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From association to function: elucidating the role of G Protein Signaling Modulator 3 and variants in its gene locus in rheumatoid arthritis

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**From association to function: elucidating the role of G Protein Signaling
Modulator 3 and variants in its gene locus in rheumatoid arthritis.**

Bryan Jeffery Gall

**Dissertation submitted to the West Virginia University School of Medicine
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Cellular & Integrative Physiology**

**Timothy Nurkiewicz, Ph.D., Chair
David Siderovski, Ph.D.
Vincent Setola, Ph.D.
Peter Stoilov, Ph.D.
William Stauber, PT, Ph.D
Christopher Cuff, Ph.D.**

Department of Physiology & Pharmacology

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complement factor 5a; monocyte chemoattractant protein-1; N-formylmethionine-
leucyl-phenylalanine; neutrophils; monocytes; SNP rs204989; promoter activity
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Abstract

From association to function: elucidating the role of G Protein Signaling Modulator 3 and variants in its gene locus in rheumatoid arthritis

Bryan Gall

Rheumatoid arthritis (RA) is an autoimmune disease that is estimated to affect 1% of Americans. RA causes significant morbidity as a result of autoimmune stimuli inducing pro-inflammatory leukocyte recruitment to the joint. Despite the large effect on quality of life and significant research into RA pathophysiology, there is still much that is not known about the etiology of RA. Although effective treatment options do exist for RA, many of them carry significant risks of compliance-limiting adverse effects, high costs, and a subset of patients do not respond to treatment. This work attempts to respond to the limitations of current therapeutics by characterizing GPSM3 as a potential genetic marker and pharmaceutical target for RA through genetic and *ex vivo* modeling.

Based on data from compelling genome-wide association studies (GWAS), and supporting evidence from knockout mouse inflammatory arthritis models, we hypothesize that the *G protein signaling modulator-3 (GPSM3)* gene and protein play a significant role in the pathogenesis of RA. Current literature has identified two single-nucleotide polymorphisms (SNPs) in humans, rs204989 and rs204991, associated with protection from multiple autoimmune diseases including RA. We show that qRT-PCR analyses of whole blood-isolated RNA from volunteers homozygous for the *GPSM3* SNPs show a decrease in *GPSM3* transcript abundance compared to volunteers without the minor SNP alleles. Results from reporter gene studies support rs204989 as the causal SNP in decreasing *GPSM3* promoter activity. In cell line models of neutrophil and monocyte physiology, *GPSM3* deficiency results in disrupted migration patterns toward LTB4 and MCP-1, respectively. We hypothesize the minor allele of rs204989 represents a genetic factor contributing to the pathogenesis of RA, acting by decreasing *GPSM3* expression and disrupting normal migration toward pro-inflammatory chemoattractants.

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Abbreviations

3'	3rd carbon in the deoxyribose molecule
5'	5th carbon in the deoxyribose molecule
6p21.3	Chromosome 6, petit arm, band 2, sub-band 1, sub-sub-band 3
A	Adenosine
aa	Amino acid
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AGS	Activator of G protein signaling
aHL	α -hemolysin
Alum	Aluminum hydroxide
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
AP-1	Activator protein-1
ASC	Apoptotic speck protein containing a card
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
B2M	Beta-2-microglobulin
BCA	Bicinchoninic acid
BMDM	Bone marrow-derived macrophage
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumen
C	Cytosine
C-terminal	Carboxy terminal
C.I.	Confidence Interval
C/EBP	CCAAT-enhancer-binding protein
C5a	Complement component 5a
CAIA	Collagen antibody-induced arthritis
CARD	Caspase activation and recruitment domains
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cDNA	Complimentary deoxyribonucleic acid
CECAM-1	Carcinoembryonic antigen-related cell adhesion molecule-1
ChIP	Chromatin immunoprecipitation
CIA	Collagen-induced arthritis
CO ₂	Carbon dioxide
Ct	Number of polymerase chain reaction cycles required to cross threshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CX3CL	C-X-X-X-C chemokine ligand

CX3CR1	C-X-X-X-C chemokine receptor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	Damage-associated molecular pattern
dbSNP	Database of single nucleotide polymorphisms
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin
DMARD	Disease modifying antirheumatic drugs
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
EMSSA	Electrophoretic mobility supershift assay
ERK	Extracellular related kinase
Fab Region	Fragment antigen-binding region
Fc Region	Fragment, crystallizable region
FcR	Fragment, crystallizable region receptor
FDA	Food and Drug Administration
fMLP	N-Formylmethionyl-leucyl-phenylalanine
FPR1	Formylpeptide receptor 1
G	Guanine
GAP	Guanosine triphosphatase activating protein
gDNA	Genomic deoxyribonucleic acid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GoLoco	G alpha i/o-loco
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
G α	Alpha subunit of the heterotrimeric G protein
G α i	"Inhibitory of adenylyl cyclase" G protein
G α o	"Other" G protein
G β γ	Beta gamma subunits of the heterotrimeric G protein
H3K27me3	Trimethylated lysine 27 of histone H3 (repressive)
H3K4me3	Trimethylated lysine 4 of histone H3 (active)
HA	Hemagglutinin
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HLA-DRB1	Human leukocyte antigen, class II, DR beta 1
HRP	Horseradish peroxidase

HSP	Heat shock protein
ICAM-1	Intracellular adhesion molecule-1
IFN γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IP	Intraperitoneal
IP3	Inositol triphosphate
IRB	Institutional review board
IU	International unit
kb	Kilobase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharride
LRR	Leucine rich repeat
LSL	Leucine-serine-leucine motif
LTB4	Leukotriene B4
LTB4R1	Leukotriene B4 receptor 1
M	Major alleles of rs204989/rs204991
m	Minor alleles of rs204989/rs204991
M/M	Homozygous for the major alleles of rs204989/rs204991
M/m	Heterozygous for the major alleles of rs204989/rs204991
m/m	Homozygous for the minor alleles of rs204989/rs204991
Mac1	Macrophage antigen 1
MAF	Minor Allele Frequency
Mcl-1	Myeloid cell leukemia sequence-1
MCP-1	Monocyte chemotactic protein-1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatability complex
MKK	Mitogen-activated protein kinase kinase
mRNA	Messenger ribonucleic acid
MTX	Methotrexate
n	Nano (10 ⁻⁹)
N-terminal	Amino terminal
NADPH	Nicotinamida adenine dinucleotide phosphate
NB4*	NB4 cells differentiated 120 hours with all-trans retinoic acid
NBD	Nucleotide binding domain (A.K.A. - NOD)
NBT	Nitroblue tetrazolium
NCBI	National center for biotechnology information
NET	Neutrophil extracellular trap
NLRC	NOD-like receptor family, CARD domain containing
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
PADI4	Peptidyl arginine deiminase, type IV

PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B (Akt)
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RF mutant	Arginine to phenylalanine point mutant of the GoLoco motif
RF	Rheumatoid factor
RFU	Relative fluorescence unit
RGS	Regulator of G protein signaling
RIPA	Radioimmunoprecipitation assay
Rluc	Renilla species luciferase
RNA	Ribonucleic acid
RPM	Rotations per minute
rs204989	<i>GPSM3</i> single nucleotide polymorphism cytosine to thymine
rs204990	<i>GPSM3</i> single nucleotide polymorphism guanine to thymine
rs204991	<i>GPSM3</i> single nucleotide polymorphism adenine to guanine
rs2812378	<i>CCL21</i> single nucleotide polymorphism thymine to cytosine
rs3096688	<i>GPSM3</i> single nucleotide polymorphism adenine to guanine
rs6457620	<i>HLA</i> single nucleotide polymorphism cytosine to guanine
shRNA	Short hairpin ribonucleic acid
SNP	Single nucleotide polymorphism
spp.	Species
T	Thymine
Th17	T-helper 17 lymphocyte
TLR	Toll like receptor
TNF- α	Tumor necrosis factor-alpha
TNFi	Tumor necrosis factor-alpha inhibitor
x g	Number times Earth's gravitational force
YC	Carboxy terminal YFP
YFP	Yellow fluorescent protein
YN	Amino terminal YFP
Δ	Change relative to controls
μ	Micro (10 ⁻⁶)

Chapter 1

General Introduction

1.1 – Rheumatoid arthritis clinical introduction

1.1.1 – Background

Rheumatoid arthritis (RA) is a chronic, autoimmune polyarthritis that, if left untreated, leads to deformity, chronic pain, and disability for patients through a process of synovial inflammation, synovial cell proliferation with angiogenesis, and joint destruction. Clinically, RA is characterized by chronic inflammation of the synovial tissue of multiple joints, and may manifest in severe joint deformity². Additional extra-articular disease co-morbidities, such as vascular disease from unchecked inflammation, further lead to increased mortality^{3–5}. Improved understanding of immune system-mediated RA pathogenesis has led to new biologic treatments that target selective elements of the RA inflammatory response; however, there are still patients who are non-responders or experience adverse effects limiting their treatment^{6,7}. This is likely a result of disease variability between the pathology of RA in different patients, characterized by differences in the frequency and severity of inflammation, duration of inflammation, and frequency of inflammatory bouts. This variability can be characterized by defining three subclasses of RA: monocyclic, polycyclic, and progressive RA. A minority of patients present with monocyclic RA, where a single inflammatory episode occurs and then remission follows. Polycyclic RA is defined as patients presenting with a continuously fluctuating level of disease activity, while progressive RA will continue to increase in severity and does not resolve².

1.1.2 – Treatment Options

1.1.2a – Non-steroidal anti-inflammatory drugs (NSAIDs):

NSAIDs are typically used to treat mild to moderate rheumatoid arthritis as a first-line therapy. The anti-inflammatory capabilities of non-selective NSAIDs are due to their antagonism of

cyclooxygenase preventing the conversion of arachidonic acids to prostaglandins. The primary effect of NSAIDs is likely due to inhibition of cyclooxygenase-2. Conversely, cyclooxygenase-1 is involved in platelet aggregation, gastric protection, and renal regulation. Therefore, inhibition of cyclooxygenase-1 in non-selective NSAIDs results in many of the reported adverse effects. Selective NSAIDs have been developed with selectivity for cyclooxygenase-2, and have similar effects to non-selective NSAIDs with lower risks of adverse effects with prolonged use (reviewed in ⁸). Drugs of this class have protective benefits over placebo in patients with RA, and show improvements in pain and stiffness scores⁹. At clinical dosages, NSAIDs do not reduce acute-phase proteins in the serum or inhibit progression of radiographic joint damage⁸.

1.1.2b – Non-biologic disease-modifying antirheumatic drugs (DMARDs):

Methotrexate (MTX): MTX is currently the first-line therapy for RA. The classical mechanism of action of MTX in malignancies is the competitive inhibition of the enzyme dihydrofolate reductase, responsible for the reduction of folic acid to metabolically active forms¹⁰. However, in the treatment of RA the weekly low-dose treatment is approximately three orders of magnitude lower than the weekly dosing in oncology¹¹, and supplementation of MTX treatment with metabolically active folates apparently does not disrupt the anti-inflammatory properties of MTX¹². Despite the approval of MTX by the FDA, researchers have yet to fully elucidate its mechanism of action for low-dose RA therapy. Some proposed hypotheses for this action include the release of anti-inflammatory adenosine^{13,14}, growth arrest, and impaired adhesion¹⁵. Although most patients respond well to low dose MTX treatment, a number of adverse effects are noted including bone marrow depression (e.g., anemia, leukopenia), hepatotoxicity, and immunosuppression¹⁶.

Azathioprine: Azathioprine is a purine synthesis inhibitor, disrupting the functions of amidophosphoribosyltransferase¹⁷. The adverse effects of azathioprine are similar to those seen in MTX treatment¹⁸.

Leflunomide: Leflunomide is a pyrimidine synthesis inhibitor, disrupting the functions of dihydroorotate dehydrogenase¹⁹. The adverse effects of Leflunomide are similar to those seen in MTX treatment, with the addition of hepatotoxicity, and a higher risk of diarrhea. Additionally Leflunomide is contraindicated during pregnancy, and inhibits the activity of the metabolically relevant proteins cytochrome P450C8 and organic anion transporter 3²⁰.

Hydroxychloroquine: Hydroxychloroquine treatment results in a decrease in a number of proinflammatory cytokines including interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor alpha (TNF α). This effect is the result of a poorly understood mechanism of action that may include inhibition of toll-like receptors²¹ and alkalization of intracellular vacuoles (disrupting pH sensitive enzymes and antigen processing)²².

Sulfasalazine: The mechanism of action of sulfasalazine is poorly understood in the context of RA. Both sulfasalazine and its metabolite are poorly absorbed in the bloodstream²³. Sulfasalazine has been shown to function in isolated colon epithelial and T-lymphocyte immortalized cell lines through inhibition of I κ B kinases. By inhibiting I κ B kinases, sulfasalazine indirectly disrupts proinflammatory Nuclear Factor κ B activity^{24,25}. Although sulfasalazine treatment is generally well tolerated, immunosuppression and megaloblastic anemia can be side effects due to inhibitory effects on folate synthesis²⁶.

1.1.2c – Biologics

TNF α Inhibitors (TNFi): TNFi are a drug class that functions by inhibition of TNF α , and result in marked decreases in inflammatory markers²⁷. There are currently five TNFi approved for treatment of RA. These are divided into four subcategories: humanized mouse monoclonal antibodies (infliximab), fully human monoclonal antibodies (adalimumab and golimumab), a recombinant soluble TNF α receptor-IgG Fc region fusion protein (etanercept), and polyethylene glycolated humanized Fab fragment (certolizumab)²⁸. Phase III clinical trials with each of the five TNFi resulted in more than half of patients experiencing a reduction in American College of Rheumatology (ACR) scores of $\geq 20\%$ (ARC20) versus placebo, except golimumab monotherapy (44%)^{29–32}. In head-to-head comparisons between TNFi and MTX, meta-analytic data show that when administered as a monotherapy, TNFi and MTX are equally efficacious in reaching ACR20 criteria; however, when TNFi are administered in combination with MTX, TNFi elicit a significant improvement in ACR20 over MTX alone³³. Noted TNFi side effects include increased risk of serious infections^{34,35}, and of lymphomas³⁶, due in part to the systemic immunomodulatory role of TNF α ^{37–39}. Additionally, the immunogenicity of TNFi can lead to increased adverse reactions and treatment failure in patients⁴⁰.

T cell co-stimulatory signal inhibitor – abatacept: Abatacept is a fusion protein composed of the Fc region of immunoglobulin G (IgG) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4). In order for T-cells to become activated by antigen presenting cells, they require interaction of the major histocompatibility complex with T-cell receptor and a co-stimulatory interaction between CD80 or CD86 and CD28⁴¹. Conversely interaction between abatacept and CD80/86 inhibits the required co-stimulatory signal and results in decreased activity of T-cells⁴¹. In head-to-head comparisons of co-administration of MTX with adalimumab or abatacept, the efficacies

of these two treatments were not statistically different, although abatacept-treated patients did report fewer adverse effects and a greater number completed the 12-month treatment⁴².

Anti-CD20 antibody – rituximab: Rituximab is a monoclonal antibody targeting CD20, a cell membrane-localized phosphoprotein expressed on B-cells but not plasma cells. Rituximab-CD20 binding results in B-cell cytotoxicity via antibody- and complement-dependent mechanisms⁴³. Another effect seems to be the inhibition of anti-apoptotic signaling pathways, further reducing circulating B-cells⁴⁴. These mechanisms are believed to reduce the production of B-cell-derived autoantibodies. In a meta-analysis of rituximab monotherapy, it was shown to have similar efficacy to other non-TNFi biologics with 51% of patients meeting ACR20 criteria⁴⁵.

Interleukin 1 (IL-1) inhibitor – anakinra: Anakinra is a recombinant form of the endogenous human IL-1-receptor antagonist. Anakinra functions by binding to and inhibiting IL-1, which acts as a proinflammatory cytokine in RA. In brief, IL-1 has several functions related to RA pathogenesis including stimulating bone resorption⁴⁶, cartilage degradation, and leukocyte activation⁴⁷. A phase III trial of MTX non-responders reported mild improvements in percent of patients receiving anakinra and MTX meeting ACR20 criteria compared to MTX monotherapy (38% vs. 22%)⁴⁸. The most frequently reported serious side effect in anakinra therapy is severe infectious episodes, when compared to placebo⁴⁹, however head-to-head comparisons are needed to assess if this is more severe than in TNFi therapy.

IL-6 receptor inhibitor – tocilizumab: Tocilizumab is a humanized monoclonal antibody targeting the IL-6 receptor, thus disrupting its signaling. Activation of the IL-6 receptor in RA promotes the pro-inflammatory differentiation of Th17 cells (with Transforming Growth Factor- β), the production of acute phase proteins⁵⁰, osteoclast differentiation⁵¹, and matrix metalloproteinase production (with IL-1)⁵². ACR20 responses in combination with MTX were reported at 61%⁵³,

and tocilizumab treatment is uniquely effective at decreasing degradation of joint tissue as well^{54,55}. Treatment with tocilizumab has been shown to increase the risk of infection⁵⁶ and immunogenicity⁵⁷, without the risk of malignancies over placebo⁵⁶.

1.1.2d – Glucocorticosteroids (GC):

Glucocorticosteroids: GC exerts multiple anti-inflammatory effects relevant to the pathophysiology of RA. One of the effects is to inhibit NF κ B-induced transcription of proinflammatory genes, such as cyclooxygenase-2, IL-1 β ⁵⁸, IL-6⁵⁹, IL-17⁶⁰, and TNF α ⁶¹. GC treatment is most effective prior to onset of tissue damage in early stages of RA (*i.e.*, 1-2 years post onset), when in combination with a DMARD, GC significantly reduced radiographic bone erosion⁶². The primary limitation of GC treatment is off-target effects in the adrenal gland (*e.g.*, adrenal hypertrophy), cardiovascular system (*e.g.*, dyslipidemia), kidney (*e.g.*, sodium retention), and nervous system (*e.g.*, glucocorticoid-induced psychoses) (reviewed in ⁶³)

1.1.3 Treatment Approach

The most common treatment approach for RA follows “treat-to-target” recommendations, which were initially proposed in 2010⁶⁴, and are now recommended by the American College of Rheumatology (ACR)⁶⁵. In summary, the overarching principles of “treat-to-target” recommendations are to maintain either remission or low disease activity by careful monitoring of disease activity and modifying treatments as deemed necessary by the rheumatologist and patient⁶⁴. The ACR has since adapted this principle into their guidelines for the treatment of RA (Fig. 1.2).

Under the ACR 2015 guidelines, the primary goal for patients presenting with early RA is disease remission, with disease activity monitored every 1-3 months. If over-the-counter non-

steroidal anti-inflammatory drugs do not control disease activity, patients are typically prescribed a non-biologic DMARD monotherapy. Of the classical non-biologic DMARDs, methotrexate (MTX) is recommended (purine synthesis inhibitor). Other non-biologic DMARDs available to rheumatologists include azathioprine, leflunomide, sulfasalazine, and hydroxychloroquine. The goal of this treatment is to enter disease remission. If patients continue to present with high disease activity following this initial treatment, then combination therapy can be prescribed. Combination therapy involves the addition of another non-biologic DMARD, a TNFi, or a non-TNF biologic DMARD at the patient and rheumatologist's discretion. In the event that remission cannot be attained, the ACR 2015 guidelines recommends an alternative goal of attaining low disease activity (Fig. 1.1)⁶⁵. Once the patient's disease activity meets the criteria for either remission or continued low disease activity, the treatment goal becomes the maintenance of the lowest possible disease severity. Typically disease activity will be monitored every 3-6 months, and treatments will be adapted as required (Fig. 1.1)^{64,65}.

1.1.4 Rheumatoid arthritis risk factors

The mechanism of RA pathogenesis remains elusive, although it is hypothesized that it is a result of both genetic and environmental factors². A non-inclusive list of RA risk factors includes age, sex, smoking, physical activity level, and genetics. It is generally accepted that onset of RA is most frequently seen in patients greater than 60-years old, with the incidence rate in women 2-3 times greater than what is seen in men. Smoking is one of the greatest modifiable risk factors with a reported risk ratio between 1.3-2.4².

The genetic risk factors for RA may have an even more important role in RA pathogenesis, as indicated by a concordance rate 4.3 times greater among monozygotic twins relative to dizygotic twins⁶⁶. Another study in twins confirms this report, estimating genetic factors contribute 60% of the risk of developing RA⁶⁷. The introduction of genetic references,

like the human genome and HapMap projects, has led to significant advances in technologies such as genome-wide association studies (GWAS). These technologies have contributed greatly to the understanding of the genetic risk factors involved in RA; however, much of the complex genetic contributions to RA pathogenesis have yet to be elucidated. An example of the complexity of this disease is reflected in the results from one of the largest GWAS meta-analyses performed to date. This study analyzed 22 different RA GWAS studies with a total of >10 million single nucleotide polymorphisms (SNPs) in >100,000 volunteers, and identified 101 risk alleles that were conserved across multiethnic individuals of Asian and European ancestries⁶⁸. Another database reports 123 risk alleles that have been confirmed in multiple independent GWAS⁶⁹. Among the identified risk alleles, some of the most significantly associated with RA include haploblocks of *HLA-DRB1*^{70–72}, *PADI4*⁷³, *PTPN22*⁷⁴, *TNFAIP3*⁷⁵, *TRAF1/C5*⁷⁶, *REL*⁷⁷, and *CCR6*⁷⁸ (reviewed in ref.⁷²). Despite the advances in GWAS, conclusions from these studies are strictly limited to disease association. To validate many of these findings, much more research needs to be performed to better understand the physiological consequences of many identified genetic variants.

1.2 Neutrophils in Rheumatoid Arthritis

This work is currently being edited for submission as a review publication in: Arthritis and Rheumatism

1.2.1 Introduction to Neutrophils

Neutrophils represent a terminally differentiated leukocyte population derived from myeloid progenitor cells, which also give rise to monocytes, basophils, and eosinophils. In humans, neutrophils are the most common circulating leukocyte in peripheral blood (40-60%)⁴¹.

These cells have multiple granules containing preformed inflammatory mediators and antimicrobial substances that are tightly regulated to ensure they are not inappropriately released. In healthy individuals, neutrophils exist in a resting state where they require additional signals to prime and subsequently release these compounds. Neutrophils require a three-step process to release pre-formed granules: (1) priming, (2) migration, and (3) activation. Priming of resting neutrophils is mediated by pathogen-associated molecular patterns (PAMPs; *e.g.*, lipopolysaccharides)⁷⁹ and cytokines (*e.g.*, TNF- α , GM-CSF, IL-8, and IFN- γ)^{80–82}. Primed neutrophils rapidly mobilize intracellular granules, containing pre-formed receptors, to the plasma membrane, and priming agents also affect transcription of many genes to alter neutrophil function and increase lifespan (discussed in detail later). Primed neutrophils can then migrate to the site of inflammation via chemotaxis and are the first leukocytes to arrive in inflamed or injured tissues⁸³. Once within the inflamed tissue, neutrophils are activated by auto-reactive immunoglobulin complexes, cytokines, and complement, inducing the release of granule contents (Table 1.1; reviewed in ref. ⁸⁴).

Once within the inflamed tissues, additional granules containing oxidant-producing enzymes and proinflammatory compounds are released into the extracellular space and contribute significantly to tissue damage in chronic disease processes like RA (Table 1.2)^{85,86}. This non-specific release of cytotoxic mediators is amplified as activated neutrophils continue to release cytokines that induce further neutrophil and pro-inflammatory leukocyte recruitment in a positive feedback loop. This positive feedback recruitment must therefore be prevented prior to any repair of tissues and resolution of the inflammatory response. In healthy individuals the acute stage of inflammation is followed by the recruitment of anti-inflammatory macrophages that inhibit neutrophil migration to tissues and removes apoptotic and necrotic cells via phagocytosis. These macrophages then continue to secrete anti-inflammatory cytokines promoting the repair of damaged tissues, and resolution of inflammation.

This review will focus on neutrophil physiology as it pertains the pathophysiology of RA, covering the recruitment to inflamed synovial tissues, the common presence of neutrophil-derived anti-granulocyte and anti-citrullinated protein antibodies in RA patients⁸⁷, the disruption of induced arthritic inflammation in transgenic mouse models⁸⁸, and the ability of neutrophils to affect other leukocytes.

1.2.2 Recruitment of neutrophils to synovial tissues and fluid

In RA, sterile tissue damage or autoantibody complexes can disrupt tissue homeostasis resulting in the production of damage-associated molecular pattern molecules (DAMPs) that can be recognized by resident antigen-presenting cells (*e.g.*, macrophages, dendritic cells) or resident stromal cells^{89,90}. The activated resident cells then release a number of pro-inflammatory mediators including IL-1 β , TNF- α , lipid mediators (*e.g.*, leukotriene B4), and chemokines. This process results in recruitment of circulating neutrophils into the synovial tissues, thereby initiating the tissue inflammation that is a hallmark of RA⁹¹.

The cascade of events by which neutrophils are recruited toward pro-inflammatory mediators includes (1) adhesion to vascular endothelial cells, (2) transendothelial migration, and (3) migration through tissue along chemokine gradients. (1) Neutrophil adhesion to vascular endothelial cells is a complex process, which can be further subdivided into (a) vascular neutrophil activation, (b) rolling adhesion, and (c) tight adhesion. (a) The activation of circulating neutrophils results in preferential association with vascular endothelial cells. Induction of this activation state is not completely characterized, but it may be the result of elevated serum TNF α , IL-1, chemoattractants (*e.g.*, formylated peptides, C5a), or anti-neutrophil autoantibodies (*e.g.*, ANCA, ACPA)^{80,92}. (b) Neutrophil rolling adhesion is a process mediated predominantly

through TNF α - and IL-1-activated⁹³ endothelial cell E- and P-selectin interaction with neutrophil adhesion molecules (e.g., P-selectin glycoprotein ligand-1 [PSGL-1], cluster of differentiation 44 [CD44], and E-selectin ligand 1 [ESL1])⁹⁴. These weak interactions result in neutrophil “rolling” in the post-capillary venules at shear stress between 1-10 dynes per cm² (⁹⁵), and the process is mediated by sequential weak binding and vascular shear stress-mediated release of complementary binding molecules. While neutrophils are rolling across the vascular endothelium, they are “sampling” chemokines and cytokines from the underlying tissue that are transported and bound to the apical/vascular surface of endothelial cells by negatively-charged glycoaminoglycans⁹⁶. Chemokine receptors on “rolling” neutrophils recognize endothelial-bound chemokines, resulting in a conformational change of β -integrins increasing their affinity and promoting (c) firm adhesion of neutrophils to activated endothelial cells proximal to the site of inflammation^{97,98}. This activation of β_1 - (e.g., VLA-4) and β_2 -integrins (e.g., LFA-1, MAC1) occurs via intracellular signaling networks – believed to be mediated through the actions of G protein $\beta\gamma$ subunits^{99,100}. Once activated, firmly adhered neutrophils can begin the process of transendothelial migration (reviewed in ref. ⁹⁴).

(2) Neutrophil transendothelial migration occurs following adhesion; however, neutrophils do not frequently transmigrate at the site of initial adhesion. Often neutrophils extend pseudopods and appear to ‘probe’ the vascular endothelial cells without moving from the initial anchor point. Under *in vivo* flow conditions, neutrophils then proceed to slowly migrate along the endothelial cells, in a MAC-1-dependent process¹⁰¹, perpendicular to forces of shear stress¹⁰². This migration pattern likely increases the odds of a neutrophil recognizing a ‘transmigration point’, because neutrophils are moving across endothelial cell axis that is elongated by vascular shear stress and therefore encounter more endothelial junctions¹⁰³. To leave the vasculature, neutrophils must first cross the endothelial cell layer through paracellular (*i.e.*, between cells) or transcellular (*i.e.*, through cells). Because paracellular migration presents the fewest barriers to migration and occurs more rapidly, neutrophils preferentially use this pathway^{101,104,105}.

Transcellular migration is observed, but is less efficient and more time consuming¹⁰⁴. The paracellular route typically occurs at endothelial tricellular junctions, where junctional proteins are at the lowest density^{105,106}. Transmigration involves the interaction of a number of neutrophil surface molecules (e.g., LFA-1, MAC-1, VLA-4, CD99, PECAM1) with endothelial surface molecules (e.g., ICAMs, VCAM1, CD99, junctional adhesion molecules) (reviewed in ⁹²).

(3) Following adhesion and transmigration, neutrophils must next migrate through a number of barriers, including the vascular basement membrane, vascular pericytes, and tissue matrix. Neutrophils preferentially migrate where the extracellular matrix is least dense¹⁰⁷, then are induced by chemoattractants¹⁰⁸ within the basement membrane to release matrix metalloproteinases (e.g., MMP-9) and proteases (e.g., elastase, collagenase; table 1.4)¹⁰⁹. These enzymes make the basement membrane more porous and allow neutrophils to migrate toward the vascular pericytes, which are less densely packed in regions with porous basement membranes, thus are readily circumnavigated¹¹⁰. Migration through additional tissue extracellular matrix is less dependent on enzymes to degrade matrix, due to the decreased density of matrix polymers. Migration through additional extracellular matrix is primarily mediated by chemokine gradients⁹². The final barrier to entry of neutrophils into the synovial tissues in early RA is a poorly vascularized layer of cartilage. This process is not well characterized, but is likely a result of the actions of neutrophil collagenase, elastase, and MMP9 (Table 1.2). There is less of a necessity to migrate through cartilage late in the RA inflammatory response, because of the formation of a neovascularized pannus, formed by synovocyte hyperplasia, within the synovial tissues¹¹¹. The additional blood vessels within the synovial tissues likely increase leukocyte recruitment by decreasing the distance and barriers to migration.

In RA, the process of neutrophil migration through these barriers to the site of inflammation is mediated by a number of chemoattractant gradients. Chou et al. highlights the

complexity of neutrophil recruitment by hypothesizing that a temporal cascade of leukotriene B4 (*i.e.*, LTB4 or 5,12-diHETE), IL-1 β , then CXCR2 agonists (*e.g.*, CXCL1/GRO α , CXCL2/GRO β , CXCL8/IL-8)¹¹² and complement orchestrates leukocyte recruitment in RA^{112–114}, a hypothesis that is widely accepted by others in the field^{86,115,116}. Initially, lipid mediators like LTB4, a highly potent chemoattractant for neutrophils, are produced at a low concentration by activated resident leukocytes. The low concentration of LTB4 induces in a mild recruitment of circulating neutrophils, which are activated in synovial tissue, leading to the release of additional LTB4, thus establishing a positive feedback loop and markedly increasing the number of neutrophils recruited. One study has shown this positive feedback on neutrophil-derived LTB4 results in concentrations as high as 400 ng/ml (1.2 μ M) in synovial fluid samples of a cohort of seropositive RA patients during inflammatory bouts¹¹⁷. The role of LTB4 in rheumatoid arthritis-like inflammation is apparent in *leukotriene B4 receptor 1* (*Ltb4r1* or *Blt1*)-deficient mice that exhibit a profound lack of detectable inflammation in both serum-transfer (K/BxN)¹¹² and collagen-induced (CIA)^{118,119} models of inflammatory arthritis. Similar protection from models of arthritis are also afforded to mice deficient in enzymes involved in LTB4 synthesis, including 5-lipoxygenase activating protein (*Flap*)¹²⁰, 5-lipoxygenase (*Alox5*)¹²¹, and leukotriene A4 hydrolase (*Lta4h*)¹²¹; protection was also reported in mouse models utilizing pharmaceutical inhibitors of many of these enzymes^{122–124}. Of note, inhibition of LTB4 receptor with the orally-active competitive antagonist, BIIL 284, did not significantly improve ACR20 scores in a 3 month clinical trial performed in patients with RA¹²⁵. Although not reported in the literature, an additional LTB4 receptor inhibitor (CP-195,543¹²⁶; clinicaltrials.gov) also failed in clinical trials due to a poor tolerability profile. These results from human trials are discrepant from positive effects seen in mouse models, but may be a result of elevated concentrations of LTB4 outcompeting the antagonist or the confounding requirement that patients cease their therapeutic regimens prior to these studies. An alternative approach to inhibit LTB4 signaling may be to target an enzyme involved in LTB4 synthesis, which had been attempted in clinical

trials with a soluble phospholipase a2 inhibitor, LY333013, but was speculated to be hampered by poor uptake into synovial fluid and tissues¹²⁷. An additional LTB4 synthetic enzyme to target may be LTA4 hydrolase, which would decrease levels of LTB4 secreted and prevent the inflammatory response in RA. Despite the inconclusive human clinical trial data, the previous mouse model studies and the elevated LTB4 levels detected in human synovial fluid suggest a role for LTB4 as an obligate mediator of early recruitment of neutrophils.

Following LTB4-induced recruitment, additional chemoattractant signals are important in the continued recruitment of neutrophils. Recruited LTB4R1-positive neutrophils are then stimulated to release IL-1 β in response to the increased synovial concentration of DAMPs (e.g., antibody complexes, purine metabolites, hyaluronan monomers, nucleic acids, interferons), and consequently, autocrine IL-1 β signaling upregulates neutrophil synthesis and secretion of proinflammatory mediators, including LTB4, CCR1 agonists, and IL-8^{112,128}. The clinical importance of IL-1 β in arthritis is highlighted by the utility of Anakinra, an FDA-approved therapeutic synthesized from human recombinant interleukin 1 receptor antagonist (IL-1Ra)⁴⁹. Of note, mouse strains deficient in IL-1 β production (*i.e.*, *pro-Il1b*-deficient and *caspase-1*-deficient) are not protected from K/BxN serum transfer-induced acute paw swelling; however, these mice are protected from cartilage damage, proteoglycan depletion, and cell influx to synovial tissue in *Streptococcal* cell wall-induced chronic arthritis¹²⁹. However, the role of IL-1 β is highlighted by the exogenous administration of recombinant IL-1 β to *Ltb4r1*-deficient mice restoring K/BxN serum transfer-induced acute arthritis, presumably via by-passing the requirement for LTB4-signaling to initiate this IL-1 β production¹¹². Therefore, it may be that the innate immune-driven models of acute arthritis may be capable of inducing inflammation primarily through lipid mediators (e.g., LTB4), but prolonged recruitment and activation of leukocytes is driven through IL-1 β signaling.

In the pathophysiology of rheumatoid arthritis, recruitment of additional lymphocytes is required, and is believed to be partially mediated by IL-1 β inducing the release of neutrophil extracellular traps (NETs)¹³⁰. NETs have been shown to contain various targets for lymphocyte-derived autoantibodies, including neutrophil cytoplasmic and histone proteins that have been citrullinated by enzymes like protein arginine deaminase 4 (PADI4). Anti-citrullinated protein antibodies (ACPA) and anti-neutrophil cytoplasmic antigen (ANCA) are hallmark RA autoantibodies that target these entities released from NETs^{131–133}. Additionally, NET release from neutrophils releases a number of lymphocyte activating components (described in detail later). Although the complete mechanism of IL-1 β in acute and chronic arthritis requires additional study; however, at least part of the role for IL-1 β is the formation of NETs, which in turn release auto-antigens necessary for the chronic inflammatory state of RA.

IL-1 β also stimulates the release of resident leukocyte-derived CXCR2 agonists, including CXCL1 (GRO- α), CXCL2 (GRO- β), and CXCL8 (IL-8), all of which amplify the recruitment of neutrophils to continue the inflammatory response within synovial tissues¹¹². In *Cxcr2*-deficient mice K/BxN serum-induced inflammatory arthritis does not appear to be different from the wild-type mice over the first 5 days. After that time, there is significant decrease in ankle swelling in the *Cxcr2*-deficient mice¹³⁴; furthermore, mice treated with a synovial injection of a dominant-negative form of CXCL8 also exhibited only partial decreases in nociception in a methylated bovine serum albumin-induced model of inflammatory arthritis¹³⁵. These data may reflect the temporal relationship of CXCR2 ligand signaling to the inflammatory process, i.e., primarily involved in late phase inflammation in RA¹¹³. Therefore, the CXCR2-deficient and -blocked models of disease may develop early LTB₄- and IL-1 β -dependent inflammation but be protected from prolonged inflammation that is not reliably demonstrated in acute inflammatory arthritis models^{112,134}. IL-1 β may also increase recruitment of monocytes/macrophages, given that treatment of human monocyte-derived macrophages with IL-1 β induces production of CCL2

(MCP-1)¹³⁶ as well as endothelial cell CX3CL1 (fractalkine) expression¹³⁷, both of which are monocyte/macrophage chemoattractants^{138–140}. The influence of CCL2-induced monocyte recruitment may however be a minor component of arthritic inflammation. This notion is supported by the lack of protection in *Ccr2*-deficient mice and moderate protection in *Cx3cr1*-deficient mice in K/BxN serum-induced inflammatory arthritis¹³⁴, as well as blockade of CCR2 and CCR5 failing to inhibit primary human monocyte recruitment *in vitro* or offer therapeutic benefits in clinical trials¹⁴¹.

Beyond the LTB4/IL1 β /CXCR2 axis, recruitment of neutrophils to synovial tissues in RA also occurs as an indirect result of autoimmune antibodies (e.g., ANCA, ACPA) or acute phase proteins (e.g., C-reactive protein) that activate the complement cascade, and additionally produce anaphylatoxins like complement component 5a (C5a)^{142,143}. The role of C5a in RA pathophysiology is supported by *C5ar*-deficient mice exhibiting protection from K/BxN serum transfer arthritis¹¹⁴, while C5a¹⁴⁴ and C5a receptor¹⁴⁵ antagonism by monoclonal antibodies protects wild type mice from collagen-induced arthritis models. Furthermore, mutations in the human C5 gene, which enhance the rate of proteolysis of the inert C5 protein into the anaphylatoxin C5a¹⁴⁶, are risk alleles for RA^{76,147}. These and other findings have led Novo Nordisk to develop a therapeutic C5a receptor inhibitor, which is currently in clinical trials¹⁴⁵. However, it has yet to be fully elucidated how C5a signaling is incorporated into the proposed temporal chemokine cascade.

In summary, these aforementioned data support the sequential recruitment of neutrophils and other leukocytes through the sequential functions of LTB4, IL-1 β , CXCR2, and complement, which emphasizes the importance of each in the pathogenesis of RA. Lack of RA protective phenotypes detected in *Ccr1-9*, *Cxcr3*, and *Cxcr5*-deficient mouse strains further support a central role for this LTB4/IL-1 β /CXCR2 chemokine axis in the development of

inflammatory arthritis¹³⁴. However, there has been recent mild success in clinical trials of the CCR1 antagonist CCX354-C¹⁴⁸. In this study, relative to placebo, patients naïve to traditional biologic medications saw an improved ACR20 response ($\geq 20\%$ improvement in 3 of 5 disease-relevant criteria; placebo - 26% vs. CCX354-C - 62%) and a trend toward improvement of ACR50 response ($\geq 50\%$ improvement in 3 of 5 disease-relevant criteria; 15% vs. 35%) following placebo or CCX354-C treatment, respectively¹⁴⁸. The greatest mRNA expression of *CCR1* is seen in both neutrophils and monocytes, important innate immune cells in RA pathogenesis¹⁴⁹, and therefore may support the additional complexity of human rheumatoid arthritis versus mouse models thereof.

Furthermore, the LTB₄/IL-1 β /CXCR2 model may not completely explain neutrophil chemotaxis, as no mouse models of arthritis completely replicate human rheumatoid arthritis. It is also noteworthy that differences in physiology exist between human and mouse neutrophils. For example neutrophils only represent 10-25% of circulating leukocytes in mice¹⁵⁰ compared to the 40-80% that human neutrophils represent⁴¹. Additionally, protein expression of the CXCR2 sister receptor, CXCR1 – that shares many agonists with CXCR2, is absent in mouse neutrophils¹⁵⁰; moreover, production of some cytokines including IL-6¹¹⁶ and IL-10¹⁵¹ is shown in mouse neutrophils, but in human neutrophils remains controversial. Furthermore, different models of inflammatory arthritis in mice may disproportionately represent certain arms of the immune response. For example, acute models of arthritis include K/BxN serum transfer and collagen antibody-induced arthritis (CAIA) that primarily model the innate immune response to autoantibody deposition. Chronic models of arthritis include as collagen-induced arthritis (CIA) and *Streptococcal* cell wall-induced arthritis (SCW), that involve both the innate and adaptive immune responses to develop autoantibodies. Stating these differences is not meant to suggest that the mouse models are non-translatable to the features of human RA, but rather these and other differences must be taken into account when interpreting mouse data. Therefore, although

a fair amount is known in human RA pathogenesis as a result of clinical studies, the need for additional clinical trials, *in vitro*, and genomic studies cannot be understated.

1.2.3 Neutrophil degranulation

Upon migration into the synovial tissues, cytokine-primed neutrophils can recognize both soluble and tissue-deposited antibodies (e.g., ACPA) via one of 3 highly expressed receptors (FcγRI, FcγRII, and FcγRIIIb), and activation of these receptors on primed neutrophils can initiate degranulation. Disruption of the normal physiology of each of these receptors may be involved in RA pathogenesis. (1) FcγRI (CD64) is a high-affinity receptor for IgG antibodies. FcγRI is not expressed at the cell surface in unprimed neutrophils from healthy individuals, but is expressed in neutrophils isolated from the synovial fluid of RA patients. The expression of this high-affinity antibody receptor may increase the sensitivity of RA neutrophils to antibody complexes resulting in increased degranulation¹⁵². (2) FcγRII (CD32) is a low-affinity constitutively expressed receptor that exists in two isoforms, the activating FcγRIIa¹⁵³ and inhibitory FcγRIIb¹⁵⁴ receptor. The importance of the balance between these isoforms is illustrated by the nearly doubled rate of radiologic joint damage in RA patients homozygous for the loss of function non-synonymous 695T>C polymorphism in the *FcγRIIb* gene¹⁵⁴. Further evidence of the importance of the balanced expression of these two isoforms is represented by infliximab (TNFi) treatment, which induces a shift in expression favoring the inhibitory isoform and may contribute to some of its therapeutic benefits in RA¹⁵³. Finally, (3) FcγRIIIb (CD16) is a low affinity receptor shown to play a role in reactive oxygen species production¹⁵⁵. This receptor is of particular interest as an RA therapeutic target, because individuals completely deficient in *FcγRIIIb* transcript do not have impairment in bacterial phagocytosis¹⁵⁶, but exhibit a functional deficit in antibody complex-mediated cytokine release¹⁵⁴. Therefore, therapeutic inhibition of

FcγRIIIb may prevent leukocyte recruitment via disruption of reactive oxygen generation and cytokine release, but not affect host defense responses to bacteria.

Following activation by immune complexes, neutrophils then degranulate and produce reactive oxygen species O_2^- , OH^- , and H_2O_2 (via NADPH oxidase) and HOCl (via myeloperoxidase). Normal resting neutrophils have little capacity to produce reactive oxygen species, because (1) the NADPH oxidase enzyme is multimeric and assembled at the plasma membrane during priming¹⁵⁷ and (2) myeloperoxidase is contained within azurophilic granules and inactive without NADPH oxidase production of its substrate H_2O_2 ¹⁵⁸. Following neutrophil activation in RA, NADPH oxidase enzyme is assembled and produces substrates for myeloperoxidase, resulting in the oxidative burst that increases oxidative stress in the synovial tissues. Increases in reactive oxygen species have been partially implicated in the pathogenesis of RA¹⁵⁹, and RA patients display increased NADPH oxidase activity compared to controls^{160,161}. The effects of reactive oxygen species in RA are likely mediated through a number of processes that result in synovial cell and tissue damage. Synovial cell damage follows oxidation of cell components and activation of NFκB transcription, both of which results in the transcription of proinflammatory genes (e.g., *COX-2*, *PLA2*, *IL-1β*, *TNFα*) and propagate the inflammatory response¹⁶². Reactive oxygen species also induce tissue damage directly through catalysis of cartilage proteoglycans by HOCl and H_2O_2 ^{85,163}, and HOCl can promote cartilage degradation by activation neutrophil collagenase and gelatinase¹⁶³.

The oxidative burst also occurs concurrent to neutrophil degranulation, which positively correlates with severity of synovial tissue inflammation and damage in RA patients¹⁶⁴. Neutrophil degranulation results in release of preformed granule enzymes that degrade structural components of the synovial tissues (Table 1.2). When neutrophils are activated these granule components are released into the synovial fluid, while autoantibodies deposited on synovial tissues result in release of granule components directly on tissues in a process known as

frustrated phagocytosis^{81,86} (Fig. 1.3). The neutrophil granule proteases, metalloproteases, catabolic enzymes, and reactive oxygen species act in concert to degrade high-molecular weight components of cartilage^{85,161,165}. Elastase¹⁶⁶, collagenase¹⁶⁶, matrix metalloproteinase^{167,168}, and gelatinase¹⁶⁸ are all present within neutrophil granules (Table 1.2), and have all been shown to degrade the cartilage matrix. The activity of these enzymes results in the direct tissue damage of arthritic synovial tissues, a hallmark of RA histopathology.

In summary, the role for antibody complex-induced activation and degranulation of neutrophils has been revealed by *in vitro* experiments in which co-culture of cell-free synovial fluid from RA patients with primary circulating neutrophils resulted in a significant increase in NADPH oxidase activity over those treated with synovial fluid from patients with other joint arthropathies. In the same study, depletion of synovial fluid antibodies resulted in a marked decrease in NADPH oxidase activity¹⁶⁹, and blockade of neutrophil FcγRIIIb and FcγRIIa disrupted reactive oxygen species formation in response to soluble and insoluble antibody complexes, respectively¹⁵⁵. These observations support the role of RA autoantibody complexes in the production of neutrophil reactive oxygen species and degranulation (Table 1.1 and 1.2) leading to the hallmark non-specific tissue damage that ensues⁸⁶.

1.2.4 Neutrophil extracellular traps in rheumatoid arthritis

Formation of neutrophil extracellular traps (NETs), first described by Brinkmann et al.¹⁷⁰, represents a unique form of neutrophil cell death distinct from apoptosis and necrosis (reviewed¹⁷¹) that may be involved in the pathophysiology of RA. Correlative evidence shows neutrophils isolated from RA patients more readily undergo the formation of NETs than neutrophils isolated from patients with other forms of arthritis¹³². NET release results from the deimination of histone arginine to citrulline by PAD4 and degradation of the nuclear envelope¹⁷². Chromatin, in association with multiple anti-microbial granule components such as

myeloperoxidase, elastase¹⁷³, and LL37^{131,174}, is then released into the extracellular space¹⁷⁵. The excessive release of citrullinated chromatin and numerous citrullinated antigens present in NETs, including elastase, cathepsin G, proteinase 3, α -defensins, and LL37^{131,176,177} act as potential autoantigens; therefore, NETs have been proposed to be a primary source of citrullinated peptides that can induce formation of ANCA autoantibodies¹³². The importance of this process in the development of inflammatory arthritis is demonstrated by protection from collagen-induced arthritis inflammation via therapeutic inhibition of a citrullinating enzyme present within NETs, PAD14¹⁷⁸. Together these findings support a role for NET formation in RA pathogenesis by presenting potential autoantibody targets during the conversion of acute inflammation into an adaptive humoral immune response that is the hallmark of autoimmune RA. Further explanation of the development of autoantibodies specific for NET-released antigens is reviewed in depth by Darrah & Andrade¹⁷⁹, and the role of NETs in autoimmunity is reviewed in depth by Dwivedi & Radic¹⁸⁰.

1.2.5 Neutrophil modulation of immune cells

Innate immune cells are a subset of leukocytes classically described by their ability to respond to generic molecular patterns (e.g., DAMPs and PAMPs) immediately but innate immune cells are incapable of inducing long-term immunity for the host, like adaptive immune cells can. Classically, neutrophils, basophils, eosinophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, and mast cells comprise the innate immune cell class of leukocytes⁴¹. Innate immune cells, like neutrophils, help to establish the chronic inflammatory response in RA and activate the adaptive immune response. Neutrophils are typically the initial leukocytes recruited, and therefore affect recruitment and activation of additional cells including macrophages, DCs, and lymphocytes (described later, reviewed in refs.^{86,115,181}).

Neutrophil-macrophage interaction: Macrophages are a subset of leukocytes derived from circulating monocytes or from tissue macrophage mitosis. Their canonical function is to recognize and phagocytize moieties that are considered dangerous, then present antigen to lymphocytes to induce an adaptive immune response⁴¹. Neutrophils and macrophages share several common functions (e.g., phagocytosis, production of reactive oxygen species, cytokine production), possibly due to their common origin from myeloid progenitor cells; however, unlike short-lived neutrophils, macrophages have a lifespan on the scale of months and reside within tissues⁴¹. While resident synovial macrophages induce the recruitment of neutrophils, neutrophils in turn recruit additional circulating monocytes through the release of chemokines like CCL2 (MCP-1), CCL3 (MIP-1 α), and CCL4 (MIP-1 β)^{136,137,182}. Neutrophils then release a number of cytokines that affect macrophage responses to inflammation. For example, activated neutrophils⁸² can produce interferon- γ ^{183,184}, which promotes polarization of monocytes toward proinflammatory M1 macrophage physiology^{185,186}. In RA the activation of macrophages can induce pathogenic generation of reactive oxygen species, cytokines, and activation of lymphocytes of the adaptive immune response^{41,187}.

Beyond the production of cytokines, neutrophils can also affect macrophages through the release of myeloperoxidase¹⁸⁸, which can directly damage synovial tissues and, in the context of alveolar inflammation, interacts with the macrophage mannose receptor to increase reactive oxygen species, TNF- α , and IL-1 β production¹⁸⁹. TNF- α and IL-1 β at sites of inflammation increase survival of recruited neutrophils, potentially prolonging and exacerbating inflammation¹⁹⁰. Further work suggests *Mpo*-deficient (myeloperoxidase-deficient) mice are protected from the inflammatory phenotypes seen in collagen-induced arthritis, although it is difficult to determine if these effects are the result of a reduction of direct tissue damage or a reduction of myeloperoxidase activation of macrophages¹⁹¹. In the rheumatic arthritic joint, this interaction between neutrophils and macrophages may both augment tissue damage via

release of proinflammatory mediators (e.g., cytokines, reactive oxygen) and further recruit additional leukocyte subsets, thereby affecting RA pathophysiology.

Neutrophil and dendritic cell (DC) interaction: DCs are another antigen-presenting cell type that functions to process antigens, migrate to proximal lymph nodes, and present antigens to lymphocytes to induce adaptive immune responses. Once activated by antigens, in this case RA-related autoantigens, DCs migrate through the lymphatic system. This process allows transport of antigen from distal sites of inflammation to lymphoid organs, which contain lymphocytes required for the establishment of adaptive immune responses⁴¹. Neutrophils are seen to co-localize with DCs when cultured together¹⁹², and their interaction is able to activate DCs naïve to antigen, which in turn elicits lymphocyte activation¹⁹³. Neutrophils also produce a number of chemokines capable of recruiting mature DCs to sites of inflammation, the most well characterized being CCL21¹⁹⁴ (see Table 1.3; neutrophil cytokine production). This capacity to recruit DCs suggests that initial neutrophil recruitment to inflammation zones may result in recruitment of local tissue DCs allowing their collection of antigens for presentation to adaptive immune cells. If this is the case, neutrophils would play an integral role in the conversion of an acute synovial inflammatory response to a chronic pathology like RA.

Activated neutrophils also release granules and NETs containing a number of alarmins (e.g., HMGB1, α -defensins, LL-37) that are capable of affecting DC migration and maturation. HMGB1-DNA complexes are one example of a neutrophil-derived alarmin that influences both migration and activation of DCs via the TLR9 receptor¹⁹⁵; this process results in the activation of a cytotoxic T_H1 response¹⁹⁶. Additionally, neutrophil granule-derived α -defensins (Human Neutrophil Peptide-1 and -2) are capable of recruiting immature DCs toward sites of inflammation¹⁹⁷, and a cathelicidin, LL-37, from neutrophil granules is observed at a higher concentration in RA synovial fluid compared to healthy controls. LL-37-targeted autoantibodies have also been detected in rat models of arthritis¹⁹⁸. Additionally, in the presence of DNA, LL-37

acts via a TLR7- and TLR8-dependent manner in DC maturation, and expression of lymphocyte co-stimulatory membrane proteins^{131,174,199}. Thus, LL-37 may be a target of pathogenic autoantibodies in RA, and neutrophil-derived alarmins influence the function of DCs in RA inflammation.

Beyond cytokine signaling, the physical interaction of neutrophils and DCs also plays a role in RA pathophysiology. Under inflammatory conditions, neutrophil cell surface expressed Mac-1 (CD11b/CD18) interacts with DC cell-surface expressed DC-SIGN resulting in (1) activation of DCs and (2) presentation of antigens to DCs¹⁹². The activation of DCs in a neutrophil-DC complex will result in DC expression of lymphocyte co-stimulatory molecules (e.g., CD80, CD86, CD40, and HLA-DR^{192,193}), that are required for activation of lymphocytes, and therefore an adaptive immune response. Another neutrophil cell surface molecule, CECAM-1 (CD66a), is also capable of interacting with DC-SIGN, resulting in a delay of neutrophil apoptosis and a prolonged inflammatory response¹⁵². In summary, an important role exists for neutrophil and DC interactions in the progression of RA.

Neutrophil activation of adaptive immune response: The adaptive immune response is defined by the activation and responses of B- and T-lymphocytes, a specialized subset of leukocytes capable of recognizing specific antigenic signals. In contrast to the innate immune response discussed until this point, the adaptive immune response requires 1-2 weeks to be fully activated, recognizes highly specific antigens (not non-specific molecular patterns), and can generate a memory response for rapid mobilization during subsequent antigenic challenges⁴¹. Activated neutrophils can affect the adaptive immune response by promoting the migration of both B- and T-lymphocytes (Table 1.3). However, the canonical role for neutrophils in adaptive immunity is indirect via the activation of antigen-presenting cells (e.g., macrophages and DCs). This activation induces expression of co-stimulatory molecules that are capable of activating antigen specific lymphocytes.

Neutrophils are also capable of expressing MHC class II molecules and thereby directly activating lymphocytes. *In vitro*, synovial fluid neutrophil activation by GM-CSF and IFN- γ has been shown to induce cell surface expression of MHC class II with low expression of co-stimulatory molecules CD80 or CD86²⁰⁰. Furthermore, *in vitro* treatment of peripheral neutrophils with GM-CSF, IL-4, and TNF- α increases cell surface protein localization of MHC class II molecules (e.g., HLA-DR and HLA-DQ), CD80, and CD86²⁰¹. Primary synovial fluid neutrophils from RA patients exhibit a similar expression profile and are capable of activating primary T-lymphocytes in co-culture^{202,203}. The activation of CD4⁺ T helper 2 (T_h2) cells can promote antibody production, which can result in the production of additional autoantibodies. In summary, the MHC class II and co-stimulatory molecule expression by neutrophils appears to be context-dependent and present within RA inflammatory attacks. The extent of MHC class II presentation may also be a factor in individual susceptibility to various autoimmune diseases. Therefore, activated RA synovial neutrophils and recruited T-lymphocytes probably interact cooperatively to propagate antibody production and inflammation in RA.

1.2.6 Resolution of neutrophil recruitment

Resolution of a normal inflammatory response, within the synovial tissues, is a highly regulated process that protects the host from prolonged inflammation and prevents the establishment of a chronic inflammatory response. A chronic disease, like RA, is the result of a decreased ability to stop the acute inflammatory response. An integral cell type involved in RA pathophysiology is neutrophils, thus prolonged lifespan of RA neutrophils and disrupted egress from inflamed tissues may lead to chronic inflammatory responses. Many recruited neutrophils enter apoptosis following a normal inflammatory response, and then are cleared from the tissues by anti-inflammatory macrophages. Apoptosis in healthy peripheral blood neutrophils typically results in a short lifespan, with a half-life of between 18 and 90 hours as shown by *in*

vivo labeling studies²⁰⁴, and cultured neutrophils from RA patients exhibited a delay of apoptosis between 6-22 hours compared to healthy controls²⁰⁵. The lifespan of neutrophils increases significantly in the pro-inflammatory RA synovial tissues, where changes in dissolved oxygen and pH significantly delay apoptosis and resolution of inflammation^{206,207}. There are also populations of neutrophils in RA that avoid apoptosis and return to the vasculature by reverse transmigration. As many as 2% of peripheral blood neutrophils from RA patients may have undergone reverse transmigration, in contrast this population make up only 0.25% of peripheral blood neutrophils are seen in healthy donors²⁰⁸. The implications of this process are not known, but it could be a protective mechanism to decrease the neutrophil load in synovial tissues, or a pathogenic mechanism that establishes a systemic state of inflammation. Although the process of neutrophil apoptosis versus reverse migration is not completely characterized, some hypotheses for the delay in neutrophil apoptosis include disrupted lipid mediator class switching^{209,210}, elevated serum and synovial fluid concentration of GM-CSF in RA patients^{207,211,212}, elevated synovial fluid lactoferrin concentrations²¹³, and hypoxic conditions within the inflamed synovial tissue²⁰⁶. Regardless of the cause, it appears there is a significant role of the anti-apoptotic protein myeloid cell leukemia sequence 1 (Mcl-1) in neutrophil apoptosis, as mRNA levels of *Mcl-1* positively correlate to GM-CSF levels in RA synovial neutrophils²¹¹ and treatment of peripheral neutrophils with GM-CSF stabilizes Mcl-1 protein following cycloheximide treatment²⁰⁷. Additionally treatment with Seliciclib, an inhibitor of cyclin-dependent kinases (CDKs), was shown to increase neutrophil apoptosis, decrease *Mcp-1* mRNA expression, and improve clinical scores in mouse models of arthritis²¹⁴. Of note, methotrexate treatment also restored normal rates of apoptosis in isolated neutrophils²⁰⁵, which may represent an additional mechanism of action in preventing RA pathophysiology. In summary, the prolonged lifespan of activated neutrophils in the synovial tissues may contribute to increased tissue damage.

Another mechanism to resolve acute inflammation is hypothesized to be initiated by a class switching of secreted lipid mediators (*i.e.*, eicosanoids), although the mechanisms and cell sources of this process are incompletely understood²⁰⁹. Part of class switching of lipid mediators is the decrease in production of neutrophil-derived proinflammatory lipids like LTB₄ and PGE₂, and an increased synthesis of lipoxins. Lipoxins antagonizes continued neutrophil recruitment^{209,215}, and promote recruitment of anti-inflammatory subsets of macrophages to remove apoptotic neutrophils, release anti-inflammatory cytokines, and initiate tissue repair²¹⁰. Additionally, endogenous glucocorticoids promote resolution of inflammation. Endogenous glucocorticoids (*e.g.*, cortisol) cause neutrophils to increase cell surface localization of annexin 1, which inhibits continued neutrophil adhesion and transmigration²¹⁶. Neutrophil-derived annexin 1 rich microparticles are released by the cell to inhibit continued neutrophil adhesion²¹⁷. In RA, the activation of endogenous glucocorticoids is decreased by lower enzymatic activity of 11 β -hydroxysteroid dehydrogenase type 1 and an increase of 11 β -hydroxysteroid dehydrogenase type 2, a glucocorticoid inactivating enzyme²¹⁸. In summary, impaired neutrophil apoptosis and response to pro-resolving molecules in RA are likely involved in the development of a chronic disease state.

1.2.7 Conclusions

Neutrophils are classically associated with the host response to pathogenic microbes, but they also are becoming more accepted as an important modulator of sterile inflammatory diseases such as arthritis. Neutrophils represent the first class of circulating immune cells to be recruited to synovial tissues in RA, and continue to recruit additional neutrophils through a cytokine positive-feedback loop. Continued recruitment and activation of neutrophils allows the release of granule products capable of damaging local tissues. Further propagation of inflammation is exerted by neutrophil interaction with additional immune cells via cytokines or

cell-cell interactions. This process supports neutrophils as a key regulator of RA inflammation, and the therapeutic potential of disruption of neutrophil physiology in RA and other sterile inflammatory diseases.

1.3 GPSM3 in cell physiology

This work below was published in ref.²¹⁹:

Billard, M. J., **Gall, B. J.**, Richards, K. L., Siderovski, D. P. & Tarrant, T. K. G protein signaling modulator-3 : a leukocyte regulator of inflammation in health and disease. *Am J Clin Exp Immunol* **3**, 97–106 (2014).

1.3.1 Signaling interactions

Elucidating the function and regulation of G protein coupled receptors (GPCRs) was recently recognized with a 2012 Nobel Prize, and these proteins continue to be the largest class of cell-surface receptors successfully targeted for the treatment of human disease^{220,221}. GPCRs are functionally associated with heterotrimeric G proteins composed of $G\alpha$, $G\beta$, and $G\gamma$ subunits. When the receptor is inactive, $G\alpha$ is bound to guanosine diphosphate (GDP) in its own inactive state; GDP binding to $G\alpha$ promotes its association with the $G\beta\gamma$ dimer, which inhibits spontaneous GDP release by $G\alpha$ and also assists in receptor coupling. After ligand binding, the GPCR acts as a guanine nucleotide exchange factor (GEF) promoting the replacement of GDP with guanosine triphosphate (GTP) and leading to conformational changes that result in dissociation of $G\beta\gamma$ from the heterotrimeric complex. Both GTP-bound $G\alpha$ and free $G\beta\gamma$ initiate signal cascades to downstream effectors. Hydrolysis of GTP to GDP returns $G\alpha$ to its inactive state, allowing re-association with $G\beta\gamma$ and signal termination^{222,223}.

Chemokine receptors are GPCR family members that signal through $G\alpha_i$ -containing heterotrimers to regulate cellular migration, survival, and angiogenesis in inflammatory conditions^{224,225}. However, as drug therapy targets in autoimmunity, chemokine receptors have had disappointing results in various neutralization strategies^{141,226} and, in particular, in the

development of chemokine signaling inhibitors as rheumatoid arthritis (RA) therapeutics^{227,228}. This failure has been attributed, in part, to redundancy of chemokine receptor/ligand interactions in inflammation²²⁴, and so it has been proposed that a more efficacious strategy should target a shared pathway regulator of chemokine receptor signaling rather than neutralization of a specific chemokine or its receptor(s) *per se*²²⁹.

GoLoco motif-containing proteins are regulators of GPCR signaling that share a signature 19-amino-acid sequence^{230,231}, as well as a hallmark biochemical activity of inhibiting G α_i release of GDP^{223,232–234}. One such member of this protein family with a highly-restricted expression pattern (Fig. 1.4), GPSM3 (*a.k.a.* AGS4²³⁵ or G18²³⁶), possesses three GoLoco motifs (but only two are functional²³⁶) and an additional LSL motif required for G β subunit binding²³⁷. The functional GoLoco motifs of GPSM3 allow the protein to bind G α_i -GDP and act as a GDP dissociation inhibitor (GDI)^{235,236} independent of G α_i -GDP interaction with the G $\beta\gamma$ dimer^{234,238}, while the LSL motif interaction with monomeric G β subunits is suspected to regulate GPCR/G-protein heterotrimer association at the level of G $\beta\gamma$ dimer assembly²³⁷ (Fig. 1.5). The cellular and biologic effects of GPSM3 continue to be explored as recent research points to its important regulatory functions in chemokine receptor signaling, monocyte phenotypes, and autoimmune disease development.

Recent studies by Giguere *et al.* have illustrated that GPSM3 deficiency affects the chemotactic response of myeloid cells activated through the chemokine receptors CCR2, CX3CR1, and CMKLR1²³⁹. Ongoing biochemical and cell biological research is helping to define the specific role that GPSM3 plays in the intracellular sequelae of chemokine receptor signaling and how this role ultimately affects cellular chemotaxis and viability.

Not only does GPSM3 interact with G α_i -GDP and monomeric G β subunits, but from studies of local energy transfer between fusion proteins expressed in cells, it is hypothesized that GPSM3 may also help position G α subunits at the cell membrane, proximal to GPCRs, and

thereby help provide a signaling substrate to ligand-activated receptors²⁴⁰; however, these latter results from ectopic overexpression of recombinant fusion proteins have yet to be confirmed in an endogenous expression context. More controversial is the suggestion that GPSM3 may regulate GPCR signaling pathways via GoLoco motif interactions that directly promote the dissociation of $G\alpha$ and $G\beta\gamma$ independent of GPCR/ligand-stimulated GEF activity²³²; evidence from both structural biology studies²³⁴ and electrophysiological recordings of $G\beta\gamma$ -gated ion channels²³⁸ suggest that the GoLoco motif/ $G\alpha_i$ -GDP interaction is mutually exclusive to the assembly of a traditional $G\alpha_i$ -GDP/ $G\beta\gamma$ heterotrimer, but that the GoLoco motif cannot itself displace a preformed $G\alpha_i$ -GDP/ $G\beta\gamma$ heterotrimer.

A common outcome of chemokine receptor signaling is the mobilization of internal calcium stores via $G\beta\gamma$ -mediated activation of phospholipase- $C\beta$ (PLC β) (Fig. 1.5). Ectopic GPSM3 expression is seen to negatively affect GPCR signaling by decreasing PLC β -mediated generation of the second messenger inositol trisphosphate (IP₃)²³⁹. This inhibitory effect requires GPSM3 interaction with $G\beta$ subunits, given that a specific loss-of-function mutation to the $G\beta$ -interaction site within GPSM3 (*i.e.*, mutation of the “LSL” motif) abrogates inhibition of IP₃ accumulation after agonist stimulation; it is important to note that mutation to the LSL mutation does not affect the GPSM3/ $G\alpha_i$ -GDP interaction²³⁹. Indeed, endogenous $G\beta$ subunits are seen to co-immunoprecipitate with the endogenous GPSM3 expressed in the human monocytic THP-1 cell line; moreover, endogenous $G\beta$ subunits co-localize with endogenous GPSM3 at the plasma membrane in THP-1 cells²³⁹. This plasma membrane colocalization is consistent with the proteomic detection of GPSM3 within cholesterol-rich, detergent-resistant, plasma membrane fragments from bovine leukocytes²⁴¹, as well as an independent report of ectopically-expressed GPSM3 being able to form a G protein-dependent complex in proximity to membrane-delimited GPCRs²⁴⁰. Additional evidence that GPSM3 interacts with $G\beta$ has been obtained by examining the direct activation of PLC β 2 via co-expression of free $G\beta$ 1 γ 2 subunits;

overexpression of wild-type GPSM3 (but not the loss-of-function LSL mutant) is seen to inhibit PLC β 2 activation by G β 1 γ 2²³⁹.

Another common G β γ -mediated signaling effector is activation of the pro-survival kinase PKB/Akt pathway (Fig. 1.5) - an event that is often measured by serine-473 phosphorylation status²⁴². When GPSM3 levels are decreased by RNA-interference in the monocytic THP-1 cell line, activation of PKB/Akt by treatment with fetal bovine serum is diminished²³⁹. The decrease in pro-survival PKB/Akt signaling in GPSM3-depleted THP-1 cells mirror the increased presence of early apoptotic markers observed in etoposide-treated GPSM3-deficient THP-1 cells²³⁹ and are consistent with the known role of GPCR/G β γ -mediated activation of class I β PI3K and resultant PIP₃-dependent activation of PKB/Akt and downstream survival signaling in leukocytes^{242,243} (Fig. 1.5).

1.3.2 GPSM3 in Immune Cell Function and Cancer

Myeloid-derived inflammation mediators and suppressors: GPSM3 expression is highly regulated during monocyte/macrophage differentiation *ex vivo*, and GPSM3 deficiency reduces monocyte survival as well as migration to specific chemokine ligands²³⁹. Ly6C^{high}CD11b⁺ monocytes are known to mobilize rapidly in response to infection and inflammation²⁴⁴; flow cytometry of splenocytes from GPSM3-deficient mice indicates that this population of Ly6C^{high}CD11b⁺ monocytes is significantly reduced in comparison to wild-type mouse controls²³⁹. Therefore, a role for GPSM3 in survival and/or differentiation of particular myeloid-lineage cells is likely, but the molecular mechanisms underlying this role remain to be elucidated.

Myeloid cells, particularly macrophages and neutrophils, are known mediators of inflammation; conversely, a heterogeneous, immature myeloid cell population, termed “myeloid-

derived suppressor cells" (MDSCs) are able to *suppress* T cell and macrophage responses in inflammatory disease^{245,246}. Work by Yan *et al.* revealed that in lysosomal acid lipase (*lal*) knockout mice, which exhibit expanded MDSC development, *GPSM3* transcript abundance is upregulated two-fold (along with other G protein-related genes) as part of a broader perturbation of myeloid development and homeostasis²⁴⁷. MDSCs have been shown to expand as a functional cell population in multiple pathological conditions including infection, autoimmunity, inflammation, and cancer²⁴⁶. In the mouse collagen-induced arthritis (CIA) model of rheumatoid arthritis (RA), MDSCs are seen to accumulate in the spleen concurrent with the apex of developed paw swelling, inhibit production of the inflammatory cytokines IFN- γ , IL-2, TNF- α , and IL-6, and suppress T helper 17 (Th17) pathogenic T cells²⁴⁸.

MDSCs also affect tumor angiogenesis and metastasis, as well as promote the development of FOXP3+ regulatory T-lymphocytes (Tregs)²⁴⁹. MDSCs expand in numbers in many tumors and are believed to be recruited from the bone marrow by tumor-derived factors²⁵⁰; these increased levels of MDSCs can interfere with innate and adaptive anti-tumor responses by depressing immune responses to tumor burden, leading to the advancement of malignancy²⁵¹. Data from mouse tumor models suggest that two populations of CD11b⁺ MDSCs predominate: "granulocytic" Ly6G⁺/Ly6C^{lo} MDSCs and "monocytic" Ly6G⁻/Ly6C⁺ MDSC²⁴⁶. Both of these MDSC subsets are decreased in the spleens of *GPSM3*-deficient mice²³⁹. The ablation of *GPSM3* protected mice from a monocyte-driven model of acute inflammatory arthritis²³⁹; however, it remains unknown whether these *GPSM3*-deficient mice possess a differential response to tumor burden.

Although *GPSM3* appears to be most highly expressed in developing monocytes and is also expressed in MDSCs, Lapan *et al.* have shown that prostatic cancer cells also increase *GPSM3* expression greater than two-fold when co-cultured *ex vivo* with endothelial cells, in a model mimicking angiogenesis within the tumor microenvironment²⁵². As a regulator of GPCR and G protein function, *GPSM3* could therefore also influence invasive or migratory

phenotypes in different cancers, or their responses to survival or angiogenic factors that might be secreted as tumors grow. Therefore, whether tumor progression in GPSM3-deficient mice may be influenced by the decreased prevalence of MDSCs or by changes that occur in adaptive immune cells or the tumor cells themselves needs to be further examined.

Lymphocytes: Although GPSM3 has its highest expression in monocytes, B- and T-lymphocytes also have detectable GPSM3^{235,239}, suggesting that GPSM3 could also regulate GPCR/G protein signaling that mediate outcomes such as survival and chemotactic trafficking within these key lymphocyte classes required for full immune function. For example, in a study by Reif and Cyster²⁵³, activated B cells dynamically regulated the expression of multiple different regulators of G protein signaling altering responses to local chemokine gradients.

Lymphocyte involvement in inflammatory diseases also involves the development of ectopic (tertiary) lymphoid structures. The architecture of these germinal center-like structures requires chemokine-driven organization and intercellular interactions²⁵⁴ likely to be impacted by GPSM3 function on GPCR signaling *in vivo*. While it is currently unknown exactly how GPSM3 functions to regulate GPCR signaling in lymphocytes, the activity of this GPCR signaling modulator could influence inflammatory function, adaptive immune responses, and/or be involved in the protection against or exacerbation of lymphocyte-mediated inflammatory disease.

1.3.3 GPSM3 Gene Variants in GWAS of Immune Diseases

Compelling genome-wide association studies (GWAS) identified protective alleles located within the *GPSM3* gene locus that are significantly less prevalent in patients with several autoimmune diseases^{76,255,256}. In the most notable examples to-date, protective alleles of two *GPSM3* single-nucleotide polymorphisms (SNPs rs204989, rs204991) have been

associated with a decreased incidence of RA^{76,255,256}. Importantly, these same SNPs associate significantly with decreased incidence of developing other autoimmune diseases that share similar immunologic mechanisms with RA: namely, ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and multiple sclerosis (MS)^{76,255,256}. Conversely, *GPSM3* SNPs show risk association with type I diabetes and autoimmune thyroid disease^{255,256}, and *GPSM3* genetic polymorphisms have also been linked with atopic dermatitis and childhood obesity, two diseases also characterized by chronic inflammation^{257,258}. The *GPSM3* locus on chromosome 6 is located very near to the *Notch4* gene locus and is found within the densely-packed and disease-relevant human leukocyte antigen (HLA) region^{76,255,256}, raising the possibility that *GPSM3* SNP variants mark functional polymorphisms in other closely associated genes. However, increasing research into *GPSM3* transcript abundance and function is revealing a consistent pattern of potential physiological involvement in immune cell types relevant to inflammatory diseases. Therefore, it is conceivable that sequence variation within *GPSM3* could alter transcript expression or splicing, and/or *GPSM3* protein sequences that could contribute directly to immune disease pathogenesis.

Two different protein isoforms of *GPSM3*, with different N-terminal polypeptide sequences (Fig. 1.6), have been predicted to date in the human genomic sequence database curated by Ensembl (<http://ensembl.org>)²⁵⁹, as encoded by three different *GPSM3* transcripts: *GPSM3-001*, *-002*, and *GPSM3-004*. It is presently unclear whether these different isoforms exert differential effects on GPCR signaling, cellular function, or disease. However, Zhao *et al.*²⁶⁰ reported that *GPSM3* differentially affects the nucleotide state of $G\alpha_i$ vs $G\alpha_o$ subunits via its N-terminal proline-rich region - a region significantly different in the open-reading frames encoded by transcripts *GPSM3-001* and *-002* versus *GPSM3-004* (Fig. 1.6).

1.3.4 GPSM3 in Autoimmune Arthritis

Pre-clinical evidence suggests that neutralization of proinflammatory, monocyte-attracting chemokines, or their cognate cell-surface receptors, would be beneficial for the treatment of RA^{224,261}; however, clinical trials have revealed disappointing results both for neutralization of chemokines or for antagonism of their GPCR counterparts^{141,228,262–264}. One explanation for this apparent discrepancy between pre-clinical evidence and clinical trial results has uses the known functional redundancy of the chemokine system in the inflammatory response, whereby many chemokines within a structural class can bind to multiple chemokine receptors sharing similar structural motifs^{224,225}. Thus, targeting monocyte/macrophage recruitment in RA may be desirable, but current strategies using single chemokine or chemokine receptor blockade have produced limited results.

GPSM3 appears restricted in its tissue transcript and protein concentration to leukocytes^{235,239,265}, therefore may play role in inflammatory cell function. In particular, monocyte viability and chemotaxis are found to be affected in a GPSM3-deficient acute inflammatory arthritis model²³⁹, and *GPSM3* SNPs are associated differentially between healthy persons and those with inflammatory arthritis from either RA, SLE, or AS^{76,255,256}. Thus, understanding the regulatory roles of GPSM3 as a GPCR signaling modulator may help to promote this protein as a desirable therapeutic target for patients with autoimmune arthritis.

In an acute, collagen antibody-induced arthritis (CAIA) mouse model, GPSM3-deficient mice (those either homozygous or heterozygous for the ablated *GPSM3* allele) exhibit decreased clinical disease scores, paw swelling, as well as reduced histopathology with less inflammation in cartilage, less synovial damage, and less bone erosion²³⁹. Additionally, a decreased disease incidence was observed in *Gpsm3*^{-/-} mice (34% for *Gpsm3*^{-/-} mice vs 95% for control mice)²³⁹. Independent studies by Schmidt *et al.* report that *GPSM3* expression decreases in the joints after anti-inflammatory treatment with dexamethasone in the chronic,

collagen-induced arthritis (CIA)²⁶⁶. Thus, both chronic and acute mouse models of inflammatory arthritis reveal a compelling role for GPSM3 involvement.

GPSM3-deficient mice have reduced CAIA that is associated with a decreased transcript expression of the proinflammatory, monocyte-derived cytokines *IL-6* and *IL-1β*, as well as of monocyte-specific chemokine receptors in the joints²³⁹. TNF-α, IL-1β, and IL-6 are all produced by monocytes/macrophages, which are recruited to the inflamed synovium by chemokines such as CCL2/MCP-1 and CX3CL1/fractalkine, among numerous others^{224,225}. GPSM3 deficiency reduces monocyte chemotaxis toward CCL2, CX3CL1 and chemerin, and enhances etoposide-induced apoptosis *ex vivo*²³⁹, providing evidence that GPSM3 affects monocytes through the regulation of chemokine-receptor signaling and its downstream intracellular sequelae. The critical nature of monocyte infiltration and pathogenesis in autoimmune arthritis²⁶⁷ is further underscored by the direct correlation between disease severity and the accumulation of monocyte-targeting nanoparticles in the inflamed joint synovium, as observed recently by Kim *et al.*²⁶⁸.

1.4 Figures and Tables

Figure 1.1 – Algorithm for treatment of RA patients according to the treat-to-target activity. This figure is adapted from Smolen *et al.* (2010)⁶⁴. The green arrows indicate the primary goal of remission, while the alternative goal is indicated in red. Prior to reaching either goal, the patient is assessed for disease activity every 1-3 months and treatment is adjusted according to the disease activity (dark background). After reaching the initial goal, the patient is monitored every 3-6 months and treatment is adjusted to maintain disease activity (light background).

Figure 1.2 – Algorithm for prescription-based therapies for RA. This figure is adapted from Singh *et al.* (2015)⁶⁵. In this diagram MTX is considered the primary non-biologic, although other non-biologic DMARD options are available. In the event of successful remission with treatment, no further therapeutics are prescribed. If remission is not reached treatments are prescribed to maintain disease activity levels. Abbreviations: MTX = methotrexate, DMARD = disease-modifying antirheumatic drug, TNFi = tumor necrosis factor α inhibitor.

Signaling molecule effects on neutrophil physiology					
	Priming	Chemotaxis	Activation	Increase Lifespan	MHCII Presentation
C5a		X			
GM-CSF	X			X	X
IFN γ	X			X	X
Ig Complex		Indirectly	X		
IL-1 β	X	Indirectly	X	X	
IL-8	X	X	X		
TNF- α	X	Indirectly	X	X	

Table 1.1 – The effects of various signaling molecules (first column) on neutrophil physiology in inflammation. Adapted from Wright et al.⁸¹.

Neutrophil granule enzymes involved in tissue damage		
Granule	Protein	Role in tissue damage
Azurophilic (primary)	Myeloperoxidase	Produces HOCl (from H ₂ O ₂ + Cl ⁻) ²⁶⁹
	Elastase	Degradation of cartilage collagen and elastin ¹⁶⁶
	Cytochrome b558 (NOX2)	An isoform of the catalytic subunit of NADPH oxidase ²⁷⁰
Specific (secondary)	Collagenase	Degradation of cartilage collagen ¹⁶⁶
	Gelatinase B (MMP9)	Degradation of cartilage collagen ¹⁶⁸
Gelatinase (tertiary)	Gelatinase B (MMP9)	Degradation of cartilage collagen ¹⁶⁸
	MMP25	Inactivates extracellular proteinases, prolonging half-life of elastase and cathepsin G ²⁷¹

Table 1.2 – A non-inclusive list of neutrophil granule molecules that affect the pathogenesis of RA. The granule (first column), granule molecule (second column), and function of the granule molecule in tissue damage (third column) are reported. Adapted from Wright, et al. 2010⁸¹

Neutrophil cytokine production and function

Chemokine	Target	Function
C-C chemokines		
CCL2 ^{136, 272}	Monocytes, dendritic cells	Chemoattractant
CCL3 ^{112, 272, 128}	T _H 1 cells	Chemoattractant
CCL4 ²⁷³	T cells and monocytes	Chemoattractant
CCL17 ^{274, 275}	T _H 2 cells and dendritic cells	Chemoattractant
CCL18 ¹²⁸	Naïve T cells	Chemoattraction of naïve T cells toward antigen presenting cells ⁴¹
CCL19 ²⁷⁶	B and T cells	Potent chemoattractant for lymphocytes ⁴¹
CCL20 ²⁷⁶	B and T cells	Potent chemoattractant for lymphocytes ²⁷⁷
C-X-C chemokines		
CXCL1 ²⁷⁸	Neutrophils and monocytes	Chemoattractant
CXCL2 ¹¹²	Neutrophils and monocytes	Chemoattractant
CXCL8 ¹²⁸ (a.k.a. IL-8)	Neutrophils and monocytes	Chemoattractant
CXCL9 ^{279, 280}	T _H 1 cells	Chemoattractant
CXCL10 ^{128, 274}	T _H 1 cells	Chemoattractant
CXCL11 ²⁸⁰	T _H 1 cells	Chemoattractant
Soluble CX3CL1 ^{*128}	Leukocytes	Chemoattractant for CX3CR1+ cells (e.g., monocytes and T cells)
Interleukins		
IL-1 β ¹¹²	Multiple	Described in text
IL-4 ²⁸¹	Naïve T cells ⁴¹	Induces differentiation of naïve T cells toward T _H 2 phenotype ⁴¹
IL-17A ²⁸²	Fibroblasts, synoviocytes, endothelial cells, and macrophages ²⁸³	Induces production of numerous cytokines (e.g., IL-6, IL-10, IL-12, CXCL1, CXCL2, CXCL8, CCL2, CCL5, PGE2; reviewed in Benedetti & Miossec ²⁸³)
IL-17B ²⁸⁴	Fibroblasts ²⁸⁴	Potentiates TNF α -induced production of cytokines (i.e., G-CSF, IL-6, IL-1 β) ²⁸⁴
IL-18 ^{*279}	NK cells, T cells, and fibroblasts	Promotes release of IFN- γ and CXCR2 agonists (reviewed in Volin & Koch ²⁸⁵)
IL-23 ²⁸⁶	Naïve T cells ²⁸⁷	Promotes differentiation of naïve T cells toward the T _H 17 lineage ²⁸⁷
Others		
TRAIL ²⁸⁸	Multiple	Induction of caspase-8-dependent apoptosis ²⁸⁸
BLyS ²⁸⁹	B cells ²⁹⁰	Promotes B cell survival and maturation to plasma cell lineage ²⁹⁰

APRIL ²⁹¹	B cells / Plasma cells ²⁹¹	Modulates late humoral immune response via induction of immunoglobulin class switching and survival signals ²⁹¹
CD40L ²⁹²	B-cells ⁴¹	Co-stimulatory ligand for B-cell activation ⁴¹
RANKL ²⁹³	Osteoclasts	Activation of bone resorption ²⁹³
Matrix metalloproteinases ^{294,295}	Extracellular matrix	Degradation of extracellular matrix promoting neutrophil migration
IFN γ ^{184, 183}	Multiple ⁴¹	Promotes macrophage activation, MHC class II expression, TH1 adaptive immune response, and antibody isotype switching to IgG ⁴¹ .
Neutrophil Granule Proteins		
Collagenase ¹⁶⁶	Cartilage collagen ¹⁶⁶	Promotes tissue damage and degradation of synovial cartilage ²⁹⁶
Elastase ¹⁶⁶	Cartilage ¹⁶⁶ and cytokines ²⁹⁷	Promotes tissue damage by cleaving collagen and elastin ²⁹⁶ ; activates CCL15 ²⁹⁸ and chemerin ²⁹⁹ .
Cathepsin G	Cytokines ²⁹⁷	N-terminal truncation of CXCL5 ³⁰⁰ , CCL15 ²⁹⁸ , and chemerin ²⁹⁹ resulting in full activation of these chemokines.
α -Defensins ¹⁹⁷	T cells, dendritic cells, and fibroblasts	Primarily an antimicrobial, but also recruits T cells and dendritic cells ¹⁹⁷ and has been shown to be a fibroblast mitogen ³⁰¹ .
LL-37 ³⁰²	Dendritic cells ^{199,303}	Antibacterial, activates dendritic cells ^{199,303}
Myeloperoxidase ¹⁸⁸	Macrophages	Upregulates reactive oxygen production in neutrophils and induces production of reactive oxygen, TNF- α , and IL-1 by macrophages ¹⁸⁹
GM-CSF and G-CSF ²⁷⁴	Myeloid progenitor cells ⁴¹	Survival, proliferation, and differentiation of pre-neutrophil progenitors in bone marrow ⁴¹ .
Adapted from ^{86,304}		Unless noted target cells and function identified in ⁴¹ , or reviewed in ^{86,304,305} .

Table 1.3 – A non-inclusive list of cytokines and signaling molecules shown to be expressed at either the transcript or protein level in neutrophils. The signaling molecule (first column), effector cell/tissue (second column), and function of signaling molecule on effector cell (third column) are reported.

Figure 1.3 – Diagram of neutrophil activation by soluble (above) or tissue-bound (below) autoimmune antibodies. Interaction between Fc receptors on the neutrophil and the Fc portion of autoimmune antibodies results in the release of granule components (Table 1.3) into the extracellular space. This release of reactive oxygen species (ROS) and proteolytic enzymes results in non-specific damage of local tissues and propagation of the inflammatory response.

Figure 1.4 – GoLoco motif-containing proteins. Expression pattern as reported by the BioGPS gene expression atlas (<http://biogps.org>)²⁶⁵.

Figure 1.5 – Model for GPSM3 function in regulating Gi-coupled chemokine receptors. Two of the three GoLoco motifs within GPSM3 (yellow) bind to $G\alpha_i$ -GDP subunits^{235,236}. Additionally, the LSL motif within GPSM3 interacts with $G\beta$ subunits toward their biosynthetic pathway in forming $G\beta\gamma$ dimers²³⁷. These interactions likely regulate ligand-activated chemokine receptor signaling pathways, including $G\alpha_i$ -mediated inhibition of cAMP production by adenylyl cyclase, inositol phosphate production from $G\beta\gamma$ -mediated activation of phospholipase-C β , and/or cell survival signaling by $G\beta\gamma$ -mediated activation of class I β phosphatidylinositol-3'-kinase (PI3K) leading to PKB/Akt phosphorylation.

	Exon 1	Exon 2
GPSM3-001	-002 MEAERPQEEEDGEQ	GPPQDEEGWPPPNSTTRPWRSAPSPPPPGRHT
GPSM3	-004 MVS R MIDIFWQH	GPPQDEEGWPPPNSTTRPWRSAPSPPPPGRHT

Figure 1.6 – GPSM3 amino acid variation between transcript isoforms. The Ensembl database²⁵⁹ predicts two distinct wild-type human GPSM3 proteins that have different N-terminal polypeptide sequences and that correlate with different mRNA transcripts.

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Chapter 2

Genetic variations in *GPSM3* associated with protection from rheumatoid arthritis affect its transcript abundance

Original Article – in press Genes & Immunity

BJ Gall¹, A Wilson², AB Schroer¹, JD Gross¹, P Stoilov³,

V Setola^{1,4}, CM Watkins², DP Siderovski¹

¹Department of Physiology & Pharmacology, ²Department of Orthopaedics,
³Department of Biochemistry, and ⁴Department of Behavioral Medicine &
Psychiatry, West Virginia University School of Medicine, Morgantown, WV, USA
26506-9229

Email: dpsiderovski@hsc.wvu.edu

Running title: SNP rs204989 decreases *GPSM3* transcription

2.1 – Abstract

G protein signaling modulator 3 (*GPSM3*) is a regulator of G protein-coupled receptor signaling, with expression restricted to leukocytes and lymphoid organs. Previous genome-wide association studies have highlighted single-nucleotide polymorphisms (SNPs rs204989, rs204991) in a region upstream of the *GPSM3* transcription start site as being inversely correlated to the prevalence of rheumatoid arthritis (RA) -- this association is supported by the protection afforded to *Gpsm3*-deficient mice in models of inflammatory arthritis. Here, we assessed the functional consequences of these polymorphisms. We collected biospecimens from 50 volunteers with RA diagnoses, 50 RA-free volunteers matched to the aforementioned group, and 100 unmatched healthy young volunteers. We genotyped these individuals for *GPSM3* (rs204989, rs204991), *CCL21* (rs2812378), and *HLA* gene region (rs6457620) polymorphisms, and found no significant differences in minor allele frequencies between the RA and disease-free cohorts. However, we identified that individuals homozygous for SNPs rs204989 and rs204991 had decreased *GPSM3* transcript abundance relative to individuals homozygous for the major allele. *In vitro* promoter activity studies suggest that SNP rs204989 is the primary cause of this decrease in transcript levels. Knockdown of *GPSM3* in THP-1 cells, a human monocytic cell line, was found to disrupt *ex vivo* migration to the chemokine MCP-1.

2.2 – INTRODUCTION

Chemokine receptors comprise a subfamily of the G protein-coupled receptor (GPCR) superfamily of transmembrane receptors that are expressed on a number of leukocyte subsets and function predominantly to regulate chemotaxis¹⁻⁵. Upon binding their cognate chemokine agonists, chemokine receptors transduce signals by inducing dissociation of their associated, intracellular G_i protein heterotrimers ($G_{i\alpha}\cdot\text{GDP}/G\beta\gamma$). This process is highly regulated through additional intracellular proteins that act upon the $G_{i\alpha}$ subunit and ultimately affect the rate of signal inactivation^{4,6,7}. In particular, proteins containing one or more conserved GoLoco motifs are capable of sequestering inactivated $G_{i\alpha}\cdot\text{GDP}$, preventing its reassociation with $G\beta\gamma$ and GPCRs and thereby disrupting continued $G_{i\alpha}$ -induced signaling without quenching $G\beta\gamma$ -mediated signaling⁶⁻¹⁰. The importance of $G\beta\gamma$ -associated signaling to chemokine actions has recently been highlighted by reports that specific $G\beta\gamma$ -activating compounds are sufficient to induce neutrophil chemotaxis¹¹ and, conversely, a $G\beta\gamma$ antagonist can inhibit fMLP-induced chemotaxis¹². GoLoco proteins may directly regulate signaling pathways required for chemotaxis by sequestering $G_{i\alpha}\cdot\text{GDP}$ and prolonging $G\beta\gamma$ -mediated signaling processes^{13,14}, thereby exacerbating inflammation.

G protein signaling modulator 3 (GPSM3) contains two functional GoLoco motifs and is restricted in its expression to leukocytes and myeloid-derived cells^{15,16}. *Gpsm3*-deficient mice exhibit a profoundly blunted course of joint damage in an acute, collagen antibody-induced model of inflammatory arthritis¹⁶. Histologic analyses of affected

synovial tissues indicate a relative acellularity of synovial cavities in *Gpsm3*-deficient mice compared to wild type mice, which suggests a potential leukocyte migration deficit. These findings suggest a role for GPSM3 in the development of inflammation in rheumatoid arthritis, most likely through a key regulatory role on leukocyte chemotaxis to the synovium¹⁷. However, a direct demonstration of such a role has yet to be exhibited with human cells.

Genome-wide association studies (GWAS) have identified two single-nucleotide polymorphisms (SNPs), rs204989 and rs204991, 5' to the *GPSM3* transcriptional start site that are significantly less prevalent in individuals with rheumatoid arthritis (and other autoimmune diseases; e.g., lupus and multiple sclerosis), implying a potential protective effect of these minor gene variations^{18–20}. Notably, rs204989 and rs204991 both exist within the chromosome 6p21.3 band, a region previously attributed to a large portion of the heritable aspect of RA; specifically, *HLA* gene region polyallelic haploblocks within the chromosome 6p21.3 region represent some of the greatest risk factors for RA²¹ (reviewed in ref. ²²). In particular, the biallelic *HLA* gene locus polymorphism, rs6457620 [C>G], has been identified as an RA risk factor in a meta-analysis of GWAS studies investigating multiple populations in the Wellcome Trust Case Control Consortium (WTCCC), North American Rheumatoid Arthritis Consortium (NARAC), and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA)^{23,24}. Thus, the potential exists for linkage disequilibrium between *GPSM3* and *HLA* gene region polymorphisms. In this study, we addressed whether *GPSM3* SNPs result in a detectable phenotype that explains their inverse association with rheumatoid arthritis.

Furthermore, we assessed whether linkage disequilibrium with the known RA risk allele in the *HLA* gene region, rs6457620^{23,24}, may affect the inverse association of *GPSM3* SNP alleles with RA. Additionally, another RA risk allele, *CCL21* rs2812378 [T>C], located on an unlinked chromosome, was analyzed as both a negative control for linkage and a positive control for RA disease risk²⁴. We recruited a group of 50 volunteers with a diagnosis of RA, 50 RA-free volunteers who were matched to the aforementioned group by a 'Bring-a-friend-to-clinic' program, and 100 unmatched healthy young volunteers to donate biospecimens for analyses. Based on the location of the polymorphisms and previous reports of protection from inflammatory phenotypes in human GWAS^{18–20} and *Gpsm3*-deficient mouse studies¹⁶, we hypothesized that individuals homozygous for the minor alleles of rs204989 and rs204991 would exhibit decreased whole-blood *GPSM3* transcript abundance. Additionally, we predicted that knockdown of *GPSM3* would result in disruption of chemokine-induced migration in a human monocytic cell line.

2.3 – RESULTS

2.3.1 – *GPSM3* SNPs rs204989 and rs204991, each previously associated by GWAS with protection from rheumatoid arthritis, form a haploblock with rs204990

The cohorts recruited for this study included an initial set of 100 unmatched healthy young volunteers, a group of 50 volunteers with a positive diagnosis of RA, and 50 RA-free volunteers matched to the aforementioned group by a ‘Bring-a-friend-to-clinic’ program. Upon genotyping all 200 volunteers recruited for this study, we found that *GPSM3* SNPs rs204989 and rs204991, originally identified to be independently^{18–20} associated with protection from RA, are in complete linkage disequilibrium within this population. Additionally, sequencing a 3.5-kb region 5’ to the *GPSM3* transcriptional start site in eight volunteers revealed a total of four polymorphisms in this region: rs204989, rs204990, rs204991, and rs3096688 (Fig. 2.1A). All of these chromosome 6 SNPs have been previously identified in the NCBI Database of Single Nucleotide Polymorphisms (dbSNPs)²⁵. In this study, three of these *GPSM3* SNPs (rs204989, rs204990, rs204991) were seen to be inherited as a haploblock in complete linkage disequilibrium; therefore, we defined all individuals homozygous for the minor (lower frequency) alleles of all three SNPs as having the “m/m” genotype and all individuals homozygous for the major (higher frequency) allele for all three SNPs as having the “M/M” genotype (Fig. 2.1A).

2.3.2 – The *GPSM3* rs204989/rs204991 haploblock is associated with decreased *GPSM3* mRNA expression in whole blood

In disease-free homozygotes for the minor alleles of *GPSM3* SNPs, whole blood-derived RNA contained only 75.9% (m/m; n = 11; 95% C.I. of $\pm 7.6\%$) of the average *GPSM3* transcript level present in homozygotes for the major alleles (M/M; n = 53; 95% C.I. of $\pm 5.6\%$). This was a decrease of 24.1% (95% C.I. of 7.1% - 41.7%; t = 3.803; df = 62; p = 0.0003) for individuals with the m/m genotype (n = 11) relative to individuals with the M/M genotype (Fig. 1B; n = 53). Heterozygous individuals (M/m genotype) exhibited an average of 82.0% of the *GPSM3* transcript abundance of the M/M genotype, but had a marked amount of variability (*data not shown*; n = 6; 95% C.I. of $\pm 31.1\%$). In our analyses of *GPSM3* mRNA abundance, we excluded RA patient samples to avoid potential confounding effects caused by anti-RA pharmacotherapy, as shown previously by RNA-seq^{26,27}. According to the 1000 Genomes Project²⁸, the minor allele frequencies (MAFs) in the general population of rs204989 and rs204991 are 23.3% and 23.4%, respectively. In our volunteer populations, the chromosome 6 rs204989 and rs204991 SNPs occurred in a haploblock with a minor allele frequency (MAF) of 29% within the unmatched healthy young cohort (n=100), 23% within the rheumatoid arthritis cohort (n = 50), and 18% within the matched control (disease-free) cohort (n = 50). The MAF of the chromosome 9 *CCL21* gene locus polymorphism rs2812378 [T>C] is 30.1% according to the 1000 Genomes Project²⁸. In our recruited populations, the *CCL21* gene polymorphism MAF was 31.5% within the unmatched healthy young population (n = 100), 30.0% within the rheumatoid arthritis cohort (n = 50), and 25.0% within the matched control cohort (n = 50). The MAF of the chromosome 6 *HLA* gene region

polymorphism rs6457620 was 56.0% within the unmatched healthy young cohort (n = 100), 35.0% within the rheumatoid arthritis cohort (n = 50), and 45.0% within the matched control population (n = 50). None of the differences in *GPSM3* (p = 0.4839), *CCL21* (p = 0.5267), and *HLA* gene region (p = 0.1938) polymorphism MAFs between the RA and matched, disease-free cohorts was statistically significant (Fig. 2.1C).

2.3.3 – The *GPSM3* SNP haploblock is in weak linkage disequilibrium with *HLA* SNP rs6457620, but the latter is unlinked to *GPSM3* transcript abundance

Both the gene for *GPSM3* and the *HLA* gene region SNP rs6457620 reside on chromosome 6, presenting the possibility of linkage disequilibrium; however, over 0.5 megabases of DNA sequence separate rs6457620 from the *GPSM3* rs204989/rs204991 SNP haploblock²⁵. In individuals homozygous for the *GPSM3* SNPs (M/M), the *HLA* gene region SNP MAF was 41.3% (Figure 2.2). In heterozygotes for the *GPSM3* SNPs (M/m), the *HLA* gene region SNP MAF was 56.3%, while homozygotes for the minor alleles of *GPSM3* SNPs (m/m) exhibited a 60.7% *HLA* gene region SNP MAF. This distribution was significantly different from chance distribution of unlinked loci (Fig. 2.2A; p = 0.0066), suggesting the presence of weak linkage disequilibrium and consistent with data from the NCBI dbSNP²⁵ (Fig. 2.2D). Within the RA cohort, there was significant variation from chance distribution of *GPSM3* SNPs MAF when stratified by *HLA* gene region SNP genotype (Fig. 2.2B; p = 0.0123), which was not observed within the matched control group (Fig. 2.2C; p = 0.2739). However, when stratified by *HLA* SNP rs6457620 genotype, *GPSM3* transcript abundance in whole blood was not

significantly different (Fig. 2.2E; $F = 1.219$; $p = 0.3050$); this result is in contrast to the significant difference in *GPSM3* transcript abundance observed when stratified by *GPSM3* SNP haploblock genotype (Fig. 2.1B).

2.3.4 – rs204989 of the *GPSM3* SNP haploblock is solely linked to decreased *GPSM3* transcript abundance

A 3.5-kb region 5' to the human *GPSM3* transcription start site, spanning the region of the *GPSM3* SNP haploblock under study (Figure 2.1A), was subcloned into a promoterless luciferase-reporter vector. Transfecting this construct into HEK293T cells, we found the normalized firefly luciferase activity induced by the “minor/polymorphic” *GPSM3* promoter (pGL3-m; containing the minor rs204989/rs204990/rs204991 haploblock as well as the minor allele of SNP rs3096688) was 26.5% less than that of the wild type promoter (pGL3-M; containing the major allele of all four SNPs) ($p < 0.0001$; Fig. 3A). To assess the relative effect(s) of each of the four minor SNP variants within this *GPSM3* promoter region, each individual polymorphism was separately introduced to the wild type promoter by site-directed mutagenesis (*i.e.*, rs204989 [C>T], rs204990 [G>T], rs204991 [A>G], and rs3096688 [A>G]). Introduction of the minor allele of rs204990 ($p = 0.1435$), rs204991 ($p = 0.2136$), or rs3096688 ($p = 0.6618$) did not significantly alter the normalized firefly luciferase activity of the wild type promoter (Fig. 2.3A). In contrast, introduction of the minor allele of rs204989 [C>T] into the wild type promoter significantly decreased promoter-induced luminescence ($p < 0.0001$; Fig. 2.3A); conversely, introduction of the major allele of rs204989 [T>C] into the polymorphic promoter (“pGL3-m-989”) was found to restore wild type promoter-induced

luminescence ($p = 0.9384$; Fig. 2.3B). Using the JASPAR transcription factor binding profile database²⁹, two potential transcription factor binding sites were found to overlap with the site of the rs204989 SNP: namely, AP1 and C/EBP β consensus binding sites (Fig. 2.4).

2.3.5 – Decreased GPSM3 abundance leads to a deficit in migration towards monocyte chemoattractant protein-1 (MCP-1) by the human monocytic THP-1 cell line

The human monocytic THP-1 cell line has previously been shown to endogenously express GPSM3¹⁶; we confirmed the presence of the *GPSM3* ancestral (“major”) alleles of rs204989 and rs204991 within this cell line (*data not shown*). We established stable *GPSM3* knockdown (and scrambled control) THP-1 cell lines (Fig. 2.5A), using previously validated shRNA-expressing lentiviral vectors directed against *GPSM3* (*i.e.*, shRNA19 and shRNA20; ref. ⁴⁷). Using these stable THP-1 cell lines in real-time Transwell plate migration assays, we measured vehicle-normalized relative fluorescence units (RFU) over a 30-minute timeframe to quantify MCP-1/CCL2-induced cell transmigration. After normalizing to vehicle-induced non-specific migration, the *GPSM3* knockdown cell lines shRNA19 and shRNA20 exhibited disrupted migration to the chemokine MCP-1 (*a.k.a.* CCL2) relative to scrambled shRNA control lines (Fig. 2.5B); these migration differences were independent of any changes in transcript abundance of the MCP-1 receptor (*i.e.*, *CCR2*) between cell lines assayed (Fig. 2.5C; $F = 2.40$; $p = 0.119$). This migratory deficit upon *GPSM3* knockdown was observed both

by a decrease in initial rate of migration (Fig. 2.5D) and by a decrease in maximum transmigration-induced fluorescence achieved (Fig. 2.5E).

2.4 – DISCUSSION

Genome-wide association studies (GWAS) have empowered researchers to investigate complex non-Mendelian genetic disorders, including RA; however, these studies can be hampered by the identification of large numbers of noncoding variants with unknown effects, reproducibility across multiple populations being uncertain, and linkage with other risk alleles obscuring results³⁰. In multiple GWAS reports, noncoding variants 5' to the transcription start site of *GPSM3* (Fig. 2.1A) have been identified as occurring *less* frequently in individuals with RA^{18–20}. However, like many identified variants, no functional consequence has been elucidated to explain this association. In the current study, we addressed (1) genotype-dependent phenotypes of the identified chromosome 6 *GPSM3* SNPs, (2) potential linkage with rs6457620, a biallelic RA risk allele with a large effect size in the *HLA* gene region on chromosome 6 (refs. ^{23,24}), and (3) a functional effect of altered *GPSM3* expression levels in a human monocytic cell line.

We recruited 50 volunteers with confirmed RA diagnoses, 50 RA-free volunteers matched to the arthritis group, and 100 younger healthy individuals to analyze genotype-dependent functional consequences of *GPSM3* SNP alleles. Our finding that homozygotes for the *GPSM3* SNPs (m/m) have lower *GPSM3* transcript abundance in

whole blood relative to homozygotes for the ancestral allele (M/M) (Fig. 2.1B) supports the hypothesis first put forth from GWAS^{18–20} that there is a potential functional consequence of the identified *GPSM3* SNPs rs204989 and rs204991, namely protection from development of rheumatoid arthritis; we have previously demonstrated¹⁶ that lowered *Gpsm3* expression in mice, as generated by gene knockout technology, results in protection from collagen-autoantibody induced inflammatory arthritis.

While our present finding of lowered *GPSM3* mRNA levels in the whole blood of homozygotes bearing the *GPSM3* SNP haploblock is consistent with GWAS and mouse gene knockout studies, the observed frequencies of the rs204989/rs204991 haploblock within the various genotyped groups are seemingly discrepant, given the trend toward decreased *GPSM3* minor allele frequency, albeit not significantly different, in the disease-free matched control (MAF = 18.0%; n = 50) compared to the RA cohort (MAF = 23.0%; n = 50). Considering the small sample size, the observed variability is not remarkable in light of the reported minor allele frequency in the general population being 23.3%. Small sample size likely also affected our ability to assign statistical significance to the trend of increased frequency of the positive RA risk allele (Fig. 2.1C), *CCL21* gene rs2812378 [T>C] on chromosome 9 (*i.e.*, unlinked to the human *GPSM3* locus on chromosome 6; ref. ²⁴).

To address whether the *GPSM3* SNP haploblock on chromosome 6 was serving as a surrogate marker for a linked and causal (yet unrelated) chromosome 6 variation

affecting *GPSM3* mRNA abundance and/or RA disease status, additional genotyping was performed on another chromosome 6 biallelic allele, specifically near the well-established risk region in the *HLA* gene region (reviewed by Holoshitz²² and Yuta, et al.³³). This RA risk allele, rs6457620 [C>G]^{23,24} is reported by RegulomeDB³⁴ (based on Hapmap2 metadata) to exist in complete linkage disequilibrium ($D' = 1.0$; $r^2 = 1.0$) with another *HLA* region RA risk allele rs6457617 [C>T]^{31,32} in a population of Americans with European ancestry (CEPH collection)³⁵ that is very similar to the demographics of our West Virginia-based sample population. Both polymorphisms (rs6457617 & rs6457620) exist ~500 kb from the *GPSM3* SNP haploblock (e.g., Fig. 2.2D). When stratifying the *HLA* region-associated SNP genotype by *GPSM3* SNP genotype, a preferential coexistence is seen between the *GPSM3* SNP haploblock minor allele and the minor allele (G) of rs6457620 (Fig. 2.2). Upon further stratification by disease state, we discovered the *GPSM3* SNP alleles were more strongly associated with the minor allele of rs6457620 (G) in our RA cohort when compared to the matched control cohort (Fig. 2.2B vs. C). These findings suggest that the protective effect of the *GPSM3* SNP haploblock minor allele is most likely masked in our small-sized cohort by the large effect size of the *HLA* gene region RA risk allele at rs6457620 (RA odds ratio of 2.55²⁴) or other RA risk-related *HLA* gene haploblocks in linkage disequilibrium^{21,33}. Previous GWAS studies have genotyped tens of thousands of ethnically-diverse volunteers to identify the two *GPSM3* SNPs rs204989 and rs204991 as inversely associated with RA^{18–20}. Therefore, we hypothesize that our inability to reproduce previous GWAS findings is most likely a consequence of the relatively small sample size we recruited for this *functional* study of *GPSM3* phenotypes and/or a founder effect among the local

recruited volunteers³⁶. Regardless, our finding of a genotype-dependent decrease in *GPSM3* mRNA abundance is supportive of the prior GWAS findings^{18–20}, especially in light of the protection from inflammatory arthritis exhibited by *Gpsm3*-deficient mice¹⁶.

To probe the molecular mechanism whereby presence of the minor allele of the rs204989/rs204991 haploblock leads to decreased *GPSM3* transcript levels in whole blood, a 3.5-kb promoter region containing the haploblock region was tested for its promoter activity upon transfection into HEK293T cells (used given low transient transfection efficiency of the THP-1 cell line). There was a significant decrease in promoter activity in the reporter construct containing all four 5' SNPs *versus* the ancestral sequence, in a direct parallel to decreased *GPSM3* transcript abundance seen in whole blood samples from individuals homozygous for the minor rs204989/rs204991 haploblock *versus* the ancestral ("major") haploblock. The presence of rs204989 [C>T] was found to be the sole determinant responsible for the reduction in *GPSM3* promoter activity. We did observe slight variability in the decrease in *GPSM3* promoter-driven luciferase activity between experiments, most likely due to experimental variability, environmental factors (e.g., temperature differentially affecting firefly and *Renilla* luciferase activity), and elapsed time between experiments. Regardless, all constructs containing the minor allele of rs204989 [C>T] were not significantly different from the construct with all 4 *GPSM3* SNP alleles. To our knowledge, this is the first reported functional connection between the *GPSM3* rs204989 polymorphism and *GPSM3* transcript abundance in human cells and human samples. The singular effect of the rs204989 cytosine-to-thymine transition on *GPSM3* promoter activity may reside in the

disruption of the consensus DNA-binding motif of AP-1 and/or C/EBP β ; this hypothesis is now the topic of future work.

A potential functional consequence of this genotype-dependent decrease in *GPSM3* abundance is dysregulated G protein-coupled receptor signaling via $G_i\alpha^{6-10}$ – a common signaling system used by chemokine receptors. Considering the restricted expression of *GPSM3* in leukocytes and lymphoid organs^{15,16} and the importance of $G_i\alpha$ -coupled chemokine receptors in leukocyte migration during RA pathogenesis³⁷, we hypothesized that a reduction in *GPSM3* expression in human monocytic THP-1 cells would result in perturbed chemokine-dependent chemotaxis. Given the role of monocyte chemoattractant protein-1 (MCP-1/CCR2) signaling in the recruitment of monocytes and macrophages during inflammatory bouts in RA patients^{38,39}, we elected to examine MCP-1-induced chemotaxis. shRNA-induced knockdown of *GPSM3* in THP-1 cells disrupted Transwell migration (both initial rate and maximal migration) toward MCP-1, but did not affect expression of its cognate receptor, C-C chemokine receptor 2 (*CCR2*) (Fig. 5). Taken together, these data support a role for *GPSM3* in supporting MCP-1-induced migration of human THP-1 cells. We note that the knockdown efficiency of the successful *GPSM3*-targeted shRNAs was significantly greater than the 24.1% decrease found in individuals homozygous for the minor alleles of the *GPSM3* SNP haploblock. Therefore, we hypothesize that the functional effect of the rs204989 *GPSM3* SNP *in vivo* is most likely more subtle, yet it likely plays a role in the pathogenesis of RA as a component of a complex genetic framework that is, to date, still being elucidated.

Based on our collective results, the *GPSM3* SNPs rs204989 and rs204991, identified as protective against RA in prior GWAS studies, likely exhibit this protective effect via linkage to rs204989 and its alteration of *GPSM3* transcription. An rs204989-mediated decrease in *GPSM3* transcript level likely decreases *GPSM3* protein levels and thereby decreases chemokine-dependent leukocyte migration in inflammation. RNA-seq ‘transcriptomic’ datasets report the *GPSM3* mRNA as being expressed at an appreciable level in all human leukocyte subsets examined (GEO accession GDS62408)⁴⁰, suggesting that decreased *GPSM3* mRNA transcript caused by SNP rs204989 may affect both the innate and adaptive immune responses that act sequentially in the pathogenesis of rheumatoid arthritis³⁷. Future work will initially address whether the presence of rs204989 is functionally relevant in primary leukocytes isolated from patient samples or in CRISPR-mediated introduction of rs204989 in human leukocyte cell lines. Additionally, we will seek to establish whether one or both of the candidate transcription factors (*i.e.*, AP1 or C/EBP β) are indeed involved in *GPSM3* promoter regulation and whether such activity is directly affected by the major or minor allele of SNP rs204989.

2.5 – MATERIALS AND METHODS

2.5.1 – Subjects: A WVU IRB-approved case-control study (IRB protocol #1304033165) recruited a total of 200 consenting local volunteers for buccal swabs and blood samples. The volunteer population was subdivided into three groups of 100 young, healthy control volunteers from among the WVU Health Sciences Center student population, 50 rheumatoid arthritis patients (ICD-9 diagnosed and anti-RF-positive), and 50 demographically matched controls. Inclusion criteria for the 100 healthy control volunteers included (1) at least 18 years of age, (2) self-reported good health, and (3) no family history of autoimmune disease. Inclusion criteria for the 50 rheumatoid arthritis individuals included (1) at least 18 years of age, (2) positive ICD-9 diagnosis and (3) rheumatoid factor positive. Inclusion criteria for the 50 demographically matched controls included (1) at least 18 years of age, (2) not a blood relative of a volunteer enrolled in the study, and (3) no family history of autoimmune disease. For buccal swab samples, volunteers were instructed to vigorously swab the inside of both cheeks for 15 seconds on each side, and resultant samples were stored in a sterile nuclease-free container at -80°C. Blood samples were collected by a trained phlebotomist sequentially into a K₂EDTA tube (Becton Dickinson) and a PAXgene Blood RNA tube (BD); as per manufacturer's suggested protocol, the PAXgene Blood RNA tube was incubated at room temperature for 120 minutes prior to archiving at -80 °C.

2.5.2 – Isolation and Quantification of gDNA: Isolation of genomic DNA (gDNA) from buccal swab samples was performed with the QIAamp DNA Blood Mini Kit (Qiagen) on

a QIAcube automated nucleic acid extraction/purification instrument (Qiagen; Germantown, MD). Briefly, buccal swabs were immersed in 400 µl of Buffer ATL (Qiagen) and treated with proteinase K by incubation at 56 °C for 10 minutes. Following incubation, buccal swabs were removed, placed into QIAamp spin columns, and centrifuged at 13,000 RPM for 1 minute on a tabletop microfuge. Buccal swabs were then discarded. Cell lysates were then loaded into separate QIAamp spin columns as per manufacturer's suggested protocols; purified gDNA was automatically collected using the custom QIAcube "buccal swab spin" protocol (Qiagen). Quantification of human gDNA was performed with the Investigator Quantiplex Kit (Qiagen) using the manufacturer's suggested protocols. Briefly, buccal swab eluate was diluted 1:1 with nuclease-free water. Quantitative PCR to measure eluate gDNA content was performed using the Investigator Quantiplex Kit on a RotorGene Q-400 thermocycler (Qiagen) with an initial denaturation phase at 95°C for 60 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 10 seconds. DNA quantity was recorded as a mean of technical replicates.

2.5.3 – SNP Genotyping of Subjects: SNP genotypes were determined using the Type-it Fast Probe PCR Kit (Qiagen), TaqMan SNP genotyping probes, and primers for rs204989 (*GPSM3*), rs204991 (*GPSM3*), rs2812378 (*CCL21*), and rs6457620 (*HLA* gene region) (Life Technologies; cat. #C_3293828_20, C_2412452_20, C_16113556_10, and C_29315329_10, respectively) using the manufacturer's suggested protocols. Briefly, equal masses of human gDNA were added to each reaction and the manufacturer's recommended cycling parameters were used to detect

both major and minor alleles in multiplex qPCR reactions performed in technical replicates on a RotorGene Q-400 thermocycler. Coded identifiers were used to blind investigators of the disease state of volunteers until all samples had been genotyped.

2.5.4 – Isolation of Whole Blood RNA: To maintain homogeneity of sample treatment, all RNA was extracted from archived whole blood collected into PAXgene Blood RNA tubes with the PAXgene Blood RNA kit (Qiagen) using a modification of the manufacturer's suggested protocol. Briefly, when possible genotypes (M/M and m/m) were prepared concurrently. Upon removal from cryostorage, PAXgene tubes were incubated on a rocker at room temperature for 15-18 hours, centrifuged at 5,000 x g for 10 minutes at 4°C, and the resulting cell pellet was resuspended in 4 ml of nuclease-free water. The solution was then centrifuged at 5,000 x g for 10 minutes at 4 °C, and the cell pellet was resuspended in 350 µl of the manufacturer's resuspension buffer (BR1). Isolation of whole blood RNA was performed using an automated QIAcube workflow according to the manufacturer's suggested protocol to minimize variability between preparations. Eluted nucleic acids were quantified by absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific) and assessed for purity using 260/280 nm ratio of ≥ 1.8 . Immediately following isolation, a sample (250 ng) of eluted nucleic acids from each subject was used to synthesize complementary DNA (cDNA) with Thermo Verso Reverse Transcriptase Kit (Thermo Scientific). cDNA was then diluted 1:5 and used immediately for qRT-PCR analyses.

2.5.5 – Quantitative Reverse Transcriptase PCR (qRT-PCR): A sample of cDNA, derived from equal masses of RNA from each volunteer, was amplified in duplicate (*with an additional* independent “no reverse transcriptase-treated” control) using the USB VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix, Cleveland, OH) and custom-designed, intron-spanning PCR primers (Supplemental Table S1 and Figure S1). Cycling conditions were modified from the manufacturer’s suggested protocol to establish linearity of transcript detection (Supplemental Figure S2A,B). Relative quantification of gene expression was performed using the $\Delta\Delta C_T$ method⁴¹, and the specificity of each amplicon was verified by gel electrophoresis and melt curve tests (e.g., Supplemental Fig. S2C).

2.5.6 – Cell lines and culture conditions: The human embryonic kidney 293T cell line (HEK293T) was obtained from the American Type Tissue Collection (ATCC). Cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The HEK293T cell line was maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU penicillin, and 100 µg/ml streptomycin. Transient transfections of HEK293T cells were performed as previously described ⁴². Briefly, transfection of monolayers grown to 60-70% confluence was performed by incubating cells 15-18 hours with Ca₃(PO₄)₂ and plasmid DNA solution. The cell monolayers were then washed twice with phosphate buffered saline (pH 7.4), then fresh medium was added. THP-1 cells, from the ATCC, were maintained in an ATCC-modification of RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin,

100 µg/ml streptomycin, and 2 µg/ml puromycin. GPSM3 expression was stably knocked-down in THP-1 cells via lentiviral transduction (under the WVU IBC protocol # 15-04-08) of Mission shRNA in the pLKO.1 vector (clones: TRCN0000036819 and TRCN0000036820). An additional two THP-1 monoclonal cell lines were established stably-expressing scrambled control (a gift from Dr. David Sabatini, ref. ⁴³; Addgene plasmid # 1864). Briefly, transduction was performed at a multiplicity of infection of 10 using the spinoculation protocol (described in ref. ⁴⁴), transduced cells were selected with 2 µg/ml puromycin, and monoclonal populations were established by limiting dilution. All THP-1 monoclonal cell lines and HEK 293T cells were confirmed free of *Mycoplasma spp.* and *Acholeplasma spp.* contamination (Supplemental Figure S3) using Lookout® Mycoplasma PCR Detection kit following manufacturer's protocols (Sigma-Aldrich, St. Louis, MO).

2.5.7 – Cloning and mutagenesis of plasmids: DNA from plasmids pGL3-basic-luc2P (Promega, Madison, WI) and pcDNA3.1(+) were digested at the *HindIII* and *XhoI* sites using FastDigest enzymes and buffers (Thermo Scientific, Pittsburgh, PA), and restricted DNA ends were phosphatase treated with calf intestinal alkaline phosphatase (Promega, Madison, WI). Vectors were purified via extraction from a 1% agarose gel (QIAquick, Qiagen). Sequences of the *GPSM3* gene locus were retrieved from the NCBI reference sequence (NC_000006.12 c32195523-32190766). A 5010-bp region of the *GPSM3* promoter was amplified from gDNA ("*GPSM3* amplification primers";

Supplemental Table S2) from one individual with the major *GPSM3* alleles (“pGL3-M”) and another individual with all minor polymorphic alleles (“pGL3-m”); nested primers (Supplemental Table S2) were used to amplify a 3512-bp span of this promoter region (NC_000006.12 32192523 - 32196035) and thereby introduce flanking *HindIII* and *XhoI* cut sites (“*GPSM3* cloning primers”). The resultant amplicons were digested with *HindIII* and *XhoI*, and ligated into both pGL3-luc2P and pcDNA3.1(+) vectors (Rapid DNA ligation kit; Roche, Basel, Switzerland). Mutagenesis was performed using the QuikChange Lightning Mutagenesis kit (Agilent Technologies, Santa Clara, CA) and manufacturer’s suggested protocols with primers (Supplemental Table S3). Briefly, mutagenesis of *GPSM3* promoter inserts was performed within the pcDNA3.1(+) vector, sequenced by the Sanger method to confirm mutagenesis, and then subcloned into pGL3-luc2P vectors.

2.5.8 – Promoter-driven luciferase assay: HEK293T cells were transiently transfected using the calcium phosphate method, as described previously^{42,45}, with 5 µg of pGL3 firefly luciferase-based reporter vector and 1 µg of pRL-TK *Renilla* luciferase-based reporter control vector (Promega, Madison, WI). Forty-eight hours following transfection, the cells were assayed for both firefly and *Renilla* luciferase activities using the manufacturer’s suggested protocol for the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Briefly, cells were harvested, washed in PBS pH 7.4, and lysed using the manufacturer’s “passive lysis” buffer. Using automatic injectors within a Flexstation-3 (Molecular Devices, Sunnyvale, CA), firefly luciferase substrate was added to the cellular lysate and luminescence was recorded in a white, clear-bottom 96-

well plate for 10 seconds. Firefly luminescence was then quenched and *Renilla* luciferase substrate was added to the lysate and luminescence was recorded for 10 seconds. Data were normalized to luminescence induced by *Renilla* luciferase activity and reported as fold-change from pGL3-M transfected HEK 293T cells. All data are representative of biologic triplicates.

2.5.9 – Transcription factor bioinformatics: The 3.5 kb of sequence 5' to the *GPSM3* transcription start site was acquired from the Genome Reference Consortium human genome 38 (GRCh38) and imported into JASPAR²⁹. Predictions of consensus binding motifs for transcription factors were cut off at 20% dissimilarity.

2.5.10 – Transwell migration assay: THP-1 cells stably expressing either *GPSM3*-knockdown shRNA or scrambled shRNA control were serum-starved in HBSS + 1% BSA at 37°C for one hour and labeled with calcein-AM cell permeant fluorescent dye (494 nm/ 517 nm). Cells were washed and suspended in HBSS + 1% BSA + 10 mM HEPES, and 5×10^7 cells/ml were loaded into the upper well of a Falcon™ HTS FluoroBlok 96-Multiwell Insert System chemotaxis chambers (Corning, NY). Cells were allowed to migrate toward vehicle or 100 ng/mL MCP-1/CCL2 (R&D Systems, Minneapolis, MN) in the lower chamber. Fluorescence was measured at 2 minute intervals over the course of 60 minutes at 37°C using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). All data are representative of biological triplicates with technical replicates (i.e., n = 6).

2.5.11 – Statistical analyses: Descriptive data from the case-control cohort were analyzed using SAS University Edition 3.1 (SAS Institute Inc., Cary, NC). Differences in 2 x 2 discrete variables were determined using Fisher's exact test, while 2 x 3 discrete variables were compared using the Fisher-Freeman-Halton exact test⁴⁶. Regression analyses in Transwell migration assays were performed using 4 parameter logistic nonlinear regression analyses over the period of migration with post-hoc comparison by one-way ANOVA of the unshared maximum and time to 50% migration parameters. All nonlinear models were confirmed to have r-squared values >0.99, and accepted the null hypothesis of the Shapiro-Wilk normality test ($p \geq 0.05$). All other analyses were performed in Prism 6.0 using a two-tailed independent t-test or one-way ANOVA with Bonnferroni or Dunnett's post-hoc tests where indicated.

2.6 – Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

2.7 – Figures and Tables:

Figure 2.1. *GPSM3* transcript abundance in whole blood from individuals homozygous for the minor allele of the rs204989/rs204991 haploblock (the “m/m” genotype) is significantly lower than in whole blood from individuals homozygous for the major allele of the haploblock (the “M/M” genotype). (A) Schematic representation of identified polymorphisms with respect to the predicted transcription start site of the human *GPSM3* gene. (B) Scatter plot representation of *GPSM3* mRNA abundance, measured in whole blood by qRT-PCR, as normalized to

the average level measured in M/M individuals. Individual results for homozygous major allele samples (M/M; n = 53) are depicted by solid circles, and homozygous minor allele samples (m/m; n=11) are depicted by open circles. An average decrease ($\Delta\mu$) in *GPSM3* transcript abundance of 24.1% ($t = 3.80$, $df = 62$, $p = 0.0003$) was observed in homozygous minor allele samples. Error bars represent S.E.M. (C) Descriptive statistics of the three cohorts of volunteers genotyped in this study: Minor allele frequencies were 23% for rs204989 and rs204991 in the rheumatoid arthritis samples versus 18% in the disease-free controls (Fisher's exact test; $p = 0.4839$). Risk/minor allele frequency for rs2812378 was 30.0% in the rheumatoid arthritis samples versus 25.0% in the disease-free controls ($p = 0.5267$). Risk/minor allele frequency for the *HLA* gene region biallelic SNP rs6457620 was 35.0% in the rheumatoid arthritis samples and 45.0% in the disease-free matched controls ($p = 0.1938$).

A	Homozygous Ancestral <i>GPSM3</i> (M/M; n = 115)			Heterozygous <i>GPSM3</i> (M/m; n = 71)			Homozygous SNP <i>GPSM3</i> (m/m; n = 14)			Significance levels of observed differences
<i>HLA</i> SNP (rs6457620) Genotype	C/C	C/G	G/G	C/C	C/G	G/G	C/C	C/G	G/G	
Genotype Occurrence	41	53	21	10	42	19	3	5	6	
Genotype Frequency	35.6%	46.1%	18.3%	14.1%	59.1%	26.8%	21.4%	35.7%	42.9%	
	Minor Allele Frequency			Minor Allele Frequency			Minor Allele Frequency			Fisher-Freeman-Halton Exact Test
<i>HLA</i> SNP (rs6457620) [G]	41.3%			56.3%			60.7%			p = 0.0066

B	Rheumatoid Arthritis (n=50)			
<i>HLA</i> SNP (rs6457620) Genotype	C/C	C/G	G/G	Significance levels of observed differences
M/M	14	15	1	
M/m	7	7	3	
m/m	0	1	2	
	<i>GPSM3</i> Minor Allele Frequency			Fisher-Freeman-Halton Exact Test
rs204989 / rs204991	16.7%	19.6%	58.3%	p = 0.0123

C	Control (n=50)			
<i>HLA</i> SNP (rs6457620) Genotype	C/C	C/G	G/G	Significance levels of observed differences
M/M	11	16	6	
M/m	2	13	1	
m/m	0	0	1	
	<i>GPSM3</i> Minor Allele Frequency			Fisher-Freeman-Halton Exact Test
rs204989 / rs204991	7.7%	22.4%	18.8%	p = 0.2739

Figure 2.2. Linkage of the *GPSM3* SNP haploblock and the known RA risk allele in the *HLA* gene region, rs6457620 [C>G], is more pronounced in RA samples than controls, but this linkage does not result in a rs6457620 genotype-dependent effect on *GPSM3* transcript abundance in whole blood. (A) Descriptive statistics of all 200 volunteers, including the RA (n = 50), disease-free matched control (n = 50), and young healthy control volunteers recruited for the current study. These data are

stratified by *GPSM3* SNP haplotype, showing that the rs6457620 [G] risk allele frequency amongst homozygous ancestral *GPSM3* haploblock individuals (M/M) is 41.3%, heterozygous individuals (M/m) is 56.3%, and homozygous minor *GPSM3* haploblock (m/m) is 60.7%. These data are significantly different from chance distribution (Fisher-Freeman-Halton exact test; $p = 0.0066$). **(B)** Analyses of *GPSM3* SNP haploblock linkage within RA samples stratified by rs6457620 genotype, again exhibiting statistically significant difference from chance distribution (Fisher-Freeman-Halton exact test; $p = 0.0123$). **(C)** Within the disease-free matched control samples, *GPSM3* SNP minor allele (m) frequency, as stratified by rs6457620 genotype, is not significantly different from chance distribution as determined by Fisher-Freeman-Halton exact test ($p = 0.2739$). **(D)** Graphical representation of minor allele frequencies (MAFs; of European ['eu'] population), physical distance (in basepairs), and linkage disequilibrium (LD) value (as quantified by heatmap) between the three linked SNPs of the *GPSM3* SNP haploblock (rs204989, rs204990, rs204991) and the *HLA* gene region SNP rs6457620, as obtained using the NIEHS SNPinfo webserver (<http://snpinfo.niehs.nih.gov/>) from NCBI dbSNP data (ref. ²⁵). **(E)** Despite modest linkage between *GPSM3* SNP minor alleles (m) and the rs6457620 risk allele (G), there is no effect of the genotype at *HLA* gene region SNP rs6457620 on *GPSM3* transcript abundance. Significance determined by one-way ANOVA with Bonferroni *post-hoc*.

Figure 2.3. Functional consequences of SNPs rs204989, rs204990, rs204991, and rs3096688 on *GPSM3* promoter activity. (A) Functional results upon subcloning a 3.5 kb-region immediately 5' to the *GPSM3* transcription start site from M/M and m/m genotype volunteers, leading to wild type (pGL3-M) and “minor”/polymorphic (pGL3-m) promoter-driven expression of firefly luciferase in HEK293T cells. Luciferase activity is reported as a ratio of *GPSM3* promoter-dependent firefly luciferase activity (FLuc) to

control *Renilla* luciferase activity (pRL-TK control vector) and normalized to the average level measured from the wild type pGL3-M vector (set to 1.00; dotted line). Introduction of independent minor alleles (denoted “+###”) into the wild type pGL3-M vector is seen to differentially affect resultant firefly luciferase activity. **(B)** Restoration of the major allele at rs204989 in the pGL3-m vector (*i.e.*, “pGL-m-989”) restores wild type *GPSM3* promoter activity. All data are compiled from three independent experiments. Error measure is S.E.M. Significance was determined using one-way ANOVA after controlling for multiple comparisons with Dunnett’s test using pGL3-M as the defined control.

Figure 2.4. Putative transcription factor binding sites within the human *GPSM3* promoter potentially disrupted by the minor allele of SNP rs204989 (arrows), as predicted by the JASPAR database: **(A)** Fos/Jun (AP1) heterodimer; **(B)** C/EBP- β .

Figure 2.5. Lentiviral-mediated shRNA knockdown of endogenous GPSM3 expression in the human monocytic THP-1 cell line disrupts migration toward MCP-1 (A) Western blot showing whole lysate (40 µg) immunoreactivity with mouse monoclonal anti-GPSM3 antibody 35.5.1 (ref. ⁴⁷) and with anti-β-actin as a loading control. (B) Real-time Transwell migration analysis of indicated calcein-AM stained THP-1 cell lines as shown by change in the ratio of MCP-1-induced to vehicle-induced relative fluorescence units (RFU) over a 30 minute timeframe. (C) qRT-PCR data from indicated stable knockdown or scrambled control THP-1 cell line total RNA preparations. There was no significant difference in *CCR2* mRNA abundance in the THP-1 cell lines as determined by one-way ANOVA (F = 2.40; p = 0.119). (D) Regression analysis-derived rate of THP-1 cell line transmigration over initial migratory period; differences between each cell line was calculated by one-way ANOVA. (E)

Regression analysis-derived maximum THP-1 cell transmigration over 30 minute timecourse; differences between each cell line were calculated by one-way ANOVA. All data are reported as means with error bars representing S.E.M.

Supplementary Information

Genetic variations in *GPSM3* associated with protection from rheumatoid arthritis affect its transcript abundance

BJ Gall, A Wilson, AB Schroer, JD Gross, P Stoilov,
V Setola, CM Watkins, DP Siderovski

Supplementary Materials and Methods:

Quantitative Reverse Transcriptase PCR (qRT-PCR): Relative quantification of gene expression was performed using the $\Delta\Delta C_T$ method using the indicated formula and primer pairs:

$$\text{Relative mRNA abundance} = 2^{\frac{(\text{Mean test gene CT of control group} - \text{Mean test gene CT of subject})}{(\text{Mean ref gene CT of control group} - \text{Mean ref gene CT of subject})}}$$

Table S2.1: Sequences of primers used in gene transcript detection

Primers:	Sequence (5'→3')
B2M Fwd	tgctgtctccatgtttgatgtatct
B2M Rev	tctctgctccccacctctaagt (product=86 bp)
GPSM3 Fwd	ctccaaactccaccactct
GPSM3 Rev	cagtctgcagggagagcgag (product=114 bp)

Table S2.2: Sequences of primers used in subcloning *GPSM3* promoter

Primers:	Sequence (5'→3')
Amplification Primer Forward	tcaagcagtggtggtgaag
Amplification Primer Reverse	tgagggtccctacaggggaatg
Cloning Primer Forward	ggtggtgaagcttccctagaggggagaaaactg
Cloning Primer Reverse	ggtggtctcgaggatcggccctggctct

Table S2.3: Sequences of primers used in mutagenizing *GPSM3* promoter

Mutagenesis Primers	Sequence (5'→3')
rs204989 (C→T) sense	gtattcactatgcatcaagtgccttattacattattaatacatcaaccc
rs204989 (C→T) anti-sense	gggttgatgtattaataatgtaataaagcacttgatgcatagtgaaac
rs204989 (T→C) sense	gtattcactatgcatcaagtgccttattacattattaatacatcaaccc
rs204989 (T→C) anti-sense	gggttgatgtattaataatgtaataaagcacttgatgcatagtgaaac
rs204990 (G→T) sense	cttcctacctcagcctctcattttccatatctatactctta
rs204990 (G→T) anti-sense	taagagatatagatatggaaaaatgagaggctgaggtaggaag
rs204991 (A→G) sense	cccctgtctgcactaaactgtaagtttccaaaatgaaccc
rs204991 (A→G) anti-sense	gggttcattttggaaacttacagtttagtgagacaagggg
rs3096688 (A→G) sense	ccgcctcagcctcccaaagtgttaggattac
rs3096688 (A→G) anti-sense	gtaatcctagcactttgggaggctgaggcgg

Supplementary Figures:

Figure S2.1. Schematic representation of the location of *GPSM3* Fwd (→) and *GPSM3* Rev (←) primers relative to the predicted exon/intron architecture described for established human *GPSM3* gene transcripts (as per ENSEMBL database record ENSG00000213654). Open rectangles, exons encoding 5' or 3' UTRs; closed rectangles, exons encoding open-reading frame sequence.

Figure S2.2. Template dilution-based tests of qRT-PCR primer efficiencies. Threshold values for custom *B2M* (A) and *GPSM3* (B) primers (Table S1) over $\log_{10}$ serial dilution of cDNA synthesized from whole blood RNA show similar primer efficiencies. (C) PCR products from whole blood cDNA following qRT-PCR amplification and electrophoretic separation performed on a 4-12% tris/borate/EDTA (TBE) gel: well 1 – DNA ladder, well 2 – *GPSM3* SYBR Green PCR amplicon (114 bp predicted), well 3 – *B2M* SYBR Green PCR amplicon (86 bp predicted).

Figure S2.3. PCR confirmation of lack of *Mycoplasma spp.* and *Acholeplasma spp.* contamination. PCR amplification of highly conserved 16S rRNA coding region (lower band) and internal control DNA (upper band) was performed using Lookout® Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO), which is sensitive to 10 genomes per 1 μ l. Performed by directly adhering to the manufacturer's instructions. Lane 1: (Positive control) PCR amplification of both the internal control (upper band) and *Mycoplasma orale* DNA fragments (lower band). Lane 2: (Negative control) PCR amplification of only the internal control. Lanes 3-7: (Culture supernatants) PCR amplification of only the internal control.

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Chapter 3

Reduction of GPSM3 expression akin to the arthritis-protective SNP rs204989 differentially affects migration in a neutrophil model

Under review – *Genes & Immunity*

BJ Gall¹, AB Schroer¹, JD Gross¹, V Setola^{1,2}, DP Siderovski¹

¹Department of Physiology & Pharmacology and

²Department of Behavioral Medicine & Psychiatry,

West Virginia University School of Medicine, Morgantown, WV, USA 26506-9229

Corresponding author:

David Siderovski, WVU Dept. of Physiology & Pharmacology
One Medical Center Drive, PO Box 9229, Morgantown, WV 26506-9229
Email: dpsiderovski@hsc.wvu.edu; Tel: 304-293-2418; Fax: 304-293-3850

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3.1 - Abstract

G Protein Signaling Modulator 3 (GPSM3) is a leukocyte-specific regulator of G protein-coupled receptors, which binds inactivated $G\alpha_i$ -GDP subunits, precluding their reassociation with $G\beta\gamma$ subunits. GPSM3 deficiency protects mice from inflammatory arthritis and, in humans, *GPSM3* single nucleotide polymorphisms (SNPs) are inversely associated with rheumatoid arthritis incidence; recently, these polymorphisms were linked to one particular SNP (rs204989) that decreases *GPSM3* transcript abundance. However, the precise role of GPSM3 in leukocyte biology is unknown. Here we show that GPSM3 is induced in the human promyelocytic leukemia NB4 cell line following retinoic acid treatment, which differentiates this cell line into a neutrophil-like cell line (NB4*). Reducing GPSM3 expression in NB4* cells disrupts their migration toward leukotriene B₄, a chemoattractant involved in initial recruitment of neutrophils to arthritic joints, but not migration toward formylated peptides. Our results suggest that the arthritis-protective *GPSM3* SNP rs204989 may act to decrease neutrophil chemoattractant responsiveness.

3.2 - Introduction

Genome-wide association studies (1-3) have highlighted single-nucleotide polymorphisms (SNPs rs204989 & rs204991) in a region upstream of the *GPSM3* transcription start site as being inversely correlated to the prevalence of rheumatoid arthritis (RA). This *GPSM3* SNP association is supported by the protection afforded to *Gpsm3*-deficient mice in an acute model of inflammatory arthritis (4). Histologic analysis of synovial tissue from *Gpsm3*-deficient mice revealed a marked acellularity (4), suggesting that protection from developing inflammation relies on a potential cellular trafficking defect.

Neutrophils are leukocytes critical to the activation of inflammation in RA and many other autoimmune-related diseases (reviewed in (5)). Neutrophils elicit this function following their initial recruitment by early-phase proinflammatory chemoattractants. In RA, this early-phase recruitment is mediated primarily by leukotriene B4 (LTB4), followed by a LTB4 positive-feedback loop in which recruited neutrophils secrete LTB4 and additional chemoattractants (reviewed in (6)). To date, work to identify the role of *GPSM3* in disease pathophysiology has exclusively focused on circulating monocytes, which compose less than 10% of circulating leukocytes (7). However, mice made deficient in one of several monocyte chemokine receptors (*i.e.*, CCR1, CCR2, and CCR5) do not exhibit significant protection from models of RA inflammation (8); furthermore, mice depleted of Ly6C^{high} monocytes have no change in histopathology (9). Moreover, the initial class of leukocytes recruited to synovial tissues in human arthritis is thought to be neutrophils, which migrate in response to a sequential cascade of LTB4, IL-1 β , and CXCR2 agonists (6). Further support for the primary role of

neutrophils in initiating RA inflammation comes from protection afforded to mouse strains that are deficient in various proteins required for the recognition or synthesis of LTB₄, including the LTB₄ receptor BLT1 (10), 5-lipoxygenase activating protein (11), 5-lipoxygenase (12), and leukotriene A₄ hydrolase (12).

The LTB₄ receptor, BLT1, is a G protein-coupled receptor linked to pertussis toxin-sensitive G_i-subfamily heterotrimers (13), and therefore sensitive to potential regulation by GoLoco motif-containing proteins (like GPSM3) that act as G α_i -selective guanine nucleotide dissociation inhibitors (14). The role of GPSM3 in neutrophil function has not previously been assessed. Here, we identify the expression of GPSM3 in an immortalized cell line that can be differentiated towards neutrophil-like function, and further identify a selective role for GPSM3 in its migration toward proinflammatory LTB₄.

3.3 - RESULTS

3.3.1 - Identification of GPSM3 expression in the ATRA-differentiated NB4 cell model of neutrophils

GPSM3 was undetectable within whole cell lysates of the human acute promyelocytic leukemia NB4 cell line. However, upon treatment of NB4 cell cultures with all-trans retinoic acid (ATRA), *GPSM3* transcript (Fig. 3.1A) and GPSM3 protein (Fig. 3.1B) increased in a differentiation-dependent manner concurrently with the established differentiation marker CD11b. This ATRA-dependent induction of GPSM3 expression also paralleled the acquisition of other markers of differentiated neutrophil function:

specifically, the activity of intracellular oxidoreductase enzymes that can reduce nitroblue tetrazolium to colored formazan dye (Fig. 3.1C) and morphologic changes (Fig. 3.1D; e.g., invagination of the nucleus), both of which have been reported previously in ATRA-differentiated NB4* cells (15).

3.3.2 - GPSM3 deficiency impairs LTB4, but not fMLP-induced migration

We established stable, monoclonal daughter lines from the parental NB4 cell line using previously validated short hairpin RNA (shRNA)-expressing lentiviral vectors directed against *GPSM3* (16). After ATRA treatment, all daughter lines exhibited equivalent upregulation of CD11b and equivalent expression of G β subunits (Fig. 2A, right panel); therefore, the established interaction between GPSM3 and neo-synthesized, free G β subunits (16) does not affect steady-state G β levels in this cell line. In addition, all daughter lines exhibited equivalent induction of intracellular oxidoreductase activity upon ATRA treatment (Fig. 3.2B, right panel). Four NB4* monoclonal lines with successful knockdown of GPSM3 expression were each found to have disrupted migration towards LTB4 in Transwell migration assays (Fig. 3.2C), compared both to scrambled shRNA control lines and to a line expressing an ineffective shRNA (*i.e.*, daughter line NB4*-shRNA22.2; Fig. 3.2A&B). Diminished migratory response to LTB4 was not correlated with any decrease in mRNA expression of the high-affinity LTB4 receptor 1 (*LTB4R1*); for example, compared to the scrambled shRNA control lines, only the NB4*-shRNA19.5 line exhibited a significant difference in *LTB4R1* mRNA expression (Fig. 3.2C inset, $\Delta = +56.5\%$, $p < 0.0001$) and this difference

was an increase in expression. In contrast to the diminished migratory response towards LTB₄ elicited by GPSM3 knockdown, no significant differences were observed in formylated peptide (fMLP)-induced migration toward a number of fMLP concentrations (e.g., Fig. 3.2D and S3.2), despite statistically significant differences observed in mRNA expression of the high-affinity, G_i-coupled formylated peptide receptor 1 (*FPR1*; Fig. 3.2D inset).

3.3.3 – The phospholipase C inhibitor, U73122, disruptes LTB₄-induced migration

Inhibition of phosphoinositide 3-kinase (PI3K) or Mitogen-activated protein kinase kinase (MKK), does not significantly affect LTB₄-induced Transwell migration; however, inhibition of phospholipase C (PLC) ameliorates LTB₄-induced migration (Fig. 3.3). When m-3M3FBS, a receptor-independent PLC activator, was added to the lower chamber it was unable to restore migration of the shRNA 19.1 monoclonal (Fig. 3.3).

3.4 - DISCUSSION

Before its mRNA was cloned (17), GPSM3 protein was first detected in neutrophil detergent-resistant fractions by mass spectrometry (18). More recently, RNA-seq datasets indicate that human neutrophils have greater *GPSM3* mRNA than all other types of circulating leukocytes (e.g., NCBI GEO accession number GSE62408 (19)). These findings sparked our interest in determining whether GPSM3 was functioning to regulate aspect(s) of neutrophil biology. Using RNA-interference to negate the *GPSM3* upregulation found to occur normally in the promyelocytic NB4 cell line upon ATRA differentiation, we have now established that GPSM3 is required for the full migratory response of a neutrophil-like cell line toward pathophysiologically relevant concentrations of LTB₄ (20). The migratory response to LTB₄ is believed to be dependent on the abundance of GPSM3 protein in these cells, given that the NB4*-shRNA20 monoclonal lines were seen to have an intermediate level of GPSM3 knockdown efficiency and, in parallel, an intermediate migratory phenotype. Given our findings here, we surmise that the synovial acellularity seen in anti-collagen antibody challenged GPSM3-deficient mice (4) and the inverse association between RA development and the *GPSM3* expression-lowering SNP rs204989 (21) could be the result of reduced neutrophil recruitment by LTB₄.

During infections, neutrophil responsiveness to bacterial-derived formylated peptides is mediated via formyl peptide receptors (22): i.e., GPCRs coupled to G α_i -containing heterotrimers in a similar fashion to LTB₄ receptors. However, GPSM3 knockdown in NB4* cells does not affect migration initiated through the formyl peptide

receptor across a gradient of concentrations (Fig. S3.2). We therefore conclude that GPSM3 knockdown does not globally affect the chemotactic machinery of NB4* cells, but instead leads to a selective abrogation of responsiveness to certain chemoattractants, like LTB4 shown here. Future investigations will be directed at identifying the specific signaling mechanism(s) regulated by GPSM3 that differentially affect LTB4- vs fMLP-induced migration. The effect of GPSM3 deficiency on NB4* LTB4-induced migration, coupled with the established role that LTB4 plays in the pathogenesis of RA, supports the therapeutic potential of GPSM3-targeted inhibitors in the treatment of RA, as we have proposed previously (21).

3.5 – MATERIALS AND METHODS

3.5.1 - Cell lines and culture conditions

The human acute promyelocytic leukemia cell line (NB4) was obtained from the Leibniz Institute German Collection of Microorganisms and Cell Cultures. Preliminary studies were performed on NB4 cells that were a generous gift from Dr. Sherman Weissman. Cells were maintained at 37°C in a humidified, 5% CO₂ atmosphere in an ATCC-modification of RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin, 100 µg/ml streptomycin, and 2 µg/ml puromycin. GPSM3 expression was stably knocked-down via lentiviral transduction (WVU IBC protocol 15-04-08) as described previously (21). Additional NB4 cell lines were established expressing a scrambled shRNA control (Addgene plasmid 1864). Briefly, transduction

was performed at a multiplicity of infection of 10 using spinoculation (ref. (23)); transduced cells were selected with 2 µg/ml puromycin and monoclonal populations established by limiting dilution. All NB4 lines were confirmed free of *Mycoplasma spp.* and *Acholeplasma spp.* contamination using the Lookout® Mycoplasma PCR Detection kit (Sigma-Aldrich, St. Louis, MO; Fig. S3.1). NB4 differentiation was performed as previously reported (24) by treatment with complete media containing 1 µM all-trans retinoic acid (Acros Organics, Geel, Belgium). Cell density was maintained between 4×10^5 and 1×10^6 cells/mL.

3.5.2 - Nitroblue Tetrazolium (NBT) reduction

Cells (5×10^5) were resuspended in PBS with 1 mg/ml NBT and 300 nM PMA, then incubated at 37°C and 5% CO₂ for 30 minutes. Cells were then washed in PBS twice and mounted on slides for visualization of 500 cells per cell population per experiment. Formazan-positive (black), -intermediate (gray), and -negative (colorless) cells were scored under brightfield microscopy.

3.5.3 - Wright-Giemsa staining

Cells were fixed with 100% methanol for 1 minute, then allowed to air-dry briefly. Cells were then stained using Wright-Giemsa stain for 2 minutes, after which 1.5 volumes of PBS, pH 6.8 was added, and the cells were allowed to incubate for another 2 minutes. Cells were washed and visualized on an EVOS FL Auto microscope (Thermo Scientific, Waltham, MA).

3.5.4 - RNA isolation, cDNA synthesis, and quantitative reverse transcriptase PCR (qRT-PCR)

RNA was extracted from ATRA-differentiated NB4 cells with the RNeasy kit (Qiagen). Briefly, cells were centrifuged at 200 x g for five minutes, and the supernatant was removed. Cells were then washed with 1X PBS and centrifuged again to remove supernatant. The cell pellet was resuspended in 600 µl of the manufacturer's lysis buffer (RLT) with 1% β-mercaptoethanol. RNA isolation was performed using an automated QIAcube workflow according to the manufacturer's suggested protocol to minimize variability between preparations. Eluted nucleic acids were quantified by absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific) and assessed for purity using a 260/280 nm cut-off ratio ≥ 1.8 . Immediately following isolation, a sample of eluted nucleic acids (250 ng) from each group of cells was used to synthesize complementary DNA (cDNA) with the Thermo Verso Reverse Transcriptase Kit (Thermo Scientific). cDNA was then diluted 1:5 and used immediately for qRT-PCR analyses. Samples of cDNA, derived from equal mass of input RNA from each sample, were amplified in duplicate (with an additional, independent, "no reverse transcriptase-treated" control) using the USB VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix, Cleveland, OH) and commercially-validated PCR primers (Qiagen, Hilden, Germany). Cycling conditions were established following the manufacturer's suggested protocol. Relative quantification of gene expression was performed using the $\Delta\Delta C_T$ method (25) with β-microglobulin chosen as the stable housekeeping gene.

3.5.5 - Transwell migration assay

shRNA-expressing NB4 cell lines were serum-starved in HBSS + 1% BSA at 37°C for one hour and labeled with calcein-AM cell-permeant fluorescent dye (ex. 494 nm/em. 517 nm). Cells were washed and suspended in HBSS + 1% BSA + 10 mM HEPES, and 2.5×10^6 cells/ml were loaded into the upper well of Falcon™ HTS FluoroBlok 96-Multiwell Insert System chemotaxis chambers (Corning, NY). Cells were allowed to migrate toward vehicle or 100 nM LTB₄ or 100 ng/ml fMLP (R&D Systems, Minneapolis, MN) in the lower chamber. Fluorescence was measured at 2-minute intervals over the course of 60 minutes at 37°C using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). All data are representative of biological triplicates with technical replicates (*i.e.*, n = 6).

3.5.6 - Antibodies and Western blot

Antibodies targeting GPSM3 (UNC Immunology Core; clone 35.5.1; 1:200 dilution), CD11b (Developmental Studies Hybridoma Bank; clone H5A4; 1:200 dilution), pan-Gbeta (T-20; Santa Cruz Biotechnology; 1:500 dilution) and β-actin (Sigma; 1:2000 dilution) were purchased from the indicated sources. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from Sigma. Cells were lysed with ice-cold RIPA buffer with protease inhibitor (ThermoScientific, Waltham, MA), sonicated on ice, then clarified by centrifugation at 20,000 X g for 5 minutes. Protein content was

quantified by the Bradford protein assay (ThermoScientific, Waltham, MA) using BSA as a standard. Lysates were diluted in Laemmli buffer (BioRad, Hercules, CA) and resolved on 8-16% pre-cast SDS-polyacrylamide gels (BioRad), transferred to nitrocellulose membranes, immunoblotted with primary (overnight, 4°C) and HRP-conjugated secondary (1:2000 dilution, 2 h at room temperature) antibodies, and visualized by chemiluminescence (ECL, ThermoScientific, Waltham, MA).

3.5.7 - Statistical analyses

Area-under-the-curve analyses of Transwell migration assay data were performed over the period of migration with post-hoc comparison by one-way ANOVA and accepted the null hypothesis of the Brown-Forsythe normality test ($p \geq 0.05$). Significance was reported as NB4*-shRNA monoclonal line response compared to those of scrambled 2 and scrambled 3 cell lines. Nonlinear modeling could not be used in these analyses because r-squared values were <0.9 . All analyses were performed in GraphPad Prism 6.0 using one-way ANOVA with Bonnferroni or Dunnett's post-hoc tests where indicated.

3.6 - ACKNOWLEDGEMENTS

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3.7 Figures

Figure 3.1 – GPSM3 transcript and protein expression are increased during all-trans retinoic acid (ATRA) differentiation of NB4 cells toward a neutrophil-like physiology.

(A) *GPSM3* mRNA abundance in NB4 cells over treatment time with 1 μ M ATRA; relative expression normalized to cells treated with vehicle (DMSO) for 120 hours. (B) Levels of *GPSM3* protein and the differentiation marker CD11b both increase with ATRA-induced differentiation. (C) ATRA-induced differentiation over time increases the neutrophil-specific ability to reduce nitroblue tetrazolium to formazan, as observed by a steady increase in formazan-positive (dark) NB4 cells over time. (D) Wright-Giemsa stained NB4* cells indicating the acquisition over time of ATRA-induced morphological changes mimicking the nuclear invagination seen in neutrophil-like precursor cells.

Figure 3.2 – GPSM3 deficiency disrupts NB4* cell migration toward LTB4.

(A) Immunoblot revealing undetectable GPSM3 and CD11b immunoreactivity within whole cell lysates from all shRNA-expressing monoclonal cell lines when these lines were mock-differentiated with vehicle only (0.01% DMSO; *left* panels). Immunoblot revealing decreased GPSM3 immunoreactivity after 120 hours of all-trans retinoic acid (ATRA) treatment of NB4* cells stably expressing GPSM3-targeted shRNAs shRNA19 or shRNA20 (*right* panels). (B) Nitroblue tetrazolium reduction assay indicating that >90% of cells within each NB4-shRNA monoclonal cell line are incapable of reducing nitroblue tetrazolium after mock-differentiation with vehicle only (0.01% DMSO; *left* bar graph). Following differentiation with all-trans retinoic acid, >87% of differentiated NB4* cells were capable of reducing nitroblue tetrazolium, irrespective of expressed shRNA species (*right* bar graph). (C) Real-time Transwell migration towards 100 nM LTB4 over 60 minutes by indicated NB4* cell lines (after 120 hours of ATRA differentiation). Significance ($p < 0.05$) determined by one-way ANOVA analyses of area under the curve with Bonferroni post-hoc. (C *insert*) Post-differentiated NB4* transcript abundance of the LTB4 receptor BLT1 (*LTB4R*); significance ($p < 0.05$) determined by Kruskal-Wallis test (unequal group variances, Brown-Forsythe $p < 0.05$) with Dunn's post-hoc. All data plotted as mean $\pm$ S.E.M. (D) Real-time Transwell migration towards 100 ng/mL fMLP over 60 minutes by indicated NB4* cell lines (after 120 hours of ATRA differentiation). (D *insert*) Post-differentiated NB4* transcript abundance of the fMLP receptor (*FPR1*); significance ($p < 0.05$) determined by Kruskal-Wallis test (unequal group variances, Brown-Forsythe $p < 0.05$) with Dunn's post-hoc. All data plotted as mean $\pm$ S.E.M.

Figure 3.3 – LTB4-induced Transwell migration is disrupted by a phospholipase C inhibitor, but not inhibitors of MEK or PI3K. Real-time Transwell migration of NB4* cells, pretreated for 30 minutes with indicated inhibitor or vehicle (DMSO), towards 100 nM LTB4 in the presence of (A) 2 μ M U73122 (PLC inhibitor), (B) 10 μ M U0126 (MEK inhibitor), or (C) 5 μ M LY294002 (PI3K inhibitor)(Mean $\pm$ S.E.M.). Closed circles indicate cells pretreated with vehicle for 30 minutes, and open circles indicate cells pretreated with indicated inhibitor.

SUPPLEMENTARY INFORMATION

Reduction of GPSM3 expression akin to the arthritis-protective SNP rs204989 differentially affects migration in a neutrophil model

BJ Gall¹, AB Schroer¹, JD Gross¹, V Setola^{1,2}, DP Siderovski¹

¹Department of Physiology & Pharmacology and

²Department of Behavioral Medicine & Psychiatry,

West Virginia University School of Medicine, Morgantown, WV, USA 26506-9229

Figure S3.1. PCR confirmation of lack of *Mycoplasma spp.* and *Acholeplasma spp.* contamination. PCR amplification of highly conserved 16S rRNA coding region (lower band) and internal control DNA (upper band) was performed using Lookout® Mycoplasma PCR Detection Kit (Sigma-Aldrich, St. Louis, MO), which is sensitive to 10 genomes per 1 µl. Performed by directly adhering to the manufacturer's instructions. Lane 1: (Positive control) PCR amplification of both the internal control (upper band) and *Mycoplasma orale* DNA fragments (lower band). Lane 2: (Negative control) PCR amplification of only the internal control. Lanes 3-9: (Culture supernatants) PCR amplification of only the internal control, including HEK 293T cells previously tested to be free of *Mycoplasma spp.* contamination. Lane 10: (Media control) PCR amplification only the internal control from fresh media.

Figure S3.2. GPSM3 deficiency does not decrease Transwell migration across a concentration gradient of fMLP. Real-time Transwell migration towards fMLP over 60 minutes by indicated NB4* cell lines (after 120 hours of ATRA differentiation). fMLP concentrations included (A) 250 ng / ml, (B) 100 ng / ml, (C) 50 ng / ml, (D) 10 ng / ml, (E) 1 ng / ml, and (F) vehicle (DMSO).

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Chapter 4

Reduced transcript abundance of G protein signaling modulator 3 by lipopolysaccharide and α hemolysin in a monocytic cell line

This work is the result of a collaborative effort with Dr. Alex Duncan's lab and is, in part, currently published in:

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4.1 – Abstract

NOD-like receptor family, pyrin domain containing 3 (NLRP3) is an inflammasome protein that is released from an inactive complex of chaperones in response to a wide variety of endogenous and pathogen-derived danger signals. G protein signaling modulator 3 (GPSM3) is another leukocyte-restricted protein that is a component of the complex that sequesters NLRP3 in the inactive state. To initiate an NLRP3-dependent inflammasome response, cells must first be ‘primed’ to increase transcription of genes coding for components (e.g., *NLRP3*) and substrates (e.g., *IL1B*) of the inflammasome. Using qRT-PCR and Western blot, we identified GPSM3 transcript and protein abundance both decrease when human and mouse monocyte cell lines and cells are primed with lipopolysaccharide (LPS). Therefore, the decrease in GPSM3 transcription probably reduces the stability of the complex of proteins that sequesters NLRP3 in an inactive state and results in an increase of NLRP3-dependent inflammasome activity.

4.2 – Introduction

G protein signaling modulator-3 (GPSM3) is a newly identified signaling regulator with prominent expression in myeloid lineage cells, and at lower relative levels in a variety of lymphoid tissues¹. GPSM3 (a.k.a. AGS4 or G18;^{2,3}) possesses two functional “GoLoco motifs”³ for binding inactive Gα_i-GDP subunits and additionally binds to Gβ

subunits during their synthetic pathway towards forming mature G $\beta\gamma$ dimers ⁴; these interactions with heterotrimeric G protein subunits are thought to underlie the effects of GPSM3 on chemokine receptor signaling that is critical to the development of inflammatory arthritis ¹. We previously investigated the role of GPSM3 in NLRP3-mediated IL-1 β secretion, and further identified GPSM3 as a component of the chaperone complex that maintains NLRP3 in an inactive state (Appendix 1). Thus, establishing a negative regulatory function of GPSM3 on NLRP3-dependent IL-1 β generation in mice ⁵. NLRP3 priming with lipopolysaccharide (LPS) and activation with *S. aureus* α hemolysin (HLA) have been shown to increase IL-1 β secretion through NLRP3-dependent caspase-1 activity ^{5,6}. Furthermore a greater increase of IL-1 β secretion is observed in *Gpsm3*-deficient mice ⁵. It is unknown how this treatment, that activates the NLRP3 inflammasome, would affect the transcription of *GPSM3* in human or mouse cells endogenously transcribing *GPSM3*. Because GPSM3 is a negative regulator of NLRP3 activity, prolonged priming and/or activation of the NLRP3 inflammasome may decrease transcript abundance of GPSM3 to increase the sensitivity of the NLRP3 inflammasome. Here, we investigate the transcriptional consequences of LPS and HLA treatment on the THP-1 human monocytic cell line.

4.3 – Results and Discussion

4.3.1 – Increased *Nlrp3* and *Il1b* transcript abundance in mouse BMDMs following LPS and HLA treatment.

Despite the involvement of GPSM3 in the migration of innate immune cells and in mouse models of RA ¹, little is known about the regulation of *GPSM3* transcription. Treatment of mouse bone marrow-derived macrophages (BMDMs) with LPS alone or HLA alone resulted in the increase in *Nlrp3* and *Il1b* transcript abundance. BMDM priming with LPS and activation with HLA resulted in an additional increase in transcript abundance of *Nlrp3* and *Il1b*. This increase was independent of *Gpsm3* genotype (Fig. 4.1A and B). Therefore, GPSM3 is not required to increase the transcript abundance of *Nlrp3* or *Il1b*. The lack of effect of GPSM3 is likely due to the G protein-coupled receptor-independent mechanisms of action of LPS and HLA, which act through toll-like receptor 4 (TLR4; *i.e.*, receptor tyrosine kinase) ^{7,8} and by forming pores in the cell membrane ⁹, respectively. Nevertheless, GPSM3 deficiency increases secretion of IL-1 β in mouse peritonitis models and isolated BMDMs ⁵; therefore, GPSM3 probably regulates IL-1 β secretion as part of a protein complex rather than by regulation of transcription.

4.3.2 – LPS and HLA treatment decreases *GPSM3* transcript and protein concentration in human monocyte-like cells.

Considering the differences in mouse and human immune systems ¹⁰, the effects of LPS on *human* THP-1 monocyte-like cell line was investigated to test similarities in response between species. In THP-1 cells, treatment with LPS increased *IL1B* (Fig. 4.1D) and trended toward increasing *NLRP3* transcript abundance (Fig. 4.1E). This mimicked the increase in transcript abundance seen in mouse BMDMs. Although not addressed, LPS probably primes THP-1 cells by increasing NLRP3 and pro-IL-1 β protein, thus increasing the amount of IL-1 β secreted following activation of the NLRP3 inflammasome. LPS treatment of THP-1 cells also significantly decreased *GPSM3* transcript abundance (Fig. 4.1C) and protein concentration (Fig. 4.1F). In the absence of a priming event, NLRP3 activity is nominal (reviewed in ¹¹); therefore, priming is required to mount an effective NLRP3-dependent response to various danger-associated molecular patterns. When priming occurs prior to activation the translation of additional proteins can potentiate NLRP3-dependent IL-1 β secretion, with pro-IL-1 β protein detection in monocytes 30 minute after LPS priming ¹². Because *GPSM3* is a negative regulator of NLRP3-dependent IL-1 β secretion ⁵, LPS-induced decreases in *GPSM3* transcript and protein abundance may result in increased sensitivity of NLRP3 to activation stimuli.

There is a greater magnitude of decrease of *GPSM3* transcript abundance by LPS pretreatment followed by HLA, a NLRP3 activator. Although, there is no difference in *GPSM3* transcript abundance of LPS or vehicle pretreated THP-1 cells prior to HLA

treatment (Fig. 4.2). The additional decrease in *GPSM3* transcript abundance is likely relevant only during prolonged treatment with HLA, and may act to further decrease the negative regulation on NLRP3 to prolong secretion of IL-1 β . It is also likely that the decrease in *GPSM3* in the presence of bacterial LPS and HLA results in impaired migration once the leukocyte reaches foci of bacterial infections¹ (Fig. 2.5 and 3.2). Whether this is protective to the host or the bacteria has not been investigated, but could act to prevent leukocytes from migrating away from bacteria.

In summary, LPS-induced increases in *NLRP3* and *IL-1 β* transcript abundance are conserved between human and mouse myeloid cells and cell lines (Fig. 4.1A-E). LPS treatment also decreases transcript and protein abundance of *GPSM3* (Fig. 4.1C and F), a negative regulator of the NLRP3 inflammasome activity. Therefore, decreases in *GPSM3* concentration likely increases the activity of the NLRP3 inflammasome by reducing the inhibitory effect of *GPSM3* (Fig. 4.3). Additional studies will be required to determine if HLA-induced decreases in *GPSM3* transcript abundance (Fig. 4.2) are relevant in IL-1 β secretion; however, induction of the NLRP3 inflammasome following HLA treatment is probably too rapid for transcriptional changes to affect protein levels. Nevertheless, continued interest in the affects of other NLRP3 priming agents will be of interest. Particularly TNF α , which is involved in the pathology of a number of chronic inflammatory states such as rheumatoid arthritis¹³.

4.4 – Materials and Methods

4.4.1 – Commercial antibodies, constructs, and other reagents. HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were from GE Healthcare (Piscataway, NJ). Monoclonal anti-GPSM3 antibody was produced by the UNC Antibody Core Facility and has been previously described ¹⁴. Recombinant *S. aureus* α hemolysin was generated as previously described ¹⁵.

4.4.2 – Cell culture. The human THP-1 cell line was each obtained from the American Type Culture Collection (ATCC) and maintained in ATCC-modification of RPMI-1640 media (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Cellgro, Manassas, VA) at 37°C in a humidified atmosphere containing 5% CO₂.

4.4.3 – Ex-vivo transcriptional activation. BMDMs and THP-1 cells were resuspended in serum-free RPMI 1640 and seeded in 24-wells plate at a density of 1×10^6 cells/ml/well. LPS (100 ng/ml) was added for 3 hours. Subsequently, cells were stimulated with α hemolysin (10 μ g/ml) for 1 hour.

4.4.4 – Quantitative RT-PCR. THP-1 or bone marrow-derived macrophage (BMDM) cells were activated as previously described with LPS (100 ng/ml) and/or α hemolysin (10 μ g/ml). Cells were harvested and lysed in buffer RLT (Qiagen) + 1% 2-mercaptoethanol (Fisher Chemical) before immediately being placed on ice. RNA extraction was performed using the Qiagen RNeasy kit following manufacturer's

instructions, and isolated RNA was DNase I treated and used to synthesize cDNA immediately (Thermo Verso cDNA synthesis kit). Relative gene expression was quantified using Veriquest Fast Probe PCR master mix (Affymetrix) and Taqman probes (Life Technologies) for GPSM3, NLPR3, and pro-IL-1 β normalized to multiplexed β 2-microglobulin. Fold change in target gene expression was calculated using threshold values by the $2^{-\Delta\Delta C_t}$ method, described by Livak & Schmittgen¹⁶, and statistical significance was determined by one-way ANOVA with Bonferroni post-hoc ($p < 0.05$).

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4.6 – Figures

Fig. 4.1. Transcriptional consequence of LPS treatment in mouse and human leukocytes. Bone marrow derived macrophages (BMDMs) were generated from wild type (WT) or *Gpsm3*^{-/-} mice and primed with LPS for 3 hours with 100 ng/ml and/or subsequently treated with α -hemolysin (HLA) for 1 hour, as indicated. Pro-IL-1 β and NLRP3 mRNA levels in each cell line were assessed using quantitative RT-PCR as described in the materials and methods (panels **A** and **B**). THP1 cells were treated with LPS (100ng/ml) or vehicle for 3 hours. GPSM3, Pro-IL-1 β , and NLRP3 mRNA levels

were assessed by quantitative RT-PCR (panels **C**, **D**, and **E**). Data are expressed in pg/ml using the means $\pm$ S.E.M. of three different experiments (n=8-10); *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA. GPSM3, NLRP3, and actin (loading control) protein levels were also assessed using immunoblot analysis with the indicated antibodies (panel **F**). Samples from 3 independent experiments are shown.

Fig. 4.2. Transcriptional consequence of a NLRP3 activation treatment on THP-1 cells. THP1 cells were treated with LPS (100ng/ml) or vehicle for 3 hours. Then treated with α hemolysin (HLA) or vehicle for 1 hour. GPSM3 mRNA levels were assessed by quantitative RT-PCR.

Fig. 4.3. Model of GPSM3 regulation by lipopolysaccharide, and proposed influence on NLRP3 inflammasome. Formation of a network between GPSM3, chaperones, and NLRP3 maintains the steady-state level of NLRP3 by stabilizing it within the complex. Activation of the inflammasome requires two independent signals. Toll-like receptors (TLR) and tumor-necrosis factor receptors (TNFR) are known to trigger priming signals leading to up-regulation of NLRP3 and pro-IL1b through a NFκB-dependent pathway as well as down regulation of GPSM3. The NLRP3 activation signal is triggered by endogenous ('danger-associated molecular patterns'; DAMPs) or exogenous ('pathogen-associated molecular patterns'; PAMPs) danger signals. Activation of NLRP3 can also be mediated by bacterial pore-forming toxins such as the alpha-hemolysin (HLA) of *S. aureus*. Activation of NLRP3 leads to formation of a

multiprotein complex containing oligomerized NLRP3, the adaptor ASC, and caspase-1. Once activated, caspase-1 cleaves pro-IL1b (and pro-IL-18), releasing the biological active form of IL-1b (and IL-18) and thereby triggering inflammation and antimicrobial activity.

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Chapter 5: Project insights and future directions

5.1 Functional consequences of *GPSM3* gene variants in the clinical setting

Although a great deal of insight has been gained since the advent of minimally biased genome-wide association studies (GWAS), there is much research that still needs to be completed to fully elucidate the complex genetics of RA. A small sampling of milestone GWAS achievements includes the identification of *HLA-DRB1* haplotype SNP tags, which are in linkage disequilibrium with risk haplotypes and may represent a diagnostic mimic for expensive direct HLA typing in the future ^{1,2}. Additionally, the discovery of risk alleles in peptidylarginine deaminase (*PADI4*) ³ supports the previous discovery that *PADI4* is involved in post-translational citrullination of proteins. Citrullination of proteins, in RA, often precedes the production of anti-citrullinated peptide antibodies (ACPA) that are often present in patient samples and are used as part of the diagnostic panel of tests for RA ⁴. Despite the successes of GWAS, there are a number of limitations of the technique, including the number of false-positive results from multiple comparison statistics and linkage disequilibrium ⁵, which necessitate additional confirmation studies, similar to what was performed in *PADI4*, to identify causal gene variants from those simply associated with causal genetic or epigenetic variants (e.g., Chapter 2). Once these causal gene variants are identified, additional studies are then required to identify their biological significance before they can properly be translated to a clinical setting as a genetic biomarker or identify the gene product as

a therapeutic target. Multiple prior GWAS publications had identified two single nucleotide polymorphisms (SNPs) within the *GPSM3* gene locus as *inversely* associated with a number of autoimmune diseases including RA, multiple sclerosis, systemic lupus erythematosus, and ankylosing spondylitis in multiple populations including European, North American, and Swedish cohorts ⁶⁻⁸. The reproducibility of these *GPSM3* gene variants warranted further investigation, because *Gpsm3*-deficient mice are afforded a significant protection from joint tissue swelling and proinflammatory cell influx into synovial tissues in an acute model of collagen autoantibody-induced inflammatory arthritis using the C57Bl/6 mouse strain ⁹, and in a chronic model of heterologous collagen-induced inflammatory arthritis within the DBA-1 background (unpublished; Billard, **Gall**, et al.). We sought to address the translational potential of *GPSM3* gene variants by initially confirming a functional consequence of inheritance of *GPSM3* gene variants (Chapters 2 & 5.1.1), and proposing additional valuable experiments to identify the mechanisms of action of the identified *GPSM3* gene variants (Chapter 5.1.2).

5.1.1 – Study of GWAS-identified *GPSM3* single nucleotide polymorphisms

To address transcriptional consequences of previously identified *GPSM3* SNPs, rs204989 ⁷ and rs204991 ^{6,8}, we addressed (1) disease state-dependent differences in minor allele frequencies within a case-control cohort of RA patients and matched controls, (2) transcriptional consequences of these SNPs, as well as (3) the potential

genetic linkage of *GPSM3* SNPs with other polymorphisms within the chromosome 6p21 region with large effect sizes (Fig. 5.1¹⁰; e.g., *HLA-DRB1* haplotypes)¹¹.

To minimize the chances of not identifying a transcriptionally relevant polymorphism in linkage disequilibrium with the previously identified *GPSM3* SNPs, we performed Sanger sequencing of a 3.5 kb region 5' to the *GPSM3* transcription start site that revealed the existence of a novel haploblock of 3 previously reported *GPSM3* SNPs, rs204989 (MAF = 0.2328¹²), rs204990 (MAF = 0.2330¹²), and rs204991 (MAF = 0.2348¹²; Fig. 2.1A). All 200 volunteers recruited in this study were confirmed to have the same minor allele (m) and the major allele (M) genotype for rs204989 and rs204991, and all 8 volunteers that were Sanger sequenced were confirmed to have the same genotype for each of the 3 *GPSM3* SNPs¹³. We elected not to sequence the coding region of the *GPSM3* gene locus, even given that NCBI dbSNP¹⁴ reports three polymorphisms with a MAF $\geq$ 0.01 in that region. Of those three rs22694420 (MAF = 0.01) and rs117877134 (MAF = 0.01) are intronic and of low frequency, supporting a lack of linkage disequilibrium. The final polymorphism, rs3134605 (MAF = 0.23), occurs in the second exon of *GPSM3* and has a similar frequency of rs204989/rs204991 haploblock, but is not predicted to be in strong linkage disequilibrium with said haploblock¹⁵. Furthermore, rs3134605 is not predicted to affect mRNA secondary structure¹⁶ (Fig. 5.2), and results in a synonymous proline-to-proline mutation. The effects of rs3134605 will need to be further investigated to better understand its effects on *GPSM3* mRNA stability, translation efficiency, and role in intron splicing. Precedence has been set for synonymous mutations affecting mRNA stability (reviewed in¹⁷). However, detecting the rs204989-dependent effect on transcription supports our

hypothesis that rs204989 is responsible for the decreased *GPSM3* transcript abundance observed in whole blood.

Individuals with the m/m genotype of the *GPSM3* SNP haploblock were shown to have a 24.1% decrease in *GPSM3* transcript abundance in whole blood relative to M/M volunteers (Fig. 2.1B). The effect the m/m genotype has in the decreased *GPSM3* transcript abundance, potentially mimics some of the protection afforded by *Gpsm3* deficiency in mouse models of RA. Both the *GPSM3* SNP haploblock and genotype-dependent decrease in transcript abundance were novel findings. From the genotype-associated decrease in *GPSM3* transcript and the 5' location of the *GPSM3* SNPs, we developed the hypothesis that the presence of the minor alleles perturbed the normal activity of DNA binding proteins, such as histones or transcription factors. We set out to first test the hypothesis that these SNPs affected transcription factor interactions with each of the *GPSM3* SNP loci.

In determining the role of non-chromosomal protein interaction with the *GPSM3* 5' region containing either the major (pGL3-M) or minor alleles (pGL3-m), we subcloned this region into a luciferase vector with minimal promoter activity (pGL3-basic) and the firefly luciferase open reading frame. When transiently transfected, the construct containing the minor alleles (pGL3-m) resulted in a decrease in firefly luciferase activity (Fig. 2.3). From these data, we hypothesize the presence of one or more *GPSM3* minor allele(s) resulted in a decrease in transcriptional activity of the subcloned region; however, the presence of the *GPSM3* SNP haploblock meant that we were unable to assess the effects of individual SNPs on *GPSM3* transcript abundance in collected

whole blood, and therefore raised the question of which SNP(s) was/were causing this identified genotype-dependent phenotype. This confounder was addressed using mutagenesis in the *in vitro* promoter-driven luciferase model of transcriptional activity (Fig. 2.3), where we identified a functional consequence for one of the SNPs, rs204989, in the impaired transcriptional activity of the *GPSM3* 5' region (Fig. 2.3A and Fig. 2.3B). We concluded that the reduced transcriptional activity of the *GPSM3* 5' region containing the minor allele of rs204989 further supports our findings of genotype-dependent decrease in *GPSM3* transcript abundance in volunteers' whole blood (Fig. 2.1B); however, these results were confounded by experimental limitations of our study. We encountered difficulties in transfection of leukocyte cell lines (endogenously expressing *GPSM3*) that are resistant to plasmid transfection, potentially via RIG-like receptor- or TLR9-dependent immune activation and degradation of plasmid dsDNA produced in *E. coli*, as reported previously in myeloid cell lines^{18,19}. This resistance resulted in low transfection efficiencies in the large reporter plasmids containing the 3.5 kb region 5' to *GPSM3* (>8 kb total size), and therefore yielded a low signal-to-noise ratio when detecting luciferase activity in THP-1 cells (Fig. 5.3). Furthermore, the pGL3 plasmid used in these experiments lacked a eukaryotic resistance gene (e.g., Neomycin-resistance [*neo*]), which prevented the establishment of stable expressing cell lines of each construct, although establishing stable monoclonal cell lines of each construct would result in plasmid incorporation into the genome that could result in chromatin-induced silencing.

To counter the difficulties in THP-1 electroporation/transfections, HEK 293T cells were used. Although HEK 293T cells do not endogenously express *GPSM3* protein or

transcript, the activity of the non-genomic plasmid containing each of the *GPSM3* promoters in HEK 293T cells suggests these cells have compatible transcription machinery (*i.e.*, transcription factors) to transcribe *GPSM3*, but there is a genomic modification that silences its expression. Correlative bioinformatic data suggests the *GPSM3* locus is associated with transcriptionally active trimethylated histone H3 lysine 4 (H3K4me3)²⁰ in CD14⁺ monocytes endogenously transcribing *GPSM3*, but not HEK 293T cells in ChIP-Seq studies (Fig. 5.4)²¹. The association of the *GPSM3* gene locus with condensed regions of chromatin likely prevents endogenous transcription of *GPSM3* in HEK 293T cells. Thus, the use of chromatin-independent plasmids for transcriptional activity in HEK 293T cells is most likely a viable alternative model that mimics the genotype-dependent decreases in *GPSM3* transcript abundance from whole blood. In cell types that do transcribe the *GPSM3* gene, we hypothesized the minor allele of rs204989 disrupts transcription factor association and result in the observed decrease in *GPSM3* transcript abundance in whole blood (Fig. 2.1B). Based on the JASPAR transcription factor database²², we predicted, the AP-1 or C/EBP β transcription factors were most likely to be affected by the minor allele of rs204989 (Fig. 2.4). AP-1 has been shown previously to undergo nuclear translocation in HEK 293T cells^{23,24}. However, a PubMed search (“[C/EBP] AND [beta] AND [HEK]”) did not return a report showing C/EBP β activity in HEK 293T cells. The paucity of findings indicating C/EBP β activity in HEK293T cells, led us to think the AP-1 transcription factor is involved in differential *GPSM3* transcription in m/m individuals. We do note that transcription factors often bind to relatively promiscuous motifs (Fig. 2.4 and²⁵) and in certain conditions act to repress transcription. Therefore, the involvement of individual

transcription factors in *GPSM3* transcription requires substantial follow-up in cells endogenously transcribing *GPSM3* (Chapter 5.1.2a).

In our case-control cohort we report the *GPSM3* minor allele frequencies (predicted to be 'protective') were trending toward being greater in the RA cohort (Fig. 2.1C). This trend was the opposite of reports in GWAS in large multiethnic cohorts ^{6–8}. We found it surprising that the genotype-dependent decreases in *GPSM3* transcript abundance and promoter activity (believed to be 'protective' in RA) were not reflected by decreased minor allele frequencies of *GPSM3* SNPs in our RA cohort (Fig. 2.1C). Our findings are confounded by the known limitations of small population genetic association studies ²⁶ and potential founders' effect within our local population ²⁷. With the IRB restrictions, we could not increase recruitment to address the small population size in what was designed to be a primarily *functional* study. Instead we attempted to address potential linkage of the *GPSM3* SNPs with rs6457620 [C>G] ^{28,29} (Odds Ratio = 2.55 ²⁹), an established RA risk locus ~500 kb from the *GPSM3* SNPs in the *HLA* region of chromosome 6p21.3 (Fig. 5.1). This *HLA* region SNP was chosen because of the repeated association of *HLA* region polymorphisms with RA, and to counter the critique that *GPSM3* SNPs may associate with RA due to linkage disequilibrium with the *HLA* region. We found an association between the *GPSM3* SNP minor alleles with the minor allele of rs6457620 that was greater than chance distribution within the RA cohort (Fig. 2.2B), but was not statistically different in the matched cohort (Fig. 2.2C). These findings are contrary to RegulomeDB metadata ³⁰ that reports minimal linkage exists between the *GPSM3* SNP loci and rs6457620. This observed association between *GPSM3* SNPs and the *HLA* SNP in our RA population was likely a result of a small

sample size or Founder's effect. Regardless, the coexistence of these two minor alleles in our RA population, but not the matched controls, may have masked the protective benefits of the *GPSM3* SNPs minor alleles by the larger effect size of rs6457620 in the RA cohort (reviewed by Holoshitz ¹¹ and Yuta, et al. ³¹).

From the combined results of: (1) decreased *GPSM3* transcript abundance in whole blood samples (Fig. 2.1B) and (2) *ex vivo* analyses of *GPSM3* 5' region transcriptional activity (Fig. 2.3), we conclude that the *GPSM3* SNP rs204989 [C>T] is impairing transcription factor binding (Fig. 2.4) likely resulting in the observed decrease in *GPSM3* transcript abundance in whole blood samples of individuals with the m/m genotype. This conclusion is based on our findings of: (1) decreases in activity of *GPSM3* promoters with the minor allele of rs204989 in HEK293T cells was markedly similar to the decrease in whole blood *GPSM3* transcript abundance, and (2) because transfection of non-genomic plasmids does not assess normal histone or epigenetic regulation of gene expression. Confirmation of this hypothesis will be the aim of future studies (Chapter 5.1.2a).

Considering the small effect size of rs204989 on *GPSM3* transcript abundance, the minor allele of rs204989 in RA may be considered as a clinical biomarker of RA risk as part of a larger genetic panel in conjunction with some of the over 100 confirmed RA risk alleles ^{32,33} (reviewed in ³¹). Regardless, rs204989 is: (1) inversely correlated with RA in multiple, large, multiethnic cohorts ⁶⁻⁸, (2) *does* result in a decrease in *GPSM3* transcript abundance in whole blood (Fig. 2.1B), (3) *Gpsm3*-deficient mice *are* protected from two models of inflammatory arthritis (unpublished and ⁹), and (4) *GPSM3* depletion

disrupts human leukocyte cell line migration toward chemoattractants relevant in RA (Figs. 2.5 and 3.2C). Therefore, *GPSM3* may rather be a target for pharmaceutical inhibition to provide therapeutic benefits in RA patients, rather than a genetic marker. This portion of my thesis represents a link between our lab group's previous findings of protection from arthritis-like paw swelling in *Gpsm3*-deficient mice and begins to translate them to humans with natural *GPSM3* polymorphisms resulting in deficiency of *GPSM3* transcript in whole blood.

5.1.2 – Future directions for rs204989

Future directions for *GPSM3* genetics research will be important to confirm our findings reported in chapter 2. To address this I propose two individual aims: to identify transcription factors affected by rs204989 (5.1.2a) and to determine the consequences of the minor allele of rs204989 in leukocyte biology (5.1.2b).

5.1.2a – Identification of transcription-relevant proteins affected by rs204989

Rationale – 5.1.2a Experiments 1-3: The plasmid-based techniques, employed to assess the role of rs204989 on transcription of *GPSM3*, focused exclusively on HEK 293T cells and factors independent of epigenetics. Although the plasmid-based model in HEK 293T cells is most likely effective in determining transcriptional activity of the *GPSM3* promoter, follow-up is required to determine which transcription-relevant protein's function is affected by the minor allele of rs204989. In an attempt to identify

potential DNA:protein interactions that are disrupted by rs204989, future studies will most likely require (5.1.2a Experiment 1) electrophoretic mobility shift assays (EMSA), (5.1.2a Experiment 2) electrophoretic mobility super-shift assays (EMSSA), and (5.1.2a Experiment 3) transcription factor pull down assays ³⁴.

5.1.2a Experiment 1: Initially EMSA will be employed to confirm that oligonucleotide probes, containing the major allele of rs204989, associate with a nuclear protein within NB4* and THP-1 cell line nuclear extracts. We will synthesize a biotin-conjugated oligonucleotide sequence that corresponds to either the major or minor allele of rs204989. This oligonucleotide will be incubated with nuclear extract from THP-1 or NB4* cells, then the mixture will be resolved on a polyacrylamide gel. Based on the decreased luciferase activity of plasmids containing the minor allele of rs204989 (Fig. 2.3), there will likely be a shift in migration patterns of the oligonucleotides incubated with nuclear extracts that correspond to a nuclear protein association with the oligonucleotide. If no transcription factor can be identified, ChIP-qPCR analyses can be performed to determine relative differences between the major and minor allele of rs204989 with respect to active (e.g., H3K4me3 ^{20,21}) *versus* inactive (e.g., H3K27me3 ³⁵) states of histones; selection of histone methylation or acetylation states to interrogate in these experiments will be based on publically-available ChIP-seq datasets. This line of experimentation would be nearly identical to 5.1.2a experiment 3, and address whether association with inactive chromatin (heterochromatin) is different in the major and minor alleles of rs204989. Therefore, explaining the decreased transcript abundance in clinical samples, without a rs204989-localized nuclear protein extract-binding event *per se*.

5.1.2a Experiment 2: Identification of the transcription-relevant proteins can be completed via EMSSA with anti-c-fos (part of AP-1 heterodimer) and anti-CEBP/ β antibodies (predicted in fig. 2.4) ²². Antibody binding with these proteins would create a supershifted band pattern that would confirm the protein associated with the oligonucleotides representing the major or minor allele of rs204989. Differential EMSA and EMSSA results from oligonucleotide probes containing the major *versus* minor alleles of rs204989 would be indicative of disruption of transcription factor binding. From the decreased promoter-driven luciferase activity of rs204989-containing promoters, we predict the oligonucleotide containing the minor allele of rs204989 would have decreased intensity. If neither antibody induces a super-shifted migration pattern, an additional bioinformatics search will be used to identify additional potential transcription-relevant proteins that may be associating with this oligonucleotide sequence.

5.1.2a Experiment 3: Primary human leukocytes can then be used to confirm differential association of the transcription-relevant protein association with the rs204989 locus can be completed with chromatin immunoprecipitation (ChIP). This immunoprecipitation would utilize antibodies specific for proteins identified in (5.1.2a Experiment 2) EMSSA to precipitate chromosomal DNA associated with that protein. The collected precipitate is then analyzed by qPCR determination of relative abundance of the rs204989 locus co-precipitated from volunteer leukocytes with either the M/M or m/m genotype³⁶. We predict the relative DNA abundance co-precipitated from whole blood of the m/m genotype will be less than the M/M genotype. Future identification of a transcription factor or chromatin interaction at this locus would validate our previous findings of an

apparent decrease in *GPSM3* promoter activity and develop a more in-depth understanding of transcriptional control of *GPSM3*.

Rationale – 5.1.2 Experiment 4: Little is known of processes that regulate the transcription of *GPSM3*. Findings in the literature suggests that lipopolysaccharide³⁷ and methotrexate (GEO: GSE35455) both decrease relative *GPSM3* transcript, but it is inconclusive as to whether that is a *GPSM3* promoter repression or a dilution of transcript. Identification of a bona fide transcription factor interaction with *GPSM3* promoter elements would provide more conclusive understanding of *GPSM3* transcriptional regulation. This would also confirm the findings in 5.1.2 experiments 1-3, by testing how activators or inhibitors of this transcription factor affect *GPSM3* transcript and protein concentration differently in the presence of the minor allele of rs204989.

5.1.2 Experiment 4: Insight into proteins involved in transcription whose binding to DNA is impaired by the minor allele of rs204989 may lead to additional follow-up experiments to determine natural and synthetic molecules that regulate transcription of *GPSM3*. To identify a molecule that affects *GPSM3* transcription, we would search previous literature for compounds that affect the activity of transcriptional protein(s), identified in the proposed EMSA and ChIP experiments, that associate with the major allele of rs204989. For example, if AP-1 is found to differentially associate with the major vs minor allele of rs204989, we could treat NB4* and THP-1 cells with an AP-1 activator, like flagellin (a TLR5 agonist)³⁸, in the presence or absence of an AP-1 inhibitor (e.g., SR 11302³⁹) to confirm AP-1 specificity. If *GPSM3* transcript or protein levels are affected by this treatment, we can then perform the same experiment using

either primary leukocytes (e.g., neutrophils and monocytes) or CRISPR/Cas9-modified NB4* and THP-1 cell lines of the M/M and m/m genotypes, to see if there are differential effects of these treatments. This experiment would test our hypothesis that the minor allele of rs204989 affects transcription factor binding, and further our understanding of regulation of *GPSM3* transcription.

5.1.2b – Determine the consequences of rs204989 in leukocyte recruitment

Rationale – 5.1.2b Experiment 1: Our current understanding is that the *GPSM3* SNP rs204989 modulates *GPSM3* gene transcription level, but we have yet to answer what effects the SNP-dependent decrease in transcription has on the function of leukocytes. Classical protein biochemistry has revealed that the GoLoco motifs within *GPSM3* act to sequester inactive Gai·GDP, preventing its reassociation into the Gαβγ heterotrimer to transact GPCR signaling^{40,41}. An important class of Gai-coupled receptors in leukocytes is the chemokine receptor class, which initiate various processes including chemotaxis⁴² and activation of adhesion molecule high affinity states^{43,44}. Previous studies indicated that *Gpsm3*^{+/-} mice (i.e., heterozygous for *GPSM3* deficiency) are afforded an intermediate level of protection from an acute model of inflammatory arthritis⁹; our conclusion, based on these data, is that a ~50% decrease in *GPSM3* protein expression is still protective from RA-like inflammation *in mice*. In unpublished data there is a time sensitive component to *GPSM3* detection by Western blot; therefore, it appears that *GPSM3* protein is fairly labile and steady-state *GPSM3* protein levels are sensitive to changes in transcript abundance. Therefore, rs204989

may decrease transcription of *GPSM3* in humans enough to (1) decrease steady-state concentrations of GPSM3 protein and (2) confer some protection from RA. However, the effect a 24% decrease in *GPSM3* mRNA will have on steady-state GPSM3 protein levels is unknown. Furthermore, we cannot directly translate the protection afforded to *Gpsm3*^{+/-} mice in CAIA to humans, because there are marked differences between mouse and human immunology that must be considered (e.g., differences in blood cell subset composition ⁴⁵ and in chemokine receptor expression ⁴⁶). Therefore, it is important to address how the minor allele of rs204989 affects normal leukocyte physiology in relevant human models (e.g., *ex vivo* leukocyte isolation or CRISPR/Cas9 genome editing).

5.1.2b Experiment 1: With prior approval from the WVU IRB, isolation of primary neutrophils and monocytes from M/M and m/m volunteers would be possible by negative selection of peripheral blood leukocyte. We will seek assistance from the WVU Flow Cytometry core to confirm leukocyte cell type purity. With primary leukocytes from these individuals, the effect that the minor allele of rs204989 has on GPSM3 protein abundance in leukocytes can be determined. Assuming the decrease in *GPSM3* transcript correlates to a decrease in GPSM3 protein, assessment of leukocyte physiology can be performed using these primary leukocytes with previously reported techniques including Transwell migration toward neutrophil chemoattractants (e.g., LTB4 and IL-8), and adhesion of chemokine-activated leukocytes (e.g., with LTB4 and IL-8) to activated vascular endothelial cells. The latter of which would require collaboration with a lab group with experience in vascular endothelial cell to leukocyte interaction, like the Nurkiewicz lab. If the isolation of primary cells were not feasible, an

alternative approach would be to use CRISPR/Cas9-mediated gene editing to introduce the minor allele of rs204989 into NB4* and THP-1 immortalized cell line models, confirmed to be M/M genotype (*unpublished*), for use in the same experiments. However, the phenotypic effects of the minor allele of rs204989 may be too small to be detected at the sensitivity of a Transwell migration assay. Additionally, as discussed previously (Chapter 1), leukocytes *in vivo* are subject to a number of additional barriers to migration including (1) vascular shear stress, (2) vascular endothelial cells, (3) extracellular matrix, (4) cartilage, and (5) synovial tissues and fluid. Alternatively, modifications to the Transwell experimental design may be more representative of physiologic conditions and quantitate any minor differences more effectively. To better mimic some of these barriers *in vitro*, modifications can be made to the current Transwell migration assay by the addition of Matrigel (a basement membrane extract) to the upper chamber of the Transwell plate. A cultured monolayer of vascular endothelial cells (*i.e.*, human umbilical vein endothelial cells [HUVEC]) will overlay the Matrigel. The addition of relevant chemoattractants to the lower chamber will mimic the transendothelial and trans-extracellular matrix migration required for these cells to migrate into inflamed synovial tissues. With the additional barriers to migration, this modified assay will also likely be more sensitive to small changes in migration rate resulting from a minor disruption in signaling owing to the rs204989 allele, potentially undetectable in static Transwell migration assays. Additionally, rs204989-dependent effects on migration may still be relevant in the context of vascular flow and/or additional RA risk factors.

5.2 – GPSM3 function in leukocytes

GPSM3 has previously been shown to function in GPCR signaling as a guanine nucleotide dissociation inhibitor for Gai/o subunits: *i.e.*, the protein's GoLoco motifs sequester inactive Gai/o-GDP subunits inhibiting their reassociation with Gβγ subunits^{41,47}. This process is believed to prolong Gβγ-mediated signaling⁹, which has been shown to be important in the migration of leukocytes toward proinflammatory signals^{44,48,49}.

The highly restricted expression pattern of GPSM3 to leukocytes and lymphoid organs led us to hypothesize that GPSM3 acts as a regulator of chemokine receptor signaling – one of the most highly expressed classes of Gai-coupled GPCRs in leukocytes⁵⁰. Furthermore, our laboratory's work resulted in the discovery that the minor allele of rs204989 (*i.e.*, one of the GWAS-identified *GPSM3* gene locus polymorphisms) resulted in a decrease in GPSM3 transcript abundance in cells from whole blood (Fig. 2.3). Although our laboratory's collaborative work points to a role for GPSM3 in the pathogenesis of RA in mouse models⁹ and in negative regulation of NLRP3 (appendix I)³⁷, the effects of GPSM3 deficiency on human leukocytes has yet to be evaluated.

5.2.1 – GPSM3 deficiency affects neutrophil and monocyte cell line migration toward select chemoattractants

Depletion of GPSM3 from the human THP-1 monocyte-like cell line disrupts migration toward a gradient of proinflammatory MCP-1 (Fig. 2.5B). The decreased migration to MCP-1 was not elicited by changes in mRNA expression of the MCP-1 cognate receptor, *C-C chemokine receptor 2 (CCR2)* (Fig. 2.5D). MCP-1 is a chemokine known to induce migration of primary monocytes in RA ^{51,52}. However, inhibition of MCP-1 signaling through CCR2 has had little success in clinical trials ⁵³, likely because of the redundant functions of CCR1 ⁵¹ and CCR5 ⁵¹ agonists in monocyte / macrophage recruitment to synovial tissues. Importantly, the role of a single chemokine in monocyte migration was studied, in isolation of the pathophysiologic conditions where many chemoattractants may be present. However, the advantage that GPSM3 deficiency has over mouse *Ccr2* knockout models ⁵⁴, is that *Gpsm3*-deficient mouse monocytes have significantly decreased migration toward multiple proinflammatory chemokines (*i.e.*, soluble fractalkine, chemerin, MCP-1) or trending toward being decreased (*i.e.*, macrophage inflammatory protein-1 α , RANTES) ⁹. Although these studies are in mouse monocytes, a similar global deficit in migration is likely replicated in human monocytes. Therefore, disruption of GPSM3 expression or function in RA patients may result in inhibition, or reduction in, monocyte migration to inflamed synovial tissues in response to multiple pathophysiologic chemoattractants. This type of inhibition has not been found with previous clinical trials with single monocyte chemokine receptor inhibitors ^{51,55}.

However, our findings of impaired *in vitro* migration in GPSM3 deficient primary mouse monocytes did not completely explain the protective phenotype seen in acute and chronic models of arthritis. In mouse models of arthritis, prior recruitment of other, more abundant, peripheral blood cell types (e.g., neutrophils) may be capable of initiating inflammation in the synovial tissues without monocyte migration ^{54,56–59}. From these data and the previous mouse model data showing *Gpsm3* deficiency protects mice from acute and chronic induced-arthritis models, we predicted neutrophils would express GPSM3 and deficiency of GPSM3 would result in an impaired GPCR-dependent migratory phenotype. Five observations led us to consider neutrophils as another leukocyte that expresses GPSM3 including (1) the established involvement of neutrophils in the development of human rheumatoid arthritis (Chapter 1.2) ^{60,61}, (2) *GPSM3* transcript in neutrophils from RNA-seq datasets ⁵⁰, (3) the detection of GPSM3 protein in bovine neutrophils ⁶², (4) higher than expected levels of *GPSM3* transcript abundance in whole blood vs isolated monocytes (Fig. 2.1B), and (5) the shared myeloid lineage of monocytes and neutrophils ⁴⁵.

In the immortalized NB4 cell line model of ATRA-differentiated neutrophil-like cells (NB4*), we showed that GPSM3 transcript and protein concentrations increase with differentiation towards a neutrophil-like state (Fig. 3.1). Similar to the THP-1 monocyte-like cell line, GPSM3-deficient NB4* cells exhibit an impaired migration toward LTB₄, a proinflammatory chemoattractant (Fig. 3.2B). The critical involvement of LTB₄ in the early migration of neutrophils in RA ^{56–58,63–66}, may represent the strongest connection between GPSM3 depletion and near acellularity of synovial tissues in mouse models of arthritis ⁹. Furthermore, disruption in neutrophil function by decreased

migration may represent another protective function for individuals with the genetic variant, rs204989, shown to influence *GPSM3* transcript abundance (Chapter 2). However, the clinical relevance of rs204989 (and its apparent ~25% reduction in *GPSM3* expression) may be small, due in part to the observed *GPSM3*-dependent disruption of migration (*i.e.*, shRNA19-mediated knockdown being more complete and phenotypically more obvious versus shRNA20-mediated knockdown being less complete and phenotypically less robust; Fig. 3.2B). Additionally, the decrease in static Transwell migration may be amplified *in vivo*. At the whole organism level, chemoattractant signaling is required to adhere to vascular endothelial cells^{44,67}, release enzymes to degrade extracellular matrix molecules⁶⁸, and to migrate. If *GPSM3* deficiency decreases each of these responses, there will likely be an additive or synergistic effect that is not modeled in this study, but will be addressed in future experiments (Chapters 5.2.1b and 5.2.2a).

Regardless, the clinical relevance of the rs204989-dependent 24.1% decrease in *GPSM3* transcript should be considered. In the THP-1 cell line, LPS treatment for 3 hours was shown to decrease transcript abundance by 25% (Fig. 4.1C) and that resulted in a reduction of protein concentration (Fig. 4.1F), thus a natural reduction of 24.1% in whole blood likely decreases protein concentration in these individuals. Such a reduction of *GPSM3* may not impair *Transwell* migration of proinflammatory leukocytes enough to be considered as a single RA-relevant genetic factor regardless of additional effects (*e.g.*, adhesion); however, rs204989 may be useful as part of a genetic screening panel in conjunction with other RA genetic risk factors³¹. Based on our present collection of data, we believe a more promising avenue for *GPSM3* is as a

target for inhibition by small molecule compounds, which could disrupt chemoattractant signaling of proinflammatory cells. This inhibition of signaling through multiple chemoattractants simultaneously would likely result in decreased migration. Inhibited signaling may also decrease other chemoattractant-dependent processes, for example tight leukocyte adhesion^{44,67} and matrix metalloprotease release⁶⁸ in RA.

However, this assumption may not pertain to all chemoattractant receptors, because GPSM3 deficiency did not decrease migration toward N-formylmethionine-leucyl-phenylalanine (fMLP; Fig. 3.2C). N-formylated peptides (e.g., fMLP) can be released from eukaryotic cell mitochondria in necrosis; therefore, may be involved only in the late-phase (e.g., once significant necrosis begins) of inflammation^{56,69}. In host defense from bacteria, N-formylated peptides are typically a bacterial-derived chemoattractant peptide involved in leukocyte recruitment. The ability of GPSM3-deficient NB4* cells to migrate toward formylated peptides may represent a novel cell response, that is likely due to the constitutively high numbers of formylated peptide receptors on neutrophils⁵⁰. This could turnover a large amount of G proteins at once, thus resulting in G $\beta\gamma$ signaling sufficient to induce migration in the absence of GPSM3 sequestering inactive G α subunits. Thus, inhibition of GPSM3 by small molecules could have little effect on neutrophil migration toward certain bacterial infections⁷⁰, and spare the patient from global immunosuppression and risk of infection as is currently found with RA biologics.

Additionally, we must consider the effects of GPSM3 deficiency in the chronic, collagen-induced antibody (CIA) model of inflammatory arthritis. It has been shown that CIA is dependent on lymphocytes (reviewed in⁷¹), which is further supported by the

model's requirement to be conducted with certain strains of mice (e.g., DBA-1) that express specific haploblocks of mouse MHC ^{72,73} (e.g., H-2^q and H-2^r) or transgenic human HLA ⁷⁴ (e.g., HLA-DR1) on lymphocytes. Considering GPSM3 is expressed in lymphocytes⁷⁵ and GPSM3 deficiency protects mice from the lymphocyte-dependent CIA model (*unpublished*), GPSM3 may also influence normal B- and T-lymphocyte physiology. Thus, the GPSM3-deficient mouse may be afforded an additional level of protection from the adaptive immune response in RA. This is supported by observations of GPSM3 expression in human lymphocytes and immortalized lymphocytic cell lines ^{9,75}, as well as the impaired migration observed in mouse lymphocytes deficient in GPSM1, a functionally similar GoLoco motif protein ⁷⁶. Research into the functions of GPSM3 in lymphocyte migration and response to autoantigens may represent another potential avenue for further investigations, which is currently beyond the scope of this project. In summary, GPSM3 may be a highly desirable target for small molecule inhibitors. In the clinical setting, GPSM3 inhibition in RA could circumnavigate the failures of previous *single* chemokine receptor-targeted therapeutics ^{51,77} by working downstream of *multiple* chemokine receptors (e.g., LTB4 and MCP-1) and cell types (e.g., neutrophils and monocytes).

Beyond the role of GPSM3 in the migratory behaviors of monocytes and neutrophils, our collaborators have shown that GPSM3 deficiency in mouse bone marrow-derived macrophages (BMDMs) results in increased IL-1 β release after priming with lipopolysaccharide (LPS) and subsequent treatment with NLRP3-activating compounds such as *S. aureus* α -hemolysin (HLA) (Fig. 4.5A-C). This inhibitory effect of GPSM3 on NLRP3 function is presumably mediated through a direct or indirect

interaction between the leucine-serine-leucine motif of GPSM3 and the 8th leucine-rich region of NLRP3 (LRR; Fig. 4.2C, 4.3, and 4.4), as part of a larger NLRP3-inactivating complex. Our experimental contributions to this work support the notion that GPSM3 deficiency does not affect the transcriptional upregulation of *Nlrp3* nor *pro-il1b* in LPS +/- HLA treated GPSM3 deficient mouse BMDMs (Fig. 4.6A and B). Thus GPSM3 acts to regulate NLRP3 protein activity independent of transcriptional changes. We also showed an increase in *NLRP3* and *pro-IL-1 β* mRNA abundance in THP-1 human monocyte-like cells upon LPS treatment, while *GPSM3* transcript and protein abundance decreased (Fig. 4.6C and D). Thus, the inhibitory effect of GPSM3 on NLRP3 function is a post-transcriptional event. The findings that GPSM3 deficiency increases release of IL-1 β supports the role of GPSM3 as a negative regulator of NLRP3 activity; however, any apparent increase in IL-1 β levels is not manifested as exacerbated inflammation in the challenged GPSM3-deficient mouse. In contrast, protection afforded GPSM3-deficient mice to collagen- and collagen-autoantibody models of inflammatory arthritis runs counter to the known proinflammatory effects of IL-1 β in RA (e.g., the clinical effectiveness of the anti-IL-1 biologic anakinra ⁷⁸ and the blunted course of chronic inflammatory arthritis exhibited by IL-1 β -/- mice ⁷⁹). Furthermore, development of collagen-induced arthritis in mice is not altered in either NLRP3-deficient or caspase-1-deficient mice ⁸⁰. Therefore, chronic arthritis models are probably dependent on IL-1 β production but not dependent on IL-1 β from the NLRP3 inflammasome. The disruption of leukocyte migration in GPSM3-deficient mice probably resulted in an apparent inability of proinflammatory cells to reach the synovial tissues. Therefore, cells releasing NLRP3-dependent IL-1 β in the synovial tissues were

presumably restricted to resident tissue leukocytes that are present in relatively small numbers compared to what is seen in inflamed tissues. This small number of cells would result in a small net change of synovial IL-1 β levels upon GPSM3 loss and would likely not overcome the effects of GPSM3 deficiency in curbing migration toward proinflammatory chemoattractants. To better understand the clinical effects of GPSM3 on NLRP3 activity, additional studies will need to be performed in human-derived cells.

5.2.2 – Future directions

In summary of 5.2.1, we have shown a role for GPSM3 in the migratory phenotypes of neutrophil- and monocyte-like leukocytes toward some (but not all) proinflammatory chemoattractants. These studies have confirmed a similarity between immune cell dysfunction in our GPSM3-deficient mouse strain and in GPSM3-depleted, human-derived cell lines. Although these are correlative data, they may represent a causal link between *GPSM3* SNP frequency and the prevalence of RA in large populations. A role for GPSM3 as a negative regulator of NLRP3 activity in mouse cells has also been identified. To address questions that still remain about the function of GPSM3 in the immune system, I propose two individual aims: to investigate cell migration-relevant phenotypes that are differentially affected by GPSM3 deficiency (5.2.2a), and to discover the effects GPSM3 deficiency has on NB4* and THP-1 adhesion to activated vascular endothelial cells (5.2.2b).

5.2.2a – Investigate chemoattractant-relevant functions that are differentially affected by GPSM3 deficiency

Rationale – experiment 1 (different migration patterns toward LTB4 and fMLP):

Our findings that GPSM3 deficiency disrupts LTB4 migration, but not fMLP-induced migration, was unexpected (Fig. 3.2B and C). Two possibilities exist here: (1) high amounts of formylated peptide receptors in neutrophils turn over enough G proteins to negate the role of GPSM3 in prolonging G $\beta\gamma$ signaling (*i.e.*, excess of signaling G $\beta\gamma$ subunits) or (2) an uncharacterized mechanism within fMLP signaling is acting that is not present for LTB4 signaling. One mechanism may be altered G β stability. In support of this hypothesis, GPSM3 has been shown to associate with neosynthesized G β subunits in intact cells, and with each G β isoform (*i.e.*, G β_{1-4}) in heterologous overexpression studies⁸¹. This observation led to the conclusion that GPSM3 acts as a chaperone for neosynthesized G β subunits⁸¹. However, GPSM3 knockdown in NB4* cells had no omnibus effect on steady-state G β protein levels. Considering the different isoforms of G β have significantly different efficacy at activating some effector molecules, like PLC β isoforms^{82,83}, GPSM3 may affect the stability and signaling availability of certain G β isoforms preferentially *in vivo*. Based on the G β isoform that couples to the receptor, some receptors may be more affected by GPSM3 depletion thereby affecting cell migration differently.

Experiment 1: To address the (1) first hypothesis (*i.e.*, high number of receptors minimizes the necessity of GPSM3) we plan to partially inhibit formylated peptide receptor 1 signaling using either shRNA or small molecule inhibition (*e.g.*,

BVT173187⁸⁴). This experiment may determine if fewer responsive receptors may result in decreased migration in GPSM3-deficient cells. To follow up on the (2) second hypothesis (*i.e.*, a GoLoco-independent function of GPSM3 is affecting signaling), we propose restoring expression of either wild-type GPSM3 or variant GPSM3 species with lentiviral transduction of various loss-of-function mutations (*e.g.*, crippling one or more of the GoLoco motifs responsible for G α i-GDP binding, or the LSL motif involved in the G β and NLRP3 interaction). Given that the shRNA19 hairpin used to knockdown *GPSM3* expression in NB4* cells targets the 3'UTR rather than the open-reading frame, we have the ability to restore plasmid-based GPSM3 expression without interference from the shRNA. Further experiments would be dependent on the results of which GPSM3 mutant(s) do(es) not restore wild-type levels of migration. Alternatively, we can investigate the G β stability hypothesis using G β -isoform specific antibodies to determine if there are any changes to specific G β isoform levels, indicative of decreased stability in the absence of GPSM3 function.

Rationale – experiments 2 and 3 (physiologic leukocyte migration): Another aspect of leukocyte migration that needs to be addressed is the role GPSM3 might play in GPCR signaling within a physiologically relevant conditions. Leukocyte recruitment to inflamed synovium in pathophysiological conditions requires migration toward multiple chemoattractants (experiment 2) and through multiple barriers that were not present in Transwell assays (experiment 3). To address this, a modified Transwell migration assay will be developed coating the Transwell membrane with Matrigel and vascular endothelial cells, to mimic basement membrane and vascular endothelial respectively. The requirement of chemoattractant signaling for adherence to vascular endothelial

cells⁴⁴ and to release enzymes to degrade extracellular matrix⁶⁸ may mean GPSM3 deficiency affects multiple processes involved in leukocyte transmigration to inflamed tissues⁸⁵. Therefore, GPSM3 deficiency could prevent proinflammatory leukocyte migration with greater magnitude than had been seen in previous simple Transwell migration assays, although this hypothesis has yet to be addressed directly.

Experiment 2: To address the role GPSM3 plays in migration toward various chemoattractants we propose sequential experiments. First, we propose investigating the effects of GPSM3 deficiency in leukocyte migration towards additional disease-relevant chemoattractants (e.g., complement factor 5a, CXCL8), to better understand the broader effects of GPSM3 function in neutrophil-like cells and their migratory responsiveness. Additional investigation in the role GPSM3 plays in multiple chemokine signaling can be performed in collaboration with the WVU Department of Orthopedics, if they are able to collect RA and osteoarthritis patient synovial fluid (SF) for future studies. Following characterization of the chemokine content of SF samples, diluted RA or osteoarthritis SF will serve as the chemoattractant on the bottom well of a Transwell plate, with control and GPSM3-deficient NB4* or THP-1 cells in the upper chamber. Alternatively, THP-1-derived macrophage conditioned media could act as another source of multiple chemokines. With knowledge of how GPSM3 affects migration toward multiple chemoattractants, we may be able to glean further knowledge of its signaling function.

Experiment 3: A modified Transwell migration assay will be used to address the effects of GPSM3 deficiency on migration through more physiologic barriers (e.g., vascular endothelial cells and basement membrane). To mimic the physiologic

vasculature, a sequential layering on the Transwell membrane of Matrigel, a basement membrane extract, and TNF- α activated vascular endothelial cells will be used. THP-1 or NB4* cells will be induced to migrate to the lower chamber by MCP-1 or LTB₄, respectively. If GPSM3 deficiency results in a decrease greater than what was observed in simple Transwell migration assays, we will further investigate the chemoattractant-induced matrix metalloprotease activity (*i.e.*, measure basement membrane degradative enzyme activity)⁶⁸ and adhesion to vascular endothelial cells (*i.e.*, interaction prior to transendothelial migration; experiment 4) (reviewed in ⁸⁵). Physiologic migration of leukocytes in RA is directly addressed in Chapter 1.2.2. A decrease in Transwell migration is expected in GPSM3 deficient cell lines based on (1) decreased migration in simple Transwell migration assays (Fig. 2.5B and 3.2C), (2) requirement of chemoattractant signaling in adhesion to vascular endothelial cells⁴⁴, and (3) involvement of chemoattractant signaling in protease release during migration through extracellular matrix⁶⁸.

Rationale – experiment 4 (adhesion to vascular endothelial cells): A cascade of events must occur for leukocyte recruitment to tissues to occur; for example, prior to any migration, circulating leukocytes must first adhere to vascular endothelial cells proximal to inflamed tissues. Initial, GPCR-independent, sequential, weak interactions with constitutively expressed binding molecules occur to promote neutrophil “rolling”. In vessels supplying inflamed tissues (*e.g.*, arthritic synovial tissues), chemokines and cytokines from the underlying tissue are transported and bound to the luminal surface of vascular endothelial cells by glycoaminoglycans ⁸⁶. Endothelial-bound chemokines

induce of β_2 -integrins to adopt a high-affinity conformation allowing firm adhesion of leukocytes, thus allowing transendothelial migration toward inflamed tissues to begin^{87,88}. This activation of β_2 -integrins occurs via intracellular signaling networks – reported in recent studies to be mediated (in part) through G protein $\beta\gamma$ signaling⁴⁴, which is hypothesized to be prolonged by the $G\alpha_i$ -GDP-sequestering action of GPSM3. It is important to address whether there is a GPSM3-dependent effect on leukocyte firm adhesion, because adhesion is an obligatory first step to leukocyte recruitment; therefore, an impairment in adhesion of GPSM3-deficient leukocytes may have an additive or even synergistic effect with the observed impairment in migration on total leukocyte recruitment *in vivo*.

Experiment 4: In collaboration with the Nurkiewicz laboratory, establishment of an *ex vivo* flow chamber is possible to assess NB4* and THP-1 cell adhesion to activated Human Umbilical Vein Endothelial Cells (HUVEC). Use of TNF- α , an activator of endothelial cells, will be used to activate HUVEC cells, and pretreatment of NB4* or THP-1 cells with a relevant chemoattractant will activate the high-affinity state of β_2 -integrins. Physiological shear stress for post-capillary endothelial cells will be established, and time-lapsed images of the flow of NB4* or THP-1 cells across the activated HUVECs will be reviewed to quantify rolling *versus* adherent cells, as shown previously in⁸⁹. Alternatively, GPSM3-deficient mice can be used for intravital microscopy to determine if there is an effect on adhesion *in vivo*. If there is not a significant effect of GPSM3 deficiency on leukocyte firm adhesion, this would be a novel finding that would be valuable to present alongside our existing data indicating the effect of GPSM3 deficiency on migration. A decrease in leukocyte cell line adhesion is

expected, because of the requirement of chemoattractant signaling in leukocyte firm adhesion⁴⁴.

Rationale – experiment 5 (phagocytosis of bacteria): Before proposing GPSM3 as a therapeutic target for RA, potential advantages over current treatments should be considered. The most effective treatment is currently “combination therapy” with a disease-modifying antirheumatic drug (DMARD), like methotrexate⁹⁰, and a biologic, the most popular being the TNFa inhibitors⁹¹ (e.g., etanercept, adalimumab). However, this therapeutic strategy is hindered by an increased risk of serious infections^{92,93}, due in part to the systemic immunomodulatory role of TNFa^{94–96}. Given this limitation, considering how GPSM3 functional deficiency (*i.e.*, shRNA) affects responses to bacterial challenges is important. In mouse models *Gpsm3*-deficient mice exhibit abrogated joint inflammation following induction of either acute collagen antibody-induced arthritis (Fig. 1) or chronic collagen-induced arthritis (Fig. 2); moreover, these mice show no overt signs of increased risk of infection during normal animal husbandry^{9,97}. GPSM3 deficient NB4* cell lines are also capable of migrating toward formylated peptides, which are involved in leukocyte migration toward bacteria (Fig. 3.2D). Thus, these data suggest that GPSM3 may represent a therapeutic target for pharmaceuticals that are both RA-specific and lack the risk of decreased bacteria-induced immune responses associated with the currently used TNFa inhibitors⁹⁸.

Experiment 5: To address the effects of GPSM3 deficiency on phagocytosis of bacteria, bacterial particles labeled with a pH-sensitive fluorescent marker will be used. To model a physiologic tissue environment, cell models will represent the differentiated

state of neutrophils (NB4*) and tissue macrophages (differentiated THP-1*) within peripheral tissues when functioning in host defense^{99,100}. The co-culture of labeled bacterial particles with NB4* or macrophage differentiated THP-1 cells (THP-1*) will allow the leukocyte cell models to phagocytize bacterial particles within phagosomes. These phagosomes will then acidify and activate pH-sensitive fluorophores on the bacterial particles. Measurement of fluorescence in real-time will allow quantification of the effect of GPSM3 deficiency on phagocytosis. Only minimal disruption of phagocytosis in GPSM3 deficient cells is expected, based on: (1) the dramatic downregulation of GPSM3 expression (to undetectable levels) seen during the differentiation pathway to mature macrophages,⁹ an important tissue phagocyte, (2) phagocytosis is driven primarily by $G\alpha_i$ -independent signaling processes (e.g., Toll-like receptors, integrins)^{101,102}, and (3) normal fMLP-induced migration in GPSM3 deficient cell lines.

5.3 – Figures

Figure 5.1 –Manhattan plot of association statistics highlighting all autosomal loci

associated with rheumatoid arthritis in this study. The P values of association to

ACPA-positive rheumatoid arthritis from the meta-analysis of the Immunochip and

GWAS data are shown. Known and newly identified rheumatoid arthritis risk loci are

shown in red and black, respectively. The *GPSM3* gene locus exists ~500 kb from the

MHC region ($P < 10^{-100}$), and may explain some of the inverse association with RA.

Three associated loci (identified by asterisks) only reach $P < 5 \times 10^{-8}$ when both ACPA-

positive and ACPA-negative cases are included in the analysis. The dashed gray line

indicates genome-wide significance ($P = 5 \times 10^{-8}$). Figure and legend adapted from

Eyre, et al.¹⁰.

GPSM3 mRNA wild-type

GPSM3 mRNA rs3134605 [A>G]

Figure 5.2 – Predicted lack of mRNA structural consequences of *GPSM3* SNP rs3134605 [A>G]. Bioinformatic prediction using RNAfold ¹⁶ of *GPSM3* mRNA secondary structure reveals there are no predicted changes in transcript structure. The location of rs3134605 is indicated in both structures by the black arrow.

Figure 5.3 – Functional consequences of SNPs rs204989, rs204990, rs204991, and rs3096688 on *GPSM3* promoter activity in THP-1 promyelocytic cells. Functional results upon subcloning a 3.5 kb-region immediately 5' to the *GPSM3* transcription start site from M/M and m/m genotype volunteers, leading to wild type (pGL3-M) and “minor”/polymorphic (pGL3-m) promoter-driven expression of firefly luciferase in THP-1 cells. Luciferase activity is reported as a ratio of *GPSM3* promoter-dependent firefly luciferase activity (FLuc) to control *Renilla* luciferase activity (pRL-TK control vector).

Figure 5.4 – Publically available HEK 293T and CD14+ monocyte H3K4me3 Chip-Seq data, from Thurman, et al.²¹, for **(A)** HEK 293T and **(B)** CD14+ monocytes, retrieved from NCBI (NC_000006.12 GRCh38.2). Red boxes highlight the *GPM3* gene locus, light green boxes are *GPM3* untranslated regions (UTR), dark green boxes represent coding regions of the *GPM3* locus, and blue peaks represent the number of fragments per kilobase of transcript per million mapped reads (FPKM). The amplitude of the blue peaks represent the likelihood that genomic regions are associated with trimethylated H3K4.

Chemoattractant	Concentration in RA SF (pg/ml) $\pm$ S.D.	Concentration in OA SF (pg/ml) $\pm$ S.D.	P value
Eotaxin / CCL11	2.6 $\pm$ 0.9	1.5 $\pm$ 1.0	0.003
Growth-related oncogene alpha (GRO α) / CXCL1	101.4 $\pm$ 164.0	4.9 $\pm$ 6.6	0.033
IL-8 / CXCL8	161.2 $\pm$ 187.4	8.2 $\pm$ 14.5	< 0.0001
Monocyte chemotactic protein 1 (MCP-1) / CCL2	75.7 $\pm$ 147.1	30.3 $\pm$ 18.9	0.073
Regulated on activation, normal T-cell expressed and secreted (RANTES) / CCL5	7.0 $\pm$ 6.6	1.4 $\pm$ 37.9	0.021
Leukotriene B4 (LTB4) / 5, 12-di-HETE	214,320 $\pm$ 190,171	52,583 $\pm$ 28,612 [#]	0.0003

= patients with non-inflammatory arthritis.

Table 5.1 – Chemoattractant concentrations in synovial fluid samples of patients with rheumatoid arthritis (RA) and osteoarthritis (OA), adapted from Hampel, et al.¹⁰³ and Klickstein, et al.¹⁰⁴. For the first 5 chemoattractants 10-20 ml of synovial fluid (“SF”) were collected during total knee alloarthroplasty. Chemokine concentration was performed with 9-plex chemokine array (Quansys Biosciences; Logan, UT, USA). For LTB4, synovial fluid was collected by aspiration from knee joints, and HETE was purified and quantified by high-performance liquid chromatography (HPLC).

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Appendix 1

G protein signaling modulator 3 inhibits the inflammasome activity of NLRP3

This work is the result of a collaborative effort with Dr. Alex Duncan's lab, to which I contributed the data and interpretation of figure 6, as well as assistance in manuscript review. I take no credit for any other experimental findings. This work is currently published in:

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A1.1 – Abstract

Inflammasomes are multi-protein complexes that regulate maturation of the interleukin 1 β -related cytokines IL-1 β and IL-18 through activation of the cysteine proteinase caspase-1. NOD-like receptor family, pyrin domain containing 3 (NLRP3) protein is a key component of inflammasomes that assemble in response to a wide variety of endogenous and pathogen-derived danger signals. Activation of the NLRP3-inflammasome and subsequent secretion of IL-1 β is highly regulated by at least three processes: transcriptional activation of both *NLRP3* and *pro-IL-1 β* genes, non-transcriptional priming of NLRP3, and final activation of NLRP3. NLRP3 is predominantly expressed in cells of the hematopoietic lineage. Using a yeast two-hybrid screen, we identified the hematopoietic-restricted protein ‘G protein signaling modulator-3’ (GPSM3) as an NLRP3-interacting protein and a negative regulator of IL-1 β production triggered by NLRP3-dependent inflammasome activators. In monocytes, GPSM3 associates with the C-terminal leucine-rich repeat domain of NLRP3. Bone marrow-derived macrophages lacking GPSM3 expression exhibit an increase in NLRP3-dependent IL-1 β , but not TNF- α , secretion. Furthermore, GPSM3-null mice have enhanced serum and peritoneal IL-1 β production following Alum-induced peritonitis. Our findings suggest that GPSM3 acts as a direct negative regulator of NLRP3 function.

A1.2 – Introduction

Phagocytic mononuclear cells such as monocytes and macrophages respond to host and environmental cues through numerous signal transduction pathways to coordinate inflammatory responses. The release of IL-1 β and IL-18 from these cells plays a major role in inflammatory responses to challenge with infectious agents and in aseptic inflammatory conditions ¹. These cytokines are produced as inactive cytoplasmic pro-cytokines and require specialized proteolytic processing and secretion mechanisms in order to be released as biologically active molecules. The proteolytic processing is carried out by a specific cysteine proteinase, caspase-1 ². Caspase-1 is synthesized as an inactive pro-enzyme and activation of procaspase-1 is tightly regulated ³. Multiprotein complexes known as inflammasomes activate caspase-1 in response to numerous pro-inflammatory stimuli ⁴. While all inflammasomes activate caspase-1, inflammasomes are assembled from various core protein components, each of which has a defined range of triggers.

Inflammasome activation of procaspase-1 is ultimately responsible for proteolytic processing of the precursors of IL-1 β and IL-18 into mature, active cytokines ⁴. In addition to procaspase-1, inflammasomes are known to contain other cellular proteins, including the adapter proteins 'Apoptotic Speck protein containing a Card' (or ASC) and Cardinal (also known as CARD8 or TUCAN), as well as a NOD-like receptor (NLR) protein family member ⁴. The mammalian genome encodes approximately 23 NLR gene family members, each defined by a conserved tripartite protein structure of a variable

number of leucine-rich repeat at the carboxyl terminus, a central nucleotide-binding oligomerization domain, and an amino-terminal effector domain. Most NLR effector domains are either *caspase activation and recruitment domains* (CARD), leading to the designation *NLR_C*, or *pyrin domains*, leading to the designation *NLR_P* ⁵. The immunologic signaling pathways within which many of these NLR proteins participate have been elucidated both through genetic disease associations and through cell biological and biochemical analyses of their function ⁶.

Of all inflammasome-forming proteins, NLRP3 is activated by the broadest range of stimuli and has been implicated in the pathogenesis of a wide array of autoinflammatory conditions, sterile inflammatory conditions, and infectious diseases. Monosodium urate and calcium pyrophosphate crystals, underlying causes of the sterile inflammatory arthritides gout and pseudogout, cause the activation of caspase-1 and secretion of IL-1 β ; macrophages from NLRP3-deficient mice fail to secrete IL-1 β in response to either stimuli ⁷. Additionally, crystals linked to various other pathologic inflammatory processes are known to signal through NLRP3, including asbestos, silica, and β -amyloid fibrils ⁸⁻¹⁰. Aluminum hydroxide (Alum), a vaccine adjuvant used in humans, also stimulates the release of NLRP3-inflammasome-processed cytokines. NLRP3-deficient mice have blunted immunologic responses to vaccinations accompanied by aluminum hydroxide, suggesting that NLRP3 plays an important role in the adaptive immune response in this setting as well ^{10,11}. In addition, several biochemical moieties produced by infectious disease agents or host inflammatory processes, named pathogen-associated molecular patterns (PAMPs) and damage-

associated molecular patterns (DAMPs), are known to activate the NLRP3-dependent inflammasome; PAMPs and DAMPs include pore-forming toxins, pathogen-related RNA and DNA species, host cell-derived ATP and DNA, and hyaluronan generated from cellular damage ¹². Activation of the NLRP3 inflammasome in response to these diverse stimuli is controlled by a series of transcriptional and post-transcriptional mechanisms that include upregulation of NLRP3 and involvement of the chaperonin HSP90 and the deubiquitinase BRCC3 ¹³⁻¹⁵.

Some of us have recently described functional studies of G protein signaling modulator-3 (GPSM3), a newly identified signaling regulator with prominent expression in myeloid lineage cells, and at lower relative levels in a variety of lymphoid tissues ¹⁶. GPSM3 (*a.k.a.* AGS4 or G18; ¹⁷⁻¹⁸) possesses two functional “GoLoco motifs” ¹⁸ for binding inactive G α_i -GDP subunits and additionally binds to G β subunits during their synthetic pathway towards forming mature G $\beta\gamma$ dimers ¹⁹; these interactions with heterotrimeric G protein subunits are thought to underlie the effects of GPSM3 on chemokine receptor signaling that is critical to the development of inflammatory arthritis ¹⁶. More recently, we have described GPSM3 as also interacting directly with the adaptor protein 14-3-3 ²⁰. To identify additional interacting partner(s) that might be involved in GPSM3-mediated signalling regulation, a yeast two-hybrid screen was performed using full-length GPSM3 as bait. This screen ¹⁹ identified one clone encoding a C-terminal fragment of NLRP3. Based on their co-expression in myeloid lineage cells and the discovery of their potential association by yeast two-hybrid screening, we investigated the role of GPSM3 in NLRP3-mediated IL-1 β screening, we investigated

the role of GPSM3 in NLRP3-mediated IL-1 β production and further characterized this association using biochemical approaches. Our data indicate a modulatory function of GPSM3 on NLRP3-dependent IL-1 β generation.

A1.3 – Results

A1.3.1 – GPSM3 interacts with the C-terminal end of NLRP3

Yeast two-hybrid screening for potential GPSM3-associated proteins within a commercial, 10⁶ clone human leukocyte cDNA library identified NLRP3, a critical component of the monocyte inflammasome complex, as a potential GPSM3-interacting protein. Fig. 4.1A shows the growth under auxotrophic selection of yeast transfected with the cDNA encoding full-length GPSM3 fused to the Gal4 DNA binding domain (bait) and the NLRP3-transcriptional activation domain fusion encoding the partial NLRP3 clone initially retrieved in the screen (prey). Sequence analysis of this prey clone revealed the last four leucine-rich repeats of NLRP3 (a.a. 831-979) in frame with the Gal4-transcriptional activation domain (Fig. 4.1B). To further characterize this interaction, cDNA encoding HA epitope-tagged NLRP3 and FLAG epitope-tagged GPSM3 were co-transfected in HEK293 cells which do not express either protein endogenously. Full-length NLRP3, as well as its C-terminal fragment (a.a. 831-979), were each efficiently co-immunoprecipitated with GPSM3 using anti-Flag antibodies (Fig. 4.1C and D, respectively).

Our group has previously described different point mutations to GPSM3 that abolish specific interactions with its known heterotrimeric G protein binding partners: $G\alpha_i$ -GDP and monomeric $G\beta$ subunits. For example, mutation to phenylalanine of two arginines at positions 81 and 152, within the critical triad sequences of the first and third GoLoco motifs, completely abolishes direct interaction with $G\alpha_i$ -GDP subunits (herein described as the 'RF mutant')²² (Fig. 4.1B). Moreover, the point mutations ⁵⁸LSL⁶⁰-to-PLP, within a leucine-rich motif upstream of the first GoLoco motif, dramatically reduce the association of GPSM3 with monomeric $G\beta$ subunits (herein described as the 'LSL mutant')¹⁹ (Fig 4.1B). While the RF mutation did not perturb co-immunoprecipitation between GPSM3 and NLRP3, the LSL mutation decreased the ability of GPSM3 to interact with full-length and truncated NLRP3 (831-979) suggesting a common interacting protein or shared binding site (Fig. 4.1C and D, last lane), in a fashion similar to that observed for the GPSM3/ $G\beta$ interaction¹⁹. To demonstrate that the GPSM3/NLRP3 association is not an artifact of protein overexpression, co-immunoprecipitation of endogenous NLRP3 and GPSM3 was performed from the human monocytic cell line, THP-1. A sheep polyclonal anti-NLRP3 antibody was used to immunoprecipitate endogenous NLRP3 and associated proteins. The immunoprecipitated complexes were analyzed for the presence of GPSM3 using immunoblot with a mouse monoclonal anti-GPSM3 antibody^{20,22}. Immunoprecipitation with anti-NLRP3 antibodies led to capture of detectable endogenous GPSM3 as well as NLRP3, while mock immunoprecipitation failed to capture either protein (Fig. 4.1E).

A1.3.2 – Mapping the GPSM3-interaction site(s) within NLRP3

To further characterize the GPSM3/NLRP3 interaction, bioluminescence resonance energy transfer (BRET) was used to identify the minimal GPSM3-interaction domain within NLRP3. Full-length GPSM3, fused to the resonance donor *Renilla* Luciferase (RLuc-GPSM3) was co-expressed with fusion proteins comprising various domains of NLRP3 fused to the acceptor fluorescent protein GFP10 (GFP10-NLRP3, Fig. 4.2A). Any interaction between RLuc-GPSM3 and the NLRP3 domain-GFP10 fusion proteins brings the luciferase donor and GFP acceptor proteins into close proximity, allowing energy transfer. Cellular co-transfection with a fixed quantity of RLuc-GPSM3 plasmid and a titration of increasing amounts of GFP10-NLRP3 (full-length) plasmid generated a saturable signal (Fig. 4.2B) with a maximum net BRET ratio of 0.3. Furthermore, when the RLuc-GPSM3 fusion contained the LSL mutation, the BRET signal with GFP10-NLRP3 was lost (Fig. 4.2B). Fig. 4.2C summarizes BRET experiment results between RLuc-GPSM3 and a variety of GFP10-NLRP3 truncation constructs (Fig. 4.2A). Expression of RLuc-GPSM3 with GFP10 fusion protein encoding solely the Pyrin domain of NLRP3 (Fig. 4.2C) gave no observable BRET signal. Truncation of individual leucine-rich repeat (LRR) domains, starting from the C-terminus, revealed the importance of the last LRR (aa 911-932) to the GPSM3/NLRP3 interaction, as ~50% of the signal was lost following its removal. However, a GFP10 fusion protein encoding both the pyrin and NBD domains (GFP10-NLRP3 536 stop) also produced ~50% of the total netBRET signal (Fig. 4.2C).

This BRET-based evidence of interaction with the N-terminal region of NLRP3 was not expected, given that the cDNA clone retrieved from the yeast two-hybrid screen contained only the last 4 LRRs of NLRP3 and interaction between GPSM3 and this C-terminal NLRP3 fragment was confirmed by reciprocal co-immunoprecipitation (Fig 4.1). One possible explanation for also detecting a GPSM3 interaction with the NLRP3 N-terminal region is that the NBD domain is known to interact with the C-terminal LRR portion, forming an intramolecular hairpin-like structure ²⁶. It is thus possible that, within this cellular context, the NBD domain of NLRP3 interacts with endogenous NLR proteins that might also interact with GPSM3. To determine whether GPSM3 could interact with the NLRP3 C-terminus without bridging by the NLRP3-NBD domain, we made a GFP10 fusion construct expressing only the C-terminal LRRs of NLRP3 (GFP10-NLRP3-LRR). Additionally, a truncation of the GFP10-NLRP3-LRR fusion lacking the last LRR (a.a. 911-932) was generated. As expected, the construct containing all the C-terminal LRRs (*i.e.*, NLRP3 without its pyrin and NBD domains) was sufficient to generate a robust BRET signal (Fig 4.2D). Removal of the final LRR abolished the BRET signal, confirming that the last LRR is required for interaction with GPSM3. Removal of the residual C-terminal extension beyond the final LRR domain (*i.e.*, “NLRP3 934 stop”) had no effect on the GPSM3/NLRP3 interaction as measured by BRET (Fig. 4.2D). The interaction between GPSM3 and the GFP10-NLRP3-LRR fusion protein was also confirmed by co-immunoprecipitation. As also observed in the BRET assay, the coimmunoprecipitation of GPSM3 and the GFP10-NLRP3-LRR fusion protein was disrupted by deletion of the last LRR domain, but not the residual C-terminal amino acids (Fig. 4.3).

A1.3.3 - Subcellular localization of the GPSM3/NLRP3 interaction

We employed a third protein-protein interaction assay, bimolecular fluorescence complementation (BiFC), to confirm the interaction between GPSM3 and NLRP3 as well as to study the localization of their complex, using previously described methods ^{20,22}. The N-terminal portion of the yellow fluorescent protein (YFP) was fused to full-length GPSM3 (“YN-GPSM3”) and the C-terminal part of YFP was fused to full-length NLRP3. When expressed alone or with the non-fused counterpart, no detectable fluorescence was measured. However, when both fusion proteins were expressed together (YN-GPSM3 + NLRP3-YC), a fluorescent signal was detectable in transfected cells (Fig. 4.4). As further confirmation of the co-immunoprecipitation and BRET results (Figs. 4.2 and 4.3), the LSL mutation in GPSM3 greatly reduced the BiFC signal (Fig. 4.4A). The YN-GPSM3/NLRP3-YC complex was seen to form punctate structures throughout transfected cells as observed by fluorescence microscopy (Fig. 4.4B). Ectopic expression of the GFP10-NLRP3 fusion, which is fluorescent without binding the GPSM3 partner, was also observed to form similar punctate structures, suggesting that the GPSM3 interaction does not trigger this subcellular localization phenomenon nor perturb the localization of overexpressed NLRP3 (Fig. 4.4B). These large dots are likely oligomers of NLRP3 similar to those observed by others using immunofluorescence based studies of overexpressed NLRP3 or activation of native NLRP3 in NLRP3-expressing cells ²⁷⁻²⁹.

A1.3.5 – Functional effects of the GPSM3/NLRP3 interaction on NLRP3-dependent IL-1 β secretion

To test the hypothesis that the GPSM3 interaction regulates NLRP3 function and inflammasome activation, bone marrow-derived macrophages (BMDMs) were generated from GPSM3-deficient mice¹⁶ and IL-1 β secretion quantified following inflammasome activation. Inflammasome-dependent IL-1 β secretion requires initial priming using lipopolysaccharide (LPS) to induce pro-IL β synthesis^{30,31}. Following LPS stimulation, different inflammasome activators were added and mature IL-1 β secreted in the medium was quantified. Activators of the NLRP3-dependent inflammasome including ATP, α -hemolysin (aHL), and Alum induced robust IL-1 β secretion with a significant increase in IL-1 β secretion in *Gpsm3*^{-/-} mice after 4 hours of stimulation compared to littermate control mice (Fig. 4.5A-C left panels). As expected, NLRP3-deficient mice showed no observable IL-1 β secretion (Fig. 4.5A-C left panels). Inflammasome activation is known to trigger release of IL-1 β but not that of cytokines, like TNF- α , not dependent on Caspase-1 activation²⁵. TNF- α was also quantified in the culture supernatants from these stimulated macrophages and found to be secreted to the same extent by all three-mouse genotypes (Fig. 4.5A-C right panels); these results suggest that GPSM3 regulation of cytokine secretion is specific for those dependent on NLRP3-mediated Caspase-1 activation.

The specificity of GPSM3 for the NLRP3 inflammasome signalling pathway was also tested using dsDNA stimulation, an AIM2-inflammasome activator³², and a flagellin

derived peptide known to activate the NLRC4-inflammasome³³. BMDMs isolated from AIM2-, NLRC4- and NLRP3-deficient mice were used as controls. At 18 hours post-treatment, dsDNA was found to induce a similar release of IL-1 β secretion in BMDMs from WT mice as compared to *Gpsm3*^{-/-} mice. As predicted, BMDMs from *Aim2*^{-/-} mice failed to secrete IL-1 β upon dsDNA stimulation; notably, NLRP3-deficient BMDMs were found to have increased IL-1 β release compared to BMDMs from WT control and *Gpsm3*^{-/-} mice (Fig. 4.5C). Similarly, BMDMs from *Nlrc4*^{-/-} mice showed a significant decrease in IL-1 β secretion in response to flagellin, as expected³³ (Fig. 4.5D). Importantly, GPSM3-deficient and WT BMDMs responded equally to dsDNA and flagellin as an inflammasome stimulator (Fig. 4.5D-E), suggesting that GPSM3 regulation of inflammasomes is limited to the NLRP3-inflammasome.

To explore the involvement of GPSM3 in NLRP3-dependent inflammasome activation in a more physiological context, we employed a model of Alum-induced peritonitis and quantified resultant IL-1 β production from peritoneal lavage and blood serum. *Gpsm3*^{-/-}, *Nlrp3*^{-/-} and wild type littermate control mice were first pretreated intraperitoneally (i.p.) with LPS and subsequently injected two hours later with Alum, an NLRP3-dependent inflammasome activator²⁵. Peritoneal exudate and peripheral blood were both collected and cytokine content quantified. We observed that mature IL-1 β secretion was higher in the lavage and serum of Alum-treated *Gpsm3*^{-/-} mice compared to WT controls, whereas blunted IL-1 β secretion was observed from *Nlrp3*^{-/-} mice (Fig. 4.5F).

To explore the mechanism by which GPSM3 regulated NLRP3-dependent IL-1 β secretion, we undertook studies of NLRP3, IL-1 β , and GPSM3 expression in macrophages and THP1 human monocytic cells. WT and *Gpsm3*^{-/-} macrophages exhibited equivalent induction of NLRP3 and Pro-IL-1 β mRNA after treatment with LPS and/or α -hemolysin (Fig. 4.6A and B), further indicating that GPSM3 alters IL-1 β secretion through post-transcriptional regulation of NLRP3. In THP1 cells, priming with LPS led to increased Pro-IL-1 β mRNA and decreased GPSM3 mRNA expression (Fig 4.6C-E). GPSM3 protein levels were also found to be decrease after treatment with LPS (Fig. 4.6F), suggesting that priming by LPS relieves GPSM3 mediated inflammasome inhibition in part through down regulation of the protein.

A1.3.5 – The GPSM3/NLRP3 complex includes HSPA8 and is independent of the GPSM3/14-3-3 interaction

The chaperonin proteins HSP90, SGT1³⁴⁻³⁶, and HSP70^{37, 38} (reviewed in³⁹) are known to be essential in regulating NLRP3-inflammasome activation. Cellular heat shock diminishes NLRP3-inflammasome mediated Caspase-1 activation, suggesting that these chaperonins are part of an inactive, basal-state NLRP3-containing complex⁴⁰. The scaffolding protein RACK1 (“receptor for activated C kinases 1”), is required for the functioning of a HSP90-, SGT1-, and HSP70-containing chaperonin complex in rice which regulates innate immune signalling through nucleotide binding leucine-rich repeat (NB-LRR)-type R proteins (the rice orthologs of mammalian NLR proteins) and other innate immune signalling systems^{36,41,42}. Our yeast two-hybrid screen identified the

heat shock protein HSPA8 (also known as HSC70 or HSP73) and RACK1 as additional GPSM3-interacting proteins, suggesting that GPSM3 may be involved in chaperonin-mediated regulation of NLRP3 function. We thus co-expressed Flag-tagged GPSM3 and HA-tagged NLRP3 in the presence or absence of HA-HSPA8 and HA-RACK1 for co-immunoprecipitation analyses. Immuno-precipitation of GPSM3 revealed formation of a complex comprising GPSM3, NLRP3 and HSPA8 but excluding RACK1 (Fig. 4.7A). Co-expression of HSPA8 increased complex formation between GPSM3 and NLRP3; surprisingly, co-expression of RACK1 also increased GPSM3-NLRP3 complex formation, and co-expression of RACK1 and HSPA8 was found to act synergistically on complex formation. The LSL mutant of GPSM3, previously found to have a greatly reduced interaction with NLRP3 (Fig. 4.1), also failed to interact with HSPA8 (Fig. 4.7A and B, lane 7), suggesting a common binding site or partner. In addition, overexpression of RACK1 and HSPA8 was found to increase fluorescence signal between YN-GPSM3 and NLRP3-YC as observed using the BiFC assay (Fig. 4.7B). This result supports the idea of a synergistic effect of RACK1 and HSPA8 on GPSM3-NLRP3 complex formation.

We recently described GPSM3 as interacting directly with the adaptor protein 14-3-3²⁰. Given that numerous heat shock proteins are known to interact or form a complex with 14-3-3 proteins, including HSPA8⁴³, we tested whether 14-3-3 proteins were involved in complex formation between GPSM3, HSPA8, and NLRP3. As previously described²⁰, mutation of a critical serine 35 within the 14-3-3-binding site of GPSM3 abolishes interaction with 14-3-3. However, the GPSM3 S35A mutant was seen

to associate with HSPA8 and NLRP3 identically as wild-type GPSM3 (Fig. 4.7C), while completely abolishing the 14-3-3 interaction, as previously reported ²⁰.

A1.4 – Discussion

Activation of NLRP3 by various danger signals contributes to pathologic inflammatory responses that drive a number of disease states ¹². Presumably the capacity to generate potent pathologic inflammatory responses has led to the evolution of a multistage activation process to rein in NLRP3 inflammasome activity in order to minimize inappropriate inflammation-driven pathologies. An unbiased screen for proteins that interact with GPSM3 identified a potential interaction with NLRP3. Our present results support an endogenous and physiologic interaction between NLRP3 and GPSM3, and the notion that GPSM3 exerts a negative regulatory effect on the function of NLRP3. GPSM3 has previously been found to contribute to the induction of acute inflammation in a collagen antibody-induced arthritis mouse model of acute inflammatory arthritis. This pro-inflammatory effect is likely mediated by the influence of GPSM3 on monocyte apoptosis and chemotaxis in response to several G protein coupled receptor-activating chemokines ¹⁶. This proinflammatory role of GPSM3 does not contradict the negative regulation of the NLRP3 inflammasome that we now describe because induction of collagen antibody-induced arthritis has previously been shown as independent of NLRP3 inflammasome activation ^{44,45}, and additional GPSM3-dependent impairment of leukocyte recruitment may offset the increase NLRP3 activity. Our current data suggests that GPSM3 has pleiotropic effects on immune signaling

pathways and that, in addition to modulation of G protein coupled receptor signaling, it is also able to regulate NLRP3 inflammasome activation.

Using a combination of methodologies, we have shown that GPSM3 interacts with the distal C-terminal region of NLRP3 and absolutely requires the last leucine-rich repeat domain (LRR8). No structural information is yet available for either protein; therefore, we cannot exclude other determinants within NLRP3 that are also important for this association. The LRR domains of NLR proteins are generally believed to serve as ligand-binding domains for these innate immune sensors. Although the structure of the LRR domain of NLRP3 has not yet been elucidated, the LRR domains of NLR proteins are believed to associate with the NBD domain forming a horseshoe tertiary structure akin to other LRR-containing proteins. Crystal structures of the LRR portion of different TLR receptors have been elucidated in the presence of different molecular ligands including dsRNA ⁴⁶, LPS ⁴⁷, and lipoprotein ⁴⁸, as well as the dsRNA-binding C-terminus of the NLR protein NLRX1 ⁴⁹. It is possible that GPSM3 interferes with the ability of NLRP3 to interact with activating ligands through interaction with the final LRR domain of NLRP3. A horseshoe tertiary structure for the LRR domains, if also present within NLRP3, might allow intramolecular interaction of the C-terminal LRR domains with the N-terminal NBD domain to facilitate the formation of a closed inactive state, similarly to that described by the NLRC4 crystal structure ⁵⁰. The observed residual BRET signal between GPSM3 and NLRP3 constructs lacking the LRR domains raises the possibility of an endogenous NLR protein bridging truncated (LRR-lacking) NLRP3 mutants and GPSM3 ⁵¹. However, one could also speculate that, if GPSM3 is capable

of simultaneously interacting with both the amino-terminus and the LRR domain of NLRP3, such an interaction could inhibit release of the NLRP3 amino terminus and LRR domain, thereby locking the protein in an inactive form. Further experiments are clearly required to determine if either one of these mechanisms is involved in the inhibition of NLRP3 by GPSM3.

A two-step regulatory circuit for cellular IL-1 β secretion, in which pro-IL-1 β is induced by TLR stimulation and NLRP3 inflammasome-mediated Caspase-1 activation is induced by a distinct second signaling event, has been a widely accepted model ⁵². The first step, or “signal 1,” of regulation of inflammasome-mediated IL-1 β secretion is a cell surface receptor-induced NF-kappaB transcriptional activation event that can be initiated by various TLR-activating stimuli or by numerous cytokine receptors. TLR stimulation primes cells for IL-1 β secretion by enhancing pro-IL-1 β production and by increasing production of NLRP3, which is a very low abundance protein under basal conditions. Our data indicate that as mRNA transcript abundance of NLRP3 and pro-IL-1 β increase, the transcript of the negative regulator GPSM3 is decreased as well (Fig. 4.6). A heterogeneous group of stimuli then provide “signal 2”, leading to post-transcriptional activation of NLRP3 (reviewed in ⁵²). The activation of NLRP3 by “signal 2” is now known to further require a post-transcriptional deubiquitination event that is catalyzed by the DUB class of deubiquitinases ⁵³. These data suggest that, in its basal state, NLRP3 is ubiquitinated. A complex containing HSP90 and the ubiquitin ligase subunit SGT1 is required for NLRP3 signaling in mammalian cells, and these proteins may be involved in the baseline ubiquitination of NLRP3 that has been previously

observed ³⁹. We have now presented data demonstrating that one other chaperonin protein, HSPA8, is also found in complex with NLRP3 and GPSM3. These data suggest the possibility of a third mechanism by which GPSM3 inhibits NLRP3 activation: namely, that GPSM3 exerts its negative regulatory effect on NLRP3 by helping to bring it to a basal Heat Shock protein ubiquitination/chaperonin complex. Based on our present study and recent advances in the field, we propose a refined model of basal NLRP3 regulation (Fig. 4.8). The role of GPSM3 in promoting NLRP3 inactivation and whether this negative regulatory pathway can be exploited therapeutically in cases of pathologic NLRP3 activation remain areas for further study.

A1.5 – Materials and Methods

A1.5.1 – Commercial antibodies, constructs, and other reagents. Horseradish peroxidase (HRP)-conjugated anti-hemagglutinin (HA) monoclonal antibody (clone 3F10) was obtained from Roche Diagnostics. Anti-Flag M2 antibody, and agarose-conjugated anti-Flag M2 antibody were purchased from Sigma. HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies were from GE Healthcare (Piscataway, NJ). Anti-GFP Abfinity antibody was from Invitrogen. Mouse monoclonal anti-NLRP3 clone cryo-2 was from Adipogen (San Diego, CA) and sheep polyclonal anti-NLRP3 from R&D systems (Minneapolis, MN). Monoclonal anti-GPSM3 antibody was produced by the UNC Antibody Core Facility and has been previously described ²⁰. All cDNAs used in this manuscript were cloned in the pcDNA3.1 backbone vector (Invitrogen, Carlsbad, CA), with Flag- or HA- epitope tag sequences included in the forward PCR primer to

produce N-terminal tagged open-reading frames as described previously²⁰. Recombinant *S. aureus* α hemolysin was generated as previously described²¹. Aluminum Hydroxide suspension (Imject Alum) was purchased from Thermo Scientific.

A1.5.2 – Cell culture and transfection. Human embryonic kidney 293 (HEK293) and THP-1 cell lines were each obtained from the American Type Culture Collection (ATCC) and maintained in DMEM or RPMI-1640 media (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum (Cellgro, Manassas, VA) at 37°C in a humidified atmosphere containing 5% CO₂. Transient transfections of cell monolayers grown to 75–90% confluence were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

A.5.3 – Immunoprecipitation and immunoblotting. Cells were lysed with ice-cold lysis buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, and Complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN)) at 4°C on a rocker platform for 30 min. Lysates were clarified by centrifugation at 16,000 x *g* for 15 min at 4°C and quantified by the bicinchoninic acid (BCA) protein content assay (Pierce, Rockford, IL). For immunoprecipitation, lysates were incubated with specific antibody for 2 hours at 4°C followed by overnight incubation with protein-A/G agarose (Santa Cruz, Santa Cruz, CA), or directly incubated with agarose-conjugated anti-Flag M2 antibody overnight. Pelleted antibody/bead complexes were then washed three times with lysis buffer and proteins eluted in Laemmli buffer. Eluted proteins or lysate samples were resolved on 4–12% precast SDS-polyacrylamide gels (Novex/Invitrogen), transferred to nitrocellulose,

immunoblotted using primary and HRP-conjugated secondary antibodies, and visualized by chemi-luminescence (ECL, GE Healthcare).

A1.5.4 – Bioluminescence resonance energy transfer (BRET). HEK293 cells were seeded in 12-well plates (3.5×10^5 cells/well) and transfected with fixed amount of RLuc-GPSM3 or RLuc-GPSM3 LSL mutant vector DNA (50 ng) and increasing amounts of GFP10-NLRP3 vector DNA (0-1500 ng) and corresponding (decreasing) amounts of pcDNA3 empty vector (1500 to 0 ng) to obtain a saturation curve. The others BRET assays were performed by transfecting cells with 50 ng of the RLuc fusion protein-expressing vector with 750 ng of GFP10-fusion protein expressing vector as indicated. 24h post transfection, cells were washed once, harvested and resuspended in BRET buffer (phosphate-buffered saline with 1 mM CaCl_2 , 0.5 mM MgCl_2 , 0.1% glucose) and distributed in white 96-wells microplates. BRET was initiated by adding coelenterazine-400a at a final concentration of 5 μM . Measurements of emitted light were collected on a Mithras LB-940 plate-reader (Berthold Technologies) using a BRET² filter set.

A1.5.5 – Bimolecular fluorescence complementation (BiFC). Fusion constructs were made similar to those previously described ²²: namely, a fusion of the N-terminal fragment (a.a. 1-158) of yellow fluorescent protein (YN) to the N-terminus of full-length GPSM3 (“YN-GPSM3”) and the C-terminal fragment (a.a. 159-238) of YFP (YC) to the C-terminus of NLRP3 (“NLRP3-YC”). HEK293 cells were transfected with an equal amount of plasmids encoding the fusion proteins YN-GPSM3 and NLRP3-YC, and cells were incubated at 37°C for 24 h. Total DNA quantity was normalized using empty

pcDNA3.1 vector DNA. To measure fluorescence from formed complexes, transfected cells were washed, harvested and resuspended in PBS. BiFC signal was acquired using a Mithras LB-940 plate-reader using an excitation/emission filter set of 485 nm and 510 nm. The level of expression of each fusion protein was quantified by western blotting using a polyclonal antibody directed against the GFP.

4.5.6 – Immunofluorescence microscopy. HEK293 cells were seeded in a 12-well plate and transfected with 0.4 μ M HEK293 cells were seeded in aNLRP3-YC. The following day, cells were transferred to a poly-D-lysine (PDL)-coated coverslip in a 6-well plate and grown overnight. Cells were then fixed with 4% paraformaldehyde plus PBS for 10 min at room temperature. Coverslips were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and examined by inverted epifluorescence microscopy (Olympus IX70) using a 40 $\times$ objective.

4.5.7 – Mice. *Gpsm3*^{-/-} mice were generated as previously described¹⁶. *Nlrp3*^{-/-} mice were a kind gift from Dr. John Bertin (Millennium Pharmaceuticals) and were further backcrossed at UNC to achieve nine generations of backcrossing onto the C57BL/6 background. *Nlrp4*^{-/-} mice were produced using C57BL/6 embryonic stem (ES) cells and were a kind gift from Dr. Vishva Dixit (Genentech)²³. *Aim2*^{-/-} mice were generated using C57BL/6 ES cells by inGenious Targeting Laboratory (Ronkonkoma, NY) (*manuscript in preparation*).

A1.5.8 – Bone marrow-derived macrophages (BMDMs) isolation and culture. BMDMs were prepared from 9-12 weeks old mice. Briefly, femurs and tibiae were removed from CO₂ euthanized mice. Both ends of the bones were cut and bone marrow cells were extracted by flushing with DMEM medium containing 10 % FBS using a syringe with a 27-gauge needle. Cell suspension was filtered through a 75 ml cell strainer and then centrifuged and resuspended in RBC lysis buffer (150 mM NH₄Cl, 10 mM KCO₃, 0.1 mM EDTA pH 7.4) for 10 minutes on ice. Cells were washed in complete medium and finally resuspended at a density of 4×10^6 cells/ml in macrophage-conditioned medium (DMEM containing 10 % FBS and 20 % L-929 culture supernatant as source of M-CSF). Cells were seeded in 10-cm tissue culture plate (4×10^6 cells/plate) in 10 ml final volume. Three days later, 10 ml of fresh macrophage conditioned medium was added and replaced with 10 ml fresh medium at day 6 and day 8. Cells were collected from culture dishes at day 8 by washing once with PBS and treated with 5 ml Accutase (Sigma) for 5 minutes at 37°C.

A1.5.9 – Ex-vivo inflammasome activation assay. BMDMs were resuspended in serum-free RPMI 1640 and seeded in 24-wells plate at a density of 1×10^6 cells/ml/well. LPS (100 ng/ml) was added for 3 hours. Subsequently, cells were stimulated with NLRP3's inflammasome activators as indicated: ATP (5 mM), α hemolysin (10 μ g/ml) and Aluminum hydroxide (130 μ g/ml Imject™, Pierce) for 1 hour. For AIM2 inflammasome activation, dsDNA (2 μ g/ml, poly(dA:dT)/Lyovect™, Invivogen) was used and cells treated for 15 hours. For NLRC4 inflammasome activation, LPS treated macrophages were then transduced with 250 ng/ml flagellin (Invivogen) using Profect-P2 (1.25 μ l/ml,

Targeting Systems) as described by Miao and colleagues ²⁴ for 1 hour. Cultures supernatant were collected after centrifugation at 13,000 xg for 10 min and cytokines (IL-1 β and TNF α) were measured by enzyme-linked immunosorbent assay (ELISA) as described by Craven et al. ²¹.

A1.5.10 – Quantitative RT-PCR. THP-1 or bone marrow-derived macrophage (BMDM) cells were activated as previously described with LPS (100 ng/ml) and/or α hemolysin (10 μ g/ml). Cells were harvested and lysed in buffer RLT (Qiagen) + 1% 2-mercaptoethanol (Fisher Chemical) before immediately being placed on ice. RNA extraction was performed using the Qiagen RNeasy kit following manufacturer's instructions, and isolated RNA was DNase I treated and used to synthesize cDNA immediately (Thermo Verso cDNA synthesis kit). Relative gene expression was quantified using Veriquest Fast Probe PCR master mix (Affymetrix) and Taqman probes (Life Technologies) for GPSM3, NLPR3, and pro-IL-1 β normalized to multiplexed β 2-microglobulin. Fold change in target gene expression was calculated using threshold values by the $2^{-\Delta\Delta C_t}$ method, described by Livak & Schmittgen, and statistical significance was determined by one-way ANOVA with Bonferroni post-hoc ($p < 0.05$).

A1.5.11 – *In vivo* peritonitis experiments. *Gpsm3*^{-/-}, *Nlrp3*^{-/-} and wild type littermate control mice were first pretreated intraperitoneally (i.p.) with LPS (20 mg/kg) and subsequently injected two hours later with 350 μ g Alum (Pierce), an NLRP3-dependent inflammasome activator ²⁵. Peritoneal exudate and peripheral blood were both collected and peritoneal cavity rinses with 3 ml of PBS (i.p. lavage) and both samples mixed

together and cytokine content quantified as described previously for *ex vivo* assay. Cardiac puncture was used to bleed mice. Serum was collected from coagulated blood that has been centrifuged.

A1.6 – Acknowledgements

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A1.7 - References

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A1.8 – Figures

Fig. A1.1. Identification of NLRP3 as a GPSM3-interacting protein. (A) *Saccharomyces cerevisiae* were co-transformed with indicated bait plasmids (either expressing the Gal4p DNA binding domain alone [pAS2-1] or as a fusion with full-length GPSM3 [pAS2-1/GPSM3]) and prey plasmids (either expressing the Gal4p activation domain alone [pGAD424] or as a fusion with the C-terminus of NLRP3 [pACT2/NLRP3]). Transformed yeast were plated onto synthetic defined agar (Yc) lacking leucine (Leu⁻, to select for the prey plasmid) and tryptophan (Trp⁻, to select for the bait plasmid); growth on Yc Leu⁻ Trp⁻ medium demonstrates incorporation of both bait and prey plasmids (*left panel*). Growth on Yc Leu⁻ Trp⁻ medium also deficient in adenine (Ade⁻) and histidine (His⁻) indicates a positive protein-protein interaction (*right panel*). (B) Domain architecture of NLRP3 comprising the pyrin domain, nucleotide-binding domain (NBD), and eight leucine-rich repeats (LRRs) in comparison to the

truncated domain structure for the NLRP3 fragment identified in the yeast two-hybrid screen. Additionally represented is the domain structure of GPSM3 comprising the three GoLoco motifs (GL1-GL3) and locations of point mutations used in this study. Amino acid numbering for each domain is based on data from UniProt identifier Q96P20 (NLRP3) and Q9Y4H4 (GPSM3). **(C-D)** HEK293 cells were transiently co-transfected with plasmids expressing HA-tagged full-length NLRP3 (a.a. 1-979; panel **C**) or an NLRP3 C-terminal fragment (a.a. 831-979; panel **D**), along with Flag-tagged GPSM3, either of wildtype sequence ('WT'), R81F + R152F double mutant ('RF'; abrogates GPSM3/Gai·GDP interaction) or the LSL mutant in which the GPSM3 amino-acids L⁵⁸SL were mutated to PLP to eliminate the GPSM3/Gb interaction as previously described. Immunoprecipitation (IP) of GPSM3 was performed using agarose-conjugated anti-Flag M2 antibody and co-immunoprecipitating proteins were detected by immunoblotting (IB) with anti-HA epitope tag antibody. **(E)** Whole cell lysates from THP-1 cells were immunoprecipitated with anti-NLRP3 goat polyclonal antibody or with Protein-A/-G agarose beads alone. GPSM3 was subsequently detected using an anti-GPSM3 mouse monoclonal antibody (clone 35.5.1) and NLRP3 was detected with anti-NLRP3 mouse monoclonal antibody. Results shown are representative of two independent experiments.

A

B

C

D

Fig. A1.2. Characterization of the GPSM3/NLRP3 interaction using bioluminescence resonance energy transfer (BRET). (A) A constant amount of wildtype (or LSL mutant) RLuc-GPSM3 fusion expression vector was co-transfected into HEK293 cells with increasing amounts of GFP10-NLRP3 fusion expression vector; the resultant net BRET ratio was plotted as a function of the acceptor/donor ratio. (B) C-terminal truncation mutants were used to identify the minimal determinants within NLRP3 required for the interaction with GPSM3. A stop codon was inserted by mutagenesis after each domain of the GFP10-NLRP3 fusion construct (as indicated) and subsequently tested in the BRET assay upon co-expression with RLuc-GPSM3. (C) The leucine-rich repeat domains were fused to GFP10 (called “GFP10-NLRP3 LRR”) and used in the same BRET experiment.

Figure A1.3. Co-immunoprecipitation of GFP10-NLRP3 protein constructs with GPSM3. Flag-tagged GPSM3 was co-transfected with the same GFP10-NLRP3 fusion proteins used to map the interaction by BRET (Figure 2) and immunoprecipitation was performed using anti-Flag M2 agarose-conjugated antibody; co-immunoprecipitated proteins were detected by immunoblotting with an anti-GFP antibody.

Figure A1.4. Analysis of the GPSM3/NLRP3 interaction using bimolecular fluorescence complementation (BiFC). (A) HEK293 cells were transfected with plasmids expressing YN-GPSM3 (a.a. 1-158 of YFP fused to GPSM3) and NLRP3-YC (NLRP3 fused to a.a. 159-238 of YFP) and the fluorescence of reconstituted YFP fluorophore was measured at 24 hours post-transfection. Cells were harvested and fluorescence quantified on a plate reader. Total DNA used for transfection was normalized using empty pcDNA3.1 vector DNA. Data are expressed as relative fluorescence units (RFU) using the means $\pm$ S.E.M. of at least three different experiments; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by one-way ANOVA. (B) Epifluorescence microscopy images of HEK293 cells transfected with plasmids expressing the two YFP protein fusions YN-GPSM3 and NLRP3-YC (top panel) or the GFP10-NLRP3 alone (bottom panel).

Fig. A1.5. GPSM3 deficiency leads to selective enhancement of the activation of the NLRP3-dependent inflammasome in macrophages and in a mouse NLRP3 dependent peritonitis. Bone marrow derived macrophages (BMDMs) were generated from wild type (WT), *Gpsm3*^{-/-} and *Nlrp3*^{-/-} mice (panels **A**, **B**, and **C**), or with wild type (WT), *Gpsm3*^{-/-}, *Nlrp3*^{-/-}, *Nlrc4*^{-/-} and *Aim2*^{-/-} mice (panels **C** and **D**), and primed with LPS for indicated times at 50 ng/ml before treatment with specific NLR inducers. ATP at 1 mM, Staphylococcus aureus α -hemolysin (HLA) at 10 ug/ml and aluminum hydroxide and potassium salts (Alum) at 130 ug/ml were used to activate the NLRP3-inflammasome. Mouse macrophages were also separately transfected with dsDNA to activate AIM2 or flagellin peptide to activate NLRC4, as described in the Materials and Methods section. Cell culture supernatant was collected following treatment and secreted IL-1 β and TNF- α quantified by ELISA as indicated. Twelve week old, wild type littermate (WT), *Gpsm3*^{-/-} and *Nlrp3*^{-/-} mice were each initially primed by an intraperitoneal injection of LPS (20 mg/kg) and subsequently injected 2 hours later with 350 mg of Alum (or saline vehicle, as indicated). Peritoneal exudate was collected and the peritoneal cavity rinsed with 3 ml of PBS (“i.p. lavage”) and IL-1 β content quantified by ELISA (panel **E**). Blood was collected and serum IL-1 β was quantified by ELISA (panel **F**). Data are expressed in pg/ml using the means $\pm$ S.E.M. of three different experiments (n=8-10); *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA.

Fig. A1.6. Pro-IL-1 β and GPSM3 are reciprocally regulated during LPS-induced inflammasome priming. Bone marrow derived macrophages (BMDMs) were generated from wild type (WT) or *Gpsm3*^{-/-} mice and primed with LPS for 3 hours with 100 ng/ml and/or subsequently treated with α -hemolysin (HLA) for 1 hour, as indicated. Pro-IL-1 β and NLRP3 mRNA levels in each cell line were assessed using quantitative RT-PCR as described in the materials and methods (panels **A** and **B**). THP1 cells were treated with LPS (100ng/ml) or vehicle for 3 hours. GPSM3, Pro-IL-1 β , and NLRP3 mRNA levels were assessed by quantitative RT-PCR (panels **C**, **D**, and **E**). Data are expressed in pg/ml using the means $\pm$ S.E.M. of three different experiments (n=8-10); *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA. GPSM3, NLRP3, and actin (loading control) protein levels were also assessed using immunoblot analysis with the indicated antibodies (panel **F**). Samples from 3 independent experiments are shown.

Fig. A1.7. The heat shock protein HSPA8 is involved in the interaction between NLRP3 and GPSM3. (A) To assess potential roles of HSPA8 and RACK1 in the interaction between NLRP3 and GPSM3, HEK293 cells were transiently co-transfected

with plasmids encoding Flag-tagged GPSM3, HA-tagged NLRP3, HA-tagged HSPA8, and/or C-terminal HA-tagged RACK1. Immunoprecipitation (IP) of GPSM3 performed using agarose-conjugate anti-Flag M2 antibody revealed the presence of HSPA8 in the complex, but the absence of RACK1, when immunoblotted with anti-HA. **(B)** HEK293 cells were transfected with plasmids expressing YN-GPSM3 and NLRP3-YC in the presence or absence of RACK1 and HSPA8 and the fluorescence of reconstituted YFP fluorophore was measured at 24 hours post-transfection. Cells were harvested and fluorescence quantified on a plate reader. Total DNA used for transfection was normalized using empty pcDNA3.1 vector DNA. Data are expressed as relative fluorescence units (RFU) using the means $\pm$ S.E.M. of at least three different experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA. **(C)** To assess the role of the known GPSM3 interactor 14-3-3, HEK293 cells were similarly co-transfected with Flag-tagged wildtype GPSM3 or GPSM3 R35A mutant (a loss-of-function mutant abolishing interaction with 14-3-3; ref. (20)) and HA-tagged HSPA8 and HA-tagged 14-3-3.

Figure 8

Fig. A1.8. Model of NLRP3 regulation by GPSM3. Formation of a network between GPSM3, chaperones, and NLRP3 maintains the steady-state level of NLRP3 by stabilizing it within the complex. Activation of the inflammasome requires two independent signals. Toll-like receptors (TLR) and tumor-necrosis factor receptors (TNFR) are known to trigger priming signals leading to up-regulation of NLRP3 and pro-IL1b through a NFkB-dependent pathway as well as down regulation of GPSM3. Non-transcriptional priming of NLRP3 allows inflammasome activation upon detection of the activation signal. The NLRP3 activation signal is triggered by endogenous ('danger-associated molecular patterns'; DAMPs) or exogenous ('pathogen-associated molecular patterns'; PAMPs) danger signals. Activation of NLRP3 can also be mediated by bacterial pore-forming toxins such as the alpha-hemolysin (HLA) of *S. aureus*. Activation of NLRP3 leads to formation of a multiprotein complex containing oligomerized NLRP3, the adaptor ASC, and caspase-1. Once activated, caspase-1 cleaves pro-IL1b (and pro-IL-18), releasing the biological active form of IL-1b (and IL-18) and thereby triggering inflammation and antimicrobial activity.