REGULATION OF THE EXPRESSION AND THE ANTI-INFLAMMATORY FUNCTION OF REGULATOR OF G-PROTEIN SIGNALING 10 (RGS10) IN ACTIVATED MICROGLIA AND MACROPHAGES

by

FARIS ABDULLAH ALMUTAIRI

(Under the Direction of Balázs Rada and Shelley B. Hooks)

ABSTRACT

Macrophage cells in different organs, such as microglia and alveolar macrophages, maintain normal organ homeostasis and become activated in response to injury and infection. Under acute activation conditions, they play an important role in phagocytosing dead cells. However, sustained activation of macrophages leads to an overproduction of inflammatory mediators, resulting in persistent inflammatory responses and inflammatory diseases. Regulator of G-protein signaling 10 (RGS10) is a small member of the R12/D RGS subfamily, which canonically modulates G-protein signaling through its GTPase-activating protein (GAP) activity. RGS10 has an abundant expression in resting microglia and macrophages, but its expression is suppressed following LPS stimulation. In the first study, we aimed to determine the inflammatory responses required for LPS-induced RGS10 suppression. Our data show that pharmacological inhibition of PI3K activity, NF-κB-dependent TNF-α and HDAC (1-3) activities stabilize RGS10 expression following LPS stimulation in alveolar macrophages, microglia and BMDMs, suggesting that these inflammatory mediators facilitate the suppressive effect of LPS on RGS10 expression. Independent of its GAP function, RGS10 acts as an anti-inflammatory protein by inhibiting LPS-induced proinflammatory mediators, such as TNF-α and COX-2. In the second study, our goal was to identify non-canonical binding partners of RGS10 in microglia and their possible roles in mediating its anti-inflammatory effects. Among multiple interacting proteins, we identify stromal

interaction molecule (STIM2) as a novel binding partner of RGS10 in BV2 microglia cells and RAW264.7 macrophage cells. Further, we demonstrate that STIM2-Orai activity is a downstream signaling pathway of TLR4 activation-stimulated COX-2, and it is essential for RGS10 action on LPS-stimulated COX-2 and TNF-α. Due to the ability of microglial RGS10 to suppress inflammatory signaling in GAP-independent mechanism, the third aim of our study was to determine whether RGS10 regulates inflammatory signaling in ovarian cancer, and if so, whether this effect is mediated by enhanced G*α*i signaling. Our data show that loss of RGS10 significantly amplifies NF-κB-p65 phosphorylation, TNF-α and COX-2 transcripts in SKOV3 cells, and upregulation of inflammatory signaling mediated by RGS10 knockdown is not affected by Gαi inhibition. The findings of our study provide novel insights into both the mechanism controlling RGS10 expression and the mechanism underlying RGS10's anti-inflammatory function in activated microglia and macrophages.

INDEX WORDS: Regulator of G-protein Signaling (RGS) proteins; RGS10; G-proteins; Microglia; Macrophages; Lipopolysaccharide (LPS); Toll-like receptor (TLR)-4; NF-κB; Cytokines; Cyclooxygenase-2; Histone deacetylases; Neuroinflammation

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DEDICATION

I dedicate this dissertation to my inspiring parents: Maha and Abdullah, for their unconditional love and endless support throughout my life. This achievement would not have been possible without their encouragement and patience. I cannot thank you enough.

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Abbreviations

AC: adenylate cyclase

AD: Alzheimer's disease

ADP: adenosine diphosphate

AlF4: aluminum fluoride

AML: acute myeloid leukemia

AngII: angiotensin II

AR: androgen receptor

Arg1: arginase-1

ATP: adenosine triphosphate

5-Aza: 5-Aza-2'-deoxycytidine

BMDMs: bone marrow-derived macrophages

Ca^{2+}: calcium ion

CaM: calmodulin

CBP: CREB-binding protein

CD: cluster of differentiation

ChIP: chromatin immunoprecipitation

CNS: central nervous system

COX-2: cyclooxygenase-2

CRAC: Ca^{2+} release-activated Ca^{2+} channels

CREB: cAMP response element-binding protein

DAMPs: damage-associated molecular patterns

DEP: disheveled EGL10-Pleckstrin

DNMTs: DNA methyltransferases

EAE: experimental autoimmune encephalomyelitis

EOC: epithelial ovarian cancer

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FSL-1: Pam2CGDPKHPKSF, synthetic diacylated lipoprotein

GAIP: G-alpha interacting protein

GAP: GTPase-activating protein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Gβ5: G-protein β subunit

GDI: guanine nucleotide dissociation inhibitor

GDP: guanosine diphosphate

GEFs: guanine nucleotide exchange factors

GFP: green fluorescent protein

GGL: G protein γ subunit-like

GIRK: G protein-coupled inwardly-rectifying potassium channel

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

HATs: histone acetyltransferases

HD: Huntington's disease

HDACs: histone deacetylases

6-OHDA: 6-hydroxydopamine

HFT: high fat diet

hNP: human neural progenitors

HRP: horseradish peroxidase

I*κ*B: inhibitor of nuclear factor-kappa B

IKK: inhibitory *κ*B kinase

IL: interleukin

iNOS: nitric oxide synthase

IP3: inositol-1,4,5-triphosphate

kDa: kilodalton

KD: knockdown

KO: knockout

LFD: low-fat diet

L/MGE: lateral/medial ganglionic

LPA: lysophosphatidic acid

LPS: lipopolysaccharide

MAP: mitogen-activated protein

M-CSF: Macrophage colony-stimulating factor

MHCII: major histocompatibility complex II

MMP: metalloproteinases

MN9D: mesencephalon neuroblastoma cell line

MS: multiple sclerosis

mTORC1: mechanistic target of rapamycin complex 1

MyD88: myeloid differentiation factor 88

N.D.: not determined

NFATc1: nuclear factor of activated T cells 1

NF-κB: nuclear factor kappa B

NO: nitric oxide

PAMPs: Pathogen-associated molecular pattern

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PGE2: prostaglandin E2

PD: Parkinson's disease

PI3K: phosphatidylinositol 3-kinases

PIP2: phosphatidylinositol-4,5-bisphosphate

PKA: cyclic AMP-dependent protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PRRs: pattern-recognition receptors

PPAR: peroxisome proliferator-activated receptor

pSNL: partial sciatic nerve ligation

PTM: post-translational modifications

PTX: pertussis toxin

RANKL: receptor activator of nuclear factor (NF)-kB-ligand

R9AP: RGS9-1 anchor protein

R7BP: R7 binding protein

RGS: regulator of G protein signaling

ROS: reactive oxygen species

RT-PCR: reverse-transcription PCR

S1P: sphingosine-1-phosphate

siRNA: small interfering RNA

SNP: single nucleotide polymorphism

SNpc: substantia nigra pars compacta

SNVs: single nucleotide variants

SOCE: store-operated Ca^{2+} entry

SPL: spinophilin

STIM2: stromal interaction molecule-2

TAK1: transforming growth factor-*β*-activated kinase 1

TG: thapsigargin

TGF-β: transforming growth factor-β

TLR: toll-like receptor

TNF-α: tumor necrosis factor-alpha

TSA: trichostatin A

TxA2: thromboxane A2

UDP: uridine 5′ diphosphate

VEGF: Vascular Endothelial Growth Factor

WT: wild-type

CHAPTER 1

INTRODUCTION

Heterotrimeric G-proteins signaling

G-protein coupled receptors (GPCRs) are composed of seven α-helical transmembrane domains (TMD) with extracellular amino and intracellular carboxy-terminal extensions. They represent the largest family of cell surface receptors and the most abundant pharmacological targets, as a third of FDA-approved drugs act on GPCRs (Lundstrom and Chiu, 2005, Garland, 2013, Sriram and Insel, 2018). GPCRs are associated with heterotrimeric G-proteins complex (Gαβγ), which function as molecular switches by coupling extracellular signals acting on the receptor to a distinct set of intracellular effectors generated a distinct set of downstream second messengers that ultimately result in regulation of cellular responses (Lambert, 2008, Syrovatkina et al., 2016, Glukhova et al., 2018, Alexander et al., 2019).

Under the resting condition, the receptor interacts via its cytoplasmic side with an inactive heterotrimeric G-protein complex consisting of a GDP bound-Gα subunit associated with the Gβγ subunits that serve as guanine nucleotide dissociation inhibitors (GDI) to prevent the release of GDP from Gα. In a general manner, a wide variety of extracellular ligands bind and activate GPRCs, which subsequently undergo conformational changes. Consequently, activated GPCRs act as guanine nucleotide exchange factors (GEFs) that trigger the exchange GDP for GTP on the Gα-binding pocket. The binding of GTP to Gα subunit causes major conformational changes in the three flexible switch regions that surround the nucleotide-binding pocket of Gα subunit and results in the dissociation of GTP-bound Gα subunit from Gβγ dimer. Subsequently, GTP-bound Gα subunit and Gβγ dimer interact with downstream effectors to transduce different signaling pathways (Gilman, 1987, Bourne et al., 1990, Simon et al., 1991, Hepler and Gilman, 1992, Hamm, 1998).

While more than 800 GPCRs are encoded in the mammalian genome, only a conserved set of 16 Gα subunits have been identified (Jacoby et al., 2006, Insel et al., 2012). These subunits are classified into four main subfamilies including Gαs, Gαi/o, Gαq, and Gα12/13 and selectively target specific effectors. The Gαs subfamily is composed of Gαs and Gαolf subunits and has a stimulatory activity by activating adenylyl cyclase (AC) and subsequently increasing cAMP production. Unlike Gαs, inhibition of AC is mediated by the Gαi/o subfamily that has eight subunits including Gαi1, Gαi2, Gαi3, Gαo, Gαz, Gαt1, Gαt2, and gustducin. The Gαq subfamily consists of Gαq, Gα11, Gα14, and Gα15 and functionally activates phospholipase C (PLC) enzyme that hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG and IP3, as second messengers, positively modulate, in turn, protein kinase C and calcium signaling, respectively. The last subfamily of Gα subunits includes Gα12 and Gα13, which is involved in cytoskeleton remodeling through activation of the small G-protein Rho family via RhoGEFs. In addition to the variety of Gα subunits, the Gβ and Gγ subunits are diverse, as Gβ encodes five subunits and Gγ encodes 12 subtypes. As a heterodimer and independent of its function as a GDI for Gα subunits, Gβγ modulates several downstream effectors, such as ion channels, PI3K signaling and a subset of AC and PLC enzymes.

The amplitude and duration of GPCRs signaling is desensitized via downregulation of ligand-bound GPCRs, which is initiated via multiple interconnected steps of signaling pathways. Following GPCRs stimulation and subsequent dissociation of Gα subunits from Gβγ, the free Gβγ dimer activates G protein-coupled receptor kinases (GRKs) and facilitates targeting of GRKs to the membrane. GRKs, in turn, phosphorylate the activated receptor and recruit β-arrestins, which hinder further G-protein coupling to the receptors, leading to the internalization of the receptor. Due to its interaction with β-arrestins, recognized now as scaffold proteins, the internalized receptors interact with other proteins, such as c-Src and start a second round of signaling independent of its cognate G-proteins. In addition, the internalized receptor can be degraded or recycled back to the membrane to start a new cycle of activation (Calebiro and Godbole, 2018).

Aside from regulatory mechanisms involved in the termination of ligand-receptor binding, deactivation of GTP-bound-Gα subunits is initiated by the hydrolysis of GTP on Gα subunits to GDP. Although there was a general acceptance that the intrinsic GTPase activity of the Gα subunits is sufficient for turning off GTP-bound Gα subunits, the discrepancy between the fast rate of GTP hydrolysis in a cellular context and the slow rate of GTP hydrolysis using the intrinsic GTPase activity of purified G-proteins has led to the discovery of the missing link, which is the founding member of the regulator of G-protein signaling (RGS) proteins. RGS proteins terminate signaling pathways downstream of GPCRs by acting as GTPase activating proteins (GAPs) on active form, GTP-bound Gα-subunits, through enhancing their intrinsic GTPase activity for GTP hydrolysis and returning G-proteins to their inactive form, GDP-bound Gα-subunits.

Regulator of G-protein signaling (RGS) proteins

In the early 1980s, the original insights into new proteins for G-protein regulation stemmed from studies in yeast. Mutated Sst2, which has demonstrated as a regulator of yeast (*Saccharomyces cerevisiae*) sensitivity to mating pheromone α-factor (Chan and Otte, 1982, Chan and Otte, 1982, Weiner et al., 1993), induced a growth arrest through acting on GPCR Ste2 coupled to Gα subunit GPA1 (Dohlman et al., 1995, Dohlman et al., 1996). Later, Sst2 was identified as a negative regulator and interacting partner of GPA1 (yeast Gα subunit) (Apanovitch et al., 1998). Subsequently, via genetic studies, multiple homologs of Sst2 termed as the regulator of G-protein signaling (RGS) proteins have been discovered in different organisms, including FlbA as a negative regulator of FadA (G-protein α-subunit) in the aspergillus (*Emericella nidulans*) (Lee and Adams, 1994, Yu et al., 1996), EGL-10 as a negative regulator of GOA-1 (G-protein α-subunit) in nematode worm (*Caenorhabditis elegans*) (Koelle and Horvitz, 1996), and mammalian cells, such as the human protein G-alpha interacting protein (GAIP) (now known as RGS19) that interacts with activated Gαi3 in yeast two-hybrid screens (De Vries et al., 1995), BL34/1R20 (now known as RGS1) (Hong et al., 1993), GOS8 (now known as RGS2), and RGS7, the most similar

mammalian protein of EGL-10 (Koelle and Horvitz, 1996, Siderovski et al., 1996). In addition to these proteins, other mammalian RGS proteins have been identified, which share a conserved 120-amino acid core domain termed as RGS domain, recognized as the hallmark of all RGS proteins (Berman;Wilkie; et al., 1996, Druey et al., 1996, Hunt et al., 1996, Watson et al., 1996, Doupnik et al., 1997, Hepler et al., 1997).

RGS domain, which is organized into a bundle of nine helices, directly binds to G-proteins α subunits in active form (Gα-GTP) and promotes GTP hydrolysis by Gα subunits through stabilization of the transition state (Gα-GDP-$AlF4^-$ as a stable mimic of Gα-GTP) for GTP hydrolysis (Berman;Kozasa; et al., 1996, Tesmer et al., 1997). This function of the RGS domain establishes RGS proteins as canonical GTPase-activating proteins (GAPs), which accelerates the hydrolysis rate of Gα-GTP (GTPase activity) up to 1000-fold, and thereby termination of G-protein signaling (Ross and Wilkie, 2000, Hollinger and Hepler, 2002). In addition, some RGS proteins act as effector antagonists to limit GPCR signaling independent of their GAP activity via competitive binding to Gα to block Gα-effector interaction (Hepler et al., 1997, Carman et al., 1999).

Twenty canonical members in mammals (**Table 1.1**), which belong to the RGS family and share the RGS domain, are subdivided into four subfamilies, including A/RZ, B/R4, C/R7, and D/R12 based on their high homology of RGS domain sequence and their structures (Ross and Wilkie, 2000, Hollinger and Hepler, 2002). While most canonical RGS-containing proteins are small, containing short N-terminal and C-terminal extensions apart from the shared RGS domain, some RGS proteins are larger and contain multiple additional functional motifs and domains(Squires et al., 2018).

The A/RZ subfamily consists of small proteins of RGS17, RGS19, and RGS20. Aside from their canonical RGS domain, these proteins have a cysteine string at their N-terminal subjected to palmitoylation reaction to promote membrane localization and interactions with binding partners (De Vries et al., 1996, Nunn et al., 2006). The members of the A/RZ subfamily show selective

GAP activity for certain subunits of both Gαi and Gαq subfamily (Tu et al., 1997, Glick et al., 1998, Mao et al., 2004). RGS17, known as RGSZ2, is abundantly expressed in the brain (Mao et al., 2004) and has a role in regulating drug dependence (Zhang et al., 2012, Hayes and Roman, 2016). While RGS17 is overexpressed in several cancers, including lung cancer, prostate cancer, and liver cancer, its expression was suppressed following chemotherapy drugs treatment in ovarian cancer cell lines, suggesting a potential role in chemoresistance (Hayes and Roman, 2016). Unlike RGS17, RGS19, known as GAIP, is expressed at low levels in the brain, where the loss of RGS19 slightly enhances analgesic effects induced by μ-opioid receptors activation (Garzon et al., 2004). RGS19 has a predominant transcript expression in the heart, in which overexpression of RGS19 in cells inhibits cardiomyocyte differentiation via suppression of the Wnt signaling. In addition, transgenic mice overexpressing RGS19 display several heart defects and elevated levels of heart failure markers, suggesting that RGS19 has negative impacts on cardiac development and functions (Ji et al., 2010). In addition, RGS19 expression is upregulated in diseases models, such as multiple sclerosis (MS) (Igci et al., 2016) and ovarian cancer (Tso et al., 2011). Similar to RGS19, RGS20, known as RGSZ1, is found in the brain and loss of RGS20 upregulates analgesia and tolerance following μ-opioid receptor stimulation by morphine (Garzon et al., 2004). Furthermore, in several types of cancer cells, including cervical, breast and lung cancers, overexpression of RGS20 leads to enhanced multiple aspects of cancer progression, such as cell aggregation, migration, invasion, and adhesion, while knockdown of RGS20 impaired these phenotypes (Yang;Lee; et al., 2016).

The C/R7 subfamily is composed of RGS6, RGS7, RGS9, and RGS11 proteins. These proteins are reported to promote GTP hydrolysis on Gαi/o subunits (Masuho et al., 2020). In addition to the RGS domain, the members of the C/R7 subfamily contain additional unique domains, including a G protein γ subunit-like (GGL) domain and disheveled EGL10-Pleckstrin (DEP) homology domain (Gold et al., 1997, Ahlers et al., 2016, Gerber et al., 2016). The GGL domain, which resembles the structure of the γ subunit of G-protein, mediates the interaction with

the G-protein β subunit (Gβ5) and stabilizes the C/R7 subfamily proteins (Snow;Krumins; et al., 1998, Anderson et al., 2009). The DEP domain is involved in interaction to anchor proteins, such as R9AP (RGS9-1 anchor protein) in the retina, and R7BP (R7 binding protein) in the brain (Anderson et al., 2009), which facilitate the C/R7 proteins to target their G-proteins (Hu et al., 2003, Drenan et al., 2006).

RGS6 has numerous splice variants and is widely expressed throughout the body, with a high expression of mRNA and protein found in the brain (Gold et al., 1997, Ahlers et al., 2016). RGS6 is enriched in the ventral tegmental area in the brain, where its expression is upregulated following chronic alcohol exposure (Stewart et al., 2015), and the brains from RGS6 knockout mice show a reduction of alcohol-seeking behaviors (Stewart et al., 2015). In addition, RGS6 is highly expressed in SNc DA neurons, where the loss of RGS6 promotes their late-age degeneration (Ahlers et al., 2016). In aged RGS6 knockout mice, degeneration of SNc DA neurons, which is a feature of PD, is correlated with reduced levels of markers that are required for DA neurons differentiation and maintenance, such as tyrosine hydroxylase (TH) and the vesicular DA transporter, Vmat2 (Ahlers et al., 2016). This suggests that RGS6 is a critical key modulator of SNc DA neuron survival. Further, RGS6 is also found in the hippocampus, a region within the brain mediating mood disorders. RGS6 KO mice spontaneously develop anxiolytic and antidepressant behaviors (Stewart et al., 2015). In addition to brain disorders, RGS6 expression is negatively linked to multiple forms of cancers, such as pancreatic cancer and breast cancer (Ahlers et al., 2016).

Like RGS6, RGS7 has an abundant expression in the brain, particularly in the ventral tegmental area and hippocampus (Larminie et al., 2004, Sutton et al., 2016). Following morphine administration, RGS7 KO mice display an enhancement of behaviors, such as reward, withdrawal, analgesia, and a delay of tolerance (Sutton et al., 2016). Some identified single nucleotide variants (SNVs) of RGS7 have been reported to be associated with disorders, such as MS (McCauley et al., 2009) and panic disorder (Hohoff et al., 2009).

The RGS9 gene generates two transcript variants, yielding two proteins: (RGS9-1) a short isoform expressed in the retina and promotes GAP activity on G-protein, such as transducin coupled to photoreceptors (He et al., 1998, Zhang et al., 1999). Functionally, loss of RGS9-1 is linked to bradyopsia (Nishiguchi et al., 2004), as well as a mutation in RGS9-1 is associated with stationary retinal dysfunction syndrome (Stockman et al., 2008, Michaelides et al., 2010). The second isoform is RGS9-2, predominantly expressed in the striatum (Zhang et al., 1999) where RGS9-2 inhibits D2 dopamine receptor signaling-mediated motor function (Cabrera-Vera et al., 2004, Kovoor et al., 2005, Celver et al., 2010). In addition, RGS9-2 is involved in regulating addiction behavior, as RGS9-2 regulates dopaminergic or μ-opioid receptor activation (Zachariou et al., 2003, Psifogeorgou et al., 2007, Hooks et al., 2008, Psifogeorgou et al., 2011). RGS9-2 expression in the striatum is downregulated following cocaine self-administration, and compared to WT mice, RGS9-2 deficient mice display locomotor sensitization acceleration and reward sensitivity enhancement (Blundell et al., 2008, Traynor et al., 2009).

RGS11 is largely expressed in the retina and has a central role in the regulation of photoreceptor signaling, as loss of Gβ5, which is required for the stability of C/R7 subfamily members, reduces retinal RGS11 expression and leads to amplification of signaling downstream of photoreceptors (Rao et al., 2007, Cao et al., 2012, Shim et al., 2012). Beyond RGS11 expression in the retina, RGS11 is overexpressed in various types of cancers, such as lung cancer (Yang;Li; et al., 2016) and colorectal cancer, where the upregulation of RGS11 expression has been linked to colorectal cancer chemoresistance (Martinez-Cardus et al., 2009).

Members of the B/R4 subfamily, representing the largest subfamily of the RGS proteins, include RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16, RGS18, and RGS21. Unlike RGS3, which is larger (~80 kDa) due to possessing the PDZ domain at its N-terminal, the remaining proteins of the B/R4 subfamily are small (~20-25 kDa) and relatively simple in structure (Squires et al., 2018). In addition to the RGS domain, these proteins contain an N-terminal amphipathic α helix (Tu et al., 2001) that facilitates plasma membrane targeting (Bernstein et al.,

2000, Heximer et al., 2001, Gu et al., 2007). With RGS2 as a selective GAP for Gαq (Heximer et al., 1997), all members of the B/R4 subfamily bind and deactivate both Gαi/o and Gαq (Hollinger and Hepler, 2002, Masuho et al., 2020).

RGS1 has important actions in immune cells, including T and B lymphocytes. In B lymphocytes, activation of the B cell receptor in response to immunoglobulin leads to the upregulation of RGS1, largely in germinal center B cells (Hong et al., 1993). RGS1 mainly regulates chemotaxis and migration of T and B lymphocytes induced by chemokine signals (Hwang et al., 2010, Gibbons et al., 2011), as RGS1 deficient mice show atypical B cell migration and more germinal center maturation (Moratz;Hayman; et al., 2004). Due to its functions in T and B lymphocytes, RGS1 has been linked to several diseases in which T and B lymphocytes have significant impacts, such as MS (Johnson et al., 2010), celiac diseases, and type I diabetes (Smyth et al., 2008).

RGS2 is ubiquitously expressed in various tissues. Loss of RGS2 reduces proliferation of T cells and IL-2 release following phorbol ester or T cell receptor activation (Oliveira-Dos-Santos et al., 2000). Various phenotypes of RGS2 deficient mice have been reported, including hypertension with increased systemic and renal resistance, renal arterial vasculature hypertrophy, extended calcium signaling in responses to AT1R and the purinergic receptor P2Y, enhanced anxiety, enhanced airway smooth muscle (ASM) contractility, enhanced airway hyperresponsiveness in response to methacholine and a model of house dust mites, enhanced β-cell apoptosis, dysregulated insulin secretion, and kidney fibrosis (McNabb;Zhang; et al., 2020). In addition, various SNPs, which are involved in RGS2 protein and function reduction, are linked to several disorders and abnormal behaviors, such as hypertension (Yang et al., 2005), anxiety, depression-like behaviors (Leygraf et al., 2006, Hettema et al., 2013), panic disorder, agoraphobia (Hettema et al., 2015, Hohoff et al., 2015), and Parkinson-like disease states (Greenbaum et al., 2009). RGS2 acts as a tumor suppressor in several cancers, such as breast cancer, ovarian cancer, and acute myeloid leukemia (AML) (McNabb;Zhang; et al., 2020).

However, the role of RGS2 in prostate cancer and lung cancer is dependent on tumor stage or cancer subtypes.

Unlike RGS2, mice deficient in RGS3 have not been reported. Despite the lack of an RGS3 KO model, RGS3 regulates cardiovascular functions, as RGS3 overexpression protects mice against cardiac hypertrophy (Liu et al., 2014) and regulates survival and maintenance of multiple cell types, such as neural progenitor cells (Nishiura et al., 2009). Further, PDZ-RGS3, the longest isoform, has physiological functions in promoting epithelial-mesenchymal transition (Shi et al., 2012) and enhancing docetaxel sensitivity in breast cancer cells (Ooe et al., 2007).

With selective distribution, the expression of RGS4 is high in the heart and the brain (Zhang et al., 1998, Ingi and Aoki, 2002). Lack of RGS4 in mice leads to cardiac conduction defects and atrial fibrillation (Opel et al., 2015). Within the brain, the transcript of RGS4 is highly expressed in the striatum, where RGS4 modulates cholinergic and dopaminergic receptors (Ding et al., 2006, Lerner and Kreitzer, 2012, Min et al., 2012). Additionally, reduced levels of RGS4 mRNA have been detected in schizophrenic patients (Mirnics et al., 2001). SNP analysis has revealed several SNPs that are associated with RGS4 expression and schizophrenia (Emilsson et al., 2006) or Alzheimer's diseases (AD) (Emilsson et al., 2006).

While RGS5 has a broad expression in different tissues, including the brain (Seki et al., 1998), its expression is found at high levels in the heart (Seki et al., 1998). RGS5 deficient mice exhibit arrhythmias and abnormal cardiac conditions (Qin et al., 2012, Qin et al., 2016). High RGS5 expression is detected in pericytes (vascular smooth muscle cells, VSMCs) (Bondjers et al., 2003, Cho et al., 2003), which critically promotes neovascularization and tumor angiogenesis (Ribeiro and Okamoto, 2015). RGS5 is highly expressed in renal carcinoma and pancreatic islet cell carcinomas (Furuya et al., 2004, Berger et al., 2005). More importantly, loss of RGS5 in mice results in normalization of tumor vascularization and tumor destruction due to influx of immune effector cells, which greatly lead to enhanced tumor-bearing mice survival (Hamzah et al., 2008). This suggests that RGS5 is a key factor in the regulation of vascular remodeling.

Like the majority of the B/R4 subfamily members, RGS8 is predominantly expressed in the brain, particularly in Purkinje cells in the granular layer of the cerebellum (Saitoh et al., 2003, Saitoh and Odagiri, 2003). Despite the availability of RGS8 knockout mice, its physiological roles have not been explored yet. However, RGS8 has been suggested to be involved in seizures, as RGS8 mRNA is regulated following acute and chronic electroconvulsive seizures (Saitoh et al., 2003).

RGS13 has relatively restricted expression in T and B lymphocytes as well as mast cells (Shi et al., 2002, Estes et al., 2004, Bansal;DiVietro; et al., 2008, Bansal;Xie; et al., 2008). Additionally, overexpression of RGS13 has been reported in Burkitt lymphoma and T cell leukemia/lymphoma (Pise-Masison et al., 2009, Sethakorn and Dulin, 2013) as well as in asthma (Raedler et al., 2015). As a GAP, RGS13 regulates chemokine receptor CXCR4 signaling mediated migration responses in B and T cells (Shi et al., 2002, Estes et al., 2004, Han et al., 2006). In mast cells, RGS13 inhibits IgE-mediated degranulation of mast cells and allergic responses (Bansal;DiVietro; et al., 2008, Bansal;Xie; et al., 2008). Consistent with these findings, RGS13 deficient mice have enhanced B cell responses (Hwang et al., 2013).

Like RGS2, RGS16, known as retinal RGS (RGS-r) because of its original cloning from the retina (Chen et al., 1996), has widespread tissue expression, including brain, heart, liver, and immune cells. In the brain, RGS16 is specifically enriched in the thalamus and the suprachiasmatic nucleus (SCN), a brain region involved in controlling circadian rhythms (Grafstein-Dunn et al., 2001, Ueda et al., 2002). Lack of RGS16 results in dysregulated circadian signaling as well as attenuated activities of circadian behavioral rhythms (Doi et al., 2011, Hayasaka et al., 2011). In the liver, RGS16 deficient mice have upregulated fatty acid oxidation rates and ketone levels (Pashkov et al., 2011). Further, expression of RGS16 has been reported to be linked with some cancers, such as pancreatic cancer (Carper et al., 2014), colorectal cancer (Miyoshi et al., 2009), and breast cancer, where knockdown of RGS16 enhances the growth of breast cancer via amplification of PI3K signaling (Liang et al., 2009).

RGS18 appears to have a selective expression in bone marrow-derived cells (Nagata et al., 2001, Park et al., 2001, Yowe et al., 2001), particularly in platelets (Gagnon et al., 2002). Activation of platelets causes RGS18 phosphorylation on serine 49 and serine 218 and thereby its association with 14-3-3γ, resulting in the inhibition of its GAP activity (Gegenbauer et al., 2012). On the other hand, inhibition of platelets by PGI2 and nitric oxide (NO) leads to phosphorylation of RGS18 on serine 216 by PKA and PKG, which displace 14-3-3γ from RGS18 and improves its canonical function on G-protein dependent signaling (Gegenbauer et al., 2012). Platelets derived from RGS18 deficient mice are hypersensitive to platelet activators *in vitro*, and RGS18 deficient mice have markedly enhanced thrombus formation (Alshbool et al., 2015) and reduced platelets recovery in response to thrombocytopenia (Delesque-Touchard et al., 2014). Importantly, the transcript of RGS18 is reported to be elevated in aspirin-resistant platelets (Mao et al., 2014). Beyond platelets, RGS18 is expressed in osteoclasts, which are fully differentiated following RANKL treatment. RGS18 is significantly suppressed in osteoclast precursor cells. Loss of RGS18 enhances RANKL-induced osteoclast differentiation and NFATc1 activation (Iwai et al., 2007).

Despite its reported wide expression, RGS21, the smallest protein of the B/R4 subfamily and the most recently discovered RGS protein, is selectively found in a subset of taste bud cells, including bitter and sweat taste cells (Li et al., 2005). Due to its potential role in the regulation of taste signaling, RGS21 inhibits bitter tastant responses to forskolin-induced cAMP production (Cohen et al., 2012). Nonetheless, its physiological and pathological roles have not been explored yet.

In addition to canonical RGS proteins, 19 RGS-like proteins have been identified that contain non-functional RGS domains. 15 of the RGS-like proteins have been categorized in four subfamilies, including E/RA subfamily (Axin1 and Axin2), F/GEF (RhoA-specific guanine nucleotide exchange factors (GEFs)), known as reg RGS, (P115-Rho GEP, PDZ-Rho GEP, and leukemia-associated Rho GEF (LARG)), G/GRK, (GPRK1,2,3,4,5,6,7), and H/SNX, known as

RGS-PX (SNX13, SNX25, and SNX14) (Siderovski and Willard, 2005). D-AKAP2, RGSL1, RGSL2, and RGSL3 (RGS22) are RGS-like proteins that fall outside the previous subfamilies (Siderovski and Willard, 2005).

Role of RGS proteins in Monocytes/Macrophages

Macrophages express several RGS proteins, including RGS1, RGS2, RGS5, RGS10, RGS16 and RGS19 (Riekenberg et al., 2009, Lee et al., 2013). Activation of macrophages via pattern-recognition receptors (PRRs), such as TLRs, regulates RGS proteins expression. As RGS proteins serve as GAPs on active Gα subunits and as Gα proteins, such as Gαi are involved in TLR4 signaling (Fan et al., 2004, Fan et al., 2007, Dauphinee et al., 2011), previous work has shown that following LPS treatment, RAW264.7 cells, macrophage-like cells, stably overexpressing Gαi2 protein have impaired pro-inflammatory cytokines compared to RAW264.7 cells transfected with an empty vector (Li et al., 2012). In addition, macrophages derived from transgenic mice with genomic knock-in (KI) of an RGS-insensitive Gαi2 (G184S/G184S, GS/GS) and treated with LPS show similar results compared to macrophages isolated from WT mice (Wiege et al., 2012). Controversially, a recent study has found that genetic deletion of Gαi2 suppresses inflammatory signaling upon LPS treatment and inflammasome activation in macrophages (Vural et al., 2019), while macrophages from (G184S/G184S, GS/GS) KI mice, which have a gain of Gαi2 signaling, significantly produce higher inflammatory cytokines and inflammasome activity (Nelson et al., 2018). Therefore, these data suggest that Gαi2 protein and RGS proteins modulate inflammatory signaling in macrophages.

Emerging evidence has demonstrated the role of several RGS proteins in macrophage activation and function. RGS10 is the most highly expressed RGS protein in microglia, brain-resident macrophages, and peripheral macrophages and has a central function in regulating inflammatory responses of macrophages (discussed in chapter 2). RGS1 is strongly expressed in monocytes (Denecke et al., 1999) and localized macrophages in blood vessels, mediating

vascular inflammation associated with atherosclerotic plaques (Patel et al., 2015). Further, RGS1 is detected at a high expression in aortic plaques-associated macrophages in an atherosclerosis mice model, apolipoprotein E ($ApoE^{-/-}$), and human macrophages derived from carotid artery plaques (Patel et al., 2015). In response to a number of chemokine ligands, including CCL2 and CCL5, macrophages isolated from RGS1 KO mice are hypersensitive and exhibit robust chemotaxis compared to WT macrophages. While $ApoE^{-/-}$ mice spontaneously develop aortic atherosclerotic plaque formation, RGS1 deficiency reduces aortic atherosclerotic plaque in double-knockout mice ($RGS1^{-/-}/ApoE^{-/-}$) due to less retention of macrophages to atherosclerotic plaques (Patel et al., 2015). Thus, RGS1 desensitizes proinflammatory chemokine signal-mediated macrophage chemotaxis and increases the numbers of retained macrophages in atherosclerotic plaques.

RGS5 is also detected in macrophages. However, in contrast to RGS1, RGS5 negatively regulates the formation of atherosclerotic plaques, as loss of RGS5 promotes the development of atherosclerosis in ApoE−/− mice and LDL receptor-deficient mice treated with AngII (Cheng et al., 2015). AngII has been shown to suppress RGS5 expression in peritoneal macrophages (Cheng et al., 2015). As a result of RGS5 deficiency, lipids and inflammatory cytokines, such as TNFα, IL-1β, and IL-6 are increased, and endothelial cells and macrophages accumulate in plaques, which exacerbate atherosclerotic plaques in mice (Cheng et al., 2015). Importantly, activation of peroxisome proliferator-activated receptor (PPAR) rescues RGS5 downregulation following AngII treatment and inhibits AngII-induced ERK phosphorylation, c-fos expression, and macrophages recruitment to plaques. Thus, this finding suggests that PPAR agonists modulate inflammatory signaling of macrophages associated with atherosclerotic plaques via enhanced RGS5 expression (Cheng et al., 2015).

RGS12 has a significant role in regulation of inflammatory responses in macrophages (Yuan;Yang;Ng; et al., 2020). Interestingly, the expression of RGS12 is upregulated in macrophages following LPS stimulation, and this upregulation in RGS12 expression is a result of

induction of TNF-*α* and COX-2-mediated PGE2 production (Yuan;Yang;Ng; et al., 2020). (TNF-*α*/TNFR) and (PGE2/EP4) signaling axis drive the transcription of RGS12 through amplification of NF-*κ*B phosphorylation and translocation to the nucleus, where NF-*κ*B binds *κ*B site at RGS12 promoter (Yuan;Yang;Ng; et al., 2020, Yuan et al., 2021). In positive feedback loop, RGS12 binds NF-*κ*B through its PTB domains, and further amplifies NF-*κ*B signaling-mediated inflammatory mediators' production, such as TNF-*α* and COX-2 (Yuan;Yang;Ng; et al., 2020, Yuan et al., 2021). Deletion of murine RGS12 globally or conditionally in macrophages inhibit the development of collagen-induced arthritis (CIA) and associated inflammatory pain, which most likely due to impaired COX-2/PGE2 signaling pathway (Yuan;Yang;Ng; et al., 2020, Yuan et al., 2021).

The impact of RGS16 on the production of proinflammatory cytokines has been explored *in vitro* in human monocytic cells, as the knockdown of RGS16 in THP-1 cells, a human promonocytic cell line, increases Pam3-induced TNF-*α*, IL-1β, and IL-6 protein levels. On the other hand, overexpression of RGS16 reduces the release of proinflammatory cytokines following Pam3 treatment (Suurvali et al., 2015).

RGS19, as a member of the A/RZ subfamily, is well expressed in macrophages. Unlike other RGS proteins, its expression in macrophages is not altered upon LPS stimulation. However, LPS induces RGS19 phosphorylation, which is inhibited in response to Notch1 knockdown or a gamma-secretase inhibitor (GSI), a Notch signaling inhibitor (Sangphech et al., 2014). Interestingly, as Akt signaling impacts macrophage survival and cell cycle, GSI treatment or RGS19 loss in RAW264.7 cells decrease LPS-stimulated phospho-Akt (Thr 308) signaling and affect the cell cycle of macrophages. This suggests that Notch signaling may control the macrophage cell cycle dependently or independently of Akt activation and may work directly or indirectly via phosphorylation of RGS19 (Sangphech et al., 2014).

TLR4-dependent inflammatory signaling

TLR-4 is highly expressed in microglia cells and macrophages and can be activated by a wide range of PAMPs or DAMPs (Kawasaki and Kawai, 2014). Of PAMPs, bacterial LPS stimulates TLR4 activation, which requires co-activators including cluster of differentiation 14 (CD14) and myeloid differentiation factor 2 (MD-2) (Hoshino et al., 2016). Activating CD14 and TLR4-MD-2 complex on the cell surface by bacterial LPS leads to receptor dimerization and initiates two distinct signaling cascades via the recruitment of distinct adaptor proteins to the plasma membrane. In the first, an activate TLR-4 via its toll-interleukin receptor (TIR) domain binds to myeloid differentiation factor 88 (MyD88) that subsequently recruits members of IL-1 receptor-associated kinase (IRAKs), promoting autophosphorylation. Phosphorylated IRAKs dissociate from MyD88 and bind to tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) that in turn forms a complex with E3 ligases and transforming growth factor-*β*-activated kinase 1 (TAK1). Ubiquitination of TRAF6 leads to TAK1 activation that in turn activates inhibitory *κ*B (I*κ*B) kinase (IKK) complex, consisting of IKK*α*/IKK*β*/IKK*γ* (known as IKK1, IKK2, and NF-κB essential modulator (NEMO), respectively). The IKK complex phosphorylates inhibitory *κ*B alpha (I*κ*B*α*) on serine residues 32 and 36 that subsequently undergoes ubiquitination followed by proteasomal degradation. As a result of the degradation of I*κ*B*α*, the p50/p65 NF-*κ*B heterodimer is released in the cytoplasm that in turn translocates to the nucleus to initiate transcription of inflammatory genes expression (**Figure 1.1**) (Giridharan and Srinivasan, 2018). In addition to the IKK complex, TAK1 also activates mitogen-activated protein kinase kinase (MAPKK) that activates downstream MAPK signaling pathways, such as extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway, and the p38 pathway (Akira and Takeda, 2004). Activation of MAPK pathways mediates the transcription factor activator protein-1 (AP-1) activation that ultimately stimulates inflammatory gene expression (Akira and Takeda, 2004). Independent of MyD88, the activated TLR4 recruits TIR domain-containing adapter protein-inducing interferon-β (TRIF) via its associated protein TRIF-

related adapter molecule (TRAM) that facilitates engaging of E3 ligase TRAF3 and TRAF6. In turn, TRAF3 and TRAF6 induce the non-canonical IKKs autoactivation, including TRAF family member-associated NF-*κ*B activator-binding kinase 1 (TBK1) and IKK-ε. Autoactivation of TBK1and IKK-ε will mediate different pathways, such as phosphorylation of the transcription factors interferon regulatory factors (IRFs), undergoing dimerization followed by nucleus translocation to induce antiviral genes as IFN-*β* as well as phosphorylation of the p50/p65 NF-*κ*B heterodimer via recruiting the adapter receptor-interacting protein 1 (RIP1) to TRIF. Like the MyD88 dependent pathway, TRIF is involved in activating p50/p65 NF-*κ*B heterodimer via TRAF6 mediated TAK1/IKK activation mechanism. For optimal transcriptional activity, the p50/p65 NF-*κ*B heterodimer needs to form a complex with co-activator like cAMP response element-binding protein CREB-binding protein (CBP) and its paralog p300. Via their histone acetyltransferase (HAT) activity, both CBP and p300 activate transcription by acetylating histone proteins around the promoter of genes for TFs access or none-histones TFs for their activation. Additionally, they also act as a bridge connecting TFs to transcriptional machinery or as a scaffold protein that integrates multiple required proteins for transcriptional activation (Mukherjee et al., 2013, Bhatt and Ghosh, 2014). Of the many genes transcribed in response to LPS-dependent p50/p65 NF-*κ*B heterodimer activation, TNF-α, a proinflammatory cytokine, further triggers inflammatory responses via stimulating its TNFR1 and TNFR2 (Webster and Vucic, 2020). TNFR1 is highly expressed in the majority of cells, in particular macrophages, and mainly mediates inflammatory signaling activation by forming a complex with the following adaptor proteins: RIP1, TRAF2 and TNFR-associated death domain (TRADD) (Webster and Vucic, 2020). In addition to TNF-α, cyclooxygenase-2 (COX-2) is induced upon LPS treatment in macrophages, including microglia and alveolar macrophages, and catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2) that is further transformed to PGE2, a crucial mediator of neuroinflammation (Hein and O'Banion, 2009).

Regulation of RGS proteins expression

RGS transcripts and protein levels are dynamically regulated in different pathological models or following numerous cues via various mechanisms ranging from epigenetic, transcriptional, and posttranslational modifications (Alqinyah and Hooks, 2018).

Emerging evidence indicates that RGS genes are subjected to epigenetic regulatory mechanisms, including DNA methylation and histone deacetylation in multiple types of cells. *In vitro* differentiation of human neural progenitors (hNP) results in upregulation of multiple RGS transcripts accompanied by a significant reduction in DNMT3 transcript (Tuggle et al., 2014). This suggests that changes in DNMT3, a major enzyme isoform involved in the methylation of DNA, may contribute to enhanced RGS transcript levels in differentiated hNP cells. Pharmacological inhibition of DNMT enzymes by 5-Aza-2'-deoxycytidine (5-Aza) proves this suggestion, in which the direct inhibition of DNA methylation by 5-Aza enhances various transcripts of RGS expression hNP cells (Tuggle et al., 2014). DNA methylation at promoters of RGS genes mediated their reduced expression and have been reported in cancers, such as prostate cancer and breast cancer, where RGS2 and RGS16 transcript levels are silenced, respectively (Wiechec et al., 2008, Wolff et al., 2012). Additionally, the expression of RGS2 is suppressed in bladder cancer due to the enhancement of DNA methylation at its promoter (Ying et al., 2015). In ovarian cancer, DNA methylation, which is involved in silencing of RGS2 transcript in chemoresistant cancer cells, is not the only epigenetic mechanism that regulates RGS2 transcript levels (Cacan, 2017). Histone deacetylation, another epigenetic mechanism executed by HDACs, also mediates RGS2 suppression in ovarian chemoresistant cells (Cacan, 2017). In addition to neural and cancer cells, the expression of RGS2, which plays a significant role in regulating hyperresponsiveness and mucin overproduction (Liu et al., 2013), is downregulated in airway epithelial cells derived from cystic fibrosis (CF) patients because of hypermethylation at its promoter (Liu et al., 2013).

Modulating RGS transcript by short endogenous non-coding RNAs (microRNAs) is another critical mechanism that regulates RGS expression in the cell. The majority of microRNAs

bind the 3' untranslated region (3'UTR) of target mRNAs and cause degradation of mRNAs followed by subsequent repression of translated proteins (O'Brien et al., 2018). RGS transcripts are regulated by many microRNAs in certain cancer types. For example, RGS17 expression is downregulated by Has-miR-182 and miR-203 in lung cancer (Sun et al., 2010) and miR-199 in hepatocellular carcinoma (Zhang et al., 2018). Overexpression of RGS17 suppresses anti-proliferative effects of Has-miR-182 in lung cancer (Sun et al., 2010) and both miR-199 and miR-203 mediated inhibition of proliferation, migration, and invasion in hepatocellular carcinoma and lung cancer, respectively. These findings suggest that these miRNAs-targeting RGS17 mRNA exert their inhibitory actions on lung cancer and hepatocellular carcinoma through RGS17 silencing (Sun et al., 2010, Zhang et al., 2018). In addition, RGS17 has been identified as a target of miR-363 in myeloid leukemia (Mosakhani et al., 2013) and miR-203 in prostate cancer (Zhang;Ma; et al., 2019). RGS16, which is identified to be targeted by miR-126 in apoptotic bodies (Mondadori dos Santos et al., 2015), has been identified to be downregulated in chondrosarcoma by miR-181a (Sun;Charbonneau; et al., 2015, Sun et al., 2019). The same miRNA has the ability to target the same RGS in different cancer types. For example, miR-126 induces silencing of RGS3 in triple-negative breast cancer (TNBC) (Hong et al., 2019) and also targets RGS3 in gastric cancer (Wang;Zhou; et al., 2017). While miR-126 inhibits different aspects of TNBC progression, these inhibitory effects are reversed by RGS3 restoration, suggesting that suppression of RGS3 is required for miR-126-induced inhibition of TNBC progression (Hong et al., 2019). RGS3, RGS1, and RGS20 are targeted by miR-25, miR-29C-3p, and miR-365 in lung cancer, melanoma, and oral squamous cell carcinoma, respectively (Chen et al., 2016, Huang et al., 2018, Wang et al., 2019). Beyond cancers, studies have shown that miRNAs regulate RGS expression in different cells and disease models. MiR-181a suppresses RGS4 during osteoblastic differentiation (Bhushan et al., 2013). MiR-22 targets and induces RGS2 suppression in *in vitro* models of HD, which causes protective effects on neurons (Bhushan et al., 2013). RGS2 is also targeted by has-miR-4717-5p that may have a potential role in anxiety-related disorders (Hommers et al., 2015).

Additionally, miR-139-5p and miR-452-5p have a negative impact on RGS13 expression in the thymus (Sengupta et al., 2018). Suppression of RGS4 is mediated by miR-21-3p, whose expression is increased following LPS treatment in retinal pigment epithelial cells (Liu;Ma; et al., 2019). RGS4 silencing results in attenuation of miR-21-3p-induced inhibition of inflammatory and apoptotic effects (Liu;Ma; et al., 2019).

The stability of RGS proteins is tightly regulated by their interaction with other proteins or posttranslational modifications, such as phosphorylation, palmitoylation, ubiquitination, and arginylation (Alqinyah and Hooks, 2018). As a result of these posttranslational modifications or RGS-protein interactions, multiple aspects of RGS proteins' function might be affected, such as GAP activity, subcellular localization, and protein stability (Tuggle et al., 2014). **Table 1.2** summarizes post-translation modifications or binding partners that affect the stability of RGS proteins, which may affect their activity or localization. Regulation of RGS10 expression and function, which is the main focus of this dissertation, is discussed in depth in chapter 2.

Alteration of RGS proteins expression by LPS

LPS is a major structural component of the outer wall of gram-negative bacterial, which stimulates TLR4 and subsequently induces inflammatory mediators that activate immune responses. LPS-mediated immune cell activation is accompanied by altering the expression of numerous genes, including RGS expression. Depending on the type of immune cells, RGS protein expression is differently regulated. In macrophages, LPS induces the downregulation of RGS2 and RGS10 (Riekenberg et al., 2009, Lee;Yeo; et al., 2010, Lee et al., 2013) and remarkable upregulation of RGS7 and RGS12 (Hausmann et al., 2002, Yuan;Yang;Ng; et al., 2020), while RGS1 expression is initially and rapidly upregulated followed by downregulation upon LPS treatment (Riekenberg et al., 2009). The expression of RGS1, RGS16, and RGS20 is increased in response to LPS treatment in dendritic cells (Shi et al., 2004) and RGS16 in pre-B cells (Li et al., 2001). On the other hand, LPS-activated dendritic cells decrease the expression of RGS14

and RGS18 (Shi et al., 2004). In addition to immune cells, LPS can affect the expression of RGS expression in other cell types. For example, LPS upregulates RGS4 in the heart (Patten et al., 2002) and downregulates its expression in ARPE-19 human retinal pigment epithelial cells (Liu;Ma; et al., 2019), respectively. This suggests that the effect of LPS on RGS4 expression is cell-dependent. RGS16 mRNA and protein levels are increased in both cardiomyocytes and vascular smooth muscle cells (Patten et al., 2003, Hendriks-Balk et al., 2009), and the increase in RGS1 expression following LPS stimulation is observed in RPMI human plasmacytoma cells (Pak et al., 2015). Altogether, LPS can affect the expression of several RGS proteins in multiple cell types, but much research is still needed to understand the mechanisms regulating their expressions by LPS.

GAP-independent functions of RGS proteins

RGS proteins regulate the duration of GPCRs signaling by deactivation of heterotrimeric G-proteins via their GAP activity that enhances the hydrolysis of GTP on Gα subunits, the canonical function mediated by their RGS domain. Aside from their RGS domain, RGS proteins contain multiple regulatory motifs and functional domains that facilitate their non-canonical functions through interaction with binding partners in different cellular compartments, including the plasma membrane, where their canonical G-proteins reside (Sethakorn et al., 2010). In addition to GAP-independent functions mediated by regions outside of the RGS domain, binding of RGS proteins with their interacting proteins has affected their GAP activity or subcellular localization. Although RGS proteins exert their GAP-dependent functions via the RGS domain, emerging evidence shows that the RGS domain interacts with binding partners distinct from its classical Gα-protein targets. In this section, a few RGS-interacting partners mediated GAP-independent functions will be discussed. However, **Table 1.3** summarizes most of the binding partners of RGS proteins that are unrelated to Gα-proteins, region of interaction, and functional effects. As mentioned above, many non-canonical functions are mediated by protein-binding

interaction via additional regulatory motifs and functional domains. For example, the GGL domain found in the C/R7 subfamily members mediates the interaction with the G-protein β subunit (Gβ5), forming a stable hetero-complex and causing localization to the plasma membrane or nucleus (Chen;Eversole-Cire; et al., 2003, Anderson et al., 2009). In addition, RGS6 protein via the GGL domain binds DMAP1 and its binding partner, DNMT1 (Liu and Fisher, 2004) and consequently suppresses DNMT1-induced gene silencing (Liu and Fisher, 2004). DEP is another domain of the C/R7 subfamily proteins that facilitates association of RGS9-1 and RGS7 with anchor proteins, R9AP and R7BP, respectively (Anderson et al., 2009), and results in localization of RGS9-1 and RGS7 at the cell membrane to regulate their G-protein targets (Hu et al., 2003, Drenan et al., 2006). Another example of non-canonical function mediated by a domain outside of the GAP-dependent region is the PDZ domain present in RGS3, where directly interacts with GSK3β (Shi et al., 2012). The direct interaction between PDZ-RGS3 and GSK3β causes inhibition of GSK3β kinase activity, resulting in Wnt3a signaling amplification (Shi et al., 2012). In addition to RGS3, RGS12 and RGS14, members of the D/R12 subfamily possess multiple motifs and domains that enable interaction of RGS12 and RGS14 with their binding proteins to exert their GAP-independent actions. Both RGS12 and RGS14 are composed of GoLoco motif, which is involved in binding the inactive form of Gα subunit (Gα-bound GDP) and prevents the exchange of GDP toward GTP or the association with Gβγ dimer (Kimple et al., 2001). They further have RBDs (Snow et al., 1997), which mediate the interaction with activated small G-proteins, such as H-Ras and H-Raf and regulate downstream MAPK signaling (Willard et al., 2007, Shu et al., 2010). Unlike RGS14, RGS12 contains two additional domains including the PTB and PDZ domains. The PTB domain interacts in a direct way with neuronal N-type calcium channels in a phosphorylation-dependent manner and accelerates the inhibition of calcium channels activity in response to GAPB stimulation (Shu et al., 2010). The PDZ domain can bind the C-terminus of the CXCR2 receptor (Snow;Hall; et al., 1998).

Structurally, the multiple functional domains apart from the RGS domain and mediate RGS proteins interaction are not always required for RGS to act as non-GAP proteins. Several reports have shown that small RGS proteins containing short N-terminal and C-terminal flanking the RGS domain interact with several proteins to exert their GAP-independent functions. For example, RGS2 via its N-terminal region interacts with several GPCRs, such as Gq-coupled muscarinic and α-adrenergic receptors (Bernstein et al., 2004, Hague et al., 2005, Roy et al., 2006), while the C-terminal region of RGS4 facilitates its binding with a couple of types of opioid receptors (Georgoussi et al., 2006, Papakonstantinou et al., 2015). Interestingly, although there are no confirmed reports on GAP activity of any RGS proteins toward Gαs, RGS proteins, such as RGS2, negatively regulate Gαs downstream signaling by interacting with adenylate cyclase (AC) through its N-terminus (Sinnarajah et al., 2001). Further, RGS2 suppresses the translation of proteins via interaction with eIF2Bε (Nguyen et al., 2009). On the other hand, through the N-terminal region, RGS2 protein is suppressed due to its interaction with E3 ligases, including FBXO44 and Teb4 (Park et al., 2015, Sjogren et al., 2015, McNabb;Gonzalez; et al., 2020). Aside from its plasma membrane localization, the primary site of its canonical function, RGS13 interacts with CREB in the nucleus and inhibits its transcription activity (Xie et al., 2008).

In addition to its ability to bind Gα-proteins and mediate GAP activity, several publications have demonstrated that the RGS domain can associate with unique binding partners to modulate their activity or function independent of GPCRs activation. For example, the RGS domain of RGS6 binds Tip60 that facilitates DNMT1 degradation (Huang et al., 2014). RGS16 is another example of RGS protein regulation of signal transduction independent of GAP activity of the RGS domain that has an anti-proliferative effect and inhibits AKT activation in response to EGF in breast cancer cells (Liang et al., 2009). The interaction between the RGS domain of RGS16 and the p85α regulatory subunit of PI3K blocks association of p85α with Gab1 and consequently causes PI3K signaling inhibition downstream of EGF stimulation (Liang et al., 2009). Further, the GAP activity of RGS proteins can be enhanced or suppressed by interacting-partners activity or function. For

example, RGS7, like several other RGS proteins, interacts with 14-3-3 that in turn inhibits RGS7 GAP activity (Benzing et al., 2000). Like RGS7, RGS3 GAP activity is inhibited by 14-3-3 interaction (Benzing et al., 2000, Niu et al., 2002, Rezabkova et al., 2010, Rezabkova et al., 2011). Another common binding partner of RGS proteins that influences their GAP activity is calmodulin. In a similar fashion of interaction with other RGS proteins, calmodulin binds RGS4 in a calcium-dependent manner that indirectly enhances GAP activity via blocking phosphatidylinositol (4,5,6)-triphosphate-induced inhibition of RGS4 GAP action (Popov et al., 2000). Collectively, RGS proteins exert non-GAP functions through modulation of activity or function of their unique binding proteins, which can affect RGS proteins' localization, stability, or GAP activity.

Alveolar macrophage

Alveolar macrophages (AMs) are specialized cells in the alveoli and are the dominant immune cells, as they represent 90-95% of the immune cells under normal conditions, making them the natural sentinels of the respiratory system (Hussell and Bell, 2014, Kopf et al., 2015). They originate from progenitors derived from the yolk sac and fetal liver that populate the lung during embryogenesis followed with a full differentiation via GM-CSF (Known as CSF-2)-mediated PPAR-γ and TGF-β signaling into alveolar macrophages in the first days after birth (Guilliams et al., 2013, Gomez Perdiguero et al., 2015, Yu et al., 2017). Under homeostatic conditions, they are long-lived and self-sustaining cells, in which distinct techniques, such as lineage-tracing and bone marrow-chimeric mice, have shown that alveolar macrophages maintain their population over a lifetime in a self-replenishing manner without any contribution from monocytes derived from bone marrow (Hashimoto et al., 2013, Yona et al., 2013). Murine AMs can be identified and distinguished from other types of lung macrophages, such as interstitial macrophages and other immune cells via the expression of CD11C (Guth et al., 2009) and sialic acid-binding immunoglobulin-like lectin F (Siglec-F) (Trapnell et al., 2003, Feng and Mao, 2012).

Like other tissue-resident macrophages, AMs are involved in maintaining lung homeostasis by phagocytosing apoptotic cells and cells debris resulting from lung infection or epithelial injury (Rubins, 2003, Westphalen et al., 2014). In particular, AMs have a key function in clearing the alveolar environment from excessive production of lipid-rich molecules (surfactants) that are produced by type II alveolar epithelial cells (AECII) and functionally prevent alveolar collapse during exhalation (Whitsett et al., 2015). However, impaired clearance function of surfactants by AMs results in pulmonary alveolar proteinosis (PAP) (Trapnell et al., 2003). The previous study has shown that mice deficient in AMs exhibit PAP due to the accumulation of surfactants in the alveolar space (Schneider et al., 2014). Further, AMs exert their lung defense mechanisms via their PRRs, including TLRs and scavenger receptors, facilitating the sensing of microorganisms or injury signals and their subsequent phagocytosis (Kopf et al., 2015). For example, CD206 (mannose receptor) mediates the recognition and phagocytosis of *Mycobacterium tuberculosis* (Rajaram et al., 2017). The upregulation of MARCO and class A scavenger receptors (SR-AI/II) on AMs are involved in the clearance of inhaled oxidized lipids and dampens pulmonary inflammation (Dahl et al., 2007). Additionally, AMs exert their bactericidal actions via induction of cytokines, such as TNF-α and IL-1β-mediated oxidative burst and chemokines, such as CXCL1 and CXCL8 mediated neutrophil recruitment (Aberdein et al., 2013, Kopf et al., 2015). In addition to bacterial clearance, AMs mainly produce type I interferons (IFN) in response to the acute infection of influenza A virus, which in turn regulates inflammatory responses and promotes viral clearance through upregulation of viral suppressant genes (Divangahi et al., 2015).

Upon chronic exposure of lung insults, including influenza A virus infection or bleomycin-induced lung fibrosis, circulating monocytes are recruited to the lung and differentiated to AMs in response to undefined lung environment signals (Misharin et al., 2017). While the resident AMs remain unchanged in terms of function and transcriptional profile, many studies have shown that monocyte-derived AMs are the dominant cells post-lung insult, in which they are immunoreactive

and pliable to change their functions based on the microenvironment (Kulikauskaite and Wack, 2020). Based on the insult type, monocyte-derived AMs may promote lung fibrosis following lung injury (Misharin et al., 2017, Jiang et al., 2020) or reduce house dust mite (HDM)-induced asthma following γ-herpesvirus (murid herpesvirus 4) infection (Machiels et al., 2018). Interestingly, influenza virus infection often leads to depletion of the resident AMs that are repopulated by the remaining resident AMs of embryonic origin and the recruited monocytes-derived AMs (Aegerter et al., 2020). In contrast to these results, another study has shown that resident AMs are expanded by the embryonically derived resident cells without any contribution from monocytic derived cells (Hashimoto et al., 2013).

Macrophage polarization

Macrophages originating embryonically from the yolk sac and fetal liver, such as microglia and alveolar macrophage or originating postnatally from bone marrow, broadly undergo two distinct types of polarization or activation in response to microenvironmental stimuli and signals (Mantovani et al., 2004, Perdiguero and Geissmann, 2016). The first type is M1-polarized macrophages, known as classically activated macrophages, which are induced upon exposure to LPS and/or T helper type 1 cells (Th1) cytokines, interferon-gamma (IFN-γ) and TNF-α. M1-polarized macrophages are mainly considered as inflammatory cells, in which they produce high levels of proinflammatory cytokines (TNF-α and interleukin (IL) (IL1*β*, IL6, IL12, and IL23) as well as CXCL(5,9,10), metalloproteinases (MMP) (such as MMP7 and MMP9), reactive oxygen species (ROS) and nitric oxide (NO). These inflammatory mediators enhance phagocytosis during acute infection to remove viruses and bacteria and facilitate tumor cell killing (**Figure 1.2**) (Martinez et al., 2008, Martinez and Gordon, 2014). On the other hand, the second type is M2-polarized macrophages, known as alternatively activated macrophages, which are induced in response to T helper type 2 cells (Th2) cytokines, IL-4 and IL-13. High expression of anti-inflammatory IL-10 is the main feature of M2-polarized macrophages that have a central role in

tissue remodeling and repair (Wynn and Vannella, 2016). Activation of M2 macrophages upregulates the murine expression of arginase-1 (Arg1), resistin-like molecule-*α* (Relm*α*, known as FIZZ1), and chitinase-3-like protein-3 (Chi3l3, known as Ym1) (**Figure 1.2**). M2-polarized macrophages also highly express CD206 and decoy IL-1 receptor (IL-R) and secrete significantly profibrotic TGF-β and vascular endothelial growth factor (VEGF), mediating angiogenesis and contributing to tumor progression (Mantovani et al., 2004). Metabolism of L-arginine is another feature to differentiate between M1 and M2 macrophages, as M1-polarized macrophages metabolize L-arginine into NO and citrulline by nitric oxide synthase (iNOS), whereas M2-polarized macrophages metabolize L-arginine into ornithine and urea by Arg1 (Modolell et al., 1995, Munder et al., 1999).

Although M1 macrophage responses are important to phagocytose dead cells and clear microbes during acute infection, uncontrolled and excessive activation of M1 macrophages leads to chronic inflammation that can cause tissue damage. For instance, in the central nervous system (CNS), prolonged inflammation characterized by an overabundance of inflammatory mediators maintain M1 macrophages activation that contributes to the pathogenesis of Parkinson's diseases (PD), AD, MS, and spinal cord injury (Du et al., 2017). In the lung, aberrant M1 macrophages activation is linked to the development of asthma, pulmonary fibrosis, and chronic obstructive pulmonary disease (COPD) (Belchamber and Donnelly, 2017). Despite the anti-inflammatory activity and immunosuppressive function of M2-polarized macrophages, dysregulated M2-macrophage activation is associated with respiratory diseases (Belchamber and Donnelly, 2017). Due to possessing some characteristics similar to M2 macrophages, tumor-associated macrophages (TAMs) are defined as a subtype of M2-macrophages. However, TAMs are established as major inflammatory cells infiltrating tumors, where they produce several pro-inflammatory mediators, such as cytokines and chemokines, fostering tumor progression (Cendrowicz et al., 2021).

Microglia

Microglia are the primary innate immune cells in the CNS that constitute 20% of the total number of glia cells and ~ 5 to 15% of the CNS cells based on their location (Lawson et al., 1990). Although microglia are recognized as CNS-resident macrophages because of features shared with peripheral macrophages, microglia are unique cells and differ from other peripheral macrophages in terms of origin. Microglia emerge from myeloid precursor cells within the yolk sac mesoderm during embryogenesis (around embryonic day (E) 7.5) and then colonize the mesenchyme of the brain (between E8.5 and E9.5 through functional and developed blood vessels), where they fully differentiate into mature microglia. By E9.5-10.5, microglia are detected in the neuroepithelium of the brain, and by E11.5, they are found within the parenchyma of the spinal cord (Ginhoux et al., 2010, Ginhoux et al., 2013). Microglia are long-lived and self-renewing cells, in which they develop and proliferate independently of circulating hematopoietic cells derived from the bone marrow (Ajami et al., 2007).

Several proteins are involved in microglial maturation and survival, including Pu.1, colony-stimulating factor 1 receptor CSF1R (activated by CSF1 or IL34), and interferon regulatory factor 8 (IRF8), as knockout of these genes results in mice devoid of microglia or a significant decline in microglia cell number (Nayak et al., 2014). During CNS development, microglia have multiple tasks, as they clear up neuron cells dying from programmed cell death (PCD), which is induced by factors released from microglia, such as TNFα and nerve growth factor (NGF) (Frade and Barde, 1998, Sedel et al., 2004). Further, superoxide ions produced by the microglial respiratory bursts are involved in Purkinje neuron apoptosis (Marin-Teva et al., 2004). Interestingly, loss of microglial CD11b and the immunoreceptor DNAX activation protein of 12kDa (DAP12) receptors control the production of superoxide ions, which consequently reduce microglial apoptotic signals causing Purkinje cells apoptosis (Wakselman et al., 2008). In addition, numerous studies have shown the involvement of microglial cells in supporting neuronal survival, proliferation, and differentiation (neurogenesis) (Rogers et al., 2011) as well as regulation of astrocyte

differentiation (Nakanishi et al., 2007, Pont-Lezica et al., 2011, Hagemeyer et al., 2017), oligodendrogenesis and myelination (Rogers et al., 2011, Hagemeyer et al., 2017). Further, microglial cells exert a crucial function in the synaptic pruning process (Paolicelli et al., 2011). They remove immature synapses via eat-me signals, as immature synapses express complement 3 protein (C3), whose function is to attract microglial cells via their complement 3 receptor (C3R) (Stevens et al., 2007). In addition to CX3C-chemokine receptor 1(CX3CR1), brain-derived neurotrophic factor (BDNF) release by microglial cells contribute to synaptogenesis, in which previous studies have demonstrated that mice lacking CX3CR1 or BDNF show deficits in synapse pruning and behavior (Rogers et al., 2011, Parkhurst et al., 2013). In adult healthy CNS, microglia continue their support of CNS surveillance via their sensing function by responding to any aberrant signs of damage or debris and their housekeeping function by promoting synapses plasticity and remodeling, important for learning and memory (Paolicelli et al., 2011).

Under physiological conditions, microglia, defined as resting (quiescent) cells, show a ramified morphology characterized by small cell bodies with multiple extended and thin processes. Through their processes, microglia are able to monitor the CNS environment and interact with neurons and other glial cells, such as astrocytes and maintain their homeostasis (Hanisch and Kettenmann, 2007). In turn, neurons are involved in keeping microglia in their resting state via secretion of different signals, such as cluster of differentiation (CD200), CD22, and CX3C-chemokine ligand 1 (CX3CL1, known as Fractalkine) that exert their effects through microglia receptors, CD200R, CD45, and CX3CR1 receptors, respectively (Chavarria and Cardenas, 2013). Several studies have demonstrated that disruption of these signaling pathways results in more microglial activation and neurotoxicity (Hoek et al., 2000, Cardona et al., 2006). In addition, astrocytes release multiple factors such as transforming growth factor (TGF-β), macrophage-colony stimulating factor (M-CSF), granulocyte/ macrophage-colony stimulating factor (GM-CSF), and purines (adenosine triphosphate, ATP) whose functions are retaining microglia in their resting state or ramification process (Schilling et al., 2001). Microglia have a high

expression of unique genes that obviously distinguish them from other cells localized in the brain, such as Cx3cr1, Hexb, Trem2, P2ry12, Tmem119, Sall 1, Gpr34, and Olfml 3 (Hickman et al., 2013, Butovsky et al., 2014, Ransohoff and El Khoury, 2015).

Upon exposure to stress, pathogens or damaged signals, microglia transform to an activated phenotype, in which they undergo morphological, functional and transcriptional changes. They adopt the amoeboid-like morphology characterized by a larger rounded soma with few retracted and thick processes (Hinwood et al., 2013). While the phagocytic activity of the resting microglia is low, activation of microglial cells leads to enhancing their phagocytic function, as they localize to the site of infection or injury and engulf cell debris, dead cells, pathogens, and toxins (Fu et al., 2014). Microglia express on their surface membrane different types of receptors known as PRRs, whose functions enable microglial cells to recognize invading microbes and sense various pathogen-associated molecular patterns (PAMPs), such as LPS or molecules released from injured tissues or cells (damage-associated molecular patterns (DAMPs)). Among PRRs, TLRs, particularly TLR4, initiate inflammatory responses as a part of CNS defense mechanisms against injury or pathogen signals. Therefore, accompanying the changes in the morphology and function, activation of microglia via their PRRs produces a wide variety of inflammatory secretory factors that contribute to phagocytic processes, such as cytokines, chemokines, nitric oxides, reactive oxygen species, cyclooxygenase-2 and prostaglandins (Rodriguez-Gomez et al., 2020). Additionally, microglia express purinergic receptors, such as the metabotropic receptors P2YRs, belonging to the GPCRs superfamily that recognizes danger signals from damaged neurons, such as ATP and uridine 5′ diphosphate (UDP) (Calovi et al., 2019). Further, upregulation of some cell surface markers is observed in activated microglia, such as cell CD14, major histocompatibility complex II (MHCII), and CD11b phagocytic-specific protein, a member of the β2-integrin family, coupled with CD18 forming C3R (known as macrophage antigen-1 (MAC-1) receptor).

Role of Microglia in neurodegenerative diseases

Depending on the time window, microglia activation can be protective or detrimental. Acute activation of microglia following PAMPs, or DAMPs, causes migration of microglia to the site of infection or neuronal damage, where they perform their normal housekeeping function by phagocytosing dead or dying neurons or debris and maintaining brain homeostasis (Fu et al., 2014). However, chronic activation of microglia leads to sustained neuroinflammatory responses and disrupted housekeeping functions, resulting in neurotoxicity and contributing to neurodegenerative diseases (Hickman et al., 2018). For example, AD is characterized by the formation of abnormal amyloid-beta (Aβ)-peptides containing plaques-causing, entangled nerve fibers and neuronal loss (Selkoe and Hardy, 2016). Previous studies have shown that microglia, which have an inflammatory M1 phenotype, accumulate around these plaques in AD patients and animal models' (Frautschy et al., 1998). Although microglia engulf intracellular Aβ peptides via phagocytosis (D'Andrea et al., 2004), overproduction of Aβ-peptides disrupts the equilibrium between Aβ-peptide production and clearance, resulting in microglia phagocytic activity decline and Aβ peptides deposition (Hickman et al., 2008). Further, Aβ-peptides directly bind TLRs on microglia cells (Hickman and El Khoury, 2013, Hickman et al., 2013), which produce more neurotoxic factors, such as ROS and NO and release more proinflammatory cytokines (Coraci et al., 2002, Venegas et al., 2017). Elevated inflammatory responses, in turn, lead to exaggeration of Aβ-peptides deposition and microglia activation and contribute to neurons loss and AD pathology (Oddo et al., 2003, Villemagne et al., 2017). Consistent with these findings, LPS treatment, which is known to polarize microglia to M1 phenotype via inflammatory mediator's induction, reduces Aβ peptides clearance (Koenigsknecht-Talboo and Landreth, 2005). On the other hand, polarizing microglia to M2 activation by IL-4 enhances Aβ-peptides phagocytosis (Michelucci et al., 2009). This suggests that targeting the chronic activation of microglia by switching their activation phenotypes from M1 to M2 could be an attractive strategy to treat AD. In addition to AD, uncontrolled activation of microglia-mediated neuroinflammation is a key factor

in the progression of PD. PD is characterized by degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta and aggregation of α-synuclein in Lewy bodies (Dickson, 2018). In a similar manner to clear Aβ-peptides, microglia localize to the α-synuclein aggregation site in an attempt to clear it. A defect in the clearance process results in the accumulation of α-synuclein, which in turn activate microglial PRPs and maintains their pro-inflammatory M1 activation phenotype by the production of inflammatory factors-mediated neurotoxicity (Croisier et al., 2005, Su et al., 2008, Halliday and Stevens, 2011, Kim et al., 2013). Samples of PD patients have demonstrated loss of DA neurons along with proinflammatory M1 microglia activation and inflammatory mediators release, such as TNF-α and NO (Wang;Liu and Zhou, 2015). Blocking of microglia activation suppresses DA neurodegeneration in the cell and animal models of PD induced by LPS (Dutta et al., 2008), suggesting that targeting microglia overactivation has neuroprotective effects.

MS is another example of neuroinflammatory disease manifested by demyelinated plaques in the white and gray matter (Milo and Kahana, 2010) and is affected by microglia depending on the stage of the lesion (Kuhlmann et al., 2017, Zrzavy et al., 2017). In the early lesions of MS, microglia have beneficial impacts by promoting remyelination, clearance of myelin debris, and supporting neurons by neurotrophic factors (Yamasaki et al., 2014). In contrast, the mouse model of advanced MS, the experimental autoimmune encephalomyelitis (EAE), reveals that antigen-presenting activity of activated microglia upregulates the MHCII expression-mediated recruiting and proliferating T cells, which leads to the destruction of myelin (Wolf et al., 2017). In addition to previous examples of neurodegenerative diseases, the overabundance of inflammatory factors released by activated microglia can contribute to the progression of other neuroinflammatory diseases, such as neuropathic pain (Chen et al., 2018), Huntington's disease (HD) (Wilton and Stevens, 2020), and prion disease (Aguzzi and Zhu, 2017). Multiple factors, such as aging, have affected microglial physiological functions and led to microglia

hyperactivation that promotes neuroinflammation and thereby exacerbates the pathogenesis and progression of neurodegenerative diseases (Xu et al., 2021).

The molecular basis of STIM-Orai activity mediated (SOCE)

The essential components involved in store-operated complex entry (SOCE) are composed of Stromal-interaction molecule (STIM) proteins and Orai family members, known as calcium-release-activated Ca^{2+} (CRAC) channels (Liou et al., 2005, Roos et al., 2005, Zhang et al., 2005, Feske et al., 2006, Mercer et al., 2006, Prakriya et al., 2006, Vig et al., 2006, Yeromin et al., 2006, Lis et al., 2007, Shim et al., 2015). STIM1 and its closely related STIM2 have been identified with a primary localization in the ER membrane, where they act as calcium sensors in response to ER calcium depletion and mediate SOCE through interaction and subsequent activation of Orai channels in the plasma membrane (Prakriya and Lewis, 2015, Fahrner et al., 2017). Structurally, STIM1 and STIM2 are single transmembrane proteins and share a high homology of their N-terminal regions, containing a calcium-binding EF-hand domain and a sterile α-motif (SAM) (Stathopulos et al., 2008, Soboloff et al., 2012). They are also highly conserved in a single transmembrane domain and a series of alpha-helices that constitute the three coiled-coil domains (CC1-CC3) (Stathopulos and Ikura, 2013). Beyond the three CC domains, STIM1 and its homolog STIM2 have divergence in their amino acid sequences except for a similar lysine-rich domain (K-rich) at their C-terminus (Liou et al., 2007, Ercan et al., 2009, Ercan et al., 2012, Berry et al., 2018). Due to its lower affinity for calcium, STIM2 is more sensitive to small declines in ER calcium levels than STIM1 (Stathopulos et al., 2006, Zheng et al., 2008, Zhou et al., 2009). However, previous studies have shown that STIM1 is more effective in binding with and activating Orai 1 than STIM2 (Stathopulos et al., 2009, Wang et al., 2014). Orai family members, forming CARC channels, consist of Orai1 and its homologs Orai 2 and Orai3 (Lis et al., 2007). Orai proteins contain tetraspanning membrane domains with cytosolic N- and C-termini (Feske, 2019).

Activation of SOCE is triggered by the association of agonist-receptor (type of receptor), such as UDP-P2Y6 (GPCR), ATP-P2Y12 (GPCR), and LPS-TLR4 (inflammatory receptor) (Sun et al., 2014, Michaelis et al., 2015) that stimulate different isoforms of PLC and subsequently cleave phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol triphosphate (IP3) and diacylglycerol (DAG) (Bird et al., 2004). IP3 diffuses through the cytosol to activate its receptors IP3R on the endoplasmic reticulum (ER) membrane, which subsequently induces calcium ion release from its intracellular ER store and results in a transient increase in cytosolic calcium and a decrease in the ER calcium ions concentration (Berridge, 2016). STIM1 and STIM2 are localized on the ER membrane and monitor calcium level change in the ER lumen through their N-terminal EF-hand Ca^{2+}-binding domain together with the SAM domain. Depleting ER calcium store causes disassociation of Ca^{2+} ions from EF-hand domains that subsequently trigger a conformation change in STIM1 and STIM2 homo or hetero-dimer, oligomerization, translocation to ER-plasma membrane junctions, where they interact with and activate Orai channels (Prakriya and Lewis, 2015). The interaction is mediated by cytoplasmic C-terminal regions of STIM proteins and C-terminal domains of Orai proteins. Activation and subsequent opening of Orai channels lead to the influx of calcium from the extracellular space into the cytoplasm. The influx of calcium through the CRAC channels is defined as SOCE, as activation of SOCE is enhanced or reduced based on the concentration of calcium ions into the ER store (Prakriya and Lewis, 2015). Elevated cytosolic Ca^{2+} concentration due to the influx of Ca^{+2} through CRAC channels leads to activated calmodulin, in which Ca^{2+} ions bind calmodulin followed by the formation of the Ca^{2+}-calmodulin complex. This complex activates different calcium-dependent effectors, such as calcineurin and calmodulin-dependent kinase CaMK. In particular, activation of calcineurin, Ca^{2+} sensitive serine/threonine phosphatase enzyme, leads to dephosphorylate its main substrate transcription factor nuclear factor of activated T cells (NFAT) that undergoes a subsequent translocation from the cytoplasm to the nucleus and upregulates transcription of its target genes (**Figure 1.3**) (Dolmetsch et al., 1997). In addition to NFAT, SOCE can modulate the transcriptional activity of

NF-κB (Berry et al., 2018). Despite the importance of the elevated cytosolic Ca^{2+} for activation of different signal transduction pathways, the rise in the concentration of Ca^{2+} in the cytosol is normalized by the movement of Ca^{2+} ion in its intracellular ER store through activation of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump that facilitates the reuptake of calcium from the cytoplasm into the lumen of ER (Primeau et al., 2018). Calcium influx or SOCE via CRAC channels is required for a wide range of physiological cellular processes, including survival, proliferation, differentiation, gene expression, and secretion (Patterson et al., 2004). However, dysregulation of SOCE is implicated and linked to several pathologies (Berna-Erro et al., 2012).

The role of STIM-Orai activity in microglia and macrophages activation

SOCE is controlled by STIM proteins, which sense the decline in the ER Ca^{2+} concentration and activate plasma membrane Orai channels, with Orai1 being the main pore-forming subunit (Prakriya et al., 2006, Yeromin et al., 2006). Compared to STIM1, STIM2 is more sensitive to a small change in the ER Ca^{2+} levels (Brandman et al., 2007, Ong et al., 2015). The increase in intracellular Ca2+ concentration modulates microglia and macrophages activation and functions, such as pro-inflammatory mediators' production, migration, and phagocytosis (Kraft, 2015, Demaurex and Nunes, 2016). Therefore, inhibition of SOCE or downregulation of STIM and Orai proteins affect their functions and activation. Pharmacological targeting of SOCE leads to inhibition of calcium influx and causes a reduction of migration and invasion of rat microglia (Ohana et al., 2009). Inhibition of CRAC channels by CM-EX-137 reduces the increase in cytoplasmic Ca^{2+} in LPS-activated microglia and suppresses the inflammatory signaling responses, including the activation of NFAT, NF-κB, and INOS expression (Mizuma et al., 2019). The reduction in NFAT activation and IL-6 release following LPS stimulation was observed in microglia cells transfected with STIM1 knockdown (Heo et al., 2015). In addition, silencing of STIM1 inhibited phagocytosis of the bacterial particles in response to UDP P2Y6 agonist and ATP-stimulated migration (Lim et al., 2017). Studies using knock-out mice have confirmed the

regulatory role of the components of SOCE in the modulation of microglia activation responses. Compared to Orai2 and Orai3, STIM1, STIM2, and Orai represent the main component of SOCE in microglia, and their abundant expression has been detected in microglia (Ohana et al., 2009, Michaelis et al., 2015). Microglia isolated from STIM1 or Orai1 knock-out mice displayed impaired SOCE following thapsigargin (TG) stimulation by using calcium imaging technique, whereas STIM2 KO microglial cells have a less inhibitory effect on TG-induced SOCE (Michaelis et al., 2015). Microglia functions, including migration and phagocytosis induced by neural danger signals, are affected by the absence of STIM1, STIM2, and Orai1 proteins. While the basal migration and phagocytosis are not affected in STIM protein or Orai1 KO microglia, purinergic receptors activation-induced microglia migration and phagocytosis are inhibited by loss of STIM1, STIM2, and Orai1 (Michaelis et al., 2015). Although the effect of Orai1 on macrophage activation is not clear, several studies have shown that STIM1 and STIM2 proteins modulate the activation of macrophages. STIM1 deficient macrophages show defective IgG-mediated phagocytosis but normal chemotaxis and LPS-induced cytokines production (Braun et al., 2009, Sogkas et al., 2015). Unlike STIM1, STIM2 knock-out macrophages have diminished phagocytosis, chemotaxis, and pro-inflammatory cytokine release in response to LPS stimulation (Braun et al., 2009, Sogkas et al., 2015). In addition, STIM1 or STIM2 protein knout-out mice display resistance against multiple inflammatory disease models, such as experimental immune thrombocytopenia, IgG-induced autoimmune hemolytic anemia (AIHA), acute pneumonitis, and thioglycolate-induced peritonitis (Braun et al., 2009, Sogkas et al., 2015). In contrast to these studies, Vaeth et al. (2015) showed that although STIM1 and STIM2 double deficient macrophages have impaired SOCE, the absence of calcium influx in these cells has no impact on phagocytosis, migration, pro-inflammatory cytokines release, and inflammasome activity (Vaeth et al., 2015). Therefore, while the discrepancies in the findings between the studies could be due to the differences in the genetic background of the mice and the source of macrophages, further studies are essential to discern the impact of STIM protein ablation on macrophages' activation and functions.

Role of RGS proteins in cancer

Dysregulation of GPCRs and G-proteins has been studied extensively in numerous cancers (Wu;Yeerna; et al., 2019), indicating their critical roles as key contributors to tumorigenic processes. As negative modulators of GPCRs signaling, regulatory roles of RGS protein have been examined in cancers (Hurst and Hooks, 2009). A significant body of evidence indicates that alteration in RGS function and/or expression alters cancer progression (Hurst and Hooks, 2009). Based on cancer type, RGS proteins either promote or suppress some features of cancer progression, such as survival, proliferation, migration, invasion, metastasis and sensitivity to chemotherapy. Overexpression of RGS proteins in various types of cancers results in induction of apoptosis and inhibition of proliferative and/or migratory and invasive effects of cancer cells, such as RGS2 in breast cancer (Lyu et al., 2015), AML (Schwable et al., 2005), and androgen-independent prostate cancer (Cao et al., 2006, Wolff et al., 2012), RGS4 in breast cancer (Xie et al., 2009, Park et al., 2017), RGS5 in ovarian and lung cancers (Altman et al., 2012, Xu et al., 2015), RGS6 in breast cancer (Maity et al., 2011, Maity et al., 2013), RGS7 in melanoma (Qutob et al., 2019), RGS12 in oral squamous cell carcinoma (OSCC) (Fu et al., 2021) and prostate cancer (Wang;Wang; et al., 2017), RGS16 in breast and pancreatic cancers (Liang et al., 2009, Carper et al., 2014), RGS17 in ovarian cancer and nasopharyngeal carcinoma (NPC) (Hayes and Roman, 2016), and RGS19 in Ras-induced tumorigenesis (Wang et al., 2013). Together, these studies show that the above examples of RGS have a suppressive role in cancer cells, where their expression is downregulated with cancer progression.

In the contrast, other studies have shown that upregulation of RGS expression has been detected in tumor tissues and several cancer cells, where knock-down of RGS expression levels leads to inhibition of cancer progression, such as RGS2 in androgen-sensitive prostate cancer (Cao et al., 2006), RGS4 in glioblastoma and non-small cell lung cancer (NSCLC) (He et al., 2019, Guda et al., 2020), RGS5 in hepatocellular carcinoma (Hu et al., 2013), RGS11 in lung cancer (Yang;Li; et al., 2016), RGS16 in glioma cells (Hong et al., 2006), RGS17 in colorectal cancer,

breast cancer, and lung cancer (Hayes and Roman, 2016), and RGS20 in bladder cancer (Li et al., 2019), cervical cancer, breast cancer, and NSCLC (Yang;Lee; et al., 2016). Collectively, these findings demonstrate that these examples of RGS listed above contribute to several malignancies and can be used as prognostic markers. In addition to the above functional studies, where gain- or loss-of function experiments can inhibit or drive cancer progression, many correlative studies have linked the changes in RGS proteins expression to the development of different cancer types (Hurst and Hooks, 2009). However, further studies are required to determine whether alteration of RGS expression can cause or impact these cancer types.

While some RGS proteins' suppressive activity in cancer cells has been connected to their GAP function, some RGS proteins mediate their inhibitory effects on cancer progression due to Gα-protein independent actions. For example, the effect of RGS4 overexpression mediated inhibition of migration and invasion of breast cancer is via GAP-dependent mechanism, as GAP-dead mutant of RGS4 protein is unable to inhibit migration and invasion (Xie et al., 2009). Unlike RGS4, both RGS6 and its GAP-deficient mutant effectively induce apoptotic pathway by doxorubicin in breast cancer, suggesting that RGS6 mediates doxorubicin-induced cytotoxicity via a non-GAP independent mechanism (Huang;Yang; et al., 2011). Finally, the expression of RGS is regulated by chemotherapeutic drugs in cancer cells, where knock-down of RGS proteins can affect the sensitivity of these cells to chemotherapy. For example, RGS17 expression is suppressed in EOC cells in response to exposure to chemotherapeutic agents, such as cisplatin, and loss of RGS17 expression reduces cell death induced by these drugs (Hooks et al., 2010). In the opposite direction, RGS6 is upregulated following doxorubicin treatment in MCF-7 breast cancer cells and facilitates apoptosis induction, whereas lack of RGS6 results in doxorubicin-resistant cells (Huang;Yang; et al., 2011).

Ovarian cancer

ovarian cancer (OC) is the deadliest cancer among gynecological malignancies. In the United States, approximately 21,410 women will be newly diagnosed with OC, and an estimated 13,770 women will die from this disease by the end of 2021 (Siegel et al., 2021). OC is mainly comprised of three types, including epithelial tumors, sex cord-stromal tumors, and germ cell tumors (Chen;Ruiz; et al., 2003). Among these types, epithelial ovarian cancer (EOC) represents the majority (~85%-90%) of all the diagnosed ovarian cancer cases (Levanon et al., 2008, Jayson et al., 2014). Based on the criteria proposed by the World Health Organization (WHO) in 2014, EOC is histopathologically classified into seven subtypes: serous, mucinous, endometrioid, clear cell, Brenner, sero-mucinous, and undifferentiated (Kurman et al., 2014). With the exception of undifferentiated carcinoma, the other subtypes of EOC are further categorized into benign, borderline, and malignant neoplasia. Serous adenocarcinoma is the most common subtype of EOC that is divided into low-grade serous carcinoma (LGSC) and high-grade serous carcinoma (HGSC) (Kurman and Shih Ie, 2016, Koshiyama et al., 2017, Labidi-Galy et al., 2017). The latter is the most prevalent and lethal EOC due to its late-stage detection and poor five-year survival (DiSaia et al., 2017).

In addition to OC classification by WHO, OC has its surgical stage system known as the International Federation of Gynecology and Obstetrics (FIGO) staging, which classifies OC based on the localization of tumor cells. The FIGO staging classification is crucial because it determines the best therapeutic options (Prat and Oncology, 2014, Javadi et al., 2016). The majority of OC patients present in the clinic with advanced stages due to the non-specific symptoms and the lack of early effective detection strategies. Unlike other cancer types, OC cells uniquely metastasize from the primary tumor site to the peritoneal cavity, where the tumor cells distribute throughout the cavity via the ascites fluid. In addition to physical examination, a couple of scan types are used to diagnose OC, such as computed tomography (CT) scan and transvaginal ultrasonography (TVU) scan. Further procedures to detect OC are laparoscopy for biopsies collection followed by

histological staining and blood tests to detect tumor markers that are commonly associated with OC, for instance, serum cancer antigen 125 (CA125) (Jacobs and Bast, 1989), carbohydrate antigen 199 (CA199) (Motoyama et al., 1990), human epididymis protein 4 (HEP4)(Karlsen et al., 2014), and carcinoembryonic antigen (CEA) (Brown and Frumovitz, 2014).

Ovarian cancer is an idiopathic disease. However, there are numerous risk factors for OC. The risk of OC is increased with age, as the majority of EOC develop in post-menopausal women, with the median age of 63 years at diagnosis (DiSaia et al., 2017). Beyond age, risk factors encompass family history, exposure to asbestos or Talk-powder (Huncharek et al., 2003), null parity, early menarche or late menopause, BRAC1/BRAC2 mutation (King et al., 2003), smoking (Tworoger et al., 2008) and excessive body weight (Makowski et al., 2014). The standard treatment for ovarian cancer patients comprises a combination of tumor debulking surgery followed by first-line chemotherapeutic agents. The first-line chemotherapy treatment includes intravenous or intraperitoneal administration of the platinum-based agents (cisplatin or carboplatin) in combination with taxanes therapy (paclitaxel or docetaxel), targeting DNA to form DNA adducts and microtubules to block mitotic spindle formation, respectively (Armstrong et al., 2006). Both platinum and taxanes based agents result in arresting cell replication.

Despite a high response rate in ovarian cancer treatment, most ovarian cancer patients frequently experience disease relapse and drug-resistant disease. For some resistant or recurrent ovarian cancer cases and depending on the OC stage, the toxicity profile of chemotherapies, and the health patient condition, other chemotherapeutic drugs agents may also be given concomitantly with platinum and taxanes therapy, such as liposomal doxorubicin and salvage therapies, like cyclophosphamide, gemcitabine, and topotecan (Kim et al., 2012). Additionally, administration of targeted chemotherapies including the Poly-(ADP-Ribose) Polymerase (PARP) inhibitors (e.g., Olaparib) and anti-VEGF (Vascular Endothelial Growth Factor) monoclonal antibody (bevacizumab) in conjunction with first-line chemotherapy have improved ovarian cancer patient overall survival (Pujade-Lauraine et al., 2014). These targeted chemotherapies alongside

salvage drugs are widely used as second-line therapy for recurrent diseases. However, OC cells can develop acquired chemoresistance by downregulation of drug targets, decreasing drugs influx, or increasing drug efflux (Samimi and Annunziata, 2020). Thus, early detection and understanding of the molecular mechanisms underlying chemotherapy resistance are critical to developing therapeutic strategies, improving current chemotherapeutic agents or developing effective targeted inhibitors, thereby improving patient survival.

The impact of NF-*κ*B, TNF-α, and COX-2 on ovarian cancer progression

Inflammation-associated cancer is characterized by the presence of inflammatory cells in the tumor microenvironment and high production of inflammatory mediators, including cytokines, chemokines, and bioactive lipids. Numerous studies have shown the contributory roles of these inflammatory mediators to ovarian cancer progression (Savant et al., 2018). Growing evidence implicates the increase in NF-*κ*B activity in promoting EOC progression and chemoresistance (Harrington and Annunziata, 2019). Like the LPS response, NF-kB is activated in response to multiple chemotherapeutic drugs, including cisplatin and paclitaxel (Nakanishi and Toi, 2005, Campbell et al., 2006, Bednarski et al., 2008, Li and Sethi, 2010). The activation of NF-*κ*B is observed in multiple chemoresistant ovarian cancer cells (Sun;Huang; et al., 2018). Blocking of NF-*κ*B activity reduces cell viability by inhibiting its anti-apoptotic target genes, such as Bcl-2 and cIAP1 and reverses chemoresistance by sensitizing cancer cells to many chemotherapies (Momeny et al., 2018).

Among inflammatory mediators, TNF-α is more highly expressed in ovarian cancer cells than in ovarian-surface epithelial cells (Szlosarek et al., 2006). In addition, high levels of TNF-α are detected in ovarian tumors compared to normal ovarian tissues (Gupta et al., 2016) and in ascites from ovarian cancer patients (Moradi et al., 1993). TNF-α acts as an autocrine and paracrine growth factor that drives the expression of numerous cytokines, chemokines, COX-2, and matrix metalloproteinases through NF-*κ*B activation (Szlosarek et al., 2006, Kulbe et al.,

2007). In mouse models, TNF-α is a key promotor of ovarian cancer growth and myeloid cells recruitment, in particular, monocytes and macrophages to the ovarian tumor microenvironment (Charles et al., 2009). While TNF-α is known to induce VEGF proangiogenic factor (Xiao et al., 2011), mice harboring ovarian cancer cells, which lack TNF-α, exhibit reduced angiogenic capacity and reduced ascites formation (Kulbe et al., 2007). In addition to ovarian cancer cells, evidence suggests that macrophages cells in the tumor microenvironment enhance TNF-α production that in turn upregulates levels of CXCL12-mediated ovarian cancer migration (Scotton et al., 2002, Kulbe et al., 2005) and TGF-α secreting stromal fibroblasts, which promotes ovarian cancer metastasis (Lau et al., 2017).

COX-2 is another inflammatory mediator that generates diverse prostaglandins (PGs), including prostaglandin E2 (PGE2) (Simmons et al., 2004), and has a predominant expression in inflammatory cells (Luskova and Draber, 2004). The latest meta-analysis (2017) suggested that the high expression of COX-2 detected in tumor tissues was correlated with the poor prognosis of ovarian cancer patients (Sun et al., 2017). *In vitro* studies have shown that upregulation of COX-2 expression through PI3K/AKT signaling pathway leads to enhanced proliferation and migration of CAOV-3 (Gu et al., 2008) and invasion of SKOV-3 and OVCAR-5 EOC cells (Uddin et al., 2010). Treatment of a xenograft model of ovarian cancer with aspirin in nude mice inhibits tumor growth via downregulation of COX-2 and AKT levels expression (Uddin et al., 2010). Activation of COX-2 expression mediated PGE2 release is also associated with angiogenesis (Masferrer et al., 2000) and chemotherapeutic drugs resistance (Ferrandina et al., 2002).

Table 1.1: RGS protein superfamily

Family	Member	Size amino acids (a.a) (Human-Canonical sequence) depending on Uniprot database	Gα GAP activity	Motifs and Domains outside of RGS domain
A/RZ	RGS17	210	Gαi/o and Gαq	C-string at N-terminal region
	RGS19	217		
	RGS20	388		
B/R4	RGS1	209		α-helix at N-terminal region
	RGS2	211		
	RGS3	519		PDZ
	RGS4	205		A-helix at N-terminal region
	RGS5	181		
	RGS8	180		
	RGS13	159		
	RGS16	202		
	RGS18	235		
	RGS21	152		
C/R7	RGS6	472	Gαi/o	DEP and GGL at N-terminal region
	RGS7	495		
	RGS9	674		
	RGS11	467		
D/R12	RGS10	181		None
	RGS12	1447		PDZ and PTB at N-terminal region RBD and GoLoco at C-terminal region
	RGS14	566		RBD and GoLoco at C-terminal region

Table 1.2: Regulation of the stability of RGS proteins

RGS proteins	PTM or (binding partner)	Residues	RGS protein stability (promoting/ inhibiting) degradation	Mechanism	Physiological effects	Reference(s)
RGS2	Acetylation	N-terminal	Promoting	(CUL4B/FBXO44/DDB 1) complex		(Sjogren et al., 2015, McNabb;Gonza lez; et al., 2020)
RGS4	Arginylation	N-terminal (Cys-2)	Promoting	ATE1 via UBR1/2		(Lee et al., 2005)
	Ubiquitination		Promoting	μ and δ opioid receptor (MOR and DOR) activation	Augmentation of δ opioid receptor-induced MAP kinase and M3-muscarinic receptor-induced cAMP accumulation	(Wang and Traynor, 2011)
	Palmitoylation	N-terminal (Cys-2)	Inhibiting	Acyltransferases	Enhancing inhibition of α-adrenergic-induced calcium signaling	(Wang et al., 2010)
RGS5	Arginylation	N-terminal (Cys-2)	Promoting	ATE1 via UBR1/2		(Lee et al., 2005)
RGS6	(Gβ5)		inhibiting			(Chen;Eversole -Cire; et al., 2003)
RGS7	(Gβ5)		inhibiting			
	Phosphorylation	Ser241, Thr245, Thr247	inhibiting	TNF-α mediated P38-MAPK activation		(Benzing et al., 1999)
RGS9	(Hsc70)		Promoting			(Posokhova et al., 2010)
	(Gβ5-R9AP)		inhibiting			(Jeffrey et al., 2010)
	(Gβ5-R7BP)		inhibiting			(Anderson et al., 2007)
	(Gβ5)		inhibiting			(Chen;Eversole -Cire; et al., 2003)
RGS11	(Gβ5)		inhibiting			
RGS13	Phosphorylation	Thr41	inhibiting	PKA	Triggering nuclear localization	(Xie;Yang; et al., 2010)
RGS16	Phosphorylation	Tyr168	inhibiting	Src	Enhancing GAP activity	(Derrien et al., 2003)
	Arginylation	N-terminal (Cys-2)	Promoting	ATE1 via UBR1/2		(Lee et al., 2005)
RGS20	Ubiquitination		Promoting	Gα o/i-coupled serotonin receptor	loss of inhibition against Gαi signaling	(Pagano et al., 2008)

Table 1.3: Non-canonical binding partners of RGS proteins

Non-canonical interacting protein	RGS protein	Domain/Residues of RGS proteins	Effect(s)	Reference(s)
R9AP	C/R7 subfamily members	DEP	Cell membrane localization and enhancing GAP activity	(Hu et al., 2003, Anderson et al., 2009)
R7BP				(Drenan et al., 2006, Anderson et al., 2007, Anderson et al., 2009)
Gβ5		GGL	Required for the members of the C/R7 subfamily RGS proteins stability	(Anderson et al., 2009)
(PI3K) p85α regulatory subunit	RGS13	N-terminal	Deactivation of PI3K signaling-mediated allergy	(Bansal;Xie; et al., 2008)
	RGS16	RGS	Inhibition of EGF-induced AKT activation and breast cancer proliferation.	(Liang et al., 2009)
Leucine rich repeat kinase 2 (LRRK2)	RGS2	N.D.	Neuroprotective role	(Boon et al., 2014, Dusonchet et al., 2014)
GSK3β	RGS3	PDZ	Inhibition of GSK3β activity and thereby enhancing Wnt3a signaling	(Shi et al., 2012)
Teb4	RGS2	N-terminal	RGS2 degradation	(Park et al., 2015)
FBXO44				(Sjogren et al., 2015, McNabb;Gonzalez; et al., 2020)
DMAP1	RGS6	GGL	Interaction with DMAP1 and its binding partner (DNMT1) and thereby enhancing DNMT1 degradation and inhibiting DNMT1-induced transcription repression	(Liu and Fisher, 2004)
Tip60		RGS	Facilitating RGS6-enhanced DNMT1 degradation	(Huang et al., 2014)
Smad3	RGS3	Residues (240-379)	Blocking Smad3/4 complex-mediated transcription	(Yau et al., 2008)
CREB	RGS13	Residues (1-33) Residues (93-117)	Blocking CREB interaction with its CBP co-activator, resulting in suppression of CREB transcriptional activity	(Xie et al., 2008)
Spinophilin (SPL)	RGS2	N-terminal	Required for RGS2-α adrenergic receptor interaction and RGS2-mediated inhibition of α adrenergic signaling	(Wang et al., 2005, Liu et al., 2006)
	RGS4	N.D.	Required for RGS4- α adrenergic receptor interaction	(Liu et al., 2006)
		N.D.	Required for RGS4-M3R interaction and RGS4-mediated inhibition M3R signaling	(Ruiz de Azua et al., 2012)
	RGS10	N.D.	Formation of a complex with SHP1 and thereby sequester RGS10 or RGS18 away from its target GPCRs	(Ma et al., 2012)
	RGS18			
	RGS8		Inhibiting the direct binding between RGS8 and M1 muscarinic acetylcholine receptor (M1-mAChR) and enhancing the inhibitory function of RGS8 on M1-mAChR signaling	(Fujii et al., 2008)
Neurabin	RGS2	N.D.	Blocking RGS2-spinophilin interaction and thereby resulting in inhibition of RGS2 action on α-Adrenergic receptor	(Wang et al., 2007)

	RGS4	N.D.	Promoting RGS4- α-Adrenergic receptor interaction and facilitating RGS4 inhibitory effect on α- Adrenergic receptor	(Chen;Liu; et al., 2012)
GIPC	RGS19	N.D.	Required for RGS9-D2 receptor interaction and thereby attenuation of D2R signaling	(Jeanneteau et al., 2004)
β-Arrestin2	RGS9-2	DEP	Mediating the assembly of RGS9-2- Gβ5 complex and thereby regulation of D3 receptor signaling	(Zheng et al., 2011)
HSC70		C-terminal	Facilitating RGS9-2 degradation	(Posokhova et al., 2010)
N-type Ca^{2+} channels	RGS12	PTB	Regulation GABA type B receptor-mediated inhibition of calcium currents	(Schiff et al., 2000, Richman and Diverse-Pierluissi, 2004)
TRPV6	RGS2	N-terminal	Inhibition Ca^{2+} currents of TRPV6	(Schoeber et al., 2006)
GIRK	RGS4	N-terminal RGS	Modulate channel activity by forming a complex with M2R-GIRK	(Jaen and Doupnik, 2006)
	Gβ5-RGS7	N.D.	Regulation GIRK channels activity	(Xie;Allen; et al., 2010, Zhou et al., 2012)
SNAPIN	RGS7	Residues (1-79)	Possible roles in synaptic vesicle exocytosis	(Hunt et al., 2003)
eIF2Bε	RGS2	Residues (79-115)	Inhibition of protein synthesis	(Nguyen et al., 2009)
H-Ras	RGS14	RBD	Inhibition of PDGF-induced Erk activation	(Shu et al., 2010)
H-Raf				
Tubulin		N.D.	Promoting tubulin polymerization	(Martin-McCaffrey et al., 2005)
Rap-2A-GTP		RBD	Possible role in modulation Rap2A signaling.	(Traver et al., 2000, Mittal and Linder, 2006)
NHERF1		PDZ	Disruption of NHERF1–NPT2A complex-mediated hormone-sensitive phosphate uptake in the kidney.	(Friedman et al., 2019)
SCG10	RGS20	N-terminal	Inhibition of SCG10-induced microtubule disassembly	(Nixon et al., 2002)
	RGS6	GGL	Promoting NGF-induced neuronal differentiation	(Liu et al., 2002)
Nek7	RGS2	N.D.	Mitotic spindle organization	(de Souza et al., 2015)
STAT3		N.D.	Inhibition of STAT3 mediated transcription	(Lee;Park; et al., 2012)
Adenylate cyclase III, V, and VI isoforms		N-terminal	Inhibition of cAMP production	(Sinnarajah et al., 2001)
Adenylate cyclase V	RGS9-2	N.D.	Inhibition of the activity of Adenylate cyclase V	(Xie et al., 2012)
Guanylyl cyclase	RGS9-1	N-terminal	Inhibition of cGMP production	(Yu et al., 2001)
H-Ras	RGS12	RBD	Formation of a complex containing TrkA/H-Ras/H-Raf/MEK2 and regulation their signaling pathway	(Willard et al., 2007)
B-Raf				
MEK2		PDZ		
Nerve growth factor (NGF) receptor tyrosine kinase (TrkA)		N.D.		
CXCR2		PDZ	Deactivation CXCR2 signaling	(Snow;Hall; et al., 1998)

COX-2			RGS12 and NF-κB(p65) are associated with COX2. Overexpression of COX2 leads to an increase in nuclear translocation of RGS12 and NF-κB(p65).	(Yuan;Yang;Ng; et al., 2020)
NF-κB(p65)		PTB	Promoting NF-κB(p65) phosphorylation and nuclear translocation	(Yuan;Yang;Ng; et al., 2020)
PDGFβ		DZ/PTB domain N-terminus	Inhibition of PDGF-induced activation of p42/p44 MAPK	(Sambi et al., 2006)
ATP5A		N.D.	Regulation of oxidative phosphorylation in chondrocytes by promotion the function of ATP5A	(Yuan;Yang;Liu; et al., 2020)
Kappa (κ) opioid receptor		N-terminal	RGS12 attenuates G protein signaling and augments β-arrestin signaling downstream of KOR *via* independent signaling mechanisms.	(Gross et al., 2019)
Ephrin β	RGS3	PDZ	Maintaining neural progenitor cells and regulation of reverse signaling	(Lu et al., 2001, Su et al., 2004)
Gβ1γ2		N-terminal	Inhibition Gβ1γ2-mediated activation of inositol phosphate production, MAPK, and AKT signaling	(Shi et al., 2001)
PLC β	RGS4	N-terminal	Inhibition of Ca^{2+} signaling	(Zeng et al., 1998, Dowal et al., 2001)
PAR-1	RGS2 RGS4	N-terminal	Inhibition of PAR1 signaling	(Ghil et al., 2014)
PAR-4	RGS2 RGS4	N-terminal	Inhibition of PAR4 signaling	(Kim and Ghil, 2020)
mu- and delta-opioid receptors	RGS4	N.D.	Modulation of DAMGO-mediated adenylyl cyclase inhibition	(Georgoussi et al., 2006)
Kappa (κ) opioid receptor	RGS2 RGS4	N.D.	Attenuation κ-agonist mediated-adenylyl cyclase inhibition and ERK phosphorylation	(Papakonstantinou et al., 2015)
Cholecystokinin receptor-2 (CCK2R)	RGS2	N-terminal	Modulation of CCK2R signaling	(Langer et al., 2009)
M1R muscarinic M1 receptor		N-terminal	Inhibition of M1R signaling	(Bernstein et al., 2004)
α1-Adrenoreceptor (α1AR)		N-terminal	Inhibition of α1AR signaling	(Hague et al., 2005)
β2-Adrenoreceptor (β2AR)		N-terminal	Inhibition of β2AR signaling	(Roy et al., 2006)
Angiotensin II type 1 receptor (AT1R)		N-terminal	Inhibition of AT1R signaling	(Matsuzaki et al., 2011)
Melanin-concentrating hormone receptor 1 (MCH1R)	RGS2 RGS8	Residues 28-80 N-terminal (9 a.a)	Inhibition of MCH1R signaling.	(Miyamoto-Matsubara et al., 2010) (Miyamoto-Matsubara et al., 2008)
cGMP-dependent protein kinase I-α (PKGI-α)	RGS2	RGS2 phosphorylation	Inhibiting RGS2 degradation and thereby promoting RGS2 membrane localization	(Tang et al., 2003, Osei-Owusu et al., 2007)
Protein kinase C (PKC)		Phosphorylation on Ser46	Inhibiting GAP activity in *vitro*	(Cunningham et al., 2001)
Ca^{2+}- dependent calmodulin	RGS4	RGS domain and N-terminal	Attenuation PIP3-induced RGS4 GAP inhibition	(Popov et al., 2000, Ishii et al., 2002)
	RGS10	N.D.	Competing with PIP3 to interact with RGS10 and enhancing Ca^{2+}oscillation-calcineurin-NFATc1 mediated osteoclast differentiation	(Yang and Li, 2007)

Ca^{2+}- dependent calmodulin and CaMKII	RGS14	R1/2 of RBD	RGS14 directly binds to Ca^{2+}/CaM and is phosphorylated by CaMKII. RGS14 regulates this signaling pathway that is essential for synaptic activity.	(Evans et al., 2018)
14-3-3	RGS7	RGS7 phosphorylation-dependent interaction to 14-3-3γ at Ser434	Inhibition RGS7 GAP activity	(Benzing et al., 2002)
	RGS3	RGS domain or N-terminal	Inhibition of the GAP activity of RGS3.	(Benzing et al., 2002, Niu et al., 2002, Rezabkova et al., 2010)
	RGS4	Gαo interacting site	Blocking Gαo interaction	(Abramow-Newerly et al., 2006)
	RGS6			
	RGS14	RGS14 phosphorylation-dependent interaction to 14-3-3γ at Ser28 RGS14:14-3-γ phosphorylation independent interaction.	Inhibition interaction with active Gαi–$AlF4^-$ to the RGS domain. Inhibition RGS14 nuclear import	(Gerber et al., 2019)
	RGS18	RGS18 phosphorylation-dependent interaction to 14-3-3γ at Ser49 and Ser218.	Inhibition of the GAP activity of RGS18 on thrombin-Gαq signaling in platelets	(Gegenbauer et al., 2012)
G-protein-signaling modulator 3 (GPSM3)	RGS5	N.D.	Enhancing RGS5 GAP activity	(Zhao and Chidiac, 2015)
Smoothened (Smo)		N.D.	Repressing Hedgehog (Hh) signaling	(Mahoney et al., 2013)
Transforming growth factor beta-activated kinase 1 (TAK1)		Residues (64-181)	Preventing TAK1 phosphorylation and the activation of the downstream JNK/p38 signaling pathway.	(Wang et al., 2021)
Ataxin-2	RGS8	N.D.	Regulation RGS8 translation in Purkinje cells	(Dansithong et al., 2015)

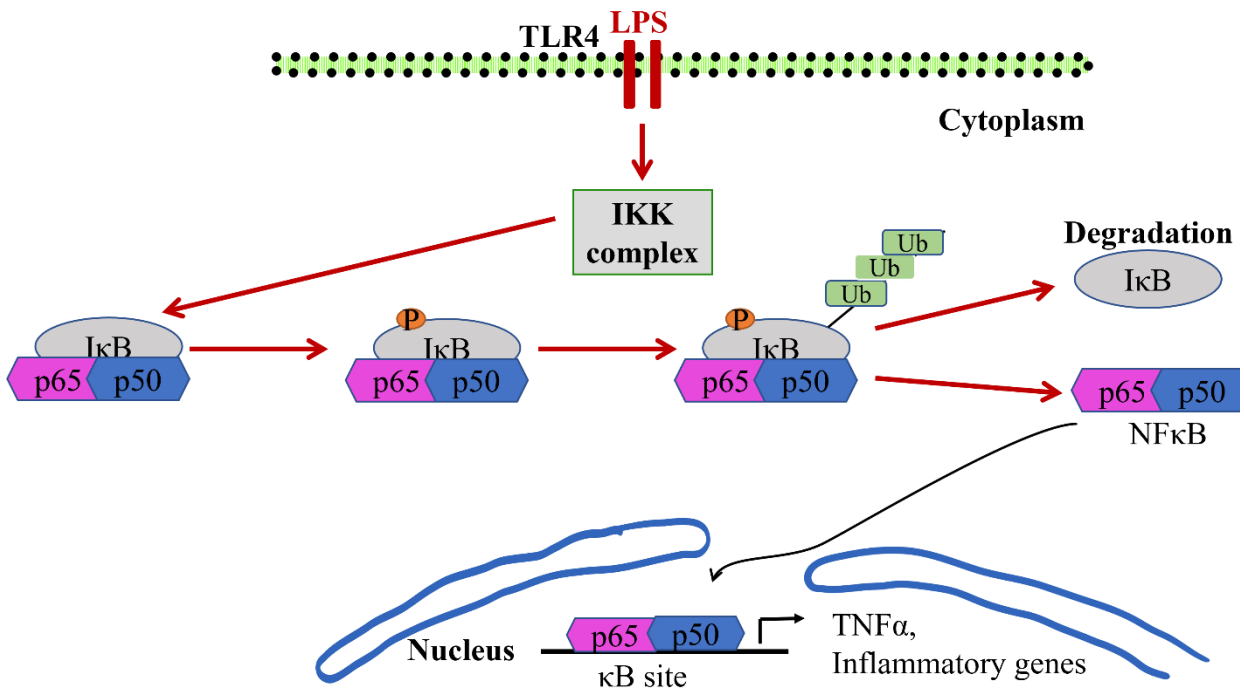

Figure 1.1: Canonical NF-κB signaling

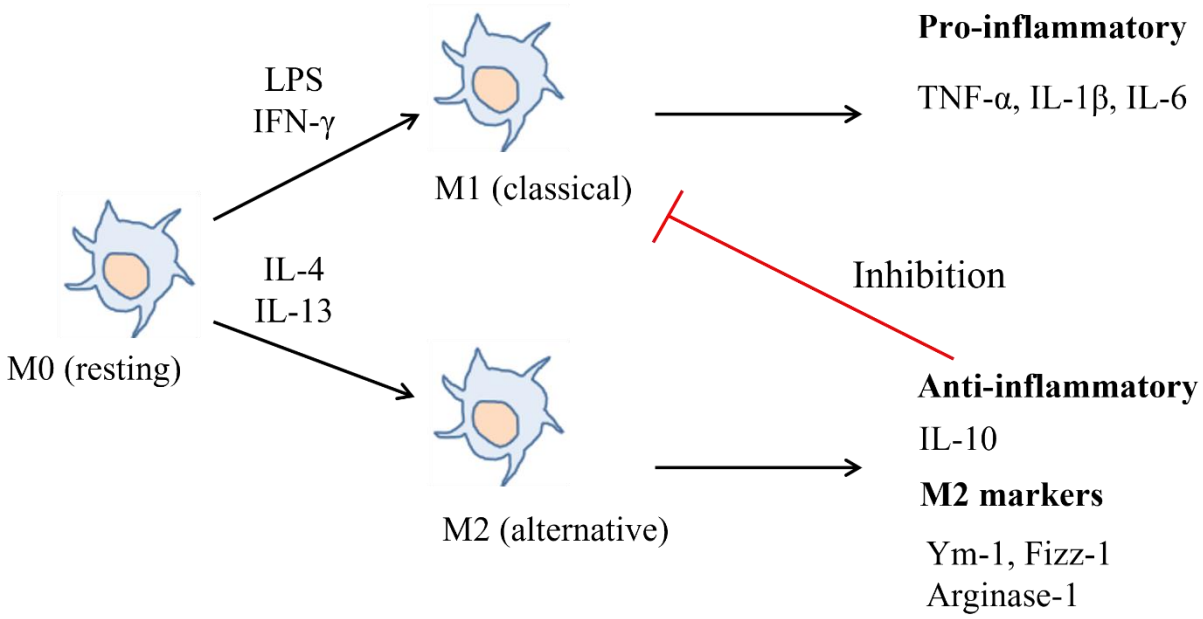

Figure 1.2: Polarization of macrophages

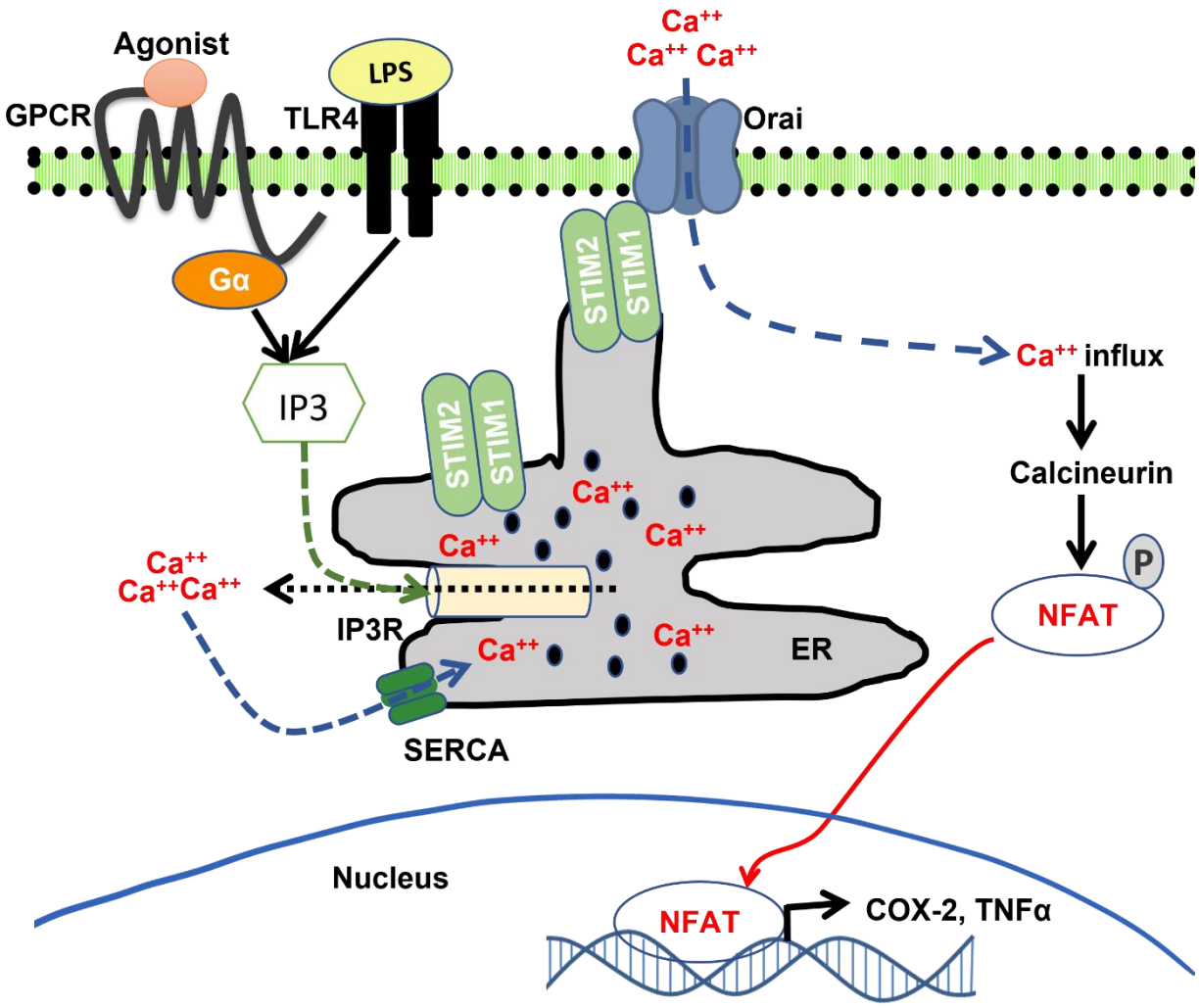

Figure 1.3: Store operated calcium entry (SOCE) machinery

CHAPTER 2

REGULATOR OF G PROTEIN SIGNALING 10: STRUCTURE, EXPRESSION AND FUNCTIONS IN CELLULAR PHYSIOLOGY AND DISEASES[1]

[1] Almutairi F, Lee JK, Rada B. *Cell Signal*. 2020 Nov; 75:109765

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Abstract.

Regulator of G protein signaling 10 (RGS10) belongs to the superfamily of RGS proteins, defined by the presence of a conserved RGS domain that canonically binds and deactivates heterotrimeric G-proteins. RGS proteins act as GTPase activating proteins (GAPs), which accelerate GTP hydrolysis on the G-protein α subunits and result in termination of signaling pathways downstream of G protein-coupled receptors. RGS10 is the smallest protein of the D/R12 subfamily and selectively interacts with Gαi proteins. It is widely expressed in many cells and tissues, with the highest expression found in the brain and immune cells. RGS10 expression is transcriptionally regulated via epigenetic mechanisms. Although RGS10 lacks multiple of the defined regulatory domains found in other RGS proteins, RGS10 contains post-translational modification sites regulating its expression, localization, and function. Additionally, RGS10 is a critical protein in the regulation of physiological processes in multiple cells, where dysregulation of its expression has been implicated in various diseases including Parkinson's disease, multiple sclerosis, osteopetrosis, chemoresistant ovarian cancer and cardiac hypertrophy. This review summarizes RGS10 features and its regulatory mechanisms, and discusses the known functions of RGS10 in cellular physiology and pathogenesis of several diseases.

Highlights

- RGS10 is dysregulated in response to multiple stimuli and various disease models.
- Epigenetic and post-translational mechanisms regulate RGS10 expression and function.
- RGS10 has emerged functions in cellular physiology and pathology.
- Stabilizing RGS10 expression represents a promising therapeutic strategy.

Introduction

GPCR/G-protein signaling

G protein-coupled receptors (GPCRs) and the transduced heterotrimeric G-proteins, which modulate a cascade of intracellular effector proteins and chemical second messengers, are essential in the regulation of various physiological functions of cells and organ systems. They represent the largest family of FDA-approved targeted drugs for the treatment of a wide range of disorders caused by dysregulation of GPCR/G-protein signaling (Lagerstrom and Schioth, 2008). In the resting state, GPCRs are bound to inactive heterotrimeric G-proteins, consisting of the Gα-GDP and Gβγ dimer. In response to a variety of extracellular stimuli, GPCRs undergo conformational changes and act as guanine nucleotide exchange factors (GEFs) by promoting the exchange of GDP for GTP on Gα. As a result, activated GTP-Gα dissociates from Gβγ dimer, and thereby both can regulate downstream effector proteins (Gilman, 1987) (**Figure 2.1**), such as enzymes, RhoGEFs, and ion channels that in turn initiate diverse signaling pathways mediating cellular responses (Clapham and Neer, 1997, Kozasa et al., 1998, Simonds, 1999, Kammermeier et al., 2000, Rhee, 2001)

The amplification and duration of signaling activity by Gα-GTP and Gβγ are tightly modulated by the intrinsic GTPase activity of Gα, in which the Gα subunit hydrolyzes GTP to GDP and promotes reassembly with the Gβγ dimer to reform the inactive Gαβγ protein complex. This, in turn, results in the deactivation of G-protein cycle. Even though Gα functions as a molecular switch and turns itself off by hydrolyzing its bound GTP to GDP, its intrinsic rate of GTP hydrolysis measured *in vitro* is very slow and does not account for the fast deactivation kinetic of G-proteins observed *in vivo* (Tsang et al., 1998). This observation has led to the discovery of proteins, named as regulators of G protein signaling that modulate the Gα-GTP hydrolysis rate, and thereby fine-tune the activation of G-proteins that mediate cellular signaling events.

RGS protein family

Regulator of G protein signaling (RGS) proteins that were initially discovered via genetic analysis in yeast and worm models (Chan and Otte, 1982, Dohlman et al., 1996, Koelle and Horvitz, 1996), represent a diverse family of structural and multifunctional intracellular proteins that canonically regulate GPCRs and heterotrimeric G-protein signal transduction. RGS proteins control the lifetime of signaling pathways mediated by G-proteins. They act as a guanosine triphosphatase (GTPase) activating proteins (GAPs), which accelerate GTP hydrolysis of the active Gα-GTP form and, in turn, convert it back to the inactive Gα-GDP form. Consequently, this results in the termination of downstream G-protein signaling pathways (**Figure 2.1**).

To date, 20 canonical RGS proteins and 19 RGS-like proteins have been identified with various regulatory roles (Ross and Wilkie, 2000, Hollinger and Hepler, 2002, Willars, 2006). All canonical RGS proteins contain the conserved, approximately 120 amino acids long RGS domain, consisting of nine α helices that are subdivided into two sub-domains. The first subdomain forms a smaller helix bundle and is comprised of helices αI, II, III, VIII, and IX, while the larger bundle subdomain comprises helices αIV, V, VI, and VII (Tesmer et al., 1997). The RGS domain selectively binds and stabilizes the Gα subunit in its transition state for GTP hydrolysis, mimicked by ($GDP+AlF4^-$), resulting in GDP production and turning off signaling cascades regulated by G-proteins (Berman;Kozasa; et al., 1996, Tesmer et al., 1997).

The structure of canonical RGS proteins ranges from small proteins comprised solely of an RGS domain responsible for GAP activity to more complex proteins containing multiple motifs and domains with functions in subcellular localization, protein stability, and protein-protein interactions. Based on the homology of the RGS domain and the presence of other common structural domains, canonical RGS proteins are categorized in four main subfamilies, consisting of A/RZ, B/R4, C/R7, and D/R12 (**Figure 2.2**). As negative regulators of G-protein signaling, RGS proteins mainly exert their GAP function on α subunits of the Gi/o and Gq families of G-proteins. Although there are no confirmed reports on GAP activity of any RGS domain toward Gαs, RGS

proteins indirectly regulate Gαs downstream signaling through interaction with subtypes of adenylate cyclase (AC) (Roy et al., 2006, Talbot et al., 2010).

RGS D/R12 proteins subfamily

RGS D/12 subfamily is composed of three distinct structural proteins (**Figure 2.2**), containing RGS10, RGS12, and RGS14, which share high sequence identity within a conserved RGS domain (**Figure 2.3A**) and act as GAP for the Gi family Gα subunits. Whereas RGS10, at 20 kDa, is a relatively simple RGS protein, RGS12 and RGS14 are much larger and more complex than RGS10, and have multiple common structural and regulatory motifs and functional domains, including a pair of tandem Ras-binding domains (RBDs) and a C-terminal G-protein regulatory (GPR) motif, also known as the GoLoco motif. Through their first RBD domain, both RGS12 and RGS14 interact with activated small G-proteins, such as H-Ras-GTP and Rap-2-GTP (Traver et al., 2000, Willard et al., 2009, Shu et al., 2010), while the two tandem RBDs of RGS14 mediate interactions with Raf kinases (Shu et al., 2010) and calcium dependent-calmodulin (CaM) (Evans et al., 2018). The GPR motif plays a role in subcellular localization and selectively binds inactive Gα proteins-GDP, thereby inhibiting the exchange of GDP toward GTP through its guanine nucleotide dissociation inhibitor (GDI) activity (Kimple et al., 2001).

Unlike RGS14, RGS12 is expressed as multiple isoforms. The longest RGS12 isoform is called trans-spliced-RGS12TS-L, is a 156 kDa protein that possesses two additional domains, including PTB and PDZ domains, making RGS12 the largest member of the RGS protein family. The PTB domain is involved in interacting with neuronal N-type calcium channels (Schiff et al., 2000, Richman et al., 2005), while the PDZ domain binds the C-terminal of the CXCR2 receptor (Snow;Hall; et al., 1998), and both PTB/PDZ domains significantly attenuate ERK phosphorylation downstream of PDGFβ receptor activation (Sambi et al., 2006). RGS12 is abundant in the brain (Snow et al., 1997), heart (Doupnik et al., 2001) and osteoclasts (Yang and Li, 2007), and is implicated in neuronal differentiation (Willard et al., 2007), bone disorders (Yuan et al., 2015),

cardiac hypertrophy (Huang et al., 2016) and lung and prostate cancers (Dai et al., 2011, Wang;Wang; et al., 2017).

RGS14, at 61 kDa, is expressed in the brain with a high enrichment in CA2 hippocampal neurons, where RGS14 naturally suppresses synaptic plasticity and limits learning and spatial memory (Evans et al., 2015). Further, RGS14 is associated with cardiac remodeling (Li et al., 2016), Parkinson's disease (PD) (Vogt et al., 2006), and kidney diseases (Urabe et al., 2012, Yasui et al., 2013, Mahajan et al., 2016). This review primarily focuses on the small RGS protein RGS10, and discusses, in-depth, its characterization, regulatory mechanisms, and the physiological and pathological roles of RGS10 in several cells and mouse models.

Characterization of RGS10 protein

Gene and protein organization

RGS10 is a small RGS protein that resembles the structure of R4 subfamily members. However, based on RGS domain sequence similarity, RGS10 is classified as a third member of the D/R12 subfamily which also includes the proteins RGS12 and RGS14 (**Figure 2.3A**) (Hunt et al., 1996, Gold et al., 1997, Popov et al., 1997). Despite the homology within their RGS domains, RGS10 is one of the smallest proteins of the entire RGS protein family and shares only a single conserved RGS domain in common with both RGS12 and RGS14 that contain more accessory domains. In addition to its conserved and functional RGS domain, RGS10 contains short disordered amino and carboxy-terminal extensions, harboring regulatory modification sites (**Figure 2.3B**).

RGS10 is highly conserved in humans and rodents and is encoded by a single gene (*Rgs10*) located at the position 10q26 on chromosome 11 in humans and on murine chromosome 7 (Haller et al., 2002, Sierra et al., 2002). Alternative splicing of the first exon of the *Rgs10* mouse gene results in two transcripts isoforms that share the last four exons and correspond to human transcripts with conserved exon structures. These two transcript variants yield two proteins,

differing only by a few amino acids in their N-terminal sequences (**Figure 2.3C**). The long isoform, called RGS10A (RGS10-1/RGS10L), is a 181 amino-acid peptide, while the short isoform, termed RGS10B (RGS10-2/RGS10S), consists of 167 amino acids (**Figure 2.3B**). The third human isoform (173 aa), arising from an alternative start site upstream of the first shared exon, has been reported and shown to have GAP activity, but its homolog in mouse has not found yet (Hunt et al., 1996, Haller et al., 2002).

RGS10A (RGS10-1/RGS10L) is the predominant and functional isoform that is naturally expressed in osteoclasts (Yang et al., 2007), microglia (Lee et al., 2008), and ovarian cancer cells (Ali et al., 2013), while RGS10B (RGS10-2/RGS10S) lacking 14 amino acids at the N-terminus has impaired GAP function (Ajit and Young, 2005).

Tissue distribution

RGS10 is ubiquitously expressed; it has been detected in several tissues of humans and other mammals. Our results shown in **Figure 2.4** confirm the wide tissue distribution of RGS10 protein expression and its lack in RGS10$^{-/-}$ mice. In human, the RGS10 mRNA is predominately expressed in the brain (Taymans et al., 2003, Larminie et al., 2004), as well as subsets of immune cells, including CD4^{+} T cells (Garcia-Bernal et al., 2011), and monocyte-derived dendritic cells (Shi et al., 2004). Also, the RGS10 transcript is highly expressed throughout the mouse and rat brains, with specific enrichment in the hippocampus, dorsal raphe, and striatum, regions of the brain that are implicated in mood disorders and anti-depressant treatment response (Gold et al., 1997, Waugh et al., 2005). Further, RGS10 is found in peripheral tissues, including the cornea (Wu et al., 2008), heart, lung, testis and immune organs including the bone marrow, lymph nodes, and spleen, but its expression is not detectable in liver, kidney, and muscles (Haller et al., 2002). Using an antibody ([C-20, sc-6206], Santa Cruz Biotechnology) raised against a synthetic peptide identical to the last 20 residues (EEDLPDAQTAAKRASRIYNT) at the C-terminus of RGS10 (Waugh et al., 2005), protein expression of RGS10 was confirmed in our laboratory by western

blotting in multiple parts of the brain including the cortex, hippocampus, cerebellum, and striatum (**Figure 2.4A**), and in several peripheral tissues including the heart, lung, spleen, stomach and intestine (**Figure 2.4B**). RGS10 expression is absent in the liver and the kidney (Data not shown).

Subcellular localization

Instead of localizing to the plasma membrane, where the canonical G protein targets reside, numerous RGS proteins are primarily accumulated in the nucleus (Chatterjee and Fisher, 2000, Dulin et al., 2000, Zmijewski et al., 2001, Chatterjee et al., 2003). The nucleus as a cellular storage compartment may serve to restrain RGS proteins' availability from regulating G-protein signaling or may allow them independently of their canonical function to regulate nuclear signal transduction through undefined nuclear interacting proteins (Sethakorn et al., 2010). Several reports (summarized in **Table 2.1** and described below) demonstrated the localization of RGS10 to both the nucleus and the cytoplasm, with no significant plasma membrane localization. Chatterjee and Fisher (2000) first reported that RGS10 that is ectopically expressed in COS-7 cells and endogenously is expressed in H4-neuroglioma cells, is mainly localized to the nucleus (Chatterjee and Fisher, 2000). In contrast to this finding, Burgon *et al.* (2001) demonstrated a predominant distribution in the cytoplasm of HEK-293 cells expressing RGS10-GFP fusion protein (Burgon et al., 2001), while Lee and her colleagues observed endogenous RGS10 throughout cytoplasmic and nuclear cellular compartments in primary microglia (Lee et al., 2008). Collectively, these findings suggest that the subcellular localization of RGS10 is cell type-specific.

Indeed, the cytoplasmic/nuclear translocation of RGS10 is a highly dynamic process. RGS10 can shuttle between the cytoplasm and the nucleus in response to cellular stimuli or signal-induced covalent modifications (**Table 2.1**). In microglia, RGS10 appears to be evenly distributed between the cytoplasm and nucleus under resting conditions. However, following lipopolysaccharide (LPS) stimulation, RGS10 is robustly enriched in the nucleus (Lee et al., 2008). Furthermore, activation of Gαi signaling by melatonin promotes RGS10 translocation from the

nucleus to the cytoplasm in PC3-AR prostate cancer cells (Rimler et al., 2006). Importantly, nuclear localization of RGS10 is induced through the cyclic AMP-dependent protein kinase A (PKA)-mediated phosphorylation of RGS10 on serine 168 (Burgon et al., 2001), a residue located outside of its common RGS domain that contains a defined putative nuclear localization sequence (Chatterjee and Fisher, 2000). Therefore, further sequence and deletion analyses are required to identify the potential nuclear localization sequences outside of the RGS domain and to elucidate the molecular mechanism underlying RGS10 nuclear localization in response to phosphorylation by PKA.

Gα binding and GAP activity

Many RGS proteins selectively target and negatively regulate a particular type of activated Gα protein signaling, while others have an affinity to interact with different Gα subtypes. RGS10 is selective to bind and terminate Gi family Gα subunits, including Gαi, Gαo, and Gαz (Hunt et al., 1996, Watson et al., 1996, Popov et al., 1997). Previous studies have defined and described the structure of RGS10 in an uncomplexed form compared to the structure of RGS10 complexed with Gαi3 (Soundararajan et al., 2008, Taylor et al., 2016). Soundararajan *et al.* (2008) demonstrated that RGS10 possesses a shorter flexible αVI helix and an extended αV-αVI loop containing 18 residues compared to typical 14 residues of αV- αVI loop for RGS domains of R4, R7, and Rz subfamilies (Soundararajan et al., 2008).

Generally, the interaction between RGS10 and Gαi3 (critical residues highlighted in **Figure 2.3C**) is similar and consistent with R4 subfamily members-Gαi complexes with observed differences in the αVI helix. Due to the disorder of the entire RGS10 αVI helix in its complex with Gαi3, a conserved arginine residue in the αVI helix (R113(99), a putative modulatory residue in RGS domain highlighted in **Figure 2.3C**) does not make a direct interaction with Gαi3 switch III region as R4 subfamily members do interact with Gαi complexes through this conserved residue (Soundararajan et al., 2008). The RGS10 RGS domain exhibits a higher GAP activity toward Gαi1

and Gαz compared to RGS domains of RGS4 and RGS19 (GAIP) (Popov et al., 1997) and approximately the same GAP activity similar to RGS domains of RGS4 and RGS16 against Gαo (Kosloff et al., 2011). However, the GAP activity of RGS10 is lower toward Gαi1 and Gαo compared to higher GAP RGS proteins, such as RGS4 and RGS16 (Asli et al., 2018), where they localize to the plasma membrane through their N-terminal amphipathic domain, efficiently contributing to their GAP activity with Gαz. Interestingly, replacement of the N-terminal domain of RGS10 with the N-terminal domain of RGS4 enhances its GAP activity toward Gαi and Gαz (Tu et al., 2001, Ajit and Young, 2005), suggesting that plasma membrane targeting is essential for its GAP activity. Moreover, unlike RGS4 and RGS16, RGS10 contains putative disruptor residues (highlighted in **Figure 2.3C**) including Q111(97), K139(125), and Y140(126) that disrupt the contact with both Gαi1 and Gαo and consequently result in reduced GAP activity (Asli et al., 2018).

RGS10 has been shown to modulate Gαi-mediated receptor signaling. Adenoviral gene delivery-mediated RGS10 expression in CHOK1 cells stably expressing the human serotonin 5-HT1A receptor results in a significant reduction of Gαi-mediated inhibition of AC and inhibition of forskolin-stimulated cAMP production (Ghavami et al., 2004). Consistent with its effect on 5-HT1A signaling, RGS10 overexpression attenuates the inhibition of AC activity mediated by μ (mu)-opioid receptor (Xie et al., 2007). Further, RGS10 blocks CXCL12- stimulated chemokine CXCR4 receptor-mediated signaling in T cells (Garcia-Bernal et al., 2011). Together, these findings support the selectivity of RGS10 in regulating GPCR-coupled Gαi signaling.

Regulation of RGS10 function and expression

Epigenetic mechanisms

Numerous studies (summarized in **Table 2.2**) have shown the regulation of RGS10 expression in response to various stimuli in several cell types and disease models. Unlike other RGS proteins, RGS10 is regulated at the transcriptional level via epigenetic mechanisms. Typically, the transcription of genes is epigenetically regulated through alteration of chromatin

structure, containing nucleosomes formed by DNA coiled around histones proteins (Venkatesh and Workman, 2015). Modification of DNA and histones leads to chromatin remodeling, which either facilitates or impedes the transcriptional machinery's access to DNA. This results in the initiation or repression of gene expression (Venkatesh and Workman, 2015). Common mechanisms underlying chromatin modifications are DNA methylation and histone acetylation/deacetylation. DNA methylation is carried out by DNA methyltransferases (DNMTs) that add a methyl group directly to the fifth carbons of cytosine residues at CpG dinucleotides and mainly leads to gene silencing (Smith and Meissner, 2013). Histone acetylation is generally associated with transcriptional activation, and is mediated by adding acetyl groups on lysine residues of histone tails by histone acetyltransferases (HATs). In contrast, histone deacetylation involves the removal of acetyl groups from histone residues by histone deacetylases (HDACs) and is linked to transcriptional repression (Bannister and Kouzarides, 2011).

Several studies have demonstrated that DNA hypermethylation and histone deacetylation are critical epigenetic mechanisms mediating the regulation of RGS10 transcription in cancer cells, as well as neurons and macrophages. RGS10 expression is suppressed in chemoresistant ovarian cancer cells due to an increase in DNA methylation and histone deacetylation at its promoter compared to chemosensitive counterparts (Ali et al., 2013). Suppression of either DNA methyltransferase 1 (DNMT1) or histone deacetylase 1 (HDAC1) using siRNA knockdown significantly increases the expression of RGS10 in chemoresistant A2780-AD ovarian cancer cells (Cacan et al., 2014). In addition, pharmacological inhibition of the activity of DNA methyltransferase with 5-Aza deoxycytidine (5-Aza) enhances the RGS10 transcript level in human neural progenitor cells (Tuggle et al., 2014). In contrast, LPS-mediated activation of microglia, where RGS10 is highly enriched in the central nervous system (Waugh et al., 2005), suppresses RGS10 expression by histone deacetylation with no significant effect in DNA methylation (Alqinyah et al., 2017). The suppression of RGS10 expression following microglia activation involving HDAC recruitment to the RGS10 promoter is blocked by Trichostatin A (TSA),

a pharmacological inhibitor of HDAC enzyme activity (Alqinyah et al., 2017). Collectively, these studies provide evidence that RGS10 is regulated in a cell type-specific manner in response to distinct epigenetic mechanisms.

The previous studies have demonstrated a dynamic regulation of RGS10 expression by epigenetic mechanisms, suggesting that modified RGS10 could be used as a biomarker. Wen *et al*. (2015) use a method called Methylated CpG Tandems Amplification and Sequencing (MCTA-Seq) to detect DNA methylation in freely circulating DNA in the blood of hepatocellular carcinoma (HCC) patients and their control subjects. Strikingly, the study finds that RGS10 is among only four biomarkers that have strongly hypermethylated CpG islands in the blood of hepatocellular carcinoma patients compared to healthy individuals. Therefore, hypermethylated RGS10 may potentially aid in detecting the early stage of hepatocellular carcinoma (Wen et al., 2015).

In addition to chromatin-modifying mechanisms regulating RGS10 expression, miRNAs, which are short non-coding RNAs, control the expression of genes via binding and degrading their transcripts and thus subsequently resulting in repression in protein translation (Nelson et al., 2003). They have been implicated in the pathogenesis of numerous diseases through the dysregulation of their target genes including RGS genes (Alqinyah and Hooks, 2018). A microarray study in multidrug-resistant (MDR) laryngeal cancer followed by miRNA Target Prediction Analysis has revealed that RGS10 is a putative target of has-miR-93 (Yin et al., 2013). Although RGS10 is suppressed in various types of chemoresistant ovarian cancer, its expression is upregulated and inversely correlated with down-regulation of has-miR-93 expression in MDR laryngeal cancer Hep-2/v resistant cells compared to chemosensitive Hep-2 cells (Yin et al., 2013). Taken together, these findings suggest that dysregulation of RGS10 expression could contribute to an acquired chemoresistant phenotype depending on the cancer cell type and its regulation by epigenetic mechanisms.

Post-translational modifications

Several RGS proteins are subjected to a wide range of post-translational modifications (PTM), which have profound impacts on their stability, GAP activity, interaction with binding partners, and subcellular localization (Kach et al., 2012, Alqinyah and Hooks, 2018). Despite the lack of multiple defined regulatory domains found in RGS12 and RGS14, RGS10 has various defined regulatory PTM sites, including phosphorylation, palmitoylation and ubiquitination (**Figure 2.3C**).

Phosphorylation of RGS10 on serine 168 at its C terminus by cAMP-dependent PKA triggers RGS10 localization from the cytosol to the nucleus. It thus results in attenuation of RGS10 availability at the plasma membrane needed to regulate G-protein-dependent activation of the G-protein-coupled inwardly-rectifying potassium (GIRK) channels without affecting its GTPase activity against the Gα protein (Burgon et al., 2001). Furthermore, a proteomic approach has identified RGS10 phosphorylation in human platelets following thrombin receptor activating peptide (TRAP) stimulation (Garcia et al., 2004). However, the site of RGS10 phosphorylation and the kinase activated by TRAP was not determined. The effects of RGS10 phosphorylation on its GAP activity and platelet activation are also unclear.

Unlike other RGS subfamily members that have the N-terminal amphipathic helix or cysteine string to localize to the cell membrane (Bernstein et al., 2000), RGS10 targets the plasma membrane by palmitoylation, which is a reversible reaction involving the attachment of a 16-carbon fatty acids palmitate to cysteine residues via a thioester linkage (Jones, 2004). RGS10 is palmitoylated on a conserved cysteine 66 residue buried inside helix 4 within the RGS domain, which influences its GAP activity based on the assay used. Palmitoylation of this residue inhibits RGS10 GTPase activity in the single turnover-GTP hydrolysis assay in detergent solution but substantially potentiates steady-state GAP activity in receptor/G-protein reconstituted proteoliposomes (Tu et al., 1997, Tu et al., 1999, Tu et al., 2001). Consistent with the previous positive impact of palmitoylation on RGS10 GAP activity in *Spodoptera frugiperda* (Sf9) insect

cells, Castro-Fernandez *et al.* (2002) show constitutive palmitoylation of RGS10 on a conserved cysteine 60 in mammalian GH3 cells stably expressing the GnRH receptor (GGH3). This constitutive palmitoylation results in the inhibition of GnRH agonist-induced inositol phosphate (I.P.) and cAMP production, while the elimination of the palmitoylation effect by substitution of cysteine 60 by alanine abolishes RGS10 GAP activity on GnRH signaling (Castro-Fernandez et al., 2002).

In addition to phosphorylation and palmitoylation, a recent study using quantitative proteomics has identified RGS10 as a substrate for ubiquitination by the E3 ubiquitin ligase tripartite motif protein 32 (TRIM32). Degraded RGS10 is detected in lateral/medial ganglionic eminence (L/MGE) progenitors and results in promoting Rheb-GTP/mTOR hyperactivity that is required for GABAergic interneuron generation via enhancing L/MGE proliferation. On the other hand, the accumulation of RGS10 in response to TRIM32 deficiency or the MG-132 proteasome inhibitor inhibits mTOR signaling and thus causes L/MGE autophagy, a feature of autism-like behaviors (Zhu et al., 2019). Since RGS10 contains undefined functional domains outside of its RGS domain, it will be interesting to determine if the RGS domain mediates the TRIM32-RGS10 interaction and whether TRIM32 also interacts with other RGS proteins.

In addition to the defined and functional regulatory modification sites mentioned above, Squires *et al.* (2018) have identified and reported a list of PTM sites found on human RGS10 (highlighted in **Figure 2.3C**), including K53(39) and K148(134) (Ubiquitination), K78(64) (Acetylation), and S24(10), S27(13), Y94(80), Y143(129), and Y179(165) (Phosphorylation). The majority of these regulatory sites are found within the canonical RGS domain, and half of these sites overlap with reported human variants in RGS10 (Squires et al., 2018). Therefore, further studies are needed to characterize the functional roles of these sites in regulating RGS10 GAP activity, subcellular localization, protein stability, and their implications in disease models.

RGS10 functions in physiology and pathological diseases

Given its broad expression in diverse cells and tissues, RGS10 has emerged as an essential regulator involving cellular processes, physiological responses, and pathological conditions. This section reviews and discusses the results from biochemical, cellular, and *in vivo* studies that have revealed the physiological roles of RGS10 and its implications in pathological states. The overview of phenotypes and disease links arising from loss of RGS10 expression is present in **Table 2.3**.

Neuroinflammation

RGS10 is normally expressed at high levels in the central nervous system, with particular enrichment in microglia (Waugh et al., 2005, Butovsky et al., 2014). Microglia are the brain's resident macrophages, which generally engulf pathogens and cell debris. However, chronic microglia activation leads to an amplified release of proinflammatory and neurotoxic mediators that eventually enhance inflammation-induced neurotoxicity. Injured neurons, in turn, produce danger signals that ultimately augment inflammatory microglial responses. This interplay between microglia and neurons results in an uncontrolled and persistent neuroinflammation, which is known to drive the progression of neurodegenerative pathogeneses, such as Alzheimer's disease and PD (Tansey and Goldberg, 2010, Subhramanyam et al., 2019). Microglia are mainly activated in response to bacterial LPS, tumor necrosis factor α (TNF-α), neurotoxins, extracellular ATP and 6-OHDA.

A polymorphism in the *Rgs10* gene has been associated with age-related maculopathy (Jakobsdottir et al., 2005) and schizophrenia (Hishimoto et al., 2004). Interestingly, activation of microglia by LPS or TNF-α triggers suppression of RGS10, which could directly amplify microglial inflammatory responses mediating neurotoxicity. Furthermore, RGS10-deficient mice exhibit enhanced CNS microglia burden, and activation. Interestingly, these mice are hypersensitive to systemic exposure of LPS-mediated substantia nigra pars compacta (SNpc) DA neuron

degeneration, a phenotype of PD (Lee et al., 2008). Activated primary microglia isolated from RGS10-deficient mice produce higher levels of inflammatory mediators, which consequently elevate media toxicity toward MN9D (mesencephalon DA neuroblastoma) cells, compared to WT mice (Lee et al., 2008, Lee et al., 2011). Similarly, down-regulation of RGS10 via siRNA in BV2 mouse microglia cells upregulates inflammation-related gene expression following LPS activation of TLR4, including TNF-α and IL-1β. Also, knockdown of RGS10 enhances MN9D cell death in response to activated BV2 cultural media incubation, which could be prevented by etanercept-mediated neutralization of TNF-α neurotoxic effects (Lee et al., 2008). In primary microglia exposed to TNF-α or LPS, RG10 deficiency leads to enhanced expression and transcriptional activity of NF-κB, a major transcription factor mediating inflammatory signaling. Reciprocally, re-expression of RGS10 in RGS10-deficient primary microglia suppresses microglia activation, NF-κB activity, proinflammatory cytokines release, and inflammatory neurotoxicity. In addition, virus-mediated gene delivery of RGS10 into SNpc of rats significantly decreases 6-OHDA neurotoxin-induced microglia activation and DA neuron degeneration (Lee et al., 2011). Consistent with these data, knockdown of RGS10 in BV2 cells enhances LPS-induced cyclooxygenase-2 (COX-2) expression and the release of its downstream effector, PGE2. Interestingly, amplification of LPS-stimulated TNF-α and COX-2 following microglial RGS10 suppression does not require Gαi activity (Alqinyah et al., 2018). Despite the importance of these results, the molecular mechanism underlying RGS10 regulation of microglial inflammatory signaling has not been clearly defined.

In addition to its higher microglial expression, RGS10 is also high and detectable within the nuclei of neurons (Waugh et al., 2005). MN9D cells transfected with direct siRNA-targeting RGS10 are more sensitive to the toxic effects of activated BV2 cultural media (Lee et al., 2008). Due to the direct role of RGS10 in promoting DA neuron survival and regulation of microglial inflammatory responses, specifically TNF-α, and the fact that TNF-α/TNFR1 signaling is involved in DA neurons loss, the prosurvival function of RGS10 in DA neurons against TNF-α-induced cytotoxicity has been addressed. Treatment of MN9D cells with TNF-α suppresses RGS10 protein

expression. As discussed, RGS10 subcellular localization in the above sections, phosphorylation of RGS10 at serine 168 triggers its translocation to the nucleus. The overexpression of WT RGS10, but not of RGS10 S168A mutant, which is resistant to PKA phosphorylation, reduces the neurotoxic effects of TNF-α in MN9D cells, through the inhibition of PARP-1 and caspase 3 cleavages and potentiation of PKA/CREB-mediated Bcl-2 anti-apoptotic expression. Consistent with these data, blocking PKA-induced RGS10 phosphorylation and the subsequent nuclear translocation limit the protective effect of RGS10 against TNF-α toxicity (Lee;Chung; et al., 2012). Together, these results suggest that phosphorylation of RGS10 by PKA is required for RGS10's neuroprotective effect against cytotoxicity induced by TNF-α. In the context of aging, as a major risk factor for neurodegenerative diseases, RGS10 expression is decreased within $Iba1^{+}$ cells in the brains of aged mice, which possibly causes changes in inflammatory responses or age-related cellular functions that could predispose to neurodegeneration (Kannarkat et al., 2015). Collectively, these findings provide evidence of the anti-inflammatory and neuroprotective roles of RGS10 in the CNS.

Immune system

Various types of leukocytes express RGS10. Within the immune system, RGS10 is strongly expressed in monocytes/macrophages (Lee et al., 2013), T lymphocytes (Garcia-Bernal et al., 2011), dendritic cells (Shi et al., 2004), and mast cells (Bansal;DiVietro; et al., 2008), with relatively low expression in B lymphocytes (Moratz;Harrison; et al., 2004) and neutrophils (Cho et al., 2012).

In peripheral macrophages, RGS10 is the most highly expressed RGS protein and has a central role in macrophage polarization. RGS10 suppresses classical M1 activation through the downregulation of NF-κB activation and inflammatory cytokines release, and promotes markers of the alternatively activated M2 phenotype (Lee et al., 2013). RGS10 also modulates NLRP3 and NLRC4 inflammasome activity in activated BMDM (Vural et al., 2019). Interestingly, long-term,

but not short-term, Gαi inhibition by PTX treatment during macrophage growth and differentiation suppresses LPS-induced M1 markers and upregulates IL-4-induced M2 markers (Vural et al., 2019). Thus, it will be interesting to test whether amplification of inflammatory M1-related genes seen in RGS10-deficient, activated BMDMs is affected by long-term PTX treatment. Therefore, RGS10 acts as an anti-inflammatory protein and a critical modulator in the regulation of peripheral macrophage activation and differentiation.

In addition to macrophages, high expression of RGS10 has been detected in human monocyte-derived dendritic cells and murine BM-derived dendritic cells (Shi et al., 2004). Although RGS10 expression is downregulated following LPS treatment in macrophages (Lee et al., 2013), LPS-mediated dendritic cell activation does not affect RGS10 expression (Shi et al., 2004). Dendritic cells are professional antigen-presenting cells, mainly inducing T cell activation. Similar to dendritic cells of wild-type mice, antigen presentation and induction of $CD4^+$ T cell proliferation are also observed in dendritic cells derived from RGS10-deficient mice (Lee et al., 2016).

A previous study has shown that RGS10 is found in human and mouse T cells, where its expression is upregulated in response to the activation of the chemokine receptor CXCR4 (Garcia-Bernal et al., 2011). Functionally, loss of RGS10 enhances chemokine-induced Vav1–Rac1 activation mediating α4β1 and αLβ1 integrin-dependent T cell adhesion, while overexpression of RGS10 reduces this adhesion response triggered by chemokine treatment. In addition, the silencing of RGS10 amplifies Cdc42 activation mediating T cell migration following chemokine stimulation (Garcia-Bernal et al., 2011). Collectively, these findings demonstrate that RGS10 is an efficient regulator that desensitizes signaling pathways controlling cellular immune responses.

Cardiac hypertrophy

Numerous recent studies have emerged novel functions of RGS proteins in cardiovascular physiology and pathology (Zhang and Mende, 2011). Among various RGS proteins, RGS10 is expressed in normal human and mouse hearts, whereas RGS10 protein is significantly expressed at lower levels in failing human hearts and hypertrophic murine hearts. In addition, RGS10 protein expression is downregulated following Angiotensin II (AngII) treatment in neonatal rat cardiomyocytes (Miao et al., 2016). However, the precise mechanism for the downregulation of RGS10 remains undefined.

Furthermore, the biological function of RGS10 in the heart has been proposed in the murine model of cardiac hypertrophy. Aortic banding mediated-pressure overload in RGS10-deficient mice displays cardiac hypertrophy, while overexpression of RGS10 alleviates this cardiac hypertrophy response (Miao et al., 2016). Moreover, in RGS10-deficient neonatal cardiomyocytes, AngII induces hypertrophy, potentially through amplification of the MEK/ERK signaling pathway (Miao et al., 2016).

In addition, RGS10 has been reported to mediate the effect of β-adrenergic activation on endogenous G-protein-gated (GIRK) current deactivation in rat atrial myocytes (Bender et al., 2008). Together, these findings strongly suggest that RGS10 is a cardioprotective protein through inhibition of the signaling downstream of AngII receptor, while its suppression may contribute to cardiac remodeling pathogenesis.

Osteoclastogenesis

Substantial evidence has revealed critical roles of RGS proteins in modulating functions of osteoclasts, bone-resorbing cells that tightly regulate and contribute to the process of bone remodeling and related pathological diseases (Jules et al., 2015). RGS10, like other RGS proteins, is highly expressed in human osteoclasts and murine osteoclast like-cells, whereas RGS10 expression is induced in osteoclasts precursors by RANKL stimulation (Yang et al., 2007,

Yang and Li, 2007), a control step required for differentiation of osteoclasts precursors into mature osteoclasts.

More importantly, the silencing of RGS10 in preosteoclasts or BMMs treated with RANKL impairs osteoclast differentiation through blocking intracellular Ca^{2+} oscillation and loss of expression of NFATc1, a master switch transcription factor in the regulation of terminal differentiation of osteoclasts (Yang et al., 2007, Yang and Li, 2007). In contrast, overexpression of RGS10 results in enhanced RANKL sensitivity leading osteoclastogenesis by regulating Ca^{2+} oscillation-NFATc1 signaling pathway. Consistent with its role in modulating osteoclast cell differentiation, RGS10-deficient mice exhibit severe osteopetrosis and osteoclast differentiation defects (Yang and Li, 2007). Interestingly, the reintroduction of either RGS10 or NFATc1 in BMMs derived from RGS10-deficient mice rescues osteoclastogenesis defects. Further elucidation of the mechanism reveals that RGS10 interacts in a competitive fashion with calmodulin (CaM) and phosphatidylinositol 3,4,5-triphosphate (PIP3) in a calcium-dependent manner leading to PLCγ activation and Ca^{2+} oscillation, which subsequently activate NFATc1 signaling required for osteoclast terminal differentiation (Yang et al., 2007, Yang and Li, 2007). Therefore, RGS10 is a crucial factor of osteoclastogenesis through potential regulation of the crosstalk between G-protein signaling and RANKL-dependent signaling pathway.

Moreover, the inhibition of RGS10 mediated by adeno-associated virus (AAV) reduces osteoclast markers and regulates the immune response in a murine model of periodontal disease, which is associated with bone loss (Yang et al., 2013). In addition to its role in osteoclasts differentiation, RGS10 is expressed in chondrocytes, the resident cells of the cartilage. Similar to RGS5 and RGS7, RGS10 promotes chondrocyte differentiation (Appleton et al., 2006), suggesting that RGS10 is essential for chondrogenesis involved in the normal function and development of cartilage.

Platelet functions

Many GPCR agonists, such as thrombin, adenosine diphosphate (ADP), and thromboxane A2 (TxA2), regulate platelet activation, which is essential for physiological hemostasis or thrombosis (Offermanns, 2006). In addition to RGS18, RGS10 is predominately expressed in quiescent platelets (Kim et al., 2006) and modulates platelet activation via binding to the spinophilin (SPL) scaffolding protein forming a complex with the SHP-1 protein tyrosine phosphatase (Ma et al., 2012). Following exposure to platelet activators, SHP-1 is phosphorylated, which subsequently triggers the dephosphorylation of SPL and, in turn, the decay of this complex (Ma et al., 2012). Dissociation of the complex increases free RGS10 levels and its availability to limit G-protein signaling and allows SPL to interact with PP1 phosphatase and inhibits its activity (Ma et al., 2015), both of which regulate platelet activation. In addition to SPL/RGS10/SHP-1 complex, RGS10 interacts with 14-3-3γ scaffolding protein in resting platelets, whereas agonists of platelets induce RGS10 release from 14-3-3γ binding (Ma et al., 2018).

Furthermore, although RGS10 is a selective GAP for Gi family Gα subunits, platelets isolated from mice lacking RGS10 are hyperresponsive to stimuli that act via receptors coupled to Gi, Gq, and G12/13 proteins (Hensch et al., 2016, Ma et al., 2018). In response to these stimuli, RGS10-deficient platelets exhibit aggregation, α-granule secretion, and integrin activation and enhance hemostasis and thrombogenesis (Hensch et al., 2016, Ma et al., 2018), without affecting platelet count and survival (DeHelian et al., 2020). Along with more thrombosis formation, RGS10-deficient mice are more susceptible to ischemia (Hensch et al., 2016). Interestingly, RGS10 expression is higher in platelets of patients with aspirin resistance and metabolic syndrome compared to aspirin-sensitive patients (Mao et al., 2014), suggesting an indirect role for RGS10 in regulating platelet functions. Collectively, these studies indicate that RGS10 serves as a molecular brake on excessive activation of platelets and the process of pathological thrombosis.

Cancers

Given the enormous implications of GPCRs and cognate G-protein signaling in cancer initiation and progression (Chaudhury et al., 2018), emerging evidences suggest a direct contribution of RGS proteins in tumor evolution, where dysregulation of RGS protein expression is observed and linked to many types of cancers (Hurst and Hooks, 2009). RGS10 has been studied in ovarian cancer cells, where its expression is lower than normal ovary cells (Ali et al., 2013). Interestingly, datasets comparing gene expression between multiple models of chemoresistant ovarian cancer cells and their parental, chemosensitive counterparts have demonstrated that the RGS10 transcript level is suppressed during the development of chemoresistance (Hooks et al., 2010). Further, RGS10 has been identified as a key modulator of ovarian cancer survival and chemoresistance, in which RGS10 knockdown via siRNA leads to enhanced ovarian cell viability and promotes chemotherapeutic drug resistance (Hooks et al., 2010), potentially through activation of Rheb-GTP/mTOR signaling mediated ovarian cancer cell survival. Altman *et al.* (2015) have shown that RGS10 interacts with the monomeric GTP-binding Rheb protein, which is known to bind and activate the mechanistic target of rapamycin complex 1 (mTORC1). Elevated levels of active GTP-bound Rheb are observed in RGS10-deficient cells, which consequently amplify phosphorylation levels of AKT, mTOR, 4EB-BP1, p70S6 kinase, eIF2a, and ribosomal protein S6 (Altman et al., 2015).

A recent study has demonstrated RGS10-mediated regulation of Inflammatory signaling in SKOV-3 ovarian cancer cells, where the expression of TNF-α and COX-2 is robustly enhanced following RGS10 knockdown compared to control cells. Interestingly, the effects of RGS10 suppression on TNF-α and COX-2 expressions are independent of amplified Gαi signaling (Alqinyah et al., 2018). Given the finding that RGS10 modulates survival and chemoresistance of ovarian cancer and regulates inflammatory mediators' expressions that have recently linked to ovarian cancer chemoresistance (Spinella et al., 2004, Mantovani et al., 2008, Maccio and Madeddu, 2012), further work is needed to investigate whether amplification of inflammatory

signaling accounts for the enhanced survival and chemoresistance observed in RGS10-deficient ovarian cancer cells.

In addition to the functional studies of RGS10 in ovarian cancer, there is a couple of correlative studies linking the change in RGS10 expression to poor prognosis of multiple cancers, including laryngeal cancer (Yin et al., 2013), hepatocellular carcinoma (Wen et al., 2015), and pediatric acute myeloid leukemia (Chaudhury et al., 2018). Therefore, these studies suggest that RGS10 may serve as a biomarker for cancer diagnosis and detection.

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating autoimmune disorder affecting the CNS. Even though the exact etiology of this disease remains unknown, several hypotheses and a variety of factors have been proposed, involving the pathological role of autoreactive infiltrating T cells attacking myelin and initiating inflammatory processes mediated by proinflammatory cytokines release, which in turn exacerbate MS disease severity (Filippi et al., 2018). The global lack of RGS10 does not alter the distribution and baseline numbers of immune cells, including B cells, $CD4^+$ and $CD8^+$ T cells, and monocytes in the brain, blood, and lymphoid tissues (Kannarkat et al., 2015). In comparison to WT cells, naïve splenic $CD4^+$ T cells derived from RGS10-deficient mice exhibit intact TCR-induced proliferation, cytokine release, and *in vitro* Th1 or Th17 effector cell differentiation following activation by polarizing stimuli (Lee et al., 2016). In contrast, in experimental autoimmune encephalomyelitis (EAE), a murine model of MS induced by myelin oligodendrocyte glycoprotein peptide fragment 35–55 (MOG35–55) immunization that mimics the immunopathological features of human MS, infiltration of both Th1 and Th17 cells in the CNS of RGS10-deficient mice (C57BL/6 background) is significantly reduced compared to WT counterparts (C57BL/6 background) (Lee et al., 2016).

Further, immunized RGS10-deficient mice have dramatically less clinical EAE symptoms associated with a significant reduction in T cell proliferation and cytokine production in response

to *in vitro* MOG35–55 immunogen reexposure. Injection of *in vitro* differentiated RGS10-deficient Th1 cells (but not Th17 cells) into WT naïve recipient mice results in less EAE clinical phenotype compared to mice receiving *in vitro* Th1 cells from WT mice (Lee et al., 2016). Due to its role in regulating T cell chemotaxis and its high expression in other immune cells, such as macrophages, further studies involving conditional knockout mice should aid in our understanding how RGS10 augments MS. Interestingly, in addition to the RGS1 protein, the transcript level of RGS10 is also higher in peripheral blood mononuclear cells (PBMCs) from MS patients compared to healthy individuals (Kemppinen et al., 2011). Therefore, this finding suggests that RGS10 modulates T cell functions that could contribute to this autoimmune disease.

Metabolic disorders

A high-fat diet (HFD), as one of the highly impactful environmental factors, induces body weight gain and central obesity, with various metabolic abnormalities. The role of RGS10 in metabolic changes related to HFD consumption has been studied in mice, where RGS10 expression is downregulated in the liver and upregulated in adipose tissues of WT mice fed with HFD compared to low-fat diet (LFD)-fed mice (Fang et al., 2019). In comparison to WT, HFD-fed RGS10-deficient mice are more susceptible to the body weight gain associated with larger white adipose and liver tissue masses. More importantly, HFD-fed RGS10-deficient mice exhibit insulin resistance, while glucose intolerance and higher leptin levels are observed in RGS10- deficient mice following either LFD or HFD feeding compared to WT mice (Fang et al., 2019). Furthermore, since RGS10 serves as an anti-inflammatory protein in macrophage activation, RGS10-deficient mice fed with HFD are more susceptible to chronic inflammation, as evidenced by higher M1 inflammatory gene transcripts in the liver and adipose tissues and lower mRNAs of Fizz1 and YM1 anti-inflammatory M2 markers (Fang et al., 2019). Thus, this study sheds light on the importance of RGS10 in managing HFD-induced body weight gain and related metabolic disorders.

Targeting RGS10 expression

Compelling evidence from several studies has shown that suppression of RGS10 in various cell types is linked to the pathogenesis of diverse diseases models, such as PD (Lee et al., 2008), cardiac hypertrophy (Miao et al., 2016), ovarian cancer chemoresistance (Hooks et al., 2010), and osteopetrosis (Yang et al., 2007, Yang and Li, 2007). More importantly, RGS10 expression is silenced in many of these cells, where the impact of RGS10 loss leads to these pathologies. For example, RGS10 expression is suppressed in microglia and neurons by inflammatory stimuli and in cardiomyocytes in response to AngII treatment. Likewise, RGS10 expression is significantly downregulated in multiple chemoresistant ovarian cancer cells compared to chemosensitive ones. As discussed above, both DNA methylation by DNMTs and histone deacetylation by HDACs mediate epigenetic silencing of RGS10 that could consequently contribute to the development of resistance to chemotherapy in ovarian cancer. Pharmacological inhibition of both enzymatic activities of DNMTs by 5-Aza and HDACs by TSA restore RGS10 expression and enhance the sensitivity of chemoresistant ovarian cancer cells to chemotherapy. Indeed, these inhibitors are not selective and have more side effects; thus, more work is needed to elucidate the molecular mechanisms and further protein targets that control RGS10 expression and function. Identification of these molecular mechanisms will provide valuable insight into the function of RGS10 and will facilitate strategies to target RGS10 in various disorders in which its function is implicated. Altogether, stabilizing RGS10 expression could potentially be a useful and promising strategy in the treatment of various ailments.

Conclusions and future perspective

Since its initial discovery, growing knowledge and considerable progress have been achieved in defining the canonical GAP functions, as well as regulatory mechanisms of RGS10 and its potential roles in a number of pathophysiological states. Despite its simple structure, the studies discussed herein suggest that RGS10 is a critical modulator of G-protein and inflammatory

signaling and a wide range of physiological cellular processes, including survival, polarization, adhesion, chemotaxis, and differentiation in multiple types of cells. Together, these findings implicate RGS10 as an attractive and promising therapeutic target for the treatment of pathological conditions in various target cells, in which alteration in its expression and function is involved. Although RGS10 has many functions in these systems, the molecular mechanisms governing its functions have not been fully defined. Therefore, further research should focus on identifying binding partners of RGS10, which will be fundamental in understating both the mechanism underlying RGS10 function and the mechanism controlling RGS10 expression in different disease states. As discussed above, RGS10 expression is suppressed following different signals in several cellular and animal models, which in turn impacts their pathophysiology. Thus, defining regulatory factors in a specific cellular environment will facilitate the development of therapeutic strategies to target RGS10 in all its implicated diseases. In addition, it will help future efforts to design and identify selective compounds that stabilize RGS10 expression using high-throughput screening libraries. Given the high expression of RGS10 in immune cells, particularly macrophages, and its role in the regulation of inflammatory responses in these cells, future studies could explore the role of RGS10 in infectious disease models in which immune and inflammatory responses have a significant influence.

Table 2.1: Subcellular localization of RGS10

Cells	Endogenous /Ectopic expression	Predominant localization under resting condition	Stimulus/ modification	Predominant localization after cellular stimulation	Reference
COS-7	Ectopic	Nucleus	N.D.	N.D.	(Chatterjee and Fisher, 2000)
Neuroglioma (H4)	Endogenous	Nucleus	ND	N.D.	
HEK293	Ectopic	Cytoplasm	Forskolin/ phosphorylation by PKA at (Ser 168)	Nucleus	(Burgon et al., 2001)
PC3-AR	Ectopic	Nucleus	Melatonin or 8-bromo-cGMP	Cytoplasm	(Rimler et al., 2006)
Primary microglia	Endogenous	Cytoplasm=Nucleus	LPS	Nucleus	(Lee et al., 2008)

Table 2.2: Regulation of RGS10 expression

Cell/Tissue	Stimuli/Disease model	mRNA/ Protein	Expression	Reference(s)
BMDM cell	FSL-1 100 nM (2&6 h)	mRNA	Decrease	(Riekenberg et al., 2009)
	LPS 10 ng/ml (3 h)	mRNA		
	LPS 100 ng/ml (48 h)	Protein	Decrease	(Lee et al., 2013)
Primary microglia cell	LPS 10 ng/ml (6 h)	mRNA	Decrease	(Alqinyah et al., 2017)
	LPS 10 ng/ml (24,48&72 h)	Protein	Decrease	(Lee et al., 2008)
BV2 cell	LPS 10 ng/ml (4-72 h)	mRNA	Decrease	(Alqinyah et al., 2017)
	LPS 10 ng/ml (48 h)	Protein		(Lee et al., 2008)
	TNF-α 10 ng/ml (24,48&72 h)	Protein		
	TSA 100,250,500 nM (24 h)	mRNA	Increase	(Alqinyah et al., 2017)
	S1P 10 μM (48 h)	mRNA		
Spinal dorsal horn (L4-L5) tissue	pSNL model of inflammatory neuropathic pain (72 h)	Protein	Decrease	
MN9D cell	TNF-α 10 ng/ml (6&24 h)	mRNA/ Protein	Decrease	(Lee;Chung; et al., 2012)
Phagocytic (MG-dNΦ) microglia cell	Injection of apoptotic neurons (dN) into the cortex and hippocampus of WT mice	Protein	Decrease	(Krasemann et al., 2017)
Chondrocytes	Differentiation (9,12&15 days)	mRNA	Increase	(Appleton et al., 2006)
Hippocampus	Acute electroconvulsive seizures ECS (24 h)	mRNA	Decrease	(Gold et al., 2002)
Ventral tegmental	Amphetamine (AMPH) self-administration	mRNA	Decrease	(Sun;Calipari; et al., 2015)
SKOV-3 cell	Cisplatin 100 μM (48 h)	mRNA	Decrease	(Hooks et al., 2010)
	5-Aza (3,5,7&9 days)	mRNA	Increase	(Ali et al., 2013)
A2780-AD cell	TSA 500 nM (48&36 h)	mRNA	Increase	(Cacan et al., 2014)
	5-Aza 10 μM&20 μM (3,5&7 days)	mRNA		
Caco-2 cell	Black tea polyphenol (Theaflavins TF-2) 50 μM (4-24 h)	mRNA	Increase	(Lu et al., 2008)
Striatum	Reserpine Acute treatment (30 min)	mRNA	Increase	(Geurts et al., 2003)
	Daily reserpine (5 days)		Decrease	
Neonatal rat cardiomyocytes	Angiotensin II 50 μmol/L (24&48 h)	protein	Decrease	(Miao et al., 2016)
Human neural progenitor (hNP)	5-Aza 5 μM (5 days)	mRNA	Increase	(Tuggle et al., 2014)
Molt-4 cell	CXCL-12 (30&60 min)	protein	Increase	(Garcia-Bernal et al., 2011)
Osteoclast derived BMM	RANKL+M-CSF (10 ng/ml) (30 min-96 h)	mRNA	Increase	(Yang and Li, 2007)
		protein		
RAW264.7 cell	RANKL+M-CSF (10 ng/ml) (30 min-96 h)	mRNA	Increase	(Yang et al., 2007)
Paw and spinal cord	Trimethylamine *N*-oxide (TMAO) (24 h)	Protein	Decrease	(Zhang;Zhang; et al., 2019)

Table 2.3: RGS10-associated loss of function phenotypes and disease links

Phenotype(s)	Proposed disease links	Year	Reference(s)
Impaired osteoclasts differentiation	Osteopetrosis	2007	(Yang et al., 2007, Yang and Li, 2007)
Neuroinflammation and neurodegeneration	Parkinson's disease and a possible link to schizophrenia	2008	(Hishimoto et al., 2004, Lee et al., 2008, Tansey and Goldberg, 2010)
Reduced chemotherapy-induced cell death	Ovarian cancer chemoresistance	2010	(Hooks et al., 2010, Altman et al., 2015)
Hypertrophy following pressure overload	Heart failure	2015	(Miao et al., 2016)
Platelets aggregation and thrombogenesis	Aspirin resistance	2016	(Mao et al., 2014, Hensch et al., 2016)
Increased body weight with insulin resistance and glucose intolerance	Obesity and related metabolic syndromes	2019	(Fang et al., 2019)

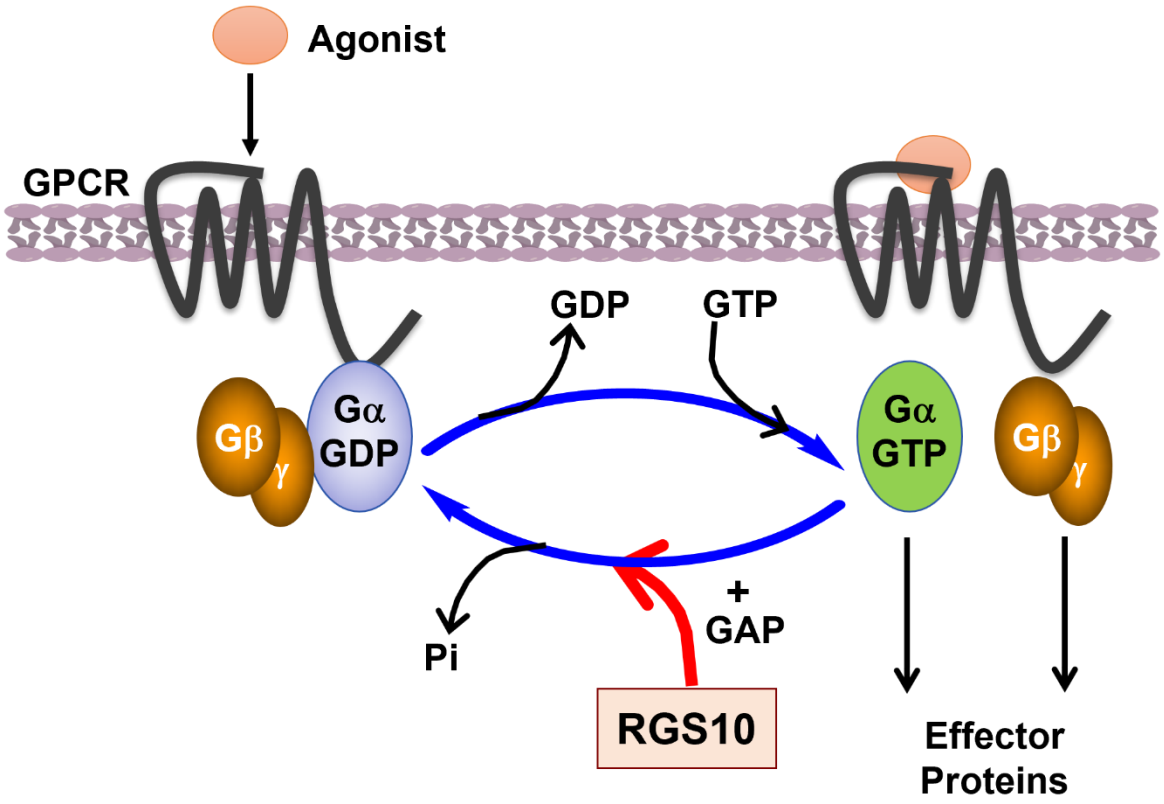

Figure 2.1: G-protein activation and RGS protein-mediated deactivation cycle.

In response to various agonists, the seven transmembrane GPCR undergoes a conformational change and induces the exchange of GDP to GTP on Gα and dissociation from Gβγ dimer, which, in turn, both activated GTP-Gα and Gβγ dimer regulate downstream effectors. This cycle is terminated by the GAP activity of RGS proteins, which accelerates hydrolysis of GTP to GDP and thus terminates GPCR signaling.

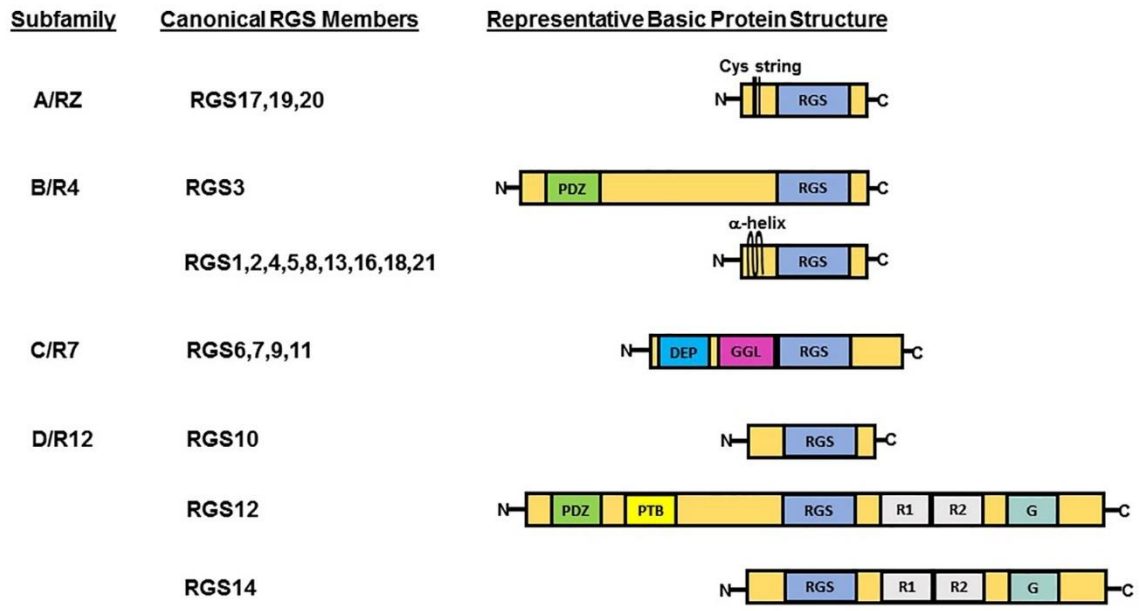

Figure 2.2: Classification and illustration of canonical RGS proteins based on the homology of the RGS domain and the presence of other common structural domains.

Protein domain and motif abbreviations are indicated as following: Cys string, cysteine string; α-helix, an amphipathic alpha–helix; RGS, regulator of G-protein signaling domain; DEP, disheveled EGL10-Pleckstrin homology domain; GGL, G protein gamma subunit-like domain; R1&R2, Ras/Rap-binding domain; G, G protein regulator motif; PTB, phosphotyrosine binding domain; PDZ, PSD95/Dlg/Z0-1/2 domain.

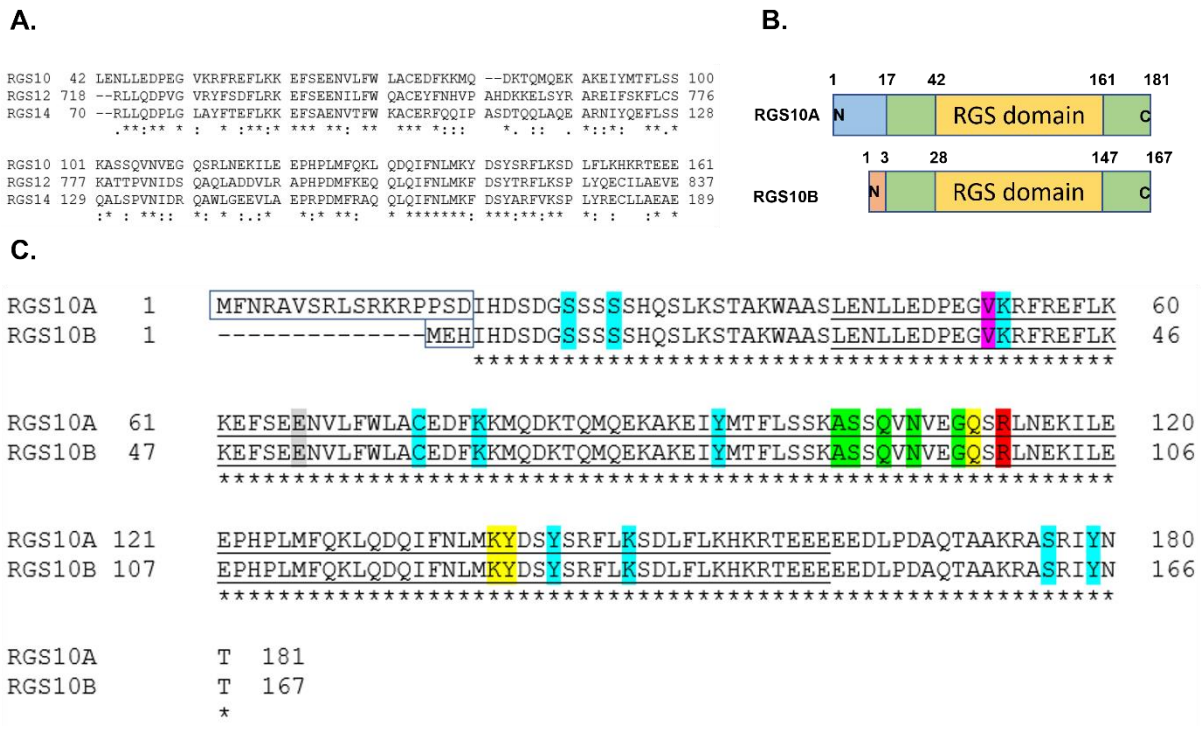

Figure 2.3: RGS10 isoforms: structure and analysis.

(A) Clustal Omega as a multiple sequence alignment program was used to align the RGS domain sequences within the D/R12 subfamily of human RGS proteins. (B) Schematic of the RGS10 isoform structures. (C) Alignment of human RGS10 isoform sequences using Clustal Omega program. Unfilled boxed amino acids represent N-terminal splice variants. Conserved catalytic RGS domains are underlined. The bright green highlighted residues, including (A102(88), S103(89), Q105(91), N107(93), G110(96)) are critical residues of RGS10 for the interaction with Gαi3 according to RGS10: Gαi3-AlF4$^-$ crystal structure (Protein Data Bank: 2IHB). The yellow-highlighted residues are putative disruptor residues, including Q111(97), K139(125), and Y140(126) that are predicted to impair the interaction with the Gα helical domain. The red-highlighted residue R113(99) is a conserved arginine residue within the RGS domain that does not contact the Gαi3 switch III region. The pink highlighted residue is a putative SNP that has been linked to schizophrenia. The gray-highlighted residue is the GAP dead mutation site based on the characterized GAP-dead mutation site in the RGS domain of RGS12 protein. Residue

positions highlighted with turquoise are reported human RGS10 PTMs sites, including phosphorylation residues (S24(10), S27(13), Y94(80), Y143(129), S176(162), Y179(165)), ubiquitination residues (K53(39), K148(134)), palmitoylation C74(60) residue, and acetylation K78(64) residue.

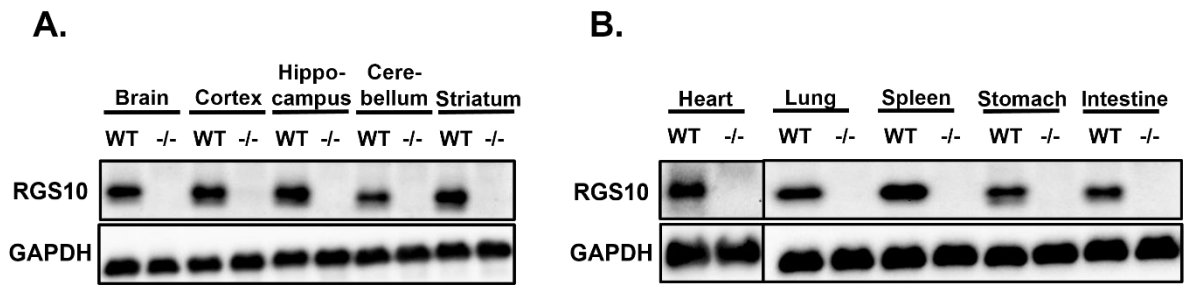

Figure 2.4: RGS10 protein expression in various tissues.

RGS10 protein expression was determined and compared by immunoblot analysis in (A) indicated brain regions, or (B) peripheral tissues (heart, lung, spleen, stomach, and intestine) of WT and RGS10-deficient mice. GAPDH was used as a loading control.

CHAPTER 3

PI3K/ NF-κB-DEPENDENT TNF-A AND HDAC ACTIVITY FACILITATE LPS-INDUCED RGS10 SUPPRESSION IN PULMONARY MACROPHAGES[2]

[2] Almutairi F, Tucker SL, Sarr D, Rada B. *Cell Signal*. 2021 Oct; 86:110099

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Abstract

Regulator of G-protein signaling 10 (RGS10) is a member of the superfamily of RGS proteins that canonically act as GTPase activating proteins (GAPs). RGS proteins accelerate GTP hydrolysis on the G-protein α subunits and result in termination of signaling pathways downstream of G protein-coupled receptors. Beyond its GAP function, RGS10 has emerged as an anti-inflammatory protein by inhibiting LPS-mediated NF-*κ*B activation and expression of inflammatory cytokines, in particular TNF-*α.* Although RGS10 is abundantly expressed in resting macrophages, previous studies have shown that RGS10 expression is suppressed in macrophages following Toll-like receptor 4 (TLR4) activation by LPS. However, the molecular mechanism by which LPS induces *Rgs10* silencing has not been clearly defined. The goal of the current study was to determine whether LPS silences *Rgs10* expression through *an* NF-*κ*B-mediated proinflammatory mechanism in pulmonary macrophages, a unique type of innate immune cells. We demonstrate that *Rgs10* transcript and RGS10 protein levels are suppressed upon LPS treatment in the murine MH-S alveolar macrophage cell line*.* We show that pharmacological inhibition of PI3K/ NF-κB/p300 (NF-κB co-activator*)/*TNF-α signaling cascade and the activities of HDAC (1-3) enzymes block LPS-induced silencing of *Rgs10* in MH-S cells as well as microglial BV2 cells and BMDMs*.* Further, loss of RGS10 generated by using CRISPR/Cas9 amplifies NF-κB phosphorylation and inflammatory gene expression following LPS treatment in MH-S cells. Together, our findings strongly provide critical insight into the molecular mechanism underlying RGS10 suppression by LPS in pulmonary macrophages*.*

Highlights

- RGS10 expression is suppressed in response to LPS-stimulated pulmonary macrophages.
- Pharmacological inhibition of inflammatory responses involving PI3K and NF-κB-dependent TNF-α stabilizes RGS10 expression following TLR4 activation by LPS.
- Blocking histone deacetylase activity restores RGS10 expression upon LPS stimulation.
- Loss of RGS10 amplifies LPS-induced NF-κB activation and pro-inflammatory gene expression in pulmonary macrophages

Introduction

Tissue-resident macrophages (TRMs) emerge from embryonic precursors that exert vital and specific functions in tissue homeostasis, inflammation, and regeneration (Ginhoux and Guilliams, 2016, Mosser et al., 2021). Among TRMs, alveolar macrophages (AMs) are the specialized cells that reside in the pulmonary alveoli and the dominant innate immune cells, as they represent 90-95% of the cellular numbers under normal conditions, making them the natural sentinels of the respiratory system (Hussell and Bell, 2014, Kopf et al., 2015). Like other TRMs, AMs are involved in maintaining lung homeostasis by phagocytosing apoptotic cells and cells debris resulting from lung infection or epithelial injury while also maintaining a dominant immunosuppressive phenotype (Rubins, 2003, Westphalen et al., 2014). In particular, AMs have a central role in clearing the alveolar environment from an excessive production of lipid-rich molecules (surfactants) that are produced by type II alveolar epithelial cells and functionally prevent alveolar collapse during exhalation (Whitsett et al., 2015). Despite these physiological functions, dysregulation of AM activation or their impaired clearance function are associated with initiation and progression of several respiratory pathologies, such as acute lung injury (ALI), pulmonary alveolar proteinosis (Trapnell et al., 2003), and chronic obstructive pulmonary disease (Vlahos and Bozinovski, 2014).

Regulator of G-protein signaling (RGS) proteins are a large family of proteins containing RGS domain that binds and deactivates heterotrimeric G-protein subunits (Watson et al., 1996, Hollinger and Hepler, 2002). Canonically, RGS proteins terminate signaling pathways downstream of G protein-coupled receptors (GPCRs) by acting as GTPase activating proteins (GAPs) on active form, GTP-bound Gα-subunits, through enhancing their intrinsic GTPase activity for GTP hydrolysis and returning G-proteins to their inactive form, GDP-bound Gα-subunits (Gilman, 1987). Due to enormous implications of GPCRs and G-protein signaling in diverse systems, RGS proteins have emerged to play a wide range of roles in regulation of physiological processes and pathologies (Squires et al., 2018).

Among RGS proteins, RGS10, a member of D/R12 subfamily of RGS proteins, is a small RGS protein that lacks structural domains and functional motifs outside of RGS domain. RGS10 has been shown to be selective to interact with and inactivate Gαi family of G-proteins via its classical GAP (Hunt et al., 1996, Popov et al., 1997, Masuho et al., 2020), and has a high enrichment in the brain (Waugh et al., 2005, Butovsky et al., 2014) and peripheral macrophages (Lee et al., 2013). Beyond its GAP function, RGS10 has an anti-inflammatory role, as loss of RGS10 in microglia and macrophages amplifies NF-κB transcriptional activity and the generation of, pro-inflammatory mediators, such as TNF-α, interleukins, and COX-2-mediated prostaglandin E2 (PGE2) upon Toll-like receptor 4 (TLR4) activation (Lee et al., 2008, Lee et al., 2011, Alqinyah et al., 2018). Further, following macrophage activation, RGS10 acts as a key regulator of macrophage polarization by suppressing classical M1 activation and promoting alternative M2 activation, a phenotype also similar to that of AMs (Lee et al., 2013).

Induction of the classically activated macrophages (pro-inflammatory M1 phenotype) is triggered by pathogen-associated molecular patterns (i.e. bacterial lipopolysaccharide (LPS)) recognized by pattern recognition receptors (i.e. TLR4). This, in turn, initiates activation of certain inflammatory events involving kinase activity of PI3K, NF-κB and MAPK signaling pathways, ultimately leading to enhance inflammatory genes expression, including TNF-α. More importantly, previous studies have implicated the direct action of TLR4 stimulation by LPS in regulating RGS10 expression. First, Lee et al. showed early (Lee et al., 2008, Lee et al., 2013) that RGS10 protein is naturally expressed at high levels in resting microglia and macrophages, but its expression is suppressed following LPS treatment in microglia and macrophages. Alqinyah et al. (Alqinyah et al., 2017) subsequently demonstrated that the inhibition in the level of RGS10 protein in response to LPS in microglia is a result of LPS-induced *Rgs10* transcript silencing. Although these studies highlight the effect of activated microglia and macrophages on RGS10 expression, the mechanistic basis for this effect have not defined yet.

In this study, we aimed to determine inflammatory responses that are involved in LPS-mediated *Rgs10* suppression in macrophages. First, we observe a reduction in the level of RGS10 expression in the MH-S alveolar macrophage cell line upon LPS stimulation, similar to what have been reported in activated microglia and bone marrow derived macrophages (BMDMs) (Lee et al., 2008, Lee et al., 2013, Alqinyah et al., 2017). Furthermore, we show for the first time that the pharmacological inhibition of PI3K activity, NF-κB-dependent TNF-α secretion, and histone deacetylase (HDAC) class I activity reverse suppression of *Rgs10* expression upon LPS stimulation in MH-S cells, as well as BV2 microglial cells and BMDMs, suggesting that these inflammatory mediators are required for the suppressive effect of LPS on RGS10 expression. Finally, consistent with its anti-inflammatory role in microglia and BMDMs, we demonstrate that loss of RGS10 in MH-S cells significantly enhances LPS-induced upregulation of NF-κB phosphorylation and inflammatory genes expression. The findings presented here provide novel insights into the molecular basis and the pharmacological approaches that regulate Rgs10 expression in activated macrophages.

Materials and Methods

Cells

MH-S mouse alveolar macrophage cell line, which is used as a model to study alveolar macrophage functions (Mbawuike and Herscowitz, 1989), was purchased from the American Type Culture Collection (ATCC® CRL-2019™). The mouse microglial BV2 cell line, which is extensively used to study microglia functions (Henn et al., 2009), was derived from primary microglial cell cultures infected with v-raf/v-myc oncogene-carrying retrovirus (J2) (Blasi et al., 1990). L-929 mouse fibroblast cell line was generously provided by Dr. Biao He lab at University of Georgia (Athens, GA). MH-S, BV2, and L-929 cells were grown in Dulbecco's modified Eagle's medium (Millipore Sigma. D6429) supplemented with 10% low-endotoxin fetal bovine serum (Thermo Fisher Scientific, cat#: 10082147) and an antibiotics combination of (1%

penicillin/streptomycin) (Thermo Fisher Scientific, cat#: 15140122) and incubated at 37 °C in a humidified atmosphere 5% CO2.

Reagents

Lipopolysaccharide (LPS) (from E. coli O111:B4 strain, cat#: L2630) was obtained from Millipore Sigma. Recombinant mouse TNF-alpha (aa 80-235) protein (410-MT) was purchased from R&D systems. Puromycin dihydrochloride (cat#: 4089) was purchased from Tocris Bioscience. LY294002 (cat#: 70920), BAY 11-7082 (cat#: 10010266), A-485 (cat#: 24119), R-7050 (cat#: 16870), PD 98059 (cat#: 10006726), SB 239063 (cat#: 19142), SP 600125 (cat#: 10010466), Trichostatin A (TSA) (cat#: 89730), JSH-23 (cat#: 15036), wortmannin (cat#: 10010591), and 5-Azacytidine (5-Aza) (cat#: 11164) were obtained from Cayman Chemical.

Isolation and culture of BMDMs

Bone marrow was isolated from tibiae and femurs of mice by flushing with 1X ice-cold PBS supplemented with 2% low-endotoxin fetal bovine serum (FBS) (Thermo Fisher Scientific, cat#: 10082147) using a 10-ml syringe and 25-gauge needle followed by centrifugation at 450 x g for 10 min at 4 °C. The cells were resuspended in 1X ice-cold PBS supplemented with 2% low-endotoxin FBS, passed through a 70 µm cell strainer to remove solid fragments and then centrifuged again at 450 x g for 10 min at 4 °C. The cell pellet was then resuspended in ammonium-chloride-potassium (ACK) lysing buffer (Thermo Fisher Scientific, cat#: A1049201) for 30 sec to lyse red blood cells. Cells were then washed in 1X ice-cold PBS (20 ml) and centrifuged again at 450 g for 10 min at 4 °C. The cell pellet was disaggregated in ice-cold complete DMEM (containing 10% low-endotoxin FBS and 1% penicillin/streptomycin) for cell counting. Differentiated macrophages were obtained by culturing 6 X 10^6 bone marrow cells on sterile petri dishes in 10 ml of complete DMDM supplemented with 20% of conditioned L-929 cell media as a source of M-CSF (conditioned media collected from confluent L-929 cells grown for

12 days) for three days at 37 °C and 5% CO2. On day 3, 5 ml of complete DMEM and supplemented with 20% of conditioned L-929 cell media were added, and the cells were grown for four additional days. On day 7, the medium of cells was removed, and the adherent cells were washed with 1X PBS and then harvested by incubating in 10 ml of cell dissociation buffer (CORNING, cat#: 25-056-CI) for 15 min at 37 °C. Plates were washed with 1X PBS to collect dislodged cells, and cells were centrifuged at 250 g for 5 min. Cell pellet was resuspended in complete DEME medium for cell counting.

Generation of CRISPR/Cas9 control and RGS10 deficient MH-S cells

Scrambled control vector (K010) and 20-bp guide RNA sequence (49 TGTTTTGCAGATATCCATGA) targeting mouse RGS10 in one-lentivector (cat#: K4107305) were purchased from ABM Inc. (Richmond, BC, Canada). 10ng of vectors were transformed into Proclone competent DH5 alpha cells and the transformants were incubated in 2 ml of LB with 100 µg/ml of carbenicillin overnight at 37 °C. The following day, glycerol stocks were prepared by resuspended 500 µl of bacterial culture in 500 µl of 50% glycerol and stored in 1.5 ml freezing tube at -80 °C. Furthermore, one ml of bacterial culture was transformed into 250 ml of LB with 100 µg/ml of carbenicillin and allowed to grow for 15 hours at 37 °C. The plasmids were extracted from the bacterial culture using the GeneJET plasmid maxiprep kit according to the manufacturer's instructions. The plasmid concentration and purity were quantified using a Nano Drop spectrophotometer. To produce lentiviral particles, 293T cells plated in two 10 cm^2 dishes transfected with 10µg of expression sgRNA CRISPER/Cas9 vectors and 10 µg of ABM Inc.'s third generation (cat#: LVO53) packaging mix in presence of Lentifectin™ transfection reagent (cat#: G074) to facilitate plasmid uptake by cells following company's lentiviral packaging protocol. 24 hours post-infection, the medium was removed and replaced with fresh complete growth medium for another 24 hours. Viral medium was collected 48 hours post-infection followed by centrifugation at 3,000 RPM for 15 minutes at 4°C. The viral supernatant was separated from cell

debris by using a low-protein binding 0.45 µm sterile filter and then subsequently concentrated by centrifugation at 25,000 RPM for two hours at 4°C (~120,000g; SW28 rotor, Beckman, Brea, C). The viral pellets were resuspended with PBS and stored at -80 °C until cells transfection. Different volumes of lentiviral particles were transfected into MH-S cells in the presence of 0.8 µg/ml of polybrene. 24 hours post-infection, the medium was removed and replaced with fresh complete culture medium. 48 hours following transfection, cells were split and selected for stable expression using 4 µg/ml puromycin selection media as determined by a kill curve assay. Genome-editing was checked using qRT-PCR and western blot analysis.

Small interfering RNA transfection

The small interfering RNA (siRNA) targeting mouse p300 (cat#: sc-29432) and non-targeting control siRNA (cat#: sc-37007) were purchased from Santa Cruz Biotechnology. The siRNA transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, cat#: 13-778-030) according to the manufacturer's recommended protocol. The mouse p300 siRNA used herein is the same as described and validated in (Lee;Lee; et al., 2010, Kadiyala et al., 2012, Jang et al., 2015, Lee et al., 2021). The final concentration of siRNA in an antibiotic-free culture medium was 60 nM. Following transfection, MH-S cells were cultured for an additional 48 hours in an antibiotic-free culture medium before cells harvesting. The efficiency of knockdown was assessed by measuring p300 mRNA using quantitative RT-PCR.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, cat#: 15596018), and cDNA was synthesized from 2µg of total RNA using the High-capacity Reverse Transcriptase cDNA kit (Applied Biosystem, cat#: 4368814). Each cDNA sample was diluted 10-fold, and a 5µl was used in a 14µl PCR reaction (SYBR™ Green PCR Master Mix.) (Thermo Fisher Scientific, cat#: 4309155) containing primers at concentration of 5µM each. All the reactions were run in

triplicates. The mRNA expression levels were normalized to the housekeeping β-actin gene and were calculated using the $2^{-\Delta\Delta CT}$ method. Mouse TNF-α, IL-1β, IL-6, INOS, RGS10 and β-actin primers were obtained from Millipore Sigma. The primer sequences used for gene amplification are listed as follows: TNF-α forward, 5′- CCTGTAGCCCACGTCGTAG-3', TNF-α reverse, 5′-GGGAGTAGACAAGGTACAACCC-3', IL-1β forward, 5′- GAAATGCCACCTTTTGACAGTG-3', IL-1β reverse, 5′-TGGATGCTCTCATCATCAGGACAG-3', IL-6 forward, 5'-CTGCAAGAGACTTCCATCCAG -3', IL-6 reverse, 5'- AGTGGTATAGACAGGTCTGTTGG -3', INOS forward, 5′-TGACGGCAAACATGACTTCAG-3', INOS reverse, 5′-GCCATCGGGCATCTGGTA-3', RGS10 forward, 5'-CCCGGAGAATCTTCTGGAAGACC-3', RGS10 reverse, 5'-CTGCTTCCTGTCCTCCGTTTTC-3', p300 forward, 5'-AGGCAGAGTAGGACAGTGAA-3', p300 reverse, 5'-CTCAGTCTGGGTCACTCAAT-3', β-actin forward, 5′- GGCTGTATTCCCCTCCATCG-3', β-actin reverse, 5′-CCAGTTGGTAACAATGCCATGT-3'.

Western Blot

Cells were washed twice with 1X cold-PBS and then were lysed with RIPA lysis buffer containing (50mM Tris HCl pH 6.8, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, and 1X proteases/phosphatase inhibitors cocktail (Cell Signaling technology, cat#: 5872S). Cell lysates were incubated for 30 minutes on ice followed by centrifugation at 20,000 g for 15 min at 4°C. Proteins concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat#: 23225). The cell lysates were normalized and mixed with an equal volume of 2X SDS-PAGE sample buffer containing (0.5 M Tris HCl pH 6.8, 10% SDS, 20% glycerol, 200 mM β-mercaptoethanol, and 1% bromophenol blue). The lysates were boiled for 10 mins at 95 °C, and 20 µl of protein sample was separated on 12% SDS-PAGE gels followed by transfer to nitrocellulose membranes (Biorad, cat#: 1620115) using standard protocol. Blotted membranes were blocked with 5% nonfat dry milk, shaking at room temperature for one hour, then incubated

overnight with the following primary antibodies: goat anti-RGS10 (diluted 1:1,000, Santa Cruz Biotechnology, cat#: sc-6206), rabbit anti-phospho-NF-*κ*B p65 (diluted 1:1000, Cell signaling technology, cat#: 93H1 #3033), rabbit-anti-NF-*κ*B p65 (diluted 1:1000, Cell Signaling technology, cat#: D14E12 #8242), and mouse anti-β-actin (diluted 1:3000, Santa Cruz Biotechnology, cat#: sc-47778). Following primary antibodies incubation, blotted membranes were washed with 1X-TBST buffer for three times (each time for 10min) and incubated at room temperature for one hour with the following suitable secondary-HRP conjugated antibodies (diluted 1:5,000) mouse anti-goat IgG-HRP (Santa Cruz Biotechnology, cat#: sc-2354), goat anti-mouse IgG HRP (Bethyl Laboratories, cat#: A90-116P), and goat anti-rabbit IgG-HRP (Millipore Sigma, cat#$: 12-348). After washing with 1X-TBS-T buffer three times, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, cat#: 34580) was applied to detect immunoreactivity of HRP. Image lab Software from Bio-Rad was used to quantify western blot bands that were normalized to the endogenous control β -actin.

TNF-α Enzyme-Linked Immunosorbent Assay (ELISA)

Culture supernatants collected from MH-S cells following LPS treatment with or without inhibitors were centrifuged at 250 g for 10 min at 4°C and assayed for TNF-α levels using mouse TNF-α DuoSet ELISA (R&D systems, cat#: DY410) according to the manufacturer's instructions. Briefly, high binding ELISA plate (CORNING, cat#: 9018) was coated with the mouse anti-TNF-α capture antibody overnight and blocked with 1X BSA for 3 hours at room temperature. Mouse TNF-α standards or samples of medium were applied to the plate for 2 hours, and then incubated with detection antibody for 2 hours followed by streptavidin HRP for 20 min at room temperature. Between steps, plate was washed with 0.05% Tween® 20 in PBS. The plate was visualized with TMB substrate (Thermo Fisher Scientific, cat#: 34021), stopped with 2N H_2SO_4, and analyzed on a microplate photometer at 450 nm (EPOCH2, BioTek Instruments, Inc).

Immunofluorescence

MH-S cells were grown overnight on 4-well glass Millicell EZ Slide (Millipore corporation, cat#: PEZGS0416) and then treated with LPS (100 ng/ml) for 60 minutes with or without a one-hour pretreatment with LY-294002 (15 μM). Following the treatment, MH-S cells were fixed with 4% paraformaldehyde for 10 min and washed with PBS. Cells were subsequently permeabilized with 0.1% Triton X100 while blocking in PBS with 5% BSA and 10% normal horse serum for 1 h. Primary antibody staining to detect rabbit-anti-NF-κB p65 was performed at a 1:250 dilution in PBS with 1% BSA, 1% normal horse serum, and 0.3% Triton X100 overnight at 4 ºC. Slides were washed in PBS, and secondary antibody staining was performed at a 1:500 dilution in PBS with 1% BSA, 1% normal horse serum for 1 h with horse anti-rabbit IgG antibody (H+L), DyLight® 488 (Vector Laboratories, cat#: DI-1088-1.5). Slides were washed with PBS and then vectashield™ anti-fade mounting medium with DAPI (Vector Laboratories, cat#: H-1200-10) was applied to the cell's prior coverslip addition. All digital images were acquired at the University of Georgia College of Veterinary Medicine Cytometry Core on a Nikon A1R confocal microscope (Nikon Eclipse Ti-E inverted microscope) and examined with NIS Element software (Nikon, Version 6.4).

Statistical Analysis

All quantified data compiled from three independent repeats, unless otherwise noted, were analyzed for statistical difference between groups using student's t-test or one-way ANOVA followed by Tukey post hoc analysis. Prism software was used to carry out statistical analyses. Data are expressed as mean ± S.E.M. where *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$ indicate the levels of significance.

Results

RGS10 expression is silenced in response to LPS stimulation in MH-S cells

RGS10 protein is expressed at high levels in resting macrophages and microglia, the brain's resident macrophages. Strikingly, RGS10 expression is silenced in microglia and BMDMs following LPS stimulation (Lee et al., 2008, Lee et al., 2013). To test whether RGS10 expression is suppressed in response to LPS in the pulmonary macrophage MH-S cells, we treated MH-S cells with vehicle or LPS (10 ng/ml) to analyze RGS10 gene and protein expression levels by quantitative RT-PCR and western blot analysis, respectively. The transcript level of *Rgs10* was silenced 6 hours or 24 hours post LPS stimulation **(Figure 3.1A)**. Consistent with the diminished *Rgs10* transcript level in activated MH-S cells, LPS treatment also triggered RGS10 protein suppression, in which the maximal effect of LPS-induced RGS10 protein suppression was observed at 48 hours **(Figure 3.1B and Figure S3.1)**. Densitometry confirmed a significant down-regulation of RGS10 protein by LPS **(Figure 3.1C)**. Therefore, polarization of alveolar macrophage to pro-inflammatory M1 phenotype upon LPS treatment negatively regulates RGS10 expression.

PI3K mediates RGS10 silencing in response to TLR-4 activation

A significant body of evidence has linked the activation of PI3K to TLR4 signaling (Cianciulli et al., 2020). To determine whether PI3K activation contributes to RGS10 suppression by LPS, we measured *Rgs10* transcript and RGS10 protein expression following LPS treatment with or without pharmacological inhibition of PI3K kinase activity. MH-S cells were treated with vehicle or the PI3K inhibitor LY294002 (15 μM) for one hour prior to LPS (10 ng/ml) stimulation for 6 hours and 48 hours, for experiments measuring transcript and protein levels, respectively. LY294002 treatment significantly increased the basal level of *Rgs10* mRNA and restored both *Rgs10* transcript expression (6 hours) **(Figure 3.2A)** and RGS10 protein levels (48 hours) upon LPS treatment **(Figure 3.2B)**. Because LPS has been shown previously to suppress *Rgs10*

transcript in microglia and BMDMs, we investigated whether inhibition of PI3K kinase activity blocks LPS-induced *Rgs10* silencing in BV2 microglial cells and BMDMs, as well. Addition of the PI3K kinase inhibitor LY294002 stabilized the transcript level of *Rgs10* upon LPS stimulation in both BV2 **(Figure 3.2C)** and BMDMs **(Figure 3.2D)**. To validate the observed effects of LY294002, we treated MH-S cells with LPS in the presence or absence of another PI3K inhibitor, wortmannin (10 μM), and observed a similar effect, in which wortmannin significantly blocked LPS-stimulated *Rgs10* suppression **(Figure 3.2E)**. Overall, these data indicate that activation of PI3K activity is required for LPS-dependent *Rgs10* silencing.

Blocking NF-κB activation restores RGS10 suppression following LPS stimulation

NF-κB is a central transcription factor that is activated upon LPS exposure and subsequently enhances the expression of inflammatory mediators, contributing to the activation and function of macrophages. Activation of PI3K has been shown to be a crucial modulator of NF-κB activation and nuclear translocation (Dilshara et al., 2014, Xu et al., 2020). To confirm whether PI3K regulates NF-κB(p65) nuclear translocation following LPS stimulation, MH-S were pretreated with LY294002 for one hour followed by LPS treatment to determine nuclear localization of NF-κB(p65) by immunofluorescence. As expected, LPS evoked the nuclear translocation of the p65 subunit of NF-κB, while LY294002 pretreatment inhibited LPS-induced NF-κB(p65) nuclear translocation **(Figure 3.3A)**. To address whether the activation of NF-κB plays a role in mediating inhibitory effects of LPS on RGS10 expression, we used the NF-κB inhibitor, BAY 11-7082. We first validated the inhibitor's effect on NF-κB by measuring phosphorylation of the p65-NF-κB subunit (a cytoplasmic event of NF-κB signal activation) following BAY 11-7082 incubation and subsequent 20 minute-long LPS stimulation. The data indicate that BAY 11-7082-inhibited LPS-enhanced phosphorylation of p65-NF-κB in MH-S cells **(Figure 3.3B)**. To assess whether NF-κB activation is needed for the LPS-induced suppression of RGS10, we next pretreated MH-S cells with the NF-κB inhibitor BAY 11-7082 (20 μM) for one

hour followed by LPS (10 ng/ml) stimulation to measure mRNA (6 hours) or protein (48 hours) levels of RGS10. Our results showed that NF-κB inhibition significantly enhanced the basal expression of RGS10 and abolished LPS-induced RGS10 downregulation **(Figure 3.3C and 3.3D)**. Consistent with its suppressive activity against LPS-mediated *Rgs10* silencing in MH-S cells, a similar effect was observed in microglia **(Figure 3.3E)** and BMDMs **(Figure 3.3F)**, as BAY 11-7082 significantly impaired the LPS-induced decrease of *Rgs10* expression in both cell types. To further confirm the involvement of NF-κB activation in LPS-induced RGS10 silencing, we examined the effect of a second and well-established NF-κB inhibitor, JSH-23 (7 μM). As expected, and demonstrated in **(Figure 3.3G)**, we observed a similar effect of Bay 11-7082, in which JSH-23 completely blunted *Rgs10* suppression in response to LPS in MH-S cells. In addition to NF-κB signaling, LPS activates downstream MAPK signaling pathways, including extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway, and the p38 pathway (Kyriakis and Avruch, 2012). Due to the importance of these pathways in regulating inflammatory responses including NF-κB activity, we examined whether inhibitors specifically targeting each of these pathways would restore decreased RGS10 expression in LPS-stimulated MH-S cells. To test this, we stimulated MH-S cells with LPS in the presence or absence of the following inhibitors (10 μM): PD 98059 (MEK1/2 inhibitor-blocking ERK activity), SB 23906 (p38 inhibitor), and SP 600125 (JNK inhibitor). At the same concentration and time point of LPS treatment described above, the inhibition of the signaling pathways of ERK **(Figure S3.2A)**, p38 **(Figure S3.2B)**, and JNK **(Figure S3.2C)** had no impact on the basal or LPS-suppressed RGS10 expression. Altogether, these findings suggest that LPS activation of TLR4 induces RGS10 suppression in a NF-κB-dependent manner that is independent of MAPK pathways.

The inhibition of p300 abrogates LPS-triggered RGS10 suppression

The p300 and its paralog CREB-binding protein (CBP) co-activators contribute to enhancement of genes transcription through their intrinsic histone acetyltransferase (HAT)

activity. They catalyze acetylation of histone proteins around the promoter of genes, resulting in chromatin relaxation for transcription factors (TFs) access and acetylation of nonhistone proteins, such as TFs for their activation (Dancy and Cole, 2015). Because of the observed involvement of NF-κB activation in LPS-induced RGS10 suppression, we next questioned whether the p300 co-activator required for full NF-kB-dependent transcriptional activity (Vanden Berghe et al., 1999, Chen et al., 2005, Hoberg et al., 2006), promotes LPS-triggered RGS10 suppression. We cultured pulmonary macrophages MH-S cells with or without A-485, a highly selective inhibitor of p-300 co-activator that has been validated to target the p-300 HAT domain (Peng et al., 2019) in multiple cells including macrophages, and treated them with LPS (10 ng/ml) for 6 hours or 48 hours. Stimulation of MH-S cells with LPS resulted in a significant decrease in the mRNA and protein expression levels of RGS10, as expected. However, 1-hour pretreatment with 10 μM A-485 abrogated the downregulation of RGS10 in response to LPS on both the mRNA **(Figure 3.4A)** and the protein levels **(Figure 3.4B)**. Similarly, A-485 treatment impaired LPS-stimulated *Rgs10* silencing in both BV2 **(Figure 3.4C)** and BMDM cells **(Figure 3.4D)**. To expand these results, and to further prove that the suppressive effect of A-485 on LPS-induced RGS10 silencing was due to targeted inhibition of p300 function, we assessed the effect of siRNA mediated knockdown of p300 on LPS-triggered TNF-α amplification and RGS10 suppression. Transient transfection of siRNA targeted at p300 in MH-S cells consistently resulted in 60% reduction of p300 mRNA level **(Figure 3.4E)**. As previously reported (Peng et al., 2019, Lee et al., 2021), p300 knockdown significantly inhibited LPS-induced upregulation of TNF-α mRNA **(Figure 3.4F)**. More importantly, siRNA-mediated p300 knockdown strongly blocked the inhibitory effect of LPS on RGS10 expression **(Figure 3.4G)**. Taken together, in line with facilitating the transcription of NF-κB-dependent inflammatory genes, our data demonstrate the essential function of the NF-κB co-activator p-300 in mediating LPS-sensitive RGS10 expression.

LPS-mediated RGS10 suppression is TNF-α-dependent

LPS is known to lead to the production of pro-inflammatory cytokines in pulmonary macrophages, such as TNF-α that is also known to act via NF-κB activation. To explore whether LPS suppresses RGS10 expression -at least in part- through a TNF-α-mediated autocrine loop, we next quantified TNF-α protein secretion in MH-S cells in response to LPS in the presence of Bay 11-7082 (NF-κB inhibitor) or A-485 (p300 inhibitor) by using ELISA. The results indicate that inhibition of either NF-κB activation or its co-activator p300 blocked LPS-stimulated TNF-α release **(Figure 3.5A)**. To explore whether TNF-α signaling affects LPS-stimulated *Rgs10* suppression, we blocked the tumor necrosis factor receptor (TNFR) by preincubating MH-S cells with R-7050 (TNFR inhibitor) followed by LPS priming. Pretreatment of MH-S cells with R-7050 upregulated endogenous RGS10 levels and fully abrogated RGS10 suppression by LPS **(Figure 3.5B and Figure 3.5C)**. In addition to MH-S cells, we also confirmed these results in BV2 cells **(Figure 3.5D)** and BMDMs **(Figure 3.5E)**, in which the TNFR inhibitor also blocked LPS-induced suppression of *Rgs10*. To further confirm that LPS downregulates RGS10 in MH-S cells through a mechanism involving TNF-α and TNFR, we treated MH-S cells with TNF-α (100 ng/ml) and examined its effect on the transcript and protein levels of RGS10. TNF-α treatment reduced *Rgs10* transcript **(Figure 3.5F)** and RGS10 protein **(Figure 3.5G)** expression levels. Given the ability of NF-κB to regulate RGS10 expression upon TLR4 activation and the fact that TNF-α is also an inducer of NF-κB signaling, we evaluated TNF-α-triggered RGS10 reduction following pharmacological inhibition of NF-κB by Bay 11-7082. Similar to the regulatory effect of NF-κB on LPS-induced RGS10 suppression, inhibition of NF-κB activation blunted RGS10 suppression by TNF-α in MH-S cells **(Figure 3.5H)**, suggesting the involvement of NF-κB activation in both LPS- and TNF-α-induced RGS10 suppression. Thus, our results collectively suggest that LPS induces the down-regulation of RGS10 expression through a mechanism that requires TNF-α.

HDAC activity is required for *Rgs10* expression silencing by LPS

Our results, along with previous studies (Lee et al., 2008, Lee et al., 2013) have demonstrated that LPS-induced reduction of RGS10 protein expression is a result of *Rgs10* transcript suppression, suggesting epigenetic mechanisms behind *Rgs10* regulation. Given that histone deacetylation mediated by HDACs is primarily implicated in epigenetic transcriptional repression, we investigated whether HDAC inhibitors reverse *Rgs10* silencing by LPS. First, to gain a global insight into the role of HDAC enzymes in promoting LPS-mediated RGS10 suppression, we treated MH-S cells with vehicle or LPS (10 ng/ml) following pretreatment with the pan-HDAC inhibitor (trichostatin, TSA, 100 nM) for one hour. We found that the inhibition of the global HDAC activity by TSA increased the basal level of RGS10 and completely blocked the suppressive effect of LPS on *Rgs10* transcript **(Figure 3.6A)** and RGS10 protein **(Figure 3.6B)** expressions. The activity or expression of HDACs 1-3 has been shown to be enhanced by LPS stimulation in macrophage cells, where they have an essential role in facilitating LPS-activated transcription of inflammatory gene expression including TNF-α (Chen;Barozzi; et al., 2012, Durham et al., 2017, Wu;Li; et al., 2019). Since TSA is a non-selective and general HDAC inhibitor, we next examined the effect of a selective inhibitor targeting class I HDACs (HDACs 1-3) on the suppression of RGS10 by LPS. We co-treated MH-S cells with LPS and the selective HDACs (1-3) inhibitor apicidin (Bradner et al., 2010). We found that apicidin treatment fully inhibited RGS10 suppression by LPS in MH-S cells **(Figure 3.6C and 3.6D)**. Consistent with these MH-S cells data, we also observed a similar effect in BV2 **(Figure 3.6E)** and BMDM **(Figure 3.6F)** cells, where apicidin significantly blocked LPS-induced *Rgs10* silencing. In addition to histone deacetylation, DNA methylation is another epigenetic mechanism that is mediated by DNA methyltransferases (DNMTs) and negatively regulate genes transcription. To determine the involvement of DNMTs in the regulation of RGS10 expression in LPS-activated MH-S cells, we pretreated the cells with the DNMTs inhibitor 5-Azacytidine (5-Aza) followed by LPS stimulation. 5-Aza pretreatment does not have a significant effect on LPS-mediated suppression of RGS10

expression **(Figure S3.3)**. These findings suggest that *Rgs10* silencing following TLR4 activation is mediated by class I HDACs (1-3), not DNMTs.

Loss of RGS10 amplifies phosphorylation of p65-NF-κB and upregulation of pro-inflammatory genes expression in pulmonary macrophages

Beyond its canonical function against Gαi-subunits, RGS10 acts as an anti-inflammatory regulator in microglia and BMDMs, where RGS10 particularly inhibits LPS-stimulated expression of various inflammatory genes, such as TNF-α and interleukins (Lee et al., 2008, Lee et al., 2013). In order to confirm the anti-inflammatory action of RGS10 in MH-S AM cells, we generated MH-S stable control and RGS10-deficient (RGS10 KO) cells using the CRISPR-Cas9 gene editing system and examined inflammatory responses in LPS-stimulated control and RGS10 KO MH-S cells. First, we confirmed that RGS10 protein expression was entirely absent in MH-S KO cells **(Figure 3.7A)**. We next treated control and RGS10 KO MH-S cells with LPS (10 ng/ml) for 20 minutes to determine the extent of phosphorylation of the p65-NF-κB subunit. Immunoblot analysis showed that loss of RGS10 enhanced LPS-induced NF-κB(p65) phosphorylation **(Figure 3.7A)**. RGS10 protein levels did not change in the parental MH-S cells during the short time incubation of LPS treatment **(Figure 3.7A)**. We subsequently measured transcript levels of pro-inflammatory genes following 24 hours of LPS stimulation and found that a significant upregulation of TNF-α **(Figure 3.7B)**, IL-1β **(Figure 3.7C)**, IL-6 **(Figure 3.7D)**, and INOS **(Figure 3.7E)** in RGS10 KO AM MH-S cells compared to control cells expressing RGS10. Our results strongly suggest that RGS10 suppresses pulmonary macrophages activation.

Discussion

RGS10 is a small protein that is implicated in multiple disease states (Almutairi et al., 2020) and controls physiology of diverse cells, including macrophages (Lee et al., 2013, Vural et al., 2019), neurons (Lee;Chung; et al., 2012), osteoclasts (Yang et al., 2007, Yang and Li, 2007, Yang et al., 2013), T-lymphocytes (Garcia-Bernal et al., 2011), cancer cells (Hooks et al., 2010, Altman et al., 2015), platelets (Hensch et al., 2016, Ma et al., 2018, DeHelian et al., 2020), and cardiomyocytes (Bender et al., 2008, Miao et al., 2016). Among these cells, the highest endogenous expression of RGS10 is found in immune cells, with abundant expression in macrophages (Lee et al., 2008, Lee et al., 2013). Macrophage cells in different organs, such as microglia and alveolar macrophage maintain normal organ homeostasis and become activated in response to injury, infection, and environmental toxins. Under acute activation conditions, they play an important role in neutralizing those assaults and engulfing dead cells and cells debris. However, sustained activation of macrophages leads to an overproduction of inflammatory mediators, such as TNF-α and interleukins, resulting in persistent inflammatory responses, inflammatory diseases, and organs damage.

Macrophages can be activated following TLR4 stimulation by microbial molecules, such as the bacterial LPS that triggers multiple inflammatory signaling pathways, leading to the transcription of inflammatory genes. RGS10 is highly enriched in macrophage cells under resting conditions, but the expression of RGS10 is silenced following stimulation with LPS (Lee et al., 2008, Lee et al., 2013). Although TLR-4 activation by LPS results in the suppression of RGS10 expression in macrophages, the inflammatory responses that are required for LPS-induced silencing of RGS10 are unknown.

In this study, we confirmed and further expanded the abundant expression of RGS10 in macrophages, as we detected its high expression in another unique type of TRMs, alveolar macrophages. While the expression of RGS10 is high in the resting state of MH-S cells, its expression is down-regulated following MH-S cells polarization to classically activated

macrophages characterized by a pro-inflammatory M1 phenotype. Consistent with the suppressive effect of LPS on RGS10 expression in microglia and BMDMs, we also showed that LPS induced suppression of *Rgs10* transcript and RGS10 protein expression levels in pulmonary macrophages.

The interaction of TLR4 with LPS results in the activation of multiple intracellular inflammatory signaling pathways, which ultimately induce inflammatory genes expression, including TNF-α. In this study, we identified the signaling steps that are essential for LPS-stimulated RGS10 suppression. In particular, we demonstrated that LPS facilitates RGS10 silencing through setting off a cascade of events: activation of PI3K, subsequent activation and initiation of NF-κB nuclear translocation, and enhancement of HDACs enzyme activity. In the nucleus, NF-κB acts as transcription factor that drives the expression of TNF-α and several other pro-inflammatory genes via an interaction with its co-activator, p-300. TNF-α, in turn, binds its TNFR receptor and in a positive feedback loop amplifies NF-κB signaling and enhances HDAC (1-3) activities, subsequently mediating histones deacetylation at *Rgs10* promoter and resulting in transcriptional silencing of *Rgs10* expression. Collectively, our data substantiate that pharmacological inhibition of the activities of these inflammatory responses enhance the basal expression of RGS10 and/or blocks LPS-mediated silencing of RGS10 **(Figure 3.8)**.

RGS10 regulates microglia and macrophages activation by acting as a critical regulator of inflammatory signaling. More specifically, following LPS stimulation, RGS10 strongly suppresses the production of pro-inflammatory mediators, in particular TNF-α and interleukins and inhibits the activity of NF-κB, a critical transcription factor and signaling link between the activation of TLR-4 by LPS and inflammatory mediator's expression (Lee et al., 2008, Lee et al., 2011, Lee et al., 2013, Ren et al., 2021). Our results expand the anti-inflammatory function of RGS10 to alveolar macrophages, where a complete loss of RGS10 expression results in a robust upregulation of LPS-induced phosphorylation of p65-NF-κB and inflammatory genes expression, such as TNF-α. Our data in combination with previous studies have showed that RGS10 expression is suppressed

following macrophages activation, which will lead to the loss of ant-inflammatory effect of RGS10 and thereby amplifies inflammatory signal responses that contribute to macrophages activation in a vicious cycle. Thus, understanding the mechanism underlying the silencing of RGS10 expression in activated macrophages is critical to identify therapeutic targets that can prevent RGS10 loss and thereby diminish multiple inflammatory signaling pathways. Our study is the first to set the basis for identifying inflammatory targets that facilitate RGS10 suppression in response to LPS-activated macrophages and provide valuable insight for guiding future efforts to screen and develop small molecule regulators of RGS10 expression that could serves as anti-inflammatory therapeutics.

Numerous studies have demonstrated the localization of RGS10 to both the cytoplasm and the nucleus of the cells, with no significant plasma membrane localization (Chatterjee and Fisher, 2000, Haller et al., 2002). In microglia, RGS10 is evenly expressed in both the cytoplasmic and the nuclear compartments under the resting condition. However, in response to LPS stimulation, much of RGS10 translocates from the cytoplasm to the nucleus (Lee et al., 2008). More importantly, an early study has reported that nuclear localization of RGS10 is driven by the cyclic AMP-dependent protein kinase A (PKA)-mediated phosphorylation of RGS10 on serine 168 (Burgon et al., 2001). In dopaminergic neurons, overexpression of RGS10-S168A (RGS10SA, resistant to phosphorylation by PKA) or blocking PKA-triggered RGS10 phosphorylation and the subsequent nuclear translocation limits the prosurvival role of RGS10 in TNF-α-induced neurotoxicity (Lee;Chung; et al., 2012). In terms of NF-κB signaling regulation, PKA is known to phosphorylate NF-κB(p65) and modulates its transcriptional activity via potentiation the interaction of the phosphorylated NF-κB(p65) and the CBP/p300 co-activators (Zhong et al., 1997, Zhong et al., 1998). Therefore, it is important to determine if PKA regulates LPS-triggered RGS10 nuclear localization and whether PKA is involved in LPS-induced RGS10 suppression in macrophages.

DNA methylation and histone modifications are epigenetic marks involved in the regulation of gene transcription (Smith and Meissner, 2013, Venkatesh and Workman, 2015). In particular, DNMTs-mediated DNA hypermethylation and loss of histone acetylation via HDAC enzymes represent critical mechanisms that alter chromatin structure, TF-DNA interactions and generally result in gene suppression. Given their role in the transcriptional repression, we tested the effect of DNA methylation and histone deacetylation inhibition on LPS-induced *Rgs10* silencing. Blocking HDACs activity by the pan-HDACs inhibitor (TSA) stabilizes the expression of RGS10 upon LPS exposure, whereas DNMTs inhibitor does not have a significant effect on LPS-induced RGS10 suppression. Stimulation of several cell types including microglia and macrophages with LPS has shown to induce expression and activity of HDAC enzymes (Zhu et al., 2010), mainly three class I HDACs (1-3), HDAC1, HDAC2, and HDAC3 (Chen;Barozzi; et al., 2012, Wu;Li; et al., 2019). We showed that the selective HDAC (1-3) enzymes inhibitor (Apicidin) blocks the ability of LPS to suppress RGS10 transcription, demonstrating that the activity of HDACs 1-3 is required for LPS silencing of RGS10. Class I HDACs (1-3) are highly homologous nuclear proteins that form homo- and heterodimers between each other and constitute the catalytic activity of multiple protein repressor complexes (Delcuve et al., 2012, Khan et al., 2013, Thomas, 2014). Ongoing studies including isoform-selective inhibitor(s) and chromatin immunoprecipitation assay are examining which class I HDAC (1-3) isoform(s) are involved in LPS-induced suppression of RGS10. Growing evidence strongly implicates class I HDACs (1-3) in the regulation of differentiation and activation of macrophages, as they support pro-inflammatory M1 responses and inhibit M2-phenotype polarization (Datta et al., 2018, Chen et al., 2020). Due to the reported anti-inflammatory HDAC inhibitors in activated macrophages and the fact that loss of RGS10 enhances inflammatory mediators' expression, it will be interesting to test whether the anti-inflammatory action of HDAC inhibitors is due to reversing RGS10 silencing in macrophages.

In addition to macrophages, suppression of RGS10 expression can occur in multiple cells, including microglia (Lee et al., 2008, Lee et al., 2011), neurons (Lee;Chung; et al., 2012),

cardiomyocytes (Miao et al., 2016), and ovarian cancer cells (Hooks et al., 2010). Loss of RGS10 expression in these cells contributes to microglia overactivation and subsequent neuroinflammation-mediated neurodegeneration (Lee et al., 2008, Lee et al., 2011, Lee;Chung; et al., 2012, Lee et al., 2016), cardiac hypertrophy (Miao et al., 2016), and ovarian cancer chemoresistance (Ali et al., 2013, Hooks and Murph, 2015), respectively. Critically, inflammatory signaling, in particular NF-κB activity and pro-inflammatory cytokines are implicated in neurodegenerative diseases (such as Parkinson's disease) (Chitnis and Weiner, 2017), heart failure (Gordon et al., 2011, Gaspar-Pereira et al., 2012), and cancer progression and chemoresistance (Harrington and Annunziata, 2019). Our findings in elucidating inflammatory responses facilitating RGS10 suppression in microglia and macrophages may have broad effects to include neurons, cardiomyocytes, and ovarian cancer cells, where loss of RGS10 expression is observed, its physiological role is protective, and the inflammatory signaling has a major influence. Therefore, RGS10 is a valuable and novel drug target, as restoring or stabilizing RGS10 expression could be an effective therapeutic strategy in the treatment of these pathologies associated with low level of RGS10 expression and high levels of inflammatory mediators.

This study has limitations, as all the data have generated in mouse cells not human cells. Further, due to the lower yield of murine primary pulmonary macrophages, the focus of this study was solely done on in vitro alveolar macrophage MH-S cells. However, we confirmed the main findings in primary mouse BMDMs and microglia, suggesting that the regulation of RGS10 expression by LPS is not macrophage type-specific.

Conclusion

This study showed the suppression of RGS10 expression in response to LPS activation in alveolar macrophages and identified the inflammatory responses that are required for the inhibitory effect of LPS on RGS10 expression. Pharmacological inhibition of PI3K activity, NF-κB-dependent TNF-α expression and HDACs (1-3) activities stabilized RGS10 expression following LPS stimulation in AMs, as well as microglia and BMDMs. MH-S cells lacking functional RGS10 are hypersensitive to LPS stimulation demonstrated by amplification of phospho-NF-κB(p65) and upregulation of pro-inflammatory gene expression. Collectively, these findings provide important information about the molecular mechanism underlying RGS10 suppression in activated macrophages and will help in the development of future small molecule RGS10 stabilizers in order to exploit its strong anti-inflammatory activity.

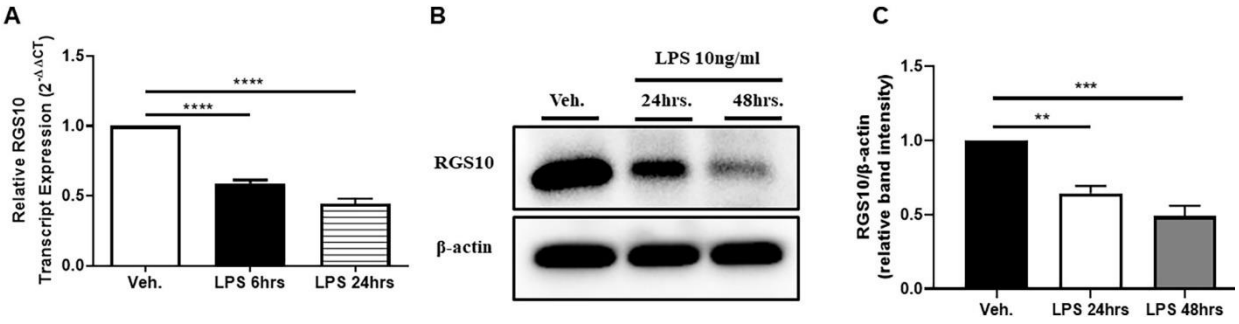

Figure 3.1. RGS10 expression is silenced in response to LPS stimulation in MH-S cells.

(A) MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 and 24 hours. RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was measured using quantitative RT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(B)** MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 24 and 48 hours. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. Fold differences of qRT-PCR and immunoblot densitometry data were calculated after normalizing to vehicle conditions and compiled from three independent experimental repeats. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

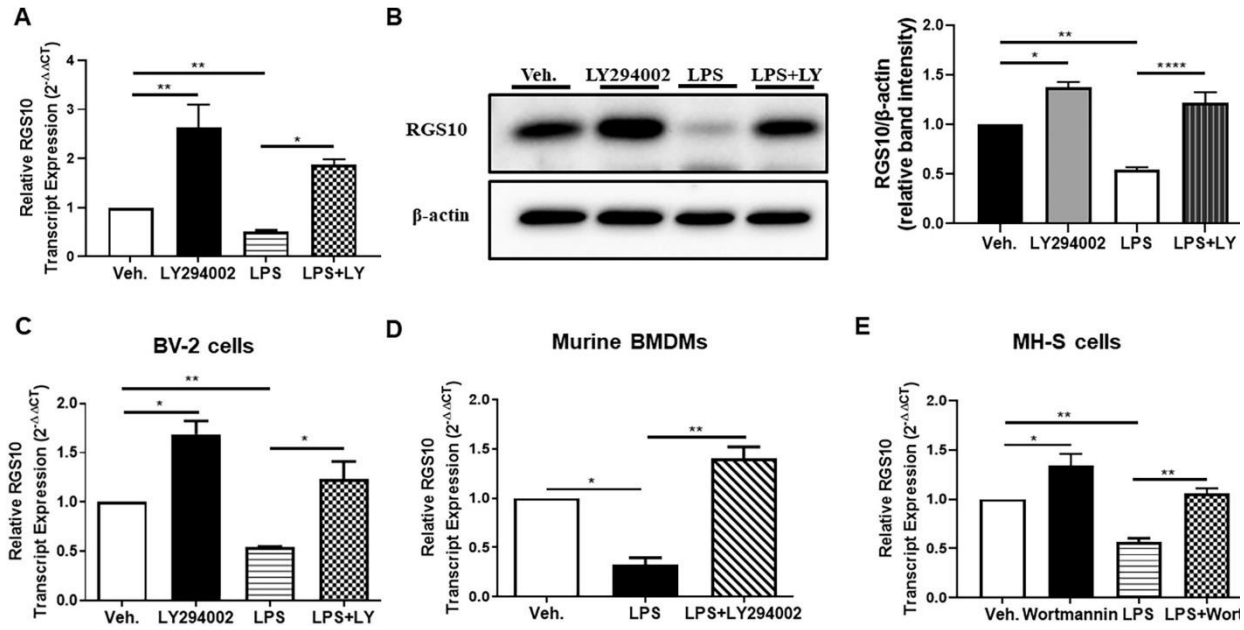

Figure 3.2. PI3K mediates RGS10 silencing in response to TLR4 activation.

(A) MH-S cells were plated in 12-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 15 μM LY294002 (LY). RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(B)** MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 15 μM LY294002 (LY). Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin**. (C)** BV2 cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 15 μM LY294002 (LY). **(D)** Murine BMDMs were treated with vehicle (serum-free media) or LPS (1 ng/ml) for 3 hours with or without LY294002 (15 μM). **(E)** MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 10 μM wortmannin (Wort.). RGS10 transcript level in **(C, D, and E)** was measured using qRT-

PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions and pooled from three independent experiments. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

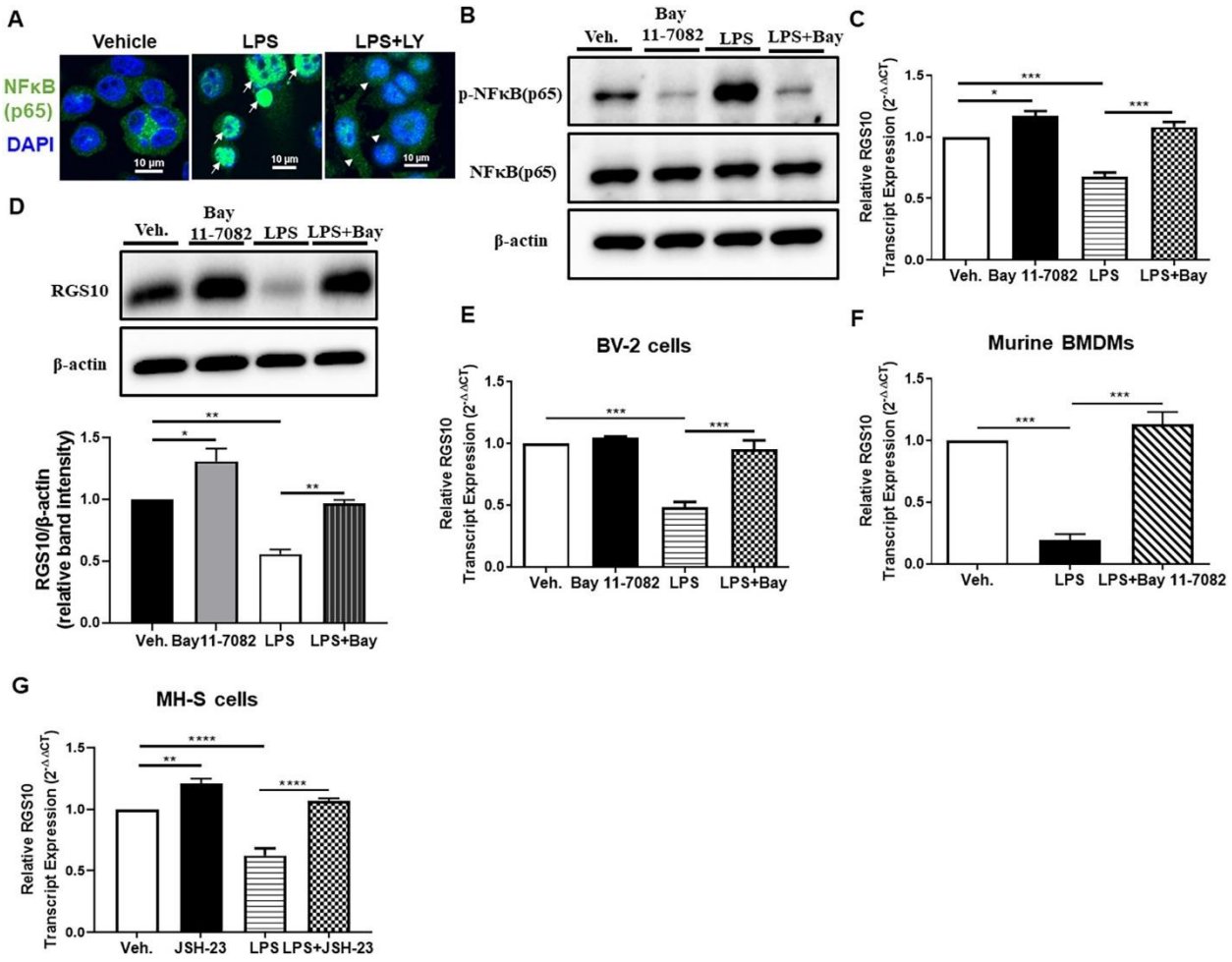

Figure 3.3. Blocking NF-κB activation restores RGS10 suppression following LPS stimulation.

(A) LY294002 inhibits LPS-induced NF-*κ*B (p65) nuclear localization. Immunofluorescence staining for NF-*κ*B (p65) in MH-S cells were treated with LPS (100 ng/ml) for 60 minutes in the presence of absence of LY294002 (15 *μ*M). IF images presented in **(A)** are representative two independent repeats. **(B)** Protein expression levels of p-NF-*κ*B (p65) (phospho-NF-*κ*B p65 (Ser536)), NF-*κ*B (p65), and β-actin following treatment of LPS (10 ng/ml) for 20 minutes with or without Bay 11-7082 in MH-S cells. Blot presented in **(B)** is representative of two independent experiments. **(C)** MH-S cells were plated in 12-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 20 *μ*M Bay 11-7082 (Bay). RNA was isolated from the cells using TRIzol

reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(D)** MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 20 μM Bay 11-7082 (Bay). Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. **(E)** BV2 cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 20 μM Bay 11-7082 (Bay). **(F)** Murine BMDMs were treated with vehicle (serum-free media) or LPS (1 ng/ml) for 3 hours with or without Bay 11-7082 (20 μM). **(G)** MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 7 μM JSH-23. RGS10 transcript level in **(E, F, and G)** was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions. All the representative data in **(C-G)** are pooled from three independent experiments. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

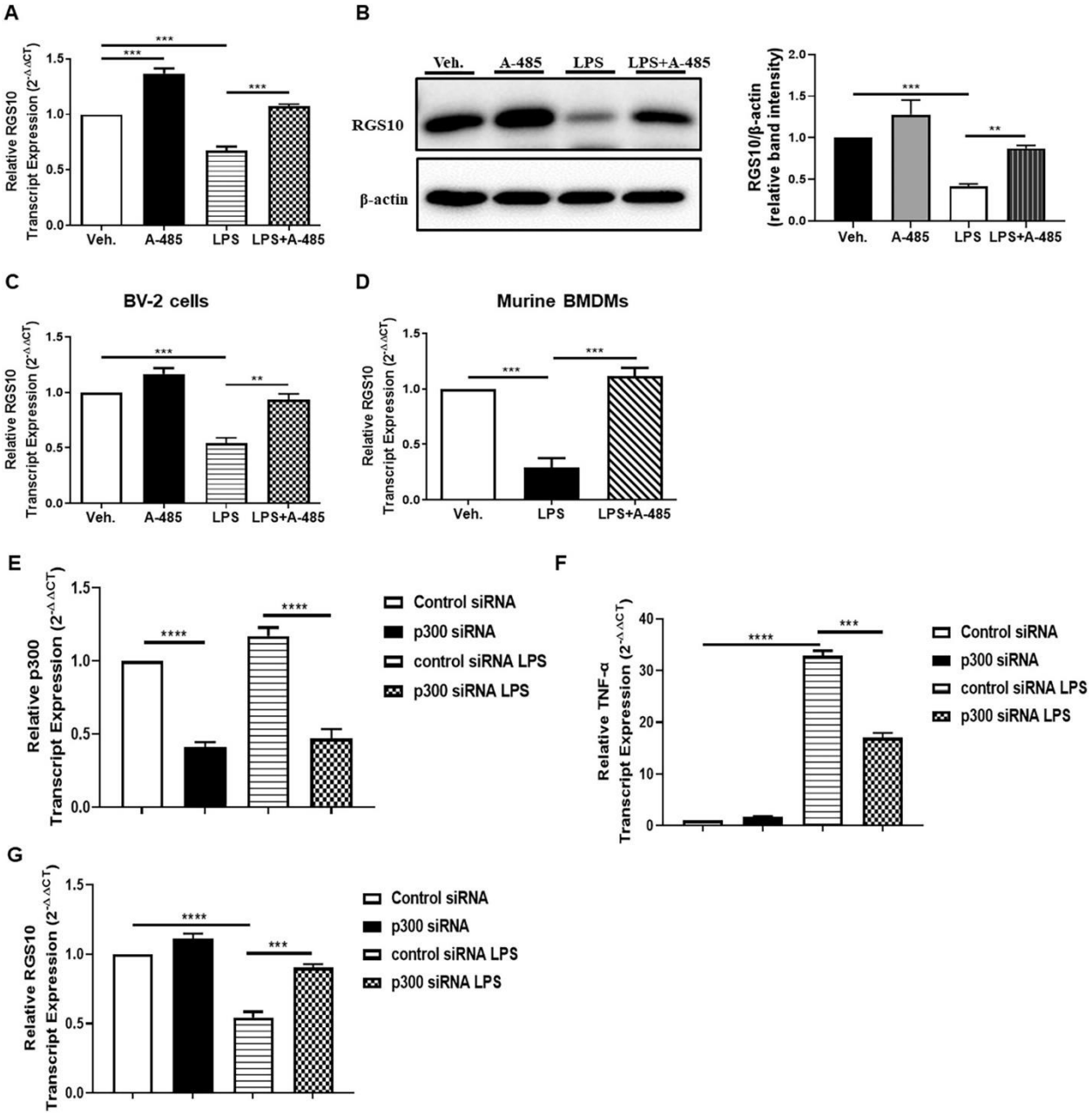

Figure 3.4. The inhibition of p300 abrogates LPS-triggered RGS10 suppression.

(A) MH-S cells were plated in 12-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 10 μM A-485. RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated

by using the $2^{-\Delta\Delta Ct}$ method. **(B)** MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 10 μM A-485. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. **(C)** BV2 cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 10 μM A-485. **(D)** Murine BMDMs were treated with vehicle (serum-free media) or LPS (1 ng/ml) for 3 hours with or without A-485 (10 μM). RGS10 transcript level in **(C and D)** was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(E-G)** MH-S cells were plated in 12-well plates and simultaneously transiently transfected with control or p300-targeting siRNA constructs. 48 hours post-transfection, cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours. The relative expression of p300 transcript **(E)**, TNF-α transcript **(F)**, and RGS10 transcript **(G)** were measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions and pooled from three independent experiments. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

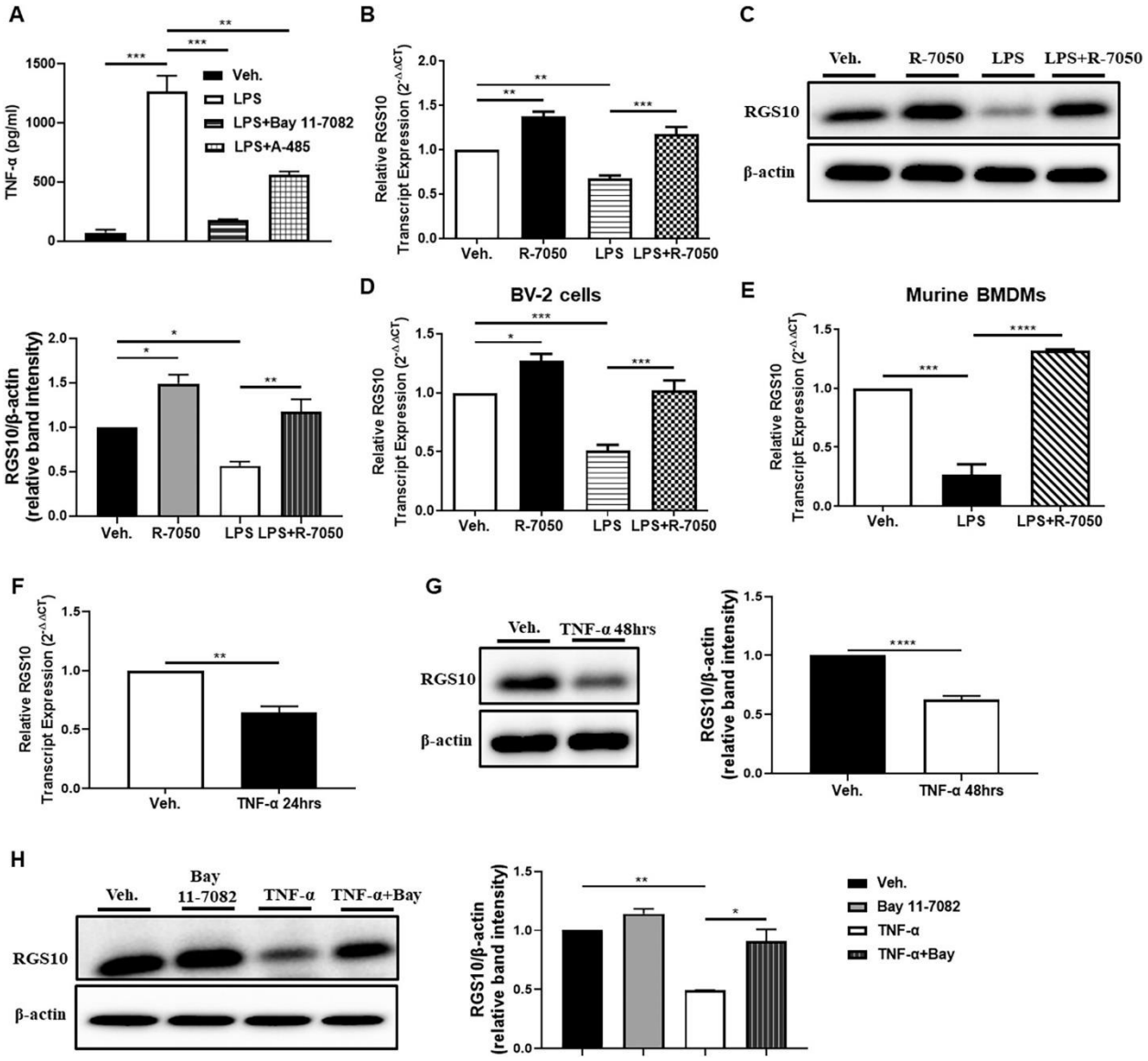

Figure 3.5. LPS-mediated RGS10 suppression is TNF-α-dependent.

(A) MH-S cells were cultured overnight and then incubated with vehicle (serum-free media) or LPS (10 ng/ml) for 24 hours with or without Bay 11-7082 (20 μM) or A-485 (10 μM). Culture medium was collected for detecting TNF-α level using ELISA. The data is representative of two independent experiments. **(B)** MH-S cells were plated in 12-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 10 μM R-7050. RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was

measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(C)** MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 10 μM R-7050. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. **(D)** BV2 cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 10 μM R-7050. **(E)** Murine BMDMs were treated with vehicle (serum-free media) or LPS (1 ng/ml) for 3 hours with or without R-7050 (10 μM). **(F)** MH-S cells were treated with vehicle or TNF-α (100 ng/ml) for 24 hours. RGS10 transcript level in **(D-F)** was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(G)** MH-S cells were treated with vehicle or TNF-α (100 ng/ml) for 48 hours. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions and pooled from three independent experiments. Data were analyzed for statistical differences using unpaired t-test or an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

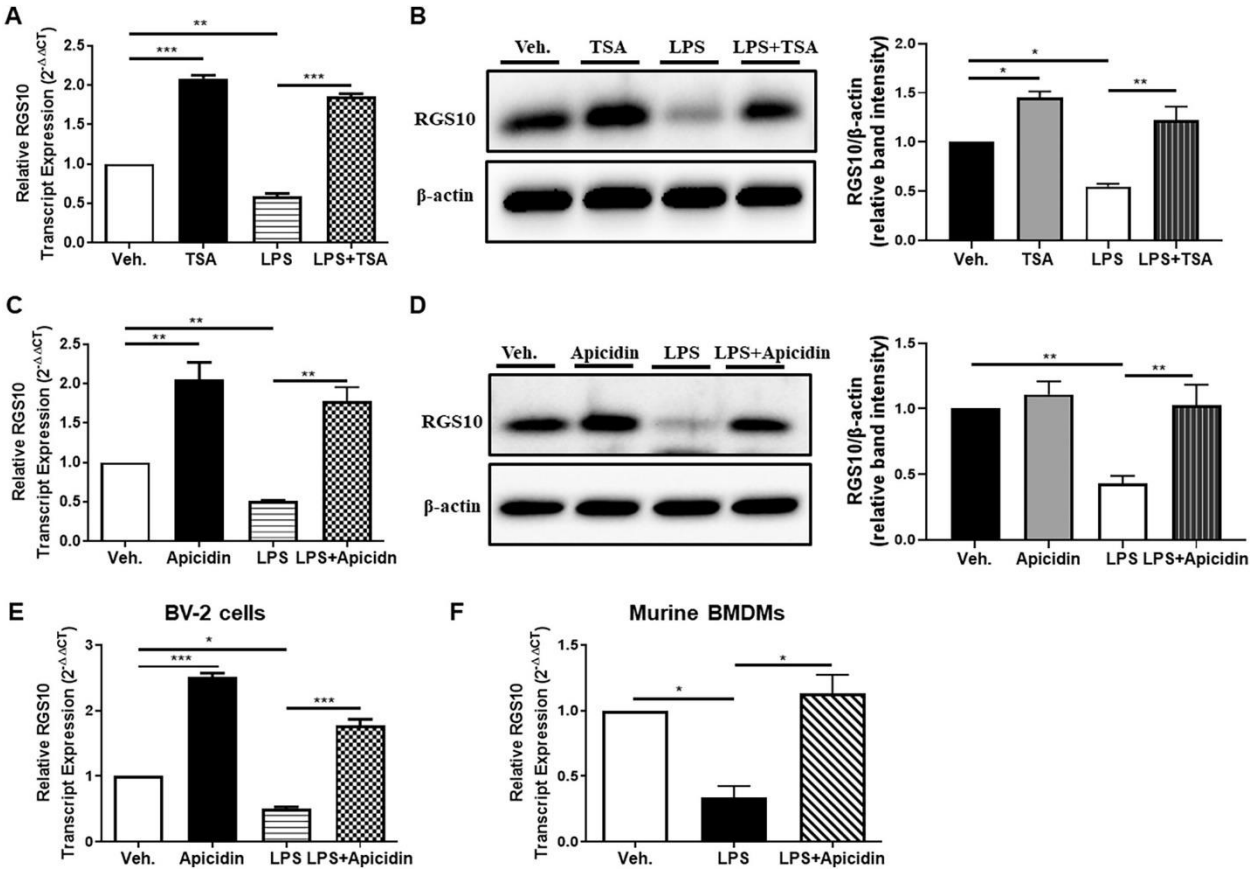

Figure 3.6. HDAC activity is required for *Rgs10* suppression by LPS.

(A and C) MH-S cells were plated in 12-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 100nM TSA **(A)** or 500nM apicidin **(C)**. RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 transcript level was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. **(B and D)** MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 100nM TSA **(B)** or 500nM apicidin **(D)**. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. Densitometry of RGS10 band was normalized to β-actin. **(E)** BV2 cells were treated with vehicle (serum-free media) or

LPS (10 ng/ml) for 6 hours with or without a one hour-pretreatment with 500nM apicidin. **(F)** Murine BMDMs were treated with vehicle (serum-free media) or LPS (1 ng/ml) for 3 hours with or without 500nM apicidin. RGS10 transcript level in **(E and F)** was measured using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions and pooled from three independent experiments. Data were analyzed for statistical differences using an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

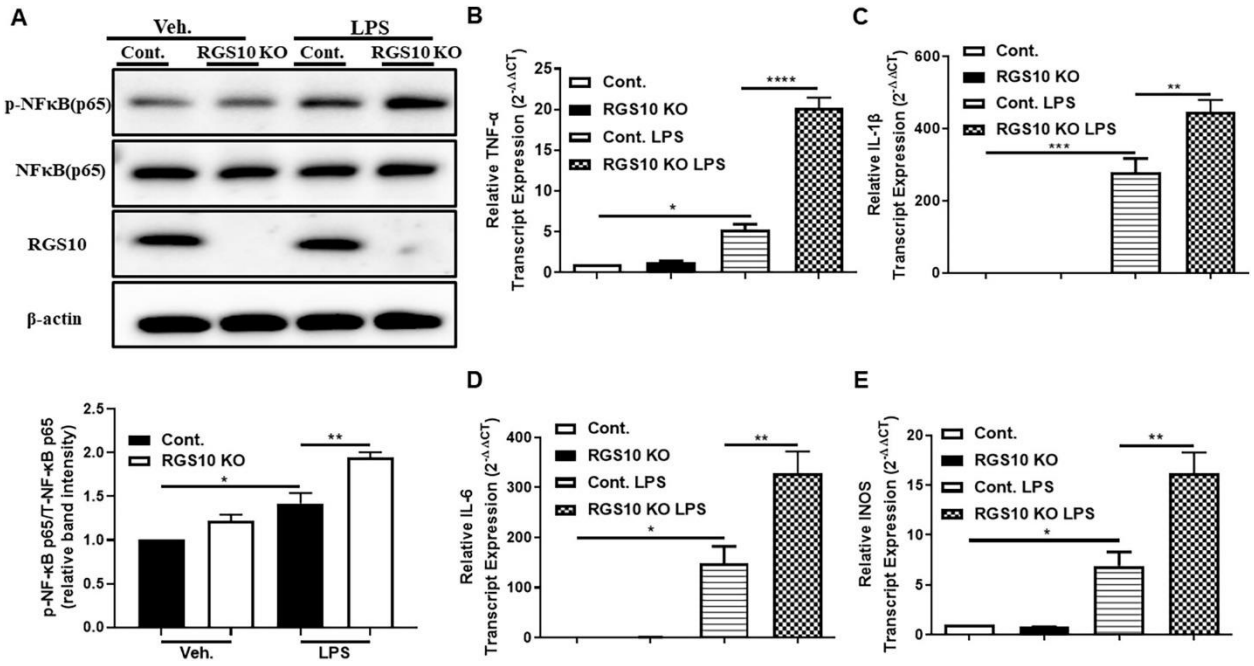

Figure 3.7. Loss of RGS10 amplifies phosphorylation of p65-NF-κB and upregulation of pro-inflammatory genes expression in pulmonary macrophages.

(A) MH-S cells were stably infected with control or RGS10 targeted CRISPR/Cas9 lentivirus as described in materials and methods. Control and RGS10 knockout (KO) MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 20 minutes. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10, p-NF-*κ*B (p65) (phospho-NF-*κ*B p65 (Ser536)), NF-*κ*B (p65), and β-actin. Densitometry of p-NF-*κ*B (p65) intensity was normalized to NF-*κ*B (p65). **(B-E)** Control and RGS10 knockout (KO) MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 24 hours. RNA was isolated from the cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. Relative expression of TNF-α transcript **(B)**, *IL*-1β transcript **(C)**, IL-6 transcript **(D)**, and INOS transcript **(E)** were analyzed using qRT-PCR and normalized to an endogenous housekeeping gene β-actin. The relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. Fold differences of qRT-PCR and IB densitometry data were calculated after normalizing to vehicle conditions and pooled from three independent experiments. Data were analyzed for statistical differences using

an analysis of variance (ANOVA) followed by Tukey post hoc test between groups. Data are presented as mean ± SEM where *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

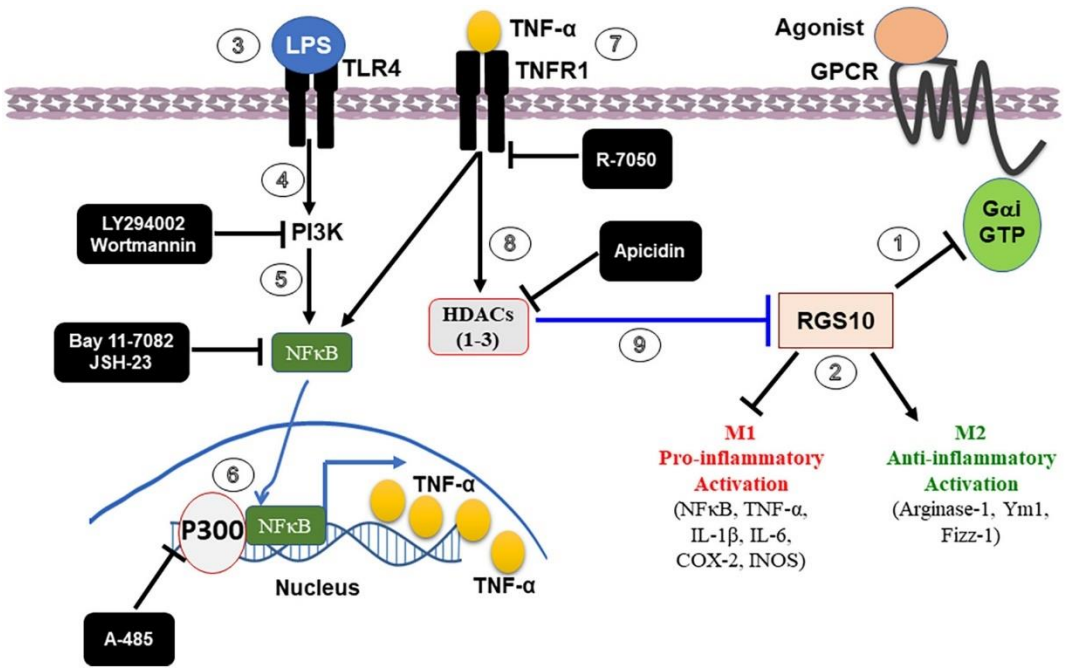

Figure 3.8. A proposed model of TLR-4 activation-induced RGS10 suppression in microglia and macrophages.

RGS10 canonically interacts with Gαi proteins and acts as GTPase-activating protein (GAP) to diminish the activation of Gαi proteins by G-protein Coupled Receptors (GPCRs) (1). Beyond its GAP activity, RGS10 is highly enriched in macrophages and plays an anti-inflammatory role by inhibiting the pro-inflammatory M1 phenotype activation by suppressing LPS-stimulated NF-κB activity, pro-inflammatory genes expression and promoting the anti-inflammatory M2 phenotype activation (2). Activation of macrophages is triggered by LPS-stimulated TLR-4 (3). Upon LPS stimulation, PI3K is activated (4), which in turn promotes NF-κB activation and its subsequent nuclear translocation (5). In the nucleus, NF-κB binds to its cognate κB DNA site and interacts with its co-activator p-300 (6) to initiate a plethora of pro-inflammatory genes expression including TNF-α. TNF-α binds its receptor (7) in an autocrine manner that further amplifies NF-κB signals in a positive feedback loop (5) and upregulates HDACs (1-3) activity (8). HDACs (1-3) mediates histones deacetylation at the *Rgs10* promoter and results in transcriptional silencing of *Rgs10* expression (9). Suppression of *Rgs10* expression will indirectly amplifies G protein-coupled

receptor signaling that is regulated by RGS10 (1) and will lead to the loss of its anti-inflammatory activity (2), resulting in an amplification of pro-inflammatory M1 activation of macrophages in a feed-forward mechanism that contribute to dysregulation of inflammatory signaling (NF-κB-dependent pro-inflammatory mediators) and inhibition of anti-inflammatory M2 activation of macrophages. Our data substantiate that pharmacological inhibition of inflammatory signal responses in (4), (5), (6), (7), and (8) enhance the basal expression of RGS10 and/or blocks LPS-mediated silencing of RGS10. The effects of pharmacological agents LY294002, wortmannin, Bay 11-7082, JSH-23, A-485, R-7050, and apicidin are shown.

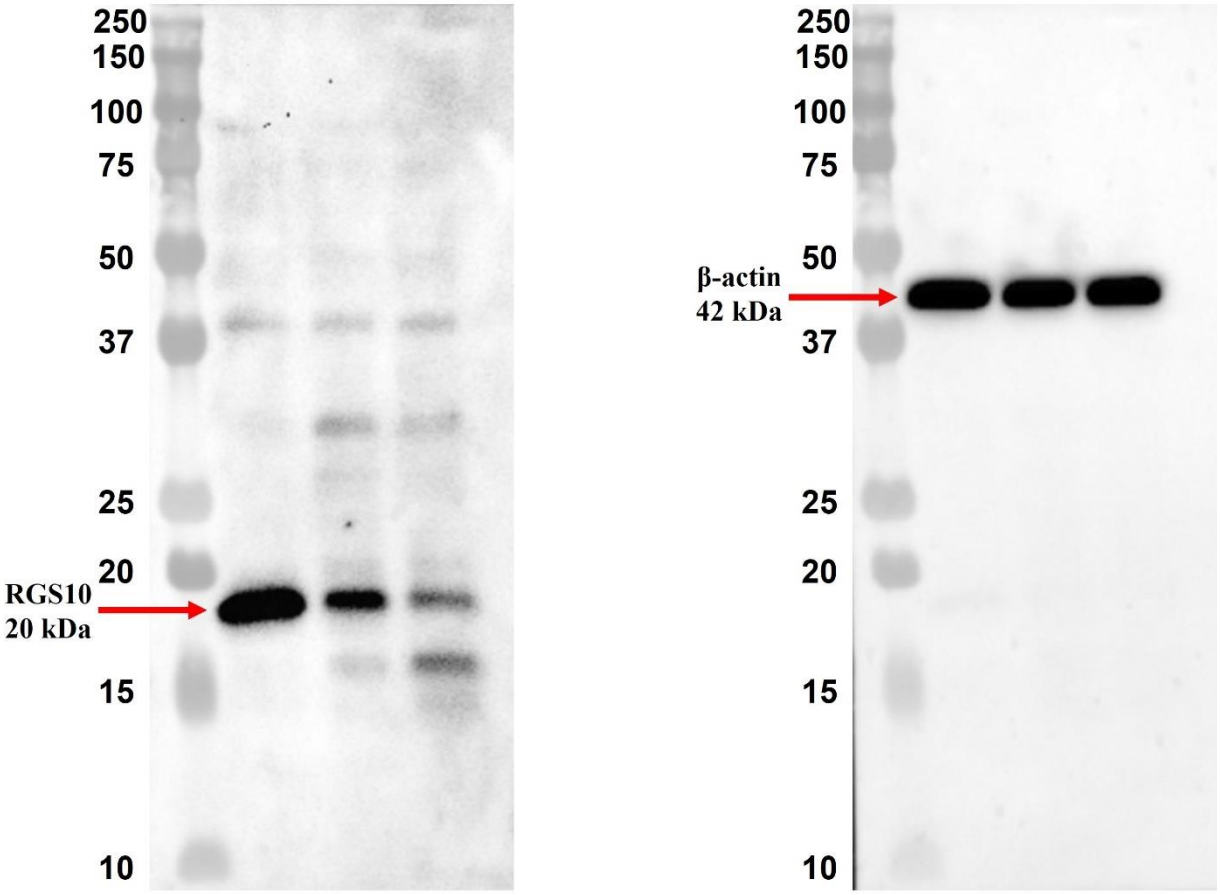

Fig. S3.1. Validation of the RGS10 antibody.

MH-S cells were treated with vehicle (serum-free media) or LPS (10 ng/ml) for 24 and 48 hours. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. The pictures represent the full-length gels with the indicated molecular weight markers (BioRad, cat#: 1610374) for Figure 1B. The picture was taken using a BioRad Molecular Imager ChemiDoc™ XRS+ System with Image Lab™ Software.

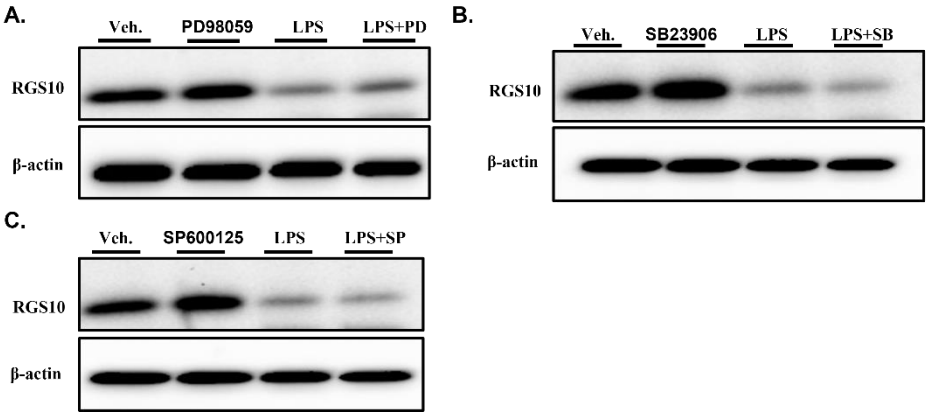

Figure S3.2. Blocking MAPK signaling pathways does not affect LPS-induced RGS10 suppression.

(A-C) MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 10 μM of ((**A)** PD98059 (PD), **(B)** SB23906 (SB), and **(C)** SP600125 (SP)) . Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. One blot of two similar independent experiments is shown in each panel.

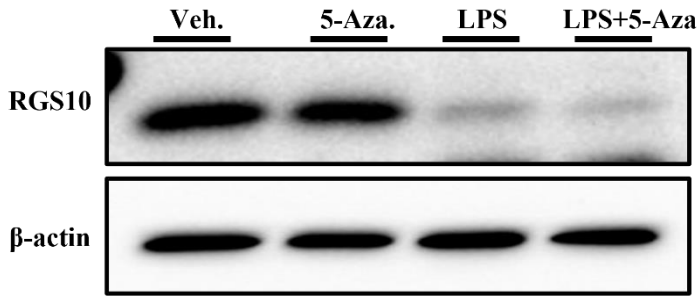

Figure S3.3. The effect of 5-Aza, DNMTs inhibitor, on LPS-stimulated silencing of RGS10. MH-S cells were plated in six-well plate and allowed to adhere overnight before treatment with vehicle (serum-free media) or LPS (10 ng/ml) for 48 hours with or without a one hour-pretreatment with 10 μM 5-Aza-2'-deoxyytidine (5-Aza). Cells were lysed and subjected to SDS-PAGE followed by immunoblotting using specific antibodies against RGS10 and β-actin. One blot of two similar independent experiments is shown.

CHAPTER 4

THE ROLE OF STIM2-ORAI ACTIVITY IN RGS10 REGULATION OF LPS-INDUCED COX-2 IN MICROGLIA[3]

[3] Almutairi F, Hooks SB. *Cell Signal*. 2021 Jul; 83:109974

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Abstract

Regulator of G-protein Signaling (RGS10) is a member of a family of proteins that canonically bind and terminate signaling downstream of G proteins. RGS10 is highly expressed in brain and immune cells, with specific enrichment in microglia. Microglial RGS10 serves an anti-inflammatory and neuroprotective role by suppressing the expression of pro-inflammatory genes, such as tumor necrosis factor-alpha (TNF-α) and cyclooxygenase (COX-2) in response to TLR4 receptor activation. We recently demonstrated that the anti-inflammatory activity of RGS10 occurs through an undefined G-protein-independent mechanism. To exploit the anti-inflammatory effects of RGS10 in the development of therapeutics for the treatment of neuroinflammation and accompanying neurodegeneration, it is essential to define the mechanism for RGS10 suppression of inflammatory gene expression. We have identified STIM2 as a novel RGS10 interacting protein in BV2 microglia cells and RAW264.7 macrophages. STIM2 is an ER-resident calcium sensor that mediates store-operated Ca^{2+} entry (SOCE) through Orai channels in the plasma membrane in response to ER calcium depletion. STIM2 activation and its coupling to Orai-mediated calcium signaling are strongly implicated in the regulation of inflammatory signaling in microglia. Therefore, we hypothesized that RGS10 suppression of TLR4-stimulated inflammatory gene expression reflects the regulation of STIM2-Orai activity. Our results demonstrate that pharmacological inhibition of Orai channels completely blocks the amplification of TLR4-stimulated COX-2 and TNF-α in response to the loss of RGS10 expression. These results suggest that RGS10 suppresses COX-2 and TNF-α expression downstream of TLR4 activation of Orai-mediated SOCE. Further, we demonstrate that thapsigargin, which triggers a transient rise in cytoplasmic calcium and ER calcium depletion, strongly enhances LPS-induced RGS10 silencing, suggesting a potential mechanism for feedback regulation of calcium signaling. These findings provide critical insight into the mechanism that RGS10 utilizes to regulate inflammatory signaling. Defining these mechanisms in microglia is essential to develop novel therapeutics that target RGS10 in treating neuroinflammatory diseases.

Highlights

- RGS STIM2, an ER-resident calcium sensor and component of the SOCE machinery, is a novel RGS10 interacting protein in BV2 microglia and RAW264.7 macrophage cells.
- Pharmacological inhibition of Orai channels and an intracellular calcium chelator (BAPTA-AM) completely block LPS-stimulated COX-2 expression.
- Orai channel activity is essential for RGS10's effect on LPS-induced COX-2 and TNF-α expression.
- TG-mediated Ca^{2+} mobilization strongly amplified LPS-stimulated COX-2 expression and enhanced LPS-mediated suppression of RGS10.

Introduction

Microglia, as resident macrophages of the central nervous system (CNS), regulate brain development and homeostasis through their participation in innate immune responses and exert protective functions in combating infection and cleaning up cellular debris (Gehrmann et al., 1995, Liu and Hong, 2003, Wolf et al., 2017). As a part of their activation in response to insult or an injury exposure, they secrete inflammatory mediators such as pro-inflammatory cytokines, chemokines, prostaglandins, nitric oxide, and reactive oxygen species (Block and Hong, 2005, Liu;Wang; et al., 2019). Although their activation is essential for brain surveillance, dysregulated microglial inflammatory responses result in chronic neuroinflammation, which is being considered the key contributor in the pathophysiology of multiple neurodegenerative diseases, such as Alzheimer's disease (Hemonnot et al., 2019), Parkinson's disease (Lee et al., 2009, Subramaniam and Federoff, 2017), neurovascular disorders (Zhao et al., 2018), and neuropathic pain (Trang et al., 2011).

Among microglial inflammatory responses, cyclooxygenases (COX) are the rate-limiting step in arachidonic acid (AA) metabolism, mediating biosynthesis of prostaglandins and other bioactive lipids (Rouzer and Marnett, 2009). Particularly, COX-2 is a central inflammatory enzyme, in which its upregulation in the brain is a key factor in neuroinflammation (Minghetti, 2004) and is linked to several neurodegenerative diseases, such as Parkinson's disease (Knott et al., 2000, Vijitruth et al., 2006), Alzheimer's disease (Hoozemans et al., 2001), stroke (Nogawa et al., 1997), and multiple sclerosis (Rose et al., 2004). COX-2 mediated prostaglandin E2 production is robustly induced following inflammatory stimuli treatment, including bacterial LPS treatment (Ikeda-Matsuo et al., 2005, Font-Nieves et al., 2012), but the mechanisms underlying its induction in LPS-activated microglia remain unclear. Therefore, identifying the molecular modulators involved in the regulation of COX-2 expression will be beneficial to find appropriate drug targets to control neuroinflammation associated with neurological disorders.

Regulator of G protein Signaling 10 (RGS10) is a member of the RGS superfamily of proteins that classically bind and deactivate heterotrimeric G-proteins by acting as GTPase activating proteins (GTPs), resulting in accelerating GTP hydrolysis and thereby termination of downstream signaling pathways of G-protein activation (Watson et al., 1996, Ross and Wilkie, 2000, Hollinger and Hepler, 2002, Willars, 2006). RGS10 is the smallest protein of the R12 subfamily, and selectivity interacts with Gαi proteins (Hunt et al., 1996) and is highly expressed in brain and immune cells, with specifically high expression in microglia (Waugh et al., 2005, Butovsky et al., 2014).

Microglial RGS10 has emerged as an anti-inflammatory protein, in which its expression is suppressed following the LPS-activated TLR4 receptor (Lee et al., 2008, Alqinyah et al., 2017), resulting in upregulation of inflammatory genes expression. Particularly, loss of RGS10 in microglia amplifies LPS-stimulated proinflammatory mediators, such as TNF-α, interleukins, COX-2 mediated PGE2 release and enhances neurotoxicity (Lee et al., 2008, Lee et al., 2011, Alqinyah et al., 2018).

Although the canonical function of RGS10 is to inhibit G-protein signaling by accelerating GTPase activity, we have recently shown that RGS10-mediated regulation of LPS-induced COX-2 and TNF-α does not require its GAP activity (Alqinyah et al., 2018), suggesting an unknown Gαi protein-independent mechanism. Therefore, the goal of this study was to identify non-canonical binding partners of microglial RGS10 and their possible roles in mediating its anti-inflammatory effects.

In this study, we identified and validated stromal interaction molecule (STIM2) as a novel binding partner of endogenous RGS10 in BV2 microglia and RAW264.7 macrophage cells. STIM2 is a calcium sensor that localizes in the endoplasmic reticulum (ER) and mediates store-operated Ca^{2+} entry (SOCE) through Orai channels in the plasma membrane upon ER-calcium depletion (Berna-Erro et al., 2017). STIM2 activation and its coupling to Orai-mediated calcium signaling are required for inflammatory genes expression in response to LPS-stimulated macrophages

(Sogkas et al., 2015) and microglial migration and phagocytosis following extracellular nucleotide treatment (Michaelis et al., 2015). However, its involvement in the regulation of LPS-induced microglial COX-2 expression has not been explored. Herein, our results demonstrate that STIM2-Orai activity is a downstream signaling pathway of TLR4 activation stimulated COX-2, and it is essential for RGS10 action on LPS-stimulated COX-2 and TNF-α.

Materials and Methods

Cells

The murine microglial BV2 cell line, which is extensively used to study microglia functions (Henn et al., 2009), was a generous gift from G. Hasko at the University of Medicine and Dentistry of New Jersey (Newark, NJ). The BV2 cell line was derived from primary microglial cell cultures infected with v-raf/v-myc oncogene-carrying retrovirus (J2) (Blasi et al., 1990). RAW264.7 mouse macrophage-like-cell was purchased from the American Type Culture Collection (ATCC® TIB-71™) (Manassas, VA). BV2 and RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (VWR. VWRL0101-0500) (Radnor, PA) supplemented with 10% low-endotoxin fetal bovine serum (Thermo Fisher Scientific. 10082147) (Waltham, MA) and antibiotics of (1% penicillin/streptomycin) (Thermo Fisher Scientific. 15140122) (Waltham, MA) and incubated at 37 °C in a humidified atmosphere 5% CO2.

Reagents

Lipopolysaccharide (LPS) (E. coli strain. L2880) was obtained from Sigma Aldrich (St. Louis, MO). YM-58483 (3939) was purchased from Tocris Bioscience (through Fisher Scientific distributor) (Pittsburgh, PA), Thapsigargin (ab120286) and BAPTA-AM (ab120503) were purchased from Abcam (Cambridge, MA).

Generation of CRISPER/Cas9 control and RGS10 Knockout BV2 cells

Scrambled control vector (K010) and 20-bp guide RNA sequence (49 TGTTTTGCAGATATCCATGA) targeting mouse RGS10 in one-lentivector (K4107305) were purchased from ABM Inc. (Richmond, BC, Canada). 10ng of vectors were transformed into Proclone competent DH5 alpha cells, and the transformants were incubated in 2ml of LB with 100 µg/ml of carbenicillin overnight at 37 °C. The following day, glycerol stocks were prepared by resuspending 500µl of bacterial culture in 500µl of 50% glycerol and stored in a 1.5ml freezing tube at -80 °C.

Furthermore, 1 ml of bacterial culture was transformed into 250ml of LB with 100 µg/ml of carbenicillin and allowed to grow for 15 hours at 37 °C. According to the manufacturer's instructions, the plasmids were extracted from the bacterial culture using the GeneJET plasmid maxiprep kit. The plasmids' concentration and purity were quantified using a Nano Drop spectrophotometer. The sequence of the construct was verified. To produce lentiviral particles, 293T cells plated in two 10 cm^2 dishes transfected with 10µg of expression sgRNA CRISPER/Cas9 vectors and 10µg of ABM Inc.'s third-generation (LVO53) packaging mix in the presence of Lentifectin™ transfection reagent (G074) to facilitate plasmid uptake by cells following company's lentiviral packaging protocol. 24 hours post-infection, the medium was removed and replaced with a fresh complete growth medium for another 24 hours. The viral medium was collected 48 hours post-infection followed by centrifugation at 3000 RPM for 15 minutes at 4°C.

The viral supernatant was separated from cell debris using a low-protein binding 0.45 µM sterile filter and then concentrated by centrifugation at 25000 RPM for two hours at 4°C (~120,000g; SW28 rotor, Beckman, Brea, C). The viral pellets were resuspended with PBS and stored at -80 °C until cell transfection. Different volumes of lentiviral particles were transfected into BV2 microglial cell line in the presence of 0.8 µg/ml of polybrene. 24 hours post-infection, the medium was removed and replaced with a fresh complete culture medium. 48 hours following

transfection, cells were split and selected for stable expression using 6 µg/ml puromycin-selection media as determined by a kill curve assay. Genome-editing was checked using qRT-PCR and western blot analysis.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen. 15596018) (Carlsbad, CA), and cDNA was synthesized from 2µg of total RNA using the High-capacity Reverse Transcriptase cDNA kit (Applied Biosystem. 4368814) (Thermo Fisher Scientific. Waltham, MA). Each cDNA sample was diluted 10-fold, and a 5µl was used in a 14µl PCR reaction (SYBR™ Green PCR Master Mix.) (Thermo Fisher Scientific. 4309155) (Waltham, MA) containing primers at a concentration of 5µM each. All the reactions were run in triplicates. The mRNA expression levels were normalized to the housekeeping β-actin gene and were calculated using the $2^{-\Delta\Delta CT}$ method. Mouse TNF-α primer was obtained from Integrated DNA Technology (IDT) (Coralville, IA), while mouse RGS10 and COX-2 were obtained from Sigma Aldrich (St. Louis, MO). The primer sequences used for gene amplification are listed as follows: TNF-α forward, 5′-CCTGTAGCCCACGTCGTAG-3', TNF-α reverse, 5′-GGGAGTAGACAAGGTACAACCC-3', COX-2 forward, 5'-TGCAAGATCCACAGCCTACC-3', COX-2 reverse, 5'-GCTCAGTTGAACGCCTTTTG-3', RGS10 forward, 5'-CCCGGAGAATCTTCTGGAAGACC-3', RGS10 reverse, 5'-CTGCTTCCTGTCCTCCGTTTTC-3', β-actin forward, 5′-GGCTGTATTCCCCTCCATCG-3', β-actin reverse, 5′-CCAGTTGGTAACAATGCCATGT-3'

Western Blotting

10^5 of plated cells were washed twice with cold-PBS and then were lysed with 120µl of RIPA lysis buffer containing 50mM Tris HCl pH 6.8, 150mM NaCl, 6mM MgCl2, 0.5% sodium deoxycholate, 1% NP-40, and proteases/phosphatase inhibitors cocktail (Cell Signaling Technology. 5872S) (Danvers, MA). Cell lysates were incubated on ice for 30 minutes, followed

by centrifugation at 14000 RPM for 15 minutes at 4°C. Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific. 23225) (Waltham, MA). The cell lysates were mixed with an equal volume of 2X SDS-PAGE sample buffer containing 0.5 M Tris HCl pH 6.8, 10% SDS, 20%Glycerol, 200mM β-mercaptoethanol, and 1%bromophenol blue. The lysates were boiled for 10 minutes at 95 °C, and 20µl of protein sample was separated on 12% SDS-PAGE gels, then transferred to nitrocellulose membranes (Biorad 1620115) (Hercules, CA) using a standard protocol.

Blotted membranes were blocked with 5% nonfat dry milk with shaking at room temperature for one hour and were incubated overnight with the following primary antibodies: goat anti-RGS10 (diluted 1:1000, Santa Cruz Biotechnology: sc-6206), mouse anti-COX-2 (diluted 1:1000, Santa Cruz Biotechnology: sc-166475) (Dallas, TX), and mouse anti-GAPDH (diluted 1:6000, Thermo Fisher Scientific: am4300) (Waltham, MA). Following primary antibodies incubation, blotted membranes were washed three times with 1X-TBST buffer (each time for 5mins) and incubated at room temperature for one hour with the following suitable secondary-HRP conjugated antibodies (diluted 1:5000) donkey anti-goat IgG-HRP (Santa Cruz Biotechnology: sc-2020) (Dallas, TX), and goat anti-mouse IgG HRP (Bethyl Laboratories: A90-116P) (Montgomery, TX). After washing three times with 1X-TBS-T buffer, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, 34580) (Waltham, MA) was applied to detect immunoreactivity of HRP. Fluorchem HD2 software (Protein Simple) (San Jose, CA) was used to quantify western blot bands normalized to the endogenous control, GAPDH.

Co-immunoprecipitation

Cells plated in 15 cm^2 dishes were washed twice with cold-PBS and then lysed in 1ml RIPA buffer (50mM Tris HCl pH 6.8, 150mM NaCl, 6mM MgCl2, 0.5% sodium deoxycholate, 1% NP-40, and proteases/phosphatase inhibitors cocktail (Cell Signaling Technology. 5872S) (Danvers, MA). Cell lysates were incubated for 30 minutes with mild shacking every 10 minutes,

followed by centrifugation at 27216g for 10 minutes at 4°C. 500µl of the collected supernatant was incubated overnight at 4°C with 2µg/ml of normal-goat IgG antibody (R&D system. AB-109-C) (Minneapolis, MN) or goat polyclonal RGS10 antibody (Santa Cruz Biotechnology: sc-6206) (Dallas, TX).

The following day, the cell lysate containing antibody was incubated with 20µl of 50% slurry of Protein G Sepharose® 4 Fast Flow (Millipore Sigma: GH: healthcare, 17-0618-01) (Burlington, MA) for two hours at 4°C and subsequently centrifugated at 2000 RPM for five minutes. The beads were then washed twice with cold-PBS and eluted with 60µl of 2X SDS-PAGE sample buffer containing 0.5 M Tris HCl pH 6.8, 10% SDS, 20%Glycerol, 200mM β-mercaptoethanol, and 1%bromophenol blue. The lysates were boiled for 10 mins at 95 °C. 20µl of eluted beads and 10µl of cell lysate mixed with 10µl of 2X SDS-PAGE sample buffer containing 0.5 M Tris HCl pH 6.8, 10% SDS, 20%Glycerol, 200mM β-mercaptoethanol, and 1%bromophenol blue, which were used to detect the input of proteins, were subjected for western blot analysis. Blotted membranes were blocked with 5% nonfat dry milk, shaking at room temperature for one hour, then incubated overnight with the following primary antibodies goat anti-RGS10 (diluted 1:1000, Santa Cruz Biotechnology: sc-6206) (Dallas, TX), rabbit anti-GNAI3 (Proteintech: 11641-1-AP), and rabbit anti-STIM2 (Proteintech: 21192-1-AP) (Rosemont, IL).

Following primary antibody incubation, blotted membranes were washed with 1X-TBST buffer three times (each time for 5mins) and incubated at room temperature for one hour with the following suitable secondary-HRP conjugated antibodies (diluted 1:5000): donkey anti-goat IgG-HRP (Santa Cruz Biotechnology: sc-2020) (Dallas, TX) and goat antirabbit IgG-HRP (Millipore Sigma: 12-348) (Burlington, MA). After washing with 1X-TBS-T buffer three times, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, 34580) (Waltham, MA) was applied to detect immunoreactivity of HRP.

Statistical Analysis

All quantified data compiled from three independent repeats, unless otherwise noted, were analyzed for statistical difference between groups using one-way ANOVA followed by Tukey post hoc analysis. Prism software was used to carry out statistical analyses. Data are expressed as mean ± SEM where *: P<0.005, **: P<0.01, and ***: P<0.001 indicate the levels of significance.

Results

STIM2 is a novel RGS10 interacting protein in BV2 microglia and RAW264.7 macrophage cells

Our recent work showed that microglial RGS10 suppresses the expression of the inflammatory gene COX-2 and TNF-α upon TLR4 stimulation by LPS via GAP-independent mechanisms (Alqinyah et al., 2018). This suggests that non-canonical RGS10 interacting partners could define the mechanism by which RGS10 regulates TLR4 induced COX-2 and TNF-α expression. To identify endogenous interacting partners associated with RGS10 in microglia, control IgG or endogenous RGS10 were immunoprecipitated from BV2 microglia cell lysate and subsequently subjected to mass spectrometry (MS). The MS analysis identified 25 specific and significant enriched RGS10 binding partners over IgG IP controls (Data not shown). These candidates contain classic RGS10 interacting proteins, guanine nucleotide-binding protein subunits Gαi2 and Gαi3, and non-classical proteins involved in molecular biological functions, such as metabolism, ion channel regulation, intracellular trafficking, and protein degradation. Among the highly enriched RGS10 interacting partners that could facilitate RGS10 regulation of inflammatory COX-2 is stromal interaction molecule (STIM2). STIM2 is an endoplasmic reticulum (ER)- resident calcium sensor that detects calcium release from the ER and mediates calcium entry through interaction and activation of plasma membrane Orai channels. Due to its critical role in the modulation of inflammatory signaling in macrophages (Sogkas et al., 2015), we characterized RGS10 interaction with STIM2. To validate the interaction and specificity of RGS10-

binding ER-calcium sensor STIM2, we immunoprecipitated endogenous RGS10 out of CRISPER/Cas9 control BV2 cells, naturally expressing RGS10 proteins and CRISPER/Cas9 RGS10 knockout BV2 cells followed by immunoblotting analysis to probe against RGS10, Gαi3, and STIM2. In addition to Gαi3 as a canonical binding partner, STIM2 was highly enriched with RGS10 in control BV2 cells with no detectable binding in RGS10 KO cells and IgG-beads IP condition, where RGS10 was not enriched **(Figure. 4.1A)**. To explore the possibility that STIM2 might bind RGS0 in other cell types, we performed RGS10 IP in RAW264.7 macrophage cells and observed interaction between RGS10 with STIM2 **(Figure. 4.1B)**, suggesting that this binding is not BV2 microglial cell-specific. Taken together, these results have identified STIM2 as a novel RGS10 binding partner in microglia and macrophages.

Orai channel-mediated calcium influx is essential for LPS-induced COX-2 expression

Previous studies have demonstrated that STIM2-Orai activity is dysregulated and implicated in the modulation of neuroinflammatory disease models (Secondo et al., 2018). In addition, STIM2 deficient macrophages produced lower proinflammatory cytokines, including TNF-α, IL-6 and IL-1β, upon LPS stimulation compared to WT macrophages (Sogkas et al., 2015). However, its effect on LPS-induced microglial inflammatory genes expression, such as COX-2, has not been addressed. To test whether STIM2/Orai-mediated SOCE deficiency affects LPS-stimulated COX-2, BV2 microglia cells were transiently transfected with STIM2 targeted siRNA or control siRNA. 24hrs following transfection, the cells were treated with LPS for an additional 24hrs. Knockdown of STIM2 significantly inhibited LPS-stimulated COX-2 protein (Data not shown). Store operated Ca^{2+} entry (SOCE) is triggered by depletion of intracellular Ca^{2+} from its ER store and subsequently followed by Ca^{2+} influx through activation and opening of plasma membrane Orai Ca^{2+} channels (Soboloff et al., 2012, Nelson et al., 2018). To assess the contribution of intracellular Ca^{2+} or influx of extracellular Ca^{2+} in the regulation of microglial COX-2 expression in response to LPS treatment, we used BAPTA-AM, a Ca^{2+} chelator, to remove

intracellular Ca^{2+} and YM58483 (also known as BTP2), an Orai inhibitor, to block the influx of extracellular Ca^{2+}. BV2 cells were treated with either YM58483 or BAPTA-AM for one hour prior to further treatment with vehicle or LPS for 24hrs. Blocking Orai channels-mediated cytoplasmic Ca^{2+} generation resulted in a significant reduction in LPS-induced COX-2 mRNA **(Figure 4.2A)** and protein levels **(Figure 4.2B)** compared to LPS treatment alone. Similar to the Orai channels blocker effect, inhibition of intracellular Ca^{2+} by BAPTA-AM suppressed LPS stimulated COX-2 protein expression **(Figure 4.2C)**. These results indicate that TLR4 stimulation by LPS mediated COX-2 induction requires STIM2 and Orai-mediated Ca^{2+} signaling.

Effect of ER-calcium store depletion on LPS regulation of COX-2 and RGS10 expression

The molecular machinery-mediated SOCE formation is triggered by the depletion of intracellular calcium from its ER store. In addition, our previous results have shown that activation of STIM2-Orai mediated calcium influx facilitates COX-2 expression in response to LPS. Since ER Ca^{2+} store depletion activates SOCE formation, we next determined whether ER Ca^{2+} store depletion affects LPS-stimulated COX-2 expression. We pretreated BV2 microglia cells with Thapsigargin (TG, a pharmacological blocker of SERCA- Ca^{2+} -ATPase) for 20 minutes to induce depletion of Ca^{2+} within ER stores prior to vehicle or LPS stimulation. TG pretreatment for 20 minutes before vehicle treatment for 24hrs induced a slight increase in COX-2 transcript **(Figure 4.3A)** and protein levels **(Figure 4.3C)**. However, pretreatment with TG for 20 minutes followed with LPS for 24hrs synergistically enhanced LPS-induced COX-2 mRNA **(Figure 4.3A)** and protein levels **(Figure 4.3C)** compared to LPS treatment alone. In addition, TG-pretreatment strongly enhanced LPS-induced RGS10 silencing **(Figure 4.3B and 4.3C)**. Taken together, these findings indicate that intracellular calcium store depletion amplifies LPS effects on COX-2 induction and RGS10 suppression.

Role of the Orai channel-mediated extracellular calcium entry signaling in RGS10 sensitive COX-2 expression

Given that STIM2 was identified as an RGS10 binding partner in microglia and its function mediating extracellular calcium entry signaling through activation of Orai channels in response to ER calcium depletion is required for LPS-induced COX-2 expression, we next sought to determine whether RGS10 anti-inflammatory effects require Orai channels activation. We measured the transcript and the protein levels of COX-2 in established control and RGS10 KO BV2 cells following LPS treatment for 24hrs in the presence or absence of an Orai channels inhibitor (YM58483). Pharmacological inhibition of Orai channel activity with YM58483 completely blocked amplification of LPS-stimulated COX-2 mRNA **(Figure 4.4A)** and protein levels **(Figure 4.4B)** in RGS10 KO cells. Therefore, these data suggest that Orai channel activity is essential for RGS10 effects on LPS-induced COX-2 expression.

Role of the Orai channel-mediated extracellular calcium entry signaling in RGS10 mediated TNF-α expression

In addition to COX-2, loss of RGS10 has been shown to enhance proinflammatory cytokine TNF-α transcript, and this effect does not require Gαi activity (Alqinyah et al., 2018). We tested whether Orai mediated calcium signaling affects RGS10-sensitive TNF-α mRNA following microglia activation by LPS. Similar to the observed COX-2 effect, LPS-stimulated TNF-α mRNA was not affected by the loss of RGS10 in the presence of Orai channels inhibitor **(Figure 4.5)**. Collectively, the result demonstrates that Orai channels activity is required for the inhibitory effect of RGS10 on LPS-induced TNF-α expression.

Discussion

The small regulator of G protein signaling, protein RGS10, has profound effects in diverse cells, including microglia (Lee et al., 2008, Lee et al., 2011), macrophages (Lee et al., 2013), neurons (Lee;Chung; et al., 2012), osteoclasts (Yang et al., 2007, Yang and Li, 2007, Yang et al., 2013), T cells (Garcia-Bernal et al., 2011), cancer cells (Hooks et al., 2010, Altman et al., 2015), and cardiomyocytes (Miao et al., 2016). Although RGS10 impacts the physiology and pathophysiology of these cells, the highest expression of RGS10 is found in the brain and immune cells, with abundant expression in microglia. Microglial RGS10 exerts strong anti-inflammatory effects, in which RGS10 suppresses LPS-induced inflammatory cytokines genes expression (Lee et al., 2008, Lee et al., 2011), including COX-2 and its production of PGE2 (Alqinyah et al., 2018). Recent work from our group has shown that RGS10 negatively regulates COX-2 and TNF-α through an unknown, Gαi protein-independent mechanism (Alqinyah et al., 2018). Despite this important finding, the mechanism that mediates RGS10 anti-inflammatory activity has not been defined. Thus, in this study, we aimed to identify binding partners of RGS10 in microglia that could facilitate its anti-inflammatory functions.

Herein, in addition to classic Gαi3 protein interaction, we identify and show a strong interaction between RGS10 and ER-localized calcium sensor STIM2 in microglial BV2 and RAW264.7 macrophage cells. As an intracellular Ca^{2+} sensor, upon ER calcium reduction, STIM2 translocates to ER-PM junction, where it interacts with plasma membrane Orai channels to mediate sustained extracellular Ca^{2+} entry (Stathopulos et al., 2009, Palty et al., 2017). Due to the implicated role of STIM2-Orai activity in LPS-stimulated pro-inflammatory cytokine production in macrophages (Sogkas et al., 2015), we demonstrate that Orai-triggered Ca^{2+} entry is required for LPS-induced COX-2 and TNF-α expression in microglia, and it is essential for microglial RGS10 suppressive effects on COX-2 induction following TLR4 activation.

In microglia, a previous study has shown even distribution of RGS10 between cytoplasm and nucleus under resting conditions. However, following LPS treatment, RGS10 is robustly

enriched in the nucleus (Lee et al., 2008). While SOCE, which is regulated by STIM2 protein, is induced by LPS in microglia (Sun et al., 2014, Wang;Liu;Tian; et al., 2015), it will be interesting to check first whether RGS10 colocalizes with ER-resident STIM2 protein under basal condition and whether LPS-induced microglia activation affects this colocalization. Further, it will be interesting to check whether RGS10 regulates STIM2 translocation to ER-PM junction to mediate calcium homeostasis.

RGS10 is a simple RGS protein, containing only a conserved RGS domain, shared among all the members of the RGS protein family (Hollinger and Hepler, 2002), and lacking regulatory motifs and functional domains that have been found in the other canonical RGS proteins (Squires et al., 2018). While other RGS proteins interact with most of their non-canonical interacting partners via functional domains beyond their RGS homology domain (Sethakorn et al., 2010), we expect the RGS10-STIM2 interaction to be mediated via the RGS domain. However, it is not known whether STIM2 has a direct interaction with RGS10 or is a part of a multiple-protein complex. Thus, further biochemical studies are required to characterize whether the RGS domain of RGS10 associates with STIM2 and whether other RGS proteins members interact with STIM2.

Store-operated calcium influx channels, also known as Ca^{2+} release-activated Ca^{2+} channels (CRAC), are mainly formed by Orai proteins in the plasma membrane and activated by STIM proteins, which coordinate calcium release from ER stores to calcium entry through Orai channels. Ca^{2+} influx via Orai channels leads to elevated cytosolic Ca^{2+}signals, which in turn activates Ca^{2+} sensitive phosphatase calcineurin. Activation of calcineurin is essential for microglial COX-2 induction by LPS and is required for the RGS10 effect on inhibiting LPS-stimulated COX-2 in microglial (Data not shown). NFAT, as a major substrate of calcineurin, is a transcriptional factor for the induction of multiple inflammatory mediators, including COX-2 and TNF-α expression (Buxade et al., 2012, Bendickova et al., 2017). Previous studies have found that STIM2 knockout suppresses NFAT activation (Oh-Hora et al., 2008) and blockage of CRAC inhibits the activation of NFkB and NFAT (Mizuma et al., 2019). In addition, a prior study showed

that knockdown of NFAT in BV2 cells suppresses TNF-α expression (Manocha et al., 2017). While the loss of RGS10 in microglia leads to enhanced expression and transcriptional activity of NF-κB, it will be interesting to determine whether NF-κB and/or NFAT are required for RGS10 regulation COX-2 and TNF-α expression. Since LPS has been recently linked to SOCE in microglia, which is triggered by ER calcium depletion and leads, in turn, to reload ER calcium stocks, our results demonstrate that depletion of ER calcium store by TG treatment amplifies LPS-induced COX-2 and LPS-induced RGS10 suppression. Thus, it will be interesting to check whether RGS10 has an impact on LPS-induced SOCE in microglia.

In addition to the anti-inflammatory function of RGS10 in microglia, RGS10 is expressed in ovarian cancer cells, where its expression is suppressed during the development of chemoresistance, and loss of RGS10 in chemosensitive ovarian cancer cells enhances cell viability and promotes chemotherapeutic drug resistance (Hooks et al., 2010). Our recent results have shown that RGS10 regulation of COX-2 and TNF-α expression is independent of amplified Gαi signaling (Alqinyah et al., 2018) (chapter 5). While COX-2 is associated with ovarian cancer chemoresistance (Ye et al., 2020), upregulation of SOCE is implicated in ovarian cancer resistance (Schmidt et al., 2014, Bonnefond et al., 2018, Huang et al., 2020) and regulation of COX-2 in cancer cells (Huang;Chai; et al., 2011, Wang;Sun; et al., 2015, Wong et al., 2017). Therefore, beyond the involvement of SOCE in RGS10's regulation of LPS-induced COX-2 upregulation in microglia, SOCE could participate in ovarian cancer chemoresistance, in which alteration of RGS10 expression is involved.

Conclusion

In this study, our proteomic analysis has identified that STIM2, which functionally binds and opens plasma membrane Orai Ca^{2+} channels to mediate SOCE upon ER stored Ca^{2+} depletion, is an endogenous RGS10 binding partner in microglia. We confirmed the interaction and showed the specificity of RGS10 binding STIM2 in control and RGS10 deficient BV2 cells and RAW264.7 macrophages. This interaction between RGS10 and STIM2, which has a functional role in calcium signaling-mediated inflammatory responses, is novel and critical in understanding RGS10 Gαi-independent anti-inflammatory mechanisms. Further, our results showed that STIM2/Orai mediated calcium signaling regulates COX-2 induction following TLR4 stimulation by LPS in microglia. In addition, the anti-inflammatory action of microglial RGS10 on LPS-induced COX-2 and TNF-α requires Orai channels activity. Finally, we demonstrated that TG-mediated Ca^{2+} mobilization strongly amplifies LPS-stimulated COX-2 expression and enhances LPS-mediated suppression of RGS10. Together, our findings shed light on the biological function of STIM2/Orai activity in mediating the inhibitory effect of RGS10 on LPS-induced COX-2 and TNF-α expression.

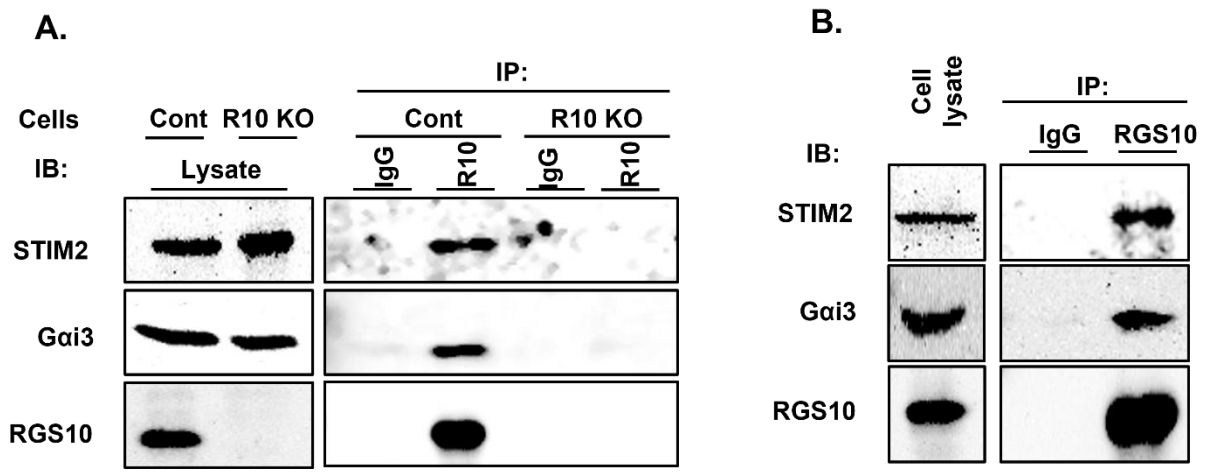

Figure 4.1. STIM2 is a novel RGS10 interacting protein in BV-2 microglia and RAW264.7 macrophage cells.

A. BV-2 microglia cells stably infected with control or RGS10-targeted CRISPR/Cas9 lentivirus and **B.** RAW264.7 macrophage cells were plated in 15cm^2 dishes and then lysed with CO-IP lysis buffer. Co-immunoprecipitation was performed using RGS10 or Control IgG antibodies. Western blotting analysis was conducted to probe for RGS10, Gαi3, and STIM2. Image in **(A)** represents only one experiment, while the image in **(B)** is representative of two independent experiments.

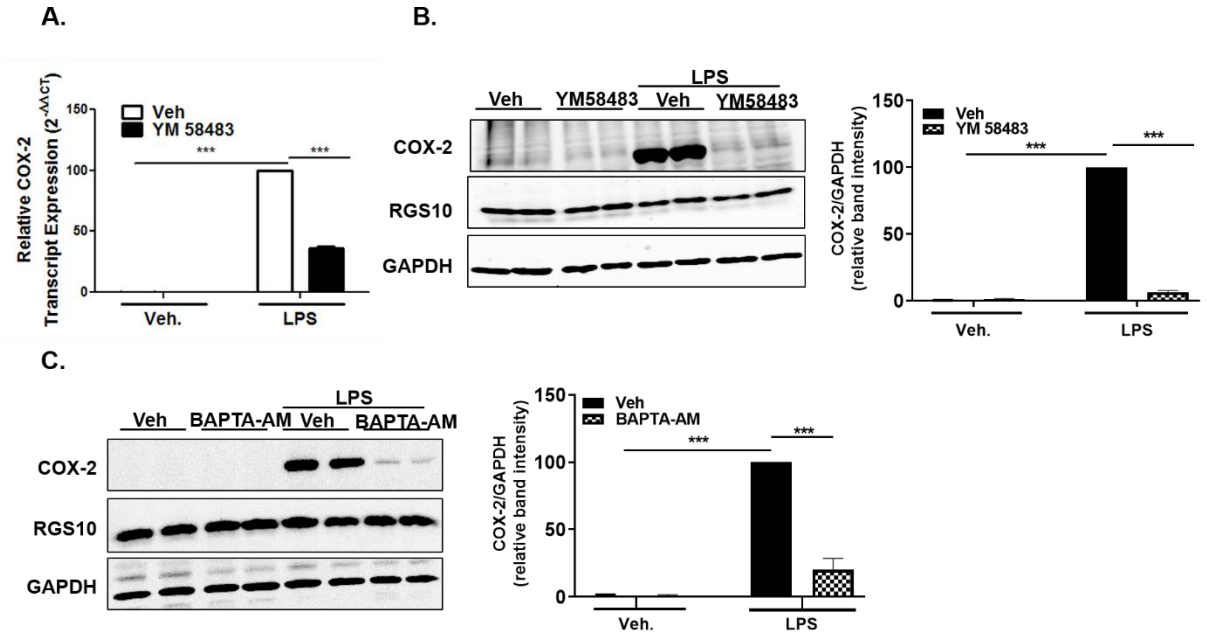

Figure 4.2. Orai channel-mediated calcium influx is essential for LPS-induced COX-2 expression.

Wild-type BV2 cells were plated in a 12-well plate and cultured overnight. Cells were then treated with serum-free media (veh.) or 10 μM YM58483 (YM) for 1 hour prior to 24-hour incubation with veh. or LPS (10 ng/mL). **A.** RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. COX-2 transcript level was quantified using qRT-PCR and was normalized to the endogenous control actin. The expression of COX-2 mRNA was calculated by the ($2^{-\Delta\Delta Ct}$) method. **B.** Cells were lysed and subjected to SDS-PAGE, and western blotting was performed using specific antibodies against COX-2, RGS10 and GAPDH. Quantified densitometry of the COX-2 band was normalized to GAPDH. **C.** Wild-type BV2 cells were plated in a 12-well plate and cultured overnight. Cells were then treated with serum-free media (veh.) or 10 μM BAPTA-AM for 1 hour before 24-hour incubation with veh. or LPS (10 ng/mL). Cells were lysed and subjected to SDS-PAGE, and western blotting was performed using specific antibodies against COX-2, RGS10 and GAPDH. Quantified densitometry of the COX-2 band was normalized to GAPDH. Data in **(A-C)** is compiled from three independent experiments. Data were analyzed

for statistical differences using analysis of variance, followed by Tukey's test between groups. Data are presented as mean ± SEM, where *** $p<0.001$ indicates the level of significance.

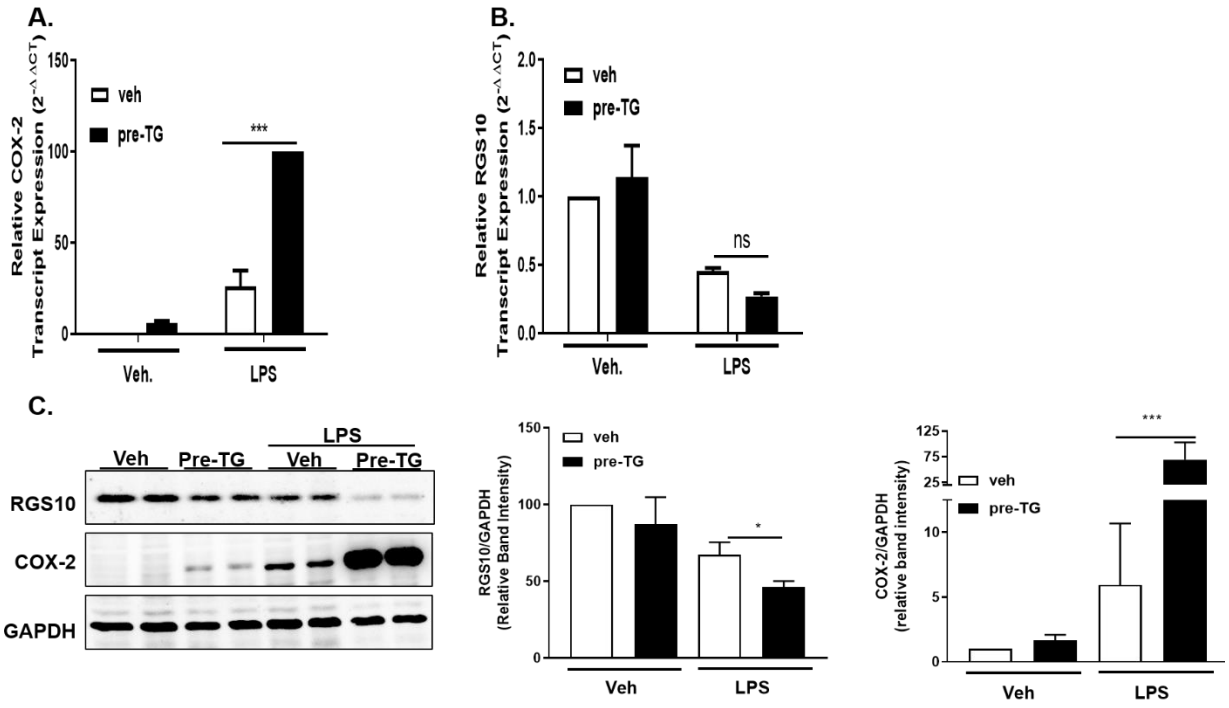

Figure 4.3. Effect of ER-calcium store depletion on LPS regulation of COX-2 and RGS10 expression.

Wild type BV2 cells were pretreated with vehicle (Veh) or 1 μM thapsigargin (TG) for 20 minutes and then removed prior to incubation with either vehicle or LPS (10ng/mL) for 24 hours. RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. COX-2 **(A)** and RGS10 **(B)** transcript levels were quantified using qRT-PCR and were normalized to the endogenous control actin. The relative expression was calculated by the ($2^{-\Delta\Delta Ct}$) method. Data in **(A&B)** were compiled from three experimental repeats. **C.** Wild type BV2 cells were pretreated with vehicle (Veh) or 1 μM thapsigargin (TG) for 20 minutes and then removed prior to incubation with either vehicle or LPS (10ng/mL) for 24 hours. Cells were lysed and subjected to SDS-PAGE, and western blotting was performed using specific antibodies against COX-2, RGS10 and GAPDH. Representative image and quantification of COX-2 and RGS10 protein levels using densitometry were compiled from three independent experiments. Data were

analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Data are presented as mean ± S.E.M., where *** $P < 0.001$, * $P < 0.05$, n.s., not significant.

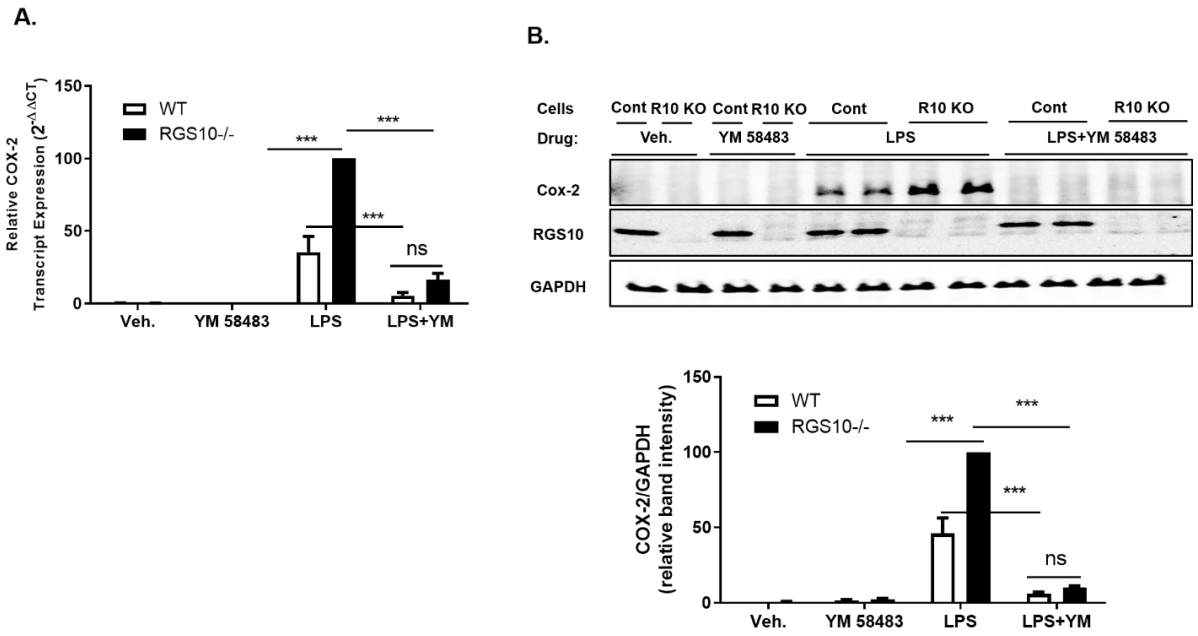

Figure 4.4. Role of the Orai channel-mediated extracellular calcium entry signaling in RGS10 sensitive COX-2 expression.

Control (Con.) and RGS10 knockout (-/-) BV2 cells were treated with serum-free media (veh.) or 10 μM YM58483 (YM) for 1 hour prior to 24-hour incubation with veh or LPS (10 ng/mL). **A.** RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. COX-2 transcript level was quantified using qRT-PCR and was normalized to the endogenous control actin. The expression of COX-2 mRNA was calculated by the ($2^{-\Delta\Delta Ct}$) method and was compiled from three experimental repeats. **B.** Cells were lysed and subjected to SDS-PAGE, and western blotting was performed using specific antibodies against COX-2, RGS10 and GAPDH. Representative image and quantification of COX-2 protein level using densitometry were compiled from three independent experiments. Data were analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Data are presented as mean ± S.E.M., where *** $P < 0.001$, n.s., not significant

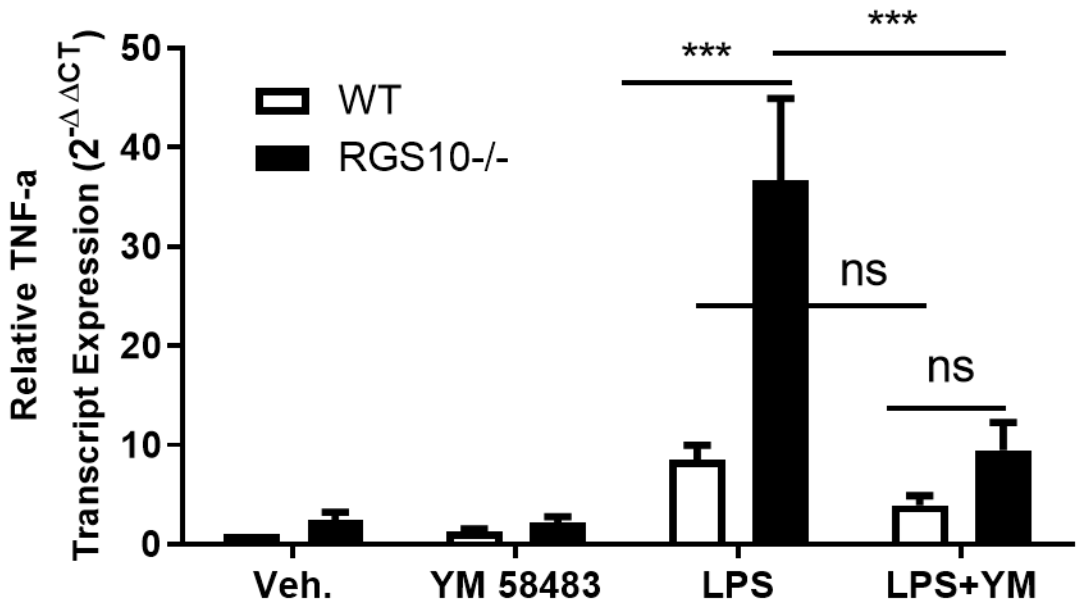

Figure 4.5. Role of the Orai channel-mediated extracellular calcium entry signaling in RGS10 mediated TNF-α expression.

Control (Con.) and RGS10 knockout (-/-) BV2 cells were treated with serum-free media (veh.) or 10 μM YM58483 (YM) for 1 hour prior to 24-hour incubation with veh or LPS (10 ng/mL). **A.** RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. TNF-α transcript level was quantified using qRT-PCR and was normalized to the endogenous control actin. The expression of TNF-α mRNA was calculated by the ($2^{-\Delta\Delta Ct}$) method and was compiled from three experimental repeats. Data were analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Data are presented as mean ± S.E.M., where *** $P < 0.001$, n.s., not significant

CHAPTER 5

RGS10 INHIBITS NF-κB SIGNALING, CYCLOOXYGENASE-2, AND TUMOR NECROSIS FACTOR-ALPHA EXPRESSION VIA A GAI PROTEIN-INDEPENDENT MECHANISM IN OVARIAN CANCER CELLS[4]

[4] Almutairi F, Hooks SB. *Mol Pharmacol*. 2018 Oct; 94(4):1103-1113

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Abstract

Epithelial Ovarian Cancer (EOC) is the fifth leading cause of cancer-related deaths among females in the United States. NF-κB signaling and the production of inflammatory mediators have been strongly linked to ovarian cancer progression and chemoresistance. Regulator of G protein Signaling 10 (RGS10) regulates ovarian cancer survival and chemoresistance and NF-κB signaling in immune cells. The goal of the current study was to determine the ability of RGS10 to inhibit NF-κB signaling and subsequent inflammatory effects in ovarian cancer. RGS10 is a negative regulator of Gαi signaling, and multiple Gi coupled GPCRs are implicated in ovarian cancer. Therefore, we predicted that RGS10 regulates NF-κB signaling and inflammatory cytokines downstream of Gαi mediated pathway. We found that knock-down of RGS10 enhanced NF-κB-p65 subunit phosphorylation and produced significantly higher levels of COX-2 and TNF-α mRNA compared to cells transfected with control siRNA in SKOV-3 EOC cells. Surprisingly, we found that Pertussis toxin (ptx), a Gαi inhibitor, had no effects on upregulation of p-NF-κB, COX-2 and TNF-α expression-mediated by RGS10 knock-down. These results provide insight into the mechanism that RGS10 utilizes to regulate ovarian cancer survival and chemoresistance

Highlights

- RGS10 regulates NF-κB activation and suppresses the production of inflammatory genes TNFα and COX2 expression in ovarian cancer cells.
- Upregulation of inflammatory signaling mediated by RGS10 knockdown is not affected by Gαi inhibition.

Introduction

Epithelial ovarian cancer (EOC), which represents 90% of ovarian cancer cases, is a deadly gynecological malignancy. Beyond the diagnosis, ovarian cancer patients will survive approximately five years due to the high percentage of metastasis into the peritoneal cavity and chemoresistance (Lheureux et al., 2019). The standard treatment for ovarian cancer patients, presented in advanced stages, comprises a combination of tumor debulking surgery followed by platinum and paclitaxel chemotherapy. Despite a high response rate in ovarian cancer treatment, most ovarian cancer patients frequently experience disease relapse and drug-resistant disease (Kim et al., 2018). Thus, understanding the molecular mechanisms underlying chemotherapy resistance is critical to developing therapeutic strategies, improving current chemotherapeutic agents, and thereby, patient survival.

Emerging evidence suggests that inflammatory signaling pathways are strongly linked to ovarian cancer progression and chemoresistance (Maccio and Madeddu, 2012, Savant et al., 2018). Inflammation-related mediators, such as cytokines, chemokines, and bioactive lipids, affect proliferation, migration, and chemoresistance (Mantovani et al., 2008, Grivennikov et al., 2010). In particular, tumor necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) promote ovarian cancer development. Tumor Necrosis Factor-alpha TNF-α, one of the major pro-inflammatory cytokines, is significantly produced by malignant and immune cells within the tumor microenvironment (Moore et al., 1999, Harrison et al., 2007, Zins et al., 2007, Egberts et al., 2008). TNF-α drives the progression of many cancers by serving as an autocrine and a paracrine factor to induce numerous cytokines, chemokines, pro-angiogenic factors, and metalloproteinases through activation of NF-κB transcription factor (Wu et al., 1993, Leber and Balkwill, 1998, Kulbe et al., 2007, Son et al., 2013). Activated NF-κB has been identified as a key factor in ovarian cancer chemoresistance (Annunziata et al., 2010) and abrogation of elevated NF-κB activity using Iκ-B phosphorylation inhibitor sensitizes ovarian cancer models to paclitaxel-induced apoptosis (Mabuchi et al., 2004). Cyclooxygenases-2 (COX-2), a key enzyme in PGE2

release, is highly upregulated in epithelial ovarian cancer (Seo et al., 2004) and is significantly correlated with poor prognosis for ovarian cancer patients (Sun et al., 2017). Overexpression of COX-2 enhances some ovarian cancer cells' viability, invasion, and chemoresistance (Spinella et al., 2004, Gu et al., 2008, Uddin et al., 2010).

Regulator of G protein signaling (RGS) proteins are a highly diverse family of proteins defined by the RGS domain that binds and deactivates heterotrimeric G-protein signaling (Watson et al., 1996). Canonically, RGS proteins terminate G-protein signaling pathways by serving as GTPase activating proteins, which enhance the hydrolysis of the active GTP-bound Gα to the inactive GDP-bound Gα proteins (Hollinger and Hepler, 2002). Dysregulation of RGS proteins has been shown to be strongly linked to various cancers, including breast (Xie et al., 2009), prostate (Cao et al., 2006), lung (Xu et al., 2015), and melanoma (Sun;Wang; et al., 2018).

RGS10, a member of the D/R12 subfamily of RGS proteins, is a small RGS protein that lacks structural domains and functional motifs outside of the RGS domain. RGS10 has been shown to preferentially bind and inactivate the Gαi family of G-proteins (Hunt et al., 1996). In ovarian cancer, RGS10 has been identified as a key factor in regulating ovarian cancer survival (Hooks et al., 2010, Hooks and Murph, 2015), where its expression is epigenetically suppressed in multiple models of chemoresistant ovarian cancer cells (Hooks et al., 2010, Ali et al., 2013). Silencing RGS10 in chemosensitive cells leads to chemoresistance (Hooks et al., 2010, Altman et al., 2015). In addition to its role in ovarian cancer, RGS10 is abundantly expressed in immune cells. In microglia and peripheral macrophages, RGS10 acts as an anti-inflammatory regulator by suppressing microglia/macrophage NF-κB activity and producing pro-inflammatory mediators (Lee et al., 2008, Lee et al., 2011, Lee et al., 2013).

This study aims to determine whether RGS10 regulates NF-κB activity, TNF-α and COX-2 expression in a GAP-dependent mechanism in ovarian cancer. We have shown for the first time that loss of RGS10 significantly amplifies NF-κB-p65 subunit phosphorylation, TNF-α and COX-2 transcript levels in SKOV3 EOC cells. Moreover, upregulation of inflammatory signaling mediated

by RGS10 knockdown was not affected by Gαi inhibition, suggesting that RGS10 suppresses NF-κB activation, TNF-α and COX-2 expression by Gαi protein-independent mechanism.

Materials and Methods

Cells

The SKOV-3 human ovarian adenocarcinoma cells were purchased from the American Type Culture Collection (ATCC® HTB-77™) (Manassas, VA). SKOV-3 cells were grown in McCoy's 5A 1X (Iwaketa &Grace Modification) medium (10-050-CV) (Corning, NY) supplemented with 10% fetal bovine serum (VWR. 89510-186) (Radnor, PA) and an antibiotics combination of (1% penicillin/streptomycin) (Thermo Fisher Scientific. 15140122) (Waltham, MA) and incubated at 37 °C in a humidified atmosphere 5% CO2 incubator.

Reagents

Pertussis toxin (ptx) (3097) and 18:1 lysophosphatidic acid (18:1 LPA) (857130) were obtained from Tocris Bioscience (through Fisher Scientific distributor) (Pittsburgh, PA) and Avanti Polar Lipids (Alabaster, AL), respectively.

Small Interfering RNA (siRNA) transfection

Human siRNA targeted RGS10 (sc-36410) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, TX). In an antibiotic-free culture medium and following the manufacture's protocol, SKOV-3 cells were cultured in a 24-well plate and were transfected using (Lipofectamine-LTX with PLUS reagent) (Thermo Fisher Scientific, 15338100) (Waltham, MA) with 60 nM/well of siRNA duplexes. 48hrs after transfection, the cells were harvested, and the knockdown efficiency was assessed by measuring RGS10 transcript and protein levels. The final concentration of siRNA duplexes is scaled up or down based on the well size of the plate.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from SKOV-3 cells using TRIzol reagent (Invitrogen. 15596018) (Carlsbad, CA). cDNA was synthesized from 2µg of total RNA using the High-capacity Reverse Transcriptase cDNA kit (Applied Biosystem. 4368814) (Thermo Fisher Scientific. Waltham, MA). Each cDNA sample was diluted 10-fold, and a 5µl was used in a 14µl PCR reaction (SYBR™ Green PCR Master Mix.) (Thermo Fisher Scientific. 4309155) (Waltham, MA) containing primers at a concentration of 5µM each. All the reactions were run in triplicates. The mRNA expression levels were normalized to the housekeeping GAPDH gene and were calculated using the $2^{-\Delta\Delta CT}$ method. Human TNF-α and GAPDH primers were obtained from Integrated DNA Technology (IDT) (Coralville, IA), while human RGS10 and COX-2 were obtained from Sigma Aldrich (St. Louis, MO). The primer sequences used for gene amplification are listed as follows: TNF-α forward, 5′-CTCTTCTGCCTGCACTTTG-3', TNF-α reverse, 5′-ATGGGCTACAGGCTTGTCACTC-3', GAPDH forward, 5′- GCCAAGGTCATCCATGACAACT-3', GAPDH reverse, 5′-GAGGGGCCATCCACAGTCTT-3', Cox-2 forward 5'-CCCTTGGGTGTCAAAGGTAA-3', Cox-2 reverse 5'-GCCCTCGCTTATGATCTGTC-3', RGS10 forward, 5'-'GACCCAGAAGGCGTGAAAAGA 3', RGS10 reverse, 5′-GCTGGACAGAAAGGTCATGTAGA-3'

Western Blotting

SKOV-3 cells (10^5) were washed twice with cold-PBS and then were lysed with 120µl of 2X SDS-PAGE sample buffer containing (0.5 M Tris HCl pH 6.8, 10% SDS, 20%Glycerol, 200mM β-mercaptoethanol, and 1%bromophenol blue). The lysates were boiled for 10 mins at 95 °C, and 20µl of protein sample was separated on 12% SDS-PAGE gels, then transferred to nitrocellulose membranes (Biorad 1620115) (Hercules, CA) using the standard protocol. Blotted membranes were blocked with 5% nonfat dry milk by shaking at room temperature for one hour, and then incubated overnight with the following primary antibodies: goat anti-RGS10 (diluted 1:1000, Santa

Cruz Biotechnology: sc-6206), mouse p-65 (diluted 1:1000, Santa Cruz Biotechnology: sc-372) (Dallas, TX), rabbit phospho NF-kB-p65 (Ser536) (diluted 1:500, Cell Signaling technology: 93H1) (Danvers, MA), and mouse anti-GAPDH (diluted 1:6000, Thermo Fisher Scientific: am4300) (Waltham, MA). Following primary antibody incubation, blotted membranes were washed with 1X-TBST buffer three times (each time for 5mins) and incubated at room temperature for one hour with the following suitable secondary-HRP conjugated antibodies (diluted 1:5000) donkey anti-goat IgG-HRP (Santa Cruz Biotechnology: sc-2020) (Dallas, TX), goat anti-rabbit IgG-HRP (Millipore Sigma, 12-348) (Burlington, MA), and goat anti-mouse IgG HRP (Bethyl Laboratories: A90-116P) (Montgomery, TX). After washing with 1X-TBS-T buffer three times, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, 34580) (Waltham, MA) was applied to detect immunoreactivity of HRP.

Statistical Analysis

Unless otherwise noted, all quantified data were compiled from three independent repeats and were analyzed for statistical difference between groups using student's t-test or one-way ANOVA followed by Tukey post hoc analysis. Prism software was used to carry out statistical analyses. Data are expressed as mean ± SEM where *: $P<0.005$, **: $P<0.01$, and ***: $P<0.001$ indicate the significance levels.

Results

RGS10 silencing amplifies phosphorylation of p65-NF-κB and TNF-α transcript in SKOV-3 EOC cells

Previous work demonstrated that RGS10 regulates LPS-induced NF-κB signaling and the expression of inflammatory mediators, including TNF-α in microglia (Lee et al., 2008, Lee et al., 2011) and macrophages (Lee et al., 2013). However, the impact of the loss of RGS10 on LPS-stimulated NF-κB signaling and TNF-α mRNA has not been tested in ovarian cancer. Firstly, we tested whether LPS induces NF-κB subunit p65 phosphorylation and TNF-α transcript level in SKOV-3 cells. We treated SKOV-3 cells with LPS (10 μg/ml) for 20 minutes to check the phosphorylation of p65 and for 6 hours to check TNF-α mRNA induction. Surprisingly, LPS does not induce NF-κB subunit p65 phosphorylation and TNF-α expression in SKOV-3 cells (Data not shown). Then, we aimed to determine whether RGS10 knockdown affects the basal levels of NF-κB signaling and the transcript level of TNF-α. We transfected SKOV-3 EOC cells with RGS10 siRNA and control siRNA for 48 hours. Following transfection, cells were harvested to check the efficiency of knockdown, which consistently resulted in a significant reduction in RGS10 protein level (**Figure. 5.1A**). Importantly, loss of RGS10 amplified NF-κB subunit p65 phosphorylation, an early event in the NF-κB signaling activation (**Figure. 5.1A**). Furthermore, following the transient siRNA-mediated knockdown, RNA was isolated, and TNF-α transcript expression was quantified using quantitative RT-PCR. SKOV-3 cells transfected with RGS10 siRNA produced a higher level of TNF-α compared to cells transfected with control siRNA (**Figure. 5.1B**). Collectively, the results indicate that endogenous RGS10 regulates p-NF-κB subunit p65 and the expression of TNF-α in ovarian cancer.

The effect of RGS10 WT and E52K mutant on Gαi protein signaling pathways

We generated a single amino acid mutation E52K in RGS10 based on the characterized GAP-dead mutation site in the RGS domain of RGS12 protein (Sambi et al., 2006). We confirmed

the inability of the E52K mutant to bind the active form of Gαi3 ($G\alpha_i3$-GDP-AlF_4^-) by co-immunoprecipitation experiment in HEK293 cells (Data not shown). To address the effect of RGS10 WT and E52K mutant on Gαi protein signaling, we transfected SKOV-3 EOC cells in the presence of serum with empty vector, RGS10 WT, and RGS10 E52K mutant for 48 hours. In these cells, ERK1/2 phosphorylation was reduced by overexpression of RGS10, while RGS10 E752K mutant could not inhibit ERK1/2 phosphorylation (**Figure. 5.2**). This result confirms the RGS10 action in regulating basal ERK1/2 phosphorylation, while the GAP-dead mutant RGS10 is non-functional to inhibit G-protein signaling.

The effect of Gαi inhibition on RGS10 knockdown-induced enhancement of NF-κB subunit p65 phosphorylation

Canonically, RGS10 as GAP is selective to bind Gαi proteins and deactivate signaling pathways mediated by Gαi proteins coupled receptors (Hunt et al., 1996, Masuho et al., 2020). Thus, we sought to determine whether amplification of p-NF-κB subunit p65 resulting from RGS10 knockdown is the result of amplified Gαi signaling. We transfected SKOV-3 EOC cells with RGS10 siRNA or control siRNA in the presence or absence of Gαi/o family inhibitor pertussis toxin (PTX). Surprisingly, PTX treatment did not affect RGS10 siRNA-mediated upregulation of phosphorylation of NF-κB subunit p65 (**Figure. 5.3A**). To confirm that the dose of PTX used in this experiment is effective in SKOV-3 cells, we treated SKOV-3 cells with LPA with or without PTX and assessed ERK phosphorylation in SKOV-3, an established Gαi-mediated event (Hurst et al., 2008). PTX treatment completely suppressed LPA-induced phosphorylation of ERK (**Figure. 5.3B**). Therefore, these data suggest that RGS10 knockdown-mediated amplification of p-NF-κB subunit p65 is not mediated by enhanced Gαi signaling.

RGS10 silencing-upregulates TNF-α and COX-2 expression and is mediated by a Gαi-independent mechanism

Based on our previous result demonstrating that induction of phosphorylation of NF-κB subunit p65 is not affected by Gαi signaling inhibition, we aimed to assess whether RGS10 regulates the transcript levels of TNF-α and COX-2 in a Gαi-independent mechanism. We transfected SKOV-3 EOC cells with RGS10 siRNA or control siRNA with or without PTX treatment. Following siRNA transfection, we first confirmed a successful knockdown using the RT-PCR method, which results in a 50% reduction in the RGS10 mRNA (**Figure. 5.4A**). p-NF-κB subunit p65 amplification and the increases in TNF-α (**Figure. 5.4B**) and COX-2 (**Figure. 5.4C**) mediated by RGS10 knockdown was completely resistant to PTX pretreatment. Taken together, these findings indicate that RGS10 inhibits the expression of TNF-α and COX-2 in a Gαi-independent mechanism.

Discussion

Regulator of G protein signaling (RGS10) belongs to a large family of proteins that canonically regulate G-protein coupled receptors signaling. GPCRs and their associated G-proteins initiate a cascade of oncogenic signaling pathways in multiple cancers, including ovarian cancer (Wu;Yeerna; et al., 2019). GPCRs-mediated activation of heterotrimeric G-protein is terminated by GAP activity of RGS proteins, where an alteration in their expression is implicated in the progression of several cancers (Hurst and Hooks, 2009).

Over the last decade, growing evidence has supported the link between inflammation and cancer, where cancer-associated inflammatory events have been shown as critical contributors to ovarian cancer pathogenesis (Savant et al., 2018). More specifically, TNF-α, a major inflammatory cytokine, and its receptors are expressed at high levels in ovarian cancers compared to normal ovarian tissues, and high levels of TNF-α have been detected in ascites from ovarian cancer patients (Moradi et al., 1993, Szlosarek et al., 2006, Gupta et al., 2016). Ovarian cancer cells produce TNF-α that, in turn, upregulates expression of TNF-α through autocrine fashion and enhances expression of numerous inflammatory mediators, such as CXCL12, CXCR-4, IL-6, VEGF, and CCL-2 that have a critical influence on migration, angiogenesis, and tumor microenvironment of ovarian cancer (Szlosarek et al., 2006, Kulbe et al., 2007). In addition to TNF-α, COX-2 mediated PGE2 production is overexpressed in biopsies from ovarian cancer patients (Lin et al., 2014); elevated expression of COX-2 enhances proliferation and migration (Gu et al., 2008) and is associated with a high incidence of chemoresistance of ovarian cancer (Ferrandina et al., 2002). As NF-κB is a major master protein in pro-inflammatory gene induction, its activation is found in cells derived from ovarian cancers and promotes platinum and taxane resistance (Gaikwad et al., 2015, Sun;Huang; et al., 2018).

Aberrant expression of RGS10 transcript and protein has correlated with poor prognosis of various cancers, including laryngeal cancer (Yin et al., 2013), hepatocellular carcinoma (Wen et al., 2015), and pediatric acute myeloid leukemia (Chaudhury et al., 2018). In ovarian cancer,

RGS10 expression is epigenetically suppressed in multiple cells lines during the development of chemotherapy resistance (Ali et al., 2013). Furthermore, this suppression contributes to amplified survival signaling pathways. Direct knock-down of RGS10 via siRNA in SKOV-3 and HEYA8 chemosensitive ovarian cancer cells amplified cell viability and reduced susceptibility of the cells to cytotoxic effects of chemotherapeutic drugs (Hooks et al., 2010). In addition to its role in ovarian cancer, RGS10 is highly expressed in microglia (Waugh et al., 2005) and peripheral macrophages (Lee et al., 2013), where RGS10 acts as a critical regulator of inflammatory signaling (Lee et al., 2008, Lee et al., 2011, Lee et al., 2013). The anti-inflammatory role of RGS10 in microglia and macrophages is observed downstream of LPS-mediated TLR-4 activation. Primary microglia and differentiated bone marrow macrophages derived from RGS10 KO mice exhibit higher levels of p-NF-κB subunit p65 expression and pro-inflammatory cytokine release in response to LPS treatment (Lee et al., 2008, Lee et al., 2011, Lee et al., 2013). Similarly, knock-down of RGS10 in BV2 cells microglial cell line amplifies the expression of pro-inflammatory TNF-α and interleukins following LPS (Lee et al., 2008, Lee et al., 2011, Lee et al., 2013). Our recent results have showed that loss of RGS10 significantly enhances LPS- induced microglial COX-2 expression (Data not shown). However, the functional impact of RGS10 in the regulation of inflammatory signaling in ovarian cancer has not been previously explored.

The results presented here introduce RGS10 as a critical modulator of inflammatory signaling in ovarian cancer. Although EOC cells do not respond to TLR-4 stimulation by LPS, our results show for the first time that RGS10 suppression strongly amplifies phosphorylation of NF-κB subunit p65 expression and enhances the basal levels of TNF-α and COX-2 in SKOV-3 EOC cells. Given the ability of RGS10 to modulate survival and chemoresistance of ovarian cancer and regulate NF-κB activation, TNF-α and COX-2 expression, and the fact that this overexpression of these inflammatory signals have recently been linked to ovarian cancer chemoresistance (Seo et al., 2004), further work is needed to investigate whether amplification of inflammatory signaling

accounts for the enhanced survival and chemoresistance observed in RGS10-deficient ovarian cancer cells.

Canonically, RGS10 selectively targets Gi/o family G-proteins (Hunt et al., 1996), accelerating GTP hydrolysis via its classic GAP activity and ultimately inactivating signaling. The initial hypothesis postulated RGS10 regulation of ovarian cancer survival through modulation signaling downstream of receptors coupling to Gαi proteins, such as LPA, identified as an autocrine ovarian cancer growth factor, which is strongly elevated in malignant ascites of ovarian cancer patients (Murph et al., 2008, Yu et al., 2008). However, RGS10 knock-down strongly enhances ovarian cancer viability and the basal level of AKT protein with no significant effect on LPA-stimulated AKT activation in SKOV-3 cells (Hooks et al., 2010). Independent of its classical role, RGS10 binds to the monomeric GTPase protein Rheb, which is known to bind and activate the mechanistic target of rapamycin complex 1 (mTORC1). RGS10 suppression leads to increase levels of active GTP-bound Rheb, which results in elevated phosphorylation levels of AKT, mTOR, 4EB-BP1, p70S6 kinase, eIF2a, and ribosomal protein S6 in Hey-A8 cells (Altman et al., 2015). Importantly, our findings reveal that amplified p-NF-κB subunit p65 and the transcript levels of TNF-α and COX-2, a result of RGS10 loss in SKOV-3 EOC cells, are not affected by Gαi inhibition with PTX treatment, suggesting that RGS10 suppresses inflammatory signaling via Gαi-independent mechanism. Therefore, identifying binding partners of RGS10 will be fundamental in understanding RGS10 regulation of inflammatory genes and will facilitate the development of therapeutic strategies to target RGS10 in ovarian cancer.

RGS10 is the most highly expressed RGS protein in macrophages and has a central role in macrophage polarization. RGS10 suppresses classical M1 activation through the downregulation of NF-κB activation and inflammatory cytokine release and promotes the alternatively activated M2 phenotype (Lee et al., 2013). Macrophages are abundant in the tumor microenvironment and produce a series of inflammatory mediators involved in the the development of tumors throughout different processes (Larionova et al., 2020). Tumor-associated

macrophages via their generated inflammatory signals have been established as key factors in ovarian cancer progression and chemotherapeutic resistance (Zhang et al., 2014). Since RGS10 has a direct role in the regulation of inflammatory mediators in macrophages and that inflammatory signaling is involved in EOC cell progression, it will be interesting to explore the effect of RGS10 expression in macrophages on ovarian cancer survival and chemoresistance.

Conclusion

In this study, we show that suppression of Regulator of G-protein 10 (RGS10) triggers phosphorylation of NF-kB (p-65) subunit and increases the transcript levels of Tumor Necrosis Factor-alpha (TNF-α) and Cyclooxygenaes-2 (COX-2). These observations are crucial because NF-kB signaling pathway, TNF-α and COX-2 are functionally dysregulated among multiple cancer cells, specifically ovarian cancer cells. Also, they could aid in identifying and understanding both the mechanism for RGS10 silencing in multiple ovarian cancer acquired chemoresistant cells and the mechanism of RGS10 regulation of ovarian cancer cell survival and chemoresistance in chemosensitive ovarian cancer cells. Finally, we demonstrate that RGS10 knockdown-mediated amplification of the NF-kB (p-65) subunit and the expression of TNF-α and COX-2 is not mediated by enhanced Gai signaling. Together, our results suggest that RGS10 controls NF-kB signaling and activation of TNF-α and COX-2, which are implicated in ovarian cancer progression and chemoresistance.

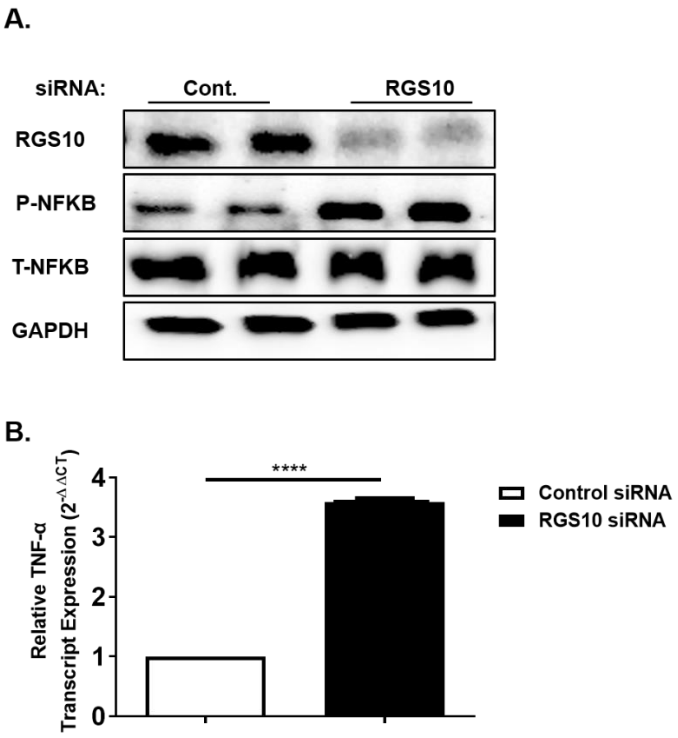

Figure 5.1. RGS10 silencing amplifies phosphorylation of p65-NF-κB and TNF-α transcript in SKOV-3 EOC cells.

A. SKOV-3 ovarian cancer cells were cultured in 24 well plates and transfected with control or RGS10 siRNA for 48 hours. Cells were lysed, and SDS-PAGE was performed, followed by immunoblotting using specific antibodies against RGS10, phospho-P65 NFκB, total P65 NFκB, and GAPDH. The ratio of phosphorylated/total of P65 NFκB was calculated. **B.** SKOV-3 cells were cultured in 24 well plates and transfected with control or RGS10 siRNA for 48 hours. RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. TNF-α transcript level was quantified using qRT-PCR and was normalized to the endogenous control GAPDH. Expression of TNF-α mRNA was calculated by the ($2^{-\Delta\Delta Ct}$) method. Data were analyzed for statistical differences using the t-student test or analysis of variance, followed by Tukey's test between groups. Data in **(A&B)** is presented and compiled from three independent experiments. Data are presented as mean ± SEM, where *** p<0.001, ** p<0.01* P < 0.05, n.s., not significant.

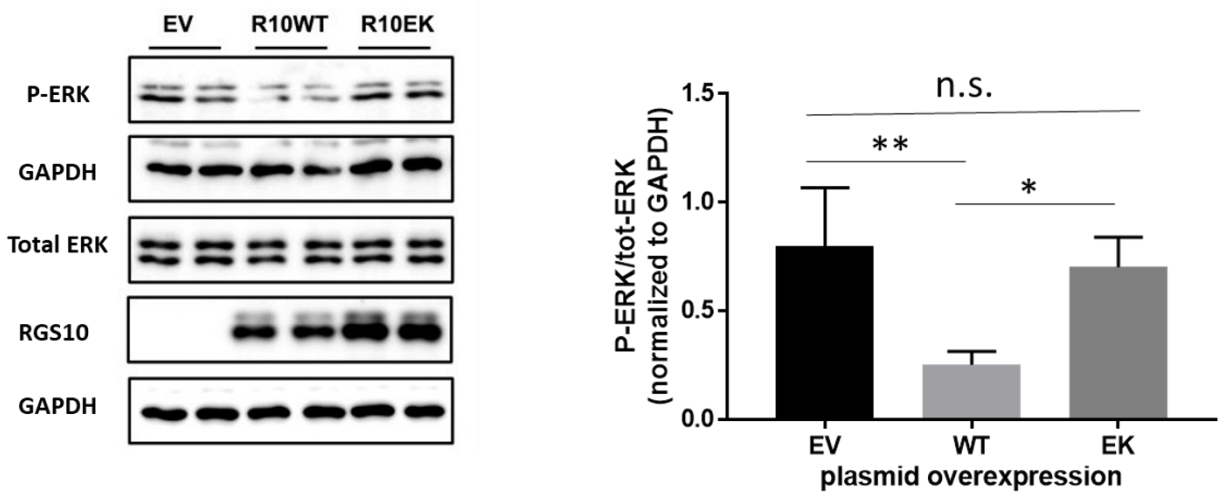

Figure 5.2. The effect of RGS10 WT and E52K mutant on Gαi protein signaling pathways.

SKOV-3 ovarian cancer cells were cultured in 24 well plates and transfected with empty vector or plasmids encoding RGS10 WT or E52K mutant RGS10 for 48 hours. Cells were lysed, and SDS-PAGE was performed, followed by immunoblotting using specific antibodies against RGS10, phospho- p42/p44 ERK MAPK, total p42/p44 ERK MAPK, and GAPDH. The ratio of phosphorylated/total of P65 NFκB was calculated. Data were analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Image is presented and compiled from two independent experiments. Data are presented as mean ± SEM, where ** $p<0.01$, * $P < 0.05$, n.s., not significant.

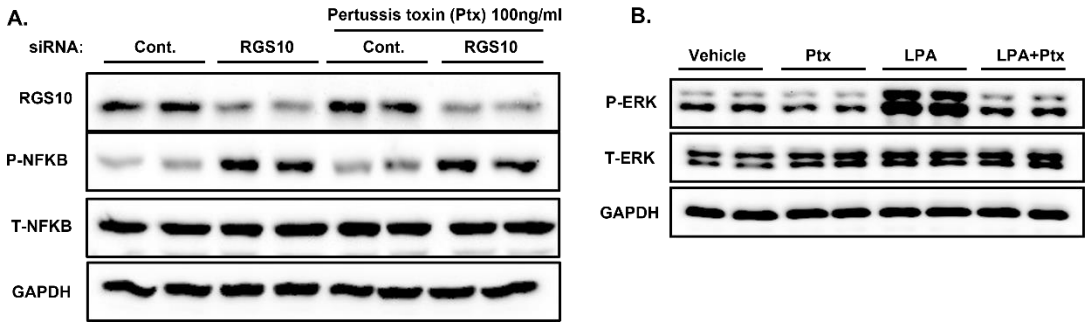

Figure 5.3. The effect of Gαi inhibition on RGS10 knockdown-induced enhancement of NF-κB subunit p65 phosphorylation.

A. SKOV-3 cells were cultured in 24 well plates and transiently transfected with control or RGS10-targeted siRNA constructs. 20 hours after transfection, cells were treated with vehicle or ptx (100ng/ml) for an additional 28 hours. Cells were lysed, and SDS-PAGE was performed, followed by immunoblotting using specific antibodies against RGS10, phospho-P65 NFκB, total P65 NFκB, and GAPDH. The ratio of phosphorylated/total of P65 NFκB was calculated. **B.** SKOV-3 cells cultured in 24 wells plate were serum-starved overnight with or without Ptx (100ng/ml) prior to treatment with vehicle or LPA (10 μM) for five minutes. Cells were lysed, and SDS-PAGE was performed, followed by immunoblotting using specific antibodies against phospho- p42/p44 ERK MAPK, total p42/p44 ERK MAPK, and GAPDH. Data were analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Image in **(A)** is presented and compiled from three independent experiments, while Image in **(B)** represents only one experiment. Data are presented as mean ± SEM, where *** $p<0.001$, ** $p<0.01$* $P < 0.05$, n.s., not significant.

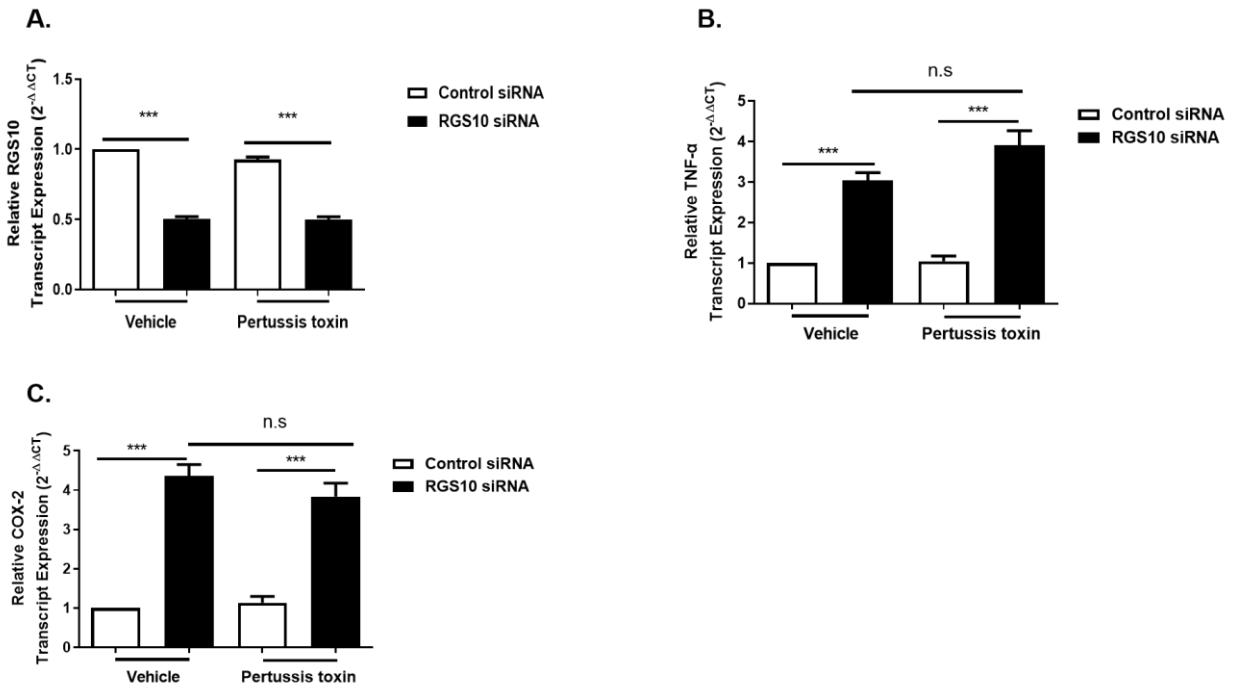

Figure 5.4. RGS10 silencing upregulates TNF-α and COX-2 expression and is mediated by a Gαi-independent mechanism.

SKOV-3 cells were cultured in a 6 well plate and transiently transfected with control or RGS10-targeted siRNA constructs. 20 hours after transfection, cells were treated with vehicle or tx (100ng/ml) for an additional 28 hours. RNA was isolated from cells using TRIzol reagent, and cDNA was synthesized from the extracted RNA. RGS10 **(A)**, TNF-α **(B)**, COX-2 **(C)** transcript levels were quantified using quantitative RT-PCR and normalized to the housekeeping gene GAPDH($2^{-\Delta\Delta Ct}$). Data were analyzed for statistical differences using analysis of variance, followed by Tukey's test between groups. Data in **(A-B)** is presented and compiled from three independent experiments. Data are presented as mean ± SEM, where *** $p<0.001$, ** $p<0.01$, * $P < 0.05$, n.s., not significant.

CHAPTER 6

Summary and Future work

The main goal of this dissertation is to define the mechanism linking TLR-4 activation by LPS to RGS10 suppression and the mechanism underlying RGS10 anti-inflammatory effects. Previous studies have shown that RGS10 is highly expressed in microglia and BMDMs under resting conditions, but the expression of *Rgs10* is silenced following stimulation with LPS (Lee et al., 2008, Lee et al., 2013). Although LPS-activated microglia and macrophages cause the suppression of RGS10 expression, the mechanistic basis responsible for silencing RGS10 has not been defined.

In chapter 3, we aimed to identify inflammatory responses essential for the suppressive effect of LPS on *Rgs10* expression. We demonstrated the abundant expression of RGS10 in MH-S alveolar macrophages and the ability of LPS to trigger a similar suppression in *Rgs10* transcript and RGS10 protein expression levels in MH-S cells to what has been reported in activated microglia and BMDMs. We further demonstrated for the first time that pharmacological inhibition of PI3K activity, NF-κB-dependent TNF-α secretion, and histone deacetylases (HDAC) class I activity reverse suppression of *Rgs10* expression upon LPS stimulation in MH-S cells as well as BV2 microglial cells and BMDMs, suggesting that these inflammatory mediators facilitate the suppressive effect of LPS on RGS10 expression. Consistent with the known role of RGS10 in activated microglia and BMDMs, we showed that loss of RGS10 in MH-S cells significantly enhances LPS-induced upregulation of NF-κB phosphorylation and inflammatory genes expression. The findings presented in this study provide novel insights into the molecular basis and the pharmacological approaches that regulate *Rgs10* expression in activated microglia and macrophages.

Stimulation of several cell types, including microglia and macrophages with LPS induces expression and activity of HDAC enzyme (Zhu et al., 2010), mainly three class I HDACs (1-3), HDAC1, HDAC2, and HDAC3 (Chen;Barozzi; et al., 2012, Wu;Li; et al., 2019). We showed that the selective HDAC (1-3) enzymes inhibitor (Apicidin) blocks the ability of LPS to suppress *Rgs10* transcription, demonstrating that the activity of HDACs 1-3 is required for LPS silencing of RGS10. Class I HDACs (1-3) are highly homologous nuclear proteins that form homo- and heterodimers between each other and constitute the catalytic activity of multiple protein repressor complexes (Delcuve et al., 2012, Khan et al., 2013). Future studies should assess which class I HDAC isoform(s) are involved in the LPS-induced suppression of RGS10. Growing evidence strongly implicates class I HDACs (1-3) in regulating differentiation and activation of macrophages, as they support pro-inflammatory M1 responses and inhibit M2-phenotype polarization (Datta et al., 2018, Chen et al., 2020). Due to the reported anti-inflammatory effect of HDAC inhibitors in activated macrophages and the fact that loss of RGS10 enhances inflammatory mediators, further work is needed to test whether the anti-inflammatory action of HDAC inhibitors is due to reversing RGS10 silencing in macrophages.

In addition to macrophages, suppression of RGS10 expression can occur in multiple cells, including microglia (Lee et al., 2008, Lee et al., 2011), neurons (Lee;Chung; et al., 2012), cardiomyocytes (Miao et al., 2016), and ovarian cancer cells (Hooks et al., 2010). Loss of RGS10 expression in these cells contributes to microglia overactivation and subsequent neuroinflammation-mediated neurodegeneration (Lee et al., 2008, Lee et al., 2011, Lee;Chung; et al., 2012, Lee et al., 2016), cardiac hypertrophy (Miao et al., 2016), and ovarian cancer chemoresistance (Ali et al., 2013, Hooks and Murph, 2015). Critically, inflammatory signaling, in particular, NF-κB activity and pro-inflammatory cytokines, are implicated in neurodegenerative diseases (such as Parkinson's disease) (Chitnis and Weiner, 2017), heart failure (Gordon et al., 2011, Gaspar-Pereira et al., 2012), and cancer progression and chemoresistance (Harrington and

Annunziata, 2019). Further studies could explore whether RGS10 suppression in these cells is due to a similar mechanism.

Numerous studies have demonstrated the localization of RGS10 to both the cytoplasm and the nucleus of the cells, with no significant plasma membrane localization (Chatterjee and Fisher, 2000, Haller et al., 2002). In microglia, RGS10 is evenly expressed in the cytoplasmic and the nuclear compartments under the resting condition. However, in response to LPS stimulation, much of RGS10 translocates from the cytoplasm to the nucleus (Lee et al., 2008). More importantly, an early study has reported that nuclear localization of RGS10 is driven by the cyclic AMP-dependent protein kinase A (PKA)-mediated phosphorylation of RGS10 on serine 168 (Burgon et al., 2001). In dopaminergic neurons, overexpression of RGS10-S168A (RGS10SA, resistant to phosphorylation by PKA) or blocking PKA-triggered RGS10 phosphorylation and the subsequent nuclear translocation limits the prosurvival role of RGS10 in TNF-α-induced neurotoxicity (Lee;Chung; et al., 2012). In terms of NF-*κ*B signaling regulation, PKA is known to phosphorylate NF-κB(p65) and modulates its transcriptional activity via potentiation of the interaction of the NF-κB(p65) and the CBP/p300 co-activators (Zhong et al., 1997, Zhong et al., 1998). Further work can investigate whether PKA regulates LPS-triggered RGS10 nuclear localization and whether PKA is involved in LPS-induced RGS10 suppression in macrophages.

Microglial RGS10 has emerged as an anti-inflammatory protein, in which loss of RGS10 in microglia amplifies LPS-stimulated proinflammatory mediators, such as TNF-α, interleukins, COX-2 mediated PGE2 release and enhances neurotoxicity (Lee et al., 2008, Lee et al., 2011, Alqinyah et al., 2018). Although the canonical function of RGS10 is to inhibit G-protein signaling by accelerating GTPase activity, we have shown that RGS10's anti-inflammatory action regulation of LPS-induced COX-2 and TNF-α does not require its GAP activity (Alqinyah et al., 2018), suggesting that unknown Gαi protein-independent mechanism.

In chapter 4, we aimed to identify non-canonical binding partners of RGS10 that could mediate its anti-inflammatory effects in microglia. We identify and validate stromal interaction

molecule-2 (STIM2) among multiple interacting proteins as a novel binding partner of endogenous RGS10 in BV2 microglia. STIM2 is a calcium sensor that localizes in the endoplasmic reticulum (ER) and mediates store-operated Ca^{2+} entry (SOCE) through Orai channels in the plasma membrane upon ER-calcium depletion (Soboloff et al., 2012, Nelson et al., 2018). STIM2 activation and its coupling to Orai-mediated calcium signaling are implicated in the regulation of LPS-induced inflammatory gene expression in microglia and macrophages (Chang, 2006, Michaelis et al., 2015, Sogkas et al., 2015). Our results have shown that pharmacological inhibition of Orai channels and an intracellular calcium chelator (BAPTA-AM) completely block LPS-stimulated COX-2 expression. More importantly, we have found that pharmacological inhibition of Orai channels completely blocks the amplification of TLR4-stimulated COX-2 and TNF-α expression in response to the loss of RGS10 expression. These results suggest that RGS10 suppresses COX-2 and TNF-α expression downstream of TLR4 activation of Orai-mediated SOCE. We further showed that ER calcium depletion triggered by thapsigargin treatment strongly enhances LPS-induced RGS10 silencing and amplifies LPS-stimulated COX-2 expression. These findings presented in this study improve our understanding of the mechanism that RGS10 utilizes to regulate inflammatory signaling that is essential to develop novel therapeutics that target RGS10 in the treatment of neuroinflammatory diseases.

RGS10 is a simple RGS protein, containing only a conserved RGS domain, shared among all the members of the RGS protein family (Hollinger and Hepler, 2002), and lacking regulatory motifs and functional domains that have been found in the other canonical RGS proteins (Squires et al., 2018). While other RGS proteins interact with most of their non-canonical interacting partners via functional domains beyond their RGS homology domain (Sethakorn et al., 2010), we expect that RGS10-STIM2 interaction is mediated via the RGS domain. However, it is not known whether STIM2 has a direct interaction with RGS10 or is a part of a multi-protein complex. Thus, future biochemical studies could characterize whether the RGS domain of RGS10 associates with STIM2 or whether RGS10-STIM2 interacts in the presence of scaffolding proteins.

In microglia, we have previously shown that LPS treatment enhances RGS10 association with its canonical binding protein Gαi3 (Alqinyah et al., 2018). Since our results have identified ER-resident STIM2 as an RGS10-binding partner under basal conditions, future work could investigate whether LPS regulates RGS10-STIM2 interaction. RGS10 is evenly distributed between cytoplasm and nucleus under resting conditions. However, following LPS treatment, RGS10 is robustly enriched in the nucleus (Lee et al., 2008). Potential future studies could define RGS10 subcellular localization with ER-resident STIM2 protein under basal conditions and following LPS stimulation.

Several studies have shown that SOCE, which is regulated by STIM2 protein, is induced by LPS in microglia (Sun et al., 2014, Wang;Liu;Tian; et al., 2015) and other cells (DebRoy et al., 2014, Velmurugan et al., 2015). Because LPS has been linked to SOCE in microglia, which is triggered by ER calcium depletion and leads, in turn, to reload ER calcium stocks, future studies could characterize the impact of RGS10 on ER-depletion induced calcium release into the cytoplasm and extracellular calcium influx.

Our results found that activation of Ca^{2+} sensitive phosphatase calcineurin is essential for microglial COX-2 induction by LPS and is required for the RGS10 effect on inhibiting LPS-stimulated COX-2 in microglial (Data not shown). As a major calcineurin substrate, NFAT is a transcriptional factor for multiple inflammatory mediators' induction, including COX-2 and TNF-α expression (Buxade et al., 2012, Bendickova et al., 2017). Previous studies have found that STIM2 knockout suppresses NFAT activation (Oh-Hora et al., 2008) and blockage of CRAC inhibits the activation of NFkB and NFAT (Mizuma et al., 2019). In addition, a prior study has shown that knockdown of NFAT in BV2 cells suppresses TNF-α expression (Manocha et al., 2017). While the loss of RGS10 in microglia leads to enhanced expression and transcriptional activity of NF-κB, it will be interesting to determine whether NF-κB and/or NFAT are required for RGS10 regulation COX-2 and TNF-α expression.

In addition to microglia, the anti-inflammatory action of RGS10 has been demonstrated in macrophages, as loss of RGS10 upregulates LPS-induced several pro-inflammatory mediators' production, including TNF-α in BMDMs and peritoneal macrophages. We have observed an association between RGS10 and STIM2 in RAW264.7 macrophage-like cells, suggesting that RGS10-STIM2 interaction is not limited to microglia. Future work could assess the effect of STIM2-Orai activity in RGS10 regulation of inflammatory gene expression in macrophages.

In ovarian cancer, RGS10 has emerged as a key factor in regulating the viability of ovarian cancer cells (Hooks et al., 2010, Hooks and Murph, 2015), where its expression is suppressed in multiple cells lines during the development of chemotherapy resistance (Hooks et al., 2010, Ali et al., 2013). Furthermore, this acquired suppression contributes to amplified survival signaling pathways, in which direct knock-down of RGS10 via siRNA in SKOV-3 and HEYA8 chemosensitive ovarian cancer cells amplifies cell viability and reduces the susceptibility of the cells to cytotoxic effects of chemotherapeutic drugs (Hooks et al., 2010, Altman et al., 2015), suggesting that endogenous RGS10 inhibits survival pathways and enhances chemotherapeutic drugs-mediated cancer cell death. Due to the RGS10 role in regulating inflammatory responses, such as TNF-α in microglia and macrophages and the fact that inflammatory mediators are implicated in ovarian cancer progression, we aimed to determine whether RGS10 can regulate inflammatory signaling in ovarian cancer. In chapter 5, while EOC cells do not respond to TLR-4 stimulation by LPS, our results show for the first time that RGS10 suppression strongly amplifies phosphorylation of NF-κB subunit p65 expression and enhances the basal levels of TNF-α and COX-2 in SKOV-3 EOC cells. Given the ability of RGS10 to modulate survival and chemoresistance of ovarian cancer and regulate NF-κB activation, TNF-α and COX-2 expression, and the fact that this overexpression of these inflammatory signals have recently been linked to ovarian cancer chemoresistance (Savant et al., 2018, Ye et al., 2020), further work can investigate whether the amplification of inflammatory signaling accounts for the enhanced survival and chemoresistance observed in RGS10-deficient ovarian cancer cells.

Importantly, our findings reveal that amplified p-NF-κB subunit p65 and the transcript levels of TNF-α and COX-2, a result of RGS10 loss in SKOV-3 EOC cells, are not affected by Gαi inhibition with ptx treatment, suggesting that RGS10 suppresses inflammatory signaling via Gαi-independent mechanism. Because STIM2 binds RGS10 in microglia and its downstream Orai activity was essential for RGS10 regulation of COX-2 and TNF-α expression (chapter 4), we checked whether STIM2 will co-precipitate with RGS10 in SKOV-3 EOC cells. In contrast to our expectation, we observe an undetectable co-immunoprecipitation between STIM2 and RGS10 in SKOV-3 cells, suggesting that STIM2/Orai signaling pathway is not essential for amplification of COX-2 and TNF-α expression in SKOV-3 cells transfected with siRNA-targeted RGS10. Thus, identifying binding partners of RGS10 will be fundamental in understanding RGS10 regulation of inflammatory genes and will facilitate the development of therapeutic strategies to target RGS10 expression and function in ovarian cancer cells.

Since TLR-4 signaling is not active in SKOV-3 cells, we explored TNF-α signaling in SKOV-3 cells and found that TNF-α treatment enhances IκB and the phosphorylation of NF-κB (Data not shown). Previous studies have shown that RGS10 modulates TNF-α-induced NF-κB activation and the expression of inflammatory cytokines TNF-α and COX-2 in macrophages (Lee et al., 2011, Ren et al., 2021). Therefore, future work can explore whether RGS10 can regulate TNF-α-stimulated phosphorylation of NF-κB and the expression of TNF-α and COX-2 in ovarian cancer cells.

The tumor microenvironment is a complex of multi-cell types, communicating with each other through extracellular mediators. Macrophage cells constituent the major immune cells infiltrating tumors and support inflammation-associated microenvironment that promotes tumor progression and therapy resistance (Solinas et al., 2009). Given that RGS10 control survival and chemoresistance of ovarian cancer and modulate inflammatory signaling in macrophages and the fact that macrophages-derived inflammatory mediators contribute to ovarian cancer progression

and chemoresistance (Takaishi et al., 2010, Lan et al., 2013, Cho et al., 2018), future studies can investigate the role of RGS10 in a syngeneic mouse model of ovarian cancer.

APPENDIX

FRONT PAGES OF PUBLISHED AND SUBMITTED MANUSCRIPTS

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Review

Regulator of G protein signaling 10: Structure, expression and functions in cellular physiology and diseases

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ABSTRACT

Regulator of G protein signaling 10 (RGS10) belongs to the superfamily of RGS proteins, defined by the presence of a conserved RGS domain that canonically binds and deactivates heterotrimeric G-proteins. RGS proteins act as GTPase activating proteins (GAPs), which accelerate GTP hydrolysis on the G-protein α subunits and result in termination of signaling pathways downstream of G protein-coupled receptors. RGS10 is the smallest protein of the D/R12 subfamily and selectively interacts with Gαi proteins. It is widely expressed in many cells and tissues, with the highest expression found in the brain and immune cells. RGS10 expression is transcriptionally regulated via epigenetic mechanisms. Although RGS10 lacks multiple of the defined regulatory domains found in other RGS proteins, RGS10 contains post-translational modification sites regulating its expression, localization, and function. Additionally, RGS10 is a critical protein in the regulation of physiological processes in multiple cells, where dysregulation of its expression has been implicated in various diseases including Parkinson's disease, multiple sclerosis, osteopetrosis, chemoresistant ovarian cancer and cardiac hypertrophy. This review summarizes RGS10 features and its regulatory mechanisms, and discusses the known functions of RGS10 in cellular physiology and pathogenesis of several diseases.

1. Introduction

1.1. GPCR/G-protein signaling

G protein-coupled receptors (GPCRs) and the transduced heterotrimeric G-proteins, which modulate a cascade of intracellular effector proteins and chemical second messengers, are essential in the regulation of various physiological functions of cells and organ systems. They represent the largest family of FDA-approved targeted drugs for the treatment of a wide range of disorders caused by dysregulation of GPCR/G-protein signaling [1]. In the resting state, GPCRs are bound to inactive heterotrimeric G-proteins, consisting of the Gα-GDP and Gβγ dimer. In response to a variety of extracellular stimuli, GPCRs undergo conformational changes and act as guanine nucleotide exchange factors (GEFs) by promoting the exchange of GDP for GTP on Gα. As a result, activated GTP-Gα dissociates from Gβγ dimer, and thereby both can regulate downstream effector proteins [2] (Fig. 1), such as enzymes, RhoGEFs, and ion channels that in turn initiate diverse signaling pathways mediating cellular responses [3–7]

The amplification and duration of signaling activity by Gα-GTP and Gβγ are tightly modulated by the intrinsic GTPase activity of Gα, in which the Gα subunit hydrolyzes GTP to GDP and promotes reassembly with the Gβγ dimer to reform the inactive Gαβγ protein complex. This, in turn, results in the deactivation of the G-protein cycle. Even though

Abbreviations: AC, adenylate cyclase; AlF4, aluminum fluoride; AngII, angiotensin II; AR, androgen receptor; ATP, adenosine triphosphate; BMDMs, bone marrow-derived macrophages; CaM, calmodulin; CNS, central nervous system; COX-2, cyclooxygenase-2; DNMTs, DNA methyltransferases; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GDP, guanosine diphosphate; GEFs, guanine nucleotide exchange factors; GFP, green fluorescent protein; GIRK, G protein-coupled inwardly-rectifying potassium channel; GPCR, G protein–coupled receptor; GTP, guanosine triphosphate; HATs, histone acetyltransferases; HDACs, histone deacetylases; 6-OHDA, 6-hydroxydopamine; HFT, high fat diet; kDa, kilodalton; KD, knockdown; KO, knockout; LFD, low-fat diet; L/MGE, lateral/medial ganglionic; LPS, lipopolysaccharide; MN9D, mesencephalon neuroblastoma cell; MS, multiple sclerosis; NFATc1, nuclear factor of activated T cells 1; NF-κB, nuclear factor kappa B; PGE2, prostaglandin E2; PD, Parkinson's disease; PKA, protein kinase A; PTM, post-translational modifications; PTX, pertussis toxin; RANKL, receptor activator of nuclear factor (NF)-kB-ligand; RGS, regulator of G protein signaling; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; SNpc, substantia nigra pars compacta; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; TSA, trichostatin A; WT, wild-type

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PI3K/ NF-κB-dependent TNF-α and HDAC activities facilitate LPS-induced RGS10 suppression in pulmonary macrophages

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ABSTRACT

Regulator of G-protein signaling 10 (RGS10) is a member of the superfamily of RGS proteins that canonically act as GTPase activating proteins (GAPs). RGS proteins accelerate GTP hydrolysis on the G-protein α subunits and result in termination of signaling pathways downstream of G protein-coupled receptors. Beyond its GAP function, RGS10 has emerged as an anti-inflammatory protein by inhibiting LPS-mediated NF-κB activation and expression of inflammatory cytokines, in particular TNF-α. Although RGS10 is abundantly expressed in resting macrophages, previous studies have shown that RGS10 expression is suppressed in macrophages following Toll-like receptor 4 (TLR4) activation by LPS. However, the molecular mechanism by which LPS induces *Rgs10* silencing has not been clearly defined. The goal of the current study was to determine whether LPS silences *Rgs10* expression through an NF-κB-mediated proinflammatory mechanism in pulmonary macrophages, a unique type of innate immune cells. We demonstrate that *Rgs10* transcript and RGS10 protein levels are suppressed upon LPS treatment in the murine MH-S alveolar macrophage cell line. We show that pharmacological inhibition of PI3K/ NF-κB/p300 (NF-κB co-activator)/TNF-α signaling cascade and the activities of HDAC (1–3) enzymes block LPS-induced silencing of *Rgs10* in MH-S cells as well as microglial BV2 cells and BMDMs. Further, loss of RGS10 generated by using CRISPR/Cas9 amplifies NF-κB phosphorylation and inflammatory gene expression following LPS treatment in MH-S cells. Together, our findings strongly provide critical insight into the molecular mechanism underlying RGS10 suppression by LPS in pulmonary macrophages.

1. Introduction

Tissue-resident macrophages (TRMs) emerge from embryonic precursors that exert vital and specific functions in tissue homeostasis, inflammation, and regeneration [1,2]. Among TRMs, alveolar macrophages (AMs) are the specialized cells that reside in the pulmonary alveoli and the dominant innate immune cells, as they represent 90–95% of the cellular numbers under normal conditions, making them the natural sentinels of the respiratory system [3,4]. Like other TRMs, AMs are involved in maintaining lung homeostasis by phagocytosing apoptotic cells and cells debris resulting from lung infection or epithelial injury while also maintaining a dominant immunosuppressive phenotype [5,6]. In particular, AMs have a central role in clearing the alveolar environment from an excessive production of lipid-rich molecules (surfactants) that are produced by type II alveolar epithelial cells and functionally prevent alveolar collapse during exhalation [7]. Despite these physiological functions, dysregulation of AM activation or their impaired clearance function are associated with initiation and progression of several respiratory pathologies, such as acute lung injury (ALI), pulmonary alveolar proteinosis [8], and chronic obstructive pulmonary disease [9].

Regulator of G-protein signaling (RGS) proteins are a large family of proteins containing RGS domain that binds and deactivates heterotrimeric G-protein subunits [10,11]. Canonically, RGS proteins terminate signaling pathways downstream of G protein-coupled receptors (GPCRs) by acting as GTPase activating proteins (GAPs) on active form,

Abbreviations: 5-Aza, 5-aza-2′-deoxycytidine; CNS, central nervous system; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GPCR, G protein coupled receptor; HDAC, histone deacetylase; IL, interleukin; INOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PGE2, prostaglandin E2; RGS, regulator of G-protein signaling; RT-PCR, reverse transcription-polymerase chain reaction; TF, transcription factor; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; TSA, trichostatin A.

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RGS10 physically and functionally interacts with STIM2 and requires store-operated calcium entry to regulate pro-inflammatory gene expression in microglia

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ABSTRACT

Chronic activation of microglia is a driving factor in the progression of neuroinflammatory diseases, and mechanisms that regulate microglial inflammatory signaling are potential targets for novel therapeutics. Regulator of G protein Signaling 10 is the most abundant RGS protein in microglia, where it suppresses inflammatory gene expression and reduces microglia mediated neurotoxicity. In particular, microglial RGS10 downregulates the expression of pro-inflammatory mediators including cyclooxygenase 2 (COX-2) following stimulation with lipopolysaccharide (LPS). However, the mechanism by which RGS10 affects inflammatory signaling is unknown and is independent of its canonical G protein targeted mechanism. Here, we sought to identify non canonical RGS10 interacting partners that mediate its anti-inflammatory mechanism. Through RGS10 co-immunoprecipitation coupled with mass spectrometry, we identified STIM2, an endoplasmic reticulum (ER) localized calcium sensor and a component of the store operated calcium entry (SOCE) machinery, as a novel RGS10 interacting protein in microglia. Direct immunoprecipitation experiments confirmed RGS10-STIM2 interaction in multiple microglia and macrophage cell lines, as well as in primary cells, with no interaction observed with the homologue STIM1. We further determined that STIM2, Orai channels, and the calcium-dependent phosphatase calcineurin are essential for LPS-induced COX-2 production in microglia, and this pathway is required for the inhibitory effect of RGS10 on COX-2. Additionally, our data demonstrated that RGS10 suppresses SOCE triggered by ER calcium depletion and that ER calcium depletion, which induces SOCE, amplifies pro inflammatory genes. In addition to COX 2, we also show that RGS10 suppresses the expression of pro-inflammatory cytokines in microglia in response to thrombin and LPS stimulation, and all of these effects require SOCE. Collectively, the physical and functional links between RGS10 and STIM2 suggest a complex regulatory network connecting RGS10, SOCE, and pro inflammatory gene expression in microglia, with broad implications in the pathogenesis and treatment of chronic neuroinflammation.

1. Introduction

Chronic inflammation is an underlying mechanism for the initiation and progression of multiple diseases [1]. Chronic activation of microglia cells is a driving factor of neuroinflammation and a hallmark of several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis [2]. Aberrant activation of microglia leads to amplified production of pro-inflammatory cytokines, prostaglandins, and other neurotoxic molecules, ultimately contributing to neuroinflammation and neurotoxocity [2,3]. Therefore, targeting novel molecular mechanisms for the regulation of microglial inflammatory signaling is a promising therapeutic strategy for neurodegenerative

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RGS10 Regulates the Expression of Cyclooxygenase-2 and Tumor Necrosis Factor Alpha through a G Protein–Independent Mechanism

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ABSTRACT

The small regulator of G protein signaling protein RGS10 is a key regulator of neuroinflammation and ovarian cancer cell survival; however, the mechanism for RGS10 function in these cells is unknown and has not been linked to specific G protein pathways. RGS10 is highly enriched in microglia, and loss of RGS10 expression in microglia amplifies production of the inflammatory cytokine tumor necrosis factor α (TNFα) and enhances microglia-induced neurotoxicity. RGS10 also regulates cell survival and chemoresistance of ovarian cancer cells. Cyclooxygenase-2 (COX-2)–mediated production of prostaglandins such as prostaglandin E_2 (PGE_2) is a key factor in both neuroinflammation and cancer chemoresistance, suggesting it may be involved in RGS10 function in both cell types, but a connection between RGS10 and COX-2 has not been reported. To address these questions, we completed a mechanistic study to characterize RGS10 regulation of TNFα and COX-2 and to determine if these effects are mediated through a G protein–dependent mechanism. Our data show for the first time that loss of RGS10 expression significantly elevates stimulated COX-2 expression and PGE_2 production in microglia. Furthermore, the elevated inflammatory signaling resulting from RGS10 loss was not affected by $G\alpha_i$ inhibition, and a RGS10 mutant that is unable to bind activated G proteins was as effective as wild type in inhibiting TNFα expression. Similarly, suppression of RGS10 in ovarian cancer cells enhanced TNFα and COX-2 expression, and this effect did not require G_i activity. Together, our data strongly indicate that RGS10 inhibits COX-2 expression by a G protein–independent mechanism to regulate inflammatory signaling in microglia and ovarian cancer cells.

Introduction

Regulators of G protein signaling (RGS) are a family of proteins that classically act as activators of the intrinsic GTPase activity of heterotrimeric Gα subunits (Watson et al., 1996). Owing to this GTPase-accelerating protein (GAP) activity and inhibition of signaling initiated by G protein–coupled receptors (GPCRs), RGS proteins play numerous roles in physiologic and pathologic conditions in diverse systems. However, multiple studies have revealed actions of RGS proteins that are independent of GTPase-accelerating activity, recently reviewed in Sethakorn et al. (2010). These noncanonical functions of RGS proteins can affect a variety of targets, including GPCRs, kinases, and transcription factors (Sethakorn et al., 2010). Therefore, to investigate the molecular mechanism of specific RGS protein actions, a critical initial question to answer is whether the RGS protein is acting in a classic GAP-dependent or noncanonical GAP-independent mechanism. The small RGS protein RGS10 regulates inflammatory and survival signaling in multiple cell types (Hooks et al., 2010; Lee et al., 2011, 2013), and has been proposed as a potential drug target for neuroinflammatory disease and ovarian cancer. However, the mechanisms by which RGS10 affects inflammatory and survival signaling are undefined, hampering the development of RGS10-targeted therapeutic strategies.

RGS10 is the smallest member of the R12 RGS subfamily with no functional domains outside of the RGS domain. RGS10 has been shown to selectively target $G\alpha_i$ family G proteins via classic GAP activity (Hunt et al., 1996), and is highly enriched in immune cells, including peripheral macrophages and microglia (Lee et al., 2008, 2013). Loss of RGS10 in microglia amplifies production of inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 1β, and enhances microglia-induced neurotoxicity triggered by the toll-like receptor (TLR) ligand lipopolysaccharide (LPS) (Lee et al., 2011). Reciprocally, activation of microglia by LPS induces epigenetic silencing of RGS10, which we predict serves to amplify inflammatory signaling (Alqinyah et al., 2017). In addition to its anti-inflammatory role in microglia, RGS10 also regulates survival of ovarian cancer cells, and loss of RGS10 induces chemoresistance in ovarian cancer cells

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ABBREVIATIONS: AlF_4^-, aluminum fluoride; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; GAP, GTPase-accelerating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein–coupled receptor; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; PTX, pertussis toxin; RGS, regulator of G protein signaling; siRNA, small interfering RNA; TLR, toll-like receptor; TNFα, tumor necrosis factor α; WT, wild type.

Dual oxidase 1 promotes antiviral innate immunity

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Dual oxidase 1 (DUOX1) is an NADPH oxidase that is highly expressed in respiratory epithelial cells and produces H_2O_2 in the airway lumen. While a line of prior in vitro observations suggested that DUOX1 works in partnership with an airway peroxidase, lactoperoxidase (LPO), to produce antimicrobial hypothiocyanite ($OSCN^-$) in the airways, the in vivo role of DUOX1 in mammalian organisms has remained unproven to date. Here, we show that Duox1 promotes antiviral innate immunity in vivo. Upon influenza airway challenge, *Duox1*$^{-/-}$ mice have enhanced mortality, morbidity, and impaired lung viral clearance. Duox1 increases the airway levels of several cytokines (IL-1β, IL-2, CCL1, CCL3, CCL11, CCL19, CCL20, CCL27, CXCL5, and CXCL11), contributes to innate immune cell recruitment, and affects epithelial apoptosis in the airways. In primary human tracheobronchial epithelial cells, $OSCN^-$ is generated by LPO using DUOX1-derived H_2O_2 and inactivates several influenza strains in vitro. We also show that $OSCN^-$ diminishes influenza replication and viral RNA synthesis in infected host cells that is inhibited by the H_2O_2 scavenger catalase. Binding of the influenza virus to host cells and viral entry are both reduced by $OSCN^-$ in an H_2O_2-dependent manner in vitro. $OSCN^-$ does not affect the neuraminidase activity or morphology of the influenza virus. Overall, this antiviral function of Duox1 identifies an in vivo role of this gene, defines the steps in the infection cycle targeted by $OSCN^-$, and proposes that boosting this mechanism in vivo can have therapeutic potential in treating viral infections.

DUOX1 | Dual oxidase 1 | influenza | hypothiocyanite | lactoperoxidase

DUOX1 is one of the seven members of the NADPH oxidase enzyme family (1). DUOX1 was first described in the thyroid gland (2) but was later also detected in several other tissues and organs including the tracheobronchial epithelium (3). DUOX1 localizes to the apical plasma membrane of ciliated respiratory epithelial cells and produces extracellular H_2O_2 into the airway lumen in a Ca^{2+}-dependent manner (3, 4). DUOX1 is the major NADPH oxidase expressed and the main source of H_2O_2 in the airway epithelium (3, 5, 6).

The respiratory epithelium employs several immune mechanisms against airborne microbes including the generation of reactive oxygen species. Respiratory epithelial cells have a proposed, rapid oxidative and extracellular antimicrobial system consisting of LPO, thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2) (7–10). LPO is found in a variety of body fluids including milk, saliva, lachrymal, and airway secretions (7, 8, 10–13). Its main substrate, SCN^-, is abundant in the airway surface liquid (7, 9, 14). LPO oxidizes SCN^- into antimicrobial hypothiocyanite ($OSCN^-$) using H_2O_2 (15). Prior in vitro data suggested that Duox1 is the epithelial H_2O_2 source that functions in partnership with LPO to produce antimicrobial $OSCN^-$(2, 3, 16). SCN^- supplementation increases bacterial clearance in mouse lung infection, supporting an antibacterial role of $OSCN^-$ in vivo (17, 18). While $OSCN^-$ kills several microorganisms in vitro, its mechanism of action and the identity of the in vivo H_2O_2 source required to generate $OSCN^-$ remained unknown. The in vivo role of Duox1 in mammals remained unproven to date (13, 19).

Influenza remains a major clinical challenge worldwide. Seasonal influenza viruses infect between three and five million people and cause 290,000 to 650,000 global deaths annually (20). In this study, we show that Duox1 promotes innate immunity in vivo against influenza infection in a mouse model. We also identify virus binding and entry into host cells as the basis for the antiviral mechanism of action of $OSCN^-$ in vitro. Overall, results shown here demonstrate the in vivo role of Duox1 and determine the steps in the influenza virus life cycle targeted by Duox1- and LPO-derived $OSCN^-$.

Results

Duox1 Improves Mortality, Morbidity, and Viral Clearance in a Murine Model of Influenza Airway Infection. We have previously shown that $OSCN^-$ produced by the Duox1/LPO-based system in vitro inactivates several influenza A and B virus strains (21, 22). To explore the in vivo role of Duox1 in anti-influenza responses, *Duox1*$^{-/-}$ mice (23) and Duox1-expressing C57BL/6 wild-type control animals (WT) (*SI Appendix*, Fig. S1*A*) were intranasally (i.n.) infected with 50 plaque-forming units (PFUs) of mouse-adapted influenza A virus strain, A/Puerto Rico/8/1934 (H1N1) (PR8). While 86% of WT mice survived the i.n. influenza challenge at day 12, only 55% of the *Duox1*$^{-/-}$ mice survived ($\chi^2 = 5.3$, $P = 0.02$) (Fig. 1*A*). *Duox1*$^{-/-}$ mice lost significantly more body weight compared with WT mice ($P < 0.05$, 5 through 7 d postinfection, dpi) (Fig. 1 *B* and *C*) during the time before the onset of mortality. The improved

Significance

Influenza infections kill millions of people worldwide. Current prophylactic treatment options are limited due to viral strain–specific vaccinations and emerging drug resistance. It is important to discover new immune mechanisms that can fight the influenza virus. Our work presented here identifies such a mechanism. The Duox1 protein helps the airways to clear influenza virus and to reduce infection-related death and sickness in an animal model. Duox1 delays the infection process by directly targeting the virus. Unlike current vaccines, this mechanism is effective against several influenza strains. Our study identifies the function of the *Duox1* gene and suggests that it has a therapeutic potential against influenza and potentially other respiratory pathogens, worth exploring in the future.

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Myeloperoxidase and Other Markers of Neutrophil Activation Associate With Malaria and Malaria/HIV Coinfection in the Human Placenta

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†This manuscript is dedicated to the memory of our dear friend and colleague

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Introduction: Placental malaria (PM) is characterized by accumulation of inflammatory leukocytes in the placenta, leading to poor pregnancy outcomes. Understanding of the underlying mechanisms remains incomplete. Neutrophils respond to malaria parasites by phagocytosis, generation of oxidants, and externalization of Neutrophil Extracellular Traps (NETs). NETs drive inflammation in malaria but evidence of NETosis in PM has not been reported. Neutrophil activity in the placenta has not been directly investigated in the context of PM and PM/HIV-co-infection.

Methods: Using peripheral and placental plasma samples and placental tissue collected from Kenyan women at risk for malaria and HIV infections, we assessed granulocyte levels across all gravidities and markers of neutrophil activation, including NET formation, in primi- and secundigravid women, by ELISA, western blot, immunohistochemistry and immunofluorescence.

Results: Reduced peripheral blood granulocyte numbers are observed with PM and PM/HIV co-infection in association with increasing parasite density and placental leukocyte hemozoin accumulation. In contrast, placental granulocyte levels are unchanged across infection groups, resulting in enhanced placental: peripheral count ratios with PM. Within individuals, PM- women have reduced granulocyte counts in placental relative to peripheral blood; in contrast, PM stabilizes these relative counts, with HIV coinfection tending to elevate placental counts relative to the periphery. In placental blood, indicators of neutrophil activation, myeloperoxidase (MPO) and proteinase 3 (PRTN3), are significantly elevated with PM and, more profoundly, with PM/HIV co-infection, in association with placental parasite density and hemozoin-bearing leukocyte accumulation. Another neutrophil marker, matrix

RGS10 reduces lethal influenza infection and associated lung inflammation in mice*

Abstract

Seasonal influenza epidemics represent a significant global health threat. The exacerbated immune response triggered by respiratory influenza virus infection causes severe pulmonary damage and contributes to substantial morbidity and mortality. Regulator of G-protein signaling 10 (RGS10) belongs to the RGS superfamily that acts as GTPase activating proteins and terminate signaling pathways downstream of G protein-coupled receptors. While RGS10 is highly expressed in immune cells, in particular monocytes and macrophages, where it has strong anti-inflammatory effects, its physiological role in the respiratory immune system has not been explored yet. Here, we show that Rgs10 negatively modulates lung immune and inflammatory responses associated with severe influenza H1N1 virus respiratory infection in a mouse model. In response to the influenza A virus challenge, mice lacking RGS10 experience enhanced weight loss, diminished viral clearance, higher mortality and significantly faster disease onset. Deficiency of *Rgs10* upregulates the levels of several proinflammatory cytokines and chemokines and increases myeloid leukocyte accumulation in the infected lung, markedly neutrophils, monocytes, and inflammatory monocytes, which is associated with more pronounced lung damage. Consistent with this, influenza-infected *Rgs10*-deficient lungs contain more neutrophil extracellular traps and exhibit higher neutrophil elastase activities than wild-type lungs. Overall, these findings propose a novel*, in vivo* role for RGS10 in the respiratory immune system controlling myeloid leukocyte infiltration, viral clearance and associated clinical symptoms following lethal influenza challenge. RGS10 also holds promise as a new, potential therapeutic target for respiratory infections.

* Faris Almutairi, Demba Sarr, Samantha L. Tucker, Kayla Fantone, Jae-Kyung Lee and Balázs Rada. Submitted to frontiers in Immunology, September 2021.

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