MOLECULAR MECHANISMS OF REGULATION OF MACROPHAGE
INFLAMMATORY RESPONSE.
(Roles for the inositol phosphatases- SHIP-1, SHIP-2 and the serine/threonine
kinase Akt)

DISSERTATION

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

By

Ruma Annabelle Pengal, M.S.

********

The Ohio State University
2005

Dissertation committee:
Susheela Tridandapani, Ph.D., Adviser
Michael Ostrowski, Ph.D.
David Saffen, Ph.D.
Larry Schlesinger, M.D.

Approved by

__________________________
Adviser
Molecular, Cellular and Developmental
Biology Graduate Program
ABSTRACT

Monocytes and macrophages express innate immune receptors such as FcγR and Toll-like receptor 4, which when engaged by their respective ligands; IgG immune complexes (ICs) and bacterial lipopolysaccharide (LPS), become activated to produce inflammatory mediators. While production of inflammatory mediators is critical for elimination of invading pathogens, this process must be tightly regulated to prevent collateral tissue damage. The goal of this thesis project is to analyze molecular mechanisms that regulate macrophage inflammatory responses to ICs and LPS.

In the first part of this project we have analyzed the role of the inositol phosphatase SHIP-2 in FcγR-mediated macrophage responses and have established for the first time that SHIP-2 regulates these responses. During the course of these studies it was found that peripheral blood monocytes (PBM) express little to no SHIP-2. However, upon LPS treatment SHIP-2 expression is robust in PBM. A similar regulation of SHIP-1 was also found in PBM. These latter findings suggested a role for inositol phosphatases in LPS signaling.

Hence, in the second part of this project we have examined the role of the inositol phosphatase SHIP-1 in LPS signaling. Our experiments established that SHIP serves to
promote macrophage inflammatory responses. Thus macrophages from SHIP-deficient animals were hyporesponsive to LPS. A comparison of the signaling events in LPS-stimulated SHIP-sufficient and SHIP-deficient cells revealed that the serine/threonine kinase Akt was hyperactivated in SHIP-deficient macrophages. These results suggested a role for Akt in dampening pro-inflammatory responses to LPS.

Therefore, in part three of this project we analyzed the role of Akt in LPS-induced cytokine responses. Here, using multiple independent experimental models we have demonstrated that Akt promotes the production of the anti-inflammatory cytokine IL-10 in response to LPS stimulation. Although Akt did not appear to have an effect on the production of pro-inflammatory cytokines, its influence on IL-10 production resulted in a survival advantage in the face of endotoxin challenge.

Taken together these studies establish novel roles for inositol phosphatases and their downstream target Akt in the regulation of macrophage inflammatory responses to ICs and LPS thus providing potential molecular targets for therapeutic intervention of inflammatory diseases.
Dedicated to my Mother
ACKNOWLEDGEMENTS

I wish to thank my adviser, Dr. Susheela Tridandapani, for the opportunity to partake in research that has been an excellent intellectual journey. Without her boundless patience, tireless guidance and infectious enthusiasm, my work would not have come to fruition.

I would like to thank the members of my lab, both past and present, for their continued support and for creating an atmosphere that has been both fun and conducive to challenging research. Dr. Latha P. Ganesan has been a constant source of inspiration and guidance. I thank Huiqing Fang, Trupti Joshi and Jing Ai for supporting me with their encouraging words and more importantly for their friendship.

I am extremely grateful to my colleagues, Dr. Christopher Baran, Dr. Tim Eubank, Dr. Judy Opalek, Dr. Melissa Hunter, Raquel Raices, Sunny Kolattukudy and Melissa Vargo for their support. I would like to thank Anthony Popkie for his unflinching rationality and for being my most trusted friend.

Finally, and most importantly, I would like to express my gratitude to my mother who has literally been my backbone. Although separated by distance, she has been with me through every step of the way. I would like to extend my gratitude to my father, my brother and his family for believing in me and supporting me in this endeavor.

This research was supported by grants from the National Institutes of Health.
VITA

October 12, 1977..........................................................Born – Mumbai, India

2000.................................................................M.S Life Sciences, University
of Mumbai.

2000 – 2003 ..........................................................Graduate Teaching Associate
The Ohio State University

2003 – 2005 ..........................................................Graduate Research Associate
The Ohio State University

PUBLICATIONS

Research Publications
   J Biol Chem Jun 20; 278(25):22657-63

* 2) Fang H, Pengal RA, Cao X, Ganesan LP, Wewers MD, Marsh CB, Tridandapani S. 

3) Ganesan LP, Wei G, Pengal RA, Moldovan L, Moldovan N, Ostrowski MC, 

* - Co-first author.

FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## Chapters:

### 1. Introduction

- The innate immune system.................................1
- Macrophages and innate immunity.........................3
- Fc receptors.............................................5
- TLR4 and innate immunity................................15
- Inositol phosphatases.....................................19
- The serine/threonine kinase Akt........................21
- Summary..................................................23
2. The Inositol Phosphatase SHIP-2 is Inducibly Expressed in Human Monocytes and Regulates FcγReceptor-Mediated Signaling
   - Abstract.................................................................26
   - Introduction.........................................................28
   - Materials and methods...........................................34
   - Results.................................................................38
   - Discussion............................................................59

3. Lipopolysaccharide-Induced Macrophage Inflammatory Response is Regulated by the SH2 Domain-Containing Inositol 5-Phosphatase SHIP.
   - Abstract.................................................................65
   - Introduction.........................................................67
   - Materials and methods...........................................71
   - Results.................................................................75
   - Discussion............................................................91

4. The serine/threonine kinase Akt promotes IL-10 production and protects from LPS-induced toxicity.
   - Abstract.................................................................96
   - Introduction.........................................................98
   - Materials and methods...........................................102
   - Results.................................................................108
   - Discussion............................................................129

Concluding Remarks and Perspectives.................................133

Bibliography.................................................................138
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 Cellular components of blood.</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Fc gamma receptors.</td>
<td>6</td>
</tr>
<tr>
<td>1.3 ITAM and ITIM of Fcγ receptors.</td>
<td>9</td>
</tr>
<tr>
<td>1.4 Fcγ receptor signaling.</td>
<td>11</td>
</tr>
<tr>
<td>1.5 Structure of TLR4</td>
<td>16</td>
</tr>
<tr>
<td>1.6 LPS-TLR4 signaling.</td>
<td>18</td>
</tr>
<tr>
<td>1.7 Inositol phosphatase function.</td>
<td>20</td>
</tr>
<tr>
<td>1.8 Cellular functions of Akt.</td>
<td>22</td>
</tr>
<tr>
<td>1.9 Activation of Akt.</td>
<td>24</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>2.1 Structure of SHIP-1 and SHIP-2.</td>
<td>30</td>
</tr>
<tr>
<td>2.2 Specificity of SHIP-2 antibody.</td>
<td>39</td>
</tr>
<tr>
<td>2.3 Expression of SHIP-2 in myeloid cells.</td>
<td>40</td>
</tr>
<tr>
<td>2.4 Induction of SHIP-2 and SHIP-1 in monocytes.</td>
<td>41</td>
</tr>
<tr>
<td>2.5 SHIP-2 is tyrosine phosphorylated upon FcγRIIa clustering.</td>
<td>43</td>
</tr>
<tr>
<td>2.6 SHIP-2 inhibits NFκB-mediated gene transcription upon FcγRIIa clustering.</td>
<td>45</td>
</tr>
<tr>
<td>2.7 SHIP-2 inhibits FcγRIIa-mediated Akt phosphorylation.</td>
<td>47</td>
</tr>
</tbody>
</table>
2.8 SHIP-2 associates with FcγRIIa ITAM peptide. 49
2.9 SHIP-2 co-immunoprecipitates with FcγRIIa. 51
2.10 Schematic of SHIP-2 constructs used in the study. 52
2.11 SH2 domain of SHIP-2 is necessary for optimal SHIP-2 association with FcγRIIa ITAM. 54
2.12 SH2 domain of SHIP-2 is necessary for optimal phosphorylation of SHIP-2 by FcγRIIa clustering. 56
2.13 SHIP-2 is tyrosine phosphorylated upon FcγRI clustering in THP-1 cells. 58
2.14 Model for SHIP-2 association with FcγRIIa and subsequent phosphorylation by src kinases. 60
2.15 Role for SHIP-2 in FcγR-mediated myeloid cell function. 62

Chapter 3
3.1 Catalytic activity of SHIP. 69
3.2 LPS stimulation leads to membrane translocation of SHIP. 76
3.3 LPS stimulation leads to tyrosine phosphorylation of SHIP. 78
3.4 SHIP positively regulates NFκB-dependent gene transcription in LPS stimulated cells. 79
3.5 LPS induced MAP Kinase phosphorylation is downregulated in SHIP−/− macrophages. 81
3.6 TLR4 expression is not altered in SHIP−/− macrophages. 83
3.7 Akt phosphorylation is enhanced in SHIP−/− BMM. 84
3.8 Inhibition of Akt restores MAPK activation in SHIP−/− BMM. 86
3.9 LPS induced TNFα and IL-6 production is suppressed in SHIP−/− macrophages. 87
3.10 SHIP negatively regulates FcγR-induced Erk phosphorylation. 89
3.11 FcγR expression in SHIP+/− and SHIP−/− BMMs. 90
Chapter 4

4.1 Immune Complexes (ICs) promote LPS-induced IL-10 production in Raw 264.7 cells.

4.2 Immune Complexes (ICs) have no effect on LPS-induced Erk activation.

4.3 Immune Complexes (ICs) have no effect on LPS-induced p38 activation.

4.4 Immune Complexes (ICs) have no effect on LPS-induced JNK activation.

4.5 Immune Complexes (ICs) promote LPS-induced Akt activation.

4.6 High-efficiency transfection of Raw 264.7 cells.

4.7 Constitutively active Akt promotes LPS-induced IL-10 production in transfected Raw 264.7 cells.

4.8 Cells with reduced Akt expression produce reduced levels of IL-10.

4.9 Myr-Akt expressing macrophages stimulated with LPS make significantly more IL-10.

4.10 TLR4 expression is unchanged in wild type vs Myr-Akt expressing mice.

4.11 Myr-Akt expressing mice challenged with E. coli have higher levels of serum IL-10.

4.12 Myr-Akt expressing mice challenged with E. coli display a survival advantage over wild type littermates.

4.13 Myr-Akt expressing mice challenged with LPS have higher levels of serum IL-10.

4.14 Wild type and Myr-Akt expressing mice challenged with LPS
show no significant difference in levels of pro-inflammatory cytokines in serum.

4.15 Proposed model for Akt influence on IL-10. 131
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fcγ receptor expression in immune cells.</td>
<td>8</td>
</tr>
<tr>
<td>2. Toll-like receptors and their ligands.</td>
<td>14</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>BMM</td>
<td>bone marrow derived macrophages</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>Egr-1</td>
<td>early growth response 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FcRn</td>
<td>neonatal Fc receptor</td>
</tr>
<tr>
<td>FcεR</td>
<td>Fc epsilon receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein-5-isothiocyanate</td>
</tr>
<tr>
<td>GAM</td>
<td>goat anti-mouse IgG</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine exchange factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>IC</td>
<td>IgG immune complexes</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin 10</td>
</tr>
<tr>
<td>IL-12</td>
<td>interleukin 12</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin 4</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor-associated kinase</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeats</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88 adapter-like protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>Myr-Akt</td>
<td>myristoylated Akt</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBM</td>
<td>peripheral blood monocytes</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 3,4-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptors</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine binding domains</td>
</tr>
<tr>
<td>PtdIns3K</td>
<td>Phosphatidyl Inositol 3-Kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3</td>
</tr>
<tr>
<td>SHIP-1</td>
<td>SH2 homology containing inositol 5'-phosphatase-1</td>
</tr>
<tr>
<td>SHIP-2</td>
<td>SH2-domain-containing inositol 5'-phosphatase-2</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2-domain-containing phosphatase-1</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGFβ-activated kinase</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF-receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-containing adapter molecule</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysates</td>
</tr>
<tr>
<td>γIFN</td>
<td>interferon gamma</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

The Innate Immune System

The vertebrate immune system mounts a response to counteract pathogenic invasions. This response can be broadly distinguished as the innate immune response and the adaptive immune response (Janeway, Jr. and Medzhitov, 2002). While the adaptive immune response chiefly entails production of antibodies directed toward a pathogen, the innate immune response is mediated by macrophages which are readily available to combat a vast array of pathogens without requiring prior exposure. Thus macrophages are often considered the primary line of defense against pathogenic invasion. The adaptive immune response is required only when a pathogen is able to evade or overwhelm the innate immune response (Ezekowitz and Hoffmann, 1996).

Macrophages are derived from myeloid progenitors which are in turn derived from pluripotent hematopoietic stem cells from which all other cellular components of
blood are also derived. Macrophages were first described by Eli Metchnikoff, as phagocytic cells capable of ingesting and destroying microbes. Neutrophils are the second major family of phagocytes that assist macrophages in eliminating pathogenic microorganisms (Underhill and Ozinsky, 2002). The first step in the phagocytic process is the recognition of the invading microbe. Macrophages and neutrophils do so by means of cell-surface receptors that are able to recognize ‘pathogen-associated molecular patterns’ (PAMPs) (Aderem and Underhill, 1999). Recognition is followed by a number of signaling events that promote actin polymerization and pseudopod extension and subsequent engulfment (phagocytosis) of the organism into a phagosome (Greenberg, 1999; Ravetch and Bolland, 2001; Underhill and Ozinsky, 2002). Within the cell, the phagosome fuses with the lysosome. This causes release of lysosomal enzymes into the phagosome. These enzymes bring about digestion of the phagosome constituents which are eventually released from the cell (Swanson and Baer, 1995). The process of phagocytosis is also accompanied by the generation of inflammatory cytokines (e.g. TNFα, IL-1 and IL-6) mediated by transcription factors like NFκB and NFAT. These cytokines are involved in cross-talk between the innate immune system and effector cells and are important in mediating clearance of the pathogen (Daeron, 1997; Ravetch and Clynes, 1998).
Macrophages and Innate Immunity

Macrophages are terminally differentiated monocytes (Fig. 1.1) and can be long-lived as ‘resident’ macrophages in contrast to other myeloid cells. Their life span can vary from a few days to even months. Macrophages can be found in most tissues of the body where they can launch a rapid response to pathogenic invasion (Aderem and Underhill, 1999). They play an important role in innate immunity and are involved in the production, activation and regulation of immune effector cells. While tissue macrophages do not normally display self-renewal properties, in certain environments (e.g. lungs), injury could lead to rapid replication of macrophages leading to formation of local foci of inflammation (Gordon, 1995). Ligation of surface receptors on macrophages induces a plethora of signaling events that ultimately result in production of lysozymes, proteinases, cytokines (both pro- and anti-inflammatory) and reactive oxygen and nitrogen species, all of which play a role in host-defense and inflammation (Underhill and Ozinsky, 2002).

Macrophages express on their cell surfaces a multitude of receptors, among which are specialized receptors called Fc receptors. These receptors recognize and bind the Fc portion of immunoglobulin molecules. Fc receptors can be broadly classified into two main classes- receptors involved in effector functions (e.g. FcγR, FcεR and FcαR) and those involved in immunoglobulin transportation (e.g. FcRn). Fc receptors are of various types depending on the class of the immunoglobulin superfamily they bind. Fc receptors for IgG, FcγR, were first identified more than thirty five years ago by Boyden and Sorkin (Ravetch and Bolland, 2001; BOYDEN and Sorkin, 1960) as being able to bind IgG
Figure 1.1: **Cellular components of Blood.** Cells of the myeloid and lymphoid lineage derive from a common precursor, the hematopoietic stem cell. Macrophages are derived from differentiated monocytes and belong to the myeloid lineage.
opsonized RBCs. Subsequent work has allowed classification of FcγRs as activating or inhibitory receptors depending on the type of intracytoplasmic motif they contain. FcγRI, FcγRIIa and FcγRIII constitute the class of activating receptors that promote phagocytosis in humans. Mice possess FcγRI and FcγRIII but lack FcRγIIa. Both human and murine macrophages express FcγRIIb which is an inhibitory receptor. The activating receptors contain an immunoreceptor tyrosine-based activation motif (ITAM), while the inhibitory receptor contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Fig. 1.2). Upon receptor clustering induced by IgG immune complexes (ICs), the tyrosine residues in the ITAMs and ITIMs becomes phosphorylated and serves as a docking site for SH2 domain containing intracellular signaling enzymes and adapter/enzyme complexes. Both classes of receptors are coexpressed and can be coengaged on the macrophage cell surface. The balance between the signaling events mediated by these two classes determines the eventual outcome of the effector response (van den Herik-Oudijk IE et al., 1995a; Unkeless and Jin, 1997).

**Fcγ Receptors**

Fc receptors are integral membrane proteins and are made up of an extracellular α subunit, a transmembrane domain and a cytoplasmic tail. The α subunit is responsible for ligand recognition and confers specificity. The α subunit of all Fc receptors have two extracellular domains except FcγRI which has three. FcγRII and FcγRIII are low-affinity receptors and can only bind aggregated immunoglobulins, while FcγRI is a high affinity
Figure 1.2: Fc gamma Receptors. Schematic representation of the activating and inhibitory FcγRs showing respective ITAM or ITIM motifs.
receptor and can bind monomeric IgG (Daeron, 1997; Unkeless, 1989; Ravetch and Bolland, 2001). The ITAM for FcγRI and FcγRIII is not intrinsic to the receptor, but instead is contained on an associated γ chain. FcγRIIa and FcγRIIb function as single chain receptors. The γ chain of FcγRI and FcγRIII plays an important role in the assembly of the α subunit and protects it from degradation in the endoplasmic reticulum. It is seen as a disulfide linked homodimer consisting of a short extracellular motif, a transmembrane domain and an intracytoplasmic domain which houses the ITAM motif. The γ chain is usually associated with the α subunit via an aspartic acid residue that interacts with a basic amino acid residue on the α subunit (Daeron, 1997; Ravetch and Kinet, 1991). The expression pattern of the FcγRs in immune cells is shown in Table I.

The ITAM motif contained within the gamma chain of FcγRI and FcγRIII and the cytoplasmic tail of FcγRIIa has two YXXL sites, both of which can be phosphorylated. The γ chain ITAM has seven amino acid residues between the two YXXL sites, while the FcγRIIa ITAM has twelve amino acids between the YXXL sites (Fig. 1.3). The ITAM is crucial for the activator functions of these receptors (Tridandapani and Anderson, 2004; Unkeless et al., 1995; van den Herik-Oudijk IE et al., 1995b; van den Herik-Oudijk IE et al., 1995a). Crosslinking of ITAM bearing receptors results in a common response, the effect of which is determined by the cell type rather than the receptor engaged. Aggregation of ITAM bearing receptors leads to activation of kinases belonging to the Src family which function to phosphorylate the YXXL motifs in the ITAM. This generates phospho-tyrosine residues that can act as docking sites for SH2-domain
<table>
<thead>
<tr>
<th>Cell type</th>
<th>FcγRI</th>
<th>FcγRIIa</th>
<th>FcγRIIb</th>
<th>FcγRIIIa</th>
<th>FcγRIIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++, a</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+/-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Basophils</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-, i</td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B cells</td>
<td>-</td>
<td>+/-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++, b</td>
<td>-</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>-, i</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

-, absent; +/-, weak expression; ; + to ++++, indication of expression level; I, inducible; a, subpopulation of cells; b, most g, d- and select a, b-cells

Table 1: Expression pattern of FcγRs in immune cells
Figure 1.3: ITAM and ITIM of FcγRs. The ITAM on FcγRIIa has two YXXL motifs separated by 12 amino acids, while the FcR γ chain ITAM has two YXXL motifs separated by 7 amino acid residues. The ITIM seen in FcγRIIb is a 13 amino acid sequence with a single YXXL motif. TM; transmembrane.
containing proteins. The tyrosine kinase Syk is recruited to the phosphorylated ITAMs via its SH2 domain and gets activated. This two-step process, tyrosine phosphorylation of ITAM followed by activation of Src kinases and recruitment and activation of Syk kinases, is essential for the transduction of the signal to bring about the desired response (Ravetch and Bolland, 2001).

Activation of Syk results in a number of key signaling events including activation of phosphatidyl inositol 3-Kinase (PtdIns3K), which in turn leads to production of phosphatidyl-inositol 3,4,5-trisphosphate (PtdIns3,4,5P$_3$) and recruitment of PH-domain containing molecules like PLC$\gamma$ and Tec kinases like Bruton’s tyrosine kinase (Btk). Activation of PLC$\gamma$ leads to generation of IP$_3$ (Inositol trisphosphate) and Diacyl glycerol (DAG) which mediate calcium mobilization and PKC activation. The Ras pathway is also activated via the Guanine exchange factor (GEF) Sos which is recruited by the adapter proteins Shc and Grb2. The Ras pathway leads to phosphorylation and subsequent activation of Raf, which in turn results in MEK kinase and MAP kinase activation. Another GEF, Vav, is activated by PtdIns3,4,5P$_3$. Vav is a GEF for the Rho family of GTPases which promote actin polymerization and cytoskeletal rearrangements which are key events in the phagocytic process (Tridandapani and Anderson, 2004). Figure 1.4 is a schematic of signaling events that are activated upon Fc$\gamma$R clustering and lead to phagocytosis.

The ITIM motif within the cytoplasmic tail of Fc$\gamma$RIIb is a 13 amino acid chain with a single YXXL motif in the center. Phosphorylation of the YXXL motif has been shown to be important for the inhibitory function of the ITIM.
Figure 1.4: Fc gamma receptor mediated signaling. Clustering of FcyRs by immune complexes leads to a multitude of signaling events within the cell that function to mediate phagocytosis coupled with gene transcription. LAT; linker for activation of T cells.
(Unkeless and Jin, 1997; Ravetch et al., 1986). The function of FcγRIIb as an inhibitory receptor was first established in B cells. Other cell types that express the ITIM include macrophages, mast cells, neutrophils and dendritic cells. The inhibitory function of FcγRIIb was elegantly demonstrated with studies in FcγRIIb knockout mice which showed an increase in the level of serum IgG in response to an antigenic challenge indicating that the receptor was involved in negative regulation of antibody production (Takai et al., 1996). Likewise, FcγRIIb-deficient macrophages display enhanced phagocytic capacity as compared to wild type macrophages. Engagement of FcγRIIb leads to tyrosine phosphorylation of the ITIM which can then recruit SH2-domain containing proteins such as SHIP-1, SHIP-2 and SHP-1. The effects triggered by ITAM activation—phagocytosis, calcium mobilization and proliferation, are inhibited by the catalytic activity of inositol phosphatases SHIP-1 and SHIP-2 which hydrolyze PtdIns3,4,5P3 (Tridandapani and Anderson, 2004; Unkeless and Jin, 1997; van den Herik-Oudijk IE et al., 1995a).

Thus, when a monocyte/macrophage encounters an immune complex it leads to clustering of both activating and inhibitory receptors in concert. The intensity of the response is determined by the ratio of the activating to inhibitory FcγR. The importance of the inhibitory receptors in down-regulating this process cannot be stressed enough because if the process is unchecked, the inflammatory mediators produced could lead to severe tissue damage. Interestingly, the ratio of activating to inhibiting FcγRs can be altered depending on the cytokines in the surrounding milieu of these cells. For example, Th1 cytokines such as γIFN up-regulate the expression of the activating receptor FcγRI,
leading to enhanced phagocytic efficiency. In contrast, Th2 cytokines such as IL-4 up-regulate expression of the inhibitory receptor FcγRIIb and dampens phagocytic efficiency (Pricop et al., 2001; Tridandapani et al., 2002a).

As described above, the first step in the process of phagocytosis is the recognition of the pathogen. Janeway and Medzhitov coined the term PAMPs to describe the motifs on the pathogen that engage the innate immune system. These motifs include peptides, mannans in the yeast cell wall and cell-wall components such as LPS (Lipopolysaccharide), teichoic acids and lipopeptides. The receptors that bind PAMPs are termed pattern-recognition receptors (PRRs) (Janeway, Jr. and Medzhitov, 1998; Janeway, Jr. and Medzhitov, 2002). There are two main types of PRRs, those that mediate phagocytosis and those that launch pro-inflammatory responses. LPS is considered the prototypic activator of innate immunity. It plays a key role in the onset of potentially lethal conditions like sepsis. LPS, dissociates from the cell wall of gram negative bacteria and binds to a serum protein called LPS binding protein (LBP). This complex is then identified by CD14 on monocytes /macrophages. CD14 then transmits the signal to TLR4 (Toll-like receptor 4) which is the main receptor involved in LPS-mediated signaling. TLR4 belongs to the family of Toll like receptors first discovered in Drosophila. Other members of the TLR family have been described as recognition receptors for heat shock proteins, viral dsRNA, CpG-DNA etc (Medzhitov and Janeway, Jr., 2002; Janeway, Jr. and Medzhitov, 1998). Presently ten coding family members have been identified in humans. These are listed in Table II.
<table>
<thead>
<tr>
<th>TLR family</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Tri-acyl lipopeptides (bacteria, mycobacteria); Soluble factors (Neisseria meningitidis)</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipotechoic acid (gram positive bacteria); Peptidoglycan (gram positive bacteria); Porins (Neisseria)</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA (virus)</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS (gram positive bacteria); Taxol (plant); Envelope proteins (MMTV)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin (bacteria)</td>
</tr>
<tr>
<td>TLR6</td>
<td>Di-acyl lipopeptides (mycoplasma)</td>
</tr>
<tr>
<td>TLR7</td>
<td>Synthetic compounds (e.g. Imidazoquinoline)</td>
</tr>
<tr>
<td>TLR8</td>
<td>?</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA (bacteria)</td>
</tr>
<tr>
<td>TLR10</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 2: Toll-like receptors and their ligands
TLR4 and innate immunity

TLR4 is crucial but not sufficient for LPS-mediated signaling. Apart from CD14, a number of other adapter molecules have been identified as playing a role in LPS signaling. For instance, expression of the small adapter molecule MD-2 is required for optimal surface expression and function of TLR4. Evidence for this came from studies with E5531, a potent LPS antagonist. E5531 exerts its effect by direct inhibition of TLR4/MD-2 complex (Akashi et al., 2003).

The structure of TLR4 (Fig.1.5) consists of extracellular leucine-rich repeats (LRR), a transmembrane domain and the intracellular TIR (Toll/IL-1 Receptor) domain. The TIR domain is shared by the IL-1 receptor family. The TIR domain consists of a conserved region of ~200 amino acids. Within the TIR domain, three conserved boxes comprise the regions of homology, which play a crucial role in signaling. The TIR domains of various TLRs share 20-30% amino acid sequence conservation (Akira and Takeda, 2004; Slack et al., 2000). The extracellular region of TLR4 consists of twenty-one copies of the LRR motif (Bell et al., 2003). TLRs in general consist of 19-25 tandem repeats of this motif. 24-29 amino acids make up each repeat, which contains the leucine-rich sequence XLXXLXLXX. The LRR contains another conserved region denoted by the sequence- ΧΦΧΧΦXXXFXXLX, where Χ denotes any amino acid and Φ a hydrophobic amino acid (Bell et al., 2003; Spitzer et al., 2002). It is believed that the LRRs are directly involved in recognition of various pathogens.

A number of other molecules are involved in TLR4 signaling- MyD88 (myeloid differentiation factor 88), Mal (MyD88 adapter-like protein), TRIF (TIR-containing
Figure 1.5: Structure of TLR4. Toll-like receptor4 (TLR4) has a conserved cytoplasmic domain, that is known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions (known as boxes 1, 2 and 3). The extracellular region of TLR4 comprises tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR).
adapter molecule) and TRAM (TRIF-related adapter molecule). The signaling pathway initiated in response to LPS binding can be briefly described- LPS binds to TLR4/MD-2 with the help of CD14. This induces homodimerization of TLR4, which recruits MyD88 and Mal to the complex. In the case of IL-1R signaling, MyD88 associates with IRAK-1 and IRAK-2 (IL-1R-associated kinase). This interaction is also seen with the TLR4/MyD88/Mal complex (Kopp and Medzhitov, 1999; Means et al., 2000; Medzhitov, 2001; Takeda et al., 2003). IRAK4 is considered a principle mediator of LPS signaling. Following association of IRAK-1 and IRAK-4 with the LPS-TLR4 complex, IRAK-1 undergoes auto-phosphorylation. Hyper-phosphorylated IRAK-1 then dissociates from the complex and associates with TRAF6 (TNF-receptor associated factor). TRAF6 activation leads to activation of the MAP kinase TAK1 (TGFβ-activated kinase). TAK1 then acts as a common activator of the p38 and JNK (c-jun N-terminal kinase) MAPK pathways as well as a number of transcription factors. NFκB has been found to play a crucial role in expression of inflammatory cytokines triggered by LPS-signaling (Kopp and Medzhitov, 1999; Takeda et al., 2003; Fitzgerald et al., 2004; Goldstein, 2004). Figure 1.6 is a simplified schematic of LPS-TLR4-mediated signaling. These events while necessary to combat the invasion, can have serious consequences if allowed to proceed unchecked. The inflammatory mediators released can lead to severe tissue damage, one of the hallmarks of sepsis. Sepsis is a leading cause of mortality, second only to cardiac arrest. Hence, a better understanding of how LPS signaling is regulated will greatly help in avoiding serious and potentially lethal conditions such as sepsis. We have investigated the regulation of both FcγR and LPS-signaling and show that the
Figure 1.6: LPS-TLR4 Signaling. LPS binds to LBP and this complex is recognized by CD14, which transduces the signal to the TLR4-MD-2 complex. This results in a plethora of signaling events within the cell which mainly lead to production of pro-inflammatory mediators via the action of transcription factors such as NFκB shown here.
inositol phosphatases SHIP-1 and SHIP-2 play a role in both signaling events (Fang et al., 2004; Pengal et al., 2003).

**Inositol Phosphatases**

SHIP-1 was discovered in 1996 as a hematopoietic cell-specific src homology 2-domain containing inositol 5’-phosphatase. More recently, a second more widely expressed protein SHIP-2 was cloned. Both proteins catalyze the hydrolysis of PtdIns3,4,5P$_3$ to PtdIns3,4P$_2$ (Phosphatidyl-inositol 3,4-bisphosphate) (Fig.1.7) and thereby act as negative regulators of PtdIns3K mediated events. SHIP-1 and SHIP-2 are multi-domain containing cytosolic proteins that contain a central inositol 5’ phosphatase domain and several protein interaction domains (Damen et al., 2001; Kavanaugh et al., 1996; Lioubin et al., 1996). Both proteins have common domains- a N-terminal SH2 domain, a central catalytic domain and a C-terminal proline rich domain (Erneux et al., 1998) The SH2 domain binds the ITIM of Fc$\gamma$RIIb with high affinity (Ono et al., 1996; Tridandapani et al., 1997c; Tridandapani et al., 1999). The proline-rich domain mediates association with SH3 domains of the Ras adapter protein Grb2 (Tridandapani et al., 1998). This region also houses NPXY motifs that bind proteins containing phosphotyrosine binding domains (PTB) such as the Ras adapter Shc (Lamkin et al., 1997; Lioubin et al., 1996; Pradhan and Coggeshall, 1997; Tridandapani et al., 1998). Since the 5’- phosphatase activity of these proteins does not change significantly following cytokine stimulation, they likely exert their downstream effects via binding to
Figure 1.7: Inositol Phosphatases and their Function. PtdIns3,4,5P$_3$ produced from PtdIns4,5P$_2$ as a result of PI3K activity is hydrolyzed by SHIP-1 and SHIP2 to PtdIns3,4P$_2$ while another phosphatase PTEN, hydrolyzes PtdIns3,4,5P$_3$ to PtdIns4,5P$_2$. PTEN; phosphatase and tensin homologue deleted on chromosome 10.
different proteins and translocating to the sites of synthesis of PtdIns3,4,5P$_3$ and I-1,3,4,5-P$_4$ (IP$_4$). The importance of SHIP came from studies in SHIP knockout mouse (Helgason et al., 2000; Huber et al., 1998a; Huber et al., 1998b; Tridandapani et al., 1998). Although these mice are viable and fertile, they overproduce granulocytes and macrophages and suffer from progressive splenomegaly, massive myeloid infiltration of the lungs, wasting and a shortened lifespan. In contrast, SHIP-2 knockout mice die perinatally from insulin-hypersensitivity induced hyperglycemia (Clement et al., 2001). Although SHIP-1 and SHIP-2 share high level of homology they are largely divergent in the C-terminal region. While SHIP-1 has two tyrosine residues in the C-terminus that conform to an NPXY motif, SHIP-2 has only one tyrosine NPXY motif (Erneux et al., 1998). Further the proline-rich region of SHIP-1 associates with Grb2, whereas the proline-rich domain of SHIP-2 does not associate with Grb2 but associates with Abl (Wisniewski et al., 1999). As a result of their catalytic activity, these proteins serve to down-regulate activation of PKB/Akt which is PtdIns3,4,5P$_3$ dependent. Indeed SHIP knockout mice showed elevated levels of Akt activation.

*The serine/threonine kinase Akt*

Akt is perhaps the best studied downstream effector of PtdIns3K signaling. It is also a critical downstream target of the inositol phosphatases SHIP-1 and SHIP-2. It is an important kinase that has anti-apoptotic properties and has been implicated in a variety of cancers apart from a multitude of cellular functions (Fig.1.8)
**Figure 1.8: Cellular functions of Akt.** Akt plays a role in multiple cellular events including growth, survival, protein synthesis and metabolism. These processes are controlled by a vast array of signaling events mediated by Akt.
Akt is a PH-domain containing protein and is activated in a PtdIns3,4,5P$_3$-dependent manner via the kinase PDK1 which is recruited as an effect of its PH-domain binding PtdIns3,4,5P$_3$. PDK1 leads to phosphorylation of Threonine-308 causing the unfolding of Akt which then allows phosphorylation of Serine-273. Akt can then go on to activate a multitude of signaling pathways within the cell (Fig.1.9) (Brazil and Hemmings, 2001; Brazil et al., 2004; Datta et al., 1999b; Scheid and Woodgett, 2003a). We have recently described a role for Akt in Fc$_\gamma$R mediated response (Ganesan et al., 2004b). Thus Akt promotes phagocytosis through the activation of p70S6 kinase. Interestingly, although a role for the PI3K/Akt pathway in LPS-mediated signaling was recently described by Guha and Mackman (Guha and Mackman, 2002b), there remain several questions with regard to the specific role for Akt in LPS-mediated signaling.

**Summary**

In the case of a gram negative bacterial invasion, the innate immune system mounts a response to combat the invasion. The Fc$_\gamma$ receptors on monocytes/macrophages are involved in the phagocytosis of the IgG-opsonized bacteria while the TLR4 is engaged by LPS that dissociates from the bacterial cell wall. The goal of this project was to investigate the role that inositol phosphatases SHIP-1 and SHIP-2 play in the regulation of Fc$_\gamma$R- and LPS-mediated signaling. The results described in ensuing chapters will demonstrate that while they serve to inhibit Fc$_\gamma$R-mediated signaling, they play a positive role...
**Figure 1.9: Activation of Akt.** The serine/threonine kinase Akt is activated in a PDK-1-dependent manner.
role in promoting LPS-induced inflammatory response. We have further extended these
studies to demonstrate an important role for Akt, a downstream target of the inositol
phosphatases, in upregulating the production of the anti-inflammatory cytokine IL-10 and
thereby imparting protection from endotoxin lethality. Thus we have demonstrated a
novel level of regulation of innate immune responses by the inositol phosphatases and
their downstream target Akt. These studies have unraveled potential molecular targets for
therapeutic intervention of inflammatory diseases such as sepsis.
CHAPTER 2

THE INOSITOL PHOSPHATASE SHIP-2 IS INDUCIBLY EXPRESSED IN HUMAN MONOCYTES AND REGULATES FC\(\gamma\) RECEPTOR-MEDIATED SIGNALING

Abstract

SHIP-2, a recently identified inositol 5’ phosphatase shares a high level homology with SHIP-1. While the role of SHIP-1 has been extensively studied, the role of SHIP-2 in myeloid cell functions is not known. Here we have analyzed expression patterns, molecular mechanism of activation and function of SHIP-2 in human myeloid cell Fc\(\gamma\)R signaling. We report that SHIP-2 is expressed in transformed myeloid cells and in primary macrophages, but not in peripheral blood monocytes (PBM). Treatment of PBM
with bacterial lipopolysaccharide induced expression of SHIP-2 in a dose-dependent manner. FcγRIIa clustering in THP-1 cells induced SHIP-2 tyrosine phosphorylation suggesting a role for SHIP-2 in modulating FcR-mediated function. Consistent with this notion, over expression of a wild-type SHIP-2 but not a catalytic-deficient SHIP-2 in THP-1 cells almost completely abrogated NFκB-mediated gene transcription in response to FcγRIIa clustering. Furthermore, FcγRIIa-induced Akt activation was blocked by wild-type SHIP-2 but not catalytic-deficient mutant of SHIP-2. Additional experiments analyzing the molecular mechanism of SHIP-2 induction by FcγRIIa revealed that SHIP-2 associates with phosphorylated FcγRIIa ITAM via the SHIP-2 SH2 domain. Thus, an SH2 domain mutant of SHIP-2 failed to associate with FcγRIIa, or become tyrosine phosphorylated upon FcγRIIa clustering. Finally, we also demonstrate that SHIP-2 phosphorylation is induced by FcγRI clustering in THP-1 cells. These findings unravel a novel level of regulation on FcγR-mediated activation of human myeloid cells by the expression and function of the inositol phosphatase SHIP-2.
**Introduction**

As described in chapter 1, clustering of FcγR on monocytes and macrophages by immune-complexes initiates a series of signaling events that result in phagocytosis of the immune-complex (Aderem and Underhill, 1999). The process of phagocytosis is accompanied by the generation of reactive oxygen and nitrogen radicals, as well as the production of inflammatory cytokines, all of which cause tissue damage. Thus, the phagocytic process must be subject to a tight regulation.

Human monocytes and macrophages express three classes of FcγR (Daeron, 1997). FcγRI, FcγRIIa and FcγRIII are activating receptors that are associated with a tyrosine-based activation motif (ITAM). In contrast, FcγRIIb is an inhibitory receptor that bears in its cytoplasmic tail a tyrosine-based inhibitory motif (ITIM) that predominantly recruits negative regulatory phosphatases, and causes a down regulation of activation events. In addition to the regulatory role of FcγRIIb, recent studies have revealed that the FcγR ITAMs are often capable of simultaneously activating positive and negative regulatory proteins such that the final biologic response is tempered. We (Tridandapani et al., 2002b), and others (Nakamura et al., 2002), have demonstrated that FcγR ITAMs are capable of recruiting and activating the inositol phosphatase SHIP-1. Other studies showed that the protein tyrosine phosphatase SHP-1 (Kant et al., 2002) and the dual phosphatase PTEN (Kim et al., 2002) also serve to regulate FcγR-mediated macrophage functions. Thus, it is clear that FcγR-mediated activation of macrophages is subject to multiple levels of regulation, which are not fully understood. In this report we
present molecular details of a novel regulatory influence exerted by the recently
identified inositol phosphatase SHIP-2.

FcγR clustering in monocytes and macrophages initiates a biochemical cascade
that begins with the activation of the src kinases that phosphorylate the ITAMs of FcγR
(Booney et al., 2001). Once phosphorylated, the ITAMs serve as docking sites for
several signaling enzymes and enzyme-adaptor complexes, including the Syk tyrosine
kinase (Chacko et al., 1994), p85 subunit of PtdIns3K (Chacko et al., 1996), and the Ras
adapter molecule Shc (William Paul, 2003; Tridandapani et al., 2002b) that serves to
recruit the Grb2/Sos complex leading to activation of the Ras pathway. Association of
PtdIns3K with the ITAM places the enzyme in proximity with its lipid substrates
resulting in the generation of the important lipid second messenger PtdIns3,4,5P₃.
PtdIns3,4,5P₃ is critical for the activation of PH domain-containing enzymes such as Btk
(Scharenberg et al., 1998), the Tec family tyrosine kinase involved in intracellular
calcium mobilization, Vav (Ma et al., 1998), the guanine nucleotide exchange factor for
Rac, and Akt, the serine/threonine kinase that is involved in cell survival (Aman et al.,
1998; Unkeless et al, 1997; Jacob et al, 1999) and in the activation of NFκB (Ozes et al.,
1999). The hydrolysis of PtdIns3,4,5P₃ by SHIP-1 has been demonstrated to down
regulate the activity of the above PH domain-containing enzymes and the down stream
functional outcomes.

SHIP-2 was originally identified as a putative phosphatidylinositol 5’-phosphatase
sequence by Drayer et al (Drayer et al., 1996). Subsequent cloning studies revealed that

29
Figure 2.1: Structure of SHIP-1 and SHIP-2. Schematic comparison of the structure of SHIP-1 and SHIP-2. % indicates percent homology.
SHIP-2 shared high sequence homology to SHIP-1 (Fig.2.1). These enzymes belong to the SHIP subgroup of the rather large family of inositol polyphosphate 5-phosphatases. The mRNA for SHIP-2 is highly expressed in the human heart, skeletal muscles and placenta. The enzymatic activity of SHIP-2 was found using anti-SHIP-2 antiserum immunoprecipitates. While SHIP-2 displays robust phosphatase activity on phosphatidyl inositol 3, 4, 5- trisphosphate, it is comparatively weak on its other substrate, Ins(1, 3, 4, 5)P$_4$ (Schurmans et al., 1999) SHIP-2 has been shown to associate with the adapter protein Shc and get tyrosine phosphorylated on stimulation of the cell with epidermal growth factor, platelet-derived growth factor, nerve growth factor, insulin-like growth factor, interleukin-3 and granulocyte/ macrophage colony stimulating factor (Krystal, 2000). Recent studies have revealed a role for SHIP-2 in regulating insulin receptor signaling and using the overexpression system it was determined that SHIP-2 played a negative role in insulin-induced mitogenesis and PKB and MAPK activation (Ishihara et al., 1999). Studies have also shown that SHIP-2 bound to the SH3 domain of Abl, indicating a potential role for SHIP-2 in myeloid proliferation seen in chronic myelogenous leukemia. Schurmans et al cloned the cDNA for murine SHIP-2 and found that it shared 95% identity with human SHIP-2. Studies of the protein structure revealed that it had a central catalytic domain (306 aa), flanked by an amino-terminal SH-2 domain (97 aa) and a carboxy terminal domain that housed three proline-rich regions. SHIP-2 was found to be expressed in all organs in the mouse embryo, with highest levels being observed in liver, brain and thymus. Due to its ubiquitous expression, SHIP-2-deficient mice display a more severe phenotype than mice lacking SHIP-1. The SHIP-2$^{-/-}$
mice die perinatally from insulin hypersensitivity-induced hypoglycemia. Mice heterozygous for SHIP-2 mutation showed increased glucose tolerance and insulin sensitivity and increased glycogen synthesis in skeletal muscles indicating that SHIP-2 is a potent negative regulator of insulin signaling (Clement et al., 2001; Sly et al., 2003).

While SHIP-2 shares high homology with SHIP-1 in its catalytic region (Giuriato et al., 1997; Pesesse et al., 2001; Pesesse et al., 1998), the proteins are largely divergent in the C-terminal region consisting of a proline-rich domain that associates with unique SH3 domain-containing proteins (Wisniewski et al., 1999). In addition, while SHIP-1 has two tyrosine residues in the C-terminus that conform to an NPXY motif shown to bind PTB (phosphotyrosine binding) domains upon phosphorylation, SHIP-2 has only one NPXY motif. Thus, these two enzymes while enzymatically similar, likely differ in functions that are related to their protein interactions via the C-terminal region. These two molecules also differ in their expression patterns: SHIP-1 is expressed predominantly in hematopoietic cells, while SHIP-2 is much more ubiquitously expressed. Recent studies have demonstrated a role for SHIP-2 in mediating the inhibitory effect of FcγRIIb in B cells (Blero et al., 2001; Brauweiler et al., 2001; Muraille et al., 1999). However, the expression and function of SHIP-2 in monocytes and macrophages is not known.

In this study, we first demonstrate that SHIP-2 is expressed in human alveolar macrophages but is almost undetectable in peripheral blood monocytes derived from the same donors. Interestingly, expression of SHIP-2 in PBM is induced by bacterial LPS in a dose-dependent manner. Second, FcγRIIa clustering in the human myeloid cell line THP-1 induced tyrosine phosphorylation of SHIP-2 suggesting that SHIP-2 may play a
role in FcγR-mediated function. Analyzing the functional consequence of SHIP-2 in FcγRIIa signaling, we report that over-expression of wild-type SHIP-2 but not catalytic-deficient SHIP-2 completely abrogated NFκB-dependent gene transcription in response to FcγRIIa clustering in THP-1 cells. Transient co-transfection experiments in COS-7 cells demonstrated that wild-type SHIP-2 down regulates FcγRIIa-induced Akt phosphorylation. Additional experiments analyzing the molecular mechanism of SHIP-2 activation by FcγRIIa demonstrated that the SH2 domain of SHIP-2 is necessary for optimal association of SHIP-2 with FcγRIIa and for optimal tyrosine phosphorylation of SHIP-2. Finally, we also demonstrate that FcγRI clustering results in phosphorylation of SHIP-2 suggesting that SHIP-2 may regulate both FcγRIIa and FcγRI-mediated myeloid cell function.
Materials and Methods

Cells, Antibodies and Reagents:

THP-1, U937, and COS-7 cells were obtained from ATCC. COS-7 cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum. All other cells were maintained in RPMI, supplemented with 10% Fetal Bovine Serum. Anti-FcγRI antibodies 197, and anti-FcγRIIa antibody IV.3 were obtained from Medarex (Annandale, NJ). Rabbit polyclonal SHIP-2 antibody was a generous gift from Dr. Bayard Clarkson (MSKCC, New York, NY) (Wisniewski et al., 1999). Goat polyclonal SHIP-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal SHIP antibody was a generous gift from Dr. K. Mark Coggeshall (OMRF, Oklahoma City, OK). pY, pAkt and Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal Syk antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Anti-Xpress antibody was purchased from Invitrogen (Carlsbad, CA)

Isolation of peripheral blood monocytes (PBM):

CD14 positive PBM were isolated as previously described (Tridandapani et al., 2000). Briefly, peripheral blood mononuclear cells (PBMCs) were first isolated by density gradient centrifugation over Histopaque (Sigma). Monocytes were then purified from the PBMCs by negative selection using the MACs Monocyte Isolation Kit (Miltenyi Biotech). For this, PBMCs were first treated with FcR blocking Reagent (hIgG), followed by a Hapten-Antibody Cocktail (cocktail of monoclonal hapten-conjugated CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies). The labeled cells were
further treated with MACS anti-hapten magnetic microbeads that were conjugated to a monoclonal anti-hapten antibody. The cells were then passed over a MACS column, and the effluent was collected as the negative fraction representing enriched monocytes.

**Preparation of human alveolar macrophages:**

Macrophages were obtained from healthy donors by bronchoalveolar lavage after signing an IRB-approved consent. Cells were washed twice with PBS and counted and analyzed by Diff-Quick staining for purity. Cell preparations were >95% positive for macrophages.

**Immunoprecipitation and western blotting:**

THP-1 cells and transfected COS-7 cells were activated by clustering FcγRI and or FcγRIIa with mAb 197 or Fab fragments of IV.3 and goat F(ab’)2 anti-mouse Ig secondary antibody (GAM). Resting and activated cells were lysed in TN1 buffer (50mM Tris pH 8.0, 10mM EDTA, 10mM Na₃P₂O₇, 10mM NaF, 1% Triton-X 100, 125mM NaCl, 10mM Na₃VO₄, 10μg/ml each aprotinin and leupeptin), and post nuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Gibco BRL) or goat F(ab’)2 anti-mouse Ig covalently linked to sepharose, depending on the antibody. Immune-complexes bound to beads were washed in TN1 and boiled in SDS sample buffer (60mM Tris pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 2% 2-ME) for 5 minutes. Proteins were separated by
SDS/PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence.

**Immunoblot Data Quantitation:**

The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). To quantitate the phosphorylation signals in the activated samples, we first subtracted background, normalized the phosphorylation signal to the amount of total precipitated protein, and plotted the values obtained by expressing them as fold increase over the value in unstimulated samples.

**Transfection:**

COS-7 cells were transfected as previously described (Tridandapani et al., 2002b). Briefly, cells were grown on culture dishes until they were 60-70% confluent. cDNA for FcγRIIa in pCEXV3, kindly provided by Dr. J. Ravetch (Rockefeller University, New York) and cDNA for wild-type SHIP-2, inactive SH2 domain-mutant of SHIP-2, and inactive catalytic domain-mutant of SHIP-2 in pcDNA3 (generously provided by Dr. Shonna Moodie, Metabolex Inc., Hayward, CA) (Taylor et al., 2000) were mixed in various combinations with Lipofectamine 2000 reagent (Gibco BRL). The DNA mix was added to cells in serum-free DMEM and incubated for 3 hours at 37°C in a CO₂ incubator. The media was then replaced by DMEM supplemented with 10% fetal bovine serum. The cells were harvested 24 hours 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 mutated SHIP-2 molecules to associate with FcγRIIa ITAM and to become tyrosine phosphorylated. In other experiments GST-Akt (a kind gift from Dr. R.D. Pearson, Melbourne, Australia) (Conus et al., 2002) was co-transfected with the above molecules (2μg FcγRIIa, 5μg SHIP-2, 1μg GST-Akt) to analyze the influence of SHIP-2 on Akt activation by FcγRIIa.

**Transfection of THP-1 cells and Luciferase assays:**

For analysis of SHIP-2 influence on NFκB transcriptional activity, THP-1 cells were transfected as previously described (Tridandapani et al., 2002b). Briefly, THP-1 cells were electroporated (310V, 950μF; Bio-Rad Gene Pulser II) with 5μg of wild-type SHIP-2 or catalytic-deficient SHIP-2 in pcDNA3, 1μg of NFκB-luc plasmid and 0.5μg of pEGFP to normalize for transfection efficiency. Transfectants were harvested 24 hours later, activated by clustering FcγRIIa by methods described above for 6h at 37°C. The cells were lysed in 100ul of cell culture lysis reagent (Promega). Luciferase activity was measured using the Promega luciferase assay reagent. Data are represented as graphs indicating the percent induction of NFκB activity in cells activated by clustering FcγRIIa over those that were not activated. Data points are expressed as mean and standard deviation of three independent experiments. Statistical analysis was performed by Student’s t test.
Results

SHIP-2 expression pattern in myeloid cells.

Expression of SHIP-2 in myeloid cells was assessed by Western blotting protein matched lysates obtained from THP-1 and U937 human monocytic cell lines, P388D1 mouse myeloid cell line, primary human PBM and alveolar macrophages. SHIP-2 shares a high level of homology with SHIP-1. First, to test the specificity of the SHIP-2 antibody, protein matched whole cell lysates from THP-1 cells were subject to immunoprecipitation with antibodies specific for SHIP-1, SHIP-2 or normal rabbit serum. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with antibodies specific for SHIP-2 or SHIP-1. The results shown in Figure 2.2 indicate the specificity of the SHIP-2 antibody as it shows no cross-reactivity with SHIP-1. The results shown in Figure 2.3 demonstrate the expression pattern of SHIP-2 in myeloid cells. SHIP-2 is expressed in the transformed myeloid cell lines and in primary human alveolar macrophages. Interestingly PBMs appeared to express very low levels of SHIP-2 compared to alveolar macrophages from the same donor. These results were consistently observed in 5 separate donors. A reprobe of the membranes with β-actin antibody (Fig.2.3, lower panels) showed equivalent loading of protein in the different lanes. Treatment of PBMs with LPS, a reagent often used to activate PBMs, resulted in increased expression of SHIP-2 in a dose-dependent manner (Fig.2.4A, upper panel). These data suggest that SHIP-2 is inducibly expressed in human monocytes. Treatment of U937 cells with LPS also increased SHIP-1 expression, indicating that SHIP-1
Figure 2.2: Specificity of SHIP-2 antibody. THP-1 whole cell lysates were subject to immunoprecipitation with antibodies specific for SHIP-1 or SHIP-2. Normal rabbit serum (NRS) immunoprecipitates were used as a control. The immunoprecipitates were analyzed by Western blotting with antibodies for SHIP-2 or SHIP-1. A. Western blot showing immunoprecipitates separated by SDS-PAGE and probed with SHIP-2 antibody. B. Western blot showing immunoprecipitates separated by SDS-PAGE and probed with SHIP-1 antibody.
Figure 2.3: Expression of SHIP-2 in myeloid cells. Protein-matched whole cell lysates (WCL) from the human monocytic cell lines U937 and THP-1, mouse myeloid cell line P388D1, human PBM and human alveolar macrophages were separated by SDS/PAGE and analyzed by Western blotting with anti-SHIP-2 antibody. The lower panels are reprobes of the same membranes with β-Actin antibody to ensure equal loading of protein in all lanes.
**Figure 2.4:** Induction of SHIP-2 and SHIP-1 in monocytes. **A.** Human PBMs were cultured overnight in the presence of increasing doses of LPS and protein-matched lysates were probed with anti-SHIP-2 antibody. The lower panel is a reprobe with anti-β-Actin antibody. These results are representative of four independent experiments. **B.** Protein matched lysates from U937 cells treated with/without LPS (50ng/ml) analyzed by Western Blotting with anti-SHIP-1 antibody. These results are representative of four independent experiments.
is also inducibly expressed in human monocytes on treatment with LPS (Fig.2.4B).

**SHIP-2 is tyrosine phosphorylated and serves to down regulate FcγRIIa-mediated function.**

In order to test whether SHIP-2 is involved in FcγR-mediated myeloid cell activation, SHIP-2 phosphorylation was assessed in THP-1 cells stimulated by clustering FcγRIIa (Fig.2.5A). For this, FcγRIIa receptors were clustered using Fab fragments of the anti-FcγRIIa-specific monoclonal antibody IV.3 followed by F(ab′)2 fragments of goat anti-mouse IgG (GAM). SHIP-2 was immunoprecipitated from resting and activated cells with a goat polyclonal SHIP-2 antibody, and analyzed by Western blotting with anti-phosphotyrosine antibody. Results indicated that FcγRIIa clustering induces SHIP-2 phosphorylation within 1’, the phosphorylation signals peak at about 7’ and subside by 30’ post stimulation (Fig.2.5A, upper panel). A reprobe of the same membrane with anti-SHIP-2 antibody revealed equal loading of SHIP-2 in all lanes. The last lane is a negative control immunoprecipitation with normal goat IgG (Fig.2.5A, lower panel). In a second set of experiments, human PBM cultured overnight with 100ng/ml LPS were stimulated by clustering FcγRIIa and SHIP-2 phosphorylation was assessed by Western blotting (Fig.2.5B). As seen in the Figure 2.5B, upper panel, SHIP-2 phosphorylation is induced by FcγRIIa clustering in PBM. The lower panel demonstrates equivalent loading of SHIP-2 in the two lanes.
Figure 2.5: SHIP-2 is tyrosine phosphorylated upon FcγRIIa clustering and inhibits NFκB-mediated gene transcription. A. THP-1 cells were activated for the various time points indicated in the figure by clustering FcγRIIa with Fab fragments of mab 1V.3 followed by GAM. SHIP-2 was immunoprecipitated from resting and activated cells and analyzed by Western blotting with anti-phosphotyrosine (pY) antibody (upper panel). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equivalent loading in all lanes. The last lane marked ‘C’ is a control immunoprecipitation with normal goat serum. These results are representative of three independent experiments. B. PBM were activated for 5’ by clustering FcγRIIa and SHIP-2 phosphorylation was assessed by Western blotting with anti-phosphotyrosine antibody (upper panel). The lower panel is a reprobe with anti-SHIP-2 antibody.
Having ensured that SHIP-2 is phosphorylated during FcγRIIa signaling we next assessed whether SHIP-2 influences FcγRIIa-mediated functional outcomes. We (Tridandapani et al., 2002b), and others (Sanchez-Mejorada and Rosales, 1998), have previously reported that FcγR clustering induces NFκB-mediated gene transcription, which is down regulated by the inositol phosphatase SHIP-1. Since SHIP-1 and SHIP-2 are both capable of hydrolyzing PtdIns3,4,5P₃ and influence downstream events we analyzed whether SHIP-2 played a role in regulating NFκB-mediated gene transcription. In these experiments we analyzed NFκB-dependent transcription from a reporter plasmid encoding the luciferase gene in the presence of excess of wild-type SHIP-2 or a catalytic-deficient (D608A) SHIP-2. Thus, THP-1 cells were transiently co-transfected with NFκB-luc plasmid and with plasmids encoding wild-type SHIP-2 or catalytic-deficient SHIP-2. The transfected cells were activated by clustering FcγRIIa for 5 hours at 37°C. Transcription of the luciferase gene was measured by a luciferase enzyme assay. Results are expressed as percent increase in luciferase activity in cells activated by clustering FcγRIIa over the activity in resting cells (Fig.2.6). Data indicate that NFκB-dependent transcription of the luciferase gene occurs upon FcγRIIa clustering. However, over expression of wild-type SHIP-2 blocks the induction of gene transcription (p-value= 0.027, presence of exogenous wild-type SHIP-2 versus pcDNA3 empty vector). Importantly, gene transcription is significantly enhanced in the presence of a dominant-negative, catalytic-deficient SHIP-2 (p-value= 0.006, presence of exogenous catalytic-deficient SHIP-2 versus pcDNA3 empty vector), suggesting that SHIP-2 serves to down regulate NFκB-
Figure 2.6: SHIP-2 inhibits NFκB-mediated gene transcription upon FcγRIIa clustering. THP-1 cells were transiently transfected with plasmids encoding the NFκB binding element coupled to luciferase gene (NFκB-luc) along with either empty vector or plasmids encoding wild-type SHIP-2 or a catalytic-deficient SHIP-2 (D408A). Cells were activated by clustering FcγRIIa for 5 hours and luciferase gene induction was measured in a luciferase enzyme assay. Results are shown as % increase of luciferase activity in the activated samples over that in resting samples. The graph represents mean and standard deviation of values from three independent experiments. Statistical analysis was performed with a paired, two-tailed student’s $t$ test.
dependent gene transcription by Fc\(\gamma\)RIIa.

Recent studies have demonstrated an upstream role for Akt in NF\(\kappa\)B activation (Ozes et al., 1999). Since Akt is a PH domain-containing enzyme whose activity is regulated by PtdIns3,4,5P\(_3\), a substrate of SHIP-2, we next analyzed Akt activation in the presence of over-expressed wild-type SHIP-2 or catalytic-deficient SHIP-2. For these experiments COS-7 fibroblasts were used in order to achieve high levels of transfection. Thus COS-7 cells were transiently transfected to express epitope (GST)-tagged Akt along with Fc\(\gamma\)RIIa (COS-7 cells do not express any endogenous Fc\(\gamma\)R) and either wild-type SHIP-2 or catalytic-deficient (D608A) SHIP-2. The transfected cells were harvested 24 hours later and activated by clustering Fc\(\gamma\)RIIa by methods described above. Whole cell lysates from resting and activated cells were separated by SDS/PAGE and analyzed by Western blotting with phospho Serine-Akt antibody (Fig.2.7A, upper panel). GST-Akt migrates around 95kDa in comparison to endogenous Akt, which is 65kDa. The slower migration of GST-Akt allows for analysis of the effect of over-expression of the SHIP-2 proteins on the co-transfected GST-Akt. The membrane was reprobed with anti-Akt antibody to detect the total amount of GST-Akt present in each lane (middle panel).

Figure 2.7A demonstrates that GST-Akt is serine phosphorylated upon Fc\(\gamma\)RIIa clustering (lane 4) in comparison to the resting sample (lane 3). Over-expression of wild-type SHIP-2 down-regulates Fc\(\gamma\)RIIa-induced Akt phosphorylation (lane 6, in comparison to lane 5). In contrast, over-expression of catalytic-deficient SHIP-2 results in enhanced phosphorylation of Akt in response to Fc\(\gamma\)RIIa clustering (lane 8), suggesting that the catalytic-deficient SHIP-2 functions in a dominant-negative manner, overcoming the
Figure 2.7: SHIP-2 inhibits FcγRIIa-mediated Akt phosphorylation. COS-7 cells were transiently transfected to express FcγRIIa and GST-Akt along with wild-type or D608A SHIP-2. A. The transfectants were activated by clustering FcγRIIa, and serine phosphorylation of Akt was assessed by Western blotting WCL with phospho-serine Akt antibody (*upper panel*). The middle panel is a reprobe of the same membrane with total Akt antibody. The lower panel is a graph representing a quantitative measure of GST-Akt phosphorylation expressed as fold increase over that in resting cells. Data represent mean and standard deviation of values obtained from three independent experiments. Statistical analysis was performed using a paired, two-tailed student’s *t* test. B. WCL from the four transfectants were analyzed for the presence of the transfected FcγRIIa by Western blotting with anti-FcγRIIa antibody. C. Expression of the transfected, Xpress-tagged SHIP-2 was assessed by probing anti-Xpress immunoprecipitates with anti-SHIP-2 antibody.
inhibition on Akt by endogenous SHIP-2 in lane 4. Akt phosphorylation was quantitated by normalizing phosho-Akt (GST) signals to total Akt (GST) signals as described in experimental methods. The results are expressed as fold increase of Akt phosphorylation in activated cells in comparison to that in resting cells. Figure 2.7A, lower panel, represents the mean and standard deviation of values obtained from three independent experiments. The inhibition of Akt phosphorylation by wild-type SHIP-2 is statistically significant (p-value 0.02). In order to ensure that transfected FcγRIIa was expressed to comparable levels in the different transfectants, WCL were probed with anti-FcγRIIa antibody (Fig.2.7B). Likewise, expression of Xpress-tagged, wild-type and catalytic-deficient SHIP-2 was comparable in the transfected cells as seen in Figure 2.7C. These data suggest that SHIP-2 down regulates FcγRIIa-induced Akt phosphorylation.

**SHIP-2 associates with the phosphorylated ITAM of FcγRIIa**

SHIP-2 is a cytoplasmic protein whose activity is regulated by subcellular localization, i.e. SHIP-2 must translocate to the membrane in order to access its lipid substrates (Erneux et al., 1998). We, therefore, tested whether FcγRIIa provides a docking site for SHIP-2 in the following experiments. First, we used biotinylated peptides derived from the ITAM of FcγRIIa that were either non-phosphorylated or doubly phosphorylated on the tyrosine residues (Fig.2.8A). These peptides were applied to THP-1 lysates and the peptide-bound material was analyzed by Western blotting with anti-SHIP-2 antibody. As seen in Figure 2.8B (upper panel), SHIP-2 is associated with
**Figure 2.8: SHIP-2 associates with FcγRIIa ITAM peptide.** A. Amino acid sequence of the ITAM peptides containing an N-terminal Biotin. B. THP-1 cells lysates were incubated with biotinylated peptides corresponding to the unphosphorylated, or the phosphorylated ITAM of FcγRIIa. The peptide bound material was analyzed for the presence of SHIP-2 by Western blotting with anti-SHIP-2 antibody (upper panel). The same membrane was reprobed with anti-Syk antibody (lower panel). The last lane is a WCL from the THP-1 cells. These data are representative of three independent experiments.
the doubly phosphorylated FcγRIIa ITAM peptide but not with the non-phosphorylated peptide. Lane 3 is a whole cell lysate from THP-1 cells. To ensure that the binding of SHIP-2 to the phospho-ITAM peptide is specific, the same membrane was reprobed with anti-Syk (lower panel). As previously reported the phospho-ITAM of FcγRIIa bound Syk (Chacko et al., 1996).

In a second set of experiments, we asked whether the native FcγRIIa receptor associates with SHIP-2 upon receptor clustering. Here, THP-1 cells were activated by clustering FcγRIIa for 5’. FcγRIIa was immunoprecipitated from resting and activated cells, separated by SDS/PAGE and analyzed for the presence of SHIP-2 by Western blotting. Results indicated that SHIP-2 is capable of associating with FcγRIIa in an activation dependent manner (Fig. 2.9, upper panel). A reprobe of the same membrane indicated equal loading of FcγRIIa in both resting and activated lanes. Taken together these results suggest that SHIP-2 translocates to the membrane upon FcγRIIa clustering by associating with the phosphorylated ITAM of FcγRIIa.

**SH2 domain of SHIP-2 is required for association with FcγRIIa ITAM**

The above findings that SHIP-2 associates with the phosphorylated ITAM of FcγRIIa suggest that the interaction may be mediated via the SH2 domain of SHIP-2. In order to formally test this possibility, COS-7 fibroblasts were transfected to express epitope (Xpress)-tagged wild-type SHIP-2, an SH2 domain mutant of SHIP-2 (R47L) or a catalytic-deficient (D608A) SHIP-2 (Fig.2.10). The tagged SHIP-2 proteins were assessed for their ability to bind FcγRIIa ITAM in a peptide-binding assay. Thus, the
**Figure 2.9:** SHIP-2 co-immunoprecipitates with FcγRIIa. FcγRIIa was immunoprecipitated from resting (R) and activated (A) THP-1 cells that had been activated for 5’ by clustering FcγRIIa. The immunoprecipitated proteins were separated by SDS/PAGE and analyzed by Western blotting with anti-SHIP-2 antibody (*upper panel*). The membrane was reprobed with anti-FcγRIIa antibody (*lower panel*). These data are representative of three independent experiments.
**Figure 2.10:** Schematic of SHIP-2 constructs used in the study.
peptide-bound material was separated by SDS/PAGE and immunoblotted with anti-Xpress antibody. Results indicated that the SH2 mutant of SHIP2 was unable to associate optimally with the phosphopeptide (Fig.2.11A, lane 3). In contrast, both the wild-type SHIP-2 and catalytic-deficient SHIP-2 were able to efficiently bind the phospho-ITAM peptide (lanes 2 and 4). No signal was seen in lane 1, as these cells were not transfected with the Xpress-tagged SHIP-2 proteins. In order to ensure that the lack of binding observed with the SH2 mutant was not due to lack of expression of the transfected protein, parallel immunoprecipitations were performed with anti-Xpress antibody and analyzed by Western blotting with SHIP-2 antibody (Fig.2.11B). Figure 2.11C is a quantitative measure of the percent of Xpress-tagged SHIP-2 that bound to the phospho-ITAM of FcγRIIa. The data represent mean and standard deviation of values obtained from three independent experiments.

**SH2 domain of SHIP-2 is necessary for FcγRIIa-induced SHIP-2 tyrosine phosphorylation**

As an additional approach to determine the importance of the SH2 domain of SHIP-2 in its association with FcγRIIa, and thereby its translocation to the membrane, we next assessed whether SH2 domain mutants of SHIP-2 would become phosphorylated upon FcγRIIa clustering. Here COS-7 cells were transfected to express FcγRIIa along with wild-type SHIP-2, SH2 domain mutant of SHIP-2 (R47L) or catalytic-deficient
**Figure 2.11: SH2 domain of SHIP-2 is necessary for optimal SHIP-2 association with FcγRIIa ITAM.**

**A.** Xpress-tagged SHIP-2 proteins (wild-type, SH2 domain mutant R47L, catalytic-deficient D608A) were expressed in COS-7 fibroblasts by transient transfection. The phospho-ITAM peptide of FcγRIIa was applied to COS-7 lysates, and binding of the SHIP-2 proteins was assessed by Western blotting with anti-Xpress antibody.

**B.** Parallel samples were immunoprecipitated with anti-Xpress antibody and probed with anti-SHIP-2 antibody to compare the level of expression of the SHIP-2 proteins in the different transfectants.

**C.** The % of SHIP-2 protein that bound the pITAM peptide was quantitated by dividing the density of the bands in figure 2.11A with the density of the bands in figure 2.11B. The graph represents the mean and standard deviation of values from three independent experiments.
mutant of SHIP-2 (D608A). SHIP-2 proteins were immunoprecipitated from resting and activated cells, which had been activated by clustering FcγRIIa, using anti-Xpress antibody and analyzed by Western blotting with anti-phosphotyrosine antibody. Results indicated that while both wild-type SHIP-2 and the catalytic-deficient SHIP-2 became tyrosine phosphorylated upon FcγRIIa clustering, the SH2 domain mutant of SHIP-2 failed to become phosphorylated (Fig.2.12A, upper panel). A reprobe of the membrane with anti-SHIP-2 antibody showed presence of SHIP-2 in all lanes (middle panel). The phosphorylation level of SHIP-2 was quantitated as described in experimental methods, 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. The graph (lower panel) represents mean and standard deviation of values obtained from three independent experiments. In order to ensure that the lack of phosphorylation of the SH2 domain mutant of SHIP-2 was not due to lack of expression of FcγRIIa in these cells, WCL from the transfectants were probed with anti-FcγRIIa antibody (Fig.2.12B). Taken together these data indicate that the SH2 domain of SHIP-2 is necessary for the association of SHIP-2 with FcγRIIa.

**SHIP-2 is phosphorylated by FcγRI clustering in THP-1 cells**

In additional experiments, THP-1 cells were activated by clustering FcγRI with the monoclonal antibody 197 followed by GAM. SHIP-2 phosphorylation was assessed
**Figure 2.12:** SH2 domain of SHIP-2 is necessary for optimal phosphorylation of SHIP-2 by FcγRIIa clustering. COS-7 cells were transiently transfected to express FcγRIIa along with either wild-type, R47L or D608A SHIP-2. **A.** The transfected cells were activated for 5’ by clustering FcγRIIa and phosphorylation of SHIP-2 was assessed by Western blotting anti-Xpress immunoprecipitates with anti-pY antibody (*upper panel*). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equal loading in the resting and activated lanes (*middle panel*). The lower panel is a quantitative measure of the phosphorylation level of the different SHIP-2 proteins normalized for the amount of total SHIP-2 present in each lane. The graph represents mean and standard deviation of values from three independent experiments. **B.** WCL from the four transfectants were analyzed for the expression of the transfected FcγRIIa by Western blotting.
by Western blotting with anti-phosphotyrosine antibody (Fig.2.13A, upper panel). The lower panel is a reprobe with anti-SHIP-2 antibody to ensure equal loading of SHIP-2 in all lanes. These results indicate that SHIP-2 is indeed invoked upon FcγRI clustering in THP-1 cells.

Since our data indicated that SHIP-2 associates with the phosphorylated ITAM of FcγRIIa, we next analyzed whether a similar association might exist between SHIP-2 and the phosphorylated ITAM of FcγR γ-chain. For this, biotinylated peptides corresponding to the ITAM of the γ-chain that were either non-phosphorylated or doubly phosphorylated were applied to THP-1 lysates. The peptide bound material was analyzed by Western blotting with anti-SHIP-2 antibody. The results in Figure.2.13B, upper panel indicate that the phospho-ITAM of the γ-chain is incapable of association with SHIP-2. In order to ensure that the phospho-peptide used in these experiments is functional, the same membrane was reprobed with anti-Syk antibody (lower panel). As seen in the figure the phospho-peptide efficiently bound Syk, consistent with earlier reports (Daeron, 1997). These data suggest that SHIP-2 translocation to the membrane in response to FcγRII clustering might involve adapter molecules or membrane-associated proteins other than the γ-chain. Further analysis is needed to resolve these issues.
**Figure 2.13: SHIP-2 is tyrosine phosphorylated upon FcγRI clustering in THP-1 cells.**

A. THP-1 cells were activated for the time points indicated in the figure by clustering FcγRI with mab 197 followed by GAM. SHIP-2 was immunoprecipitated from resting and activated cells, and analyzed for phosphorylation by Western blotting with anti-pY antibody (upper panel). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equivalent loading of SHIP-2 in all lanes. The last lane is a control immunoprecipitation with isotype-matched antibody. B. Unphosphorylated and doubly phosphorylated peptides corresponding to the ITAM of the FcγRγ-subunit were applied to THP-1 lysates. Peptide bound material was separated by SDS/PAGE and analyzed for binding of SHIP-2 by Western blotting with anti-SHIP-2 antibody (upper panel). The same membrane was reprobed with anti-Syk (lower panel). These data are representative of three independent experiments.
Discussion

Fcγ Receptor-mediated phagocytosis is regulated by a complex set of phosphatases, each using a distinct molecular mechanism for mediating its inhibitory function. Thus recent reports have implicated the inositol phosphatase SHIP-1 in modulating FcγR-mediated macrophage function including phagocytosis of IgG-opsonized particles (Cox et al., 2001; Nakamura et al., 2002) and induction of gene transcription (Tridandapani et al., 2002b). Likewise, Durden and colleagues have demonstrated a role for the protein tyrosine phosphatase SHP-1 (Kant et al., 2002) and for PTEN (Kim et al., 2002) in down regulating phagocytosis. While it is clear that in order to limit the tissue damage caused by the reactive oxygen and nitrogen radicals and the inflammatory cytokines that accompany phagocytosis this process must be subject to a tight regulation, the mechanisms involved in this regulation are not fully understood.

In this study we have analyzed the molecular details of a novel level of regulation exerted by the inositol phosphatase SHIP-2. Our results indicate that SHIP-2 associates with the ITAM of FcγRIIa via the SH2 domain of SHIP2 and becomes tyrosine phosphorylated. This is schematically represented in Figure.2.14. As in the case of SHIP-1, tyrosine phosphorylation of SHIP-2 does not influence SHIP-2 enzyme activity. However, since membrane translocation of SHIP-2 is necessary for SHIP-2 tyrosine phosphorylation by src kinases (Prasad et al., 2002), and since membrane translocation
**Figure 2.14:** Model for SHIP-2 association with FcγRIIa and subsequent phosphorylation by src kinases. Clustering of FcγRIIa leads to phosphorylation of tyrosine residues in the ITAM. SHIP-2 associates with the phosphorylated ITAM via its SH2 domain and is itself tyrosine phosphorylated by src kinases.
places SHIP-2 in the proximity of its lipid substrates, tyrosine phosphorylation of SHIP-2 serves as a correlate of SHIP-2 activation. Our data also demonstrate that SHIP-2 serves to down regulate FcγRIIa-induced activation of Akt and NFκB-dependent gene transcription, as over-expression of wild-type SHIP-2 but not catalytic-deficient SHIP-2 almost completely abrogates these two events (Fig.2.15). Both SHIP-1 and SHIP-2 mediate their inhibitory effects via the SH2 domains, which are reported to have 54% homology. Thus, it must be noted that the enhanced activation of NFκB-dependent luciferase gene induction by the dominant-negative SHIP-2 (D608A) used in the transfection studies in THP-1 cells (Fig.2.6) may be the result of competition of D608A with both endogenous SHIP-1 and SHIP-2.

In contrast to the association of SHIP-2 with the FcγRIIa ITAM, we were unable to detect any binding of SHIP-2 to the phosphorylated ITAM of the FcγRI-associated γ-chain, despite the fact that SHIP-2 is efficiently phosphorylated upon FcγRI clustering (Fig.2.13). Co-immunoprecipitation experiments revealed a very weak association of SHIP-2 with the γ-chain that became apparent only when the western blot was over-exposed (data not shown). Based on the results of Figure 2.13, we would suggest that SHIP-2 association with the γ-chain is likely mediated via adapter molecules, or that the association may be weak and transient and is disrupted by the detergent-based lysis buffer. In a recent report Mitchell and colleagues have demonstrated an association of SHIP-2 with the actin binding protein filamin (Dyson et al., 2001). This association is mediated via the C-terminal proline-rich domain of SHIP-2 and the SH3 domain of filamin. Since filamin is known to be associated with FcγRI (Stossel et al., 2001) in
Figure 2.15: Role for SHIP-2 in FcγR-mediated myeloid cell function. SHIP-2 serves to down-regulate FcγR-mediated Akt activation. It also inhibits NFκB-driven gene transcription. Dashed line indicates possible additional signaling intermediates.
macrophages, it is possible that FcγRI clustering recruits SHIP-2 via filamin.

Nonetheless, our data indicate that FcγRI clustering induces tyrosine phosphorylation of SHIP-2 suggesting a role for SHIP-2 in regulating FcγRI-mediated events.

Interestingly, SHIP-2 expression in human PBM is almost undetectable but is induced upon activation of these cells with bacterial lipopolysaccharide. Similar findings were recently reported by Cambier and colleagues in murine B cells (Brauweiler et al., 2001). It is tempting to speculate that the induction of SHIP-2 by LPS is a mechanism employed by gram-negative bacteria to suppress the host immune response in order that the pathogen may evade the phagocytic machinery. Numerous earlier studies have reported that LPS attenuates FcγR-mediated phagocytosis (Sundaram et al., 1993; Wonderling et al., 1996). However, the molecular mechanism by which LPS mediates its suppressive effect has thus far remained elusive. Further studies are underway to investigate the details of LPS induction of SHIP-2 expression, and the functional consequence on FcγR-mediated phagocytosis.

It is not clear why myeloid cells express two inositol 5’ phosphatases, SHIP-1 and SHIP-2. Our data would suggest that these two molecules may not be co-expressed at all times. Furthermore, although the results of this study demonstrate overlapping functions for SHIP-1 and SHIP-2, i.e. inhibition of Akt and NFκB-activation, it is likely that these two phosphatases mediate additional and non-overlapping effects on signaling pathways. Indeed, previous studies have demonstrated that the proline-rich C-terminal region of SHIP-1 and SHIP-2 are quite different, leading to association of distinct set of SH3 domain-containing molecules (Wisniewski et al., 1999), which likely influence distinct
signaling pathways. We are currently investigating the functional differences between SHIP-1 and SHIP-2 in FcγR-mediated activation of monocytes and macrophages.

Of note are our findings that both SHIP-1 and SHIP-2 proteins are upregulated in monocytes by LPS. These findings suggest a role for the inositol phosphatases in LPS-induced macrophage responses. A role for inositol phosphatases in LPS signaling has not been described previously. Our pioneering studies on the role of SHIP in LPS signaling are described in chapter 3.
CHAPTER 3
LIPOPOLYSACCHARIDE-INDUCED MACROPHAGE
INFLAMMATORY RESPONSE IS REGULATED BY THE
SH2 DOMAIN-CONTAINING INOSITOL 5-PHOSPHATASE
SHIP

Abstract
Lipopolysaccharide stimulates monocytes/macrophages through TLR4 resulting in the activation of a series of signaling events that potentiate the production of inflammatory mediators. Recent reports indicated that the inflammatory response to LPS is diminished by PtdIns3K, through the activation of the serine/threonine kinase Akt. SHIP is an inositol phosphatase that can reverse the activation events initiated by PtdIns3K, including the activation of Akt. However, it is not known if SHIP is involved in TLR4 signaling. In this study, we demonstrate that LPS stimulation of Raw 264.7
mouse macrophage cells induces the association of SHIP with lipid rafts, along with IRAK. In addition, SHIP is tyrosine phosphorylated upon LPS stimulation. Transient transfection experiments analyzing the function of SHIP indicated that overexpression of a wild type SHIP, but not the SHIP SH2 domain lacking catalytic activity, upregulates NFκB-dependent gene transcription in response to LPS stimulation. These results suggest that SHIP positively regulates LPS-induced activation of Raw 264.7 cells. To test the validity of these observations in primary macrophages, LPS-induced events were compared in bone marrow macrophages derived from SHIP+/+ and SHIP−/− mice. Results indicated that LPS-induced MAP Kinase phosphorylation is enhanced in cells expressing SHIP. In contrast, Akt phosphorylation was enhanced in SHIP−/− cells. Finally, LPS-induced TNFα and IL-6 production was significantly lower in SHIP−/− BMM. These results are the first to demonstrate a role for SHIP in TLR4 signaling, and propose that SHIP is a positive regulator of LPS-induced inflammation.
**Introduction**

Lipopolysaccharide-induced activation of monocytes and macrophages involves the Toll-like receptor 4 (TLR4) and results in the production of proinflammatory cytokines including TNFα (Beutler and Rietschel, 2003; Medzhitov and Janeway, Jr., 2000). The receptor proximal signaling events include the association of the adapter MyD88 with TLR4, followed by the recruitment and activation of IRAK-1. More distal to the receptor, the MAP Kinases Erk1/2, p38 and JNK and the transcription factors NFκB and AP-1 become activated and promote gene transcription. The signaling events bridging receptor proximal events to the activation of MAP Kinases are not fully understood.

PtdIns3K is activated in response to LPS stimulation and has been shown to play a role in the signaling cascades triggered by LPS (Monick et al., 2001). In a recent study, Guha and Mackman demonstrated that inhibition of PtdIns3K results in enhanced activation of LPS-induced MAP Kinase activation, and gene transcription driven by the transcription factors NFκB, AP-1 and Egr-1 (Guha and Mackman, 2002b). The inhibitory effect of PtdIns3K was shown to be mediated via the serine/threonine kinase Akt. In other reports it has been shown that PtdIns3K negatively regulates the stability of COX2 mRNA in LPS stimulated human alveolar macrophages (Monick et al., 2002), LPS-induced nitric oxide synthase production in glial cell (Pahan et al., 1999), and LPS-induced NO production from murine peritoneal macrophages. Finally, in a recent study Williams and colleagues demonstrated that inhibition of PtdIns3K in vivo, resulted in increased inflammatory cytokine levels in the serum, as well as early mortality in septic
mice, in a murine model of cecal ligation and puncture (Williams et al., 2004a). Interestingly, while the above reports all indicate that PtdIns3K dampens LPS signaling, other groups have found that PtdIns3K activity promotes LPS signaling [reviewed in (Fukao and Koyasu, 2003)]. These disparate observations may reflect cell-type specific influences of PtdIns3K, which are currently not fully understood [reviewed in (Fukao and Koyasu, 2003)].

The SH2 domain-containing inositol phosphatase SHIP is specifically expressed in hematopoietic cells and is capable of reversing the effects of PtdIns3K by hydrolyzing the 5’ phosphate of its product PtdIns3,4,5P$_3$ (Krystal, 2000) (Fig.3.1). The inhibitory role of SHIP in immune receptor and growth factor/cytokine receptor signaling is well-established [reviewed in (Krystal, 2000)]. Thus, the catalytic activity of SHIP results in the consumption of PtdIns3,4,5P$_3$ and leads to inhibition of downstream PH domain-containing enzymes that are dependent on PtdIns3,4,5P$_3$ for their activation (Aman et al., 1998; Jacob et al., 1999; Scharenberg et al., 1998). In addition, the non-catalytic regions of SHIP have been shown to influence signaling pathways by association with critical proteins that regulate these pathways (Aman et al., 1998; Jacob et al., 1999; Tamir et al., 2000; Tridandapani et al., 1997a; Tridandapani et al., 1997b). However, it is not known if SHIP is involved in regulating LPS signaling.

In this study we have analyzed the influence of SHIP in LPS-induced activation of murine macrophages. We report that SHIP becomes tyrosine phosphorylated upon LPS stimulation in Raw 264.7 cells, and translocates to lipid rafts along with IRAK. Transient co-transfection experiments testing the functional consequence of SHIP revealed that
Figure 3.1: Catalytic activity of SHIP. The inositol phosphatase SHIP hydrolyzes the 5’ phosphate product of PtdIns3K, PtdIns3,4,5P₃, to give PtdIns3,4P₂.
NFκB-driven gene transcription in response to LPS is upregulated in the presence of wild-type SHIP but not an isolated SH2 domain of SHIP lacking in enzyme activity. These results suggest that SHIP is a positive regulator of LPS-induced activation of macrophages. Consistent with this notion, BMMs from SHIP-deficient mice stimulated with LPS displayed reduced activation of Erk and p38 MAP Kinases in comparison to wild-type BMM. Likewise, LPS-induced TNFα and IL-6 production was lower in SHIP-deficient BMM. In contrast, Akt phosphorylation was enhanced in SHIP−/− BMM. Taken together, these data demonstrate that SHIP promotes LPS-induced macrophage inflammatory response, at least in part through the inactivation of Akt. These results are in contrast to the previously reported inhibitory role of SHIP in immune receptor and growth factor receptor signaling (Krystal, 2000). However, these findings are consistent with the reported negative regulatory role of PtdIns3K in TLR4 signaling (Guha and Mackman, 2002b; Monick et al., 2002; Pahan et al., 1999; Park et al., 1997).
Materials and Methods

**Cells and Reagents:**
Raw 264.7 murine macrophage cell line was obtained from ATCC, and maintained in RPMI with 3.5% Fetal Bovine Serum. Antibodies specific for phospho-Erk, phospho-Akt and phospho-p38 were purchased from Cell Signaling Technology (Beverly, MA). Actin, Akt and TLR4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). SHIP antibody and IRAK antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-mouse CD16/32 (FcγRIII/II) was purchased from BD Pharmingen (San Diego, CA). Rabbit polyclonal SHIP antibody was a generous gift from Dr. K. Mark Coggeshall (OMRF, Oklahoma City, OK). LPS from *E. coli* strain 0127:B8 was obtained from Difco (Detroit, MI).

**Culture of murine bone marrow-derived macrophages:**
Bone marrow macrophages (BMM) were derived from SHIP\(^{+/+}\) and \(^{-/-}\) male littermates as previously described (Tridandapani et al., 2002b). Briefly, bone marrow cells were cultured in RPMI containing 5% fetal bovine serum, 10μg/ml polymixin B, and supplemented with 20ng/ml CSF-1 for 7 days, before they were used in the experiments.

**Immunoprecipitation and Western blotting:**
Macrophages were stimulated with or without 500ng/ml LPS, or by clustering FcγR as previously described (Tridandapani et al., 2002b). 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$_3$VO$_4$, 10ug/ml each aprotinin and leupeptin). Proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes, probed with the antibody of interest and appropriate horseradish peroxidase-conjugated secondary antibodies. The filters were then developed by enhanced chemiluminescence (ECL).

**Immunoblot Data Quantitation:**

The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). To quantitate the phospho-specific signal in the activated samples, we first subtracted background, normalized the signal to the amount of actin in the lysate, and plotted the values as fold increase over unstimulated samples, as previously described (Tridandapani et al., 2002b).

**Transfection and Luciferase Assays:**

Raw 264.7 cells were transfected with the appropriate plasmid DNA using the Amaxa Nucleofector apparatus (Amaxa biosystems, Germany). Briefly, 2x10$^6$ cells were resuspended in 100ul Cell Line Nucleofector Solution V and were nucleofected with 1ug of NFκB-luc and/or 5ug of WT-SHIP or SHIP SH2 domain alone. Immediately post nucleofection, 500ul of pre-warmed RPMI was added to the transfection mix before transferring to 12-well plates containing 1.5ml pre-warmed RPMI per well. Plates were incubated for 24hr at 37°C.
Transfected cells were left untreated or stimulated with 500ng/ml LPS for 2hr. The cells were lysed in 100μl of Luciferase Cell Culture Lysis 5X Reagent (Promega). Luciferase activity was then measured using Luciferase Assay Reagent (Promega), as previously described (Tridandapani et al., 2002b).

**Sucrose Density Gradients:**

Triton-soluble and triton-insoluble cell fractions were prepared using methods described previously (Triantafilou et al., 2002). Briefly, Raw 264.7 cells starved in serum-free medium were left untreated or stimulated with 100ng/ml LPS for 30min and then lysed in TN1 buffer containing 0.5% Triton X-100. 1ml of the lysate was mixed with 1ml of 85% sucrose and loaded at the bottom of a Beckman centrifuge tube, and overlaid with 7ml of 30% sucrose followed by 3.5ml of 5% sucrose. The gradients were centrifuged for 17hr at 38000rpm at 4°C in a Beckman SW40Ti rotor. 9 fractions (~ 1.4ml each) were collected from the top of the gradient and were used for analysis by Western blotting.

**ELISA determination of cytokine production:**

Cells were cultured for varying time points ranging from 2 hours to 14.5 hours (overnight), in the presence or absence of 500ng/ml LPS. Cell supernatants were harvested, centrifuged to remove dead cells and analyzed by ELISA using cytokine specific kits from R & D Systems (Minneapolis, MN). Data were analyzed using a paired Student’s *t*-test.
Measurement of receptor expression by flow cytometry:

Murine BMMs were tested for expression of FcγRs by incubating with anti-FcγRII/III mAb 2.4G2 (Pharmingen), at a concentration of 10μg/ml for 30 minutes at 4°C. The cells were washed and incubated with FITC-labeled goat anti-rat Ig secondary antibody for 30 minutes at 4°C. Cells were subsequently washed, fixed in 1% paraformaldehyde and analyzed by flow cytometry on an Elite EPICS fluorescence-activated cell sorter (Coulter, Hialeah, FL). Data from 10,000 cells per condition were recorded to yield the percentage of cells expressing receptors (Figure 3.6B).
**Results**

*LPS stimulation of murine macrophages induces SHIP membrane translocation.*

Previous studies indicated that LPS stimulation results in the movement of TLR4 into the cholesterol-enriched, detergent-insoluble, lipid-rafts, where the receptor complex is assembled (Triantafilou et al., 2002). SHIP is a cytosolic enzyme that must translocate to the plasma membrane in order to access its lipid substrates (Krystal, 2000). Thus, in order to test whether SHIP is involved in LPS signaling, triton-soluble and triton-insoluble fractions were isolated from Raw 264.7 murine macrophage cells using sucrose density gradients. The fractions were separated by SDS/PAGE and examined for the presence of SHIP by Western blotting. As previously reported (Triantafilou et al., 2002), results indicated that IRAK becomes associated with lipid rafts following LPS stimulation (Fig.3.2A, lower panel). Likewise, SHIP translocated to the lipid rafts upon LPS stimulation (Fig.3.2B, lower panel). Neither SHIP nor IRAK were detected in the lipid rafts in unstimulated cells (Fig.3.2A and B, upper panels).

Membrane translocation of SHIP during immune receptor and growth factor receptor signaling is accompanied by phosphorylation of SHIP on tyrosine residues (Krystal, 2000). Thus, as a second test of membrane translocation of SHIP during TLR4 signaling, tyrosine phosphorylation of SHIP was assessed in Raw 264.7 cells stimulated for varying time points with LPS. Here, SHIP immunoprecipitates were separated by SDS/PAGE and analyzed by Western blotting with anti-phosphotyrosine antibody.
Figure 3.2: LPS stimulation leads to membrane translocation of SHIP. A and B. Western blotting of triton-soluble and -insoluble fractions showing translocation of IRAK and SHIP respectively. The upper and lower panels indicate unstimulated and cells stimulated with LPS for 30min. These data are representative of four independent experiments.
Results indicated that SHIP phosphorylation is induced in Raw 264.7 cells in response to LPS stimulation. A reprobe of the same membrane with anti-SHIP antibody (Fig.3.3, lower panel) demonstrated equal loading of SHIP in all lanes. The lane marked ‘C’ is an immunoprecipitate using normal rabbit serum in activated Raw 264.7 lysates. Taken together, these data indicate that SHIP is involved in TLR4 signaling.

**SHIP positively regulates NFκB-dependent gene transcription in response to LPS stimulation.**

LPS stimulation of macrophages results in the activation of transcription factors such as NFκB. We, and others, have previously reported that SHIP negatively regulates Fc receptor and growth factor receptor-mediated activation of NFκB (Tridandapani et al., 2002b; Baran et al., 2003; Kalesnikoff et al., 2002a). Therefore, we asked whether SHIP influenced gene transcription driven by NFκB in response to LPS stimulation. In these experiments Raw 264.7 cells were transiently transfected with luciferase reporter plasmids that were dependent on NFκB binding (NFκB-luc). Stimulation of these transfected cells with LPS induced NFκB-dependent transcription of luciferase, as previously reported (Fig.3.4A)(Guha and Mackman, 2002b). Surprisingly, the induction of luciferase gene transcription was significantly enhanced in the presence of overexpressed wild type SHIP (*p value* 0.04). In contrast, the overexpression of SHIP
Figure 3.3: LPS stimulation leads to tyrosine phosphorylation of SHIP. Raw 264.7 cells were stimulated with 500ng/ml LPS for the time points indicated, followed by immunoprecipitation with anti-SHIP antibody. Tyrosine phosphorylation of SHIP was assessed by immunoblotting with anti-phosphotyrosine (upper panel). The membrane was reprobed with anti-SHIP to ensure equal loading. The lane marked ‘C’ is an immunoprecipitate using normal rabbit serum. The last lane is loaded with whole cell lysate (WCL). These data are representative of four independent experiments.
Figure 3.4: SHIP positively regulates NFκB-dependent gene transcription in LPS stimulated cells. Raw 264.7 cells were transfected with plasmids encoding the NFκB-dependent luciferase, along with WT-SHIP or SHIP-SH2. Cells were left unstimulated or were stimulated with 500ng/ml LPS for 2hrs. A. Cells were lysed and luciferase gene induction was measured. Black bars indicate fold increase in luciferase activity in activated cells over that in resting cells. The graph represents mean and S.D. of values obtained from three independent experiments. B. Protein-matched lysates from parallel experiments were separated by either 10% SDS/PAGE (upper panel) or 15% SDS/PAGE (lower panel), and Western blotted with anti-SHIP antibody to detect the overexpressed proteins.
SH2 domain alone, that lacks the catalytic domain, failed to influence NFκB-driven luciferase gene transcription (p value 0.25). Cell lysates from the transfectants were analyzed by Western blotting to ensure that the transfected wild-type SHIP and the SHIP SH2 domain were indeed expressed (Fig.3.4B). These results suggest that SHIP enzyme activity positively regulates TLR4 induced activation of the NFκB transcription factor. Since SHIP and PtdIns3K are opposing enzymes, these results are consistent with the findings of Guha and Mackman demonstrating that PtdIns3K negatively regulates LPS-induced NFκB activity (Guha and Mackman, 2002b).

LPS-induced MAP Kinase phosphorylation is downregulated in SHIP\(^{-/-}\) macrophages.

To examine the molecular details of SHIP influence on TLR4 signaling, we next assessed MAP Kinase activation in bone marrow macrophages derived from SHIP\(^{+/+}\) and SHIP\(^{-/-}\) littermates. In these experiments BMM were serum starved overnight and then stimulated with 500ng/ml LPS for varying time points. Phosphorylation of the MAP Kinases Erk and p38 was analyzed by Western blotting protein-matched lysates with phospho-specific Erk and p38 antibodies (Fig.3.5A and B, upper panels). Results indicated that robust Erk and p38 phosphorylation was induced in SHIP\(^{+/+}\) BMM. In contrast, Erk and p38 phosphorylation were reduced in SHIP\(^{-/-}\) BMM. The same membranes were reprobed for actin, to ensure equal loading in all lanes (Fig.3.5A and B, middle panels).
Figure 3.5- LPS induced MAP Kinase phosphorylation is downregulated in SHIP\(^{-/-}\) macrophages. BMM from SHIP\(^{+/+}\) and SHIP\(^{-/-}\) animals were plated in 6 well plates, serum starved overnight, and then stimulated with 500ng/ml LPS for the time points indicated in the figure. Whole cell lysates were analyzed by Western blotting with A. phospho-Erk antibody, and B. phospho-p38 antibody (upper panels). The middle panels are reprobes of the same membrane with Actin antibody. Phosphorylation levels were quantitated by measuring band intensities in the upper panels and normalizing these values to the Actin signals in the respective lanes. The histograms indicate fold increase of phosphorylation over resting samples in SHIP\(^{+/+}\) BMM. These data are representative of four independent experiments.
In parallel experiments to confirm that the SHIP\(^{+/+}\) cells were indeed deficient in SHIP expression, whole cell lysates were probed with anti-SHIP antibody (Figure 3.6A). To ensure that the signaling differences observed in the SHIP\(^{+/+}\) and SHIP\(^{-/-}\) cells were not due to a difference in the expression of TLR4, TLR4 immunoprecipitates were probed with anti-TLR4 antibody (Fig.3.6B). These results indicate that the influence of SHIP on TLR4-induced signaling occurs upstream of Erk and p38.

**Akt phosphorylation is enhanced in SHIP\(^{-/-}\) BMM.**

Recent studies indicated that Akt activation leads to a downregulation of LPS-induced MAP Kinase activation (Guha and Mackman, 2002b). Overexpression of a dominant-negative Akt in these studies lead to enhanced transcriptional activity driven by NF\(\kappa\)B, AP-1 and Egr-1. To test whether the suppression of MAP Kinase phosphorylation in the SHIP\(^{-/-}\) macrophages is accompanied by enhanced Akt activation, serine phosphorylation of Akt was compared in SHIP\(^{+/+}\) and SHIP\(^{-/-}\) BMM stimulated with LPS. The results shown in Figure 3.7 indicate that Akt phosphorylation is induced by LPS in SHIP\(^{+/+}\) BMM by about 15 minutes post stimulation. In contrast, Akt phosphorylation in SHIP\(^{-/-}\) BMM is constitutively high, and remains enhanced post LPS stimulation. These results suggest that SHIP may positively regulate LPS-induced MAP Kinase activation by suppressing the activation of Akt.

To test whether inhibition of Akt in SHIP\(^{-/-}\) BMM would restore LPS-induced
Figure 3.6: TLR4 expression is not altered in SHIP−/− macrophages. A. Whole cell lysates from SHIP+/+ and SHIP−/− cells were probed with anti-SHIP antibody. B. TLR4 immunoprecipitates were probed with anti-TLR4 antibody. These data are representative of four independent experiments.
Figure 3.7: Akt phosphorylation is enhanced in SHIP\(^{+/−}\) BMM. SHIP\(^{+/+}\) and SHIP\(^{−/−}\) BMM were stimulated with 500ng/ml LPS. Protein matched lysates were separated SDS/PAGE and analyzed by Western blotting with antibodies specific for serine phosphorylated Akt (upper panel). The middle panel is a reprobe of the same membrane with actin antibody. These data are representative of four independent experiments. Phosphorylation levels were quantitated by measuring band intensities in the upper panels and normalizing these values to the actin signals in the respective lanes. The histograms indicate fold increase of phosphorylation over resting samples in SHIP\(^{+/+}\) BMM.
MAP Kinase phosphorylation SHIP−/−, BMM were treated with the PtdIns3K inhibitor wortmannin (100nM) for one hour prior to stimulation with LPS. The results shown in Figure 3.8 indicate that indeed MAP Kinase phosphorylation in restored in SHIP+/− BMM treated with wortmannin. Reprobes of the same membranes with antibodies to phospho-Serine Akt confirmed that Akt phosphorylation was suppressed with wortmannin treatment. These data further support the notion that the PtdIn3-Kinase/Akt pathway negatively regulates LPS-induced MAP Kinase activation.

**LPS-induced inflammatory cytokine production is downregulated in SHIP-deficient macrophages.**

To address whether the suppression of LPS-induced signaling events in SHIP−/− BMM is reflected in functional outcomes, we next assessed cytokine production induced by LPS. For this, SHIP+/+ and SHIP−/− BMM were stimulated for varying time points with 500ng/ml LPS. Cell supernatants were harvested and analyzed for TNFα by ELISA. Results indicated that TNFα production was suppressed significantly in SHIP−/− BMM compared to SHIP+/+ BMM (Fig.3.9A). Parallel experiments revealed that LPS-induced IL-6 production was also significantly lower in SHIP−/− macrophages than in SHIP+/+ macrophages (Fig.3.9B). These results are consistent with our above observations that SHIP serves to positively regulate TLR4 function.
Figure 3.8: Inhibition of Akt restores MAPK activation in SHIP\(^{-/-}\) BMM. SHIP\(^{+/+}\) and SHIP\(^{-/-}\) BMM were incubated for 1 hour with either DMSO or Wortmannin (100nM), prior to stimulation with 500ng/ml LPS for the time points indicated in the Figure. Protein-matched lysates were analyzed by first Western blotting with phospho-Erk antibody. The same membranes were reprobed with antibodies to Actin, phospho-Serine Akt, and SHIP.
Figure 3.9: LPS induced TNFα and IL-6 production is suppressed in SHIP\textsuperscript{-/-} macrophages. BMM from SHIP\textsuperscript{++} and SHIP\textsuperscript{-/-} animals were stimulated for the time points indicated in the figure with 500ng/ml LPS. The amount of TNFα and IL-6 in the supernatants were measured by ELISA. A. The graph represents the mean and SD from three independent experiments. B. The graph represents IL-6 production from three independent experiments. Data were analyzed by a paired Student’s \textit{t}-test.
SHIP is a negative regulator of FcγR-mediated signaling.

The above findings are in contrast to previous reports demonstrating a negative regulatory role for SHIP in immune receptor and growth factor receptor signaling (Krystal, 2000; Nakamura et al., 2002; Tridandapani et al., 1997a; Tridandapani et al., 2002b; Huber et al., 1998a; Ono et al., 1996). Thus, in order to test whether the macrophages used in our experiments would respond differently to other stimuli, we stimulated the cells by clustering Fcγ receptors. To cluster FcγR, BMM were first incubated with mAb 2.4G2 anti murine FcγRII/III, followed by crosslinking with mouse F(ab’)2 anti-rat IgG secondary antibody, and the resultant Erk phosphorylation was analyzed. Consistent with other reports (Helgason et al., 2000), results indicated that Erk phosphorylation is higher in SHIP−/− macrophages in comparison to SHIP+/+ macrophages (Fig.3.10). Both wild type and knockout macrophages showed equivalent FcγR expression as determined by flow cytometry using mAb2.4G2 by methods described previously (Fig.3.11) (Tridandapani et al., 2002b). These findings indicate that the role of SHIP as a positive or negative regulator of signaling events is stimulus-specific.
**Figure 3.10:** SHIP negatively regulates FcγR-induced Erk phosphorylation. SHIP\(^{+/+}\) and SHIP\(^{-/-}\) macrophages were activated by clustering FcγRII/III using mAb 2.4G2 followed by a secondary goat F(ab')2 anti-rat IgG antibody. Whole cell lysates were probed first with phospho-Erk antibody (*upper panel*), and second with Actin antibody (*middle panel*). Phosphorylation levels were quantitated by measuring band intensities in the upper panels and normalizing these values to the Actin signals in the respective lanes. The histograms indicate fold increase of phosphorylation over resting samples. These data are representative of four independent experiments.
Figure 3.11: FcγR expression in SHIP^{+/+} and SHIP^{−/−} BMMs. FcγR expression on the SHIP^{+/+} and SHIP^{−/−} BMMs was analyzed by flow cytometry. For this, the cells were labeled with anti-FcγRII/III mAb 2.4G2 followed by FITC-labeled goat anti-rat IgG secondary antibody (solid line). Cells were also labeled with secondary antibody alone (dashed line).
Discussion

LPS-induced macrophage inflammatory response involves the activation of the MAP Kinases Erk, p38 and JNK (Guha and Mackman, 2001b). The role of PtdIns3K in LPS signaling is somewhat controversial. While there are several reports demonstrating a positive role for PtdIns3K, there are several others that demonstrate a negative role for PtdIns3K in LPS-induced inflammatory responses [reviewed in (Fukao and Koyasu, 2003)]. Our current findings support a negative regulatory role for PtdInd3 kinase.

PtdIns3K is reported to suppress the LPS-induced inflammatory response through the activation of Akt (Guha and Mackman, 2002b). Akt is a serine/threonine kinase that is activated in a PtdIns3,4,5P$_3$-dependent manner (Franke et al., 1997) and appears to exert its inhibitory influence on LPS-induced activation events at two different levels (Figure 3.12). First, Akt activation leads to the suppression of MAP Kinase activation. Thus, Akt has been shown to phosphorylate and inactivate MEKK3 resulting in the suppression of p38 (Blum et al., 2001; Gratton et al., 2001). Other studies indicate that Akt suppresses Erk by inactivating the upstream Raf-1 kinase (Rommel et al., 1999). A second mechanism of inhibition by Akt is reported to occur through the phosphorylation and inactivation of GSK-3β, resulting in downregulation of NFκB transcriptional activity (Guha and Mackman, 2002b). Our current observations that in SHIP$^{-/-}$ BMM, where Akt activation is constitutively enhanced, LPS-induced MAP Kinase phosphorylation and inflammatory cytokine production are downregulated support these previous reports.
Figure 3.12: Proposed model for role of SHIP in TLR4 signaling. LPS stimulation induces translocation of SHIP to membrane lipid rafts. SHIP enhances LPS induced activation of MAP Kinases, NFκB, and the downstream cytokine production, at least in part by downregulating Akt.
Our study demonstrates that SHIP associates with membrane lipid rafts after LPS stimulation. SHIP is a cytosolic enzyme that translocates to the membrane to hydrolyze its membrane-associated lipid substrates. Work from several groups indicated that during immune receptor and growth factor receptor signaling the movement of SHIP to the membrane is facilitated by its association with membrane-associated receptors, either directly or indirectly through adapter molecules (Baran et al., 2003; Nakamura et al., 2002; Tridandapani et al., 1997c; Tridandapani et al., 2002b). In these studies the SH2 domain of SHIP was shown to be necessary for SHIP association with Fcγ receptors. Other studies have indicated an essential role for the carboxy-terminal region of SHIP in membrane association and function of SHIP (Aman et al., 2000; Damen et al., 2001). Our findings that overexpression of SHIP SH2 domain had no influence on LPS-induced NFκB activation suggests that the SH2 domain may not be involved in membrane translocation of SHIP during TLR4 signaling (Fig.3.4). The same SHIP SH2 domain construct has been shown by our group and others to function in a dominant-negative manner in influencing FcyR-mediated function. In the latter case the SH2 domain is critical for membrane translocation of SHIP. Further studies are needed to understand the molecular mechanism of SHIP translocation to the membrane in response to LPS stimulation.

We, and others, have previously reported a negative regulatory role for SHIP in the activation of NFκB and the Ras/Erk pathway by immune receptors and growth factor receptors (Baran et al., 2003; Kalesnikoff et al., 2002b; Tamir et al., 2000; Tridandapani et al., 1998; Tridandapani et al., 2002b). Paradoxically, our current findings show that
SHIP positively regulates MAP Kinase and NFκB activation during LPS signaling.

Indeed, a positive role for SHIP has been previously reported by Rothman and colleagues in 32D myeloid cells stimulated with IL-4 (Giallourakis et al., 2000). These investigators found that IL-4 induced proliferative response is enhanced in the presence of SHIP. Thus it would seem that the role of SHIP as a negative or a positive regulator of signaling events is stimulus-specific, and likely involves as yet unidentified downstream targets that are distinct for the various stimuli.

In summary, we have investigated the role of the inositol phosphatase SHIP in LPS-mediated activation of macrophages. Our results demonstrate that SHIP serves to positively regulate LPS-induced MAP Kinase activation and inflammatory cytokine production. These results demonstrate a role for inositol phosphatases in LPS-induced inflammatory response, and are consistent with a recent review article by Rauh et al. that proposed a role for SHIP in LPS-induced macrophage responses based on their unpublished observations (Rauh et al., 2003). Also consistent with this we have recently observed that the inositol 3-phosphatase PTEN, which like SHIP consumes PtdIns3,4,5P3 and suppresses Akt activation, promotes LPS-induced signaling in macrophages (Cao et al., 2004). In contrast to the negative regulatory role of inositol phosphatases in immune receptor signaling, our data indicate that SHIP positively regulates TLR4 signaling. Based on our observations, we propose that macrophage inflammatory responses to bacterial lipopolysaccharide are finely tuned by the concerted actions of lipid kinases and phosphatases.
Our current studies also propose that Akt, a downstream effector of PtdIns3K and a target of SHIP, may play a role in modulating LPS-induced inflammation. Thus, in the next chapter we describe our studies addressing the role of Akt in LPS-induced macrophage inflammatory response.
CHAPTER 4

THE SERINE/THREONINE KINASE AKT PROMOTES IL-10 PRODUCTION AND PROTECTS FROM LPS-INDUCED TOXICITY

Abstract

The bacterial endotoxin lipopolysaccharide (LPS) is a potent inducer of the inflammatory response. Previous studies demonstrated that this LPS-induced toxicity is reversed upon FcγR clustering by IgG immune complexes (IC) through upregulation of the anti-inflammatory cytokine IL-10. However, the signaling events that lead to the enhanced production of IL-10 are not known. In this study, we have compared signaling events in macrophages stimulated with either LPS alone, or with a combination of LPS and ICs. Our experiments revealed that while Akt was activated under both conditions, the level of activation was significantly higher in cells stimulated with LPS and ICs.
suggesting that Akt may be involved in IC-induced upregulation of IL-10 production. Using several independent models we have then tested the notion that enhanced Akt activation may lead to enhanced LPS-induced IL-10 production. Over-expression of constitutively active Myr-Akt in the mouse macrophage cell line Raw 264.7 led to significant increase in IL-10 production in response to LPS. In addition, downregulation of Akt by siRNA resulted in a decrease in LPS-induced IL-10 production. Peritoneal macrophages from transgenic mice with macrophage-specific expression of Myr-Akt produced significantly higher levels of IL-10 when stimulated with LPS, compared to their wild-type counterparts. Consistent with this observation, serum levels of IL-10, post *E. coli* challenge, was higher in the Myr-Akt transgenic mice compared to the wild-type mice. Finally, the Myr-Akt transgenic mice displayed a survival advantage over wild-type mice when challenged with lethal doses of *E. coli*. Taken together, these data strongly suggest that Akt activation may enhance IL-10 production in macrophages stimulated with LPS and provide protection from LPS-induced toxicity.
Introduction

Lipopolysaccharide (LPS), a component of the gram negative bacterial cell wall, is one of the major causative agents of gram negative sepsis (Meng and Lowell, 1997). It is a potent inducer of monocytes and macrophages, which are key mediators of the innate immune response. Stimulation of these cells by LPS leads to a cascade of intracellular signaling events that ultimately result in production and secretion of cytokines and other inflammatory mediators that constitute the pro-inflammatory response. While the pro-inflammatory response is crucial for effective clearance of the pathogen, the inflammatory mediators produced cause tissue damage and inflammation. Hence, a balance needs to be maintained between the activation and down-regulation of this response in order to avoid severe tissue damage (Cohen, 2002c). Dysregulation of this response could lead to potentially lethal conditions like septic shock and systemic inflammatory response syndrome (SIRS).

LPS induces the production of pro-inflammatory mediators through the CD14-TLR4-MD-2 complex (Bochud and Calandra, 2003). Binding of LPS to this complex leads to homodimerization of TLR4 and association of adapter proteins MyD88 and Mal with TLR4. This is followed by recruitment and activation of IL-1R-associated kinase-1 (IRAK-1). Signaling events distal to the receptor include activation of MAPKs- Erk 1/2, p38 and JNK, and transcription factors- NFκB and AP-1, which promote gene transcription (Guha and Mackman, 2001a; Fujihara et al., 2003). While the signaling cascade that mediate these events are rather well characterized, the down-regulation of these events are not fully understood.
IL-10 is a potent anti-inflammatory cytokine produced by monocytes, macrophages and lymphocytes. Tumor necrosis factor α (TNFα), IL-12, IL-1 and IL-6 are the major pro-inflammatory cytokines produced by monocytes and macrophages in response to LPS. IL-10 has been shown to inhibit the expression of these pro-inflammatory cytokines and thereby limit the inflammatory response (Grutz, 2004b). Fiorentino et al demonstrated that IL-10 inhibited the production of TNFα, IL-1 and IL-6 in macrophages stimulated by LPS (Fiorentino et al., 1991b). Similarly, IL-10 deficient mice display an elevated Th1 response and develop chronic enterocolitis and are hypersensitive to LPS (Takakura et al., 2002). Pajkrt et al have demonstrated that administration of IL-10 led to a diminished inflammatory cytokine response in human models of endotoxemia (Pajkrt et al., 1997). IL-10 administration also decreases TNFα secretion in vivo and can protect from LPS-induced lethality in a murine model of septic shock (Howard et al., 1993a; Gerard et al., 1993b).

FcγR ligation by IgG-immune complexes (ICs) has been shown to regulate LPS-induced cytokine production by macrophages (Sutterwala et al., 1997a; Sutterwala et al., 1998). For example, work by Gerber and Mosser demonstrated that FcγR ligation leads to reversal of the toxic effects of LPS. Specifically, co-stimulation with LPS and ICs was shown to result in a dramatic increase in IL-10 production and diminished levels of the pro-inflammatory cytokine IL-12, in murine bone-marrow-derived macrophages (BMMs). Increased IL-10 production was a specific response to FcγR ligation, as ligation of complement receptors did not result in a similar effect. Finally, in an elegant in vivo model, these investigators demonstrated that, mice receiving macrophages primed in the
presence of LPS and ICs prior to LPS administration, displayed a survival advantage over those that received LPS alone due to enhanced production of IL-10 (Gerber and Mosser, 2001b).

Phosphatidylinositol 3-kinase (PtdIns3K) is an important enzyme, activated in response to LPS stimulation (Lee et al., 2000; Pahan et al., 1999; Park et al., 1997), however its role in the regulation of LPS-mediated inflammation is controversial. While some reports indicate that PtdIns3K serves to up-regulate the inflammatory response, others have demonstrated that it may actually play a negative role and thereby diminish LPS-induced inflammation. A recent study by Guha and Mackman indicated that inhibition of the PtdIns3K-pathway resulted in an increase in LPS-induced TNFα and Tissue Factor (TF) gene expression (Guha and Mackman, 2002a). In a separate study, Mackman and colleagues have shown that the PtdIns3K-Akt pathway suppresses LPS-induced inflammation and coagulation in endotoxemic mice (Schabbauer et al., 2004). PtdIns3K activity results in production of PtdIns3,4,5P3, a crucial second messenger in cell signaling. Accumulation of PtdIns3,4,5P3 leads to recruitment of PH-domain containing enzymes that serve to activate Akt/PKB, which is one of the best characterized downstream effectors of PtdIns3K (Toker, 2000; Rameh and Cantley, 1999; Scheid and Woodgett, 2003b). Recent studies by Bommhardt et al have demonstrated that activation of Akt leads to a decrease in lymphocyte apoptosis and improved survival in a cecal ligation and puncture (CLP) model of sepsis (Bommhardt et al., 2004b). These studies together suggest a role for Akt in LPS-induced inflammation.
In this study we have analyzed the role of Akt in LPS-induced signaling in macrophages. We first investigated the signaling events that mediate up-regulation of IL-10 production in macrophages co-stimulated with LPS and ICs. Our results indicated that Akt activity was significantly higher in cells that were co-stimulated compared to cells stimulated with LPS alone. This suggested a role for Akt in IL-10 production in response to LPS. We have used several independent models to test this hypothesis. First, using transient transfection, we demonstrate that Raw 264.7 macrophages over-expressing a constitutively active form of Akt (Myr-Akt) but not a catalytically inactive CAAX-Akt, produce significantly higher levels of IL-10 in response to LPS. Second, using specific siRNA to down-regulate expression of Akt, we showed that macrophages stimulated with LPS produce lower levels of IL-10 in comparison to macrophages that received non-specific control siRNA. Third, peritoneal macrophages from transgenic mice having macrophage-specific expression of Myr-Akt produced significantly higher levels of IL-10 in response to LPS compared to macrophages obtained from their wild-type counterparts. Consistent with these findings serum levels of IL-10 post E. coli challenge were higher in the Myr-Akt transgenic mice in comparison to wild-type mice. Finally, the Myr-Akt transgenic mice displayed a survival advantage over wild-type mice when challenged with lethal doses of E. coli. Taken together, our data suggest that, Akt mediates an increase in IL-10 production on LPS challenge and provides protection from potentially lethal gram negative infections.
Materials and Methods

Cells, antibodies and reagents:

Raw 264.7 murine macrophage cells were obtained from ATCC (Manassas, VA) and maintained in RPMI with 3.5% heat-inactivated fetal bovine serum. Erk and Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Phospho-Serine Akt antibody, from Cell Signaling Technology (Beverly, MA). LPS from Escherichia coli strain 0127:B8 was obtained from Difco (Detroit, MI). Akt siRNA was obtained from Ambion Inc (Austin, TX). Non-specific Control Duplexes XIII (pool of 4) was obtained from Dharmacon (Lafayette, CO). Chromopure Mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA). Plasmids encoding Myr-Akt and CAAX-Akt were kindly provided by Dr. D. Stokoe (UCSF, CA) and Dr. Burgering (Utrecht University, Netherlands) respectively.

Preparation of heat-aggregated IgG:

Heat aggregated IgG was prepared according to methods described previously (Ptak et al., 1998). In brief Chromopure mouse IgG was heated at 62°C for 30 minutes, immediately cooled on ice and used at a final concentration of 750 μg /ml to stimulate the cells.

Akt in vitro kinase assay:

Kinase assays were performed using the Akt Kinase Assay kit from Cell Signaling (Beverly, MA). Briefly, cells were lysed for 10 minutes in ice-cold lysis buffer (20mM...
Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 10 μg/ml leupeptin, and 1 mM PMSF). Nuclear material was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C. The amount of protein in the lysates was estimated using the DC Protein Assay kit from Biorad (Hercules, CA). Equal amount of the protein extracts were immunoprecipitated with Akt antibody and the appropriate control antibody. The immune complexes bound to beads were washed two times each with lysis buffer and kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2] and subsequently incubated with 40 μl of Kinase buffer supplemented with 20 μM ATP and 1 μg of GSK-3 fusion protein as a substrate. Reaction was carried out for 30 minutes at 30°C as per the manufacturers’ protocol. The reaction was terminated by boiling in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% β-mercaptoethanol) for 5 minutes. 12% SDS–PAGE was used to separate the proteins which were then transferred to nitrocellulose filters, probed with phospho-GSK-3 antibody and developed by enhanced chemiluminescence.

**Generation of Myr-Akt transgenic mice:**

Transgenic mice with macrophage-specific expression were generated by methods previously described (Datta et al., 1999a). The Myr-Akt-HA plasmid used for this purpose was a kind gift from Dr. P. Tsichlis (Thomas Jefferson University, PA). The fms-Myr-Akt plasmid was constructed such that the GFP cassette between Apa I and Not I
site of 6.7fmsGFP plasmid described previously was replaced by Myr-Akt-HA. The fms-
Myr-Akt was linearized with Sal I and Pvu I, and transgenic mice were generated in the
OSU Comprehensive Cancer Center transgenic facility, Columbus, Ohio. Transgenic
mice were identified by Southern blots with Akt1 cDNA as a probe, and Western blots
with anti-HA antibody. Subsequent transgenic offspring were identified by PCR with
three primers (Forward primer for the transgene Myr-Akt:
5’-CCAAAGCATGGTCCCAGTGTTGGG-3’, common reverse primer:
5’- GAAGTAGCGTGGCCGCCAGG-3’, and forward primer for endogenous Akt:
5’-AGCGGTGGCACCTCCTGGG-3’).

Isolation of peritoneal macrophages:
Peritoneal macrophages were isolated as previously described (Conrad et al., 1977).
Briefly, peritoneal macrophages were induced by i.p injection of 1ml 2.9% Brewer’s
complete thioglycolate broth. Macrophages were harvested 5 days post injection by
peritoneal lavage using PBS. Cells were plated in DMEM with 10% Fetal Bovine Serum.
Adherent cells were used in experiments.

Transfection:
Raw 264.7 cells were transfected using the Amaza Nucleofector apparatus (Amaza
Biosystems, Cologne, Germany). Briefly, 6 x 10^6 cells were resuspended in 100 μl of
Cell Line Nucleofector Solution V (Amaza Biosystems) and were nucleofected with 6 μg
of plasmid DNA or 8 μM siRNA. Post nucleofection, 500 μl of prewarmed RPMI 1640
was added to the transfection mix, and cells were transferred to 12-well plates containing 1.5 ml of prewarmed RPMI 1640 per well. Cells were incubated for 24 hours at 37°C and then used in experiments.

**Cell stimulation, lysis and Western blotting:**
Raw 264.7 cells were stimulated with 500 ng/ml LPS alone, or co-stimulated with 500 ng/ml LPS and heat-aggregated IgG. Peritoneal macrophages were activated with 500 ng/ml LPS. Cells were lysed in TN1 buffer (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na\textsubscript{3}VO\textsubscript{4}, 10 g/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.

**Western blot data quantitation:**
A densitometry program (Scion Image, Scion, Frederick, MD) was used to quantitate the ECL signal. The background was subtracted, and the signal normalized to the amount of Erk or total target protein. The values were plotted as fold increase over mock, control and wild-type samples.

**ELISA determination of cytokine production:**
Cells were stimulated with 500 ng/ml LPS alone or with 500 ng/ml LPS and heat-aggregated IgG. Dead cells were removed from the cell supernatants by centrifugation
and IL-10 was measured by ELISA using kits R&D Systems (Minneapolis, MN). Data were analyzed using a paired Student’s t test.

**Measurement of serum cytokines:**

*E. coli* BL21 (DE3) [a kind gift from Dr. M. D. Wewers, Ohio State University. OH] was cultured overnight in Luria Bertani Medium (Sigma Aldrich, MO) and harvested by centrifugation at 13,000 rpm for 5 minutes. The cells were washed 3x with PBS to clear the bacterial medium and resuspended in sterile PBS. Age-matched mice were injected intraperitoneally (i.p.) with live *E. coli* (5 x 10^8 cfu in 200 μl PBS). 5 hours after the injection the mice were sacrificed and blood was collected from the posterior vena cava (Hoff, 2000). To obtain serum, blood was centrifuged at 13,000 rpm for 10 minutes at 4°C. IL-10 levels were measured using ELISA kits from R&D Systems. In independent experiments mice were challenged i.p with LPS (5mg/kg). Five hours following injection, serum was obtained and levels of cytokines (IL-10, TNFα, IL-1b and IL-6) were analyzed by ELISA. All mouse experiments were performed with ILACUC approved protocols.

**Survival Assays:**

*E. coli* inoculum was prepared as mentioned above. Age-matched mice were injected i.p. with *E. coli* BL21 (DE3) [5 x 10^8 and 3 x 10^8 cfu/200μl] and returned to their cages. Control mice were injected with 200 μl sterile PBS. Time of death was recorded for mice injected with *E. coli*. Survival curves were plotted using Sigma Plot software.
Statistical Analysis:

Kaplan-Meier survival curves were generated for each experiment, using Sigma Plot software. We tested for differences in survival between groups using the log-rank test to compute the $p$-values. Data were analyzed to determine the median survival times for the two genotypes.
Results

*Immune Complexes (ICs) promote LPS-induced IL-10 production and augment Akt activation*

Previous studies have shown that ligation of FcγRs by ICs induced an increase in IL-10 production in bone marrow-derived macrophages stimulated with LPS (Gerber and Mosser, 2001a; Sutterwala et al., 1997b; Sutterwala et al., 1998). However, the signaling events leading to the increased IL-10 production are not known. In order to explore the latter, we used the mouse macrophage cell line Raw 264.7. These cells were stimulated with either LPS alone or with ICs in combination with LPS, for varying time points, and the level of IL-10 in the supernatants was measured by ELISA. Macrophages co-stimulated with ICs and LPS produced significantly higher levels of IL-10 compared to cells stimulated with LPS alone (Fig.4.1).

Having ensured that the model used here is consistent with the previously reported studies, we next investigated the signaling events that mediate the up-regulation of IL-10. We examined a number of signaling molecules known to be activated by FcγR ligation as well as LPS stimulation including, MAPKs- Erk 1/2, p38 and JNK and the serine-threonine kinase, Akt (Rose et al., 1997; Ganesan et al., 2004a; Ganesan et al., 2003). To analyze the effect of co-stimulation on MAPK activation, Raw 264.7 cells were stimulated with LPS alone or IC+LPS for the time-points indicated and protein-matched lysates were separated by Western blotting with appropriate phospho- antibodies to determine MAPK phosphorylation. The results indicated that, there was no significant
Figure 4.1: Immune Complexes (ICs) promote LPS-induced IL-10 production in Raw 264.7 cells. Raw 264.7 cells were stimulated with LPS (500 ng/ml) alone or in combination with heat-aggregated IgG (IC+LPS) for the time points indicated. Supernatants were harvested and analyzed for IL-10 production by ELISA. These data are representative of four independent experiments.
difference in phosphorylation level of the three MAPK’s- Erk 1/2 (Fig.4.2), p38 (Fig.4.3) and JNK (Fig.4.4).

We next examined the effect of co-stimulation on Akt activity in Raw 264.7 cells. Interestingly, our experiments revealed that while Akt was activated under both conditions, the level of activation in cells co-stimulated with ICs and LPS was significantly higher compared to the level of activation in cells stimulated with LPS alone (Fig.4.5). In these experiments, Raw 264.7 cells were stimulated for the time points indicated in the figure, cells were lysed and Akt was immunoprecipitated from protein-matched lysates. The immunoprecipitates were used in an in vitro kinase assay and Akt activity was measured by its ability to phosphorylate GSK-3, a known substrate of Akt. The samples were separated by SDS-PAGE and analyzed by Western blotting using an antibody specific for phosphorylated GSK-3. Akt activity was more robust in cells co-stimulated with ICs + LPS (Fig. 4.5, third panel) compared to cells stimulated with LPS alone (Fig. 4.5, first panel). The membranes were reprobed with anti-Akt antibody to ensure equal loading (Fig.4.5, second and fourth panel). The fold induction in Akt activity in co-stimulated cells was plotted over time and compared to fold induction in Akt activity seen in cells stimulated with LPS alone (Fig. 4.5B, bottom panel). The graph represents the mean and SD of values obtained from three independent experiments. These results suggest a role for Akt in mediating enhanced IL-10 production on FcγR ligation in macrophages stimulated with LPS.
Figure 4.2: Immune Complexes (ICs) have no effect on LPS-induced Erk activation. Raw 264.7 cells were stimulated with LPS (500 ng/ml) or IC+LPS. Whole cell lysates (WCL) were analyzed by Western blotting with antibodies specific for phospho-Erk (*top panel*). Membranes were reprobed with antibody specific for actin (*lower panel*).
Figure 4.3: Immune Complexes (ICs) have no effect on LPS-induced p38 activation. Raw 264.7 cells were stimulated with LPS (500 ng/ml) or IC+LPS. Whole cell lysates (WCL) were analyzed by Western blotting with antibodies specific for phospho-p38 (top panel). Membranes were reprobed with antibody specific for actin (lower panel).
Figure 4.4: Immune Complexes (ICs) have no effect on LPS-induced JNK activation. Raw 264.7 cells were stimulated with LPS (500 ng/ml) or IC+LPS. Whole cell lysates (WCL) were analyzed by Western blotting with antibodies specific for phospho-JNK (*top panel*). Membranes were reprobed with antibody specific for actin (*lower panel*).
Figure 4.5: Immune Complexes (ICs) promote LPS-induced Akt activation. Raw 264.7 cells were stimulated with LPS or IC+LPS. Whole cell lysates (WCL) were used to immunoprecipitate Akt. Immunoprecipitates were subjected to in vitro kinase assay using GSK-3 as substrate. GSK-3 phosphorylation was assessed using anti-phospho-GSK-3 antibody (first and third panels). Membranes were reprobed with antibody specific for Akt (second and fourth panels). IgH represents the heavy chain of the immunoprecipitating antibody. The graph (bottom panel) represents fold induction of Akt activity seen in first and third panels. Levels of GSK 3 phosphorylation were normalized to Akt in each lane.
Constitutively active Akt, but not dominant negative Akt, promotes LPS-induced IL-10 production

As our results above indicated that stimulation with ICs and LPS promotes IL-10 production and augments Akt activity, we next wanted to address whether Akt was involved in promoting IL-10 production in cells stimulated with LPS. As a first approach, we used an over-expression system in which Raw 264.7 cells were transiently transfected with vector alone or plasmids encoding Myr-Akt or CAAX-Akt. Myr-Akt is a constitutively active form of Akt which is myristoylated and thereby anchored in the membrane (Stokoe et al., 1997). CAAX-Akt is also anchored in the membrane due to the CAAX motif, which is the membrane targeting signal of Ki-Ras, but is a functionally inactive form of Akt (van Weeren et al., 1998). High-efficiency transfection of Raw 264.7 cells was achieved using the Amaza Nucleofector. Raw 264.7 cells were transfected (Program: U-14; Solution: V) with 5 μg of a plasmid encoding EGFP. We were able to achieve 70-80% transfection efficiency as seen in the fluorescence image of GFP-transfected cells (Fig.4.6). To examine the role of Akt in promoting IL-10 production, Raw 264.7 cells transfected with the Akt constructs were stimulated with LPS for time points indicated (Fig.4.7A) and cell supernatants were collected and the level of IL-10 was assessed by ELISA. Cells expressing Myr-Akt produced significantly higher levels of IL-10 compared to CAAX-Akt expressing cells. In order to confirm over-expression of the transfected molecules, lysates from these cells were separated by SDS-PAGE and analyzed by Western blotting using an antibody specific for Akt (Fig.4.7B). The upper band represents the over-expressed transfected Akt, while the lower band is
Figure 4.6: High-efficiency transfection of Raw 264.7 cells. Raw 264.7 cells transfected with 5μg EGFP using the Amaxa Biosystems Nucleofector (Program:U-14; Solution:V).
**Figure 4.7:** Constitutively active Akt promotes LPS-induced IL-10 production in transfected Raw 264.7 cells. 

**A.** Raw 264.7 cells were transiently transfected with 6 μg of plasmid DNA as indicated (Either vector alone, Myr-Akt or CAAX-Akt) followed by stimulation with LPS (500 ng/ml) for varying time points. Amount of IL-10 in the supernatants was determined by ELISA. **B.** Presence of endogenous and transfected Akt was analyzed by Western blotting lysates with Anti-Akt antibody. These data are representative of three independent experiments.
the endogenous Akt. These results suggested that enhanced Akt activity can potentiate increased IL-10 production in macrophages stimulated with LPS.

**Downregulation of Akt expression leads to decrease in LPS-induced IL-10 production**

The above results indicated that over-expression of constitutively active Akt promoted LPS-induced IL-10 production. As an alternate approach to confirm the involvement of Akt in mediating enhanced IL-10 production by cells stimulated with LPS, Akt expression was downregulated using siRNA. For this analysis, Raw 264.7 cells were transiently transfected with siRNA specific for Akt or non-specific control siRNA. Cells were cultured for 24 hours followed by stimulation with LPS for 8 hours. The level of IL-10 in the supernatants was measured by ELISA. IL-10 production by cells with siRNA-mediated Akt depletion was significantly lower in comparison to cells expressing non-specific control siRNA (Fig.4.8A). In order to confirm the efficacy of the siRNA, protein-matched lysates from the above samples were separated by SDS-PAGE and analyzed by Western blotting with anti-Akt antibody (Fig.4.8B upper panel). The same membrane was reprobed with an antibody specific for Erk to ensure specificity of the siRNA (Fig.4.8B middle panel). Cells transfected with Akt-siRNA showed approximately 40% reduction in IL-10 production compared to cells transfected with non-specific siRNA. This change was similar to the 30% reduction in Akt protein expression seen in Figure 4.8B (lower panel).
Figure 4.8: Cells with reduced Akt expression produce reduced levels of IL-10. Raw 264.7 cells were transiently transfected with siRNA specific for Akt or non-specific control duplexes. Post-transfection, the cells were cultured for 24 hours. Cells were then stimulated with LPS (500 ng/ml) for 8 hours. A. IL-10 levels were measured by ELISA. The black bar indicates IL-10 production in cells transfected with control siRNA and the white bars represent IL-10 production in cells transfected with Akt siRNA (p-value=0.02). B. To ensure down-regulation of Akt protein and specificity of siRNA, lysates were analyzed by Western blotting using an antibody specific for Akt (upper panel) and then reprobed with Anti-Erk antibody (lower panel). C. Akt protein level was normalized to Erk level in each lane and plotted as % Akt expression. All data represent mean of three independent experiments.
Myr-Akt expressing murine macrophages produce more IL-10 when stimulated with LPS

Our results in Raw 264.7 cells suggest a role for Akt in enhancing LPS-induced IL-10 production. In order to confirm the above findings in primary cells, we utilized mice that are transgenic for macrophage-specific expression of Myr-Akt (Sasmono et al., 2003). Thioglycollate-elicited peritoneal macrophages from wild type and Myr-Akt expressing mice were isolated and adherent cells were stimulated with LPS for 8 hours or left unstimulated. Cell culture supernatants were harvested and analyzed for IL-10 production. Myr-Akt expressing macrophages stimulated with LPS produced significantly more IL-10 than wild type macrophages (Fig.4.9A). Interestingly, the presence of Myr-Akt alone was not sufficient to induce IL-10 production as indicated by the level of IL-10 in the untreated macrophages, suggesting that additional molecules activated by LPS stimulation are required in this process.

To confirm presence of Myr-Akt, protein-matched lysates from these cells were separated by SDS-PAGE and analyzed by Western blotting using an antibody specific for phospho-Ser Akt. Myr-Akt macrophages showed presence of Myr-Akt in addition to wild type Akt (Fig.4.9B). To address the possibility that the enhanced production of IL-10 may be due to enhanced TLR4 expression in the Myr-Akt macrophages, TLR4 expression was examined by Western blot analysis. Here, TLR4 was immunoprecipitated using anti-TLR4 antibody and subsequently Western blotted with an antibody specific for TLR4 (Fig.4.10). Results indicated that both wild-type and Myr-Akt expressing peritoneal macrophages expressed equivalent levels of TLR4. These results strongly
Figure 4.9: Myr-Akt expressing macrophages stimulated with LPS make significantly more IL-10. Peritoneal macrophages from wild-type and Myr-Akt expressing mice were stimulated with LPS (500 ng/ml) for 8 hours. A. Level of IL-10 in cells from wild-type and Myr-Akt mice. The white bars indicate the amount of IL-10 produced by unstimulated cells and the black bars indicate amount of IL-10 produced by LPS-stimulated cells. B. Protein-matched lysates were analyzed by Western blot using an antibody specific for phospho-Serine Akt.
**Figure 4.10:** TLR4 expression is unchanged in wild type vs Myr-Akt expressing mice. TLR4 was immunoprecipitated from macrophage whole cell lysates from wild type and Myr-Akt expressing mice. The immunoprecipitates were then analyzed by Western blotting with anti-TLR4 antibody.
suggest that, enhanced IL-10 production is due to enhanced expression of constitutively active Akt.

**Myr-Akt expressing mice have higher serum IL-10 levels post E. coli challenge.**

Our results, both in Raw 264.7 cells and primary macrophages, strongly suggested that Akt is responsible for mediating up-regulation of IL-10 production on LPS stimulation. To examine whether these findings could be extended to an *in vivo* LPS response, wild-type and Myr-Akt mice were challenged with intraperitoneal injection of *E. coli*. Mice were sacrificed 5 hours post challenge and serum levels of IL-10 were analyzed by ELISA. Myr-Akt expressing mice showed significantly higher levels of IL-10 in serum compared to wild-type mice (Fig. 4.11A), further strengthening our hypothesis that Akt mediates enhanced IL-10 production in response to LPS. Analysis of TNFα levels in serum showed no significant difference (Fig. 4.11B).

**Expression of Myr-Akt confers a survival advantage in mice challenged with E. coli.**

The results indicated an *in vivo* function for Akt in IL-10 production post *E. coli* challenge. Administration of IL-10 has been shown to protect from endotoxin-mediated lethality in several murine models of septic shock (Howard et al., 1993b; Gerard et al., 1993a; Santucci et al., 1996; Berg et al., 1995; Smith et al., 1994; Marchant et al., 1994). To determine if Akt-mediated increase in IL-10 production protects from *E. coli* toxicity, we monitored survival in wild type and Myr-Akt expressing mice challenged with two
Figure 4.11: Myr-Akt expressing mice challenged with *E. coli* have higher levels of serum IL-10. A. Wild-type and Myr-Akt expressing mice were injected i.p. with $3 \times 10^8$ cfu live *E. coli*. Five hours post-injection, level of IL-10 in serum was determined by ELISA. Data represent the mean and SD of values obtained from five pairs of animals ($p$-value=0.00116). B. Wild-type and Myr-Akt expressing mice were injected i.p. with $3 \times 10^8$ cfu live *E. coli*. Five hours post-injection, level of TNFα in serum was determined by ELISA. Data represent the mean and SD of values obtained from five pairs of animals ($p$-value=0.352145).
different doses of live *E. coli* in independent experiments (5×10⁸ and 3×10⁸ cfu/mouse). In the first experiment, where mice were challenged with 5×10⁸ cfu of *E. coli*, the median survival time for the wild-type mice was 27 hours, while that for the Myr-Akt mice was 39 hours (Fig.4.12A). Although the Myr-Akt mice displayed a survival advantage over the wild-type mice, the difference in the median survival time was only 12 hours. To determine if this co-related to the dose, we used a lower dose (3×10⁸ cfu of *E. coli*) and monitored survival. The wild-type mice in this experiment had a median survival time of 29 hours while the median survival time for the Myr-Akt-expressing mice increased to 70.5 hours (Fig.4.12B). Taken together our data strongly suggest that activation of Akt in macrophages results in enhanced IL-10 production and confers protection from *E. coli*-induced lethality.

*Analysis of serum cytokines in wild-type and Myr-Akt mice challenged with LPS*

Myr-Akt expressing mice showed significantly higher levels of IL-10 in serum following *E. coli* challenge. To determine the effect of LPS challenge, wild-type and Myr-Akt mice were injected i.p. with LPS (5mg/kg). Five hours post-injection serum was obtained and levels of cytokines were analyzed by ELISA. Results indicated that while serum IL-10 levels were significantly higher in Myr-Akt expressing mice (Fig.4.13), there was no significant difference in the levels of TNFα, IL-1β and IL-6 (Fig.4.14), suggesting that Akt may only effect production of IL-10 and may not influence pro-inflammatory cytokines.
Figure 4.12: Myr-Akt expressing mice challenged with *E. coli* display a survival advantage over wild type littermates. Myr-Akt and wild-type littermates were challenged with two different doses of live *E. coli* in independent experiments. Animals were monitored every 12 hours. Results are expressed as Kaplan-Meier curves. Differences in survival between the two groups, was tested using the log-rank test. **A.** Mice received $5 \times 10^8$ cfu of *E. coli*. Wild-type and Myr-Akt mice had different survival characteristics (log-rank *p*-value = 0.0076). **B.** Mice received $3 \times 10^8$ cfu of *E. coli*. Wild-type and Myr-Akt mice had different survival characteristics (log-rank *p*-value = 0.0273).
Figure 4.13: Myr-Akt expressing mice challenged with LPS have higher levels of serum IL-10. Wild-type and Myr-Akt expressing mice were injected i.p. with LPS (5mg/kg). Five hours post-injection, level of IL-10 in serum was determined by ELISA. Data represent the mean and SD of values obtained from six pairs of animals ($p$-value=0.0382).
Figure 4.14: Wild type and Myr-Akt expressing mice challenged with LPS show no significant difference in levels of pro-inflammatory cytokines in serum. Wild-type and Myr-Akt expressing mice were injected i.p. with LPS (5mg/kg). Five hours post-injection, level of TNFα, IL-1β and IL-6 in serum was determined by ELISA. Data represent the mean and SD of values obtained from six pairs of animals. P values-TNFα= 0.191, IL-1β= 0.104 and IL-6= 0.95.
**Discussion**

LPS induces monocytes and macrophages to produce a vast array of cytokines and inflammatory mediators. While the signaling events that mediate this response have been extensively studied, the molecular mechanisms that serve to down-regulate the inflammatory response have not been well characterized. IL-10 is one among the multitude of cytokines produced on LPS stimulation. It is a potent anti-inflammatory cytokine and its function in limiting the production of pro-inflammatory mediators, and imparting protection from LPS-induced septic shock, has been well documented (Grutz, 2004a). Our findings suggest a novel role for the serine-threonine kinase Akt in enhancing LPS-induced IL-10 production. We further extended these studies to demonstrate that Akt has a protective effect in a murine model of *E. coli*-induced toxicity. Akt is a down-stream effector of PtdIns3K and is activated in a PtdIns3,4,5P$_3$-dependent manner. The role of PtdIns3K in regulation of the inflammatory response has thus far been controversial. PtdIns3K has been shown to be a potent inducer of NFκB-mediated gene transcription (Reddy et al., 2000; Reddy et al., 1997; Madrid et al., 2001; Li et al., 2003). NFκB is one of the major transcription factors involved in production of pro-inflammatory cytokines. However, a recent study by Guha and Mackman indicated that PtdIns3K may play a negative role in LPS-mediated inflammatory response. Several other studies have since lent support to this theory. Veres *et al* postulated that the protective effect of a PARP [poly-(ADP-ribose) polymerase 1] inhibitor tested in a murine model of LPS-induced septic shock was mediated by activation of the PtdIns3K/Akt pathway (Veres et al., 2003). Similarly, several independent studies
suggest that, stimulation of the PtdIns3K/Akt pathway may be an effective approach for preventing or treating sepsis and/or septic shock (Williams et al., 2004b; Martin et al., 2003). Finally, a recent study showed that Akt over-expression in lymphocytes prevents sepsis-induced apoptosis and improves survival in a cecal ligation and puncture model of poly-microbial sepsis (Bommhardt et al., 2004a). In this study, using several independent experimental approaches, we provide evidence that, Akt mediates up-regulation of LPS-induced IL-10 production. Our data support the studies that indicate a role for the PtdIns3K/Akt pathway in improving survival in sepsis, and further, specifically indicating a role for Akt in mediating protection from LPS-induced toxicity by enhancing IL-10 production.

While we show that Akt promotes IL-10 production. However analysis of serum levels of other cytokines in mice challenged with LPS showed that Akt had no significant effect on pro-inflammatory cytokines. Further studies need to be conducted to test the effect of Akt on other anti-inflammatory cytokines. In this study we have examined TLR4-mediated signaling which is predominantly seen in gram negative bacterial infections. Whether Akt plays a similar role in signaling via other TLRs, such as TLR-2 in the case of gram positive infections, could be the subject of further studies. Similarly, the molecular mechanism for Akt-mediated upregulation of IL-10 production remains to be investigated. One possible mechanism is by Akt-mediated inhibition of GSK-3β. GSK-3β phosphorylates and inactivates C/EBPα which co-operates with Sp1, a transcription factor that has been shown to mediate IL-10 gene expression (Fig.4.15)
**Figure 4.15: Proposed model for Akt influence on IL-10.** LPS stimulation of macrophages leads to Akt activation. Akt phosphorylates and inhibits GSK-3β. Akt-mediated inhibition of GSK-3β relieves inhibitory phosphorylation of C/EBPα, which can now co-operate with Sp1 to drive IL-10 gene transcription.
(Ross et al., 1999; Wick et al., 2004; Tone et al., 2000). We have recently shown that Akt promotes FcγR-mediated phagocytosis in murine macrophages through the activation of p70S6K. As Mosser and colleagues have already shown that co-stimulation of FcγRs by ICs and LPS imparts protection from LPS-induced toxicity, it would also be interesting to investigate the role of p70S6K in LPS-mediated signaling.

Severe sepsis and septic shock are often associated with a high mortality rate (Cohen, 2002a). Activation of the PtdIns3K pathway has been suggested as a possible therapeutic approach in the treatment of sepsis (Schabbauer et al., 2004). Our data reveal a novel level of regulation of the signaling events that mediate the inflammatory response. Hence, a better understanding of the molecular events that lead to Akt up-regulation of IL-10 may prove invaluable in the development of effective therapeutic strategies for the treatment of potentially lethal conditions like sepsis.
Macrophages are often described as the front line of host defense. They mature from circulating monocytes and are found in practically every tissue in the body where they participate in a vast array of biological processes (Morrissette et al., 1999). However, it is their role as sentinels of the immune system that has been most extensively studied and characterized. As key mediators of host defense, they exert their signature effects of recognition, ingestion and destruction of invading pathogens. In fulfilling their role as sentinels, they also form a crucial bridge between innate and adaptive immunity (Aderem and Underhill, 1999; Underhill and Ozinsky, 2002). Hence it is not surprising that macrophage function is tightly regulated. An imbalance in these controls could result in a wide array of inflammatory diseases.

Although the role of macrophages as key players in host immunity has been studied for many years, molecular mechanisms that regulate these functions are not fully understood. In this study we have analyzed macrophage inflammatory response initiated by distinct stimuli. Our study has unraveled novel roles for the inositol phosphatases
SHIP-1 and SHIP-2, and their downstream target Akt, in the regulation of macrophage responses induced by FcγR clustering and LPS stimulation.

In the first part of this study we have investigated the regulation of macrophage inflammatory response induced by clustering Fcγ receptors. Fcγ receptors play a crucial role in the phagocytic arm of macrophage function. In addition, recent work has uncovered the significant contribution of Fcγ receptors to the efficacy of antibody therapy for the treatment of cancers (Clynes et al., 2000; Green et al., 2002; Stockmeyer et al., 2001; Clynes et al., 1998). In an elegant study, Clynes et al described the importance of Fc-receptor-dependent mechanisms in the action of cytotoxic antibodies against tumors (Clynes et al., 2000). They utilized mice deficient in the common γ chain and mice deficient in FcγRIIb as models to address the roles of FcγRs in the anti-tumor response. The Fcγ−/− mice lacked the activation receptors, FcγRI and FcγRIII. Administration of the protective antibody, trastuzumab in Fcγ+/+ mice resulted in a 90-96% reduction in tumor mass. However, this protective effect was reduced in Fcγ−/− mice, indicating the importance of activation receptors in the anti-tumor response. In contrast, FcγRIIb−/− mice were significantly more effective than FcγRIIb+/+ mice in tumor clearance following antibody treatment. This study clearly indicates the significant contribution of FcγRs in effective cytotoxic activity of antibodies against tumors and also exemplifies the importance of understanding the signaling events that regulate FcγR-mediated function.

Our study on the role of the inositol phosphatase SHIP-2 in FcγR-mediated signaling reveals a novel mode of regulation of macrophage function mediated by FcγRs
(described in chapter 2). We have demonstrated that SHIP-2 down-regulates the signaling events mediated by the activation receptor FcγRIIa (Pengal et al., 2003). In addition, our data also indicate a role for SHIP-2 in regulating FcγRI-mediated events. Taken together this study has major implications in designing more effective antibody-based therapeutic strategies for the treatment of cancers.

We have also investigated another facet of macrophage inflammatory response, that induced by the bacterial product lipopolysaccharide. Our initial studies on SHIP-2 (described in chapter 2) revealed a possible involvement of inositol phosphatases in LPS-mediated inflammation. The results described in chapter 3 indicate a role for SHIP-1 as a pro-inflammatory molecule. Thus inositol phosphatases regulate both FcγR and TLR4-mediated macrophage responses, but they seem to do so in opposing ways. In addition, we described similar findings with another phosphatase, PTEN (Cao et al., 2004). Consistent with our results, in a recent review Rauh et al reported that peritoneal macrophages derived from SHIP-deficient animals produce significantly lower levels of LPS-induced inflammatory cytokines in comparison to SHIP\(^+/\) animals (Rauh et al., 2003). Further, LPS-induced NO production was also lower in SHIP\(^{-/-}\) macrophages. Taken together, these findings suggest that inositol phosphatases may be mediating their pro-inflammatory effect via their consumption of PtdIns3,4,5P\(_3\), a product of PtdIns3K activity. Rauh et al postulated an elegant model in which they hypothesize that, the enhanced activation of the PtdIns3K pathway seen in SHIP\(^{-/-}\) macrophages may result in hyporesponsiveness/tolerance to LPS stimulation.
Our results described in chapters 2 and 3 suggest that PtdIns3,4,5P3-dependent downstream enzymes may be suppressing the pro-inflammatory response induced by LPS. Consistent with this theory, recent studies have clearly implicated the PtdIns3K/Akt pathway as having anti-inflammatory properties (Bommhardt et al., 2004c; Guha and Mackman, 2002b; Williams et al., 2004a). We have investigated the role of the serine/threonine kinase Akt, a downstream target of inositol phosphatases, in regulating LPS-TLR4-induced inflammation. The TLR4 on the surface of macrophages is a crucial player in innate sensing by macrophages (Means et al., 2000; Medzhitov, 2001). Gram negative bacterial infections are a major cause of sepsis and septic shock (Cohen, 2002b). While the pro-inflammatory response is crucial in activating signaling events to mediate clearance of the pathogen, dysregulation of this response could have a potentially lethal outcome. The high mortality rate associated with septic shock has warranted extensive studies on the signaling events mediated by LPS.

Our studies have unraveled a novel role for Akt in mediating up-regulation of the anti-inflammatory cytokine IL-10. This indicates an anti-inflammatory function for Akt. Administration of IL-10 has been shown to provide protection from LPS-induced lethality (Fiorentino et al., 1991a; Howard et al., 1993c). We have demonstrated that Akt can provide protection, at least in part, from E. coli-induced lethality, by up-regulating the production of IL-10. Indeed, Schabbauer et al have proposed that activation of the PtdIns3K pathway could be used as a therapeutic approach in the treatment of sepsis (Schabbauer et al., 2004). Our data indicate the possibility of using a similar strategy (activation of Akt) in the treatment of sepsis. However, care should be taken as Akt has
also been implicated in a variety of human cancers (Datta et al., 1999). Manipulating Akt could make the patient more vulnerable to the oncogenic effect of Akt. Further studies elucidating the down-stream effectors of Akt in IL-10 up-regulation could avoid such undesired outcomes and on the other hand may prove invaluable in the development of effective therapeutic strategies for the treatment of potentially lethal conditions like sepsis.
BIBLIOGRAPHY


phosphatase (SHIP) in immunity: aberrant development and enhanced function of b lymphocytes in ship -/- mice. J. Exp. Med. 191, 781-794.


143


Ono,M., Bolland,S., Tempst,P., and Ravetch,J.V. (1996). Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. Nature 383, 263-266.


Tridandapani, S., Wang, Y., Marsh, C.B., and Anderson, C.L. (2002b). Src homology 2 domain-containing inositol polyphosphate phosphatase regulates NF-kappa B-mediated...


