THE ROLE OF SH2-DOMAIN INOSITOL 5’ PHOSPHATASE IN THE INHIBITION OF MACROPHAGE ACTIVATION

by

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ABSTRACT

Interleukin-10 (IL-10) is an anti-inflammatory cytokine essential for maintaining immune homeostasis. One of its major targets is the macrophage where it inhibits production of pro-inflammatory cytokines, chemokines and other soluble mediators. However, the intracellular signaling mechanisms by which IL-10 achieves macrophage deactivation remain under intense investigation. Our studies suggest that in addition to canonical STAT3 signaling, IL-10 mediates its early phase anti-inflammatory response through SHIP1 in a STAT3-independent manner. Upon macrophage activation by bacterial lipopolysaccharide, the phosphoinositide 3’ kinase (PI-3 kinase) pathway is activated to produce cytokines such as tumor necrosis factor α (TNFα). SHIP1 is a negative regulator of the PI-3 kinase pathway and its activation downstream of the IL-10 receptor suppresses PI-3 kinase-initiated signals that trigger transcriptional elongation of TNFα and other pro-inflammatory related genes. We next investigated whether SHIP1 activation could mimic the anti-inflammatory actions of IL-10. We screened for small-molecule activators of SHIP1 and isolated the meroterpenoid compound Pelorol. Pelorol and its derivatives specifically enhanced SHIP1’s phosphatase activity and thus suppressed inflammation in macrophage cultures, and in murine models of endotoxic shock, acute anaphylaxis, and inflammatory bowel disease. Closer examination of SHIP1’s enzyme kinetics indicated that SHIP1 is subject to allosteric activation by its product phosphatidylinositol-3,4-bisphosphate (PI-3,4-P₂). We subsequently identified a previously unrecognized C2 domain residing C-terminal of SHIP1’s phosphatase domain which is required for its allosteric activation and is the binding site for both PI-3,4-P₂ and the small-molecule SHIP1 agonists. Bioinformatic and structural analyses also revealed
another previously unappreciated domain located N-terminal of SHIP1’s catalytic domain. Using NMR spectroscopy, we characterized this domain as having pleckstrin homology (PH) domain-like topology. We demonstrate that SHIP1’s PH-related (PH-R) domain participates in recruiting SHIP1 to the plasma membrane upon cell stimulation via direct interactions with phosphatidylinositol-3,4,5-trisphosphate. The PH-R domain is essential for SHIP1 inhibition of FcR-dependent phagocytosis and represents another target to which to develop modulators of SHIP1 function. Together, this work suggests that IL-10 activation of SHIP1 is important in its inhibition of macrophage activation, and that mimicking IL-10 with small-molecule SHIP1 agonists could be an effective and viable approach to treating various inflammatory and autoimmune conditions.


**PREFACE**

**Contributions of Collaborators:**

Design of all research, data analysis and manuscript preparation were completed with the assistance of Dr. Alice Mui.

All experiments were performed solely by the author with the following exceptions:

*Chapter 2:*

Real-time quantitative PCR analysis of primary response genes were performed with the assistance of Erin McCarrell.

*Chapter 3:*

Structural identification of Pelorol and the synthesis of its structural analogues, AQX-016A and AQX-MN100 (Figure 3.1A), were performed by our collaborators Drs. Raymond J. Andersen and Matthew Nodwell.

Myeloperoxidase assays were performed with the assistance of Loutfig Demirjian (Figure 3.2B).

Dosing and tissue harvest of IL-10−/− mice was performed with the assistance of Michael Kennah.

Scoring of H&E stained tissue sections was performed with the assistance of Eva So (Figure 3.1 C).
Expression of recombinant C2 domain and Protein-Lipid Overlay assays were performed with the assistance of Joseph Kim

Chapter 4:

NMR-spectroscopy structural determinations of the SHIP1 PH-R domain were performed by our collaborators Drs. Lawrence McIntosh and Shaheen Shojania (Figures 4.2, 4.3, and 4.7). The corresponding materials and methods were prepared with their assistance.

Confocal microscopy image capture and scoring were performed with the assistance of Eileen Shaw, Eva So, Erin McCarrell and Tina Chang (Figures 4.5B and C, 4.9B, and 4.10)

Bacterial expression of recombinant PH-R mutant domains and Protein-Lipid Overlay assays were performed with the assistance of Ida Wang and Eileen Shaw.

Appendices:

Figure A.1 were provided courtesy of Gary Golds.

Data in Figures B.1 and E.1 were provided courtesy of Dr. Ali Ghanipour.

List of Publications:

The data presented in Chapter 2 are contained in the following manuscript that is currently under review for publication:

The majority of studies and figures described in Chapter 3 was originally published in *Blood*. Ong, CJ, Ming-Lum, A, Nodwell, M, Ghanipour, A, Yang, L, Williams, DE, Demirjian, L, Qasimi, P, Ruschmann, J, Cao, L, Ma, K, Chung, SW, Duronio, V, Andersen, RJ, Krystal, G, Mui, AL. Activation of inositol phosphatases to inhibit the phosphoinositide 3-kinase pathway. *Blood*. 2007 Sep 15;110(6):1942-9. © the American Society of Hematology.

The data presented in Chapter 4 are contained in the following manuscript that is currently under review for publication:

Ming-Lum, A, Shojania, S, So, E, McCarrell, E, Shaw, E, Vu, D, Wang, I, Chang, T, Andersen, R.J., Ong, C.J., Krystal, G, McIntosh, L.P., Mui, A.L. A pleckstrin homology-related domain in SHIP1 mediates membrane localization in Fcγ receptor induced phagocytosis.

**Ethics Approval:**

All animal experiments were performed in accordance with the UBC Animal Care Committee guidelines under the following protocols:

- **SHIP1**<sup>-/-</sup> Mouse Colony: A06-0336
- **IL-10**<sup>-/-</sup> Mouse Colitis Model: A08-0875-R001
- Endotoxemia Model: A11-0216

All biohazardous experiments were performed in accordance with the Health Canada, Laboratory Biosafety Guidelines under protocol B06-0140.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-HT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>72-5ptase</td>
<td>72-kDa-5’ phosphatase</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>adenylate-uridylate rich element</td>
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<tr>
<td>AREBP</td>
<td>ARE-binding protein</td>
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<td>ATF-2</td>
<td>activating transcription factor 2</td>
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<td>BCL-3</td>
<td>B-cell lymphoma 3-encoded protein</td>
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<td>BCR-ABL</td>
<td>breakpoint cluster region – Abelson virus oncogene</td>
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<td>BMDM</td>
<td>bone marrow derived macrophages</td>
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<td>Brd4</td>
<td>bromodomain-containing protein 4</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>CCR</td>
<td>chemokine (C-C motif) receptor (e.g. CCR2, CCR5)</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation (e.g. CD11b, CD45)</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
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<td>CR3</td>
<td>complement receptor 3</td>
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<tr>
<td>CX3CR</td>
<td>CX3C chemokine receptor</td>
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<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
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<td>DAP10/DAP12</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>Acronym</td>
<td>Term</td>
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<tr>
<td>DNFB</td>
<td>2,4-dinitro-1-fluorobenzene</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulphate</td>
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<tr>
<td>EC50</td>
<td>effective concentration (half maximal)</td>
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<tr>
<td>EGR-1</td>
<td>early growth response protein 1</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
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<td>ETV3</td>
<td>Ets (E26)-variant 3</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<td>Fcε-receptor</td>
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<td>FcgR</td>
<td>Fcγ-receptor</td>
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<td>fetal calf serum</td>
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<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
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<td>Gab1</td>
<td>Grb2-associated binding protein 1</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GM-CFU</td>
<td>granulocyte/macrophage-colony forming unit</td>
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<td>GM-CSF</td>
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<tr>
<td>Grb2</td>
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<td>HO-1</td>
<td>heme oxygenase 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>HSC</td>
<td>hemopoietic stem cell</td>
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<tr>
<td>HTS</td>
<td>high throughput screen</td>
</tr>
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<td>HuR</td>
<td>Hu-antigen R</td>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>inter-cellular adhesion molecule 1</td>
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</table>
IFNγ  interferon γ
IKK  inhibitor of κB (IκB) kinase
IL  interleukin (e.g. IL-10, IL-23)
IP4  inositol-1,3,4,5-tetrakisphosphate
Irgm1  immunity-related GTPase family M member 1
ITAM  immunoreceptor tyrosine-based activation motif
ITIM  immunoreceptor tyrosine-based inhibitory motif
Jak1  janus kinase 1
Jnk  c-Jun N-terminal kinase
KC  keratinocyte chemoattractant
KO  knockout
LFA1  leukocyte function-associated antigen 1
LIF  leukemia inhibitory factor
LPS  lipopolysaccharide
Ly6C  lymphocyte antigen 6C
LysM  lysozyme M
M-CFU  macrophage-colony forming unit
M-CSF  macrophage-colony stimulating factor
M1  classically activated type 1 macrophage
M2  alternatively activated type 2 macrophage
Mac1  macrophage antigen 1
MAPK  mitogen-activated protein kinase
MCP-1  monocyte chemotactic protein 1
MHC  major histocompatibility complex
MIP1  macrophage inflammatory protein 1
MIR  myeloid immunoregulatory cells
miRNA  micro-RNA
<table>
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<tr>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>myeloid suppressor cell</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κ B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer T-cell</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>PIP₃</td>
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<td>PKC</td>
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<td>PMΦ</td>
<td>peritoneal macrophage</td>
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<tr>
<td>PRG</td>
<td>primary response gene</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>proline-rich region</td>
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<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<td>PTPN1</td>
<td>protein tyrosine phosphatase, non-receptor type 1</td>
</tr>
<tr>
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<tr>
<td>PX</td>
<td>phox homology</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation normal T-cell expressed and secreted</td>
</tr>
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<td>RNAPolII</td>
<td>RNA polymerase II</td>
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<td>RT</td>
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<td>RT-qPCR</td>
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<tr>
<td>s-SHIP</td>
<td>stem-cell (short) - SHIP</td>
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<td>SHIP1</td>
<td>SH2 domain-containing inositol 5′-phosphatase</td>
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<td>SHP-1</td>
<td>SH2 containing phosphatase (e.g. SHP-1, SHP-2)</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SMAD</td>
<td>Sma and Mad related protein</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling (e.g. SOCS1, SOCS3)</td>
</tr>
<tr>
<td>Sp1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>SRG</td>
<td>secondary response gene</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription (e.g. STAT3)</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα converting enzyme</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGFB</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TH</td>
<td>T-cell helper (e.g. TH1, TH2, TH17)</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell intracytoplasmic antigen 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFA</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNFα receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor-inducing interferon β</td>
</tr>
<tr>
<td>TTP</td>
<td>tristetrapolin</td>
</tr>
<tr>
<td>Tyk2</td>
<td>tyrosine kinase 2</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>vIL-10</td>
<td>viral IL-10</td>
</tr>
<tr>
<td>VLA4</td>
<td>very-late antigen 4</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Foremost, I would like to thank my supervisor, Dr. Alice Mui for her mentorship, encouragement, guidance, and unwavering support. This thesis would not have been possible without her steadfast belief in my abilities as a student and scientist for which I am eternally grateful. My sincere thanks also to members of the Mui Lab, past and present, for all their help, support and friendship. Many thanks to the members of my supervisory committee, Drs. Michael Gold and Ken Harder for their input, recommendations, and guidance throughout my Ph.D. studies. I would also like to express my utmost gratitude to my program director, Dr. Vincent Duronio, for his kind help and support. To my friends at the Jack Bell Research Centre and neighbouring research institutes, you have made the years fly by. Thanks for the memories. Lastly, I dedicate this thesis to my parents who have, as always, provided their unconditional love and support.
CHAPTER 1: INTRODUCTION
1.1 Inflammation

Rubor et tumor cum calore et dolore – “redness and swelling with heat and pain” - were the four cardinal signs of inflammation first recorded by the ancient Roman encyclopedist, Aulus Cornelius Celsus (ca 25 – ca 50) \(^1\). Many years later, Rudolph Virchow (1821-1902) added a fifth sign, functio laesa (disturbance of function), which highlighted the cellular nature of the inflammatory process and is the only sign common to all presentations of inflammation \(^1,2\). Today, we recognize inflammation as a complex physiological process elicited in response to microbial pathogens, damaged cells, foreign cells, or chemical irritants. The inflammatory response aims to combat and/or remove the insulting stimulus and initiate the reparative pathways leading to restoration of normal tissue structure and function. However, despite having over two thousand years passed since the 4 cardinal symptoms were documented in Celsus’ *De medicina*, we are still unraveling the cellular and molecular mechanisms governing the inflammatory process.

Inflammation begins with the activation of differentiated mast cells or macrophages residing in the tissues. When these cells encounter allergens, pathogen associated molecular patterns (PAMPs) expressed on the surface of microbe \(^3–6\), or damage associated molecular patterns (DAMPs) (intracellular molecules released into the extracellular environment by disrupted or dying cells) \(^7–9\), they release a variety of soluble mediators. These mediators include: cytokines such as tumor necrosis factor \(\alpha\) (TNF\(\alpha\)), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-12 (IL-12); chemokines such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP1); and chemical factors such as histamine, prostaglandins, and reactive oxygen and nitrogen species (reviewed in \(^10\)). Collectively, these molecules
promote the activation of surrounding cells, the recruitment of other immune cells from circulating blood to the sites of infection or injury, and the direct killing of microbial pathogens.

These molecules can also have local effects causing vasodilation and increased permeability of the local blood vessels\textsuperscript{11-15}. This increased blood flow to the site of inflammation or infection is concurrent with the up-regulated expression of adhesion molecules on the luminal surface of the endothelial layer. This, in turn, promotes the cell-to-cell contact between circulating leukocytes (primarily neutrophils) and endothelial cells—slowing their movement and causing them to “roll” along the endothelial wall. Once this occurs, leukocytes can then be sufficiently exposed to local inflammatory mediators – initiating cell activation, leukocyte spreading and firm adhesion to the endothelial cell lining. Following a chemotactic gradient, leukocytes then transmigrate across the endothelial layer to the site of infection. The recruited neutrophils, in concert with differentiated macrophages, mast cells, and plasma components such as antibodies and complement, then kill invading microorganisms or clear damaged cells and debris\textsuperscript{11-15}.

While inflammatory responses are beneficial to clear the body of invading microbes and pathogens, they must be quickly terminated to prevent inadvertent damage to normal, healthy cells and tissues. Regulation of inflammation is achieved by several mechanisms. One means of inhibiting inflammation is through the release of anti-inflammatory molecules such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and glucocorticoids (reviewed in\textsuperscript{10,16}). These molecules can function at multiple levels including: inhibiting the production of pro-inflammatory mediators,
inducing changes in the target cells that the pro-inflammatory mediators act upon (e.g. down-regulating the expression of a surface receptor), or interfering with the intracellular signaling pathways that pro-inflammatory mediators activate. Altering signaling pathways, for example, can switch the conversion of arachadonic acid from pro-inflammatory prostaglandins to lipoxins. Lipoxins can reduce vascular permeability, inhibit extravasation of neutrophils, and promote the recruitment of “healing” macrophages \(^{16}\). If these mechanisms fail to properly resolve an inflammatory response, various pathologies can arise including acute systemic inflammation in the form of sepsis \(^{17}\), or chronic inflammatory diseases such as inflammatory bowel disease \(^{18,19}\).

### 1.2 Macrophages

Macrophages are the sentinels of the innate immune system whose various roles include phagocytosis of microbes, pathogens and cellular debris; activation of other cells to facilitate combat and clearance of the immune stimulus; and initiation of the pathways leading towards the development of an adaptive immune response (reviewed in \(^{20-23}\)). Macrophages are a component of the mononuclear-phagocyte system, which additionally includes the circulating blood monocytes from which they are differentiated and the lineage-committed, common myeloid progenitors residing in the bone marrow. It is well recognized that macrophages are an incredibly heterogeneous population whose phenotype and function are profoundly influenced by the tissues within which they reside and the immunological microenvironment.
1.2.1 Monocyte/Macrophage heterogeneity

Monocytes comprise approximately 5-10% of peripheral leukocytes in human blood and exhibit a high degree of variability with respect to size and granularity. Further, the identification of monocyte subsets with differential expression of cell surface markers has suggested that there may be monocyte subsets with specific physiologic functions. One of the earliest monocyte classification schemes separated cells based on their expression of CD14, a component of the lipopolysaccharide (LPS) receptor complex, and CD16 (also known as FcγRIII). The CD14\(^{\text{high}}\)CD16\(^{-}\) and CD14\(^{-}\)CD16\(^{+}\) monocytes defined in this classification were further identified to have differential expression of other surface molecules. CD14\(^{\text{high}}\)CD16\(^{-}\) cells expressed CCR2 while CD14\(^{+}\)CD16\(^{+}\) cells expressed higher levels of CD32 (also known as FcγRII) and CCR5. CD14\(^{\text{high}}\)CD16\(^{-}\) monocytes are frequently referred to as classic “inflammatory” monocytes while CD14\(^{+}\)CD16\(^{+}\) cells are considered “resident” monocytes and more closely resemble tissue-residing macrophages. Over the years, additional subsets of monocytes have been defined including classification based on the expression of CD64 (also known as FcγRI). CD14\(^{+}\)CD16\(^{-}\)CD64\(^{+}\) monocytes produce larger quantities of TNFα and IL-6 and have greater phagocytic activity than CD14\(^{+}\)CD16\(^{+}\)CD64\(^{-}\) cells and are able to stimulate lymphocytes to a greater degree than CD14\(^{\text{high}}\)CD16\(^{-}\) monocytes.

Adding to the difficulty in characterizing distinct monocyte subsets, was the differing expression of markers between humans and mice. It was not until 2003, when Geissmann et al. defined a murine classification analogous to the human CD14\(^{\text{high}}\)CD16\(^{-}\)/CD14\(^{-}\)CD16\(^{+}\) scheme. Classic “inflammatory” monocytes were characterized as
CCR2^+CD62L^+CX3CR1^{low}Ly6C^+ while the equivalent of “resident” monocytes were CCR2^−CD62L^−CX3CR1^{high}Ly6C^− (See Figure 1.1). At the functional level, the “inflammatory” monocytes are believed to be released into the circulation from the bone marrow and are recruited by chemokines and other pro-inflammatory signals to inflamed tissues where they subsequently differentiate into mature macrophages or dendritic cells (DCs) and combat the inflammatory stimulus. In contrast however, many questions still persist as to the origin of tissue-resident macrophages, which are believed to have multiple roles in the clearance of dead or damaged cells, repair and tissue remodeling after an inflammatory response. Whether resident macrophages are differentiated from a specific subset of circulating monocyte, derived from the proliferation of pre-existing tissue macrophages, or if recruited “inflammatory” macrophages are capable of further differentiating into reparative “resident” macrophages, are all possibilities being investigated 38-41.

Regardless of their origin, “resident” macrophages adopt a phenotype unique and specialized to the tissue. For example, alveolar macrophages and peritoneal macrophages express high levels of pattern recognition receptors (PRRs) and scavenger receptors 42-47, osteoclasts develop the ability to resorb bone 48, while macrophages residing in the lamina propria of the gut exhibit high phagocytic activity but low levels of secreted pro-inflammatory cytokines 49-51.
Figure 1.1   The development of macrophages in mice.

Monocytes are derived from a common myeloid progenitor cell that is shared with neutrophils. Ly6C$^+$ monocytes are released into the blood where they adopt a Ly6C$^{\text{Mid}}$ phenotype and express CCR2, CCR7 and CCR8. Under steady-state conditions, circulating monocytes lose their expression of Ly6C and upregulate their expression of CX3CR1. These Ly6C$^-$ cells can migrate into the peripheral tissues to replenish resident macrophage populations. Ly6C$^+$ and Ly6C$^{\text{Mid}}$ monocytes can respond to CCL2 and extravasate into the tissues towards sites of inflammation. HSC, hemopoietic stem cell, GM-CFU, granulocyte/macrophage colony forming unit, M-CFU, macrophage colony forming unit, CCR, CC-chemokine receptor, CCL, CC-chemokine ligand, CD, cluster of differentiation, CX3CR, CX3C-Chemokine Receptor, CX3CL, CX3C-chemokine ligand. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] Gordon, S. & Taylor, P.R. Monocyte and macrophage heterogeneity. Nat Rev Immunol 5, 953-964 (2005). Copyright © (2005) 52.
1.2.2 Macrophage activation

Circulating “inflammatory” monocytes, defined as being CCR2$^+$ CD62L$^+$ Ly6C$^+$, express LFA1, Mac1, Pecam1, and VLA4, which facilitate its endothelial adhesion and migration towards the site of inflammation $^{15}$. The so-called “classical” activation of M1 macrophages is typically elicited in response to toll-like receptor (TLR) ligands (e.g. LPS) and interferon-γ (IFNγ) and yields an IL-12$^{\text{high}}$, IL-23$^{\text{high}}$, IL-10$^{\text{low}}$ phenotype$^{53-58}$. Classically activated macrophages exhibit increased expression of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNFα) and reactive-oxygen species, enhanced microbicidal activity, and upregulation of major histocompatibility complex (MHC) class II and antigen presentation molecules$^{53-58}$. However, in the presence of IL-4, IL-13, glucocorticoids, or TGFβ, macrophages become “alternatively” activated. Unlike the M1 macrophages, these alternatively activated M2 macrophages are associated with humoral immunity and tissue repair$^{53-58}$. They have an IL-12$^{\text{low}}$, IL-23$^{\text{low}}$, IL-10$^{\text{high}}$, Fizz$^+$, YM-1$^+$ phenotype and have differing pro-inflammatory cytokine expression profiles depending on the instigating stimulus. M2 macrophages also display enhanced endocytosis with increased expression of scavenger mannose and galactose-type receptors, and a switch in arginine metabolism from citrulline and nitric oxide (NO) production (as in M1 macrophages) to ornithine and polyamines. The characteristic differences between M1 and M2 macrophages are described in Figure 1.2. Much like the T$_H$1 and T$_H$2 paradigm in T-cells, the M1 and M2 classification of macrophage activation is an oversimplification and used more as a conceptual tool with the added appreciation that there is a far greater diversity in differentiation and activation states and that
macrophages may have the ability to move through these states through the course of an inflammatory event \(^{59}\).
Figure 1.2 Characteristics of M1 and M2 polarized macrophages.

Classically activated “M1” macrophages are induced through TLR stimulation such as LPS. They are characterized by the production of pro-inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species. M1 macrophages preferentially use transcription factors IRF5 and the p65 subunit of NFκB. Alternatively activated “M2” macrophages are associated with immunosuppressive responses and tissue remodeling. They produce high levels of the anti-inflammatory cytokine IL-10 and have high activity of Arg1. M2 macrophages additionally have high expression of the chitinases Ym1 and Ym2, and the resistin-like protein, Fizz1. M2 macrophages preferentially utilize transcription factors c-Maf, the p50 subunit of NFκB, STAT6, and C/EBPβ. LPS, lipopolysaccharide, IFNγ, interferon-γ, iNOS, inducible nitric oxide synthase, ROI, reactive oxygen intermediates, RNI, reactive nitrogen intermediates, IL, interleukin, TNFα, tumor necrosis factor α, CXCL, chemokine (C-X-C motif) ligand, IRF, interferon regulatory factor, MHC, major histocompatibility complex, CD, cluster of differentiation, TLR, Toll-like receptor, MR, mannose receptor, SR, scavenger receptor. Adapted by permission from John Wiley & Sons Inc.: [European Journal or Immunology] Mantovani, A., et al. New Vistas on macrophage differentiation and activation. Eur J Immunol 37, 14-16 (2007). Copyright © (2007) 52.
1.3 LPS

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria where it functions to structurally stabilize and protect the bacteria from certain forms of chemical attack. LPS is a mammalian endotoxin and is a potent stimulator of the immune system by virtue of its ability to bind the CD14/TLR4/MD2 receptor complex to induce the production of pro-inflammatory cytokines and mediators.

Structurally, as depicted in Figure 1.3, LPS is composed of three parts: the O-antigen, the core oligosaccharide, and Lipid A. The O-antigen forms the outermost domain of an LPS molecule. It is an oligosaccharide chain whose structure and composition is specific for each bacterial strain and confers the immunogenicity of LPS. The O-antigen is covalently attached to the LPS core oligosaccharide with commonly contains multiple ketodeoxyoctocnic acids, heptose sugars, and non-carbohydrate components including phosphates and amino acids. The core oligosaccharide, in turn, is covalently attached to Lipid A, which is typically a glucosamine disaccharide with varying numbers of fatty acid chains attached. The fatty acid chains of Lipid A allow LPS to be inserted and anchored into the bacterial membrane. It is the Lipid A component of LPS that interacts with TLR4 and stimulates the activation of immune cells.
Figure 1.3    The structure of LPS.

1.3.1 LPS/Toll-like receptor 4 signaling

TLRs are one of the major families of PRRs, which recognize a variety of PAMPs including: lipopeptides (TLR1, TLR2, TLR6), double-stranded RNA (TLR3), LPS (TLR4), bacterial flagellin (TLR5), guanosine or uridine-rich single-stranded viral RNA (TLR7, TLR8), and unmethylated CpG DNA (TLR9)\textsuperscript{64,65}. TLRs are type-1 membrane proteins with a leucine-rich extracellular domain and an intracellular signaling domain comprised of a conserved toll-IL-1 receptor (TIR) domain\textsuperscript{65,66}. Upon receptor stimulation, the TIR domain facilitates protein-protein interactions with other TIR domain-containing adaptor proteins such as MyD88, Mal, TRIF, TRAM and SARM. Different TLRs employ various combinations of these adaptor proteins to mediate their pro-inflammatory signaling\textsuperscript{64-68}.

TLR4 is the signaling receptor for LPS but additionally requires other proteins in order to properly bind its ligand. LPS binding protein (LBP) is a serum lipid transferase that facilitates the transfer of LPS from the bacteria to the membrane glycoprotein CD14 on the surface of host cells. CD14, in turn, presents LPS to TLR4. MD2 is another membrane bound glycoprotein, which associates with TLR4 and also assists in LPS presentation\textsuperscript{69-72}.

TLR4 is known to signal through two main pathways – the MyD88-dependent and independent pathways. In the MyD88-dependent pathway, LPS binding promotes the interaction of the adaptor proteins Mal (also known as TIRAP) and MyD88 to TLR4. MyD88 contains a death domain, which mediates protein-protein interactions with the death domain containing IL-1 receptor associated kinase 4 (IRAK4). At the membrane, IRAK4 becomes activated by phosphorylation and in turn, recruits IRAK1 and IRAK2 to
the receptor complex where they also become phosphorylated and activated. The IRAKs then dissociate from the TLR4 receptor complex and interact with TNF receptor-associated factor 6 (TRAF6) in the cytoplasm. TRAF6 is an E3 ubiquitin ligase that interacts with ubiquitin-conjugating enzyme (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A), which together, promote the synthesis of lysine 63-linked polyubiquitin chains and activates TGFβ-activated protein kinase 1 (TAK1). TAK1, a member of the MAP kinase kinase kinase (MAP3K) family, in addition with TAB1, TAB2, and TAB3 then activate kinases upstream of p38, JNK, and the IKK complex. The IKK complex is responsible for the phosphorylation of IkB, promoting its degradation and the subsequent liberation of NFκB, which translocates to the nucleus to upregulate the expression of pro-inflammatory genes. Additional TRAF6-independent pathways have been described where, upon LPS stimulation, IRAK1 and IRAK4 activate the UBC-conjugating enzymes Pellino-1 and Pellino-2. Pellino-1 and Pellino-2 then lysine 63-polyubiquitinate IRAK1, causing the recruitment and activation of the IKK complex 65,66,69-74.

In the MyD88-independent pathway, LPS binding induces the association of TLR4 with TIR-domain-containing adapter-inducing interferon β (TRIF) via TRIF-related adaptor molecule (TRAM). TRIF contains a C-terminal RIP homotypic interaction motif (RHIM) allowing it to bind and activate receptor-interacting protein 1 (RIP1). RIP1, in turn, is able to activate the IKK complex leading to the expression of NFκB regulated gene products. TRIF also contains an N-terminal region capable of binding TRAF3. TRAF3 serves as an adaptor for the association of TRIF with TANK, TBK1 and IKKe. These three kinases are then able to phosphorylate and activate
interferon regulatory factor 3 (IRF3), a transcription factor that induces the expression of type I interferon genes \(^{65,66,69-74}\) (Figure 1.4).
LBP, CD14 and MD2 facilitate LPS interaction with TLR4. Ligand binding activates the MyD88-dependent signaling pathway, which begins with recruitment of TIRAP and MyD88 to the receptor complex, which initiate signaling cascades leading to activation of the MAPKs p38 and Jnk as well as the NFκB transcription factor. LPS binding also activates MyD88-independent signaling, which starts with recruitment of TRIF and TRAM and ends with activation of IRF transcription factors. TLR, Toll-like receptor, CD, cluster of differentiation, LBP, LPS binding protein, TRIF, TIR-domain-containing adaptor-inducing interferon-β, TRAM, TRIF-related adaptor molecule, RIP, receptor
1.3.2 Negative regulation of LPS/TLR4 signaling

Although TLR4 signaling is essential for initiating protective inflammatory processes in response to an invading pathogen, its activity must be tightly controlled as exaggerated and/or sustained TLR4 signaling gives rise to various chronic and autoimmune pathologies. There are numerous negative regulators of TLR4 signaling at every level of the signaling cascade. This diversity in regulation allows for the fine-tuning of a cellular response so that it is tailored and appropriate for a given inflammatory stimulus. Table 1.1 presents a summary of the reported negative regulators of the TLR4 signaling pathway.
Table 1.1: Negative regulators of TLR4 signaling

<table>
<thead>
<tr>
<th>Cellular Compartment</th>
<th>Protein Name</th>
<th>Mechanism of Inhibition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>Soluble CD14</td>
<td>Soluble decoy. Blocks interaction between LPS and membrane CD14.</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Soluble MD2</td>
<td>Soluble decoy. Blocks interaction between TLR4 and membrane MD2.</td>
<td>76, 77</td>
</tr>
<tr>
<td></td>
<td>Soluble TLR4</td>
<td>Soluble decoy receptor. Blocks interaction between TLR4 and CD14/MD2.</td>
<td>78-80</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>CD32A</td>
<td>May inhibit TLR4 signaling by activating PI-3 kinases and preventing TIRAP recruitment by PI-4,5-P.</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>DAP12/TREM1</td>
<td>Interacts with and activates PI-3 kinase and PLCγ. Depletes PI-4,5-P and prevents TIRAP membrane localization.</td>
<td>82, 83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLR4 homolog. Binds and sequester CD14/MD2.</td>
<td>84, 85</td>
</tr>
<tr>
<td></td>
<td>ST2</td>
<td>Sequesters MyD88 and TIRAP/Mal. Soluble ST2 binds to a putative receptor and suppresses NFκB binding to IL-6 promoter.</td>
<td>86-89</td>
</tr>
<tr>
<td></td>
<td>SIGIRR</td>
<td>Inhibits MyD88 signaling. Prevents TLR4 dimerization. Complexes with IRAKs and TRAF6.</td>
<td>90-92</td>
</tr>
<tr>
<td></td>
<td>TRAILR</td>
<td>Binds and stabilizes IκB.</td>
<td>93</td>
</tr>
<tr>
<td>Intracellular</td>
<td>A20 (TNFAIP3)</td>
<td>Inhibits Ubc13 and UEV1A interactions with TRAF6 and subsequent recruitment of TAK1/Tab1 and Tab2.</td>
<td>94-96</td>
</tr>
<tr>
<td></td>
<td>ATF3</td>
<td>CREB family transcription factor. Binds to acetylated histones and prevents NFκB binding.</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>β-Arrestin</td>
<td>Binds to IκB and prevents its phosphorylation and ubiquitination. Binds to TRAF6 and prevents its autophosphorylation and activation.</td>
<td>98-100</td>
</tr>
<tr>
<td></td>
<td>DUSP</td>
<td>Negatively regulates MAPK activation.</td>
<td>101-104</td>
</tr>
<tr>
<td></td>
<td>FLIIH</td>
<td>Binds TIR domains of TLR4 and MyD88 and prevents their mutual interaction.</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>FLN29</td>
<td>Binds TRAF6 and inhibits downstream signaling.</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>IRAK1c</td>
<td>Splice variant. Sequesters MyD88, IRAK2 and TRAF6 away from IRAK1.</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>IRAK-M</td>
<td>Prevents dissociation of IRAK1/IRAK4 from the MyD88 complex and prevents the activation of TRAF6.</td>
<td>108-110</td>
</tr>
<tr>
<td></td>
<td>MyD88s</td>
<td>MyD88 splice variant. Forms MyD88/MyD88s dimmers that bind and sequester IRAK1.</td>
<td>111-113</td>
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<tr>
<td></td>
<td>PIN1</td>
<td>Binds phosphorylated IκB3 and promotes its ubiquitination and degradation.</td>
<td>114-116</td>
</tr>
<tr>
<td></td>
<td>RIP3</td>
<td>Inhibits RIP1 activity by sequestering it from TRIF.</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Rab7b</td>
<td>Small GTPase that promotes the lysosomal degradation of TLR4.</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>SARM</td>
<td>Competitive inhibitor of TRIF.</td>
<td>119-121</td>
</tr>
<tr>
<td></td>
<td>SHP-2</td>
<td>Negatively regulates MyD88 independent signaling by binding to and inhibiting TBK-1.</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>SOCS1</td>
<td>Interacts with and potentially inhibits p65 subunit of NFκB. Binds Mal and promotes its proteasomal degradation.</td>
<td>116, 123</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>Splice variant of TRAM. Disrupts TRAM-TRIF association.</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>TOLLIP</td>
<td>Binds to TLR4 and IRAK and prevents IRAK phosphorylation.</td>
<td>125, 126</td>
</tr>
<tr>
<td></td>
<td>TRAF4</td>
<td>Interacts with p47&lt;sub&gt;Phox&lt;/sub&gt;, TRAF6, TRIF and IRAK1 and inhibits NFκB activation.</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>TRIAD3A</td>
<td>E3 ubiquitin ligase. Targets RIP1, TRIF, TIRAP/Mal for proteasomal degradation.</td>
<td>128, 129</td>
</tr>
<tr>
<td></td>
<td>TRIM30α</td>
<td>Targets TAB2/TAB3 for degradation via the lysosomal pathway.</td>
<td>130</td>
</tr>
</tbody>
</table>
1.4 Tumor necrosis factor α

One of the hallmarks of inflammatory disease is heightened levels of pro-inflammatory cytokines such as TNFα. TNFα was first described by Carswell et al. in 1975 as a factor in the serum of mice treated with endotoxin that was capable of inducing hemorrhagic necrosis of tumors. Since that time, TNFα has become recognized as a potent activator of immune cells and as one of the earliest detected and most highly expressed cytokines in an inflammatory reaction.

The TNFα gene is located on chromosome 6 (human) and 17 (mouse) and consists of 4 exons and 3 introns. TNFα production is induced in a variety of cell types but most robustly in monocytes/macrophages. In macrophages, the signaling pathways downstream of TLR4 activation that lead to increased transcription of TNFα have yet to be fully elucidated. LPS stimulation has been reported to activate various kinases including IKK and MAPKs, which then go on to initiate the activity of multiple transcription factors including NFκB, AP-1, ATF-2 and Egr-1. Once activated, these transcription factors then bind to cis-acting DNA promoter elements upstream of the TNFα gene. Both the signaling pathways and transcription factors employed appear to be macrophage type-specific. TNFα is additionally subject to post-transcriptional mechanisms of regulation, which are dependent on the 5’ and 3’ untranslated regions (UTRs) of the TNFα mRNA. Elements in these regions can interact with various RNA binding proteins to regulate both mRNA stability and, in the case of 3’ UTR AU-rich elements (ARE), translation.
TNFα is first synthesized as a membrane-anchored, 27 kDa pre-protein which is then cleaved by TNFα-converting enzyme (TACE) to produce the mature 17 kDa protein α161-166. Interestingly, it is believed that both membrane-bound and soluble TNFα are biologically active molecules with both shared and exclusive physiologic functions 167-174.

TNFα mainly signals through two cysteine-rich receptors: TNF receptor 1 (TNFR1), which is constitutively expressed in almost all cell types; and TNF receptor 2 (TNFR2), which is expressed primarily on particular subsets of immune cells 175-178. Both receptors bind to TNFα with high affinity but with differing kinetics. TNFR1 binds irreversibly to its ligand while TNFR2 binds with rapid association/dissociation kinetics 172,179,180. The two receptors also differ in the structure of their cytoplasmic domains. While TNFR1 and TNFR2 both have cysteine rich extracellular domains, TNFR1 alone contains a cytoplasmic death domain capable of initiating apoptosis through the TNFR1-associated death domain (TRADD) and Fas-associated death domain (FADD) adaptor proteins 181,182. TNFR2, alternatively, signals the transcription of cell survival genes via TNF receptor-associated factors (TRAFs) and activation of the NFκB pathway 183,184. Additionally, TNFR2 is most strongly activated by uncleaved, membrane-bound TNFα and to a lesser degree by the soluble form of TNFα 172,179,180. The signaling pathways employed by TNFR1 and TNFR2 are represented in Figure 1.5.

TNFα is produced by macrophages in response to TLR stimulation. TNFα, in turn, is capable of activating macrophages with concomitant priming by interferon-γ (IFNγ). IFNγ signaling promotes the nuclear sequestration of the signal transducer and activator of transcription 1α (STAT1α) and prevents it from interacting with TNFR1,
thereby potentiating TNFα stimulation of the NFκB pathway. Similarly, it has been reported that TNFα can induce the sustained but low-level expression of Interferon-β, which synergizes with TNFα activation of the NFκB pathway and promotes the sustained expression of pro-inflammatory genes such as cxcl9, cxcl10, and cxcl11 and delayed expression of interferon response genes such as interferon response factor-1 (IRF-1) and STAT1. Collectively, it is thought that these molecules establish the autocrine feedback loops that contribute to chronic inflammation such as those observed in various autoimmune diseases.
Figure 1.5  The TNFα signaling pathway.

1.4.2 TNFα in disease

Despite TNFα’s integral role in defense against microbial infection, its overabundant production from macrophages has been attributed to various inflammatory diseases including IBD\(^{186-188}\), sepsis\(^{189-193}\), rheumatoid arthritis\(^{194-197}\), and atherosclerosis\(^{198-201}\). Highlighting the central role that TNFα has in disease pathogenesis, anti-TNFα antibodies have shown clinical efficacy in the treatment of some of these conditions. For example, in patients with Crohn’s disease or ulcerative colitis, administration of the murine anti-human TNFα monoclonal antibody, infliximab, resulted in reduced clinical inflammation and patients were able to discontinue use of standard corticosteroids\(^{202-206}\). Further, anti-TNFα antibody treatment has been reported to promote healing of the intestinal mucosa in patients who have become unresponsive to corticosteroid treatment\(^{207,208}\). Side-effects associated with anti-TNFα therapy, while rare, have been reported including an increased susceptibility to bacterial infection (particularly *Mycobacterium tuberculosis*), hematopoietic malignancies and disorders, congestive heart failure, and demyelinating diseases (reviewed in\(^{209}\)).

1.5 Transcriptional programs

Stimulation with LPS causes the differential regulation of over 200 hundred genes within 1 or 2 hours\(^{210-212}\). These genes can be further grouped into clusters of genes that contribute towards a shared biological function such as phagocytosis, cell migration, and anti-microbial defense. Genes grouped in these clusters are often subject to regulation by a defined and shared set of transcription factors. Thus, modulated expression of groups of genes can be simultaneously coordinated based upon which transcription factors are activated. Medzhitov and Horng\(^{213}\) categorized transcription factors involved in the
inflammatory response into 3 categories. Class I transcription factors are constitutively expressed as proteins and are regulated post-translationally by TLR stimulation, typically by phosphorylation events that regulate their nuclear translocation. Class I transcription factors regulate the primary response genes (PRGs), which are rapidly induced upon stimulation. Class II transcription factors are produced during the primary response by Class I transcription factors and regulate the expression of secondary response genes (SRGs). Class III transcription factors are active during macrophage differentiation and are not directly regulated by inflammatory stimuli. These transcription factors regulate the expression of genes involved in chromatin remodeling. These 3 classes of transcription factors, in concert with covalent modifications in chromatin structure, and transcriptional co-activators and co-repressors, collectively form transcriptional programs capable of determining the type of stimulus-specific response elicited, duration of response and modes of regulation.

1.5.1 Primary response genes

According to the transcriptional program classification of genes by Medzhitov and colleagues, TNFα is a PRG whose early increased expression following LPS stimulation is mediated by Class I transcription factors such as NFκB. PRGs also have distinguishing characteristics with regard to their chromatin structure. As opposed to SRGs, such as IL-6, PRGs have highly permissive chromatin in resting cells with high degrees of histone H3K4 trimethylation and H3K9 acetylation. These covalent modifications correlate with high promoter GC content and facilitate the association of RNA polymerase II (RNAPolII) in the basal state of unstimulated cells. When bound to PRG promoters in resting cells, RNAPolII is phosphorylated at Ser5 and is capable of
transcribing low levels of full-length, unspliced, unstable mRNA. Upon LPS stimulation, bromodomain-containing protein 4 (Brd4) is recruited to the PRG promoters, which in turn recruits the P-TEFb complex composed of cyclin T1 and cyclin-dependent kinase 9 (CDK9). The P-TEFb complex is responsible for the phosphorylation of RNAPolII at Ser2 in its C-terminal domain, which signals a switch of RNAPolII to a highly productive, elongation-competent producer of mature mRNA transcripts. A graphical representation of PRG transcriptional elongation control is depicted in Figure 1.6.

A report by Smallie et al. further characterized a mechanism by which PRGs can be negatively regulated by the anti-inflammatory cytokine IL-10. IL-10 was shown to inhibit the recruitment of the RelA (p65) component of the NFκB complex to κB sites in the TNFα promoter. They additionally demonstrated that in a gene-specific manner, RelA recruits CDK9 to the promoters of PRGs. Thus, by inhibiting RelA recruitment, IL-10 also inhibits the recruitment of CDK9 and the subsequent Ser2 phosphorylation of RNAPolII. This prevents the signal switch to transcriptional elongation and inhibits the production of mature PRG mRNAs.
Figure 1.6 PRG transcriptional elongation control.

In a basal state, primary response genes have GC-rich promoters that have high degrees of covalent histone modifications. RNApolIII is constitutively associated with the promoters of PRGs and is phosphorylated at Ser5, which activates low-level production of unspliced RNA. Upon LPS stimulation, the P-TEFb complex composed of CyclinT1 and CDK9 is recruited, which phosphorylates RNApolII at Ser2 switching it to produce high levels of mature, spliced RNA. Secondary response genes have inaccessible chromatin and must undergo chromatin remodeling before transcription factors are capable of binding and subsequent recruitment of the transcriptional complex. Met, methyl, Ac, acetyl, HAT, histone acetyltransferase, SP1, specificity protein 1, RNApolIII, RNA polymerase II, LPS, lipopolysaccharide, NFκB, nuclear factor κB, CDK9, cyclin dependent kinase 9, BRG1, BRM/SWI2-related gene 1, IRF, interferon-regulatory factor. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] Medzhitov, R. & Horng, T. Transcriptional control of the inflammatory response. Nat Rev Immunol 9, 962-703 (2019). Copyright © (2009) 213.
1.6 Interleukin-10

The discovery of interleukin-10 (IL-10) was first reported by researchers at the DNAX Research Institute in 1989 as a factor secreted by Th2 cells that inhibited Th1 cell cytokine production\textsuperscript{216,217}. It was later shown that IL-10’s inhibition of T-cells and NK cells was via an indirect mechanism involving macrophages and that IL-10 was able to potently inhibit macrophage production of pro-inflammatory cytokines and chemokines, and surface expression of MHC Class II and co-stimulatory molecules\textsuperscript{218}.

IL-10 is a glycosylated, 178 amino acid protein composed of an 18 amino acid signal sequence and the 160 amino acid mature polypeptide\textsuperscript{219,220}. Structurally, IL-10 is composed of six $\alpha$-helices. Four amino-terminal helices form the core protein and two carboxy-terminal helices are necessary for protein-protein interactions with another IL-10 molecule, forming the fully-active, non-covalent, IL-10 homodimer (reviewed in\textsuperscript{221}).

IL-10 is expressed by a wide variety of cells usually in response to a stimulus, such as LPS\textsuperscript{222-225}, and mediated by a number of transcription factors including Specificity protein-1 (Sp1)\textsuperscript{226}, Sp3\textsuperscript{226,227}, C/EBPs\textsuperscript{228}, and NFkB\textsuperscript{229}. Evidence suggests that IL-10 is constitutively expressed in a number of cell types and that its expression is additionally regulated at the post-transcriptional level via mechanisms affecting IL-10 mRNA stability\textsuperscript{230}.

The functional IL-10 receptor is a tetramer composed of two ligand-binding subunits, IL-10R1 (also referred to as IL-10R$\alpha$)\textsuperscript{231,232}, and two signaling subunits, IL-10R2 (also referred to as IL-10R-$\beta$)\textsuperscript{233,234}. IL-10R1 is expressed by almost all hemopoietic cells but most abundantly on monocytes/macrophages\textsuperscript{222,235}. Further, while
IL-10R1 expression is downregulated by stimulation in T-cells, its expression is upregulated in macrophages upon activation by inflammatory stimuli consistent with IL-10’s potent inhibitory activity on these cells. IL-10R2 does not participate in interaction with IL-10 ligand, rather it initiates downstream signaling primarily through activation of the janus family kinases, Jak1 and tyrosine kinase-2 (Tyk2). Unlike IL-10R1, IL-10R2 is constitutively expressed in almost all cells and its levels are unaffected by stimulation. IL-10R2 is the shared signaling chain for at least 4 other cytokine receptors including IL-22R, IL-26R, IL-28R and IL-29R. Interestingly, the pleiotropic activities of these cytokines are generally associated with pro-inflammatory responses as opposed to exerting anti-inflammatory activities, despite sharing the common IL-10R2.

1.6.1 IL-10 signaling

In the current literature, it is believed that IL-10 signals solely through the canonical janus kinase (Jak)/STAT pathway. Jak1 and Tyk2, which are constitutively associated with IL-10R1 and IL-10R2 respectively, become phosphorylated and activated upon IL-10/IL-10R binding. In turn, these kinases then phosphorylate residues Y446 and Y496 on the intracellular domain of human IL-10R1 (Y427 and Y477 on murine IL-10Rα). These phosphorylated tyrosines serve as docking sites for STAT3, which interact with IL-10R1 through its Src-homolgy 2 (SH2) domain. STAT3 then becomes phosphorylated by the receptor-associated Jaks at Y705, which facilitates STAT3 dimerization, and S727, which enhances STAT3 transcriptional activity. Upon dimerization and activation, STAT3 translocates to the nucleus where it interacts with STAT3 binding elements in the promoters of IL-10.
responsive genes and thereby enhances the expression of genes associated with an anti-inflammatory response (e.g. SOCS3, HO-1, ETV3, SBNO2) and anti-apoptosis/cell-cycle progression (e.g. Bcl-3, c-Myc, Cyclins) 239,242,245-252.

However, there is compelling evidence in the literature that suggests that IL-10 signals through pathways independent of the STAT3 transcription factor. In a report published by Lang et al., STAT3 Wildtype bone marrow-derived macrophages (BMDMs) stimulated with LPS and treated with IL-10, exhibited a marginally reduced production of TNFα as compared to macrophages stimulated with LPS alone 247. BMDMs derived from STAT3^flox/- LysMcre mice, however, produced significantly higher levels of TNFα when stimulated with LPS. These higher levels of TNFα production additionally appeared to be subject to a greater fold inhibition by IL-10 than the STAT3 WT BMDMs, suggesting that IL-10 employs non-STAT3 dependent pathways to regulate TNFα expression. Using a dominant negative STAT3, O’Farrell et al. demonstrated that IL-10 was still capable of inhibiting TNFα production in LPS-stimulated J774.1 cells 244. In a similar study, using an adenovirally delivered STAT3 dominant negative, Williams et al. observed that STAT3 only contributed to a partial inhibition of TNFα production 242. Further, when comparing macrophages stimulated with LPS for 1 hour and 2 hours, IL-10’s inhibition of TNFα production appears to have a greater dependence on STAT3 at longer timepoints whereas inhibition appears to be STAT3-independent at earlier, 1-hour timepoints. In the same study, Williams et al. also demonstrated that at early timepoints, IL-10 does not require de novo protein synthesis in order to inhibit TNFα mRNA levels 242. These data collectively suggest that there are
additional, STAT3-independent pathways utilized by IL-10, particularly at early timepoints.

### 1.6.2 IL-10 biological activity

The biological activity of IL-10 is essential for regulating both the extent and duration of an inflammatory response. Due to their high expression of IL-10R1, macrophages are generally considered to be the immune cell that is most sensitive to the effects of IL-10. IL-10 is able to potently inhibit the production of cytokines (e.g. IL-1α, IL-1β, IL-6, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, PAF)\(^ {224,253-256}\), chemokines (e.g. MCP1, MIP1α, RANTES, IL-8, KC)\(^ {257-260}\) and other soluble factors (e.g. prostaglandin E2, MMP2, MMP9)\(^ {261-267}\). Additionally, IL-10 can alter the expression of molecules on the surface of macrophages. For example, it has been demonstrated that treatment of macrophages with IL-10 results in reduced expression of molecules involved in antigen presentation including MHCII and co-stimulatory molecules CD80 and CD86\(^ {268-271}\). IL-10 has also been reported to inhibit the surface expression of the LPS receptor TLR4\(^ {272}\), as well as CD54 (aka ICAM-1)\(^ {271}\) which is necessary for establishing cell-cell contacts between monocytes and endothelial cells during immune cell transmigration. In contrast, IL-10 enhances the expression of CD16 and CD64 Fcγ-receptors (FcγR)\(^ {255,273,274}\), which promotes the phagocytic uptake of immunoglobulin (Ig)-opsonized microbes.

#### 1.6.2.1 IL-10’s transcriptional and post-transcriptional regulation of TNFα

IL-10’s regulation of TNFα production is an area of intense research and at the molecular level, still not entirely clear. IL-10 has been reported to regulate TNFα at both
transcriptional \textsuperscript{275,276} and post-transcriptional levels \textsuperscript{157,277,278}. With the current belief that STAT3 is required for mediating all activities of IL-10, much research has been invested in characterizing the gene products upregulated by IL-10 treatment. IL-10 is known to induce expression of Suppressor of Cytokine Signaling 1 and 3 (SOCS1 and SOCS3) in a STAT3-dependent manner \textsuperscript{235,248,269}. Both these proteins have been suggested to negatively regulate LPS-induced TNF\(\alpha\) production directly by targeting and inhibiting components of the TLR4 signaling pathway \textsuperscript{249,279} thereby preventing activation of pro-inflammatory transcription factors, as well as indirectly, by suppressing pathway components of the potentiating IFN\(\alpha\) and IFN\(\gamma\) signaling pathways \textsuperscript{280-282}. Tyrosine-protein phosphatase non-receptor Type 1 (PTPN1) is another gene upregulated by IL-10 treatment, which has been proposed to inhibit LPS-induced TNF\(\alpha\) production due to its broad-spectrum phosphatase activity \textsuperscript{283,284}. IL-10 has also been reported to induce the expression of transcriptional repressors such as Bcl-3 \textsuperscript{246,247}, ETV3, and SBNO\textsubscript{2} \textsuperscript{245}. Bcl-3’s ability to associate with the p50 subunit of NF\(\kappa\)B has been proposed as the mechanism by which it mediates IL-10’s inhibition of TNF\(\alpha\) production. By recruiting transcriptionally inactive p50 homodimers to the promoter of TNF\(\alpha\), Bcl-3 may prevent the recruitment of transcriptionally functional NF\(\kappa\)B p50/p65 heterodimers \textsuperscript{246,247,285,286}. ETV3, which associates with the helicase co-repressor DDX20, and SBNO\textsubscript{2}, which contains a DExD/H helicase domain, are suggested to modify the chromatin structure of the TNF\(\alpha\) promoter, thus inhibiting its transcription \textsuperscript{245}. 

There are also indications that IL-10 is able to regulate TNF\(\alpha\) production via post-transcriptional mechanisms. The TNF\(\alpha\) mRNA contains AU-rich elements (AREs) in its
3′- untranslated region (UTR). AREs confer a propensity for degradation unless the mRNA is stabilized by signals such as LPS stimulation \(^{287}\). IL-10 has been reported to inhibit translation \(^{157}\) and stability \(^{278}\) of TNFα mRNA by targeting the AREs. By inhibiting the p38 MAPK/MK2 signaling axis, IL-10 is proposed to inhibit: the stabilizing effect of ARE binding proteins (AREBPs), such as TIA-1 and HuR, and the AREBP-mediated recruitment of TNFα mRNA to actively translating polysome complexes \(^{157,277,288}\). IL-10 has also been shown to induce the expression of the mRNA destabilizing factor, tristetraprolin (TTP) \(^{278}\) in a STAT3 dependent manner. The multiple points of IL-10 regulation are depicted in Figure 1.7.
Figure 1.7 IL-10 inhibition of the TLR4 signaling pathway and TNFα production.

IL-10 has been reported to inhibit LPS-induced pathways at a number of points (indicated by red stars). IL-10 and LPS both induce the expression of SOCS1 and SOCS3, which are capable of targeting proteins downstream of TLR4 (e.g. IRAK4) and IFNγR (e.g. STAT1/2) for proteasomal degradation. IL-10 has also been reported to inhibit activation of p38 MAPK thereby preventing translation of LPS-induced pro-inflammatory proteins. Additionally, IL-10-induced activation of phosphatases such as Protein tyrosine phosphatase nonreceptor Type I (PTPN1) and SHIP1 are capable of antagonizing the PI-3 kinase pathway, preventing activation of NFκB and transcription of pro-inflammatory mediators. IL-10 has also been proposed to regulate LPS-induced pro-inflammatory mediators at the post-transcriptional level by influencing the proteins that regulate mRNA stability and decay, such as TIA-1, TTP and other AREBPs. IL-10 can additionally upregulate the expression of various transcription factors such as Bcl-3, c-Maf and B-ATF, which can compete with NFκB for promoter binding. IFN-R, interferon-receptor, STAT, signal transducer and activator of transcription, SOCS, suppressor of cytokine signaling, TLR, Toll-like receptor, CD, cluster of differentiation,
1.6.3 IL-10 in disease

Due to its potent anti-inflammatory actions, defects giving rise to either increased or decreased activity of IL-10 are associated with disease. Through evolution, many pathogens have developed mechanisms of exploiting IL-10’s activity of inhibiting the immune system in order to establish a state of chronic infection. *M. tuberculosis* \(^{289,290}\), *Candida albicans* \(^{291}\), *Shistosoma mansoni* \(^{292}\), *Toxoplasma gondii* \(^{293,294}\), *Leishmania major* \(^{295}\) and lymphocytic choriomeningitis virus \(^{296}\) are all infectious pathogens that have been demonstrated to manipulate the increased production of IL-10 from macrophages and DCs to prevent immune clearance of the infectious pathogen. Of note, several viruses including Epstein-Barr virus and human cytomegalovirus are capable of producing viral IL-10 (vIL-10), which mimics the biological activity of mammalian IL-10 and can inhibit immune activation \(^{220,297}\).

1.6.3.1 IL-10 in inflammatory bowel disease

The vital importance of IL-10 in maintaining proper homeostasis is underscored by genome-wide association studies that have identified polymorphisms in both the IL-10 and IL-10R loci \(^{298,299}\), which confer an increased susceptibility to the development of IBD. These observations are consistent with the phenotype of the *IL10*\(^{-/-}\) and *IL10rb*\(^{-/-}\) mice which spontaneously develop colitis \(^{234,300}\). IL-10 is essential for the negative regulation of mucosal inflammation since it inhibits the infiltration and activation of leukocytes, suppresses the production of pro-inflammatory cytokines from the gut-resident immune cells, and prevents epithelial cell damage. Further, characterization of the phenotype of *IL10*\(^{-/-}\) mice revealed that the lack of IL-10 fails to suppress the production of the key cytokine, IL-23, from macrophages and DCs in the gut in response
to intestinal flora\(^\text{301}\). IL-23 is then thought to mediate many of the pathogenic responses leading to colitis. IL-23 induces the expression of IL-17 and IL-6, which promote the development of pathogenic T\(_{H}17\) cells.

While recombinant IL-10 has shown some efficacy in treating IBD in animal models\(^\text{302,303}\), treating human IBD using this strategy has not been as successful\(^\text{304}\). Several reasons for this difference have been postulated. It is possible that systemic delivery of recombinant protein is insufficient for achieving high enough concentrations in the gut in order for IL-10 to perform its anti-inflammatory action. To address these issues, researchers have investigated the use of lactobacilli modified to express human IL-10 as a novel means of protein delivery with indications that biologically functional levels of IL-10 are achieved to reduce Crohn’s disease activity index scores\(^\text{305}\). There has also been a report to suggest that systemic administration of recombinant IL-10 stimulates a pro-inflammatory response marked by an increase in IFN\(_{\gamma}\) levels\(^\text{306}\). Furthermore, patients who harbor specific single nucleotide polymorphisms in the IL-10R may be unresponsive to IL-10 treatment\(^\text{299}\).

### 1.7 The PI-3 kinase pathway

The phosphoinositide 3’-kinases (PI-3 kinases) are a group of enzymes, which phosphorylate membrane phosphatidyl inositol lipids (PIs) at the 3’-hydroxyl position. The resulting 3’-PI products then act as second messengers to recruit proteins containing lipid interacting domains such as pleckstrin homology (PH) and phox homology (PX) domains. Once localized at the membrane, these effector proteins become activated and initiate signaling cascades that mediate a broad range of cellular functions including
cellular activation, proliferation, growth, and motility. Defects in PI-3 kinase pathway signaling have been implicated in a variety of diseases including cancer, inflammatory diseases, cardiovascular diseases and metabolic disorders (reviewed in 307,308).

There are 8 PI-3 kinase isoforms that are categorized into 3 classes based upon their PI substrate specificities and their domain structure. The class I PI-3 kinases are further sub-categorized into Class IA, which includes p110α, p110β and p110δ, and Class IB, comprised of p110γ. Each of these PI-3 kinase catalytic subunits are associated with a regulatory subunit, p85 and p101 for Class IA and Class IB respectively. While all Class I isoforms are expressed in mammalian cells, p110δ and p110γ exhibit particularly enriched expression in immune cells (reviewed in 307-312). Class II and Class III PI-3 kinases are less well characterized and their biological roles yet to be clearly defined. Class II PI-3 kinases have been reported to be activated downstream of integrins and certain growth factor and chemokine receptors. Class III PI-3 kinase exclusively generates PI-3-P as its product and has been reported to have roles in vesicular trafficking (reviewed in 307-312).

1.7.1 The PI-3 kinase pathway in inflammation

The PI-3 kinase pathway is known to have important roles in the immune system, however, delineating its contribution to various cellular responses has been difficult for a number of reasons. Firstly, the PI-3 kinase pathway appears to be activated by nearly all receptors on immune cells, which poses the challenging question of how such a commonly used signaling pathway can give rise to such a diverse array of biological activities in response to different stimuli. Secondly, different PI-3 kinase isoforms are activated depending on the cell type and stimulus and may signal in isolation or in
parallel with other isoforms in a redundant or non-redundant fashion. Thirdly, the tools that we have used to study the PI-3 kinase pathway have confounded our understanding due to issues with specificity, in the case of small molecule PI-3 kinase inhibitors \cite{313-315}, and compensatory actions of other PI-3 kinase isoforms, in the case of isoform-specific knock-out models \cite{308,316}.

These difficulties have contributed towards the debate regarding the pro- or anti-inflammatory effects of LPS-triggered PI-3 kinase. Several groups have demonstrated with the use of inhibitors or PI-3 kinase isoform-specific knockout cells that the PI-3 kinase pathway positively contributes to cellular activation \cite{317-325}. In contrast, other groups, primarily through the use of pan-PI-3 kinase inhibitors, have reported that the PI-3 kinase pathway attenuates TLR4-induced activation \cite{326-337}. One potential source for these discrepancies is the contributing effects of autocrine IL-10 production; these would vary according to which cell types are used and the timepoint at which indices of inflammation and activation are being assessed. Studies in our lab and others have observed that in vitro cultured BMDMs are high producers of IL-10 in comparison to more mature macrophages such as peritoneal elicited macrophages or the RAW264.7 or J774.1 cell lines \cite{247}. It is possible that the autocrine effects of these high IL-10 levels could alter both the basal activation and responsiveness of the PI-3 kinase pathway downstream of TLR4 stimulation. The timepoint at which measurements are made – the time post-LPS stimulation that supernatant TNFα-levels are measured, for example – is also a critical parameter to consider when interpreting the role of the PI-3 kinase pathway in TLR4 signaling. LPS stimulation, in addition to inducing the production of pro-inflammatory cytokines like TNFα, IL-1β, and IL-12, also induces the expression of IL-
Thus, it is possible that the effects of autocrine IL-10 could confound measurements of pro-inflammatory cytokine production or macrophage activation taken past 2 hours of LPS stimulation and mask the positive role of the PI-3 kinase pathway in LPS signaling. The concentration of LPS used in experiments is another possible source for the controversy. Data from our lab suggests that macrophages respond quite differently in response to low amounts versus supra-physiological amounts of LPS, which may induce additional signaling pathways that are not biologically relevant in vivo or render cells less capable of activating the PI-3 kinase pathway in response to TLR4 agonist. The specificity of the inhibitors used to evaluate the role of the PI-3 kinase pathway must also be considered as a source for the differing results. Wortmannin and LY-294002 have predominantly been used to investigate PI-3 kinase contributions to TLR signaling. It has been shown that both inhibitors have off target effects and the advent of Class I isoform-specific PI-3 kinase inhibitors, knockout animals, and siRNA targeting strategies has demonstrated that broad-spectrum inhibitors can mask the biological role of the individual isoforms. Of note, a report from Kevan Shokat’s group has highlighted the anti-inflammatory actions of PI-3 kinase γ, δ, and dual γ/δ inhibitors and has suggested that additional inhibition of other isoforms may decrease the inhibitory capacity of these compounds. However, despite the advent of these improved isoform-specific tools, controversy still exists in the literature with conflicting reports attributing both positive and negative roles of particular PI-3 kinase isoforms to immune cell activation.
1.8 SHIP1

The PI-3 kinase pathway is tightly controlled in cells by mechanisms regulating PI-3 kinase enzyme activation as well as the activity of the PIP₃ metabolizing enzymes phosphatase and tensin homolog (PTEN), which degrades PIP₃ into PI-4,5-P₂, and SH2 domain-containing inositol 5’-phosphatases 1 and 2 (SHIP1 and SHIP2), which degrade PIP₃ into PI-3,4-P₂. Of these inositol phosphatases, SHIP1 is of particular interest with regards to immune regulation as it has been suggested that its activity is elicited only in the context of immune cell stimulation, whereas PTEN is believed to have constitutive, low-level enzymatic activity (reviewed in 343). Further, unlike PTEN and SHIP2, which exhibit ubiquitous expression in virtually all cell types, SHIP1’s expression is predominantly restricted to hemopoeitic cells making it an attractive target for the development of immune-cell specific anti-inflammatory therapeutics.

3 independent research groups first cloned SHIP1 in 1996 as a 145 kDa protein that interacted with the Shc adaptor protein following cytokine and growth factor stimulation of immune cells 344-346. SHIP1 contains an amino-terminal SH2 domain, a 5’-phosphatase domain, and a carboxy-terminal proline rich region (PRR) containing 2 NPXY motifs. The central phosphatase domain performs SHIP1’s enzymatic activity of degrading PIP₃ to PI-3,4-P₂. SHIP1 has been demonstrated to specifically degrade inositol-1,3,4,5-tertrakisphosphatase (IP₄) in vitro 347 and PIP₃ in vitro and in vivo 348. SHIP1’s SH2 domain facilitates interactions with various tyrosine-phosphorylated proteins including FcεRI, FcγRIIa, and CD3 via immune receptor tyrosine-based activating motifs (ITAMs) 349-351, FcγRIIb via immune receptor tyrosine-based inhibition motifs (ITIMs) 352-355, Shc and Grb2 associated proteins (GABs) 344,345,356-358. Likewise,
SHIP1’s PRR mediates interactions with numerous proteins capable of binding to its phosphorylated NPXY motifs including Shc, Doks 1 and 2, Grb2, and the p85α regulatory subunit of PI-3 kinase \(^{356,359-362}\). The domain structure of SHIP1 and its enzymatic reaction are illustrated in Figure 1.8. In addition to the 145 kDa SHIP1 protein, SHIP1 variants have been reported that are generated via alternative splicing mechanisms or truncations, which effectively remove either the SH2 domain or PRR \(^{361-365}\). Of note, a 104 kDa s-SHIP is also generated by transcription from an internal promoter between exons 5 and 6 of the SHIP1 gene. This isoform lacks an SH2 domain and has been suggested to be necessary for pluripotent stem cell growth and survival \(^{366-369}\).

While SHIP1 expression is restricted to hemopoietic cell lineages, there is evidence that the degree of SHIP1 expression can differ depending on the cell type and that these levels can contribute greatly to the biological function of different immune cell subsets. SHIP1 expression can be regulated at multiple levels. The SMAD transcription factors have been implicated in the transcriptional regulation of SHIP1 expression \(^{347,370}\). Sly et al. have further demonstrated that LPS can induce SHIP1 expression via the autocrine production of TGFβ \(^{371}\). Additionally, SHIP1 levels are subject to post-transcriptional regulation by microRNAs, particularly mIR-155 \(^{372-376}\), as well as post-translational control by proteosomal degradation \(^{377}\).
Figure 1.8  The domain structure of SHIP1 and its enzymatic reaction.

SHIP1 is structurally comprised of an N-terminal SH2 domain, a central phosphatase domain and a C-terminal proline rich region containing NPYX and PXXP motifs. PI-3 kinase catalyzes the conversion of PI-4,5-P$_2$ to PIP$_3$. The lipid phosphatasles PTEN and SHIP1 antagonize PI-3 kinase activity by degrading PIP$_3$ into PI-4,5-P$_2$ and PI-3,4-P$_2$ respectively.
1.8.1 SHIP1’s biological activity

SHIP1 is a negative regulator of immune cell signaling. By opposing the PI-3 kinase pathway, SHIP1 had been demonstrated to inhibit the membrane recruitment and activation of Tec kinases \(^{378-383}\), Akt, and PLC\(\gamma\). It has also been shown to inhibit the Erk, Jnk, and p38 MAPK pathways and the activation of transcription factors such as NF\(\kappa\)B and NFAT \(^{382,384-389}\). As well, SHIP1 negatively regulates immune cell activation by virtue of its ability to act as an adaptor protein \(^{381,390,391}\). For example, by acting as an adapter between the inhibitory Fc\(\gamma\)RIIb and SHP-2, SHIP-1 is able to negatively regulate the formation of the PI-3 kinase signaling complex by dephosphorylating Gab1 \(^{390}\). SHIP1 can also function by blocking the recruitment of other signaling enzymes such as SHP-1 to the 2B4 receptor in NK-cells and PI-3 kinase recruitment to DAP10 and DAP12 in osteoclasts and BMDMs \(^{82,392,393}\).

1.8.1.1 SHIP1’s role in myeloid cells

Much of our knowledge with regards to SHIP1’s role in different immune cell lineages has been derived from experiments using SHIP1\(^{+/−}\) mice and conditional SHIP1 knock-outs. The phenotype of SHIP1\(^{+/−}\) mice includes a shortened life-span, a Paget’s-like osteoporosis, splenomegaly, and an asthma-like syndrome (reviewed in \(^{394}\)). These pathologies can be attributed to the profound defects in the myeloid cell compartment in germline SHIP\(^{+/−}\) mice. Enhanced osteoclast proliferation and activity results in accelerated bone resorption, myeloid proliferation in the bone marrow causes extramedullary hemopoiesis in the spleen, and myeloid cells accumulate in the lungs of SHIP1\(^{+/−}\) mice impairing respiratory function due to increased fibrosis and deposition of Ym1 crystals \(^{395}\).
SHIP1 is also involved in regulating myeloid cell phagocytic activity where it inhibits FcγR and CR3-mediated phagocytosis in macrophages. Interestingly, a report by Tiwari et al. demonstrated that SHIP1-mediated generation of PI-3,4-P₂ was necessary for phagosome maturation and uptake of *M. tuberculosis* via the recruitment of the GTPase, Irgm1. These results, in addition to other reports suggest that while SHIP1’s overall net effect may be the negative regulation of phagocytosis, its presence at the phagocytic cup may be necessary for the proper formation and membrane sealing of the mature phagosome.

SHIP1⁻/⁻ mice are also hypersensitive to immune stimulation such as when they are challenged with bacterial LPS. SHIP1⁻/⁻ macrophages produce elevated levels of IFNβ, IL-1β, IL-6, TNFα and reactive oxygen species. This phenotype led to the discovery that SHIP1 is necessary for establishing endotoxin tolerance by inhibiting the production of IFNβ and arresting the propagation of the pro-inflammatory response. Rauh et al. also made the discovery that SHIP1⁻/⁻ macrophages are skewed towards an alternatively activated (or M2) phenotype, suggesting that SHIP1 is necessary for suppressing the development of these so-called “healing” macrophages. Reports from William Kerr’s lab have observed the expansion of a similar macrophage subset in SHIP⁻/⁻ mice that they referred to as “myeloid immuno regulatory” cells or MIR, which were able to suppress Th1 responses in Graft-Versus-Host Disease. Weisser et al. have also demonstrated that SHIP1⁻/⁻ M2 macrophages confer protection against an experimental model of colitis induced by dextran sodium sulfate (DSS).

It is worthy of mention that two independent groups have generated LysMCre conditional SHIP1 KO mice and have reported differing phenotypes. In a review, Kerr
reports unpublished findings that LysMCre SHIP1\(^{-/-}\) mice do not exhibit a myeloproliferative disorder, which suggests that the expansion of myeloid cells observed in germline SHIP1 KO cells is due to disturbances in the stromal microenvironment and myeloid growth factor production\(^{405}\). Leung et al., however, observe that their LysMCre SHIP1\(^{-/-}\) mice suffer from a milder myeloproliferative disease as compared to germline SHIP1 KO mice and that the T-cells are T\(_{H17}\) skewed due to the increased levels of IL-6 being produced by SHIP1-deficient macrophages\(^{406}\). Leung et al. also note that their LysMCre SHIP1\(^{-/-}\) lack suppressor macrophage cells\(^{406}\). Further studies will need to be performed in order to identify the source of these apparently disparate phenotypes.

1.8.1.2 SHIP1’s role in other immune cells

*In vitro* studies in T-cell lines have shown that SHIP1 inhibits T-Cell receptor (TCR) signaling but unlike the behaviour of SHIP1\(^{-/-}\) myeloid cells, enhanced T-cell proliferation is not observed in germline SHIP1\(^{-/-}\) mice\(^{382,407-412}\). Kashiwada et al. reported elevated levels of CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\) regulatory T-cells (T\(_{reg}\)) in germline SHIP1\(^{-/-}\) mice, however, studies using mice with T-cell restricted SHIP1 deletion have suggested that the increased T\(_{reg}\) population is not attributable to inherent T-cell defects but via a myeloid cell intermediate\(^{405,406,413}\).

SHIP1 has been shown to be necessary for Fc\(\gamma\)RIIB inhibition of activating signals initiated by immune complex binding to the B-Cell Receptor (BCR)\(^{387,414}\). Germline SHIP1\(^{-/-}\) mice show reduced numbers of circulating B-Cells, spontaneous formation of germinal centres and increased isotype switching towards low-affinity receptors\(^{410,415}\). These phenotypes were also observed in CD19cre conditional KO mice but like the T-cell compartment, there does not appear to be any intrinsic defect in B-cell
development in the bone marrow. Rather, reduced circulating B-cell numbers are attributed to alterations in CD22-mediated homing of IgM⁺IgD⁺ cells from the spleen to the bone marrow. SHIP1 has been reported to directly interact with CD22 ITIMs.

Work by Kerr et al. have demonstrated a significant role of SHIP1 in the signaling pathways downstream of NK cell inhibitory receptors such as 2B4/CD244. In SHIP1−/− mice, there is increased expression of 2B4 that predisposes NK cells to a hypo-responsive state. SHIP1−/− NK cells also exhibit impaired production of IFNγ. Together, these effects impair NK cell mediated rejection in MHC-I mismatched bone marrow reconstitution experiments leading the authors to suggest SHIP1 knockdown as a potential strategy for bone marrow transplant therapy.

In neutrophils, Nishio et al. have demonstrated that SHIP1 is required for the coordination of polarization and motility during chemotaxis in response to fMLP. SHIP1 has been shown to accumulate at the leading edge of the migrating neutrophil where the accumulation of PIP₃ and PI-3,4-P₂ is coupled to cytoskeletal rearrangements and actin polymerization.

By opposing the PI-3 kinase pathway, SHIP1 has also been demonstrated to inhibit mast cell proliferation in response to FcεRI and cytokine stimulation in vitro. Reconstitution of mast cell-deficient mice with wildtype and KO SHIP1 mast cells has further demonstrated that loss of SHIP1 in mast cells results in an enhanced anaphylactic allergic response characterized by mast cell hyperplasia and elevated levels of IL-6, TNFα and IL-5.
Two studies by Kuroda et al. have elegantly described the role of SHIP1 in basophils \(^{424,425}\). By suppressing IL-3 activated PI-3 kinase, particularly the p110\(\alpha\) isoform, SHIP1 inhibits the production of IL-4 from basophils, which is capable of skewing macrophages and T-cells towards M2 and T\(_{H2}\) phenotypes respectively.

The multiple roles of SHIP1 in immune cells are described in Table 1.2.

**Table 1.2:** The roles of SHIP1 in immune cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes/Macrophages</td>
<td>Negatively regulates M-CSF induced proliferation</td>
<td>405,426</td>
</tr>
<tr>
<td></td>
<td>Inhibits Fc(\gamma) and CR3-mediated phagocytosis</td>
<td>396,397</td>
</tr>
<tr>
<td></td>
<td>Required for phagosome maturation</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>Negatively regulates oxidative burst</td>
<td>397,400</td>
</tr>
<tr>
<td></td>
<td>Inhibits pro-inflammatory cytokine production</td>
<td>397</td>
</tr>
<tr>
<td></td>
<td>Required for endotoxin tolerance</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>Required for development of marginal zone macrophages</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>Required for development of myeloid suppressor cells</td>
<td>402,403,427</td>
</tr>
<tr>
<td>T-Cells</td>
<td>Negatively regulates TCR stimulation</td>
<td>407,408,428</td>
</tr>
<tr>
<td></td>
<td>Negatively regulates Treg development</td>
<td>413,429</td>
</tr>
<tr>
<td></td>
<td>Promotes T(_{H17}) development</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>Suppresses T(_{H1}) skewing</td>
<td>406,431</td>
</tr>
<tr>
<td>B-Cells</td>
<td>Inhibits Fc(\gamma)RIIb signaling</td>
<td>355,414,417</td>
</tr>
<tr>
<td></td>
<td>Contributes to the positive selection of high-affinity receptor B-Cells</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>in germinal centres</td>
<td>446,416,412</td>
</tr>
<tr>
<td></td>
<td>Participates in CD22-mediated homing of IgM(^+)IgD(^-) cells from spleen to bone marrow</td>
<td>413,434</td>
</tr>
<tr>
<td>NK Cells</td>
<td>Necessary for CD16-mediated cytotoxicity</td>
<td>369,392,435</td>
</tr>
<tr>
<td></td>
<td>Required for NKG2D and Ly49H-mediated cytotoxicity</td>
<td>420</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Involved in establishing polarization necessary for chemotaxis</td>
<td>379,380,415,421</td>
</tr>
<tr>
<td>Mast Cells</td>
<td>Negatively regulates IgE-induced degranulation</td>
<td>424,425</td>
</tr>
<tr>
<td>Basophils</td>
<td>Inhibits IL-4 production</td>
<td></td>
</tr>
</tbody>
</table>
1.8.2 SHIP1 in disease

Mutations or altered expression of SHIP1 have been implicated in a number of human disorders. In acute myelogenous leukemia (AML) and acute lymphoblastic leukemia inactivating mutations have been identified in the phosphatase domain and proline-rich regions of SHIP1 suggesting that SHIP1 functions as a tumour suppressor in hemopoietic progenitor cells \(^{436}\). SHIP1 has also been reported to be constitutively phosphorylated and associated with Shc in CD34\(^+\) chronic myelogenous leukemia (CML) progenitors \(^{437}\). Further, inverse relationships have been noted between oncogenic BCR-ABL and KIT\(^{K641E}\) with SHIP1 indicating that reduced SHIP1 levels may be a requirement for tumour progression in CML and AML \(^{438,440}\). Heterozygosity at the 2q36 locus, the chromosomal location of SHIP1, has also been reported in a subset of patients with familial Paget-like osteoporosis supporting the animal model observations that reduced levels of SHIP1 results in increased bone resorption due to enhanced activity and proliferation of osteoclasts \(^{441}\). Patients with chronic idiopathic urticaria (hives) exhibit reduced expression of SHIP1 in their basophils, which potentiates Fc\(\varepsilon\)RI mediated histamine release and allergic responses \(^{442,443}\). SHIP1 levels are elevated in the oral mucosa of patients with chronic periodontitis, which the authors of the study propose is a compensatory mechanism to induce tolerance towards the Gram negative bacteria in the dental plaques \(^{444}\). In knockout mouse models, deficiency of SHIP1 has been implicated in the development of inflammatory bowel disease \(^{445,446}\). However, Arijs et al. have reported a significantly increased level of SHIP1 mRNA expression and SHIP1 immunohistological staining in mucosal biopsies taken from IBD patients with active
Whether these increased levels contribute to disease pathogenesis or are a compensatory reaction to increased gut inflammation has yet to be determined.

1.9 Phagocytosis

Phagocytosis is the cellular ingestion of particles greater than 0.5 µm in diameter. The phagocytic process functions in two major capacities: 1) microbial killing as microbes are digested through successive phagosome/phagolysosome stages and 2) antigen presentation to cells of the adaptive immune system. Various cell surface receptors, signaling pathways, and cytoskeletal proteins are known to be involved in phagocytosis but how all these components are coordinated to achieve particle ingestion is still an area of active research. Numerous cell surface receptors are capable of initiating phagocytosis including the complement receptor 3 (CR3), the mannose receptor, scavenger receptors AI and AII, and Dectin I but FcγR-mediated phagocytosis has been the most thoroughly characterized (reviewed in 448-450).

1.9.1 FcR-mediated phagocytosis

Opsonization of particles with immunoglobulins (Ig) marks them for rapid uptake and clearance by professional phagocytes such as neutrophils and macrophages. The conserved Fc domain of the immunoglobulin molecule binds to its cognate Fc-receptor, initiating signaling pathways that trigger the formation of actin-dependent pseudopodial extensions around the particle being ingested as well as the coupled production of reactive oxygen species and inflammatory cytokines 451,452. While the less abundant IgA and IgE immunoglobulins are capable of initiating phagocytosis 453,454, IgG is the primary opsonin, which is capable of binding the five isoforms of the FcγR: FcγRI (CD64), FcγRIIa (CD32), FcγRIIb1, FcγRIIb2, and FcγRIII (CD16) on the surface of phagocytes.
All the FcγRs can support phagocytosis with the exception of FcγRIIb, which negatively regulates phagocytosis and phagocyte activation. Upon ligand binding, FcγRs cluster within cholesterol-rich microdomains and are rapidly phosphorylated within their ITAMs by Src family kinases. Phosphorylated ITAMs then serve as docking sites for Syk. Syk, in turn, facilitates the recruitment of the Grb2 and Gab2 adaptor proteins, PLCγ1 and the Class I PI-3 kinases. Generation of PIP3 by Class I PI-3 kinases, recruits a number of downstream signaling proteins involved in actin cytoskeleton rearrangement including the Rho family GTPases Cdc42 and Rac1. Cdc42 localizes to the tips of advancing pseudopodia and stimulates localized actin polymerization through interactions with WASP. When in its GTP-bound form, Rac activates a number of downstream proteins including Pak1, p67phox, WAVE and PI-4P-kinase, which collectively contribute towards phagosome closure.

Coupled to the activation of the cytoskeletal machinery are dynamic changes in the lipid composition at the phagocytic cup. PI-4P-kinase, PI-3 kinase, PLA2, PLC, PLD, and SHIP1 are lipid modifying enzymes whose products have been demonstrated to regulate the phagocytic process either by coordinating actin polymerization or the rate at which phagocytosis occurs (Figure 1.9).
Figure 1.9  FcγR-mediated phagocytosis.

Binding of opsonized particles causes clustering of FcγRs and phosphorylation of ITAM motifs within their cytoplasmic domains. Src family kinases and Syk are recruited and activated, which then initiate a number of downstream signaling pathways including activation of PLCγ, PLD, PKC and PI-3 kinase. These pathways promote actin reorganization and polymerization, which is required for particle uptake and/or contributes to regulating the rate at which phagocytosis occurs. Ig, immunoglobulin, ITAM, immunoreceptor tyrosine-based activation motif, FcγR, Fcγ-receptor, PL, phospholipase, IP₃, inositol-trisphosphate, DAG, diacylglycerol, PA, phosphatidic acid, PAP1, PA-phosphatase 1, AA, arachidonic acid, PI3K, Phosphoinositide 3’-kinase, CDC42, cell division control protein 42, ERK, extracellular signal-regulated kinase, MLCK, myosin light chain kinase. Adapted by permission from The Journal of Leukocyte Biology: Garcia-Garcia, E. & Rosales, C. Signal transduction during Fc receptor-mediated phagocytosis. J Leukoc Biol 72, 1092-1108 (2002). Copyright © (2002) 454.
1.10 Objectives and aims

The overall objective of this thesis was to further characterize the molecular mechanisms regulating SHIP1 activity and its contributions to the inhibition of macrophage activation and function. Previous preliminary studies from our lab identified that treatment of macrophages with IL-10 resulted in a rapid increase in SHIP1 tyrosine phosphorylation. This increase in phosphorylation correlated to enhanced SHIP1 phosphatase activity. We hypothesized that in addition to the canonical STAT3-mediated anti-inflammatory response, IL-10 is also capable of signaling through SHIP1 in a STAT3-independent manner to inhibit the production of pro-inflammatory cytokines such as TNFα. We also hypothesized that unlike STAT3-dependent signaling, which requires de novo gene transcription, signaling though SHIP1 would be more immediate and serve to inhibit the early phases of pro-inflammatory cytokine production. Work from our lab also identified small molecule SHIP1 agonists that were able to enhance SHIP1 activity in in vitro phosphatase assays. We hypothesized that these small molecule activators could be mimicking IL-10 activation of SHIP1 and sought to determine the mechanism by which these compounds regulated SHIP1 phosphatase activity.

The first aim of my thesis was to characterize IL-10’s SHIP1-dependent regulation of TNFα production. Work performed by past students in our lab suggested that IL-10 was able to regulate TNFα mRNA post-transcriptional association with polysomes in a SHIP1-dependent manner. However, we specifically asked whether SHIP1 was able to mediate IL-10’s negative regulation of TNFα production at the transcriptional level. The second aim of my thesis was to characterize the mechanism by
which small molecule activators of SHIP1 enhanced its phosphatase activity and the resulting impact on macrophage activation. Further, we wanted to determine the efficacy of these drugs in treating a number of inflammatory disorders using *in vivo* murine models. Through bioinformatic and structural analyses of how the SHIP1 activators stimulated enzyme activity, we identified two previously unrecognized structural domains flanking SHIP1’s phosphatase domain. The final chapter of my thesis aims to characterize the structure and function of the newly identified domain amino-terminal of the catalytic phosphatase domain. In particular, we investigated its potential role in regulating SHIP1 activity by facilitating SHIP1’s membrane recruitment upon macrophage activation. Together, the findings from this thesis lend deeper insight into the function of SHIP1 in macrophages and highlights that SHIP1 is subject to more mechanisms of regulation than has been previously appreciated. In addition, we have demonstrated that small molecule activators of SHIP1 can be used to mimic the activity of IL-10 and inhibit inflammation *in vivo*. 
CHAPTER 2: SHIP1 IS REQUIRED FOR MEDIATING IL-10’S ANTI-INFLAMMATORY RESPONSE
2.1 Introduction

IL-10 limits the magnitude and duration of the inflammatory response and loss of normal levels of IL-10 production or defects in its signaling results in immune dysfunction and inflammatory diseases such as colitis. These defects are clearly evident as was first observed in IL-10 deficient mice, which develop spontaneous colon inflammation in response to normal gut flora. In humans, polymorphisms in the IL-10 gene are associated with ulcerative colitis, while homozygous loss-of-function mutations in the IL-10R subunits results in early onset colitis. Thus, understanding the mechanism by which IL-10 exerts its action on target cells may provide insight for the development of therapeutics to treat inflammatory disease.

One of the core *in vivo* functions of IL-10 is inhibition of macrophage activation and it is the loss of IL-10 inhibition of myeloid cells, such as macrophages, which contributes most to the development of colitis in IL-10 mice. The IL-10R in macrophages consists of a ligand-specific IL-10R1 subunit and a second subunit, IL-10R2, which is also found in other cytokine receptor complexes. Analysis of intracellular signaling pathways downstream of the receptor has lead to the suggestion that activation of the STAT3 transcription factor and expression of STAT3-regulated gene products is sufficient to mediate the anti-inflammatory actions of IL-10. However, as detailed in this chapter, we now show that LPS-stimulated activation of PI-3 kinase pathway signaling in macrophages is additionally countered by IL-10 activation of the SHIP1 inositol phosphatase. SHIP1 is expressed predominantly in hemopoietic cells and negatively regulates PI-3 kinase signaling by dephosphorylating the PI-3 kinase product PIP3. Robust production of pro-inflammatory cytokines, such as TNFα, is one
of the hallmarks of TLR stimulation of macrophages. Using continuous flow cultures we found that LPS-induced TNFα is produced in two distinct phases, and that IL-10 differentially inhibits the first and second phases by signaling through SHIP1 and STAT3 respectively.

TNFα is a PRG, as defined and classified by Hargreaves et al. PRGs are a group of genes whose expression are quickly induced upon exposure to LPS. Rapid expression of PRGs is facilitated by virtue of the GC content and epigenetic modifications at their promoters, which maintain their permissivity and allow RNAPolII to be constitutively associated with them, even when cells are in a resting state. In un-activated cells, RNAPolII at PRG promoters is phosphorylated on Ser5 but not Ser2. Phosphorylation of Ser2 by the P-TEFb kinase complex is necessary for successful transcription elongation and proper processing of the primary transcript. We now show that IL-10 treatment reduces the amount of Ser2 phosphorylated RNAPolII bound to the TNFα promoter within minutes of IL-10 addition and that this is dependent on SHIP1. Furthermore, we found that IL-10 inhibited the expression of 17 out of the approximate 50 LPS-induced PRGs defined by Medzhitov’s group. These 17 include TNFα, other pro-inflammatory cytokines, chemokines, and transcription factors associated with macrophage activation. Remarkably, in SHIP1 deficient cells, IL-10 enhanced rather than inhibited the expression of the majority of these genes.
2.2 Materials and methods

2.2.1 Mice

Balb/c SHIP1\textsuperscript{+/+} and SHIP1\textsuperscript{−/−} mice (kindly provided by Dr. Gerald Krystal, BC Cancer Research Centre, Vancouver, B.C.) used for the experiments described in this chapter were housed and maintained in accordance with ethics protocols approved by the University of British Columbia Animal Care Committee.

2.2.2 Lentiviral constructs

Small interfering RNA (siRNA) constructs specifically targeting STAT3 and SHIP1, and a scrambled control siRNA sequence (Scrmb) were designed and generated using the BLOCK-iT RNAi Expression system (Invitrogen, Mississauga, ON) with a protocol modification whereby, using Invitrogen’s Gateway technology, the shRNA sequences were recombined into a modified form of the tetracycline-inducible pTRIPZ lentiviral vector (Thermo Fisher Scientific, Nepean, ON). Please refer to Figure A.1 in Appendices for a more detailed description of the lentiviral constructs and cloning strategy. A plasmid construct containing a modified form of human AKT was kindly provided by Dr. Megan Levings (University of British Columbia, Vancouver, B.C.). In this construct, the PH domain is removed from AKT, a src myristoylation signal sequence is added to the amino terminal end and the steroid binding domain of the estrogen receptor (ER) and a hemagglutinin tag are added to the carboxy terminal end\textsuperscript{481}. This AKT-ER sequence was sub-cloned into the pENTR-1A vector (Invitrogen, Mississauga, ON) and recombined into a modified lentiviral vector. VSV-pseudotyped second-generation lentiviruses were produced by transient 3-plasmid co-transfection into HEK293T cells and concentrated by ultracentrifugation.
2.2.3 Cells

RAW264.7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 9% fetal calf serum (FCS, Thermo Fisher Scientific, Nepean, ON). To generate stably transduced cell lines expressing SCRMB, STAT3, and SHIP1 siRNA constructs and AKT-ER, RAW264.7 cells were transduced with concentrated lentivirus in the presence of 8 µg/ml of protamine sulfate (Sigma, Burlington, ON). Transduced cells were selected according to the antibiotic resistances conferred by their respective lentiviral vectors or further purified by fluorescence activated cell sorting 24-hour doxycycline-treated cells for green-fluorescent protein (GFP) positive events. For experiments, siRNA lentivirus-transduced cells were treated with 2 µg/ml of doxycycline (Sigma, Burlington, ON) for 48 hours prior to stimulation. AKT-ER lentivirus-transduced cells were treated with 150 nM 4-hydroxy tamoxifen (4-HT, Sigma, Burlington, ON) prior to stimulation. For in vitro signaling and cytokine production experiments, primary cells from male and female Balb/c SHIP1+/+ or SHIP1−/− mice aged 6-12 weeks were isolated by peritoneal lavage with 3-5 ml of sterile phosphate buffered saline (PBS, Thermo Fisher Scientific, Nepean, ON). Cells were transferred to Iscove’s modified Dulbecco’s medium (Thermo Fisher Scientific, Nepean, ON), supplemented with 10% FBS, 10 µM β-mercaptoethanol, 150 µM monothioglycolate, and 1 mM L-glutamine, and allowed to adhere to tissue-culture treated plates for 4 hours in a 37°C, 5% CO2, 95% humidity incubator, prior to stimulation.
2.2.4 Immunoblot analysis of proteins

Cells plated at a density of $1.5 \times 10^6$ cells per well on a 6-well tissue culture plate were stimulated with 10 ng/ml of LPS (E. coli Serotype 0111:B4, Sigma, Burlington, ON) +/- 100 ng/ml IL-10 (Recombinant murine IL-10 protein was cloned, expressed, and purified in house). Cells were then lysed with 500 µl of Nonidet P-40 (NP-40) lysis buffer (50 mM HEPES, 2 mM EDTA, 1 mM NaVO₄, 100 mM NaF, 50 mM NaPP₄, 1% NP40, supplemented with Complete Protease Inhibitor Cocktail, Roche Diagnostics, Laval, QC). Lysates were rocked at 4°C for 30 minutes and clarified by centrifugation for 20 minutes at 12,000 G. Lysates were then made 1X in Laemmli buffer, boiled 3 minutes, loaded onto 7.5% SDS-polyacrylamide gels and run under constant voltage. Alternatively, proteins were extracted using TRIzol reagent (Invitrogen, Mississauga, ON) concurrently with total RNA preparation as per the manufacturer’s protocol. Resolved proteins were immobilized onto PVDF membrane (Millipore, Etobicoke, ON) using a semi-dry blotting apparatus (Biorad, Mississauga, ON), blocked with 3% bovine serum albumin (BSA, Sigma, Burlington, ON) in Tris-Buffered Saline, probed with specific protein or phospho-protein antibodies, detected with fluorescence-conjugated secondary antibodies, and developed using a Li-Cor Odyssey Infrared Imager (Lincoln, NE). Antibodies used in this chapter include: anti-Phospho AKT (Ser473), anti-AKT, anti-Phospho CDK9 (Thr186) purchased from Cell Signaling Technologies (Pickering, ON), anti-SHIP1 purchased from BD Biosciences (Mississauga, ON), anti-STAT3 purchased from Upstate Biotechnology (Lake Placid, NY), and anti-Actin purchased from Sigma (Burlington, ON).
2.2.5 Analysis of AKT phosphorylation by flow cytometry

Peritoneal macrophages (PMΦs) were treated with 10 ng/ml LPS +/- 100 ng/ml IL-10 for times indicated ranging between 10 and 30 minutes. Stimulation media was removed and cells were immediately washed in ice-cold PBS. Cells were incubated in Fixation/Permeabilization solution (eBioscience, San Diego, CA) for 30 minutes at 4°C, washed 2 times in permeabilization buffer (eBioscience, San Diego, CA), then incubated with anti-CD16/32 antibody (Fc Block, BD Biosciences, Mississauga, ON) for 30 minutes at 4°C. Cells were then incubated with anti-CD11b-PE and anti-Phospho AKT (Ser473)- FITC (BD Biosciences, Mississauga, ON) for 45 minutes at 4°C. Cells were washed 3 times in permeabilization buffer then samples were read on a BD FACS Canto and analyzed with FlowJo Version 8.7 (Ashland, OR).

2.2.6 Measurement of TNFα protein production

For standard stimulations, RAW264.7 cells were plated in a 24-well plate (2.5X10^5 cells per well in 500 µl volume) and incubated overnight at 37°C, 5% CO₂, 95% humidity in complete growth medium. Following overnight incubation, media was replaced with fresh growth medium and stimulated with 10 ng/ml of LPS +/- 10 ng/ml of IL-10. Cell supernatants were collected after 1, 2 and 24 hours post-stimulation and analyzed using a BD OptEIA Mouse TNFα Enzyme-Linked Immunosorbent Assay (ELISA) kit (BD Biosciences, Mississauga, ON). AKT-ER transduced cells were similarly plated and stimulated with the added modification that cells were treated with or without 150 nM of 4-hydroxytamoxifen (4-HT, Sigma, Burlington, ON) 20 minutes prior to LPS stimulation. SCRMB, STAT3, and SHIP siRNA transduced cells were similarly plated and stimulated as RAW264.7 parental cells with the exception that these cells were treated for 48 hours
with 2 µg/ml doxycycline (Sigma, Burlington, ON) prior to LPS stimulation. For PMΦ stimulation, cells were plated in a 24-well plate (2.5X10⁵ macrophages/well) in supplemented IMDM for 4 hours prior to LPS +/- IL-10 treatment. In continuous flow experiments, RAW264.7 parental, siRNA and AKT-ER transduced, and PMΦs were plated as per standard stimulation conditions. Cells were removed from culture media and equilibrated in Leibovitz’s L-15 media (Invitrogen, Mississauga, ON) supplemented with 9% FCS for 1 hour at 37°C with some wells being treated with 25 µM LY294002 (Sigma, Burlington, ON) for the last 30 minutes of the hour equilibration and AKT-ER cells being treated with 150nM 4-HT for the last 20 minutes of equilibration. Following equilibration, cells were placed in a continuous flow apparatus where 37°C stimulation media was passed through a modified well inlet over cell monolayers by a syringe pump (New Era Syringe Pumps Inc., Farmingdale, NY) set to a constant flow rate of 150 µl per min. Concurrently, cell supernatants were removed at a constant flow rate of 150 µl per minute and fractions were collected at 5-minute intervals over the course of 4 hours. Fractions were analyzed for TNFα levels using a BD OptEIA ELISA kit (BD Biosciences, Mississauga, ON).

2.2.7 Measurement of mRNA expression

Cells were stimulated with LPS +/- IL-10 for 0.5 or 1 hour, lysed in TRIzol reagent (Invitrogen, Mississauga, ON) and total RNA extracted as per the manufacturer’s recommended protocol. Purified RNA was then treated with DNase (Roche Diagnostics, Laval, QC) reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) and the resulting cDNA analyzed by Sybrgreen-based real-time quantitative PCR (RT-qPCR) using a 7300 Real-Time PCR
apparatus (Applied Biosystems, Foster City, CA) and gene specific primers for PRGs, and a GAPDH normalization control (See Table A.1 in Appendices for a list of primer sequences).

2.2.8 Chromatin immunoprecipitation

Cells plated at a density of 1 X 10^7 cells on a 100 mm tissue culture dish were treated with vehicle, IL-10 (100 ng/ml), or LY294002 (25 μM) for 30 minutes prior to LPS stimulation for 30 minutes. Following LPS stimulation, proteins were cross-linked by adding freshly prepared formaldehyde to a final concentration of 1% for 10 minutes at room temperature (RT). A final concentration of 125 mM glycine was added for 5 minutes at RT to quench the cross-linking reaction. Cells were then washed with ice-cold PBS and lysed with SDS Lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, 150 mM NaCl, and Protease Inhibitor Cocktail, Roche Diagnostics, Laval, QC) for 10 minutes on ice. Lysates were sonicated to shear cross-linked DNA into 200-1000 bp fragments. Insoluble materials were then removed from lysates by centrifuging samples at 10,000X G for 10 minutes at 4°C. Clarified lysates were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl). Samples were then pre-cleared with Protein G Agarose beads (Sigma, Burlington, ON) that had already been pre-adsorbed with sonicated salmon sperm DNA (Invitrogen, Mississauga, ON) for 1 hour at 4°C with rotation. Beads were removed by centrifugation at 1500X G for 5 minutes. Supernatants were transferred to fresh microfuge tubes and 1 μg of Phospho-Ser2 RNA PolII (Abcam, Cambridge, MA) or isotype control antibody (Sigma, Burlington, ON) was added and incubated for 18 hours with rotation at 4°C. Following overnight incubation, Protein-G Agarose beads were
added to the samples and incubated for an additional hour at 4°C with rotation. Protein G beads were pelleted and washed with low-salt wash buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% NP-40, 2 mM EDTA, and Protease Inhibitor Cocktail), high-salt wash buffer (low-salt wash buffer with 500 mM NaCl), and LiCl wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 mM LiCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA and Protease Inhibitor Cocktail) for 5 minutes each with rotation at 4°C. Beads were then washed with 2X 5 minute TE washes with rotation at 4°C. Protein/DNA complexes were eluted from the beads by incubating beads in elution buffer (25 mM Tris-HCl, pH 10, 1 mM EDTA, and 1 mM CaCl$_2$) for 15 minutes at RT with rotation. A final 200 mM concentration of NaCl was added to samples and incubated at 65°C for 18 hours to reverse the DNA-protein crosslinks. RNase A (Sigma, Burlington, ON) was added and incubated for 30 minutes at 37°C followed by addition of 10 mM EDTA, 50 mM Tris-HCl, pH 10, and Proteinase K and further incubation for 1 hour at 45°C. DNA was purified using standard phenol-chloroform extraction techniques then used as template for Sybrgreen-based RT-qPCR with murine TNFα promoter-specific primers (See Table A.1 in Appendices for sequence).

2.2.9 Mouse endotoxemia model

Groups of 6-8 week old Male and Female Balb/C SHIP1$^{+/+}$ and SHIP1$^{-/-}$ mice were intraperitoneally injected with either 1 or 5 mg/kg of LPS with or without co-administration of 1 mg/kg of IL-10. Blood was drawn 1 hour later by cardiac puncture for determination of plasma cytokine levels by ELISA. ELISA kits were purchased from BD Biosciences (Mississauga, ON) and eBioscience (San Diego, CA) for TNFα and CCL2 respectively.
2.3 Results

2.3.1 IL-10 inhibits LPS activation of PI-3 kinase through SHIP1

The PI-3 kinases are a family of enzymes which are activated in response to specific extracellular signals to phosphorylate the membrane lipid PI-4,5-P₂ to generate the second messenger PIP₃. PIP₃ interacts with and activates PH domain-containing proteins such as the protein kinase Akt. As part of signal downregulation, the PIP₃ is then degraded through the action of inositol phosphatases that remove either the 3' or 5' phosphate to produce PI-4,5-P₂ or PI-3,4-P₂ (reviewed in [343,482,483]). Previous studies in our lab investigating the contribution of IL-10 in regulating LPS-induced PI-3 kinase signaling, observed that treatment of P-orthophosphate labeled J2M macrophages with LPS resulted in an anticipated increase in membrane PIP₃ within 15 minutes of stimulation, as determined by methanol/chloroform lipid extraction and subsequent HPLC analysis. In cells stimulated with LPS and IL-10, PIP₃ levels were dramatically reduced with a corresponding increase in membrane PI-3,4-P₂. These initial results suggested that LPS activates PI-3 kinase to produce PIP₃ and that treatment with IL-10 promotes its dephosphorylation to PI-3,4-P₂. (See Figure B.1 in Appendices). Since the LPS and IL-10 induced PIP₃ and PI-3,4-P₂ expression patterns indicated the action of a 5'-phosphatase, we examined whether IL-10 might activate SHIP1 to antagonize the PI-3 kinase pathway. We found that IL-10 treatment of RAW264.7 macrophage cells results in rapid phosphorylation of SHIP1 on tyrosine 1020 and this phosphorylation event required tyrosines 446 and 496 on the IL10R2 cytoplasmic domain (See Figure B.1 in Appendices). These residues were previously described to be required for the anti-inflammatory action of IL10 and involved in recruiting STAT3.
However, we found that the phosphorylated, but not unphosphorylated, form of these residues can also interact with SHIP1 (See Figure B.1 in Appendices). These data suggest that IL10R signaling includes recruitment and activation of SHIP1.

Continuing this line of investigation, we then wanted to confirm if activation of SHIP1 and alterations in membrane PIP3 levels correlated with modifications in downstream PI-3 kinase pathway signaling. We measured the phosphorylation state of Akt at residue serine 473 (S473) as an indication of Akt activation. Consistent with the previous data, LPS induction of Akt phosphorylation and the inhibition of this by IL-10 was observed in both peritoneal macrophages (PMΦs, Figure 2.1A) and in the RAW264.7 macrophage cell line (Figure 2.1B).

We next compared the ability of IL-10 to inhibit LPS-induced Akt phosphorylation and TNFα production in PMΦs isolated from wild-type (SHIP1+/+) or SHIP1 deficient (SHIP1−/) mice. Cells were stimulated with LPS +/- IL-10 for 0-30 minutes and Akt activation status assessed by intracellular staining with phospho-AKT S473 antibodies and subsequent analysis by flow cytometry. In SHIP1+/+ cells, LPS treatment induced phospho-AKT S473 staining, and the presence of IL-10 reduced the induction (Figure 2.1C Left). In contrast, IL-10 was not able to inhibit LPS-induced phospho-Akt S473 induction in SHIP1−/− cells (Figure 2.1C Right).

Next, SHIP1+/+ and SHIP1−/− PMΦs were treated with LPS and 0-100 ng/mL of IL-10 for 1 hour prior to collection of culture supernatant for TNFα protein determination. As shown in Figure 2.1D, SHIP1+/+ cells were significantly more sensitive to inhibition by IL-10 than SHIP1−/− cells with the greatest difference between
SHIP1<sup>+/+</sup> and SHIP1<sup>−/−</sup> cells observed at ~10 ng/mL of IL-10. Treatment with IL-10 also resulted in a greater inhibition of LPS-induced TNFα mRNA levels in SHIP1<sup>+/+</sup> than SHIP1<sup>−/−</sup> cells (Figure 2.1E). Interestingly, in SHIP1<sup>−/−</sup> cells, low concentrations (0.01 and 0.1 ng/mL) of IL-10 enhanced rather than inhibited LPS-induced TNFα mRNA levels.
Figure 2.1
Figure 2.1 IL-10 inhibits PI-3 kinase pathway activation via SHIP1.

Immunoblot analysis of Balb/C PMΦs (A) or RAW264.7 cells (B) stimulated with LPS (10 ng/ml) or LPS + IL-10 (100 ng/ml) for the indicated times, probed with anti-Phospho-AKT (Ser473) and protein AKT (Loading Control). (C) Flow cytometric analysis of wildtype (Left) or SHIP1−/− PMΦs (Right) stimulated with LPS (10 ng/ml) (■) or LPS + IL-10 (100 ng/ml) (Δ) for the times indicated. Data represent differences between geometric means of treated and untreated samples gated on CD11b/CD86 positive events ± s.d. (n=3). Geometric Means of SHIP+/+ LPS treated, SHIP1+/+ LPS + IL-10 Treated, SHIP1−/− LPS treated and SHIP1−/− LPS + IL-10 treated samples at 30 minutes were 26.0 ± 0.2, 22.8 ± 0.2, 18.6 ± 0.4, 18.2 ± 0.3 respectively. ***p<0.001 when comparing LPS treated to LPS + IL-10 treated cells (Two-way ANOVA). (D) TNFα Enzyme-linked immunosorbent assay (ELISA) of cell supernatants collected from SHIP1+/+ or SHIP1−/− PMΦs stimulated with LPS (10 ng/ml) and IL-10 at the various concentrations indicated for 1 hour. Data represent mean TNFα levels as a percentage of LPS alone treated samples ± s.d. (n=3). TNFα levels of LPS alone treated samples were 49.16 ± 2.41 and 41.69 ± 1.54 pg/ml for SHIP1+/+ and SHIP1−/− cells respectively. * p<0.05, ***p<0.001 when comparing SHIP1 wildtype to knockout (Two-way ANOVA). (E) Real-Time quantitative PCR of cDNA prepared from SHIP1+/+ (■) or SHIP1−/− (Δ) PMΦs stimulated for 1 hour with LPS (10 ng/ml) with or without IL-10 at the concentrations indicated. Data represent mean TNFα expression levels relative to GAPDH ± s.d. (n=3). *p<0.05, **p<0.01 when comparing SHIP1 wildtype to knockout (Two-way ANOVA).
2.3.2 Macrophage production of TNFα occurs in two phases

To probe the relative contribution of STAT3 and SHIP1 to IL-10’s inhibition of LPS-induced TNFα production, we generated RAW264.7 cell lines in which STAT3 and SHIP1 protein expression were reduced by RNA silencing. siRNA sequences targeting STAT3, SHIP1 or a scrambled sequence were cloned into a lentiviral vector which contained microRNA (miRNA)-like processing elements to express these siRNA sequences in the context of a doxycycline (Dox) regulated promoter (See Figure A.1 in Appendices). As shown in Figure 2.2A, the addition of Dox to the SHIP1 siRNA knockdown cells inhibited SHIP1 protein expression by 98% and reduced STAT3 expression by 57% in STAT3 siRNA knock-down cells. Several targeting sequences for STAT3 were tested, however this was the greatest degree of knockdown that could be achieved, perhaps because STAT3 appears to be necessary for cell survival.

These siRNA-transduced cells were treated for 48 hour with Dox, then stimulated with LPS + IL-10 for 1, 2 and 24 hours. TNFα levels in the culture supernatant were determined and graphed in Figure 2.2B as a percent of the TNFα levels in parallel cultures stimulated with LPS alone. We found that IL-10 inhibited TNFα protein expression similarly at all timepoints in parental and control scrambled (Scrmb) siRNA expressing cells. Consistent with the reported resistance of macrophages from STAT3 deficient mice to the inhibitory effect of IL-10 \(^{238}\), we observed IL-10 hypo-responsiveness in the STAT3 siRNA knockdown cells, but notably, this was only observed at the longer 2 and 24-hour timepoints. At 1 hour, the STAT3 knockdown cells responded to IL-10 equally well as the parental or SCRMB siRNA expressing cells.
Conversely, SHIP1 knock-down cells were hypo-responsive to IL-10 at the 1 and 2 hr timepoints, but responded normally to IL-10 at 24 hours.

We investigated the kinetics of IL-10 inhibition of TNFα protein more precisely using a continuous-flow culture system. This apparatus (see Figure C.1 in Appendices) allows for cytokine production to be monitored over a continuous period of time. Figure 2.3 shows that LPS induces two peaks of TNFα protein expression with the first peaking around 50 minutes and the second peaking around 110 minutes. This bi-phasic TNFα production profile was observed in all 4 RAW264.7 cell types (parental, SCRMB siRNA, STAT3 siRNA and SHIP1 siRNA, Figure 2.3A), as well as in SHIP+/+ PMΦs (Figure 2.3B). The 2nd peak of TNFα production may result, in part, from the action of autocrine factors produced during the first 90 min of stimulation since diluted culture supernatants from cells treated with low-dose LPS (0.25 ng/ml) collected at 45 or 90 minutes stimulated TNFα production when added to naïve cells (Figure 2.3C).

IL-10 inhibited both peaks of TNFα production in: parental and SCRMB siRNA expressing RAW264.7 cells, and SHIP1+/+ PMΦs. However, only the 1st peak was inhibited by IL-10 in STAT3 knockdown cells. Alternately, IL-10 did not inhibit the 1st peak of TNFα in either SHIP1 siRNA-expressing RAW264.7 cells or SHIP1−/− PMΦs. We also examined the effect of adding the PI-3 kinase inhibitor LY294002 and found that it profoundly inhibited both peaks of LPS-induced TNFα (Figure 2.3D). Collectively, these data demonstrate that IL-10 inhibits the initial phase of LPS-stimulated TNFα production through a SHIP1-dependent mechanism. Interestingly, inhibition of the 2nd peak was impaired in both SHIP1 and STAT3 knock-down cells, suggesting that although IL-10 can use SHIP1-independent (i.e. STAT3-dependent) mechanisms to
inhibit the 2nd phase of TNF\(\alpha\) protein expression, the degree of inhibition is impaired in the absence of 1st phase inhibition.

Since IL-10 inhibits Akt activation through SHIP1, we hypothesized that ectopic activation of Akt would overcome the inhibitory action of IL-10. To test this, we expressed an Akt-estrogen receptor (ER) fusion protein in RAW264.7 cells. The addition of 4-hydroxytamoxifen (4-HT) activates Akt-ER by displacing Hsp90 bound to the ER ligand-binding domain, relieving steric hindrance and allowing the constitutively active Akt access to its substrates\(^{481,488}\). We found that IL-10 could not inhibit TNF\(\alpha\) protein (Figure 2.3E) expression in 4-HT treated Akt-ER cells. 4-HT did not alter the IL-10 responsiveness of parental RAW264.7 cells (data not shown). Interestingly, the presence of IL-10 enhanced TNF\(\alpha\) production in 4-HT treated Akt-ER cells.
Figure 2.2 SHIP1 is required for IL-10’s early phase inhibition of TNFα production.

(A) Immunoblot analysis and densitometry quantification of RAW264.7 Parental cells and cells stably transduced with inducible lentiviral constructs containing a scrambled siRNA sequence (SCRMB), or siRNA sequences targeting STAT3 (STAT3) or SHIP1 (SHIP1), cultured in the presence or absence of doxycycline (2 µg/ml) for 48 hours. Blot was probed with anti-SHIP1 and anti-STAT3. Data are representative of 3 independent experiments. (B) TNFα ELISA of cell supernatants from RAW264.7 Parental or SCRMB, STAT3 or SHIP1 siRNA transduced cells cultured in the presence of doxycycline (2 µg/ml) for 48 hours then treated with LPS (10 ng/ml) or LPS + IL-10 (100 ng/ml) for the times indicated. Data represent mean TNFα levels as a percentage of LPS alone treated samples ± s.d. (n=3). TNFα levels of LPS alone stimulated samples were 1.59 ± 0.20, 5.09 ± 0.13, and 56.43 ± 0.91 ng/ml for RAW264.7 parental cells at 1, 2 and 24 hours respectively, 1.16 ± 0.015, 4.02 ± 0.10, and 55.55 ± 0.093 ng/ml for SCRMB siRNA cells at 1, 2 and 24 hours respectively, 0.94 ± 0.013, 2.84 ± 0.073, 47.2 ± 0.091 ng/ml for STAT3 siRNA cells at 1, 2 and 24 hours respectively, and 1.27 ± 0.018, 3.07 ± 0.054, and 51.4 ± 0.37 ng/ml for SHIP1 siRNA cells at 1, 2 and 24 hours respectively. ***p<0.001 when comparing siRNA transduced cell lines to RAW264.7 parental cells. (Two-Way ANOVA)
Figure 2.3
Figure 2.3 SHIP1 is required for IL-10 inhibition of the first peak of TNFα production but not the second.

(A) TNFα ELISA of fractions collected from RAW 264.7 Parental or SCRMB, STAT3 or SHIP1 siRNA transduced cells treated with doxycycline (2 µg/ml) for 48 hours prior to continuous-flow apparatus stimulation with LPS (10 ng/ml) (■) or LPS + IL-10 (10 ng/ml) (Δ). Data represent TNFα concentrations of each 5 min fraction over the course of 4 hours stimulation. Data are representative of 2 independent experiments. (B) TNFα ELISA of fractions collected from SHIP1+/+ (Left) or SHIP1−/− (Right) PMΦs stimulated with LPS (10 ng/ml) (■) or LPS + IL-10 (10 ng/ml) (Δ). Data represent TNFα concentrations of each 5 min fraction over the course of 4 hours stimulation. Data are representative of 2 independent experiments. (C) RAW264.7 macrophages were stimulated with low dose LPS (250 pg/ml), a 1:100 LPS dilution (2.5 pg/ml, LPS Dil.) or 1:100 dilutions of continuous-flow fractions collected at 45 and 90 minutes following stimulation with 250 pg/ml of LPS (45 min. Fraction Dil. and 90 min. Fraction Dil., respectively). Cells were stimulated overnight and supernatants analyzed for TNFα production by ELISA. (D) TNFα ELISA of fractions collected from RAW 264.7 Parental cells treated with LY-294002 (25 µM) for 30 minutes prior to continuous flow apparatus stimulation with LPS (10 ng/ml) + Vehicle (■) or LPS + LY294002 (Δ). Data represent TNFα concentrations of each 5 min fraction over the course of 4 hours stimulation in the continuous flow apparatus. Data are representative of 2 independent experiments. (E) TNFα ELISA of fractions collected from AKT-ER transduced RAW264.7 cells treated with 4-HT (150 nM) for 20 minutes prior to continuous-flow apparatus stimulation with LPS (10 ng/ml) (■) or LPS + IL-10 (10 ng/ml) (Δ). Data represent TNFα concentrations of each 5 min fraction over the course of 4 hours stimulation in the continuous flow apparatus. Data are representative of 2 independent experiments.
2.3.3 IL-10 inhibits Ser2 phosphorylation of RNA polymerase II associated with the TNFα promoter

Hargreaves et al. classified TNFα as belonging to a subclass of PRGs, termed PRG-I, whose expression are controlled by signals initiating transcriptional elongation rather than through the classical mechanism of RNAPolIII recruitment to gene promoters. PRG-I genes have pre-assembled RNAPolIII at their promoters and a high degree of H3K4 trimethylation and H3K9 acetylation in basal, resting cells. RNAPolIII associated with PRG-I promoters is phosphorylated at Ser 5 (but not Ser 2) and supports the production of low levels of full-length, unspliced transcripts which fail to make mature, protein-coding mRNAs. Stimulus-induced phosphorylation at Ser 2 by the P-TEFb kinase then activates RNAPolII transcriptional elongation and processing of functional PRG-I mRNAs. This preassembly of RNAPolIII at PRG-I promoters is thought to allow rapid mRNA expression of these genes. Since IL-10 appears to regulate the rapid, initial phase of TNFα expression through SHIP1, we examined whether IL-10 controlled RNAPolII Ser 2 phosphorylation and whether this occurred in a SHIP1 dependent manner.

Parental or SHIP1 siRNA-expressing cells were stimulated for 15 minutes with LPS +/- IL-10 prior to genomic DNA extraction for chromatin immunoprecipitation (ChIP) with an antibody against the Ser2 phosphorylated form of RNAPolIII and qPCR primers specific for the TNFα promoter region. As shown in Figure 2.4A, LPS stimulated an increased amount of phospho-Ser2 RNAPolIII associated with the TNFα promoter in both parental and SHIP1 siRNA cells. IL-10 reduced this to levels comparable to un-stimulated cells in parental cells but not SHIP1 knock-down cells. In
contrast, treatment with LY294002 reduced the amount of phospho-Ser2 RNAPolIII associated with the TNFα promoter in both parental and SHIP1 siRNA-expressing cells.

We next determined whether IL-10 regulated RNAPolIII Ser2 phosphorylation by altering the activity of the P-TEFb complex. Using a phospho-specific antibody for CDK9, the catalytic component of the P-TEFb complex, we observed that IL-10 treatment of LPS-stimulated macrophages was able to inhibit CDK9 phosphorylation at Thr186 in parental RAW264.7 cells but SHIP1 siRNA-expressing cells appeared resistant to this inhibition (Figure 2.4B). However, CDK9 phosphorylation was inhibited in both parental and SHIP1 knock-down cells when treated with LY294002 (Figure 2.4C). Together, these data suggest that PI-3 kinase activation is upstream of CDK9 phosphorylation of RNAPolII and that IL-10 inhibition of Ser 2 phosphorylation during at least the first 15 minutes of LPS stimulation is mediated by SHIP1.

We then examined whether other LPS-induced PRGs were inhibited by IL-10 through a SHIP1-dependent mechanism. Of the PRG-Is defined by Hargreaves et al. in BMDMs, 17 (including TNFα) were induced by LPS in both PMΦs and RAW264.7 cells, and inhibited by IL-10 in SHIP1+/+ PMΦs, parental and Scrmb siRNA-expressing RAW264.7 cells (Figures 2.5A and 2.5B). However, IL-10 increased rather than inhibited the expression of 16 of these PRGs (including TNFα) in SHIP1−/− PMΦs and 11 PRGs in SHIP1 knock-down RAW264.7 cells. We investigated the IL-10 enhancement of TNFα expression in SHIP1−/− PMΦs further and found that the increase in mRNA was reflected in increases in protein during a specific phase of TNFα production around 100 minutes after LPS stimulation (Figures 2.6A, 2.6B and Figure D.1 in Appendices).
Figure 2.4 IL-10 inhibits initiation of transcriptional elongation

(A) Chromatin Immunoprecipitation analysis of RAW264.7 parental or SHIP1 siRNA transduced cells treated with LY-294002 (25 µM) or vehicle for 30 minutes prior to stimulation with LPS (10 ng/ml) with or without IL-10 (10 ng/ml) using antibody that recognizes phosphorylated RNApolIII at Ser2. Data represent mean TNFα promoter association with Phospho Ser2 RNA PolII relative to GAPDH promoter association ± s.d. (n=3) as determined by Real-time quantitative PCR using promoter specific primers. ***p<0.001 when comparing to LPS stimulated cells (One-way ANOVA). (B) Immunoblot analysis of RAW264.7 parental or SHIP1 siRNA cells stimulated with LPS (10 ng/ml) with or without IL-10 (10 ng/ml) for the indicated times, probed with anti-Phospho CDK9 (Thr186) and actin (Loading Control). (C) Immunoblot analysis of RAW264.7 parental or SHIP siRNA cells treated with LY-294002 (25 µM) or vehicle for 30 minutes prior to LPS (10 ng/ml) stimulation of 1 hour, probed with anti-Phospho CDK9 (Thr186) and actin (Loading Control).
Figure 2.5 IL-10 suppression of primary response genes switches to enhancement in the absence of SHIP1

SHIP1 WT or KO PMΦs (A) and RAW264.7 parental, SCRMB, and SHIP1 siRNA expressing cells (B) were treated with LPS (10 ng/ml) with or without IL-10 (100 pg/ml) for 30 minutes. Data represent mean mRNA expression relative to GAPDH as determined by RT-qPCR and values expressed as a percentage of LPS alone treated samples ± s.d. (n=3).
Figure 2.6  Absence of SHIP1 results in IL-10 enhancement of TNFα production during a specific phase of the first peak of production.

(A) TNFα ELISA of fractions collected from SHIP1+/+ and SHIP1−/− PMΦs stimulated with LPS (1 ng/ml) with or without IL-10 (100 pg/ml) under continuous-flow conditions. Mean TNFα levels in fractions collected at 100 minutes post-stimulation from 3 independent experiments are presented in (B) ***p<0.001 when comparing to LPS stimulated cells (Two-way ANOVA).
2.3.4 IL-10 inhibition of TNFα and CCL2 expression in mice requires SHIP1

To study the contribution of SHIP1 to IL-10 action in vivo, we compared the ability of IL-10 to inhibit LPS-induced increases in two PRG-Is, TNFα and CCL2, in SHIP1+/+ and SHIP1−/− mice. Since SHIP1−/− mice are hypersensitive to LPS, we first determined the dose of LPS required in each of the two strains of mice to give similar levels of serum TNFα and CCL2. As shown in Figures 2.7A and 2.7B, injection of 5 mg/kg and 1 mg/kg of LPS into SHIP1+/+ and SHIP1−/− mice respectively, induces similar levels of TNFα and CCL2 detectable in serum. Co-administration of IL-10 at 1 mg/kg reduced TNFα and CCL2 in the SHIP1+/+ but not the SHIP1−/− mice. These findings are consistent with our in vitro observations that TNFα and CCL2 are both PRGs whose transcription are regulated by IL-10 in a SHIP1-dependent manner.
Figure 2.7   IL-10 requires the presence of SHIP1 in order to inhibit inflammation in an *in vivo* model of endotoxemia.

TNFα (A) and CCL2 (B) ELISA of serum samples prepared from wildtype or SHIP1−/− mice intra-peritoneally injected with LPS or co-administered LPS + IL-10 at the concentrations indicated for 1 hour. Data represent means of n=6. **p<0.01 when comparing to LPS alone stimulated mice (One-way ANOVA)
2.4 Discussion

The anti-inflammatory actions of IL-10 are essential for maintaining proper immune homeostasis. In its absence, stimulatory pathways are left unchecked and lead to various acute and chronic pathologies\textsuperscript{221}. It is currently believed that the transcription factor, STAT3, mediates all of IL-10’s anti-inflammatory signaling where it upregulates the expression of various genes whose products then go on to inhibit pro-inflammatory pathways at the level of transcription\textsuperscript{242,276}. This can be regarded as a relatively lengthy process, requiring translocation of STAT3 from the membrane to the nucleus, \textit{de novo} gene transcription, and translation of the nascent polypeptide. However, data from our lab and others have shown that IL-10 is able to inhibit LPS-induced production of pro-inflammatory cytokines, such as TNF\textalpha, within the first 30 minutes of IL-10 treatment (data not shown). This time scale is not in agreement with STAT3-exclusive regulation. We thus investigated other signaling pathways that could potentially mediate IL-10’s early phase anti-inflammatory activity. In this chapter, we characterize IL-10’s signaling through the lipid phosphatase SHIP1, a key negative regulator of the PI-3 kinase pathway.

Using regulation of TNF\textalpha production as a model for similar pro-inflammatory PRGs as described by Hargreaves \textit{et al.}\textsuperscript{214}, we made the observation that TNF\textalpha production occurs in two phases within the first 2 hours of LPS stimulation. Of note, a bi-phasic profile for TNF\textalpha production has also been reported for macrophages stimulated by Staphylococcal enterotoxins B (SEB) with similar kinetics\textsuperscript{489}. Whereas STAT3 was required for mediating IL-10 inhibition during the second phase of TNF\textalpha production, it was not necessary for IL-10 mediated inhibition of the first peak of TNF\textalpha. In contrast,
the presence of SHIP1 was required for inhibition of the first peak of TNFα. These results provide evidence for STAT3-independent signaling pathways utilized by IL-10, which predominate during IL-10’s early phase anti-inflammatory action.

To further characterize the mechanism by which SHIP1 was mediating IL-10’s early phase regulation, we compared downstream signaling pathways between parental RAW264.7 macrophages and SHIP1 siRNA expressing macrophages. Using ChIP and phospho-specific antibodies, we determined that in the presence of SHIP1, IL-10 inhibited CDK9 phosphorylation, which prevented Ser2 phosphorylation on RNAPolII. TNFα transcription is not regulated by RNAPolII recruitment to the *tnfα* promoter, rather, it is constitutively associated with the promoter in resting cells. Phosphorylation of RNAPolII at Ser2 is required for signaling a switch from basal, low-level production of full-length non-spliced transcripts to high levels of mature, full-length, spliced mRNAs. Smallie *et al.* have reported a unique requirement for NFκB binding motifs within TNFα’s 3’ UTR that is necessary for both LPS induction of TNFα and IL-10’s inhibition of TNFα transcription. These studies demonstrated that IL-10 acts as a general block of RelA (the p65 subunit of NFκB) recruitment to TNFα’s 3’ UTR and thus prevents NFκB-dependent CDK9 recruitment. Whether SHIP1 is also necessary for mediating IL-10’s inhibition of NFκB recruitment is an intriguing hypothesis worth pursuing in future investigation.

Medzhitov’s group has classified a number of genes induced upon LPS-stimulation based upon the transcription factors and chromatin modifications that regulate their expression. For PRGs, TLR stimulation post-translationally activates
transcription factors such as NFκB and IRFs, which bind to promoter regions of genes that are enriched with histone H3K4 trimethylations and H3K9 acetylations. These covalent modifications in chromatin structure maintain the promoter regions in a permissive state allowing constitutive association of RNA PolIII and thus rapid transcription upon cell stimulation. SRGs, on the other hand, are regulated by transcription factors that are de novo transcribed and translated upon TLR stimulation, such as ATF3 and C/EBPδ. These transcription factors then bind to promoter elements and recruit chromatin modifying enzymes and the transcriptional machinery. Due to these limitations, SRG expression typically occurs at later timepoints (2-8 hours post-stimulation).

17 of the genes classified by Hargreaves et al. as PRGs were both upregulated by LPS and inhibited by IL-10 in SHIP1 WT PMφs, Parental RAW264.7 cells and RAW264.7 cells expressing a control scrambled siRNA sequence. In SHIP1 KO PMφs or SHIP1 specific siRNA expressing cells, however, treatment with 100 pg/mL IL-10 had the reverse effect on the majority of these IL-10 regulated genes in that their expression was enhanced. These findings were surprising as IL-10 is the prototypical anti-inflammatory cytokine and the capacity for IL-10 to stimulate macrophage pro-inflammatory responses has never before been directly addressed in the literature. It is worthy of note that IL-6, an archetypal pro-inflammatory cytokine, has conversely been reported to switch to an anti-inflammatory response when SOCS3 is deleted. Although STAT3 is activated downstream of both the IL-6 and IL-10 receptors, SOCS3 only binds to the IL-6R. Consequently, IL-10R stimulation results in sustained STAT3 activation while IL-6R stimulation transiently activates STAT3 before being targeted for
proteasomal degradation by SOCS3. Akin to SOCS3 being the molecular switch determining whether IL-6 exerts a pro or anti-inflammatory effect, SHIP1 may be a molecular determinant of whether IL-10 acts as an anti-inflammatory or pro-inflammatory agent.

We also investigated the contribution of SHIP1 to IL-10 activity in vivo. The presence of SHIP1 was necessary for mediating IL-10’s inhibition of two PRGs, TNFα and CCL2, in a mouse model of endotoxemia. These results suggest that despite having intact STAT3, the lack of SHIP1 renders SHIP−/− mice incapable of suppressing the initial phase of TNFα production. Furthermore, inhibition of this early phase appears to be necessary for IL-10’s attenuation of subsequent phases of pro-inflammatory cytokine production via STAT3.

In summary, we have demonstrated the existence and biological relevance of STAT3-independent signaling pathways utilized by IL-10 through SHIP1. This regulatory pathway predominates during the early phases of IL-10 signaling and acts to suppress the expression of a sub-set of PRGs by preventing their transcription by RNAPolIII. In so doing, IL-10 is able to temper and fine-tune the activity of a transcriptional program in a more expedient manner than would be achievable by STAT3-mediated transcriptional regulation alone. Thus, targeted manipulation of SHIP1 activity may represent a potential strategy for treatment of IL-10 deficiency-related diseases.
CHAPTER 3: ALLOSTERIC ACTIVATION OF SHIP1 INHIBITS INFLAMMATION
3.1 Introduction

As described in the previous chapter, in response to extracellular signals, PI-3 kinase becomes activated to phosphorylate PI-4,5-P₂ within the plasma membrane to generate PIP₃. PIP₃ then initiates a cascade of downstream signaling pathways by interacting with PH domain-containing proteins, such as Akt, that regulate cellular activation, proliferation or survival, depending on the cell type and stimulus. Cellular levels of PIP₃ are normally tightly regulated by modulation of: (i) PI-3 kinase activity, (ii) the 5′ inositol phosphatases SHIP1, SHIP2, and 72-kDa 5′-phosphatase (72-5ptase) and (iii) the 3′ inositol phosphatase PTEN. Of these, SHIP1 is unique in that its expression is restricted mainly to immune and hemopoietic cells. SHIP1’s role in immune cell homeostasis is shown both by the myeloproliferative syndrome observed in SHIP1⁻/⁻ mice, as well as the hypersensitivity of SHIP1⁻/⁻ mice and cells to immune stimulation. SHIP1 mediates signaling from the inhibitory FcγRIIB receptor and is important in terminating signal transduction from activating immune/hemopoietic cell receptor systems. Diminished SHIP1 activity or expression has been observed in human inflammatory diseases and hemopoietic malignancies.

Since dysregulated activation of the PI-3 kinase pathway contributes to inflammatory/immune disorders and cancer, much effort has been invested into the development of inhibitors of PI-3 kinase itself, as well as downstream protein kinases. The precedent for discovery and biological efficacy of kinase inhibitors is well established and a number of promising new PI-3 kinase isoform specific inhibitors have recently been developed and used in mouse models of inflammatory disease and glioma with minimal toxicities. However, because of the dynamic interplay between
phosphatases and kinases in regulating biological processes, inositol phosphatase activators may provide an alternate and complementary approach to inhibit PIP₃ levels (discussed in Knight and Shokat⁵⁰²). Of the four phosphatidylinositol phosphatases that have been reported to degrade PIP₃, SHIP1 is a particularly ideal target for development of potential therapeutics for treating immune and hemopoietic disorders because its hemopoietic-restricted expression would limit their action to only these cell types.

Having observed in the experiments described in Chapter 2 that IL-10 signals through SHIP1 to mediate its early-phase anti-inflammatory action, we hypothesized that activating SHIP1 using small-molecule agonists could mimic the biological effects of IL-10. In search of small molecule modifiers of SHIP1, our lab previously developed a chromogenic enzyme assay to monitor SHIP1 phosphatase activity. We identified the meroterpenoid Pelorol, isolated from a marine invertebrate extract library, as a potent SHIP1 activator (See Figure E.1 in Appendices). Structural analogues of Pelorol, AQX-016A and AQX-MN100, were synthesized by our collaborators, Drs. Raymond Andersen and Matthew Nodwell (University of British Columbia, Departments of Chemistry and Earth & Ocean Sciences, Vancouver, B.C.)⁵¹⁰, which exhibited greater SHIP1 activating activities than the parent compound. The data presented in this chapter show that these small molecule agonists could selectively activate SHIP1 in intact cells and were protective when administered in mouse models of inflammatory disease. We further applied these SHIP1 activating compounds as molecular tools to further characterize SHIP1 enzyme activity and revealed that SHIP1 is subject to a previously unrecognized allosteric regulation by its product, PI-3,4-P₂.
3.2 Materials and methods

3.2.1 Formulation of compounds

For in vitro testing in the SHIP1 enzyme assay, AQX-016A and AQX-MN100 were dissolved in EtOH and diluted into aqueous buffer (20 mM Tris-HCl, pH 7.5 and 10 mM MgCl₂). The actual concentration of drug in solution was determined by optical density measurement at 280 nM (λ<sub>max</sub> of both compounds) after high speed centrifugation at 14 000 X g for 30 min to remove precipitated drug. For testing on cells, compounds were formulated in the carrier cyclodextrin (Cyclodex Technologies, High Springs, FL) at 6 mM (2 mg/mL). For oral administration to animals, compounds were dissolved in 100% cremophore EL (Sigma-Aldrich Canada, Oakville, Ontario) at 150 mM (50 mg/mL) prior to dilution to 6 mM in phosphate buffer saline. Compounds caged in cyclodextrin or formulated in cremophore EL micelles are very soluble in aqueous solution, however they could not be used in the SHIP1 enzyme assays because of interference from both cyclodextrin and cremophore EL.

3.2.2 Production of recombinant SHIP1 enzyme and SHIP1 C2 domain

Recombinant, N-terminal His₆ tagged SHIP1 enzyme was expressed in mammalian 293T cells by transient transfection with pME18S-His-SHIP1 plasmid and purified to >95% homogeneity by Ni-chelating bead chromatography (Qiagen, Mississauga, Ontario) as assessed by Coomassie Blue visualization of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated material. Recombinant SHIP1 C2 domain (amino acid residues 725 to 863) was expressed in E. coli transformed with a pET28C expression vector constructed as described below. Recombinant protein purified from the
cell lysates by Ni-chelating bead chromatography was >95% pure by SDS-PAGE analysis.

3.2.3 in vitro SHIP1 enzyme assay

The SHIP1 enzyme assay was performed in 96-well microtitre plates with 10 ng of enzyme/well in a total volume of 25 µL of 20 mM Tris-HCl, pH 7.5 and 10 mM MgCl₂. SHIP1 enzyme was incubated with test extracts (provided in DMSO) or vehicle for 15 min at 23°C before the addition of 100 µM inositol-1,3,4,5-tetrakisphosphate (Echelon Biosciences Inc, Salt Lake City, Utah). The reaction was allowed to proceed for 20 min at 37°C and the amount of inorganic phosphate released assessed by the addition of malachite green reagent followed by an absorbance measurement at 650 nm \(^{511}\). SHIP2 enzyme was purchased from Echelon Biosciences (Salt Lake City, Utah) and an equivalent amount of inositol phosphatase activity was used in the in vitro enzyme assay. Enzyme data are expressed as the mean of triplicates +/- SEM. Experiments were performed at least 3 times.

3.2.4 Production of SHIP1\(^{+/+}\) and SHIP1\(^{-/-}\) in bone marrow derived macrophages

Bone marrow derived macrophages from SHIP1\(^{+/+}\) and SHIP1\(^{-/-}\) mice were obtained as described previously \(^{371}\) and maintained in IMDM supplemented with 10% FCS, 150 µM MTG, 2% C127 cell conditioned medium as a source of macrophage colony stimulating factor (M-CSF) (macrophage medium).

3.2.5 LPS stimulation of macrophages

For the analysis of LPS-stimulated TNFα production, 2 x10⁵ cells were plated the night before in 24 well plates in macrophage medium. The next day, the medium was changed
and AQX-016A or carrier was added to cells at the indicated concentrations for 30 min prior to the addition of 10 ng/mL LPS. Supernatants were collected after 1 hr for TNFα determination by ELISA (BD Biosciences, Mississauga, ON, Canada). For analysis of intracellular signaling, 2 x 10^6 cells were plated the night before in 6 cm tissue culture plates. The next day, the cells were cultured in macrophage medium without M-CSF for 1 hr at 37°C and then pretreated with AQX-016A or carrier for 30 min prior to the addition of 10 ng/mL LPS for 15 min. Cells were washed with 4°C PBS and resuspended in lysis buffer (50 mM Hepes, 2 mM EDTA, 1 mM NaVO₄, 100 mM NaF, 50 mM NaPPi and 1% NP40) supplemented with Complete Protease Inhibitor Cocktail (Roche, Montreal, Canada). Lysates were rocked at 4°C for 30 min and clarified by centrifuging 20 min at 12000 x g. Lysates were then made 1 x in Laemmli’s buffer, boiled 2 min and loaded onto 7.5% SDS polyacrylamide cells. Immunoblot analysis for phospho PKB (Cell Signaling Technology, Pickering, Ont), SHIP1 and actin (Santa Cruz, Santa Cruz, CA) were carried out as described previously.

3.2.6 Mouse endotoxemia model

6-8 week old C57Bl/6 mice (VCHRI Mammalian Model of Human Disease Core Facility, Vancouver, BC) were orally administered the indicated dose of AQX-016A, AQX-MN100 or dexamethasone or carrier 30 min prior to an IP injection of 2 mg/kg of LPS (E. Coli serotype 0111: B4, Sigma, Oakville, Ont). Blood was drawn 2 hrs later for determination of plasma TNFα by ELISA. Results are representative of 3 independent experiments.
3.2.7 Mouse acute cutaneous anaphylaxis model

6-8 week old CD1 mice (VCHRI Mammalian Model of Human Disease Core Facility, Vancouver, BC) were sensitized to the hapten DNP by cutaneous application of 25 µL of 0.5% dinitrofluorobenzene (DNFB) (Sigma, Oakville, Ont) in acetone to the shaved abdomen of mice for two consecutive days. One week later, test substances (dissolved in 10 µL of 1:2 DMSO:MeOH) were painted on the right ear while the left ear received vehicle control. 30 min after drug application, DNFB was applied to both ears to induce mast cell degranulation. A 6 mm punch was taken from the ear and immediately frozen on dry ice for subsequent determination of neutrophil myeloperoxidase (MPO) activity as described.

3.2.8 Mouse colitis model

Colitis was induced in 6-8 week old Balb/c IL-10−/− mice (VCHRI Mammalian Model of Human Disease Core Facility, Vancouver, BC) by administering the colonic contents of conventional C57Bl/6 mice diluted 1:10 in PBS by oral gavage. Mouse weights and fecal consistencies were monitored and colitis allowed to develop for 4 weeks. Ethanol (Vehicle) and AQX-MN100 (3 mg/kg) was diluted in cage drinking water and dexamethasone (0.4 mg/kg) was administered every 2 days by oral gavage for 3 weeks. At the end of the dosing period, proximal, medial and distal colon sections were collected for paraffin embedding or stored in RNALater (Invitrogen, Mississauga, ON) for RNA extraction. Slides were prepared, stained with hematoxylin and eosin, and mounted by the UBC Department of Pathology and Laboratory Medicine Histochemistry Facility. Specimens were assigned pathological scores by 3, independent, blinded investigators according to a method described by Madsen et al. In brief, colonic inflammation was
graded using a 4-point system assessing submucosal edema, immune cell infiltration, goblet cell ablation, and integrity of the epithelial layer. For analysis of mRNA expression, colon sections were homogenized and total RNA extracted using TriZOL Reagent (Invitrogen, Mississauga, ON) as per the manufacturer’s protocol. Purified RNA was then treated with DNase (Roche Diagnostics, Laval, QC) reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) and the resulting cDNA analyzed by Sybrgreen-based real-time quantitative PCR (RT-qPCR) using a 7300 Real-Time PCR apparatus (Applied Biosystems, Foster City, CA) and gene specific primers for IL-17, CCL2, and a GAPDH normalization control (See Table A.1 in Appendices for a list of primer sequences).

3.2.9 Construction of the SHIP1 ΔC2 mutant and isolated C2 domain

A His₆ tagged SHIP1 ΔC2 domain deletion mutant (deleted amino acid residues 725 to 863) in the mammalian expression vector pME18S was generated by a standard PCR-based methodology. An N-terminal His₆ C2 domain construct was also generated by PCR inserted into the pET28C bacterial expression vector using EcoRI and NdeI restriction sites.

3.2.10 Protein lipid overlay assays

Protein lipid overlay (PLO) assays were performed essentially as described with minor modifications. Lyophilized phosphatidylinositol-3,4-bisphosphate diC16 (PIP₂, Echelon Biosciences, Salt Lake City, UT) was reconstituted in a 2:1.8 solution of methanol and water. PVDF membranes (Millipore, Missisauga, ON) were initially wetted in methanol for 1 minute, and washed 3 X 5 min with water, and gently agitated in TBST buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl (TBS) with 0.05% Tween 20) at 23°C overnight. The
treated membranes were air-dried and dilutions of reconstituted lipids were spotted in 1 µl aliquots to give the indicated amount of PIP$_2$ per membrane spot. The membranes were dried completely and blocked with blocking buffer (3% BSA in TBS with 0.05% NaN$_3$) for 1 h at 23°C. Purified, recombinant C2 domain was diluted into blocking buffer (5 µM final) and treated with 4 µM AQX-MN100 or EtOH control for 30 min at 23°C prior to overnight incubation with the PIP$_2$ spotted membranes. Membranes were washed 10 times over 50 min in TBST buffer at 23°C and incubated with anti-His$_6$ mouse IgG (Qiagen, Missisauga, ON) for 1 h at 23°C. Membranes were washed as above and incubated with Alexa Fluor 660 anti-mouse goat anti-mouse IgG (Invitrogen, Mississauga, ON) for 1 h at 23°C. After washing, bound proteins were detected and quantified on a Li-Cor Odyssey scanner (Lincoln, NE).

### 3.2.11 Scintillation proximity assays

AQX-MN100 was radiolabelled with tritium by GE Healthcare (Piscataway, NJ) to a specific activity of 42 Ci/mmole. Copper chelate (His-Tag) YSi SPA scintillation beads (GE Healthcare, Piscataway, NJ) were diluted in 0.25% BSA/TBS to 1.5 mg/mL and recombinant, His$_6$-tagged protein added at the indicated concentrations: wild-type (1 pM), ΔC2 SHIP1 enzyme (1 pM) or C2 domain (10 nM). BSA or human serum albumin (HSA) (10 nM) were used as controls. Protein was allowed to bind 1 h at 23°C, and 250 µg of beads were aliquoted per well of a 96-well plate. 5 µCi of [$^3$H]-AQX-MN100 was added per well, the plate gently agitated for 30 min and the amount of bead associated radioactivity quantified by counting in a Wallac BetaPlate plate scintillation counter.
3.3 Results

3.3.1 AQX-MN100 is as biologically active as AQX-016A

Previous studies in our lab have demonstrated that the pelorol analogue, AQX-016A was able to elicit a 3-fold higher activation of SHIP1 enzyme activity than the parent compound and inhibited macrophage production of TNFα in vitro and in vivo (See Figure E.1 in Appendices). It was observed that SHIP1+/+ cells and mice were far more sensitive to AQX-016A than cells and mice deficient in SHIP1, suggesting that the compound acts by specifically targeting SHIP1. These observations were corroborated using in vitro phosphatase assays with SHIP1’s most closely related enzyme, SHIP2, where AQX-016A exhibited no enhancement of phosphatase activity (See Figure E.1 in Appendices). However, the presence of a catechol moiety within AQX-016A (Figure 3.1A) is potentially problematic since catechols can exhibit activities independent of their specific protein pocket-binding interaction. For example, catechols can bind metals or be oxidized to an ortho-quinone which can lead to covalent modification of proteins through redox reactions. To rule out these possibilities a non-catechol version of AQX-016A designated AQX-MN100 was synthesized. Analogous to AQX-016A, AQX-MN100 enhanced SHIP1 enzyme activity in vitro (Figure 3.1B). Like AQX-016A, AQX-MN100 also selectively inhibited TNFα production from SHIP1+/+ but not SHIP1−/− macrophages (Figure 3.1C). The EC50 for this inhibition was 0.3 – 0.6 µM. Additionally, AQX-MN100, similarly to IL-10, is able to potently inhibit both peaks of TNFα production in RAW264.7 cells stimulated under continuous flow conditions (Figure 3.1D).
AQX-MN100 specifically targets SHIP1 to inhibit TNFα production.

(A) Structures of Pelorol, AQX-016A and AQX-MN100. (B) *in vitro* phosphatase assays were performed with recombinant SHIP1 enzyme with 50 µM IP₄ substrate in the presence or absence of AQX-016A (50 µM) or AQX-MN100 (50 µM). (C) TNFα ELISA of cell supernatants from SHIP1⁺/⁺ (■) and SHIP1⁻/⁻ (Δ) BMDMs stimulated with LPS (10 ng/ml) in the presence or absence of AQX-MN100 at the concentrations indicated for 2 hours. (D) TNFα ELISA of fractions collected from RAW 264.7 Parental cells treated with AQX-MN100 (10 µM) for 30 minutes prior to continuous flow apparatus stimulation with LPS (10 ng/ml) + Vehicle (■) or LPS + AQX-MN100 (10 µM) (Δ). Data represent TNFα concentrations of each 5 min fraction over the course of 4 hours stimulation in the continuous flow apparatus. Data are representative of 2 independent experiments.
3.3.2 AQX-MN100 is protective in *in vivo* models of inflammation

We went on to test whether AQX-MN100 would be effective in inhibiting inflammatory reactions *in vivo* by assessing its ability to confer protection in mouse models or endotoxemia, allergy and colitis. The mouse model of endotoxic shock involves IP injection of bacterial LPS and measurement of serum TNFα 2 hours post-challenge. As shown in Figure 3.2A, oral administration of AQX-MN100 30 minutes prior to IP injection of LPS markedly inhibited concentrations of serum TNFα to levels comparable to the steroidal drug, dexamethasone.

We also tested AQX-MN100’s ability to inhibit cutaneous anaphylaxis. Anaphylactic or allergic responses are mediated by allergen-induced degranulation of pre-sensitized mast cells. The mouse ear edema/cutaneous anaphylaxis model involves pre-sensitization of mice with the haptenizing agent dinitrofluorobenzene (DNFB). One week later, the allergic reaction is elicited by painting DNFB onto the ears of the mice. The efficacy of potential anti-inflammatory compounds is tested by topical application of the test substance to one ear and comparing the resulting ear edema or inflammation of the two ears. By measuring the amount of myeloperoxidase (MPO) activity (an abundant enzyme in neutrophils) in ear punch homogenates as an indicator for immune cell recruitment and inflammation, Figure 3.2B shows that topically applied AQX-MN100 dramatically inhibited allergen-induced inflammation compared to the vehicle control-treated ear.

As described in Chapter 2, we demonstrated that SHIP1 is required for certain IL-10 actions but not whether activation of SHIP1 itself might be sufficient to alleviate inflammation resulting from the loss of normal IL-10 function. One model in which to
test this possibility is the IL-10 KO mouse model of colitis. IL-10−/− mice develop colitis when colonized with normal intestinal flora because the lack of IL-10 eliminates the normal immunosuppressive mechanisms needed to temper the host immune response. If SHIP1 activation is important in the anti-inflammatory action of IL-10, then SHIP1 activation by the small molecule SHIP1 agonist, AQX-MN100, might reduce disease severity in colitic IL-10 KO animals.

We initiated colitis in IL-10 KO mice by inoculating them with freshly isolated colon contents of normal, specific pathogen-free mice and allowed inflammation to develop for 6 weeks. Mice were then treated for an additional 6 weeks with 2 mg/kg AQX-MN100, 0.4 mg/kg dexamethasone or vehicle prior to colon tissue collection for histological and mRNA expression analyses. Hematoxylin and eosin (H&E) stained sections were prepared from the proximal, medial and distal colons, as well as from mice that were not inoculated with normal fecal contents (no colitis group). Two independent blinded investigators scored the sections using a colitis scoring scheme described by Stecher et al., based on submucosal edema, immune cell infiltration, goblet cell ablation and epithelial integrity. In the three groups in which colitis was induced, the dexamethasone and AQX-MN100 groups had significantly lower pathology scores than the vehicle group. Total mRNA was isolated from colon sections from all four groups for analysis of TNFα, IL-17 and CCL2 expression. TNFα mRNA expression was too low for reliable determinations, but IL-17 and CCL2 mRNA were readily detectable. As shown in Figure 3.2E, both AQX-MN100 and dexamethasone treatment significantly reduced the levels of IL-17 and CCL2 mRNA. These data suggest
that AQX-MN100 treatment can reduce the inflammation in colitis resulting from the loss of IL-10.
Figure 3.2
Figure 3.2  AQX-MN100 inhibits inflammation in in vivo mouse models of inflammation.

(A) Mice were administered 20 mg/kg AQX-MN100 or 0.4 mg/kg dexamethasone orally 30 min prior to an IP injection of 2 mg/kg LPS. Blood was collected 2 hours later for TNFα determination by ELISA. Each symbol indicates one mouse and data are representative of three independent experiments. (B) Mice were topically sensitized with DNFB, and vehicle or AQX-MN100 applied to pairs of ears prior to acute DNFB challenge. Some mice were not challenged with DNFB (no DNFB). Ears were harvested and MPO levels determined. P-value <0.05 for the AQX-MN100 vs the vehicle treated groups. All data are representative of three independent experiments. (C) Representative H&E stained colon sections and pathological scores (D) of normal (n=6) and colitic IL-10−/− mice treated with vehicle (Veh, n=9), AQX-MN100 (3 mg/kg) (n=8) or dexamethasone (Dex, 0.4 mg/kg) (n=3) for 3 weeks. P=proximal colon, M=mid colon, D=distal colon. (E) RT-qPCR of cDNA prepared from colonic sections of non-colitic and colitic IL-10−/− mice treated with vehicle, AQX-MN100 (3mg/kg), or Dexamethasone (0.4 mg/kg). Data represent mean IL-17 and CCL2 expression relative to GAPDH. ** p<0.01, *** p<0.001 when comparing drug treatment to vehicle alone (One-way ANOVA)
3.3.3 SHIP1 is an allosterically activated enzyme

The allosteric regulation of enzymes has remained under-appreciated primarily because allosteric effectors are not easy to find. While the majority of allosteric regulators have been discovered through serendipity, a few allosteric regulators have been deduced from discovery of activators or inhibitors of enzymes as a result of high-throughput chemical screens (HTS) \(^{522}\). For example, the allostery of glucokinase was discovered from an HTS in search of activators for treatment of diabetes \(^{522}\). By analogy, our discovery of small molecule activators of SHIP1 led us to postulate that SHIP1 might in fact be allosterically regulated. To this end, we investigated the molecular mechanism by which AQX-MN100 activates SHIP1, first by performing kinetic analysis of its enzyme activity. Activity measurements were performed with substrate concentrations ranging from \(10^{-100} \mu M\). Plots of the initial reaction velocity at each substrate concentration should be hyperbolic if SHIP1 obeys conventional Michaelis-Menten kinetics \(^{523}\). However, we found SHIP1 displayed sigmoidal reaction kinetics, which suggests allosteric activation by its end-product (Figure 3.3A). Indeed, addition of the SHIP1 product PI-3,4-P\(_2\) to the enzyme reaction activated wild-type SHIP1 enzyme to the same extent as AQX-MN100 (Figure 3.3B). Interestingly, the 3’ inositol phosphatases PTEN \(^{524}\) and myotubularin (MTM) \(^{525}\) have also been recently shown to be allosterically activated by their phosphatidylinositol products (PI-4,5-P\(_2\) and PI-5-P respectively).

In PTEN and MTM, the allosteric binding sites for their products were mapped to lipid-binding motifs in each protein. Since our experiments examining the enzymatic properties of SHIP1 revealed that it is allosterically activated by its product PI-3,4-P\(_2\), we searched for potential PI-3,4-P\(_2\) binding domains within SHIP1. Alignment of amino
acid sequences of SHIP1 and SHIP2 from multiple species, and secondary structure predictions led to the identification of a predicted C2 domain residing C-terminal of SHIP1’s enzymatic phosphatase domain (Figure 3.3C). C2 domains were first described in protein kinase C (PKC) where it serves to bind Ca^{2+}, but C2 domains have since been identified in other proteins where they have been shown to bind to a variety of ligands including lipids\textsuperscript{526,527}. To test this possibility, we produced SHIP1 enzyme in which the C2 domain (ΔC2 SHIP1) was deleted. As shown in Figure 3.3B, although the ΔC2 SHIP1 enzyme was as active as the wild-type molecule, its activity could not be enhanced by the addition of either PI-3,4-P\textsubscript{2} or AQX-MN100. This indicates that the C2 domain is required for allosteric activation of SHIP1 activity and that it may be the binding site for its allosteric activators PI-3,4-P\textsubscript{2} and AQX-MN100.

In order to examine whether the C2 domain could bind PI-3,4-P\textsubscript{2}, we expressed recombinant, His\textsubscript{6}-tagged C2 domain and determined its PI-3,4-P\textsubscript{2} binding ability using protein lipid overlay assays\textsuperscript{515}. Purified C2 domain was incubated with membrane strips spotted with PI-3,4-P\textsubscript{2} and bound protein detected using an anti-His\textsubscript{6} antibody. Figure 3.3D shows the C2 domain binds PI-3,4-P\textsubscript{2} and that this binding is inhibited by AQX-MN100, consistent with the hypothesis that both AQX-MN100 and PI-3,4-P\textsubscript{2} interact with the C2 domain at a common binding site.

AQX-MN100 was verified to directly bind the C2 domain using scintillation proximity assays (SPAs) in which SPA beads were coated with either the C2 domain or control protein (BSA) prior to incubation with $[^3]$H-AQX-MN100. Figure 3.3E shows that the C2 domain does indeed interact with $[^3]$H-AQX-MN100. In complementary studies, we observed that $[^3]$H-AQX-MN100 bound to wild-type SHIP1 but not to SHIP1
lacking its C2 domain (Figure 3.3F). Together, these data are consistent with AQX-MN100 binding to SHIP1’s C2 domain, resulting in allosteric activation of the enzyme.
Figure 3.3
**Figure 3.3  The C2 domain is required for end-product allostERIC activation of SHIP1 and binding of AQX-MN100**

SHIP1 enzyme initial velocities were determined at the indicated concentration of inositol-1,3,4,5-tetrakisphosphate (IP$_4$) substrate. (B) The ability of PI-3,4-P$_2$ (20 µM) or AQX-MN100 (30 µM) to activate wild-type (WT) and C2 domain deleted (ΔC2) SHIP1 enzyme was determined at 30 µM IP$_4$. (C) ClustalW alignment of all SHIP1 and SHIP2 sequences deposited in the NCBI database corresponding to the regions 160 amino acids C-terminal of the putative phosphatase domains. (D) Recombinant C2 domain was pre-incubated for 30 min at 23°C with 200 µM AQX-MN100 or Vehicle control (EtOH) and allowed to bind to PI-3,4-P$_2$ immobilized on membrane strips in a protein overlay assay as previously described. (E) Recombinant C2 domain (10 nM) or Full-length WT or ΔC2 SHIP1 enzyme (1 pM) (F) was coated onto Copper chelate (His-Tag) YSi SPA Scintillation Beads in the presence of 0.25% BSA. Beads were then incubated with 5 µCi of [³H]-AQX-MN100 and the bead-associated radioactivity measured as described in Materials and Methods. BSA = bovine serum albumin, HSA = human serum albumin. Data are expressed as mean +/- SEM and are representative of at least three independent experiments. **p<0.01, *** p<0.001 (One-way ANOVA)
3.4 Discussion

The PI-3 kinase pathway has been the target of intense efforts for the development of therapeutics \(499-504,528\). The PI-3 kinase family consists of multiple isoforms which vary in tissue distribution and receptor systems to which they are coupled \(501\). The classic PI-3 kinase inhibitors wortmannin and LY292004 have been useful experimental tools for probing PI-3 kinase function, but they have not been successful in clinical development partly because they globally inhibit all members of the PI-3 kinase family and are known to have off-target effects \(313,314,338-340,529\). Recently however, isoform specific PI-3 kinase inhibitors are emerging as a promising class of therapeutic agents. For example, a dual PI-3 kinase \(\alpha/\text{mTOR}\) inhibitor \(502\) was found to have efficacy in a human glioma xenograft model \(509\) without any undue toxicities even though PI3K\(\alpha\) is expressed in all tissues and is important in insulin signaling \(500,530\). It is postulated that a sufficient therapeutic window exists because cancer cells become very dependent on particular signaling pathways (termed “oncogene addiction” \(499\)) and thus pharmacological inhibitors show selectivity towards cancer vs normal cells \(499\). Similarly, PI-3 kinase \(\delta\) and \(\gamma\)-specific inhibitors are actively being investigated for treatment of inflammatory diseases due to the enrichment of these isoforms in immune cells \(307-312\). PI-3 kinase \(\gamma\) mediates signaling from G-protein coupled receptors (GPCR) and although its expression can be detected in endothelium, heart and brain, it is mainly expressed in immune cells \(529\). PI-3 kinase \(\gamma\) inhibitors thus benefit from its relatively restricted expression compared to the other PI-3 kinase isoforms, and the fact that many (though not all \(531\)) inflammatory processes involve GPCR-dependent steps \(529\). PI-3 kinase \(\gamma\) inhibitors have been found to be protective in mouse models of rheumatoid arthritis \(505\).
and glomerulonephritis. In addition to PI-3 kinase itself, downstream protein kinases are also being targeted with mixed results. Work is continuing on Akt inhibitors with limited success perhaps because of dose limitations due to toxicities. The mTORC1 inhibitor rapamycin, on the other hand, is currently approved as an immunosuppressive agent with manageable side effects. The toxicities of both Akt and mTORC1 inhibitors are partly related to the ubiquitous expression of both targets.

As an alternative to inhibiting PI-3 kinase and downstream protein kinases, we describe a novel paradigm for inhibiting PI-3 kinase signaling through activation of the phosphatases that negatively regulate this pathway. The SHIP1 phosphatidylinositol phosphatase is a particularly good target for immune/hemopoietic disorders because of its restricted expression to hemopoietic cells. Because the relative activity of phosphatases present in a cell will influence the efficacy of kinase inhibitors, as discussed by Knight and Shokat, SHIP1 agonists could also be used to potentiate the activation of PI-3 kinase inhibitors and target non-tissue specific PI-3 kinase inhibitors to the hemopoietic/immune cell compartment. The experiments described in this chapter demonstrate that the small molecule SHIP1 agonist, AQX-MN100, can be used to inhibit immune cell activation in vitro, and in vivo mouse models of inflammatory disease.

Our results suggest that the current model for SHIP1 activation, involving translocation of SHIP1, via its SH2 or its phosphorylated NPXY motifs, from the cytoplasm to the plasma membrane without any change in its intrinsic phosphatase activity needs to be modified. Specifically, we postulate that upon recruitment to the plasma membrane, SHIP1 hydrolyzes a small amount of PIP3 at a low, basal rate. This generates some PI-3,4-P2 which then binds to the C2 domain, leading to a
conformation change which enhances its catalytic activity. Interestingly, end-product activation has also been reported for two 3′ inositol lipid phosphatases. PTEN binds its product (PI-4,5-P₂) using an N-terminal lipid binding motif resulting in enhancement of phosphatase activity. Similarly, the MTM phosphatase, binds its product (PI-5-P) via a divergent PH domain, which allosterically activates its function. In the case of PTEN, the requirement for PI-4,5-P₂ helps localize PTEN protein to specific regions of the membrane. The binding of SHIP1 by its product PI-3,4-P₂ may also similarly serve to localize SHIP1 to the plasma membrane in addition to allosterically activating its intrinsic inositol phosphatase activity providing a positive feedback mechanism to rapidly reduce membrane PIP₃ levels. Regardless of whether or not PI-3,4-P₂ binding regulates SHIP1 localization, this newly described allosteric activation domain within SHIP1 may be exploited therapeutically by pharmacological agents such as AQX-MN100 that bind to the allosteric activation site to stimulate SHIP1 activity.

From their first description, allosteric sites have been considered to be more important as drug targets than active sites and allosteric regulators have been predicted to possess more selectivity than active site modulators. While it remains challenging to prove that a certain drug-target interaction is responsible for mediating its biological effects, our observation that AQX-MN100 had minimal effects on macrophages lacking the SHIP1 target provides compelling support that the PI-3 kinase pathway inhibitory effects observed are mediated by SHIP1 itself. Furthermore, our observation that AQX-MN100 exhibits efficacy having a submicromolar EC₅₀ suggests that this class of compounds possesses a low likelihood of off-target effects based on calculations by Knight and Shokat. Indeed, it has been determined that AQX-MN100 has minimal
off-target effects when screened against 100 other kinases and phosphatases (Figure F.1 in Appendices).

The studies in this chapter offer proof-of-principle that small molecule activators of lipid phosphatases exist and that they provide a new paradigm for inhibition of PI-3 kinase-dependent processes. Small molecule agonists of the hemopoietic cell-specific SHIP1 enzyme, in particular, represent potential therapeutics for treatment of immune/hemopoietic disorders in which the PI-3 kinase pathway is dysregulated. Due to their unique target and mechanism of action, these compounds may also be powerful synergistic agents in combination with current therapies. Agonists of other allosterically regulated phosphatases, such as PTEN \textsuperscript{524} and MTM \textsuperscript{525}, may similarly be useful for diseases in which their impaired activity has been implicated.
CHAPTER 4: A PLECKSTRIN HOMOLOGY-RELATED DOMAIN IN SHIP1 MEDIATES MEMBRANE LOCALIZATION IN FcγR-MEDIATED PHAGOCYTOSIS
4.1 Introduction

Lipid phosphatases have a central role in regulating a vast array of cellular processes induced by extracellular signals, including phagocytosis, cell migration, proliferation, and survival. In particular, inositol lipid phosphatases such as SHIP1 and SHIP2, PTEN and MTM, are known to be essential for maintaining cellular homeostasis and mutations in these genes are attributed to various hematologic cancers, solid organ tumors, and skeletal myopathy respectively. The cellular function of these phosphatases requires their physical recruitment to the intracellular membrane compartments containing their phosphatidyl inositol substrates. Thus, understanding the mechanisms by which these enzymes are recruited to the membrane is important.

Phagocytosis is a dynamic process involving the coordinated recruitment and activation of signaling proteins, lipid-modifying enzymes, and components of the cytoskeletal machinery. How all these elements are spatially and temporally regulated to achieve particle engulfment is still not fully understood. In FcγR-induced phagocytosis, Ig-opsonized particles bind to FcγRs on the surface of phagocytic cells. Clustering of ligand-bound FcγRs triggers the activation of Src and Syk family kinases and subsequent stimulation of signaling proteins including the PI-3 kinases and Rho-family GTPases. Together, these pathways facilitate the membrane modifications and actin remodeling required for pseudopod extension and formation of the phagosome around the particle being ingested.

One of the principle events committing a cell towards phagocytosis of a particle is generation of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) by PI-3 kinases through the phosphorylation of PI-4,5-P₂. Pharmacological inhibition of PI-3 kinase by agents such
as wortmannin and LY294002 prematurely arrests phagocytosis and demonstrate that PI-3 kinase is essential for engulfment of particles greater than 2 μm in size \(^{471,548}\). Synthesis and hydrolysis of PIP\(_3\) follows an ordered sequence upon particle binding to and clustering of FcγRs. Beginning at the base of the phagocytic cup where PI-4,5-P\(_2\) is converted to PIP\(_3\), the wave of PIP\(_3\) generation proceeds up the lateral edges of membrane surrounding the particle, and finally disappears immediately upon phagosome sealing \(^{450,552-554}\). Cellular levels of PIP\(_3\) are tightly regulated under normal conditions, both by controlling PI-3 kinase activation and by the presence of the 5′-inositol phosphatases (SHIP1, SHIP2, and 72-kDa 5′-phosphatase, 72-5ptase), which hydrolyze PIP\(_3\) to PI-3,4-P\(_2\), and the 3′-inositol phosphatase PTEN, which generates PI-4,5-P\(_2\) \(^{492-494}\). Of the four phosphatases, only SHIP1, SHIP2 and 72-5ptase are reported to localize to the phagocytic cup where they are thought to be responsible for the observed hydrolysis of PIP\(_3\) to PI-3,4-P\(_2\) \(^{396,494,555}\). Whether these three phosphatases have redundant roles or are regulated independently to perform specific functions during the phagocytic process has yet to be determined.

SHIP1 negatively regulates phagocytosis as shown by over-expression \(^{396}\) and gene knock-out studies \(^{396,555}\). FcγRI and FcγRIIa receptors have been reported to recruit SHIP1 to the phagocytic cup via interactions between phosphotyrosines within ITAMs on the receptors and the SHIP1 SH2 domain \(^{349,350}\). Once at the membrane, SHIP1 is brought into the vicinity of its substrate where it negatively regulates FcγRI/FcγRIIa signaling and phagocytosis through degradation of PIP3. Imaging of inositol lipids during phagosome formation has shown that membrane recruitment of SHIP1 to the leading edge correlates with a decreasing gradient of PIP\(_3\) and a corresponding increase
of the SHIP1 product PI-3,4-P$_2$ along the developing phagosome$^{450,554,556}$. Additionally, SHIP1 has also been reported to be recruited to the phagocytic cup via the inhibitory Fc$\gamma$RIIb receptor through interactions between its SH2 domain and phosphotyrosines within immunoreceptor tyrosine inhibitory motifs (ITIMs) on Fc$\gamma$RIIb$^{352,414,557}$. The membrane proximal SHIP1 then negatively regulates the activating signals generated by Fc$\gamma$RI/Fc$\gamma$RIIa.

This chapter describes a previously unrecognized domain in SHIP1 that mediates direct binding to membrane lipids and that this lipid mediated interaction is the major mechanism by which SHIP1 is recruited to the phagocytic cup. Using nuclear magnetic resonance (NMR) spectroscopy, we demonstrate that this segment of SHIP1 adopts an independently folded structure predicted to have pleckstrin homology (PH) domain-like topology. This PH-related (PH-R) domain binds PIP$_3$ and is required for SHIP1 localization of SHIP1 to the phagocytic cup to inhibit Fc$\gamma$R-mediated phagocytosis. Site directed mutagenesis of candidate amino acid residues reveals two critical lysine residues involved in the binding. Replacement of these lysines with alanines abrogates the ability of recombinant PH-R domain to interact with PIP$_3$ in in vitro lipid binding assays and the ability of SHIP1 protein to translocate to the phagocytic cup in macrophages to negatively regulate particle uptake. These studies provide further insight into mechanisms regulating SHIP1 function in cells and indicate that phosphoinositol lipid-mediated recruitment of proteins is an important step in phagosome maturation. These findings also suggest that small molecules, which alter PH-R domain interactions with PIP$_3$, may be another means of modifying SHIP1 activity for therapeutic purposes.
4.2 Materials and methods

4.2.1 SHIP1 sequence domain identification

Secondary structure and order/disorder prediction algorithms were used to identify a potentially folded region in SHIP1 adjacent to the phosphatase domain (Figure 4.2). Alignments performed using ClustalW \(^{558}\) confirmed that this region is conserved amongst all deposited SHIP1 sequences of the various species represented in the National Center for Biotechnology Information database (Figure 4.1). To define the boundaries of the structured region more precisely, truncation and extension constructs were expressed and tested for their lipid binding ability as described below.

4.2.2 Expression and purification of SHIP1 PH-R domain and K397A/K370A (KAKA) mutant domain

A cDNA encoding residues 292-401 of SHIP1 (mouse) was generated by PCR and cloned into pET28c (Novagen, Madison, WI). The cDNA was inserted such that a 21 amino acid segment containing a 6×His epitope tag was fused to the N-terminus of the 111-residue protein (full sequence in Supplemental Methods). Constructs were transformed into \(E.\ coli\) BL21 (DE3) competent cells (Promega, Madison, WI). Protein was expressed and purified using a method as described previously \(^{559}\). Standard site-directed mutagenesis methodologies were used to generate mutant PH-R constructs in which candidate PIP binding residues were replaced with alanines. Recombinant protein from these constructs, including the KAKA variant with K370A/K397A were expressed and purified as described above.
4.2.3 Sequence of 6×His affinity tagged SHIP1 PH-R domain

MGSSHHHHHHHSSGLVPRGSMSSTNRRSLIPPVTFEVKSESLEIPQKMKHDV
ESGKLIVKKSKDSDSEQYSHKHILQLIKSGQFKNKLQILVETEKEKILRKEVFA
DSKKREGFCQLQQMKNKHSQ

The 21 amino acid segment containing the 6×His epitope tag is indicated in bold face.

4.2.4 Expression and purification of the isotopically labeled PH-R domain

Uniformly labeled SHIP1 PH-R domain was expressed in *E. coli* Rosetta (λDE3) cells (Novagen, Madison, WI) in M9 minimal medium supplemented with $^{15}$NH$_4$Cl and $^{13}$C$_6$-D-glucose (Cambridge Isotope Laboratories Inc., Andover, MA). The expression protocol was modified from the methods of Marley *et al.* 560,561 to reduce the consumption of isotopically-labeled ingredients. Briefly, the cells were initially grown in 4×1 L terrific broth (TB) at 37 °C to an OD$_{600}$ of 0.7 and then harvested at 2,600×g at 4 °C for 15 min. Pelleted cells were resuspended and pooled in a wash solution of the M9 buffer salts (50 mL) and allowed to stand at 4 °C for 20 min followed by centrifuging again at 2,600×g at 4 °C for 15 min. This cleaning step was repeated once more to remove any residual rich media. The washed pellet was resuspended in 10 mL of the M9 salt solution and added to 500 mL of pre-incubated (at 37 °C) 2×M9 media with 0.7 g $^{15}$NH$_4$Cl and 2 g $^{13}$C$_6$-D-glucose as the sole sources of nitrogen and carbon. The M9 media was also supplemented with nutrients and vitamins according to Neidhardt *et al.* 562. Expression of the recombinant PH-R domain was initiated after 30 min by the addition of 240 mg of isopropyl-β-D-thiogalactopyranoside (IPTG) (BioShop, Burlington, ON). Expression was halted after 16 hours by chilling the cells on ice for 30
min, followed by centrifugation at 2,600×g at 4 °C for 30 min. Harvested cells were flash-frozen in liquid nitrogen and stored at -80 °C.

The majority of the expressed PH-R domain was found in the insoluble fraction. Protein purified from the soluble fraction under non-denaturing conditions produced the same 2-dimensional $^1$H–$^{15}$N HSQC correlation spectrum as the PH-R domain purified from the inclusion bodies under strong denaturing and reducing conditions. For this reason all further purifications of the PH-R domain were done under denaturing conditions to maximize the protein yield.

Pellets from the protein expression (approximately 10 g wet weight) were thawed and resuspended in 40 mL of extraction buffer (6 M guanidine hydrochloride (Gdn-HCl), 100 mM Tris-HCl, 500 mM NaCl, 5 mM tris(2-carboxyethyl) phosphine (TCEP) at pH 8.2). The suspended cells were microprobe sonicated (3 mm tapered tip) on a Branson Sonifier 250 ultra sonic cell disruptor for 15 min at power 4 and a 50% duty cycle to reduce sample heating. The sonicated sample was then flushed through a 22 gauge, hypodermic needle twice and then a 26 gauge needle once to shear DNA and reduce the sample viscosity. The lysed cells were centrifuged at 30,600×g for 30 min to remove the insoluble material. The supernatant was then injected onto 5 mL HisTrap column (GE Heathcare, Piscataway, NJ) that was pre-equilibrated with the extraction buffer on a ÄKTA purifier (GE Healthcare, Piscataway, NJ) running at a flow rate of 4 mL/min. Once the entire sample was injected (in 10 mL increments), the column was washed with an additional 40 mL of the extraction buffer. A two-step imidazole gradient was used to wash and elute the protein from the column by combing the extraction buffer and the elution buffer (6 M Gdn, 100 mM Tris-HCl, 300 mM NaCl, 5 mM TCEP and 200 mM
imidazole). The first gradient had an increasing imidazole concentration rate of 4 mM/min for 15 min (12 column volumes), followed by a second gradient increase at a rate of 20 mM/min for 7 min reaching 100% of the elution buffer with 200 mM imidazole. The column was then washed with an additional 8 column volumes of the 100% elution buffer. The PH-R domain began eluting with approximately 50 mM imidazole. The appropriate fractions were pooled, concentrated with an Amicon Ultra-15 (Millipore, Billerica, MA) centrifugal filter to a final volume of 10 mL. The sample was then serially dialyzed at room temperature (5 hours each) in 3,500 MWCO SnakeSkin (Pierce, Rockford, IL) to remove the Gdn and salts against 0.1, 0.05, and 0.01 M ammonium acetate at pH 4. A final dialysis step was done against water. The dialysate was then frozen and lyophilized. The protein yield was in general between 15 and 20 mg by this method.

The K370A/K397A double mutant was transformed and expressed under identical conditions as the wild-type PH-R domain except that the M9 media was isotopically enriched with only $^{15}$NH$_4$Cl. All other aspects of the expression and purification of the K370A/K397A mutant are identical.

4.2.5 NMR sample preparation

Samples of the His$_6$-tagged PH-R domain were initially prepared at 0.3 mM concentration at pH 7 in 20 mM Tris-HCl, with 150 NaCl and 10 mM β-mercaptoethanol (βME). Although showing well dispersed spectra, there were a number of missing or weak resonances. At 0.3 mM, the PH-R samples also seemed to reach a critical concentration as the sample would show signs of precipitation within a week. To overcome this stability issue, subsequent samples were prepared by dissolving the
lyophilized protein at pH 5.8 in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer with 200 mM Gdn-HCl, and 5 mM TCEP. Under these conditions protein samples at 0.5 mM remained soluble for months.

4.2.6 NMR spectral assignments

Spectra of the 131-residue $^{13}$C/$^{15}$N-labeled His$_6$-tagged PH-R domain were recorded at 25 °C on a 600 MHz Varian INOVA spectrometer equipped with a cryogenic triple resonance probe head, using standard sensitivity-enhanced gradient Varian BioPack pulse sequences $^{563-567}$ including $^1$H-$^{15}$N HSQC, HNCA CB $^{564}$, CBCA(CO)NH $^{565}$, HNCO $^{566}$ and HN(CA)CO $^{567}$. Spectra were processed with NMRPipe $^{568}$ and subsequently exported to Sparky for analysis $^{569}$. $^1$H chemical shifts were referenced to external 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 25 °C $^{570}$. $^{15}$N and $^{13}$C referenced using recommended magnetogyrical ratios $^{571}$.

4.2.7 NMR-monitored titrations

The interaction of phosphatidylinositol-3,4,5-trisphosphate diC8 (Echelon Biosciences, Salt Lake City, UT) with the PH-R domain was monitored by $^1$H-$^{15}$N HSQC spectroscopy. Aliquots of saturated PIP3 solution were titrated to a final concentration of 0.17 mM into ~0.1 mM $^{15}$N-labeled PH-R domain. The protein was in 20 mM Tris-HCl pH 7 with 150 mM NaCl, as initial studies of the PH-R domain with PIP3 in PLO assays showed that the ligand did not interact in the presence of Gdn-HCl and at pH 5.8.

4.2.8 Phosphoinositol binding (PLO assay)

diC16 lipids (Echelon Biosciences, Salt Lake City, UT) reconstituted in a 2:1.8 solution of methanol and water were spotted onto dry PVDF membranes at the indicated
quantities. Membranes were blocked in blocking buffer (3% BSA in TBS with 0.05% NaN₃) for 1 hour at 23°C with gentle agitation. Membranes were then incubated with 625 nM of recombinant PH-R domain protein in a 6 ml volume of blocking buffer for 6 hours, washed 10 times over 50 minutes in TBST buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20), followed by incubation with 1 µg/mL anti-His₆ mouse IgG (Sigma, Mississauga, ON) for 1 hour at 23°C. Membranes were washed an additional 10 times with TBST buffer, and incubated with AlexaFluor660 goat anti-mouse IgG (Invitrogen, Burlington, ON) for 1 hour at room temperature. After washing, bound domain was detected and quantified on a LiCor Odyssey Infrared Scanner (Lincoln, NE).

4.2.9 Cells and reagents

RAW264.7 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified eagle’s medium (Thermo Scientific, Logan, UT) supplemented with 9% (v/v) fetal bovine serum (FBS) (Thermo Scientific, Logan, UT). J16 (SHIP₁⁺/⁺) and J17 (SHIP₁⁻/⁻) cell lines were generated by infecting BMMΦs from C57BL/6 SHIP₁⁺/⁺ and SHIP₁⁻/⁻ mice respectively, with the J2 virus and maintained in Iscove’s Modified Dulbecco’s Medium (Thermo Scientific, Logan, UT), supplemented with 10% FBS, 10 µM β-mercaptoethanol, 150 µM monothioglycolate, and 1 mM L-glutamine. J17 cells were transduced with lentiviruses harbouring expression constructs for wild-type (WT) SHIP1 or SHIP1 with residues K370 and K397 mutated to alanines (KAKA) under the control of a tetracycline-responsive promoter. Transduced cells were separated from non-transduced cells by fluorescence-activated cell sorting based on the co-expression of mCherry on the lentiviral transfer plasmid. Transduced cells were treated with 2 µg/ml of doxycycline (Sigma, Mississauga, ON) for 48 hours to induce
WT or KAKA SHIP1 expression prior to use. Expression of WT and KAKA SHIP1 were confirmed by resolving cell lysates by SDS-PAGE and immunoblotting using anti-SHIP1 (P1C1) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).

4.2.10 GFP-tagged SHIP1 PH-R domains

eGFP fusion constructs were generated by inserting PCR-generated cDNA fragments corresponding to SHIP1 residues 292-401 and AKT1 residues 1-165 into the pEGFP-C1 vector (Clontech, Mountain View, CA) using EcoRI and BamHI restriction cut sites. Plasmid constructs were then transiently transfected into RAW264.7 cells using FuGENE HD (Roche, Laval, QC) as per the manufacturer’s protocol.

4.2.11 Scintillation proximity assays (SPA)

Copper chelate YSi SPA Scintillation Beads (GE Healthcare, Piscataway, NJ) were diluted in 0.25% BSA in TBS to 1.5 mg/ml. 10 nM of each His6-tagged SHIP1 C2 domain or PH-R domain were allowed to bind to the beads by incubating at 23°C for 1 hour. 250 µg of domain-coated beads were aliquoted per well of a 96-well plate. 0.185 MBq (5 µCi) of \[^{3}H\]-AQX-MN100 was then added to each well and incubated for 30 minutes with gentle agitation. The amount of bead-associated radioactivity was quantified by counting in a Wallac (Perkin-Elmer, Waltham, MA) Betaplate scintillation counter.

4.2.12 Phagocytosis assays

_Bead preparation_ - 3 µm or 15 µm latex beads (Polysciences Inc, Warrington, PA) were opsonized with 100 µg/ml human IgG (Sigma, Mississauga, ON), or 100 µg/ml of human IgG labeled with a Dylight 680 conjugating kit (Thermo Scientific, Rockford, IL),
respectively, overnight at 37°C with gentle agitation. Beads were washed extensively with PBS containing 3% FBS and resuspended in cold DMEM supplemented with 9% FBS. **Cell Preparation** – Transfected RAW264.7 cells, J16 SHIP1<sup>+/+</sup> (SHIP1<sup>+</sup>), J17 SHIP1<sup>−/−</sup> (SHIP1<sup>−</sup>), J17 SHIP1<sup>−/−</sup> cells reconstituted with SHIP1 WT (SHIP1<sup>−/−</sup>:WT) or J17 SHIP1<sup>−/−</sup> cells reconstituted with SHIP1 KAKA (SHIP1<sup>−/−</sup>:KAKA) were plated onto poly-L-Lysine treated glass coverslips and allowed to adhere overnight at 37°C, 5% CO₂.

**Bead stimulation** – Overnight supernatants were removed from the cells and replaced with opsonized beads at a 10 beads:1 cell ratio. Plates were centrifuged at 4°C to allow beads to settle onto the coverslips, then plates were immediately placed in a 37°C, 5% CO₂ incubator for phagocytosis to proceed. **Slide preparation** – Stimulated cells were washed thoroughly with cold PBS, fixed in 2% paraformaldehyde in PBS for 30 minutes at 37°C, washed again with PBS, then mounted onto glass slides using Prolong® Gold antifade reagent with DAPI (Invitrogen, Burlington, ON). Images were acquired using a Zeiss Axioplan2 Fluorescence microscope equipped with a 63X oil-immersion lens and analyzed using Zeiss AxioVision4.8 software. Phagocytic indices were calculated where the phagocytic index was defined as the average number of beads phagocytosed by one cell. For experiments involving localization of SHIP1 by immunocytochemistry, cells on coverslips were stimulated with 15 µm beads prior to 2% paraformaldehyde in PBS fixation and permeabilization with 0.5% TritonX-100 in PBS. The coverslips were blocked with 3% BSA, stained with primary mouse anti-SHIP1 (P1C1) antibody (SantaCruz Biotechnologies, SantaCruz, CA), followed by FITC-conjugated goat anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, Westgrove, PA). The coverslips were then mounted onto glass slides and images acquired using a Zeiss
LSM780 confocal microscope equipped with a 63X oil-immersion lens. Confocal images were analyzed using Zeiss Zen 2009 software. Images were scored using a 4-point grading system by 3 independent, blinded investigators.

4.2.13 Production of recombinant SHIP1 WT and SHIP1 KAKA
Full-length N-terminal His$_6$-tagged WT or KAKA SHIP1 were produced in Sf9 cells using a baculovirus expression system (Invitrogen, Burlington, ON). Cells were pelleted, lysed, and recombinant enzyme was purified from lysates using TALON cobalt affinity chromatography resin (Clontech, Mountain View, CA) and determined to be more than 95% pure as described above.

4.2.14 in vitro phosphatase assays
SHIP1 enzyme assays were performed by diluting 10 ng of recombinant WT of KAKA SHIP1 enzyme in 25 µl of dilution buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$) in each well of a 96-well microtiter plate. Enzymes were incubated with vehicle, PI-3,4-P$_2$ or AQX-MN100 for 15 minutes at 23°C prior to the addition of 100 µM inositol 1,3,4,5-tetrakisphosphate (IP$_4$, Echelon Biosciences, Salt Lake City, UT). After 10 minutes incubation at 37°C, malachite green reagent was added and the amount of inorganic phosphate released was measured by an absorbance reading at 650 nm. For enzyme kinetics determinations, enzyme reactions with IP$_4$ substrate concentrations ranging from 10-200 µM IP$_4$ were sampled at 1 minute intervals for 10 minutes. Enzyme initial rates were determined from the slope of the linear portion of the resulting time courses and plotted against IP$_4$ concentrations.
4.3 Results

4.3.1 Identification of a PH related (PH-R) domain in SHIP1

Since our study of the enzymatic properties of SHIP1 described in Chapter 3 revealed that it is allosterically activated by its product PI-3,4-P₂, we searched for potential PI-3,4-P₂ binding domains within SHIP1. In addition to the C2 domain, amino acid alignments (Figure 4.1) and secondary structure predictions revealed a second 110 amino acid region N-terminal to the phosphatase domain that had features of a PH domain (Figure 4.2).

In order to characterize this newly recognized region of SHIP1, residues 290-410 were cloned and expressed for NMR spectroscopic analysis. As shown in Figure 4.3A (red), the resulting protein fragment yielded a well-dispersed \(^1\text{H}-^{15}\text{N}\) heteronuclear single quantum coherence (HSQC) spectrum, confirming that this segment of SHIP1 indeed adopts an independently folded structure. After assigning the signals from the mainchain \(^1\text{H}, ^{13}\text{C}\) and \(^{15}\text{N}\) nuclei of this species (Figure 4.3B), the chemical shift-based secondary structure propensity (SSP) algorithm was used to identify its secondary structural elements (Figure 4.3C). Consistent with the sequence-based secondary structure predictions, the SHIP1 domain contains several \(\beta\)-strands as well as a clear C-terminal helix.

Unfortunately, although having an assignable \(^1\text{H}-^{15}\text{N}\) HSQC spectrum, residues 290-410 of SHIP1 also exhibited unfavorable dynamic properties leading to extensive conformational exchange broadening of the NMR signals from sidechain nuclei. This prevented us from determining its tertiary structure using NMR measured restraints. Therefore, we used the THRIFTY webserver to predict the fold of the SHIP1 domain.
based upon chemical shift-guided homology threading. This program essentially finds residues in the database of NMR derived structures that have similar chemical shifts to a query protein. With two perpendicular β-sheets followed by a C-terminal α-helix, the resulting model has the overall fold characteristic of a PH domain (Figure 4.3D). However, it is important to stress that PH domains show significant variation in sequence, structure and function, reviewed in 576,577. Accordingly, in the absence of a more detailed structural analysis, we conservatively denote this region of SHIP1 as a PH related (PH-R) domain.
Figure 4.1  Sequence alignment and phylogram of the PH-R domain.

(A) ClustalW alignment and (B) phylogram of all SHIP1 and SHIP2 sequences deposited in the NCBI database corresponding to the regions 120 amino acids N-terminal of the putative phosphatase domains.
Figure 4.2
Figure 4.2  SHIP1 contains an independently folded Pleckstrin Homolgy-Related (PH-R) domain.

(A) Schematic of the domain structure of SHIP1. Following the N-terminal SH2 domain, there is a 300-residue segment leading up to the catalytic phosphatase domain that contains the newly recognized PH-R domain. Following the phosphatase domain, the C-terminal region is a stretch of approximately 350 residues that contains a calcium-dependent binding domain (C2) 559, 2 phosphotyrosine motifs (NPXY) and 3 potential SH3 domain binding motifs (PXXP). (B) To identify the PIP3 lipid-binding region more precisely in order to clone the domain, the program PONDR 578-580 was used to predict the ordered and disordered regions of SHIP1. Shown are PONDR L-XT scores for the full length 1191 residue murine SHIP1 sequence (solid green line) and for the isolated putative PH-R domain (residues 292-401; dashed magenta line). The lower panel is an expanded view for residues 290-410. Regions above/below the threshold of 0.5 are considered disordered/ordered. (C) Secondary structure predictions (helices, red cylinders; strands, blue arrows) for residues 292-401 were obtained using the webserver algorithms HNN 581, GOR4 582, PSIPRED 583,584, SOPMA 585, Jpred3 586, PORTER 587, PROF 588, Sspro 589, and JUFO 590,591. (D) A consensus score was generated for each residue with predicted helices (red) being valued as +1, strands (blue) as -1, and coils as 0. Also shown for comparison in panel B are the SSP (Secondary Structure Propensity 574) scores based upon the observed 13Cα and 13Cβ chemical shifts of the isolated PH-R domain.
Figure 4.3
Figure 4.3  SHIP1 contains an independently folded PH-R domain.

(A) Overlaid $^1$H-$^{15}$N HSQC spectra of the His$_6$-tagged PH-R in the absence (red) and presence of 170 µM PIP$_3$ (blue). The excellent dispersion of the signals from the amide $^1$H-$^{15}$N groups indicates that the isolated PH-R domain is folded and the spectral changes upon addition of PIP$_3$ confirm the binding of this ligand. (B) Assigned amide backbone region of the $^1$H–$^{15}$N HSQC spectrum of His$_6$-tagged PH-R domain from SHIP1 (0.5 mM) at pH 5.8 in 20 mM MES, 5 mM TCEP, and 200 mM Gdn-HCl. The spectrum was recorded at 25°C on a 600 MHz Varian INOVA spectrometer equipped with a cryogenic triple resonance probehead using 612×128 complex points ($^1$H sw = 12 ppm; $^{15}$N sw = 32 ppm) and 16 scans per increment. The dashed and solid ellipses encompass the signals from sidechains of Asn/Gln and Arg (aliased), respectively. The high degree of signal intensity variation is attributed to a range of conformational dynamic exhibited the isolated PH-R domain. (C) The SSP (Secondary Structure Propensity) scores determined from $^{13}$C$^\alpha$ and $^{13}$C$^\beta$ chemical shifts identifies experimentally the helical (+1) and strand (-1) regions in the PH-R domain. (D) A model generated with THRIFTY using chemical shift information to guide molecular homology threading, demonstrates that residues 292-401 of SHIP1 adopt a PH domain-like fold. Highlighted in yellow is a possible PIP binding motif similar to that proposed in $^{592}$. Also shown in magenta are the side chains of K370 and K397.
4.3.2 Functional characterization of the PH-R domain

To test the ability of this PH-R domain within SHIP1 to bind PIPs, we expressed and purified the N-terminal His₆-tagged PH-R protein in E. coli for in vitro studies. Much of the PH-R protein was found as insoluble inclusion bodies but ~10% existed in a soluble form that we purified by cobalt-chelating affinity chromatography from Nonidet P-40 lysates. We tested the ability of the PH-R domain to bind PIPs in a PLO assay. PVDF membranes were spotted with 0 to 50 pmoles of PI-4,5-P₂, PIP₃, or PI-3,4-P₂ and incubated with 0 to 625 nM of His₆-PH-R protein. Figure 4.4A shows a representative PLO blot and the quantification is summarized in Figure 4.4B. The SHIP1 PH-R domain bound to PIP₃ more strongly than either PI-4,5-P₂ or PI-3,4-P₂. To calculate the binding affinities of the PH-R domain to each of the phosphatidyl inositol lipids, we plotted double-reciprocal graphs of the data shown in Figure 4.4B (Figure 4.4C). Based on this analysis, the equilibrium dissociation constant, Kₐ, of the PH-R domain/PIP₃ complex was found to be 1.9 ± 0.2 nM. The Kₐ values for the PI-4,5-P₂ and PI-3,4-P₂ could not be determined with precision by this approach giving estimates of ~10 nM for both lipids. In parallel, we also used NMR spectroscopy to confirm that the isolated PH-R domain binds PIP₃ (Figure 4.3A, blue).

As described in Chapter 3, SHIP1 is allosterically activated by its product, PI-3,4-P₂, and by the small molecule agonist, AQX-MN100 via SHIP1’s C2 domain. Because of the observed PI-3,4-P₂ binding of the PH-R domain, we tested whether it might also bind AQX-MN100. We compared the ability of the PH-R and C2 domains to bind [³H]-AQX-MN100 using SPA beads coated with recombinant PH-R or C2 protein. Figure 4.4D shows that the C2 domain binds AQX-MN100 as previously described. In contrast,
the PH-R domain and BSA negative control protein exhibit similar levels of [$^3$H]-AQX-MN100 binding. Thus, the PH-R domain does not measurably bind this compound.

We next determined whether the PH-R domain behaved like other PIP$_3$-binding PH domains in cell-based assays. Fc$\gamma$R-mediated phagocytosis is associated with activation of PI-3 kinase, which causes a rapid and transient accumulation of PIP$_3$ at the phagocytic cup $^{471,548}$. The Akt-PH domain preferentially interacts with 3$'$-phosphoinositides and binding of PIP$_3$ and PI-3,4-P$_2$ by an N-terminal GFP fusion of this domain has been well characterized in cells $^{554,594}$. We constructed a similar N-terminal fusion protein of the SHIP1 PH-R domain and transiently expressed either Akt-PH domain-GFP, SHIP1 PH-R domain-GFP, or GFP alone in RAW264.7 macrophage cells. These transfected cells were plated onto coverslips and exposed to Ig-coated 3 $\mu$m latex beads (Ig-opsonized beads) for the indicated length of time (Figures 4.5B and 4.5C). GFP fluorescence associated with fusion domain recruitment from the cytoplasm to the phagocytic cup was then quantified by scoring micrographs with a 4 point scoring key (Figure 4.5A). As shown in Figure 4.5C, the SHIP1-PH-R domain translocated to the forming phagocytic cup with similar kinetics to the Akt-PH domain whereas GFP alone did not exhibit any defined localization.
Figure 4.4
Figure 4.4  SHIP1’s PH-R domain preferentially binds PIP₃ but does not bind the allosteric activator AQX-MN100.

(A) Ability of recombinant PH-R domain to bind to PI-4,5-P₂, PI-3,4-P₂ or PIP₃ in protein lipid overlay (PLO) assays. PVDF membranes were spotted with the indicated amount of PIP and incubated PH-R domain as described in Materials and Methods. The PLO membrane scan presented is representative of 3 independent experiments. (B) The spots in (A) were quantified and data are expressed as mean intensities ± standard deviations (n=3) ***P <0.001 when comparing PIP₃ binding to PI-4,5-P₂ or PI-3,4-P₂ [Two-way ANOVA]. (C) Reciprocal of the mean intensities in (B) were plotted against the reciprocal of the amount of lipid spotted. K_D values were calculated from the slope of the lines as determined by linear regression (GraphPad Prism, San Diego, CA). (D) Ability of PH-R domain to bind [³H]-AQX-MN100. Recombinant SHIP1 PH-R or C2 domains were coated onto copper chelating (His-Tag) YSi SPA Scintillation Beads in the presence of 0.25% BSA. Beads were incubated with 0.185 MBq (5 µCi) of [³H]-AQX-MN100 and the bead-associated radioactivity measured. Data are expressed as scintillation count means ± standard deviations (n=3). **P <0.01 [One-way ANOVA]
Figure 4.5
Figure 4.5   The SHIP1 PH-R domain localizes to the phagocytic cup.

RAW264.7 cells transfected with SHIP1 PH-R or AKT1 PH domain GFP fusion constructs or GFP alone were stimulated with human IgG opsonized 3 μm Latex Beads for the times indicated. An asterisk indicates the position of the latex bead on the micrographs. Scale bars = 10 μm. Micrographs were scored by 3 independent, blinded investigators according to the four-point scoring key depicted (A). Representative brightfield and confocal micrographs are shown in (B) and the mean scores of 100 phagocytosis events at each time point are presented ± standard deviations (C). **P <0.01, ***P <0.001 when comparing Akt-PH-GFP or SHIP1-PH-R-GFP to GFP alone [Two-way ANOVA]
4.3.3 Identification of amino acid residues involved in PIP₃ binding

To identify the specific amino acid residues involved in PIP₃ binding, we expressed PH-R mutant proteins in which alanines were substituted for candidate residues (Figures 4.6A and Figure G.1 in Appendices). These residues were chosen based upon their conservation among the 14 SHIP1 orthologs and their charge and/or sequence proximity to positively-charged residues, which could potentially mediate an interaction with the negatively-charged PIP₃ inositol headgroup. Unfortunately, few clues were provided from the NMR-monitored titrations shown in Figure 4.3A, as residues showing chemical shift perturbations upon PIP₃ binding were distributed broadly over the model of the PH-R domain. The lack of a clearly defined binding site could reflect indirect conformational changes of the rather dynamic protein domain (Figure 4.3B), as well as the possibility of multiple binding interfaces.

As before, His₆-tagged proteins were expressed in E. coli, purified, and tested for their ability to bind PIPs in a PLO assay (Figure 4.6). Of the 10 mutant domains analyzed, two lysine to alanine mutants, K370A and K397A, exhibited an impaired ability to bind PIP₃ and PI-3,4-P₂ (Figure 4.6A and Figure G.1 in Appendices). The $K_D$ values for the mutant domains were indeterminable as they bound PIP₃ and PI-3,4-P₂ with such low affinity that we were not able to saturate binding (Figure 4.6A and Figure H.1 in Appendices). We then constructed a double mutant domain where both K370 and K397 residues were substituted with alanines (KAKA). The resulting KAKA PH-R mutant exhibited a further reduction in PIP₃ and PI-3,4-P₂ binding as compared to either of the individual point mutation domains (Figure 4.6B). Interestingly, the wild-type (WT) and KAKA mutant proteins bound PI-4,5-P₂ equally well. The $^1$H-$^{15}$N-HSQC
spectrum of the KAKA PH-R domain was also well-dispersed, indicating that the loss of PIP_3 and PI-3,4-P_2 binding ability is not due to a disruption of global protein structure (Figure 4.7). Dowler et al. suggest a PIP binding motif for PH domains of the form K-X-small-X(6-11)-R/K-X-R-hydrophobic-hydrophobic. The SHIP1 PH-R domain has a region including one of the critical lysines (K370) and spanning residues 334-372 that has a similar form of KDG-X(31)-JEKIL (Figure 4.3D). The second critical lysine residue, K397 is not involved in this motif, but rather is in the C-terminal helix. It is possible that K397 is part of a second binding interaction that binds PIP_3 via basic residues between the C-terminal helix and the adjacent β-strand in a manner similar to phox homology (PX) domain binding.

4.3.4 The KAKA PH-R protein has impaired localization to the phagocytic cup

We then examined the ability of the KAKA PH-R domain to be recruited to the phagocytic cup in RAW264.7 macrophages stimulated with Ig-opsonized beads. Using the same scoring key described in Figure 4.5A, we found that the KAKA PH-R protein was unable to translocate to the phagocytic cup, suggesting that residues K370 and K397 were important in mediating the translocation and/or association of the PH-R domain with the membrane (Figure 4.6D).
Figure 4.6
Figure 4.6  Mutation of SHIP1 PH-R domain residues K370 and K397 to alanines impairs its ability to interact with PIP₃.

PLO assays were performed and quantified as previously described for each of PI-(4,5)-P₂, PIP₃, and PI-(3,4)-P₂ (spotted at the amounts indicated) with 625 nM of (A) recombinant WT SHIP1 PH-R domain, K370A, or K397A mutant PH-R domains or (B) the double-mutant KAKA PH-R domain. Data are expressed as mean intensities ± standard deviations and are representative of 3 independent experiments. *P <0.05, **P <0.01, ***P <0.001 [Two-way ANOVA]. (C) RAW264.7 cells were transiently transfected with WT PH-R-GFP or KAKA PH-R-GFP fusion constructs and imaged as in Figure 4.5. Micrographs were scored by 3 independent, blinded investigators according to the four-point scoring key depicted Figure 4A. *P <0.05, ***P <0.001 [Two-way ANOVA].
Figure 4.7  Overlaid $^1$H–$^{15}$N HSQC spectra of the His$_6$-tagged wild type (blue) and K370A/K397A double mutant (red) PH-R domains at pH 5.8 and 25°C in 20 mM MES, 5 mM TCEP, and 200 mM Gdn-HCl.

The wild type sample was 0.5 mM, whereas the less soluble mutant was ~0.4 mM. Both domains yield well-dispersed spectra and hence adopt defined global, tertiary structures. However, the spectral differences suggest that the mutations cause changes in the conformation of the PH-R domain.
4.3.5 SHIP1 with the KAKA substitution is still subject to allosteric regulation

To further define the consequence of K370/K397 mutations on SHIP1 function, we expressed full-length WT SHIP1 and SHIP1 containing the KAKA mutations and compared their ability to bind PIP₃ in vitro. His₆-tagged proteins were expressed and purified from Sf9 cells and their PIP₃ binding ability assessed by PLO as previously described. As seen in Figure 4.8A, KAKA SHIP1 had significantly lower PIP₃ binding than WT SHIP1. This confirms that the PH-R domain and the K370/K397 residues in particular, contribute to SHIP1’s interaction with PIP₃.

We next compared the enzymatic properties of WT and KAKA SHIP1 and found their catalytic rates and specific activities indistinguishable (Figure 4.8B). We also compared the ability of the KAKA SHIP1 enzyme to be stimulated by SHIP1’s allosteric activators, PI-3,4-P₂ and AQX-MN100. Addition of either PI-3,4-P₂ or AQX-MN100 enhanced the phosphatase activity of both WT and KAKA SHIP1 (Figure 4.8C). Altogether, while K370A/K397A mutations interfere with SHIP1’s ability to interact with PIP₃, its catalytic activity and allosteric activation remain unaltered, suggesting that these two residues only facilitate SHIP1’s localization to PIP₃ and that this process is independent of SHIP1’s catalytic domain.
Figure 4.8
Figure 4.8 Mutation of residues K370 and K397 to alanines in full-length SHIP1 abrogates *in vitro* PIP₃ binding ability but does not affect its phosphatase activity.

(A) PLO assays were preformed and quantified as previously described for PIP₃ spotted at 50 pmols with a titration of recombinant full-length WT or KAKA SHIP1 enzyme. Data are expressed as mean intensities ± standard deviations (n=3) *P < 0.05*. (B) Initial rate enzyme kinetics for full-length WT or KAKA SHIP1 enzyme were determined using an *in vitro* phosphatase assay and increasing concentrations of inositol-1,3,4,5-tetrakisphosphate (IP₄). Data are representative of 3 independent experiments. (C) *in vitro* phosphatase assays were performed with recombinant, full-length WT or KAKA SHIP1 with 50 µM IP₄ substrate in the presence of increasing concentrations of the allosteric activators PI-(3,4)-P₂ and AQX-MN100. Data are expressed as the mean fold-increases of enzyme activity ± standard deviations (n=3).
4.3.6 SHIP1 with the KAKA substitution is not able to inhibit Fcγ-R mediated phagocytosis

We next investigated the impact of the KAKA substitutions on the function of SHIP1 protein in FcγR-mediated phagocytosis. Using lentivirus mediated gene transfer, we reconstituted the J2M SHIP1−/− macrophages with either full length WT or KAKA SHIP1 protein. SHIP1 expression in the SHIP1+/+, SHIP1−/−, and SHIP1−/− cells reconstituted with WT and KAKA SHIP1 is shown in Figure 4.9A. Note that the expression level of WT and KAKA SHIP1 are similar, but both are ~50% less than that of endogenous SHIP1 in the SHIP1+/+ cell line. Difficulty in obtaining high-level expression of SHIP1 has been reported previously and is attributed to the fact that ectopic/over-expression of SHIP1 inhibits growth and survival.

Using immunofluorescence confocal microscopy, we tested the ability of full length WT and KAKA SHIP to be recruited to the phagocytic cup in the reconstituted cell lines. Cells were incubated with latex beads opsonized with fluorescently labeled human IgG (red) for the times indicated, fixed, permeabilized and stained with anti-SHIP1 antibody followed by a fluorescently labeled secondary detection antibody (green) (Figure 4.9B). Similar to SHIP1+/+ cells, cells reconstituted with WT enzyme exhibited pronounced fluorescence at the phagocytic cup consistent with SHIP1’s translocation from the cytoplasm to accumulating levels of PIP3 at the cell membrane. However, cells reconstituted with KAKA enzyme had significantly lower mean fluorescence scores than SHIP1+/+ cells or cells reconstituted with WT enzyme, indicating that K370A and K397A mutations impair SHIP1’s localization to PIP3 being produced at the phagocytic cup.
As described by others, SHIP1⁻/⁻ cells phagocytose more rapidly and ingest more latex beads per cell than SHIP1⁺/⁺ cells. This suggests that SHIP1 has a role in the negative regulation of FcγR-mediated phagocytosis. To correlate our observations of impaired SHIP1 recruitment in KAKA SHIP1 reconstituted cells with phagocytic function, we measured the phagocytic index (defined as the average number of beads phagocytosed per cell) of our SHIP1⁺/⁺, SHIP1⁻/⁻ cells, and WT and KAKA SHIP1 reconstituted cells. As seen in the representative micrographs and quantification in Figure 4.10, reconstitution of SHIP1⁻/⁻ cells with KAKA SHIP1 failed to reduce phagocytic activity to WT levels. In comparison, cells reconstituted with WT SHIP1 had a partial reduction of phagocytic activity to WT levels. This partial phenotype is likely attributed to the fact that reconstituted cells had overall lower expression levels of SHIP1 than SHIP1⁺/⁺ cells.
Figure 4.9
**Figure 4.9**  SHIP1 with residues K370 and K397 mutated to alanines has impaired recruitment to the phagocytic cup.

(A) Lysates from J16 SHIP\(^{+/+}\) (SHIP\(^{+/+}\)), J17 SHIP\(^{-/-}\) (SHIP\(^{-/-}\)), and cells transduced with lentivirus encoding WT SHIP1 (SHIP1\(^{-/-}\)::WT) or mutant SHIP 1 (SHIP1\(^{-/-}\)::KAKA) were subjected to immunoblot analysis with anti-SHIP1 (P1C1) primary antibody and anti-STAT3 as a loading control. Band intensities were determined using Biorad Quantity One Software.  

(B) SHIP1\(^{+/+}\), SHIP1\(^{-/-}\), SHIP1\(^{-/-}\):: WT and SHIP1\(^{-/-}\)::KAKA cells were stimulated with AlexaFluor680-labelled, human IgG-opsonized 15 µm Latex Beads (Red) for the times indicated. Cells were fixed, permeabilized and stained with anti-SHIP1 (P1C1) primary followed by FITC-labelled secondary detection antibody (green) and counterstained with the DNA binding dye DAPI (blue). Scale bars = 10 µm. Micrographs were scored by 3 independent, blinded investigators according to the four-point scoring key previously described. Representative micrographs are presented (left) and the mean scores of 100 phagocytosis events at each time point are presented ± standard deviations (right). *\(P < 0.05\) when comparing to either SHIP1\(^{+/+}\) or SHIP1\(^{-/-}\):: WT cells [Two-way ANOVA].
**Figure 4.10**  
SHIP1 with residues K370 and K397 mutated to alanines cannot restore normal regulation of FcγR-mediated phagocytosis.

SHIP1\(^{+/+}\), SHIP1\(^{-/-}\), SHIP1\(^{-/-}\): WT and SHIP1\(^{-/-}\): KAKA cells were stimulated with human IgG-opsonized 3 µm latex beads for the times indicated. Representative micrographs are presented (upper) and phagocytic indices at each time point quantified ± standard deviations (lower) Scale bars = 10 µm. Micrographs were scored by 3 independent, blinded investigators. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) [Two-way ANOVA].
4.4 Discussion

FcγR-mediated phagocytosis is essential for removal of antibody-opsonized foreign particles. Upon ligation of FcγR on the macrophage cell membrane, ITAMs on the cytoplasmic tail of the FcγR become phosphorylated and provide docking sites for SH2-domain containing signaling proteins including Syk, PI-3 kinase, and PKC. Once recruited to the membrane, these proteins initiate dynamic changes in membrane lipid composition and the actin cytoskeleton necessary for phagocytic cup formation, particle internalization, and phagosome maturation. SHIP1 has also been demonstrated to be recruited to the phagocytic cup via interaction of its SH2 domain with phosphotyrosines within ITAMs where it negatively regulates phagocytosis, presumably by degrading PIP₃ though there may also be contributions of other signaling proteins recruited by virtue of SHIP1’s capacity to act as an adapter protein via its multiple protein interaction domains. SHIP1 has additionally been shown to interact with the tyrosine phosphorylated ITIMs on the inhibitory FcγRIIb.

We have characterized a new domain in SHIP1, termed the PH-R domain, which possesses structural and functional similarities with members of the PH domain family. Although PH domains have been described to bind a variety of ligands, their interaction with phosphoinositol lipids are the best characterized. As shown herein, SHIP1’s PH-R domain binds to PIP₃ and also to PI-3,4-P₂ and PI-4,5-P₂, albeit with lower affinity, and the interaction with PIP₃ is mediated either directly or indirectly by at least 2 key lysine residues at positions 370 and 397. The ¹H-¹⁵N-HSQC NMR spectra of wild-type and KAKA PH-R domains confirmed that mutation of these two lysines does not interfere with global conformation or proper folding. However, both we and Edlich et al. have
observed numerous spectral differences between wild-type and lysine residue substituted domains which suggest that the substitutions themselves cause some conformational perturbations. Interestingly, while mutation of these two residues to alanines significantly reduces the ability of the PH-R domain to interact with PIP$_3$ and PI-3,4-P$_2$, binding to PI-4,5-P$_2$ remains relatively unaltered. If K370 and K397 are directly involved in lipid binding, then these positively-charged lysine residues could possibly facilitate stable interaction with PIP$_3$ via co-ordination with the negatively-charged phosphate at the 3’ position of the inositol headgroup whereas other residues in the PH-R domain are required for binding to PI-4,5-P$_2$.

The binding of PIP$_3$ and PI-3,4-P$_2$ by the PH-R domain could serve several functions. PIP$_3$ is a SHIP1 substrate so the PH-R domain might recruit PIP$_3$ to the enzyme and transfer it to the phosphatase domain. However, this seems unlikely since although substituting lysine residues 370 and 397 with alanine significantly impairs the ability of the domain to bind PIP$_3$, this does not alter SHIP1’s basal phosphatase activity. PI-3,4-P$_2$, a product of SHIP1 hydrolysis of PIP$_3$, is an allosteric activator of SHIP1 phosphatase activity $^{559}$ so the PH-R domain might be involved in PI-3,4-P$_2$-mediated activation of SHIP1’s enzymatic activity. However, this also seems unlikely since although the KAKA mutant is impaired in its ability to bind PI-3,4-P$_2$, it retains its ability to be activated by both PI-3,4-P$_2$ and AQX-MN100.

The observation that mutations in the PH-R domain have no impact on SHIP1’s phosphatase activity is consistent with the literature reports on mutations, deletions, and truncations made in regions outside of the core 5’-phosphatase domain $^{360,387,601-604}$. In
a study by Aman et al. in which a series of SHIP1 truncations and deletions were expressed, they found the non-catalytic C-terminal region extending beyond amino acid residue 900 was necessary for FcγRIIB-mediated inhibition of calcium flux in DT40 chicken B-cells. Interestingly, reconstitution of SHIP1−/− cells with a SHIP1 C-terminal truncation at residue 900 (SHIP1_{900}) was able to partially restore inhibition of calcium flux whereas a construct lacking both the presently characterized PH-R domain and the C-terminal domain (SH2-18aa-401-900) was not. These results support our identification of SHIP1’s PH-R domain and suggest that in addition to the non-catalytic C-terminal region, SHIP1’s PH-R domain may also contribute to stabilizing the interaction between SHIP1 and FcγRIIB indirectly via binding to membrane PIP₃.

We have additionally demonstrated that the PH-R domain contributes to SHIP1’s localization to the phagocytic cup during FcγR-mediated phagocytosis. SHIP1 transiently localizes to the phagocytic cup at the early stages of cup formation and is sequestered at the leading edge of the phagosome. Our data show that despite having a functional SH2 domain capable of interacting with FcγR ITAM, KAKA SHIP1 has a significantly impaired ability to translocate to the cell membrane in cells stimulated with Ig-opsonized beads. These findings suggest that SHIP1’s SH2 domain is not sufficient to mediate SHIP1’s recruitment to the phagocytic cup. Zhang et al. have recently proposed a model whereby the density of FcγR activation determines early-stage signals such as the recruitment of Syk, while later stages of FcγR signaling, i.e. those that ultimately commit the cell to complete phagocytosis of a particle, are dependent on 3’ PI levels (e.g. PIP₃ and PI-3,4-P₂) in unclosed phagocytic cups. It is thus possible that, similarly to Syk, relatively few molecules of SHIP1 are recruited to the
FcγR upon ligand binding via SHIP1’s SH2 domain interacting with tyrosine phosphorylated ITAM/ITIM motifs. The number of SHIP1 molecules recruited during the early stage is thus directly related to the number of FcγRs engaged, which in turn is determined by the density of IgG opsonization on the particles. Subsequently, only phagocytic cups having accumulated a threshold number of PIP₃ molecules are able to successfully commit to the formation of a phagosome around a particle and concomitantly recruit large amounts of SHIP1 to the phagocytic cup possibly via its newly identified PH-R domain.

Our current understanding of phagocytosis has moved beyond the classical “zipper” model where engulfment occurs as receptors bind ligands sequentially along the surface of the particle being ingested. A review by Jaumouillé and Grinstein eloquently described a model that considers the actin cytoskeletal contributions to the regulation of phagocytosis by partitioning or “fencing-off” regions of membrane to limit the lateral movement of FcγRs. While actin polymerization is required for the initial filopodial extensions that are necessary for the identification and attachment of opsonized particles, their model suggests that underlying cortical actin barriers must be broken down in order for FcγR to move laterally and form the receptor clusters responsible for initiating the signaling cascade and actin remodeling that drives particle internalization. Applying this model to our own observations of the PH-R domain dependent translocation of SHIP1 to the plasma membrane, we suggest that SHIP1 can be recruited to the membrane through both SHIP1-SH2/FcγR phosphotyrosine and SHIP1-PH-R/PIP₃ interactions. However, the actin partitions, which restrict lateral movement of transmembrane proteins, prevent the aggregation of FcγR molecules and thus limit the
amount of FcγR ITAM/ITIM-bound SHIP1 to densities too low to be detected by conventional immunofluorescence microscopy. On the other hand, it is possible that similar to what has been reported for PI-4,5-P₂, PIP₃ may not be subject to the same restrictions in lateral motion by actin and thus the density of PIP₃ would be high enough such that the SHIP1 recruited (via its PH-R domain) is easily visualized at the phagocytic cup.

Identification and characterization of SHIP1’s PH-R domain now provides a more detailed illustration of the mechanism whereby SHIP1 translocates to the membrane upon FcγR ligation and lends further insight into how SHIP1 contributes to modifications in membrane lipid composition and actin rearrangements that collectively promote particle engulfment. By directly interacting with PIP₃ being generated at the phagocytic cup, the PH-R domain facilitates SHIP1’s rapid enrichment at the leading edge where it can hydrolyze PIP₃ to PI-3,4-P₂ and thus regulate the progression of stages through phagosome maturation.

The degradation of PIP₃ at the phagocytic cup may be only one of many mechanisms by which SHIP1 negatively regulates FcγR mediated signaling and phagocytosis. A study by Ganesan et al. has shown that SHIP1’s non-catalytic function regulates Ras/Erk dependent induction of Interleukin-1β upon FcγR stimulation.³⁹⁷ Mehta et al. recently described the association of SHIP1 with LyGD1, a Rho guanidine dissociation inhibitor that prevents the membrane association of the small GTPase Rac and thus negatively influences actin assembly needed for phagosome formation.⁶⁰⁸ In the same study, the authors reported the identification of several other proteins that uniquely interacted with SHIP1 and that many had known functions in regulating actin
re-organization. The PIP₃–mediated membrane localization of SHIP1 may not be limited solely to FcγR signaling and future studies will seek to determine whether the PH-R domain also mediates SHIP1’s recruitment to other cellular compartments and in response to other cell stimuli. It will also be interesting to find small molecules which modify PH-R domain interactions with PIP₃ as these may have potential therapeutic applications.
CHAPTER 5: CONCLUSION
5.1 Conclusion

The work presented in this thesis describes hitherto unappreciated mechanisms by which SHIP1 mediates IL-10’s anti-inflammatory action and how SHIP1 activity itself is regulated within immune cells. Both IL-10 and SHIP1 are known to have significant contributions to proper immune cell function as exemplified by animal knock-out models and in human pathologies associated with mutations within the loci of these genes. Thus, a better understanding of how IL-10 signals in cells, the nature of the role SHIP1 plays in IL-10 signaling, and how SHIP1 activity can be modulated, will provide insights towards developing targeted therapeutics with application in treating a variety of inflammatory diseases.

In the current literature, all of IL-10’s signaling is believed to be mediated by STAT3, which then upregulates the expression of specific gene products. These IL-10 induced products, in turn, negatively regulate immune cell activation at the level of transcription. However, expanding upon work initiated by previous graduate students in our lab, the experiments described in Chapter 2 characterize a STAT3-independent signaling pathway utilized by IL-10 whereby IL-10 activates SHIP1, the predominant lipid phosphatase in immune cells responsible for countering PI-3 kinase-generated PIP₃ via degradation of PIP₃ to PI-3,4-P₂. By opposing the PI-3 kinase pathway, IL-10 induced activation of SHIP1 negatively regulates the activation of downstream proteins, such as Akt, and inhibits the transcription of a number of PRGs, which are among the earliest genes whose expressions are upregulated in response to immune cell stimulation by TLR-agonists.
The role of the PI-3 kinase pathway in immune cell activation - macrophages especially - has been a point of contention for many researchers. Data from our lab strongly supports a positive role for the PI-3 kinase pathway in macrophage activation and we propose that differences in experimental design account for the contrary observations made by other groups. One of the major factors contributing to this discrepancy is the fact that LPS stimulation of macrophages, in addition to up-regulating the expression of PRGs, also induces the expression of IL-10 within 2 hours. The autocrine action of IL-10 can potentially confound interpretation of pro-inflammatory cytokine production and the role of PI-3 kinase signaling in regulating their production (particularly evident at longer timepoints). To address the issue of autocrine cytokine production and signaling, we have adapted a perifusion system frequently employed in diabetes research to observe glucose-induced changes in pancreatic beta-cell insulin production, to supply continuous stimulation to macrophage monolayers and effectively remove cytokines that they produce from the extracellular milieu. This continuous-flow apparatus allows us to profile changes in cytokine production over time.

Use of the continuous-flow apparatus revealed several fascinating features of LPS-stimulated TNFα production. Firstly, within the first 2 hours of stimulation, TNFα production occurs in 2 phases with peak concentrations at approximately 50 and 110 minutes post-stimulation. Secondly, in conjunction with SHIP1 KO cells and inducible siRNA knockdown cell lines, we observed that IL-10 was able to inhibit the initial phase of TNFα production only in the presence of SHIP1. STAT3 siRNA knockdown cells, however, were fully responsive to IL-10 mediated inhibition of this first peak indicating that STAT3 is not required for inhibition of this first influx of TNFα. While, we concede
that the ~57% knockdown of STAT3 achieved using the inducible siRNAs is modest, it
nevertheless appears to be sufficient to significantly impair IL-10’s ability to inhibit the
second peak of TNFα production. Thus, IL-10 appears to inhibit the first and second
phases of TNFα differentially via a SHIP1-dependent and STAT3-dependent pathways
respectively.

In the second chapter, we have also shown that via the SHIP1-dependent pathway,
IL-10 inhibits the phosphorylation and activation of CDK9, and the subsequent
phosphorylation of RNAPolII at Ser2. Phosphorylation at Ser2 is necessary for switching
RNAPolII from synthesizing low levels of full-length unspliced transcripts to producing
high levels of mature, spliced, protein-coding mRNAs. However, IL-10 was unable to
inhibit CDK9 phosphorylation or subsequent RNAPolII Ser2 phosphorylation in cells
expressing SHIP1 siRNA. SHIP1 thus appears to be necessary for mediating IL-10’s
inhibition of transcriptional elongation of PRGs like TNFα. Interestingly, we made the
additional observation that in SHIP1 KO PMΦs, and SHIP1 siRNA expressing cells, low-
dose IL-10 treatment of LPS-stimulated cells enhanced rather than inhibited the mRNA
expression of 11/17 PRGs. These findings suggest that IL-10 signaling through SHIP1 is
not only necessary for mediating IL-10’s early phase anti-inflammatory action but is
required for suppressing IL-10’s augmentation of pro-inflammatory gene expression as
well. The enhanced production of TNFα mRNA in SHIP1 deficient cells is reflected by
enhanced TNFα protein production during the first peak of expression detected in
continuous-flow culture macrophages. Our observations of the requirement for SHIP1 in
IL-10 action in vitro were recapitulated in vivo in the mouse endotoxemia model. IL-10
inhibited LPS induced production of TNFα and CCL2 levels in wild-type but not SHIP1 KO mice.

Taken together, these results recommend a revision of the current, prevailing opinion in the literature for the absolute requirement of STAT3 in IL-10 signaling. While our results are consistent with STAT3 being necessary for mediating IL-10’s inhibition of TNFα at longer timepoints post-LPS stimulation - corresponding with the second phase of TNFα production – we have demonstrated that STAT3 is dispensable for IL-10’s inhibition of the first peak of TNFα production within the first hour post-stimulation. However, the presence of SHIP1 is required for IL-10 mediated inhibition of this first phase of TNFα production, and in SHIP1’s absence, IL-10 actually has the capacity to enhance the expression of TNFα and other PRGs.

The results described in Chapter 2 open a number of avenues for future investigation. Firstly, of the potential sources of discrepancy with regards to differing accounts for the role of PI-3 kinase signaling in macrophage activation, we have yet to investigate the contribution of the individual PI-3 kinase p110 isoforms. It is entirely possible that specific p110 isoforms predominate in response to LPS stimulation as has been previously reported by other groups who have employed the new isoform specific PI-3 kinase inhibitors to delineate their respective roles [401,610]. It is equally feasible that SHIP1 may specifically target different cellular pools of PIP3 being generated by a particular p110 isoform. In this manner, SHIP1 activity may serve to negatively regulate some PI-3 kinase signaling events which positively contribute to macrophage activation, while others are independent of SHIP1 regulation. Combinatorial use of the isoform specific inhibitors and/or siRNAs in conjunction with SHIP1 KO or siRNA expressing
cells may facilitate a better understanding of the relationship between p110 isoforms and SHIP1 activity. Secondly, by using the continuous-flow culture apparatus, we were able to collect culture supernatant fractions at specific time points and apply them to naïve cultures. We observed that there is a soluble factor produced within the first phase of TNFα production that is necessary for maximal induction of the second peak. Identification of this factor may provide further insight into the dynamic sequence of events during the early stages of LPS stimulation. Thirdly, we discovered that a subset of PRGs as described by Hargreaves et al. are induced by low-dose treatment of IL-10 in SHIP1 KO cells rather than inhibited as they are in WT cells. These data lead to the question of whether there are shared elements amongst the promoters of these PRGs – a shared sequence that recruits a “master” transcription factor, for example, that in the absence of SHIP1, mediates a pro-inflammatory response to IL-10 instead of a deactivating response. Bioinformatic analysis of the promoter regions of the PRGs and luciferase reporter assays may help to elucidate these possibilities. The studies proposed are in line with the growing appreciation for the organization of innate immune responses into transcriptional programs. Such programs allow for rapid and elegant control of genes that together perform similar or related functions. Thus, it is possible that SHIP1 may serve as a key regulator of an arm of the TLR-induced transcriptional program. Alternatively, these PRGs may be differentially regulated at the post-transcriptional level. miRNAs are known to have integral roles in controlling the inflammatory response. High through-put microarray analysis of the mRNA and microRNA profiles of LPS +/- IL-10 treated macrophages from SHIP1+/+ and SHIP1-/-. 
mice may reveal if IL-10 is capable of regulating these immunoregulatory molecules in a SHIP1-dependent manner.

Having observed that IL-10 does indeed signal through STAT3-independent pathways and that SHIP1 is necessary for IL-10’s early phase signal transduction, in Chapter 3 I investigated whether it was possible to activate SHIP1 using small molecule agonists and whether by doing so, we could mimic the biological activity of IL-10 to inhibit inflammation in mouse models where IL-10 is lacking. Pelorol, a compound that enhanced SHIP1 activity in a high throughput in vitro SHIP1 assay, was identified from a marine invertebrate extract library as a SHIP1 agonist and used as a starting point for derivatization to produce more active compounds. One of these derivatives, AQX-MN100 exhibited approximately 8-fold higher enhancement of SHIP1 phosphatase activity than its parent compound and inhibited inflammatory cell activation in vitro. AQX-MN100 is specific for SHIP1 as it does not inhibit TNFα production in macrophages lacking SHIP1 nor does it exhibit any appreciable effects when screened against an in vitro panel of 100 other kinases and phosphatases. AQX-MN100 was also effective at reducing inflammation in several in vivo murine models including LPS-induced septic shock, DNFB-induced cutaneous anaphylaxis, and in IL-10−/− mouse colitis.

Through the course of the in vitro studies described in Chapter 3, we noticed that SHIP1 exhibits a unique enzyme kinetics profile. Unlike most enzymes, which typically display standard Michaelis-Menton saturation kinetics, SHIP1 exhibited a sigmoidal profile, which is the hallmark of enzymes subject to end-product allosteric activation. Indeed, when we added exogenous PI-3,4-P₂, SHIP1’s product, to the in vitro enzyme
reaction, SHIP1’s phosphatase activity is increased to the same degree as the enhancement observed when AQX-MN100. Bioinformatic analysis of SHIP1’s sequence revealed a previously unidentified structured region immediately C-terminal of SHIP1’s central catalytic domain with predicted features similar to lipid-binding C2 domains. We determined that the C2 domain binds both SHIP1’s end-product, PI-3,4-P₂, and AQX-MN100 in a manner similar to the allosteric activation mechanisms described for other lipid phosphatases like PTEN and MTM,

The data presented in Chapter 3 illustrate an additional means of regulating SHIP1’s activity that has not previously been appreciated. In the current literature, it is believed that SHIP1’s activity is constitutively active and is regulated solely by its subcellular localization. In response to activation of membrane receptors, SHIP1 is believed to translocate to the membrane via protein-protein interactions mediated by its SH2 domain and C-terminal PRR with adaptor proteins or membrane receptors directly. At the membrane, SHIP1 then has access to its PIP₃ substrate. However, we have discovered that SHIP1 can be allosterically activated by PI-3,4-P₂ and by small molecule SHIP1 agonists thus providing an additional level of regulation. We propose a model whereby SHIP1 is recruited by phosphorylated membrane receptors and adaptor proteins to the localized site of PIP₃ accumulation. At this stage, SHIP1 activity is modest and is only capable of converting small amounts of PIP₃ to PI-3,4-P₂. These small amounts of PI-3,4-P₂ however, are capable of binding to the C2 domain, inducing a conformational change that makes the enzyme active site more accessible to substrate. SHIP1 is then able to rapidly degrade the remaining PIP₃ from the membrane.
Identification of SHIP1’s C2 domain and allosteric activation mechanism has exciting implications towards the development of a new class of anti-inflammatory compounds similar to AQX-MN100 that manipulate SHIP1’s activity in cells. Not only can AQX-MN100 effectively suppress immune cell activation and pro-inflammatory cytokine production in inflammatory disease models, but they would also possess an added advantage over conventional immunosuppressive agents. By specifically targeting SHIP1, an enzyme primarily expressed in hemopoietic cells, SHIP1 agonists would avoid many of the side-effects attributed with the more conventional immunosuppressive agents such as glucocorticoids and mTOR inhibitors (e.g. Rapamycin/Sirolimus) whose drug targets are ubiquitously expressed throughout the body. Further, targeting an allosteric regulatory site confers even greater drug specificity as most small molecule drugs are enzyme inhibitors, which target enzyme active sites that are required to be more structurally conserved amongst related enzymes in order to perform a catalytic function. Allosteric regulatory sites, on the other hand, are not as structurally constrained.

While our PLO and SPA assays provide strong support for direct binding of PI-3,4-P\(_2\) and AQX-MN100 to SHIP1’s C2 domain, we still lack structural information as to the exact nature of these interactions. We have made several attempts to analyze the C2 domain’s structure using NMR spectroscopy, however, the domain is insoluble under the buffer conditions required for NMR analysis. Thus, future studies may investigate the structure of the C2 domain using other techniques such as X-ray crystallography. By co-crystallizing the C2 domain with and without PI-3,4-P\(_2\) or AQX-MN100, we may identify the specific amino acid residues required for mediating binding between SHIP1 and its allosteric activators. Further, we could express the C2 domain in context with SHIP1’s
phosphatase domain to characterize the conformational changes induced by allosteric binding of PI-3,4-P₂ or AQX-MN100 and how these structural modifications lead to enhanced phosphatase activity. These data in turn may allow us to perform rational drug design to generate more efficacious small molecule SHIP1 activators.

However, characterization of AQX-MN100 as a SHIP1 allosteric activator itself opens many opportunities for future investigation. Having a specific small molecule SHIP1 agonist allows us to further dissect the role of SHIP1 in inflammatory and immune cell processes. Of note, the immune defects associated with SHIP1 KO mice are primarily attributed to perturbations in the myeloid cell compartment.⁴⁰⁵,⁴⁰⁶ Therefore, we can employ AQX-MN100 as a tool to further characterize the function of SHIP1 in myeloid cell development and its function in response to various inflammatory stimuli. One area of particular interest is SHIP1’s role in the development of myeloid suppressor cells (aka MSCs, Myeloid-Derived Suppressor Cells, Myeloid Immune Suppressor Cells, MIR). Several reports have provided evidence that SHIP1 deficiency gives rise to increased numbers of MSCs.³⁹⁵,⁴⁰²,⁴⁰³ In the IL-10⁻/⁻ mouse colitis experiments described in Chapter 3, in addition to colon sections, we collected lymph nodes and spleens and preliminary flow cytometry experiments indicated that MSC numbers (defined by gating on CD11b⁺Gr1⁺ events) were modulated upon treatment with AQX-MN100 (data not shown). However, further experimentation needs to be performed to confirm these observations and to confirm the suppressive function of these cells. Intriguingly, it has also been reported that SHIP1 is required for T₁₁7 cell development, a T-cell sub-type known to have a positive role in the development of IBD.⁴³⁰,⁶¹⁹. Because of our data indicating that SHIP1 activation via treatment with AQX-MN100 is protective in colitis.
and inhibits production of IL-17, it would be of great interest to determine if, similarly to its action on myeloid suppressor cells, AQX-MN100 modulates T_{H}17 populations as well.

In addition to the C2 domain, I have characterized a previously unrecognized PH-like domain residing N-terminal of SHIP1’s phosphatase domain. Results described in Chapter 4 indicate that SHIP1’s PH-like domain exhibits a binding specificity for PIP_{3} and that it is necessary for regulating SHIP1’s membrane localization to the phagocytic cup during FcγR-mediated phagocytosis. Generation of SHIP1 enzyme where point mutations were made that abrogated PIP_{3} binding activity further demonstrated that despite having intact, functional, SH2 domains and PRRs, mutant SHIP1 recruitment to the phagocytic cup was still impaired. These data suggest that SHIP1’s PH-like domain is one of the primary contributors to its rapid recruitment to the PIP_{3}-rich phagocytic cup. Thus, similarly to the C2 domain, it may be possible to develop small molecule drugs targeting the PH-like domain to regulate SHIP1 activity by modulating its membrane recruitment to membrane PIP_{3} pools.

Although PH domains have been best characterized for their interactions with lipids, the PH domains of several other signaling proteins have been reported to mediate protein-protein interactions as well (reviewed in \textsuperscript{620}). In particular, the PH domains of Btk and PLCβ have been reported to interact with filamentous actin \textsuperscript{621,622}. Initial studies with SHIP1’s PH-R domain have similarly demonstrated an ability to bind actin (data not shown). Future NMR studies of SHIP1’s PH-R domain in the presence and absence of filamentous actin could further characterize this interaction. Perhaps of greater interest, however, is investigation into whether PH-R domain/Actin interactions are required for
proper SHIP1 activity. Co-localization studies using confocal microscopy may yield
added insight into an actin-dependent regulatory mechanism of SHIP1 function possibly
by coordinating its shuttling from cytoplasmic compartments to the membrane or by
influencing its lateral movement in membrane corrals as described by Jamouillé and
Grinstein\textsuperscript{606}.

In summary, the contents of this thesis describe a number of previously
unrecognized mechanisms of immune cell regulation namely: IL-10’s inhibition of the
early phase of pro-inflammatory cytokine production via SHIP1, allosteric activation of
SHIP1 by binding of its newly identified C2 domain with its natural end-product, and
membrane recruitment of SHIP1 mediated by direct interactions with its newly
characterized PH-R domain and membrane lipids. Data presented also demonstrate that
some of these mechanisms can be exploited by small molecule drug agonists. SHIP1
activators thus represent an exciting new class of anti-inflammatory compounds, which
would have obvious clinical applications but could additionally be used as a molecular
tool to further characterize the role of this important regulatory phosphatase in immune
cell function.
BIBLIOGRAPHY


136. Clark, I.A. How TNF was recognized as a key mechanism of disease. Cytokine Growth Factor Rev 18, 335-343 (2007).


229. Liu, Y.W., Chen, C.C., Tseng, H.P. & Chang, W.C. Lipopolysaccharide-induced transcriptional activation of interleukin-10 is mediated by MAPK- and NF-


358. Liu, L., *et al.* The Src homology 2 (SH2) domain of SH2-containing inositol phosphatase (SHIP) is essential for tyrosine phosphorylation of SHIP, its


398. Tiwari, S., Choi, H.P., Matsuzawa, T., Pypaert, M. & MacMicking, J.D. Targeting of the GTPase Irgm1 to the phagosomal membrane via PtdIns(3,4)P(2) and PtdIns(3,4,5)P(3) promotes immunity to mycobacteria. Nat Immunol 10, 907-917 (2009).


APPENDICES
Double stranded siRNA designed using Invitrogen’s BLOCK-iT design software was annealed into linearized pcDNA 6.2-GW/EmGFP-miR vector via complementary overhanging nucleotides to create pcDNA 6.2-GW/EmGFP-miR-siRNA. Using Invitrogen’s Gateway system, a BP recombination reaction was then performed between pcDNA 6.2-GW/EmGFP-miR-siRNA and pDONR-221 to generate the pENTR-221-siRNA vector. A LR recombination reaction between the generated pENTR-221-siRNA vector and pTRIPZ-Dest vector was then performed to create the pTRIPZ-siRNA vector used for lentiviral production. attB = attB recombination site. EmGFP = GFP gene. miR = microRNA-like sequence. attP = attP recombination site. attL = attL recombination site. attR = attR recombination site. TRE = tetracycline response element. Figure was provided courtesy of Gary Golds.
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**Table A.1:** Primer sequences for real-time quantitative PCR using SYBR green detection.
Figure B.1  IL-10 activates SHIP1

(A) Immunoblot analysis of J774.1 macrophage cells retrovirally transduced to express wildtype hIL-10R or mutant hIL-10R-TyrFF stimulated with hIL-10 (50 ng/ml) in the presence of 10 µg/ml IBI.2 rat anti-mIL-10R blocking antibody for the indicated times, probed with anti-Phospho Tyrosine antibody (4G10) and protein SHIP1 (Loading Control).  (B) Immunoblot analysis of J774.1 cell lysates precipitated with a phosphorylated synthetic hIL-10R peptide (p-446/496) or an unphosphorylated hIL-10R peptide (446/496) and probed for SHIP1. HPLC Inositol phospholipid analysis of orthophosphate labeled J16 BMDMs treated with control buffer, LPS (50 ng/ml) or co-treated with LPS + IL-10 (100ng/ml) for 15 minutes. Data represent mean CPMs ± s.d. (n=3).  *p<0.05, **p<0.01 (One-way ANOVA).  Figure provided courtesy of Dr. Ali Ghanipour.
Figure C.1  Continuous Flow Apparatus.

Continuous-flow apparatus facilitates constant stimulation and removal of cell supernatants to determine kinetic profiles of cytokine production over time. A=Syringe Pump set to dispense at a constant rate of 150 µl stimulation media/min injects stimulation media through “Inlet Line”. B=Continuous-flow chamber plate. Cells plated in a 24-well tissue culture plate are fitted with rubber stoppers adapted with 18 gauge needles. Stimulation media volume within each well is maintained at a volume of 500 µl. Pressure generated by stimulation media injection forces the simultaneous removal of cell supernatants through the “Outlet line”. C=Fraction collector set to advance every 5 minutes. Component A and B are maintained in a 37°C incubator.
Figure D.1  Replicate experiments demonstrating IL-10 enhancement of TNFα production during a specific phase of the first peak of production in the absence of SHIP1.

(A) TNFα ELISA of fractions collected from SHIP1<sup>+/+</sup> and SHIP1<sup>-/-</sup> PMΦs stimulated with LPS (1 ng/ml) with or without IL-10 (100 pg/ml) under continuous-flow conditions. (B) Replicate experiment as (A) but only fractions collected at 75 through 150 minutes were analyzed by TNFα ELISA.
Figure E.1  Pelorol and AQX-016A enhance SHIP1 phosphatase activity.

(A) Purified Pelorol and AQX-016A were tested at 2 µM for their ability to enhance recombinant SHIP1 enzyme activity.  (B) HPLC Inositol phospholipid analysis of orthophosphate labeled J16 BMDMs treated with vehicle, AQX-016A (15 µM) or LY294002 (25 µM) 30 minutes prior to stimulation with LPS (50 ng/ml).  (C) The effect of AQX-016A on SHIP1 (■) and SHIP2 (Δ) enzyme activity was compared in in vitro enzyme assays.  (D) (A) SHIP^{+/+} and SHIP^{−/−} macrophages were pre-treated with AQX-016A or vehicle for 30 minutes prior to stimulation with 10 ng/mL of LPS at 37°C for 2 h and TNFα production determination by ELISA.  Absolute TNFα levels for SHIP^{+/+} and SHIP^{−/−} cells were 623 +/- 30 and 812 +/- 20 pg/ml, respectively. Data are expressed as mean +/- SEM and are representative of three independent experiments.  Figure provided courtesy of Dr. Ali Ghanipour.
Figure F.1  AQX-MN100 specifically enhances SHIP1 phosphatase activity and has minimal off-target effects

Compound profiling activity was undertaken using 100 protein kinase and phosphatase targets by SignalChem (Richmond, BC) against compound AQX-MN100 (2 µM final concentration). Protein kinase assays were performed in the presence of 50 µM ATP at 30°C for 15 min. Protein phosphatase activities were determined using pNPP as substrate and were also performed at 37°C for 15 min. The activity of the enzymes in the presence of AQX-MN100 was compared to that in the vehicle control and expressed as a % change in activity relative to that observed in the vehicle control. Changes in activity of <25% were not considered significant. Enzymes affected by AQX-MN100 are plotted in an expanded graph in B.
Figure G.1  Phosphatidylinositol lipid binding ability of single mutant SHIP1 PH-R domains.

(A) Initial screen of SHIP1 PH-R domain point mutant constructs made using a standard PCR-based site directed mutagenesis method. Mutant constructs were expressed in BL21 (DE3) competent cells and purified as previously described. Recombinant domains at a concentration of 625 nM in 6 ml of blocking buffer were tested in PLO assays for their ability to bind (A) PI-4,5-P₂, (B) PIP₃ or (C) PI-3,4-P₂ spotted onto PVDF membrane in amounts ranging from 0 to 50 pmols (n=1).
Figure H.1  Double reciprocal plots of phosphatidyl inositol lipid binding ability of mutant SHIP1 PH-R domains

(A) Recombinant K370A or K397A single mutant domains or (B) KAKA SHIP1 PH-R domain were expressed in BL21 (DE3) competent cells and purified as previously described. Recombinant domains at a concentration of 625 nM in 6 ml of blocking buffer were tested in PLO assays for their ability to bind PI-4,5-P₂, PIP₃, or PI-3,4-P₂ spotted onto PVDF membrane in amounts ranging from 0 to 50 pmols. Blots were probed with primary and secondary antibodies, and quantified as in Materials and Methods. The reciprocal of the mean intensities were plotted against the reciprocal of the amount of lipid spotted. K_D values were calculated from the slope of the lines \(^{593}\) as determined by linear regression (GraphPad Prism, San Diego, CA).