MOLECULAR STUDIES ON G-CSF RECEPTOR SIGNALING IN GRANULOCYTES & REGULATION OF F_{C\gamma} RECEPTOR FUNCTION IN MACROPHAGES

(Roles for a novel protein LRG and inositol phosphatase SHIP-2 respectively)

DISSERTATION

 Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2006

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Neutrophils and macrophages are two major cell types involved in innate immune response. They both have specific surface receptors, which, in the presence of corresponding stimulants, will transduce unique signals that are critical for their survival, differentiation, and functions.

In the first part, we characterized the physiological roles of a novel protein, LRG, in G-CSFR signaling pathways in granulocytes. We showed that the transcription factors PU.1 and C/EBPε that regulate the expression of multiple myeloid-specific genes also bind to the LRG promoter, and LRG localizes to the same cytoplasmic compartment as myeloperoxidase. Stable transfection of LRG into 32Dcl3 cells resulted in accelerated G-CSF-mediated neutrophil differentiation and induction of CD11b and CD13 expression, which was found to correlate with increased level of phospho-Stat3 but not with PU.1 or p27kip1 levels. In contrast, constitutive expression of LRG in 32Dwt18 cells expressing a chimeric Epo/G-CSF receptor consisting of the EpoR extracellular domain fused to the G-CSFR transmembrane and cytoplasmic domains failed to do so in response to Epo stimulation. Collectively, these findings suggest a role for LRG in modulating neutrophil differentiation and activation via non-redundant G-CSFR signals.
In the second part, we studied the regulation of FcγR-mediated phagocytosis of IgG-coated particles in macrophages, which involve inositol phosphatases PTEN and SHIP-1. We have analyzed the role of SHIP-2, an inositol phosphatase with high level homology to SHIP-1, in phagocytosis using independent cell models that allow for manipulation of SHIP-2 function without influencing SHIP-1. We presented evidence that SHIP-2 translocates to the site of phagocytosis and downregulates FcγR-mediated phagocytosis. Our data indicated that SHIP-2 must contain both the N-terminal SH2 domain and the C-terminal proline-rich domain to mediate its inhibitory effect. The effect of SHIP-2 is independent of SHIP-1, as overexpression of dominant-negative SHIP-2 in SHIP-1 deficient primary macrophages resulted in enhanced phagocytic efficiency. Likewise, specific knockdown of SHIP-2 expression using siRNA resulted in enhanced phagocytosis. Finally, analysis of the molecular mechanism of SHIP-2 downregulation of phagocytosis revealed that SHIP-2 downregulates upstream activation of Rac. These data suggest that SHIP-2 is a novel negative regulator of FcγR-mediated phagocytosis independent of SHIP-1.

Taken together, we concluded that specific receptors have unique downstream signaling events which are under tight control.
Dedicated to my family
ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Susheela Tridandapani, who provided me the precious opportunity to work in such an exciting research field. Her enthusiasm, constant encouragement and guidance, especially constructive criticism, have been invaluable to the completion of this work. Dr. Belinda Avalos, my co-advisor, who led me into science four years ago, has always been my mentor and friend. Her inspiration and trust made me believe that I can be a successful scientist. I am particularly grateful to her endeavors during the transition period before I joined my current lab.

I would like to thank my committee members, Dr. Jas Lang, Dr. John Robinson, and Dr. Chris Weghorst, for their support and insightful remarks. Their generosity of sharing lab equipment is greatly appreciated.

I want to thank all my previous and current lab members, Pam, Tammy, Huiqing, Latha, Trupti, Kishore, and Ruma, for their unselfish help and friendship, which made every day much more comfortable. Special thanks go to Drs. Melissa Hunter and Larry Druhan, who guided my way through the first two years, which was a great learning experience.

I am grateful to Dr. David Bisaro, Director of MCDB program, for his support during the difficult times. I also want to thank Jan Zinaich of MCDB program, whose efforts made my life as smooth as possible.
I want to say thank-you to all my colleagues on campus, especially Dr. Wenrui Duan, Dr. Weiguo Zhu, Dr. Tom Knobloch, Alan Bakaletz, Yijie Wang, and Dr. Shengying Bao, for kindly providing reagents or technique trainings. I also want to thank Dr. Dan Link, Dr. Gary Bokoch, and Dr. Stephane Schurmans for generously providing cell line, plasmids, and animal model.

Last, but not least, I would like to express my gratitude to my family. My parents have given me absolute love, trust and support throughout my study. Every tiny bit of accomplishment that I have ever achieved belongs to them. Gang, my dear husband and best friend, has certainly made me a better person. Irene, my beautiful daughter, has become my utmost motivation. She changed my life with magic. My gratitude also extends to my brother and my in-laws, for supporting me in their own ways.

This research was funded by grants from NIH/NCI and fellowship from The Ohio State University.
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<td>AML</td>
<td>acute myelogenous leukemia</td>
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<tr>
<td>ANC</td>
<td>absolute neutrophil count</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst-forming-unit erythroid</td>
</tr>
<tr>
<td>BMM</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CFU-E</td>
<td>CFU erythroid</td>
</tr>
<tr>
<td>CFU-Eo</td>
<td>CFU eosinophil</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>CFU granulocyte-erythroid-monocyte-megakaryocyte</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>CFU granulocyte-monocyte</td>
</tr>
<tr>
<td>CFU-me</td>
<td>CFU megakaryocyte</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromosome immunoprecipitation assay</td>
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<td>CML</td>
<td>chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CRH</td>
<td>cytokine receptor homology</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor-1</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>Epo</td>
<td>erythropoietin</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>G-CSFR</td>
<td>granulocyte colony stimulating factor receptor</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide exchange inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GMP</td>
<td>granulocyte/macrophage progenitor</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activating motif</td>
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<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>LF</td>
<td>lactoferrin</td>
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<td>LRG</td>
<td>leucine rich α2-glycoprotein</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<tr>
<td>MPO</td>
<td>meloperoxidase</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
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<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PBM</td>
<td>peripheral blood monocyte</td>
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<td>PH</td>
<td>pleckstrin homology</td>
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<td>PMN</td>
<td>polymorphonuclear cells</td>
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<tr>
<td>PRD</td>
<td>proline-rich domain</td>
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<tr>
<td>PtdIns3,4,5P₃</td>
<td>Phosphatidyl inositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin homolog deleted on chromosome Ten</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
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<tr>
<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TPO</td>
<td>thrombopoietin</td>
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<td>WCL</td>
<td>whole cell lysate</td>
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PART I

LRG-ACCELERATED DIFFERENTIATION DEFINES UNIQUE G-CSFR SIGNALING PATHWAYS IN MURINE GRANULOCYTES

CHAPTER 1

INTRODUCTION

Granulocytes and macrophages are two major cell types in innate immune system. They function as first-line host defense by recognizing, ingesting, and destroying foreign
particles and infectious organisms. In response to external stimuli, specific receptors on cell surface mediate a series of complex, yet finely tuned intracellular events, which play critical roles in the survival, proliferation, differentiation, and biological functions of these cells. In Part I we will discuss the role of a novel protein in the unique G-CSFR-mediated signaling pathways in murine granulocytes. The subsequent part will discuss the regulation of FcγR-mediated phagocytosis in murine macrophages.

1.1 Granulopoiesis

There are eight principal cell types in blood (Figure 1.1). Despite their diversity in morphology and function, they all have a limited life span and they are unable to replicate themselves. In order to keep the number of blood cells steady, a small pool of pluripotent hematopoietic stem cells in fetal liver and adult bone marrow must give rise to all the cell types through the process hematopoiesis. This is accomplished through exponential expansion of progenitor cells coupled with simultaneous lineage commitment and progressive terminal differentiation (Figure 1.1). Numerous cytokines have been found to play essential roles during hematopoiesis. While some of them have common stimulating effects on the progeny of many blood cell types, others have more specified actions on a single lineage, such as erythropoietin (Epo) and granulocyte-colony stimulating factor (G-CSF).
Figure 1.1 Schematic diagram of hematopoietic compartment structure. From reference 1.

Myeloid cells, including granulocytes and monocytes, arise from the common granulocyte/macrophage progenitors (GMPs), with the expression of CD34 and Fcγ receptor II/III\textsuperscript{2,3}. These progenitor cells in turn give rise to granulocyte-, monocyte-, and granulocyte/monocyte-colony forming units (CFU-G, CFU-M, and CFU-GM). G-CSF promotes the growth of CFU-G, macrophage-colony stimulating factor (M-CSF, CSF-1) enables the growth of CFU-M, and granulocyte/macrophage-colony stimulating factor (GM-CSF) or interleukin-3 (IL-3) stimulates CFU-G, CFU-M, and CFU-GM. Most of these cytokines support the proliferation of these early myeloid progenitor cells and have a limited ability to induce their differentiation and maturation. However, G-CSF is unique among the regulators of granulopoiesis in that it not only stimulates the proliferation but also potently induces the terminal maturation of myeloid progenitor cells to neutrophilic granulocytes. Specific cell surface receptors for G-CSF enable cells to respond to this cytokine. Details about G-CSF and G-CSF receptor (G-CSFR) will be discussed in 1.2.

There are three types of granulocytes: neutrophils, eosinophils, and basophils. Neutrophil granulocytes are the most common among these, constituting about 99% of the mature circulating polymorphonuclear cells (PMNs). Neutrophil maturation is accompanied by the appearance of distinct morphologic characteristics (Figure 1.2), which are often used to determine the differentiation stages. Myeloblast is the most immature type, with clearly visible nucleoli and a rim of cytoplasm without granules.
Figure 1.2 Morphologic characteristics during granulopoiesis. Maturation starts from myeloblast and ends with segment neutrophils. Alterations in nuclear morphology and cytoplasmic granules are most distinct.
Promyelocytes are considerably larger, and many azurophilic (primary) granules appear in the cytoplasm. Cell division ceases and secondary or specific granules appear in myelocytes. As the cells differentiate towards the terminal stages, changes in nuclear morphology become more apparent. The nucleus of a metamyelocyte is bean or kidney shaped, it turns to a narrower C or S shape in band cells, and finally becomes multilobulated with three to five segments.

The primary function of neutrophils is the phagocytosis and destruction of microorganisms. Granules contained within neutrophils are lysosome-like organelles that store bactericidal proteins and enzymes. These proteins are critical to the phagocytic function of neutrophils and are released upon neutrophil activation. Four distinct classes of neutrophil granules have been identified and are referred to as primary, secondary, tertiary granules, and secretory vesicles. Myeloperoxidase (MPO), neutrophil elastase (NE), azurocidin (AZU), proteinase 3 (PR3), and defensins localize to the primary granule compartment. Lactoferrin (LF) is the best characterized of the secondary or specific granule class. Gelatinase (GEL) is a key enzyme contained within tertiary or gelatinase granules. The presence of plasma proteins distinguishes secretory vesicles from other granule classes. Notably, the genes encoding the primary granule proteins AZU, PR3, and NE are organized as a gene cluster on chromosome 19p13.3, with each gene in the cluster having the same transcriptional orientation.
Granule proteins are sorted into their respective granule types by temporal regulation of their expression during neutrophilic granulocyte differentiation \(^4,^8\). The expression of myeloid-specific genes such as neutrophil granule genes is tightly regulated by several key transcription factors. Disruption of this transcriptional machinery has been shown to result in profound, sometimes even lethal hematopoietic abnormalities. Recurring chromosomal translocations involving transcription factors are common in human leukemias. Transcription factors, including PEBP2/CBF, the C/EBP family, the ets family protein PU.1, and c-Myb, regulate the expression of cytokine receptors and neutrophil granule enzymes \(^8,^9\). PU.1 activates transcription of multiple receptors including those for G-CSF and GM-CSF. PU.1 has also been shown to activate transcription of the primary neutrophil granule enzymes NE, AZU and PR3 \(^{10-12}\). PU.1 knock-out mice lack B lymphoid cells and monocytes and have greatly reduced neutrophil development \(^{13-16}\). Interestingly, it was recently reported that PU.1 promotes lymphopoiesis but restricts granulopoiesis in adult mice \(^{17}\). The C/EBP family of transcription factors includes C/EBP\(\alpha\) and C/EBP\(\epsilon\) \(^{18}\), both of which have been shown to critically regulate neutrophil production and function \(^{19-21}\). C/EBP\(\alpha\) is the predominant C/EBP family member in immature myeloid cells and appears to play an important role in regulation of early granulopoiesis \(^{20,22-24}\). C/EBP\(\alpha\) null mice exhibit a block in granulocytic differentiation at the myeloblast stage and also have undetectable G-CSF receptor protein \(^{21,23,24}\). Recent studies also indicated that C/EBP\(\alpha\) normally serves to
limit hematopoietic stem cell self-renewal, and to inhibit E2F activity and its target gene c-Myc \textsuperscript{25-27}. C/EBPε null mice have increased number of immature myeloid cells in their bone marrow and hypossegmented dysfunctional neutrophils \textsuperscript{18,20,23,24,28}, and its functions have also been related to the repression of E2F1 and the interaction with Rb \textsuperscript{29}. These observations underscore the importance of transcription factors in regulation of normal granulopoiesis as well as control on leukemogenesis.

Aberrant granulopoiesis may have significant clinical manifestations. Severe congenital neutropenia (SCN) is a disorder of ineffective neutrophil production (absolute neutrophil count [ANC] < 0.5×10\(^9\)/L) associated with recurrent infections and an increased risk for acute myelogenous leukemia (AML) \textsuperscript{30-33}. A majority of patients bear mutations in the ela2 gene encoding neutrophil elastase (NE) \textsuperscript{34,35}, most of which cluster around exons 4 and 5. A severe phenotype with an ANC typically less than 0.1×10\(^9\)/L, also associating with an increased risk for leukemic transformation, was shown to result from the substitution of glycine to arginine at position 185 (G185R) \textsuperscript{36}. It was recently shown that this mutant enzyme accelerates apoptosis of differentiating but not of proliferating cells \textsuperscript{37}. The G185R mutant is abnormally processed and localizes predominantly to the nuclear and plasma membranes rather than the cytoplasmic compartment observed with the wild-type enzyme. It also appears to alter the subcellular distribution and expression of adaptor protein 3 (AP3). These observations suggest that abnormal protein trafficking and accelerated apoptosis of differentiating
myeloid cells contribute to the severe SCN phenotype. On the other hand, an increasing body of evidence indicates that SCN is genetically heterogeneous. Mutations in the G-CSFR gene resulting in truncation of the carboxy-terminal region have been detected in the subset of patients with SCN transforming to AML. Although the absence of G-CSFR mutations in the majority of SCN patients and the presence of NE mutations in most patients (20% vs. 80%) suggest that NE mutations and not G-CSFR mutations cause SCN, G-CSFR mutations may still contribute to transformation of SCN to AML.

1.2 G-CSF and G-CSFR

The cloning and functional characterization of hematopoietic growth factors and their cognate surface receptors represent milestones in understanding the molecular basis of hematopoiesis. More importantly, these findings lead to the introduction of hematopoietic growth factor-based therapies, which contribute significantly to clinical hematology.

G-CSF was originally identified as a distinct activity that induced differentiation of the murine myelomonocytic leukemic cell line WEHI-3B+. This activity led to its purification and subsequent molecular cloning. G-CSF belongs to the cytokine class I superfamily, its structure is characterized by four antiparallel α-helices. Recombinant
human and murine G-CSF were found to be cross-reactive and capable of supporting the normal proliferation and neutrophilic differentiation in vitro\textsuperscript{48}.

Like other cytokines, G-CSF has pleiotropic effects on different cell types, including normal hematopoietic progenitor cells, myeloid leukemic cells and cell lines, and mature neutrophils, as well as some nonhematopoietic cell types, such as human vascular endothelial cells\textsuperscript{49}. G-CSF is indispensable for normal granulopoiesis in vivo. It induces a much higher peripheral blood (PB) granulocyte count than does either GM-CSF or IL-3\textsuperscript{50}. This potent granulopoietic effect has been reproduced in humans and has led to its widespread clinical application in the settings of chemotherapy-induced neutropenia and bone marrow transplantation (BMT)\textsuperscript{51-53}. The growth of granulocytic progenitor cells (CFU-G) in vitro is absolutely dependent upon the presence of G-CSF and sigmoidally increases as a function of increasing G-CSF concentration\textsuperscript{54-56}. G-CSF not only maintains a steady-state neutrophil production, but also acts as an “emergency” factor for augmenting granulopoiesis\textsuperscript{57-60}. In addition, G-CSF enhances neutrophil effector functions, such as release of arachidonic acid and production of leukocyte alkaline phosphatase and myeloperoxidase by mature neutrophils\textsuperscript{61-63}. It also primes neutrophils for enhanced superoxide anion production and antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{61,64}.

Mice lacking G-CSF due to targeted disruption of the G-CSF gene (\textit{gcsf}\textsuperscript{−/−}) were reported to develop congenital chronic neutropenia\textsuperscript{65}. Those mice were viable and
fertile, but their PB neutrophil levels were only 20%-30% of those in wild-type littermates. Granulocyte precursors were found to be reduced by 50% in the marrows of $gcsf^{-/-}$ mice. Heterozygous mice ($gcsf^{+/-}$) had intermediate neutrophil levels, suggesting a gene-dosage effect. In addition, the granulopoietic response to infection was severely impaired in G-CSF-deficient mice. These mice had a markedly impaired ability to control infection with *Listeria monocytogenes* and exhibited diminished increments in PB neutrophil and monocyte numbers and reduced infection-driven granulopoiesis, indicating that G-CSF also has an important role in regulating granulopoiesis in stress conditions.

Major clinical applications of G-CSF include the treatment of various forms of neutropenia, including SCN. G-CSF treatment ameliorates the neutropenia and associated infections in a large majority of cases $^{33,66,67}$. In addition, G-CSF is also capable of inducing the egress of hematopoietic stem and progenitor cells from the bone marrow into the peripheral blood, which has resulted in utilization of G-CSF in the mobilization and isolation of peripheral hematopoietic stem cells for transplantation purposes $^{33,66-68}$. Although still in debate, G-CSF is also believed to induce the mobilization of neutrophils from the bone marrow $^{69,70}$. The mechanism is not fully understood, but it has been suggested to involve multiple effector pathways, including proteolytic enzyme release, activation of chemokine receptors, and modulation of adhesion molecules $^{71,72}$. 

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The biological activities of G-CSF are mediated through specific receptors on the surface of responsive cells. The G-CSFR is expressed on myeloid progenitor cells, myeloid leukemia cells and cell lines, mature neutrophils, platelets, monocytes, and some T- and B-lymphoid cell lines. It has also been detected on several nonhematopoietic cell types, such as endothelial cells, placenta, trophoblastic cells and some small cell lung carcinoma cell lines. G-CSFR expression has been reported to be a marker of normal myeloid differentiation and is upregulated in immature myeloid cells in response to G-CSF, with mature human neutrophils displaying approximately 200 to 1000 receptors per cell. The human G-CSFR gene which encodes a 3.7 kb mRNA has been mapped to chromosome 1p32-35, while the gene for the murine G-CSFR to the distal region of mouse chromosome 4. The murine and human G-CSFR proteins are single transmembrane peptides of 812 and 813 amino acids, respectively, with an overall homology of 72.5% at the nucleotide level and 62.5% at the amino acid level. The molecular mass is 130 kD to 150 kD. In addition to the wild-type G-CSFR, at least six isoforms have been described, all of which are products of alternative mRNA splicing. Compared to wild-type G-CSFR, these alternate isoforms only have minimal expression level in bone marrow progenitor cells, suggesting that they do not have a significant role in normal myelopoiesis. However, overexpression of certain isoforms has been found in AML cases.
The G-CSFR is a member of the well-characterized hematopoietin receptor superfamily $^{87,88}$, which is structurally characterized by four highly conserved cysteine residues and a tryptophan-serine repeat (WSXWS) in the extracellular domain. Both motifs are located within the so-called cytokine receptor homology (CRH) region. G-CSFR contains a single extracellular, a transmembrane, and a cytoplasmic domain (Figure 1.3). The extracellular region has a composite structure containing an Ig-like domain, a CRH region, and three fibronectin type III domains. Data from crystallography studies of receptor/ligand complexes, epitope mapping with monoclonal antibodies, and alanine scanning mutagenesis have suggested that G-CSF and the G-CSFR form a 2:2 tetrameric complex $^{89}$. It appears that G-CSF binds to one site within the CRH domain, and to one site within the Ig domain $^{90}$, similar to that found in the IL-6/gp130 complex $^{91}$. The role of the fibronectin type III domains is not clear, although it is suggested that they may be involved in active receptor complex formation $^{92}$. The intracellular domain of the G-CSFR has limited sequence homology to other members within the superfamily. The membrane-proximal region of 53 amino acids of human G-CSFR, containing the conserved “box1” and “box2” motifs, has been shown to be sufficient for generating mitogenic signals $^{93}$. Within box1, there is a proline (P)-rich motif that is conserved in the members of the cytokine receptor family $^{94}$. The PXP sequence within this motif is essential for mitogenic signaling by the G-CSFR. Substitution of proline by alanine at amino acid positions 639 and 641 completely
Figure 1.3 Schematic diagram of human G-CSFR structure. The extracellular region (EX) contains an Ig-like domain, a CRH region with conserved cysteine (C) residues and the WSXWS motif, and three fibronectin type III (Fn3) domains. It has a single transmembrane domain (TM). Within the intracellular domain (ID), there are three conserved subdomains referred to as box1, box2, and box3. The proliferation and maturation domains are indicated. Numbers correspond to amino acid residues, and tyrosine (Y) residues are marked.
abolished growth signal transduction by the human G-CSFR. P→A substitutions of the corresponding residues in the murine G-CSFR also inactivated the mouse receptor for G-CSF-triggered growth signal transduction. Termination of the G-CSFR between the conserved box1 and box2 sequences was also reported to inactivate proliferative signal of G-CSFR. These observations indicate that both box1 and box2 are required for the transduction of proliferation signals, although they lack tyrosine residues. The carboxy-terminal region of 98 amino acids of human G-CSFR has been found to strongly inhibit mitogenic signaling in myeloid but not lymphoid cell lines. It is implicated that this region controls G-CSF-induced differentiation of myeloid progenitor cell lines and more recently also in the transduction of phagocytic signals in mature neutrophils. It is possible that the box3 subdomain plays a direct role in maturation induction. There are also four tyrosine residues within the cytoplasmic tail, at postitions 704, 729, 744, and 764 of human G-CSFR (equivalent to 703, 728, 743, and 763 in the murine G-CSFR), which can serve as possible docking sites for Src homology 2 (SH2)-containing proteins upon phosphorylation stimulated by G-CSF binding. The presence of distinct functional regions in the G-CSFR suggests the existence of multiple signaling pathways that elicit biological effects.

A rudimentary framework for signaling through the G-CSFR has emerged, which includes the canonical Jak/Stat pathway, the Ras/MAPK pathway, and the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway (Figure 1.4). The initial event
Figure 1.4 Schematic diagram of G-CSFR-mediated signaling pathways and their contribution to cellular responses of myeloid progenitor cells to G-CSF.
appears to be ligand-dependent receptor aggregation, although more recent data support a mechanism whereby ligand binding produces a conformational change in a preformed receptor dimer (or oligomer), and it is the conformational change itself that activates signal transduction\(^{101,102}\). One of the earliest events in the G-CSFR signaling cascade is activation of Jak kinases, which associate with the membrane-proximal cytoplasmic region of the receptor and become activated upon ligand binding\(^86,103\). This family has four members, Jak1, Jak2, Jak3, and Tyk2. These kinases, either alone or in conjunction with each other, appear to be responsible for the effects mediated by most cytokines and neurokines\(^{104,105}\). Jak activation leads to phosphorylation of STATs (signal transduction and activation of transcription), which subsequently dimerize by phosphotyrosine/SH2 interactions and translocate to the nucleus to activate target gene transcription. Among the different Stat family members, Stat1 is only weakly and transiently activated by G-CSF and it is considered redundant for granulopoiesis\(^{106-108}\). In contrast, Stat3 is mainly recruited to \(Y^{704}\) and \(Y^{744}\) of G-CSFR and is robustly activated by it\(^{106,109,110}\). Stat3 can also be activated via a tyrosine-independent route, which requires the membrane-distal region of the G-CSFR\(^{110,111}\). Overexpression of dominant negative forms of Stat3 resulted in a lack of growth arrest and a block in neutrophilic differentiation\(^{112,113}\). Importantly, following forced G1 arrest, those cells fully regained their ability to differentiate, suggesting that Stat3 is required for cell cycle exit, a prerequisite for myeloid differentiation, but not for execution of the differentiation program itself\(^{113}\).
Although transgenic mice with a targeted mutation of G-CSFR (d715F) that abrogates Stat3 activation displayed severe neutropenia with an accumulation of immature myeloid precursors in their bone marrow, the mutant receptor was also severely truncated in its cytoplasmic domain, possibly complicating the phenotype of this mouse. Instead, Stat3 may not be essential for in vivo neutrophil differentiation per se either, since it was recently reported that conditional knock-out mice with selective deletion of Stat3 or Stat3 deficiency in hematopoietic system are still producing functional neutrophils, or even exhibiting granulocytosis. Stat5 has also been implicated in G-CSFR-mediated proliferation and survival signal transduction.

Y\textsuperscript{764} of the G-CSFR plays a major role in proliferation signaling. Phosphorylated Y\textsuperscript{764} serves as a binding site for SH2 domains of Shc, which further recruits Grb2 and Sos to activate the Ras/MAPK signaling pathway. The Raf-Mek-Erk cascade is the major effector pathway downstream from Ras, and it is responsible for proliferative signaling in cell lines as well as in primary myeloid progenitor cells. It was reported that Stat3-null bone marrow cells displayed a significant activation of Erk1/2 under basal conditions, and the activation of Erk was enhanced and sustained by G-CSF stimulation, indicating that Erk activation might be the major factor responsible for inducing the proliferation of hematopoietic cells in response to G-CSF. The p38MAPK and Jun N-terminal kinase (JNK) are also controlled mainly via Y\textsuperscript{764}, although their roles in G-CSF signaling are still unclear. The PI-3K/Akt pathway also
contributes to G-CSF-induced survival and proliferation \(^{120,122,124,127,128}\). The Src kinase Lyn, which constitutively associates with the G-CSFR \(^{128}\), functions upstream of PI-3K through a mechanism involving c-Cbl and Shc \(^{129}\). It is suggested that Lyn associates with c-Cbl, which subsequently recruits the p85 subunit of PI-3K \(^{130-133}\).

Like other signaling pathways, the G-CSF signaling must be somehow downregulated. Negative regulation of G-CSF signaling is governed by multiple mechanisms, including dephosphorylation of signaling molecules by phosphatases, receptor endocytosis, and proteasomal targeting \(^{41}\). Mice with a mutation in the SH2 domain-containing protein tyrosine phosphatase SHP-1 exhibit aberrant regulation in several myeloid and lymphoid lineages, including substantial increases in the number of immature granulocytes \(^{134}\), indicating that SHP-1 is a negative regulator of granulopoiesis. It has also been suggested that intermediate signaling molecules may be involved in the recruitment of SHP-1 into the G-CSFR complex \(^{134,135}\). The SH2-containing inositol phosphatase SHIP-1 is also recruited to G-CSFR in an Y\(^{764}\)-dependent way \(^{119,127}\), and it is suggested that SHIP-1 is a negative regulator of growth factor-mediated PI-3K/Akt activation and survival of myeloid cells \(^{136}\). Suppressor of cytokine signaling (SOCS) proteins, especially SOCS3, is also involved in downregulation of G-CSFR-driven normal and emergency granulopoiesis by binding to G-CSFR Y\(^{729}\), and is under direct transcriptional control of Stats, in particular Stat3 \(^{137-141}\). Ligand-binding has been shown to trigger receptor endocytosis, internalization, and subsequent degradation.
intracellularly which effectively decreases the number of receptors on the cell surface and attenuates receptor signaling to protect cells from over-stimulation \textsuperscript{142-144}. Interestingly, increasing evidence suggests that receptor internalization and intracellular trafficking not only inhibit signaling, but may also be required for appropriate spatio-temporal activation of the full complement of signal transduction proteins \textsuperscript{145,146}.

A number of mutations or rare polymorphisms in the \textit{gcsfr} gene have been reported in myeloid disorders, such as previously described SCN, and less commonly in myelodysplasia (MDS) and \textit{de novo} AML. Mice carrying a targeted knock-in mutation (\textit{\(\Delta 715\)}) in their G-CSFR that reproduces the most commonly found G-CSFR mutation in patients with AML preceded by SCN (\textit{\(\Delta 716\)}) have been generated as an \textit{in vivo} model system to study the role of G-CSFR mutations in the pathogenesis of SCN and/or the development of AML \textsuperscript{147,148}. The mutation truncates the C-terminal tail that is required for growth arrest, differentiation signaling, and down-modulation of receptor expression. In these mice, the absolute number of G-CSF-responsive progenitors in the bone marrow was found to be increased and these progenitors had an increased proliferative response to G-CSF. These studies as well as the finding that G-CSFR mutations are acquired in humans with SCN transforming to AML provide strong evidence that these mutations are not responsible for SCN but likely contribute to transformation to AML. Very recently, a novel truncation mutation in the extracellular domain of the \textit{gcsfr} gene in a patient with SCN unresponsive to G-CSF therapy was reported. This mutation disrupts the WSXWS
motif after the first tryptophan and localizes to the same region of the G-CSFR where mutations were identified in two previous patients with SCN who were also unresponsive to G-CSF \(^{101,149,150}\), suggesting a common mechanism underlying G-CSF refractoriness in patients with SCN. An acquired G-CSFR mutation (C2390T) from another SCN patient was reported to result in increased proliferation of chronic myelomonocytic leukemia (CMML) cells \(^{151}\). These findings provide new insights into the basic mechanisms of G-CSFR processing and signaling, and the role of G-CSFR in normal granulopoiesis vs. malignant transformation.

1.3 Leucine-rich \(\alpha 2\) glycoprotein (LRG)

LRG was purified from human serum in 1977 \(^{152}\). The primary sequence of the purified protein was determined using Edman degradation, which revealed the presence of eight repeating 24 amino acid segments with a notable consensus sequence \(^{153}\). This 24 amino acid consensus sequence, termed the leucine-rich repeat (LRR), has since been identified in a large family of proteins \(^{154,155}\). LRRs are short protein modules characterized by a periodic distribution of hydrophobic amino acids, especially leucine residues, separated by more hydrophilic residues.

Experiments using a synthetic LRR peptide derived from the *Drosophila* Toll receptor suggested that groups of repeats may form amphipathic \(\beta\)-structures and these can form intermolecular \(\beta\)-sheets \(^{156}\), supporting the view that LRRs can participate in
protein-protein interactions. LRR-containing proteins are also frequently involved in signal transduction. LRR can serve as ligand binding region, as well as mediators of receptor dimerization or oligomerization. Such aggregation has been shown, for a number of receptor types, to correlate with their activation. These include the receptor tyrosine kinases (RTKs) and serine/threonine kinases. Ligand binding in these receptors causes a conformational change that favors dimerization of the extracellular domains so that the cytoplasmic regions can phosphorylate, and thereby activate, one another. Therefore, some LRR-containing receptors, such as gp150, may utilize LRR for this purpose. Certain LRR proteins may serve as adhesive receptors that play important roles in morphogenesis and development. The adhesive LRR proteins all have similar 24-amino acid LRRs with at least one conserved cysteine-rich flank. Many proteins with an LRR region also contain other modules which are implicated in intermolecular recognition, such as EGF repeats, fibronectin III repeats, zinc fingers, and leucine-zippers. It is thus plausible that in some cases one motif acts adhesively while others act in an “anti-adhesive” or repulsive fashion to prevent promiscuous interactions. Interestingly, many LRR proteins are implicated in nervous system development during which repulsive interactions are probably of widespread importance.

It has become increasingly clear that LRR proteins play a role in a wide range of cellular processes. However, the function of the founding member of this superfamily, LRG, remains largely unknown.
CHAPTER 2

CHARACTERIZATION OF LEUCINE-RICH α2 GLYCOPROTEIN (LRG) IN MURINE GRANULOCYTIC CELL LINES

2.1 Abstract

Leucine-rich α2 glycoprotein (LRG) was purified from serum more than 25 years ago, yet its function remains largely unknown. We have previously isolated cDNA and genomic clones for murine and human LRG, and showed that expression of LRG is upregulated during neutrophilic granulocyte differentiation. LRG expression appeared to follow the expression of myeloperoxidase but precedes lactoferrin expression in developing granulocytes. We also mapped the gene for human LRG to chromosome 19p13.3, the same region to which the genes for multiple neutrophil granule proteins also localize. Within the putative promoter region of the human LRG gene, we have identified potential binding sites for the PU.1 and C/EBP transcription factors. In order
to further characterize the physiological roles of LRG, we stably transfected the murine LRG cDNA tagged with a V5 epitope into 32Dcl3 cells. 32Dcl3 cells do not constitutively express LRG but upon neutrophilic granulocyte differentiation in response to G-CSF, expression of LRG is induced. Results from immunofluorescent staining and multiphoton confocal microscopy indicate that LRG resides in the cytoplasmic compartment in a diffuse granular pattern that overlaps with the pattern observed for myeloperoxidase. This would suggest that LRG localizes to primary or azurophilic neutrophil granules. We next made stable murine LRG transfectants in another 32D subline, the 32Dwt18 cell line. 32Dwt18 cells are stably transfected with a chimeric G-CSFR in which the extracellular domain of the G-CSFR has been replaced with the extracellular domain of the erythropoietin (Epo) receptor. In response to Epo, 32Dwt18 cells differentiate into neutrophils that appear normal by morphology. Epo-treated 32Dwt18 cells do not undergo massive cell death following initial transfer from IL-3- to Epo-containing media as observed with 32Dcl3 cells. Similar to 32Dcl3 cells, 32Dwt18 cells do not constitutively express LRG, but treatment with Epo to stimulate neutrophil differentiation induces the expression of LRG. Results from nitrogen cavitation confirmed that LRG co-localizes with myeloperoxidase in both proliferating and differentiating cells. Using chromatin immunoprecipitation (ChIP) assay, both PU.1 and C/EBPε were observed to bind at very low levels to the LRG promoter in parental 32Dwt18 cells and treatment with Epo to induce neutrophil differentiation resulted in
increased binding of both transcription factors. Our cumulative data suggest that LRG may be a novel neutrophil granule protein that is also regulated by PU.1 and C/EBPs.

2.2 Introduction

Leucine-rich α2 glycoprotein (LRG) was purified from human serum almost three decades ago\textsuperscript{152}, establishing a novel family of proteins characterized by the presence of leucine-rich repeats in their amino acid sequences. Although several family members have been shown to be involved in signal transduction, protein-protein interaction or cell adhesion and development\textsuperscript{154}, the function for LRG has remained elusive.

Considerable efforts have been put in elucidating the mechanisms by which G-CSF induces granulocytic differentiation. The murine interleukin 3 (IL-3)-dependent 32Dcl3 cell line was established from normal murine dipoid bone marrow cells\textsuperscript{161}. Replacement of IL-3 with recombinant human G-CSF (rhG-CSF) triggers an initial proliferation for 4-5 days followed by growth arrest and apparent neutrophil-like morphology by 12 days (Figure 2.1A). It has also been demonstrated that 32Dcl3 cells acquire mature neutrophil functions during terminal differentiation\textsuperscript{162}, suggesting that this cell line can serve as an effective model system. Therefore, it has been extensively used to study signaling for myeloid proliferation and differentiation\textsuperscript{122,163}. Although 32Dcl3 cell line is the ideal in\textit{vitro} model to study granulopoiesis, it has also been observed that there is overwhelming cell death within the initial IL-3 removal and G-CSF induction, which proved difficult to
study the induced 32Dcl3 cells. To overcome this problem, 32Dwt18 cells (an IL-3-dependent subline of 32Dcl3) have been used instead. This cell line carries a chimeric form of the G-CSFR containing the extracellular domain of the Epo receptor and the transmembrane and cytoplasmic signaling domain of the G-CSFR (Figure 2.1B). These cells differentiate in response to Epo and express secondary granule protein lactoferrin (LF) at a level comparable to that seen with the 32Dcl3/G-CSF system. The main advantage is that constitutive cytokine receptor expression prevents the high rate of cell death encountered upon induction of 32Dcl3 with G-CSF.

To identify genes induced by G-CSF during neutrophilic granulocyte differentiation, we have previously used cDNA representational difference analysis (RDA) to generate a representational cDNA library enriched for neutrophil-specific transcripts from G-CSF-treated 32Dcl3 cells. Several clones were obtained and confirmed to be differentially expressed in G-CSF treated cells by Northern blot analysis. Further characterization of one of the positive clones revealed its identity as LRG. 32Dcl3 cells do not constitutively express LRG, but LRG expression is induced upon G-CSF treatment, and its expression preceded the expression of LF and gelatinase but followed myeloperoxidase (MPO). LRG transcripts were also detected in human neutrophils and progenitor cells but not in peripheral blood mononuclear cells. Notably, LRG expression was upregulated during neutrophilic differentiation but downregulated during monocytic differentiation. The human LRG gene was localized to chromosome 19p13.3, a region to which the genes
Figure 2.1 Cell lines to study granulocytic differentiation. (A) 32Dcl3 cells are maintained in IL-3-containing media, and terminally differentiate into mature neutrophils after transfer to IL-3-free and G-CSF-containing media. (B) 32Dwt18 cells are stably transfected with a chimeric form of G-CSFR. These cells differentiate along the granulocytic lineage after removal of IL-3 and induction with Epo.
for several neutrophil granule enzymes also map. The putative promoter region of LRG was found to contain consensus-binding sites for PU.1, C/EBP, and Stat transcription factors, which are essential for commitment and differentiation to the neutrophil granulocyte lineage and which also regulate the expression of neutrophil granule proteins. Collectively, these data suggest that LRG is a novel marker for early neutrophilic granulocyte differentiation. In this chapter, we have further characterized LRG using stable transfectants from both 32Dcl3 and 32Dwt18 cells.

2.3 Materials and methods

Cells, antibodies, and reagents

G-CSF responsive murine 32Dcl3 cell line was kindly provided by Dr. Giovanni Rovera (The Wistar Institute, Philadelphia, PA), and were maintained in RPMI-1640 medium supplemented with 10% FBS and 10% WEHI 3B conditioned medium, as a source of IL-3. The 32Dwt18 cells was a generous gift from Dr. Dan Link (Washington University, St Louis, MO), and were maintained in IMDM medium supplemented with 10% FBS and 10% WEHI 3B conditioned medium. The vectors pcDNA3.1D/V5-TOPO in frame with the V5 epitope tag and pcDNA6/HisB were purchased from Invitrogen (Carlsbad, CA). G418 and blasticidin were from Invitrogen. Precast Bis-Tris acrylamide gels and MOPS running buffer were purchased from Invitrogen. Anti Raf-1 antibody and a goat anti-human MPO antibody (cross reactive with mouse) were
purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to PU.1, C/EBPα, C/EBPε, and a normal rabbit IgG used in ChIP assay were purchased from Santa Cruz Biotechnology. A rabbit polyclonal antibody to the p85 subunit of PI-3K was purchased from Upstate Biotechnology (Lake Placid, NY). Protein G-agarose and anti-V5 tag antibody were from Invitrogen. TRITC-conjugated donkey anti-mouse F(ab)’₂ and Cy5-conjugated donkey anti-goat F(ab)’₂ antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hoechst solution and ProLong anti-fade mounting media were from Molecular Probes (Eugene, OR). Trizol, Superscript II reverse transcriptase and Taq polymerase were from Invitrogen. Cocktail protease inhibitors were purchased from Roche (Indianapolis, IN). The bicinchoninic acid (BCA) reagent was from Pierce (Rockford, IL). Enhanced chemiluminescence (ECL) reagent was from Amersham (Piscataway, NJ). Purified recombinant human G-CSF (rhG-CSF) was generously provided by Amgen Inc. (Thousand Oaks, CA), and purified human erythropoietin (Procrit, Epo) by Ortho Biotech Inc. (Bridgewater, NJ).

Stable transfection

Murine LRG (mLRG) cDNA was cloned into the pcDNA3.1D/V5-TOPO vector and transfected into 32Dcl3 cells by electroporation (300V, 960μF, single pulse). mLRG cDNA in frame with the V5 epitope tag was also cloned into the pcDNA6/HisB vector, and transfected into 32Dwt18 cells by electroporation as described above. Clones
expressing mLRG were selected by growing them in media containing either G418 (300μg/ml, for transfected 32Dcl3 cells) or blasticidin (10μg/ml, for transfected 32Dwt18 cells) 48 hours after transfection. Expression of mLRG mRNA in positive clones was confirmed by RT-PCR. Western blotting and immunoprecipitation assay (IP) were used to confirm the expression of mLRG protein.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified with Trizol and subsequently incubated with Superscript II transcriptase to generate cDNA. PCR with gene-specific primers was performed using Taq DNA polymerase at a final MgCl$_2$ concentration of 2mM. The PCR products were size-fractionated on agarose/TAE gels (1% w/v) and visualized with ethidium bromide staining. The primers used are listed below:

Endogenous mLRG:  
Forward: 5’-TCAAGGAAGCCTCCAGGATCTC-3’;  
Reverse: 5’-GACTCCAGCAGGTTGTACCCAAG-3’

Ectopic mLRG:  
Forward: 5’-AGTACCCTTCACCATGGTC-3’;  
Reverse: 5’-GACTCCAGCAGGTTGTACC-3’

Murine MPO:  
Forward: 5’-AACCAGCTGGGGCTGGCTTGCTGTAATAC-3’;  
Reverse: 5’-AACTCCAGGTTCCTTCAGCACCCTGCGC-3’

Murine LF:  
Forward: 5’-GCCAGTCACAGGAGAAGTTTGG-3’;  
Reverse: 5’-GCCATTTGCTTTTGAGAGATTC-3’
**Immunoprecipitation assay (IP) and Western blotting**

Cells (2×10^7) were lysed in lysis buffer (1.5% triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.1 mM sodium deoxycholate, 500 mM NaCl, 5 mM EDTA, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH 7.8) containing a cocktail of protease inhibitors. A total of 150 μg protein was incubated with 2 μg anti-V5 antibody and 30 μl protein G-agarose beads overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2000×g at 4°C, washed 3 times with IP buffer (10% glycerol, 100 mM KCl, 5 mM MgCl_2, 50 mM Tris, pH 8), and resuspended in reducing lithium dodecyl sulfate (LDS) sample buffer. The samples were then heated at 70°C for 10 min, resolved on precast 10% Bis-Tris acrylamide gels using a morpholinepropanesulfonic acid (MOPS) running buffer, and transferred to nitrocellulose. For Western blotting, 50 μg of protein WCL was separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the respective primary antibodies, followed by corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were visualized using the ECL reagent.

**Cytoplasmic and nuclear extraction**

Cytoplasmic and nuclear extraction was performed following the protocol for NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL), using buffers supplied in the kit. Briefly, 32Dcl3 cells stably transfected with V5-tagged mLRG cDNA...
were washed out of IL-3-containing media, and transferred to G-CSF (10ng/ml)-
containing media. At different time point after transfer, 6x10^6 cells were spun down,
resuspended in Cytoplasmic Extraction Reagent I (CER I), and incubated on ice for 10
min. The samples were then mixed thoroughly with Cytoplasmic Extraction Reagent II
(CER II), and centrifuged at 16,000g for 5 min. The resulting supernatant fraction
(cytoplasmic extraction) was separated from the pellet containing nuclei. The pellet was
resuspended in Nuclear Extraction Reagent (NER), incubated on ice for 40 min, and then
centrifuged at 16,000g for 10 min. The resulting supernatant fraction (nuclear extraction)
was removed and kept on ice. Cytoplasmic and nuclear fractions were next subjected to
Western blot analysis.

**Immunofluorescence staining and confocal microscopy**

A total of 5x10^4 32Dcl3 cells per clone were spun onto non-charged slides, and fixed
in 4% formaldehyde. The fixed cells were washed in PBS, and then permeabilized with
1% TX-100 in TBS containing 0.1% sodium azide for 30 min at room temperature. To
minimize non-specific antibody binding, the slides were incubated with 1% BSA in TBS
for 1 hr at room temperature. To detect expression of the V5-tagged mLRG protein, a
murine anti-V5 antibody was used as the primary antibody in conjunction with the
TRITC-conjugated donkey anti-mouse F(ab')2 secondary antibody. To detect mouse
myeloperoxidase, goat anti-human myeloperoxidase antibody (cross reactive with mouse)
and donkey anti-goat F(ab)’2 antibody conjugated with Cy5 were used. Hoechst was used for nuclear staining. ProLong anti-fade mounting media was placed on each slide after staining. Slides were examined under confocal microscopy using a Zeiss LSM510 multiphoton confocal microscope (Zeiss, Oberkochen, Germany). Zeiss LSM5 Image software was used for image processing.

**Subcellular fractionation**

Subcellular fractionation of 32Dwt18 transfectants was performed as described. Briefly, cells were washed with PBS, resuspended in cold KRG buffer (130mM NaCl, 5mM KCl, 1.27mM MgSO₄, 0.95mM CaCl₂, 5mM Glucose, 10mM NaH₂PO₄/Na₂HPO₄, pH 7.4, 5mM PMSF), and incubated on ice for 5 min. Cells were then spun down, resuspended at 10⁸ cells/ml in disruption buffer (100 mM KCl, 3mM NaCl, 3.5mM MgCl₂, 10mM Piperazine N,N′-bis2 [ethan-sulfonic acid], pH 7.2, 1mM ATPNa₂, 0.5mM PMSF) with one complete mini protease inhibitor cocktail tablet, and disrupted by nitrogen cavitation (pressurized under nitrogen for 5 min at 380 psi). The cavitate was collected drop-wise into disruption buffer containing a final concentration of 0.5mM EGTA. Nuclei and intact cells were pelleted by centrifugation at 400×g for 15 min. The postnuclear supernatant was applied onto a three-layer Percoll gradient solution (densities 1.050, 1.065, and 1.090 g/ml), and was centrifuged at 37,000×g for 30 min at 4°C. After centrifugation, six fractions of 5ml each were collected. Percoll was removed by
ultracentrifugation at 100,000×g for 90 min. Biological materials above the Percoll pellet were collected, and were next subjected to Western blot analysis.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed following the protocol for the acetyl-histone H4 ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY), using buffers supplied in the kit. Briefly, 5×10^6 cells were cross-linked with 1% formaldehyde for 30 min at 37°C. Prior to cross-linking, an aliquot of the cells was removed for analysis of input chromatin DNA. After cross-linking, cells were washed twice in cold PBS, resuspended in lysis buffer, and incubated on ice for 10 min. The samples were then subjected to sonication to shear protein-cross-linked DNA into fragments of 1-3 kb, and diluted with IP buffer. After pre-clearing with a salmon sperm DNA/protein A agarose slurry, samples were then incubated with antibodies to either PU.1 (4 μg), C/EBPα (8 μg), C/EBPε (4 μg), or with normal rabbit IgG (4 μg) at 4°C overnight. Samples containing no antibody were also included as controls. After sequential washing with low salt, high salt, LiCl and TE buffers, the protein-DNA complex was eluted with freshly made elution buffer (1% SDS, 0.1M NaHCO₃). Cross-linking was then reversed by addition of 5M NaCl at 65°C for 4 hrs. After protease K treatment, DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. Equal volumes of DNA products were used as PCR templates. Primers were designed to specifically amplify the sequences within the
mLRG promoter corresponding to consensus binding sites for C/EBPα, C/EBPε and PU.1 (nt. 794-990, 197 nt product). The primer sequences are listed below:

Forward: 5’-AATCCCCACCTCACCCCTTAATG-3’
Reverse: 5’-CTATGTCTGTCCTCAAGACCCAG-3’

2.4. Results

Constitutive expression of murine LRG in 32Dcl3 cells

Our preliminary data have shown that the expression of LRG is upregulated during G-CSF-induced granulocytic differentiation. The gene for human LRG was mapped to the same chromosome region to which the genes for multiple neutrophil granule proteins also localize. Within the putative promoter region of the human LRG gene, potential binding sites for the PU.1 and C/EBP transcription factors were identified. These results suggest that LRG might be a novel neutrophil granule protein and a differentiation marker. To further characterize the physiological roles of LRG in neutrophilic differentiation, we cloned the murine LRG cDNA into pcDNA3.1D/V5-His-TOPO vector, and stably transfected the mLRG tagged with a V5 epitope into the murine IL-3-dependent 32Dcl3 cell line using electroporation. 48 hours after transfection, cells were transferred to selective media containing G-418. Single clones were obtained by limiting dilution. The expression of the transfected LRG protein was confirmed by Western blotting using an anti-V5 tag antibody (Figure 2.2B) and immunoprecipitation
assay using the same antibody (Figure 2.2C). Whole cell lysate from mLRG transfected Cos-7 cells was used as a positive control. The transfected LRG protein appeared as a single band of approximately 45 kDa on SDS-PAGE. Three single clones with equivalent high expression level were used for the following experiments. A mixpool from three single clones transfected with empty vector was used as control.

**LRG resides within the cytoplasmic compartment in 32Dcl3 cells**

To begin to study the subcellular localization of LRG, we performed cytosol/nuclear separation. Immunoblotting analysis of cytoplasmic and nuclear extracts purified from 32Dcl3 transfectants with an anti-V5 antibody that recognizes the ectopic LRG detected a band of appropriate size that is present exclusively in the cytoplasmic fraction (Figure 2.3, upper panel, lanes 1 and 4). To confirm the purity of the cytoplasmic and nuclear extracts examined and to control for equal protein loading, the same blots were stripped and re-blotted with antibodies to Raf-1 and the p85 subunit of PI-3K, respectively (Figure 2.3, middle and lower panels). The Raf-1 kinase is the entry point to the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) signaling pathway, and it has been shown to locate to the cytoplasmic compartment only

167 The PI-3K, consisting of a p110 catalytic domain and a p85 regulatory domain, is tightly linked to signaling mediated by growth factor receptors. Although its function requires largely a cytoplasmic localization, several reports provided evidence that the
Figure 2.2 Stable transfection of mLRG into 32Dcl3 cells. (A) pcDNA3.1D/V5-His-TOPO vector used for transfection. (B) Positive clones after G-418 selection were further screened for LRG expression. Whole cell lysates were separated on SDS-PAGE analyzed by immunoblotting with an anti-V5 antibody. A mixpool of three single clones transfected with empty vector was used as negative control. WCL from mLRG-transfected Cos-7 cells was used as positive control. The transfected LRG was shown to be a single band of about 45 kDa. (C) Results from (B) were further confirmed by immunoprecipitation with anti-V5 antibody.
p85-associated PI-3K can translocate to the cell nucleus\textsuperscript{168,169}. Interestingly, LRG was also found to rapidly translocate to nucleus in response to G-CSF. 4 hrs after transfer from IL-3- to G-CSF-containing media, LRG could be detected in nuclear fraction (Fig 2.3, upper panel, lane 5), and its level increased after 8 hrs culture in G-CSF (Fig 2.3, upper panel, lane 6).

To confirm the nuclear/cytosol extraction results, we examined the intracellular localization of constitutively expressed V5-tagged LRG and endogenously expressed MPO in 32Del3 transfectants by immunofluorescence staining and multiphoton confocal microscopy. In LRG clones, LRG (green, Figure 2.4A) and MPO (red, Figure 2.4B) were detected by anti-V5 and anti-MPO primary antibodies, which were bound to secondary antibodies conjugated with TRITC and Cy5, respectively. Nuclei were stained with Hoechst (blue, Figure 2.4C). LRG was found to localize to the cytoplasmic compartment in a diffuse granular pattern that overlaps with the pattern observed for MPO (Figure 2.4D). Cells transfected with empty vector alone displayed MPO staining only, although both antibodies were used (Figure 2.4E). When stained with secondary antibodies only, LRG clone displayed nuclear staining with a clear background (Figure 2.4F). These results suggest that LRG may localize to the primary granules together with MPO.
Figure 2.3 Cytoplasmic localization of LRG and its rapid translocation to nucleus in response of G-CSF. Cytoplasmic and nuclear fractions were isolated from 32Dc13 cells stably transfected with a V5-tagged murine LRG cDNA using the NE-PER Nuclear and Cytoplasmic Extraction Kit. The purified fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody to V5 that recognizes the V5-tagged LRG (upper panel). To confirm that the nuclear fraction was free of cytoplasmic protein contamination, the membrane was stripped and reblotted with a rabbit polyclonal antibody to Raf-1 (middle panel). The membrane was subsequently stripped and rebotted with a rabbit polyclonal antibody to the p85 subunit of PI-3K, which is found in both nucleus and cytosol (lower panel). LRG was found to reside within the cytoplasmic compartment without G-CSF treatment. In response to G-CSF, however, LRG rapidly (after 4 hrs) translocates to nucleus.
Figure 2.4 LRG exhibits the same subcellular localization as that of MPO. 32Dc13 cells stably transfected with mLRG or vector alone were cytospun onto slides and fixed with 4% formaldehyde. LRG and MPO were detected by mouse monoclonal anti-V5 and goat anti-human MPO primary antibodies, which were detected using TRITC-conjugated donkey anti-mouse IgG and Cy5-conjugated donkey anti-goat IgG secondary antibodies, respectively. Nuclei were stained with Hoechst. (A) Cellular localization of V5-LRG in LRG clone. (B) Cellular localization of MPO in LRG clone. (C) Nuclear staining with Hoechst in LRG clone. (D) Merged figure of A, B and C. (E) Cells transfected with empty vector alone stained positively for MPO only. (F) LRG clone stained with secondary antibodies only.
Constitutive expression of murine LRG in 32Dwt18 cells

We were interested to follow up with the confocal immunofluorescence microscopy study to examine the localization of ectopic LRG in differentiated cells. However, after initial transfer from IL-3- to G-CSF-containing media, there was a massive cell death (Figure 2.5A). The cell viability decreased by more than 85% within 24 hours (Figure 2.5B), even with high concentration of G-CSF. The surviving cells slowly recovered, and subsequently underwent terminal differentiation into mature neutrophils. Notably, LRG clones displayed higher viability compared to cells transfected with vector alone despite the massive cell death (Figure 2.5B), suggesting that constitutive expression of LRG provides a survival advantage. Still, the massive cell death after initial transfer to G-CSF-containing media proved to be problematic.

To overcome this, we stably transfected the murine LRG cDNA tagged with a V5 epitope into the 32Dwt18 cells by electroporation, and used this subline as an alternative model system for our studies. Like 32Dcl3 cells, 32Dwt18 cells do not constitutively express LRG, but its expression is induced in response to Epo along with other neutrophilic granulocyte differentiation markers (Figure 2.6), but without the massive cell death observed in 32Dcl3 cells (Figure 2.7). Positive clones from transfection were selected in blasticidin-containing media, and single clones were obtained by limiting dilution. The expression of LRG protein was verified by Western blotting (Figure 2.8), using WCL from mLRG-transfected Cos-7 cells as positive control. Three single clones
Figure 2.5 Massive cell death after initial transfer to G-CSF-containing media in stably transfected 32Dcl3 cells. Cells stably transfected with vector alone (black, triangles) or mLRG cDNA (grey, squares) were washed out of IL-3 and put in G-CSF-containing media. Cell viability was monitored using trypan blue exclusion. (A) Total live cell numbers at different time points. (B) Cell viability at different time points. Data from three independent experiments were shown.
Figure 2.6 Epo-induced differentiation of 32Dwt18 cells. Parental 32Dwt18 cells were removed out of IL-3-containing media and transferred to Epo-containing media (1μ/ml) for indicated time. At each time point, mRNA was isolated and reverse transcription-PCR was performed with specific primer sets designed for endogenous LRG, granulocytic differentiation markers MPO and LF, and the house-keeping gene GAPDH. +RT: with reverse transcriptase; -RT: without reverse transcriptase.
Figure 2.7 Better survival after initial transfer to differentiation media in 32Dwt18 cells. Cells stably transfected with vector alone (black, triangles) or mLRG cDNA (grey, squares) were washed out of IL-3 and put in Epo-containing (1u/ml) media. Cell viability was monitored using trypan blue exclusion. No massive cell death was observed as seen in 32Dcl3 cells. (A) Total live cell numbers at different time points. (B) Cell viability at different time points. Data from three independent experiments were shown.
Figure 2.8 Stable transfection of mLRG into 32Dwt18 cell line. (A) pcDNA6/HisB vector used for transfection. (B) Positive clones after blasticidin selection were further screened for LRG expression. Whole cell lysates were separated on SDS-PAGE and analyzed by immunoblotting with an anti-V5 antibody. A mixpool of three single clones transfected with empty vector was used as negative control. WCL from mLRG-transfected Cos-7 cells was used as positive control.
with equivalent high LRG expression levels were used for the following experiments. A mixpool of three single clones transfected with empty vector was used as negative control.

**LRG has the same subcellular distribution with MPO in 32Dwt18 cells**

To further confirm that LRG indeed localizes to a granular compartment within the cytoplasm of myeloid cells that also corresponds to the same compartment to which MPO is targeted, namely, the primary neutrophil granules, we analyzed different subcellular fractions from the 32Dwt18 transfectants. Using standard methods employing nitrogen cavitation and Percoll density gradient centrifugation, we isolated six continuous subcellular fractions from these transfectants for analysis by Western blotting with antibodies to MPO and V5. Both LRG and MPO were found to localize to the same fractions, mainly fractions 3-5 (Figure 2.9A). Notably, when these cells were cultured in Epo-containing media for seven days to induce differentiation and maturation, LRG, together with MPO, was found to localize more to the lower density fractions, mainly fractions 4-6 (Figure 2.9A). Cells transfected with vector alone was used as a control, showing the localization of MPO only (Figure 2.9B). Thus, our preliminary data suggest that LRG localizes to the subcellular fractions corresponding to the primary neutrophil granules. These findings, along with results from confocal imaging studies and
Figure 2.9 Analysis of subcellular fractions from 32Dwt18 transfectants. 32Dwt18 cells stably transfected with (A) V5-tagged mLRG (undifferentiated and differentiated in Epo) or (B) with empty vector only (undifferentiated) were resuspended in disruption buffer, and disrupted by nitrogen cavitation. The nuclei and intact cells were pelleted by centrifugation, and the resultant postnuclear supernatants were loaded onto Percoll gradients. After centrifugation, six continuous fractions from bottom to top were collected. Following ultracentrifugation to remove Percoll, the biological materials were collected, and were then subjected to SDS-PAGE analysis. Samples were immunoblotted with an antibody to the V5 epitope or murine MPO as indicated.
cytoplasmic/nuclear extraction analysis, suggest that LRG, like MPO, is targeted to the primary (azurophilic) granule compartment within the cytoplasm of myeloid cells.

**LRG expression is regulated by PU.1 and C/EBP transcription factors**

Within the putative promoter region of LRG, consensus binding sequences for PU.1 and C/EBP are present. To determine whether these sequences are functional binding sites for these transcription factors, we performed chromatin immunoprecipitation (ChIP) assays on parental 32Dwt18 cells. As shown in Figure 2.10, a low basal level of binding of both PU.1 and C/EBP to the amplified sequence within the putative LRG promoter could be detected (Figure 2.10, lanes 5 and 9) which increases significantly after three days of culture in Epo-containing media to induce differentiation (Figure 2.10, lanes 6 and 10). In contrast, no significant changes in binding of C/EBPα to the same region in the LRG promoter was observed during the same time period following transfer to Epo-containing media. As negative controls, reactions were included in which either no antibody (Figure 2.10, lanes 1 and 2) or normal rabbit IgG (Figure 2.10, lanes 3 and 4) was added. As expected, no PCR products from either untreated or Epo-treated cells were detected. As an additional negative control, PCR reactions were also performed in the absence of input DNA (Figure 2.10, lane 11). As a positive control, isolated DNA (Input, Figure 2.10, lane 12) was subjected to PCR amplification prior to immunoprecipitation using the same primers.
Figure 2.10 Chromatin immunoprecipitation assay (ChIP) indicated functional binding sites for PU.1 and C/EBPε within the promoter region of mLRG gene. 32Dwt18 cells were cross-linked with 1% formaldehyde, washed and resuspended in lysis buffer. Samples were sonicated to shear the protein-DNA complexes and immunoprecipitated with antibodies for PU.1, C/EBPα and C/EBPε. The complexes were washed, eluted, and the cross-linkage was reversed. Isolated DNA was then subjected to PCR using primer sets specific for the consensus binding sites of PU.1, C/EBPα and C/EBPε within the putative promoter region of mLRG gene. Normal rabbit IgG was used as a negative control. No-antibody control and input DNA control were also included.
2.5 Discussion

Although LRG was first identified almost three decades ago and is the founding member of the LRR superfamily, its functions have never been studied thoroughly. It is interesting, therefore, to find that the expression of LRG is upregulated during neutrophilic granulocyte differentiation. This finding led to the subsequent isolation of the genomic and cDNA clones of human and murine LRG\(^{165}\).

The gene for human LRG localizes to the same chromosome region to which genes for multiple primary neutrophil granule enzymes also localize, suggesting that LRG might be another primary granule protein. Lack of commercially available antibodies to LRG has hindered the determination of its cellular localization. We have thus stably transfected a V5-tagged LRG cDNA into either 32Dcl3 or 32Dwt18 cells, so that specific antibody to V5 epitope tag could be utilized. Cytoplasmic and nuclear extraction experiments clearly showed that the presence of LRG was strictly limited to the cytoplasmic fraction in unstimulated 32Dcl3 transfectants. Immunofluorescence staining and confocal microscopy results indicated that both LRG and MPO have similar diffuse granular distribution patterns within the cytoplasmic compartment, suggesting that LRG localizes to primary neutrophil granules. Subcellular fractionation experiments further confirmed the co-localization of both LRG and MPO within the same subcellular fractions. Notably, LRG in differentiated cells tends to move to fractions of lower density, indicating its progression on the post-synthesis modification and secretion
pathways\textsuperscript{166}, which is possibly related to its functions in neutrophil maturation. A comparison between the distribution profiles of LRG and that of markers for secretory vesicles (such as latent alkaline phosphatase or albumin) should clarify this issue. In addition, this result suggests that LRG might be secreted, which is consistent with the notion that LRG was originally identified as a serum protein\textsuperscript{152}. Collectively, these data suggest that LRG localizes to the primary neutrophil granules, and like several other primary neutrophil granule proteins it may be released upon neutrophil degranulation.

Heparin-binding protein (HBP, also termed azurocidin or CAP37), another primary neutrophil granule protein that belongs to a family of serine proteases, was also recently shown to localize to secretory vesicles in addition to primary granules\textsuperscript{170}. In human mature neutrophils, it was shown that approximately 74\% of the HBP is stored in the azurophil granules, whereas the remaining part is divided between the secretory vesicles (18\%) and the plasma membrane (8\%). HL-60 cells, on the other hand, contained less HBP than mature neutrophils, and the low amount of HBP appeared to be exclusively stored in azurophil granules. It is plausible that LRG, similar to HBP, has dual localization. In undifferentiated 32Dcl3 cells, LRG was produced and stored in primary granules. When its expression is upregulated during differentiation and maturation, however, LRG could also be targeted to other compartments, such as the secretory vesicles. This dual localization may imply that LRG has multiple functions in granulopoiesis.
One interesting finding was that LRG rapidly translocates to nucleus upon G-CSF stimulation. Nuclear protein import is known to be regulated by cellular signal transduction pathways. Signal molecules, such as Stat proteins, become phosphorylated within cytoplasm in response to cytokine receptor activation, translocate to the nucleus to bind to target gene promoters and thus activate their transcription. LRG might also be a molecule involved in signaling transduction (discussed in Chapter 3), which requires its transportation between cytoplasm and nucleus. The mechanism of this transportation is still unknown. Although LRG expression is upregulated in response to G-CSF, it is unlikely that the over-expressed protein could just passively diffuse and pass through the nuclear pore complexes (NPC) and appears within nucleus, since LRG is found to have a granular localization. On the other hand, specific nuclear localization signal (NLS) has not yet been identified in LRG. Conventional signal-dependent nuclear protein import requires cytosolic NLS-binding receptor importin α/β heterodimer, the monomeric GTP-binding protein Ran, Ran GAP1 (Ran GTPase activating protein), the guanine nucleotide exchange factor RCC1, and ATP. β-catenin can translocate to the nucleus through its Armadillo repeats, bypassing the requirement for cytosolic receptor, but still need ATP and Ran. Another pathway has been found in the nuclear import of granzymes A and B during cytotoxic T lymphocyte (CTL)-mediated apoptosis of target cells. The Granzymes are released from CTL by exocytosis, and then enter target cells and translocate to nucleus. This nuclear uptake is independent of ATP and Ran.
Recently, it was reported that proteinase-3 (PR3), which belongs to the myeloid serine proteinase family including HBP and cathepsin G, localizes to both azurophil granules and secretory vesicles. The same group further demonstrated the role of PR3 in the proliferation of myeloid cells via the proteolytic regulation of the cyclin-dependent kinase inhibitor p21^waf1. Using subcellular fractionation, they detected the presence of both p21 and PR3 in the granular or cytosolic fractions, where they could interact. Interestingly, p21 was present in the nuclear fraction in control RBL cells, but not in the nuclear fraction in PR3 transfected cells (RBL/PR3). This leads to the question whether PR3 could transport to nucleus to cleave p21 (although PR3 activity was not detected in the nuclear fraction), also via an undermined nuclear import pathway.

We previously identified DNA-binding motifs for PU.1 and C/EBP transcription factors within the putative LRG promoter region. Similar to the regulatory mechanisms that modulate the expression of other neutrophil granule proteins, the PU.1 and C/EBP transcription factors could also bind to the promoter region of LRG to regulate its expression. To test this possibility, we performed chromatin immunoprecipitation (ChIP) analysis with untreated and Epo-treated 32Dwt18 cells using specific antibodies and primers to amplify the regions corresponding to the binding sites for PU.1 or C/EBP within the putative LRG promoter. Induction of 32Dwt18 cells towards neutrophil maturation by Epo treatment appears to change the dynamics of protein binding to both the LRG PU.1 and C/EBP binding sites. Binding of PU.1 and C/EBPε to their respective
LRG binding sites dramatically increased within the initial three days of Epo-induced neutrophil maturation, while the binding pattern for C/EBPα did not vary significantly. As described previously, both PU.1 and C/EBP family members have been shown to activate transcription of the primary neutrophil granule enzymes such as neutrophil elastase. Our results indicate that PU.1 and C/EBPε bind to the putative LRG promoter region that we previously identified, and binding of these transcription factors appears to modulate expression of LRG during neutrophil maturation. The binding of C/EBPα to LRG promoter also slightly increased in differentiating cells, but not as significant as that of C/EBPε. This may suggest that the expression of LRG is not an immediate event during granulopoiesis.
CHAPTER 3

LRG-ACCELERATED DIFFERENTIATION DEFINES UNIQUE G-CSFR SIGNALING PATHWAYS

3.1 Abstract

In previous studies, we have shown that the expression of LRG is upregulated during neutrophilic granulocyte differentiation. LRG is shown to localize to the cytoplasmic compartment and is targeted to the primary granules like MPO, and its expression is also shown to be regulated by PU.1 and C/EBPε transcription factors that also regulate the expression of several other granule proteins. These results suggest that LRG may be a novel marker for neutrophilic granulocyte differentiation. To further characterize the physiological roles of LRG during granulopoiesis, we again utilized the stable transfected LRG clones in both 32Dcl3 and 32Dwt18 cells. We initially investigated the effects of constitutive expression of LRG on the growth and differentiation of 32Dcl3 cells. Notably, constitutive expression of LRG significantly accelerates differentiation of the
cells into mature neutrophils in 32Dc13 cells. LRG clones also display accelerated expression of lactoferrin mRNA and surface expression of CD11b (Mac-1) and CD13. Phosphorylation of Stat3 appears to be enhanced in LRG clones as well. When we next studied the differentiation pattern of LRG-transfected 32Dwt18 cells, however, there was no evidence that Epo stimulation of 32Dwt18 cells constitutively expressing LRG induces accelerated differentiation either by morphology, cell cycle analysis, or expression of CD11b and CD13. These results suggest that LRG accelerates neutrophilic differentiation via non-redundant signaling pathways activated by the full-length G-CSFR, which probably involves Stat3. In addition, we studied a novel mutation (Δ319) in the extracellular portion of the G-CSFR identified from an SCN patient, and showed that the mutant G-CSFR decreases the surface expression of the wild-type receptor and thereby inhibits proliferative signaling by the wild-type G-CSFR, suggesting a common mechanism underlying G-CSF refractoriness in SCN patients.

3.2 Introduction

We have shown previously that LRG may be a novel marker for neutrophilic granulocyte differentiation (Chapter 2). However, specific role for LRG in granulopoiesis has not been elucidated. G-CSF has been shown to be the major regulator of granulopoiesis and supports not only the survival, proliferation, and neutrophilic differentiation of myeloid progenitor cells but also activates certain functions of mature,
terminally differentiated neutrophils $^{56,61,178}$. The biological activities of G-CSF are mediated by specific receptors on the surface of responsive cells. G-CSF binds to the extracellular portion of the G-CSFR resulting in activation of a complex signaling cascade that includes the Jak/Stat signaling pathway. Expression of the G-CSFR, like many other myeloid-specific genes including genes for several neutrophil granule proteins, has been shown to be regulated by PU.1 and the C/EBP family of transcription factors.

Studies in mice with knock-out or knock-in mutations in the G-CSFR gene suggest that the G-CSFR generates unique nonredundant signals required for polymorphonuclear (PMN) cell production and marrow egress to maintain homeostatic levels of circulating PMN $^{49,179,180}$. G-CSFR knock-out mice have chronic neutropenia with a uniform decrease in myeloid cells in the bone marrow. PMN from these mice also exhibit selective defects in activation $^{181,182}$. These observations suggest that the G-CSFR is a critical regulator of both PMN differentiation and activation.

To further characterize the physiological role of LRG in granulopoiesis, we have stably transfected the murine LRG cDNA into 32Dcl3 cells endogenously expressing the wild-type murine G-CSFR, and also into 32Dwt18 cells stably transfected with a chimeric Epo/G-CSF receptor, and examined the effects of constitutive expression of LRG on their proliferation, differentiation and activation. Other investigators have previously utilized the 32Dwt18 cell line in place of 32Dcl3 cells to study myeloid
maturation since 32Dwt18 cells transferred to Epo-containing media do not undergo the early massive cell death observed when 32Dcl3 cells are transferred to G-CSF-containing media. We report here significant differences in these cell lines and provide further evidence that unique signaling pathways are activated by the full-length G-CSFR which involves LRG. Our data also suggest a role for the extracellular domain of the G-CSFR in signal transduction.

In extension of the above results, we studied a novel mutation identified in the extracellular portion of the G-CSFR in a patient with SCN without AML who was refractory to G-CSF treatment. As described in Chapter 1, the majority of SCN patients bear the mutations in the gene encoding neutrophil elastase (NE). However, in the subset of patients with SCN developing AML, nonsense mutations in the gcsfr gene that are heterozygously expressed have been identified. These mutations truncate the C-terminal tail that is required for growth arrest, differentiation signaling, and down-modulation of receptor expression, and produce a dominant-negative phenotype both in vivo and in vitro that is postulated to be mediated by heterodimerization of wild-type G-CSFR and mutant G-CSFR forms. This novel mutation that we identified (designated Δ319) disrupts the WSXWS motif after the first tryptophan and localizes to the same region of the G-CSFR where mutations were identified in two previous SCN patients who were also unresponsive to G-CSF treatment. Both the surface expression of wild-type G-CSFR and the proliferation signals elicited by the wild-type G-CSFR appear
to be hampered by the mutant G-CSFR, suggesting a possible mechanism underlining the refractoriness to G-CSF treatment.

3.3 Materials and methods

Cells, antibodies, and reagents

G-CSF responsive murine 32Dcl3 cell line and the 32Dwt18 cells were maintained as described in Chapter 2. Ba/F3 cells were maintained in PRMI 1640 medium supplemented with 2mM glutamine, 10% FBS, and 10% WEHI 3B conditioned media as a source of interleukin 3 (IL-3). The pcDNA3.1D/V5-His6 and pcDNA6/HisB vectors were purchased from Invitrogen (Carlsbad, CA). Blasticidin and G418 were from Invitrogen. Antibodies to PU.1 and Stat3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Trizol, Superscript II reverse transcriptase and Taq polymerase were from Invitrogen. Cocktail protease inhibitors were purchased from Roche (Indianapolis, IN). The bicinechonic acid (BCA) reagent was from Pierce (Rockford, IL). Enhanced chemiluminescence (ECL) reagent was from Amersham (Piscataway, NJ). Antibody to V5 tag was purchased from Invitrogen. FITC-conjugated rat anti-mouse CD11b and CD13 antibodies were purchased from Pharmingen (San Diego, CA). Biotinylated mouse anti-human G-CSFR monoclonal antibody recognizing the FnIII domains was purchased from BD Pharmingen (San Diego, CA), and Cy5-conjugated streptavidin was from Caltag (San Francisco, CA). Mouse anti-human G-
CSFR antibody recognizing an amino terminal fragment of the receptor (aa. 25-200) used in Western blotting was from Santa Cruz.

**Stable transfection**

Murine LRG was stably transfected into 32Dcl3 cells and 32Dwt18 cells as described in Chapter 2. Human wild-type G-CSFR cDNA was cloned into the pcDNA6/HisB vector and transfected into 32Dcl3 cells by electroporation (300V, 960μF, single pulse). Clones expressing human G-CSFR were selected by growing in media containing blasticidin (10μg/ml) 48 hours after transfection. The Myc-tagged Δ319 G-CSFR construct was generated and cloned into pcDNA3.1D/V5-His6 vector \(^{101}\). Ba/F3 cells were stably transfected with the various G-CSFR forms using electroporation (300V, 25μF, single pulse), and selected by growing in media containing either blasticidin (10μg/ml for wild-type G-CSFR transfection) or G418 (300μg/ml for Δ319 G-CSFR transfection), or both (for co-transfection of both constructs) 48 hrs post-transfection. Single clones were obtained by limiting dilution and screened by immunoblotting.

*Reverse transcription-polymerase chain reaction (RT-PCR)*

This has been described in Chapter 2. Briefly, total RNA was purified and incubated with Superscript II transcriptase to generate cDNA. PCR with gene-specific primers was performed using *Taq* DNA polymerase, and PCR products were size-fractionated on 1%
agarose/TAE gel and visualized with ethidium bromide staining. The primers used are listed below:

**Endogenous mLRG:**
- **Forward:** 5’-TCAAGGAAGCCTCCAGGATCTC-3’
- **Reverse:** 5’-GACTCCAGCAGGTTGTACCCAAG-3’

**Ectopic mLRG:**
- **Forward:** 5’-AGTACCCTTCACCATGGTC-3’
- **Reverse:** 5’-GACTCCAGCAGGTTGTACC-3’

**Murine MPO:**
- **Forward:** 5’-AACCAAGCTGGGGCTGCTGGCTGTCAATAC-3’
- **Reverse:** 5’-AACTCCAGGTTCTTCAGCACCGTGCCG-3’

**Murine LF:**
- **Forward:** 5’-GCCAGTCACAGGAGAAGTTTGG-3’
- **Reverse:** 5’-GCCATTGCTTTTGGAGGATTTC-3’

*Western blotting*

1×10⁷ cells were lysed in lysis buffer (1.5% triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.1 mM sodium deoxycholate, 500mM NaCl, 5mM EDTA, 25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH7.8) containing a cocktail of protease inhibitors. A total of 50μg protein was mixed with reducing lithium dodecyl sulfate (LDS) sample buffer. The samples were then heated at 70°C for 10 min, resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the respective primary antibodies, followed by corresponding horseradish
peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were visualized using the ECL reagent.

**Differentiation Assay**

Purified recombinant human G-CSF (rhG-CSF) was generously provided by Amgen Inc. (Thousand Oaks, CA), and purified human erythropoietin (Procrit, epoitin alfa, Epo) was a kind gift from Ortho Biotech Inc. (Bridgewater, NJ). 32Dcl3 and 32Dwt18 cells were removed from IL-3-containing media, washed twice in PBS to remove residual IL-3, and transferred to G-CSF (10ng/ml, for 32Dcl3 cells)-containing or Epo (0.1-1u/ml, for 32Dwt18 cells)-containing media to induce neutrophilic granulocyte differentiation. At varying time points, aliquots of cells were removed for analysis of differentiation. Differentiation was monitored by morphology (Wright-Giemsa staining) and flow cytometric analysis.

**Flow Cytometric Analysis**

Cells (1×10⁶) were washed twice with cold HBSS solution supplemented with 1% BSA and 0.1% sodium azide, resuspended in 500μl of cold HBSS/BSA/Azide solution, and incubated with FITC-conjugated rat anti-mouse CD11b or FITC conjugated rat anti-mouse CD13 antibody for 1hr at 4°C. After incubation, cells were washed twice with cold HBSS/BSA/sodium azide solution, and fixed in 500μl of cold 1% paraformaldehyde.
solution. Cells were then analyzed by flow cytometry on FACS Calibur (Becton-Dickinson Immunocytometry System, Manassas VA) using Cell Quest software.

For detection of human wild-type G-CSFR, $5 \times 10^5$ cells were washed in PSA (PBS supplemented with 1% FBS and 0.02% sodium azide), resuspended in 50$\mu$l PSA, and incubated with 0.5$\mu$g biotinylated mouse anti-human G-CSFR antibody on ice for 1hr. After incubation, cells were washed and subsequently incubated with 2$\mu$l Cy5-conjugated streptavidin. After a final wash in PSA, the cells were resuspended in 500$\mu$l PSA, and 10$\mu$g/ml propidium iodide (PI) was added to exclude dead cells. The cells were subsequently analyzed on FACS Calibur.

For cell cycle analysis, cells were washed in PBS and fixed by adding cold ethanol to a final concentration of 80%. Cells were stained by adding one-tenth volume of a 38mM solution of PI and analyzed on FACS Calibur.

### 3.4 Results

*Constitutive expression of LRG accelerates neutrophilic differentiation in 32Dcl3 cells*

Our previous results suggest that LRG is a novel marker for granulopoiesis. To further investigate the role of LRG in this process, we again utilized the stably transfected LRG clones. 32Dcl3/LRG and cells transfected with empty vector alone were removed out of IL-3-containing media and transferred to G-CSF-containing media. As described in Chapter 2, there was a massive cell death following initial transfer from IL-3- to G-
CSF-containing media (Figure 2.5). The surviving cells then slowly recovered, and underwent terminal differentiation into mature neutrophils. Notably, morphological analysis of Wright-Giemsa stained cells demonstrated that by day 7 of culture with G-CSF, more than 50% of LRG transfectants displayed segmented nuclei consistent with the nuclear morphology of mature neutrophils (Figure 3.1). In contrast, only about 5%-10% of cells transfected with vector alone exhibited segmented nuclei (Figure 3.1), suggesting that constitutive expression of LRG significantly accelerates their differentiation into mature neutrophils.

To confirm neutrophil maturation, we examined the surface expression levels of CD11b and CD13, markers for neutrophil differentiation, by flow cytometry. Compared to untransfected parental 32Dc13 cells, CD11b and CD13 expression are both accelerated in LRG clones (Figure 3.2). We also examined the mRNA expression of MPO and LF, two common markers for granulocytic differentiation, in both vector- and LRG-transfected cells (Figure 3.3). LRG expression is not detectable in undifferentiated vector-transfected cells but is induced upon G-CSF stimulation. In comparison, LRG clones have accelerated expression of LF at Day3 after G-CSF treatment. Taken together, these results indicate that constitutive expression of LRG accelerates neutrophil differentiation in 32Dc13 cells. Interestingly, undifferentiated LRG clones appear to have a slightly higher expression of G-CSFR, compared to parental 32Dc13 cells where G-CSFR expression is undetectable (Figure 3.4).
Figure 3.1 Accelerated neutrophilic differentiation in LRG transfected 32Dc13 cells in response to G-CSF treatment. Cells stably transfected with vector alone or mLRG cDNA were washed out of IL-3 and transferred to G-CSF-containing media. At indicated time points, aliquots of cells were cytospun onto slides. (A) Neutrophilic differentiation was monitored by Wright-Giemsa staining. Arrows: morphologically mature neutrophils. (B) Bar graphs indicate the fraction of the total population of cells transfected with vector alone or LRG at each stage of differentiation at the indicated time points.
Figure 3.2 Accelerated expression of CD11b and CD13 in 32Dcl3 cell constitutively expressing LRG. Parental or LRG-transfected 32Dcl3 cells were washed out of IL-3 and transferred to G-CSF-containing media. At indicated time points, cells were incubated with FITC-conjugated antibodies to (A) CD11b and (B) CD13, and subjected to FACS analysis.
Figure 3.3 Accelerated expression of LF mRNA in LRG-transfected 32Dcl3 cells. Cells stably transfected with vector alone or mLRG cDNA were washed out of IL-3 and transferred to G-CSF-containing media. At indicated time points, mRNA was isolated from cell aliquots, and reverse transcription-PCR was performed with specific primers for LRG, MPO, LF and GAPDH.
Figure 3.4 Expression of G-CSFR mRNA in undifferentiated parental 32Dcl3 cells and 32Dcl3/LRG clones. mRNA from undifferentiated parental 32Dcl3 cells and 32Dcl3/LRG clones was isolated, and subjected to reverse transcription-PCR analysis using specific primers for murine G-CSFR. PCR product amplified from water was used as negative control. PCR product amplified from neutrophils was used as positive control. +RT: with reverse transcriptase, -RT: without reverse transcriptase.
Constitutive expression of LRG do not accelerate neutrophilic differentiation in 32Dwt18 cells

Further investigation in LRG-transfected cells has been hindered due to the massive cell death after initial transfer from IL-3-containing- to G-CSF-containing media. For this reason, we used the stably transfected 32Dwt18 cells as an alternative model. Initially, we used a higher concentration of Epo (1u/ml) as previously reported. No massive cell death was observed after transfer from IL-3- to Epo-containing media (Figure 2.7). However, at this concentration, we did not observe accelerated neutrophil differentiation in LRG clones. As we were concerned that the accelerated differentiation, if any, might be masked by the strong Epo signaling due to its high concentration, we decreased the Epo concentration to as low as 0.1u/ml. At this lower concentration, we started to see the similar die-off as what we observed in 32Dcl3 cells following transfer to G-CSF-containing media (Figure 3.5). Interestingly, LRG clone still displayed higher viability compared to cells transfected with vector alone (Figure 3.5). Even at the lower concentration of Epo, however, we still did not observe accelerated differentiation in LRG clones compared to cells transfected with vector alone, either by morphology (Figure 3.6), or by CD11b and CD13 expressions (Figure 3.7). These results suggest that different signaling pathways exist for 32Dcl3 and 32Dwt18 cell systems upon neutrophilic differentiation.
Figure 3.5 Growth curve and viability of 32Dwt18 cells stably transfected with empty vector alone or LRG in lower Epo concentration. Cells stably transfected with vector alone (black, triangles) or mLRG cDNA (grey, squares) were washed out of IL-3 and put in Epo-containing (0.1u/ml) media. Cell viability was monitored using trypan blue exclusion. Similar cell death was observed as seen in 32Dcl3 cells. (A) Total live cell numbers at different time points. (B) Cell viability at different time points. Data from three independent experiments were shown.
Figure 3.6 Neutrophilic differentiation is not accelerated in LRG transfected 32Dwt18 cells in response to Epo treatment. Cells stably transfected with vector alone or mLRG cDNA were washed out of IL-3 and transferred to Epo (0.1u/ml)-containing media. At indicated time points, aliquots of cells were cytospun onto slides. (A) Neutrophilic differentiation was monitored by Wright-Giemsa staining. (B) Bar graphs indicate the fraction of the total population of cells transfected with vector alone and LRG clone at each stage of differentiation at the indicated time points.
Figure 3.7 Expression of CD11b and CD13 is not accelerated in 32Dwt18 cell constitutively expressing LRG. 32Dwt18 cells stably transfected with empty vector alone or LRG were washed out of IL-3 and transferred to G-CSF-containing media. At indicated time points, cells were incubated with FITC-conjugated antibodies to (A) CD11b and (B) CD13, and subjected to FACS analysis.
Overexpression of LRG does not promote growth arrest in G1 phase of the cell cycle in 32Dwt18 cells

It has been shown that the acceleration of granulocytic differentiation of Jak3-overexpressing 32Dcl3 cells in response to the G-CSF stimulus was preceded by cell cycle arrest in the G1 phase of the cell cycle within 48 hrs of G-CSF simulation. Since constitutive expression of LRG in 32Dwt18 cells did not show accelerated differentiation by either morphology or flow cytometric analyses, we examined the cell cycle characteristics of these cells within 48 hrs of Epo treatment. Three single LRG clones were used for analysis. A mixpool from three clones transfected with empty vector alone was used as control. There was no evidence of accelerated cell growth arrest in LRG clones compared to cells transfected with empty vector alone (Table 3.1), consistent with the result that LRG does not accelerate granulocytic differentiation in these cells.

The expression pattern of phospho-Stat3, but not PU.1 or p27kip1, differs between 32Dcl3 and 32Dwt18 cells

Constitutive expression of LRG accelerates granulocytic differentiation in 32Dcl3 cells, but not in 32Dwt18 cells. To understand the discrepancy between these two cell systems, we put them both in differentiation media, and immunoblotted for proteins involved in signal transduction, cell cycle regulation and gene transcription. We first
Table 3.1 Constitutive expression of LRG does not promote G1 arrest in cell cycle in 32Dwt18 cells (% of cells in each phase ± SD)

<table>
<thead>
<tr>
<th>Time</th>
<th>Phase</th>
<th>Vector</th>
<th>LRG clone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>59.73 ± 2.35</td>
<td>60.51 ± 3.94</td>
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<tr>
<td></td>
<td>S</td>
<td>32.96 ± 3.12</td>
<td>28.70 ± 4.17</td>
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<tr>
<td></td>
<td>G2</td>
<td>7.31 ± 1.02</td>
<td>10.79 ± 0.48</td>
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<tr>
<td>0 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>69.93 ± 1.26</td>
<td>66.28 ± 3.43</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>24.21 ± 2.15</td>
<td>25.18 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>5.86 ± 1.22</td>
<td>8.54 ± 3.29</td>
</tr>
<tr>
<td>24 hrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>64.85 ± 0.12</td>
<td>59.55 ± 4.36</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>27.12 ± 1.25</td>
<td>28.78 ± 4.57</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>8.02 ± 1.56</td>
<td>11.67 ± 0.94</td>
</tr>
<tr>
<td>48 hrs</td>
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investigated the activation of Stat3, which has been reported to be required for G-CSF-induced neutrophilic differentiation. In 32Dcl3 cell system, we detected a rather weak signal of phospho-Stat3 (pStat3) in cells transfected with vector alone at 24 hours in G-CSF treatment; in contrast, strong signals of pStat3 could be detected as early as 8 hours in LRG clone. As a control for equal loading, the membrane was stripped and rebotted with anti-Stat3 antibody. The expression level of Stat3 remained the same (Figure 3.8A).
This result suggests that Stat3 may be involved in LRG-accelerated differentiation in 32Dcl3 cells. In 32Dwt18 cells, on the other hand, we detected similar patterns of pStat3 signals in both vector-transfected cells and LRG clone (Figure 3.8B). This might be one of the reasons why we did not observe LRG-accelerated differentiation in these cells. We further put the 32Dwt18 cells in G-CSF-containing media. Surprisingly, we did not detect any pStat3 signals within 24 hours of G-CSF treatment (Figure 3.8C). In addition, the expression level of Stat3 significantly decreased at 24 hours in G-CSF in both LRG clone and cells transfected with vector alone (Figure 3.8C), suggesting that the endogenous full-length G-CSFR in the 32Dwt18 cells might not be functional.

We next investigated the expression levels of the cyclin-dependent kinase inhibitor p27kip1 and the transcription factor PU.1. In 32Dcl3 cells, we detected a decreased expression p27kip1 in cells transfected with vector alone at 24 hours after G-CSF treatment, but the decrease was not seen in LRG clone (Figure 3.9A). The expression of PU.1 disappeared in cells transfected with vector alone at 24 hours in G-CSF, but its expression was still detectable in LRG clone at 24 hours, though decreased (Figure 3.9A). In 32Dwt18 cells, we detected the same trends for the expression of both PU.1 and p27kip1 as we observed in 32Dcl3 cells (Figure 3.9B). Taken together, these results suggest that Stat3, but probably not p27kip1 or PU.1, is involved in LRG-accelerated neutrophil differentiation in 32Dcl3 cells.
### Figure 3.8 Stat3 is activated at an early stage in LRG-transfected 32Dcl3 cells but not in LRG-transfected 32Dwt18 cells.

Cells were washed out of IL-3-containing media and transferred to media containing G-CSF or Epo as indicated. Whole cell lysates were made from cells harvested at indicated time points and used for immunoblotting with Stat3 and phospho-Stat3 antibodies. (A) Vector- or LRG-transfected 32Dcl3 cells in G-CSF-containing media. (B) Vector- or LRG-transfected 32Dwt18 cells in Epo-containing media. (C) Vector- or LRG-transfected 32Dwt18 cells in G-CSF-containing media.
Figure 3.9  p27<sup>kip1</sup> and PU.1 display the same trends of expression in LRG clones established from both 32Dcl3 and 32Dwt18 cells. Cells were washed out of IL-3-containing media and transferred to media containing G-CSF or Epo as indicated. Whole cell lysates were made from cells harvested at indicated time points and used for immunoblotting with p27<sup>kip1</sup> antibody. The membrane was stripped and rebotted with PU.1 antibody. (A) Vector- or LRG-transfected 32Dcl3 cells in G-CSF-containing media. (B) Vector- or LRG-transfected 32Dwt18 cells in Epo-containing media.
Stable transfection of G-CSFR in 32Dcl3 cells

Since 32Dwt18 cells appear to have quite a few diversions from 32Dcl3 cells, including the activation pattern of Stat3, we need to return to the 32Dcl3 model system. The massive cell death of 32Dcl3 cells after initial transfer to G-CSF-containing media is probably due to the low expression level of endogenous mouse G-CSFR on cell surface (Figure 3.4). The expression of G-CSFR on granulocyte is upregulated during terminal differentiation 49. However, after initial removal of IL-3 and addition of G-CSF, the number of G-CSFR may not be sufficient to support the survival and proliferation of 32Dcl3 cells. Therefore, it is plausible that the survival of 32Dcl3 may be improved by increasing the number of receptors on cell surface. We thus cloned the human wild-type G-CSFR in pcDNA6/HisB vector and stably transfected it into 32Dcl3 cells by electroporation. G-CSFR transfectants were obtained by selecting the cells in blasticidin-containing media. Single clones were further isolated by limiting dilution. The single clones were shown to have different expression levels which were consistently confirmed by both Western blotting (Figure 3.10A) and flow cytometry (Figure 3.10B). Although the proliferative responses to G-CSF vary among different clones, again consistent with the different receptor expression levels, G-CSFR transfectants have an obvious survival advantage over the parental 32Dcl3 cells (Figure 3.11).
Figure 3.10 Stable transfection of human wild-type G-CSFR into 32Dcl3 cells. Human wild-type G-CSFR was cloned into pcDNA6/HisB vector and stably transfected into 32Dcl3 cells by electroporation. Cells were selected in blasticidin-containing media and single clones were obtained from limiting dilution. (A) WCL from single clones or vector-transfected cells was made and 50μg of protein was used for Western blotting. Variable expression of G-CSFR was detected from individual clone. WCL from G-CSFR-transfected Ba/F3 cells was used as positive control. (B) Cells were sequentially incubated with biotinylated mouse anti-human G-CSFR antibody and Cy5-conjugated streptavidin antibody, and were subjected to FACS analysis. Variable surface expression of G-CSFR was detected. Ba/F3 cells transfected with G-CSFR were used as positive control.
Figure 3.11 Growth curve and viability of 32Dcl3 cells stably transfected with human G-CSFR in G-CSF. Cells stably transfected with human G-CSFR were washed out of IL-3-containing and put in G-CSF-containing (10ng/ml) media. Cell viability was monitored using trypan blue exclusion. (A) Total live cell numbers at different time points. (B) Cell viability at different time points.
Coexpression of the wild-type G-CSFR with the Δ319 mutant abrogates G-CSF-stimulated proliferative signaling by the wild-type G-CSFR and reduces its surface expression

The differences between 32Dcl3 cells and 32Dwt18 cells suggest that the full-length G-CSFR generates unique signaling pathways in which the extracellular domain of the receptor may play an important role. To further study the function of G-CSFR, we stably transfected either a wild-type G-CSFR, a novel mutant G-CSFR (Δ319), or both, into Ba/F3 cells. Ba/F3 cell is a pre-B cell line that does not have endogenous G-CSFR. The novel mutant G-CSFR was identified from a patient with SCN who was refractory to even high doses of G-CSF (100μg/kg/d) treatment. It was shown to be a deletion mutation, which produces a frame-shift immediately distal to the W318 codon, resulting in the introduction of an additional 29 missense codons followed by a premature stop codon. The mutation generates a truncated G-CSFR form containing the first 318 amino acids of the wild-type G-CSFR followed by the missense amino acids, hence designated as Δ319 (Figure 3.12), with the entire transmembrane and cytoplasmic regions and part of the extracellular domain deleted. Stable transfection was confirmed by immunoblotting assay, using an anti-G-CSFR antibody that recognizes the N-terminus of the receptor (Figure 3.13).
Figure 3.12 Schematic diagram of the Δ319 mutant. The deletion disrupts the WSXWS motif and deletes the three terminal FnIII domains in the extracellular region and the entire transmembrane and cytoplasmic domains.
Figure 3.13 Stable transfection of wild-type and/or Δ319 G-CSFR constructs into Ba/F3 cells. Ba/F3 cells were transfected with wild-type G-CSFR alone, Δ319 G-CSFR alone, or both by electroporation. 48 hrs after transfection, cells were selected in media containing either blasticidin (for wild-type G-CSFR) or G418 (for Δ319 G-CSFR), or both. (A) WCL from mixpools of transfectants was separated on 10% SDS-PAGE and blotted with anti-human G-CSFR antibody recognizing the N-terminus of the receptor (upper panel). The membrane was reprobed with actin antibody to confirm equal loading (lower panel). (B) Single clones were isolated from mixpools. In cells coexpressing the wild-type and the Δ319 G-CSFR constructs, the expression levels of Δ319 relative to the wild-type G-CSFR are variable in different single clones (upper panel). The membrane was reprobed with actin antibody to confirm equal loading (lower panel).
We first examined the proliferation of Ba/F3 cells stably expressing the wild-type G-CSFR only, the Δ319 G-CSFR only, or coexpressing both the wild-type and Δ319 G-CSFR forms in response to G-CSF. As expected, Ba/F3 cells expressing the wild-type G-CSFR grew exponentially in the presence of G-CSF (Figure 3.14). In contrast, cells expressing only the Δ319 G-CSFR died following transfer to G-CSF-containing media, similar to the behavior of parental Ba/F3 cells and Ba/F3 cells transfected with the empty vector alone. Coexpression of the truncated receptor with the wild-type G-CSFR (WT/Δ319 clones) inhibited the growth of cells in response to G-CSF. Inhibition of the proliferative signaling correlates with the level of expression of the truncated receptor and increases proportionally with increasing expression of the Δ319 G-CSFR. Following culture for seven days in G-CSF, cells expressing the highest levels of the Δ319 G-CSFR relative to the wild-type G-CSFR exhibited the greatest inhibition of cell growth (Figure 3.13 and 3.14). In consistency with the growth curve, flow cytometry revealed a decrease in surface expression of the wild-type G-CSFR when the Δ319 mutant was coexpressed with it (Figure 3.15).

3.5 Discussion

In Chapter 2, we discussed the subcellular localization of LRG and the regulation of its expression. In this chapter, we set out to investigate the functional roles of LRG in neutrophilic granulocyte differentiation. Previous work showed that the expression of
Figure 3.14 The Δ319 mutant abrogates proliferative signaling by the wild-type G-CSFR. Ba/F3 cells transfected with the wild-type G-CSFR (WT), Δ319 G-CSFR, or both receptor forms (WT/Δ319) were grown in G-CSF-containing media (10ng/ml) for the indicated times. Results with four independent Δ319 clones with varying levels of expression of the mutant receptor versus the wild-type receptor are shown. (A) Growth curve of individual clones. (B) Viability of cells was determined by trypan blue exclusion.
Figure 3.15 Decreased surface expression of the wild-type G-CSFR in cells coexpressing the wild-type G-CSFR and the Δ319 mutant G-CSFR. Flow cytometry was used to analyze surface expression of the G-CSFR in Ba/F3 cells transfected with empty vector (Vector), the Δ319 G-CSFR, the wild-type G-CSFR (WT), or both receptor forms (WT/Δ319). Cells were incubated with biotin-conjugated anti-G-CSFR antibody, washed and then incubated with Cy5-conjugated streptavidin, and analyzed on a FACS Calibur with a total of 10,000 gated events analyzed.
LRG is induced during granulopoiesis in response to G-CSF, leading us to the hypothesis that LRG may be involved in this important biological process. Constitutive expression of LRG significantly accelerates neutrophilic differentiation in 32Dc13 cells, demonstrated by both nuclear morphologic changes and cell surface expression levels of CD11b and CD13. Proliferation of LRG transfectants is decreased compared to cells transfected with empty vector alone, consistent with an expected decrease in proliferation as cells undergo differentiation and also consistent with our observation that LRG transfectants undergo accelerated neutrophilic differentiation (data not shown). Preliminary studies from our group to examine primary cells from patients with AML and other disorders of granulopoiesis showed an approximate 60% decrease in expression of LRG in cells from patients with AML compared to normal donors (Figure 3.16).

The mechanisms of this accelerated differentiation are still under investigation. By immunoblotting analysis, LRG-transfected 32Dc13 cells displayed a stronger phospho-Stat3 signal at an earlier time point in response to G-CSF treatment. Jak kinases and Stat proteins have been shown to be activated in hematopoietic cells by the majority of hematopoietic cytokines. Over-expression of Jak3 in 32Dc13 cells was recently reported to accelerate neutrophil differentiation in response to G-CSF. This effect was mediated in part through Stats and cell cycle regulatory proteins. It is possible that accelerated differentiation induced by over-expression of LRG is mediated by similar signaling pathways involving Stat3. Whether LRG facilitates the phosphorylation and
Figure 3.16 LRG expression is lower in AML patients. Total mRNA was isolated from bone marrows of four AML patients and four normal donors. (A) Reverse-transcription-PCR was performed using specific primers for human endogenous LRG. HPRT gene was used as control for equal cDNA loading. (B) The intensities of bands from (A) were quantitated using Scion Image software. The ratios from LRG/HPRT band intensities were presented as bar graph.
activation of Stat3 or LRG directly interacts with other genes involved in granulocytic differentiation after it translocates to nucleus upon G-CSF stimulation remains to be determined. On the other hand, our results suggested that PU.1 or the cyclin-dependent kinase inhibitor p27kip1 might not be involved in the accelerated differentiation of LRG-transfected 32Dcl3 cells, since we did not detect any difference in the expression patterns of either p27kip1 or PU.1 between 32Dcl3 and 32Dwt18 cells (constitutive expression of LRG does not accelerate neutrophilic differentiation in the latter). This is in contrast with previous reports on Stat3-mediated activation of p27kip1 113 and PU.1 189. One explanation for this is that Stat3 may be necessary, but not sufficient for the activation of p27kip1 and PU.1.

In these experiments, we have been using two model systems: 32Dcl3 and 32Dwt18 cells. The 32Dcl3 cells have endogenous wild-type G-CSFR, and respond to G-CSF to differentiate. The 32Dwt18 cells, on the other hand, respond to Epo, because they are stably transfected with an Epo/G-CSF chimeric receptor. They both differentiate into morphologically mature neutrophils when transferred from IL-3-containing media to either G-CSF- or Epo-containing media. It would have been expected that the signaling pathways mediated by the same cytoplasmic domain of G-CSFR should lead to the same cellular processes in the two cell systems. However, the effects of constitutive expression of LRG in the two systems are quite different. It accelerates neutrophilic
differentiation in 32Dcl3 cells, but not in 32Dwt18 cells, suggesting significant
differences in their cellular differentiation and maturation signaling pathways.

G-CSFR-specific signals are required for complete neutrophil development, including
changes in cell adhesion and migration capabilities. In vivo studies\textsuperscript{190} demonstrated
that in homozygous mutant mice expressing a G-CSF/Epo receptor in which the
extracellular and transmembrane domains of G-CSFR are fused to the cytoplasmic
domain of Epo receptor, morphologically mature neutrophils were produced, but their
mobilization was greatly impaired. A similar phenotype is found in G-CSFR d715F
mice, which have impaired activation of Stat3 in response to G-CSF\textsuperscript{114}. It was further
shown that the membrane-proximal 87 amino acids of the G-CSFR cytoplasmic region
are sufficient to mediate neutrophil release from bone marrow\textsuperscript{69}. It has also been
observed that G-CSFR-dependent Stat3 signals are required for induction of β2 integrins
expression and β2 integrins function\textsuperscript{191,192}. The β2 integrins leukocyte function antigen-
1 (LFA-1, CD11a) and macrophage antigen-1 (Mac-1, CD11b) have been reported to
play a role in the attachment of CD34\textsuperscript{+} cells to stromal cells in the bone marrow through
their ligand, intercellular adhesion molecule-1 (ICAM-1), and through heparin sulfate\textsuperscript{193,195}. Interestingly, we showed that CD11b, the major β2 integrin expressed on
neutrophils, is only induced by the full-length G-CSFR but not the chimeric Epo/G-
CSFR; and its expression is accelerated by the constitutive expression of LRG. These
data indicate that the full-length G-CSFR (including the extracellular portion) plays a
critical role in neutrophil activation, in addition to neutrophilic differentiation. Considering that the activation of Stat3 is promoted in 32Dcl3 cells constitutively expressing LRG, LRG may regulate the expression of CD11b through the action of Stat3 in response to G-CSF. A two-step model of G-CSF-induced neutrophil mobilization has been proposed: a yet undetermined subset of hematopoietic cells is activated by nonredundant G-CSFR signals first, and they subsequently generate secondary signals that act in trans to induce neutrophil release from the bone marrow. How LRG might fit into this model still awaits further study.

In conventional view, the role of the extracellular domain of cytokine receptors is simply to specifically bind to the cytokine and mediate receptor dimerization. It is the cytoplasmic domains that play critical roles in signal transduction, as they bear the receptor tyrosine kinase (RTK) activity or serve as docking sites for cytoplasmic tyrosine kinases. Here we presented evidence that the extracellular domain of G-CSFR is also indispensable for the unique signaling pathways mediated by the full-length G-CSFR. We thus proposed a model, as shown in Figure 3.17. In response to G-CSF, the wild-type, full-length G-CSFR undergoes unique conformational changes in the cytoplasmic domain, activating the associated tyrosine kinases. This results in the recruitment and activation of Stat proteins, including Stat3. Other unknown molecules may also be involved in this step. The activated Stats (phosphorylated form) then translocate to the nucleus and activate the transcription of target genes. The cell undergoes terminal
Figure 3.17 A model for the role of LRG in the unique signaling pathway mediated by the full-length G-CSFR.
differentiation, and CD11b expression is induced. On the other hand, in response to Epo, the chimeric Epo/GCSFR may undergo different conformational changes within the cytoplasmic domain. This may lead to the activation of different Stat molecules or other factors. When these factors translocate to the nucleus, they may activate some different target genes that are not seen in the signaling pathway described above. LRG may be an important factor involved in the non-redundant signaling pathways mediated by the full-length G-CSFR only, and its constitutive expression accelerates neutrophilic differentiation in 32DeI3 cells. Studies on more details about these possible signaling pathways should help further clarify the functions of LRG in granulopoiesis.

The study on the Δ319 G-CSFR mutation identified from an SCN patient extended our knowledge about G-CSFR signaling. This mutation disrupts the WSXWS motif and deletes the extracellular portion distal to the first tryptophan and the entire transmembrane and cytoplasmic domains. Expression of this mutant in Ba/F3 cells, either alone or in combination with the wild-type G-CSFR, reproduces the dominant-negative phenotype observed in the patient. The proper targeting of the receptor to the cell membrane is disrupted, and the response to G-CSF is also abrogated. Interestingly, the intensity of growth inhibition quantitatively correlates with the level of expression of the mutant receptor form so that greater inhibition is observed as the level of expression of the Δ319 mutant relative to the wild-type G-CSFR increases. It has also been shown that the Δ319 G-CSFR accumulates intracellularly, and it forms oligomers in vivo with
the wild-type G-CSFR in the absence of ligand to inhibit signaling by the wild-type G-CSFR. Taken together, these data suggest a novel mechanism underlying G-CSF refractoriness in SCN patients. Both the wild-type and the mutant G-CSFR forms may be transcribed and oligomerize during intracellular processing, leading to accumulation of the heterodimeric complexes intracellularly disrupting transport of the G-CSFR to the cell surface. This results in insufficient signal generation to sustain survival, growth, and/or differentiation in response to G-CSF stimulation. Similar defects in receptor processing and assembly have been reported with truncation mutants of the Epo receptor as well, indicating that sequences in the extracellular domain are critical for correct expression and sorting of both mature receptor complexes to the plasma membrane. Therefore, together with previous findings with C-terminal truncated G-CSFR mutants that are defective in internalization and hyperresponsive to G-CSF, these results provide contrasting models of G-CSF responsiveness, and underscore the importance of receptor intactness and proper receptor trafficking in the control of cytokine signaling.
Neutrophils are the most abundant circulating human phagocytes and constitute a critical component of host defense. Granules within neutrophils store proteins that have important antimicrobial and enzymatic properties. Deficiencies in several of the neutrophil granule proteins in humans have been shown to lead to severe immunodeficiency and recurrent infections. We have identified LRG as a protein that is upregulated during neutrophilic granulocyte differentiation. Our data suggested that LRG, like MPO, localizes to the primary granules of neutrophils. Interestingly, we also showed that constitutive expression of LRG accelerates neutrophilic granulocyte differentiation in 32Dcl3 cells. More than a quarter of a century has passed since LRG was first purified. Although its functions remain largely unknown, our research provided new insight into its roles in hematopoietic cell system.
There are two tantalizing possibilities for additional functions of LRG. First, LRG may modulate the activity of transforming growth factor beta (TGF-β)\(^{201}\). LRG expression was reported to be increased in hepatoma cells sensitive to the growth inhibitory action of TGF-β. LRG was also shown to be coordinately expressed with the TGF-β1 RII receptor, and it was suggested that LRG interacted with the receptor\(^{202}\). Notably, TGF-β has been shown to modulate hematopoietic cell proliferation, with both inhibitory and stimulatory activities. TGF-β was reported to increase GM-CSF-driven granulocyte differentiation that was postulated to occur via increased surface expression of the GM-CSF receptor induced by TGF-β\(^{203}\). Recently, Saito et al. reported isolation of LRG from high endothelial venule cells and proposed a role for LRG in adhesive interactions between lymphocytes and the endothelium\(^{204}\). They also reported that LRG immobilized on plastic wells could bind to TGF-β\textit{ in vitro}. Therefore, LRG may aid in the control of granulopoiesis by facilitating the modulation of the surface expression of different receptor types, including TGF-β receptor, GM-CSFR, and maybe G-CSFR. It is possible that LRG increases the surface expression of G-CSFR by either stimulating its production or inhibiting its downregulation, or both, so that the differentiation process is accelerated. Second, the primary structure of LRG shows significant homology (35% identical; 51% similarity) to β-type phospholipase A2 (PLA2) inhibitors purified from the plasma of two snake species\(^{205,206}\). PLA2 catalyzes the hydrolysis of glycerophospholipids\(^{207}\). Multiple phospholipase inhibitors (PLIs) have been purified
from the serum of venomous reptiles and amphibians, and they are postulated to provide animals protection from their own phospholipase-rich venom\textsuperscript{208}. In addition to being a component of venom, PLA2 has also been shown to play a role in phospholipid turnover and the production of pro-inflammatory lipids\textsuperscript{209,210}. Multiple mammalian PLA2 enzymes have been identified, some of which are retained intracellularly and others are secreted\textsuperscript{211,212}. At least ten different groups of secretory phospholipase A2s (sPLA2) have been reported in humans\textsuperscript{210-212}. To date, no naturally occurring PLA2 inhibitors have been identified in humans. So, LRG might function as a naturally occurring inhibitor of phospholipase A2.

In addition, our study emphasized the importance of the intactness of G-CSFR in granulocytic proliferation, maturation and activation. Not only the cytoplasmic signal-transduction portion, but also the extracellular ligand-binding region and the transmembrane domain are indispensable. Various molecules, including LRG, may interact with G-CSFR and modulate its functions by different mechanisms. In Part II of this dissertation, we will discuss the regulation of Fc\(\gamma\) receptor in the context of macrophage, another important phagocyte type in human immune system.
PART II

THE INOSITOL PHOSPHATASE SHIP-2 DOWNREGULATES FγR-MEDIATED PHAGOCYTOSIS IN MURINE MACROPHAGES

CHAPTER 5
INTRODUCTION

During the evolutionary process, constant battles were fought between host and invading pathogens. Multiple defense systems have evolved to help host survive. For
vertebrates, including mice and humans, the immune system plays a critical role in defending against infections. The normal functions of this system depend on the cooperation of various cell types, cell surface receptors, and a complex network of cytokines and signaling pathways, which ensure that the immune response generated is highly effective and also well-regulated. In this part, we will focus on the regulation of functions of another cell type in innate immune system, macrophages.

5.1 Innate immunity

Innate immunity is an ancient system, presented in most organisms from plants to animals. In contrast to adaptive immunity, which involves more slowly developing, long-lived, and highly evolved antigen-specific protective responses, innate immunity is activated very quickly after infection, it has receptors recognizing conserved structures present in a large group of pathogens, and the reactions generated are non-specific. Although quite different from each other, the components of innate and adaptive immunity have remarkably diverse and complex interactions. Innate immunity is involved in cytokine-dependent antiviral and antibacterial activities, pathogen phagocytosis, and production of cytokines. Several cell types are engaged in innate immunity, including monocytes/macrophages, neutrophils, natural killer cells, and dendritic cells.
Mononuclear phagocytes, including circulating monocytes and tissue macrophages, and many epithelial cells, express a family of receptors that is highly homologous to the *Drosophila* receptor Toll. The *toll* gene was originally identified to be responsible for segmentation and polarity of *Drosophila* and later found to direct anti-fungal resistance in the fly. The products of genes similar to Toll were named Toll-like receptors (TLRs). These TLRs are currently considered the main receptors recognizing pathogen-associated molecular patterns (PAMPs). Following the cloning of TLR-4, a total of ten TLRs have been cloned and studied, with the same intracellular domains and similar pathways of intracellular signaling. TLRs can recognize not only bacteria but also viruses, and they may play a role in anti-tumor immunity as well.

Common ligands include Gram-negative bacterial lipopolysaccharides (LPS), bacterial lipoproteins, lipoteichoic acids of Gram-positive bacteria, bacterial cell-wall peptidoglycans, and viral RNAs. Signaling through TLRs has been well described. Ligand binding generates a signal through an adaptor molecule MyD88, which leads to intracellular association with IL-1 receptor-associated kinase. This subsequently activates TNF receptor-associated factor 6, which in turn results in nuclear translocation of nuclear factor κB (NF-κB). NF-κB then activates the promoters of the genes for a broad range of cytokines and other proinflammatory products, including TNF-α, IL-1, IL-6, and IL-8.
In addition to TLRs, other receptors are also presented in cells involved in innate immunity. The mannose receptor (MR) belongs to the lectin family, and it was the first structure found to play a role in antiviral innate immunity by producing interferon. The scavenger receptors (SRs) bind and internalize microorganisms such as Gram-positive and -negative bacteria, and mice with knocked-out genes for SR are more sensitive to bacterial infections. The structures and functions of another important receptor, Fc receptor (especially Fcγ receptor), will be discussed below.

5.2 Macrophages and Fcγ receptors

Like neutrophils, macrophages are the major effector cells of the innate immune system. Macrophages are derived from blood monocytes, and are highly distributed throughout the body at the sites where organisms interact with environment, such as lymph nodes, liver, lung, gastrointestinal tract, bone, skin, etc. The functions of macrophages depend largely on their surface Fcγ receptors (FcγRs), which bind to the Fc portion of IgG and are essential mediators of the inflammatory effect of immune complexes and cytotoxic antibodies. The function of FcγR is not only critical for effective clearance of pathogens, but also plays a central role in the therapeutic use of cytotoxic antibodies for the treatment of infectious and neoplastic diseases.

Macrophages express three classes of FcγRs: FcγRI, FcγRII, and FcγRIII. While human macrophages have FcγRI, FcγRIIa, FcγRIIb, and FcγRIII, mouse macrophages do
not express FcγRIIa because they lack the gene for it \(^{227,228}\) (Figure 5.1). Very recently, a novel murine Fc receptor with preference for the IgG2a subclass was discovered and named FcγRIV \(^{229}\). FcγRs can be generally divided into activation receptors and inhibitory receptors. Activation receptors are characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) either intrinsic to the receptor, as in the case of FcγRIIa, or as part of an associated \(\gamma\) subunit dimer found in FcγRI and FcγRIIIa (A decoy receptor, FcγIIIb, is found in humans neutrophils that binds IgG immune complexes without inducing activation) \(^{228}\). Ligand binding triggers the tyrosine phosphorylation of the ITAM by receptor-associated Src-family kinases. The phosphorylated ITAM in turn serves as docking sites for cytoplasmic SH2 domain-containing signaling molecules such as Syk, which becomes activated and phosphorylates a number of downstream signaling proteins \(^{230-232}\). Besides ITAMs, the lipid raft-associated adapter protein LAT which constitutively associates with FcγRs can also recruit and activate SH2 domain-containing molecules once it is phosphorylated upon receptor clustering \(^{233}\). Proteins thus recruited and activated include PLC\(\gamma\), the Ras activating guanine exchange factor Sos through adapter proteins Shc and Grb2, and the PtdIns3-kinase (PI-3K) through its p85 adapter subunit \(^{234}\). Recruitment of these effector proteins to the plasma membrane not only delivers them to the proximity of their activating kinases, but also facilitates them to access their substrates to initiate a variety of functional outcomes, including the phagocytosis of IgG-opsonized particles, the
Figure 5.1 Diagram of different Fcγ receptors expressed on macrophages. Both human and mouse macrophages express FcγI, FcγIIb, and FcγIIIa, while mouse macrophages lack FcγIIa. These Fcγ receptors are generally divided into activation and inhibitory receptors, depending on the ITAM or ITIM that they bear. The very recently discovered FcγRIV is not included.
generation of reactive oxygen and nitrogen species, and the production of inflammatory cytokines such as IL-1, IL-8 and TNF-α (Figure 5.2).

FcγRIIb is the single inhibitory FcγR for both human and mouse macrophages. The inhibitory activity of FcγRIIb was contained within the cytoplasmic immunoreceptor tyrosin-based inhibitory motif (ITIM), which is a 13 amino acid sequence AENTITYSLLKHP. It was shown to be both necessary and sufficient to mediate the inhibition of B cell receptor (BCR)-generated calcium mobilization and cellular proliferation. Co-transfection of FcγRIIb was also shown to significantly decrease the phagocytic efficiency in Cos-1 cells transfected with ITAM-containing-FcγR. These results were confirmed by *in vivo* studies where FcγRIIb knockout mice display elevated levels of serum IgG in response to antigenic challenge, and the finding that phagocytic capacity of FcγRIIb-deficient macrophages is enhanced compared to wild-type macrophages. Human hematopoietic cells express two forms of this receptor, FcγRIIb1 and FcγRIIb2, which result from the splicing of a 19 amino acid insertion into the cytoplasmic tail of FcγRIIb1. Although they exhibit differential expression patterns, it has been shown that they both are functional inhibitory receptors in the phagocytic process. The mechanism by which FcγRIIb mediates its inhibitory effects was originally proposed to involve the recruitment of SH2 domain-containing enzymes such as protein tyrosine phosphatase SHP-1 and the inositol phosphatase SHIP-1 to the
Figure 5.2 Diagram of FcγR-mediated signaling pathways. Cross-linking of FcγR by immune complex leads to the phosphorylation of the receptor ITAM by associated Src-family kinases. The phosphorylated receptor serves as docking sites for multiple cytoplasmic signaling molecules, such as Syk, PI-3K, and Shc. The functional outcomes include cytokine production, ROS generation, and phagocytosis.
phosphorylated ITIM$^{240-242}$, although it was later revealed that SHIP-1, but not SHP-1, is the effector molecule of FcγRIIb-mediated inhibition$^{243-245}$.

When a monocyte/macrophage encounters an immune complex, both the activating and the inhibitory FcγRs are clustered so that the magnitude of the resulting phagocytic response is dictated by the ratio of the activating to inhibitory FcγR$^{234}$. Interestingly, clustering of ITAM-FcγR on human monocytes induces phosphorylation of inhibitory enzymes SHIP-1 and SHIP-2, leading to a down regulation of both phagocytosis and cytokine gene expression$^{246-250}$. These observations suggest that the activation process initiated by ITAM-FcγR clustering is simultaneously subject to the inhibitory influence to make sure that the biological response is modulated.

5.3 Phagocytosis

A major function of monocyte/macrophage is phagocytosis, which is to internalize, degrade and eventually present peptides derived from particulate antigens$^{251}$. This seemingly smooth process actually relies on profound rearrangements of the actin cytoskeleton and the plasma membrane to engulf particles. Rho GTPases have integral roles in regulating the actin rearrangements that mediate particle uptake by phagocytes. Rho GTPases, GTP-binding proteins of about 22kDa that cycle between GDP-bound inactive and GTP-bound active states, act as molecular switches that have important functions$^{252}$. The activation cycle is modulated by three classes of regulatory proteins
(Figure 5.3). Guanine nucleotide exchange inhibitors (GDIs) sequester the GDP-bound GTPases, thereby preventing the dissociation of GDP. GTPase-activating proteins (GAPs) stimulate the low intrinsic GTP hydrolytic activity of the GTPases, resulting in their conversion back to the inactive state. Guanine nucleotide exchange factors (GEFs) catalyze the release of bound GDP, resulting in the formation of the GTP-bound active proteins.

Phagocytosis by macrophages can be initiated by Fcγ receptors in membranes. As previously described, activated ITAMs recruit the tyrosine kinase Syk and PI-3K, which are required for phagocytosis. PI-3K phosphorylates its lipid substrates to generate three phosphoinositides, particularly PtnIns3,4,5P3 (PIP3), which recruits and activates pleckstrin homology (PH) domain-containing proteins, such as Vav253,254. Vav is a GEF for the Rho family GTPases Cdc42 and Rac, which regulate actin cytoskeleton during FcγR-mediated phagocytosis255,256. The role of Rac as a major regulator of phagocytosis is established257, GTP-bound Rac can activate a number of proteins important for phagocytosis, while Cdc42 can stimulate localized actin polymerization258. It has been suggested that these two molecules contribute to phagocytosis in different ways, in which Cdc42 may regulate pseudopod extension and Rac may regulate phagosome closure258. In addition, the ADP-ribosylation factor (ARF)6 GTP-binding protein is also activated during FcγR-mediated phagocytosis255.
Figure 5.3 The Rho GTPase family. Proteins from the Rho GTPase family exist in two forms: a GDP-bound inactive form and a GTP-bound active form. Guanine nucleotide exchange factor (GEF) helps convert Rho-GDP to Rho-GTP, while GTPase activating protein (GAP) facilitates the reversion of the reaction. Guanine nucleotide exchange inhibitor (GDI) sequesters the Rho proteins in their GDP-bound form. Rho-GTP proteins are involved in several important cellular functions, including cytoskeletal effects such as chemotaxis and phagocytosis, NADPH oxidase activation, and transcription of genes for proinflammatory cytokines and NF-κB, etc.
Great efforts have been put into the investigation of signaling events leading to phagocytosis. Phagocytosis starts from the formation of phagocytic cup, which is the cup-shaped plasma membrane extension from the macrophage around the IgG-opsonized particle. Actin cytoskeleton remodeling on forming phagosomes has been observed by fluorescence microscopy, which demonstrated a robust and intricate series of regulatory interactions governing phagosome formation. Actin accumulates at sites of particle attachment and is present in pseudopods extending around the target; it transiently surrounds the whole phagosome on phagosome closure, and subsequently disassembles asymmetrically from the base of the phagosome until the actin “cap” between the internalizing phagosome and the plasma membrane is dissolved. Fluorescence microscopy of live macrophages expressing GFP chimeras has shown that phagocytosis consists of temporally and spatially distinct stages (Figure 5.4), whose sequence corresponds to the distinct, morphological stages of phagosome formation. These stages, pseudopod extension, contraction, phagosome closure, and the generation of reactive oxygen and nitrogen species, reflect the changing molecular regulatory networks associated with the FcγR. It was shown that Cdc42 occurs early and preferentially at the tips of extending pseudopodia. Rac-1 activation occurs around the phagocytic cup and persists during closure, while Rac-2 is active both during and after phagosome closure. On the other hand, the sequence of FcγR signals could reflect the continued advance of the phagocyte membrane over the particle. Therefore, the FcγR activated at
Figure 5.4 Diagram of fluorescent protein chimera association with forming phagosomes. The movements of macrophage membrane (black and red lines) were shown at different time points after initial binding of the IgG-opsonized sheep erythrocyte (grey shape). Red lines show the approximate distribution of fluorescent protein chimeras. AktPH indicates PIP₃ plus PI(3,4)P₂, 2xFYVE indicates PI(3)P, PLCδ1PH indicates PI(4,5)P₂, C1δ indicates diacylglycerol, Cdc42:p21-binding domain of PAK1 (PBD) indicates fluorescence resonance energy transfer (FRET)-based localization of GTP-Cdc42, Rac1:PBD and Rac2:PBD indicate FRET-based localization of GTP-Rac1 and GTP-Rac2, respectively. From reference 258.

Journal of Leukocyte Biology, 2004; 76: 1093
the distal regions of the particle surface would be initiating actin polymerization at the same time as the first activated FcγR at the base of the phagosome, which may have already begun to generate reactive intermediates. This is consistent with the proposed “zipper model” to explain how phagocytosis is achieved. According to this model, the advance of the phagocytic cup occurs as an ordered progression of local FcγR-IgG interactions, in which particle-bound Fc portion of IgG are continually engaged by macrophage FcγR, and each FcγR signals autonomously and governs the membrane and cytoskeletal activities in one small segment of the membrane.

After closure, the phagosome matures via a series of fusion events with organelles of the endocytic pathways, which ultimately leads to the insertion of lysosomal membrane proteins in the phagosomal membrane and to the delivery of lysosomal lytic enzymes into the lumen of the phagosomes. During the course of maturation, phagosomes migrate on microtubules from the cell periphery to a perinuclear location. Hydrolases delivered to the phagosomal lumen by late endosomes and lysosomes contribute to the degradation of the ingested material.

5.4 Regulation of phagocytosis

There is no doubt that the activation of innate immunity is very important, since this will stimulate the production of cytokines to kill infected or transformed cells, or stimulate phagocytosis to clear the infectious particles. Although inflammatory and
immune reactions protect the host from invasion by microorganisms and eliminate debris at the site of tissue injury, they can also be responsible for significant tissue damage or may even stimulate the development of autoimmunity \(^{213}\). Therefore, innate immunity must be under strict control. The phagocytic process itself is accompanied by the generation of reactive oxygen and nitrogen radicals and inflammatory cytokines, byproducts that have the potential to cause severe collateral tissue damage. This means that phagocytosis must also be subject to tight regulation (downregulation), so that it returns to basal level once the infectious agent is eliminated. Indeed, phagocytosis is under several levels of regulation, including negative feedback mechanism, regulatory cytokines, and cytoplasmic phosphatases such as SHIP-1, SHIP-2, SHP-1, and PTEN.

The SH2 domain-containing inositol phosphatase, SHIP-1, was cloned in 1996 by three independent groups \(^{266-268}\). SHIP-1 is a multi-domain protein, containing an N-terminal SH2 domain that has high affinity for the phosphorylated ITIM of FcγRIIb \(^{242,269,270}\), a central catalytic domain which selectively hydrolyzes the 5’ phosphate from IP\(_4\) and PIP\(_3\) \(^{266}\), two tyrosine phosphorylation sites in a motif (NPXY) that preferentially binds to phosphotyrosine binding (PTB) domain-containing proteins such as the Ras adapter Shc \(^{267,271,272}\) and the RasGAP-binding protein Dok \(^{273}\), and a C-terminal proline-rich domain (PRD) that is constitutively associated with the SH3 domains of the Ras adapter protein Grb2 \(^{274}\) (Figure 5.5). As stated above, PIP\(_3\) is required for the activation of several key enzymes that contain a PH domain, such as Btk, Akt, and Vav \(^{275-279}\).
Figure 5.5 Diagram of SHIP-1 structure. SHIP-1 is a multi-domain protein, with an N-terminal SH2 domain, a central catalytic domain which can hydrolyze PIP$_3$ to PI(3,4)P$_2$, and a C-terminal proline rich domain. There are also two C-terminal NPXY motifs that can interact with PTB domain-containing molecules.
SHIP-1 stays within cytoplasm in resting state and translocates to cell membrane upon activation of the cell, so that it can access to its lipid substrates. Membrane association of SHIP-1 is mediated by the interaction between its SH2 domain and the phosphorylated receptor either directly or via the adapter molecule Shc, and the C-terminal of SHIP-1 appears to stabilize this interaction at the membrane.\textsuperscript{280-282}

The inhibitory effect of SHIP-1 on phagocytosis was first identified in macrophages derived from SHIP-1 knockout mice.\textsuperscript{283} Phagocytic efficiency was enhanced in these cells compared to macrophages from the wild-type littermates. Analyses on the molecular details of SHIP-1 activation by macrophage FcγR soon followed, demonstrating that SHIP-1 associates not only with phosphorylated ITIMs, but also with phosphorylated ITAMs either directly or through the adapter protein Shc,\textsuperscript{246,248} suggesting that SHIP-1 downregulates phagocytosis through both activating and inhibitory FcγRs. Besides its ability to hydrolyze PIP\textsubscript{3} to PI(3,4)P\textsubscript{2}, SHIP-1 also influence signaling pathways through interaction with key signaling molecules (Figure 5.6). For example, SHIP-1 appears to inhibit the Ras signaling pathways in B cells by at least two mechanisms that do not require its catalytic function. SHIP-1 competes with the Grb2/Sos complex for the binding of Shc, thus preventing Grb2/Sos membrane translocation and the subsequent activation of Ras.\textsuperscript{284,285} In addition, SHIP-1 was also reported to associate with p62Dok, a Ras GAP-binding protein, thus promote the
Figure 5.6 Diagram of the regulation of FcγR functions by SHIP-1. SHIP-1 hydrolyzes PIP₃ which is critical for multiple downstream signaling molecules. The role of SHIP-1 in regulating the MAPK pathway has been suggested in B cells, and similar mechanisms may exist in the regulation of FcγR-mediated macrophage functions.
inactivation of Ras. Whether similar mechanisms exist in the downregulation of macrophage FcγR functions by SHIP-1 awaits further studies.

Following the cloning of SHIP-1, another SH2 domain-containing inositol 5’-phosphatase was identified and named SHIP-2. Unlike the hematopoietic-specific expression of SHIP-1, SHIP-2 is much more ubiquitously expressed. SHIP-2 has high level of homology with SHIP-1 in the N-terminal SH2 domain and the central catalytic region, but has a quite different C-terminal proline rich domain (Figure 5.7), which associates with specific SH3 domain-containing proteins such as Abl but not Grb2. Additionally, SHIP-2 has only one NPXY motif that binds to PTB domain-containing molecules upon phosphorylation. SHIP-2 is also reported to associate with actin-binding proteins such as filamin and p130Cas, an interaction that is not shared by SHIP-1. Thus, these two molecules may have different functions that are related to their unique protein-protein interactions, but not their catalytic abilities.

Many lines of evidence suggest that SHIP-2 regulates PI-3K-dependent insulin signaling. To date, two different SHIP-2 knockout mice have been generated with different phenotypes. The first model bears a deletion of exons 19-29 of the ship-2 gene. Although much of the 5’-phosphatase domain may not have been inactivated by this gene-targeting construct, it did delete the active site Asp encoded by exon 20 which has been shown to be essential for enzyme activity. A truncated protein should have been inactive if produced in this case. These mice are hypoglycemic and do not survive.
Figure 5.7 Diagram of the structures of SHIP-1 and SHIP-2. SHIP-1 and SHIP-2 have high level of homology in their SH2 domain as well as catalytic domain. However, they differ largely in their C-terminal PRD. In addition, SHIP-2 has only one NPXY motif while SHIP-1 has two.
past the first day. However, it was recently shown that the third and final exon of the *phox2a* gene was also inadvertently deleted, which may result in a truncated and non-functional Phox2a protein\(^{299}\), thus further complicated the explanation of the phenotype.

A new SHIP-2 knockout mouse model has been generated by deleting the first 18 exons of the *ship-2* gene which encodes the SH2 and the catalytic domains\(^{299}\). These mice survive to adult life and surprisingly show no abnormalities in insulin or glucose homeostasis. Significantly, these mice do not become obese on a high fat diet, showing obesity resistance. Further studies are needed to reconcile the differences between these two *ship-2\(^{-/-}\)* phenotypes and elucidate the underlining molecular mechanisms. The role of SHIP-2 in FcγR-mediated phagocytosis will be discussed in details in the following chapters.

Apart from the inositol phosphatases SHIP-1 and SHIP-2, protein tyrosine phosphatase SHP-1 may also play important roles in the regulation of phagocytosis. Like SHIP-1, SHP-1 is a multi-domain protein and is expressed predominantly in hematopoietic cells. SHP-1 contains two N-terminal SH2 domains, a catalytic domain and two tyrosine phosphorylation sites in the C-terminal region\(^{300-302}\). In resting cells, the N-terminal SH2 domain is folded over the catalytic domain, locking it in inactive state\(^{303,304}\). Phosphopeptide engagement onto the SH2 domain opens the phosphatase domain so that the enzyme becomes activated. The C-terminal SH2 domain, on the other hand, serves to recruit SHP-1 substrates. Of the two tyrosine phosphorylation sites, it has
been reported that Y536 contributes to activation of the enzyme \(^{305}\). Studies from two mouse models bearing point mutations in the \(shp-1\) gene led to the identification of SHP-1 as a negative regulator. Motheaten viable (Mev) mice express a catalytically inactive splice variant of \(shp-1\) gene, while motheaten (Me) mice have no SHP-1 protein expression at all \(^{306-309}\). The two types of mice both have severe phenotypes, with uncontrolled expansion of myeloid cells and a shortened life span of about 5-10 weeks. Although it has been conclusively demonstrated that Fc\(\gamma\)RIIb-mediated inhibition occurs via SHIP-1 and not SHP-1 \(^{243-245}\), recent studies have implied that SHP-1 is activated in macrophages during Fc\(\gamma\)R-mediated phagocytosis, perhaps in a non-Fc\(\gamma\)RIIb-dependent manner \(^{234}\). SHP-1 was found to downregulate Fc\(\gamma\)RIIa-mediated phagocytosis in a transfected fibroblast model \(^{234,310}\). In monocytic cells, SHP-1 was reported to associate specifically with the phosphorylated N-terminal ITAM tyrosine of Fc\(\gamma\)RIIa and downregulate NF-\(\kappa\)B-dependent gene transcription in response to Fc\(\gamma\)RIIa clustering \(^{311}\).

PTEN (Phosphatase and tensin homologue on chromosome 10) is a dual phosphatase that can dephosphorylate both phospholipids (at 3’ position) and tyrosine-phosphorylated proteins \(^{312,313}\), the former activity being required for most of its biological effects. PTEN has been reported to negatively regulate immune receptor and growth factor receptor-mediated events \(^{234}\). The role of PTEN in phagocytosis, however, remains largely unknown, although it has been suggested to suppress phagocytosis through the downregulation of Rac activation in a transfected fibroblast model \(^{314}\).
Taken together, the clearance of immune complex is a highly regulated process, and multiple regulation mechanisms exist to prevent excessive inflammation and potential tissue damage. Additional work is needed to define the exact molecular details of these mechanisms.
CHAPTER 6
SHIP-2 DOWNREGULATES FcγR-MEDIATED PHAGOCYTOSIS
INDEPENDENTLY OF SHIP-1

6.1 Abstract

FcγR-mediated phagocytosis of IgG-coated particles is a complex process involving the activation of multiple signaling enzymes, and is regulated by the inositol phosphatases SHIP-1 and PTEN. In a recent study we have demonstrated that SHIP-2, an inositol phosphatase with high level homology to SHIP-1, is involved in FcγR signaling. However, it is not known whether SHIP-2 plays a role in modulating phagocytosis. In this chapter we have analyzed the role of SHIP-2 in FcγR-mediated phagocytosis using independent cell models that allow for manipulation of SHIP-2 function without influencing the highly homologous SHIP-1. Our data indicate that SHIP-2 must contain both the N-terminal SH2 domain and the C-terminal proline-rich domain to mediate its
inhibitory effect. The effect of SHIP-2 is independent of SHIP-1, as overexpression of dominant-negative SHIP-2 in SHIP-1 deficient primary macrophages resulted in enhanced phagocytic efficiency. Likewise, specific knockdown of SHIP-2 expression using siRNA resulted in enhanced phagocytosis. Fetal liver macrophages from SHIP-2 +/- mice exhibited elevated phagocytic ability compared to cells derived from the wild-type littermates. Finally, analysis of the molecular mechanism of SHIP-2 downregulation of phagocytosis revealed that SHIP-2 suppresses upstream activation of Rac. Thus, we conclude that SHIP-2 is a novel negative regulator of FcγR-mediated phagocytosis independently of SHIP-1.

6.2 Introduction

IgG-coated infectious particles are cleared by macrophages via FcγR by phagocytosis. FcγR clustering by immune complex (IC) initiates a signaling cascade that begins with the activation of the Src kinases that activate the ITAMs within the receptor by phosphorylation\(^{315}\). These phosphorylated ITAMs then serve as docking sites to recruit multiple cellular signaling enzymes and enzyme-adapter complexes, including the p85 subunit of phosphatidylinositol 3’-kinase (PI-3K)\(^ {316}\). Association of PI-3K with phosphorylated ITAMs places the enzyme in proximity with its lipid substrates in the plasma membrane, thus allowing for the generation of the important lipid second messenger PIP\(_3\). Additionally, PIP\(_3\) recruits the adapter molecule Gab2 via the Gab2 PH
Association of Gab2 with the plasma membrane results in the tyrosine phosphorylation of Gab2 and the recruitment of PI-3K and consequently enhancement of PIP$_3$ in the membrane. The importance of PI-3K in phagocytosis is well established. Inhibition of PI-3K results in incomplete phagocytosis$^{318-321}$. It has been demonstrated that there is an accumulation of PIP$_3$ in the phagocytic cup$^{322}$. In addition to the recruitment of Gab2, PIP$_3$ recruits other PH domain-containing enzymes including Vav and Akt. Vav is a guanine nucleotide exchange factor for Rac, which is necessary for actin polymerization and cytoskeletal rearrangement that are required for phagocytosis$^{276,323}$. Akt promotes Fc$\gamma$R-mediated phagocytosis working through the downstream molecule p70S6K$^{324}$.

Fc$\gamma$R-mediated activation of macrophages is subject to multiple levels of regulation. It can be regulated by the inhibitory Fc$\gamma$RIIb that predominantly recruits negative regulatory phosphatases$^{224,246,325,326}$. Interestingly, recent studies by us and others have revealed that in addition to positive signals, the Fc$\gamma$R ITAMs are often capable of simultaneously activating negative regulatory proteins including the inositol phosphatase SHIP-1 so that the final biologic response is tempered$^{311,327,328}$. The negative regulatory function of SHIP-1 in various hematopoietic cell functions has been well described$^{327,328}$, but the analysis of SHIP-2 function in hematopoietic cells has lagged since transfection systems have the potential of confounding effects of the overexpressed SHIP-2 molecules on SHIP-1. We have previously reported$^{329}$ that SHIP-2 is expressed in human alveolar
macrophages, but is almost undetectable in peripheral blood monocytes (PBM) derived from the same donors. The expression of SHIP-2 in PBM, however, can be induced by bacterial LPS in a dose-dependent manner. In the human myeloid cell line THP-1, tyrosine phosphorylation of SHIP-2 is induced upon FcγRIIa clustering, and overexpression of wild-type SHIP-2 but not catalytically deficient SHIP-2 completely abrogated NF-κB-dependent gene transcription. The SH2 domain of SHIP-2 is required for its optimal association with FcγRIIa and optimal tyrosine phosphorylation of SHIP-2. We have also shown that FcγRII clustering can result in phosphorylation of SHIP-2 in THP-1 cells. In addition, transient cotransfection experiments in Cos-7 cells demonstrated that wild-type SHIP-2 down-regulated FcγRIIa-induced Akt phosphorylation. However, it is still not clear whether SHIP-2 influences phagocytosis, and if yes, how SHIP-2 does it.

In this study, we have used independent cell models in which the function/expression of SHIP-2 is modulated without interfering with SHIP-1. We demonstrate here that SHIP-2 is a negative regulator of FcγR-mediated phagocytosis in murine macrophages. SHIP-2-mediated downregulation of phagocytosis is dependent on an intact SH2 as well as the C-terminal proline-rich domain of SHIP-2. Our data also indicate that SHIP-2 downregulates phagocytosis by suppressing the upstream activation of Rac.
6.3 Materials and Methods

Cells, antibodies and reagents: Raw 264.7 murine macrophage cells were obtained from ATCC and maintained in RPMI with 3.5% heat-inactivated fetal bovine serum (FBS). Rabbit polyclonal anti-SHIP-2 antibody was a generous gift from Dr. Bayard Clarkson (Memorial Sloan Kettering Cancer Center, New York, NY)\textsuperscript{290}. Goat polyclonal anti-SHIP-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-SHIP-1 antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse anti-Rac antibody was purchased from CHEMICON International, Inc. (Temecula, CA). Rat anti-hemagglutinin (anti-HA) mAb cross-reacting with mouse was from Roche (Indianapolis, IN). Xpress-tagged cDNAs of murine full-length, wild-type SHIP-2 (SHIP-2 WT) and catalytic-inactive SHIP-2 (SHIP-2 D608A) cloned into pcDNA3 vector were provided by Dr. S. Moodie (Metabolex, Hayward, CA)\textsuperscript{330}. HA-tagged cDNAs of human full-length SHIP-2 (HA-SHIP-2 WT), and truncation mutations of PRD (HA-SHIP-2 ΔPRD) and SH2 domains (HA-SHIP-2 ΔSH2) cloned into the pCGN vector were a kind gift from Dr. C. Mitchell (Monash University, Victoria, Australia)\textsuperscript{291}. pmaxGFP was from Amaza Inc. (Gaithersburg, MD). Constitutively active Rac (CA-Rac, Q61L), and GST PAK1 PBD constructs were kind gifts from Dr. Gary Bokoch (La Jolla, CA).
Culture of murine bone marrow macrophages: SHIP-1^{+/−} animals were generously provided by Dr. G. Krystal (B.C. Cancer Agency, Vancouver, BC, Canada). Heterozygotes were bred to obtain SHIP-1^{+/+} and SHIP-1^{+/−} mice. Bone marrow macrophages (BMMs) were derived from these animals as previously described. Briefly, bone marrow cells were cultured in RPMI containing 10% FBS plus 10 μg/ml polymixin B and supplemented with 20ng/ml CSF-1 for 7 days.

Fetal liver macrophage isolation and culture: SHIP-2 gene knockout mice were generated as previously reported. Fetal liver macrophages were isolated and cultured as described. Briefly, fetal livers were harvested, and single-cell suspensions were obtained by passing the minced liver through an 18-gauge TW syringe. Cells were cultured at 37° C for 6 days, with the addition of 20ng/ml M-CSF each day to allow differentiation into macrophages.

Cell stimulation, lysis, and Western blotting: Raw 264.7 cells were activated by clustering FcγR as previously described. Briefly, cells were incubated with 2.4G2 anti-FcγRII/III mAb for 30 minutes on ice. Unbound antibody was washed away, cells were moved to 37° C, and stimulated for varying time points by adding the secondary crosslinking antibody F(ab’')2 mouse anti-rat IgG. Resting and activated cells were lysed in TN1 buffer (50mM Tris pH 8.0, 10mM EDTA, 10mM Na₄P₂O₇, 10mM NaF, 1%
Triton-X 100, 125mM NaCl, 10mM Na<sub>3</sub>VO<sub>4</sub>, 10μg/ml each aprotinin and leupeptin). Proteins were separated by SDS/PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence (ECL).

In other experiments transfected Raw 264.7 cell lysates were subjected to immunoprecipitation with anti-HA antibody and protein G-agarose beads (Invitrogen, Carlsbad, CA) at 4°C overnight, washed in TN1 buffer, and eluted from beads using SDS loading buffer. The samples were further analyzed by Western blotting with the appropriate antibodies.

*Western blot data quantitation:* The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). Background pixel values were subtracted, and the values were plotted as fold increase over unstimulated samples.

*Transient transfection:* To study the influence of SHIP-2 on phagocytosis, Raw 264.7 cells or SHIP-1<sup>−/−</sup> BMMs were transfected using the Nucleofector (Amaxa, Gaithersburg, MD). In brief, 5×10<sup>6</sup> cells in 100 μl of buffer (“Kit V” for Raw 264.7 cells, “Kit T” for BMMs, Amaxa Biosystems) at room temperature were mixed with 5 μg of the SHIP-2 constructs, along with 1 μg EGFP encoding plasmids. The cells were transfected using the program U-14 (for Raw 264.7 cells) or T-20 (for BMMs). After transfection the
samples were transferred to 10cm round dishes containing pre-warmed complete media. Transfectants were cultured for 24 hours and then used in phagocytosis assays.

**SHIP-2 downregulation by siRNA:** Two separate siRNA oligos (SHIP-2 siRNA1, 5’-GGACUUCAUCUUUGUCAGUtt-3’; SHIP-2 siRNA2, 5’-GGUGUUUGACCAGCAG AGCtt-3’) directed against exon 7 and 10 respectively were purchased from Ambion (Austin, TX), along with a control siRNA, which encodes a scrambled sequence with no particular homology to any known sequence. These siRNAs were introduced at a concentration of 100nM into Raw 264.7 cells using the nucleofector. Cells were harvested 24 hours later and protein-matched lysates were analyzed by Western blotting with anti-SHIP-2 antibody. Parallel lysates were probed with anti-SHIP-1 antibody.

**Preparation of IgG-coated sheep RBCs:** Sheep RBCs (SRBCs) (Colorado Serum, Denver, CO) were washed in PBS, and labeled with PKH26 Red (Sigma, St. Louis, MO). Labeled cells were then washed in PBS and incubated with a sub-agglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37°C for 1 hour. Unbound IgG was removed by washing the cells with PBS.

**Phagocytosis Assays:** Phagocytosis assays were performed as previously described. Briefly, IgG-coated SRBCs described above were added to transfected BMMs or
transfected Raw 264.7 cells. The cells were pelleted by low speed centrifugation to increase contact between SRBCs and phagocytes. The samples were incubated for 1 hour at 37°C to study phagocytosis. Cells were then subjected to brief hypotonic lysis with water to get rid of externally bound RBCs prior to fixation in paraformaldehyde to be viewed under a fluorescence microscope. That phagocytosis analyzed was via Fc receptors was confirmed by the lack of ingested particles observed in samples incubated with fluoresceinated RBCs that were not opsonized with IgG. Phagocytosis was measured by counting the total number of SRBCs ingested by 100 transfectants (GFP-positive BMMs, or transfected Raw 264.7 cells). To measure binding, IgG-coated SRBCs were added to transfected Raw 264.7 cells, centrifuged briefly to increase contact, and incubated for 1 hour at 4°C. Cells were then fixed and the ‘binding index’ was measured by counting the total number of SRBCs bound by 100 rosetting (cells that bind 3 or more SRBCs) cells. No binding was seen in samples treated with non-opsonized SRBCs. All experiments were repeated at least three times. Statistical analysis was performed using the Student’s t test, setting a p value of < 0.05 as significant.

Rac activity assay: pGEX-2T encoding PAK1-PBD (p21 binding domain) was a kind gift from Dr. Gary Bokoch (The Scripps Research Institute, La Jolla, CA). Glutathione agarose beads coated with GST-PAK1-PBD were prepared, as described previously. siRNA treated Raw 264.7 cells were activated by clustering FcγR with anti-mouse
FcγRIII/II 2.4G2 antibody followed by mouse F(ab’)2 anti-rat antibody for indicated time points. Cells were lysed in TN1 buffer. Protein-matched cell lysates were incubated with GST-PAK1-PBD beads for 1 hour at 4 °C. After 1 hour, beads were washed with TN1 and then boiled in 1× SDS sample buffer for 10 minutes. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, probed with anti-Rac antibody and developed by ECL. The membranes were reprobed with anti-GST antibody to ensure equal loading of the bait protein.

Flow cytometry of Raw 264.7 cells transfected with GFP: 5×10^6 cells in 100 μl of buffer “Kit V” at room temperature were mixed with 1 μg EGFP encoding plasmids. The cells were transfected using the program U-14. After transfection the samples were transferred to 10cm round dishes containing pre-warmed media. Transfectants were cultured for 24 hours and then analyzed on FACS Calibur (Becton-Dickinson Immunocytometry System, Manassas VA) using Cell Quest software.

6.4 Results

Nucleofection efficiently introduces plasmid DNA into both cell lines and primary myeloid cells

Nucleofection is a recent technological advance that has made transfection of myeloid cells (both primary and transformed) feasible. This system directly delivers DNA into
the nucleus, while maintaining the viability of the cells. We have standardized the transfection protocols for our cells, so that Raw 264.7 cells are transfected using solution “V” and program “U-14”, and BMMs are transfected using solution “T” and program “T-20”. 24 hours after transfection with EGFP plasmid, cells were assessed by flow cytometric analysis and immunofluorescence microscopy. Over 90% transfection efficiency is achieved in Raw 264.7 cells (Figure 6.1A). For BMMs, the transfection efficiency is about 30% (Figure 6.1B, left panel: immunofluorescence image; right panel: phase contrast image of the same field).

**SHIP-2 downregulates FcγR-mediated phagocytosis in Raw 264.7 cells**

We have previously reported that SHIP-2 is recruited to FcγRIIa in human monocytic cells in a manner that is dependent on an intact SH2 domain, and downregulates NF-κB-driven gene transcription 247. Here, to examine the role of SHIP-2 in phagocytosis, we transiently transfected into Raw 264.7 cells plasmids encoding HA-tagged wild-type SHIP-2, SHIP-2 with the N-terminal SH2 domain deleted, or SHIP-2 with C-terminal proline-rich domain deleted (Figure 6.2A). All transfectants also received plasmids encoding GFP as a marker for successful transfection. Phagocytic efficiency of these transfectants was assessed 24 hours after transfection. Phagocytosis was measured by counting the total number of SRBCs ingested by 100 GFP-positive cells. The
Figure 6.1 Nucleofection efficiently introduces plasmid DNA into both cell lines and primary myeloid cells. Cells were transfected using Nucleofector™ with EGFP plasmids. 24 hours after transfection, cells were analyzed for transfection efficiency. (A) Over 90% transfection efficiency can be achieved in Raw 264.7 cells assessed by flow cytometry. (B) The transfection efficiency for BMMs is about 30% (left panel: immunofluorescence image; right panel: phase contrast image of the same field).
experiments were repeated three times, each time analyzing phagocytosis in triplicate. The results are shown in Figure 6.2B and Table 6.1. Compared to cells transfected with empty vector alone (164.67 ± 2.4), cells overexpressing wild-type SHIP-2 showed significantly lower phagocytic ability (101.22 ± 1.02, p<0.05), indicating that SHIP-2 downregulates phagocytosis in Raw 264.7 cells. This downregulation is dependent on an intact SH2 domain as well as an intact PRD, as overpression of the SHIP-2 constructs deplete of either the SH2 domain or the PRD failed to downregulate phagocytosis. To ensure that all transfected molecules were equally expressed, aliquots of cells were lysed, and the whole cells lysates were subjected to immunoprecipitation using anti-HA antibody. The immunoprecipitated proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane, which was subsequently probed with anti-HA antibody (Figure 6.2C, upper panel). The same membrane was washed and reprobed with anti-SHIP-2 antibody (Figure 6.2C, lower panel). As seen in Figure 6.2C, the transfected HA-SHIP-2 constructs were expressed equivalently, and they were of the expected sizes. Note that in the lower panel, the SHIP-2 antibody does not recognize the ΔPRD SHIP-2 as the antibody was raised against the proline-rich domain of SHIP-2. These results suggest that both SH2 domain and the PRD of SHIP-2 are required for SHIP-2 to mediate its inhibitory effect on phagocytosis.
Figure 6.2 SHIP-2 downregulation of FcγR-mediated phagocytosis is dependent on an intact SH2 domain as well as the C-terminal proline-rich domain. Raw 264.7 cells were transiently transfected with (A) empty vector alone, wild-type SHIP-2 (HA-SHIP-2 WT), HA-SHIP-2 ΔSH2, or HA-SHIP-2 ΔPRD. GFP was co-transfected as a marker for transfection. (B) IgG-coated SRBCs were added to the transfectants 24 hours post transfection. Phagocytosis was measured by counting the total number of SRBCs ingested by 100 transfectants (GFP-positive) each time for a total of three readings per sample in each experiment. *: p<0.05 compared to cells transfected with empty vector alone. (C) Whole cell lysates from the transfectants were incubated with anti-HA antibody and protein G-agarose beads overnight. The precipitated proteins were next separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane, which was Western blotted with anti-HA antibody. The same membrane was washed and reprobed with anti-SHIP-2 antibody. These results are representative of three independent experiments.
Table 6.1 SHIP-2 downregulation of FcγR-mediated phagocytosis is dependent on an intact SH2 domain as well as PRD

<table>
<thead>
<tr>
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<th>Phagocytic Index (Mean of three experiments ± SD)</th>
<th>p Value (compared to empty vector transfectants)</th>
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</thead>
<tbody>
<tr>
<td>Vec</td>
<td>164.67 ± 2.40</td>
<td></td>
</tr>
<tr>
<td>SHIP-2 WT</td>
<td>101.22 ± 1.02</td>
<td>0.0008</td>
</tr>
<tr>
<td>SHIP-2 ΔSH2</td>
<td>171.78 ± 3.91</td>
<td>0.1835</td>
</tr>
<tr>
<td>SHIP-2 ΔPRD</td>
<td>170.33 ± 3.00</td>
<td>0.2088</td>
</tr>
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SHIP-2 downregulates FcγR-mediated phagocytosis in SHIP-1−/− BMMs

Although the above experiments demonstrate a role for SHIP-2 in downregulating phagocytosis there are potential problems with this interpretation. The reduction in phagocytosis observed in the cells transfected with wild-type SHIP-2 merely suggests that when SHIP-2 is overexpressed, phagocytosis is downregulated. These experiments did not test whether there is a role for endogenous SHIP-2 in these cells. We reasoned that the use of dominant-negative constructs of SHIP-2 could resolve this issue. However, due to the high level of homology of SHIP-2 with SHIP-1 it was necessary to exclude the possibility that the dominant-negative SHIP-2 constructs interfere with
endogenous SHIP-1. Therefore, to address this question, we have used three independent models, described below, in which expression of SHIP-2 was manipulated without influencing SHIP-1. Thus, in the first model we utilized BMMs isolated from SHIP-1−/− mice to specifically study the function of SHIP-2 in FcγR-mediated phagocytosis. First, we ensured that the BMMs used were indeed deficient for SHIP-1 expression by Western blotting protein-matched lysates from SHIP-1+/+ and SHIP-1−/− BMMs (Figure 6.3A, upper panel). Parallel lysates were probed with anti-SHIP-2 antibody (Figure 6.3A, lower panel). Results indicated that both SHIP-1+/+ and SHIP-1−/− expressed equivalent levels of SHIP-2.

Next, we transiently transfected the SHIP-1−/− BMMs using the Amaxa Nucleofector™ with either empty vector alone, wild-type SHIP-2 (SHIP-2 WT), or a catalytically-inactive SHIP-2 (SHIP-2 D608A) which is a dominant negative form of SHIP-2. Plasmids encoding GFP were co-transfected as a marker for transfection. 24 hours later, phagocytosis assays were performed. Phagocytosis was measured by counting the total number of SRBCs ingested by 100 GFP-positive BMMs. In parallel, aliquots of cells were lysed and whole cell lysates were analyzed by Western blotting. Results from Western blotting confirmed overexpression of SHIP-2 constructs in the transfectants (Figure 6.3B, upper panel), and the transfection efficiency was equivalent as seen by the expression of GFP (Figure 6.3B, lower panel). Phagocytosis was measured in triplicates for each experiment and the experiment was repeated three times.
Figure 6.3 Ectopic expression of SHIP-2 in SHIP-1−/− BMMs. (A) BMMs from SHIP-1+/+ and SHIP-1−/− animals were tested for the expression of SHIP-1 and SHIP-2 by Western blotting. (B) SHIP-1 knock-out BMMs were transiently transfected with empty vector alone, wild-type SHIP-2 (SHIP-2 WT), or catalytically deficient SHIP-2 (SHIP-2 D608A). GFP was co-transfected as a marker for transfection. Whole cell lysates from the transfectants were separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane, which was blotted with anti-SHIP-2 or anti-GFP antibody. These results are representative of three independent experiments.
The data are shown in Table 6.2. Compared to cells transfected with empty vector alone, overexpression of wild-type SHIP-2 significantly reduced the phagocytic ability of these SHIP-1⁻/⁻ BMMs (p<0.05). On the other hand, overexpression of the dominant-negative form of SHIP-2 significantly enhanced phagocytic ability (p<0.05), suggesting that SHIP-2 does indeed downregulate phagocytosis.

SHIP-2 knockdown by siRNA results in enhanced FcγR-function

As an additional approach to confirm the role of SHIP-2 in downregulating phagocytosis, and to overcome any confounding effects of overexpression systems, we next specifically knocked down the expression level of SHIP-2 in Raw 264.7 cells. For this, two separate siRNA oligonucleotides (SHIP-2 siRNA1 and SHIP-2 siRNA2) directed against exons 7 and 10 respectively and a control siRNA encoding a scrambled sequence were transiently transfected into Raw 264.7 cells at a concentration of 100nM using the Nucleofector. Cells were harvested 24 hours later and protein-matched lysates were analyzed by Western blotting with anti-SHIP2 antibody (Figure 6.4A, upper panel). SHIP-2 band intensity was quantitated by laser densitometry and is shown in the graph in the middle panel. Parallel lysates were probed with anti-SHIP-1 antibody (Figure 6.4 A, lower panel). Results indicated that both SHIP-2 siRNAs were capable of specifically downregulating the expression of SHIP-2 with equal efficiency, and did not influence the expression of the highly homologous SHIP-1. The control siRNA had no effect on either
Table 6.2 SHIP-2 downregulates FcγR-mediated phagocytosis in SHIP-1⁻/⁻ BMMs

<table>
<thead>
<tr>
<th></th>
<th>Phagocytic Index (Mean of three readings ± SD)</th>
<th>p Value (compared to empty vector transfectants)</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vec</td>
<td>163.00 ± 20.66</td>
<td></td>
</tr>
<tr>
<td>SHIP-2 WT</td>
<td>122.33 ± 11.24</td>
<td>0.0177</td>
</tr>
<tr>
<td>SHIP-2 D608A</td>
<td>261.00 ± 16.09</td>
<td>0.0168</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vec</td>
<td>247.67 ± 23.12</td>
<td></td>
</tr>
<tr>
<td>SHIP-2 WT</td>
<td>185.67 ± 8.74</td>
<td>0.0193</td>
</tr>
<tr>
<td>SHIP-2 D608A</td>
<td>355.33 ± 28.22</td>
<td>0.0009</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vec</td>
<td>202.00 ± 3.00</td>
<td></td>
</tr>
<tr>
<td>SHIP-2 WT</td>
<td>151.33 ± 3.75</td>
<td>0.0021</td>
</tr>
<tr>
<td>SHIP-2 D608A</td>
<td>299.00 ± 9.97</td>
<td>0.0004</td>
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Three representative experiments are shown.
SHIP-2 or SHIP-1 expression, as expected. Time course experiments revealed that downregulation of SHIP-2 occurs around 18 hours post introduction of siRNA, and persists until around 48 hrs (data not shown).

SHIP-2 siRNA2 was then used in experiments examining phagocytosis. Compared to cells transfected with control siRNA (161.75 ± 10.9), SHIP-2 siRNA transfected Raw 264.7 cells displayed significantly higher phagocytic ability (210.25 ± 6.34) (Figure 6.4B, p< 0.05). To examine whether downregulation of SHIP-2 influences FcγR expression, binding capacity of the cells to IgG-coated SRBC was measured. Cells transfected with either control siRNA or SHIP-2 siRNA displayed equivalent binding ability (398.67 ± 10.69 vs 394.67 ± 6.03, p value 0.314) (Figure 6.4C). Taken together, these data strongly suggest that SHIP-2 is a negative regulator of phagocytosis in macrophages independently of the influence of SHIP-1.

*Phagocytosis is significantly enhanced in fetal liver macrophages derived from SHIP-2 knockout mice*

We have also studied phagocytosis in fetal liver macrophages isolated from either SHIP-2+/− or SHIP-2+/+ mice. Compared to wild-type cells (286.33 ± 34.27), cells derived from mice heterozygous for *ship-2* gene displays significantly higher phagocytic ability (398.33 ± 60.80, p<0.05, Figure 6.5A), which is consistent with the results from SHIP-1.
Figure 6.4 SHIP-2 knockdown by siRNA results in enhanced FcγR-function.

(A) Raw 264.7 cells were transiently transfected with (1) control siRNA, (2) SHIP-2 siRNA1, and (3) SHIP-2 siRNA2. Cells were harvested 24 hrs after transfection and protein-matched lysated were analyzed by Western blotting with anti-SHIP-2 antibody (upper panel) and anti-SHIP-1 antibody (lower panel). The middle panel is a quantitative measurement of SHIP-2 band intensities. (B) Raw 264.7 cells were transiently transfected with control siRNA or SHIP-2 siRNA2. 24 hours after transfection, the IgG-coated SRBCs were added. The samples were incubated for 1 h at 37 °C to study phagocytosis. Phagocytosis was measured by counting the total number of RBCs ingested by 100 transfectants. (C) Raw 264.7 cells transfected with control siRNA or SHIP-2 siRNA2 were incubated with IgG-coated SRBCs for 1 h at 4°C to study binding. The binding activity was expressed as the total number of bound SRBCs on 100 rosetting Raw cells that each bound three or more SRBCs ("Binding Index").
knockout BMMs and SHIP-2 siRNA treated Raw 264.7 cells, indicating that SHIP-2 is a negative regulator of FcγR-mediated phagocytosis. To confirm that SHIP-2 expression is indeed deficient in macrophages derived from SHIP-2+/− mice, protein-matched lysates from SHIP-2+/+ and SHIP-2+/− fetal liver macrophages were analyzed by Western blotting (Figure 6.5B, upper panel).

**SHIP-2 downregulates phagocytosis by suppressing upstream Rac activation**

To understand the mechanism of SHIP-2 downregulation of phagocytosis, we set out to test whether SHIP-2 had an effect on signaling molecules involved in phagocytosis. The low molecular weight GTP-binding protein Rac has been shown to be critical for cytoskeletal remodeling necessary for phagocytosis\(^{276,323}\). Rac GTP binding is facilitated by the guanine nucleotide exchange factor Vav, which in turn is reported to be activated by the binding of Vav PH domain to PIP\(_3\). Thus, we theorized that SHIP-2 with its ability to hydrolyze PIP\(_3\) would likely have a negative influence on Rac activation. To test this notion, we used SHIP-2 siRNA2 to knock down the expression of SHIP-2 in Raw 264.7 cells, and then activated the cells by clustering FcγR. Active GTP-bound Rac was captured from whole cell lysates using glutathione agarose beads coated with GST-PAK1-PBD (p21 binding domain), and resolved by SDS-PAGE. As seen in Figure 6.6A, Rac activation was prominent at 5 minutes after stimulation, and went down slightly at 15 minutes in cells transfected with control siRNA (upper panel). The same activation...
Figure 6.5 Phagocytosis is significantly enhanced in fetal liver macrophages derived from SHIP-2 knockout mice. (A) IgG-coated SRBCs were added to fetal liver macrophages derived from SHIP-2 knockout mice. The samples were incubated for 1 h at 37 °C to study phagocytosis. Cells were then subjected to brief hypotonic lysis prior to fixation in paraformaldehyde to be viewed under a fluorescence microscope. Phagocytosis was measured by counting the total number of RBCs ingested by 100 transfectants (GFP-positive). *: p<0.05. (B) Protein-matched lysates from SHIP-2 +/- and SHIP-2 +/+ fetal liver macrophages were separated on 10% SDS-PAGE, and analyzed by Western blotting with anti SHIP-2 antibody.
pattern was observed in cells transfected with SHIP-2 siRNA, but the activation levels were significantly higher (Figure 6.6A, p<0.05). The membranes were subsequently reprobed with anti-GST antibody to ensure equal loading of the bait protein (middle panel). Band intensities of Rac were quantitated and converted to the fold increases over unstimulated control sample. Data from at least three independent experiments are presented in Figure 6.6A. Compared to cells transfected with control siRNA, downregulation of SHIP-2 expression significantly enhanced Rac activation, suggesting that SHIP-2 may negatively regulate phagocytosis by suppressing upstream Rac activation. This effect was specific for SHIP-2, since the expression of SHIP-1 was not altered by siRNA treatment (Figure 6.6B).

To further analyze whether the effect of SHIP-2 on phagocytosis is mediated via its influence on Rac activation, SHIP-2 expression was downregulated in cells expressing constitutively active Rac (CA-Rac). Phagocytic efficiency of these transfectants was then assessed. Figure 6.7A is a Rac GTP-binding assay to ensure that CA-Rac is indeed constitutively active. Figure 6.7B and 6.7C are Western blots of protein-matched lysates from the transfectants indicating the presence of the CA-Rac, and SHIP-2 downregulation by siRNA respectively. Knock-down of SHIP-2 resulted in a significant enhancement of phagocytosis, as expected (p<0.05, Table 6.3 and Figure 6.7D). In contrast, although cells expressing CA-Rac showed enhanced phagocytosis compared to cells transfected with vector alone, SHIP-2 downregulation in these cells conferred no further
Figure 6.6 SHIP-2 downregulates Rac activation. Raw 264.7 cells were transiently transfected with either a non-specific control siRNA or SHIP-2 siRNA. (A) 8 hrs after transfection, cells were starved in incomplete RPMI media for 16 hrs. After starvation, cells were stimulated with 2.4G2 followed by MAR (mouse anti-rat IgG) for indicated time. Protein-matched cell lysates were incubated with GST-PAK1 PBD-agarose beads for 1 hr at 4 °C. Active Rac bound to the beads was eluted and loaded on 12% SDS-PAGE (upper panel). Equal volume of lysis buffer only and whole cell lysate were loaded as negative and positive controls (Neg & WCL). The membranes were reprobed with anti-GST antibody (middle panel). Rac band intensities were quantitated, and are presented as fold-increase over the resting control siRNA transfected sample (lower panel). (B) Whole cell lysates from the resting samples were separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane, which was then probed with anti-SHIP-2 (upper panel) or anti-SHIP-1 antibody (lower panel).
enhancement in phagocytic efficiency (239.67 ± 12.22 vs 234.33 ± 9.71), strongly suggesting that SHIP-2 influences phagocytosis by downregulating Rac activation.

Table 6.3 SHIP-2 downregulates phagocytosis by suppressing upstream Rac activation

<table>
<thead>
<tr>
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<th>Phagocytosis Index (Mean of three experiments ± SD)</th>
<th>p Value (compared to control siRNA transfectants)</th>
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<tbody>
<tr>
<td>Control siRNA</td>
<td>151.00 ± 4.58</td>
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</tr>
<tr>
<td>SHIP-2 siRNA</td>
<td>192.67 ± 5.03</td>
<td>0.0050</td>
</tr>
<tr>
<td>Control siRNA +</td>
<td>239.67 ± 11.24</td>
<td>0.0104</td>
</tr>
<tr>
<td>CA-Rac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHIP-2 siRNA +</td>
<td>234.33 ± 9.71</td>
<td>0.0088</td>
</tr>
<tr>
<td>CA-Rac</td>
<td></td>
<td></td>
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</tbody>
</table>

6.5 Discussion

Macrophages play an important role in innate immune response by phagocytosing IgG-coated infectious particles. However, collateral tissue damage can occur if this process goes unchecked. Regulation of phagocytosis is rather complicated. It not only
Figure 6.7 SHIP-2 downregulates phagocytosis by suppressing upstream Rac activation. (A) Raw 264.7 cells were transiently transfected with constitutively active Rac (CA-Rac). 8 hrs after transfection, cells were starved in incomplete RPMI media for 16 hrs, and then stimulated with 2.4G2 followed by MAR for 5 min. Protein-matched cell lysates were incubated with GST-PAK1 PBD-agarose beads for 1 hr at 4 °C. Equal volume of lysis buffer was incubated with beads as a negative control (Neg). Active Rac bound to the beads was eluted and loaded on 12% SDS-PAGE (upper panel), and analyzed by Western blotting with anti-Rac antibody. The membrane was washed and reprobed with an anti-GST antibody (lower panel). (B) Raw 264.7 cells were co-transfected with either a non-specific control siRNA or SHIP-2 siRNA with CA-Rac. 24 hours after transfection, WCLs were made and separated on 12% SDS-PAGE, and probed with anti-Rac antibody. (C) Parallel samples were separated by 10% SDS-PAGE, and probed with anti-SHIP-2 (upper panel) or anti-SHIP-1 antibody (lower panel). (D) IgG-coated SRBCs were incubated with the transfectants for 1 h at 37 °C to assess phagocytosis.
involves the opposing actions of activating vs. inhibitory FcγRs, which can be affected by
the microenvironment (e.g. proinflammatory/anti-inflammatory cytokines) \(^{325,326,336}\), but
also engages multiple cytoplasmic phosphatases \(^{310,314,333,337}\). While the role of SHIP-1 in
regulation of phagocytosis has been well studied \(^{246,283}\), the functions of SHIP-2 in this
critical immune reaction remain largely unknown.

In this chapter, we have demonstrated that SHIP-2 downregulates FcγR-mediated
phagocytosis using multiple cell models. We have observed similar downregulatory
effect of SHIP-2 in SHIP-2\(^{+/+}\) compared to SHIP-2\(^{+/−}\) fetal liver macrophages. These
latter data need to be taken with more caution since the mice used in these studies, which
were initially thought to be SHIP-2 knockouts, were subsequently shown to express
fragments of SHIP-2 and are also deleted in other adjacent genes \(^{294,338}\). However, results
from SHIP-1\(^{-/−}\) BMMs overexpressing wild-type and dominant negative forms of SHIP-2,
and from Raw 264.7 cells treated with specific SHIP-2 siRNA provided convincing proof
that SHIP-2 is a negative regulator of phagocytosis independently of SHIP-1.

Our data showing that overexpression of wild-type SHIP-2 significantly reduced
phagocytosis in Raw 264.7 cells whereas overexpression of ΔSH2-SHIP-2 or ΔPRD-
SHIP2 did not have this downregulation effect are consistent with our previous finding
that SH2 domain is required for the recruitment and activation of SHIP-2 \(^{247}\). Other
studies have reported that SHIP-2 translocates to the cell membrane upon stimulation of
macrophages with CSF-1 or fibroblasts with insulin, and this translocation depends on
the interaction between its C-terminal proline-rich domain with the actin-binding protein Filamin\textsuperscript{291,332}. Our current studies suggest that SHIP-2 is likely recruited to the activated Fc\(\gamma\)R via its SH2 domain, and this membrane localization is further stabilized through its PRD domain. Indeed, a similar role for the PRD of SHIP-1 has been described\textsuperscript{280-282,339}. Thus, mutation of either the SH2 domain or the PRD of SHIP-2 would prevent stable association of SHIP-2 with the plasma membrane, making either mutant incapable of downregulating phagocytosis and acting in a dominant-negative fashion.

Our results indicate that SHIP-2 downregulates phagocytosis possibly through suppression of the upstream Rac activation. Once recruited to the phosphorylated Fc\(\gamma\)Rs, SHIP-2 depletes the key secondary messenger PIP\(_3\) by hydrolyzing it to PI(3,4)P\(_2\), leading to the inhibition of several PH domain-containing molecules that require PIP\(_3\) such as Vav and Akt, which can initiate signaling pathways that are critical for actin polymerization. There are multiple Rac GEFs identified, and many are expressed in macrophages\textsuperscript{340}. The Vav family of proteins act as GEFs for Rac, and its activity towards Rac is regulated by both tyrosine phosphorylation and PIP\(_3\)\textsuperscript{341,342}. It has been suggested that Vav acts as a PI-3K-dependent activator for Rac activation in macrophages stimulated with M-CSF\textsuperscript{343}. In SHIP-1\textsuperscript{-/-} BMMs, Rac was shown to be constitutively active and Vav was constitutively phosphorylated. Whether SHIP-2 suppresses Rac activity similarly through Vav need to be further clarified.
SHIP-1 and SHIP-2 both serve to downregulate phagocytosis, yet they must have non-overlapping functions as well. In addition to the divergence of the two molecules in their C-terminal proline-rich domain, SHIP-1 and SHIP-2 also have different number of tyrosine residues in the C-terminus that conform to an NPXY motif shown to bind PTB domains upon phosphorylation, all of which lead to distinct protein-protein interactions. For example, the proline-rich domain of SHIP-1 associates with Grb2, whereas the proline-rich domain of SHIP-2 fails to associate with Grb2 but associates with Abl 287. These two molecules also differ in their expression pattern: SHIP-1 is expressed predominantly in hematopoietic cells, while SHIP-2 is much more ubiquitously expressed, suggesting that SHIP-2 has more generalized cellular functions. In addition, the expression of SHIP-2 is more prominent in functionally mature cells such as macrophages but not monocytes, although its expression in monocytes can be upregulated by bacterial LPS stimulation. SHIP-2 has been reported to associate both with the phosphorylated ITIM of FcγRIIb and the ITAMs of FcγRI and FcγRIIa 247,344. Interestingly, unpublished data from us showed that SHIP-2 associates with the phosphorylated ITAM of FcγRIIa with equivalent efficiency as it does with the phosphorylated ITIM of FcγRIIb. This is in contrast to the binding ability of SHIP-1, which is highly efficient for the ITIM but comparatively weaker for the ITAM. In fact, work from our group and others indicated that the association of SHIP-1 with phosphorylated ITAMs is predominantly indirect and is mediated by the Ras adapter Shc
Although it can associate to some extent directly to phosphorylated ITAMs, taken together, these observations suggest that SHIP-2 may be the dominant regulator of phagocytosis when both SHIP-1 and SHIP-2 are present. More studies are needed to elucidate the functional differences between SHIP-1 and SHIP-2 in phagocytosis as well as other FcγR-mediated functions.
CHAPTER 7

SUBCELLULAR LOCALIZATION OF SHIP-2 IN MURINE MACROPHAGES

7.1 Abstract

We have previously shown that SHIP-2 downregulates FcγR-mediated phagocytosis of IgG-coated particles independently of SHIP-1. To directly examine the localization of SHIP-2 during phagocytosis, we utilized immunofluorescence staining and confocal microscopy analyses in both fibroblasts and macrophages. Using GFP-tagged constructs, SHIP-2 was shown to concentrate around the IgG-coated SRBCs in Cos-7 cells during phagocytosis. In Raw 264.7 cells, we presented evidence that SHIP-2 translocates to the site of phagocytosis, and this translocation requires both an intact SH2 domain and a PRD. SHIP-2 and SHIP-1 colocalize in resting cells as well as during phagocytosis in Raw 264.7 cells. In Cos-7 cells cotransfected with FcγRIIa and GFP-tagged SHIP-2
constructs, FcγRIIa clustering induces the phosphorylation of SHIP-2. This phosphorylation again requires both SH2 and PRD domains, consistent with the translocation results from Raw 264.7 cells. Thus, we conclude that SHIP-2, like SHIP-1, is recruited directly to the site of phagocytosis to function, and both SH2 domain and PRD are indispensable for the translocation of SHIP-2.

7.2 Introduction

The biological functions of intracellular proteins are closely related to their specific subcellular localizations. SHIP-1, a well-studied negative regulator of phagocytosis, has been shown to translocate to the phagocytic cup during phagocytosis (Figure 7.1). Once there, SHIP-1 can hydrolyze PIP3, which plays important roles in the assembly of actin cytoskeleton required for phagocytosis. In previous chapter, we have shown that SHIP-2 also downregulates phagocytosis, and its function is independent of SHIP-1. However, it is not clear whether SHIP-2 can be recruited to the site of phagocytosis as well.

The subcellular localization of SHIP-2 has been investigated in various cell types. In quiescent cells, SHIP-2 localizes to the cytosol and it relocalizes to membrane ruffles following cell adhesion and/or growth factor stimulation (Figure 7.2), which is associated with SHIP-2 tyrosine phosphorylation. SHIP-2 has been shown to facilitate cytoskeletal dynamics through the interactions with the actin-binding protein filamin,
**Figure 7.1** SHIP-1 is recruited to the phagocytic cup during phagocytosis. Adherent thio-macrophages obtained from SHIP-1<sup>+/+</sup> mice were incubated with ElgG2a for 7 min at 37°C. After fixation, cells were stained for the presence of SHIP-1 and ElgG2a and subjected to confocal microscopy. From reference 273.
Figure 7.2 SHIP-2 localizes to actin-rich regions in Cos-7 cells. Cos-7 cells were transiently transfected with GFP-SHIP-2 and either left unstimulated or serum-starved followed by stimulation with epithelial growth factor (EGF) (100ng/ml) for the indicated times, and fixed and phalloidin stained. Arrows indicate SHIP-2 membrane localization. Bar: 10μm. From reference 281.
p130Cas and Shc. SHIP-2 can also complex directly with the hepatocyte growth factor (HGF/SF) receptor c-Met, via phosphotyrosine 1356 to regulate cell migration. It has also been shown that SHIP-2 associates with the c-Cbl-associated protein (CAP) and c-Cbl and coimmunoprecipitates with the insulin receptor, although the functional consequences are unknown.

In this chapter, we have examined the subcellular localization of SHIP-2 in both fibroblasts and macrophages. In Cos-7 cells transiently transfected with GFP-tagged constructs, SHIP-2 was shown to concentrate around the IgG-coated SRBCs during phagocytosis. This result is confirmed in Raw 264.7 cells, that SHIP-2 translocates to the site of phagocytosis. By using HA-tagged SHIP-2 constructs, we further showed that this translocation requires both an intact SH2 domain and a PRD. SHIP-2 and SHIP-1 colocalize in resting cells as well as during phagocytosis in Raw 264.7 cells. In addition, we cotransfected Cos-7 cells with FcγRIIa and GFP-tagged wild-type SHIP-2. Immunoprecipitation assay (IP) indicated that FcγRIIa clustering induces the phosphorylation of SHIP-2, which again requires both SH2 and PRD domains. Collectively, our results suggest that SHIP-2 is recruited directly to the site of phagocytosis to function, and both SH2 domain and PRD are indispensable for the translocation of SHIP-2.
7.3 Materials and methods

Cells, antibodies and reagents: Raw 264.7 murine macrophage cells were obtained from ATCC and maintained in RPMI with 3.5% FBS. Cos-7 fibroblast cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% FBS. Rabbit polyclonal anti-SHIP-2 antibody was a generous gift from Dr. Bayard Clarkson (Memorial Sloan Kettering Cancer Center, New York, NY). Goat polyclonal anti-SHIP-2 antibody and mouse anti-SHIP-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- FcγRIIa antibody was obtained from Medarex (Annandale, NJ). Anti-phospho-tyrosine antibody 4G10 was from Upstate (Charlottesville, VA). Mouse anti-GFP antibody was purchased from BD Bioscience (San Diego, CA). Alexa Fluor 647 rabbit anti-goat IgG and alexa Fluor 594-conjugated F(ab’)2 fragment goat anti-mouse IgG were from Molecular Probes, Inc. (Eugene, OR). Cy5-conjugated F(ab’)2 fragment donkey anti-goat IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hoechst nuclear stain was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-HA mAb was from Roche (Indianapolis, IN). Mouse anti-HA antibody was from Cell Signaling (Beverly, MA). HA-tagged cDNAs of human full-length SHIP-2 (HA-SHIP-2 WT), and truncation mutations of PRD (HA-SHIP-2 ΔPRD) and SH2 domains (HA-SHIP-2-ΔSH2) cloned into the pCGN vector were a kind gift from Dr. C. Mitchell (Monash University, Victoria, Australia). pmaxGFP was from Amaxa Inc. (Gaithersburg, MD).
**Culture of murine bone marrow macrophages:** As described in Chapter 6, SHIP-1\(^{+/−}\) animals were bred to obtain SHIP-1\(^{++}\) and SHIP-1\(^{−−}\) mice. Bone marrow cells were cultured in RPMI containing 10% fetal bovine serum plus 10 μg/ml polymixin B and supplemented with 20ng/ml CSF-1 for 7 days.

**Cell stimulation, lysis, immunoprecipitation and Western blotting:** transfected Cos-7 cells were activated by clustering Fc\(γ\)RIIa with monoclonal antibody FLI8 (BD Pharmingen, San Diego, CA) for 30 min at 4°C followed by goat F(ab’\(^{2}\)) anti-mouse IgG secondary antibody for 5 min at 37°C. Resting and activated cells were lysed in TN1 buffer (50mM Tris pH 8.0, 10mM EDTA, 10mM Na\(_4\)P\(_2\)O\(_7\), 10mM NaF, 1% Triton-X 100, 125mM NaCl, 10mM Na\(_3\)VO\(_4\), 10μg/ml each aprotinin and leupeptin), and post-nuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Invitrogen). Immune complexes bound to beads were washed with lysis buffer and boiled in SDS sample buffer (60 mM Tris [pH 6.8], 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 2% 2-mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the antibodies of interest, and developed by ECL. Aliquotes of whole cell lysates were also directly subjected to immunoblotting analysis with desired antibodies.
**Western blot data quantitation:** The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). Background pixel values were subtracted, and the values were plotted as fold increase over unstimulated samples.

**Transient transfection of HA-SHIP-2 in Raw 264.7 cells:** $5 \times 10^6$ cells in 100 μl of buffer ("Kit V") at room temperature were mixed with 5 μg of the SHIP-2 constructs. The cells were transfected using the program U-14. After transfection the samples were transferred to 10cm round dishes containing pre-warmed media for 24 hrs. Aliquots of transfected cells were placed in 12-well plate with coverglass in the well.

Cos-7 cells were transfected as described previously\(^{248}\). Briefly, cells were grown on culture dishes to 90% confluency. cDNA for FcγRIIα in pCEXV3 (kindly provided by Dr. J. Ravetch, Rockefeller University, New York) and cDNAs for GFP-SHIP-2 constructs (wild-type, the inactive SH2 domain mutant of SHIP-2 [R47K], the inactive catalytic domain mutant of SHIP-2 [D608A], SHIP-2 with PRD deleted, and PRD only) were mixed in various combinations with Lipofectamine 2000 reagent (Invitrogen). The DNA mixture was added to cells in serum-free DMEM medium and incubated for 4 hrs at 37 °C in a CO₂ incubator. The medium was then replaced with DMEM medium supplemented with 10% FBS. The cells were harvested 24 hrs later and analyzed for expression of the transfected cDNAs by Western blotting. Having ensured that the various transfectants expressed comparable levels of protein, we then examined the
ability of the wild-type and mutant SHIP-2 molecules to become tyrosine-phosphorylated upon FcγRIIa clustering.

Preparation of IgG-coated sheep RBCs: Sheep RBCs (SRBCs) (Colorado Serum, Denver, CO) were washed in PBS, and labeled with PKH26 Red (Sigma, St.Louis, MO). Labeled cells were then washed in PBS and incubated with a sub-agglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37°C for 1 hour. Unbound IgG was removed by washing the cells with PBS.

Immunofluorescence staining and Confocal Microscopy: BMMs from SHIP-1−/− mice, or Untransfected or HA-SHIP-2 transfected Raw 264.7 cells were grown on coverslips, and incubated with IgG-coated SRBCs for 5 minutes at 37 °C. Cells were then fixed in 4% paraformaldehyde and permeabilized in 0.2% TritonX-100. After blocking in serum, untransfected cells were stained with a goat anti-SHIP-2 antibody, followed by a Cy5 conjugated donkey anti-goat F(ab’)2 fragment to detect endogenous SHIP-2. Endogenous SHIP-1 was stained with a mouse anti-SHIP-1 antibody, followed by an Alexa Fluor 594 conjugated goat anti-mouse F(ab’)2 fragment. Transfectants were stained with a mouse anti-HA antibody, followed by an Alexa Fluor 594 conjugated goat anti-mouse F(ab’)2 fragment to detect HA-SHIP-2. F-Actin was stained with FITC-phalloidin. Nuclei were stained with Hoechst. Coverslips were mounted on slides using mounting media
(DakoCytomation, Carpinteria, CA), and read using Zeiss LSM510 multiphoton confocal microscope (Zeiss, Oberkochen, Germany). Zeiss LSM5 Image software was used for image processing.

For immunofluorescence staining of transfected Cos-7 cells, cells were grown on coverslips overnight and transfected with desired plasmids. 24 hrs after transfection, IgG-coated SRBCs were added and incubated for different time at 37 °C. For ΔIgG stimulation, human IgGs (whole molecule) were heated at 62 °C for 30 min, quickly cooled down on ice for 2 min, and used at a concentration of 300μg/ml. Cells were then fixed and permeabilized. Nuclei were stained with Hoechst. Coverslips were mounted on slides using mounting media, and read using Olympus IX50 immunofluorescence microscope or Zeiss LSM510 confocal microscope.

### 7.4 Results

**SHIP-2 is recruited to the site of phagocytosis in transfected Cos-7 cells**

To begin to study the subcellular localization of SHIP-2 during phagocytosis, we first utilized the Cos-7 fibroblast cells. As these cells do not express endogenous FcγRs, plasmids encoding human FcγRIIa are transiently transfected into them. 24 hrs after transfection, IgG-coated SRBCs were added. Compared to mock-transfected cells, cells transfected with FcγRIIa displayed rosetting, indicating that the transfected FcγRIIa are expressed on cell surface and are functional (Figure 7.3). To localize SHIP-2 during
Figure 7.3 Transfection of FcγRIIa into Cos-7 cells. Cos-7 cells were transiently transfected with human FcγRIIa. 24 hrs after transfection, IgG-coated SRBCs were added. Compared to mock-transfected cells (upper panel), cells transfected with FcγRIIa displayed rosetting, indicating that the transfected FcγRIIa are expressed on cell surface and are functional.
phagocytosis, GFP-tagged wild-type SHIP-2 was cotransfected with FcγRIIa into Cos-7 cells. Cells cotransfected with plasmid encoding GFP only were used as a control, and GFP was shown to have an evenly distributed cytoplasmic expression pattern (Figure 7.4, upper panel). The transfected GFP-SHIP-2 also localizes to the cytoplasm, but with bright spots near the nucleus (Figure 7.4, lower panel).

These cotransfected Cos-7 cells were then incubated with IgG-coated SRBCs for different time, and the localization of GFP-SHIP-2 was determined by immunofluorescence microscopy. As shown in Figure 7.5, GFP-SHIP-2 has a cytoplasmic expression pattern which is enhanced around nucleus in resting cells. Five minutes after incubation with IgG-SRBCs, SHIP-2 can be seen to be recruited to the site of phagocytosis, and the enhanced bright spots around nucleus disappeared. This translocation is still detectable at ten minutes after IgG-SRBC addition.

SHIP-2 is recruited to the phagocytic cup in Raw 264.7 cells

Previous studies demonstrated that during FcγR-mediated phagocytosis SHIP-1 localized to the phagocytic cup. To examine whether SHIP-2 would likewise move to the phagocytic cup in macrophages, we next examined the cellular localization of endogenous SHIP-2 in Raw 264.7 cells using confocal immunofluorescence microscopy. Endogenous SHIP-2 was labeled by a goat anti-SHIP-2 primary antibody, followed by a Cy5-conjugated secondary antibody. In resting cells, SHIP-2 (yellow) was seen to
Figure 7.4 Cotransfection of GFP-SHIP-2 and FcγRIIa into Cos-7 cells. Cos-7 cells grown on coverslips were cotransfected with GFP-tagged wild-type SHIP-2 and FcγRIIa. 24 hrs after transfection, coverslips were fixed, washed, mounted on slides, and read with an immunofluorescence microscope. The expression of GFP was evenly distributed within the cytoplasm in cells transfected with GFP only (upper panel). GFP-SHIP-2 also displayed cytoplasmic localization, but with bright spots around nucleus (lower panel).
Figure 7.5 SHIP-2 is recruited to the site of phagocytosis in transfected Cos-7 cells. Cos-7 cells cotransfected with GFP-SHIP-2 and FcγRIIa were incubated with IgG-SRBCs for indicated time. SHIP-2 is recruited to the site of phagocytosis (arrows). Left panel shows images with filters set for GFP only. Right panel shows overlay images with also nuclear and RBC stainings. Bar: 10μm.
localize to the cytoplasm in a diffuse pattern, with no apparent membrane staining (Figure 7.6, upper panel). However, 5 min after incubation with IgG-coated SRBCs, SHIP-2 was seen to translocate to the site of phagocytosis, around the ingested SRBCs (red) and co-localize with F-actin (green) (middle panel), suggesting that SHIP-2 is involved in regulating early phagocytic events including actin remodeling. The observed distribution pattern was specific for SHIP-2, since slides stained with normal goat IgG followed by the same fluorescence-labeled secondary antibody showed a clean background (lower panel).

**SHIP-2 translocates to the phagocytic cup independently of SHIP-1**

Raw 264.7 cells express both endogenous SHIP-2 and SHIP-1 molecules. Cells stained simultaneously for the two molecules showed that SHIP-2 colocalizes with SHIP-1 during phagocytic cup formation, confirming that SHIP-2 and SHIP-1 are both involved in early actin cytoskeleton remodeling necessary for phagocytosis (Figure 7.7).

To further test whether the translocation of SHIP-2 is independent of SHIP-1, and to test the specificity of the staining antibodies, we examined the cellular localization of SHIP-2 in SHIP-1−/− BMMs. Indeed, we detected the same recruitment of SHIP-2 from cytoplasm to the phagocytic cup in these BMMs (Figure 7.8), consistent with the finding that SHIP-2 downregulates FcγR-mediated phagocytosis independently of SHIP-1.
Figure 7.6 SHIP-2 is recruited to the site of phagocytosis. Raw 264.7 cells were grown on coverslips, and incubated with IgG-coated SRBCs (red) for 5 minutes at 37 °C. Cells were then fixed in 4% paraformaldehyde and permeabilized in 0.2% TritonX-100. After blocking, cells were stained with a goat anti-SHIP-2 antibody, followed by a Cy5-conjugated donkey anti-goat F(ab’)2 fragment (yellow). F-actin was stained with FITC-phalloidin (green), and nuclei were stained with Hoechst (blue). Coverslips were mounted on slides, and read using Zeiss 510 confocal microscope. SHIP-2 has a diffused cytoplasmic pattern in resting cells (upper panel). SHIP-2 localizes to the site of phagocytosis (middle panel), where it co-localizes with F-actin. Cells stained with normal goat IgG followed by Cy5-conjugated donkey anti-goat F(ab’)2 fragment showed clean background (lower panel).
A. Resting

B. IgG-SRBC phagocytosis

Figure 7.7 SHIP-2 colocalizes with SHIP-1 during phagocytosis. Raw 264.7 cells were grown on coverslips, and either (A) left untreated or (B) incubated with IgG-coated SRBCs for 5 minutes at 37 °C. Cells were then fixed and permeabilized. Endogenous SHIP-2 (yellow) and SHIP-1 (red) were stained with their specific antibodies, followed by either an Alexa Fluor 647-conjugated or an Alexa Fluor 594-conjugated secondary antibody. F-actin was stained with FITC-phalloidin (green), and nuclei were stained with Hoechst (blue). Coverslips were mounted on slides, and read using Zeiss 510 confocal microscope. SHIP-2 was shown to colocalize with SHIP-1 in both resting and phagocytosing cells. In phagocytic cup, SHIP-1 and SHIP-2 also colocalize with F-actin.
Figure 7.8 SHIP-2 is recruited to the site of phagocytosis independently of SHIP-1. SHIP-1−/− BMMs were grown on coverslips, and incubated with IgG-coated SRBCs (red) for 5 minutes at 37 °C. Cells were then fixed, permeabilized, and stained with fluorescence-conjugated specific antibodies for both SHIP-1 and SHIP-2 (yellow). Nuclei were stained with Hoechst (blue). Coverslips were mounted on slides, and read using Zeiss 510 confocal microscope. SHIP-2 has a diffuse cytoplasmic pattern in resting cells (upper panel), and translocates to the site of phagocytosis (lower panel). SHIP-1 staining is negative.
**SH2 domain and PRD are both required for the translocation of SHIP-2**

To further test whether the recruitment to the phagocytic cup has any domain requirements, we transfected HA-tagged SHIP-2 constructs into Raw 264.7 cells, and examined their subcellular localization using confocal microscopy. In resting cells, wild-type SHIP-2 (SHIP-2 WT), SHIP-2 with SH2 domain or PRD deleted (SHIP-2 ΔSH2 or SHIP-2 ΔPRD) all have a diffuse cytoplasmic localization. The transfected wild-type SHIP-2 was recruited to the phagocytic cup as expected (Figure 7.9B). However, there appeared to be no such translocation for SHIP-2 with either SH2 domain or PRD deleted (Figure 7.9C, 7.9D). Empty vector transfected cells were used as a negative control (Figure 7.9A). Immunoprecipitation of whole cell lysates with anti-HA antibody showed that the transfected constructs were expressed, and were of the expected sizes (Figure 7.9E). These results are consistent with the finding that both the SH2 domain and the PRD are required for SHIP-2 to downregulate phagocytosis (Figure 6.2).

**SHIP-2 translocates to plasma membrane upon FcγR clustering**

It has been reported that SHIP-2 translocates to the cell membrane upon stimulation of macrophages with CSF-1 or fibroblasts with EGF, and this translocation depends on the interaction between its C-terminal proline-rich domain with the actin-binding protein filamin. To test whether SHIP-2 translocates to cell membrane upon FcγR activation, we cotransfected FcγRIIa and GFP-SHIP-2 into Cos-7 cells. 24 hrs after
**Figure 7.9** SH2 and PRD domains are both required for translocation of SHIP-2 to the phagocytosis site. Raw 264.7 cells transfected with HA-tagged SHIP-2 constructs were grown on coverslips, and incubated with IgG-coated SRBCs (red) for 5 minutes at 37 °C. Transfected SHIP-2 constructs were stained with a mouse anti-HA antibody, followed by an Alexa Fluor 594 conjugated goat anti-mouse F(ab’2)2 fragment (yellow). (A)-(D) show images of cells transfected with empty vector, HA-SHIP-2 WT, HA-SHIP-2 ΔSH2, or HA-SHIP-2 ΔPRD. (E) Whole cell lysates from the transfectants were analyzed by immunoprecipitation with anti-HA antibody.
transfection, cells were stimulated with heat-aggregated human IgG to cluster, and thus activate the transfected FcγRIIa. Confocal immunofluorescence microscopy analysis showed that membrane stain for GFP-SHIP-2 started to appear as early as 1 min upon FcγRIIa activation, and it continued for at least 10 min (Figure 7.10), suggesting that SHIP-2 translocates to plasma membrane upon FcγR clustering.

SHIP-2 is phosphorylated upon FcγRIIa clustering in an SH2- and PRD-dependent way

It has been previously shown that SHIP-2 phosphorylation can be induced by FcγRI clustering in THP-1 cells as well as FcγRIIa clustering in transfected Cos-7 cells, and the latter requires an intact SH2 domain. To further test the involvement of other domains in SHIP-2 phosphorylation, and thereby its translocation to the membrane, we cotransfected plasmid encoding FcγRIIa along with plasmids encoding various GFP-tagged SHIP-2 constructs (wild-type SHIP-2 [WT], catalytic domain mutant of SHIP-2 [D608A], SH2 domain mutant of SHIP-2 [R47K], SHIP-2 with PRD deleted [ΔPRD], or PRD only) into Cos-7 cells (Figure 7.11). FcγRIIa receptors were clustered using specific mouse antibody FLI8 followed by GAM (goat anti-mouse IgG). SHIP-2 proteins were immunoprecipitated from resting and activated cells using anti-GFP antibody and analyzed by Western blotting with anti-phosphotyrosine antibody 4G10. Results indicated that while both WT and D608A SHIP-2 become tyrosine phosphorylated upon
Figure 7.10 SHIP-2 translocates to cell membrane upon FcγRIIa clustering in transfected Cos-7 cells. Cos-7 cells grown on coverslips were cotransfected with GFP-SHIP-2 and FcγRIIa, and were stimulated with heat-aggregated IgG for indicated time. Cells were then fixed and stained for nuclei. The coverslips were mounted onto slides and read by confocal microscope. SHIP-2 translocates to cell membrane upon FcγRIIa clustering. Bar: 10μm.
Figure 7.11 Diagram showing GFP-tagged SHIP-2 constructs used in Cos-7 cell transfection.
FcγRIIa clustering, the SH2 domain mutant of SHIP-2 or the PRD-deleted SHIP-2 failed to do so (Figure 7.12A, upper panel). PRD only did not get phosphorylated either. Parallel blotting with anti-GFP antibody showed that all the SHIP-2 constructs were expressed (Figure 7.12A, middle panel). The phosphorylation levels of SHIP-2 were quantitated and normalized for the amount of total SHIP-2 present in each lane and expressed as fold increase of SHIP-2 phosphorylation in the activated samples over that in resting samples (Figure 7.12A, lower panel). To ensure that the difference of phosphorylation levels was not due to unequal expression of the SHIP-2 constructs or lack of expression of FcγRIIa, aliquots of WCL were probed with anti-SHIP-2 (Figure 7.12B, upper panel, note that a faint endogenous SHIP-2 band was detected in each lane, indicated by the second arrow from top), anti-GFP (Figure 7.12B, middle panel), or anti-FcγRIIa antibody (Figure 7.12B, lower panel). Taken together, these data indicate that both SH2 domain and the PRD are necessary for the phosphorylation of SHIP-2, hence its membrane translocation.

7.5 Discussion

Phagocytosis is a highly coordinated process which involves multiple players including the Rho GTPase family protein, PI-3K, actin, and regulators such as inositol phosphatases SHIP-1 and SHIP-2. The activated FcγRs recruit all these molecules to a limited location where phagocytosis takes place. It has been shown that SHIP-1
Figure 7.12 SHIP-2 is phosphorylated upon FcγRIIa clustering in an SH2- and PRD-dependent way in transfected Cos-7 cells. Cos-7 cells were cotransfected with FcγRII and various GFP-tagged SHIP-2 constructs. Transfectants were stimulated for 5 min by clustering FcγRIIa. (A) SHIP-2 phosphorylation was assessed by Western blotting GFP immunoprecipitates with anti-phosphotyrosine antibody (upper panel). Parallel immunoprecipitates were blotted with anti-GFP antibody (middle panel). The lower panel is a quantitative estimation of SHIP-2 phosphorylation, normalized to total SHIP-2 present in each sample. (B) Aliquots of WCL were probed with either anti-SHIP-2 (upper panel), anti-GFP (middle panel), or anti-FcγRIIa antibody (lower panel).
translocates to the phagocytic cup to regulate actin remodeling in macrophages. In previous chapter, we have demonstrated that SHIP-2, a molecule with high level of homology to SHIP-1, downregulates FcγR-mediated phagocytosis independently of SHIP-1. However, it is not known whether SHIP-2 can also be recruited to the site of phagocytosis.

In this chapter, we first used transfected fibroblast Cos-7 cells to show that GFP-tagged SHIP-2 translocates to the site of phagocytosis. Although detectable, the translocation of GFP-SHIP-2 was rather faint in these cells. We next confirmed this result in Raw 264.7 cells, demonstrating that the endogenous SHIP-2 acts in a similar way, and it clearly colocalizes with F-acin at phagocytic cup. SHIP-2 colocalizes with SHIP-1 during phagocytosis, but the translocation of SHIP-2 does not rely on the presence of SHIP-1, as SHIP-2 still goes to the phagocytic cup in SHIP-1−/− BMMs. It has been actually reported by others that compared to SHIP-1, SHIP-2 exhibited a higher affinity for the cytoskeletal network in platelets. Using HA-tagged SHIP-2 constructs, we identified that both SH2 and PRD domains are required for this translocation, consistent with our previous results that they are both necessary for the negative regulation function of SHIP-2 in phagocytosis. This is further supported by additional results from transfected Cos-7 cells, in which the phosphorylation of SHIP-2 by FcγRIIa clustering, hence the membrane translocation of SHIP-2, is dependent on an intact SH2 domain as well as PRD. Thus, our results suggest that SHIP-2 is recruited via its SH2
domain to the activated FcγR, where phagocytosis starts, and once recruited, it is further stabilized through its PRD. Anchored on site, SHIP-2 hydrolyzes PIP₃ back to PI(3,4)P₂, shutting down the PIP₃-dependent Vav/Rac signaling pathway that is necessary for actin polymerization required for phagocytosis (Figure 7.13). This is consistent with results from previous chapter that SHIP-2 downregulates phagocytosis by suppressing the upstream Rac activation, and is also consistent with previous report that Rac localizes within phagocytic cup²⁵⁹.

In other studies, it has been suggested that SHIP-2 localization to membrane ruffles in fibroblast is mediated by its C-terminal PRD to the actin-binding protein filamin²⁹¹. Filamin forms a scaffold for the binding of Rho GTPases and their activators. This arrangement may allow spatial coordination of actin nucleation at sites where newly assembled actin filaments are cross-linked. SHIP-2 was further reported to localize within a protein complex including filamin, actin, and GPI-IX-V in human platelets³⁴⁹. Whether SHIP-2 translocation in macrophages is also mediated by its interaction with filamin still needs to be investigated.

We have identified bright spots of transfected GFP-SHIP-2 around the nucleus of Cos-7 cells, some of the spots even overlaps with nuclear staining. Although it might be due to overproduction of transfected proteins in endoplasmic reticulum attached to the nuclear membrane, this localization pattern of SHIP-2 may also indicate its specific functions. Indeed, it has been reported that SHIP-2 localizes within nuclei isolated from
Figure 7.13 Diagram of SHIP-2 translocation during phagocytosis. SHIP-2 is recruited to the activated FcγR via its SH2 domain, and is further stabilized through its PRD. Once at the site, SHIP-2 hydrolyzes PIP3 to PI(3,4)P2, shutting off the downstream Vav/Rac signaling pathway that is required for actin polymerization necessary for phagocytosis. Whether SHIP-2 can be directly recruited to the site of actin polymerization through the interaction between PRD of SHIP-2 and the actin-binding protein filamin remains to be elucidated.
pig aorta vascular smooth muscle cells (VSMCs), and it appears to be the primary enzyme responsible for hydrolyzing PIP₃ in these cells. In addition, SHIP-2 concentrates in nuclear speckles, suggesting that SHIP-2 may be involved in pre-mRNA processing. More detailed studies into the subcellular localization of SHIP-2 in macrophages may provide new insight into its biological roles.
Like neutrophils, macrophages play important roles in innate immunity. They help protect the host by eliminating IgG-coated infectious foreign bodies through FcγR-mediated functions. Activation of these receptors elicits a cascade of signaling pathways, which lead to the clearance of the pathogens by phagocytosis, and the generation of inflammatory cytokines as well as reactive species along the course. Although less well-studied compared to the activation process, inhibition (downregulation) of FcγR-mediated events has significant impacts on infection, inflammation, and even tumor therapy

SHIP-2 is a novel 5’ inositol phosphatase with high level of homology to SHIP-1, which has been well-described as a negative regulator of FcγR-mediated phagocytosis and related functions. Here, we used various cell models to demonstrate that SHIP-2 downregulates FcγR-mediated phagocytosis independently of SHIP-1. Directly recruited
to the site of phagocytosis, SHIP-2 works its way through suppressing the activation of the Rho GTPase family Rac, which in turn is required for the actin reorganization necessary for phagocytosis. The above functions depend on an intact SH2 domain as well as PRD, the two domains possibly anchoring and stabilizing the membrane localization of SHIP-2.

Phosphoinositides are ubiquitous components of cell membranes that act as signaling molecules, regulating actin polymerization, apoptosis, cellular proliferation, insulin signaling and vesicular membrane trafficking. As SHIP-2 is also ubiquitously expressed, it must have more biological roles other than downregulating phagocytosis in macrophages. Many lines of evidence suggest that SHIP-2 regulates PI-3K-dependent insulin signaling. The insulin receptor is a tyrosine kinase that phosphorylates different substrates including PI-3K, which subsequently activates effecter protein kinases such as Akt to carry out many of the downstream effects of insulin and other ligands. Potential negative regulators of PI-3K signaling have received more attention, since their malfunction might contribute to insulin resistance and subsequently to the development of diabetes. A new SHIP-2 knockout mouse model provides novel insights into this process. These mice displayed normal glucose homeostasis despite increased insulin-dependent activation of Akt, and they stayed thin on a high-fat diet. It is possible that constitutive activation of Akt and increased p70 S6 kinase activity, as a consequence of sustained PIP3 signaling in insulin-sensitive tissues by SHIP-2 loss, might
produce a negative feedback loop to somewhat attenuate insulin signaling, and thus masking the phenotype \(^{338,353}\). In addition, SHIP-2 is highly expressed in the brain. Therefore, SHIP-2 knockout may enhance PI-3K signaling important for anorexic hormones like leptin or other hormones that regulate feeding or energy expenditure.

Besides SHIP-2 and SHIP-1, other phosphatases also involve in the negative regulation network. Their roles may be redundant in some ways, but are largely independent. For example, PTEN, a tumor suppressor gene, is a 3’ phosphatase that suppresses the PI-3K/Akt pathway by hydrolyzing PIP\(_3\). However, there is little evidence to date implicating SHIP-2 in the regulation of PI-3K-dependent proliferation or cell death pathways that control tumorigenesis \(^{298}\), although it has been suggested that SHIP-2 is a direct substrate for BCR/ABL tyrosine kinase in chronic myelogenous leukemia (CML) \(^{290}\). More works are needed to clarify the differences among phosphatases and to identify new biological roles for SHIP-2.
CONCLUDING REMARKS

This dissertation is divided into two separate but still related parts. In Part I, we discussed the physiological roles of a novel protein, LRG, in G-CSFR-mediated neutrophilic granulocyte differentiation. In Part II, we provided evidence that SHIP-2, an inositol phosphatase with high level of homology to SHIP-1, negatively regulates FcγR-mediated phagocytosis in macrophages. Granulopoiesis and phagocytosis, two complicated processes seemingly different in every angle, do actually have something in common. They both belong to innate immunity, and they are both induced by specific surface receptors. But more importantly, they are both highly regulated and finely tuned. It is fascinating to imagine that the cell, a tiny biological structure that is invisible without a microscope, has a “micro-universe” in itself. As an old Chinese saying indicates, the world is dominated by both positive and negative forces, the “Yin” and “Yang”. Applied to research, this philosophy may help us discover a lot of treasure that are still sleeping somewhere.
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