Regulation of Human Monocyte Functional Properties by Phosphatidylinositol 3-Kinase

by

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ABSTRACT

Mononuclear phagocytes are important regulators and effectors of both the innate and acquired immune responses. Extensive research has highlighted an important role for phosphoinositides in monocyte cell regulation. The 3'-phosphoinositide metabolites, produced by phosphatidylinositide 3-kinase (PI3K) family of lipid kinases are known to be involved in regulating numerous monocyte activities including phagocytosis, adherence, oxidative burst, and cytokine secretion. An important research objective is to develop an understanding of how specificity is achieved in PI3K signaling for these diverse biological functions. The goal of this thesis was to determine when particular monocyte functional properties are governed by PI3K in an isoform-specific manner versus situations in which PI3K family members have redundant functions.

Studying mononuclear phagocyte cell biology through genetic manipulation by non-viral transfection methods has been challenging due to the dual problems of low transfection efficiency and the difficulty in obtaining stable transfection. To overcome this problem, we developed a system for mediating RNA interference in monocytic cells. The p110α isoform of PI3K was silenced using a lentiviral vector expressing short hairpin RNA. This resulted in the generation of stable THP-1 and U-937 human monocytic cell lines deficient in p110α. The role of p110α in regulating cell adherence, phagocytosis, the phagocyte oxidative burst, and LPS-induced cytokine secretion was examined.

Monocyte adherence induced in response to either LPS or vitamin D₃ was blocked by PI3K inhibitor LY294002. However, while adherence induced in response to D₃ was
sensitive to silencing of p110α, LPS-induced adherence was not. These findings
demonstrate that LPS and vitamin D₃ use distinct isoforms of class IA-PI3K to induce
functional responses. We also observed that p110α was required for phagocytosis of IgG
and serum opsonized particles in differentiated U-937 cells. The phagocyte oxidative
burst induced in response to either PMA or opsonized zymosan in differentiated THP-1
cells was also found to be dependent on p110α. Furthermore, p110α was observed to
exert selective and bi-directional effects on the secretion of pivotal cytokines apparently
independently of other PI3K isoforms. LPS stimulation of p110α deficient THP-1 cells
demonstrated that p110α was required for inducing IL-12 and IL-6 production, whereas
this isoform of PI3K appeared to negatively regulate the production of TNF-α and IL-10.

The results reported in this thesis demonstrate that lentiviral-mediated delivery of shRNA
is a powerful approach to study monocyte biology. Furthermore, taken together, the data
suggest that p110α PI3K is involved in regulating important monocyte effector functions
independently of other PI3K family members.
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<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich elements</td>
</tr>
<tr>
<td>ARF6</td>
<td>ADP ribosylation factor 6</td>
</tr>
<tr>
<td>ARNO</td>
<td>ARF nucleotide binding site opener</td>
</tr>
<tr>
<td>ASK-1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>ATF-2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton tyrosine kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>D₃</td>
<td>1α, 25-dihydroxycholecalciferol</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>FGD1</td>
<td>Faciogenital dysplasia</td>
</tr>
<tr>
<td>Gab</td>
<td>Grb2-associated binder</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GRP</td>
<td>General receptor for phosphoinositides</td>
</tr>
<tr>
<td>Hrs</td>
<td>Hepatocyte growth factor regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of nuclear factor-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor-κB-kinase complex</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response elements</td>
</tr>
<tr>
<td>ITK</td>
<td>Inducible T-cell kinase</td>
</tr>
<tr>
<td>JIP</td>
<td>JNK-interacting protein</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88-adaptor like protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP-K2</td>
<td>MAP-kinase-activated protein kinase-2</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAP kinase kinase kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAPK/ERK kinase kinase</td>
</tr>
<tr>
<td>MK2</td>
<td>MAP-kinase-activated protein kinase-2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary-response protein 88</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
</tr>
<tr>
<td>NIK</td>
<td>NFκB inducing kinase</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>OPZ</td>
<td>Opsonized zymosan</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PDE3B</td>
<td>Phosphodiesterase 3B</td>
</tr>
<tr>
<td>PDK</td>
<td>3'-phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHLPP</td>
<td>PH domain leucine-rich repeat protein phosphatase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositides</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinases A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinases B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinases C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PtdIns(4)P</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PRK</td>
<td>PKC-related kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome ten</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology</td>
</tr>
<tr>
<td>RAC</td>
<td>Related to A and C kinases</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>Regulatory associated protein of mTOR</td>
</tr>
<tr>
<td>Ras-GRP</td>
<td>Ras guanyl nucleotide-releasing proteins</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RhoGDI</td>
<td>Rho GDP dissociation inhibitor</td>
</tr>
<tr>
<td>RICTOR</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S6K1</td>
<td>p70 S6 kinase 1</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing 5'-inositol phosphatase</td>
</tr>
</tbody>
</table>
shRNA Short hairpin RNA
siRNA Small interference RNA
TAB TAK-binding protein
TAK TGF-β activated kinase
TANK TRAF-family-member-associated NFκB activator kinase
TAPP Tandem PH domain-containing protein
TBK-1 TANK-binding kinase 1
TGF-β Transforming growth factor-β
Th T helper
TIR Toll/IL-1 receptor
TIRAP TIR-domain-containing adaptor protein
TLR Toll-like receptors
TNF-α Tumor necrosis factor-α
TRAF TNF receptor associated factor
TRAM TRIF-related adaptor molecule
TRIF Toll-IL-1 receptor domain-containing adaptor inducing IFN-β
TSC Tuberous sclerosis complex
UTR Untranslated region
VAMP3 Vesicle-associated membrane protein 3
VDR Vitamin D receptor
Z Zymosan
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CHAPTER I: INTRODUCTION

1.1 Mononuclear phagocytes

1.1.1 Macrophage origin, morphology, and heterogeneity

Cells of the mononuclear phagocyte lineage are heterogeneous in their phenotype due to their widespread tissue distribution, morphology, and function. Among these cells are included blood monocytes, Kupffer cells in liver, Langerhans cells in the skin, osteoclasts in bone, microglia in the central nervous system, alveolar macrophages in the lung, and tissue macrophages in lymph nodes. To emphasize their common lineage, the term mononuclear phagocyte system (MPS) includes progenitor cells in the bone marrow, blood monocytes, and resident tissue macrophages\textsuperscript{1,2} (Fig. 1-1). Even though not all cells in the MPS are truly mononuclear—osteoclasts, epithelioid cells, and giant cells are all multinucleated—the term places the emphasis on the fact that the majority of cell division occurs in the monoblast and promonocyte stage, while tissue macrophages have very limited roles in the maintenance of resident tissue macrophage populations\textsuperscript{2}. Macrophages are phylogenetically primitive, being found in all vertebrates, conserved throughout metazoa, and even some protozoa exhibit features similar to the mammalian macrophage. Although cells of the macrophage system are usually thought of as principally immune cells, it has been postulated that cells of the MPS originally evolved to meet a fundamental need for the integrity of multicellular organisms\textsuperscript{3}. In this model, multicellular organisms required cells like macrophages to eliminate damaged or abnormal cells and their products, thus maintaining homeostasis and structural integrity.
Host defense functions of these cells evolved as a secondary gain to this requirement for multicellularity.

Figure 1-1A

![Diagram showing differentiation, distribution, and activation of macrophages and multinucleated giant cells.](image)

1-1B

![Diagram showing differentiation, distribution, and activation of macrophages and multinucleated giant cells.](image)

Figure 1-1. Schematic diagram showing differentiation, distribution, and activation of macrophages and multinucleated giant cells. A. Tissue macrophages originate from bone marrow hematopoietic stem cells (HSC) that have the capacity for self-renewal. Under the influence of lineage-determining cytokines (e.g. GM-CSF, M-CSF) and stromal interactions, HSC give rise to short-term HSC which differentiate through common
myeloid progenitors and then granulocyte macrophage progenitors (GM-CFU). Further
differentiation and expansion then gives rise to monoblasts, promonocytes, and finally
monocytes that leave the bone marrow to enter the blood. In the blood, monocytes are
recruited to tissue via a multistep process: margination due to hemodynamic changes,
routing of monocytes on endothelium occurs through low affinity selectin-ligand
interactions, activation of monocytes by chemokines leads to expression of high-affinity
integrins resulting in stable adherence to endothelium, and finally transmigration through
the vessel wall at inter-endothelial cell junctions. Resident macrophages in different
organs adapt to the local microenvironment, and differentiate into sub-lineages. B,
Tissue macrophages fuse and become multinucleated, and then differentiate into
osteoclasts or giant cells. Tissue macrophages must undergo clonal expansion first in
response to M-CSF. Adapted from Ref. 4,5.
Monocytopenesis begins in the bone marrow in humans, and the most immature cell of the MPS is the monoblast, which like all cells of the hematopoietic system has its ultimate origin from pluripotent hematopoietic stem cells \(^6\) (Fig. 1-1A). Various cytokines (e.g. GM-CSF, M-CSF, and IL-3) have been identified as having a regulatory role in monocytopenesis. The presence of these mediators and stromal interactions leads to further differentiation and proliferation into promonocytes and then monocytes. These monocytes leave the bone marrow to enter the bloodstream. Studies in mice have shown that the half-life of monocytes in blood is about 17h under normal circumstances \(^7\), while longer times of up to 70h have been reported in human \(^8\). In adult humans, about 1-6% of total circulating white blood cells are monocytes, rarely exceeding 10% under normal conditions \(^6\). Monocytes enter the tissues by first rolling along the endothelium via low-affinity interactions, followed by high-affinity adherence to endothelium, and then diapedesis between endothelial cells (Fig. 1-1A). In the tissues, depending on the microenvironment monocytes differentiate into specific subpopulations with distinct functions, thus generating heterogeneity. This seeding of monocytes to different tissues is thought to be a random process \(^6\).

A particularly interesting ability of macrophages is syncytium formation, which is known to be mediated in part through CD47 and macrophage fusion receptor (MFR) interactions (Fig. 1-1B) \(^5\). Multinucleation has two main functions. First, the increase in cell size permits the elimination of large targets via extracellular degradation, a process that has been described as an 'extracellular lysosome', analogous to intracellular lysosomes \(^9\). This can occur when the macrophages are not able to ingest the particle. For example in
chronic inflammatory states directed at large foreign bodies, macrophages fuse and differentiate into multinucleated giant cells in an attempt to clear the debris. A second function of multinucleation is that it endows macrophages with enhanced capacity, such as amplified bone resorption by osteoclasts. This contrasts with mononucleated macrophages which cannot resorb bone efficiently. Differentiation into osteoclasts is highly regulated and involves a vast array of genes.

1.1.2 Macrophages respond to heterogeneous stimuli through a group of diverse cell surface receptors

Macrophages are able respond to diverse stimulus due to the expression of a large number of cell surface receptors. This ability to recognize a wide range of exogenous and endogenous ligands and to respond to them appropriately is fundamental to macrophage function in host defense (both innate and adaptive immunity), homeostasis, inflammation, autoimmunity, and immunopathology. Immune recognition receptors are diverse, and in addition to receptors for numerous cytokines, macrophages express receptors for non-opsonic recognition of microbes (CD14/LBP, TLR, CR3), phagocytosis of opsonized particles (FcγR, CR3), adherence (integrins), chemokines (CCR2), and recognition of apoptotic cells (CD36) (Table 1-1). Among innate immune receptors, pattern recognition receptors (PRR) have been studied extensively in recent years. PRR are used by the host to detect microbial infection, and the structures that these receptors recognize are called pathogen-associated microbial patterns (PAMP). Examples of these include bacterial lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, lipopeptide, dsRNA, ssRNA, bacterial flagellin, CpG DNA and others. Some investigators have
argued that the terms PRR and PAMP are a bit misleading, since there is no absolute requirement for a true molecular pattern \textit{per se}, such as multivalent crosslinking, or the presence of any repeating units $^{13}$. Rather, PAMP may be more aptly characterized as particular molecular sequences found within microbial macromolecules and conserved amongst diverse species which may or may not be pathogenic. Furthermore, some PRR have been shown to bind endogenous ligands like fibrinogen or heat shock proteins $^{14}$. However, when looking at the Toll-like receptors (TLR) family as a whole, each particular microbe—based on its array of PAMPs—has the potential to activate several different TLR simultaneously, such that an innate "immune signature" is formed $^{13}$. Such a pattern may allow the host to modulate an appropriate immune response towards a given type or class of microorganism.

The discovery of PRR began with research on the \textit{Drosophila} Toll protein. Toll was known to be involved in establishing dorsal-ventral polarity during embryogenesis, and was later found to be important in host defense against fungal infections in adult flies [reviewed in Ref. $^{15}$]. The cytoplasmic domain of the \textit{Drosophila} Toll is very similar to the mammalian IL-1 receptor, and these domains are referred to as Toll/IL-1 receptor (TIR) domain. It was actually this similarity that prompted investigation of the Toll pathway in regulating immune responses $^{16}$.

Since the discovery of \textit{Drosophila} Toll's role in host defense, subsequent research has identified a family of structurally related proteins related to Toll, and these collectively have been referred to as Toll-like receptors (TLR) (\textbf{Fig. 1-2}). Of the thirteen mammalian
TLR genes that have been identified, ten are expressed in human. Each of these germline-encoded, non-clonal receptors can recognize a particular set of PAMPs, and thus they represent a primary group of sensors used by the host to detect pathogens. Two key, common structural features of TLRs are an extracellular leucine-rich repeat (LRR) domain and an intracellular TIR domain. Recent crystal structure analysis of TLR3 revealed that the ligand binding site resides in the C-terminal half of the LRR, while the N-terminal half of LRR is believed to interact with other coreceptor or accessory molecules. Monocytes and macrophages express mRNA for most TLR except TLR3, which is expressed in dendritic cells. The major ligands for most of the TLR have been identified. A partial list is shown in Table 1-2. Besides structural specificity achieved from the existence of diverse TLR, ligand specificity and diversity can be further broadened through heterodimerization between TLR. For example TLR2 can heterodimerize with either TLR1 or TLR6, resulting in different ligand specificity (Fig. 1-2). Some TLR like TLR1, 2, 4, and 5 are expressed on the cell surface, while some are endosomal (TLR3, 7, 8) and can detect ligands inside endosomes. Another way diversity can be achieved is through associations with non-TLR receptors, such as the situation in which TLR2 dimerizes with dectin-1, a C-type lectin that enables TLR2/dectin-1 to recognize yeast wall particles called zymosans. Activation of TLRs results in overlapping and distinct cellular responses in macrophages, dendritic cells, and B cells. The downstream responses include production of inflammatory mediators, secretion of cytokines and chemokines, upregulation of costimulatory molecules, and transcription of antiviral genes.
While TLR are clearly a major focus of innate immunity research, it should be noted that other non-TLR PRRs are known to be important in innate recognition. These include f-methionyl-leucyl-phenylalanyl (fMLP) receptor, a G protein coupled receptor (GPCR) on neutrophils involved in chemotaxis. A deficiency of fMLP receptors in mice increases susceptibility to *Listeria* infections \(^{24}\). Another important class of sensors is the intracellular pathogen sensors belonging to the nucleotide-binding oligomerization domain (NOD) protein family \(^{21}\). NOD proteins are structurally similar to the plant R proteins, that are involved in resistance to infection. Missense mutations in the human *nod2* gene are associated with the pathogenesis of inflammatory diseases like Crohn’s disease and Blau syndrome \(^{25}\). These highlight how dysregulation of the innate immune recognition system can lead to inflammation, autoimmunity and impaired pathogen clearance.
Table 1-1

<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Example</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scavenger (collagenous)</td>
<td>SR-A</td>
<td>Phagocytosis of bacteria and apoptotic cells, endocytosis of modified LDL, adhesion</td>
</tr>
<tr>
<td>Scavenger (noncollagenous)</td>
<td>CD36</td>
<td>Phagocytosis of apoptotic cells, diacyl lipid recognition of bacteria</td>
</tr>
<tr>
<td>GPI-anchored</td>
<td>CD14</td>
<td>LPS-binding protein, TLR/MD2/MyD88 interactions, apoptotic cell recognition</td>
</tr>
<tr>
<td>Integrin</td>
<td>CR3 (CD11b/CD18)</td>
<td>CR-mediated phagocytosis, adhesion to endothelium</td>
</tr>
<tr>
<td>Ig Superfamily</td>
<td>FcR (ITAM/ITIM)</td>
<td>Antibody-dependent binding, phagocytosis, killing</td>
</tr>
<tr>
<td>Seven transmembrane</td>
<td>CCR2, C5aR</td>
<td>Receptor for chemokine MCP-1, Chemotaxis, degranulation</td>
</tr>
<tr>
<td>NK-like C-type lectin-like</td>
<td>Dectin-1</td>
<td>β-glucan receptor, fungal particle ingestion</td>
</tr>
<tr>
<td>C-type lectin (single CTLD)</td>
<td>DC-SIGN</td>
<td>Dendritic cell pathogen recognition, ICAM adhesion</td>
</tr>
<tr>
<td>Multiple CTLD</td>
<td>Mannose Receptor</td>
<td>Clearance, alternative activation, antigen transport</td>
</tr>
<tr>
<td>Toll-like receptors</td>
<td>TLR2, TLR4</td>
<td>Response to peptidoglycan, zymosan, Response to LPS</td>
</tr>
</tbody>
</table>

Table 1-1. Overview of macrophage receptors in immune recognition. Adapted from Ref. 11.
Figure 1-2. **Toll-like receptor family and their principle ligands.** TLR2 can heterodimerize with either TLR1 or TLR6, resulting in different ligand specificity. TLR2 can also dimerize with non-TLR receptors, such as the lectin, dectin-1, to recognize zymosan. Murine TLR11 recognizes uropathogenic bacterial products. TLR11 is a pseudogene in humans. TLR3, TLR7, TLR8 and TLR9 are localized to endosomal membranes, and recognize nucleic acids. TLR9 can also recognize non-nucleic acids such as hemozoin, a breakdown product of hemoglobin produced by *Plasmodia spp.* Only representative ligands are shown. TLR10 is an orphan receptor that is able to homodimerize and also heterodimerize with TLR1 and TLR2. Adapted from Ref. 21.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Peptidoglycans, Lipoteichoic acid Lipoarabinomannan Atypical LPS</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS Taxol HSP 60, 70 Fibrinogen</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>Zymosan Diacyl lipopeptides</td>
</tr>
<tr>
<td>TLR7</td>
<td>Imidazoquinoline ssRNA Loxorebine</td>
</tr>
<tr>
<td>TLR8</td>
<td>Imidazoquinoline ssRNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA</td>
</tr>
<tr>
<td>TLR10</td>
<td>unknown</td>
</tr>
<tr>
<td>TLR11</td>
<td>unknown, from uropathogenic bacteria</td>
</tr>
</tbody>
</table>

Table 1-2. Toll-like receptors and their ligands. Adapted from Ref. 14.
1.1.3 Activation of macrophages

Macrophages can acquire new or enhanced functional capacities when "activated" in response to various agonists \(^{27}\). This may lead to enhanced cytokine and chemokine expression, phagocytic capacity, cellular metabolism, responses to chemoattractant signals as well as acquisition of antimicrobial or antitumor functions, and modulation of the capacity to process and present antigens \(^{4,27}\). In contrast to "activation", the term "priming" refers to prior exposure of a cell to a soluble agonist that alters its response to a subsequent stimulus \(^{28,29}\). Priming stimuli do not result in an activated phenotype \(\textit{per se}\), but rather sensitize the cell such that it can then respond to subthreshold stimuli. The classical macrophage priming stimulus is low dose IFN-\(\gamma\) \(^{30}\), but exposures to other mediators such as TNF-\(\alpha\) or proteinases can also prime \(^{28,30,31}\). Generally, it is not the chemical nature of the stimulus that determines if it activates or primes, but rather it is the concentration of the stimulus along with other factors such as co-secreted molecules or adhesive interactions with neighboring cells \(^{32}\). Priming is not restricted to cells of the MPS, as it has also been observed in neutrophils, basophils, lymphocytes, and eosinophils \(^{32}\).

In general macrophage activation may be rapid and take effect within seconds to minutes, such as with the generation of reactive oxygen species and release of arachidonic acid metabolites \(^{27,33}\). Activation can also lead to adaptive changes that require gene expression and this process occurs over periods of hours to days \(^{27}\). Macrophage activation has been classified into five types: innate activation, humoral activation, classical activation, alternate activation, and deactivation [reviewed in Ref. \(^{4,11,30}\)]. Innate
activation involves stimulation by microbial products like LPS, through Toll-like and other pattern-recognition receptors. This may result in secretion of pro-inflammatory cytokines, reactive oxygen species and nitric oxide followed by a regulated anti-inflammatory response. Humoral activation occurs via Fc receptors or complement receptors, and results in cytolytic activity and cytokine secretion. Classical activation is mediated by initial stimulation with IFN-γ followed by a microbial trigger (e.g. LPS), which results in the secretion of pro-inflammatory cytokines, upregulation of MHC class II, and an oxidative burst. Alternate activation involves IL-4 and IL-13, which mediate their actions through the IL-4/IL-13 common receptor α-chain (IL-4Rα). Alternate activation of macrophages biases towards humoral immunity (Th2) favoring allergic and anti-parasite responses, upregulation of MHC class II molecules and mannose receptor expression and increased endocytic activity. The fifth type of “activation” is actually “deactivation”, and can be brought about by deactivating cytokines (e.g. IL-10, TGF-β), phagocytosis of apoptotic cells, or some pathogens. Macrophage deactivation results in secretion of anti-inflammatory cytokines, and downregulation of MHC class II expression. Deactivation plays a role in the resolution of inflammation and in some pathological conditions.

1.1.1.4 Functional properties of macrophages

Macrophages have diverse roles in both immunity and tissue homeostasis. These functions can be broadly classified into several areas: phagocytosis and destruction of microorganisms, removal of cellular products including dead and dying cells, chemotaxis, antigen processing and presentation, and secretion of multiple effectors and regulators.
Phagocytosis is the receptor-mediated uptake of large particles (>0.5 μm in diameter) into cells. Cells with phagocytic capabilities are of myeloid lineage and include cells of the MPS and neutrophils. Recognition of particles is mediated by phagocytic receptors expressed on macrophages, and this enables binding to a wide range of particles leading to phagocytosis. Phagocytosis is a cytoskeletal-based process that requires membrane insertion into nascent phagocytic cups, and the engulfment of particles leads to the generation of the phagosome. Phagosomes are dynamic organelles that undergo extensive luminal and membrane modifications via interactions with diverse endocytic vesicles and the endoplasmic reticulum. The process is termed phagosome maturation, and culminates in the formation of a phagolysosome, also termed secondary lysosome. The microbicidal capacities of phagolysosomes have been attributed to their low luminal pH and abundance of hydrolytic enzymes and reactive oxygen intermediates (ROI) [reviewed in Ref. 34-37]. Recent evidence, however, has led to the suggestion that K+ influx into the phagosome is a more important contributor to killing through the activation of proteolytic enzymes through hypertonicity and elevated pH. In this model, the action of superoxide and other ROI is not proposed to be direct attack on microbial structures, but rather as a trigger that leads to protease degradation. The actual relative contributions of these two disparate models are controversial, and are likely to be resolved with further research. Phagosomes also provide a site for antigen processing and presentation, as well as signaling through PRRs. In this manner, phagosomes are organelles that provide a link between innate and adaptive immunity. Numerous important pathogens have evolved to survive within macrophages by either escaping the
phagosome (listeria, shigella, rickettsia) or modifying it in such a manner that they are able to survive within it (mycobacteria, salmonella, brucella, leishmania, toxoplasma). How pathogens escape killing by macrophages is an important research focus since further understanding will be useful in developing novel antimicrobial strategies.

Macrophages have important roles in the initiation, maintenance, and resolution of inflammation. Injury or microbial invasion stimulates local connective tissue macrophages to secrete cytokines, such as TNF-α and IL-1, which induce rapid changes in endothelial surfaces essential for margination, arrest, and diapedesis of leukocytes. The first leukocytes that are recruited from the blood to sites of inflammation are neutrophils, followed by monocytes. After extravasation into tissues, monocytes differentiate into macrophages and migrate towards the site of injury by chemotaxis. Monocytic cells are able to sense and respond to chemotactic gradients produced during inflammation. Through GPCR, monocytes and macrophages can migrate towards the source of chemokine production. Both endogenous and exogenous substances can act as chemoattractants. The bacterial product fMLP (N-formyl-methionyl-leucyl-phenylalanine), as well as endogenous mediators such as C5a, leukotriene B4 (LTB4), and chemokines (IL-8) are some examples. Activation of macrophages results in secretion of cytokines, chemokines, vasodilators, metalloproteinases, and enhanced phagocytosis and microbicidal killing. All of these events contribute to the inflammatory response.
Macrophages are also involved in embryogenesis, morphogenesis, and tissue repair. Remodeling of tissues and resolution of inflammation or injury requires clearing of cellular debris, and macrophages are involved in these processes partly through phagocytosis. Phagocytosis of apoptotic cells is different from that of microorganisms in that inflammatory responses are not induced with the former. In addition, production of anti-inflammatory cytokines such as TGF-β has been demonstrated to accompany ingestion of apoptotic cells. Recognition of apoptotic cells requires some additional receptors that are distinct from pathogen recognition, such as phosphatidylserine receptors. Macrophages also perform functions such as the removal of necrotic tissue, fibrin dissolution, regulation of fibroblast recruitment and growth, connective tissue remodeling, and bone remodeling through resorption by osteoclasts. In this manner, macrophages contribute to tissue homeostasis in both normal and pathogenic states through the engulfment of foreign material and the removal of altered endogenous products.

Macrophages and dendritic cells function as antigen-presenting cells, by presenting antigenic peptides to stimulate clonal expansion of B and T cells. In this capacity, cells of the MPS act as bridge to link innate and adaptive immunity responses. Mature DCs are unique in their ability to sensitize naïve T cells to peptide antigens, whereas a number of different cell types including macrophages are able to bring about a secondary response by presenting the corresponding peptide to primed T cells. Immature DCs are present in most tissues and are efficient at capturing and processing antigens due to their
high endocytic activity. Capturing of antigen signals them to mature and migrate towards lymphoid organs.  

Macrophages have an extraordinary secretory capability. Over one hundred substances have been identified to be secreted by macrophages [reviewed in Ref. 3]. These include enzymes (lysozyme, proteases), enzyme inhibitors (α2-macroglobulin, α1-antitrypsin inhibitor), complement proteins (C1-C5), reactive oxygen intermediates (superoxide, H₂O₂), cytokines (TNF-α, IL-1, IL-6, IL-10, IL-12, M-CSF, chemokines), coagulation factors (tissue factor, factors II, VII, IX, X, XIII), and arachidonic acid intermediates (PGE₂, leukotrienes, PAF) to name only a few. This ability to secrete a diverse and large group of mediators enables the macrophage to participate in a large variety of normal cellular processes, as well as pathological ones.

1.2 Phosphatidylinositide 3-kinase

1.2.1 The PI3K family and 3'-phosphoinositides

Phosphatidylinositol 3-kinases (PI3K) constitute a family of lipid kinases that phosphorylate the 3’-hydroxyl of the inositol group of D-myophosphatidylinositol or its derivatives (Fig. 1-3A). Phosphatidylinositol (PtdIns) is an inositol derivative of phosphatidic acid, and PtdIns is the most abundant inositol lipid in unstimulated mammalian cells. The term phosphoinositides (PI) applies to PtdIns that has been phosphorylated at one or more position on the inositol ring. The inositol head group has five free hydroxyl groups, but positions 2 and 6 have not been documented to be esterified with phosphate. The 3'-PI metabolites produced as a result of PI3K activity
are known to be involved in regulating a multitude of cellular events such as mitogenic responses, insulin signaling, differentiation, apoptosis, cytoskeletal organization, membrane traffic along the exocytic and endocytic pathways (reviewed in Ref. 47,49,50), autophagy 51, and others. The ability of PI3K to affect such diverse cellular functions can be partially explained by the existence of multiple isoforms of PI3K, multiple levels of regulation, and likely subcellular localization of PI3K itself, its metabolites, effectors and targets.

PI3K family members have been sub classified based upon their structure and substrate specificity in vitro (Fig. 1-4). Class I isoforms exhibit the broadest degree of substrate selectivity in vitro and phosphorylate multiple forms of PtdIns, including PtdIns, PtdIns(4)P, PtdIns(4,5)P2 (Fig. 1-3B). In vivo, PtdIns(4,5)P2 is reported to be the most likely substrate leading to the formation of PtdIns(3,4,5)P3 52,53. Resting mammalian cells contain a significant level of PtdIns(3)P, but very little in the way of other 3'-PIs 47,54. Upon stimulation by agonists such as platelet-derived growth factor (PDGF), PtdIns(3,4,5)P3 and PtdIns(3,4,)P2 levels rise rapidly 54,55. Class I PI3K is further divided into two subclasses, both of which are known to be activated by cell surface receptors. Class IA PI3K are heterodimers consisting of a regulatory subunit (p85α and its splice variants, and p85β or p55γ,) and a 110 kDa (p110α, p110β, or p110δ isoforms) catalytic subunit (Fig. 1-4) 49,50. Both p110α and p110β are widely expressed in various tissues, whereas p110δ is more restricted to leukocytes 56. Class I PI3K catalytic subunits have four characteristic domains or homology regions (HR) 1 to 4, which correspond to the
kinase domain, helical domain, C2 domain, and the Ras binding domain, respectively (Fig. 1-4) 47.

Class IA PI3K contains different regulatory subunits encoded by three distinct genes (PIK3R1, PIK3R2, and PIK3R3) which encode proteins that share similar structures (Fig. 1-4) 50. The PIK3R1 gene product gives rise to at least 5 different splice forms (only 3 are shown in Fig. 1-4) 57,58. Full length p85α and p85β have N-terminal Src-homology 3 (SH3) domains, and Rac-binding domains (also called breakpoint cluster region homology (BH) domain) flanked by two proline rich regions. The Rac-binding domain is homologous to GTPase accelerating factors (GAP) for Rho family small G proteins, however it lacks GAP activity 50. At the C-termini, the two Src-homology 2 (SH2) domains flank the inter-SH2 (iSH2) region, which contains the binding site for the p110 catalytic subunit 47. p85 subunits function as both adaptors and regulators through their multiple domains (Fig. 1-5A) 59. Both p85α and p85β exhibit a wide tissue distribution 57,60, although splice variants of p85α are predominantly expressed in skeletal muscles and brain 57. PIK3R3 encodes p55γ, which differs from p85α and p85β in that it lacks the N-terminal SH3 and the Rac-binding domain, and has very low expression in peripheral blood leukocytes 61. PI3KR3 can generate two forms of the p55γ, p55PIK and p50PIK, through alternative initiation of translation 62. Overall, each distinct regulatory subunit (p85α, p85β, or p55γ) is not known to selectively associate with a particular p110 catalytic isoform 60.
Class IB PI3K is composed of a p110γ catalytic subunit that associates with a p101 adapter encoded by the PIK3R5 gene (Fig. 1-4). The overall structure of p110γ is similar to that of class IA p110 isoforms except that instead of the regulatory binding domain at the amino terminus, a p101 binding domain exists 50. Other differences include the presence of Gβγ binding domains in p110γ 63. Association with p101 is required for activation of p110γ by βγ subunits of heterotrimeric G proteins 64, although it has been reported that it can also be directly stimulated by Gβγ through binding to distinct sites on p110γ (Fig. 1-4) 65,66. p110γ is expressed mainly in leukocytes, platelets, and cardiomyocytes 67,68.
Figure 1-3

A. Structure of phosphatidylinositol. The phosphatidic acid component consists of a glycerol backbone with esterified fatty acids at position 1 and 2, and phosphoric acid at position 3. Arrow indicates the site of PI3K action, and scissor indicates the cleavage site by PLC.

B. Pathways of D-3'-phosphoinositides and select PtdIns kinases and PtdIns phosphatases. Adapted from Ref. 47,48,50.

Figure 1-3. Structure of phosphoinositides and pathways of 3-phosphoinositide synthesis and degradation. A, Structure of phosphatidylinositol. The phosphatidic acid component consists of a glycerol backbone with esterified fatty acids at position 1 and 2, and phosphoric acid at position 3. Arrow indicates the site of PI3K action, and scissor indicates the cleavage site by PLC. B, Pathways of D-3'-phosphoinositides and select PtdIns kinases and PtdIns phosphatases. Adapted from Ref. 47,48,50.
Figure 1-4. Mammalian PI3K catalytic and regulatory subunits. The diagrams are schematic and not drawn to scale. Further splice forms with insertions into the inter-SH2
region of p85α and p55α are not shown. Also not shown are the two different forms of p55γ subunits. Adapted from Ref. 50,60,63.

Class II PI3Ks contain two C2 domains and are not known to be associated with a regulatory subunit 50. The C2 domain was initially thought to provide membrane binding and Ca\(^{2+}\) regulation 69, although subsequent analysis revealed that C2 domains in class II PI3K lack the critical aspartate residues that form the calcium binding pocket 70. In vitro, Class II PI3K phosphorylates PtdIns and PtdIns(4)P 69. They have also been shown to phosphorylate PtdIns(4,5)P\(_2\) at low levels when lipid substrates were presented together with phosphatidylserine acting as a carrier 69. Three isoforms (C2α, C2β, and C2γ) of mammalian class II PI3K have been characterized 69,71,72. Both the C2α and C2β isoforms are expressed ubiquitously, while the C2γ is predominantly expressed in the liver 72. In contrast to class I PI3Ks, which are mainly cytosolic, class II PI3K enzymes are predominantly associated with membrane fractions of cells 48. C2α and C2β differ in divalent cation dependence in that C2α has a preference for Mg\(^{2+}\) over Mn\(^{2+}\) and Ca\(^{2+}\) and their sensitivity to PI3K inhibitors wortmannin (isolated from Penicillium wortmanni) and the structurally unrelated LY294002 73,74. Like the rest of the PI3K family members, C2β is sensitive to wortmannin and LY294002 whereas C2α is resistant to similar concentrations of either inhibitor 69.

Class III PI3K is the orthologue of yeast PI3K Vps34p (vacuolar protein sorting), the only type of PI3K in yeast, and phosphorylates only PtdIns 75. In mammalian cells, hVps34 is associated with p150, a myristylated serine/threonine kinase. The
myristoylation of p150 likely helps target hVps34 to membranes, as is the case in yeast. Yeast strains with mutations in the gene for Vps34p are defective in directing soluble hydrolases to the vacuole, the yeast equivalent of the lysosome. In mammalian cells, hVps34 also plays a role in vesicle trafficking. Fusion events between early endosomes require the product of hVps34. hVps34 is also implicated in regulating degradative pathways in the cell. For example, vesicular trafficking of internalized immune complexes for degradation in the lysosome, macroautophagy, and phagosome maturation have all been shown to depend on class III PI3K. In addition, inhibitory antibodies to hVps34 disrupt post-endocytic trafficking of PDGF receptor.

Comparisons between major model organisms suggest that class I and II PI3K are present mainly in animals, and the number of isoforms increases with the complexity of the organism (Table 1-3). The yeast genome encodes only class III, and class II PI3K is not found in plants or yeasts, or in the unicellular eukaryotic social amoebae Dictyostelium discoideum. Invertebrates generally encode a single isoform of class IA, II, and III, usually with no class IB PI3K. Caenorhabditis elegans and Drosophila melanogaster each have a single regulatory subunit of class I PI3K which share characteristics with the shorter mammalian p50α and p55α subunits. It has been suggested that the longer molecules p85α and p85β may have evolved to provide mammals with distinct functions. Together, these findings suggest that duplication and diversification has occurred in mammalian evolution with respect to PI3Ks. It has been hypothesized that PI3K diversity in vertebrates was driven by the complex regulatory circuits in the adaptive immune system.
<table>
<thead>
<tr>
<th>Table 1-3. PI3K family in eukaryotes.</th>
</tr>
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<tbody>
<tr>
<td><em>No ortholog found as yet. Adapted form ref</em>[^47].</td>
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</tbody>
</table>
1.2.2 Activation and regulation of PI3K

Through their regulatory subunits Class IA catalytic isoforms of PI3K are recruited to and activated by cell surface receptors with intrinsic protein tyrosine kinase activity (e.g. growth factor receptors) or receptors coupled to non-receptor tyrosine kinases such as src-family kinases (e.g. B-cell receptor, T-cell receptor, Fc receptors) or Janus kinases (e.g. IL-6 receptors) \(^{47}\). However, the p110 catalytic subunits can also be directly regulated by other mediators. For example, GTP-bound Ras can further augment class IA PI3K activation through direct binding to p110 \(^{82-84}\). The class IA PI3K p110\(\beta\) isoform has also been shown to be activated downstream of G protein-coupled receptor (GPCR), such as chemokine receptors \(^{85,86}\), and be activated directly by G\(\beta\gamma\) subunits of heterotrimeric G proteins \textit{in vitro} \(^{66,87,88}\). Class IB PI3K p110\(\gamma/p101\) can be activated by G\(\beta\gamma\) subunits following stimulation of GPCRs such as fMLP receptor \(^{64,66,88}\). The p110\(\gamma\) catalytic subunit can also be directly regulated by Ras, as is the case for class IA p110 isoforms \(^{89,90}\).

Comparisons of the kinetic properties between p110\(\alpha\) and p110\(\beta\) revealed interesting differences \(^{91}\). In terms of lipid kinase activity, p110\(\alpha\) has a higher \(V_{\text{max}}\) and 25-fold higher \(K_m\) for PtdIns than p110\(\beta\). When PtdIns(4,5)P\(_2\) was used as a substrate, similar results were obtained. In thymocyte lysates, p110\(\delta\) lipid kinase activity was intermediate between p110\(\alpha\) and p110\(\beta\) when PtdIns(4,5)P\(_2\) was used as a substrate \(^{92}\). These findings suggest that kinetic differences, in addition to differential tissue distribution, provide a mechanism to explain differential activity of class IA PI3K isoforms.
The diversity of protein interaction domains on the regulatory subunits likely contributes to the ability of multiple signaling proteins to activate class IA PI3K (Fig. 1-5A). Through their SH2-containing regulatory subunits, class IA PI3Ks bind to phosphorylated tyrosine residues that are generated by activated tyrosine kinases in receptors and various receptor-associated adaptor proteins. The class IA regulatory subunits prefer to bind to proteins containing the consensus phosphotyrosine motif pYxxM. Phosphotyrosine binding promotes translocation of the cytosolic PI3Ks to the plasma membrane, where they encounter their lipid substrates and Ras. Non-phosphotyrosine-based recruitment mechanisms may also contribute to PI3K activation. Some membrane receptors, such as CD2 and IL-2 receptor, show constitutive association with class IA PI3Ks. All mammalian cell types investigated express at least one class IA PI3K isoform, and stimulation of diverse receptor that induce tyrosine kinase activity also leads to class IA PI3K activation.

Under resting conditions, the regulatory subunits of class IA PI3K have been shown to inhibit the catalytic activity of p110 subunits, as well as to protect them from thermal inactivation in vitro. Dimerization with p85 increases the half-life of p110, compared to monomeric p110. Tonic inhibition of p110 by p85 is removed by binding of phosphotyrosine-containing peptides to SH2 domains of p85. Specifically, the iSH2 domain of p85 is sufficient to bind p110α but inhibition of p110α requires the presence of both the N-terminal SH2 domain linked to the iSH2 domain. Binding of phosphopeptides to the N-terminal SH2 results in increased kinase activity. Thus the N-terminal SH2 domain mediates both inhibition of p110α and disinhibition by
phosphopeptides. In contrast, phosphopeptide binding to the C-terminal SH2 relieves tonic inhibition only in the context of an intact full length p85. It has also been shown that regulation of class IA PI3K can be mediated by phosphorylation of the Tyr residue within the C-terminal SH2 domain of p85 by Src family tyrosine kinases (Lck and Abl). Phosphorylation of Tyr leads to increased class IA PI3K activity. The N-terminal SH2 domain is able to bind to phospho-Tyr in the C-terminal SH2, thus an intramolecular interaction has been proposed. Furthermore, the C-terminal region of p85 has been demonstrated to block Ras-induced class IA p110 activation. An emerging model (Fig. 1-5B) is that phosphotyrosyl-proteins recruit PI3K through interactions with the two SH2 domains of the regulatory subunits, and binding to the N-terminal SH2 domain results in conformational changes that releases inhibition of p110 by the regulatory subunit. Binding of phosphotyrosyl-proteins to the C-terminal SH2 also allows p110 to be activated by Ras. The phosphorylation of Tyr in the C-terminal SH2 results in the formation of an intramolecular association, also promoting activation of PI3K. This intramolecular interaction may also promote amplification of the PI3K signal by facilitating the removal of phosphorylated PI3K and freeing the receptor for subsequent association with a new PI3K heterodimer. Since SH2 domain-containing phosphatase 1 (SHP-1) has been shown to de phosphorylate Tyr, the newly detached, phosphorylated PI3K may then be dephosphorylated by SHP-1 (Fig. 1-5B) and return to basal state. This would allow PI3K to be recycled for recruitment to a phosphorylated receptor.
In addition to the direct inhibitory effect by the regulatory subunit on p110 subunits, free monomeric p85 has also been reported to mediate a signaling function, independent of its regulation on p110 subunits. Kahn and coworkers demonstrated that p85 is more abundant than p110 subunits, and at least 30% of p85 exists as a monomer in murine embryonic fibroblasts. This results in competition between the p85 monomer and the PI3K heterodimer in mediating signaling downstream of insulin-like growth factor-1 (IGF-1). Studies in p85 knockout (KO) cells also revealed that activation of Jun N-terminal kinase (JNK) by insulin is independent of the associated p110 catalytic activity. Instead, this ability to activate JNK is dependent on the N-terminal regions of the p85α or p85β, thus indicating the existence of a p85-dependent but PI3 kinase-independent signaling pathways.

More recently, the molecular balance or ratio, between the regulatory and catalytic subunits of PI3K has been shown to regulate insulin sensitivity in mice. PI3K activity is required for insulin-induced glucose uptake into muscle and fat cells and inhibition by insulin of glucose production by the liver. It was a surprising finding, therefore, when mice lacking any isoform of p85 exhibited increased insulin signaling even under conditions where total PI3K levels were reduced. These findings raised the possibility that p85 subunits mediate a negative role in insulin signaling. Cantley et al. examined mice that were heterozygous knock outs for either p110α, p110β or both to examine the effects of reduced p110 subunits expression on insulin signaling. Double heterozygous mutants were used because homozygous knockout for either p110α or p110β resulted in embryonic death. They discovered that double heterozygous loss
of PI3K catalytic subunits p110α and p110β resulted in mice with mild glucose intolerance and mild hyperinsulinemia in the fasting state, suggesting slightly impaired insulin signaling \(^{106}\). This correlated with a 50% reduction in class IA PI3K protein levels in the cells. Thus, deletion of p85 versus p110 subunits can have opposing effects on insulin sensitivity and their ratios have a critical role in determining the set point for \textit{in vivo} insulin sensitivity (Fig. 1-5C). These findings highlight the importance of not considering p85 knockout to be models of class IA PI3K deficiency.

In insulin signaling, both mammalian target of rapamycin (mTOR) and p70\(^{S6}\)kinase 1 (S6K1) have been implicated in the negative regulation of the PI3K-Akt pathway [reviewed in Ref. \(^{116}\)]. mTOR is a highly conserved protein kinase and is crucial in promoting ribosome biogenesis and cell growth \(^{117}\). mTOR activity is regulated by at least three main inputs: nutrients, energy metabolism, and growth factors \(^{117}\). S6K1 is a serine/threonine kinase involved in cell growth and proliferation and its activity is regulated by mTOR \(^{117}\). mTOR exists as a complex with GβL and regulatory associated protein of mTOR (RAPTOR) \(^{117}\) (Fig. 1-6). This complex is sensitive to the compound rapamycin, hence the name of mTOR \(^{116}\). S6K1 has been implicated in phosphorylation of a specific serine residue on insulin receptor substrate-1 (IRS-1), which leads to increased degradation IRS-1 and decreased insulin receptor interaction with IRS-1 \(^{116,118}\). S6K1 also has a negative effect on IRS-1 gene transcription \(^{119}\). In this model, insulin or IGF-1 binding to the insulin receptor leads to the recruitment of IRS and activation of class IA PI3K. The PtdIns(3,4,5)P\(_3\) generated as a result then recruits and activates PDK-1 and Akt. Akt phosphorylates and inactivates tuberous sclerosis complex 1 and 2 (TSC-
1/2), a protein complex with a GAP activity that inhibits the mTOR/S6K1 pathway through inhibition of the upstream activator Rheb GTPase (Fig. 1-6) \(^{120}\). By inactivating TSC1/2, inhibition of the mTOR/S6K1 pathway is removed leading to serine phosphorylation of IRS-1 by S6K1. The resulting degradation of IRS-1 leads to reduced recruitment and activation of PI3K. This negative regulation of PI3K activity selectively uncouples the stimulatory effects of insulin, and does not affect PI3K activation by other stimuli such as PDGF \(^{116}\). This example provides a mechanistic and conceptual framework of how class I PI3K can be regulated at the level of activation by a specific negative feedback pathway, while leaving intact the activation of PI3K by other stimuli.

Recently, the class III PI3K hVps34 has been demonstrated as a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to the mTOR and S6K1 pathway \(^{121,122}\). In this model, hVps34 is also required for insulin stimulation of S6K1 in addition to class I PI3K, but hVps34 itself is not regulated by insulin \(^{121}\). Instead, nutrient status such as low amino acid or glucose starvation, and activation of AMP-activated protein kinase (AMPK) inhibits hVps34 (Fig. 1-6). This inhibition prevents hVPS34 from activating mTOR, and hence activation of S6K1 as well. Thus, the two main inputs to S6K1 activation are independently regulated by distinct PI3K classes: class I regulates the growth factor stimulation component, and class III regulates the amino acid and glucose sensing component. This illustrates how different classes of PI3K can cooperatively regulate a common pathway.
The catalytic subunits of class I and III PI3K also have in vitro protein-serine kinase functions, in addition to lipid kinase activities. The potential roles of these protein kinase activities have not been investigated to the same extent as has the lipid kinase function of PI3K. Since classical PI3K inhibitors wortmannin and LY294002 interfere equally with both the lipid kinase and protein kinase activities, it cannot be assumed that PI3K signals solely by generating 3'-phosphoinositides. In p110δ, autophosphorylation of the catalytic subunit on serine residues results in inhibition of the lipid kinase activity, and this activity was also blocked by PI3K inhibitors. Similar autophosphorylation has also been reported for p110β and p110γ. Autophosphorylation of p110β resulted in down-regulated PI3K lipid kinase activity. However, no inhibitory effect of p110γ autophosphorylation on its lipid kinase activity was observed. Protein kinase activity has also been shown for p110α, but rather than autophosphorylation, it phosphorylates p85α at the p110-binding domain. This inhibitory phosphorylation is increased following insulin stimulation and provides a negative feedback mechanism to regulate PI3K activity. Serine phosphorylation of the p85 subunit caused a 3 to 7 fold decrease in PI3K lipid kinase activity. In terms of protein kinase kinetics, p110α has a higher $V_{\text{max}}$ and $K_m$ towards peptide substrates than does p110β. Taken together, these findings support a role for inter-subunit and intracatalytic subunit serine phosphorylation in the regulation of the PI3K, and differences amongst various isoforms may contribute to differential regulation (Fig. 1-5A). Although IRS-1 has been reported to be a substrate of PI3K protein kinase activity downstream of insulin and IFN-α signaling, further research is required to clarify the functional importance of these modification. A role for protein kinase activity in...
class IB PI3K has also been described. Using COS-7 cells expressing lipid kinase inactive p110γ, MAP kinase activation in response to lysophosphatidic acid was shown to be dependent on p110γ protein kinase activity. The importance and physiological relevance of the protein kinase activities of various PI3K isoforms remains to be clarified further.

Class II PI3Ks are activated by numerous cell surface receptors. For example, the CC chemokine receptor-2 (CCR2) and the insulin receptor activate C2α, while epidermal growth factor receptor (EGFR) activates both C2α and C2β. C2α can also be activated by TNF-α and leptin. Insulin preferentially activates C2α rather than C2β isoform of PI3K. The precise mechanism of class II PI3K regulation is not well understood. Although there is a Ras binding domain on the N-terminal region (Fig. 1-4), there is no evidence for binding or regulation by Ras. However, deletion of the C2 domain of C2β increased the lipid kinase activity suggesting that it functions as a negative regulator of the catalytic domain.

Considerably more is known about regulation of class III PI3K. hVps34 is complexed with the protein kinase p150, and association with p150 is required for activation of hVps34. The product of class III PI3K, PtdIns(3)P, is constitutively produced in both yeast and mammalian cells and total levels do not change significantly in response to cell stimulation with PDGF. In contrast, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are almost absent in resting cells. Nearly constant levels of PtdIns(3)P suggest that the subcellular localization of PtdIns(3)P production regulates various hVPS34 dependent
cellular processes. In addition to regulation by amino acid or AMPK in the context of mTOR/S6K1 signaling, as discussed previously, class III hVPS34 is also regulated by the Rab family of GTPases in several vesicle trafficking processes. On early endosomes, hVps34 interacts with Rab5 GTPase to generate PtdIns(3)P, and the resultant lipid then recruits tethering and docking machinery to facilitate the fusion of early endosomes.

In late endosomal trafficking, the late endosomal protein Rab7 GTPase is known to influence interactions between early and late endosomes. It has been demonstrated that the inactive GDP-bound Rab7 colocalizes and interacts with hVps34 via p150, while the active GTP-bound Rab7 dissociates from hVps34/p150. Dissociated hVps34/p150 then becomes active on late endosomes, and mediates late endosomal transport. Therefore membrane recruitment of p150/hVps34 to the late endosome does not appear to be the point of regulation, rather it is the dissociation from Rab7 that determines the activity of hVps34. Since a larger fraction of hVps34 is complexed with Rab7 than Rab5, it has been proposed that Rab7 is the critical regulator in the localized control of hVps34 function.
Figure 1-5A

Rab4, 5, 6
Rac1
cdc42

Other binding partners:
SHIP, Cbl, ezrin, Src family

Inhibition of kinase activity

Ras
Membranes

Inhibition of Ras activation

PtdIns(4,5)P₂

Auto and trans protein phosphorylation

Figure 1-5B

Growth factor receptor

Inhibited

Permissive

Permissive
Figure 1-5C

Figure 1-5. Regulation of class IA PI3K. A, The intermolecular and intramolecular regulation, catalytic activities, and substrate interactions of class IA PI3K heterodimers. The protein kinase activity of p110 subunits can lead to the downregulation of the lipid kinase activity of the complex. This occurs either by phosphorylation of the inter-SH2 region of p85 (in the case of p110α) or autophosphorylation (in the case of p110β and p110δ). Class IA regulatory subunits stabilize the p110 subunit and inhibit their catalytic activities. This inhibition can be relieved by engagement of the SH2 domains of the regulatory subunits with phosphotyrosine residues in receptors or adaptors. Some of the known proteins that are known to bind p85 through interactions via various domains of p85 are shown. Adapted from Ref. 60. B, Model of the effect of phosphorylation of Tyr^{688} in p85 on PI3K activity, and formation of an intramolecular interaction between the two SH2 domains. In the basal state, Tyr^{688} is not phosphorylated. Once recruited by phosphotyrosine residues on receptors or adaptors, conformational changes lead to an
active PI3K. Binding of the C-terminal SH2 also relieves the inhibition on the Ras binding domain, such that PI3K can be further activated by Ras, which is membrane bound (not shown). Src-family kinases such as Lck can also phosphorylate Tyr\textsuperscript{688} and induce an intramolecular complex between the N-SH2 and C-SH2 domains. This form is active, and becomes detached from the receptors such that other PI3K heterodimers can become recruited, thus amplifying the PI3K signaling. SHP-1 can dephosphorylate Tyr\textsuperscript{688}, restoring PI3K to its basal state. Adapted from Ref.\textsuperscript{83,101,102}.

Model of the molecular balance of p110 and p85 in mediating insulin signaling. Insulin sensitivity is regulated by the ratio of the positive acting heterodimer p85/p110, and the negative regulator free p85. A given ratio between p85/p110 and free p85 defines the normal state of insulin sensitivity in wild-type animals. In p85\textsuperscript{a+/−} or p85\textsuperscript{β−/−} mice, free p85 is decreased preferentially. This causes the balance between p85/p110 and free p85 to shift towards the positive mediator p85/p110, which causes increased insulin sensitivity in mutant mice. In contrast, in p110\textsuperscript{α+/−} p110\textsuperscript{β+/−} mice the p85/p110 pool is preferentially decreased over free p85. In this case the balance is shifted towards the negative regulator free p85, resulting in decreased insulin sensitivity. Alternatively, overexpression of p85 leads to increase in free p85 and therefore also shifts the balance towards negative regulation, resulting in decreased insulin sensitivity. Adapted from Ref.\textsuperscript{106}.
Figure 1-6. Control of insulin and PI3K signaling by mTOR and S6K1. Insulin or IGF-1 stimulates the activation of class IA PI3K/PDK1 pathway via IRS-1, leading to the activation of Akt by a Thr\(^{308}\) phosphorylation. Full activation of Akt requires Ser\(^{473}\) phosphorylation as well, mediated by the mTOR/GβL/RICTOR complex. Activated Akt then phosphorylates and inactivates TSC1/2, leading to the activation of Rheb GTPase, mTOR/GβL/RACTOR, and then S6K. Thr\(^{308}\) and Ser\(^{473}\) can be dephosphorylated by protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP), respectively\(^{143,144}\). S6K negatively regulates the ability of IRS-1 to transduce
the insulin or IGF-1 signal. Class III PI3K hVps34 can integrate nutrient input, such as amino acids, to mTOR/S6K. Adapted from Ref. 116,121,122,144-147.
1.2.3 PI3K Effectors

The products of class I PI3K are lipid second messengers that control a wide range of cellular responses via diverse downstream effectors. Levels of PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ rise sharply in cells after cell stimulation, and they interact with an array of protein effectors that contain pleckstrin homology (PH) domains. PH domains are modular segments of approximately 100 amino acids found in many signaling proteins, and a subgroup of PH domains show specificity for PtdIns(3,4)P$_2$, PtdIns(3,4,5)P$_3$, or both. PH domains that selectively bind PtdIns(3)P have also been identified. The empirical finding that some PH domains interact specifically in vitro with PtdIns(3,4)P$_2$ and/or PtdIns(3,4,5)P$_3$ correlates with in vivo data defining the same PH domain-containing proteins as PI3K effectors. PH domain-containing effectors include serine kinases (e.g. 3'-phosphoinositide-dependent kinase-1 [PDK1] and protein kinase B [PKB/Akt]), Tec family tyrosine kinases (Bruton tyrosine kinase [Btk] and inducible T-cell kinase [Itk]), phospholipases (PLCy2), adaptor proteins (Gab1/2 [Grb2-associated binder]), guanine nucleotide exchange factors (GEF) for Rho and ARF family GTPases, and GTPase-activating proteins (GAP) such as centaurins and GAP1m (Fig. 1-7). Several PH domains exhibit high affinity for distinct 3'-PI lipids. These include the PH domains of Btk and centaurin-1 which recognize PtdIns(3,4,5)P$_3$ with high affinity and specificity, whereas other PH domains-containing proteins (e.g. tandem PH domain-containing protein-1 [TAPP1/TAPP2]) will only interact with PtdIns(3,4)P$_2$. Collectively, PH domain-containing proteins are able to propagate and drive downstream signaling events (Fig. 1-7).
One of the most thoroughly studied PI3K effectors is the protein Ser/Thr kinase Akt/PKB (also called RAC [related to A and C kinases]), which translocates to the plasma membrane by binding PtdIns(3,4)P$_2$ or PtdIns(3,4,5)P$_3$. Full Akt activation requires both specific threonine (Thr$^{308}$) phosphorylation by PDK1 and serine phosphorylation (Ser$^{473}$) by PDK2. Activated Akt is then able to participate in diverse signaling pathways including those involved in regulation of cell survival, insulin signaling, cell cycle control, activation of endothelial nitric oxide synthase and others.

It has been shown that the activity of PDK1 is also specifically controlled through the binding of PtdIns(3,4,5)P$_3$ or PtdIns(3,4)P$_2$ to its PH domain. Furthermore, PDK1 has recently been shown to phosphorylate and activate protein kinases C (PKC) and δ directly. The exact identity of PDK2 is not clear, and at least ten kinases have been proposed to serve this function. One of the candidates with strong evidence is mTOR when it is complexed with GβL and rapamycin-insensitive companion of mTOR (RICTOR) (Fig. 1-6). The mTOR/GβL/RICTOR complex is not sensitive to rapamycin like the mTOR/GβL/RAPTOR complex, and can phosphorylate the critical Ser$^{473}$ on Akt, instead of Thr$^{389}$ on S6K1. Thus through an interaction with either RAPTOR or RICTOR, mTOR switches its specificity towards S6K1 or Akt, respectively.

Phox homology (PX) domains are distinct from PH domains, but are also able to bind various PI. PX domains are approximately 120 AA in length and were initially identified through a database search for proteins homologous to the C-terminal region of the class II PI3K C2γ. Examples of PX domain containing proteins with 3'-PI specific
binding include the p40\textsubscript{phox} subunit of NADPH oxidase and Vam7 which selectively bind PtdIns(3)P, and the p47\textsubscript{phox} subunit of NADPH oxidase that selectively binds PtdIns(3,4)P\textsubscript{2} \textsuperscript{160,162}. The PX domain containing class II PI3K binds to PtdIns(4,5)P\textsubscript{2}, but not its 3'-PI product \textsuperscript{162}.

FYVE domains selectively bind PtdIns(3)P, and are named after the first four proteins found to contain this motif (Fab1p, YOTB, Vac1p, and Early endosomal antigen-1) \textsuperscript{48}. The majority of FYVE domain-containing proteins identified to date are involved in membrane trafficking (e.g. EEA1 mediates early endosome fusion) (Fig. 1-6) \textsuperscript{47}. Some FYVE domain-containing proteins are involved in signaling, such as SARA (Smad anchor for receptor activation), which can recruit smad transcription factors to the TGFβ receptor \textsuperscript{163}.
Figure 1-7. PI3K signal transduction pathways showing interaction of various 3'-PI with PH or FYVE domain-containing proteins. The exact type of 3'-PI preferred by the PH domain-containing protein is not shown. Examples of GEFs for Rac GTPases include Vav and Tiam1, and ARF GTPases include ARNO, cytohesin-1, and GRP1. GAP proteins include GAP1m and GAP1IP4BP, which are GAPs for Ras GTPase. Abbreviations: ARNO (ARF nucleotide binding site opener), GRP1 (general receptor for phosphoinositides), Hrs (hepatocyte growth factor regulated tyrosine kinase substrate), FGD1 (faciogenital dysplasia). Adapted from Ref. 48,153,164.
1.2.4 Negative regulation of PI3K products: Phosphatases

The products of class I PI3K are metabolized by two important phosphatases. The tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) and SH2-containing inositol-5'-phosphatase-1 (SHIP-1) are important in regulating the levels of PtdIns(3,4,5)P$_3$ in immune cells. PTEN removes the 3-phosphate of PtdIns(3,4,5)P$_3$ to generate PtdIns(4,5)P$_2$, while SHIP-1 removes the 5-phosphate to generate PtdIns(3,4)P$_2$ (Fig. 1-3B). Knockouts of either of these phosphatases have been created in mice. Mice deficient in SHIP-1 have shorter life spans, and mortality was associated with extensive consolidation of the lungs resulting from infiltration by myeloid cells. Recent reports of SHIP-1 mutations in acute human leukemias suggests that SHIP has a potential to act as a tumor suppressor by negatively regulating the PI3K/Akt pathway. SHIP-2 is an isoform that differs from SHIP-1 in its tissue distribution. SHIP-1 is predominantly expressed in hematopoietic cells, while SHIP-2 is broadly expressed and participates in growth factor stimulated signaling downstream of receptor tyrosine kinases. SHIP-2 has been reported to be important in insulin signaling and diabetes. Knockout of SHIP-2 resulted in increased sensitivity to insulin, which was characterized by severe neonatal hypoglycemia, dysregulated expression of the genes involved in gluconeogenesis, and perinatal death. However, SHIP-2 has also been shown to play a role in myeloid cells. SHIP-2 is expressed in human alveolar macrophages, and is inducible by LPS in human peripheral blood monocytes. Studies in transfected cell lines suggest that SHIP-2 down regulates NFκB-dependent gene expression and Akt activation in response to Fcγ receptor IIa (FcγRIIa) clustering.
While PTEN\textsuperscript{+/−} was found to be embryonically lethal \textsuperscript{170}, PTEN\textsuperscript{+/−} mice are viable but have a propensity to form tumors, indicating a balance between PI3K and PTEN is crucial for regulating cell growth \textit{in vivo} \textsuperscript{171}. Cells and animals deficient in either PTEN or SHIP have revealed important roles for PI3K in a variety of functions. These models are limited, however, in that they cannot be used to directly address which class I isoform is responsible for a particular phenotype since the products of all 4 isoforms of class I PI3K are subject to regulation by PTEN and SHIP.

Other 3'-phosphatases include the FYVE domain containing myotubularin-like phosphatase \textsuperscript{172}, and the yeast Sac1p phosphatase \textsuperscript{173}. Both of these have been reported to be specific for PtdIns(3)P [reviewed in Ref. \textsuperscript{47}]. The precise roles of these phosphatases in regulating PI3K pathways remain to be defined.

\textbf{1.2.5 Multiple isoforms of PI3K confer diversity in regulation of cellular processes}

A considerable amount of research has led to the conclusion that flexibility and diversity of cellular control is differentially mediated by distinct PI3K isoforms \textsuperscript{47,50}. Gene targeting of various PI3K isoforms in mice has identified non-overlapping functions \textsuperscript{60,63,67,81,167,174}. All four class I PI3K catalytic subunits and the regulatory subunits have been genetically manipulated in mice (Table 1-4). Overall, an interesting disparity was observed with regards to the viability of the resulting embryos. Knockouts of either p110α or p110β resulted in embryonic death \textsuperscript{114,115}, while that of p110δ or p110γ did not \textsuperscript{92,175}. These distinctions were likely due to the ubiquitous expression of p110α and p110β, while p110δ and p110γ are more restricted to hematopoietic cells \textsuperscript{67}. Instead of
gene knockouts, mutant p110δ mice have also been created and are useful alternatives. Thus far, however, no phenotypes in monocytic cells from either knockout or mutant p110δ animals have been reported. Macrophages from class IB PI3K p110γ knockout mice, on the other hand, displayed impaired chemotaxis but normal phagocytosis.

Knocking out the regulatory subunit p85 has not turned out to be particularly informative in developing an understanding the roles of distinct class IA p110 isoforms. For example, in cells from p85α or p85β knockout mice, expression of p110 isoforms α, β, and δ were either reduced or normal depending on the cells studied. Furthermore, although deficient in p85, these mice did not appear to be bona fide PI3K knockouts since rather than showing loss-of-function, PI3K signaling in some pathways was apparently elevated. For example, p85α knockout mice were paradoxically found to be hypoglycemic and exhibited increased insulin sensitivity. These results were surprising since class IA PI3K is known to be essential for mediating the effects of insulin on glucose homeostasis. In p85β knockout mice, similar hypoinsulinemia, hypoglycemia, and improved insulin sensitivity were also observed. Moreover, insulin-induced activation of PKB/Akt, a downstream effector of PI3K, was upregulated in p85β knockout mice when compared to wild-type counterparts.

In the case of p85α knockout mice, it was shown that alternative splice forms of p85α (p55α and p50α), were expressed at higher levels than in wild type controls and this may have accounted for increased insulin-induced generation of PtdIns(3,4,5)P3. However,
mice that were deleted for p85α and all of its splice variants (pan-85α) still displayed enhanced insulin action similar to mice in which only p85α alone had been deleted (Table 1-4) \textsuperscript{110}. The paradoxical phenotype of increased insulin sensitivity and hypoglycemia in p85 KO may be explained by the complex interaction between p85 regulatory subunits and p110 catalytic subunits \textsuperscript{83,105,167}. For example, since p85 normally inhibits the kinase activity of its associated p110 subunit in the basal state, removing p85 could result in hyperactive p110 subunits \textsuperscript{112,174}. Furthermore, p85 has been shown to interfere with Ras activation of p110 \textsuperscript{83}. Once again, removal of the influence of p85 has the potential to make p110 monomers more sensitive to activation by Ras \textsuperscript{174}. Taken together, these findings indicate that studies targeting the class I regulatory subunits have substantial limitations when the aim is to investigate the specific contributions of p110 catalytic isoforms to cellular control.
<table>
<thead>
<tr>
<th>Subunit disrupted</th>
<th>KO Viability</th>
<th>Immunological phenotypes</th>
<th>Non-immune phenotypes</th>
<th>Alteration in PI3K subunit expression</th>
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<tr>
<td>p85α, p56κ, p50α (still express p85α)</td>
<td>Viable</td>
<td>Impaired B cell development and Xid-like phenotype</td>
<td>Increased insulin sensitivity, hypoglycemia</td>
<td>T p85α and p56κ in muscle and fat cells, p110α and p110β not affected in insulin sensitive cells. B cells: 4p110δ, p55α and p50α unaffected T cells: T p55α and p50α. Mast cells: p55α and p50α unaffected, 4p110α. Dendritic cells: p55α and p50α unaffected, 4p110α. Pancreatic β cells: p55α and p50α unaffected, 4p110α, p110α and p110δ</td>
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<td></td>
<td></td>
<td>92,177,180, 160-166</td>
</tr>
<tr>
<td>p110l</td>
<td>Viable</td>
<td></td>
<td></td>
<td></td>
<td>92,177,180, 160-166</td>
</tr>
</tbody>
</table>
1.3 RESEARCH OBJECTIVES

Mononuclear phagocytes are important regulators and effectors of both the innate and acquired immune responses and they are also prominently involved in inflammation. Consequently, regulation of monocyte function is an intense area of research interest and recent studies have highlighted an important role for phosphoinositides in monocyte cell regulation. The 3'-PI metabolites produced by PI3K family members are known to be involved in regulating a multitude of cellular events such as mitogenic responses, differentiation, apoptosis, cytoskeletal organization, membrane traffic along the exocytic and endocytic pathways, and aspects that are more specific to monocyte function such as phagocytosis, adherence, the phagocyte oxidase, and cytokine secretion.

A central question posed at the outset of this thesis was to determine how specificity is achieved in PI3K signaling for these diverse biological functions in human monocytic cells. An important goal was to determine when these functions are dependent on isoform-mediated specificity or when the isoforms have redundant functions. It was anticipated that the results of these investigations would help toward understanding the complex and diverse signaling pathways mediated by PI3K in monocytes, and possibly provide new ideas toward novel therapeutic approaches for inflammatory and immunological disorders.

The first objective of this thesis was to address the development of a new approach to stable gene silencing in human monocytic cell lines using lentiviral-delivered small interfering RNA (siRNA). The second objective was to identify the role of the p110α isoform in mediating monocyte adherence and phagocytosis. The final objective dealt
with examining the role of p110α in regulating LPS-induced cytokine production and the monocyte oxidative burst.

1.3.1 Objective 1: To selectively downregulate a distinct PI3K isoform using lentiviral-delivered siRNA.

**Rationale**

Despite recent progress in understanding the functions of PI3K family members in various cell types, study of distinct functions of individual PI3K isoform in monocytes has been difficult due to their resistance to genetic manipulation. The traditional PI3K inhibitors that are available including LY294002, wortmannin, and 3-methyladenine (3-MA), are not useful in assigning function to specific isoforms because at effective concentrations they inhibit virtually all classes of the PI3K family except for class II PI3K C2α. One way to obviate this problem has been to rescue PI3K inhibitor-induced phenotypes by delivering either class I PI3K or class III products such as PtdIns(3,4,5)P3 and PtdIns(3)P respectively, by lipid carriers into cells. However, this approach also cannot provide insight into the roles of distinct class I PI3K isoforms.

Another non-genetic approach to study the function of specific PI3K enzymes has been microinjection of inhibitory antibodies. This has proven to be useful for examining the roles of PI3K isoforms when combined with imaging studies of single cells, such as the murine macrophage cell line J774. However, not every type of cell can be subjected to this technique and due to the limited number of cells that can be studied, biochemical characterization is virtually impossible. In addition, this method is transient.
in nature, and cannot generate a stable cell population for phenotype analysis for a prolonged period of time.

Genetic approaches to assigning function to individual class I PI3K p110 isoforms have been limited as well since gene knockouts of p110α or p110β in mice were found to be embryonically lethal \(^{114,115}\). Consequently, it has not been possible to determine with precision the roles of these isoforms in immune cells. Due to the close and complex relationships between class IA regulatory and catalytic subunits \(^{83,105,167}\), an ideal strategy in studying the functions of specific PI3Ks would be to reduce the expression of individual isoforms without disturbing the molecular balance of the regulatory and catalytic subunits. This has not always been achievable. For example, p110α embryonic knockout cells had increased p85α expression \(^{114}\), and the massive accumulation of p85α monomers observed was suggested to exert a dominant negative effect on the remaining class IA p110 isoforms by binding non-productively to receptors \(^{105,111}\). Conversely, in cells from p85α or p85β knockout mice, expression of p110 isoforms α, β, and δ was either reduced or normal depending on the cells studied \(^{180-183}\).

A genetic approach that has had success in monocytic cell lines is viral-mediated transduction. Lentiviruses in particular, have been shown to transduce at >90% efficiency \(^{196-198}\). RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into target cells \(^{199}\). By using a lentiviral vector, we attempted to generate monocytic cell lines that stably expressed siRNAs targeting the p110α isoform of PI3K. This approach
allowed us to utilize an efficient gene delivery method coupled with the ability to stably silence a specific PI3K isoform.

1.3.2 Objective 2: Examining the role of p110α isoform in monocyte adherence, FcγR- and CR3-mediated phagocytosis.

Rationale

1α, 25-dihydroxycholecalciferol (D₃) is a biologically active form of vitamin D and plays an important role in numerous cellular and physiological processes such as calcium homeostasis and regulates cells of the hematopoietic system [reviewed in Ref. 200-202]. For example, D₃ induces maturation markers such as CD11b and CD14 in monocytic cell lines such as THP-1, U-937 and HL-60 187,203-206. In previous work from this laboratory, D₃ was observed to activate PI3K, and PI3K activity was shown to be required for the induction of CD11b and CD14 expression by D₃ 187. D₃ also induced adherence in cells of the human promonocytic cell line THP-1 203,207,208, although it is not known whether this involved PI3K. Bacterial lipopolysaccharide (LPS) is also known to enhance adherence of leukocytes in vitro 209,210 and it also activates PI3K in monocytic cells 188,211. We and others have previously shown that LPS induced adherence in THP-1 cells 192,212-214 and that this was PI3K dependent 192. However, the roles of individual PI3K isoforms in this phenotype have not yet been defined. Using THP-1 cells deficient in p110α, in this thesis we investigated whether p110α regulates D₃ or LPS-induced adherence or both, and whether CD11b upregulation by D₃ is dependent on p110α.
Phagocytosis plays a pivotal role in host defense against infection. Several lines of strong evidence have indicated a role for PI3K, particularly class IA, in mediating phagocytosis in macrophages. Pharmacologic inactivation of PI3K using either wortmannin or LY294002, blocked particle internalization during FcγR- or complement receptor 3 (CR3)-mediated phagocytosis. Since wortmannin and LY294002 inhibit all isoforms of PI3Ks with the exception of class II PI3K C2α, it was not possible based upon these studies to specifically assign this function to one particular enzyme class. Another question is whether regulation of FcγR-mediated phagocytosis by PI3K is receptor-specific, or whether phagocytosis mediated by CR3 is similarly regulated. Several observations favor the involvement of class IA PI3K in regulating FcγR-mediated phagocytosis. Non-phagocytic cells transfected with PI3K p85-FcγRIα chimeric receptor were rendered phagocytic, further supporting a role of class IA PI3K in regulating FcγRI-mediated phagocytosis. Furthermore, phagocytosis via FcγR was associated with increased activity of the class IA enzyme and with increased levels of the class I product PtdIns(3,4,5)P3 in nascent phagosomal cups. More recent evidence supporting a role for p110β in mediating phagocytosis in murine macrophages was provided using microinjection of inhibitory antibodies. The specific roles of different class I PI3K isoforms in regulating phagocytosis in human monocytic cells are uncertain. While indirect evidence suggests that PI3K may also regulate CR3-mediated phagocytosis, this remains to be studied directly while at the same time examining the specific isoforms involved.
1.3.3 Objective 3: Role of p110α isoform in LPS-induced cytokine secretion and in oxidative burst.

Rationale

Prior research from this laboratory and others demonstrated that the bacterial lipopolysaccharide (LPS), activated PI3K. Otherwise known as endotoxin, LPS is recognized to be a potent agonist leading to cytokine production by monocytes, including TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-8 and many other chemokines [reviewed in Ref. 223]. Several reports in the literature demonstrated a role for PI3K in regulating LPS-induced cytokine production 191, 224-228. For example, pre-treatment of human monocytic cells with PI3K inhibitors prior to LPS suggested that PI3K negatively regulates TNF-α production 191. Bone marrow derived dendritic cells from p85α knockout mice were found to have enhanced IL-12 secretion in response to LPS, here again suggesting that class IA PI3K limits proinflammatory cytokine production downstream of TLR4 228. These findings, however, are based upon global inhibition of nearly all PI3K isoforms and may also be limited by non-specific effects of inhibitors and knockouts. In fact, knowledge about the roles of individual PI3K isoform in regulating LPS-induced cytokine production in human monocytes is not available thus far. Here, we investigated the role of PI3K p110α isoform in regulating monocyte cytokine production in response to LPS.

One important component of the host innate immune response to infection is the phagocyte oxidative burst. This results in the generation of reactive oxygen intermediates (ROI) and microbicidal activity 229. ROI in phagocytes include OCl-, OH,
and H$_2$O$_2$, which are derived from the superoxide (O$_2^-$) generated by phagocyte NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase $^{229}$. Mutations in the genes encoding the NADPH oxidase subunits in humans results in chronic granulomatous disease (CGD), which is characterized by the absence or very low levels of superoxide production in phagocytes and susceptibility to recurrent bacterial and fungal infections $^{230}$. Pharmacological inhibition or genetic studies have demonstrated that PI3K is required for the activation of the neutrophil NADPH oxidase when stimulated by fMLP, C5a, IL-8, or opsonized zymosan $^{175,178,179,231-234}$. When PI3K p110$\gamma^{-/-}$ neutrophils were incubated with chemoattractant they did not produce PtdIns(3,4,5)P$_3$, did not activate Akt, and displayed both impaired oxidative burst activity and chemotaxis $^{175}$. On the other hand, superoxide production in response to the particulate agonist opsonized zymosan was intact in p110$\gamma$ KO neutrophils. Notably, the opsonized zymosan-induced oxidative burst in p110$\gamma$ KO neutrophils was sensitive to PI3K inhibitors $^{175,231}$. This finding, along with increased PI3K activity in phosphotyrosine immunoprecipitates from zymosan stimulated monocytic cells, suggested that a class IA PI3K is required for particle induced oxidative burst responses $^{235,236}$. Using monocytic cells deficient in p110$\alpha$, we examined directly whether the oxidative burst responses to either opsonized zymosan or the soluble agonist phorbol 12-myristate 13-acetate are dependent upon this class IA PI3K isoform.
CHAPTER II: MATERIAL AND METHODS

2.1 Reagents, chemicals, and cell lines

Reagents—RPMI 1640, DMEM, Hanks' balanced salt solution (HBSS), penicillin/streptomycin and 1 M HEPES solution were from Stem Cell Technologies (Vancouver, BC). PMSF, anti-BSA antibody (B-7276), human IgM (I-8260), LPS from Escherichia coli O111:B4 (L-3012), Polybrene, poly-L-lysine, wortmannin, cytochrome c from horse heart (C-7752), superoxide dismutase (S-8160), and phorbol 12-myristate 13-acetate (P-1585) were obtained from Sigma-Aldrich (Oakville, ON). LY294002, 1α, 25-dihydroxycholecalciferol, and antibodies to human p110δ were from Calbiochem (San Diego, CA). Antibody to human p110α (clone 19) was from BD Biosciences (Mississauga, ON). Antibodies to human p110β and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-p85-N-SH3 antibody was from Upstate Biotechnology (Lake Placid, NY). RPE conjugated anti-CD11b antibody and RPE conjugated isotype-matched control antibodies were from Caltag Laboratories (San Francisco, CA). Anti-phospho-p38 (Thr180/Tyr182), phospho-Erk (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-NFκB p65 (Ser536), and phospho-Akt (Thr308) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH antibody was from Research Diagnostics (Concord, MA). HRPO conjugated anti-Rabbit, anti-mouse, and anti-goat secondary antibodies were from Cedarlane Laboratories (Hornby, ON). Zymosan A and bovine serum albumin (BSA) were from MP Biomedicals, LLC (Irvine, CA). Protein G Sepharose was from Amersham Biosciences (Piscataway, NJ).
Cell lines—The promonocytic cell lines THP-1 and U-937 (ATCC, Rockville, MD) were cultured in RPMI 1640 supplemented with 10% FBS (Life Technologies, Burlington, ON), 2mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cultures were maintained without exceeding 0.5 x 10^6 cells/ml. 293T human embryonic kidney (HEK) cells, were also from ATCC and were cultured in DMEM, supplemented with 10% FBS, 2mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml), and 20mM HEPES.

2.2 Monocyte transfection and electroporation with oligonucleotides and siRNA

Sense and antisense phosphorothioate-modified oligonucleotides, siRNA duplex—Phosphorothioate-modified oligonucleotides were prepared and incorporated into cells as described previously 237. Briefly, phosphorothioate-modified oligonucleotides to the α-isoform of the p110 subunit synthesized by Life Technologies, Inc. Oligonucleotides were phosphorothioate-modified to prevent intracellular degradation and purified by HPLC to remove incomplete synthesis products. 21-mers were produced to the human α isoform of the p110α subunit of PI3K, including the presumed translation initiation site in both sense and antisense directions with the following sequences: sense (5'-ATG CCT CCA AGA CCA TCA TCA-3') and antisense (5'-TGA TGATGG TCT TGG AGG CAT-3'). THP-1 cells (5 x 10^6) were resuspended in 500 µl of Opti-MEM I Reduced Serum Medium containing 2.5% Oligofectamine (Life Technologies) and 5 µM phosphorothioate-modified oligonucleotides and incubated on a rotary shaker for 4 h at 37 °C prior to adherence and differentiation. The 21mer siRNA targeting p110α (5'-AAU GCC UCC GUG AGG CUA CAU- 3') with dTdT 3'-overhangs and the 5'-
Electroporation of siRNA—Cultured THP-1 cells were first washed twice with RPMI at room temperature, and each 0.4 cm cuvette (BioRad) contained 5 x 10^6 cells/400 µl of RPMI. Control siRNA labeled with FITC (Dharmacon, Chicago, IL) was added to a final concentration of 100 nM. All electroporations were done in a BioRad electroporation device, set at 975µF, with the voltage varying from 200 to 500V. Cells were chilled on ice for 10 minutes prior to electroporation. After electroporation, cells were transferred to wells containing 5 ml of complete culture medium, and cultured overnight.

2.3 Lentiviral preparation and transduction of monocytic cells

Constructs—The U6 promoter vector pSHAG-1 and pSHAG-Ffl were kind gifts from Dr. G.J. Hannon (Cold Spring Harbour Laboratory, Cold Spring Harbour, NY). pSHAG-1 contains the attL1/L2 transposition elements that are compatible with Gateway Cloning Technology (Invitrogen Canada Inc., Burlington, ON). Antisense to p110α mRNA (GenBank NM_006218) was targeted to two nucleotide segments: 5'-ATATACATTTCCTGATCTTCCTCGTGCTG-3' (nucleotide positions 1171 to 1198, referred to as α3), and 5'-CAAGACCATCATCAGGTGAACTGTGGGG-3' (nucleotide positions 8 to 35, referred to as α1). The hairpin containing sequence was created as described 238. Oligonucleotides p110α1 and p110α3 listed in Table I were synthesized by Qiagen Inc. (Valencia, CA). All of the sequences contained a HindIII site in the
hairpin region, a site that is not present in the native pSHAG-1 vector, and BamHI and BseRI ends to enable directional cloning. The oligonucleotides were annealed and then ligated into pSHAG-1 via the BamHI/BseRI site. DH5α E. coli (Invitrogen) were transformed and clones were screened by HindIII digestion. pSHAG-Ffl contains a U6 driven sequence that generates an hairpin RNA that targets GL3 firefly luciferase at nucleotide positions 1619 to 1647 (GenBank U47296). Construction of the lentiviral transducing plasmid, pHR-CMV-EGFP, packaging vector pCMVΔR8.2, and VSV envelope vector pMD.G have been described elsewhere. Purified pSHAG-1, pSHAG-p110α1, and pSHAG-p110α3 served as entry clones. The lentiviral transducing vector pHR-CMV-EGFP was modified by inserting the Gateway vector conversion cassette (Invitrogen) in the Clal site, that is located downstream of 5’LTR, but upstream of the CMV promoter. The resulting pHR-Gateway served as a destination vector since it contained attR1/2 sites. The various entry clones were transposed to the pHR-Gateway by Gateway LR Clonase Enzyme Mix (Invitrogen). Positive clones were then isolated and the plasmids (pHR-U6, pHR-p110α1, pHR-p110α3) purified. All plasmid purifications were carried out using Qiagen Endofree Plasmid kits.
Table 2-1

<table>
<thead>
<tr>
<th>Construct</th>
<th>5'-Antisense sequence (28nt)</th>
<th>harpin loop (8 nt)</th>
<th>Sense sequence (28nt)</th>
<th>Termination (6 T's)-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110α1</td>
<td>CCCACA GTTACCTGATGTGTCTTGGAAGCTGGCGAGACCGTACGTTGGGCTGGTGGGCATTTTTTTT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATGAAAAAATGCCACAGCTGACCCGATGACGCTTCGCA4GGCTCCAAAGACCACATCAGGGTGAAGCTGGGGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p110α3</td>
<td>CAGCACGAAAGAAGAATTATATGATATGATAACACGTTCTCCTGCCGATCCTGGCTGTTGCTCTTTTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATCAAAAAGAGCAACACGGAAGAGACCAATGATGATGATAACACGTTCTCCTGCCGATCCTGGCTGTTGCTCTTTTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1. Short Hairpin RNA encoding sequences targeting human p110α mRNA.
Hairpin sequences with the *HindIII* site are underlined and italicized. For each construct, the two strands of DNA were annealed and ligated into the pSHAG-1 vector. The underlined sequences at the 5' and 3' ends are for directional cloning into pSHAG-1, which was cut with *BseRI* and *BamHI*.
Lentivirus packaging—The packaging cell line 293T HEK (5 x 10^6) was plated on poly-L-Lysine coated 100 mm tissue culture plates (Corning) and transfected the following day. Ten μg of the transducing vector pHr (pHR-U6, pHr-p110α1, or pHr-p110α3), 7.5 μg of the packaging vector pCMVΔR8.2, and 2.5 μg of the VSV envelope pMD.G were co-transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The media were changed the next day, and cells were cultured for another 24 hr. Conditioned media were then collected and cleared of debris by low speed centrifugation (2,500g for 5 min) filtered through a 0.45 μm filter and stored at -70°C. This collection was repeated daily for three more days, and media from the 4 days were pooled and ultracentrifuged at 100,000 x g, at 4°C for 2 hr. Pellets were resuspended in 500 μl of medium (overnight on a nutator at 4°C), and aliquots were stored at -70°C. Viral stocks were assayed for the p24 core antigen using the Vironostika HIV-1 Antigen ELISA kit (bioMérieux, Inc., Durham, NC) according to the manufacturer’s instructions.

Titration of lentiviral vectors—1 x 10^5 293T cells were plated in each well of a six-well plate. On the following day, cells from three wells were removed with cell dissociation solution (Sigma), and counted in order to determine the average number of cells at the time of titration. Three dilutions (1/50,000, 1/5,000, and 1/500) of the concentrated viral stocks were used to transduce the cells. Transduction of 293T cells was done in the presence of transduction adjuvant Polybrene (8 μg/mL). The media were changed 24 hr after transduction. Cells were removed 48 hr post-transduction and analyzed by flow cytometry for GFP expression. The calculation used to determine the titer was transduction units/ml = average cell number at the time of transduction x % of GFP positive cells/100 x dilution factor.
Transduction of target cells—$1 \times 10^5$ THP-1 or U-937 cells were seeded in each well of a 12-well plate in 500 µl of complete media and transduced by lentiviral vectors at a multiplicity of infection of 10:1. Transduction was carried out in the presence of Polybrene (8 µg/mL). Transduced cells were analyzed by flow cytometry after six days. For transduction of 293T HEK cells, $1 \times 10^5$ cells were seeded in each well of a 12-well plate and transduced the next day. Cells were verified for p110α deficiency and viral transduction by western blot and flow cytometry analysis, respectively, prior to use in each experiment.

2.4 Western blot analysis

Cells were washed once with PBS and lysed in boiling lysis buffer (1% SDS, 50 mM Tris pH7.4, 0.15 M NaCl, 1mM NaF, 10 mM PMSF, 1 mM sodium ortho-vanadate, 1 mM EDTA) for 5 min and passed through a 27 gauge needle. Lysates were cleared by centrifugation at 12,000 x g for 1 min and protein concentration was determined using Bio-Rad DC Protein Assay. Equal amounts of protein were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membranes. Membranes were blocked with 5% skim milk or 3% BSA in TBST for 1 hr at room temperature depending on the antibody. Primary and secondary antibodies were used according to manufacturer’s instructions, followed by detection with enhanced chemiluminescence (ECL) technique (Amersham Biosciences, Piscataway, NJ).

2.5 Adherence assays

Adherence assay were performed as reported previously with some modifications. Briefly, 96-well flat bottom culture plates were filled with $5 \times 10^4$ THP-1 cells in 200 µl culture medium.
Cells were either treated or not with 25 μM LY294002 for 30 min at 37°C, followed by LPS (12.5-500 ng/ml), D₃ (100 nM), or PMA (20 ng/mL). All treatments were done in triplicate. LPS was opsonized with 50% normal human serum for 30 min at 37°C prior to use. Plates were incubated for 24 hr (for LPS treatment) or 48 hr (D₃ treatment) at 37°C, 5% CO₂. Non-adherent cells were removed by three washes with 200 μl of warm culture medium (37°C). The remaining cells were then fixed with 2% paraformaldehyde/PBS, for 15 min at 37°C. Fixed cells were then washed once and stained with 0.05% crystal violet in 20% methanol for 10 min at room temperature. Dye was removed and the wells were rinsed with water three times. The plates were allowed to dry at room temperature. Cell associated dye was eluted in 100 μl per well of 100% methanol and absorbance was measured at 570 nm in a microtiter plate reader (BioRad Laboratories, Hercules, CA). Absorbance of adherent cells was normalized to PMA treated cells.

2.6 Dual luciferase assays

Each well of a 12-well culture plate was seeded with 2 x 10⁵ 293T HEK cells and transfected the following day with 0.05 μg pGL3-SV40 and 0.02 μg pRL-TK reporter plasmids (Promega Corp., Madison, WI) using Lipofectamine 2000 (Invitrogen). Cells were lysed 48 hr later and assayed for luciferase and renilla activity using Dual Luciferase Assay reagent (Promega). Activities are reported as ratios of *Photinus pyralis* GL3 luciferase (Pp-luc) to *Renilla reniformis* RL luciferase (Rr-luc).

2.7 Flow cytometry analysis
For staining of surface receptors, 1 x 10^6 cells were washed once with binding buffer (HBSS, 1% FCS, and 0.1% NaN_3), and then stained with anti-CD11b, FcγRI, FcγRII, or FcγRIII-RPE conjugated antibody, or isotype-matched control-RPE conjugated antibody for 20 min at room temperature. Cells were then washed once with binding buffer and resuspended in 1 ml of binding buffer containing 1.85% paraformaldehyde. Ten thousand cells were analyzed using a Becton Dickinson FACSCalibur flow cytometer. Data were acquired using BD CellQuest software and analyzed using Summit V3.1 software (Cytomation Inc., Fort Collins, CO).

2.8 Cytokine Measurement

THP-1 cells at 1 x 10^6 /ml were stimulated by serum opsonized LPS at 100 ng/ml for the indicated times at 37 °C, 5% CO_2. Cultures were subsequently centrifuged at 400 x g for 10 min at 4°C and cell free supernatants were collected and stored at -70°C until use. TNF-α, IL-6, IL-12p40 (BD Biosciences Pharmingen), and IL-10 (eBioscience) in culture supernatants were measured using sandwich enzyme-linked immunosorbent assay (ELISA) using paired cytokine-specific monoclonal Abs according to the manufacturer's instructions.

2.9 RT-PCR

Total RNA was prepared using RNeasy kit (Qiagen, Valencia, CA), and RT-PCR was performed using the Superscript First-strand Synthesis System for first strand cDNA synthesis (Life Technologies, Grand Island, NY). Taq DNA polymerase was from Fermentas (Burlington, ON). The primer sequences for the various cytokines are listed in Table 2-2. An initial denaturation for 5 min at 94°C was followed by a cycle of 94°C for 1 min, the desired annealing temperature (see Table 2-2) for 50 seconds, and 74°C for 1 min for extension, with the number
of cycles depending on the primer used. The end of the cycle was followed by 10 min at 72°C. PCR products were run on 1% agarose gel in TAE buffer, visualized with ethidium bromide.

### Table 2-2. Primer sequences and conditions for RT-PCR.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequences (forward/reverse)</th>
<th>Number of cycles</th>
<th>Annealing Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TCTCGAACCCCGAGTGACA / GGCCCGGCCTTCA</td>
<td>35</td>
<td>55°C</td>
</tr>
<tr>
<td>IL-6</td>
<td>ATGAACTCCTTCTCCACAAGCGC/ GAAGAAGCCCTCAGGCTGACTG</td>
<td>35</td>
<td>67°C</td>
</tr>
<tr>
<td>IL-10</td>
<td>ACGGCCGTGTACGATTT/ TTGGGAGCTTATAAAGGCATTCTTC</td>
<td>37</td>
<td>56°C</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>GGATGCCGTTCCAACAG/ CCCATCGCTCCAAAGA</td>
<td>30</td>
<td>60°C</td>
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<tr>
<td>Actin</td>
<td>TGACGGGGCTCACCACACTGTGGCCCATCTA/ CTAGAAGCATCGCGTGGACGATGGAGGG</td>
<td>31</td>
<td>55°C</td>
</tr>
</tbody>
</table>

2.10 Phagocytosis assay

U-937 cells were incubated in 6-well plates at 1 x10⁶/ml, and differentiated with PMA (10 ng/mL) for 48h. Fluorescent Nile Red CML 6 µM polystyrene latex beads (Interfacial Dynamics Corp., Oregon) were opsonized as follows: For IgG opsonization, rinsed beads were incubated with BSA, followed by anti-BSA IgG. For complement opsonization, human serum was first treated with protein G Sepharose for 1h at 4°C with rotation and centrifuged prior to use. The beads were coated with human IgM followed by incubation with protein G-treated and centrifuged serum for 30 min at 37°C. Complement coating was verified by anti-iC3b monoclonal antibody (Quidel Corporation, Mountain View, CA). Prior to adding the particles, the medium was replaced with fresh culture medium and cells were chilled at 4°C for 30 min. After a further 30 min at 4°C, particles were added and phagocytosis was allowed to proceed at 37°C, for 1h. Plates were then transferred on ice and non-ingested beads were removed by
washing three times with ice cold PBS. Cells were then washed once with binding buffer and resuspended in 1 ml of binding buffer containing 1.85% paraformaldehyde. Ten thousand cells were analyzed using a Becton Dickinson FACSCalibur flow cytometer. Data were acquired using BD CellQuest software and analyzed using Summit V3.1 software (Cytomation Inc., Fort Collins, CO).

2.11 Superoxide assay
Superoxide assays were performed as described previously by measuring superoxide dismutase inhibitable reduction of ferricytochrome c $^{242,243}$, but with several modifications. Briefly, 0.5 x $10^6$ THP-1 cells were differentiated overnight with 10ng/ml (1.6 nM) of PMA. Prior to stimulation, all cells were washed with HBSS twice, and rested in RPMI for 4h at 37°C. Zymosan particles were opsonized or not with pooled human serum for 30 min at 37°C prior to use. Cells were either untreated or stimulated with either 1 μM PMA or zymosan (20:1) in assay-buffer (5.5 mM glucose, 79 μM cytochrome c), in the presence or absence of 30 μg/ml SOD. Each sample was mixed well, and incubated at 37°C. Absorbance readings at 550 nm were done at 30 min post-stimulation, on a BioRad SmartSpec. PBS was used as blank control.

2.12 Statistical analysis
For comparison of two treatment groups, two-tailed $t$ test was performed. A $p$-value <0.05 was considered significant. For three or more treatment groups, one-way ANOVA was performed on each group and followed by Tukey test for multiple comparisons. A $p$-value <0.05 was considered significant. All statistics and graphs were performed using GraphPad Prism software, version 3.0 (GraphPad Software, San Diego, CA).
CHAPTER III: LENTIVIRAL-MEDIATED DELIVERY OF siRNA INTO HUMAN MONOCYtic CELL LINES

3.1 Introduction

Cells of the mononuclear phagocyte series respond to a wide range of diverse stimuli and show complex cell regulation. From the perspectives of cell biology, understanding disease causation and developing novel therapeutics, there continues to be a great deal of interest in understanding how the responses of these cells are regulated. However, study of monocyte and macrophage biology through genetic manipulation by non-viral transfection methods has been challenging [reviewed in Ref. 245]. Methods involving cationic lipid and liposome-mediated delivery of DNA or physical methods such as electroporation result in low transfection efficiency in monocytic cells, loss of viability, and the difficulty of obtaining stable transfection 246,247. An approach that has met with greater success in monocytic cell lines is viral-mediated transduction. While not all viruses can transduce monocytic cells efficiently, lentiviruses have been shown to do so at >90% efficiency 196-198.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into target cells 199. RNAi is a natural regulatory mechanism that occurs in many organisms including plants, Caenorhabditis elegans, Drosophila, and mammalian cells [reviewed in Ref. 199]. The RNAi pathway begins by processing dsRNA into short (< 30 bp) dsRNA duplexes called small-interfering RNA (siRNA) by a host RNAse Dicer. The siRNA then becomes incorporated into a multi-component nuclease

1 Most of material presented in this chapter is derived from that published in Ref. 244.
complex called the RNA-induced silencing complex (RISC). RISC then uses the siRNA sequence as a guide to recognize cognate mRNAs for degradation.

Delivery of siRNAs into mammalian cells by transfection of siRNA or DNA vectors expressing short hairpin RNA (shRNA) has been shown to mediate RNAi successfully. Transfection of siRNA is transient, lasting only for a week or so, although DNA-based vectors may last longer with drug selection. In contrast, viral vectors have also been used to deliver siRNA successfully, and these methods tend to provide more stable gene silencing. This section describes how human monocytic cell lines can be effectively transduced using a lentiviral vector to stably silence an endogenous lipid kinase. Other methods such as electroporation or cation lipid–mediated delivery of oligonucleotides or siRNA are presented here for comparison.

3.2 Transfection of anti-sense oligonucleotides and siRNAs into monocytic cell lines using cationic lipids and electroporation

Although the delivery of the anti-sense oligonucleotides using Oligofectamine was moderately efficient as assessed using FITC-labeled oligonucleotides, silencing of PI3K p110α isoform was only modest, achieving around 14 to 45% reduction in protein levels on average. Not surprisingly this did not result in a detectable phenotype in either phagocytosis or adherence (data not shown). We also tried to transflect siRNA duplexes to initiate RNA silencing of PI3K isoforms. This did not silence PI3K to any greater extent than had the anti-sense oligonucleotide approach (data not shown).
Electroporation of siRNA into THP-1 cells was also attempted in order to reduce p110α protein levels. Using FITC-labeled siRNA, the efficiency of electroporation was evaluated. Despite evaluating various settings known to be optimal for mammalian cells, the best transfection efficiency achieved was 9.3%, at 400V and 975μF. No silencing of p110α protein was observed using Western blot analysis (data not shown).
Figure 3-1A

Lipofectamine only

FITC-labeled Oligonucleotides

52%

Fluorescence

Figure 3-1B

p110α Sense

p110α Anti-sense

Anti-p110α

Anti-actin
Figure 3-1. Transfection of antisense oligonucleotide into THP-1 cells by Oligofectamine.  

A, Flow cytometry analysis of THP-1 cells transfected with FITC-labeled anti-sense oligonucleotides (shaded histogram) and with Oligofectamine only (clear histogram). 10,000 cells were analyzed, and the FITC fluorescence intensity was measured on the FL1 channel. Approximately 52% of transfected THP-1 cells were FITC positive.  

B, Western blot analysis of class IA PI3K p110α catalytic subunit in THP-1 cells transfected with anti-sense oligonucleotides. Actin was used as protein loading control. All figures shown are representatives of at least three independent experiments.
3.3 Lentiviral-mediated delivery of siRNA

Construction of VSV-pseudotyped lentiviral vector expressing shRNA—In order to deliver siRNA into monocytic cell lines, we developed a lentiviral-based vector that expresses a short-hairpin RNA (shRNA) (Fig. 3-2A and B). Vesicular stomatitis virus-pseudotyped lentiviral vectors have been shown previously to be able to transduce GFP into monocytic cell lines \(^{196}\). The VSV glycoprotein G is a substitute for the lentiviral gp120/gp41 as the viral coat protein since it broadens the range of cell types that can be infected by the virus and also helps to stabilize the virion, yielding higher titers of the virus \(^{240}\). Furthermore, by stabilizing the virion, VSV G protein also allows the viral particles to be concentrated by ultracentrifugation, thereby providing higher titers for transduction \(^{240,254}\). The synthesized sense and anti-sense oligonucleotides encoding the shRNA (Table 2-1) were annealed and then ligated into the BamH1/BseRI site of pSHAG-1 downstream of the RNA Pol III-specific U6 promoter (not shown) \(^{239}\). The U6 promoter and the sequence encoding shRNA were then subcloned into the viral transducing vector (pHR-U6-shRNA) using Gateway cloning. The resulting plasmid was then used for transient transfection of the packaging cell line HEK 293T (Fig. 3-2A). Transfection of separate plasmids encoding viral structural genes (gag-pol gene products and accessory proteins on pCMVΔR8.2, and VSV G glycoprotein on pMD.G) and non-structural sequences (packaging sequence \(\Psi\), Rev Responsive Element, and LTRs on pHR-U6-shRNA), ensures that progeny virus and the target cell will not contain any genes that encode viral proteins \(^{255}\). Recombinant viruses were released into the medium and collected every 24 hr for four days. Viral particles were concentrated by ultracentrifugation. Verification of viral production was done by performing p24 antigen ELISA on concentrated viral stocks. All viral samples gave high p24 levels (>>160 pg/ml). Viral stocks were titered on 293T HEK cells.
which became GFP positive when transduced. We routinely obtained $1 \times 10^8 - 1 \times 10^9$ transducing units/ml.
Figure 3-2A

Transient co-transfection of HEK 293T packaging cell

Transcription of vector RNA

Viral structural proteins

Packaging of vector RNAs, assembly and budding of progeny virus

Harvest conditioned media every 24 hours for 4 days
Figure 3-2. Construction of a lentiviral vector for transduction of shRNA into target cells.

A, To produce the recombinant lentiviral vectors, the packaging cell line HEK 293T was co-transfected by the vector plasmid (pHR-U6-shRNA), helper plasmid (pCMVΔR8.2), and envelope plasmid (pMD.G). The general strategy in the production of lentiviral vector-delivered siRNA is to segregate the trans-acting sequences that encode for viral proteins from the cis-acting sequences (regions recognized by viral proteins) involved in the transfer of vector sequences encoding the shRNA [reviewed in Ref. 255]. The vector plasmid contained a U6 promoter-driven shRNA coding sequence, followed by a CMV-driven enhanced green fluorescent protein (EGFP) reporter. The shRNA nucleotide sequence shown is not specific and is only intended to illustrate a generic shRNA. These elements were flanked by long terminal
repeats (LTR), and also contained *cis*-acting sequences that allowed the vector RNA to be packaged and subsequently, to be reverse transcribed and integrated in the target cell. The packaging sequence (Ψ) was only present in the vector plasmid, and not in the other two plasmids. Following transfection, the plasmids pCMVΔR8.2 and pMD.G were transcribed downstream of CMV promoters, and they provided the viral structural proteins in *trans*. These included viral integrase, protease, reverse transcriptase, capsid and matrix proteins, and vesicular stomatitis virus G protein. Together, these proteins act to *trans*-complement the vector by assembling the progeny viral particles, which are limited to a single round of infection. Vector proteins were produced as well, so transfected cells were GFP positive. Conditioned medium was then harvested, concentrated by ultracentrifugation, and stored at -70°C. *B*, Transduction of target cells was done at a MOI of 10:1. Virions attach at the cell surface via VSV G proteins, fuse with the cell membrane, and release the viral core. Reverse transcription and uncoating of the viral core occurs in the cytoplasm. The dsDNA is then transported into the nucleus where it integrates randomly into the target cell genome. Following integration, the U6 and CMV promoters transcribe their respective genes and this results in shRNA production and mRNA for the GFP reporter. The shRNA and mRNAs are exported to the cytoplasm. GFP is then translated, and the shRNA is processed by Dicer and then incorporated into the RNA induced silencing complex (RISC) [reviewed in Ref. 238]. RISC then targets and degrades cognate mRNAs.
High efficiency transduction of human monocytic cell lines—THP-1 and U-937 cells were either mock-transduced or transduced with HR-U6, HR-p110α1, or HR-p110α3 viral stocks at a multiplicity of infection at 10:1. The viral vector RNAs, after being reverse transcribed into dsDNA in the cytosol entered the nucleus and randomly integrated into the genome, thus generating stable cell lines (Fig. 3-2B). Transduced cells expressed GFP and shRNA (GFP only in HR-U6 vector). Six days post-transduction, cells were analyzed for GFP expression using flow cytometry (Fig. 3-3A). The lentiviral vectors were able to consistently transduce >90% of the target cells.
Figure 3-3

A

THP-1

97.1%

U-937

96.9%

GFP

B

C

Mock

HR-Δ6

HR-p110α1

HR-p110α3

Mock

Anti-p110α

Anti-p110β

Anti-p110δ

Anti-p85 N-SH3

Anti-actin

Anti-p110α

Anti-p110β

Anti-p110δ

Anti-p85 N-SH3

Anti-actin

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Figure 3-3. Transduction of monocytic cell lines by lentiviral vectors is efficient and generates stable cell lines deficient in p110α. A, Flow cytometry analysis of transduced (solid histogram) or mock transduced cells (clear histogram) was performed 6 days after viral transduction. 10,000 cells were analyzed, and the GFP fluorescence intensity was measured on the FL1 channel. Approximately 97% of the THP-1 and U-937 cells were GFP positive. The mean fluorescence intensity (MFI) for mock-infected cells was 8.5 for THP-1 and 6.6 for U-937. Transduced cells had MFIs of 38.3 for THP-1 and 132.3 for U-937. B and C, Western blot analysis of class IA PI3K p110 catalytic subunit isoforms (α, β, and δ), and p85 regulatory subunit in THP-1 cells and U-937 cells. Actin was used as protein loading control.
3.4 Silencing of PI3K p110α isoform in human monocytic cell lines

*Stable and specific silencing of Class IA PI 3-kinase p110α isoform*—shRNA specific to PI3K p110α mRNA was used to induce RNA silencing by a mechanism involving the RISC (Fig. 3-2B). Transduced cells were expanded and examined by Western blotting. Figure 3-3B and C show that transduction of THP-1 and U-937 with the HR-p110α3 viral vectors resulted in nearly complete elimination of PI3K p110α isoform expression. In contrast, transduction of cells with either lentiviral vector HR-p110α1 expressing a second α1 shRNA sequence, U6-promoter control virus, or mock transduction did not affect p110α protein levels. The effect of HR-p110α3 was specific in that levels of other Class IA PI3K catalytic subunits p110β and p110δ, or the p85α regulatory subunits were not affected. The stability of p110α silencing was confirmed by Western blotting of cells that had been in continuous culture for more than six weeks and of cells stored in liquid nitrogen for at least 2 years.

3.5 Transduced HEK 293T cells can be further transfected with reporter plasmids

*Stable and specific silencing of luciferase activity in HEK 293T cells*—To verify that the hairpin construct from HR-p110α3 does not nonspecifically interfere with the transcription of reporter plasmids, we transduced HEK 293T cells with either HR-p110α3 or HR-Ffl, which produces a shRNA targeting GL3 firefly luciferase. HEK 293T cells were chosen because they are much more receptive to transfection than are monocytic cell lines. Western blot analysis demonstrated that the level of p110α in HEK 293T cells transduced with HR-p110α3 viruses was markedly reduced similar to what was observed in similarly treated THP-1 and U-937 cells. As expected transduction of cells with HR-Ffl virus had no effect on p110α expression (data not shown). HR-p110α3 transduced cells or HR-Ffl transduced cells were then co-transfected with
firefly luciferase (pGL3-SV40) and *Renilla* luciferase (pRL-TK) plasmids, the latter serving as an internal control. Forty-eight hours post-transfection, cells were analyzed using dual luciferase assay (Fig. 3-4). Cell transduction with HR-Ffl reduced firefly luciferase expression by more than 80%, while HR-p110α3 had no effect when compared to mock transduced cells. These results indicate that in cells expressing shRNA targeting p110α, non-specific interference with the function of a reporter plasmid is not a problem. The data also show that lentiviral-mediated RNAi is capable of silencing exogenous genes driven by a strong promoter.
Figure 3-4. Silencing of an exogenous gene in HEK 293T cells expressing shRNA. HEK 293T cells were transduced or not with viruses targeting PI3K p110α (HR-p110α3) or firefly luciferase GL3 (HR-Ffl). After seven days, cells were transfected with firefly luciferase pGL3-SV40 and renilla luciferase pRL-TK reporter plasmids. Forty-eight hours after transfection, cells were lysed and analyzed for luciferase activity using Promega's Dual Luciferase Assay. The activities are reported as firefly luciferase (Pp-Luc) /Renilla luciferase (Rr-Luc). HR-Ffl transduced cells gave a Pp-Luc/Rr-Luc ratio < 20% of either mock or HR-p110α3 transduced cells ($p < 0.01$, post-ANOVA Tukey test). Cells transduced with shRNA targeting p110α had similar ratios as non-transduced cells ($p > 0.05$, post-ANOVA Tukey test). One-way ANOVA for all three cells lines $p = 0.0027$. Error bars indicate standard deviation, $n = 3$. 

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3.6 Discussion

A major obstacle in studying monocyte cell biology has been the resistance of these cells to genetic manipulation, particularly when using non-viral methods. In the studies described in this thesis, we report a strategy for stable gene silencing in monocytic cells. Using a VSV-pseudotyped lentiviral vector, monocytic cell lines that stably expressed shRNAs targeting an endogenous gene were generated resulting in silencing of the p110α isoform of PI3K. Historically, lentiviral vectors have been shown to be superior to non-viral methods such as cationic lipid-mediated delivery of DNA vectors to monocytic cells because of their much higher transduction efficiency and longer period of transgene expression. Although the mechanism underlying the resistance of monocytic cells to DNA transfection is not known precisely, it has been proposed that much of the exogenous DNA enters the cell via endocytosis resulting in degradation of the DNA by abundant lysosomal nucleases. VSV-pseudotyped lentiviral vectors obviate this problem, since the viral core containing the genetic elements of interest is delivered directly into the cytosol after the viral envelope fuses with the plasma membrane of the target cell. Another physical approach for transfection that has not been very successful in monocytic cells is electroporation. Poor success here has been related to low viability of cells after electroporation, typically below 10-22% survival.

Amongst the various viral-based approaches, lentiviral-based vectors seem to be the most promising for transduction of monocytic cells. Onco-retroviruses are similar to lentiviruses, but the latter have a more complex genome, and consequently more complex replication cycle [reviewed in Ref. 255]. One advantage of lentiviral vectors over onco-retroviral vectors lies in their ability to transduce both proliferating and non-proliferating cells, such as liver, muscle,
retina, and neurons\textsuperscript{257-259}. This has been attributed to the presence of nuclear localization signal sequences present in lentiviral gene products\textsuperscript{260} which are absent from onco-retroviral vectors. Interestingly, onco-retroviruses transduce monocytic cell lines THP-1, U-937, and HL-60 at lower efficiencies (1-31\%)\textsuperscript{197,198}, even though these are proliferating cells. Furthermore, compared to onco-retroviruses, lentiviral vectors are also much less susceptible to transcriptional silencing of the viral transgene, an event that may result from methylation of foreign DNA in the vicinity of the promoter, as well as by integration of the viral elements into condensed chromatin regions\textsuperscript{245,261}. Taken together, all of the above differences make lentiviruses potentially superior vectors for the delivery of siRNAs into monocytic cells.

Other viruses such as adenoviruses and adeno-associated viruses have also been used to transduce monocytic cells. Adenoviruses do not integrate into the host genome and as a result are not useful for long term expression of the exogenous sequences\textsuperscript{245}. Adeno-associated virus (AAV) in contrast, do integrate into the host genome, and have been used successfully in transducing primary human monocytes and dendritic cells\textsuperscript{262,263}, although their efficacy in transducing human monocytic cell lines has been low (<1\%)\textsuperscript{264}. By combining the ability of lentiviral vectors to stably transduce monocytic cell lines at a high efficiency and the potential for siRNA to mediate RNA interference, we have shown that stable gene silencing in human monocytic cell lines is achievable (Fig. 3-3). Transduced cells can be propagated under normal conditions without drug selection. The silenced phenotype appears to be stable during 6-8 weeks of continuous culture, and transduced cells may be used after long periods of storage in liquid nitrogen.
In the study of PI3K function, gene-targeting studies in mice have revealed important roles for specific PI3K isoforms in immunity, metabolism and cardiac function. However, a particular problem with targeting individual PI3K isoforms has been that silencing of a regulatory or catalytic subunit often results in altered expression of the non-targeted isoforms (Table 1-4). Altered subunit expression often leads to changes in signaling, and thus makes interpretation of the observed phenotypes complicated. In this context, lentiviral-mediated RNA silencing of p110 isoforms appears to be a superior approach, since we were able to specifically reduce p110α expression while not affecting levels of either other class IA p110 isoforms or p85 (Fig. 3-3B and C). However, it can be argued that since p85 normally heterodimerizes with p110, the lack of reduction in p85 in p110α deficient cells may suggest that p85 is in relative excess to the total p110 in these cells. We cannot exclude this possibility at the present time. Nevertheless, when taken together the results shown indicate that lentiviral-delivered siRNA is an efficient method for specific gene silencing in monocytic cells. Furthermore, by virtue of the specificity offered by RNAi, studies of individual isoforms from protein families can be done with relative ease.

In contrast to lentiviral vector HR-p110α3, transduction of the HR-p110α1 vector did not reduce cellular levels of p110α (Fig. 3-3B and C). While HR-p110α1 was originally designed as a candidate shRNA to mediate RNAi, the sequence did not bring about the desired result. Nevertheless, these cells transduced with HR-p110α1 served as useful controls for non-specific effects of transduction and shRNA expression. There are several possible explanations for why this candidate siRNA might not have been effective. It has been suggested that target mRNA regions where hydrogen bonds form in secondary and tertiary structures can impede silencing.
Another possible explanation for the lack of effect of the HR-p110α1 vector may be that this construct targeted a region close to the AUG start codon. It has been suggested that these regions may be richer in sequences that bind regulatory proteins and this may limit the ability of the RISC complex to access the RNA target sequence. Nevertheless, this is not an absolute restriction since it has been shown that targeting sequences close to the start codon may successfully induce RNA silencing.

The experiments reported above demonstrated the ability of VSV-pseudotyped lentiviral vectors to stably silence the PI3K p110α isoform in the monocytic cell lines THP-1 and U-937. The ability of lentiviral vectors to transduce both dividing and non-dividing cells and stable expression of the transgene make this a versatile strategy for gene silencing based on RNAi. Moreover, the finding that lentiviral transduced cells expressing shRNA can be further manipulated by transfection with reporter plasmids, for instance luciferase, significantly expands the utility of this approach. One important application of this technique may be in gene therapy research, such as silencing genes in myeloid leukemia cells or other difficult to transfect cells in order to better understand their biology and to identify potential therapeutic targets.
CHAPTER IV: ROLE OF p110α PI3K IN REGULATING MONOCYTE ADHERENCE AND PHAGOCYTOSIS

4.1 LPS and vitamin D₃ induced adherence

Vitamin D₃ (D₃) is well known for its role in regulating calcium homeostasis, however, it also has other important actions in the regulation of hematopoietic cells. D₃ has been shown to induce differentiation of monocytc cells, and augment maturation markers such as CD11b and CD14 in monocytic cell lines such as THP-1, U-937 and HL-60. Previous work from this laboratory showed that D₃ activated PI3K, and PI3K activity was required for the induction of CD11b and CD14 expression by D₃. Adherence of monocytes to endothelial cells is an essential requirement for the localization of these cells to sites of tissue inflammation. Adherence in cells of the human promonocytic cell line THP-1 can also be induced by D₃, although it is not clear whether this also involves PI3K.

Bacterial LPS is another agonist known to enhance adherence of leukocytes *in vitro*, and it also activates PI3K in monocytc cells. Numerous reports have previously shown that LPS induces adherence in THP-1 cells, and that this is a PI3K dependent process. The roles of individual PI3K isoforms involved in this phenotype have not been defined and therefore we sought to use THP-1 cells deficient in p110α isoform to investigate whether this PI3K isoform is required for either LPS-induced or D₃-induced adherence.

²Some material presented in this chapter are derived from that published in Ref. 244.
CD11b is the α chain of the β2 integrin Mac-1 (also called complement receptor 3), and Mac-1 initiates multiple cellular processes including adherence, phagocytosis, degranulation, and migration. We have previously reported that CD11b induction by D3 in THP-1 cells was sensitive to inhibition by PI3K inhibitors. The role of PI3K p110α isoform in D3 induced up-regulation of CD11b will also be examined in this chapter.

4.1.1 Monocyte adherence induced by D3, but not LPS is dependent on p110α.

We have previously shown that LPS-induced adherence of THP-1 cells is dependent upon PI3K. However, use of pharmacologic inhibitors did not permit determination of whether this involved class IA PI3K and if so which p110 isoform. To address this question, we examined LPS-induced adherence in THP-1 cells rendered p110α deficient using shRNA (Fig. 4A). Consistent with previous findings that PMA-induced monocyte adherence is resistant to PI3K inhibitors, p110α deficient cells showed normal PMA-induced adherence (data not shown). This indicated that differentiation of p110α deficient cells appear to be normal with PMA stimulation, compared to control transduced cells. Using PMA-treated cells as a control, adherence induced by LPS was determined as percentage of PMA-induced adherence by a colorimetric based adherence assay as described in Experimental Procedures. Figure 4-1A shows that while PI3K inhibitor LY294002 significantly reduced LPS-induced adherence in all transduced cells, silencing of p110α did not affect LPS-induced adherence, and this was true over a range of LPS concentrations (12.5-500 ng/ml). LPS was opsonized by human serum prior to use, however the presence of human serum alone did not induce adherence (data not shown). Similar to LPS, LY294002 inhibited D3-induced adherence in all types of transduced cells (Fig. 4-1B). In contrast to LPS, however, monocyte adherence induced by D3 was found to be
attenuated in p110α-deficient cells. Moreover, this inhibitory effect was comparable to that observed in LY294002-treated cells.
Figure 4-1

A  LPS-induced adherence

B  D₃-induced adherence
Figure 4-1. Monocyte adherence induced by D3, but not LPS is dependent on p110α. 

A, LPS-induced adherence. Cells were either treated or not with LY294002 (25 μM) for 30 min. at 37°C, before treatment with human serum opsonized LPS (12.5-500 ng/ml). For each treatment group, incubation with PMA (20 ng/ml) was also done in parallel. After 24 hr, non-adherent cells were rinsed away, and adherent cells stained with crystal violet. Dye was then eluted with 100% methanol, and absorbance measured at 570nm. To control for cell numbers plated per cell type, adherence was expressed as a percentage of PMA-induced adherence. PMA-induced adherence was similar among all cell types (p > 0.05, data not shown). All samples treated with LPS alone were not significantly different from each other (one-way ANOVA p = 0.2827). The same applies to the LY294002 plus LPS treated group (p = 0.1962). All of the LY294002 plus LPS treated samples were significantly different from LPS treated control samples (post-ANOVA Tukey test, p < 0.01 for all pairs), and the values observed were approximately 40% of the LPS only group. 

B, D3-induced adherence. Cells were either treated or not with LY294002 (25 μM) for 30 min at 37°C, before treatment with D3 (100 nM). Cells were incubated with D3 for 48 hr before being assayed for adherence. All samples treated with LY294002 plus D3 were not significantly different from each other (one-way ANOVA p = 0.5945), while the D3 treatment alone samples were significantly different from each other (one-way ANOVA p = 0.0006). All of the LY294002 plus D3 treated samples were significantly different from cells treated with D3 only (post-ANOVA Tukey test, p < 0.05 for all pairs), with the exception of HR-p110α3 (p>0.05). D3-induced adherence in p110α deficient cells is approximately 50% of control, and equivalent to treatment with LY294002. All treatments were done in triplicate. Error bars indicate standard deviation, n = 3.
4.1.2 CD11b expression in response to vitamin D3

_D3-induced CD11b expression is attenuated in p110α deficient cells_—We have previously shown that CD11b induction by D3 in THP-1 cells was sensitive to inhibition by PI3K inhibitors. To determine whether this is p110α-dependent, we incubated cells with LY294002 or not, followed by D3 for 72 hr after which the cells were collected and stained with anti-CD11b RPE-conjugated antibody or isotype matched control RPE-conjugated antibody. After washing and resuspension in binding buffer, cells were analyzed by flow cytometry. Viable cells were gated, and analyzed for RPE signals. For transduced cells, only GFP positive cells were analyzed. As shown in Figure 4-2, D3-induced CD11b expression was reduced significantly in p110α deficient cells when compared to control cells (p < 0.05). The results shown are expressed as mean fluorescence intensity (MFI) index which is the ratio of [MFI of D3 treated samples stained with anti-CD11b antibody – MFI of isotype-matched control antibody)/ (MFI of anti-CD11b antibody stained, untreated samples - MFI isotype-matched control antibody]. Therefore, a MFI index of 1 indicates that there was no induction of CD11b by D3. To calculate % reduction of D3-induced CD11b expression in p110α deficient cells relative to D3-treated control cells, the following calculation was used: % reduction = [(MFI index of control cells – MFI index of HR-p110α3 cells)/ (MFI index of control cells - 1)] x 100. The % reduction of D3-induced CD11b expression in p110α deficient cells relative to controls cells was approximately 59-63%, similar to that observed in LY294002-treated cells.
Figure 4-2

Attenuation of D₃-induced CD11b expression in p110α deficient THP-1 cells

![Graph showing MFI index for different conditions.]

Figure 4-2. CD11b induction by D₃ is attenuated in p110α deficient THP-1 cells. THP-1 cells were either incubated or not with LY294002 (25 μM) for 30 min, followed by 100 nM D₃ for 72 h at 37° C and 5% CO₂. Cells were then washed once in binding buffer and stained with anti-CD11b RPE-conjugated antibody or isotype matched RPE-conjugated control antibody, according to the manufacturer’s instructions. After washing and resuspension in binding buffer containing 1.85% paraformaldehyde, 10,000 cells were analyzed for RPE fluorescence. For transduced cells, the analysis was restricted to GFP positive cells. Data were collected after compensation of GFP and RPE fluorochromes on channels FL1 and FL2. Mean Fluorescence Intensity (MFI) index is the ratio of (MFI of D₃ treated samples stained with anti-CD11b antibody − MFI of isotype-matched control antibody)/ (MFI of anti-CD11b antibody stained, untreated samples − MFI isotype-matched control antibody). Therefore, a MFI index of 1 indicates that there was no induction of CD11b by D₃, or complete inhibition by LY294002. All
of the LY294002 plus D₃ treated samples were significantly different from control cells treated with D₃ alone (post-ANOVA Tukey test, p < 0.05 for all pairs), but not from HR-p110α3 plus D₃ alone (p > 0.05). Error bars indicate standard deviation, n = 3.
4.2 Regulation of phagocytosis by p110α PI3K

Phagocytosis is the uptake of large (>0.5 μm) particles by cells [269]. This process is triggered by the recognition of ligands exposed on the particle surface by specific phagocyte cell surface receptors. Following receptor binding and crosslinking, actin polymerizes around the phagocytic cup. Phagocytosis also involves bringing about membrane extension or addition to surround the particle, thereby creating an intracellular, particle-containing phagosome [36]. In lower eukaryotes such as the slime mold Dictyostelium, phagocytosis is a process primarily for the acquisition of food [270]. In mammals, the ability to phagocytose efficiently is limited to professional phagocytes (neutrophils and macrophages), cells which play critical roles in host defense, homeostasis, and tissue remodeling. These cells have numerous types of receptors that can mediate binding to a wide variety of ligands. The most well studied type is the Fcγ receptor (FcγR). Two main classes of these receptors exist, one group activates effector functions, and the second group inhibits these functions. In humans, the former group includes FcγRI, FcγRIIA, and FcγRIIIA [reviewed in Ref. 271]. These receptors differ somewhat in their structures, but they have in common a cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) present either in the cytoplasmic domain of the receptor itself (FcγRIIA), or in an associated γ subunits (FcγRI and FcγRIIIA). It has been shown that clustering of the FcγRIIA leads to tyrosine phosphorylation of the ITAM motif, present within its cytoplasmic domain, by Src family tyrosine kinases (e.g. Hck and Lyn) [272]. The phosphorylated ITAM then recruits the non-receptor tyrosine kinase Syk via its two SH2 domains. Studies in Syk-deficient mice have shown that Syk is required for completion of FcγR-mediated phagocytosis [273]. Tyrosine phosphorylated
Syk has been shown to recruit PI3K via the SH2 domain of the p85 subunit. The Grb2-associated binder 2 (Gab2), a scaffolding adaptor containing a PH domain, has been proposed to further amplify the PI3K signal by recruiting more PI3K via Gab2’s phosphotyrosine residues. This provides at least one mechanism to physically link class IA PI3K to downstream signaling during FcγR mediated phagocytosis. Additional evidence linking PI3K to FcγR-mediated phagocytosis is the ability of PI3K inhibitors wortmannin and LY294002 to inhibit uptake of IgG-coated RBC or latex beads by macrophages and neutrophils. Inhibition of PI3K did not affect actin polymerization, suggesting that pseudopod extension for particle engulfment is not just an actin driven event. Although class IA PI3K has been implicated in regulating phagocytosis based upon its association with Syk in FcγR mediated uptake, other evidence suggests that additional isoforms are also likely to be involved in regulating FcγRI-mediated phagocytosis. For example, Allen and coworkers showed that following FcγRI cross-linking in human monocytes, the activities of both class IA p85/p110 and class IB p110γ/101 PI3K were increased. More recently, using microinjection of inhibitory antibodies revealed that p110β isoform was required for phagocytosis of IgG-coated RBC and apoptotic cells in murine macrophages. It is unclear whether this finding also applies to human phagocytes or for CR3-mediated phagocytosis as well.

The events downstream of PI3K leading to phagosome formation are not well understood. However, current data suggest that PI3K may play a role in mediating contractile activity and membrane insertion required for phagocytosis. Greenberg and
colleagues proposed that myosin X provides a molecular link between PI3K and pseudopod extension during phagocytosis. Myosin X is an unconventional myosin with three PH domains that accumulates in the phagocytic cup in a PI3K dependent manner and is also required for membrane spreading on IgG opsonized particles. It has been shown that wortmannin and LY294002 sensitivity in phagocytosis of IgG-coated beads is dependent on particle size. Thus, internalization of particles larger than 4μm, but not smaller, was attenuated by PI3K inhibitors and this correlated with a reduction in exocytic plasma membrane insertion. One hypothesis proposed to explain these findings was that larger particles require more membrane to become enclosed, and a specific PI3K may be involved in regulating the delivery of membranes from vesicles to the plasma membrane during phagocytosis. Consistent with the model of targeted delivery of endomembranes contributing to the elongation of pseudopods is that recycling endocytic vesicles bearing the VAMP3 (vesicle-associated membrane protein 3) marker have been shown to be delivered at the site of phagocytosis. Furthermore, the small GTP-binding protein ARF6 (ADP ribosylation factor 6) has been implicated in membrane delivery during phagocytosis and expression of dominant negative ARF6 inhibited the recruitment of VAMP3 compartments at the site of phagocytosis. A possible link between ARF6 and PI3K is that an exchange factor for ARF6, ARNO (ARF nucleotide binding site opener), is known to depend on PI3K for activation. ARNO also contains a PH domain, and has been shown to bind PI3K products. Thus, in addition to recruiting myosin X, PI3K products may also act to recruit and activate PI-binding proteins that can stimulate vesicle trafficking required for
pseudopod extension. This would provide a unified and elegant mechanism for PI3K to couple exocytosis of endomembrane with myosin X-mediated pseudopod extension.

Phagocytosis mediated by other receptors, such as complement receptor 3 (CR3), has also been shown to be inhibited by PI3K inhibitors. The mechanism by which PI3K regulates CR3-mediated phagocytosis is less clear, however, vesicles have been observed to accumulate around bound particles during CR3-mediated phagocytosis suggesting the possibility of a similar role in exocytosis of membranes. It is not known which isoform of PI3K regulates CR3-mediated phagocytosis.

4.2.1 Effect of p110α silencing on phagocytosis

To investigate whether p110α regulates FcγR-, CR3-mediated phagocytosis or both, we first determined if PI3K p110α knockdown had any effect on the expression of these phagocytic receptors in our model system. To examine expression of phagocytic receptors FcγRI, FcγRII, FcγRIIIA, and CR3 (CD11b), flow cytometry analysis was used. The expression of FcγRI, FcγRIIA, FcγRIIIA, and CD11b were approximately 83%, 83%, 6.5%, and 87%, respectively, with control isotype matched antibody having <7% staining (Fig. 4-3A). These findings are consistent with previous reports that—with the exception of FcγRIIIA—these receptors are highly expressed on human monocytic cell lines. No significant differences were observed between control cells and p110α knockdown cells (p>0.05). Flow cytometry analysis of latex beads was performed to verify their opsonization with either IgG or iC3b (Fig. 4-3B and C).
PMA differentiated U-937 cells deficient in p110α had reduced FcγR- and CR3-mediated phagocytosis compared to PMA differentiated control cells (Fig. 4-3D and E). On average, the reduction in FcγR-mediated phagocytosis was 66.5% (SD ±14.3%) compared to control cells, while CR3-mediated was reduced by 55.9% (±20.9%) compared to control cells. Binding of particles to target cells as assessed by exposing the cells to the particles at 4°C for 1.5 h. No differences between the groups were observed ($p > 0.05$, data not shown).
Figure 4-3. Surface receptor expression, opsonization of latex beads, and phagocytosis of IgG and serum opsonized latex beads by PMA-differentiated U-937 cells deficient in PI3K p110α subunit. A, Receptor expression of FcγRI, FcγRII and FcγRIIIA and CD11b was not affected by silencing PI3K p110α. Isotype control antibodies (ctl Ab), anti-FcγRI (aCD64), anti-FcγRIIIA (aCD32), anti-FcγRIIIA (aCD16), or anti-CD11b were used to assess the surface expression of these receptors. The %
expression on the Y-axis is the fraction of cells that express the receptor prior to differentiation. No significant difference was seen between p110α deficient cells and shRNA control cells (p>0.05). B and C: 6.6 μm latex beads were opsonized with serum or IgG using methods previously described with minor modifications. B, IgG opsonization was achieved using anti-BSA antibodies against BSA-coated latex beads. Verification by flow cytometry was done by using secondary, anti-IgG antibody conjugated to FITC. Clear histogram represents secondary antibody alone, and solid histogram represents anti-BSA IgG followed by secondary antibody. C, IgM coated latex beads were opsonized with complement using fresh serum and this was confirmed with anti-iC3b antibody and FITC-conjugated secondary antibody. Latex beads incubated in the presence of human serum (solid histogram) or incubated in the absence of serum (clear histogram) are shown. D and E: U-937 cells transduced with HR-p110α1 or HR-p110α3 were differentiated with PMA for 48 h before exposure to opsonized latex beads for 1h. D, Phagocytosis of IgG opsonized latex beads was significantly (p=0.0165, two-tailed paired t test) diminished in p110α knockdown cells (transduced by HR-p110α3 virus) compared to control cells (HR-p110α1). E, Phagocytosis of serum opsonized latex beads was significantly (p=0.0225, two-tailed paired t test) diminished in p110α knockdown cells (HR-p110α3) compared to control cells (HR-p110α1). Error bars indicate standard deviation, n = 3.
4.3 Discussion

Vitamin D₃ and LPS are both known to induce adherence in monocytic cells and to activate PI3K. Through the use of in vitro kinase assays, we and others have previously shown that both LPS and D₃ activate PI3K in human monocytes and macrophages. Since in these studies the basic approach used was an anti-p85 antibody to immunoprecipitate the kinase, the results led to the conclusion that class IA PI3Ks are activated in response to LPS or D₃, although the involvement of other PI3K family members could not be ruled out. Since p110α is a more robust PI3K in terms of biochemical kinetics than either p110β or p110δ, we hypothesized that p110α might be the dominant class IA PI3K in mediating LPS and D₃ signaling. Based on this assumption, we predicted that by silencing PI3K p110α in THP-1 cells, a phenotype of diminished adherence induced by either LPS, D₃, or both would be observed. As shown in Figures 4-1A and B, whereas adherence in response to both LPS and D₃ was sensitive to PI3K inhibitor LY294002, LPS-induced adherence was resistant to silencing of p110α while D₃-induced adherence was not. This was examined over a range of LPS concentrations to control for the possibility that LPS might only utilize p110α at lower concentrations. Thus, these findings suggest that differential utilization of p110α reflects qualitative differences between LPS and D₃. These results lead to the somewhat surprising and interesting conclusion that these two agonists use signaling pathways in monocytes that activate distinct isoforms of class IA PI3Ks at least for some functional responses. It would appear most likely that LPS-induced adherence is mediated by p110β or p110δ, since LPS is known to activate class IA PI3K, and LPS-induced adherence was inhibited by LY294002 (Fig. 4-1A). At this point, we
cannot rule out the possibility that LPS activates p110α for other signaling pathways not related to adherence.

Prior studies from this laboratory showed that in THP-1 cells treated with D₃, the vitamin D receptor (VDR) associated with the p85 subunit of PI3K in a ligand-dependent manner. In addition, within 20 minutes of exposure to D₃, a corresponding rise in PI3K activity was observed when PI3K assays were performed on either anti-p85 or anti-VDR immunoprecipitates. Furthermore, activation of PI3K by D₃ was linked to changes in gene expression after 24 hr. The findings in the latter and the present reports are consistent with a model in which class IA PI3K is activated through a steroid receptor. This model differs from the conventional paradigm in which class IA PI3K activation occurs downstream of transmembrane receptors such as growth factor receptors, immunoreceptors [reviewed in Ref. 63], and toll-like receptor 2. However, recent progress in D₃ signaling research has resulted in the detection of a putative membrane bound receptor (VDR_mem), that based upon differences in binding properties, appears to be distinct from the nuclear VDR (VDR_nuc) [reviewed in Ref. 288]. For example, selective binding of synthetic D₃ analogs to VDR_mem and not VDR_nuc has shown that the former mediates non-genomic rapid signaling effects of D₃, but not delayed classical genomic responses. Taken together, these findings suggest the interesting possibility that class IA PI3K may be activated by both VDR_mem and VDR_nuc, such that PI3K may regulate both rapid, non-genomic signaling as well as at least some delayed genomic effects of D₃. Clearly in this model there would be ample opportunity for activation of PI3K through VDR_mem to influence cellular responses to D₃ brought about through the classical VDR_nuc.
Further studies will be required to identify whether VDR\textsubscript{mem} complexes with and activates PI3K p85/p110α.

The β\textsubscript{2} integrin receptor CR3 (CD11b/CD18, also known as α\textsubscript{M}β\textsubscript{2}), is a marker of monocyte differentiation and can mediate adherence, phagocytosis and leukocyte transmigration [reviewed in Ref. \textsuperscript{290}]. CD11b is the α subunit of CR3 and associates non-covalently with its partner CD18. Expression of CR3 is restricted to myeloid cells, and its level of expression depends on the state of differentiation with mature neutrophils and macrophages having the highest levels \textsuperscript{291-293}. THP-1 cells are known to express relatively low levels of CR3 in the basal state \textsuperscript{283,294-296}, and D\textsubscript{3} is known to augment CR3 expression \textsuperscript{187,203,204}. We have previously reported that CR3 induction by D\textsubscript{3} in both THP-1 cells and human monocytes is sensitive to PI3K inhibitors \textsuperscript{187}. Using THP-1 cells made deficient in p110α by RNAi, we observed a significant reduction (59 to 63%) in D\textsubscript{3}-induced CD11b expression compared to several negative control cell populations (Fig. 4-2). Adherence of monocytes to plastic induced by PMA has been shown to be dependent on CR3 \textsuperscript{297}. Therefore, the defect in D\textsubscript{3}-induced adherence we observed in p110α deficient THP-1 cells (Fig. 4-1B) may be partially due to the attenuation of CD11b expression. Alternatively, reduced adherence in response to D\textsubscript{3} by p110α deficient cells, may be related to defective activation of CR3 since the active conformation of CR3 is required for optimal function \textsuperscript{298,299}. In regard to LPS-induced adherence, we have previously reported that another β\textsubscript{2} integrin, LFA-1 (CD11a/CD18), played an important role in mediating LPS-adherence to ICAM-1 coated plates \textsuperscript{192}. Using neutralizing monoclonal antibodies, we found that blocking CD11a and CD18 resulted in 50% and
65% decrease in adherence, respectively. When the two antibodies were used in combination, the reduction in LPS-induced adherence was ~85%. Since CR3 also shares CD18 with LFA-1 and can bind to ICAM-1 as well, this suggests the possibility that CR3 may also mediate LPS-induced adherence to some degree. This hypothesis is strengthened by the fact that LPS also induces CD11b expression in monocytic cells. Further studies are required to investigate the relative roles of various integrins in mediating adherence induced by LPS and D₃.

Phagocytic cells have crucial roles in innate immunity and host defense by virtue of their abilities to recognize, ingest and destroy invading microbes. PI3K has been demonstrated to be an important regulator of both FcγR and CR3-mediated phagocytosis. The specific isoform of PI3K involved in mediating these events in human monocytic cells is not clear. Using microinjection of inhibitory antibodies, it was shown that p110β and to a lesser extent p110δ, but not p110α, were required for apoptotic cell and FcγR-mediated phagocytosis of IgG-opsonized RBC in murine macrophages. These results are at variance with our results in human monocytic cells where we observed that both FcγR- and CR3-mediated phagocytosis were p110α PI3K-dependent (Fig. 4-3D and E).

There are at least three possible explanations for these discordant findings. First, this inconsistency may reflect species-specific differences in PI3K isoform usage. This possibility is supported by the finding of such species-dependent effects on PI3K isoform usage with effects on phagocyte function in neutrophils. Using second generation isoform specific PI3K inhibitors, it was shown that TNF-α primed oxidative burst in...
human neutrophils in response to fMLP required the sequential activation of p110γ followed by p110δ. In contrast, the oxidative burst in murine neutrophils required only p110γ and was independent of p110δ.

A second possible explanation may be the differentiation state of the monocytic cells used in our experiments. For example, it has been shown that the PKC isoforms activated by FcyR varies depending on the differentiation state of the cells. In IFN-γ primed U-937 monocytes for example, FcyRI engagement led to increased PKC activity and translocation of PKC isoforms δ, ε, and ζ, while in dibutyryl cAMP differentiated U-937 cells the conventional PKC isoforms (α, β, and γ) were recruited. Therefore it may be possible that the PMA-differentiated U-937 cells in our model may use a different PI3K isoform for phagocytosis than in murine bone-marrow derived or peritoneal macrophages, based upon differences in differentiation state.

A third possibility to account for these discordant findings is the difference in the particle system used. It has been shown that mechanical properties of the particle can affect the efficiency of phagocytosis and signaling, independent of size. For example, rigid versus soft polyacrylamide beads showed differential dependence on Rac1 GTPase. This variable could be controlled for by comparing experiments using IgG-opsonized RBC as targets.

The results shown in Figure 4-3D show that like Fcy-mediated bead ingestion, CR3-mediated phagocytosis also appear to require p110α since the uptake of serum-opsonized
beads was significantly reduced in p110α knockdown cells. These conclusions need to be tempered somewhat by the caveat that in the model systems we used—whereas events were heavily biased towards either CR3 or FcγR—it was not possible to completely exclude the potential involvement of other receptors. Furthermore, it has been reported that CR3 can form functional associations with other receptors such as CD14, FcγRIIIB, and CD87. An interesting observation by Grinstein and coworkers was that FcγR crosslinking brought about increased avidity of CR3, and CR3 was mobilized into phagocytic cups during FcγR-mediated phagocytosis. It has been reported that in neutrophils FcγR crosslinking activated CR3 via a PI3K-dependent pathway. Thus, CR3 may participate in FcγR-mediated phagocytosis, even when the particles specifically bind FcγR. This suggests the hypothesis that crosstalk between these two receptors may be altered in p110α deficient cells and the possible amplification of a phenotype of defective phagocytosis. Further studies will be required to address this question.

Residual phagocytic activity in p110α deficient cells (Fig. 4-3D and E) may be due to usage of other PI3K isoforms or may be PI3K-independent. Various studies have suggested that not all phagocytosis requires PI3K. PI3K-independent phagocytosis of a variety of microbial organisms, including Helicobacter pylori, Yersinia enterocolitica, and Legionella pneumophila has been described. PI3K-independent contractile activity has also been reported as well. The differentiation state of the cells may also influence whether PI3K regulates phagocytosis. For example, it has been shown that undifferentiated THP-1 cells ingested particles independent of PI3K, while FcγR-mediated phagocytosis by retinoic acid/IFN-γ differentiated THP-1 cells is inhibitable by...
PI3K inhibitors. These findings suggest that PI3K may contribute to more efficient phagocytosis in macrophages compared to monocytes. Basal, PI3K-independent phagocytosis is likely to coexist with the more efficient PI3K-dependent phagocytosis pathways.

In summary, using transduced THP-1 cells deficient in PI3K p110α, we found that D3-induced, but not LPS-induced adherence is dependent on p110α. D3-induced upregulation of CD11b was also found to be dependent on p110α. Therefore our data suggests that p110α is required for D3-induced differentiation. Our data also demonstrated a prominent role of p110α in mediating FcγR- and CR3-mediated phagocytosis in PMA-differentiated U-937 cells. It should be noted that while PMA-induced differentiation appear to be normal in p110α deficient cells, our current data cannot exclude the possibility that other aspects of PMA-induced differentiation besides adherence were affected by p110α silencing.
CHAPTER V: ROLE OF PI3K p110α IN REGULATING CYTOKINE PRODUCTION AND THE OXIDATIVE BURST

5.1 Toll-like Receptor family

Mononuclear phagocytes can detect infection through PRR, which enable these cells to sense diverse microbial ligands (PAMP). These germline encoded, non-clonal receptors are critical aspects of host defense against pathogenic microorganisms. The innate immune response is rapid and can modulate the development of the adaptive immune response. A related group of PRR that has sparked tremendous research interest in the last several years is the Toll-like receptor (TLR) family, which was discovered through studies of the Drosophila Toll protein involved in ontogenesis and antimicrobial resistance [reviewed in Ref. 15]. TLRs are type I transmembrane receptors with leucine-rich repeats in their extracellular domains, while their cytoplasmic domains have homology to the mammalian IL-1 receptor called TIR (Toll/IL-1 receptor) domain 16.

TLR stimulation induces a wide variety of genes in macrophages and dendritic cells, including those encoding cytokines, chemokines, proteolytic enzymes, extracellular matrix proteins, and proteins involved in antigen presentation such as MHC class II, and co-stimulatory molecules amongst others [reviewed in Ref. 223]. Balancing the induction of pro- and anti-inflammatory responses is an important aspect of TLR signaling, since these are amongst the first receptors stimulated on innate immune cells. For example induction of IL-12 promotes proinflammatory responses, while IL-10 is predominantly anti-inflammatory 223.

*TLR4 signaling in macrophages*
The first mammalian TLR discovered was TLR4, and this was accomplished through both a forward genetic approach by positional cloning in a strain of mice that was LPS hyporesponsive and through reverse genetics via TLR4 knockout mice [reviewed in Ref. 312]. Active TLR4 is a homodimer, and similar to other TLRs it has a TIR domain in the cytoplasmic region. LPS induced TLR4 signaling is initiated when LPS binds to LPS binding protein (LBP)- in serum, and then to a glycosylphosphatidylinositol (GPI)-anchored CD14 on the plasma membrane. This complex then presents LPS to the TLR4/MD2 complex on the cell surface. Although currently there is no evidence that LPS binds directly to TLR4 313, LPS-induced signaling leading to inflammatory responses clearly involves TLR4. One model proposed is that LPS is recognized by a cluster of receptors associated with lipid rafts 314. CD14 and other receptors are constitutively found in lipid rafts, while TLR4 is recruited to lipid rafts upon LPS binding. Following formation of a complex between LPS/LBP/CD14 and TLR4, the latter dimerize and its TIR domains can recruit four TIR domain-containing adaptors through homophilic TIR-TIR domain interactions. These adaptors include MyD88 (myeloid differentiation primary-response protein 88), MAL (MyD88-adaptor like protein, also known as TIR-domain-containing adaptor domain, TIRAP), TRIF (Toll-IL-1 receptor domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule).

Conceptually, two main pathways have been defined downstream of TLR4, one of which is MyD88-dependent pathway and the other TRIF-dependent (Fig. 5-1). It has been shown that induction of proinflammatory cytokine gene expression downstream of TLR4 requires both pathways 315,316. In contrast, activation of the transcription factor interferon
regulatory factor (IRF3) and the subsequent induction of IFN-β and IFN-inducible genes require only the TRIF-dependent pathway (Fig. 5-1) \(^{316}\). MAL likely acts upstream of MyD88, since overexpression of MyD88 in embryonic fibroblasts deficient in both MyD88 and Mal was able to activate NFκB-dependent promoter activity, whereas overexpression of only Mal was not \(^{14}\).

MyD88 has both a carboxy-terminal TIR domain and an amino-terminal death domain (DD). Through TIR-TIR and DD-DD interactions, MyD88 forms homodimers when recruited to the receptor complex \(^{317}\). The DD allows recruitment of members of the IRAK family of serine/threonine kinases (IL-1R associated kinase), which also have a DD in their amino-termini. Among the IRAK family, IRAK4 is recruited first to MyD88, followed by IRAKI. After recruitment, IRAKs undergo autophosphorylation and cross-phosphorylation and as a consequence their affinity for MyD88 decreases. This results in recruitment and activation of the adaptor TRAF6 (TNF receptor-associated factor 6). IRAK1 and TRAF6 then dissociate from the receptor complex and activate TAK1 (TGF-β activated kinase 1) at the plasma membrane. ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) is an adaptor protein that bridges TRAF6 to MEKK-1 (mitogen-activated protein kinase/ERK kinase kinase-1), a member of the MAP kinase kinase kinase (MAPKKK) (Fig. 5-1) \(^{318}\). Activation of MEKK1 leads to subsequent phosphorylation and activation of MKKs (MAP kinase kinase), JNK, and p38 \(^{319}\). The translocation of JNK and p38 to the nucleus then leads to the activation of AP-1-or ATF-2-like complexes that also regulate pro-inflammatory genes \(^{319}\).
TAK1 is also a member of the MAPKKK family that is essential for LPS-induced NFκB activation through the IκB kinase (IKK) complex (Fig. 5-1). TAK1 is associated with three TAK-binding proteins (TAB): TAB1 is an activator of TAK1, while the other two act as adaptors for TRAF6 (TAB2 and 3). IRAK1 is degraded at the membrane, and the remaining TRAF6/TAK1/TAB complex translocates to the cytosol to activate the IKK (α, β, and γ) complex and MAPK pathways by phosphorylation. The IKK complex then phosphorylates IκB leading to its ubiquitination and degradation. This allows NFκB dimers to translocate to the nucleus to drive the transcription of target genes.

Activation of MAP kinase pathways as described above by TAK1 results in an early phase of MAPK phosphorylation, which is distinct from the slow phase mediated by the TRIF-dependent pathway in MyD88 deficient mice. TAK1 kinase activity has been demonstrated to be required for LPS-induced p38 and JNK phosphorylation, but not for that of ERK. Phosphorylation and activation of MAPK has important roles in the inflammatory response, which include histone modification that unmask binding sites for NFκB (also referred to as κB sites) in the promoters of some inflammatory cytokine genes. p38 MAPK also is involved in post-transcriptional control of cytokine mRNAs like TNF-α.

A member of the IFN regulatory factor (IRF) family of transcription factors, IRAF5, has been demonstrated to act downstream of TLR4-MyD88 as a master transcription factor leading to activation of genes encoding inflammatory cytokines. In macrophages and
dendritic cells from IRF5<sup>−/−</sup> mice challenged with either TLR4 or TLR9 ligands, the production of IL-12p40 was severely impaired. Levels of TNF-α and IL-6 were also significantly diminished, though not to the same extent as that of IL-12p40. IRF5 binds to MyD88 and TRAF6 directly (Fig. 5-1) and once activated translocates to the nucleus where it binds to interferon-stimulated response elements (ISRE) of IL-12p40. IRF5 is believed to cooperate with other transcription factors, because the IL-12p40 promoter has binding sites for transcription factors activated by JNK and p38 MAPK, as well as κB binding sites. A similar mechanism has been proposed for IL-6 and TNF-α transcription. Although binding of IRF5 to the promoters of these cytokine genes was not examined, multiple ISREs have been identified in the promoters of IL-6 and TNF-α<sup>327</sup>. Recently, IRF4 has been shown to negatively regulate the production of proinflammatory cytokines (TNF-α, IL-6, IL-12p40) by macrophages in response to TLR stimulation including LPS, by competing with IRF5 for MyD88 binding (Fig. 5-1)<sup>328</sup>. IRF-4 is expressed in lymphocytes and macrophage and dendritic cells and negatively regulates TLR4 signaling through the MyD88 pathway, thereby attenuating the production of proinflammatory cytokines.

It is important to note that the influences of IRF4 and IRF5 on cytokine production have been shown to be cell-type specific in mice. They have been observed in peritoneal and splenic macrophages<sup>328</sup>. Bone marrow derived macrophages and dendritic cells from either IRF4<sup>−/−</sup> or IRF5<sup>−/−</sup> mice, on the other hand, show normal secretion of inflammatory cytokines compared to control cells<sup>328</sup>. Therefore in different conditions or cell types, IRF5 is not an absolute requirement for LPS-induced proinflammatory cytokine
production, and IRF4 is not a consistent inhibitor. The roles of IRF4 and IRF5 in regulating the production of inflammatory cytokines in human monocytic cells are as yet unknown.

For TLR2, TLR5, TLR7, and TLR9, activation of the MyD88 pathway alone is sufficient to result in the expression of inflammatory cytokines. In contrast, activation of the MyD88 pathway alone downstream of TLR4 results in IRAK phosphorylation and early phase NFκB activation, but these events are not sufficient to activate the expression of inflammatory cytokines. In order for TLR4 to bring about the expression of inflammatory cytokines, the TRIF-dependent pathway is also required. It is currently not clear why TLR4 signaling evolved to require both of these pathways.

Activation of the TRIF-dependent pathway requires the adaptor TRAM (Fig. 5-1). Following TLR4 stimulation, TRIF can recruit TRAF6 and activate NFκB and the MAPK pathways, although at a slower rate than the MyD88-dependent pathway. Since mice deficient in both MyD88 and TRAF6 still can partially activate NFκB in response to LPS, this has led to the suggestion that TRIF must have access to at least one alternative pathway leading to NFκB activation (i.e. TRAF6-independent activation of NFκB downstream of TRIF). The N-terminal region of TRIF can bind to both TRAF6 and TBK1 (TANK-binding kinase 1), while the C-terminus has been shown to bind RIP-1 (Receptor-interacting protein-1). Embryonic fibroblasts from RIP-1 deficient mice showed impaired NFκB activation in response to TLR3 ligands. Both TRAF6 and RIP-1 independently are involved in NFκB activation, although it is unclear
if RIP-1 mediates this in a manner similar to TRAF6. In addition, TRIF can activate TBK1, which is associated with IKKe and TANK (TRAF-family-member-associated NFκB activator kinase) \textsuperscript{334}. TBK1 and IKKe phosphorylate IRF3, leading to its nuclear translocation and transcription of IFN-β and other IFN-inducible genes (Fig. 5-1) \textsuperscript{316}. Transcription of IFN-β also requires NFκB for full activation \textsuperscript{335,336}. Recently through the use of inhibitors of Src tyrosine kinase family, these tyrosine kinases have been shown to be required for LPS-induced IFN-β expression in the TRIF-dependent pathway \textsuperscript{337}. How these kinases are activated by the TRIF-dependent pathway remains to be determined, however.

Even in the simplified schematic diagram shown in Figure 5-1, it is evident there are at least three parallel pathways downstream of TLR4 activation that can lead to the induction of pro-inflammatory cytokine gene expression. Furthermore, two of these lead to the transcription of IFN-related genes. The existence of these multiple pathways, the possibility of other yet to be discovered pathways combined with post-transcriptional regulation, indicates the control of TLR4-induced cytokine production is complex and tightly regulated.

\textit{PI3K and TLR}

Class IA PI3K has been shown to be involved in the innate immune response through TLRs in macrophages, dendritic cells, endothelial cells, and B-cells \textsuperscript{224,225,228,338}. The p85 subunit has been shown to bind to phosphotyrosine residues in MyD88 \textsuperscript{339} in murine macrophages, and this association is significantly increased after LPS treatment. This
interaction with MyD88 explains how PI3K may be activated downstream of TLR4 even though the TIR domain of TLR4 does not contain a Y-x-x-M motif. This is in contrast to TLR2 or IL-1R, both of which do have Y-x-x-M motifs in their TIR domains. In the context of TLR2, the PI3K p85 subunit has been shown to bind directly to TLR2 via two phosphotyrosine residues. For TLR4, studies using an IRAK deficient embryonic fibroblast cell line demonstrated that IRAK is required for LPS-induced Akt phosphorylation. Several mechanisms have been proposed to explain this. These include the possibilities that IRAK may either be involved enzymatically in the activation of PI3K or its downstream effectors. Alternatively, IRAK may have a general stabilizing effect on the TLR4/MyD88/IRAK receptor complex, thus promoting downstream signaling.

There are two lines of evidence to suggest that PI3K is also linked to the TRIF-dependent, MyD88 independent pathway (Fig. 5-2) leading to negative regulation of the IFN-β response. In human PBMC-derived dendritic cells, wortmannin or LY294002 enhanced IFN-β expression upon TLR4 stimulation. PI3K inhibitors also increased the levels of IKKα/β phosphorylation and IκB-α degradation with a concomitant increase in NFκB nuclear translocation. NFκB DNA-binding was also increased with PI3K inhibitors, while IRF3 DNA-binding was not affected. This suggests that NFκB is required to cooperate with IRF3 to transcribe IFN-β and that PI3K inhibits the activation of NFκB but not IRF3 (Fig. 5-2). In this same study, using exogenous TRIF expression in HEK293T cells, it was demonstrated that TRIF can enhance NFκB transcriptional activity and the introduction of a catalytically active PI3K p110α attenuated this effect.
In this same system, p110α was shown to bind TRIF directly, although this association has yet to be reported to occur under native conditions. Together, these results suggest that PI3K p110α may be involved in attenuating TRIF-dependent activation of NFκB leading to restriction of the IFNβ response.

In studies concerned with TLR3 signaling in HEK 293 cells, PI3K was found to be required for full IRF3 activation. In this study using HEK 293 cells expressing TLR3, PI3K was found to be necessary for a critical phosphorylation of IRF3 in order for it to be activated and bind to the ISG56 (interferon-stimulated gene 56) promoter. ISG56 is an IFN-β inducible gene involved in the inhibition of translation initiation, and ISG56 promoter does not have κB sites. Furthermore, in this study it was shown that in the TLR3 pathway, TRIF acted downstream of PI3K, and that PI3K was able to bind to TLR3 via phosphotyrosine-p85 interactions. PI3K/Akt pathway was demonstrated to be essential for the serine phosphorylation of IRF3, an event necessary for IRF3 dimerization and binding to the promoter of IGF56. In this model of two-step activation of IRF3 (Fig. 5-2, right side), TRIF activated TBK1/IKKe first phosphorylates IRF3 such that it can dimerize, but not bind DNA. Subsequently, via the PI3K/Akt pathway the dimer undergoes additional serine phosphorylation, such that IRF3 can bind to DNA and interact with transcriptional coactivators such as CREB binding protein (CBP). Taken together, these results show that PI3K can participate in TRIF-dependent pathways in TLR3 signaling. It is uncertain if this positive role for PI3K on IRF3 occurs in TLR4 signaling, or if this occurs at all in monocytic cells.
Taken together these two studies provide evidence that PI3K may be involved in TRIF-dependent pathways. In the context of TLR4 signaling, PI3K serves to decrease the transcription of IFN-β by restricting NFκB activation.

Figure 5-1

Figure 5-1. Schematic diagram of TLR4 signaling pathways. TRIF is utilized in the MyD88-independent pathway, and activates IRF3 and the subsequent induction of expression of IRF3-dependent genes such as IFN-β. IRF5 associates with MyD88 and TRAF6 to induce inflammatory cytokine genes, while IRF4 competes with IRF5 binding.
to MyD88 to oppose IRF5 actions. Mal and TRAM serve as bridging adaptors to recruit MyD88 and TRIF, respectively, in TLR4 signaling. TAK1 is a member of MAPKKK family, and can activate the NFκB pathway and the MAPK pathway. TAB 2/3 acts as an adaptor linking TAK to TRAF6, while TAB1 functions as an activator of TAK1. ECSIT interacts specifically with TRAF6 and MEKK-1 and leads to activation of MAPK pathway \(^{318}\). The two branches also modulate each other, as TAK1 can activate MKKs and their downstream effectors and MEKK1 can activate the IKK complex and NF-κB \(^{319}\). TRIF can activate the NFκB pathway through both TRAF6 and RIP-1 \(^{14,332,333,343}\). How RIP-1 leads to NFκB activation is not clearly understood (not shown). Not shown are dimerization of TLR4 and some adaptors, LPS complex with CD14/LBP, MD2 and other extracellular interactions. Adapted from Ref. \(^{21,327,328}\).
Figure 5-2. Model of PI3K involvement in TLR4-TRIF pathway and PI3K-dependent IRF3 activation in TLR3 signaling. The left side of the figure shows the TRIF-dependent pathway downstream of TLR4 activation. The TRIF pathway is capable
of mediating the late (delayed) phase of NFκB activation as well as activating IRF3. The N-terminal region of TRIF can bind to both TRAF6 and TBK1, while the C-terminus binds only RIP-1. Both TRAF6 and RIP-1 are involved in NFκB activation, although it is unclear if RIP-1 mediates this in a manner similar to TRAF6. The TBK1 pathway leads to IRF3 activation. Together, these processes lead to the transcription of IFNβ. PI3K may inhibit activation of the NFκB pathway by inhibiting IKK phosphorylation through the action of Akt, as has been previously proposed. The right side of diagram shows a two step model for IRF3 activation by TLR3, proposed by Sarkar et al. TRIF can recruit TBK complex and this leads to phosphorylation of IRF3 at Ser, causing IRF3 to form a dimer. This IRF3 dimer requires further phosphorylation in order to bind the promoter and to CBP more efficiently. PI3K activity provides this phosphorylation, possibly via Akt. Fully activated IRF3 can then drive the transcription of ISG56. Adapted from Ref. 14,332,342.
5.2 PI3K and expression of pro-inflammatory cytokines in TLR4 signaling

Various studies have examined the role of PI3K in the secretion of pro-inflammatory cytokines induced by bacterial LPS through TLR4 as well as other TLR and the related IL-1R\textsuperscript{344,345}. The results obtained are not entirely consistent since both positive and negative influences have been reported\textsuperscript{224-227}. For example, some of the evidence that focused on NFκB activation suggested a positive role for PI3K in mediating LPS signaling through TLR4. Thus, PI3K inhibitors or expression of a dominant negative of the PI3K effector Akt/PKB caused a partial defect in NFκB transcriptional activity, but not DNA binding activity\textsuperscript{224,339}. Similarly, expression of a dominant negative mutant PI3K p85 subunit also resulted in inhibition of NFκB activity and IL-6 secretion in LPS stimulated human endothelial cells\textsuperscript{224}. Others have reported a decrease in NO production in the presence of PI3K inhibitors\textsuperscript{227}. Consistent with these findings, bone marrow derived macrophages (BMM) from SHIP-1\textsuperscript{−/−} mice [enhance levels of PtdIns(3,4,5)P\textsubscript{3}] were reported to secrete more TNF-α, IL-6, IL-1β, and NO in response to LPS, compared to cells from SHIP-1\textsuperscript{+/+} mice\textsuperscript{346}. The issue is somewhat muddied, however, by the fact that another report using similar cells reached opposite conclusions\textsuperscript{347}. It has also been reported that BMM from SHIP-1 KO mice have a reduced capacity to develop tolerance to LPS, again suggesting a role for PtdIns(3,4,5)P\textsubscript{3} in mediating inflammatory pathways\textsuperscript{346}.

While the findings described above indicate that PI3K can play a positive role in regulating cytokine production, evidence to the contrary has also been reported. For
example, PI3K has been shown to promote IRAK1 degradation in the early phase after LPS exposure. PKC-ζ is known to associate with IRAK1, and inhibitors of PKC prevented IRAK1 degradation. Since PKC-ζ has been shown to be activated downstream of PI3K in LPS treated cells, the IRAK/PI3K/PKC axis may provide a negative feedback loop, acting to attenuate signals mediated by IRAK1. In this regard, PI3K may serve as a negative regulator of TLR4 signaling.

There are other reports that suggest PI3K plays an anti-inflammatory role such as limiting the production of TNF-α and IL-12 following LPS stimulation in human monocytes and murine dendritic cells, respectively. An increase in NO production in the presence of PI3K inhibitors has also been reported. Recently, it was shown that primary cells from SHIP-" mice displayed an anti-inflammatory phenotype that required exposure to TGF-β and aging of the animals. In addition, it was also shown that a pathway involving PI3K mediates anti-inflammatory signaling through inhibition of glycogen synthase kinase 3β (GSK3) following LPS challenge. GSK3 is a target of Akt, and when phosphorylated it loses its ability to phosphorylate and inactivate the transcription factor cyclic AMP response element binding protein (CREB). Activated CREB then continues to sequester CREB binding protein (CBP) from NFκB, limiting activation of the latter. Consequently, secretion of IL-10 is enhanced by increased CREB activity, and proinflammatory responses including secretion of TNF and IL-6 are reduced due to suboptimal NFκB activity.
Inconsistent data from the literature regarding whether PI3K is predominantly pro- or anti-inflammatory with respect to regulating cytokine production, may reflect species-specific or cell type-specific differences, or different in vitro conditions. Another likely possibility is that distinct PI3K isoforms regulate different pathways, some positively and others negatively. In the context of either species- or cell type-specific differences, inhibition of all PI3K isoforms using conventional inhibitors may therefore result in conflicting outcomes.

A limitation common to most of the studies that investigated PI3K and TLR4 signaling was that the roles of individual PI3K isoforms were not directly assessed. An example is the use of first generation PI3K inhibitors, which also do not address individual isoform specific pathways as they inhibit all PI3K isoforms except class II PI3K C2α. These inhibitors also have been shown to be non-specific for PI3K, even at relatively low concentrations. Another example which may have limited specificity is the SHIP knockout model, which reveals the effects of relative higher PtdIns(3,4,5)P3 levels compared to levels of PtdIns(3,4)P2. It is possible, therefore, that phenotypic changes observed in this model may reflect the effects of reduced concentrations of PtdIns(3,4)P2 rather than increased levels of PtdIns(3,4,5)P3. A lack of SHIP in cells could also potentially dysregulate all of the class I PI3K pathways, and mask contributions by individual isoforms. Given the evidence of non-redundancy in function amongst some of the p110 isoforms (e.g. p110α or p110β knockouts are embryonically lethal), it may not be possible to assign a function to any isoform-specific PI3K pathway through this approach alone. In the interest of overcoming some of these limitations, in this chapter,
we report the results of studies that investigated LPS-induced TNF-α, IL-6, IL-10, and IL-12 secretion in p110α deficient THP-1 cells using cytokine ELISAs, qualitative RT-PCR, and Western blot analysis.

5.3 Effects of p110α silencing on LPS-induced cytokine expression

*THP-1 cells deficient in PI3K p110α have an altered LPS-induced cytokine production profile.*

To examine the role of p110α on LPS-induced cytokine production, THP-1 cells deficient in p110α or control cells were incubated with serum opsonized LPS. Cytokine ELISAs were performed on supernatants collected at 5 h and 18 h post-exposure to LPS. THP-1 p110α deficient cells showed enhanced production of TNF-α (*Fig. 5-3A*), and IL-10 (*Fig.5-3B*) at both time points, compared to control cells. In contrast, LPS-induced levels of IL-12 and IL-6 were significantly diminished in p110α deficient cells (*Fig. 5-3C* and *Fig. 5-3D*, respectively).
Figure 5-3

A

**TNF-α 5h**

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

**PG/ml**

1000 2000 3000 4000 5000 6000 7000

**TNF-α 18h**

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

**PG/ml**

0 1000 2000 3000 4000
Figure 5-3

B

IL-10 5h

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

pg/ml

IL-10 18h

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control

pg/ml
Figure 5-3 C

**IL-12p40 5h**

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

**IL-12p40 18h**

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS
Figure 5-3

D

IL-6 5h

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

pg/ml

0 50 100 150 200 250 300 350

IL-6 18h

- p110α knockdown
- shRNA control
- p110α knockdown + LPS
- shRNA control + LPS

pg/ml

0 50 100 150 200 250 300 350
Figure 5-3. Results of ELISA for TNF-α, IL-6, IL-10, and IL-12p40 at 5h and 18h post LPS-stimulation of THP-1 cells. Cells, either transduced with control siRNA virus (shRNA control) or virus containing siRNA targeting p110α (p110α knockdown), were either incubated or not with LPS at 100 ng/ml for 5 or 18h. Error bars indicate standard deviation, n=5. A, LPS-induced TNF-α production was significantly higher in p110α deficient cells compared to controls at 5h (post-ANOVA Tukey test, p<0.01) and 18h (post-ANOVA Tukey test, p<0.05). B, LPS-induced IL-10 production was significantly higher in p110α deficient cells compared to controls at 5h (post-ANOVA Tukey test, p<0.001) and 18h (post-ANOVA Tukey test, p<0.001). C, LPS-induced IL-12p40 production was significantly lower in p110α deficient cells compared to controls at 5h (post-ANOVA Tukey test, p<0.05) and 18h (post-ANOVA Tukey test, p<0.001), and was not significantly different from unstimulated cells (p>0.05, for both time points). D, LPS-induced IL-6 production was significantly lower in p110α deficient cells compared to controls at 5h (post-ANOVA Tukey test, p<0.05) and 18h (post-ANOVA Tukey test, p<0.001), and was not significantly different from unstimulated cells (p>0.05, for both time points).
*LPS-induced cytokine production in THP-1 cells is mediated through TLR-4.*

To verify that in this model LPS was signaling through TLR-4, THP-1 cells were preincubated for 1 h at 37°C with neutralizing antibodies to either TLR-4 or TLR-2, prior to LPS stimulation. At 18h post-LPS exposure, IL-12p40 or IL-6 ELISAs were performed on the supernatants (Fig. 5-4). Pre-incubation with anti-TLR-4 antibody reduced IL-12p40 and IL-6 production by 54% \((p<0.001)\) and 76% \((p<0.01)\), respectively, relative to preincubation with anti-TLR-2 antibody (Fig. 5-4A and 5-4B). The quantities of LPS-induced IL-12p40 and IL-6 production in the presence of anti-TLR2 antibody were not significantly different from LPS-stimulated control cells incubated in the absence of antibody \((+LPS, p>0.05)\). In contrast, preincubation with anti-TLR-2 antibody only diminished zymosan-induced IL-12p40 (44%, \(p<0.01\)), but not IL-6 \((p>0.05)\) production relative to preincubation with anti-TLR4 antibody, suggesting zymosan might trigger IL-6 production through other receptors as well. The amounts of IL-12p40 and IL-6 produced in response to zymosan in the presence of anti-TLR4 antibody were not significantly different from those produced by zymosan-treated control cells without neutralizing antibody preincubation \((+Zy, p>0.05)\).
Figure 5-4. Effect of neutralizing antibodies to TLR-4 and TLR-2 on LPS and zymosan induced IL-12p40 and IL-6 production in control shRNA transduced THP-1 cells. A, IL-12p40 and B, IL-6 ELISA were performed on supernatants derived from THP-1 cells that had been transduced with control siRNA virus, preincubated for 5h with either TLR-4 (aTLR4) or TLR-2 (aTLR2) neutralizing antibody, and then incubated with LPS (100 ng/ml) or serum opsonized zymosan for 18h. Pre-incubation with anti-TLR-4 antibody reduced IL-12p40 and IL-6 production by 54% (post-ANOVA Tukey test \( p<0.001 \)) and 76% (post-ANOVA Tukey test \( p<0.01 \)), respectively, relative to preincubation with anti-TLR-2 antibody. In contrast, preincubation with anti-TLR-2 antibody only diminished zymosan-induced IL-12p40 (44%, post-ANOVA Tukey test \( p<0.01 \)), but not IL-6 (post-ANOVA Tukey test \( p>0.05 \)) production. There was no significant difference between LPS stimulation alone vs anti-TLR2 with LPS, or between zymosan alone and anti-TLR4 plus zymosan (\( p>0.05 \)). Error bars indicate standard deviation. The data are representative of two independent experiments.
The presence of neutralizing antibodies to TNF-α or IL-10 did not normalize the cytokine response of p110α deficient THP-1 cells.

Exogenous TNF-α has been reported to augment IL-10 production in monocytes. We considered the possibility that enhanced TNF-α production by p110α deficient cells might bring about augmented IL-10 production via a paracrine effect. To examine this possibility, p110α deficient THP-1 cells were preincubated or not with anti-TNF-α or isotype-matched control antibodies. Figure 5-5 shows that anti-TNF-α neutralizing antibodies restored neither LPS-induced IL-10, IL-12p40, nor IL-6 production by p110α deficient cells to control levels. However, anti-TNF-α antibodies did moderately suppress IL-6 production in p110α deficient cells compared to isotype matched controls.

IL-10 has also been reported to suppress LPS-induced IL-6 and IL-12 production. To investigate whether diminished LPS-induced IL-6 and IL-12 secretion by p110α deficient THP-1 cells was due to enhanced IL-10 production, neutralizing antibody to IL-10 was added or not prior to stimulation of cell with LPS. Preincubation with neutralizing anti-IL-10 antibody did not restore IL-6 or IL-12 production to levels seen in control cells. Finally, coincubation of cells with both anti-TNF-α and anti-IL-10 neutralizing antibodies together also did not restore IL-6 or IL-12 to control levels, ruling out a possible synergism between enhanced TNF-α and IL-10 levels in the suppression of IL-12 and IL-6 production by p110α deficient cells.
Figure 5-5

A

B
Figure 5-5

C

IL-12p40 19h

D

IL-6 13h
Figure 5-5. Effect of neutralizing anti-TNF-α or anti-IL-10 antibodies on LPS-induced IL-12p40 and IL-6 production by THP-1 cells. THP-1 cells transduced with control siRNA virus (shRNA control, clear bar graphs) or virus containing siRNA targeting p110α (p110α knockdown, filled bar graphs) were preincubated or not with anti-TNF-α antibody (aTNFα), anti-IL-10 (aIL-10), or isotype matched control antibody (mlgG or rlgG) at the indicated concentration for 5h. Cells were then either stimulated or not (No Tx) with LPS at 100 ng/ml for 5 or 18h. ELISAs for TNF-α (A), IL-10 (B), IL-12p40 (C), or IL-6 (D) were then performed on supernatants. For A and B, samples treated with neutralizing antibodies TNF-α (A) or IL-10 (B) were all significantly reduced compared to control antibody plus LPS (post-ANOVA Tukey test p<0.001). For C and D, shRNA control cells stimulated with LPS was significantly higher than all other treatment groups (post-ANOVA Tukey test p<0.001), while the remaining groups were not significantly different from each other (post-ANOVA Tukey test p>0.05). Error bars indicate standard deviation. The data are representative of two independent experiments.
Figure 5-6

A

IL-12p40 19hr

shRNA control + LPS
(-) shRNA control
lgG + LPS
IgG ctl + LPS
aTNFα + aIL-10 + LPS
aIL-10 + LPS
aTNFα + LPS
+ LPS

IL-12p40 48hr

shRNA control + LPS
(-) shRNA control
lgG + LPS
IgG ctl + LPS
aTNFα + aIL-10 + LPS
aIL-10 + LPS
aTNFα + LPS
+ LPS

pg/ml
Figure 5-6

B

IL-6 19hr

- shRNA control + LPS
- (-) shRNA control
- rlgG + mlgG + LPS
- lgG + LPS
- aTNFα + all-10 + LPS
- all-10 + LPS
- aTNFα + LPS
- + LPS

IL-6 48hr

- shRNA control + LPS
- (-) shRNA control
- rlgG + mlgG + LPS
- lgG ctrl + LPS
- aTNFα + all-10 + LPS
- all-10 + LPS
- aTNFα + LPS
- + LPS

pg/ml

140
Figure 5-6. Coincubation of neutralizing anti-TNF-α and anti-IL-10 antibodies did not restore LPS-induced IL-12p40 and IL-6 production. THP-1 cells transduced with control siRNA virus (shRNA control, clear bar graphs) or virus containing siRNA targeting p110α (p110α knockdown, filled bar graphs) were preincubated or not with anti-TNF-α antibody (aTNFα), anti-IL-10 (aIL-10) antibody, or isotype matched control antibodies (mlgG, IgG) at the indicated doses for 5h. The cells were then either stimulated or not (-) with LPS at 100 ng/ml for 19 or 48h. ELISAs for IL-12p40 (A) or IL-6 (B) were then performed on supernatants. Error bars indicate standard deviation. The data are representative of two independent experiments.
5.4 Activation of signaling pathways by p110α in response to LPS

Activation of NFκB and p38 MAP kinase have been shown to be important in the regulation of LPS-induced cytokines such as TNF-α, IL-6 and IL-12. LPS is known to activate all three MAP kinases p38, JNK, and ERK. Since LPS stimulated p110α deficient cells had an enhanced ability to secrete TNF-α but diminished capacity to secrete IL-6 and IL-12, we examined the phosphorylation status of MAP kinases, as well as that of NFκB p65. In addition, to determine if the changes in cytokine profile observed were due to transcriptional or post-transcriptional regulation, semi-quantitative RT-PCR of cytokine mRNA was examined.

5.4.1 Western blot analysis

*LPS-induced phosphorylation of p38, but not JNK or ERK, is enhanced in p110α deficient THP-1 cells.*

It has been reported that LY294002 pretreated monocytic cells have enhanced LPS-induced TNF-α production and this correlates with increased phosphorylation of p38, ERK, and JNK kinases. We carried out Western blot analyses to determine if these changes would be observed in p110α deficient THP-1 cells. Cells transduced with shRNA against p110α were markedly deficient in p110α as compared to control shRNA transduced cells (Fig. 5-7A). Of interest, anti-phospho-Akt antibodies revealed that whereas LPS treatment resulted in increased phosphorylation of Akt in control cells, no significant increase in phospho-Akt was observed in p110α deficient cells (Fig. 5-7B). Since Akt is activated as a downstream target of PI3K, these findings suggest that the
only PI3K responsible for bringing about Akt phosphorylation—indirectly through PDK1—in response to LPS cell treatment is p85/p110α.

We also observed that phosphorylation of p38 was enhanced in p110α deficient cells after 20 and 30 min post-LPS stimulation compared to control cells. In contrast, the phosphorylation of neither JNK (Fig. 5-7B) nor ERK was significantly altered in p110α deficient cells when compared to controls and after normalization for protein loading (Fig. 5-7C). On the other hand, the results shown in Figure 5-7D indicate that like p38, phosphorylation of NFκB p65 was also clearly enhanced in p110α deficient THP-1 cells relative to controls.
Figure 5-7

A

p110α  
Actin

B

Phospho-Akt  
Phospho-JNK  
Phospho-p38  
GAPDH

LPS  -  5  10  20  30  60  -  5  10  20  30  60

PI3K p110α1  PI3K p110α3

C

Phospho-ERK  
GAPDH

LPS  -  5  20  30  -  5  20  30

PI3K p110α1  PI3K p110α3

D

Phospho-p65/NFκB  
GAPDH

LPS  -  5  20  30

PI3K p110α1  PI3K p110α3

Figure 5-7. Western blot analysis. Antibodies to: A, PI3K p110α, B, phospho-Akt, phospho-JNK, phospho-p38, C, phospho-ERK, and D, phospho-p65 NFκB, in THP-1
cells that were either transduced with control siRNA virus (PI3Kp110α1) or virus containing siRNA targeting p110α (PI3Kp110α3). Cells were either incubated or not (-) with LPS at 100 ng/ml at various times (minutes). GAPDH was used as protein loading control. Anti-phospho-p65 NFκB antibody was assessed on total cell lysates. Results are representative of three independent experiments.
5.4.2 mRNA levels for TNF-α, IL-6, IL-10, and IL-12

Using RT-PCR, we examined whether the changes observed in LPS-induced cytokine expression in p110α deficient cells could be explained by corresponding alterations in mRNA levels (Fig.5-8). Consistent with reports that TNF-α is regulated both transcriptionally and post-transcriptionally, TNF-α mRNA levels from cells deficient in p110α were not significantly enhanced compared to controls, despite the fact that these cells produced more TNF-α (Fig. 5-3A). Likewise increased secretion of IL-10 by p110α deficient cells was not reflected by increased IL-10 mRNA levels after LPS-stimulation. In contrast, IL-12p40 mRNA levels in p110α deficient cells were significantly less than those of control cells, particularly at 4h post-LPS stimulation. This was also found to be the case for IL-6 where mRNA levels were lower in p110α deficient cells when compared to control cells at 4 and 16h. These results suggest that diminished IL-12 and IL-6 production by p110α deficient cells may in part be explained by events at the level of mRNA accumulation, but this does not appear to be the case for enhanced TNF-α and IL-10 production.
Figure 5-8. RT-PCR for TNF-α, IL-12p40, IL-6, and IL-10, mRNA in LPS-stimulated THP-1 cells deficient in PI3K p110α. THP-1 cells either transduced with control siRNA virus (α1) or virus containing siRNA targeting p110α (α3) were stimulated with 100 ng/ml LPS (+) or not (-) for the times indicated. Cells were then collected and cDNA were generated from the RNA preparations. Actin was used as loading control. Results are representative of three independent experiments.
5.5 NADPH-dependent oxidase and PI3K

Professional phagocytes have critical roles in innate immunity and the production of microbicidal oxidants is a key component of host defense. This is exemplified by a genetic disease in humans called chronic granulomatous disease (CGD), characterized by severe recurrent bacterial and fungal infections. The generation of microbicidal oxidants by monocytes and neutrophils requires the activation of a multi-protein complex called the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Genetic defects in CGD patients result in either absent or defective components of the NADPH oxidase. Cells from these patients are normal in terms of phagocytosis, chemotaxis, and degranulation, but are unable to generate an oxidative burst.

5.5.1 Assembly of the NADPH-dependent oxidase complex is highly regulated and requires PI3K activity

The regulation of NADPH oxidase function is tightly regulated, as unregulated production of reactive oxygen species (ROS) can result in deleterious effects on the host. Control is achieved through both spatial and temporal regulation of the NADPH oxidase complex assembly and activation. The multi-component NADPH oxidase is unassembled and inactive in resting cells, but assembles at the plasma or phagosomal membrane upon phagocyte activation (Fig. 5-9A). The NADPH oxidase complex consists of two membrane-bound components (gp91phox and p22phox), and at least four cytosolic components (p40phox, p47phox, p67phox, and Rac GTPase). The phox family of proteins is named for phagocyte oxidase. The NADPH oxidase in human
monocytes uses Rac1, while neutrophils use Rac2. The membrane bound components form a heterodimer called flavocytochrome b₅₅₈, which contains all the redox components used by the NADPH oxidase for transmembrane electron transport.

Assembly of the NADPH-dependent oxidase requires PI3K activity and 3'-phosphoinositides. PI3K serves at least two important roles in NADPH oxidase activation including recruitment and localization of p47phox and p40phox to the membrane, and activation of p47phox. In this regard, phagosomal lipid rafts have been proposed to be platforms for the recruitment of cytosolic NADPH oxidase components and for their assembly into an active NADPH oxidase complex. Binding or phagocytosis of particulate agonists such as opsonized zymosan (OPZ) to CR3 leads to activation of class IA PI3K via Src family phosphotyrosine kinases such as Hck. The PI3K product PtdIns(3,4,5)P₃ generated on the plasma membrane is then able to recruit PH domain containing effectors such as PDK1 and PLCγ₂, leading to activation of Akt and PKC, respectively (Fig. 5-9A). Depending on the agonist, a number of kinases have been proposed to phosphorylate p47phox, an event that is critical in the activation of NADPH oxidase assembly. These kinases include PKC, MAPK p38, Erk1/2, p21 activated kinase (PAK), Akt, and phosphatidic acid-activated kinase. The most convincing physiological evidence has been shown for PKC, while the physiological roles of the other kinases are less well understood. Both classical PKC (α, βI, βII) and novel PKC isoform (δ) have been reported to mediate p47phox phosphorylation. Soluble agonists like phorbol esters (PMA) can activate cPKC and nPKC. Activated PKC is then able to phosphorylate critical serine residues on p47phox.
which in the unstimulated state assumes an inactive intramolecular conformation (Fig. 5-9A) 360. Akt has also been reported to phosphorylate p47\textsuperscript{phox} in a cell-free system derived from neutrophils 373, but experiments in monocytic cells using Akt inhibitors show that fMLP-induced NADPH activation is independent of Akt 190. It is not clear whether this also applies to the OPZ-induced oxidative burst.

Once phosphorylated by PKC, a conformational change results which exposes the various domains of p47\textsuperscript{phox} such as the PX and SH3 domains (Fig. 5-9A) 360. Phosphorylated p47\textsuperscript{phox} is then able to interact with p67\textsuperscript{phox}, p40\textsuperscript{phox}, and p22\textsuperscript{phox} through interactions with their SH3 and PxxP (proline–rich motif) domains (Fig. 5-9A) 360. The two cytosolic components, p40\textsuperscript{phox} and p47\textsuperscript{phox}, contain a Phox homology (PX) domain 374. PX domains are known to bind various phosphoinositides 160,162,364, and the PX domain of p40\textsuperscript{phox} specifically binds PtdIns(3)P 374,375, while the PX domain of p47\textsuperscript{phox} specifically binds PtdIns(3,4)P\textsubscript{2} 374. Studies have identified two lipid phosphatases, SHIP-1 and PtdIns(3,4)P\textsubscript{2} 4-phosphatase, that are required to metabolize class I PI3K product PtdIns(3,4,5)P\textsubscript{3} in order to recruit cytosolic oxidase components to the membrane [reviewed in Ref. 364,376]. SHIP-1 removes the 5' phosphate from PtdIns(3,4,5)P\textsubscript{3} to generate PtdIns(3,4)P\textsubscript{2}, and the latter recruits p47\textsuperscript{phox}375. Subsequently, the PtdIns(3,4)P\textsubscript{2} 4-phosphatase can generate PtdIns(3)P from PtdIns(3,4)P\textsubscript{2} 375. This results in the recruitment of p40\textsuperscript{phox} to the membrane. Other interactions between the membrane components (gp91\textsuperscript{phox}, p22\textsuperscript{phox}, Rac1) and the cytosolic components are shown in Figure 5-8A. Oxidase activity requires p67\textsuperscript{phox} to associate directly with flavocytochrome b\textsubscript{558}.
(gp91\textsuperscript{phox}/p22\textsuperscript{phox}) and this has been proposed to mediate allosteric effects on the catalytic activity of flavocytochrome b\textsubscript{558}\textsuperscript{360}.

Rac translocation to the membrane is also an essential element of NADPH oxidase activation\textsuperscript{361}. In the basal state, Rac is associated with RhoGDI (Rho GDP dissociation inhibitor) in an inactive GDP-bound form. Upon phagocyte activation, Rac dissociates from RhoGDI and exchanges GDP for GTP and translocates to the NADPH oxidase complex at the membrane. Active Rac can then bind the TPR (tetratricopeptide repeat) domain of p67\textsuperscript{phox} and interact directly with gp91\textsuperscript{phox}. PI3K may activate Rac through activation of Vav, a PH-domain containing Rac-GEF\textsuperscript{236,377}. However, in OPZ-stimulated bovine neutrophils, MAPK p38 has also been shown to activate Rac and promote its translocation to the membrane, and p38 activation was PI3K dependent\textsuperscript{378}. Yamamori \textit{et al} proposed that p38 can activate cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}), leading to the release of arachidonic acid\textsuperscript{378}. Arachidonic acid is then able to induce the dissociation of Rac and RhoGDI, resulting in Rac activation\textsuperscript{378}. Further studies will be required to clarify the exact mechanism of Rac activation.

Overall, Rac, p47\textsuperscript{phox} and p40\textsuperscript{phox} seem to function as adaptors to provide a stable platform for the interaction between the catalytic component (gp91\textsuperscript{phox} and p22\textsuperscript{phox}) and the regulatory component (p67\textsuperscript{phox}) of the NADPH oxidase\textsuperscript{360}. Once the oxidase is assembled at the membrane, electrons are transferred from NADPH in the cytoplasm through the gp91\textsuperscript{phox} component across the membrane leading to the reduction of intraphagosomal or extracellular O\textsubscript{2} to superoxide anion O\textsubscript{2}\textsuperscript{-} (\textbf{Fig. 5-9B}). Protons that exit the
cytosol via voltage-gated proton channels are then able to react with $O_2^-$ to generate hydrogen peroxide ($H_2O_2$). This can occur either spontaneously or from the action of superoxide dismutase (SOD). $H_2O_2$ may be converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). MPO is expressed in monocytes and neutrophils [379], but not normally expressed in murine macrophages and cultured human macrophages [380]. Termination of NADPH oxidase activity is less well understood, but it correlates with a loss of $p47/67_{phox}$ from the membrane [243], and phosphorylation of $p47_{phox}$ by casein kinase 2 has been shown to deactivate the oxidase [381].
Figure 5-9. Model of NADPH oxidase assembly and activation. A, Assembly of the NADPH-dependent oxidase requires PI3K activity and 3'-phosphoinositides. Binding of...
particulate agonists such as opsonized zymosan (OPZ) to CR3 leads to activation of class IA PI3K via Src family phosphotyrosine kinases (PTK), possibly Hck. PtdIns(3,4,5)P$_3$ generated on the plasma membrane is able to recruit PH domain containing effectors such as PDK1 and PLCγ2, leading to activation of Akt and PKC, respectively. Both classical PKC (cPKC) and novel PKC isoform (nPKC) have been reported to mediate p47$^{phox}$ phosphorylation. Soluble agonists like PMA can activate both cPKC and nPKC. In the unstimulated state, p47$^{phox}$ forms an intramolecular complex. Activated PKC is able to phosphorylate serine residues on p47$^{phox}$ and induce a conformational change which exposes the various domains of p47$^{phox}$ thereby facilitating interactions with other proteins. The role of Akt in p47$^{phox}$ phosphorylation in vivo is uncertain. Rho GTPase has also been reported to be essential for p47$^{phox}$ phosphorylation downstream of OPZ stimulation, although the mechanism of this is not clear. Phosphorylated p47$^{phox}$ is then able to interact with p67$^{phox}$, p40$^{phox}$, and p22$^{phox}$ through various SH3-PxxP (proline-rich motif) interactions (shown by red bidirectional arrows). SHIP-1 removes the 5' phosphate from PtdIns(3,4,5)P$_3$ to generate PtdIns(3,4)P$_2$, which then recruits p47$^{phox}$. The PtdIns(3,4)P$_2$ 4-phosphatase can generate PtdIns(3)P from PtdIns(3,4)$_2$. This results in the recruitment of p40$^{phox}$ to the membrane as well. Other interactions between the membrane components (gp91$^{phox}$, p22$^{phox}$, Rac1) and the cytosolic components are shown in black bidirectional arrows. Rac is associated with RhoGDI (Rho GDP dissociation inhibitor) in the inactive GDP-bound form. Upon phagocyte activation, Rac dissociates from RhoGDI and exchanges GDP for GTP and translocates to the NADPH oxidase complex at the membrane. Active Rac can bind TPR (tetratricopeptide repeat) domain of p67$^{phox}$ and interact directly with gp91$^{phox}$. PI3K
may active Rac through activation of Vav, a PH-domain containing Rac-GEF. MAPK may also regulate the dissociation of Rac/RhoGDI complex via arachidonic acid (AA) by activation of cPLA₂. Not shown are the interactions between the three cytosolic components (p40⁵⁺, p47⁵⁺, and p67⁵⁺) in the resting state, and the contribution of class IB PI3K downstream of GPCR (e.g. fMLP receptor). Adapted from ref. 190, 236, 360, 364, 376.

B. Generation of ROS in phagosomes contributes to microbicidal activity. Electrons are removed from NADPH in the cytosol and transferred through the gp91⁵⁺ component across the membrane to reduce intraphagosomal/extracellular molecular O₂ to O₂⁻. Protons exit the cytosol via voltage-gated proton channels. Hydrogen peroxide (H₂O₂) is generated from O₂⁻ and H⁺ either spontaneously or through the action of superoxide dismutase (SOD). H₂O₂ may be converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). Adapted from Ref. 39.
5.5.2 Effect of p110α silencing on activation of the phagocyte oxidase by PMA and opsonized zymosan

To examine the role of p110α in the oxidative burst, both soluble and particulate agonists were used. THP-1 cells deficient in p110α or transduced with control shRNA were differentiated with low dose PMA (1.6 nM) for 20 hr, and rested in fresh media for 4 hr before stimulation by either high dose PMA (1 μM), serum opsonized zymosan (OPZ), or unopsonized zymosan (Z) (Fig. 5-10). PMA induced robust superoxide production in control cells, while cells deficient in p110α were significantly impaired (Fig. 5-10A) with a mean reduction of 76% (p<0.001). For OPZ, control cells similarly produced significant superoxide compared to unstimulated cells (p<0.001). In contrast, OPZ-stimulated THP-1 cells deficient in p110α had a significant reduction in superoxide production (Fig. 5-10B) with a mean decrease of 54% (p<0.01). In contrast to either PMA or OPZ, no significant production of superoxide was detected in control cells when stimulated by unopsonized zymosan (Fig. 5-10C). These results indicate that p110α is required for superoxide production in response to both the soluble agonist PMA and the particulate agonist OPZ. In this system, non-opsonized zymosan did not appear to bring about oxidase activation.
Figure 5-10. p85/p110α PI3K is required for PMA- and opsonized zymosan-induced oxidase activation. THP-1 cells deficient in p110α or transduced with control shRNA were differentiated overnight with 10 ng/ml (1.6 nM) of PMA, and rested in RPMI for 4h prior to stimulation. A, PMA-induced superoxide production. THP-1 cells were stimulated or not with 1 μM PMA for 30 min. In response to PMA stimulation, cells
deficient in p110α were significantly reduced in superoxide production compared to control cells (post-ANOVA Tukey test, p<0.001). Unstimulated control cells were not significantly different from stimulated p110α deficient THP-1 cells (post-ANOVA Tukey test, p>0.05), n=11. B, Opsonized zymosan (OPZ) induced superoxide production. Zymosan particles were opsonized with human serum for 30 min at 37°C prior to use. The ratio of particles to cells was 20:1. In response to OPZ, cells deficient in p110α were significantly reduced in superoxide production compared to control cells (post-ANOVA Tukey test, p<0.01). Unstimulated cells were not significantly different from OPZ-stimulated p110α deficient THP-1 cells (post-ANOVA Tukey test, p>0.05), n=7. C, Unopsonized zymosan (Z) did not induce superoxide production significantly in THP-1 cells (one-way ANOVA, p=0.3437). Cells were stimulated or not by zymosan particles at a ratio of 20:1, n=3. Data are expressed in nanomoles of O₂⁻ produced by 0.5 x 10⁶ cells in 30 min. A p-value <0.05 was considered significant. Error bars indicate SEM.
5.6 Discussion

The data presented here suggest that the p85/110α isoform of PI3K positively regulates the production of the proinflammatory cytokines IL-12 and IL-6, and concurrently negatively regulates the production of TNF-α and a major anti-inflammatory cytokine IL-10 in response to LPS. In control THP-1 cells, LPS-induced TNF-α rose rapidly and reached a maximum by five hours (Fig. 5-3A), while IL-6, IL-12p40, and IL-10 rose more slowly reaching plateaus between 19h to 48h post LPS stimulation (Fig. 5-6A to C). These dynamics of cytokine production in response to LPS were consistent with what has been reported previously for human monocytic cells.356,357

It has been shown previously that the use of PI3K inhibitors resulted in augmented production of TNF-α by LPS-treated human monocytic cells.191 However, because LY294002 and wortmannin are neither PI3K class- nor isoform-specific, it has not been possible to assign this function to an individual PI3K. The results of the present study, show for the first time in human monocytic cells that an individual isoform of PI3K—in this case p85/p110α—is able to regulate the production of TNF-α and other cytokines. This suggests that the other class IA isoforms have non-redundant functions at least in terms of this cellular response to LPS.

In the report by Guha and Mackman referred to above where biochemical inhibitors of PI3K kinase were used, enhanced production of TNF-α correlated with enhanced p38 phosphorylation and concurrently increased in NFκB activation and binding. These investigators proposed that PI3K serves to limit the production of inflammatory cytokines.
such as TNF-α, so that its expression is only transient in nature. We now present data to show that this pathway appears to be regulated by the p110α isoform of PI3K since phosphorylation of NFκB p65 was clearly enhanced in p110α deficient THP-1 cells relative to controls (Fig. 5-7C). In addition, we also found that activation of p38 MAPK in response to LPS was clearly under the control of p85/p110α. Unlike the results obtained using PI3K inhibitors, however, we did not find that the phosphorylation of JNK or ERK were significantly enhanced relative to controls (Fig. 5-7A and B). Consistent with our findings, however, DN-p85 or DN-Akt had no effect on LPS-induced JNK activation in human endothelial cells. Differences between p85/p110α-deficient THP-1 cells and those obtained using non-selective inhibitors may reflect other p110 isoform-specific effects, differences in cell-type, or alternatively non-specific effects of inhibitors.

Although we did not assess mRNA stability, results of semi-quantitative RT-PCR (Fig. 5-8) suggested that the early augmentation of LPS-induced TNF-α secretion in p110α deficient cells (Fig. 5-3) was likely due to a mechanism unrelated to mRNA accumulation and was likely post-transcriptional. In fact, post-transcriptional regulation of TNF-α production in murine splenic cells has been reported to be positively regulated by p38 MAPK. This is consistent with our finding of enhanced phosphorylation of p38 MAPK in LPS-treated p110α deficient cells (Fig. 5-7B). LPS-induced TNF-α production by murine splenic cells has also been shown to be regulated post-transcriptionally. Kotlyarov et al demonstrated that the mechanism of translational control of LPS-induced TNF-α production involved the p38 substrate MAP-kinase-
activated protein kinase-2 (MAPKAP-K2, or MK2)\textsuperscript{326}. The mechanism involved activation of the p38 pathway which contributed to upregulation of cytokine genes at multiple levels\textsuperscript{326,383}. p38 and other signaling pathways can regulate cytokine genes at the transcriptional level through activation of transcription factors\textsuperscript{383}. In addition, regulation of cytokine translation by the p38 pathway is mediated by a downstream kinase, MK2. MK2 can phosphorylate proteins that bind to AU-rich elements (ARE) located in the 3'-untranslated region of cytokine mRNAs to regulate cytokine translation\textsuperscript{383}. Phosphorylation of these ARE-binding proteins results in the release of translational repression of TNF-α mRNA. Consistent with this model, IL-10 has been shown to negatively regulate TNF-α by inhibiting TNF-α translation, and this action involves inhibition of the p38/MK2 pathway\textsuperscript{384}. Therefore, the increased p38 phosphorylation we observed may contribute to enhanced TNF-α and IL-10 production in p110α deficient cells, despite the findings that mRNA levels for these cytokines were not significantly elevated compared to control cells (Fig. 5-8).

Studies in knockout mice had previously suggested that PI3K can regulate cytokine production, but the isoforms involved were not clearly identified. Thus, Fukao et al demonstrated that LPS-induced IL-12 production was enhanced in dendritic cells isolated form p85α KO mice\textsuperscript{228}. In addition, when enteric bacteria from the ceca of control mice were inoculated into the peritoneal cavities of p85α KO mice, the peritoneal washings showed diminished TNF-α production compared to cells from wild-type mice\textsuperscript{183}. Notably, these findings are the converse of those we described above, where we found that p85/p110α negatively regulated TNF-α and positively regulated IL-12. The
observation that bacteria-induced TNF-α production was diminished in the p85α KO mice is also the converse of what Ghua and Mackman’s found when they examined TNF-α production in response to LPS in human monocytic cells pretreated with LY294002 to inhibit PI3K. Several possibilities may account for these differences. First, the wild-type DC used in the murine model did not express p110α or p110δ, but only p110β. It is conceivable that the absence of both p110δ and p110α from these cells might have contributed to different outcomes. Second, cells from the p85α KO mice were not uniform in their expression of either the regulatory subunits or the catalytic subunits of PI3K, and this varied with cell type. For example, while the catalytic subunits p110α and p110δ were absent from bone marrow derived-dendritic cells (BMDC), bone marrow derived mast cells (BMMC) were only deficient in p110α and they were normal for other isoforms. Moreover, p55α and p50α regulatory subunits were found to be increased in T-cells and adipocytes, but normal in BMMC and BMDC. An additional major concern about these cells from knockout mice is that they were derived from animals that had been reported by numerous groups to exhibit increased PI3K activity despite being null for p85α. For example, PI3K signaling downstream of insulin receptor is known to be dependent on class IA PI3K. Based upon this, it would be predicted that a true PI3K KO would exhibit a diabetic phenotype. Surprisingly, PI3K signaling was enhanced and sustained for a longer time in p85α KO mice than in wild-type mice, and as a result these p85α KO mice were hypoglycemic. Moreover, heterozygous disruptions of either p85α or p85β knockout mice were observed to have improved insulin signaling. Taken together these results indicate that p85α knockouts cannot be considered as null for class IA PI3K.
Okkenhaug and Vanhaesebroeck suggested that defective regulation of catalytic subunits due to the absence of regulatory subunits in p85 knockout cells likely contributes to enhanced signaling in some contexts and diminished PI3K signaling in others. One example of this is the finding in p85α KO cells of increased PI3K activity associated with the p85β isoform, and this may account for the paradoxical observation of increased PI3K signaling in p85α KO cells. Since p85 normally inhibits the kinase activity of its associated p110 subunit in the basal state, disruption of p85 could result in hyperactive p110 subunits.

To return to the issue of addressing how our results diverge from those of Fukao et al, it is important to note that in their murine, dendritic cell p85α KO model Akt phosphorylation was only mildly diminished following LPS stimulation. This indicates that significant class IA PI3K activity was still present in these p85α KO dendritic cells. Thus, the simplest way to reconcile the reports of enhanced IL-12 and reduced TNF-α production by cells from p85α knockout mice with our converse findings is to conclude that these mice were not true class IA PI3K knockouts. On the other hand, the superiority of using siRNA to selectively silence the class IA isoform p110α was amply shown based upon two important criteria: (1) deficiency of p110α expression while preserving normal expression of residual catalytic and regulatory isoforms, and (2) demonstration of a bona fide and unambiguous defect in PI3K signaling. Whether or not other variables such as species-specificity or differences in experimental...
models also may have contributed to these contrasting phenotypes remains to be determined.

Exogenous TNF-α has been reported to augment IL-10 production in monocytes $^{356}$, and IL-10 has also been demonstrated to suppress LPS-induced IL-6 and IL-12 production $^{357}$. We considered the possibility that enhanced production of TNF-α by π110α deficient cells might have led to augmented IL-10 production through a paracrine effect and that this may have led suppression of IL-6 and IL-12. Our data indicate that suppression of IL-12 and IL-6 was in fact not explained on this basis (Fig. 5-5 and 5-6). These experiments did not, however, rule out potential paracrine effects from other cytokines not assessed in this study. Interestingly, anti-TNF-α antibodies did moderately suppress IL-6 production in π110α deficient cells compared to isotype matched controls (Fig. 5-5D and 5-6B) and this suggested that TNF-α maybe be required for optimal IL-6 production to some extent. Since we did not examine the effects of these antibodies on shRNA control cells, we cannot not conclude that TNF-α is similarly required for the control levels of IL-6 production. The independent effects of silencing π110α PI3K on the several cytokine studies suggest that this class IA PI3K cannot be classified either as strictly pro-inflammatory or anti-inflammatory in regulating LPS-induced cytokine production. What appears clear, however, is that PI3K π110α is required for IL-12 and IL-6 production, while it restricts to limit the production of both TNF-α and IL-10.

IL-10 is a potent anti-inflammatory and immunosuppressive cytokine, with effects on T cells and mononuclear phagocytes [reviewed in Ref. $^{385}$]. IL-10 regulation is controlled
mRNA-destabilizing motifs like AU-rich' elements (ARE) have been identified in the 3'-untranslated region (UTR) of murine IL-10 mRNA. It has been suggested that many cell types constitutively transcribe IL-10 mRNA and much of the regulation is determined post-transcriptionally. LPS induces IL-10 in macrophages, and this requires p38 MAPK and the transcription factor Sp1 in human monocytes.

The results reported in this thesis show that IL-10 cytokine secretion was significantly enhanced in p110α deficient THP-1 cells particularly at later time points. This effect did not appear to be explained by corresponding changes in levels of mRNA (Fig. 5-8). These observations suggest that p110α may regulate IL-10 production through a negative effect on the translation of IL-10 mRNA, although this was not directly examined. It is also possible p110α PI3K may be acting post-translationally. Our finding of enhanced phosphorylation of p38 MAPK in p110α deficient cells (Fig. 5-7B) was also consistent with increased IL-10 secretion, since IL-10 production is known to be p38 MAPK-dependent. As discussed above, like TNF-α, IL-10 mRNA also has 3' ARE, however, translational control of IL-10 has not been shown to be dependent on the same mechanism of regulation described for TNF-α by p38 MAPK/MK2. This does not, however, rule out the possibility of other mechanisms of translational or post-translational regulation of IL-10 under the control of PI3K.

In summary, the results presented in this chapter demonstrate that a specific PI3K isoform can regulate multiple cytokines in response to LPS, with effects that are
cytokine-specific. Since both pro-inflammatory and anti-inflammatory signaling pathways have been reported to operate downstream of PI3K [reviewed in Ref. 390], we propose that both of these effects may be regulated by p110α isoform. PI3K subunits can physically bind to both key adaptors of TLR4 complex. Thus, p110α is able to bind TRIF 341, and the p85 subunit can bind to MyD88 339. These interactions support a model in which PI3K can regulate signaling via both TRIF-dependent and MyD88-dependent pathways. As one example, IRF5 has been shown to regulate LPS-induced IL-6 production downstream of MyD88 327 and endothelial cells expressing DNp85 had defective LPS-induced IL-6 production 224. Taken together, these findings suggest the possibility that positive regulation of IRF5 by PI3K p110α downstream of TLR4 could account for our observations of decreased LPS-induced IL-6 production in THP-1 cells where p110α has been silenced.

PI3K may regulate IRF5 by a mechanism similar to that described for TLR3 and IRF3 (Fig. 5-2). In HEK 293T cells expressing TLR3, it was demonstrated that PI3K p110α activated IRF3 downstream of TLR3 by a specific serine phosphorylation of the IRF3 dimer, leading to transcription of IFN-β 342. Dominant negative Akt abrogated this, leading to the conclusion that Akt was responsible for direct serine phosphorylation of the IRF3 dimer (Fig. 5-2) 342. This phosphorylation of IRF3 combined with a second phosphorylation event mediated by TBK1/IKKe, converted IRF3 into an active conformation capable of recruiting higher levels of CBP and binding ISRE 342,391. Thus p85/p110α PI3K might regulate IRF5 in a similar fashion, to account for the data reported in this thesis. Thus, we propose a model in which activation of TLR4 leads to
the recruitment of p85/p110α PI3K to MyD88 via phosphotyrosine residues, following which p110α brings about phosphorylation of IRF5, possibly through Akt. IRF5 subsequently binds to the ISRE and transcriptional coactivators more efficiently leading to the transcription of proinflammatory cytokines. Further investigations are required to determine whether PI3K p110α modulates IRF5 activation in this manner.

A model incorporating the present findings on the role of PI3K p110α in LPS-induced cytokine expression in human monocytic cells is shown on Figure 5-11. We propose that p110α may activate IRF5, similar to the mechanism described for TLR3/IRF-3 signaling in HEK293 cells in which IRF3 is activated through phosphorylation by the PI3K effector Akt. The overall effect of IRF5 activation is the induction of proinflammatory cytokines such IL-12p40 and IL-6. This would be consistent with diminished IL-12p40 and IL-6 cytokine mRNA levels seen in LPS-stimulated p110α deficient THP-1 cells. So far it is not known what activates IRF5, but our findings suggest one potential candidate may be PI3K p110α. TNF-α may also be positively induced by IRF5, but its transcription in THP-1 cells may be less dependent on IRF5 than on NFκB, as suggested by the fact that IRF5-/- mice were still able to secrete TNF-α albeit at a reduced level. In this model we propose, therefore, despite reduced IRF5 activation, increased NFκB and p38 phosphorylation in LPS-stimulated p110α deficient cells may overcompensate and lead to enhanced TNF-α production.

Other potential mechanisms may explain the decreased responses to LPS by p110α deficient cells for IL-6 and IL-12 secretion in the face of increased TNF-α production.
PI3K may limit LPS-induced TNF-α production at the transcriptional level, post-transcriptional, or both. In the MyD88-dependent pathway, PI3K may activate PKCζ, which may then bring about rapid degradation of IRAK. This would dampen the
Figure 5-11. Model of PI3K in LPS-induced monocyte signaling. LPS and LBP bind to CD14 and presents it to TLR4. TLR4/MD2 undergoes dimerization and activation,
which promotes the tyrosine phosphorylation of MyD88 by an unknown kinase. This then leads to the recruitment of p85-p110α. TRIF has also been reported to be able to bind p110α directly, and recruitment increases with LPS stimulation \(^{341}\). IRF5 can bind directly to TRAF6 and MyD88, and stimulate TNF-α, IL-12p40 and IL-6 through binding to ISRE \(^{327}\). These effects are independent of p38 or JNK phosphorylation status. On the other hand, IRF4 can mediate negative regulation of cytokine production via direct binding to MyD88 thereby competing with IRF5 binding \(^{328}\). In this model p110α via its effectors stimulate IRF5 such that downstream IL-12 and IL-6 transcription can occur more efficiently. TNF-α is also activated via NFκB, independent of IRF5, since IRF5\(^{-/-}\) mice still can secrete TNF-α albeit at a much lower level \(^{327}\). PI3K p110α can limit, but not inhibit TNF-α production. Activation of Akt by PI3K/PDK1 will lead to lower p38 MAPK phosphorylation through inhibition of MAPKKK like ASK-1 or MEKK-3 \(^{255,392}\). This leads to less phosphorylation of ARE binding proteins by MK2, such that TNF-α mRNA is more transcriptionally repressed. TAK1 maybe a target of PI3K effectors, and inhibition of TAK1 will dampen NFκB activation and p38/JNK phosphorylation. The reduced p38 also limits IL-10 transcription. IRF3 activation for ISFG56 transcription requires PI3K p110α in TLR3 signaling \(^{342}\), but its role in TLR4 signaling is not certain. Dashed lines indicate how ECSIT interacts specifically with TRAF6 and MEKK-1 (another MAPKKK) and appears to function in this pathway by facilitating the processing of MEKK-1 \(^{318}\). JIP3 (JNK-interacting protein 3) is a TLR4 associated protein that acts as a scaffold protein for JNK \(^{393}\), and this is illustrated to show how a MAPK localizes relative to the TLR4 complex. Arrows indicate activation
and blunt arrows indicate inhibition. NFκB activation downstream of RIP-1 is not illustrated. See text for more details. Adapted from Ref. 14,318,338,339,341,342,390,394.
signal through IRAK/TRAF6/IKK pathway, and reduce NFκB activation and TNF-α transcription. In IRAK1 KO mice, however, TNF-α production in response to LPS was diminished only at low concentrations of LPS (<5 ng/ml)\(^{395}\), and in our experiments mRNA levels in LPS-stimulated p110α silenced cells were not significantly higher than in control cells (Fig. 5-8). These findings would suggest that the ability of PI3K to limit TNF-α production is not likely mediated solely through IRAK degradation. A more dominant pathway for limiting TNF-α might involve Akt phosphorylation and inhibition of a MAPKKK such as ASK-1 (apoptosis signal-regulating kinase 1) or MEKK-3 (MAPK/ERK kinase kinase 3)\(^{255,392}\). This could lead to restricted activation of p38, less MK2 activity and correspondingly diminished phosphorylation of ARE binding proteins. As a result these proteins would retain their ability to inhibit translation. Limited activation of p38 MAPK could also restrict IL-10 production to some extent, by restraining activation of Spl\(^ {389}\).

In the model described above and shown schematically in Figure 5-11, PI3K p110α through Akt serves to limit overproduction of the early pro-inflammatory cytokine TNF-α, while providing signals to maintain the inflammatory response such as IL-12 that are needed to drive the immune response towards Th1. Interestingly, phospho-JNK and phospho-ERK were not significantly elevated in LPS-stimulated p110α deficient cells compared to control cells, suggesting that p110α may selectively regulate p38 MAPK. How this may come about remains to be determined. PI3K effectors like Akt may inhibit TAK1 leading to both reduced activation of p38 and NFκB, since human monocytic cells expressing kinase dead TAK1 showed reduced IKKβ activity in response to LPS.
treatment, as well as a reduction in p38 and JNK phosphorylation, but not that of ERK. It is possible that in our system the effect of p110α silencing on JNK was below the level of detection.

As shown in Figures 5-2 and 5-11 and discussed elsewhere in this chapter, inhibition of PI3K activity has been shown to bring about enhanced IFNβ gene expression in response to LPS via the TRIF-dependent pathway. In addition, PI3K inhibitors have been demonstrated to enhance IKKα/β phosphorylation, IκB-α degradation and NFκB nuclear translocation in response to LPS in a model of TRIF-mediated NFκB activation. Based upon these findings, we propose a model in which p110α exerts negative regulation upstream of the IKK complex in a TRIF-dependent pathway. Perhaps through inhibition of TAK1, PI3K may negatively regulate the late phase of NFκB activation as well, which then limits IFN-β production because efficient transcription of IFN-β requires NFκB activation in addition to IRF3. It remains to be determined whether IRF3 activation in TLR4 signaling is modulated by PI3K, as in the case for TLR3 signaling. Since we did not examine the status of either IRF3 or IFNβ in our experiments, we cannot determine if any of the effects on cytokines by silencing p110α is mediated through the TRIF-dependent pathway. Further research will be required to clarify this.

The model shown in Figure 5-11 provides a number of working hypotheses for future investigations. Although many of the potential mechanisms illustrated are not established yet, the model highlights the complexity of the roles of PI3K in regulating diverse
responses downstream of TLR4. Our data show that p110α can regulate several important cytokines independent of other PI3K isoforms. This observation may have been masked by other approaches such as those that used global PI3K inhibitors or mice with targeted disruptions of PI3K genes. The inconsistent data from various studies may also reflect cell-type specific effects, in addition to the use of different methodologies and experimental conditions. Many genes are differentially controlled by transcription factors and this depends on the type and differentiation status of the cells.

Augmentation of IL-10 and inhibition of IL-6 and IL-12 in p110α deficient monocytic cells suggests that p110α, its effectors, or both may be potential targets for modulating the inflammatory response secondary to LPS exposure or TLR4 activation. Future research focusing on the mechanisms of selective, monocyte cell regulation by p110α and other isoforms should reveal candidates for therapeutic targeting. PI3K catalytic isoform specific inhibitors have recently been described. These new second generation inhibitors may be useful tools to examine further the role of specific PI3K isoforms in animal model of sepsis. In addition, selective silencing of other PI3K isoforms by lentiviral delivered siRNA should be informative in respect to assigning specific functions to distinct PI3K isoforms.

The NADPH-dependent oxidase is important in host defense, and is also involved in the pathogenesis of inflammatory disorders such as arthritis, ischemic/reperfusion injury, and others. Understanding the mechanism and regulation of NADPH oxidase activation is of medical interest because of the potential to help advance therapeutics for
modulating the inflammatory response. Experimentally, serum opsonized zymosan is known to fix complement and to bind to CR3 leading to the induction of superoxide production by macrophages and neutrophils. Studies from class IB PI3K p110γ knockout mice have suggested that fMLP-induced superoxide production is regulated by p110γ. While OPZ-induced oxidase activation was normal in neutrophils from these p110γ knockout mice, experiments using PI3K inhibitors showed that the oxidative burst to OPZ-zymosan nevertheless required PI3K activity. Using THP-1 cells deficient in the class IA PI3K p110α isoform, we have now shown that this isoform is required for the OPZ-induced oxidative burst in monocytic cells (Fig. 5-10B). This finding suggests that NADPH oxidase activation induced by a particulate agonist is brought about by a distinct PI3K isoform from that induced by agonists that stimulate GPCRs, such as fMLP.

In our system, unopsonized zymosan did not induce significant superoxide production (Fig. 5-10C). Non-opsonic recognition of zymosan by CR3 is mediated through the lectin domain of CR3, which is distinct from the I/A domain essential for binding and phagocytosis of iC3b-coated particles. It has been shown that binding zymosan and opsonized zymosan results in differential signaling and mechanisms of phagocytosis. La Cabec et al showed that uptake of zymosan, in contrast to that of OPZ depends on Rac and cdc42, but not Rho activity. Furthermore, transmission electron microscopy showed that zymosan uptake resembled FcγR-mediated phagocytosis with the appearance of filopodia formation around the particles, while OPZ uptake displayed the classical sinking appearance characteristic of iC3b-coated particle phagocytosis. Lastly, it was
found that CR3-mediated phagocytosis of unopsonized zymosan depended on Hck tyrosine kinase, while internalization of OPZ did not. Taken together, these observations suggest that depending on the binding site involved, CR3 can take on different conformations leading to activation of distinct signaling pathways. This may explain why non-opsonized zymosan was unable to induce significant superoxide production in PMA-differentiated THP-1 cells (Fig. 5-10C). However, unopsonized zymosan has been reported to induce superoxide production in other contexts, such as in retinoic acid/vitamin D3 differentiated U-937 cells. In this system, OPZ-induced superoxide production was about 1.5 fold over that induced by zymosan. It is possible that use of a distinct differentiation agent or cell type in our experiments may account of these differences.

Phorbol esters such as PMA mimic the action of the second messenger diacylglycerol (DAG) and can activate both PKC (classical and novel isoforms) and non-PKC phorbol ester/DAG receptors. In this thesis, high dose PMA induced a strong oxidative burst in control cells, while cells deficient in p110α were effectively non-responders (Fig. 5-10A). These findings indicate that—contradictory to the traditional view that PI3K acts upstream of PKC—p110α acts downstream of PKC in triggering the oxidative burst. This observation was not entirely unexpected since PI3K products are known to be required to recruit the cytosolic components p47phox and p40phox to the membrane. Studies of trans-resveratrol (t-RVT)—a naturally occurring polyphenolic compound found in grapes—are consistent with a model in which p110α acts downstream of PKC in activating the oxidase and provide support for this finding. It has been reported that t-
RVT inhibited the PMA-induced oxidative burst in human monocytes and an in vitro kinase screen revealed that t-RVT inhibited PI3K by more than 40%, while having no effect on either PDK1 or Akt. fMLP-stimulated U-937 cells also had increased PI3K activity in phosphotyrosine immunoprecipitates, implicating class IA PI3K activation, and this activity was inhibited by over 70% with t-RVT. The ability of t-RVT to negatively modulate class IA PI3K activity coupled with its ability to inhibit PMA-induced oxidase activation again suggests that the latter requires class IA PI3K activity. These findings are consistent with our data showing that p110α deficient THP-1 cells were unable to activate the oxidase in response to PMA treatment (Fig. 5-10A), and reveal a novel role for p85/p110α PI3K downstream of PKC. It is interesting to consider these results in the context of a recent report that focused on hepatic growth factor signaling. Here it was shown that conventional PKCα acted as a negative regulator of class IA PI3K lipid kinase activity through phosphorylation of the p110α subunit. Whether PKC activates or inactivates PI3K in monocytic cells or can do both, depending upon the context, remains to be determined. Nevertheless, our findings provide clear evidence that PKC is upstream of PI3K in a pathway leading to activation of the monocyte oxidase. It is interesting to compare and contrast this finding with other reported actions of PMA and PKC such as the induction of adherence in monocytic cells independent of PI3K.

While we believe the results presented in Figure 5-10A are most likely explained by p85/p110α acting downstream of PKC, alternative models could be proposed. For example, a yet unidentified non-PKC phorbol ester/DAG receptor may be involved.
upstream of PI3K p110α, leading to PMA-induced oxidase activation. Several non-PKC phorbol ester/DAG receptors have thus far been identified such as: Ras-GRP (guanyl nucleotide-releasing proteins that act as GEFs for Ras and Rap1), Munc13 isoforms (a family of proteins involved in neurotransmitter release/exocytosis), and chimaerins (GTPase-activating proteins for Rac GTPase)\(^{401}\). The latter are particularly interesting candidates since Rac is a component of the NADPH oxidase\(^{360}\). It is not clear if any of these proteins or other yet unidentified non-PKC phorbol ester/DAG receptors mediate PMA-induced oxidative burst through PI3K (Fig. 5-10A). Further studies will be required to elucidate the mechanism of p85/p110α PI3K-dependent oxidase activation by PMA.

The results of our studies focusing on activation of the oxidase are particularly informative when viewed in the context of results obtained from class IB PI3K p110γ\(^{−/−}\) neutrophils\(^{175}\). Neutrophils from PI3K p110γ knockout mice had normal PMA- and OPZ-induced oxidative burst responses, but LPS-primed neutrophils from these mice had defective fMLP-induced burst activity when compared to control cells\(^{175}\). Although similar experiments in macrophages from these mice were not reported, these findings together with our data suggests that class IA PI3K p110α isoform is required for oxidase activation downstream of CR3 and PMA receptors, while class IB PI3K p110γ is required for the GPCR stimulated oxidative burst. Therefore regulation of the oxidase by PI3K provides an example where distinct PI3K isoforms can be coupled to distinct receptors to mediate a common biological function. The reasons for this segregation of isoform usage is not entirely clear, but the fact that p110α can also mediate other aspects of monocyte
biology such as cytokine signaling and phagocytosis suggest that distinct isoform usage allows different cellular processes to be coupled together depending on the physiological context.
CHAPTER VI: DISCUSSION

The signaling pathways in which PI3K family members are involved are complex. This is due to several reasons including: the existence of multiple isoforms with different regulation and enzymatic activities, diverse actions of multiple effectors, and the different tissue distribution and intracellular localization among the different isoforms. Furthermore, PI3K family members are linked to numerous receptors as well as to diverse regulatory subunits and this also contributes to the challenge of studying their roles in regulating biological processes. This thesis describes a novel approach to silencing PI3K isoforms in human monocytic cells and also identifies several phenotypes brought about by this manipulation. Thus, we show that a deficiency of class IA PI3K p110α isoform results in defects involving several important mononuclear phagocyte functions including: adherence, expression of cell surface receptors, phagocytosis, activation of the phagocyte oxidase, and cytokine secretion in response to bacterial endotoxin. The data show that in respect to these properties p85/p110α PI3K appears to have both redundant and non-redundant roles. Several generalizations of this isoform specificity as well as the limitations of the gene-silencing approach used are discussed below.

6.1 Choice of gene silencing strategy may affect phenotype.

Different PI3K gene silencing strategies can result in different signaling and functional outcomes. The non-specific or bystander effects of alterations in PI3K subunit expression seen in some PI3K knockout experiments were discussed in Chapter III. We
show here that—in contrast to the non-specific effects observed with the generation of p85α knock-out mice \(^{183,228}\)—lentiviral-delivered siRNA provides a means to achieve specific silencing of a PI3K family member without altering the expression of other isoforms.

The particular gene targeting strategy used may also affect experimental outcomes by virtue of the occurrence of PI3K kinase-independent effects. Many functions attributed to PI3K signaling have been discovered through the use of PI3K inhibitors, which inhibit the kinase function of the catalytic p110 subunit. The p110 subunit, however, has multiple domains that can mediate non-kinase dependent effects, and therefore gene knockouts that remove the entire catalytic subunit will not be able to distinguish between kinase and non-kinase dependent functions \(^{404}\). A good example illustrating this is the effect on cardiac phenotype by class IB PI3K p110γ \(^{68}\). PI3K p110γ KO mice had, in addition to its immunological phenotypes (Table 1-4), increased cardiac contractility and developed cardiac tissue damage under pressure overload \(^{68}\). One mechanism proposed to suggest how p110γ regulates contractility is by reducing basal levels of cAMP \(^{405}\). In experiments by Hirsch and colleagues, mice with a complete knockout of PI3K p110γ isoform were compared with mice expressing a kinase-dead p110γ, created by “knocking in” an inactivating point mutation of the catalytic domain. They discovered that p110γ kinase-dead mice did not develop myocardial damage and did not have elevated cAMP concentrations \(^{68}\). Kinase dead PI3K p110γ was shown to bind and activate phosphodiesterase 3B (PDE3B), which mediated cAMP hydrolysis. Therefore, the cardiac PI3K p110γ is able to participate in a kinase-independent activity that relies on
protein interactions to regulate PDE3B activity and negatively modulate cardiac contractility. This example illustrates the importance of protein-protein interactions and scaffolding in the control of signaling pathways, and highlights the limitation of gene silencing strategies. This kinase-independent effect of p110γ isoforms have not been identified in class IA PI3K, and was not explored using the lentiviral approach presented in this thesis. However, we cannot exclude the possibility that kinase-independent functions of p110α isoform may be partly responsible for the phenotypes observed. A recent report of newly developed PI3K class I isoform specific inhibitors may be useful tools in clarifying this issue by doing studies using a combination of specific enzymatic inhibitors with approaches that down-regulate protein levels.

Another limitation of the results presented in this thesis is that experiments did not address changes in 3'-PI levels or location in the cells brought about as a result of silencing p110α by lentiviral delivered siRNA. Overall levels of PtdIns(3,4,5)P₃ in the cell after stimulation could be normal even in p110α silenced cells since other class I isoforms are still active, and may be activated through other pathways. Therefore, phenotypic features attributed to p110α may be due to alterations in subcellular localization of PtdIns(3,4,5)P₃ or other metabolites rather than to overall reductions in their levels. Studies using GFP tagged probes with PH or PX domains might help to localize changes in the subcellular distribution of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ and allow further insight into mechanisms.
6.2 A single receptor complex can activate multiple PI3K isoforms, and different receptors can lead to the same biologic response by using different PI3K isoforms

Results from Chapter 4 on LPS and D₃-induced adherence illustrate how different PI3K isoforms can lead to a common function through associations with different receptors (Fig. 6-1A). Our data show that p110α was required for D₃ but not LPS-induced adherence, even though the latter was also a PI3K dependent process (Fig. 4-1). The PI3K isoform mediating LPS-induced adherence may be a distinct p110 enzymatic subunit or it may belong to another class of PI3K. Similar results to these have been observed in other systems. For example in mast cells, kit-induced exocytosis of secretory granules and Akt phosphorylation used class IA PI3K, while FceRI-initiated exocytosis and Akt phosphorylation was dependent on PI3K activity, but independent of class IA PI3K. These findings suggest a common theme of receptor-specific usage of different PI3K isoforms to regulate identical cellular functions. Future experiments using siRNA targeting of other PI3K isoforms will help to determine which isoform mediates LPS-induced adherence.

It is interesting and informative to contrast these results with our studies of cytokine production in response to LPS. We found that unlike the case with LPS-induced adherence, p110α was required for LPS-induced cytokine production (Chapter 5). Taken together, these findings suggest that a given receptor/receptor complex can use multiple PI3K isoforms to bring about diverse biological responses to the same agonist. The factors that govern isoform recruitment by any specific receptor are likely to be complex.
and are a focus of particular interest. For example it was recently shown that p110β but not p110α, was able to bind the early endosomal marker Rab5 through amino acid residues that are specific to p110β. This suggests that under both basal and stimulated conditions, various class IA PI3K isoforms may have different subcellular localizations influenced at least in part by their sequence differences. These differences may partially account for the mechanism of differential isoform recruitment by receptor complexes and even for recruitment of distinct isoforms to separate regions of the same receptor.

6.3 Control of diverse monocyte effector functions by a single isoform of PI3K

Phagocytosis and the subsequent killing of microbes in the phagosome form the basis of innate immunity against pathogens. Assembly of an activated NADPH oxidase on the phagosomal membrane is crucial for the microbicidal function of phagocytes and host defense against infections as has been made evident by patients with chronic granulomatous disease. Findings reported in Chapters 4 and 5 show that both phagocytosis and activation of the NADPH oxidase is dependent p85/p110α PI3K. This suggests that common isoform usage may allow different cellular processes to be coupled together (Fig. 6-1B). Depending on the context, however, the pathways and functions described here are not exclusive to p85/p110α PI3K. For example, class IB p110γ PI3K was shown to regulate the oxidative burst downstream of fMLP receptors, and phagocytosis of apoptotic cells required p110β in murine macrophages. Although in the latter study NADPH oxidase activation was not examined, it has been reported that phagocytosis of apoptotic Jurkat cells by murine macrophages had attenuated PMA-
induced oxidative burst. Taken together, these findings suggest that inflammatory responses may or may not be coupled to phagocytosis. Furthermore, when coupling occurs it may be mediated by p85/p110α PI3K downstream of specific phagocytic receptors, as in the case of FcγR- or CR3-mediated phagocytosis. In addition, other receptors that activate PI3K like TLR also contribute to proinflammatory signals. Further research will be required to test this hypothesis.
Figure 6-1. Model of PI3K isoform usage linked to monocyte receptors and functional responses. A, Different receptors use distinct PI3K isoforms to regulate a common cellular function, and a single receptor complex can activate multiple PI3K isoforms. Abbreviations: LY, LY294002. Wm, wortmannin. B, A single PI3K isoform regulates multiple monocyte effector functions. In addition to adherence and cytokine production (panel A) PI3K p110α isoform is required for efficient phagocytosis through FcγR. This may be mediated through myosin X and ARNO for respectively pseudopod extension and focal endomembrane insertion. It is not clear if myosin X is also required for CR3-mediated phagocytosis. The same PI3K isoform is then able to activate the NADPH oxidase, an important component in the microbicidal activity against ingested microbes.
6.4 Perspectives and significance

Mononuclear phagocytes play crucial roles in both the innate and acquired immune responses. The 3'-PI produced by PI3K regulate many processes in monocytic cells. In addition, impaired regulation of PI3K pathways in immune cells has been demonstrated in several immunological processes and diseases. Knockouts of p110γ and p110δ in mice have revealed defective recruitment of neutrophils and macrophages to inflammatory sites, and indicated that these PI3K isoforms are involved in inflammatory bowel disease and autoimmune renal disease (Table 1-4). Because gene targeting of class 1A p110α and p110β was found to be lethal in mice, less has been known about their contribution to both normal and pathological conditions. Using lentiviral-delivered siRNA, we were able to identify important roles for p110α in the regulation of adherence, phagocytosis, the phagocyte oxidase, and cytokine production in human mononuclear phagocytes. The fine specificity of how these critical effector functions are regulated is a major focus for future investigations. Elucidation of the precise contribution of PI3K isoforms could lead to the development of isoform specific approaches to promote a selective therapeutic action while minimizing deleterious side effects.

A clear understanding of the contribution of PI3K enzymes to monocyte cell regulation—through the use of siRNA—has important implications beyond the immunological sciences. For example monocytes and macrophages are critical to the pathogenesis of atherosclerosis and lentiviral based strategies for gene silencing of PI3K should provide important insights here. Furthermore, PI3K has been implicated in a variety of
non-immune diseases such as cardiac failure, type II diabetes, hypertension, and thromboembolism where mononuclear phagocytes are found at sites of tissue damage. Therefore, PI3Ks are promising targets for therapeutic intervention.

PI3K is known to promote diverse cellular properties such as proliferation, anti-apoptosis, and migration which can contribute to oncogenesis. Thus, it is not surprising that heterogeneous defects in the PI3K-Akt signaling pathway have been identified with a high frequency in human tumors. In fact, somatic mutations in the PIK3CA gene, which encodes p110α isoform, has been implicated in a variety of tumors. For example, studies have shown a PIK3CA mutation in up to 32% of colorectal cancers, 5-27% of glioblastomas, 6.5-25% of gastric cancers, 36% of hepatocellular carcinomas, 8-40% of breast cancers, 4-12% of ovarian cancers, and 1.3-4% of lung cancers. Functional analyses of these PIK3CA mutations have shown that they uniformly result in increased enzymatic activity leading to enhanced Akt signaling and high efficiency oncogenic transformation when they are expressed in chicken embryo fibroblasts. To date, none of the other PI3K catalytic isoforms have been found to carry somatic mutations in tumors.

These findings highlight the importance of understanding features that are specific to PI3K p110α signaling and cell regulation. The use of multiple approaches including siRNA as described in this thesis and isoform specific inhibitors provide a promising opportunity to broaden knowledge of how this pathway contributes to both normal cell function and to the pathogenesis of a wide spectrum of diseases including autoimmune, inflammatory, infectious and malignant.
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