype observed in monocytes of MKD patients (218). The same study showed that aberrant signaling of RAC1 causes caspase-1 dependent maturation of IL-1β production (218). Importantly, targeting RAC1 blunts cytokine production induced by statins (218). In support of the notion that these effects are caused by reduced prenylation, the increased IL-1β production was rescued by addition of GGPP. The rescue of increased cytokine production with GGPP suggests that defective RHO protein prenylation might be involved in the progression of MKD (219, 220). Some studies report that increased caspase-1-dependent IL-1β production was due to inactivation of RHOA signaling by statins (221). However, researchers failed to address how increased RAC1 activation and inactivation of RHOA communicated with each other in the development of MKD.

Until now, no studies have proposed a convincing model for how statins and MKD leads to increased cytokine production. And many other questions remain: Why do mice lacking GGTase-I in macrophages develop hyperinflammation and arthritis? Why do RHO family proteins become GTP-bound in cells with inhibited GGTase-I or cells treated with statins? Is one or many of GGTase-I's 60+ substrates involved in arthritis development? In this thesis I have addressed these questions and provided clear answers to a few of them.

3 EXPERIMENTAL STRATEGY

In this section, I describe the transgenic mouse models that were used to uncover the cellular and molecular mechanisms behind the hyperinflammation and erosive arthritis in mice lacking GGTase-I in macrophages. I will also provide a rationale for the use of mice in research and describe techniques used to manipulate its genes.

Transgenic Mice

The mouse (*Mus musculus*) is one of the most popular biomedical research tools that has been used to understand both basic biology and pathogenesis of human diseases. This due to the close similarity between mice and humans in terms of molecular behaviour compared to other model systems, e.g. rats and flies (222). The main reasons for using mouse models include anatomy and physiology similarity to humans, easy handling, short breeding cycle (three weeks) and early sexual maturity (five weeks) (223, 224). From the last three decades, the use of mouse models has dramatically increased due to the emergence of recombinant DNA technology. Advances in the field have given researchers more opportunities to introduce foreign DNA elements (transgene) into the mouse genome (225). These transgenic mice give us sophisticated tools to study the effect of deletions or mutations of specific genes in the whole mouse or in specific tissues and the opportunity to evaluate their role in health and disease development. For example, mice lacking low-density lipoprotein receptor (LDLr) are used to study the development of atherosclerosis (226). A common way to generate transgenic mice is by pronuclear microinjection of a transgene into the zygote. Unfortunately, the method has an increased risk of random incorporation of foreign DNA elements into the host genome. To overcome that, a method based on the targeted manipulation of embryonic stem cells (ESCs) to more reliably produce transgenics with correct insertions/substitutions. In this method, the transgene is inserted by homologous recombination into a specific locus of a predetermined target DNA region. Upon successful integration, the manipulated ESCs are injected into blastocysts, and the embryos are implanted into a pseudopregnant female. The resulting pups are called chimaeras. This particular method allows us to delete or inactivate the desired gene, i.e., create a knockout (225).

Cre-*loxP* techniques

The Cre-LoxP technique allows for the excision of an engineered DNA sequence from genomic DNA. This tool uses Cre recombinase, an enzyme derived from P1 bacteriophages that recognises *loxP* sites present in the inserted engineered DNA. P1 plasmids contain sequences called *loxP* (locus of X-over P1 bacteriophage), consisting of a 34-bp DNA sequence, that includes 13 symmetric base pair sequences in both ends and 8 asymmetric base pairs sequences at the center. The Cre recombinase identifies an engineered sequence that is flanked by *loxP* sites (this sequence is chosen to be an essential or important part of the activity of target gene) and executes excision of DNA sequence between the *loxP* sites (Fig. 5) (227).

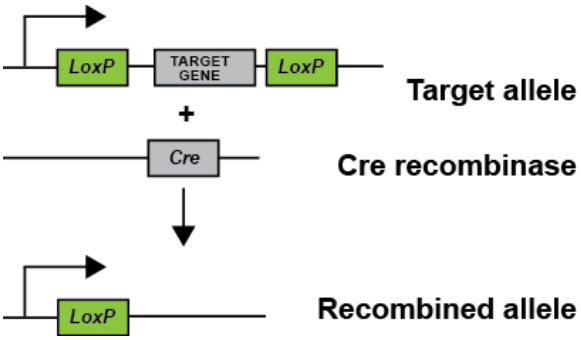

Fig. 5. Critical DNA sequence, which is vital for target gene activity was flanked by LoxP sites. Cre recombinase recognize and then bind to the LoxP sites. Once Cre recombinase binds to LoxP sites, it cuts out the target gene sequence present between the LoxP sites resulting in inactivation of the target gene.

There are several ways to express Cre recombinase in cells. First, it is common to simply transfect a plasmid encoding the recombinase, another way is by using a virus to introduce Cre recombinase in cells or animals, and a third is to engineer transgenic mice harbouring Cre recombinase driven by a specific promoter (228, 229). This promoter is expressed in either ubiquitously or is constrained to a specific cell type/tissue. The main advantage of using these mouse models is the ability to recapitulate human disease conditions in mice by introducing transgene mutations and thus find targets that are responsible for disease progression. For example, mice expressing mutant $KRAS^{G12D}$ are used to study the development of different cancers including lung, skin and pancreatic cancers (230).

In the present study, we used the Cre-*loxP* technique to inactivate GGTase-I, RAC1, RHOA and CDC42 in mice macrophages; and we used a conventional knockout/null allele for IQGAP1.

Macrophage-specific knockout of GGTase-I and RHO proteins

To generate a macrophage-specific knockout mouse for GGTase-I (*Pggt1b*$^{\Delta/\Delta}$), we first generated a conditional *Pggt1b* allele (*Pggt1b*$^{fl/fl}$) by inserting *loxP* sites flanking exon 7. Exon 7 encodes amino acids that are essential for the catalytic activity of the beta subunit of GGTase-I. The *Pggt1b*$^{fl/fl}$ mice were bred with transgenic mice expressing Cre recombinase under the control of Lysozyme-M-promoter (LysM-Cre). The resultant heterozygous offspring lack 50% GGTase-I activity in myeloid cells, which is enough to effectively prenylate RHO family proteins in myeloid cells (heterozygous GGTase-I knockout mice are indistinguishable from WT). We further intercrossed these mice to get 100% deletion of GGTase-I in macrophages. These mice were termed as *Pggt1b*$^{\Delta/\Delta}$ mice (30) (Δ = D = deleted)

In Paper I, using these mice, we studied general cellular and molecular mechanisms underlying inflammation in *Pggt1b*$^{\Delta/\Delta}$ mice and found that GGTase-I-deficiency activates the pyrin inflammasome, caspase-1, and IL-1β production.

In Paper II, we studied the hypothesis that one of the main GGTase-I targets RAC1, RHOA, and CDC42 become hyperactivated in the absence of prenylation and underlie the increased cytokine production *in vitro* and arthritis *in vivo* in *Pggt1b*$^{\Delta/\Delta}$ mice (mice harbouring conditional knockout alleles for *Rac1* (231), *Rhoa* (232) and *Cdc42* (233) were generated as described). To accomplish this, we knocked out one copy of *Rac1*, *Rhoa* or *Cdc42* genes in *Pggt1b*$^{\Delta/\Delta}$ mice in the same way we generated *Pggt1b*$^{\Delta/\Delta}$ mice (Figure 6). These mice were designated *Rac1*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$, *Rhoa*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$, and *Cdc42*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$. We also evaluated mechanisms underlying the accumulation of RAC1, RHOA, and CDC42 in the active GTP-bound state in *Pggt1b*$^{\Delta/\Delta}$ macrophages. As outlined in Paper II, we found that non-prenylated RAC1 binds strongly to IQGAP1. We also tested the role of IQGAP1 in the increased inflammation in *Pggt1b*$^{\Delta/\Delta}$ mice. For that, we knocked out of IQGAP1 (*Iqgap1*$^{-/-}$) (148) by breeding *Iqgap1*$^{-/-}$ mice with *Pggt1b*$^{\Delta/\Delta}$ mice. These mice were designated *Iqgap1*$^{-/-}$*Pggt1b*$^{\Delta/\Delta}$ (Fig. 6).

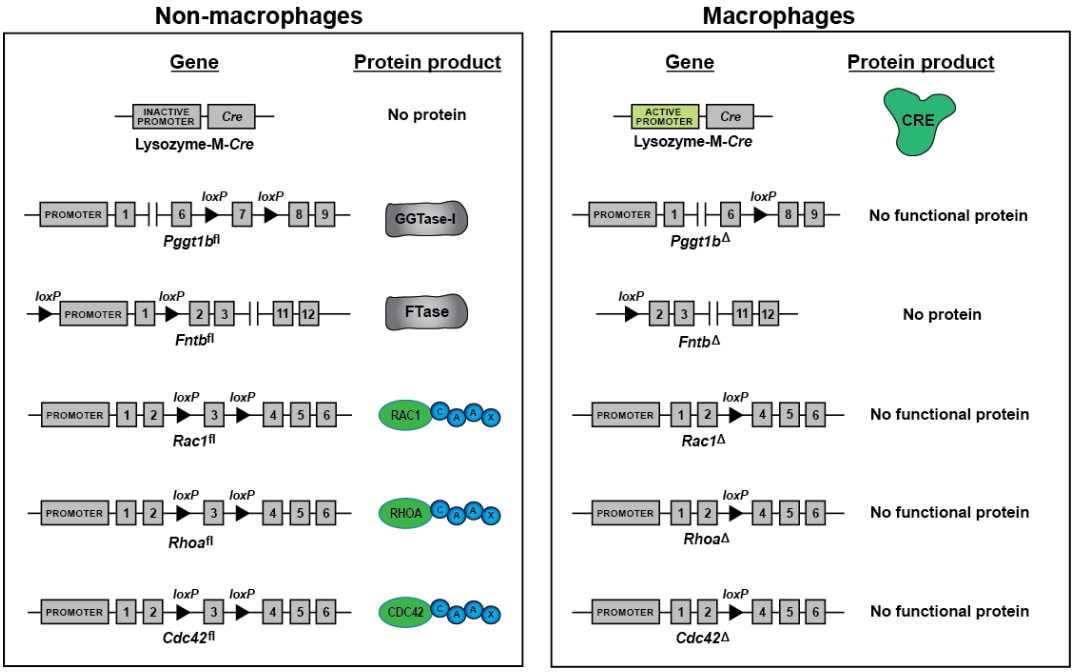

Fig. 6. Generation of macrophage-specific knockout of GGTase-I and RhoGTPases in mice.

4 BACKGROUND AND PREVIOUS RESULTS

Several studies have reported that RHO family proteins are important for tumor cell metastasis, so targeting RHO family proteins has been considered a potential strategy to treat cancer. Furthermore, some studies have found that RHO family proteins, most importantly RAC1, RHOA, and CDC42 are important for immune cells to perform their functions (234, 235). RHO family proteins are important in innate immunity functions such as the response to bacterial toxins.

As outlined earlier, RHO family proteins are substrates of GGTase-I during their maturation process, and geranylgeranylation has been widely assumed to be essential for RHO-protein activity. Therefore, targeting GGTase-I, to prevent prenylation of RHO proteins, has been proposed as a potential strategy to treat inflammatory diseases such as atherosclerosis and multiple sclerosis (236). To evaluate this hypothesis, our lab has mice with a conditional knockout allele for *Pggt1b*, the gene encoding the essential beta-subunit of GGTase-I, and then inactivated the enzyme in macrophages (48).

The knockout of macrophage GGTase-I clearly blocked the prenylation of RHO family proteins. However, contrary to expectations, the mice developed severe, spontaneous, and chronic joint inflammation, which resembles erosive arthritis in humans (Fig. 7A) (48). Even more surprisingly, GGTase-I-deficient macrophages had increased levels of RHO proteins in their active GTP-bound form compared to controls (Fig. 7B), and they also produced higher amounts of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Fig. 7C). Moreover, non-prenylated RAC1 still interacted well with the plasma membrane. Inhibition of RAC1 signaling with a small-molecule inhibitor or knocking down RAC1 expression with shRNAs in GGTase-I-deficient macrophages reduced the secretion of pro-inflammatory cytokines. These results suggest the interesting but at the time farfetched, possibility that a single GGTase-I substrate (out of many dozens) may be responsible for the inflammatory phenotype. But we had not performed similar experiments with RHOA and CDC42.

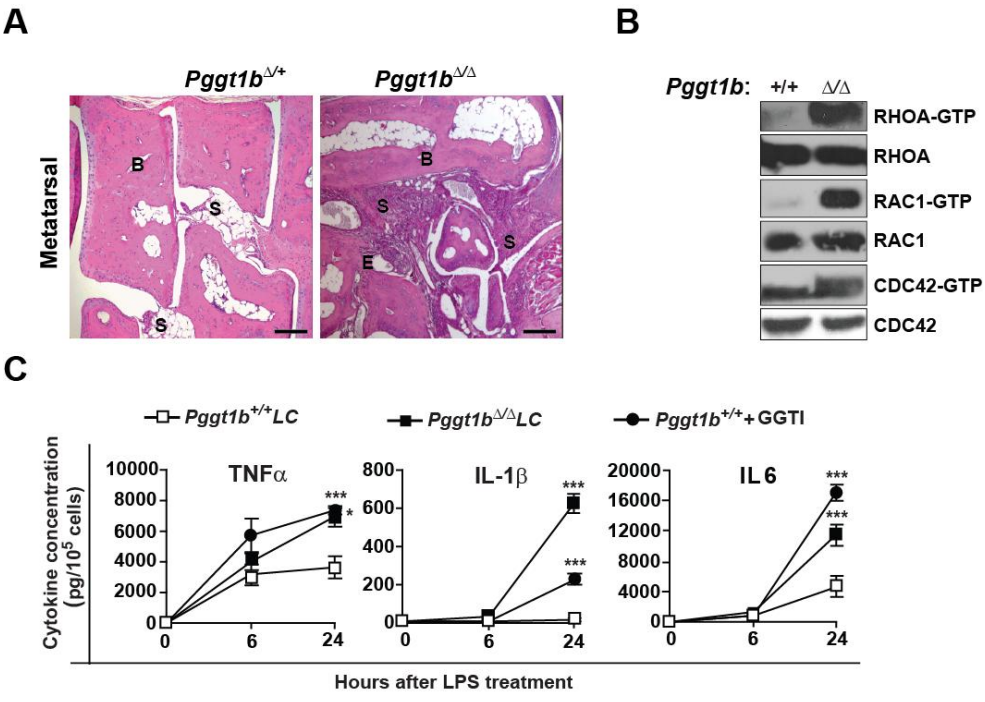

Fig. 7. Knockout of GGTase-I results in erosive arthritis in mice. (A). Synovitis (S) and Erosion (E) in bone metatarsal joint inflammation in control and knockout mice. (B). Accumulation of GTP-bound form of non-prenylated RHO proteins in GGTase-I knock macrophages. (C). Increased production of inflammatory cytokine IL-1β, IL-6 and TNF-α in response to LPS in knockout macrophages and wild-type control macrophages treated with GGTI-298.

5 AIMS

Our long-term goals are to define the biochemical and medical importance of the posttranslational processing of *CAAX* proteins. The aim of my PhD thesis was to define cellular and molecular mechanisms underlying inflammation and arthritis in mice lacking GGTase-I in macrophages.

Specific Aims

Project I: To define the molecular mechanisms involved in the excessive innate immune responses of GGTase-I-knockout macrophages.

Project II: To test the hypothesis that RAC1, RHOA, or CDC42 is responsible for inflammation and arthritis in GGTase-I knockout mice, and to define the mechanism behind the increased GTP-loading of non-prenylated RHO family proteins.

Knockout of GGTase-I in mouse macrophages causes inflammation and erosive arthritis. GGTase-I-deficient macrophages contain increased levels of RHO proteins in their active GTP-bound form, and they secreted higher amounts of pro-inflammatory cytokines in two different pathways: an NFκB-mediated pathway resulting in IL-6 and TNFα secretion, and an inflammasome-mediated pathway resulting in IL-1β maturation. However, there are many gaps in our understanding of the biochemical and physiologic consequences of blocking prenylation. For example, is the increased signaling triggered by specific Toll-like receptors (TLR)? Which pathways are essential for the pro-inflammatory responses? Is the inflammasome activated in *Pggt1b*$^{\Delta/\Delta}$ macrophages and if so, which part? By what mechanism does non-prenylated RHO proteins become constitutively GTP bound? In my PhD studies, I have addressed these questions and present the results in two manuscripts.

6 SUMMARY OF RESULTS

Project I

To define the molecular mechanisms involved in the excessive innate immune responses of GGTase-I-knockout macrophages (237).

Pggt1b is important for cytokine production in macrophages

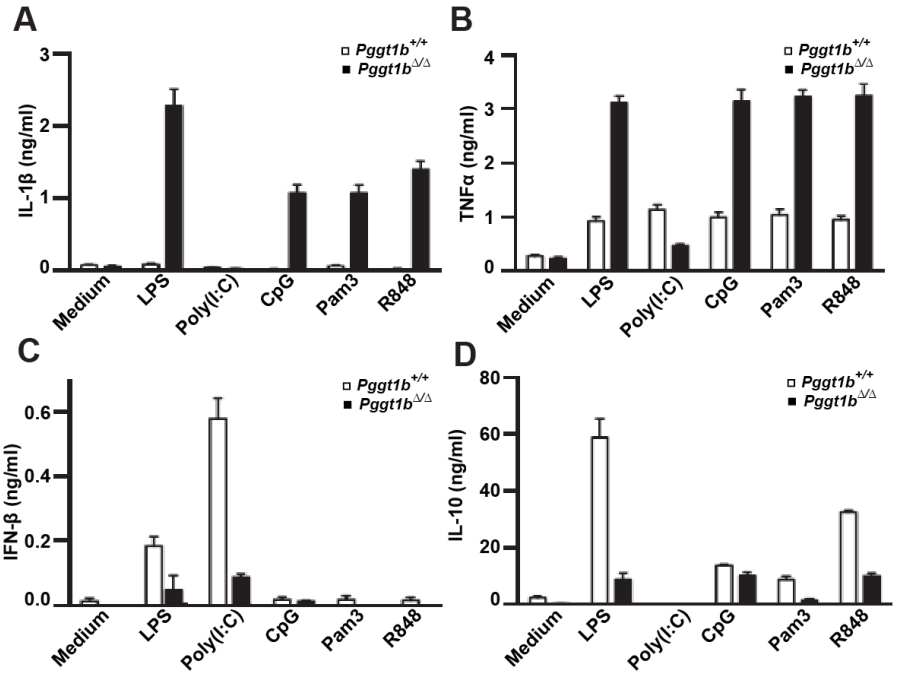

Fig. 8. **Knockout of *Pggt1b* altered the balance between inflammatory and anti-inflammatory cytokine production.** (A—D) Semi-quantification of IL-1β, TNF-α, IFN-1β, IL-10 cytokines by ELISA shows in control and GGTase-I knockout macrophages supernatants stimulated with TLR ligands for 8 h. Data are from three different experiments.

Toll-like receptors (TLRs), a class of pattern recognition receptors (PRRs), detect and mount responses to pathogen-associated molecular patterns (PAMPs) that are generated by invading pathogens at a site of infection (238). To find out more about the role of *Pggt1b* in the regulation of TLR-induced cytokine production, we incubated bone marrow-derived macrophages with the TLR4-agonist lipopolysaccharide (LPS), TLR9-agonist CpG, TLR2-agonist Pam3CSK4, TLR3-

agonist poly (I:C), and TLR7-agonist R848. *Pggt1b*$^{\Delta/\Delta}$ macrophages showed increased production of pro-inflammatory cytokines (Fig. 8A & 8B) and reduced production of anti-inflammatory cytokines in response to TLR agonists (Fig. 8C & 8D). Altered balance between pro-inflammatory and anti-inflammatory cytokines in *Pggt1b*$^{\Delta/\Delta}$ macrophages suggests that GGTase-I has a central role in the regulation of cytokine production.

Pggt1b is important for activation of PI(3)K signaling in macrophages

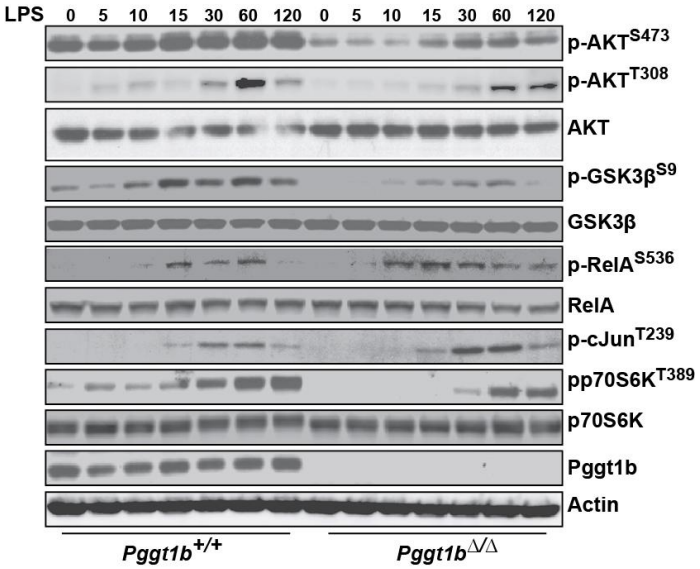

Fig. 9. **Knockout of *Pggt1b* reduced signaling of the PI3K-AKT-GSK3β pathway.** Western blot analysis of phospho-AKT^{S473}, phospho-GSK3β, phospho-RELA^{S536}, phospho-c-jun^{T239} and p70-S6K in bone marrow-derived macrophages stimulated with LPS (10 ng/ml) for 0, 5, 10, 15, 30, 60 and 120 Min.

Next, we investigated which signalling pathway was responsible for the altered balance of cytokine production in *Pggt1b*$^{\Delta/\Delta}$ macrophages. *Pggt1b*$^{\Delta/\Delta}$ macrophages exhibited the reduced activity of pAKT^{S473} and increased the activity of GSK3β in response to LPS treatment compared to the controls used in this project. Two well-known targets of GSK3β, RELA and c-JUN, had increased levels of phosphorylated S536 and Thr239 respectively in *Pggt1b*$^{\Delta/\Delta}$ macrophages, which further confirms

increased GSK3β activity (Fig. 9). Furthermore, reduced activity of P70S6K, one important downstream target of mTORC1 (Fig. 9), suggests that GGTase-I controls the signaling of the mTORC1-pAKT-GSK3β pathway or the PI3K-AKT pathway.

Pggt1b enzyme increases association of KRAS and P110δ and thereby controls cytokine productions

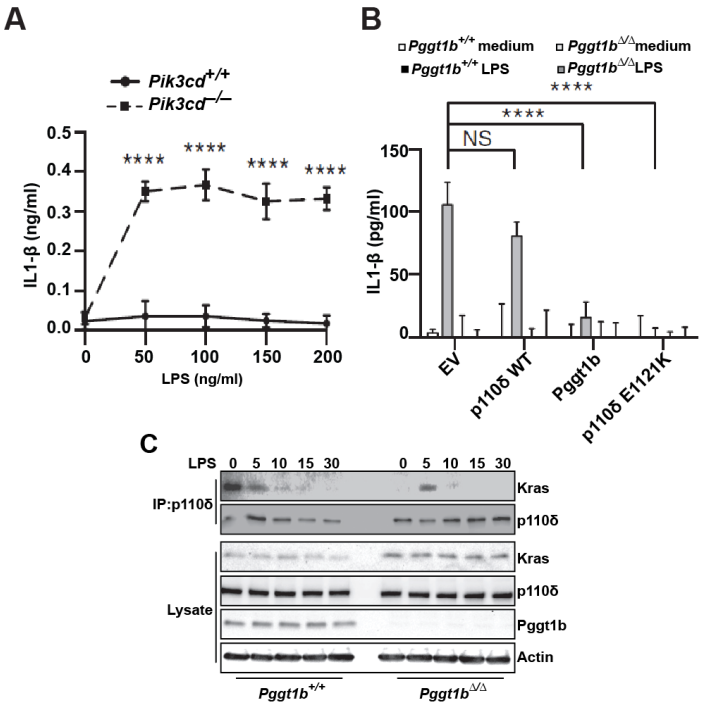

Fig. 10. ***Knockout of Pggt1b increased KRAS-p110δ dissociation and thereby increased inflammatory cytokine production.*** **(**A) IL1β ELISA levels from control and *Pi3kcd*$^{-/-}$ macrophage supernatants after stimulation with different concentrations of LPS for 8hrs. (B). ELISA levels of IL1β from the macrophages after transfection with active p100δ, p110wt and *Pggt1b* in *Pggt1b*$^{\Delta/\Delta}$ macrophages. (C). Immunoblots showing interaction levels of KRAS with different PI3K catalytic subunits from the lysates collected from macrophages after stimulation with LPS for 0, 5, 10, 15 and 30 min. NS (not significant), **** $P < 0.0001$.

As stated above, *Pggt1b*$^{\Delta/\Delta}$ macrophages showed deregulation of PI(3)K-AKT-GSK3β pathway in response to LPS treatment. Recent results have shown that

inactivation of p110δ, a class-1 PI3K catalytic subunit, makes mice more prone to LPS-induced endotoxin death (239). Moreover, *Pik3cd*$^{-/-}$ (p110δ knockout) macrophages produced more IL-1β (Fig. 10A), which pheno-copied *Pggt1b*$^{\Delta/\Delta}$ macrophages. Forced expression of active p110δ in *Pggt1b* knockout macrophages normalized the levels of pro-inflammatory cytokine secretion, suggesting that deregulation of p110δ signaling contributes to the inflammatory cytokine production (Fig. 10B). We found a disturbance in the interaction between KRAS and P110δ (Fig. 10C) in *Pggt1b*$^{\Delta/\Delta}$ macrophages. LPS stimulation made this interaction stronger for a short time (5 min), but the effect is not sustained for a longer period.

Pyrin (MEFV) is responsible for the increased inflammasome in *Pggt1b* knockout macrophages

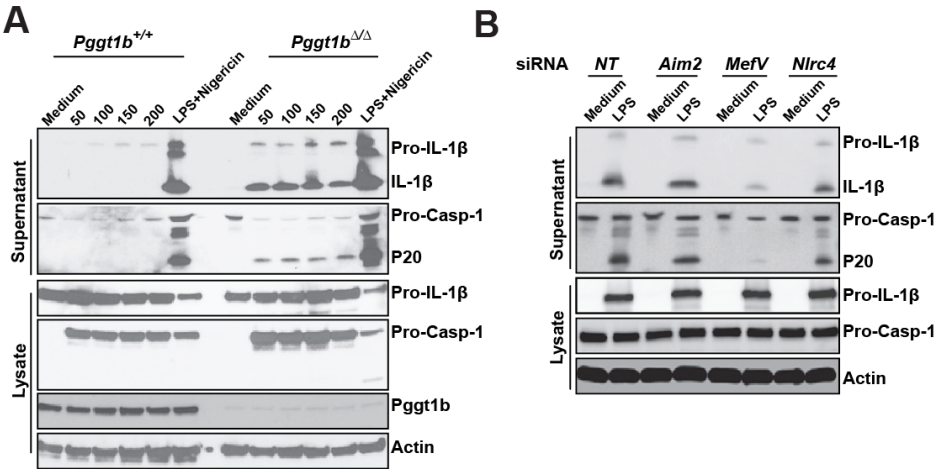

Fig. 11. Knockout of *Pggt1b* caused increased activation of the pyrin-dependent inflammasome. (A) Immunoblot analysis for mature IL-1β and Caspase-1 in supernatants, and pro-IL-1β and pro-Caspase-1 in lysates of LPS-stimulated BM macrophages. (B) Immunoblot analysis for mature IL-1β and Caspase-1 in supernatants, and pro-IL-1β and pro-Caspase-1 in lysates of macrophages incubated with siRNAs targeted against *Aim2*, *Mefv* and *Nlrc4.*

Many of the inflammatory cytokines are regulated through inflammasome activity by activating caspase1 signaling (240). We therefore examined the role of inflammasome activation in *Pggt1b*$^{\Delta/\Delta}$ macrophages in response to LPS. Increase in inflammasome activity contributes to the production of more IL-1β cytokines.

Pggt1b$^{\Delta/\Delta}$ macrophages produced more active IL-1β and had increased activation of caspase-1 signaling, which indicates that caspase-1 is important for active IL-1β production in the GGTase-I-deficient cells (Fig. 11A). We further examined which inflammasome receptor responsible for the increased caspase1 activity in *Pggt1b*$^{\Delta/\Delta}$ macrophages. We found that knockdown of pyrin (*Mefv)* but not *Aim2* and *Nlrc4* in *Pggt1b*$^{\Delta/\Delta}$ macrophages reduced IL-1β production and Caspase-1 signaling (Fig. 11B).

Conclusion

Knockout of GGTase-I results in an impaired PI3K signaling pathway that caused increased inflammatory signaling in macrophages. Impaired PI3K signaling contributed to the increased production of inflammatory cytokines and increased activation of pyrin-dependent inflammasome signaling in *Pggt1b*$^{\Delta/\Delta}$ macrophages.

Project II

To test the hypothesis that RAC1, RHOA, or CDC42 is responsible for inflammation and arthritis in GGTase-I knockout mice, and to define the mechanism behind the increased GTP-loading of non-prenylated RHO family proteins (Submitted manuscript).

RAC1 mediated erosive arthritis in *Pggt1b*$^{\Delta/\Delta}$ mice (Manuscript)

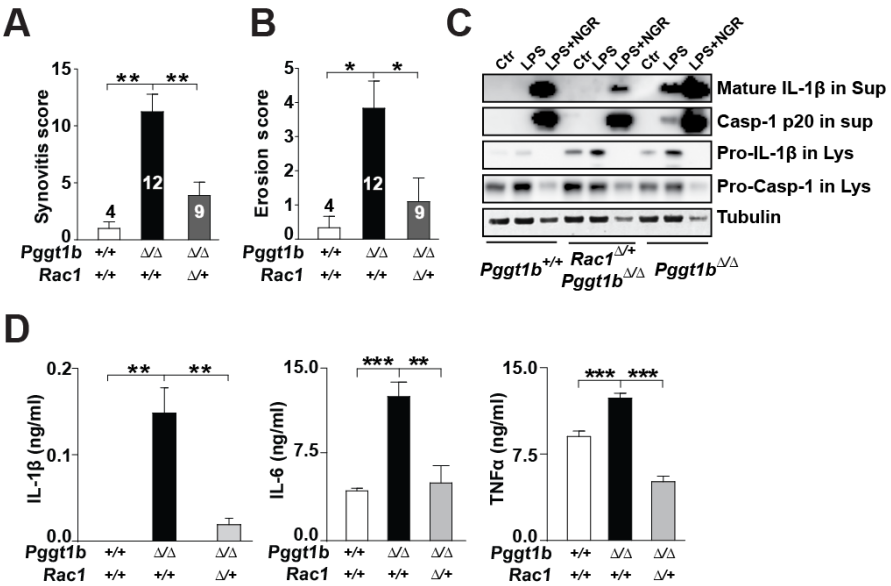

Fig. 12. Knockout of RAC1 in *Pggt1b*$^{\Delta/\Delta}$ mice reduced erosive arthritis, inflammasome activation and inflammatory cytokine production. (A—B) Synovitis and erosion scores from joints of *Pggt1b*$^{\Delta/+}$ *(n* = 4*)*, *Pggt1b*$^{\Delta/\Delta}$ *(n* = 12*)*, and *Rac1*$^{\Delta/+}$ *Pggt1b*$^{\Delta/\Delta}$ *(n* = 9*)* mice at 12 week age. (C) Immunoblots showing levels of mature IL-1β and Caspase-1 in supernatants (Sup), and pro-IL-1β and pro-Caspase-1 in lysates (Lys) of LPS-stimulated BM macrophages; Beta-Tubulin was used as a loading control. Nigericin (NGR) was used as a positive control to induce inflammasome-mediated Caspase-1 activation and IL-1β production. (D) ELISA cytokine levels of IL-1β, IL-6 and TNF-α on bone marrow-derived macrophage supernatants from *Pggt1b*$^{\Delta/+}$ *(n* = 3*)*, *Pggt1b*$^{\Delta/\Delta}$ *(n* = 4*) and Rac1*$^{\Delta/+}$ *Pggt1b*$^{\Delta/\Delta}$ *(n* = 3*)*. n.s not significant, ** $P < 0.01$, *** $P < 0.001$.

Next, we examined the role of three *CAAX* proteins in the development of arthritis in *Pggt1b*$^{\Delta/\Delta}$ mice. For that, we knocked out one copy of *Rac1* (*Rac1*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$), one copy of *Rhoa* (*RhoA*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$) and one copy of *Cdc42* (*Cdc42*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$)

in *Pggt1b*$^{\Delta/\Delta}$ mice. Histological analysis on joints revealed that knocking out of one copy *Rac1* reduced synovitis and erosion scores in *Pggt1b*$^{\Delta/\Delta}$ mice at 12 weeks age (Fig. 12A & 12B).

We further asked whether knocking out one copy of *Rac1* was enough to reduce caspase-1 mediated mature IL-1β production in the absence of *Pggt1b* in macrophages (Fig. 12C). Deletion of one copy of RAC1 significantly reduced the production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 (Fig. 12D) in *Pggt1b*$^{\Delta/\Delta}$ macrophages in response to LPS, while knocking out one copy *Rhoa* and *Cdc42* did not have any effect on cytokine secretion. From the above results, we conclude that RAC1 mediates the production of cytokines which lead to the development of erosive arthritis in *Pggt1b*$^{\Delta/\Delta}$ mice.

Non-prenylated RAC1 bound strongly to IQGAP1 which contributed to GTP loading and inflammatory signaling

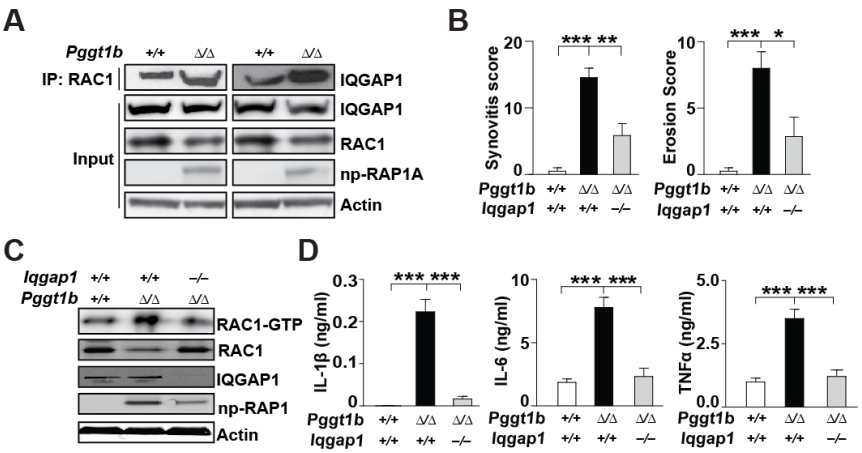

Fig. 13. Non-prenylated RAC1 associated strongly with IQGAP1 and contributed to TLR-induced pro-inflammatory cytokine secretion. (A) Immunoprecipitation analysis showing RAC1 interaction with IQGAP1 in *Pggt1b*$^{\Delta/+}$ and *Pggt1b*$^{\Delta/\Delta}$ macrophage lysates. **(**B) Synovitis and erosion scores from joints of the *Pggt1b*$^{\Delta/+}$ *(n* = 4*), Pggt1b*$^{\Delta/\Delta}$ *(n* = 9*), Iqgap1*$^{-/-}$ *Pggt1b*$^{\Delta/\Delta}$ *(n* = 7*)* mice at 12 weeks of age. (C) Immunoblots showing levels of RAC1-GTP, RAC1, IQGAP1 from *Pggt1b*$^{\Delta//+}$ and *Pggt1b*$^{\Delta/\Delta}$ *and Iqgap1*$^{-/-}$ *Pggt1b*$^{\Delta/\Delta}$ macrophage lysates. Actin was used as loading control, and np-RAP1 was used as a marker for the absence of GGTase-I. **(**D) IL-1β, IL-6 and TNFα cytokine levels in supernatants of bone marrow-derived macrophages from *Pggt1b*$^{\Delta/+}$ *(n* = 3*), Pggt1b*$^{\Delta/\Delta}$ *(n* =

3*) and Iqgap1*$^{-/-}$ *Pggt1b*$^{\Delta/\Delta}$ *(n* = 3*)* mice after stimulation with LPS (10ng/ml) for 8 hrs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, we investigated possible reasons for the conversion of non-prenylated RAC1 into its active GTP-bound form in *Pggt1b*$^{\Delta/\Delta}$ macrophages. In order to do so, we used mass spectroscopy to find RAC1-specific GAPs in GGTase-I knockout macrophages. We identified a list of 717 proteins, whose levels were significantly different in GGTase-I knockout macrophages compared to control macrophages (Table 3). Out of 717 proteins, five of them were specific GAPs for RAC1 and the top hit was RAS GTPase activating-like protein 1 (IQGAP1). Instead of exhibiting conventional GAP activity, IQGAP1 is known to stabilize RAC1 in its GTP-bound conformation. We further corroborated this finding with IP-western blots and found an increased association between non-prenylated RAC1 and IQGAP1 (Fig. 13A).

To determine the role of IQGAP1 in inflammation, we knocked out *Iqgap1* in GGTase-I knockout mice. Knockout of *Iqgap1* markedly reduced inflammation in mouse joints (Fig. 13B), and essentially normalized the levels of RAC1-GTP (Fig. 13C) along with a significant reduction of inflammatory cytokine production (Fig. 13D). We can therefore conclude from the results presented above that IQGAP1 is required for the increased RAC1-GTP levels, the high inflammatory signaling cascade, and the arthritis of in *Pggt1b*$^{\Delta/\Delta}$ macrophages.

TIAM1 contributes to increased RAC1-GTP loading and cytokine production of GGTase-I-deficient macrophages

Because IQGAP1 does not have GEF or GAP activity, we next assessed whether another RAC1-specific GEF is involved. We found that non-prenylated RAC1 binds more strongly with T-cell Lymphoma and metastasis 1 (TIAM1), a known guanine exchange factor (GEF) which converts RAC1 to its GTP-bound form (Fig. 14A). Furthermore, we investigated the functional importance of this increased interaction. Knockdown of TIAM1 expression with small-interfering RNAs (siRNAs) reduced RAC1-GTP levels (Fig. 14B) and also reduced IL-1β cytokine production in GGTase-I knockout macrophages (Fig. 14C). We also demonstrate that TIAM1 binds to IQGAP1 and that this interaction was increased in *Pggt1b*$^{\Delta/\Delta}$ macrophages (Fig. 14D).

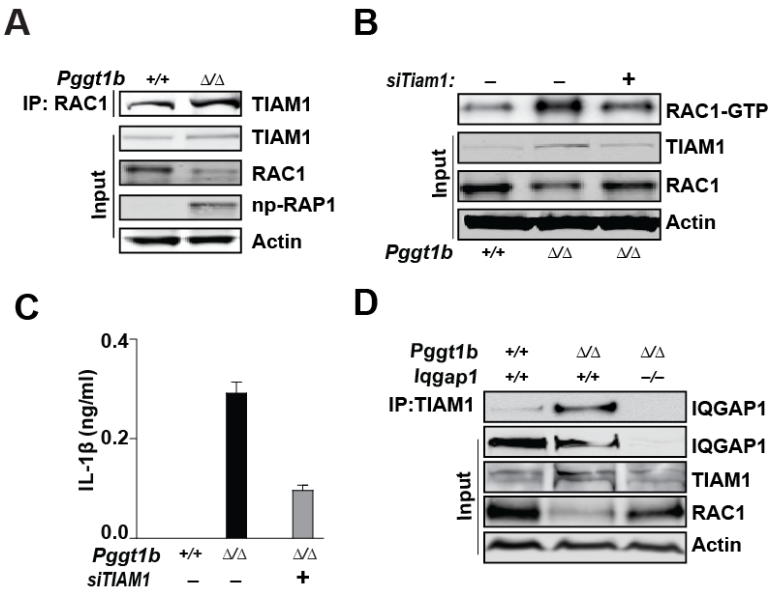

Fig. 14. TIAM1 associated strongly with np-RAC1 and IQGAP1 in GGTase-I knockout macrophages and then contributed to RAC1-GTP loading and cytokine production (A) Immunoprecipitation analysis showing RAC1 interaction with TIAM1 in *Pggt1b*$^{\Delta/+}$ and *Pggt1b*$^{\Delta/\Delta}$ macrophage lysates. **(**B) Immunoblots showing the levels of RAC1-GTP from *Pggt1b*$^{\Delta//+}$ and *Pggt1b*$^{\Delta/\Delta}$ *and TIAM1 knockdown Pggt1b*$^{\Delta/\Delta}$ macrophage lysates. Actin was used as loading control, and np-RAC1 was used as a marker to indicate the absence of GGTase-I in macrophages. (C) IL-1β cytokine levels, after 8 hours of stimulation with LPS, from supernatants of *Pggt1b*$^{\Delta/+}$, *Pggt1b*$^{\Delta/\Delta}$ *and Tiam1 knockdown Pggt1b*$^{\Delta/\Delta}$ bone marrow-derived macrophages. **(**D) Immunoprecipitation analysis showing TIAM1 interaction with IQGAP1 in *Pggt1b*$^{\Delta/+}$ and *Pggt1b*$^{\Delta/\Delta}$ macrophage lysates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Conclusion

Hyperactive RAC1 mediated both pro-inflammatory signaling in vitro and inflammation and arthritis in mice lacking *Pggt1b* in macrophages in vivo. Non-prenylated RAC1 became GTP-bound through an increased interaction with IQGAP1 and TIAM1. The results suggest that IQGAP1 and RAC1 could drive inflammatory processes in conditions where prenylation is inhibited and raise the possibility that inflammatory disorders, such as MKD, that exhibit high levels of activated RAC1, could be treated with IQGAP1 or RAC1 inhibitors.

Statistics

Values are represented to mean and SEM. Significance difference between the groups are analyzed by student t-test or one-way ANOVA with Tukey´s post hoc test. Significance difference less than 0.05 are considered as significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

7 DISCUSSION

In paper I, we showed the underlying molecular mechanisms involved in the control and regulation of excessive innate immune responses in GGTase-I-deficient macrophages. In paper II, we showed the importance of RAC1 in inflammatory phenotypes in mice lacking GGTase-I in macrophages and also investigated potential mechanisms on how non-prenylated RHO proteins become GTP-bound. These studies increase our understanding of the biochemical and medical importance of GGTase-I-mediated prenylation. Our data suggested that one role of prenylation in macrophages is to restrain innate immune reactions by limiting RAC1 effector interactions.

The role of GGTase-I in macrophages for innate immunity

Macrophages are considered as crucial mediators to trigger innate immune responses (186). In the first part of my PhD, I evaluated the importance of macrophage-specific GGTase-I in innate immune responses. The PI(3)K-Akt-Gsk3β and mTOR signaling pathways are important contributors for TLR-induced immune responses via productions of inflammatory mediators, and they emerged as crucial regulators in sensing of immune cells (241-243). However, the underlying molecular mechanisms behind inflammatory signaling of PI3K-AKT-GSK3β and mTOR kinase cascades are not clearly understood. Previous studies identified B-cell adaptor protein for PI3K (BCAP) required for activation of PI3K signaling triggered by Myd88-dependent TLRs (TLR4, TLR7 and TLR9) (244, 245). It is unclear whether TLR3 follows similar mechanisms to activate PI3K signaling. TLR3 directly recruits PI3K to its cytoplasmic tails via specific phosphorylation of tyrosine residues present in cytoplasmic domain of TLR3 (246). We found that there is an altered balance between the production of inflammatory cytokines (IL-1β, IL-6 and TNFα) and anti-inflammatory cytokines (IL-10 and IFN-β) in response to TLR ligands (LPS and Poly:IC) in GGTase-I knockout macrophages. We further noticed impaired signaling of PI3K-AKT-GSK3β, mTOR kinase cascades in response to TLR ligands in GGTase-I knockout macrophages. These results are consistent with previous published data, in which PI3K is essential for production of IFN-β via reduced activation of GSK3β (247-249). These results suggested that GGTase-I is required for PI3K signaling and for the maintenance of the balance

between inflammatory and anti-inflammatory cytokine production via Myd88-dependent and -independent signaling.

Previous studies have shown that RAS, an oncogene, can directly interact with RBD (Ras-binding domain) of PI3K and this interaction is essential for normal and malignant cell growth (250, 251). In addition to that, a few studies reported that RAS and PI3K interaction is responsible for both physiological and pathological consequences of PI3Ks (251). However, it is unclear yet, how this interaction contributes to TLR induced innate immune responses. Three decades ago, the finding that farnesylation of RAS is required for RAS oncogenic transformation caught much attention in scientific world and led to the development of FTIs as discussed earlier. By contrast, clinical trials with FTIs showed no promise against treating cancers and it mainly due to change in prenylation status of KRAS when cells lack FTase I. (59, 252, 253). In this study, we found that reduced RAS activation of PI3K could explain the reduced AKT activation and increased inflammasome activation in GGTase-I-deficient macrophages. And one potential explanation for that could be that a specific pool of geranylgeranylated KRAS normally stimulates PI3K-AKT signaling in macrophages and that this pool in inactivated in GGTase-I-deficient macrophages. Previously, our lab showed that knockout FTase-I in myeloid cells had no effect on KRAS functional activity (29), and more importantly lack of FTase-I had no aberrant effect on immune cell function (48). Our lab showed myeloid cell-specific knockout of both FTase-I and GGTase-I in mice had inflammatory phenotype similar to GGTase-I knockout in macrophages (48). Together with the above results and our data indicated that GGTase-I controls the interaction between KRAS and p110δ and this interaction responsible for controlling signaling cascades responsible for inflammation.

Previous research has shown that defects in LPS induced internalization and subcellular trafficking of TLR4 in dendritic cells lacking p110δ (244). We found that no such defects in GGTase-I deficient macrophages even though PI3K-AKT signaling was impaired in GGTase-I-deficient macrophages and this mainly due to difference in cell types that we examined. Moreover, p110δ-knockout macrophages produced high amounts of inflammatory cytokines and lower amounts of IFN-β, suggesting that those macrophages phenocopy GGTase-I knockout macrophages. We noticed similar results of increased inflammatory cytokine productions in

statin-treated macrophages suggested that the role of unprenylated proteins in increased inflammatory cytokine productions.

We noticed that increased basal activity of IκBα in GGTase-I-deficient macrophages indicating increased NFκB activity via increased activity of RELA. Furthermore, increased GSK3β activity in GGTase-I-deficient macrophages supports activation of NFκB via reduced activation of AKT signaling. The PI3K-AKT-GSK3β and mTOR signaling pathways are pivotal for normal cellular functions that includes proliferation, survival and metabolism. Aberrant signaling of PI3K are noticed in several human diseases, and our data shed at least some light on the importance of the mevalonate pathway in these signaling pathways.

The main criticism of this paper is that we provide no conclusive evidence that KRAS is geranylgeranylated under normal circumstances. Moreover, there is no solid evidence in the literature of KRAS geranylgeranylation, other than in FTI-treated cells. And although absence of evidence is not evidence of absence; clearly, more studies are required to understand the role of p110δ, KRAS, and AKT-signaling for the inflammatory phenotypes of GGTase-I-deficient macrophages.

RAC1 contributes to erosive arthritis in GGTase-I knockout mice

During the second part of my PhD, we set out to identify the cellular mechanisms behind erosive arthritis in mice lacking GGTase-I in macrophages. Our data revealed new insights of physiological and biochemical consequences of blocking GGTase-I in immune cells. GGTase-I prenylates several dozen *CAAX* proteins (perhaps more than 60) and our data revealed that only one of them, RAC1, is responsible for the main phenotypes. Even though arthritis was significantly reduced in RAC1 knockout mice (to a level that was actually statistically indistinguishable from wild-type controls), it was not eliminated completely, indicating that other RAC isoforms, such as RAC2, might be involved in the residual inflammation.

Prenylation is believed to be important for RHO family protein function such as membrane trafficking, activation of RHO proteins, and their interactions with effectors (1). Many studies supported the argument that prenylation is required for

RHO family function. Contrary to that, our study identified several important things on how unprenylated proteins behave in cells that goes against previous arguments.

First, in our earlier studies non-prenylated RAC1 was hyperactive, and its membrane targeting was functional in GGTase-I knockout macrophages (and we proved with metabolic labeling that RAC1 was not prenylated) (48). In support of that, we found in the current study that RAC1 is hyperactive and targeted to membranes in HEK cells harbouring nonprenylated RAC1 mutations in the presence of normal GGTase-I activity. The only main deviation in cellular localization is that we consistently find RAC1 in the nucleus of normal macrophages, but in multiple studies, we see that endogenous nonprenylated RAC1 is excluded from the nucleus. This finding goes against a wide-spread belief that non-prenylated RAC1 accumulates in the nucleus, a result that comes from expressing GFP-tagged exogenous forms of the protein (254).

Second, consistent with previous results, we showed that non-prenylated RAC1 accumulate in its active GTP-bound form in GGTase-I knockout macrophages; and in the present study, we add that total levels of RAC1 are consistently reduced – which leads to a marked increase in RAC1 specific activity. The reduction of total RAC1 is likely due to increased ubiquitin-mediated degradation of RAC1-GTP in GGTase-I knockout macrophages, an effect that was controlled by IQGAP1.

Third, our studies showed that increased levels of GTP-bound and total nonprenylated RHOA and CDC42, even though these proteins did not appear to contribute to the inflammatory phenotype in GGTase-I knockout mice. These results suggested that blocking geranylgeranylation differentially regulates degradation of RHO proteins; the precise mechanisms underlying stabilization of total levels of RHOA and CDC42 needs to be investigated further, but again we found clear evidence that it is regulated by IQGAP1. It is possible that IQGAP1 can target some GTP-bound RHO proteins to degradation and protect others.

Fourth, our studies showed that blocking prenylation leads to sustained activation of RAC1. Activation of RAC1 triggered production of inflammatory cytokines activated pyrin and caspase-1-dependent inflammasome signaling (Figure 14). These increased inflammasome signaling clearly underlies the increased production

of mature IL1β. Altogether, our data suggest that blocking RAC1 geranylgeranylation leads to inflammatory phenotypes in GGTase-I knockout mice.

Previous studies reported a reduced association between nonprenylated RAC1 and RHOGDI1 (48). Another earlier study showed that RAC1-GTP levels are increased in cells where RHOGDI1 expression was suppressed by siRNAs (255). This raises the possibility that RAC1 in GGTase-I-deficient cells interacts less with RHOGDI1 and that this explains the increased GTP-loading. We tested this possibility in great detail in three different macrophage cell lines by both siRNAs and CRISPR/CAS9-mediated knockout of RHOGDI1. We found that knockdown of RHOGDI1 increased RAC1-GTP in one of the three cell lines and that this led to increased production of IL-6 and TNFα in response to LPS, but IL1β was not produced. Thus, we concluded that RHOGDI1 is likely not involved in the main phenotypes of GGTase-I deficiency. Instead, the phenotypes are explained by an increased association between RAC1, IQGAP1, and TIAM1.

Previous studies reported that IQGAP1 preferentially binds and stabilize to RAC1 in its GTP form (147) (256). We found that IQGAP1 binds stronger to non-prenylated RAC1 and that TIAM1 is the main GEF involved. We also found that IQGAP1 is essential for development of inflammatory phenotypes in macrophage-specific GGTase-I knockout mice. Indeed, knockout of IQGAP1 reduced inflammatory phenotypes; reduced levels of GTP-bound RHO proteins; and normalized total levels of RHO proteins in GGTase-I knockout macrophages.

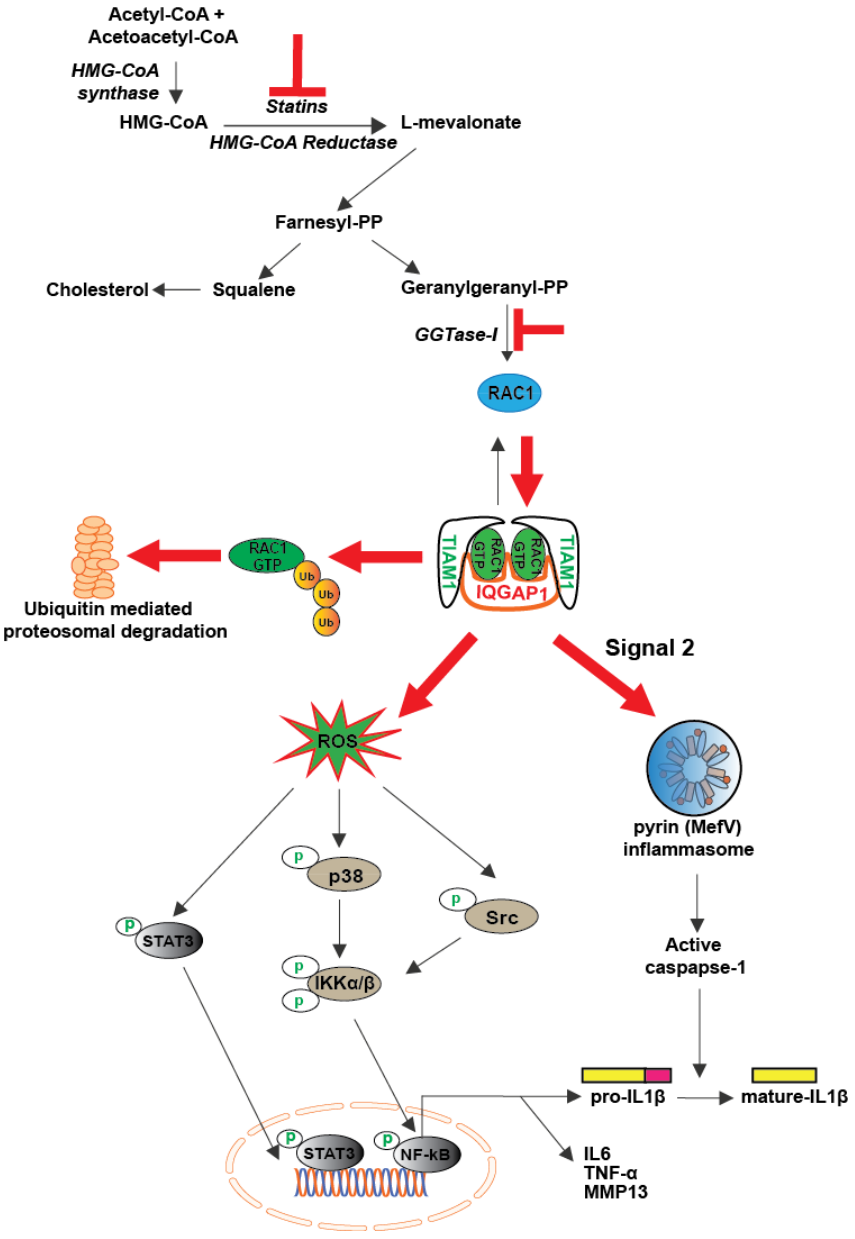

Fig. 15. The mevalonate pathway controls the innate immune response through geranylgeranylation of RAC. Geranylgeranyl pyrophosphate is an important intermediate produced by the mevalonate pathway. GGTase-I uses GGPP for prenylation of RHO family proteins. GGTase-I limits RAC1signaling (RAC1-p38-Nf-KB) to control cytokine production and *Mefv* inflammasome formation.

Nonprenylated RAC1 had a high affinity for IQGAP1, and this interaction was essential for in vitro and in vivo phenotypes. This raises the interesting possibility that targeting IQGAP1 might be an effective strategy to treat auto-inflammatory disorders like HIDS and MKD in which there is evidence of reduced prenylation. Earlier research suggests RAC1 inhibitors might be useful in the treatment of MKD patients but knockout of RAC1 is lethal, and it causes several cellular phenotypes in mice. Thus, we propose that IQGAP1 might be worthwhile testing. IQGAP1 is not essential for mouse development and in our studies, we find that cytokine production by IQGAP1-deficient macrophages was normal (Figure S5).

Statin treatment of macrophages consistently produced similar phenotypes as GGTase-I deficiency in our experiments. Some of those statin effects have been observed in earlier studies, but no clear mechanism has been established (218, 257). Here we clearly link statin effects to IQGAP1 because statin effects were blunted or abolished in *Iqgap1*-deficient cells. Most studies conclude that statins have anti-inflammatory rather than pro-inflammatory effects. We discuss this issue in the Paper II and propose that statins anti-inflammatory effects might be caused by blocking prenylation in lymphocytes rather than macrophages. Moreover, we propose that side-effects of statin therapy might be caused by reduced prenylation and hyperactivation of RHO family proteins. Side effects are observed in more than 10% of people treated with statins (i.e., many hundred thousand patients) and range from muscle pain (myositis, common) to rhabdomyolysis (rare) and death.

The main criticism of this study is that we didn't reveal whether RAC1 becomes GTP-bound before or after binding to IQGAP1. On one hand, RAC1 could be GTP-loaded by TIAM1 immediately after synthesis and then become bound to IQGAP1. On the other hand, RAC1 could first be recruited to IQGAP1, encounter TIAM1 and then become GTP-bound. Our results do not clearly distinguish between those possibilities. It would also be interesting to create a RAC1 mutant that is incapable of binding IQGAP1 (or vice versa) and determines if this would still lead to increased RAC1-GTP loading and pro-inflammatory signaling. It would be informative to analyze mice expressing constitutively active RAC1 in macrophages (or ubiquitously) and determine if increased RAC1-GTP levels are sufficient to produce arthritis or if RAC1 also has to be unprenylated.

8 FUTURE DIRECTIONS

Previously, we reported that mice lacking *Pggt1b* in macrophages develop erosive arthritis; and now we have shown that knockout of one copy of *Rac1* significantly reduces disease symptoms in joints of *Pggt1b*$^{\Delta/\Delta}$ mice.

One of the most prominent hallmarks of *Pggt1b*-deficient macrophages is the accumulation of non-prenylated proteins such as RAC1, RHOA and CDC42 in the active GTP-bound form; and the associated TLR-agonist-induced production of pro-inflammatory cytokines. To further determine if RAC1 alone mediates inflammation, we need to eliminate the possibility that any one of the other 50-100 GGTase-I substrates (that also accumulates in the non-prenylated form in the cells) are not involved. To accomplish this, we will engineer an endogenous *CAAX*-mutant form of *Rac1* and express it ubiquitously in mice or only in macrophages or lymphocytes. For this, we will use CRISPR/CAS9 or conventional gene-targeting in ES cells to create a mutation in the *Rac1* gene that will lead to the production of RAC1^{C190S} and RAC1$^{C190\Delta}$ which will not be prenylated. If such "*Rac1*-SLLL" mice develop arthritis, we would have proven this point. If they don't, we need to understand why.

We also need to address an important unanswered question relating to Paper I: how could the absence of GGTase-I reduce the interaction between KRAS and p110δ given that neither of these two proteins should be affected by the absence of GGTase-I? Although KRAS can be geranylgeranylated by GGTase-I, it is a better substrate for FTase. One theory, proposed from Paper I, is that there is a pool of KRAS that is normally exclusively prenylated by GGTase-I and that this pool becomes non-prenylated in the GGTase-I-deficient macrophages—and that non-prenylated KRAS interacts poorly with p110δ. Another theory would be that hyperactive RAC1 influences the KRAS-p110δ interaction in the absence of GGTase-I. In the future, we plan to address these issues in a definitive fashion and further increase our understanding of the role of *CAAX* protein prenylation in mammalian cells.

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